Current Biology, Vol. 15, 804–814, May 10, 2005, ©2005 Elsevier Ltd All rights reserved. DOI 10.1016/j.cub.2005.03.048

Repulsive Interactions Shape the Morphologies and Functional Arrangement of Zebrafish Peripheral Sensory Arbors

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Summary

Background: Trigeminal sensory neurons detect thermal and mechanical stimuli in the skin through their elaborately arborized peripheral axons. We investigated the developmental mechanisms that determine the size and shape of individual trigeminal arbors in zebrafish and analyzed how these interactions affect the functional organization of the peripheral sensory system.
Results: Time-lapse imaging indicated that direct repulsion between growing axons restricts arbor territories. Removal of one trigeminal ganglion allowed axons of the contralateral ganglion to cross the midline, and removal of both resulted in the expansion of spinal cord sensory neuron arbors. Generation of embryos with single, isolated sensory neurons resulted in axon arbors that possessed a vast capacity for growth and expanded to encompass the entire head. Embryos in which arbors were allowed to aberrantly cross the midline were unable to respond in a spatially appropriate way to mechanical stimuli.
Conclusions: Direct repulsive interactions between developing trigeminal and spinal cord sensory axon arbors determine sensory neuron organization and control the shapes and sizes of individual arbors. This spatial organization is crucial for sensing the location of objects in the environment. Thus, a combination of undirected growth and mutual repulsion results in the formation of a functionally organized system of peripheral sensory arbors.

Introduction

Vertebrates employ trigeminal sensory neurons to sense mechanical and thermal stimuli in the head. Trigeminal neurons are part of the peripheral nervous system and reside in two ganglia located lateral to the anterior hindbrain. These neurons possess a bipolar or pseudo-unipolar morphology—they receive sensory information via an elaborate peripheral axon that arborizes extensively under the skin and transmit that information through an unbranched central axon that innervates the hindbrain. Trigeminal sensory neurons are among the first vertebrate neurons to develop. In zebrafish, they begin to elaborate their axons at approximately 16 hr postfertilization (hpf) and animals respond specifically to touch around 21 hpf [1, 2]. Even at these very early stages, embryos are able to discern the location of touch stimuli and respond appropriately. For example, touching the left side of the head causes embryos to escape to the right, touching the right causes them to escape to the left, and after 27 hpf, touching the tail causes embryos to swim forward [2]. Embryonic sensory neurons are thus faced with the task of rapidly elaborating peripheral axon arbors that blanket the skin with a dense network of fibers, making every part of the head sensitive to touch, while simultaneously arranging themselves in a way that allows appropriate spatial discrimination. We have investigated how the rapidly developing arbors of trigeminal neurons are shaped, limited in size, and coordinated with the growing peripheral axons of their neighbors to create a functional arrangement of arbors.

Analogous to the role played by trigeminal neurons in the head, neurons in the dorsal root ganglia (DRG) sense mechanical and thermal stimuli in the body. In fish, a transient population of cells called Rohon-Beard neurons subserve this function until DRG neurons replace them later in development [1]. Rohon-Beard neurons are located in the dorsal neural tube, and the neural-crest-derived DRGs are organized into peripheral ganglia located along the length of the spinal cord. Trigeminal, Rohon-Beard, and DRG neurons are together responsible for all touch sensation in the skin of zebrafish.

Interactions between developing neuronal arbors can influence the morphologies of arborizing neurites in several systems. These interactions have been best examined among dendrites in the mammalian retina and among the arbors of peripheral sensory neurons in leech and *Drosophila* larvae [3–11]. For example, lesioning the retina causes retinal ganglion cells near the wound to reorient their arbors toward the lesion [12]. This result was interpreted as a disposition of neurons to fill in the space lacking innervation. Ablation of mechanosensory or nociceptive neurons that innervate the leech epidermis caused other neurons of the same subclass to expand their arbor territories [5]. Similarly, ablation of *Drosophila* sensory neurons causes other neurons of the same subclass to expand their dendritic territories [9, 11]. The role of homotypic interactions in limiting arbor territories is particularly clear at the dorsal midline, where neurites from opposite sides of the animal meet. Among both sensory neurons in *Drosophila* larvae and trigeminal neurons in *Xenopus*, ablation of neurons on one side of the midline causes contralateral neurons to expand their peripheral arbors aberrantly past the midline, suggesting that arbors from opposite sides interact competitively with one another to confine themselves to one side of the midline [6, 13]. The minimally overlapping arrangement that could result from these competitive interactions is often referred to as "tiling" and ensures thorough surface cov-

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erage while preventing multiple cells from innervating the same area [14]. In several systems, neuronal subclasses innervating the same surface tile independently so that dendrites tile within but not between subclasses [5, 7–9, 11, 15].

Despite evidence that developing arbors employ homotypic interactions to minimize arbor territories, a recent study by Lin et al. has challenged the notion that these interactions are absolutely required for determining arbor morphologies [16]. These authors examined retinal ganglion cell dendrites in two mouse mutants that reduce the total number of neurons without affecting their specification. Despite the much lower density of retinal ganglion cells in these mutants, neither the sizes nor shapes of dendritic territories were significantly affected, suggesting that homotypic interactions are not required for determining individual arbor morphologies. Instead, neuron-neuron interactions might fine tune borders between dendritic territories.

Here, we investigate how the morphologies of zebrafish trigeminal and spinal cord sensory neuron peripheral axons are limited, shaped, and arranged. Using time-lapse analysis, we demonstrate that subsets of neurons directly repel one another. Interactions between axons determine the behavior of arbors at the midline, the outgrowth trajectory of arbors, their topographic organization, and the respective territories of trigeminal and spinal cord sensory neuron arbors. By creating embryos with single trigeminal neurons, we reveal that isolated cells possess a vast capacity to grow and arborize. Thus, interactions between developing axons are not employed simply for fine tuning but, rather, are the chief mechanism regulating trigeminal sensory neuron shape and size. Behavioral experiments demonstrate that the spatial organization of arbors that results from these interactions is crucial for allowing the organism to correctly sense the location of environmental stimuli and respond appropriately.

Results

Trigeminal and Spinal Cord Sensory Neurons Possess Elaborate, Heterogeneous Peripheral Arbors that Are Topographically Organized

To study the development of sensory neurons in vivo, we created a transgenic line that drives strong GFP expression in trigeminal sensory neurons and the closely related Rohon-Beard neurons (for details, see Experimental Procedures). This transgene revealed an elaborate, heterogeneous, and fast-growing constellation of trigeminal and Rohon-Beard peripheral axon arbors (Figure 1). Unexpectedly, lines with the stably-integrated Tg(sensory:GFP) transgene expressed GFP in a variegated manner—between one fifth and one half of the neurons in a trigeminal ganglion of a transgenic animal expressed GFP. The reason for this variegation is unknown, but as with similarly variegated transgenes, this feature facilitates the visualization of dynamic behaviors of single cells [17]. We visualized several hundred single trigeminal neurons in individual animals with transient transgenics (Figure 1). Each neuron extended a peripheral axon that arborized over a discrete region of the face. The majority of axons (89%, n = 230) extended at least 50 μm away from the ganglion before arborizing. Sensory axon arbors tended to be largely confined to one side of the midline (see below). As expected from previous descriptions [1], trigeminal neuron arbors were confined to the head and anterior yolk, whereas arbors of Rohon-Beard neurons were confined to the body and posterior yolk. Rohon-Beard neurons never extended axons as far as the eyes or to any part of the face anterior to them, and trigeminal neurons never sent axons over the somites.

We determined the correspondence between cell-body position within a ganglion and the direction of peripheral arbor projection with variegated Tg(sensory:GFP) transgenics (Figures 2A and 2B) by simultaneously staining for the GFP transgene and HuC, a marker that reveals all trigeminal neuron cell bodies [18]. Cells were assigned to a column based on their anterior/posterior position in the ganglion (Figure 2A), and arbors were scored for their projection to one of four zones (anterior, dorsal, posterior, or ventral) (Figure 2B). Cell-body position and projection zone strongly correlated (Figure 2C). For example, although only 49% of scored cells resided in the two most anterior columns, 93% of axons innervating the anterior zone came from cells in one of these two columns ($p = 0.05$). Conversely, although only 40% of cells resided in column 4 or more posterior columns, 100% of axons innervating the posterior zone originated from these columns ($p < 0.001$). The distribution of axons projecting to the dorsal or ventral zones did not differ significantly from the total anterior-posterior distribution of cells within the ganglion ($p > 0.9$ for the dorsal zone and $p > 0.99$ for ventral zone). Thus, a rough topographic map relating cell-body position within a ganglion to the direction of axon trajectory develops early in the zebrafish trigeminal sensory ganglion, similar to the organization described in rat [19, 20].

Developing Peripheral Sensory Arbors often Repel One Another upon Contact

To gain insight into the mechanisms by which arbor architecture develops, we made time-lapse movies of growing arbors expressing the Tg(sensory:GFP) transgene (e.g., Movie S1 available with this article online). These movies revealed that most branches form as collaterals emerging from an axon shaft, but growth-cone bifurcations also occasionally occur (Figures S1A and S1B and Movies S2 and S3). Unexpectedly, rapid local axon degeneration was occasionally observed (Figure S1C and Movie S4).

Time-lapse analysis of growing axon arbors revealed another striking behavior: the reluctance of axons to cross over one another (Figure S2 and Movies S5–S7; Table 1). Often when a growth cone encountered another axon, it collapsed or changed direction. Occasionally when a growth cone did cross another axon branch, it appeared to trigger local degeneration in the crossed axon. These interactions were observed isoneurally (between branches of the same cell) (Figure S2A and Movie S5), between axons of different cells residing in the same ganglion, between axons growing from the two opposite ganglia (Figure S2B and Movie S6), and between trigeminal and spinal cord sensory axons (Figure S2C and Movie S7). We analyzed 573 in-

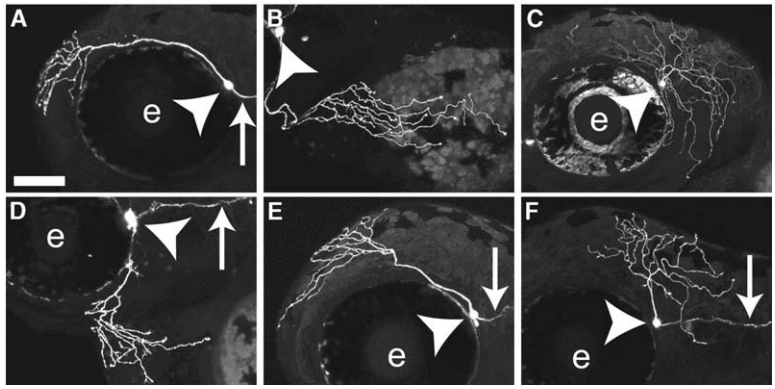

Figure 1. Trigeminal Peripheral Axon Arbors Possess Elaborate and Diverse Morphologies

Confocal projections of peripheral arbors of single trigeminal sensory neurons revealed by transient expression of the Tg(sensory:GFP) transgene at 4 dpf. Anterior is left, and dorsal is up in all panels. The scale bar in (A) represents approximately 100 μm in (A) and (D), 80 μm in (B), 85 μm in (C), and 105 μm in (E) and (F). Arrowhead indicates cell body, and arrow indicates central projection where visible. "e" indicates center of eye where visible. Axons project anteriorly over the head (A), ventroposteriorly over the yolk (B), dorsoposteriorly over the head (C), ventrally over the pericardium (D), anterodorsally over the head (E), and dorsally over the head (F).

dividual encounters between growth cones and axons in 18 different time-lapse movies. Isoneural encounters exhibited the highest rate of repulsion, with branches avoiding one another in roughly 84% of encounters; all other types of encounters resulted in similar repulsion frequencies (45%–56%) (Table 1). To determine whether all the branches of a single neuron behave in a consistent way with respect to other neurons, we analyzed thirteen pairs of sensory neuron arbors that encountered each other at least four times at different growth cones or branches. Six of these neuron pairs repelled each other consistently (84% of encounters resulted in repulsion, range = 67% to 100%). The seven other pairs consistently ignored each other (2% repulsion, range = 0% to 17%). Thus, pairs of neurons either consistently repelled or consistently ignored each other.

In the Absence of Contralateral Neurons, Peripheral Sensory Arbors Grow Too Far Past the Midline

Because axons growing from the two trigeminal ganglia reach the midline at roughly the same time, we wondered whether repulsive interactions between axons

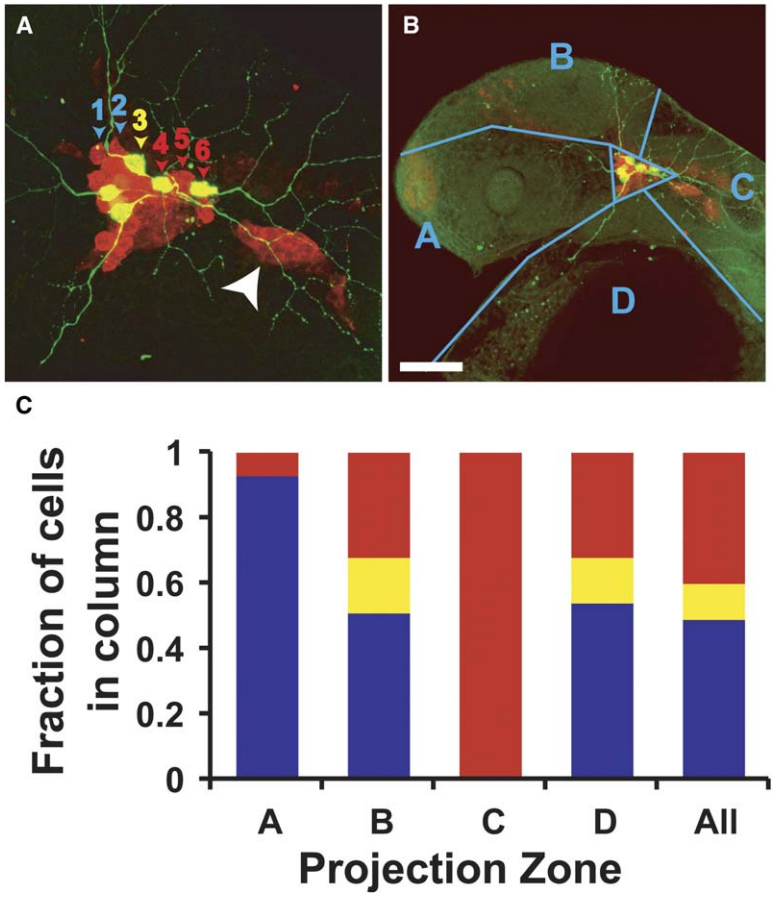

Figure 2. Axon Trajectory Predicts Cell-Body Location within a Ganglion

Anterior is left and dorsal is up in (A) and (B). Bar in (B) represents 100 μm; (A) is 2.7 times higher magnification image of the same embryo. Arrowhead in (A) indicates anterior lateral line ganglion. (A) Confocal projection of trigeminal ganglion revealed by HuC staining (red), and Tg(sensory:GFP) expression (green) at 30 hpf. Numbers above cells indicate column assignments for cells below them. (B) Zonal assignments for arbors. Area "A" is anterior, over the eye; "B" is dorsal, from the eye to the rhombic lip; "C" is posterior, from the rhombic lip to the yolk; and "D" is ventral, over the yolk. (C) Quantification of correspondence between projection zone and cell-body position (n = 135 neurons in 24 embryos). X axis shows projection zones and total distribution of cells scored for the analysis (All); y axis shows fraction of cells residing in columns 1 and 2 (blue), 3 (yellow), and 4 or greater (red).

Table 1. Outcomes of Axonal Encounters

Interaction	Percent Repulsion	Percent Degeneration	Percent Not Responding	n
Isoneural trigeminal	86	1	13	229
Isoneural Rohon-Beard	70	0	30	27
Isoneural total	84	1	15	256
Trigeminal/trigeminal (same ganglion)	48	2	50	187
Trigeminal/trigeminal (different ganglia)	56	0	44	59
Trigeminal/Rohon-Beard	48	7	45	29
Rohon-Beard/Rohon-Beard	45	5	50	42
Different cells total	49	2	49	317

Encounters between growth cones and axons were scored for whether they resulted in the growth cone collapsing or changing direction (repulsion), local degeneration of an encountered axon (degeneration), or the two axons progressing past one another (no response). Data were compiled from 18 movies of individual embryos that each spanned some subset of the developmental time period between 16 hpf to about 48 hpf.

from the two ganglia are responsible for arbors stopping near the dorsal midline. We created animals with a single ganglion by transplanting wild-type cells carrying the Tg(sensory:GFP) transgene into hosts injected with a morpholino antisense oligonucleotide against *neurogenin-1* (*ngn-1MO*) during pregastrulation stages. Depletion of *ngn-1* prevents the differentiation of trigeminal and Rohon-Beard sensory neurons [21, 22]. We transplanted wild-type cells from an embryo bearing the Tg(sensory:GFP) transgene into wild-type embryos without a transgene, as a control. Axons crossed an average of 26.6 ± 5.3 μm over the midline in wild-type animals (n = 46 axon tips in 17 embryos) (Figures 3A and 3D), whereas axons transplanted into *ngn-1* morphants crossed an average of 85.9 ± 3.9 μm (n = 232 axon tips in 22 embryos) (Figures 3B and 3D). This measurement is an underestimate because axons were analyzed at a stage when they still have the capacity to grow further in *ngn-1* morphants (see below).

Embryos with one trigeminal ganglion were created surgically to rule out the possibility that injection of *ngn-1MO* caused transplanted neurons to behave aberrantly. Trigeminal ganglia in Tg(sensory:GFP)-bearing but otherwise wild-type fish were removed with a micropipette at approximately 24 hpf, several hours before axons reach the midline. Axons of unpaired ganglia crossed an average of 91.1 ± 5.0 μm over the midline, confirming that interactions between arbors originating from opposite ganglia limit the extent of growth past the midline (n = 166 axon tips in 15 embryos) (Figures

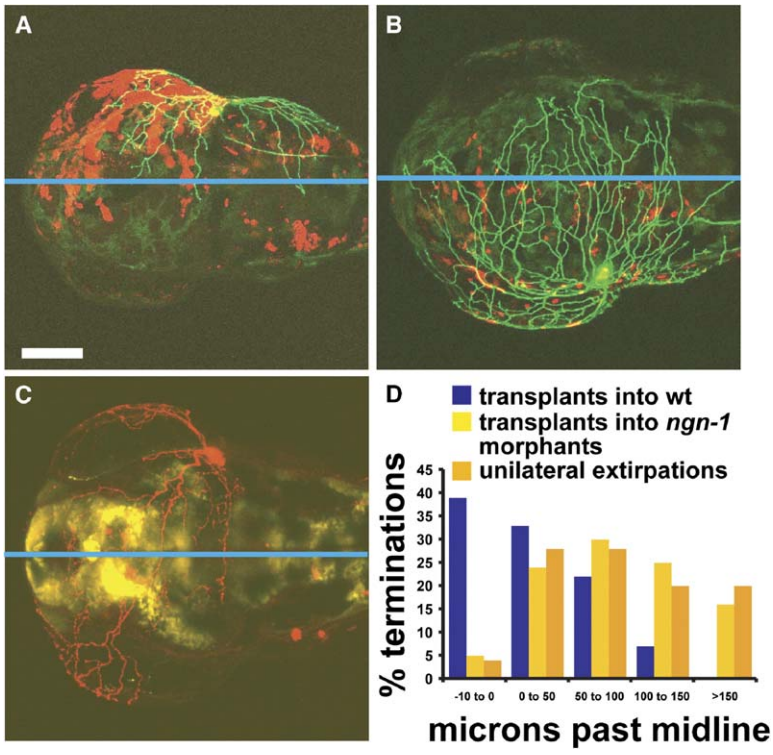

Figure 3. Interactions between Axons from Opposite Trigeminal Ganglia Stop Arbor Growth Near the Midline

Confocal projections of dorsal views of 3 dpf embryos. Anterior is left, and right is up in all panels. Bar in (A) represents 100 μm. (A and B) Wild-type rhodamine-labeled cells (red) were transplanted into wild-type (A) or *ngn-1* morphants (B), giving rise to trigeminal neurons (green). (C) Trigeminal cells were manually removed with a pipette from the left side of the embryo. Yellow indicates *ngn1:GFP* expression, used to identify neurons for extirpation, transient expression of Tg(sensory:RFP) (red) labels cells in the remaining ganglion. (D) Location of axon branch terminations with respect to the midline (compilation of data from all embryos; wild-type, 46 axon tips in 17 embryos; transplants into *ngn-1* morphants, 232 axon tips in 22 embryos; extirpations, 166 axon tips in 15 embryos). X axis indicates distance from the midline in microns, and y axis indicates percent of axons terminating in each region. Blue bars indicate distribution of axon terminations in cells transplanted into wild-type, yellow indicates distribution of axon terminations in cells transplanted into *ngn-1* morphants, and orange indicates distribution of axon terminations in embryos in which the opposite ganglion was extirpated. Distributions of axon terminations in each of the three situations were compared to one another with chi-square tests. Wild-type distribution was significantly different from the two experimental situations ($p < 0.001$ for both comparisons), but extirpation distribution was not significantly different from transplants into *ngn-1* morphants ($p > 0.10$).

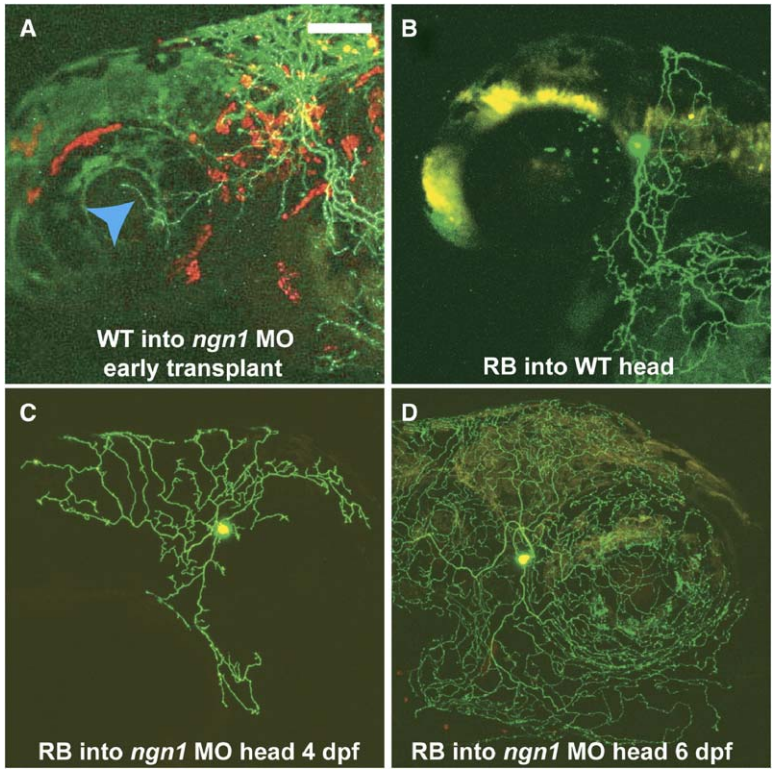

Figure 4. Rohon-Beard and Trigeminal Sensory Neuron Arbors Are Developmentally Equivalent

Anterior is left and dorsal is up in (A) and (B); anterior is right in (C) and (D). Bar in (A) represents 100 μm. (A) Rhodamine-labeled cells (red) were transplanted into *ngn-1* morphants during pregastrulation stages and gave rise to Rohon-Beard neurons (green) but not trigeminal neurons. In the absence of a trigeminal ganglion, Rohon-Beard neuron peripheral arbors extended over the eye (blue arrowhead) by 5 dpf. (B–D) Single Rohon-Beard neurons were transplanted to the region of the trigeminal ganglion during midsomitogenesis stages in wild-type (B) and *ngn-1* morphant embryos (C and D). In wild-type, cells grew normal arbors that projected to one part of the face and arborized over a discrete region (B), whereas isolated cells in a *ngn-1* morphant background extended unconstrained arbors (C and D) that continued growing at 4 dpf (~2.5 days after transplantation) (C) and 6 dpf (D). Like the trigeminal transplants (Figure 5), seven out of the eight spinal cord cells transplanted into wild-type or morphant hosts failed to extend central axons. One of the cells transplanted into wild-type ganglia did, however, extend a morphologically normal central axon, and this was neither the cell with the "less-constrained" morphology nor the one that survived past 5 dpf (see text and Table 2).

3C and 3D). Thus, like *Drosophila* and *Xenopus* sensory arbors, competitive developmental interactions among arbors growing from opposite sides of the embryo confined each other to one half of the head [6, 13]. This is likely the only mechanism that stops axons near the midline because in the absence of contralateral neighbors, growth cones traverse the midline at a continuous rate (Figure S3).

Interactions between Developing Rohon-Beard and Trigeminal Sensory Neurons Limit Each Other's Territories

Trigeminal and Rohon-Beard neurons innervate head and body, respectively, but are genetically similar. Every marker known to be expressed by them is found in both cell types [1, 23–25], and every genetic intervention that affects their development affects both populations [21, 22, 26]. These similarities led us to investigate whether interactions between developing arbors of these two populations limit the territories of their peripheral arbors. To test this, we transplanted wild-type cells before gastrulation stages into *ngn-1* morphants, which lack both populations. Transplanted wild-type cells occasionally gave rise to Rohon-Beard clones populating the anterior spinal cord but not any trigeminal neurons. In four out of five such cases, spinal cord sensory neurons extended peripheral arbors anteriorly as far as the eye by 5 days postfertilization (dpf), a phenomenon never observed in wild-type embryos (Figure 4A).

Interactions between Sensory Axon Arbors Control Their Sizes, Shapes, and Outgrowth Trajectories

We wondered whether interactions between axons emerging from the same ganglion also confined arbor territories or determined the direction of outgrowth. Arbor morphologies and their orderly topographic organization might be determined in at least three ways. First, each cell may be born with intrinsic genetic instructions determining axon direction, size, and shape. Second, patterned molecular cues in the environment could tell trigeminal neurons where to extend their axons. Finally, interactions between axons during development could determine outgrowth trajectories and organize their topographic arrangement de novo. The latter hypothesis can be distinguished from the first two by monitoring the behavior of isolated neurons. If neurons possess genetic instructions determining their direction of outgrowth or if they respond to environmental cues, an isolated neuron should grow a normal arbor, but if neurons require interactions with their neighbors to choose their outgrowth trajectory, an isolated neuron should behave abnormally, perhaps sending its peripheral arbor symmetrically about its cell body.

We assessed the behavior of single, isolated neurons by transplanting neurons from Tg(sensory:GFP) embryos into *ngn-1* morphant embryos [21, 22]. We first transplanted cells from trigeminal-GFP embryos into wild-type and *ngn-1* morphant embryos during pregastrulation stages and screened for embryos in which transplanted cells gave rise to a single trigeminal neuron. In twenty-three cases, control transplants gave rise to a single trigeminal neuron. In all of these cases, the neuron derived from a transplant was morphologically normal, extended a single peripheral axon in a particular direction that correlated with its position in the ganglion, arborized over a discrete region of the head, and ceased growing after 2 dpf (Figure 5A and Table 2). Transplants into a *ngn-1* background usually gave rise

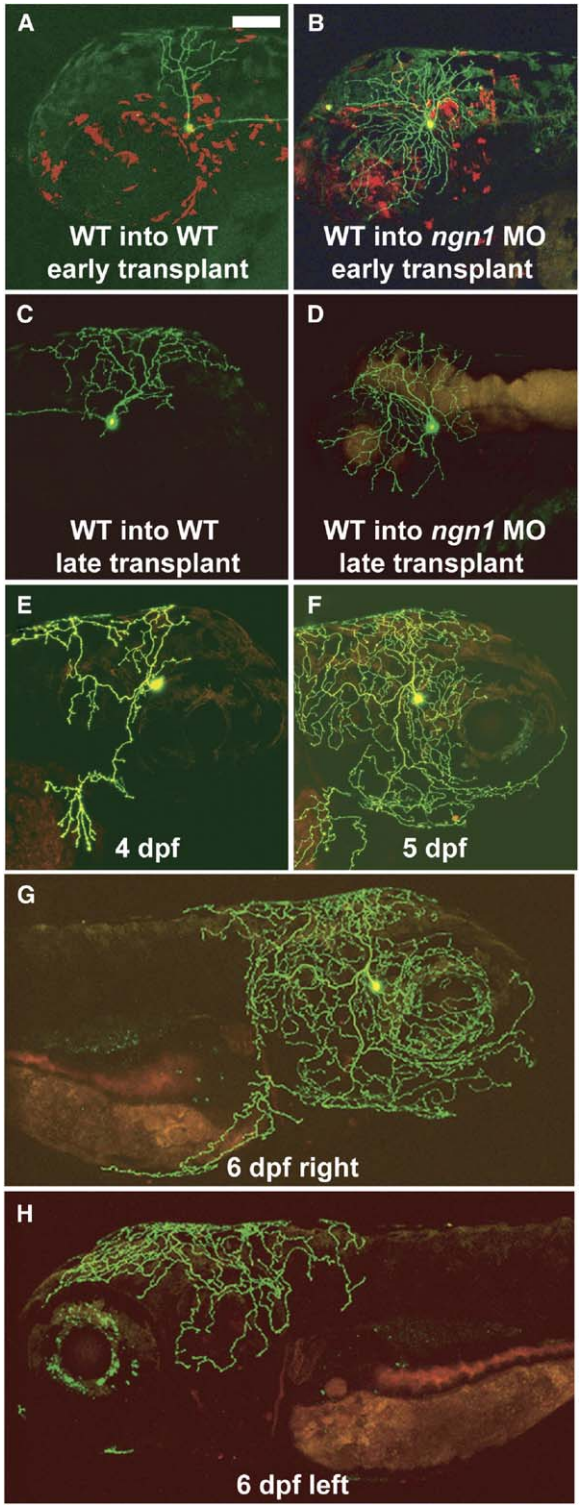

Figure 5. Trigeminal Sensory Neurons Influence One Another's Outgrowth Trajectory and Size

Anterior is left and dorsal is up in (A), (B), (D), and (H); anterior is right in (C) and (E)–(G). Bar in (A) represents 100 μm. (A and B) Rhodamine-labeled cells (red) were transplanted into wild-type (A) or *ngn-1* morphants (B) during pregastrulation stages and gave rise to trigeminal neurons (green). Single trigeminal neurons were transplanted during midsomitogenesis stages into wild-type (C) and *ngn-1* morphant embryos (D). For the experiment in (D), a Tg(sensory:RFP)-expressing cell was transplanted into a neurogenin::GFP-expressing wild-type embryo; colors were switched to make the figure consistent. In wild-type, cells grew normal arbors that projected to one part of the face and arborized over a discrete region (A and C), whereas isolated cells in a *ngn-1* morphant background extended unconstrained, symmetrical arbors (B and D). A single neuron was transplanted into a *ngn-1* morphant at midsomitogenesis stages and visualized at 4 dpf (E), 5 dpf (F), and 6 dpf (G and H). Unexpectedly, two of the three single-neuron clones derived from pregastrulation transplants into *ngn-1* morphants, and all of the single neurons transplanted at midsomitogenesis stages (whether into wild-type or *ngn-1* morphants) failed to extend central axons (Table 2). These observations indicate that a critical number of trigeminal neurons may be required for the formation of central projections and that something about late-stage single-neuron transplants compromises a cell's ability to extend a central projection. The lack of central projections, however, does not alter our conclusions about peripheral arbor outgrowth because central projections were missing in single-cell transplants into both wild-type and *ngn-1* morphants, which exhibited strikingly different peripheral axon morphologies.

to larger trigeminal clones, but three single neuron clones were found (Figure 5B). In contrast to control, the three isolated trigeminal neurons derived from pregastrulation stage transplants into *ngn-1* morphants developed arbors that grew out from the cell body in an apparently unconstrained fashion. Their peripheral arbors began branching immediately proximal to the cell body, rather than extending for a distance before arborization. Branching did not appear to be limited to any particular part of the head, ultimately resulting in an arbor with a more symmetrical spread around its cell body.

To rule out the possibility that precursor cells transplanted early in development gave rise to aberrantly specified trigeminal neurons, we created embryos with only one trigeminal neuron by directly transplanting single, specified neurons during midsomitogenesis stages (12–18 somites), when axonogenesis is just beginning. Single neurons transplanted into three wild-type embryos, and two *ngn-1* morphants extended peripheral arbors (Figures 5C–5H; Table 2). As in the previous experiment, all three neurons successfully transplanted into wild-type extended normal peripheral axons that projected to one part of the head and elaborated an arbor over a discrete region—one anteriorly to the front of the face, one dorsally to the top of the head, and one ventrally over the heart. Moreover, the trajectory of their projections corresponded to that expected from their cell-body position. Unexpectedly, although the single neurons transplanted into wild-type embryos during somitogenesis stages appeared normal 24 hr after transplantation, all three began rapidly degenerating within the next 12 hr. In contrast, the two neurons successfully transplanted into *ngn-1* morphants survived for as long as they were observed (5 days after transplantation), suggesting that transplanted wild-type neurons are compromised, potentially for neurotrophic support, in a competitive interaction with endogenous neurons. Strikingly, the isolated trigeminal neurons in *ngn-1* morphants elaborated peripheral arbors that grew and arborized for as long as they were monitored, well after wild-type neurons have stopped growing and

Table 2. Summary of Transplantation Experiments

Condition		Unconstrained axon Growth[a]	Normal Central Projection	Survival Past Day 3 Postfertilization
Early-stage transplants	Into wt	0/23	23/23	23/23
	Into *ngn-1* morphants	3/3	1/3	3/3
Single trigeminal neuron transplants	Into wt	0/3	0/3	0/3
	Into *ngn-1* morphants	2/2	0/2	2/2
Single Rohon-Beard neuron transplants	Into wt	1/3[b]	1/3[b]	1/3[b]
	Into *ngn-1* morphants	5/5	0/5	5/5

[a] Axons were placed in this category if they branched immediate adjacent to the cell body and their arbor extended over more than two of the territories defined for the topography analysis by 3 dpf (Figure 2).
[b] The neuron with the unconstrained axon, the neuron with a normal central projection, and the neuron that survived past 3 dpf were three different neurons.

neurons transplanted into wild-type ganglia have died (Figures 5E–5H and Table 2). 5 days after transplantation, the arbors of these isolated neurons covered almost the entire side of the head onto which they were transplanted as well as a large portion of the opposite side. These results indicate that in the absence of interactions with other axons, peripheral arbors possess a vast capacity for growth (Figures 5G and 5H).

Rohon-Beard Neurons Can Populate Trigeminal Ganglia and Their Peripheral Arbors Display a Vast Growth Potential Similar to Trigeminal Neurons

The mutual repulsion between sensory neurons in the body and those in the head suggests that these two cell types have similar developmental potentials. To test this idea, we transplanted single Rohon-Beard neurons during midsomitogenesis stages into the face of both wild-type embryos and *ngn-1* morphants, in the position normally occupied by trigeminal neurons. Two of the three spinal cord neurons successfully transplanted into wild-type trigeminal ganglia extended normal peripheral arbors. The third extended an axon that branched close to the cell body and invaded several parts of the face (Figure 4B and Table 2), but even in wild-type embryos, a rare neuron exhibits such a morphology. Similar to transplanted trigeminal neurons, two of the three spinal cord neurons transplanted into wild-type died between 24 and 48 hr after the operation. The neuron that survived until day five postfertilization stopped extending its arbor by 48 hpf, similar to wild-type trigeminal neurons in unoperated animals. In contrast to the transplants into wild-type hosts, five out of five spinal cord neurons successfully transplanted into the faces of *ngn-1* morphant embryos extended unconstrained arbors, survived, and continued to extend their peripheral arbors for as long as they were tracked (Figures 4C and 4D). Transplanted trigeminal and Rohon-Beard neurons thus behaved indistinguishably, arguing for a high degree of similarity between the two cell types (Table 2). Together, these data strongly indicate that sensory neuron peripheral arbors have a capacity for growth and arborization limited mainly, if not exclusively, by interactions with like neurons.

Trigeminal Sensory Arbor Expansion across the Midline Results in the Reversal of Touch Response Laterality

The anatomical limits of peripheral arbor territories that result from repulsive interactions (e.g., at the midline, between the head and body) may have important functional implications. We therefore tested the behavioral consequences of the failure of repulsive interactions to limit arbor territories.

Fish embryos possess a robust escape response to mechanical stimuli. When touched on the right side of their head, they respond with a stereotyped reflex that causes them to bend away from the stimulus toward the left. Conversely, if touched on the left, they escape toward the right. The neuronal circuit that mediates this response has been well characterized in goldfish and zebrafish [27, 28]. Stimulation of sensory neurons leads to activation of hindbrain interneurons that project to the contralateral side of the spinal cord, where they activate motor neurons and cause muscles on one side of the animal to contract, thus turning the fish away from the stimulus. This robust behavioral response provides an opportunity to test the functional importance of limiting axon arbor territories.

The relative confinement of trigeminal sensory neuron arbors to the ipsilateral half of the face could provide a simple mechanism determining the laterality of the escape response. If the spatial organization of trigeminal peripheral arbors is used to interpret the location of environmental stimuli, then embryos with axons that aberrantly cross the midline should exhibit abnormal touch response laterality. To test this, we scored the response of embryos at 3 dpf to touch on the left and right sides of the head in wild-type embryos and embryos with single trigeminal ganglia. As we have shown, removal of one trigeminal ganglion allows the other to extend axons across the midline. In these experiments, we removed the trigeminal ganglion by laser ablation. Ablations were performed at approximately 24 hpf, and embryos were assessed for the laterality of their response to touch between 50 and 56 hpf. Because laser ablations were variably effective, we stained embryos after behavioral testing with antibodies to the neuronal marker HuC to determine the suc-

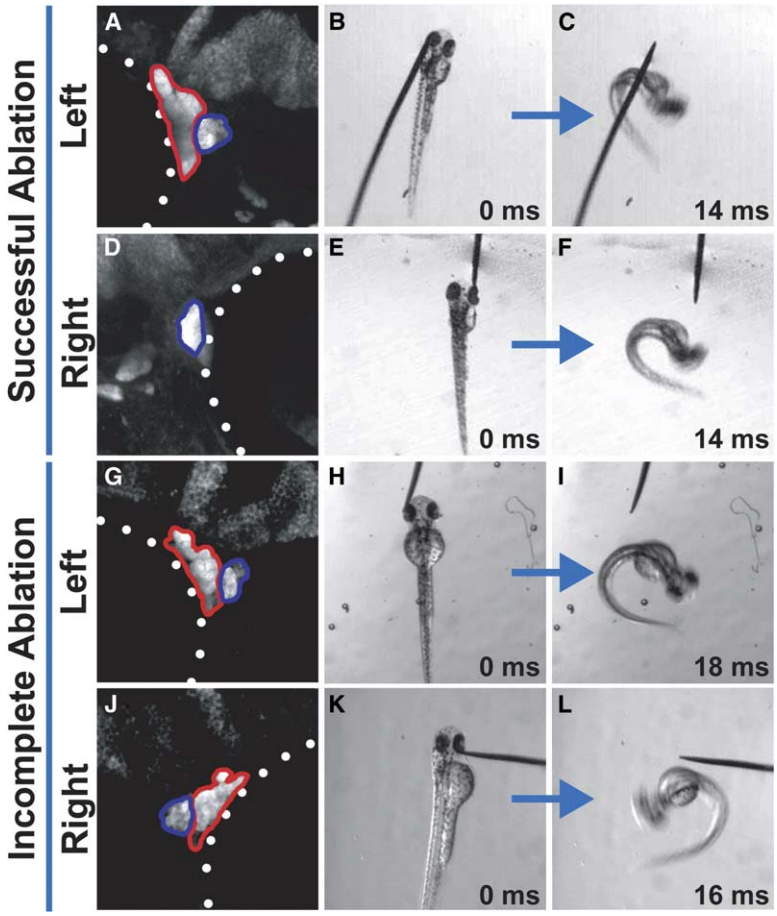

Figure 6. Embryos with Only One Trigeminal Ganglion Exhibit Behavioral Defects in the Laterality of Their Response to Touch Stimuli

Embryos were subjected to laser ablation of the trigeminal ganglion and tested for the laterality of their response to mechanical touch to the head. (A–F) Embryo in which the ablation appeared to be complete. (G–L) Embryo in which the ablation was at best partial. (A), (D), (G), and (J) show close-up confocal images of HuC stains to highlight neuronal cell bodies. In (A) and (G), anterior is left and dorsal is up, and in (D) and (J), anterior is right and dorsal is up. Dotted line indicates approximate location of the eye. Red outline indicates cells of the trigeminal ganglion; blue outline indicates cells of another cranial ganglion, the anterior lateral line ganglion. The trigeminal ganglion is fully (D) or partially (J) ablated on the right side. When touched on the left side (unoperated side) of the head with an insect pin, animals bend away from the stimulus (B,C, H, and I). When touched on the right side (operated side) of the head animals bend toward (E and F) (full ablation) or away (H and I) (partial ablation) from the stimulus. Complete data for this experiment is presented in Figure S4.

cess of the operations (Figures 6A, 6D, 6G, and 6J and Figure S4).

As expected, wild-type fish responded with perfect laterality: when touched on the right, they always escaped to the left, and when touched on the left, they escaped to the right (64/64 responses, Figure S4). All embryos subjected to the ablation procedure responded with proper laterality when touched on the side with the unperturbed ganglion (88/88 responses, Figures 6B–6C and 6H–6I and Movies S8 and S10). In contrast, when embryos were touched on the side subjected to ablation, they often responded inappropriately, "escaping" toward the touch stimulus, a behavior never observed in control (39/88 reversed and 49/88 normal responses). Some animals responded with completely reversed laterality (for example see Figures 6E–6F and Movie S9), responding to touch on any part of their head by moving away from the side with the intact ganglion. Others responded with apparently wild-type laterality (for example see Figures 6K–6L and Movie S11), moving appropriately away from the stimulus, and some responded with mixed laterality, sometimes moving toward and sometimes away from the stimulus when touched on the operated side. Staining ganglia with a HuC antibody revealed a strong correlation between the effectiveness of the ablation procedure and reversal of laterality behavior (Figure 6 and Figure S4). These results indicate that the spatial organization of peripheral arbors is important for animals to determine the spatial location of stimuli in their environment.

Discussion

To investigate the mechanisms that control arborization of peripheral sensory neurons, we have employed embryological, behavioral, and imaging approaches in zebrafish. Although the arbors of trigeminal sensory neurons are highly elaborate and diverse, their territories are limited to discrete regions of the head and their outgrowth trajectories are topographically organized. Our embryological and imaging experiments indicate that repulsive interactions between developing axons organize sensory arbors into an orderly arrangement that allows them to partition the receptive field. These developmental interactions are crucial for the arrangement of arbors in a functionally coherent manner, allowing animals to discern the location of stimuli in their environment.

Repulsive Interactions Organize and Shape Trigeminal Sensory Arbors

Our high-resolution, real-time imaging experiments suggest that homotypic repulsion limits sensory axon arborization. The rapid kinetics of the cellular behaviors observed during axon encounters are most consistent with direct-contact-mediated repulsion. The repulsive

events we observed result in distinct growth-cone behaviors—growth cones often collapse upon encountering another axon and either cease to grow forward or turn in another direction. These mutually repulsive interactions among trigeminal and spinal cord sensory neuron peripheral arbors could be sufficient to endow them with a spatially self-organizing character. All that might be required to create an organized pattern of peripheral projections is for each neuron to possess an undirected internal drive to grow and branch and a repulsive system that restricts this drive when two peripheral arbors collide with one another. In this "growth-and-repulsion" model no intricate genetic blueprint or complex environmental patterns need be invoked to explain the final organization of sensory arbors. The spatial locations of trigeminal and spinal cord sensory neurons in the embryo create natural collision zones for growing arbors—the midline, the area between the head and body, the center of a ganglion—allowing them to form a system of arbors with a predictable, orderly, and functionally significant arrangement. Our model can account for the topographic restriction of axon arbors and is supported by the results of embryological manipulations. First, ablation of sensory neurons on one side allows processes of neurons on the other side to cross into contralateral regions, similar to studies in *Drosophila* and *Xenopus* [6, 13]. Second, single isolated neurons can project in all directions. In the absence of neighboring neurons, an isolated neuron possesses a vast capacity for growth—axon arbors of these cells continued growing long after wild-type cells have stopped growing. The arbors of isolated neurons had both abnormal sizes (too big) and shapes (too symmetrical), demonstrating that interactions with neighboring neurons regulate both aspects of their morphologies. Third, ablation of trigeminal sensory neurons allows Rohon-Beard neurons to innervate the head, and transplanted Rohon-Beard neurons possess identical arborization and repulsive properties as trigeminal neurons. Previous studies in chick and frog have shown that DRG neurons, a population that serves a similar function to Rohon-Beard neurons, compete amongst themselves for axonal territories in the skin of the body [29–31]. Thus, one common system might allow vertebrate peripheral sensory neurons to interact while innervating the epidermis.

This model does not exclude additional mechanisms that might control arborization. Although direct repulsion is the predominant force sculpting axon arbors, we occasionally observed that a growth cone grew past an axon and appeared to trigger local axon degeneration in the axon that had been crossed. In addition, our results do not rule out the possibility that competition for positive factors such as neurotrophins also plays a role in limiting trigeminal arbor size [32]. It is possible that in our single-cell experiments not only are neuronal arbors freed of repulsive influences from their neighbors but also from competition for a limited source of neurotrophins.

Different Subsets of Trigeminal Sensory Neurons Might Interact Independently

Not all interactions between two axons observed in our time-lapse analyses resulted in a repulsive event (Table 1). Isoneural branches failed to repel one another roughly 15% of the time. This may reflect the fact that their target territory is not truly two-dimensional, allowing axons to slip occasionally past one another without making contact. Pairs of neurons seemed to interact in a consistent manner, either ignoring each other wherever they met or repelling each other at a rate similar to isoneural repulsion. This observation raises the possibility that there are different subclasses of trigeminal neurons that use different molecular repulsive systems. This idea is consistent with observations in other systems that neuronal subclasses innervating the same area tile independently of one another [5, 7–9, 11, 15]. Indeed, trigeminal neurons are functionally and molecularly heterogeneous, mediating benign or noxious mechanical, chemical, and thermal sensation and expressing different genes involved in their development or function. Individual subsets of trigeminal neurons subserving different modalities might therefore repel only neurons of the same subclass, allowing the whole body to be covered efficiently by the processes of each sensory neuron type.

Diverse Mechanisms Control Neurite Arborization in Different Systems

Together with previous studies, our results reveal mechanistic diversity among different systems of neurite arborization. Similar to our zebrafish system, ablation of sensory neurons in leech allows others to expand their territories, and in *Drosophila*, at least one subclass of sensory neurons invades territories after the ablation of neighboring neurons [5, 9, 11]. It remains unclear, however, whether neurons in any of these other systems possess the vast capacity for growth of zebrafish trigeminal neurons revealed in our single-cell experiments.

In contrast to the repulsive interactions between sensory neurons innervating the skin, the morphologies of at least some classes of retinal ganglion cells seem to be determined by different mechanisms. When genetically deprived of close-by neighbors, these neurons do not expand their dendritic arbors, suggesting that in the retina homotypic interactions between dendritic arbors are only employed to fine tune the borders between the territories of adjacent arbors [16]. Even in *Drosophila*, some classes of peripheral sensory neurons do not interact with their neighbors to limit dendritic territories [9, 11]. At least two mechanisms, therefore, limit arbor territories: repulsive interactions in the case of many sensory neurons that innervate epidermal territories in *Drosophila*, leech, and zebrafish and a yet-to-be-determined mechanism in the case of retinal neurons and nontiling *Drosophila* sensory neurons.

An Orderly Spatial Arrangement of Sensory Arbors Is Required for Animals to Respond Appropriately to Their Environment

The morphological and organizational features that emerge from repulsive interactions between trigeminal neurons have important implications for the functional logic of the sensory system and, ultimately, the behavior of the animal. Experiments in *Drosophila*, leech, and *Xenopus* have shown that in the absence of neigh-

boring neurons, neurite arbors physically overextend [5, 9, 11, 13], and experiments in leech and *Xenopus* have demonstrated that these aberrant arbors are physiologically active [5, 13]. Our behavioral experiments extend these observations to demonstrate that overgrowth of arbors impairs the ability of animals to perceive correctly the location of stimuli in their environment. Fish completely missing one ganglion were capable of escaping only in one direction. Touch on the side with an intact trigeminal ganglion elicited a normal escape response toward the opposite side, but touch on the ablated side caused animals to "escape" inappropriately toward the touch stimulus. These results demonstrate that spatial discrimination takes place at the level of the peripheral arbors—the central connections are incapable of adjusting to change in peripheral arbor territories.

The dramatic behavioral consequences of the failure of trigeminal axons to stop at the midline raise the possibility that there are other important functional consequences of mutual repulsion. For example, early in zebrafish development, embryos respond with distinct behaviors when touched on the head or tail—turning away from the stimulus when touched on the head and swimming forward when touched on the tail [2]. It is likely that a failure of axons to repel one another at the border between the head and tail would impair the ability of fish to distinguish between touch to these two body regions. It is tempting to speculate that the topographic organization of trigeminal ganglia that results from repulsive interactions has analogous implications for the ability of animals to sense the location of touch to different parts of the head.

Experimental Procedures

Tg(sensory:GFP)

To create the Tg(sensory:GFP) construct (as well as a Tg(sensory:RFP) construct), we used a previously described enhancer from the *islet-1* gene to drive expression of GAL4-VP16, which in turn activated eGFP under the control of fourteen copies of the Gal4 upstream activating sequences (UAS) [33, 34]. We do not make reference to the *islet-1* gene in the transgene name to avoid confusion with the widely used Isl1-GFP transgene, which uses a different enhancer and is expressed primarily in motor neurons [33]. Our transgene also drove GFP expression in a few forebrain cells. Approximately 15 pg of the transgene were injected into the one-cell-stage embryo to obtain transient transgenics expressing GFP in single trigeminal neurons. 30 to 50 pg of the Tg(sensory:GFP) were injected, along with the I-SceI endonuclease, according to the published method [35] to obtain stable transgenics. Nineteen transgenic lines were obtained and all displayed variegated GFP expression. Embryos from four of these lines (sk31–sk34) were used for further study.

Topographic Organization

Tg(sensory:GFP) transgenic embryos were fixed at 30 hpf and stained with anti-GFP (Clontech; 1:500 dilution) and anti-HuC (Molecular Probes; 1:2000 dilution) primary antibodies by standard methods and with fluorescein-conjugated anti-rabbit and rhodamine-conjugated anti-mouse secondary antibodies (Jackson ImmunoResearch). 135 neurons in 24 embryos were included in this analysis. Chi-square analysis was employed to determine the significance of the results. Eleven additional neurons appeared to have two axons emerging from the cell body projecting to different zones and were excluded from the analysis for the sake of simplicity.

Time-Lapse Analysis

Embryos were mounted in agarose in a sealed chamber and imaged with confocal microscopy by using a 10× objective. Approximately 20–40 confocal sections that were between 5 and 10 μm thick were compiled into a 3D projection at each time point. Time points were recorded either every 5 or 10 min for 10 to 20 hr durations. A heated stage was employed to keep the embryos at approximately 28°C.

Early-Stage Transplants

Cells were transplanted into the animal pole of control or *ngn-1* morphant embryos between sphere and 30% epiboly stages by standard methods. Rhodamine-dextran was used to track transplanted cells. *Ngn-1* morphants were created by injecting 3–5 ng of *ngn-1MO* (5′-CGATCTCCATTGTTGATAACCTTA-3′) between the one- and four-cell stages of development. Only those axons that reached at least 10 μm from the dorsal midline were counted to assess midline crossing. Standard error of the mean was calculated.

Ganglion Extirpations and Single-Neuron Transplants

Ganglion extirpations and single cell transplants were modeled on previous experiments [36]. Extirpations were performed at approximately 24 hpf. Trigeminal neurons were identified by Nomarski optics and GFP expression in a *ngn1:GFP* or *HuC-GFP* line [37, 38]. Embryos were injected with approximately 30 pg of a Tg(sensory:RFP) transgene to visualize trigeminal axon arbors. Embryos for single-cell transplants were between 12 and 18 somite stages. Tg(sensory:GFP) transgenic embryos or embryos injected with Tg(sensory:RFP) were used as donors, and wild-type embryos or *ngn1:GFP* embryos were used as hosts.

Laser Ablations and Behavioral Analysis

Laser ablations were performed between 20 and 26 hpf. Embryos were mounted in 2%–3% methylcellulose. Ablations were performed with a Micropoint Laser System (Photonics) in *ngn1:GFP* transgenics to facilitate the identification of trigeminal neurons. Behavior was recorded between 50 and 60 hpf with a high-speed video camera mounted on a standard dissecting scope, and frames were recorded every 2 ms. Embryos were touched with an insect pin to elicit a response. Embryos were fixed after behavioral testing at approximately 60 hpf and stained with anti-HuC primary antibodies. A biotin-conjugated secondary antibody (1:2000 dilution) was used and coupled to horseradish peroxidase with the Vectastain ABC kit (Vector Laboratories), developed with fluorescein-conjugated tyramides (PerkinElmer), and imaged on a confocal microscope.

Supplemental Data

Supplemental Data include eleven movies and four figures and can be found with this article online at http://www.current-biology.com/cgi/content/full/15/9/804/DC1/.

Acknowledgments

We are grateful to Hitoshi Okomoto for providing the *islet-1* promoter fragment, Uwe Straehle and Patrick Blader for *ngn1:GFP* transgenics, and Reinhard Köster and Scott Fraser for the Gal4/UAS vector. We thank Harry Burgess and Michael Granato for access to their high-speed video camera and helpful discussions, Hernan Lopez-Schier and Jim Hudspeth for the use of their laser ablation set up, Joe Frey for help editing movies, and Fan Wang, Gord Fishell, Jason Rihel, and Sophie Caron for comments on the manuscript. This work was supported by grants from the National Institutes of Health to A.F.S., who was a Scholar of the McKnight Foundation for Neuroscience and is an Irma T. Hirschl Trust Career Scientist and an Established Investigator of the American Heart Association. A.S. was supported by a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research and is supported by a Burroughs-Wellcome Fund Career Award in the Biomedical Sciences.

Received: February 11, 2005
Revised: March 25, 2005
Accepted: March 29, 2005
Published: May 10, 2005

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