

Activity-independent specification of synaptic targets in the posterior lateral line of the larval zebrafish

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The development of functional neural circuits requires that connections between neurons be established in a precise manner. The mechanisms by which complex nervous systems perform this daunting task remain largely unknown. In the posterior lateral line of larval zebrafish, each afferent neuron forms synaptic contacts with hair cells of a common hair-bundle polarity. We investigated whether afferent neurons distinguish hair-cell polarities by analyzing differences in the synaptic signaling between oppositely polarized hair cells. By examining two mutant zebrafish lines with defects in mechano-electrical transduction, and by blocking transduction during the development of wild-type fish, we found that afferent neurons could form specific synapses in the absence of stimulus-evoked patterns of synaptic release. Asking next whether this specificity arises through intrinsically generated patterns of synaptic release, we found that the polarity preference persisted in two mutant lines lacking essential synaptic proteins. These results indicate that lateral-line afferent neurons do not require synaptic activity to distinguish hair-cell polarities and suggest that molecular labels of hair-cell polarity guide prepatterned afferents to form the appropriate synapses.

calcium channel | hair cell | neuromast | planar cell polarity | protocadherin

An essential feature of neural development is the establishment of specific synaptic connections. To form the appropriate contacts, each growing axon must respond to guidance cues, find its target region, and then establish synapses with specific target cells (1, 2). The first two of these steps—axonal guidance and target recognition—rely predominantly on molecular signposts that attract or repulse growth cones in a manner independent of neuronal activity (3, 4). How neurons decide to form stable synapses with particular target cells, however, remains unclear. Activity serves an important role in regulating the growth of axonal arbors and in selectively stabilizing synapses (5–8). In several vertebrate systems, axons form synapses diffusely within the target region and then undergo activity-dependent pruning to eliminate inappropriate synapses (9–14). Hebb's postulate, by which correlated activity between synaptic partners strengthens connections (15, 16), offers an attractive model to explain this phenomenon (17). Nevertheless, the evidence for an activity-dependent process must be reconciled with data suggesting that normal brain architecture can form in the absence of synaptic transmission (18–20). In this case, synaptic specificity could derive from a combinatorial code of cell-surface molecules such as cadherins (21) or members of the immunoglobulin superfamily (22). These fundamental uncertainties highlight the need for *in vivo* studies in an experimentally tractable vertebrate system.

The posterior lateral line of zebrafish permits a detailed analysis of the role of activity in establishing synaptic specificity. The larval posterior lateral line consists of superficial clusters of hair cells, the neuromasts, that respond to water-borne mechanical stimuli (23). To transduce water motions into electrical signals, each hair cell bears an apical hair bundle comprising a staircase-like array of stereocilia with the kinocilium, a true cilium, at the tall edge (24). The planar-cell-polarity pathway

(25) controls the polarization of the hair bundle and determines its axis of mechanical sensitivity, such that bundle deflection toward the kinocilium causes depolarization whereas deflection in the opposite direction hyperpolarizes the hair cell (26). Each neuromast contains two groups of hair cells of opposite hair-bundle polarity arranged across a plane of mirror symmetry (27). In the posterior lateral line, most neuromasts contain anteriorly and posteriorly polarized hair cells, whereas a particular few neuromasts contain dorsally and ventrally polarized cells (28).

Upon innervating a neuromast, each afferent neuron forms synapses almost exclusively with hair cells of one orientation (29, 30). One possible explanation for this result is that afferent neurons distinguish hair-cell polarities by analyzing the temporal pattern of synaptic activity. Another possibility is that the specificity arises from an intrinsic affinity of afferent neurons for particular hair-cell polarities through direct molecular interactions. In this study, we have investigated the role of synaptic activity in target cell choice and in doing so shed light on the mechanisms by which neurons form the appropriate connections.

Results

Afferent Neurons Selectively Innervate Hair Cells of a Common Polarity. In a transgenic line of zebrafish that expresses membrane-targeted GFP in hair cells (31), we labeled individual afferent neurons *in vivo* with a membrane-targeted form of the fluorescent protein mCherry. Upon innervating a neuromast containing two groups of oppositely polarized hair cells, a fluorescently labeled afferent fiber reliably contacts hair cells of a common polarity revealed by staining with fluorescent phalloidin (Fig. 1 *A* and *B*). This specificity in target choice is remarkably robust and is thought to occur through direct sensing of hair-cell polarity by the afferent neurons (29).

We considered three models to explain the observed specificity (Fig. 1C). The first posits that an afferent neuron innervates hair cells randomly but then eliminates certain contacts by analyzing the temporal pattern of synaptic release elicited by sensory experience. A unidirectional stimulus should simultaneously intensify synaptic release from hair cells of one polarity and suppress release from cells of the opposite orientation (31). If afferent neurites serve as coincidence detectors, they could strengthen synapses with hair cells of a particular polarity and eliminate synapses with those of the opposite polarity through a Hebbian mechanism. A second activity-dependent model requires oppositely polarized hair cells to possess different patterns of spontaneous synaptic activity. This model differs from the first in that the distinguishing quality is a spontaneous rather than an experience-evoked pattern of neurotransmitter release. The third model asserts that hair cells of opposite polarity express distinct membrane-associated or secreted proteins that are recognized by prepatterned afferent neurons with intrinsic affinities

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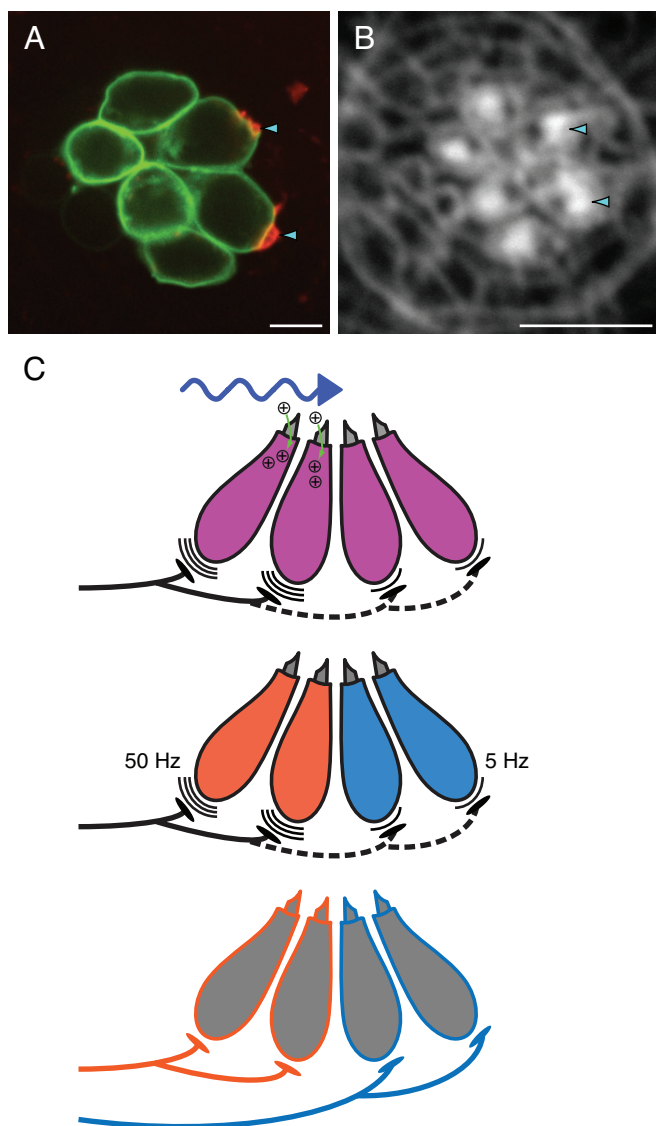


Fig. 1. Afferent terminals synapse specifically with hair cells of one orientation. (A) In this anteroposteriorly oriented neuromast of the posterior lateral line in a zebrafish larva at 5 dpf, the axonal terminal of an mCherry-labeled afferent neuron (red) contacts two of the six hair cells marked by GFP (green). Each site of innervation is marked by an arrowhead oriented in the direction of the associated hair cell's direction of excitatory stimulation. (B) Staining of the same neuromast with fluorescent phalloidin reveals the hair-bundle polarities: the unlabeled kinocilia appear as dark notches in the bright, actin-rich cuticular plates. The two labeled terminals contact hair cells sensitive to anteriorly directed stimuli. Arrowheads mark the hair bundles corresponding to the innervated somata in the two previous panels. In this and all subsequent morphological illustrations, the same labeling convention applies; anterior is to the left and dorsal to the top. The same labeling reagents are used in Figs. 2 and 3. (All scale bars, 5 μm .) (C) Three models might explain the ability of afferent neurons to distinguish between hair-cell polarities. (Top) A posteriorly directed stimulus depolarizes posteriorly polarized hair cells while hyperpolarizing anteriorly polarized cells. Afferents might form synapses diffusely but, after detecting temporal differences in synaptic release from oppositely polarized hair cells, eliminate synapses with hair cells firing out of phase with the rest of their synaptic repertoire (dashed neuronal segment). (Middle) Oppositely polarized hair cells express different complements of ion channels that produce distinct patterns of spontaneous synaptic release. In this example, hair cells of the two orientations release neurotransmitter at different frequencies, allowing neurites to distinguish them. (Bottom) Hair cells express distinct membrane-bound or secreted proteins that attract prepatterned afferents with intrinsic affinities for particular molecular markers. The afferents then detect hair-cell polarities independently of synaptic activity.

for particular hair-cell polarities. Although this mechanism might require activity for refining connections or for long-term synaptic maintenance, it requires no synaptic input to achieve initial specificity. We used these three models to develop an experimental framework for deducing the mechanism operative in the lateral line.

Sensory Experience Is Not Required for Synaptic Specificity. We first tested whether afferent neurons can distinguish hair-cell polarity in the absence of experience-evoked patterns of synaptic release. We examined two mutant lines with defects in mechanotransduction that prevent sensory stimuli from eliciting membrane depolarization and synaptic-vesicle exocytosis. Larvae lacking hair-bundle function characteristically display auditory and vestibular deficits, lack microphonic potentials, and exhibit no uptake of fluorophores through their mechanotransduction channels (32).

We examined zebrafish mutants lacking *Tmie*, a transmembrane protein required for hair-cell mechanotransduction (33), at 5 days postfertilization (5 dpf). In seven anteroposteriorly oriented neuromasts of *tmie* mutant larvae, each afferent fiber consistently innervated hair cells of only a single polarity (Fig. 2 A–C). Specific innervation was also characteristic of the four *tmie* neuromasts we examined that contained dorsally and ventrally polarized hair cells (Fig. 2 D–F).

We next examined synaptic specificity in *protocadherin 15a* mutants, which lack a component of the stereociliary tip link essential for transducing mechanical force into hair-cell depolarization (34). In each of the 19 neuromasts studied, the axonal terminals formed synaptic boutons on hair cells of only one particular orientation (Fig. 2 G–I).

We also raised wild-type zebrafish with GFP-labeled hair cells from 2 dpf to 5 dpf in system water supplemented with 1 mM amiloride to block mechanotransduction. In 12 amiloride-treated neuromasts, each mCherry-labeled afferent formed contacts with hair cells of identical polarity (Fig. 2 J–L). Although acutely applied amiloride is a reversible inhibitor of the hair cell's mechanosensitive channels, incubation of zebrafish larvae from 2 dpf to 5 dpf resulted in an irreversible interruption of transduction, as verified by microphonic recordings (Fig. 2M). Fluorescence microscopy revealed that the hair cells of treated animals had accumulated amiloride, which may have blocked the mechano-electrical-transduction channels from their cytoplasmic surfaces.

Synaptic Specificity Is Preserved in the Absence of Spontaneous Synaptic Transmission. Because the foregoing experiments demonstrated that the preference of afferents for hair-cell polarity remains robust in the absence of sensory input, we evaluated the possibility that an intrinsically generated pattern of synaptic release by hair cells reveals their polarity to afferents. Oppositely polarized hair cells might differ, for example, in their frequency or pattern of spontaneous neurotransmitter release, and afferents might display complementary preferences.

We studied two mutant lines with defects in essential synaptic components and consequent loss of auditory and vestibular function. The *cav1.3a* mutation disrupts the L-type voltage-gated Ca^{2+} channels that couple membrane depolarization to transmitter release at the hair cell's afferent synapse (35). In each of the 21 *cav1.3a* mutant neuromasts that we analyzed, the labeled afferent fiber made synapses onto hair cells of only a single polarity (Fig. 3 A–C).

We additionally examined *vglut3* mutants, which lack the vesicular glutamate transporter type 3 responsible for filling synaptic vesicles with the afferent neurotransmitter glutamate (36, 37). In each of 15 *vglut3* mutant neuromasts, a labeled afferent neuron formed specific synapses onto hair cells of a

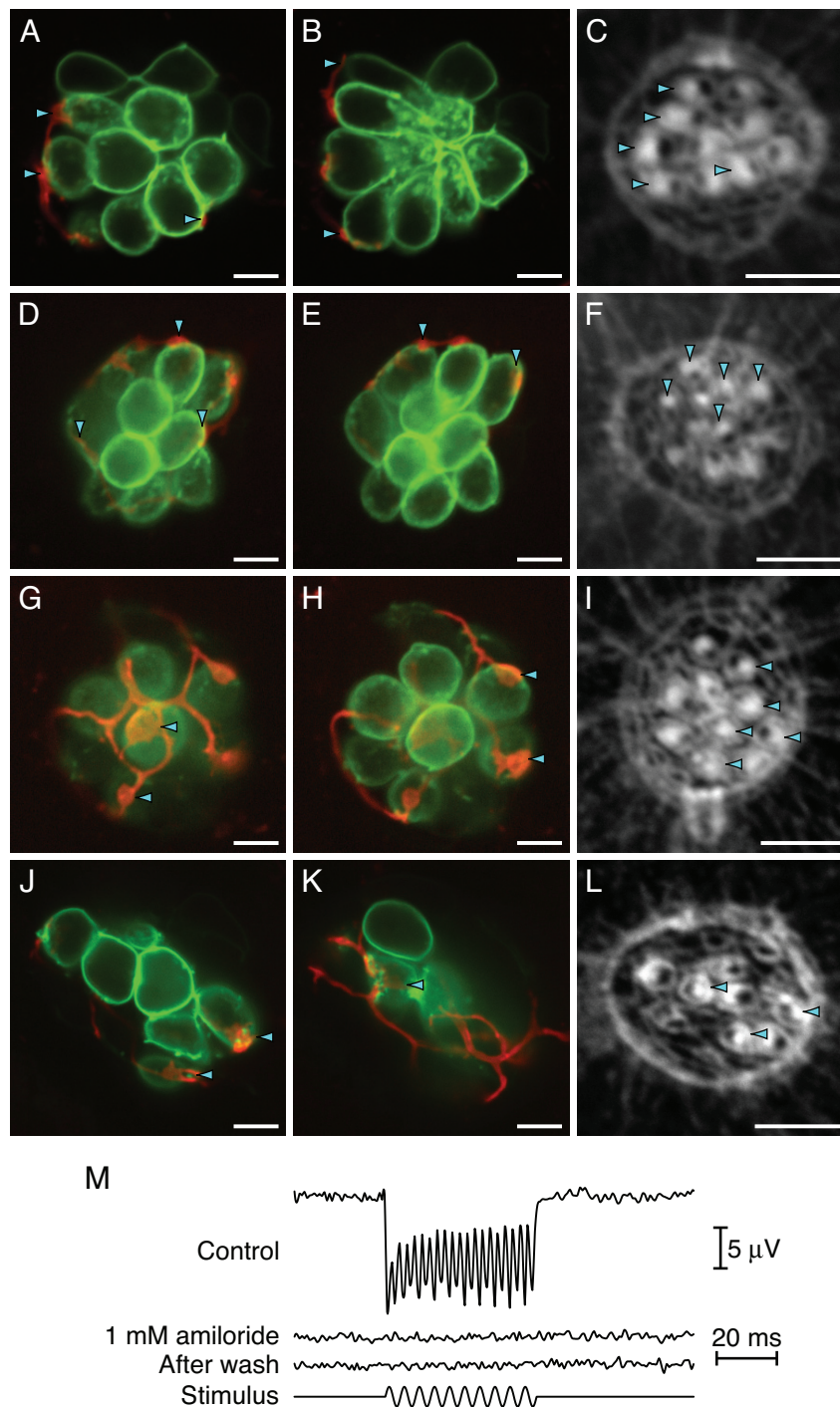


Fig. 2. Stimulus-evoked patterns of synaptic release are not required for polarity choice. (A and B) In an anteroposterior neuromast of a *tmie* mutant larva, a labeled afferent fiber synapses with five of the ten hair cells. In this and the subsequent morphological illustrations, the two micrographs represent different planes of focus. (C) The hair-bundle polarities of this neuromast reveal that the neuron innervates all five posteriorly polarized hair cells and none of the opposite polarity. (D–F) In a dorsoventral neuromast of a *tmie* mutant, an afferent neuron innervates only the five ventrally polarized hair cells. (G–I) An afferent fiber in a *pcdh15a* mutant forms synapses with four of the five anteriorly polarized hair cells but with none of the five cells of the opposite polarity. (J–L) In a neuromast of a larva treated with 1 mM amiloride from 2 dpf to 5 dpf, the labeled fiber forms synapses with only the three anteriorly polarized hair cells. (M) The microphonic potential recorded from a neuromast of a 5-dpf larva under control conditions (Top trace) reveals a response at twice the frequency of the 200-Hz, $\pm 8\text{-}\mu\text{m}$ stimulus (Bottom trace). Stimulation of a neuromast from a sibling maintained for 3 days in 1 mM amiloride reveals no microphonic signal (Second trace). Even after extensive washout of the amiloride, the neuromast fails to respond (Third trace).

common polarity (Fig. 3 D–F). Taken together, our study of zebrafish lacking hair-bundle or synaptic function provides evidence that synaptic specificity persists in the absence of specific patterns of synaptic signaling.

Polarity Preference and Synapse Maintenance Are Activity-Independent. Although the mutants and amiloride-treated larvae displayed severe loss-of-function phenotypes, they might conceivably have retained sufficient synaptic activity to signal their polarities to

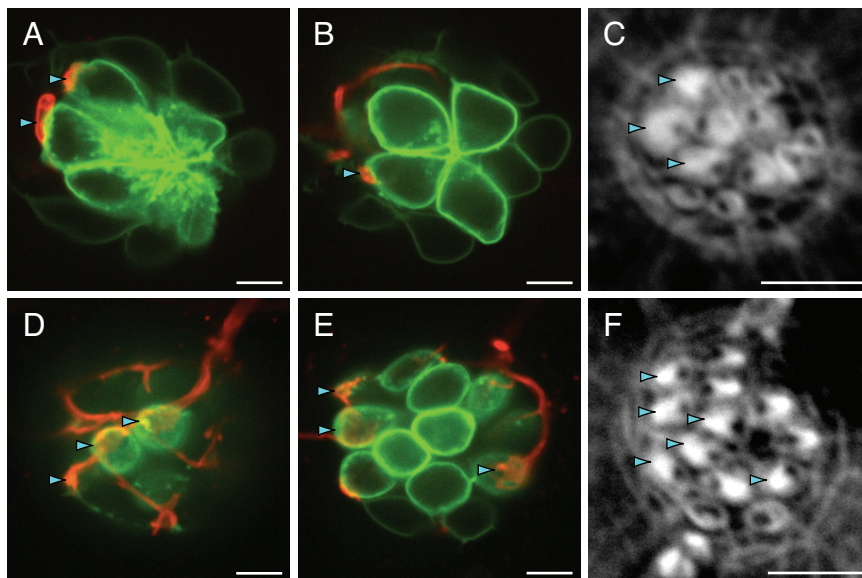


Fig. 3. Synaptic transmission is dispensable for hair-cell polarity preference. (A–C) In an anteroposteriorly oriented neuromast of a *cav1.3a* mutant lacking functional L-type voltage-gated Ca^{2+} channels, the three mature posteriorly polarized hair cells bear labeled afferent synapses; none of the opposite polarity does. (D–F) This *vglut3*-deficient neuromast contains six posteriorly polarized hair cells, all of which are innervated by the labeled afferent fiber.

afferents. If this were the case, we would nevertheless expect the afferent neurons to have exhibited a diminished capacity to distinguish between polarities. To rigorously detect small changes in polarity preference, we analyzed synapse formation in wild-type, mutant, and amiloride-treated neuromasts and then applied a statistical model of polarity preference (29). The model contains a bias parameter ω that expresses the neuron's preference for one polarity over another. To represent neuronal bias independently of the particular polarity being preferred, we calculated the mean of the probability of $|\omega - 0.5| + 0.5$.

For the mutant and amiloride-treated fish, the weight of evidence (29) favored selective as opposed to random innervation by decisive factors ranging from 10^5 to 10^8 . The afferent neurons of these larvae displayed an ability to distinguish polarities to a degree commensurate with that of wild-type afferents (Fig. 4A). Our statistical analysis thus points to an activity-independent specification of synaptic targets, but it does not address whether afferent synapses require activity for long-term maintenance. To answer this question, we calculated the fraction of a neuromast's hair cells innervated by a single afferent fiber. Because neuromasts comprise two equal populations of oppositely polarized hair cells, we expected no more than half of a neuromast to be innervated by a labeled fiber. The mean fraction innervated was similar for mutant, amiloride-treated, and wild-type animals (Fig. 4B), suggesting that neurotransmitter release is not essential for synaptic maintenance during the first week of life.

Discussion

We have assessed the role of synaptic activity in ensuring specific connectivity between afferent neurons and plane-polarized hair cells in the posterior lateral line of larval zebrafish. In two mutant lines with defects in mechanotransduction, wild-type animals with blocked mechanotransduction, and two mutant lines with deficiencies of synaptic signaling, lateral-line afferents correctly synapsed with hair cells of a common polarity. By applying a statistical model of polarity preference to data from each mutant line, we confirmed that afferent synaptogenesis remained highly biased for one polarity over the other at a level matching that observed for wild-type animals. In addition, the

fraction of each mutant or amiloride-treated neuromast innervated by the labeled afferent fiber was comparable to that in wild-type neuromasts, indicating that synaptic transmission is not essential for synaptic maintenance. These results imply that afferent neurons do not interpret a pattern of evoked or spontaneous neurotransmitter release but instead use intrinsic molecular cues to identify and synapse with the appropriately polarized hair cells.

This conclusion accords with two previous observations (29, 30). First, when an afferent fiber innervates multiple neuromasts, it is consistent in its polarity preference both within each innervated neuromast and between neuromasts. It seems improbable that unbiased branches of a fiber would consistently prefer the same polarity by analyzing experience-evoked patterns of coincident synaptic release. Second, afferent fibers retain their polarity preference following hair-cell death and regeneration. If unbiased afferents use patterns of coincident synaptic release to restrict themselves to a single polarity, one would instead expect the preference to depend on the polarity of the first hair cell innervated. Both of these observations contradict a model whereby initially unbiased afferent neurons use experience-dependent patterns of synaptic release to restrict themselves to a single polarity. These findings are nevertheless compatible with an activity-dependent mechanism in which prepatterned afferent neurons prefer a polarity-specific pattern of spontaneous synaptic release. Our present results with *cav1.3a* and *vglut3* mutant fish speak against this mechanism, however, favoring instead activity-independent specification.

Before a role for synaptic activity can be excluded altogether, three important issues should be addressed in future studies. The first is that our experimental strategy involved loss-of-function approaches. The unlikely possibility exists that patterned neurotransmitter release ordinarily overrides the default molecular mechanism that confers specificity in the mutant and amiloride-treated animals. The second issue is that synaptic activity could play other, more subtle roles in neuronal morphology and behavior. Despite their ability to correctly identify hair-cell polarities in the absence of synaptic signaling, afferent neurons might exhibit increased exploratory behavior manifested by a greater spread of axonal arbors or accelerated dynamics of axonal extension and retraction. Consistent with this idea, we

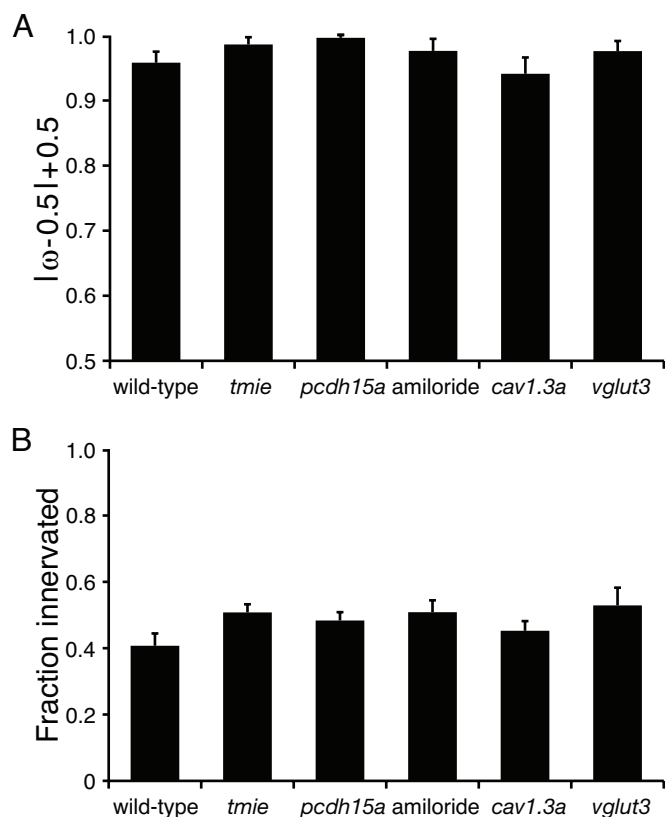


Fig. 4. Statistical analysis confirms the polarity preference of afferent terminals. (A) The parameter ω , which ranges from 0 to 1, represents the degree to which a neuron's choice of hair cells is biased toward one polarity; a value of 0.5 represents a lack of bias. The results are expressed as the means and standard deviations of the probability distribution of $|\omega - 0.5| + 0.5$, so the ordinate reflects increasing bias. Values of ω above about 0.95 represent near certainty: in these populations, a neuron makes less than one error per three neuromasts innervated. (B) The mean fractions of the hair cells that were innervated by a labeled afferent fiber were similar for neuromasts of each genotype. The error bars represent standard errors of the means for the following numbers of observations: wild-type, $n = 21$; *tmie*, $n = 11$; *pcdh15a*, $n = 19$; amiloride, $n = 12$; *cav1.3a*, $n = 21$; *vglut3*, $n = 15$.

observed more extensive branching of axonal terminals in amiloride-treated neuromasts and to a lesser degree in those of mutants. The final obstacle to rejecting a role for synaptic activity in this system is that we have not identified the molecular mechanism that endows afferents with the ability to distinguish hair-cell polarity. A likely possibility is that oppositely polarized hair cells express distinct membrane or secreted proteins that attract or repel afferent neurites bearing appropriate receptors. The difficulty in identifying these molecular polarity cues stems from the fact that oppositely oriented hair cells are commingled within neuromasts and lack distinguishing morphological characteristics after isolation.

Why might the posterior lateral line have evolved a hard-wired approach to distinguish between oppositely polarized hair cells? Perhaps the sheer simplicity of the system lends itself to a molecular code. Each afferent neuron faces a simple binary choice in its selection of synaptic targets. Moreover, it is a choice that the neuron must continue to make throughout life as new hair cells are produced to replace dying ones. What this system foregoes in activity-dependent refinement and plasticity, it gains in reproducibility and speed.

One interesting possibility is that the planar cell polarity of a neuromast depends upon the direction of migration of the

primordium that deposited the neuromast (28, but see ref. 38) and that the signals responsible for this feature specify neuronal connectivity as well. It is possible, for example, that posteriorly and ventrally polarized hair cells bear an identical or a similar polarity identity, whereas anteriorly and dorsally polarized hair cells manifest the opposite identity. Each of these coteries of hair cells originates, respectively, more proximally or more distally with respect to the origin of the relevant primordium; for instance, both posteriorly and ventrally polarized hair cells arise on the sides of their respective neuromasts that were proximal to the source of primordial migration. Dorsoventral and antero-posterior neuromasts might even use the same code to differentiate hair-cell polarities. Because single afferents ordinarily do not innervate both dorsoventral and anteroposterior neuromasts (29), a single code could suffice.

Peripheral mechanisms that ensure wiring specificity do not function alone, but rather act in concert with central components in generating somatotopy and organizing sensory and behavioral circuits. An important question arising from this work is whether the degree of predetermination that we have observed peripherally also extends to the central projections (39). If afferent neurons use a molecular code to distinguish between hair-cell polarities, does this same code function in the hindbrain to organize polarity-specific sensory pathways (40)? If so, how are afferents encoding anteriorly and posteriorly directed stimuli distinguished from those representing dorsally and ventrally directed stimuli? Another fascinating issue is how somatotopy, the mapping of neuromast position along the body to the corresponding projection zone in the brain, relates to the polarity pathway. Because an afferent's choice of neuromast can be predicted from its hindbrain projection and from the morphology of its growth cone (41, 42), afferent neuronal differentiation might involve the concerted specification of polarity and target-neuromast position through a multimodal molecular code. The use of hard-wired molecular mechanisms to ensure synaptic specificity in the periphery may provide the foundation upon which to build complex yet flexible circuits in the central nervous system.

Materials and Methods

Zebrafish Strains and Husbandry. Zebrafish were maintained under standard conditions. Naturally spawned eggs were collected, cleaned, staged (43), and maintained in system water at 28.5 °C at a density of 50 per 100-mm-diameter Petri dish. Embryos were raised in system water with the addition of 200 μ M 1-phenyl-2-thiourea at 1 day postfertilization (dpf) to inhibit pigment formation. In the case of amiloride-treated fish, 1 mM amiloride (Sigma) was added at 2 dpf to block mechanotransduction until microphonic recording or live imaging was performed at 5 dpf.

The wild-type strain used was *Tübingen Long Fin* (TL). The relevant transgenic and mutant strains include *Pou4f3:gap43-GFP* (formerly known as *Brn3c:gap43-GFP*), *Tg(Pou4f3:gap43-mGFP)^{356t}*; *tmie*, *tmie^{u1000}*; *protocadherin 15a*, *pcdh15a^{th263b}*; *vglut3*, *slc17a8^{v01}*; and *cav1.3a*, *cacna1d^{tc323d}*.

DNA Injection and Screening of Transgenic and Mutant Fish. The *HuC:gap43-mCherry* plasmid was created as described (29). One- and two-cell embryos were pressure-injected with supercoiled plasmid DNA at a concentration of 50 ng/ μ l. Deaf mutant larvae were identified at 5 dpf by the startle-response assay (32) and screened for mCherry expression in the posterior lateral-line nerve with a Zeiss Axioplan 2 wide-field fluorescence microscope.

Live Imaging of Larvae. For confocal imaging, specimens were embedded under anesthesia in 1% low-melting-point agarose on a glass coverslip. Images were acquired with an Ultramer Perkin-Elmer spinning-disk system on a Zeiss Axiovert 200M microscope equipped with a 63 \times , 1.4 NA objective lens, a Hamamatsu Orca-ER cooled CCD camera, and MetaMorph software (Molecular Devices/MDS). Z-stacks were acquired at 1 μ m intervals, imaging GFP (488 nm excitation, 500–550 nm emission) and mCherry (568 nm excitation, 590–650 nm emission). After each examination, the larvae were excised from the agarose and returned to individually marked dishes. At the conclusion of live imaging, larvae were genotyped to confirm their status as mutants.

Phalloidin Staining and Imaging. Fish were fixed overnight at 4 °C in PBS containing 1% Tween-20 (PBST) and 4% paraformaldehyde, then were washed thrice in 1% PBST for 1 h and stained overnight at 4 °C with a 1:20 dilution of Alexa Fluor 568 phalloidin (Invitrogen) in 0.2% PBST. They were next washed twice for 4 h and mounted in Vectashield (Vector Laboratories). Samples were imaged at a scan rate of 8 μ s per pixel with Kalman averaging on an Olympus FV1000 laser-scanning confocal microscope with a 60 \times , 1.42 NA objective lens.

Microphonic Recordings. Each wild-type zebrafish larva of 5–6 dpf was anesthetized with 650 μ M benzoic acid ethyl ester in saline solution containing 116 mM NaCl, 2.9 mM KCl, 1.8 mM CaCl₂, and 5.0 mM Hepes at pH 7.2. Secured on its side with 100–200 μ l droplets of cyanoacrylate glue (Nexaband Topical Tissue Adhesive, Abbott Laboratories) at the head and tail, the larva was observed under differential-interference-contrast optics with a 60 \times , 0.9 NA objective lens.

Stimuli were presented and data acquired with programs written in LabVIEW (National Instruments). Sinusoidal stimuli with an amplitude of 8 μ m were delivered to anteroposteriorly oriented neuromasts through a stiff glass probe attached near the cupula's tip and driven by a piezoelectric stimulator. Recordings were obtained at room temperature with a capacitively coupled amplifier (P55, Grass Technologies) at a gain of 10,000 \times . Borosilicate-glass electrodes, which displayed resistances of 2–3 M Ω when filled with the bathing solution, were placed within 1 μ m of a neuromast's aperture. Signals were

acquired at 50- μ s sampling intervals and digitally low-pass filtered at 600 Hz. The displayed records represent averages of 200 stimulus presentations.

Statistical Modeling of Polarity Preference. We modeled a neuron's ability to distinguish between opposing polarities by Fisher's noncentral hypergeometric distribution (29). Using a beta (1, 1) prior, we calculated $P(\omega | D)$, the posterior of the parameter ω for an observed distribution D of synaptic contacts. A neuron innervating only anteriorly polarized hair cells is assigned the parameter value $\omega = 0$ whereas a posteriorly selective neuron has $\omega = 1$. A neuron with no ability to distinguish polarization has $\omega = 0.5$. Because we sought to quantify each neuron's ability to distinguish polarity in a way that was independent of its specific polarization preference, we made a change of variables to $|\omega - 0.5| + 0.5$. The new distribution, $0.5|P(\omega | D) + P(1 - \omega | D)|$, which we characterized by its mean and standard deviation, satisfies the symmetry requirement.

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