Hox2 Genes Are Required for Tonotopic Map Precision and Sound Discrimination in the Mouse Auditory Brainstem

Highlights
- Hox2 genes are expressed in glutamatergic cells of the anteroventral cochlear nucleus
- Hox2 deletion results in impaired refinement of endbulb of Held presynaptic input
- Hox2 deletion results in impaired tonotopic precision of sound transmission
- Hox2 mutant mice fail to discriminate close sound frequencies in behavioral tests

Graphical Abstract

In Brief
Karmakar et al. find that the Hoxa2 and Hoxb2 transcription factors are required in mouse brainstem cochlear neurons for the tonotopic maturation of synaptic targeting from their peripheral auditory afferents and for the precision of sound discrimination in adult mice.

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**SUMMARY**

Tonotopy is a hallmark of auditory pathways and provides the basis for sound discrimination. Little is known about the involvement of transcription factors in brainstem cochlear neurons orchestrating the tonotopic precision of pre-synaptic input. We found that in the absence of Hoxa2 and Hoxb2 function in Atoh1-derived glutamatergic bushy cells of the anterior ventral cochlear nucleus, broad input topography and sound transmission were largely preserved. However, fine-scale synaptic refinement and sharpening of isofrequency bands of cochlear neuron activation upon pure tone stimulation were impaired in Hox2 mutants, resulting in defective sound-frequency discrimination in behavioral tests. These results establish a role for Hox factors in tonotopic refinement of connectivity and in ensuring the precision of sound transmission in the mammalian auditory circuit.

**INTRODUCTION**

Tonotopy is a major organizing principle of auditory circuits (Kandler et al., 2009). Tonotopic arrangement of neuronal connectivity along the auditory pathway first arises from the orderly encoding of sound frequencies along the apical to basal axis of the inner ear cochlea that in turn activates specific subsets of spiral ganglion (SG) primary sensory neurons of the vestibulocochlear nerve. SG afferents relaying low to high sound frequencies topographically target the ventral-to-dorsal axis of the brainstem cochlear nuclear complex, forming isofrequency bands of activation of neighbor target cochlear neurons. Sound information is in turn relayed from brainstem cochlear nuclei to midbrain, thalamus, and cortex, maintaining a tonotopic organization of connectivity at all levels of the pathway (Kandler et al., 2009).

The brainstem cochlear nuclear complex is composed of the anterior ventral cochlear nucleus (AVCN), posterior ventral cochlear nucleus (PVCN), and dorsal cochlear nucleus (DCN). Distinct cochlear nuclei are generated by different subsets of progenitors spatially segregated along the brainstem rostral-caudal axis termed rhombomeres (Di Bonito et al., 2013; Farago et al., 2006; Kiecker and Lumsden, 2005; Wang et al., 2005). The AVCN, involved in binaural sound localization circuitry, is mostly derived from rhombomere 3 (r3) with a small contribution from r2 (Farago et al., 2006; Maricich et al., 2009; Wang et al., 2005). Moreover, AVCN excitatory, glutamatergic cells originate from the dorsal Atoh1-expressing rhombic (auditory) lip progenitor domain (Fujiyama et al., 2009; Maricich et al., 2009; Wang et al., 2005). The auditory nerve fibers innervating the Atoh1-derived glutamatergic bushy cells in the AVCN form large axosomatic synaptic endings, the endbulbs of Held, ensuring the fidelity of sound transmission (Limb and Ryugo, 2000; Ryugo et al., 2006). SG axon topographic input to cochlear nuclei is developmentally hardwired before birth (Kandler et al., 2009; Koundakjian et al., 2007). Nonetheless, pre-hearing and post-hearing axon refinement and synaptic elimination play critical roles in achieving the high tonotopic precision of the mature brainstem auditory circuits (Clause et al., 2014; Kandler et al., 2009; Leake et al., 2002). Although the morphological and physiological processes leading to the development of tonotopic maps have been studied, to date, no transcription factors have been identified in post-synaptic cochlear neurons that are necessary to orchestrate the fine-tuned tonotopic precision of SG afferent connectivity and synaptic development.

The homeodomain transcription factors of the Hox gene family are expressed in rhombomere-derived mitotic progenitors and are essential for providing rostral-caudal subtype identity to neuronal subsets contributing to sensory nuclei of multi-rhombomeric origin (Bechara et al., 2015; Di Bonito et al., 2013; Di Meglio et al., 2013; Narita and Rijli, 2009; Oury et al., 2006;
Philippidou and Dasen, 2013). Hox gene expression is maintained in post-mitotic neurons during stereotypic migration, nuclear assembly, and establishment of broad topographic connectivity (Philippidou and Dasen, 2013). However, whether Hox factors are also required during sensory circuit refinement and synapse maturation is unknown.

Hox paralogous group 2 (Hox2) genes, Hoxa2 and Hoxb2, are the only Hox genes expressed in the AVCN, whereas in the PVCN and DCN, their expression overlaps with additional Hox factors (Di Bonito et al., 2013; Narita and Rijli, 2009). In the AVCN, Hox2 genes are expressed throughout pre-natal and post-natal development and at least up to 2 months of age (Narita and Rijli, 2009; Di Bonito et al., 2013). Connectivity (Philippidou and Dasen, 2013). However, whether Hox2 factors might regulate expression of genes involved in target-derived signaling, adhesion, synaptogenesis, and synaptic transmission. These results provide important insights into the role of Hox transcription factors during the development and maturation of highly topographic brainstem sensory circuits.

RESULTS

Hox2 Genes Are Expressed in Glutamatergic Bushy Cells of the Anterior Ventral Cochlear Nucleus through Postnatal Stages

We generated an Atoh1::Cre (Atoh1Cre) transgenic line in which Cre is driven by the Atoh1 enhancer (Helms et al., 2000; Machold and Fishell, 2005) (Figure S2A). In Atoh1Cre::ROSA1SLSZsGreen1 (Atoh1Cre::ROSA1SLSZsGreen1) or Atoh1Cre::ROSA1SLSldTomato (Atoh1Cre::ROSA1SLSldTomato), mice, prenatal and postnatal distribution of ZsGreen1 or RFP reporter expression was in keeping with the previously reported pattern for rhombic lip Atoh1 derivatives, including cerebellar, cochlear, precerebellar, and dorsal spinal cord neurons, as well as inner ear hair cells (Figures 1, S2B, and S2G) (Machold and Fishell, 2005; Wang et al., 2005). Moreover, ZsGreen1+ or RFP+ auditory neurons were glutamatergic (vGlut2+) (Figure 1A), as expected for the Atoh1-derived lineage (Fujisawa et al., 2009). Notably, in Atoh1ROSA::ZsGreen1 or Atoh1ROSA::tdTomato mice, no ZsGreen1+ or RFP+ cells were found at any stage analyzed in the auditory lip progenitor domain (Figures S2C–S2E). Cre expression was barely detectable in the Atoh1+ auditory lip and mostly restricted to postmitotic neurons ingressing the cochlear nucleus (compare Figures S2F and S2G). Atoh1Cre is therefore a suitable driver to conditionally inactivate Hox2 genes in AVCN glutamatergic neurons while bypassing their early requirements in rhombomere patterning and mitotic progenitor specification (Di Bonito et al., 2013; Gavalas et al., 1997).

To identify Atoh1-derived Hox2-expressing AVCN neurons through prenatal and postnatal stages, we used an intersectional strategy and crossed Atoh1Cre with the Hoxa2flox-neo-lox:EGFP knockin allele expressing EGFP from the Hoxa2 locus upon Cre-mediated recombination (Pasqualetti et al., 2002) (Atoh1Hoxa2flox-neo-lox:EGFP) (Figures 1B–1D, S2H, S2I, and S2K). In Atoh1Hoxa2flox-neo-lox:EGFP mice, Cre-induced Hox2-driven EGFP expression was only present in postmitotic AVCN neurons and not in the progenitor domain (Figures S2H and S2I), in keeping with the Atoh1Cre activity profile described above. Atoh1-derived Hoxa2-expressing EGFP+ AVCN neurons co-expressed Hoxb2 and vGlut2 mRNA, and their soma was surrounded, as assessed by immunohistochemistry, by large presynaptic VGLUT1+ endings characteristic of endbulb of Held synapses (Figures 1B–1D), thus identifying Atoh1-derived Hoxa2+/Hoxb2+ AVCN neurons as glutamatergic bushy cells. Neither Hoxa2/EGFP nor Hoxb2 expression was detected in inner ear Atoh1-derived hair cells (Figures S2L–S2O). Moreover, in Atoh1ROSA::tdTomato mice, no Cre-mediated excision was detected in the MafB+ spiral ganglion of the inner ear, as assessed by the lack of RFP+ cells (Figures S2P–S2R). Thus, the Atoh1Cre line is suitable for studying the consequences of Hox2 deletion in brainstem cochlear glutamatergic postmitotic neurons.

AVCN Size, Patterning, and Broad Connectivity Are Not Affected in Atoh1Hoxa2cKO Mutants

Next, we crossed Atoh1Cre with Hoxa2flox and Hoxb2flox conditional alleles (Di Bonito et al., 2013; Ren et al., 2002) and generated single Atoh1Hoxa2cKO, Atoh1Hoxb2cKO, and compound Atoh1Hoxa2cKO conditional mutants, respectively. qPCR analysis of the extent of the targeted deletion in dissected cochlear nuclei from Atoh1Hoxa2cKO compound mutants (see Experimental Procedures) confirmed nearly complete Cre-mediated excision of both Hoxa2 and Hoxb2 conditional alleles (Figures S3A and S3B). Atoh1Cre-driven Hox2 single or double conditional deletions did not significantly affect AVCN size or AVCN cell density when compared to wild-type (Figures 1E and S1C). Moreover, in single or compound Atoh1Hoxa2cKO mutant fetuses, Atoh1 was normally expressed at the rhombic lip, abutting the Lmx1b-expressing choroid plexus domain, and maintained in the developing AVCN (Figures S3C–S3F). Atoh1-derived AVCN neuron specification and postmitotic differentiation proceeded normally
Figure 1. Characterization of Atoh1-Derived Hox2-Expressing Cells in the Anterior Ventral Cochlear Nucleus

(A) E18.5 Atoh1^{ROSA::tdTomato}-positive cells, detected by anti-RFP (red) immunohistochemistry, are vGlut2^{+} (green) glutamatergic, as assessed by fluorescent in situ hybridization (FISH).

(B–D) Hoxa2-expressing Atoh1-derived cells, as detected by anti-EGFP (green) in E18.5 Atoh1^{Hoxa2::EGFP} animals, are vGlut2^{+} glutamatergic (red) (B) and coexpress Hoxb2 (red) (C) as assessed by FISH. (D) Anti-VGLUT1 (red) immunostaining labels developing endbulb of Held synapses (white arrows) onto P14 Atoh1^{Hoxa2::EGFP} (green) cells, identifying Atoh1-derived Hoxa2-expressing AVCN bushy cells.

(E) The size of the AVCN is not affected in Atoh1^{Hox2cKO} (n = 4), Atoh1^{Hoxa2cKO} (n = 3), and Atoh1^{Hoxb2cKO} (n = 3), as compared to control (n = 7) animals (one-way ANOVA analysis, p value = 0.2448). Values are mean ± SEM. Statistical analysis is described in Experimental Procedures. AVCN, anterior ventral cochlear nucleus. N.S, not significant.

Scale bar, 5 μm. See also Figures S1–S3.
as assessed by expression of the bushy cell-specific markers Math5 (Atoh7) (Saul et al., 2008) and MafB (Figures S3G–S3J). Notably, unlike when using a mouse line expressing Cre at early progenitor stages (see Figures 5D and 5E in Di Bonito et al., 2013), in Atoh1<sup>Hox2cKO</sup> animals, Rig1 was also normally expressed and AVCN axons projected normally to contralateral MNTB and ipsilateral lateral superior olive (LSO) nuclei (Figures S3K–S3L and S3S–S3V). Furthermore, mutant AVCN displayed a normal distribution of excitatory and inhibitory neurons as assessed by expression of vGlut1, vGlut2, and vGat mRNA when compared to wild-type (Figures S3M–S3R). Thus, Atoh1<sup>Cre</sup>-driven conditional inactivation of Hox2 genes did not affect specification, broad connectivity, or neurotransmitter phenotype of AVCN glutamatergic neurons.

**Broad Tonotopic Organization of Inner Ear Afferents Is Preserved at Birth in the AVCN of Single and Compound Atoh1<sup>Hox2cKO</sup> Mutants**

We then asked whether Hox2 genes might be required in AVCN neurons to organize topographic precision of inner ear afferent tonotopic input. We inserted tiny strips of Neurovue filters coated with different lipophilic fluorescent dyes at distinct positions in the cochlear middle and basal turns of embryonic day 18.5 (E18.5) wild-type, single, and Atoh1<sup>Hox2cKO</sup> conditional mutants (Figure 2A). This allowed for focal tracing of distinct afferent subsets spatially segregated within the auditory nerve tract. We evaluated the correlation between dye angular spread in the cochlea (Figure 2A) and corresponding targeted area of labeled SG afferents along the AVCN tonotopic axis (Figure 2B). For each injection, the surface fraction of AVCN targeted by the SG afferents labeled from basal or middle cochlear turn (S<sub>b</sub>/SAVCN<sup>100</sup> and S<sub>m</sub>/SAVCN<sup>100</sup>, respectively) was normalized according to the corresponding dye angular spread in the cochlea basal (q<sub>b</sub>) or middle (q<sub>m</sub>) turns ([S<sub>b</sub>/SAVCN<sup>100</sup>/q<sub>b</sub>]) and ([S<sub>m</sub>/SAVCN<sup>100</sup>/q<sub>m</sub>]), respectively (Figures 2C and S2D).

Comparison of quantifications did not show significant alteration of cochleotopic arrangement of afferent input into E18.5 AVCN of Atoh1<sup>Hoxa2cKO</sup> or Atoh1<sup>Hoxb2cKO</sup> single mutants when compared to control littermates (Figures 2C and 2D). In compound Atoh1<sup>Hox2cKO</sup> E18.5 mutants, SG axons traced from basal or middle cochlear turn projected topographically along the tonotopic AVCN axis. Thus, cochleotopic organization of incoming SG fibers is not dependent on Hox gene expression in second-order AVCN neurons, in keeping with the pre-targeting topographic ordering of SG projections (Koundakjian et al., 2007; Yu and Goodrich, 2014). However, SG axons targeted >50% broader dorsoventral areas in Atoh1<sup>Hox2cKO</sup> AVCN than in single mutants or controls (Figures 2C and 2D), suggesting that

![Figure 2. Altered Precision of Tonotopic Input Targeting the Atoh1<sup>Hox2cKO</sup> Anterior Ventral Cochlear Nucleus](image-url)

(A) Neurovue-labeled basal (green) or middle (red) turn of E18.5 cochlea in control mice.

(B) The Neurovue labeled axon terminals of E18.5 spiral ganglion neuron afferents innervating the cochlear basal (green) or middle (red) turns, target distinct areas along the tonotopic (dorsoventral) axis of AVGN, immunostained with anti-MafB (white).

(C) Comparison of tracing from basal turn ([S<sub>b</sub>/SAVCN<sup>100</sup>]) in E18.5 control (n = 5), Atoh1<sup>Hox2cKO</sup> (n = 5), Atoh1<sup>Hoxa2cKO</sup> (n = 5), and Atoh1<sup>Hoxb2cKO</sup> (n = 3) fetuses. Kruskal-Wallis test shows significant differences among the samples (p < 0.0001). Multiple comparison shows significant differences between control and Atoh1<sup>Hox2cKO</sup> but non-significant differences between control and Atoh1<sup>Hoxa2cKO</sup> or Atoh1<sup>Hoxb2cKO</sup>. Values are mean ± SEM.

(D) Comparison of tracing from the middle turn (gray bars) ([S<sub>m</sub>/SAVCN<sup>100</sup>]) in E18.5 control (n = 5), Atoh1<sup>Hox2cKO</sup> (n = 5), Atoh1<sup>Hoxa2cKO</sup> (n = 5), and Atoh1<sup>Hoxb2cKO</sup> (n = 3) fetuses. Kruskal-Wallis test shows significant differences among the samples (p < 0.0001). Multiple comparison shows significant differences between control and Atoh1<sup>Hox2cKO</sup> but non-significant differences between control and Atoh1<sup>Hoxa2cKO</sup> or Atoh1<sup>Hoxb2cKO</sup>. Values are mean ± SEM.
Figure 3. Altered Isofrequency Bands in Anterior Ventral Cochlear Nucleus and Impaired Tone Discrimination in Hox2 Mutants

(A and B) Activated neurons in adult control and Atoh1<sup>Hox2cKO</sup> mutant AVCN labeled by anti-c-fos antibody staining (in white) in response to 15 kHz (A) or 8 kHz (B) pure tone stimulation.

(C) c-fos activated neurons detected by in situ hybridization in response to 15 kHz stimulation in control and Atoh1<sup>Hox2cKO</sup> mutant AVCN.

(D) Anti-c-fos-labeled AVCN activated neurons (white) in response to simultaneous 15 and 8 kHz pure tone stimulation. In controls, a separation area between the two activated isofrequency bands is readily detected (white double-headed arrow), whereas no obvious band separation is observed in Atoh1<sup>Hox2cKO</sup> mutants.

(E) Proportional area activated by 15 kHz in AVCN.

(F) Proportional area activated by 8 kHz in AVCN.

(G) Area of separation between the activated bands.

(legend continued on next page)
Quantification of the area of band separation normalized to total AVCN area in control (n = 4) and Atoh1Hox2cKO (n = 3) mutants (p value = 0.0194, Mann-Whitney test) stimulations. Values are mean ± SEM.

Figure 4. Atoh1Hox2cKO Mutant Mice Fail to Discriminate between Close Pure-Tone Frequencies in Discriminative Auditory Fear Conditioning Setup

(A) Schematic for the fear conditioning setup. Adult control or Hox2 mutant mice were subjected to discriminative fear conditioning behavioral test. During the training (acquisition) phase, the animals were subjected to a pure tone (CS+) at the end of which a foot-shock was administered; after a period of silence, another pure tone (CS−) was played, but not coupled to any foot shock. Training consisted of ten pairings of CS+ and CS−.

(B) In the recall test, four sets of CS− were played, followed by four sets of CS+. Freezing response was compared with response to no sound (baseline).

(C) Atoh1Hox2cKO mutants show impaired discriminative (CS+/CS− freezing ratio) fear response; control (n = 30) and Atoh1Hox2cKO (n = 28) mutants (p value = 0.0004, Mann-Whitney test). AVCN, anterior ventral cochlear nucleus. Scale bar, 50 μm. Values are mean ± SEM.

Next, we asked whether the Atoh1Hox2cKO mutant animals were capable of discriminating two close yet distinct frequencies (8 kHz and 15 kHz). We subjected control and mutant adult animals to a discriminative auditory fear conditioning paradigm (Experimental Procedures; Figure 4 A and 4B). During the acquisition phase, freely moving mice were exposed in an experimental chamber to ten pairings of two pure tones (8 kHz and 15 kHz, 30 s each), one paired with a foot shock (conditioned stimulus) (CS+) and the other without any foot shock (CS−). To avoid any bias due to the frequency used, both 8 kHz and 15 kHz tones were used as CS+ in a counterbalanced manner for different animals. Moreover, Atoh1Hox2cKO mutant animals and controls did not show any significant difference in behaviors that could affect the readout of the conditioning experiment, such as general mobility (i.e., distance traveled or speed of movement) or the freezing behavior itself (Figures S4G–S4I).

To assess the specificity of fear memory, animals were exposed to the CS+ and CS− 24 hr after acquisition, and stimulus-evoked freezing behavior was quantified (Experimental Procedures; Figures 4A and 4B). The ratio of the percentage of freezing during CS+ and CS− (freezing CS+/CS−) was calculated as an indication of tone discrimination (Figure 4C). Notably, the conditioned control animals exhibited significantly higher freezing behavior (Figure 4C).
Endbulb of Held Synapse Maturation is Impaired in Atoh1^Hox2cKO Mutant AVCN Bushy Cells

In the mouse, endbulb of Held synapse development takes place postnatally during pre-hearing and post-hearing stages. At birth, endbulbs appear as small boutons, which subsequently grow in size to a large calyceal or club-shaped terminal, reaching their mature volume and shape by ~2 months of age (Limb and Ryugo, 2000). These maturation steps are accompanied by loss of axon terminal branches and reduction to only up to one to four terminals on average contacting individual bushy cells at the end of the process (Jackson and Parks, 1982; Limb and Ryugo, 2000; Lu and Trussell, 2007). This process is fundamental to achieve tonotopic sound transmission.

VGLUT1 (labeling endbulbs) and parvalbumin (labeling AVCN bushy cell somata) antibody immunostaining revealed endbulb synapse morphological abnormalities in Atoh1^Hox2cKO mutants when compared to control adult mice (Figure 5A). In order to carry out a 3D reconstruction at cellular resolution of single axonal synaptic input to individual AVCN bushy cells, we carried out serial block face-scanning electron microscopy. In control bushy cells, a single axonal input generated a large-volume calyceal terminal surrounding most of the soma surface with additional smaller endings (two to six) from distinct axon terminals (Figures 5B and 5C). In contrast, Atoh1^Hox2cKO mutant bushy cells were targeted by up to 12–15 distinct axon terminals bearing small-volume endings (Figures 5B and 5C).

Thus, the observed overlapping and broadening of c-fos+-responsive bands induced upon tone stimulation could be explained by multiple inputs relaying distinct frequencies and...
converging on individual bushy cells (Figures 3 and 4; model, Figure 5D), resulting in degradation of tonotopic tuning of mutant AVCN bushy cells and failure to discriminate two close pure-tone frequencies.

**Hox2 Factors Regulate Expression of Genes Involved in Target-Derived Signaling, Adhesion, Synaptogenesis, and Synaptic Transmission**

Presynaptic afferent refinement resulting in precise frequency tuning requires not only pruning of axon branches, synapse strengthening, and elimination but also interactions with postsynaptic molecules and cell-membrane-bound receptors in the target nuclei (Brenowitz and Trussell, 2001; Kandler et al., 2009; Jackson and Parks, 1982; Leake et al., 2002; Ryugo et al., 2006; Yu and Goodrich, 2014).

To gain insights into the molecular programs regulated by Hox2 transcription factors that might influence early steps of pre-synaptic maturation, we carried out transcriptome analysis in newborns by RNA sequencing (RNA-seq). We dissected out the AVCN and isolated neurons by fluorescence-activated cell sorting (FACS) from control (Atoh1<sup>Rosa::tdTomato</sup>; n = 4 replicates, three or four AVCNs per replicate) and mutant (Atoh1<sup>Hoxa2hetHoxb2cKO</sup>;Rosa::tdTomato; n = 4 replicates, three or four AVCNs per replicate) newborn animals recovered at E18.5 by cesarean sections. We identified 400 differentially expressed genes (fold change [FC] > 1.5; p value: < 0.01) (Figures 6A and 6B; Table S1). Among them, Gene Ontology (GO) analysis (Table S2) identified molecules involved in calcium signaling pathway regulating synaptogenesis and synaptic transmission, cell adhesion molecules, and target-derived cues involved in axon pruning and synapse development (Figures 6C).

For example, genes upregulated in mutant AVCN neurons included the voltage-sensitive Ca<sup>2+</sup> channels Cacna1g (Cav.3.1) and Cacna1i (Cav.3.3), calcium homeostasis regulating Mctp1, calcium modulator Pcp4, Ca<sup>2+</sup>/calmodulin-dependent protein kinase Camk2b (whereas Camk4 was downregulated), calcium binding protein of the calmodulin family (Cahn1), Tspan7 (involved in morphological and functional maturation of glutamatergic synapses), the synaptic adhesion-like molecule Lrtn5 (binding postsynaptic density 95 [PSD95] and regulating synapse formation), the core protein of the glutamatergic receptor postsynaptic scaffold complexDlgap1, neuron-specific tyrosine phosphatase (Ptpn5; regulating glutamatergic synaptic plasticity), and the NMDA receptor subunit Grin1. Several cell adhesion molecules, potentially involved in cell-cell recognition during synapse formation, were also upregulated, including Ajap1, Lgsf5, and Svep1, members of the cadherin superfamily such as Cdh1, Cdh4, Cdh7, and Cdh13, and the protocadherin Pcdh11x.

Target-derived cues and retrograde signaling by secreted molecules may also be involved in pre-synaptic axon branch pruning, synapse strengthening, and elimination (Bagri et al., 2003; Kalinovsky et al., 2011; Umemori et al., 2004; Xiao et al., 2013). We detected an upregulation of Bmp2, Bmp6, and Fgf10 and concomitant downregulation of the repulsive molecules Sema6a and Slit2, whereas we did not detect significant expression changes in ephrin (Eph) and/or Eph receptors, which are involved in axon guidance during early auditory circuit development (Cramer and Gabriele, 2014).

Lastly, a panel of differentially expressed genes from the RNA-seq analysis, namely Cai1, Ptpn5, Dlgap1, Cdh1, Cdh7, Camk4, and Sema6a, was further validated by qRT-PCR of RNA from dissected and FACS-isolated AVCN neurons from Atoh1<sup>Rosa::tdTomato</sup> control, Atoh1<sup>Hoxa2hetHoxb2cKO</sup>;Rosa::tdTomato, and Atoh1<sup>Hoxb2cKO</sup>;Rosa::tdTomato double homozygous mutant newborns (Figures 6D and 6E).

Altogether, the transcriptional changes detected in AVCN glutamatergic neurons deficient in Hox2 function provide a post-synaptic molecular correlate to understand the multiple innervation of AVCN bushy cells, impairment of endbulb synapse development, and lack of tonotopic precision of AVCN neuron response (Figures 3, 4, and 5).

**DISCUSSION**

This study further expands the repertoire of emerging roles of Hox genes in orchestrating topographic circuit assembly (reviewed in Philippidou and Dasen, 2013). Hox genes are key regulators of neuronal anteroposterior identity, but the links among Hox-mediated neuronal diversification, assembly of regionally specialized circuits, and refinement of topographic precision of connectivity are still poorly understood. We have recently shown that in the principal trigeminal nucleus of the somatosensory brainstem, Hoxa2 is sufficient to organize topographic input-output connectivity of barrelette neurons underlying whisker map formation (Bechara et al., 2015). Here, we found that Hox2 genes are required in Atoh1-derived glutamatergic bushy cells of the AVCN, which receive cochleotopic input from SG afferents. In the absence of Hox2 function, broad input topography and sound transmission is largely preserved as supported by the c-fos activation patterns in AVCN and behavioral functional tests. However, the fine-scale tonotopic refinement and maturation of the brainstem auditory circuit is impaired, resulting in defective frequency tuning in the mutant animals. These findings point to an important role of Hox2 genes during the development of highly topographic brainstem sensory circuits.

In this study, we did not attempt to investigate whether Hoxa2 or Hoxb2 have distinct roles or whether they are fully or only...
partially redundant at maturation. Nonetheless, molecular, anatomical, and functional analyses indicated that Hox2 transcription factors collectively orchestrate the refinement of pre-synaptic input tonotopic precision in the post-synaptic neuron through transcriptional regulation of target-derived signaling molecules, activity-dependent calcium signaling molecules, neurotransmitter receptor expression, axon pruning, and endbulb synapse maturation. Tonotopic map refinement of brainstem circuits has been shown to be dependent on presynaptic activity bursts generated by cochlear hair cells and propagated by SG neurons to brainstem auditory nuclei (Clause et al., 2014). It will be interesting to investigate whether late-stage Hox gene expression might be involved in activity-dependent transcriptional regulation. Altogether, these results provide important insights into the multifaceted involvement of Hox transcription factors in CNS and circuit development. They establish a non-cell-autonomous role for Hox factors in the post-synaptic cochlear neuron regulating pre-synaptic endbulb of Held development and the precision of sound transmission in the first central station of the auditory circuit, with their absence resulting in central auditory discrimination deficits.

Lastly, these findings may also advance our understanding of the genetic basis of central auditory processing disorders (CAPDs) in children (Bellis and Ferre, 1999). CAPDs are detected of the genetic basis of central auditory processing disorders.

Atoh1Cre

The Atoh1Cre mouse line was generated by subcloning the 1.7-kb Atoh1 (Helms et al., 2000; Machold and Fishell, 2005) enhancer in the kPS-β-globin-Cre-SV40pa plasmid. The Atoh1 enhancer was PCR amplified from genomic DNA using the primers: 5′-AGTTGTGGGCTGTCATAAGGTC-3′ and 5′-ATCTACTAGTGGCTGTTCTAATACC-3′. The PCR band was purified and inserted 5′ to the β-globin promoter using restriction sites SacI and Spel. The resulting construct consisted of the Atoh1 enhancer, β-globin minimal promoter, and Cre recombinase encoding sequence. The construct was linearized, purified, and microinjected into the pronuclei of mouse zygotes. Founders were identified by PCR using the following primers: 5′-AGTGGA GAATGGTTAATACC-3′ and 5′-ATCAGTGGCTTGGAACGCTA-3′.

Historical Analysis, Immunostaining, and In Situ Hybridization

Prenatal or postnatal brains, dissected when necessary, were fixed in 4% paraformaldehyde (PFA) diluted in phosphate buffer (PBS 1×) from 30 min at room temperature to overnight at 4°C. For cryostat sections, tissues were cryoprotected in 20% sucrose (Fluka)/PBS 1× and embedded in gelatine 7.5% (Sigma)/10% sucrose/PBS 1× before being frozen at −80°C. Cryostat (Microm HM560) sections (20 μm and 30 μm) were cut in coronal and sagittal planes. For immunohistochemistry, cryoprotected tissues were mounted with normal water and frozen to −50°C and cut at 40 μm thickness. Vibratome (Leica VT1000S) sections (60 μm) were prepared from postnatal brains after embedding in 4% agarose (Promega)/0.1 M phosphate buffer (pH 7.4). Immuno- histochemistry was performed as described previously (Di Meglio et al., 2013) using the antibodies ZsGreen (rabbit, Clontech, 632474), BAFHL1 (rabbit, Sigma, HPA04809), MafB P-20: sc-10022 (goat, Santa Cruz Biotechnology), Parvalbumin 28 (rabbit, Swant), Pax6 (Millipore, AB2237), streptavidin Alexa 488 conjugates (Life Technologies, S-11223), GFP (rabbit, Life Technologies, A6455), GFP (chicken, Invitrogen, A10262), RFP (rabbit, Rockland, 600-401-379), c-Fos Ab-5, 4-17 (rabbit, Calbiochem, PC38), and VGLUT1 (mouse, Chemicon, AB56502). Standard in situ hybridization was performed as described (Di Meglio et al., 2013). Fluorescence in situ hybridizations were performed as described in the Perkin Elmer, TSA Plus Fluorescence kit manual. The following probes were used: Barh1, Hoxa2, Hoxb2, Rig1, Math5, and MafB (Di Bonito et al., 2013; Di Meglio et al., 2013). Other probes were synthesized by amplifying the region of interest by PCR from template cDNA prepared from whole RNA extracted from E14.5 brain and by cloning the amplicons into pCRII-TOPO vector using a TOPO TA cloning kit (Life Technologies); namely, Vglut1 (forward: 5′-CTG TCTGTTGGTTGTCTC-3′; reverse: 5′-TGCTCTTCATTCACTTT-3′, Xhol, Sp6 promoter), Vglut2 (forward: 5′-CCAAATTCTAGCTGCTACCTC-3′; reverse: 5′-TAGCCATCTTTCTGTTCTACT-3′, Xohl, Sp6 promoter), c-fos (forward: 5′-AGATCGAACGGGGG-3′; reverse: 5′-GGAGGCCAGAT GTGAGT-3′, XbaI, Sp6 promoter). For double in situ hybridization and immuno- histochemistry staining, in situ hybridization was carried out prior to immuno- histochemistry. X-galactosidase staining on whole embryos, whole brains, or cryostat sections was performed as described previously (Di Meglio et al., 2013).

RT-PCR Analysis of Hox2 Gene Expression at Postnatal Stages

To average variability between individuals, gene expression analysis was performed on two sets (duplicates) of wild-type pups or animals at postnatal day 0 (P0), P2, P6, P11, P16, P21, P30, and P60. In brief, the AVCN and somatosensory cortex (negative control) were hand-dissected from brains at different stages under a microscope (Leica, MZ7s) and used for total RNA extraction using the manufacturer’s instructions, A6455, and 501-379), c-Fos Ab-5, 4-17 (rabbit, Calbiochem, PC38), and VGLUT1 (mouse, Chemicon, AB56502). Standard in situ hybridization was performed as described (Di Meglio et al., 2013). Fluorescence in situ hybridizations were performed as described in the Perkin Elmer, TSA Plus Fluorescence kit manual. The following probes were used: Barh1, Hoxa2, Hoxb2, Rig1, Math5, and MafB (Di Bonito et al., 2013; Di Meglio et al., 2013). Other probes were synthesized by amplifying the region of interest by PCR from template cDNA prepared from whole RNA extracted from E14.5 brain and by cloning the amplicons into pCRII-TOPO vector using a TOPO TA cloning kit (Life Technologies); namely, Vglut1 (forward: 5′-CTG TCTGTTGGTTGTCTC-3′; reverse: 5′-TGCTCTTCATTCACTTT-3′, Xhol, Sp6 promoter), Vglut2 (forward: 5′-CCAAATTCTAGCTGCTACCTC-3′; reverse: 5′-TAGCCATCTTTCTGTTCTACT-3′, Xohl, Sp6 promoter), c-fos (forward: 5′-AGATCGAACGGGGG-3′; reverse: 5′-GGAGGCCAGAT GTGAGT-3′, XbaI, Sp6 promoter). For double in situ hybridization and immuno- histochemistry staining, in situ hybridization was carried out prior to immuno- histochemistry. X-galactosidase staining on whole embryos, whole brains, or cryostat sections was performed as described previously (Di Meglio et al., 2013).

Analysis of Hox2 Gene Deletion Mosaicism by qPCR

Mosaicism analysis was performed on E16.5 Atoh1Hox2CreKO;Rosa::tdTomato mutant fetuses. In brief, the fluorescent cochlear nuclei from each mutant were hand-dissected, pooled, and collected in individual tubes and incubated in DMEM (GIBCO)/0.1% trypsin (GIBCO) for 8 min at 37°C. Tissue was then transferred to and rinsed four times in DMEM/10% FBS before mechanical dissociation. Fluorescent cells were collected by fluorescence-activated cell sorting (FACS) (Becton Dickson). The sorted cells were digested with proteinase K, and the genomic DNA was extracted with phenol/chloroform/isooamyl alcohol, precipitated with ethanol/sodium acetate, and resuspended in water. To serve as reference values, we also extracted genomic DNA from E12.5 littermate control (carrying two wild-type alleles) and Hoxa2 (Pasqualetti et al., 2002) or Hoxb2 (Di Bonito et al., 2013) heterozygous mutant (carrying only one Hoxa2 or Hoxb2 wild-type allele, respectively) embryos. qPCR analysis was carried out on each Atoh1Hox2CreKO;Rosa::tdTomato double-homozygous and Hoxa2 and Hoxb2 single-heterozygous mutant and control samples using the StepOne Real Time PCR System (Applied Biosystems) and primers located within the floxed alleles for Hoxa2 and Hoxb2 (target genes) and in Hoxa5 (reference gene) for the normalization. The mosaicism was calculated using the 2−ΔΔCt calculation (Tesson et al., 2010).
Neurovue Labeling from Cochlea to the AVCN and Quantification

The heads of E18.5 fetuses were fixed in 4% PFA diluted in phosphate buffer (PBS 1 x) overnight at 4°C. After removing the heads from the PFA, the lower jaw, tongue, and attached tissue on the ventral side of the head were removed to reveal the bony labyrinth. Dissecting through the bony labyrinth, the cochlea was revealed. Small incisions were made into the cochlea with microscissors to reveal the bony labyrinth. Dissecting through the bony labyrinth, the cochlea was hand-dissected fluorescent cochlear nuclei were incubated in DMEM (GIBCO)/0.1% trypsin (GIBCO) for 8 min at 37°C. Tissue was then transferred to and rinsed four times in DMEM/10% FBS before mechanical dissociation. Fluorescent cells were collected by fluorescence activated cell sorting (FACS)Calibur, Becton Dickson). RNA was extracted using the PicoPure RNA Isolation Kit (Applied Biosystems) and retro-transcribed. Libraries were prepared using the Total RNA Sequencing TotalScript Kit (Epicerent), and sequencing was performed using Hi-Seq 2500 Illumina solid sequencer. Data analysis was performed using the Bioconductor Quasar package (release 3.0). Gene Ontology analysis was performed using the GO Enrichment Analysis tool powered by PANTHER (https://geneontology.org/page/go-enrichment-analysis). For validation of gene expression changes between control and mutants, we performed qRT-PCR. Fluorescent cochlear nuclei were collected from E18.5 Atoh1flkOsa::tdTomato or Atoh1flh+Mm0056135_m1), Cdhr1 (Mm00479063_m1), (Mm00556135_m1), Cdh1 (Mm00499988_m1), Dlgap1 (Mm00510688_m1), Camk4 (Mm00135291_m1), Smad6 (Mm00444441_m1), and Gapdh (Mm09999915_g1). Gapdh was used for the normalization, and the relative gene expression was calculated using the 2^-ΔΔCt calculation, where ΔΔCt = (Ctgene target - Ctgene reference)/(Ctgene target - Ctgene reference), and “known” refers to heterozygous controls. The final values are expressed in percentage of remaining gene = (2^-ΔΔCt) x 100. Primers for qPCR are Hoxa2 (forward: 5'TAGAATGCGAGGATGGTG-3'; reverse: 5'-AAGCGTGGAGTCTGTTTGA-3'); Hoxb2 (forward: 5'-CCTACACCAACGCGAATCGT-3'; reverse: 5'-CTTGTGTTCATGGCTCGTTC-3'); Hoxa5 (forward: 5'-AAAAGAAATATGCAGCGGCCA-3'; reverse: 5'-GAAAGCTCAAGAACGGCAGTAC-3').

Biotinylated Dextran Amine Axonal Tracing

Biotinylated dextran amine (BDA) labeling from the AVCN was performed as described previously (Di Bont et al., 2015) in P7 control and mutant dissected brains. Following incubation for 6 hr at room temperature, the brains were fixed as described before, and vibratome sections at 60 μm were made the following day. The sections were stained with streptavidin Alexa 488 (Invitrogen) and immunohistochemistry was carried out with anti-VGLUT1. The sections were counterstained in DAPI and imaged with confocal microscope LSM 700 (Axio Imager Z2, upright microscope and LSM 700 scanning head; Software: ZEN Black 2012).

SBF-SEM and 3D Reconstructions

Adult mice were fixed by cardiac perfusion with paraformaldehyde and glutaraldehyde, and brain areas including the AVCN were sectioned with a vibratome (Leica). Sections were stained according to National Center for Microscopy and Imaging Research (NCMIR) protocol (https://ncmir.ucsd.edu/sbsem-protocol.pdf). The slices were further rinsed in 0.1 M cacodylate buffer (pH 7.4) and post-fixed with 1.5% formaldehyde in 2% osmium for 1 hr. After extensive rinsing in double-distilled water (ddH2O), sections were stained in 1% thiocarbohydrazide for 20 min at 60°C followed by rinsing in ddH2O. A second osmium post-fixation in 2% osmium was done for 30 min. Sections were then stained in uranyl acetate 1% overnight. The next day, sections were stained for 20 min in Walton’s lead aspartate before dehydration and embedding in epoxy resin. Serial BlockFace scanning electron microscopy (SBFSEM) was done as described (Denk and Horstmann, 2004) using the 3View microscope (Gatan) mounted on a QUANTA 200 VP-FEG (FEI). The AVCN was identified on vibratome sections, cut out of the section, and mounted on aluminum stubs for the sample holder of the 3View. The blocks were then trimmed on pyramidal shape and sputter-coated with gold. Once placed on the microtome, the surface of the block was imaged (4 kV beam, spot size 3, low vacuum mode with 0.22 Torr of water pressure in the chamber). Two fields of view with an overlap of 15% were determined in the center of the AVCN. Each field of view represents a surface of 51 x 51 μm (8,192 x 8,192 pixels, 6nm pixel size). After imaging of the block face using the back-scattered electron detector of 3View (Gatan), a diamond knife removed 100 nm from the surface of the block, and the new surface was imaged using the same parameters. This cycle was repeated 1,000 times (150 hr of image acquisition per block). Images were then preprocessed to equalize the contrast and to be registered (TrakEM2, Fiji). Further 3D reconstruction was done using TrakEM2 in Fiji. The volumes of the presynaptic axonal terminals were measured using Fiji software.

RNA-Sequencing Analysis and qRT-PCR

To average variability between individuals, RNA-seq expression analysis was performed on four sets of control and mutant embryos, each consisting of pooled hand-dissected fluorescent cochlear nuclei from three or four Atoh1flkOsa::tdTomato or Atoh1flh+Mm0056135_m1), Cdhr1 (Mm00479063_m1), (Mm00556135_m1), Cdh1 (Mm00499988_m1), Dlgap1 (Mm00510688_m1), Camk4 (Mm00135291_m1), Smad6 (Mm00444441_m1), and Gapdh (Mm09999915_g1). Gapdh was used for the normalization, and the relative gene expression was calculated using the 2^-ΔΔCt calculation, where ΔΔCt = (Ctgene target - Ctgene reference)/(Ctgene target - Ctgene reference), and “known” refers to heterozygous controls. The final values are expressed in percentage of remaining gene = (2^-ΔΔCt) x 100.
17 (rabbit, Calbiochem, PC38) was discontinued; further experiments were carried out by in situ hybridization with c-fos antisense mRNA probe that resulted in similar results as the c-fos antibody staining (Figure 3C). Quantification of the c-Fos-labeled cells was done using Cell Counter plugin in ImageJ software (http://fiji.sc/Fiji). For calculation, the number of cells or the area covered by the labeled cells was normalized over the size of the AVCN for each experiment and the ratio was used to plot the graphs using GraphPad prism software (version 6). The area size of the AVCN on each section was measured using ImageJ software. The ratio between c-Fos-labeled cells and the AVCN area was fed into a R-script for automated quantification. Samples were randomized before quantification to enable quantification blind to genotype.

Auditory Fear Conditioning Experiments
We carried out a discriminative auditory fear conditioning paradigm (Ciocchi et al., 2010). Before behavioral experiments, control and mutant 2- to 3-month-old mice were individually housed in a 12-hr light/dark cycle. Behavioral experiments were performed during the light cycle. Fear conditioning experiments were performed as described previously (Ciocchi et al., 2010). Fear conditioning and fear testing took place in two different contexts (context A and B). The conditioning and test boxes and the floor were cleaned with 70% ethanol or 1% acetic acid before and after each session, respectively. Freezing behavior was scored blind to genotype using an automatic infrared beam detection system placed on the bottom of the experimental chambers (Coulbourn Instruments). Mice were considered to be freezing if no movement was detected for 2 s and the measure was expressed as a percentage of time spent freezing. Discriminative fear conditioning was performed on day 1 in context A by pairing the CS– with a foot shock (1-s foot shock, 0.6 mA). The onset of the foot shock coincided with the offset of the CS+. The CS– was presented after each CS+/foot shock association but was never reinforced (inter-trial interval: 20–180 s). Each animal was trained to ten pairings of CS+/foot shock and CS–. Both 8 kHz and 15 kHz were used as CS+ or CS– to avoid any bias due to the tone used, and the CS+ was counterbalanced across animals. On day 2, conditioned mice were submitted to fear retrieval in context B. Following a 3-min baseline period in which no tones were played, mice received four presentations of the CS– and the CS+ (each 30 s long, variable inter-trial interval), respectively and the freezing responses were compared. To compare the discriminative fear response, the ratio of freezing response in CS+ to freezing response in CS– was calculated.

Quantification of Behavior
All behavioral sessions were recorded to video using Cineplex software (Plexon), and mice were tracked using contour tracking. Behavior was scored from the video recording by an observer blind to the condition, using the assistance of a frame-by-frame analysis of pixel changes (Cineplex Editor, Plexon). By calibrating the known size of the chambers with the pixel dimensions of the camera to determine a calibration coefficient, speed (centimeters per second) was extracted using the animal center of gravity. To assess general motility traits in control and mutant mice, we used the speed calibration to quantify the average speed and distance traveled during the 3-min baseline period of the retrieval session.

Image Processing
Fluorescent images were acquired with LSM700 (Laser Scanning Microscope; Axio Imager Z2, upright microscope plus LSM 700 scanning head; Software: Zen Black 2012) unless mentioned otherwise. The chromogenic images of the in situ hybridization were obtained with a bright-field Microscope (Nikon E600 Eclipse). Image analysis was performed using Fiji imaging analysis software (http://fiji.sc/Fiji).

Statistical Analysis
Statistical analyses were done with GraphPad Prism 6 software. All results are expressed as the mean ± SEM. For statistical analyses, normality tests were done for all datasets. A nonpaired, two-tailed Student’s t test and one-way ANOVA were performed for datasets that passed normality tests and were accepted at the p < 0.05 level. For datasets that did not pass the normality tests, the non-parametric Mann-Whitney and Kruskal-Wallis tests were performed, as mentioned.

ACCESSION NUMBERS
The accession number for all high-throughput sequencing datasets reported in this paper is GEO: GSE64092.

SUPPLEMENTAL INFORMATION
Supplemental Information includes four figures and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2016.12.021.

AUTHOR CONTRIBUTIONS
K.K. carried out anatomical and molecular assays, auditory stimulation and fear conditioning tests, and data analysis. Y.N. started the project and carried out afferent tracing assays as well as initial auditory stimulation and fear conditioning tests. A. Loche and T.K. carried out RNA-seq and analysis. J.F., J.B., and S.D. set up fear conditioning experiments. S.D. generated and characterized transgenic lines and carried out mosaic analysis. C.G. helped with data collection and supervised EM data analysis. R.T. created MATLAB scripts. T.D.M. carried out some in situ hybridization experiments. A. Lüthi supervised fear conditioning tests and provided input to the manuscript draft. F.M.R. conceived the project, supervised data analysis, and wrote the manuscript based on a first draft by K.K.

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