Maturation of Ribbon Synapses in Hair Cells Is Driven by Thyroid Hormone

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Ribbon synapses of inner hair cells (IHCs) undergo developmental maturation until after the onset of hearing. Here, we studied whether IHC synaptogenesis is regulated by thyroid hormone (TH). We performed perforated patch-clamp recordings of Ca^{2+} currents and exocytic membrane capacitance changes in IHCs of athyroid and TH-substituted *Pax8*^{−/−} mice during postnatal development. Ca^{2+} currents remained elevated in athyroid IHCs at the end of the second postnatal week, when it had developmentally declined in wild-type and TH-rescued mutant IHCs. The efficiency of Ca^{2+} influx in triggering exocytosis of the readily releasable vesicle pool was reduced in athyroid IHCs. Ribbon synapses were formed despite the TH deficiency. However, different from wild type, in which synapse elimination takes place at approximately the onset of hearing, the number of ribbon synapses remained elevated in 2-week-old athyroid IHCs. Moreover, the ultrastructure of these synapses appeared immature. Using quantitative reverse transcription-PCR, we found a TH-dependent developmental upregulation of the mRNAs for the neuronal SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins, SNAP25 (synaptosomal-associated protein of 25 kDa) and synaptobrevin 1, in the organ of Corti. These molecular changes probably contribute to the improvement of exocytosis efficiency in mature IHCs. IHCs of 2-week-old athyroid *Pax8*^{−/−} mice maintained the normally temporary efferent innervation. Moreover, they lacked large-conductance Ca^{2+} -activated K^{+} channels and KCNQ4 channels. This together with the persistently increased Ca^{2+} influx permitted continued action potential generation. We conclude that TH regulates IHC differentiation and is essential for morphological and functional maturation of their ribbon synapses. We suggest that presynaptic dysfunction of IHCs is a mechanism in congenital hypothyroid deafness.

Key words: thyroid hormone; ion channel; ribbon synapse; exocytosis; hair cell; capacitance; Pax8

Introduction

Mice are born deaf and start to hear toward the end of the second postnatal week (Mikaelian and Ruben, 1965; Ehret, 1985). Inner hair cell (IHC) afferent synaptic transmission is one of several auditory processes maturing at approximately the onset of hearing. Synapse number (Sobkowicz et al., 1982) and Ca^{2+} current density (Beutner and Moser, 2001; Brandt et al., 2003; Johnson et al., 2005) reach a peak at the end of the first postnatal week, when action potentials in IHCs drive presynaptic transmitter release (Beutner and Moser, 2001; Glowatzki and Fuchs, 2002; Johnson et al., 2005). Morphologically, this stage is characterized by large

active zones anchoring multiple ribbons and branched afferent fibers, giving rise to multiple postsynaptic terminals (Shnerson et al., 1981; Sobkowicz et al., 1982). The number of synapses declines and active zones assume a more confined appearance anchoring a single ribbon at approximately the onset of hearing (Shnerson et al., 1981; Sobkowicz et al., 1982; Pujol et al., 1997; Nemzou et al., 2006). This structural maturation goes along with a decline of the presynaptic Ca^{2+} current and an improvement of stimulus secretion coupling (Beutner and Moser, 2001; Johnson et al., 2005).

Little is known about the regulation of hair cell afferent synapse development and accompanying molecular changes. Here, we tested whether thyroid hormone (TH) contributes to the regulation of afferent synaptogenesis. TH regulates neural and sensory development through nuclear TH receptor-mediated transcriptional control (for review, see Forrest et al., 2002; Bernal, 2005). In the inner ear, TH regulates various aspects of development, including the formation of the inner sulcus, the tunnel of Corti, and a proper tectorial membrane, as well as the postnatal hair cell differentiation [e.g., acquisition of large-conductance calcium-activated (BK) channels] (Deol, 1973b; Uziel et al., 1981; Knipper et al., 1998; Li et al., 1999; Rusch et al., 2001; Christ et al., 2004; Ng et al., 2004) (for review, see Bryant et al., 2002; Forrest et al., 2002). As a consequence, congenital or endemic hypothyroidism causes, besides mental retardation, profound deafness (Deol,

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Table 1. Passive electrical properties

		num _{IHCs}	C_m (pF)	R_s (M Ω)	I_{rest} (pA)	V_m (mV)
P7	<i>Pax8</i> ^{+/+}	<i>n</i> = 12	8.17 $\pm$ 0.17	22.5 $\pm$ 1.42	−47.98 $\pm$ 2.64	−70.96 $\pm$ 2.04
	<i>Pax8</i> ^{−/−}	<i>n</i> = 7	6.60 $\pm$ 0.22	23.81 $\pm$ 1.50	−43.44 $\pm$ 3.88	ND
P15	<i>Pax8</i> ^{+/+}	<i>n</i> = 12	8.80 $\pm$ 0.30	17.79 $\pm$ 0.90	−31.53 $\pm$ 2.96	−78.28 $\pm$ 2.24
	<i>Pax8</i> ^{−/−}	<i>n</i> = 7	7.39 $\pm$ 0.25	22.88 $\pm$ 2.06	−46.13 $\pm$ 2.78	−68.60 $\pm$ 1.71

C_m and R_s of mutant and wild-type IHCs were repetitively estimated by time domain analysis of the resistor–capacitor circuit of the cell during the recording in the perforated patch mode. I_{rest} is the current at the holding potential of −84 mV, and V_m represents the membrane potential of the IHC measured in whole-cell mode (*n* = 3 for each group in these experiments; ND, not determined). V_m values were corrected for the liquid junction potential of the KCl intracellular solution (−4.4 mV). Data are presented as means $\pm$ SEM of the averages of each individual IHC (grand average).

1973a; Glorieux et al., 1983) (for review, see Bernal, 2005; Rose et al., 2006).

For our study of TH function in postnatal IHC differentiation, we used mice lacking the transcription factor Pax8 (Mansouri et al., 1998). These mice serve as a model of human primary congenital hypothyroidism (Macchia, 2000; Kopp, 2002). They are athyroid because they do not form thyroid follicular cells (Mansouri et al., 1998). Their auditory system has been characterized recently in detail (Christ et al., 2004). Pax transcription factors (Pax2, Pax6, and Pax8) are also involved in early ear development, in which the other Pax isoforms seem to compensate the lack of Pax8 (Xu et al., 1999). Here, we studied the presynaptic function of TH-deficient IHCs and used confocal and electron microscopy to investigate the number and morphology of their ribbon synapses. Using quantitative reverse transcription (RT)-PCR, we determined the mRNA levels of synaptic proteins in the organ of Corti during normal development and in case of TH deficiency. Our study shows that synapse maturation in IHCs is severely impaired in the absence of TH.

Materials and Methods

Animals

The animals were maintained according to the animal welfare guidelines of the University of Göttingen and the State of Lower Saxony. *Pax8*^{+/−} male and female mice were used for mating. For the TH-substitution experiment, *Pax8*^{−/−} mice received daily intraperitoneal injections of 0.3 μ g of levo-thyroxine (cell-culture tested; Sigma, St. Louis, MO) per gram body weight starting from the day of birth until the day of experiment (Weber et al., 2002). Levo-thyroxine was dissolved in isotonic saline at a concentration of 6 ng/ μ l, stored frozen and used only freshly after thawing.

Patch-clamp of IHCs

IHCs from the apical coil of freshly dissected organs of Corti from *Pax8*^{−/−} mice [mutant (mut)] and their wild-type (wt) littermates or C57BL/6J mice (results of the latter two groups were pooled because they were statistically not distinguishable) were patch clamped at room temperature (20–25°C) on postnatal day 6 (P6) to P8 and P14–P16.

Chemicals were obtained from Sigma unless otherwise stated. The dissection solution (HEPES–HBSS on ice) contained the following (in mM): 5.36 KCl, 141.7 NaCl, 1 MgCl₂, 0.5 MgSO₄, 0.1 CaCl₂, 10 Na-HEPES, 10 D-glucose, and 3.4 L-glutamine. The pH was adjusted to 7.2 with NaOH, and the osmolality was 290 mmol/kg.

Perforated-patch recordings of Ca²⁺ current and exocytosis. (1) The pipette solution contained the following: 130 mM Cs-gluconate, 10 mM tetraethylammonium-Cl (TEA-Cl), 10 mM Cs-HEPES, 10 mM 4-aminopyridine, 1 mM MgCl₂, and 250 μ g/ml amphotericin B (Calbiochem, La Jolla, CA). The osmolality was 290 mmol/kg, and pH was adjusted to 7.2 with HCl. With this solution, pipette resistances were ~4–6 M Ω .

(2) The extracellular solution contained the following (in mM): 113 NaCl, 2 CaCl₂, 35 TEA-Cl, 2.8 KCl, 1 MgCl₂, 10 Na-HEPES, and 10 D-glucose. Osmolality was in the range of 300–320 mmol/kg, and pH was adjusted to 7.2 with NaOH.

Standard whole-cell recordings of K⁺ current and membrane potential. (1) The pipette solution contained the following (in mM): 135 KCl, 1 MgCl₂, 10 K-HEPES, 2 Mg-ATP, 0.3 Na-GTP, and 0.1 EGTA. Osmolality

was 290 mmol/kg, and pH was adjusted to 7.2 with KOH. Pipettes filled with this solution had resistances in the range of 2.5–4 M Ω .

(2) The extracellular solution contained the following (in mM): 144 NaCl, 5.8 KCl, 2 CaCl₂, 0.9 MgCl₂, 10 HEPES, and 10 D-glucose. Osmolality was 300–320 mmol/kg, and pH was adjusted to 7.2 with NaOH.

EPC-9 amplifiers (HEKA Elektronik, Lambrecht, Germany) controlled by Pulse software (HEKA Elektronik) were used for measurements. All voltages were corrected for liquid junction potentials (−14 mV for Cs-gluconate and −4.4 mV for KCl intracellular solutions). For perforated-patch recordings of Ca²⁺ currents and exocytic ΔC_m , no series resistance (R_s) compensation was performed, but rather we discarded recordings with R_s > 30 M Ω . Currents were low-pass filtered at 2 kHz and sampled at 10 kHz. Cells that displayed a membrane current exceeding −50 pA at our standard holding potential of −84 mV were discarded. Ca²⁺ currents were further isolated from background current by (1) subtracting a linear fit to the current–voltage relationships (*I*–*V*) between −74 and −64 mV (for correction of Ca²⁺ current *I*–*V* relationships) or (2) a P/6 protocol (for Ca²⁺ currents used to elicit exocytosis). C_m was measured as described previously using the Lindau–Neher technique (Lindau and Neher, 1988; Moser and Beutner, 2000). ΔC_m was estimated as the difference of the mean C_m over 400 ms after the depolarization (the initial 200 ms were skipped) and the mean prepulse capacitance (400 ms). Ca²⁺ current integrals were calculated from the total depolarization-evoked inward current, including Ca²⁺ tail currents.

For standard whole-cell recordings of K⁺ currents and membrane potentials, R_s was compensated on-line (50–80%; τ = 10 μ s), and the remaining voltage error was corrected off-line. Currents were low-pass filtered at 5 kHz and sampled at 40 kHz. A P/10 protocol was used to subtract capacitive and ohmic background currents. For average values of R_s , resting C_m , and resting current (at −84 mV), see Table 1.

Data analysis was performed using Igor Pro software (WaveMetrics, Lake Oswego, OR). Means $\pm$ SEM are provided and were compared using an unpaired two-tailed *t* test with equal or unequal variances dependent on the result of Fisher's variance analysis.

Immunocytochemistry

Immunostaining procedure. The freshly dissected apical cochlear turns were fixed with 4% paraformaldehyde (PFA) for 1 h on ice (standard), with 99.9% ethanol (Merck, Darmstadt, Germany) for 20 min at −20°C, or with 99.9% methanol (Merck) for 20 min at −20°C (when specified in figure legends). Thereafter, the preparations were washed three times for 10 min in PBS and incubated for 1 h in goat serum dilution buffer (GSDB) (containing 16% normal goat serum, 450 mM NaCl, 0.3% Triton X-100, and 20 mM phosphate buffer, pH 7.4), in a wet chamber at room temperature. Primary antibodies were dissolved in GSDB and applied overnight at +4°C in a wet chamber. After washing with wash buffer (three times for 10 min), the organs of Corti were incubated with secondary antibodies in GSDB in a wet light-protected chamber for 1 h at room temperature. Then the preparations were washed three times for 10 min in wash buffer (450 mM NaCl, 20 mM phosphate buffer, and 0.3% Triton X-100) and one time for 10 min in 5 mM phosphate buffer, placed onto the glass microscope slides with a drop of fluorescence mounting medium (DakoCytomation, Glostrup, Denmark), and covered with thin glass coverslips. The following antibodies were used: mouse IgG₁ anti-C-terminal binding protein 2 (CtBP2) (1:100; BD Biosciences, San Jose, CA), rabbit anti-glutamate receptor subunit 2/3 (GluR2/3) (1:100; Chemicon, Temecula, CA), mouse anti-parvalbumin (1:500; Swant, Bellinzona, Switzerland), rabbit anti-BK (1:200; Sigma), mouse anti-

synaptophysin (1:200; Synaptic Systems, Göttingen, Germany), mouse anti-synaptosomal-associated protein of 25 kDa (SNAP25) (1:100; Synaptic Systems), rabbit anti-small-conductance calcium-activated potassium channel (SK2) (1:200; Sigma) and secondary AlexaFluor488- and AlexaFluor568-labeled antibodies (1:200; Invitrogen, Carlsbad, CA).

Confocal microscopy. Confocal images were acquired using a laser scanning confocal microscope LSM 510 (Zeiss, Jena, Germany) with 488 nm (argon) and 543 nm (helium–neon) lasers for excitation. We used 40× oil or water immersion objectives and the bandpass filters 500–550 and 565–615 nm. To produce three-dimensional (3D) reconstructions of the specimen, a z-axis stack of 2D images was taken with a step size of 0.2 μ m. The pixel size was 0.09 $\times$ 0.09 μ m.

Image analysis. Images were processed using LSM 510 software (Zeiss) or NIH ImageJ and assembled for display in Adobe Photoshop and Illustrator software (Adobe Systems, San Jose, CA). Whole-mount preparations of the organ of Corti provided the possibility to analyze several IHCs in a row (Khimich et al., 2005). The RIBEYE immunofluorescence spots in the basolateral portion of the IHCs (up to the apical end of the CtBP2-stained nucleus) were counted in 3D reconstructions of the organ of Corti and divided by the number of IHCs (number of nuclei in the field of view). Likewise, we counted SK2 immunospots and related them to the number of IHCs in the field of view (identified by their halo of synaptophysin fluorescence) or to the average count of 5.2 ± 0.72 IHCs in the field of view (40× objective lens and 5× optical zoom (Nemzou et al., 2006)). Quantitative data are presented as mean $\pm$ SEM.

Electron microscopy

The organs of Corti were fixed immediately after dissection with 2.5% glutaraldehyde in HEPES–HBSS (see above) for 30 min at room temperature. Thereafter, the samples were fixed overnight at 4°C in 2% glutaraldehyde in 0.1 M cacodylic buffer at pH 7.4. After an additional fixation in 0.1% OsO₄, the samples were stained with 1% uranyl acetate and dehydrated in a series of EtOH and finally in propylene oxide. They were then embedded in Agar 100 (purchased through Science Services, Munich, Germany). Thin sections (80 nm) were counterstained with lead citrate and examined using a Philips (Eindhoven, The Netherlands) CM 120 BioTwin transmission electron microscope. Pictures were taken with a TemCam F224A camera (TVIPS, Gauting, Germany) at 20,000-fold magnification.

Real-time RT-PCR

We isolated total RNA from six organs of Corti (as well as six modiol, six retinas, and three cerebella) from P14–P17 *Pax8*^{+/+} and *Pax8*^{-/-} littermates (three mice each), as well as three P6 *Pax8*^{+/+} mice for each experiment using TRIzol Reagent (Invitrogen). Reverse transcription (10 min at 25°C, 50 min at 42°C, and 15 min at 70°C) of the total RNA (adjusted to ~ 1.5 μ g per reaction, except for the organ of Corti, in which we used all isolated RNA; see Results) was performed in first-strand cDNA synthesis mix containing the following (after the final dilution): 50 mM Tris–HCl, 75 mM KCl, 5 mM MgCl₂, 5 mM DTT adjusted to pH 8.3, 100 U of SuperScript II Reverse Transcriptase (Invitrogen), 40 U of RNaseOUT Ribonuclease inhibitor (Invitrogen), as well as 2.5 ng/ μ l random hexamers (Applied Biosystems, Foster City, CA). After the RT reaction, cDNA was subjected to real-time PCR using ABI Prism 7000 or 7500 Sequence Detection Systems (Applied Biosystems). cDNAs for otoferlin, KCNMA1, α 1D (Ca_v1.3), RIBEYE, Bassoon, SNAP25, SNAP23, synaptobrevin 1 (vesicle-associated membrane protein 1) and TBP (TATA-binding protein as a housekeeping gene) were selectively amplified (in triplicates) using commercially available TaqMan Gene Expression Assays (Mm00453306_m1, Mm00516078_m1, Mm01209910_m1, Mm01163439_m1, Mm01330351_mH, Mm00464451_m1, Mm01336180_m1, Mm00772307_m1, and Mm00446973_m1, respectively; purchased from Applied Biosystems) in separate reactions (20 μ l volume) according to the protocol of the manufacturer. Relative amounts of target mRNAs, normalized to that of TBP, were calculated using the comparative 2^{− $\Delta\Delta$ Ct} method (Applied Biosystems) and reported as population estimate of the mRNA abundance $\pm$ 95% confidence interval. Significance of expression differences was tested at the level of Δ Ct values using a paired two-tailed *t* test. The efficiency of

amplification, as determined by linear regression of standard curves, was >90% for all assays in all tissues. The slopes of the standard curves for the organ of Corti amounted to −3.49, −3.56, −3.33, −3.54, −3.41, −3.43, −3.45, −3.50, and −3.40 for otoferlin, KCNMA1, α 1D (Ca_v1.3), RIBEYE, Bassoon, SNAP23, SNAP25, synaptobrevin 1, and TBP, respectively.

Results

Impaired maturation of presynaptic function in IHCs of athyroid mice

To investigate the presynaptic function of *Pax8*^{-/-} IHCs, we performed perforated patch-clamp measurements of Ca²⁺ currents and exocytic capacitance changes (ΔC_m) in athyroid *Pax8*^{-/-} and wt IHCs on P6–P8 (before onset of hearing in wt) and P14–P16 (after onset of hearing in wt). Ca²⁺ currents in wt IHCs were larger before than after onset of hearing (Fig. 1*A–D*), which is in line with previous data obtained from IHCs of Naval Medical Research Institute [NMRI (Beutner and Moser, 2001), C57BL/6 (Brandt et al., 2003), and CD1 mice (Johnson et al., 2005)]. Ca²⁺ currents of *Pax8*^{-/-} IHCs were smaller than wt currents when compared on P6–P8 ($p = 0.0004$; $n = 7$ for *Pax8*^{-/-} IHCs and $n = 12$ for wt IHCs). However, the Ca²⁺ currents of athyroid *Pax8*^{-/-} IHCs were ~ 2.5 -fold larger than those of wt IHCs when compared 2 weeks after birth (Fig. 1*A–D*) ($p = 0.0001$; $n = 7$ for *Pax8*^{-/-} IHCs and $n = 12$ for wt IHCs). These organs of Corti appeared immature also in their light microscopical structure. In contrast, normal Ca²⁺ current amplitudes of IHCs and mature light microscopical appearance of the organ of Corti were observed in P15 *Pax8*^{-/-} mice that had been TH-substituted after birth (Fig. 1*B–D*) ($n = 3$ IHCs, 3 additional recordings that did not meet our quality criteria showed similar findings). This suggests that the normal developmental reduction of Ca_v1.3 current had not taken place in the absence of TH at that time. This is further supported by similar findings obtained in the companion study in a pharmacological hypothyroidism model (Brandt et al., 2007). The voltage dependence of the Ca²⁺ current in athyroid IHCs was comparable with wt IHCs on P14–P16 but showed a somewhat more hyperpolarized peak potential in athyroid IHCs on P6–P8 (Fig. 1*C*).

Figure 1*D* plots the Ca²⁺ current integrals as a function of age for athyroid mut and wt IHCs [evoked by 25 ms depolarization to −14 (−19 mV for P6–P8 mutant IHCs) in 2 mM [Ca²⁺]_e] together with results from NMRI IHCs (20 ms depolarization to −5 mV in 10 mM [Ca²⁺]_e) after normalization to the P14–P16 wt data. It emphasizes the delayed Ca²⁺ current upregulation and lack of downregulation up to P15 in *Pax8*^{-/-} IHCs unless TH substituted.

Figure 1*D* also compares the corresponding exocytic ΔC_m responses that were again normalized to the P14–P16 *Pax8*^{+/+} data. The large Ca²⁺ current in P14–P16 athyroid IHCs elicited robust exocytosis exceeding the responses of wt mice when probing ΔC_m with step depolarizations of 25 ms or longer duration (Fig. 1*B,D,E*) ($p = 0.002$ for 50 ms stimuli; $n = 7$ for *Pax8*^{-/-} IHCs and $n = 12$ for wt IHCs). Sustained exocytosis, involving serial resupply of vesicles to the active zones and parallel extrasynaptic turnover of synaptic vesicles, depends on long-distance Ca²⁺ signaling (for review, see Novian et al., 2006) and was sufficiently recruited by the large Ca²⁺ currents in P14–P16 athyroid IHCs. Shorter stimuli, preferentially recruiting the readily releasable vesicle pool in mature wt IHCs (Moser and Beutner, 2000), did not elicit more exocytosis in P14–P16 athyroid IHCs of *Pax8*^{-/-} ($p = 0.21$ for 10 ms stimuli; $n = 7$ for *Pax8*^{-/-} IHCs and $n = 12$ for wt IHCs) despite the large Ca²⁺

current amplitudes. This indicates that Ca^{2+} influx is less efficient in causing fast exocytosis in athyroid *Pax8*^{−/−} IHCs at P14–P16 than in mature wt IHCs. Figure 1*F* demonstrates this more directly by relating ΔC_m of P14–P16 *Pax8*^{−/−} and wt IHCs to the integrated Ca^{2+} influx showing a clear segregation of the two datasets for small Ca^{2+} current integrals. The exocytic efficiency was lower also in immature wt IHCs than in mature wt IHCs, which is in line with previous work (Beutner and Moser, 2001; Johnson et al., 2005). Interestingly, the efficiency of sustained exocytosis in P14–P16 athyroid IHCs exceeded that of immature wt IHCs (Fig. 1*F*).

Despite substantial Ca^{2+} currents, we observed very little exocytosis in athyroid IHCs at P6–P8 (Fig. 1*A,D,E*) for all but very long stimulus durations (e.g., 1 s; data not shown), resulting in the lowest exocytosis efficiency in our sample. The presynaptic dysfunction observed in IHCs of *Pax8*^{−/−} mice was specifically caused by the TH deficiency, because TH substitution restored normal Ca^{2+} currents and exocytic ΔC_m on postnatal day 15 in *Pax8*^{−/−} IHCs. This is consistent with the (partial) restoration of hearing in *Pax8*^{−/−} mice after TH substitution (Christ et al., 2004).

IHCs ribbon synapses are morphologically immature in athyroid *Pax8*^{−/−} mice at P15

We analyzed the morphology of IHC ribbon synapses by confocal microscopy of immunolabeled organs of Corti as well as by electron microscopy. Ribbon synapses of mature IHCs can be readily identified as juxtaposed pairs of sharply delimited immunofluorescence spots of presynaptic RIBEYE (marking the ribbon) and postsynaptic glutamate receptors (GluR2/3, marking the postsynaptic density) by confocal microscopy (Khimich et al., 2005) (Fig. 2*A*). Different from this mature staining pattern in P14–P15 wt and heterozygote IHCs (Fig. 2*A*), the GluR2/3 immunofluorescence was less confined in immature wt IHCs (P6–P8) (Fig. 2*C*) and athyroid IHCs (Fig. 2, *B*, p14–15, *D*, p6). Their GluR2/3 immunofluorescence assumed a more confluent pattern, enwrapping the basolateral pole of the IHCs and lacking the clear one-to-one juxtaposition to a corresponding ribbon (Fig. 2*B–D*) (for a more detailed analysis throughout wt development, see Nemzou et al., 2006). Therefore, we did not attempt to quantify the number of postsynaptic boutons based on GluR immunohistochemistry but rather focused on counting the number of RIBEYE immunofluorescence spots (Fig. 2*E,F*) (Nemzou et al., 2006). IHCs of immature wt, P6, and P14–P15 *Pax8*^{−/−} organs of Corti showed comparable numbers of RIBEYE spots (P6–P8 wt, 18.8 ± 0.9 per IHC, $n = 47$ IHCs of 4 cochleae of 4 mice; P6 *Pax8*^{−/−}, ~ 18 per IHC, $n = 7.5$ IHCs of 2 cochleae of 1 mouse; and P14–P15 *Pax8*^{−/−}, 22.5 ± 0.5 per IHC, $n = 39.5$ IHCs, of 4

Figure 1. Impaired maturation of presynaptic function in IHCs of *Pax8*^{−/−} mice. **A** and **B** plot representative Ca^{2+} current traces ($I_{\text{Ca}^{2+}}$; top) and exocytic capacitance responses (ΔC_m ; bottom) for P7 (**A**) and P15 (**B**) mut (gray), rescued mut (dark gray), and wt (black) IHCs elicited by step depolarization (50 ms) to -14 mV (peak Ca^{2+} current potential). The R_s values were as follows: 18.1 M Ω for wt P7, 20.1 M Ω for mut P7, 16.4 M Ω for wt P15, 20.8 M Ω for untreated mut P15, and 17.4 M Ω for TH-treated mut P15. In **C**, average current–voltage relationships recorded from wt ($n = 11$ for P6–P8; $n = 9$ for P14–P16) and *Pax8*^{−/−} IHCs (athyroid, $n = 7$ IHCs for P6–P8 and $n = 7$ IHCs for P14–P16; rescued, $n = 3$ for P14–P15) are displayed. **D** shows developmental changes of ΔC_m (top) and Ca^{2+} current integral ($Q_{\text{Ca}^{2+}}$; bottom) in response to short depolarizations in IHCs from NMRI mice (white bars) (data from Beutner and Moser, 2001), *Pax8*^{+/+} (or C57BL/6; black, data as in **E**, **F**), and *Pax8*^{−/−} mice without (gray) and with (dark gray) TH substitution. All data have been normalized to the P14–P16 *Pax8*^{+/+} results. **E**, **F**, ΔC_m to depolarizations to the peak Ca^{2+} current potential having variable duration were recorded for wt ($n = 12$ for P6–P8, $n = 12$ for P14–P16) and *Pax8*^{−/−} (athyroid, $n = 7$ for P6–P8 and $n = 7$ for P14–P16; $n = 3$ for TH-rescued P14–P16) IHCs were plotted versus the stimulus duration (**E**). **F** displays binned ΔC_m data versus their corresponding $Q_{\text{Ca}^{2+}}$ of P6–P8 and P14–P16 wt and mut (untreated) IHCs. Intervals between the pulses were at least 30 s to allow for complete recovery of the readily releasable pool (Moser and Beutner, 2000).

cochleae of 4 mice). These numbers clearly exceeded the counts in mature wt IHCs (P14–P15, 14.11 ± 0.27 per IHC, $n = 47$ IHCs of 3 cochleae of 3 mice). We interpret this as a larger number of ribbons in the P7 wt and P6 as well as P15 *Pax8*^{−/−} IHCs. In conclusion, the afferent synaptic organization of P14–P15 *Pax8*^{−/−} IHCs was found to be immature with higher numbers of ribbons and less confined postsynaptic glutamate receptor immunoreactivity. A normal maturation of afferent synaptic organization could be essentially restored in IHCs of P14–P15 *Pax8*^{−/−} mice by TH substitution (confined juxtaposed presynaptic and postsynaptic immunofluorescence, 13.3 ribbons per IHC, $n = 11.5$ IHC, $n = 2$ organs of Corti of 2 mice; data not shown).

Using electron microscopy, we explored the ultrastructure of afferent synapses in IHCs of P14–P15 wt (nine cochleae of six mice) and athyroid *Pax8*^{−/−} (six cochleae of four mice) mice. Qualitatively, we encountered many more afferent as well as ef-

Figure 2. IHC ribbon synapses are morphologically immature in athyroid *Pax8*^{−/−} mice at p15. **A–D**, Representative projections of confocal sections obtained from P15 heterozygote (**A**), athyroid P14 mut (**B**), P8 wt (**C**), and P6 athyroid mut (**D**) organs of Corti stained for RIBEYE/CtBP2 (red) and GluR2/3 (green). Synaptic ribbons were identified as small RIBEYE-positive spots juxtaposing GluR2/3 immunofluorescence spots in P15 wt IHCs (**A**). GluR2/3 immunofluorescence was less confined in athyroid P6 and P14 mut as well as P8 wt IHCs (**A**). **E–G**, Red channel only, of comparable projections as used for counts of small RIBEYE-positive spots in P15 wt (**E**), P14 athyroid mut (**F**), and P8 wt (**G**) organs of Corti. In **A–C** and **E**, specimens were fixed with 4% paraformaldehyde at room temperature. In **D**, **F**, and **G**, MetOH (99%) at -20°C was used. **H**, **I**, Electron micrographs of IHC ribbon synapses from P15 wt (**H**) and P15 athyroid mut (**I**) mice. Synaptic ribbons are seen as electron-dense bodies, each with a halo of synaptic vesicles and positioned close to the presynaptic membrane, opposing the postsynaptic density of the afferent fiber (aff). **I** shows an active zone representative for its large extension holding two ribbons and showing additional small vesicle clusters (asterisks). The arrowhead points toward a probably endocytic membrane invagination. In addition to the synaptic vesicles (small vesicles with rather homogeneous size), cisternae and tubes of varying size and shape as well as mitochondria were observed. Scale bars: **A–G**, 5 μm ; **H**, **I**, 200 nm.

ferent IHC synapses in the *Pax8*^{−/−} organs of Corti than in wt while sectioning the basolateral pole of IHCs. The afferent synaptic contacts of wt IHCs appeared clearly confined in space with one presynaptic vesicle cluster, and, when hit, the corresponding ribbon facing a clearly delimited postsynaptic density (representative example in Fig. 2H). In contrast, the sections of athyroid IHC afferent synapses showed extended synaptic contacts more frequently displaying more than one presynaptic vesicle cluster and ribbon (Fig. 2I, 11 multi-ribbon active zones of 45 synapses in athyroid *Pax8*^{−/−} mice vs 1 multi-ribbon active zone in 33 wt synapses). Provided a sufficient separation, the presence of multiple ribbons at the active zones might also contribute to the increased number of RIBEYE spots in athyroid IHCs. The postsynaptic membrane of mutant synapses often showed more than one density, each facing a presynaptic ribbon/vesicle cluster. It is likely that these patchy postsynaptic densities indicate the presence of multiple glutamate receptor clusters of variable size in a given postsynaptic terminal. This, together with an increased total number of synapses, most likely accounts for the more confluent GluR2/3 immunofluorescence described above (Fig. 2B,D), which is also typical for immature wt IHCs (Fig. 2C) (Nemzou et al., 2006).

TH regulates the expression of genes encoding synaptic proteins in the organ of Corti

Little is known about the changes in molecular composition of the presynaptic active zone of the IHC during development. Here, we used quantitative RT-PCR to explore whether and how

the mRNA levels of synaptic proteins change in the wt organ of Corti from before (P6) to after (P14–P17) the onset of hearing. The analysis included otoferlin [synaptic vesicle C₂-domain protein (Roux et al., 2006)], Bassoon [cytomatrix protein of the active zone (tom Dieck et al., 1998; Khimich et al., 2005)], RIBEYE [major ribbon component (Schmitz et al., 2000; Khimich et al., 2005)], SNAP25 [neuronal target SNARE (tSNARE) (Safieddine and Wenthold, 1999)], SNAP23 [ubiquitously expressed tSNARE (Ravichandran et al., 1996)], synaptobrevin 1 [vesicle SNARE (Safieddine and Wenthold, 1999)], and the Ca_v1.3 L-type Ca²⁺ channel (Platzter et al., 2000; Brandt et al., 2005). The relative RNA abundance of the individual genes differed to varying degrees among the tissues (differences were largest for RIBEYE and otoferlin mRNAs and smallest for SNAP25; data not shown).

In parallel, the analysis was run on organs of Corti from athyroid P14–P17 *Pax8*^{−/−} mice to investigate potential regulatory effects of TH on the expression of our genes of interest. We reasoned that the expression of genes encoding synaptic proteins in the IHC, which presents the major presynaptic element in the organ of Corti, would be represented well in these assays. We compared the results obtained in the organ of Corti with findings in the modiolus (mostly representing spiral ganglion neurons), the retina (containing ribbon and conventional synapses), and the cerebellum to evaluate how general effects of development and/or thyroid hormone signaling on the expression level of these genes might be. Several commonly used housekeeping genes (e.g., β -actin and glyceraldehyde-3-phosphate dehydrogenase) had been shown previously to be regulated by TH (Poddar et al., 1996; Barroso et al., 1999) and, hence, could not be used here. TBP mRNA was found to be comparably abundant in P6 and P14–P17 wt as well as P14–P17 *Pax8*^{−/−} organs of Corti (mean total RNA and Ct values of TBP: 1.8 μg and 29.1 for P6 wt, 0.8 μg and 30.0 for P14–P17 wt, and 1.4 μg and 29.6 for P14–P17 *Pax8*^{−/−}). The differences in total RNA and TBP Ct values most likely resulted from the varying efficiency of harvesting the organ of Corti between the three groups of animals. Therefore, we normalized the mRNA abundance for each gene of interest to TBP mRNA.

Figure 3A displays the mRNA levels of P14–P17 organs of Corti of wt mice (black; $n = 24$ ears of 12 mice) and their *Pax8*^{−/−} littermates (white; $n = 24$ ears of 12 mice) relative to those of P6 *Pax8*^{+/+} mice (set to 100%, gray; $n = 24$ ears of 12 mice). During normal development, we found a significant upregulation of SNAP25 ($p = 0.006$), synaptobrevin 1 ($p = 0.007$), and Bassoon ($p = 0.030$) mRNAs as well as a trend toward higher mRNA levels in mature organs for otoferlin ($p = 0.055$). SNAP23 mRNA levels remained unchanged [P6: 100% (95% confidence interval [CI], 71...141%), P14–P17: 90% (95% CI, 82...100%)]. The mRNA levels of RIBEYE and Ca_v1.3 did not significantly drop despite the observed reduction of ribbon synapse

number and of Ca^{2+} current (see above). The developmental Ca^{2+} current reduction may, therefore, be mediated by a regulation of $\text{Ca}_v1.3$ channel abundance on the posttranscriptional level. This could for example be achieved by changes of β -subunit expression affecting the $\text{Ca}_v1.3$ targeting to the plasma membrane (Herlitze et al., 2003). A developmental up-regulation of SNAP25, synaptobrevin 1, and Bassoon was found also for the modiolus (Fig. 3B) ($p = 0.009$, $p = 0.006$, and $p = 0.007$, respectively) and the cerebellum (Fig. 3D) ($p = 0.006$, $p = 0.012$, and $p = 0.014$, respectively). The cerebellum further showed an increase in otoferlin mRNA levels during development ($p = 0.027$). In the retina (Fig. 3C), synaptobrevin 1 and RIBEYE were upregulated ($p = 0.012$ and $p = 0.025$, respectively).

Organs of Corti of P14–P17 $\text{Pax8}^{-/-}$ mice showed lower mRNA levels for synaptobrevin 1 ($p = 0.011$), SNAP25 ($p = 0.036$), and Bassoon ($p = 0.037$) when compared with those of age-matched littermates. In contrast, the abundance of RIBEYE mRNA was increased ($p = 0.008$), which, most likely, relates to the supernumerous presence of ribbons in IHCs (and outer hair cells; data not shown). The SNAP23 mRNA abundance was unchanged [95% (95% CI, 76... 111%)]. The TH deficiency did not affect the abundance of the mRNAs of interest in the retina. However, the mRNA levels of SNAP25 and synaptobrevin 1 tended to be reduced in the cerebellum of P14–P17 $\text{Pax8}^{-/-}$ mice as were those of Bassoon and synaptobrevin 1 in modiolus, suggesting a delayed synaptic maturation of the conventional synapses in these tissues in the absence of TH.

Lack of large-conductance Ca^{2+} -activated K^{+} currents and KCNQ_4 currents: persistence of action potentials

IHC sound coding requires rapid and graded transduction of the mechanical stimulus into a receptor potential. BK channels activate rapidly during depolarization and increase the conductance of the cell to several nanosiemens for fast dynamics of the membrane potential of the cell in mature IHCs (Kros and Crawford, 1990; Oliver et al., 2006). BK channels are only acquired by IHCs at approximately the onset of hearing (Kros et al., 1998; Langer et al., 2003), and this requires the presence of functional TH receptors (Rusch et al., 1998; Rusch et al., 2001). Figure 4 demonstrates the rapidly activating outward currents ($I_{K,f}$) characteristic for currents mediated by BK channels in wt IHCs after the onset of hearing (A; $n = 6$, P14–P16), which were absent in P14–P16 $\text{Pax8}^{-/-}$ (B; $n = 4$), and P6–P8 wt (C; $n = 6$) IHCs. All three groups of IHCs displayed delayed rectifier potassium currents (A–C, middle), which were responsible for the residual current in P14–P16 $\text{Pax8}^{-/-}$ and P6–P8 wt IHCs measured during 3-ms-long depolarizations (Fig. 4A–C, top, D).

We then used current-clamp recordings to study the resting membrane potential and the membrane potential responses to current injections (Table 1, Fig. 4A–C, bottom). The resting potentials were more depolarized in P14–P16 $\text{Pax8}^{-/-}$ and P6–P8 wt IHCs than in P14–P16 wt IHCs. Action potentials occurred

Figure 3. Molecular changes accompanying postnatal maturation of IHC presynaptic morphology and function. **A–D** show relative amounts of otoferlin, RIBEYE, Cav1.3, Bassoon, SNAP25, and synaptobrevin 1 mRNA estimated by quantitative RT-PCR. Total RNA was isolated from organ of Corti (**A**), modiolus (**B**), retina (**C**), and cerebellum (**D**) of P6 $\text{Pax8}^{+/+}$, P14–P17 $\text{Pax8}^{+/+}$, and P14–P17 $\text{Pax8}^{-/-}$ mice ($n = 12$ mice each). The expression of the target mRNAs was normalized first to that of TBP and then to the values obtained for corresponding mRNA and tissue of P6 $\text{Pax8}^{+/+}$ mice. The data are represented as means from four independent experiments in which all samples were analyzed in triplicates (error bars indicate 95% confidence intervals of the population estimate of mRNA abundance).

spontaneously and/or after injection of small depolarizing currents in P14–P16 $\text{Pax8}^{-/-}$ and P6–P8 wt IHCs but not in P14–P16 wt IHCs. Ca^{2+} action potentials persisted also in a pharmacological rat model of congenital hypothyroidism (Brandt et al., 2007). KCNQ_4 currents, setting the resting membrane potential in mature IHCs (Oliver et al., 2003), were readily identified as slowly deactivating currents during hyperpolarization in P14–P16 wt IHCs ($n = 3$). We failed to detect KCNQ_4 currents in P14–P16 $\text{Pax8}^{-/-}$ IHCs (Fig. 4E) ($n = 3$ IHCs). This probably accounts for the depolarized resting membrane potential in the mutant P14–P16 IHCs. In addition to showing immature membrane currents, mutant P14–P16 IHCs also displayed immature passive electrical properties (Table 1).

Immunoreactivity for the pore-forming subunit KCNMA1 with the typical spot-like staining at the “neck” of IHCs (Pyott et al., 2004; Hafidi et al., 2005; Nemzou et al., 2006) was detected in organs of Corti of P15 wt mice (Fig. 4F) ($n = 3$ cochleae of 2 mice). Consistent with the lack of functional BK channels in the mutant IHCs, we did not observe KCNMA1 immunoreactivity in organs of Corti from P15 $\text{Pax8}^{-/-}$ mice (Fig. 4G) (three cochleae of two mice). However, we found the typical spot-like KCNMA1 staining in P14–P15 IHCs of TH-substituted $\text{Pax8}^{-/-}$ mice (Fig. 4H) ($n = 2$ cochleae of 2 mice), lending additional support for the TH dependency of BK abundance in IHCs. Using quantitative RT-PCR, we failed to detect significant differences between KCNMA1 mRNA levels in organs of Corti of P14–P17 wt [100% (95% CI, 75... 133%)] and P14–P17 $\text{Pax8}^{-/-}$ [92% (95% CI, 53... 137%)] mice ($n = 24$ organs of Corti from 12 mice). Follow-up experiments with higher IHC specificity, including single IHC RT-PCR for KCNMA1 or *in situ* hybridization, should be performed in the future. KCNMA1 mRNA abundance was TH dependent in the cerebellum (data not shown).

Figure 4. Lack of large-conductance Ca^{2+} -activated K^{+} currents and KCNMA1 immunoreactivity: persistence of action potentials. **A–C** display representative membrane currents (top two rows) and potentials (bottom row) of P15 wt and $\text{Pax8}^{-/-}$ as well as P7 wt IHCs. Top row, Three millisecond depolarizations (maximum potential indicated) elicited fast outward currents (BK currents) in this P15 wt IHC but in neither P15 mut nor P7 wt IHCs, which showed slower activation of delayed rectifier currents only. R_s values were as follows: 4.92, 4.48, and 2.48 $\text{M}\Omega$ for wt P15, mut P15, and wt P7, respectively. Middle row, Large currents in all three groups in response to 100 ms voltage steps but lack of BK current in mut and wt P7 IHCs. R_s values were as follows: 3.16,

Prolonged presence of efferent IHC synapses in $\text{Pax8}^{-/-}$ mice

Current-clamp recordings from P15 $\text{Pax8}^{-/-}$ IHCs revealed small biphasic potentials (Fig. 5A) that closely resembled the previously described cholinergic postsynaptic potentials of immature IHCs (Glowatzki and Fuchs, 2000; Brandt et al., 2003; Katz et al., 2004). We then analyzed the efferent IHC innervation by immunohistochemistry for the efferent presynaptic marker synaptophysin (IHCs are devoid of synaptophysin) and for SK2 as postsynaptic efferent marker. In P15 $\text{Pax8}^{-/-}$ mice, we readily observed juxtaposed synaptophysin and SK2 immunofluorescence spots indicative of efferent IHC synapses (Fig. 5B) (~ 11.7 spots per IHC; $n = 20$ IHCs, 4 cochleae of 4 mice). We observed a lower number of juxtaposed synaptophysin and SK2 immunofluorescence spots in P15 wt IHCs (Fig. 5C) (4.1 spots per IHC; $n = 3$ cochleae of 2 mice), which is consistent with the description of a developmental decline of efferent IHC innervation in wt rats and mice (Katz et al., 2004; Nemzou et al., 2006). Although SK2 expression was robust in athyroid IHCs, it was more variable in IHCs of wt organs of Corti ranging from a mosaic pattern (Fig. 5C) to a complete lack of SK2 staining (Fig. 5D). The synaptophysin staining remaining in mature wt organs of Corti after loss of IHC SK2 immunoreactivity most likely reflects efferent presynaptic terminals of the lateral olivocochlear bundle making synapses onto afferent fibers (Simmons, 2002; Nemzou et al., 2006). Vesiculated efferent terminals facing a postsynaptic density and its nearby synap-

4.27, and 3.96 $\text{M}\Omega$ for wt P15, mut P15, and wt P7, respectively. Bottom row, Membrane potentials in response to injection of 50 pA (P15 wt) and 10 pA (P15 mut and P7 wt) depolarizing current. Action potentials were observed in P15 mut and P7 wt IHCs but not in P15 wt despite stronger current injection. **D**, Mean $I-V$ relationship for 3 ms depolarizations, obtained by averaging currents for 1 ms (starting 1 ms after stimulus onset) in IHCs of P14–P16 wt (black squares; $n = 6$) and $\text{Pax8}^{-/-}$ (gray squares; $n = 4$) as well as P6–P8 wt IHCs (circles; $n = 6$). **E**, Representative currents of a P15 wt IHC (black) and a P15 mut IHC (gray) during repolarization to -154 mV from -64 mV (200 ms), clearly showing a deactivating current characteristic for KCNQ_4 channels in the wt but not in the mut IHC; no P/n correction was applied. R_s values were as follows: 2.49 and 5.24 $\text{M}\Omega$ for wt P15 and mut P15. **F–H** show representative projections of confocal sections obtained from organs of Corti of P15 WT (**F**), mut (**G**), and rescued mut (**H**) mice after KCNMA1 (red) and parvalbumin (green) immunostaining; note spots of KCNMA1 immunofluorescence at the neck of wt and rescued mut IHCs, which are lacking in athyroid mut IHCs. PFA (4%) was used as fixative. Scale bars, 5 μm .

Figure 5. Prolonged presence of efferent IHC synapses in *Pax8*^{-/-} mice. **A** shows a membrane potential recording from a P15 *Pax8*^{-/-} IHC displaying spontaneous action potentials and small biphasic potentials (arrowheads; example enlarged in the inset) characteristic for cholinergic postsynaptic potentials. **B–D** show representative projections of confocal sections

Figure 6. Schematic representation of basolateral IHC properties before and after the onset of hearing in wt and in 2-week-old TH-deficient *Pax8*^{-/-} mice. **A** sketches the immature IHC morphological and functional properties found in wt IHCs before the onset of hearing and in 2-week-old *Pax8*^{-/-} IHCs. **B**, Mature basolateral makeup of IHCs after the onset of hearing. For simplicity, we only drew one afferent and efferent synapse each (at the same size) and did not depict the branching of afferent fibers seen in immature organs of Corti. Structures (ion channels and synapses) are not drawn to scale.

toplasmic cistern (Lioudyno et al., 2004) were much more frequently encountered in electron micrographs of mut P14–P15 IHCs (Fig. 5D) than in IHCs of wt littermates.

Discussion

In the present study, we investigated the postnatal maturation of cochlear IHCs and their synapses in normal mice, athyroid *Pax8*^{-/-} mice, and TH-rescued *Pax8*^{-/-} mice. We demonstrate that TH signaling is required for the normal molecular, morphological, and functional maturation of IHC ribbon synapses. IHC action potential firing and efferent innervation of IHCs by olivocochlear fibers persisted in athyroid animals at least up to the end of the second postnatal week (Fig. 6 summarizes TH-dependent developmental changes of IHCs). Congenital hypothyroidism has been shown to impair multiple developmental processes within the inner ear, offering much mechanistic explanation for hypothyroid deafness. The presynaptic dysfunction of IHCs in athyroid mice demonstrated here seems incompatible with coding of the temporal structure and graded intensity of sounds, even if cochlear amplification, transduction, and other upstream processes were intact.

Ribbon synapses are formed, but their maturation is retarded in IHCs of athyroid mice

Our morphological investigation of athyroid *Pax8*^{-/-} mice revealed that IHC ribbon synapses are formed despite congenital

obtained from organs of Corti of a P15 mut (**B**) and a P15 wt (**C**, **D**) mice after synaptophysin (red) and SK2 (green) immunostaining. **E**, Representative electron micrograph showing the basal pole of a P15 mut IHC with an efferent synapse onto the IHC featuring the presynaptic terminal with synaptic vesicles and the characteristic postsynaptic cistern in the IHC (arrowheads). The IHC forms a ribbon synapse with an afferent fiber next to the efferent IHC synapse. Scale bars: **B–D**, 2 μm; **E**, 500 nm.

Table 2. Comparison of developmental IHC defects in mutants with TH signaling defects in comparison to $\text{Ca}_v1.3$ knock-out and *Tmc1* mutants

	Acquisition of BK and KCNQ_4 current	Ribbon synapse morphology	Ribbon synapse function (Ca^{2+} current and exocytosis)	Efferent IHC innervation
TH signaling defect	BK delayed and reduced^{a,b}, BK and KCNQ_4 not detectable up to P15 in Pax8 KO	Retarded synaptogenesis in <i>Pax8</i> knock-outs	Late upregulation and no maturation at least up to P15 in Pax8 knock-outs	Maintained at least up to P15 in Pax8 knock-outs
<i>Tmc1</i> defect ^{c,d}	BK and KCNQ_4 not detectable up to P58	Only few nerve endings found at P15	Maintained immature Ca^{2+} current and exocytosis	Not evaluated
$\text{Ca}_v1.3$ deletion ^{e,f}	BK not detectable up to P35; KCNQ_4 present	Normal synaptogenesis, secondary loss	Stimulus–secretion coupling blocked; normal exocytosis during Ca^{2+} uncaging	Maintained at least up to P45

The table compares the developmental abnormalities of hair cells and their synapses between hypothyroid mutants, *Tmc1* mutants, and $\text{Ca}_v1.3^{-/-}$ mice. Common phenotypes are in bold.

^aRusch et al., 1998.

^bRusch et al., 2001.

^cBock and Steel, 1983.

^dMarcotti et al., 2006.

^eBrandt et al., 2003.

^fNemzou et al., 2006.

TH deficiency. However, the maturation of their molecular anatomy and physiology was impaired. Our quantitative PCR revealed that the mRNA levels for the SNAREs SNAP25 and synaptobrevin 1 in the organ of Corti increase during normal postnatal synaptic maturation in a TH-dependent manner. We take this as an indication for a developmental upregulation of SNAREs in IHCs, which is in line with a preliminary immunohistochemical report (Eybalin et al., 2002). This adds to previous work describing a developmental upregulation of cysteine string protein in IHCs (Eybalin et al., 2002), which serves as a presynaptic co-chaperone (Schmitz et al., 2006). The TH-dependent increase of mRNA abundance for the two SNAREs in the cerebellum is in line with previous reports of a developmental SNAP25 protein upregulation accompanying synaptogenesis (Mayanil and Knepper, 1993; Bark et al., 1995) and a TH dependence of cerebellar development (Vincent et al., 1982; Mayanil and Knepper, 1993; Hashimoto et al., 2001; Bernal, 2005).

Morphologically, IHCs of TH-deficient organs of Corti continued to have large numbers of ribbons and immature synaptic contacts (e.g., showing several ribbons) at least up to P15, when normal maturation and pruning have resulted in fewer and confined single ribbon synapses (Pujol et al., 1980; Shnerson et al., 1981; Sobkowicz et al., 1982; Pujol et al., 1997; this study). The IHCs of 2-week-old *Pax8*^{-/-} mice were also functionally comparable with immature wt IHCs (Fig. 6, Table 2) (see the below section), except for showing more sustained exocytosis. Normal maturation of ribbon synapse morphology and function could be restored on TH substitution, proving that the TH deficiency was at the origin of our findings in the athyroid *Pax8*^{-/-} mice.

Toward deciphering the developmental improvement of stimulus-secretion coupling in IHCs

The ribbon synapses of mature IHCs code sound with extraordinary temporal precision (Fuchs, 2005; Moser et al., 2006). Two of the mechanisms contributing to this capability (rapid but graded membrane potential responses to sensory input and efficient Ca^{2+} influx–exocytosis coupling) are lacking from immature wt as well as from P14–P16 mice with impaired TH signaling (Kros et al., 1998; Rusch et al., 1998; Beutner and Moser, 2001; the present study).

First, their IHCs lack BK currents, and hence their membrane potential cannot follow transduction with graded responses but fire Ca^{2+} action potentials instead. Large Ca^{2+} currents and the less hyperpolarized resting potential attributable to the absence of KCNQ_4 channels also contribute to the firing in 2-week-old

Pax8^{-/-} IHCs. The lack of IHC BK currents of 2-week-old athyroid *Pax8*^{-/-} mice is consistent with the previously described delayed BK acquisition in IHCs of TH receptor mouse mutants (Rusch et al., 1998; Rusch et al., 2001). We could not test for a potential later acquisition of BK channels because *Pax8*^{-/-} mice die before weaning (Mansouri et al., 1998). The absence of KC-NMA1 immunoreactivity confirms our electrophysiological finding. However, our study cannot definitively tell which step toward the acquisition of functional BK channels by IHCs is promoted by TH (see below).

Second, more Ca^{2+} influx is required for the same amount of fast exocytosis in IHCs of immature wt as well as of P14–P15 *Pax8*^{-/-} mice. Potential mechanisms include impaired priming of readily releasable vesicles for exocytosis and a less favorable positional arrangement of $\text{Ca}_v1.3$ Ca^{2+} channels and vesicular release sites. Developmental changes in the molecular composition of the exocytic machinery (see above) could well improve the fusion competence of the vesicle and/or the topography of the active zone. A lower expression of SNAP25 and synaptobrevin 1 in immature wt and *Pax8*^{-/-} IHCs could imply that vesicles have a lower probability of becoming primed (Sorensen et al., 2003; Borisovska et al., 2005). SNAP23 may then compete more successfully with SNAP25 for the formation of SNARE complexes in immature wt and 2-week-old *Pax8*^{-/-} IHC, resulting in reduced vesicle priming and exocytosis efficiency. The efficiency of Ca^{2+} influx to trigger sustained exocytosis in 2-week-old *Pax8*^{-/-} IHCs (Fig. 2F) exceeded that of immature wt IHCs. This might argue that 2-week-old *Pax8*^{-/-} IHC have matured to some extent beyond what is normally achieved at the end of the first postnatal week.

In addition or alternatively, immature wt and *Pax8*^{-/-} IHCs may have not yet achieved the tight spatial coupling of vesicles to Ca^{2+} channels of mature IHCs, which gives rise to a “ Ca^{2+} nanodomain” control of exocytosis (Brandt et al., 2005). Let us assume that the so far uncharacterized “intrinsic” Ca^{2+} cooperativity of exocytosis in immature IHCs was as high as estimated in mature IHCs by Ca^{2+} uncaging (four to five) (Beutner et al., 2001). Then, we would expect their (“apparent”) Ca^{2+} cooperativity during Ca^{2+} influx evoked by depolarizations of varying strength to approach the “intrinsic” Ca^{2+} cooperativity, if many channels cooperated to control exocytosis of a given vesicle a immature active zone (“ Ca^{2+} microdomain” control). Indeed, the apparent Ca^{2+} cooperativity of exocytosis was shown to be higher in immature than in mature IHCs, when manipulating Ca^{2+} influx by changing the strength of depolarization (Johnson et al., 2005). A developmental switch from Ca^{2+} microdomain to

Ca^{2+} nanodomain control of exocytosis as suggested for IHCs has been indicated recently for the calyx of Held (Fedchyshyn and Wang, 2005).

Regulation of postnatal IHC development

Recently, insights into the regulation of postnatal hair cell development have been obtained from analyzing mouse mutants with impaired TH signaling (for review, see Forrest et al., 2002) (for another recent study, see the companion paper by Brandt et al., 2007), deficiency for the $\text{Ca}_v1.3$ L-type Ca^{2+} channel (Brandt et al., 2003; Nemzou et al., 2006), and mutations of the transmembrane cochlear-expressed gene 1 (*Tmc1*) (Marcotti et al., 2006). Besides showing various other inner ear defects, all of these mutants share a prolonged immaturity of two or more aspects of IHC biology (Table 2). It is exciting to think about how the signaling pathways targeted in these different mutants converge to mediate normal development. However, to date, an integrative view has yet to be established.

TH regulation of gene transcription is a well established mechanism and has been implicated in various neurodevelopmental processes (Bernal, 2005). TH binding to its nuclear receptors modulates their function as transcription factors (for review, see Forrest et al., 2002). TH receptors bind to DNA regulatory elements [TH response elements (TREs)] as homodimers or heterodimers with retinoid-X receptors to regulate gene transcription. In outer hair cells of the cochlea, for example, it was shown that TH promotes expression of the motor protein Prestin through TH receptor β TRE binding (Weber et al., 2002) and at the same time derepresses transcription of the *Kcnq4* gene, because TH binding to TH receptor α dissociates it from its TRE. The latter mechanism may also be at work for the TH-dependent expression of KCNQ_4 channels during IHC development. We consider it possible that TH, rather than acting on a TRE upstream of each individual affected gene, may trigger an entire transcriptional program, for example by interacting with other transcription factors, including activity-dependent factors (Kim et al., 2005) or a Ca^{2+} channel-derived transcription factor (Gomez-Ospina et al., 2006). This could offer an explanation for common effects of TH deficiency and lack of $\text{Ca}_v1.3$ channels and consequently absent regenerative Ca^{2+} signaling in neonatal IHC (Table 2).

In addition, TH may promote hair cell differentiation by regulating expression of target proteins at steps downstream of transcription (Sampson et al., 2002). Such a mechanism may account for the lack of functional BK channels in IHCs of *Pax8*^{−/−} mice, because, in contrast to our findings in *Ca_v1.3*^{−/−} mice (Nemzou et al., 2006), we failed to detect a reduction of *KCNMA* mRNA levels in their organs of Corti. Interestingly, a role of TH in the regulation of the developmental redistribution of prestin in outer hair cells has been demonstrated recently (Weber et al., 2002). The putative trafficking protein *Tmc1* is an attractive candidate for mediating posttranscriptional TH effects in IHC differentiation because IHCs of *Tmc1* and hypothyroid mutants share a number of developmental defects (Table 2). Additional experiments, including the investigation of *Tmc1* expression in IHCs of hypothyroid mice, will be required to further dissect the complex signaling driving postnatal IHC differentiation.

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