Cytochrome P450 26b1-mediated specification of vestibular striola and central zones is required for transient responses in linear acceleration

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Competing interests

The authors have no competing interests.

ABSTRACT

Each vestibular sensory epithelia of the inner ear is divided into two zones, the striola and extrastriola in maculae of otolith organs and the central and peripheral zones in cristae of semicircular canals, that differ in morphology and physiology. We found that formation of striolar/central zones during embryogenesis requires Cytochrome P450 26b1 (*Cyp26b1*)-mediated degradation of retinoic acid (RA). In *Cyp26b1* conditional knockout mice, the identities of the striolar/central zones were compromised, including abnormal innervating neurons and otoconia in otolith organs. Vestibular evoked potentials (VsEP) in response to jerk stimuli were largely absent. Vestibulo-ocular reflexes and standard motor performances such as forced swimming were unaffected, but mutants had head tremors and deficits in balance beam tests that were consistent with abnormal vestibular input. Thus, degradation of RA during embryogenesis is required for patterning highly specialized regions of the vestibular sensory epithelia that may provide acute feedback about head motion.

INTRODUCTION

The sense of balance is mediated by the integration of vestibular, visual, and proprioceptive systems. While unilateral vestibular deficits can largely be compensated by sensorimotor reorganization, bilateral vestibular damage, caused by systemic insults such as aminoglycoside ototoxicity, often cannot¹. As a consequence, patients with chronic vestibulopathy are disabled by imbalance and oscillopsia. Understanding how vestibular inputs are encoded by vestibular organs to maintain gaze and head stability is important from basic science and therapeutic perspectives.

Vestibular sensory epithelia are comprised of the maculae of the utricle and saccule, which detect linear acceleration, as well as three canal cristae, which detect angular acceleration. These sensory epithelia each have two types of mechanosensitive hair cells (HCs), type I and type II, which are surrounded by supporting cells (SCs) and innervated by afferent neurons of the vestibular ganglion (Fig. 1a). Type I and II HCs are contacted by different afferent synaptic terminals: large calyceal endings on type I HCs and small bouton endings on type II HCs. The mechanosensitive hair stereociliary bundles of HCs couple to an otolithic membrane in the utricle and saccule and to a cupula in the cristae. Head accelerations deflect these accessory structures and the coupled stereociliary bundles, modulating mechanotransduction channels in the bundles and ultimately changing the firing rate of afferent neurons².

Each vestibular organ has near its center a conserved, specialized region called the striola in the maculae and the central zone in the cristae³⁻⁵. Striolas and central zones differ from extrastriolas and peripheral zones in many features including stereociliary bundle morphology, ion channel expression and otoconia size⁶⁻⁸.

Additionally, afferents form complex calyces around multiple type I HCs in greater proportion in striolar/central zones (Fig. 1a)^{4,5,9,10}. Such differences give rise to afferent nerve populations with very different spontaneous and evoked physiological responses. Striolar/central zone afferents have more irregular spike timing and more sensitive to higher-frequency head motion than extrastriolar/peripheral afferents¹¹⁻¹³. Higher densities of low-voltage-activated K (K_{LV}) channels are expressed in striolar/central zone afferents, making them less excitable - less likely to fire in response to small currents – and more directly driven by synaptic inputs, which contributes to their irregular firing patterns¹⁴. By virtue of their different regularities, afferents from the two zones encode head motion into spike trains by different strategies: temporal pattern of spikes for the striolar/central zones vs. spike rate for extrastriolar/peripheral zones¹⁵, which are optimal for different kinds of sensory information. The distinct anatomy and physiology of the vestibular epithelia zones motivated our interest in understanding how the zones are established during development and their distinct contributions to key vestibular-driven functions such as the vestibulo-ocular and vestibulo-spinal reflexes (VOR and VSR).

We evaluated the importance of the striola to the VsEP evoked by linear jerk stimulation, a method that allows *in vivo* assessment of macular function independently of other sensory and motor contributions¹⁶⁻¹⁸. Several lines of indirect evidence implicate irregular afferents, which innervate the striola, in generating the VsEP^{2,16,19,20}. Here we directly tested this notion by eliminating striolar specializations. We show that specification of the striolar/central zones of vestibular organs requires degradation of retinoic acid (RA) by the zone-specific expression of *Cyp26b1*, a gene encoding a RA

degradation enzyme. RA is the bioactive form of vitamin A (retinol) and it serves as a transcription factor that regulates many cellular events during embryonic development²¹. The availability of RA during embryogenesis is controlled by restricted expression of RA synthesizing enzymes such as class 1A aldehyde dehydrogenases (Aldh1a), as well as degradation enzymes such as Cyp26s²¹. The complementary expression patterns of these enzymes during embryogenesis are important in patterning many tissues including the anterior-posterior axis of the inner ear²²⁻²⁵. We show that *Cyp26b1* conditional knockout mice (cKO) exhibit a severe reduction of striolar/central zones as manifested by a number of morphological, molecular and physiological properties. These mice have normal horizontal angular vestibular ocular reflexes (aVOR), derived from the horizontal cristae, and normal responses to off-vertical axis rotation (OVAR), derived from the macular organs, but they lack VsEP. They appear normal in their daily activities but display a characteristic head tremor and have difficulty traversing a balance beam. Together, our results suggest that the striola and central zones are not essential for mediating vestibular ocular reflexes. Rather, striola and central zones are important for responding to changes in linear acceleration and are likely important for controlling head stability and performing challenging vestibulo-motor activities.

RESULTS

Complementary expression patterns of *Cyp26b1* and *Aldh1a3* in the vestibular sensory organs

We observed complementary expression patterns of transcripts for the RA degrading enzyme *Cyp26b1* and the RA synthesizing enzyme *Aldh1a3* in developing vestibular organs that were not described previously. During embryogenesis, the sensory epithelia of the utricle (Fig. 1b-d), saccule (Fig. 1b, Supplemental Fig.1) and cristae (Fig. 1b-d) show *Cyp26b1* expression in the center surrounded by *Aldh1a3* expression in the periphery. The *Cyp26b1* expression domain in the maculae appears comparable to that of *β-tectorin*, a striolar SC marker (Fig. 1c,e)²⁶. Adjacent cryo-sections processed for *in situ* hybridization confirmed the complementary relationships among *Aldh1a3*, *Cyp26b1* and *β-tectorin* in both maculae and in the lateral crista (Fig. 1f-h, Supplemental Fig.1). Expression of all three genes is concentrated in the basal epithelium corresponding to the layer of SC. Together, these expression patterns suggest that differential expression of RA could establish the central and peripheral zones of vestibular organs.

Specification of the striolar/central zone in vestibular organs requires *Cyp26b1*

To test the hypothesis that decreased RA levels specify the striolar/central zone of each vestibular sensory organ, we analyzed vestibular organs in gain- and loss-of RA function mutants, *Cyp26b1* and *Aldh1a3* knockout mice respectively. The Ca²⁺-binding protein oncomodulin (*Ocm*) is expressed only in the type I HCs located within the striolar/central zone (Fig. 2a)²⁷. In *Cyp26b1*^{-/-} inner ears, *Ocm* expression is reduced in

both maculae and cristae (Fig. 2b, Supplemental Fig. 2). Quantification of HC number showed a significant decrease in the percentages of Ocm⁺ HCs in *Cyp26b1*^{-/-} utricles (Fig. 2d), saccules (Supplemental Fig. 2) and lateral cristae (Fig. 2e), without apparent loss in the total number of Myo7a-positive HCs in each sensory organ (Fig. 2b).

In *Aldh1a3*^{-/-} mutants, in which RA synthesis is reduced, the Ocm expression domain is increased in the maculae but not cristae (Fig. 2c, Supplemental Fig. 2). In *Aldh1a3*^{-/-} utricles, the expression domain of Ocm expands towards the medial extrastrilar (MES) region (Fig. 1a) but not the lateral extrastrilar (LES) region of utricles (Fig. 2c, Supplemental Fig. 3); in *Aldh1a3*^{-/-} saccules, in contrast, the Ocm expression domain is broadly expanded (Supplemental Fig. 3). The percentages of Ocm⁺ HCs are increased per utricle (Fig 2d) and saccule (Supplemental Fig. 2), compared to controls. The increased Ocm⁺ HC numbers in *Aldh1a3*^{-/-} ears are consistent with the decreased numbers in *Cyp26b1*^{-/-} ears. In contrast, no increase in Ocm⁺ HCs is evident for the lateral crista (Fig. 2e), suggesting the presence of other compensatory sources of RA in the cristae. Nevertheless, these combined results (Table 1) suggest that formation of the striolar/central zone-specific HCs in vestibular organs is regulated by *Cyp26b1*-mediated degradation of RA.

We next examined whether the identity of SCs in the striolar/central zone of vestibular organs is also affected in mutants deficient for enzymes in the RA pathway. In normal maculae, β -tectorin is specifically expressed in striolar SCs (Fig. 2f, i)²⁶. Consistent with the changes in Ocm expression by HCs, the extent of β -tectorin expression is reduced in *Cyp26b1*^{-/-} utricles (Fig. 2g, i) and increased in *Aldh1a3*^{-/-} utricles (Fig. 2h, i). These results show that striolar SC identity in the utricle requires the

decrease in striolar RA levels that is mediated by *Cyp26b1*. In the saccule, in contrast, no significant difference in the β -tectorin positive-region was detected in either *Cyp26b1*^{-/-} or *Aldh1a3*^{-/-} ears (Table 1, Supplemental Fig. 2), suggesting that changes in RA levels may not affect SC formation in the saccule. In cristae, which have different accessory structures than the maculae, β -tectorin is not expressed (Fig. 2f) and there is no known SC marker for the central zone, preventing the evaluation of RA effects on SC identity.

Another prominent feature of mammalian vestibular epithelia is the postnatal development of afferent nerve endings (calyces) encasing single or multiple type I HC bodies (Fig. 1a)^{6,9,10}. In order to investigate the role of RA in calyx formation, we bypassed the lethality of *Cyp26b1*^{-/-} and *Aldh1a3*^{-/-} mice^{28,29} by first generating cKO of *Cyp26b1* (*Foxg1*^{Cre}; *Cyp26b1*^{lox/-}) using *Foxg1*^{Cre}, which is expressed in tissues such as the otic placode and forebrain during early embryogenesis³⁰. We also generated viable *Aldh1a3*^{-/-} mice by supplementing maternal RA during Embryonic day(E) 8.5 to E14.5 of development (*Aldh1a3*^{-/-} (em RA)). This RA supplementation has been demonstrated to rescue the RA requirements of *Aldh1a3*^{-/-} mice during early embryogenesis without reducing the inner ear defects, which develop later^{29,31}. In *Cyp26b1* cKO mice, Ocm and β -tectorin phenotypes are similar to those of the *Cyp26b1* knockouts (data not shown). We used calbindin-D28K immunoreactivity to selectively stain for striolar/central zone afferents³² in control and mis-regulated RA mutants at post-natal day (P) 40 or P45. Similar to what was observed for HCs and SCs, the calbindin expression domain is decreased in all *Cyp26b1* cKO vestibular sensory organs compared to controls (Fig. 2j, k, m, n, Supplemental Fig. 2). These results indicate

that the development of calyceal-bearing afferents of striolar/central zones requires relatively lower levels of RA signaling regulated by *Cyp26b1*.

In *Aldh1a3*^{-/-} (em RA) mice, the calbindin-expressing domain is increased in the utricle but not in the lateral crista (Fig. 2j, l, m, n, Table 1), consistent with the expanded striolar region in the *Aldh1a3*^{-/-} mutants. Together, these results suggest that formation of the striolar/central zone in the vestibular organs requires Cyp26b1 enzyme to degrade the RA emanating from the surrounding sensory tissue, which is largely generated by Aldh1a3 in the maculae, though not in the cristae (Table 1).

Anatomical features of the striola are affected by *Cyp26b1* cKO mice during embryogenesis

Next, we investigated the mature anatomy of the striola in *Cyp26b1* cKO utricles as a result of altered RA signaling during embryogenesis. A significant characteristic of the maculae is the presense of the otoconia, mineralized calcium carbonate crystals embedded above the otolithic membrane (Fig. 1a)⁷. Under low magnification, the otoconial layer in the utricle is more transparent in the striola than the rest of the organ due to the smaller size and number of otoconia (Fig. 1a, 3a). This clear zone is absent in *Cyp26b1* cKO utricles (Fig. 3b). Scanning electron micrographs (SEM) of control utricles show smaller otoconia and more extensive perforations of the underlying meshwork otoconia in the striola (Fig. 3c-c'')⁷ compared to the MES (Fig. 3c'''). In *Cyp26b1* cKO utricles, SEM revealed no regional differences in otoconial crystals (Fig. 3d-d''), consistent with low-power brightfield images (Fig. 3b). Crystals throughout

Cyp26b1 cKO utricles were of similar size to control MES crystals (Fig. 3e) indicating that the zonal difference in otoconial size was absent in the *Cyp26b1* cKO utricles.

At the sensory epithelium level, the striola has a lower density of HCs (number per surface area) than in the rest of the sensory organ⁴, reflecting larger apical cell surfaces. To investigate whether this regional marker is also affected by RA signaling, the density of HCs in the striolar region was compared between controls and *Cyp26b1* cKO mutants. In *Cyp26b1* cKO utricles, which lack normal striolar identifying features, we delineated a comparable region to the control striola (see Materials and Methods) for comparison. In control utricles, HC density was lower in striola than in LES (Supplemental Fig. 4). This regional difference in HC density was not detected in mutant utricles (Supplemental Fig. 4). Striolar HC density in *Cyp26b1* cKO mutant epithelia was significantly higher than in control striola and comparable to HC density in control LES. Consistently, the ratio of HC number in striola to LES was greater in *Cyp26b1* cKO mutants than in controls (Supplemental Fig. 4), indicating that *Cyp26b1* cKO utricles had more HCs in the striolar region and lacked a zonal difference in HC density.

In control utricles, the ratio of the length of the kinocilium to that of the tallest stereocilium (K/S ratio) is significantly smaller in the striola than in the extrastriola⁸. We therefore measured whether RA signaling affected the K/S. For this analysis, we referenced hair bundle location to the line of polarity reversal (LPR) of stereociliary bundles, which is closely associated with the striola in macular organs (Fig. 1a), and stereociliary bundle orientation was identified by phalloidin staining of actin-filled stereocilia or anti-spectrin staining of the cuticular plate in which stereocilia insert³³. In

the mouse, the utricular striola is largely medial to the LPR whereas the saccular striola straddles the LPR (Fig. 1a, Supplemental Fig. 5)³⁴. We determined that the relative position of the LPR was not altered in either the mutant utricle or saccule (Supplemental Fig. 5). In control utricles, K/S ratio is higher in the LES than in the striolar region (Supplemental Fig. 6). In contrast, the K/S ratio in the striolar region of *Cyp26b1* cKO utricles was comparable to those of the control utricular LES (Supplemental Fig. 6). These results suggest that the zonal difference in the K/S ratio is absent in the *Cyp26b1* cKO utricles.

Visual inspection of living utricular epithelia with differential contrast microscopy suggested that complex calyces, a prominent features of the striolar/central zone^{4,5,8}, were less numerous in *Cyp26b1* cKO utricles. To quantitatively examine this impression, we stained neurons with Tuj1 antibody and counted calyces in control and *Cyp26b1* cKO utricular striolas. Complex calyces encasing two or three type I HCs (double or triple calyces, respectively) were present in significantly higher numbers in control striolas (Fig. 4a, c) than in *Cyp26b1* cKO striolar regions (Fig. 4b, c).

Together, our results showed that the lack of *Cyp26b1* during embryogenesis affects many mature features of the striola including the otoconia, HC density, hair bundle morphology, expression of Ca^{2+} -binding proteins, and incidence of complex calyces, suggesting that striolar maturation depends on low levels of RA signaling.

Loss of functional properties of the striola in *Cyp26b1* cKO utricles

To assess whether zonal differences in physiology were affected by the mutation, we recorded from HCs and afferent calyces in both controls and *Cyp26b1* cKO mice

using a semi-intact preparation of the utricle and the distal nerve. We visualized the epithelium from its apical surface with differential-interference-contrast optics, which allow resolution of hair bundles, HC bodies, and calyceal afferent terminals. Because the *Cyp26b1* cKO utricles had lost the zonal markers that we usually use (hair bundle size, HC density as viewed from above, the proportion of calyces that are complex), we relied on the LPR, assigning recordings in the first 10 HC rows medial to the LPR as “striolar”. We performed the whole-cell recording configuration on visually identified type I or type II HCs or on calyceal afferent terminals that surround type I HCs, and measured voltage-gated currents in voltage clamp mode and resting potential and step-evoked voltage changes in current clamp mode. Recorded HCs and calyceal terminals were filled with fluorescent dye (sulforhodamine 101) in the pipette solution, allowing their visualization with fluorescence optics.

In HCs ($n = 64$, from 21 mice), there were no obvious effects of the *Cyp26b1* cKO manipulation on whole-cell voltage-sensitive conductances, as revealed by currents evoked by voltage steps or voltage responses to current steps. Wildtype type I and type II cells from mice and other amniotes³⁵ have outwardly rectifying K^+ currents with very different voltage dependence: the voltage of half-maximal activation ($V_{1/2}$) is 40-70 mV more negative in type I HCs compared to type II HCs. The unusually negative voltage dependence of the type I-specific K^+ conductance ($g_{K,L}$) has significant consequences for the size and speed of the receptor potential and appears to be essential for non-quantal transmission at type I-calyx synapses³⁶. This key difference was preserved in the striolar zones of *Cyp26b1* cKO utricles: 3-factor ANOVAs on $V_{1/2}$ for HC type (I vs. II), genotype, and epithelial zone yielded a highly significant difference

for HC type ($F(1,46) = 1348.33$, $P = 0$) but for no other comparison. Thus, the *Cyp26b1* cKO mutation did not appear to affect the electrophysiological differentiation of type I and type II HCs.

Differences were seen, however, when we compared the spiking activity of striolar and LES calyx-bearing afferents in *Cyp26b1* cKO and *Foxg1^{Cre}; Cyp26b1^{lox/+}* controls. In the LES, calyx-bearing afferents are dimorphic afferents (forming both calyx and bouton terminals, Fig. 1A). In the striola, calyx-bearing afferents are either dimorphic or pure-calyx afferents. We measured the spiking activity of dimorphic (a nerve with both calyx and bouton endings, Fig. 1A) or pure-calyx vestibular afferent neurons via ruptured-patch recordings from their calyceal terminals; spikes initiate on the afferent neurite below the base of the calyx⁶. Striolar afferent neurons were more excitable in *Cyp26b1* cKO mice compared to *Foxg1^{Cre}; Cyp26b1^{lox/+}* controls, as measured by a lower current threshold for spiking and a tendency to fire a longer train of spikes in response to supra-threshold current steps. Threshold current was defined as the first level at which spiking occurred as applied currents were incremented in 50-pA steps (red traces, Fig. 4d,e). In comparing threshold currents across genotype and zone with 2-factor ANOVA, we saw significant main effects of genotype (control vs. cKO, $F(1,41) = 6.1$, $P = 0.02$), zone (striolar vs. extrastriolar, $F(1,41) = 10.0$, $P = 0.003$) and their interaction ($F(1,41) = 11.2$, $P = 0.002$). The significant differences were all between control striolar afferents (400 ± 90 pA, $n = 8$) and each of the other groups (Fig. 4f), including control ES afferents (127.3 ± 12 pA, $n = 11$; Tukey means comparison: $p = 0.0003$, effect size (Hedge's g) 1.9) and *Cyp26b1* cKO striolar afferents (156.3 ± 23 pA, $n = 16$; $P = 0.0005$, effect size (Hedge's g) 1.62). Threshold currents did not differ

significantly between *Cyp26b1* cKO striolar afferents and control ES afferents (Tukey means comparison, $P = 0.94$) or cKO ES afferents (164 ± 32 pA, $n = 7$, Tukey means comparison, $P > 0.99$). Thus, the *Cyp26b1* cKO manipulation reduced threshold current for spiking in striolar afferents to levels typical for extrastriolar afferents.

Afferent firing patterns were classified for current steps at 2-3 times threshold current as either *transient* (1-3 onset spikes) or *sustained* (>4 spikes). The tendency of isolated neurons to respond to injected current steps with transient or sustained responses correlates with their tendency to fire more irregularly or more regularly *in vivo*, when they are driven by synaptic inputs from HCs¹⁴. In control utricles, striolar afferents were more likely to have transient than sustained responses and extrastriolar afferents were more likely to have sustained than transient responses (Fig. 4g), consistent with previous data from rat vestibular ganglion neurons¹⁴ and calyceal terminals³⁷. In *Cyp26b1* cKO utricles, the normal zonal difference in firing pattern disappeared: most striolar afferents, like extrastriolar afferents, had sustained responses to current steps (Fig. 4g).

These changes in excitability in the cKO zone corresponding to the striola (reduced current threshold, more sustained firing) likely involves loss of striolar-specific ion channel expression. Candidates include low-voltage-activated K (K_{LV}) channels, which reduce neuronal excitability by increasing K^+ conductance around resting potential and, in normal utricular afferents, are expressed more in striola than extrastriola^{6,14,37,38}. Preliminary comparisons suggest that *Cyp26b1* cKO afferents lack this zonal difference: mean I_{KLV} did not differ significantly across zones (striola: 870 ± 114 pA, $n = 16$; extrastriola: 1192 ± 151 pA, $n = 6$; $P = 0.14$, $n = 22$, estimated power

0.3). Thus, *Cyp26b1* cKO utricles showed changes in afferent physiology that are consistent with a loss of striolar identity.

Absent vestibular-evoked potential in *Cyp26b1* cKO mice

To assess the functional consequences of loss of the striola *in vivo*, we recorded with scalp electrodes the vestibular-evoked potential (VsEP), which are thought to represent summed far-field afferent responses to transient linear accelerations, in control and *Cyp26b1* cKO animals. Control animals (*Cyp26b1*^{lox/+} and *Foxg1*^{Cre}; *Cyp26b1*^{lox/+} heterozygous animals) had normal VsEP responses, whereas *Cyp26b1* cKO mice showed either absent or remnant VsEP responses (Fig. 5a). VsEP thresholds between the *Cyp26b1*^{lox/+} and *Foxg1*^{Cre}; *Cyp26b1*^{lox/+} heterozygotes were comparable (Fig. 5b). There were also no differences in VsEP response activation latencies (P1, N1) or amplitude (P1-N1) at the highest stimulus level (+6 dB re: 1g/ms) across the two genotypes (data not shown). Taken together, these data show that VsEP was severely affected in *Cyp26b1* cKO mice, indicating that the loss of striola effectively abolishes most, if not all, VsEP response, supporting the existing hypothesis² that the VsEP is a summated potential reflecting the activity of striolar afferents and their downstream targets.

Normal angular vestibulo-ocular reflex and off-vertical axis rotation response in *Cyp26b1* cKO mice

Next, we tested whether the VOR was also affected in the *Cyp26b1* cKO mutants based on the hypothesis that the striolar/central zone is important for vestibular-reflex

function^{13,20}. The VOR plays an important role in ensuring gaze stabilization during everyday activities by producing compensatory eye movements in the opposite direction of the head movement³⁹. Because the dynamic response properties (i.e., gain and phase) of the VOR can be precisely quantified, it has also become a valuable tool for accessing vestibular function in mice. The best characterized VOR is the horizontal angular VOR (aVOR), which counter-rotates eyes in the horizontal plane and is largely driven by signals from the lateral cristae. As a control for eye muscle function, we also examined the optokinetic reflex (OKR), which uses visual signals to control eye motion, allowing tracking of a moving visual scene or objects. Normally, OKR functions in concert with VOR and vestibular neck reflexes to achieve the correct head and eye motion to stabilize visual field³⁹. Both the *Foxg1*^{Cre};*Cyp26b1*^{lox/+} heterozygotes and *Cyp26b1* cKO mice showed robust OKR responses (data not shown). Although aVOR gain increased and aVOR phase decreased systematically with increasing frequency of stimulus, there were no significant differences between *Foxg1*^{Cre};*Cyp26b1*^{lox/+} heterozygotes and *Cyp26b1* cKO mice in gain, phase change, model gain or model time constant up to 10 Hz (Fig. 6a-e; $P > 0.05$ for all comparisons).

Next, to detect reflex responses derived from macular organs, off-vertical axis rotation (OVAR) testing was conducted⁴⁰. For this purpose, we analyzed the recorded eye movements from the mice with heads fixed to a rotating platform (50 deg/s constant velocity), tilted 17 degrees with respect to the ground (Fig 6f). The eye velocity comprises two different responses: (1) an initial transient increase followed by a gradual exponential decrease and (2) steady-state response, in which eye velocity was oscillating around a constant bias with a sinusoidal waveform. We quantified the

difference between the two groups by comparing the amplitude and time constant of the transient response as well as the amplitude and bias of the steady-state response.

Similar to aVOR results, both transient steady-state OVAR responses were comparable for *Cyp26b1* cKO vs. controls (Fig. 6f, g). Together, these results indicate that despite the loss of striolar/central zone identity in vestibular sensory organs, horizontal aVOR and OVAR responses were normal in the *Cyp26b1* cKO mice, at least up to the maximal testable frequencies, which largely span the physiologically relevant range of head motion for mice in their natural environments⁴¹.

***Cyp26b1* cKO mice performed better on rotarod but worse on balance beam tests**

The vestibular system plays a vital role in balance control. To ensure stable body posture, vestibulo-spinal reflexes (VSR) produce compensatory movements of the neck and body to maintain the head in an upright position. Thus, we next tested *Cyp26b1* cKO mutants on a number of vestibular tasks used to test for balance and postural defects in mice. First, we found that these mice did not exhibit either circling or head tilting behaviors that are often associated with loss of vestibular function in rodents. Additionally, open-field testing revealed no hyperactivity in *Cyp26b1* cKO mutants (Supplemental video 1, Supplemental Fig.7), and the swimming ability of mutants was not affected under either light or dark conditions (data not shown). We further subjected animals to rotarod testing⁴² and quantified their performance based on the time the animal remained on the rotating rod with increasing acceleration. *Cyp26b1* cKO and *Foxg1^{Cre};Cyp26b1^{lox/+}* controls exhibited similar motor performance on day 1 of the trial (Fig. 7a). However, *Cyp26b1* cKO mutants performed better (took longer time to fall)

than controls on the second and third days (Fig. 7a). Whether this improved performance on the rotarod is due to replacement of striolar/central zone with extra-striolar/peripheral zone tissue or other mechanisms is not clear. Nevertheless, these collective behavioral results support the conclusion that the *Cyp26b1* cKO mutants exhibit no balance deficits during many of the tests commonly used to assess balance and motor control functions in mice.

The balance beam test can detect subtle deficits in motor skills and balance that may not be detected by other standard motor and balance tests such as the rotarod test⁴³. Accordingly, we tested the performance of *Cyp26b1* cKO mice on the balance beam. We found that *Cyp26b1* cKO mice walked slower than controls on a 20 mm wide beam over 60 cm length (Fig.7b). On a narrower beam (6 mm) over a distance of 40 cm, *Cyp26b1* cKO mice tended to freeze and stop walking (3 out of 6 vs. 1 out of 5 in controls) or moved slower than controls (Fig. 7c; Supplemental video 2). These results indicate that *Cyp26b1* cKO mice show a deficit in coordinating challenging vestibulomotor functions.

Increased head tremor in *Cyp26b1* cKO mice

Despite normal aVOR, OVAR, and performance for all standard motor and balance tests other than balance beam testing, we observed that it was possible to distinguish *Cyp26b1* cKO mice from controls in their cages, because the former demonstrated head tremors. This distinctive feature of *Cyp26b1* cKO mice was particularly obvious at early postnatal ages (Supplemental video 3). Quantitative analysis at P9 showed that the incidence of head tremors per 100 mm distance traveled

was higher in mutants than controls (Fig. 8a) and each head tremor episode also lasted longer in mutants (Fig. 8b). These head tremors became less noticeable during normal activities in a cage as the mutants matured (Supplemental video 1), suggesting some level of compensation. To quantify head tremors in adult *Cyp26b1* cKO mutants, we affixed a 6D MEMS module consisting of three gyroscopes and three linear accelerometers (see Materials and Methods) to the mouse's head implant. We confirmed that, when mice were not actively moving through their environment, *Cyp26b1* cKO adults exhibit head tremors (Fig. 8c, d). Power spectra of head movements revealed higher power for *Cyp26b1* cKO mutants than controls at high frequency (5-20 Hz) for all six dimensions (Fig. 8e, f). For mid-frequency (1-5 Hz), *Cyp26b1* cKO mutants have higher power for yaw and roll head velocity (Fig. 8f). Taken together, these results indicate that the ability to maintain head stabilization in *Cyp26b1* cKO mice is compromised.

DISCUSSION

Striolar/central zones of vestibular organs require reduced RA signaling

Based on molecular, cellular and physiological evidence, we demonstrated that striolar/central zones of vestibular sensory organs in the *Cyp26b1* mutants (gain-of RA signaling) are severely reduced. We concluded that reduced RA signaling mediated by the expression of *Cyp26b1* in the prospective striolar/central zones is required for this regional formation. This conclusion is supported by results from a complementary mouse model, *Aldh1a3*^{-/-} mice, in which endogenous RA signaling is reduced in the peripheral region of sensory organs and striolar HCs are expanded in the two maculae (Fig. 2, Supplemental Fig. 2). The expansion of Ocm⁺ HC in the central zone is not observed in the cristae (Supplemental Fig. 3). We attributed this difference between maculae and cristae to possible functional redundancy provided by *Aldh1a1* and *Aldh1a2*, which are expressed in the adjacent nonsensory tissues or in the roof of the sensory organs⁴⁴. Additionally, the extent of expansion of Ocm⁺ HCs is different between the utricle and saccule (Fig. 2c and S2c). Therefore, it is possible that intrinsic molecular differences across different regions of a sensory organ could also affect the response to the loss of RA signaling.

It is unclear how lower RA signaling mediates the unique cellular and anatomical features of the central regions during development. Given that expression of both *Cyp26b1* and *Aldh1a3* is concentrated in the SCs (Fig. 1f, g), it is possible that RA levels regulated by SCs provide a niche for the HCs and afferent nerve endings contained within the sensory epithelium. Receptors of RA are known to be expressed in the otic vesicle and sensory epithelium of the inner ear⁴⁵⁻⁴⁷. Alternatively, the reduced

RA in striolar SCs could regulate downstream genes within SC, which indirectly affect the development of HCs and their afferents. Previous results have suggested such a function for SCs⁴⁸. Activated erbB receptors in SCs of the utricle lead to production of BDNF, which promotes synaptogenesis between HCs and afferent neurons.

The striola generates the vestibular evoked potential (VsEP)

VsEP measure utricular and saccular function and are produced in response to transient changes in the linear head acceleration (jerk)^{17,18}. Several lines of evidence have implicated the irregular afferents of the striola in the generation of VsEP. First, the phase-locking and transient firing properties of irregular neurons render them the most likely candidates to respond in a synchronized manner to linear jerk stimuli and produce the signature compound action potentials^{2,20}. Second, VsEP is impaired by inhibitors of KCNQ, which are subunits of potassium channels that are most abundant in the calyceal endings of the striola¹⁹. Third, the linear pulse stimuli used to evoke VsEP are likely to activate irregular, striolar, afferents based on experiments with sound and bone vibration stimuli in the 500-1000 Hz range^{20,49}.

Our results provide strong evidence that the VsEP originates in the striola. The loss of morphological and physiological features in the striola of *Cyp26b1* cKO mice together with the VsEP suggests that the striola is necessary for the generation of VsEP. Earlier it was established that otoconia are essential for generating VsEP^{17,50}; here we show that the presence of otoconia is not sufficient for VsEP. In *Cyp26b1* cKO mice, otoconia are present but have extrastriolar-like properties throughout the epithelium. In control otolith organs, mechanical input to HCs in different zones is differentially shaped

by striking differences in otoconia, otolithic gel layers, stereocilia- bundle morphology and bundle coupling to the otoconia⁷. These differences are likely to contribute to the greater sensitivity of striolar afferents to high-frequency and transient head motion, including the linear pulse stimuli used to evoke VsEPs. Other likely factors are the zonal differences in calcium binding proteins, afferent calyceal synapses and excitability, all absent in *Cyp26b1* cKO.

Striolar/central zones are not essential for vestibulo-ocular reflexes

Given the larger cell bodies, higher conduction velocities^{51,52} and better phase-lead properties⁵³⁻⁵⁵ of the irregular neurons in the striolar/central zones than regular neurons in the extra-striola/peripheral zones, these regions have been postulated to be important for mediating fast vestibular reflexes⁵⁶. The aVOR in mice is fast with a latency as low as < 7 milliseconds⁵⁷. However, we did not detect any deficit in aVOR and OVAR in *Cyp26b1* cKO mutants when lateral cristae and macular organs were inertially stimulated respectively, suggesting that the striolar/central zone of vestibular organs (preferentially innervated by irregular afferents) are dispensable for these functions, at least in the adults. We cannot rule out the possibility that vestibular reflexes at a younger age or stimulations beyond frequencies tested (10 Hz in the case of aVOR) may require normal function of the central zones. These results further suggest that regular afferents concentrated in the extrastriolar/peripheral zones of the vestibular organs are important for mediating VOR. It was reported that *Aldh1a3*^{-/-} (em RA) mice show absence of aVOR and OVAR responses³¹, and our results show that the striolar/central zones in the vestibular organs are expanded based on Ocm staining (Fig.

2). Thus, these results are consistent with the notion that the extrastriolar/peripheral zones are more important than striolar/central zones for aVOR and OVAR. When irregular afferents in squirrel monkeys were selectively affected using galvanic currents, aVOR was not changed⁵⁸. Together, these results suggest that regular afferents in the extrastriolar/peripheral zone rather than irregular afferents in the striolar/central zone are more important for mediating angular and linear VOR in mice.

***Cyp26b1* cKO mice show deficits in precise balance control and self-motion**

A long-standing view is that irregular afferents, which innervate the striolar/central zone, preferentially contribute to VSR^{59,60}. Our finding that loss of striolar/central zones leads to a deficit in coordinating challenging postural control on the balance beam is consistent with this proposal^{59,60}. Surprisingly, however, *Cyp26b1* cKO mice performed well on other less sensitive tests commonly used for accessing changes in balance and motor control function in vestibularly deficient mice. Further, we did not observe classical vestibular behavioral deficits such as head tilt and circling in the *Cyp26b1* cKO mutants. Together these two observations suggest that the extrastriolar/peripheral zones, which constitute 75-80% of the sensory epithelia^{4,5,11,12} and are innervated by regular afferents, play a critical role in vestibulo-spinal as well as vestibulo-ocular reflexes in mice. In support of this hypothesis, regular afferents with their sustained firing properties (less adapting) are thought to be more important in conveying head positional information in steady state such as head tilts⁶¹.

A distinguishing characteristic of *Cyp26b1* cKO mice was that they demonstrated head tremor. Similar head movement behaviors have been reported in both primates

and humans with compromised vestibular functions. For example, squirrel monkeys show transient head tremor and postural instability after plugging one of the lateral semicircular canals⁶². Patients with chronic bilateral vestibular loss that exhibit gaze variability and oscillopsia also show pronounced head oscillations in response to weighted head mass, compared to healthy individuals⁶³. This head tremor behavior is attributed to a failure of vestibular input that is normally required to maintain head stability^{64,65} and ensure movement accuracy during active goal-directed behaviors^{63,66}. It is tempting to speculate that in the *Cyp26b1* cKO mouse, poor or abnormal vestibular inputs from the striolar/central regions cause head tremors or oscillations, which affected challenging vestibulo-motor activities such as traversing a narrow balance beam when rapid vestibular input to motor centers is required. One caveat is that *Cyp26b1* cKO mice show some loss of *Cyp26b1* expression in the brain (data not shown), suggesting that the behavioral phenotype may result in part from central as well as peripheral *Cyp26b1* loss.

In summary, using a genetic approach we generated a viable mouse mutant that largely lacks the striolar/central zone of vestibular organs. By disrupting RA signaling during embryogenesis, the entire axis of this specialized zone failed to develop properly, including associated components such as the otoconia and innervating neurons. As a result, the loss of this highly specialized and conserved region⁶⁷ selectively affected the ability of afferents to respond to transient changes in linear acceleration and may affect vestibular input. Interestingly, low levels of RA signaling mediated by two of the RA degradation enzymes, *Cyp26a1* and *Cyp26c1*, are also required for the formation of the fovea, a high acuity area of the retina²³. Therefore, the same developmental strategy is

being employed to generate complexity and specialization in two different sensory systems. A better understanding of the striolar/central zone-specific function has clinical and therapeutic relevance as HCs in this region are more susceptible to ototoxic insults in animal models⁶⁸.

METHODS

All of the methods are described in the supplementary information.

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LEGENDS

Fig. 1. Complementary expression patterns of *Cyp26b1* and *Aldh1a3*.

(a) Schematic illustration of the inner ear and sectional view of the utricle (ut) across the striola and lateral extrastriola region (LES). Pear-shaped type I HCs are innervated by calyces and cylindrical-shaped type II HCs are innervated by bouton type endings. Pure/complex calyces are exclusively present in the striolar/central zone, whereas dimorphic nerve endings are found across the entire organ. Otoconia are smaller in size and less abundant in striola than extra-striola of the utricle. Asterisk indicates that the relationship between stereocilia bundles and the otoconial membrane is not clear, but evidence suggests that hair bundles of striolar HCs are less firmly embedded in the otolithic membrane than their counterparts in the extra-striolar region^{7,69}. Yellow line represents the line of polarity reversal (LPR), which separates each macula into two regions, LES and MES (medial extrastriola), with opposite hair bundle orientation. The striola of maculae and the central zone of anterior (ac) and lateral cristae (lc) are in blue color. The LPR is at the lateral edge of the striola in the ut but it bisects the striola in the saccule (sac). (b) Schematic summary of the expression pattern of *Cyp26b1* in the striolar/central zones (light blue) and *Aldh1a3* in the extrastriolar/peripheral zones (dark blue) of vestibular organs described in (c-h). (c-e) Whole mount *in situ* hybridization analysis of *Cyp26b1*, *Aldh1a3*, and β -*tectorin* transcripts at E18.5 mouse ut and ac and lc. Expression of *Cyp26b1* (c) is restricted to the central zone of the two cristae and striolar region of the ut that is β -*tectorin*-positive (e), whereas *Aldh1a3* (d) is predominantly expressed in the peripheral regions. (f-h) Adjacent tissue sections at the levels of ut and lc at E15.5. (f) *Cyp26b1* expression is concentrated in the supporting

cell (SC) layer of the central zone of the lc and striola of the ut, comparable to the β -*tectorin* domain (**h**, bracket). (**g**) *Aldh1a3* expression is complementary to that of *Cyp26b1* (**f**) in each organ. Scale bars for both whole mount and section images equal 200 μ m. RA, retinoic acid; D, dorsal; A, anterior; L, lateral; pc, posterior crista.

Fig. 2. Disruption of RA signaling alters striola and central zone formation in the vestibular sensory organs.

(**a-c**) Immunostaining using antibodies against myosin7a (magenta) and oncomodulin (Ocm; green) on HCs of E18.5 utricle (ut), anterior crista (ac), and lateral crista (lc). (**a**) In *Cyp26b1*^{+/-} or *Aldh1a3*^{+/-} controls, Ocm is expressed in type I HCs in striola and central zones only, whereas all HCs are positive for Myosin7a. (**b-e**) Ocm expression is severely reduced in *Cyp26b1*^{-/-} utricles (**b, d**, 2.3 ± 0.2 %, $n = 6$, $P < 0.0001$) but expanded in utricles of *Aldh1a3*^{-/-} mutants (**c, d**, 28.4 ± 1.4 %, $n = 5$, $P < 0.0001$) when compared to controls (14.3 ± 0.5 % in controls, $n = 6$). Ocm⁺ HCs in lateral cristae are reduced in *Cyp26b1*^{-/-} mutants (**b, e**, 3.2 ± 1.1 , $n = 3$, $P = 0.0005$) but remain unchanged in *Aldh1a3*^{-/-} mutants (**c, e**, 44.1 ± 5.6 %, $n = 4$, $P = 0.302$), compared to controls (36.2 ± 2.7 %, $n = 5$). (**f**) Immunostaining of β -tectorin that labels striolar supporting cells in control utricles only. (**g-i**) β -tectorin-positive domain is reduced in *Cyp26b1*^{-/-} (**g, i**, 7.4 ± 0.6 %, $n = 4$, $P = 0.0002$) but increased in *Aldh1a3*^{-/-} (**h, i**, 26.4 ± 1.7 %, $n = 4$, $P = 0.0003$) mutant utricles, compared to controls (17.4 ± 0.9 % in controls, $n = 5$). (**j-l**) Immunostaining of afferent nerve endings in the striola and central zones with anti-calbindin antibodies. (**k-n**) Calbindin immunoreactivity is severely reduced in *Cyp26b1* cKO (**k, m**, 3.8 ± 0.3 %, $n = 3$, $P = 0.0005$), but increased in *Aldh1a3*^{-/-} (em RA) (**l, m**,

36.0 ± 6.6 %, n = 4, P = 0.0006) mutant utricles, compared to controls (**j, m**, 19.7 ± 1.7 %, n = 5). Similar to Ocm-positive region, calbindin-positive area is decreased in *Cyp26b1* cKO (**k, n**, 4.7 ± 1.4 %, n = 3, P < 0.0001), but unchanged in cristae of *Aldh1a3*^{-/-} (em RA) mutant ears (**l, n**, 34.4 ± 0.5, n = 3, P = 0.3021), compared to controls (**j, n**, 34.2 ± 1.1 n = 3). The one-way ANOVA with multiple comparisons was applied. A, anterior; L, lateral. **P < 0.01 and ***P < 0.001. Scale bar; 200 μm.

Fig. 3. Loss of regional differences in the otoconia of *Cyp26b1* cKO utricles.

(**a, b**) A dissected utricle (ut), anterior (ac) and lateral cristae (lc) of *Foxg1*^{Cre};*Cyp26b1*^{lox/+} control (**a**) and *Cyp26b1* cKO (**b**) ears at E18.5. In control utricle, the striola region shows a clearance of the otoconia (arrow in **a**), which is missing in *Cyp26b1* cKO utricles. (**c-e**) SEM of otoconia in *Foxg1*^{Cre};*Cyp26b1*^{lox/+} controls (**c-c'''**) and *Cyp26b1* cKO (**d-d''**) utricles at P75. Insets show low power views of respective utricles. In controls (**c', c'', c'''**), the striolar region (dotted outline) shows otoconia with smaller crystals (**c'', e**, 19.7 ± 1.5 μm, n = 22 crystals) and perforated holes in the subcupular meshwork layer (**c''**), whereas the medial extrastriolar region (MES) shows larger otoconia (**c'''**, **e**, 40.2 ± 2.2 μm, n = 23, P < 0.0001). There is no clear regional difference in the size of the otoconia in the *Cyp26b1* cKO mutant utricle (**d, d'**), and the otoconia crystals in the presumptive striolar region are larger in size (**d'', e**, 42.9 ± 3.1 μm, n = 21), comparable to those found in MES of controls (**c'''**, P = 0.6509). The one-way ANOVA with multiple comparisons was applied. ***P < 0.001. Scale bars; 200 μm for (**a, b**), 300 μm for (**c, d**) and (**c', d'**), and 100 μm for (**c'', c'''**, **d''**).

Fig. 4. Afferent calyces in *Cyp26b1* cKO utricles showed a reduction in striolar-like morphology and neural activity.

(a-c) P45 wholemount utricles from controls (**a, a'**) and *Cyp26b1* cKO (**b, b'**) immunolabeled with anti-Tuj1 (green) and anti-calbindin (magenta) antibodies. (**a, b**) Maximum intensity projection of the entire utricle. (**a', c**) Enlarged, single plane image of the rectangular region in control striola (**a**), showing the presence of large number of double (white circles, 42.6 ± 1.8 double calyces/ utricle) and triple (cyan circles, 10.0 ± 2.0 triple/utricle, $n = 3$ utricles) calyces at the cell body level. (**b', c**) Fewer double calyces (white circles, 13.6 ± 2.8 double calyces/utricle, $P = 0.001$) and no triple calyx (0.6 ± 0.6 triple calyces/utricle, $P = 0.006$, $n = 3$) are found in the corresponding region of *Cyp26b1* cKO mutants. Scale bars; 200 μm (**b**); 30 μm (**b'**). Unpaired t-test. ** $P < 0.01$. A, anterior; L, lateral. **(d-g)** *Cyp26b1* cKO striolar afferents, like control ES afferents, were more excitable than control striolar afferents, as shown by their tendency to fire more spikes and by reduced current threshold for spiking. **(d, e)** Whole-cell current clamp records from patched calyces of a control striolar afferent (**d**) and a *Cyp26b1* cKO striolar afferent (**e**). 500-ms current steps were delivered in 50-pA increments from -200 pA to >1 nA relative to zero holding current; a subset is shown including the response at threshold current (red). Three times (3x) threshold current (blue) evoked transient spiking in a control striolar afferent but sustained spiking in *Cyp26b1* cKO striolar afferent. **(f)** Mean threshold current was significantly higher in Control-S calyces than in all other categories – i.e., the cKO-S calyces resembled cKO-LES calyces and Control-LES calyces. See text for details on statistics. **(g)** Transient

firing was more common in Control-S afferents; sustained firing was more common in Control-LES, *Cyp26b1* cKO-S and *Cyp26b1* cKO-LES afferents.

Fig. 5. Absence of linear vestibular-evoked potential (VsEP) in *Cyp26b1* cKO mutant mice.

(a) Three representative VsEP waveforms for each genotype recorded at maximal jerk stimulus (+6 dB). In the *Cyp26b1* cKO mutants, distinct peaks of P1-P2 and N1-N2 are not detectable. (b) Summary of thresholds for VsEP determined by various jerk magnitudes. There was no significant difference in VsEP thresholds between the *Cyp26b1*^{lox/+} (-10.5 ± 1.34 dB re:1g/ms, n = 5) and *Foxg1*^{Cre}; *Cyp26b1*^{lox/+} heterozygotes (-8.1 ± 1.17 dB re:1g/ms; n = 10, P = 0.1026). *Cyp26b1* cKO mutants generated only a remnant response (RR).

Fig. 6. Normal horizontal angular vestibulo-ocular reflex and off-vertical axis rotation response in *Cyp26b1* cKO mice.

(a) Schematic view of horizontal angular vestibulo-ocular reflex (aVOR) apparatus. Constant-acceleration step gain G_A (b) and latency (c) for aVOR responses to whole-body, 3000°/s² whole-body passive yaw rotations in darkness about an Earth-vertical axis through the head. Open markers denote individual mice; thick markers and lines show mean ± SEM. Gain (d) and phase lead (e) for yaw slow-phase aVOR responses to 0.02-10 Hz, 100°/s peak velocity sinusoidal, whole-body passive yaw rotations. Solid and dashed lines show first-order high-pass filter model fits to control and *Cyp26b1* cKO mouse population data, respectively. The high variability of phase and 0.02 Hz and relatively poor fit to gain data at 0.02Hz are due to the small amplitude of responses at

that frequency. Differences between control and *Cyp26b1* cKO mice were not significant for G_A , latency, model gain or model time constant ($P < 0.05$ for all comparisons). (f) Schematic view of apparatus for off-vertical axis rotation (OVAR). Pitch of the table was maintained at 17° . (g) No significant difference in the eye velocity was observed between control and *Cyp26b1* cKO mice during transient response (~ 20 sec; amplitude: 43.8 ± 8.3 vs. 38.7 ± 7.4 deg ($P = 0.66$), time constant: 4.1 ± 1.1 vs. 5.1 ± 2.3 s ($P = 0.68$), when both semicircular canal and otolith organs are stimulated, and steady-state response (20 sec~; amplitude: 5.3 ± 0.72 vs. 5.8 ± 0.75 deg ($P = 0.64$), bias: 10.5 ± 1.3 vs. 8.5 ± 1.3 ($P = 0.33$)), time when only responses from otolith organs are expected to be measured.

Fig. 7. Impaired balance of *Cyp26b1* cKO mice during challenging vestibular-motor activities.

(a) Quantification of rotarod tests. Each mouse was placed on a rotating rod, which accelerated from 5 to 40 rpm over a 5 min period. *Cyp26b1* cKO and *Foxg1^{Cre};Cyp26b1^{lox/+}* controls exhibited similar motor performance on day 1 of the trial (90.3 ± 15.9 s in mutants, $n = 10$, vs. 65.3 ± 6.5 s in controls, $n = 10$, $P = 0.2573$). *Cyp26b1* cKO mutants were able to stay on rod longer than controls on the second day of testing (123.3 ± 11.0 s mutants vs. 71.7 ± 7.5 s controls, $P = 0.0003$) and third day (141.2 ± 17.8 s mutants vs 85.7 ± 9.5 s controls, $P = 0.0037$, Fig. 7a). (b) Quantification of speed to traverse 60 cm distance on a 20 mm-wide beam. *Cyp26b1* cKO mutants moved slower (0.083 ± 0.014 m/s, $n = 6$) than controls (0.148 ± 0.011 m/s, $n = 5$, $P = 0.0056$). (c) Quantification of time taken to cross 40 cm distance on a 6 mm-wide beam.

Half of *Cyp26b1* cKO mutants failed to traverse the beam in 2 min (3/6), whereas most of controls reached the endpoints within 30 sec (4/5). By assigning 2 min for all the mice that failed to complete the 40 cm distance, *Cyp26b1* cKO mutants moved slower (taking 84.0 ± 19.24 s to traverse the beam) than controls (33.0 ± 22.04 s for controls, $P = 0.0099$). $**P < 0.01$ and $***P < 0.001$. T.O., time-out.

Fig. 8. Increased head tremors in *Cyp26b1* cKO mice.

(a, b) Quantification of head-tremors for P9 pups. The number of bouts normalized over a 100 mm distance traveled (a) and average duration of each head-tremor episode over a 10-min period are shown (b). *Cyp26b1* cKO pups showed more frequent (a, 4.8 ± 1.1 in mutants, $n = 6$, vs. 1.2 ± 0.2 in controls, $n = 8$, $P = 0.0059$) and longer duration (b, 398 ± 38 ms in mutants vs. 192 ± 9 ms in controls, $n = 8$, $P < 0.0001$) head tremors, compared to *Foxg1^{Cre};Cyp26b1^{lox/+}* controls. (c) Schematic of the apparatus for measuring head tremors of an adult mouse at rest with a miniture head motion sensor affixed on the top of the skull. (d) *Cyp26b1* cKO showed characteristic head tremors (5/6), which were not present in controls. (e, f) Comparison of power spectra density of head movements in translational axes (e) and rotational axes (f) between controls (blue) and *Cyp26b1* cKO mutants (red). *Cyp26b1* cKO exhibit significantly higher power than controls at high frequencies (5-20 Hz, $P = 0.006$ for right/left, $P = 0.005$ for fore/aft, $P = 0.01$ for up/down, $P = 0.035$ for pitch, $P = 0.014$ for roll, and $P = 0.011$ for yaw axis, $n = 6$ /group). Angular head velocity for yaw and roll axes of *Cyp26b1* cKO also had significantly higher power for frequencies between 1 and 5 Hz (upper graphs, $P = 0.05$

for yaw, and $P = 0.028$ for roll axis). Unpaired t-test was applied for all tests. $*P < 0.05$.

$**P < 0.01$ and $***P < 0.001$.

Fig.1

Cyp26b1

Aldh1a3

β -tectorin

Fig.2

Fig.3

Control

***Cyp26b1* cKO**

Fig.4

Fig.5

a

b

Fig.6

Horizontal angular vestibulo-ocular reflex (aVOR)

a

d

Sinusoidal rotation

b

Constant-acceleration step rotation

c

e

Off-vertical axis rotation (OVAR)

f

g

Fig.7

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a

b

**Speed traversing across
a 20 mm-wide balance beam**

c

6 mm balance beam tests

Fig.8

Measurements at P9

Measurements at adults

Table 1. Phenotypic summary of *Cyp26b1* cKO and *Aldh1a3* (emRA) KO

<i>Cyp26b1</i> cKO	utricle	sacculle	cristae
Ocm (HCs)	↓	↓	↓
β-tectorin (SCs)	↓	no change	N/A
Calbindin (afferents)	↓	↓	↓

<i>Aldh1a3</i> (emRA) KO	utricle	sacculle	cristae
Ocm (HCs)	↑	↑	no change
β-tectorin (SCs)	↑	no change	N/A
Calbindin (afferents)	↑	N/D	no change