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Research Article

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Preserved Vestibular Function in Mice with Loss of α-9 Subunit of the α-9/10 Nicotinic Acetylcholine Receptor (α-9/10 nAChR)

Abstract

Background: The α-9/10 nicotinic acetylcholine receptor is known to be the primary channel through which both vestibular and auditory efferents mediate the inhibition of their respective peripheral hair cells and afferents. With respect to the auditory system, the deletion of the α-9 subunit results in abnormalities in the development of properly functioning cochlear hair cells. Given the high degree of similarity between the auditory and the vestibular systems, we hypothesize that α-9 knockout mice should have impaired vestibular hair cell development and consequently compromised vestibular-mediated functions.

Methods: In order to characterize vestibular function in α-9 knockout alert mice, we quantified the vestibulo-ocular reflex (VOR) through both gain and phase. Additionally, the optokinetic nystagmus (OKN) was similarly assessed as a control. VOR in light (VORl) was also quantified to further evaluate VOR and OKN efficacy. Furthermore, as information from the vestibular system mediates postural regulation and head stabilization, we assessed these properties through rotor rod and balance beam paradigms.

Results: Surprisingly, the loss of the α-9 subunit in knockout mice did not result in any attenuation in VOR gain nor deviations in phase compared to wild type. OKN and VORl's gain and phase values remain similarly unchanged, confirming preserved function within the vestibular nucleus. Descending vestibulospinal information seems to be unaltered as well, as no significant difference was observed in postural testing.

Limitations: The α-9 knockout mice used specifically had exon 1 and exon 2 of the α-9 gene targeted, which could potentially limit generalizability. Also, frequencies greater than 3Hz were not tested.

Conclusions: Our findings demonstrate that α-9 knockout mice still maintain normal vestibular function.

Introduction

The vestibular system plays a fundamental role in the maintenance of posture, the stabilization of gaze, and our innate ability to sense self-motion. The physiological basis of this system is two distinct categories of sensory organs within the inner ear: the semicircular canals, which detect angular acceleration of the head, and the otoliths, which detect linear acceleration of the head. (1) Within these sensors, there are two types of vestibular hair cells: Type I and Type II. While the two types vary by morphology and afferent innervation, they both synapse onto primary vestibular afferents which project to neurons within the vestibular nuclei. The vestibular neurons innervate the abducens nucleus which then project onto oculomotor neurons, prompting eye movements that are involved in the vestibulo-ocular reflex. (2) The vestibulo-ocular reflex (VOR) stabilizes gaze in the presence of head movement by producing equal and opposite eye rotations to that of the head rotation, preserving image location in the center of the visual receptive field. Due to its simplicity and response properties, the VOR is often used as a method to test the functionality of the vestibular system in various species. Additionally, the vestibular nuclei project onto the spinal cord, where they mediate postural stabilization and higher order centers. (3)

The efferent vestibular system (EVS) is composed of neurons that synapse directly onto multiple hair cells and/or primary vestibular afferents in the vestibular end organs. (4-5) While many neuromodulators regulate the vestibular efferent system, acetylcholine is the primary neurotransmitter of this system. (3) Activation of the α-9/10 nicotinic acetylcholine receptor (nAChR) in both the auditory and vestibular systems provides a means of efferent mediated inhibition. When activated, the α-9/10 nAchR allows a transient influx of calcium followed by a coupling to the SK channel, causing an efflux of potassium which then hyperpolarizes the cell. This hyperpolarization reduces further calcium influx and thus inhibits the release of glutamate onto afferents, causing overall cellular inhibition. (6-8) Many parallels are often drawn between the vestibular and auditory systems; it has been postulated that the auditory system evolved from the vestibular system. (9) As such, cross-system inferences can act as initial directives for determining potentially functional components in either system. To understand the role of the α-9/10 nAChR subunits in the auditory system, Johnson (10) tested transgenic mice which lacked the α-9 nAChR subunit gene in inner hair cells (IHCs). Evaluating the sensitivity of calcium influx through whole cell voltage-clamp recordings, measuring calcium current, and determining changes in cellular membrane capacitance showed that the IHCs of α-9 knockout mice exhibited dependence similar to that of immature IHCs. This is indicative of a lack of normal maturation of the synaptic machinery. Inner hair cells were unable to respond to the inhibitory efferent input in the absence of the α-9 subunit, a direct result of the failure in development of the auditory efferent system. While the α-9/10 nAchR is known to play an important role in the auditory and vestibular efferent system, specifically in the IHCs, little is known about whether or how the loss of the α-9 subunit influences overall vestibular function.

To that end, we assess the functional role of the α-9 subunit of the α-9/10 nAchR using the α-9 null mouse strain. We first characterized the VOR and found no significant attenuation of VOR gain or phase in the α-9 knockouts relative to wild type. Behavioral assays further demonstrated no significant difference in postural regulation between the α-9 knockout and the α-9 wild type mice. To assess the functionality of downstream innervations within the vestibular nuclei, we characterized the optokinetic nystagmus (OKN) response – a visually driven eye movement which works in conjunction with the VOR to stabilize gaze by producing eye motion in the same direction as visual motion. No significant deficit was found. Our

results suggest that vestibular efficacy is maintained even after removal of the α-9 subunit of the α-9/10 nAchR.

Methods

Animals

The α-9 wild-type (+/+) and knockout (-/-) transgenic mice used in this study were generously provided by the laboratory of Dr. Barbara Morley of Boys Town National Research Hospital. These mice were created by targeting exon 1 and 2 of the α-9 gene. The mice were then shipped to McGill University for eye movement and vestibular testing. A total of 16 mice were used, aged 5-6 months: 9 α-9 knockouts (5 female, 4 male) and 7 α-9 wild type (4 female, 3 male). The McGill University Animal Care Committee approved the use and care of the animals in accordance with the Canadian Council on Animal Care.

Head Post Surgery

An aluminum head post was constructed to accommodate head restraint during eye recordings. Mice were anesthetized with an intraperitoneal injection of ketamine (100 mg/mL), xylazine (20 mg/mL), and acepromazine (10 mg/mL) in sterile isotonic saline. Once anesthetized, mice were given an analgesic subcutaneous injection of carprofen (concentration: 4 mg/ml; dosage: 5 µL/g) prior to surgery. An incision was made and the skin was held back to expose the skull. An aluminum head post was secured to the skull using methods described by Muniak et al. (11) The incision was then sutured closed and Polysporin was applied to prevent infection. To prevent hypothermia and dehydration throughout surgery, a heating pad was placed underneath the mice and subcutaneous injections of isotonic saline (dosage: 0.2-0.5mL/10g body weight) were given when necessary. Analgesic injections of carprofen (concentration: 4 mg/mL; dosage: 5 µL/g) were given every 24 hours during the next 24-48 hours of recovery.

Eye Movement Data Acquisition

Mice were briefly anesthetized using gas isoflourane in order to restrain them in a plexiglass tube. Their heads were secured by screws in the surgically attached head post and pitched at ~30° to align the semicircular canals with the horizontal plane. Once alert, each animal was then placed onto the center of the turntable, concentric with the drum, and secured (Fig.1). Eye movement data was recorded using an infrared video system (ISCAN). Head velocity was recorded using an angular velocity sensor. All signals were combined in REX (Real-Time Executive), a QNX-based (Unix-like operating system) real-time data acquisition system. (12) OKN eye movement responses were evoked by sinusoidal rotations of a drum placed around the turntable at frequencies 0.1, 0.2, 0.4, 0.8, 1, 2, and 3 Hz with peak velocities of both ±8°/s and ±16°/s. High contrast stripes (5°) were placed on the inside of the drum to ensure a response. To record VOR, the turntable was rotated at sinusoidal frequencies 0.1, 0.2, 0.4, 0.8, 1, 2, and 3 Hz with peak velocities of both ±8°/s and ±16°/s in both the light and dark. In the light condition, the drum remained stationary, while in the dark condition both the drum and turntable rotated in phase.

Postural Data Acquisition

For the rotor rod task, mice were trained with trials of 5, 10, and 20 rpm for 120 seconds each, with 15 minutes rest periods between trials and a 1 minute acclimation period. Mice were allowed to acclimate for 5 minutes on the rotor rod during training and test trials. For each test trial, the rotor rod was programmed to accelerate from 4 rpm to 40 rpm with a ramp of 300 seconds, the maximum duration of the test. 3 trials were performed each day for 3 consecutive days.

For the balance beam task, a beam one meter in length and 14 mm in diameter, inclined 52.5-88.3 cm above the ground, was used. The mouse was held in a cardboard box at the end of the beam to acclimate to the goal location. During training, the mouse was placed directly outside of the box on the beam and allowed to walk in. A reward period of 1 minute was given after the mouse had reached the box. Once the mouse was trained, it was placed 60 cm away from the box and allowed to move across to the goal position. This was repeated 5 times with 1 minute rest periods in between.

Analysis

MATLAB by Mathworks was used to analyze all data. Least-square optimization (13) determined the VOR and OKN gains and phases, plotted as mean ± standard deviation (SD) against all frequencies for all mice. For each rotor rod trial, the time at which the mouse fell from the rod was recorded. If the mouse resisted falling by grasping onto the rod, this was considered a failure and time was noted. The mean times ± SD were then plotted. Traversal time of the balance beam was recorded for each trial and mean times ± SD were plotted. To determine the statistical significance between the wild type and null knockout mouse a two-way ANOVA test with Bonferroni post hoc tests was used.

Results

α-9 Knockouts Demonstrate Unaltered VORd, OKN, and VOR1 Response Dynamics

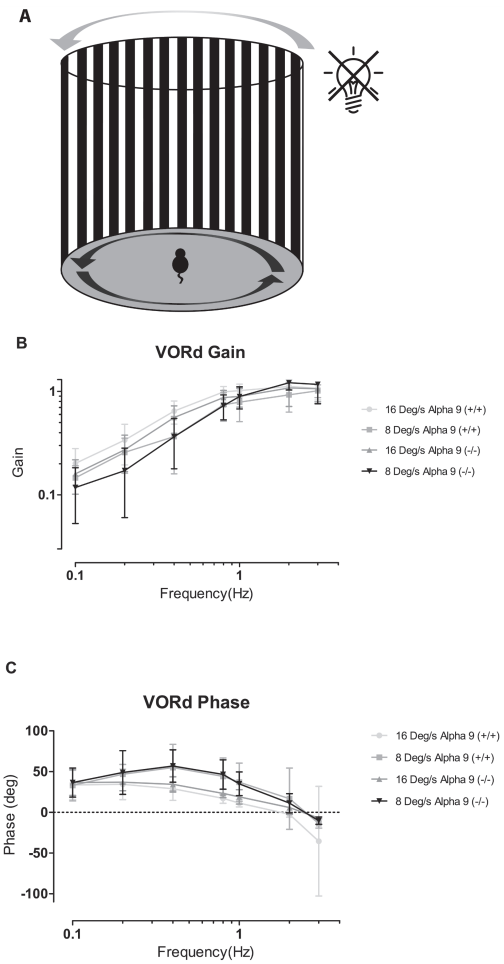

Fig. 1. Characterization of VORd. α-9 (-/-) mice demonstrate normal VORd at peak velocities of 16 deg/s and 8 deg/s. (A) Schematic of recording set-up for VORd stimulation; movement of turntable and drum. (B, C) VORd gain and phase (mean±SD) plotted as a function of frequency for α-9 (+/+) (n=7) and α-9 (-/-) (n=8).

In our study, we subjected both α-9 control and α-9 knockout mice to three conditions: VORd (whole body rotation in darkness (Fig. 1A)), OKN (rotation of optokinetic drum with stationary mouse (Fig. 2A)), and VORl (whole body rotation in a lit environment with the optokinetic drum held stationary (Fig. 3A)). As the majority of head movements of mice during

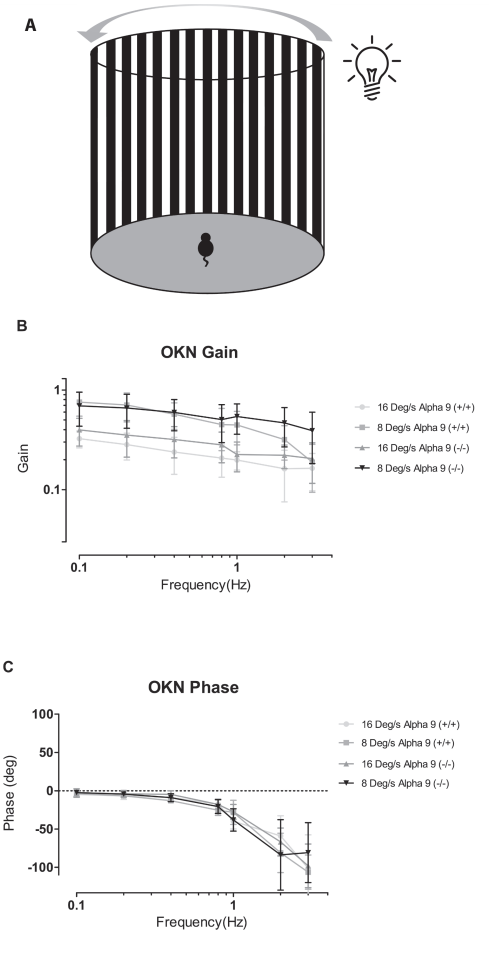

Fig. 2. Characterization of OKN. α-9 (-/-) mice demonstrate normal OKN at peak velocities of 16 deg/s and 8 deg/s. (A) Schematic of recording set-up for VORd stimulation; movement of drum only. (B, C) OKN gain and phase (mean±SD) plotted as a function of frequency for α-9 (+/+) (n=7) and α-9 (-/-) (n=8).

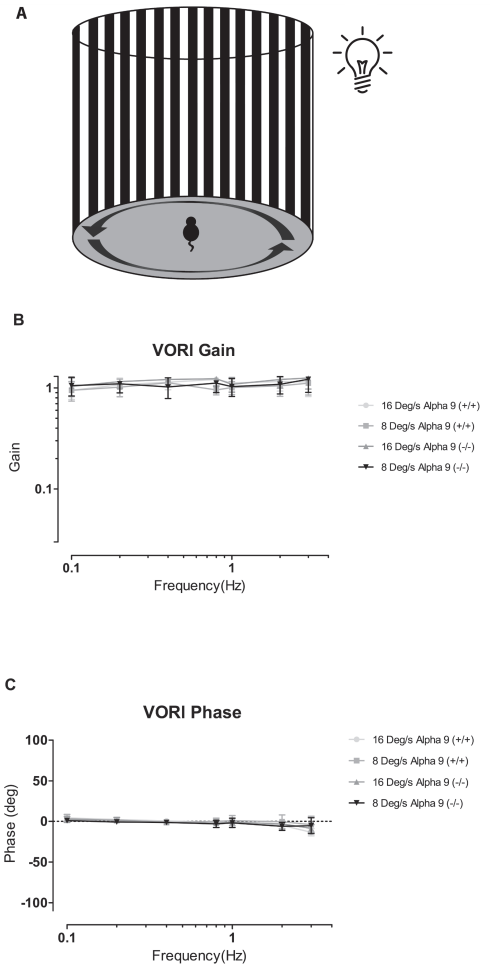

Fig. 3. Characterization of VORl. α-9 (-/-) mice demonstrate normal VORd at peak velocities of 16 deg/s and 8 deg/s. (A) Schematic of recording set-up for VORl stimulation; movement of turntable only. (B, C) VORl gain and phase (mean±SD) plotted as a function of frequency for α-9 (+/+) (n=7) and α-9 (-/-) (n=8).

exploration have component frequencies less than 4 Hz, (13) all sinusoidal rotations were done at frequencies within this physiologically relevant range (0.1 Hz to 3 Hz). Furthermore, each condition was tested using peak angular velocities of 8°/s and 16°/s in order to evaluate any potential non-linearity in response differences between the strains.

Figures 1B, C depict the Bode plots for gain and phase respectively during the VORd condition. In accordance with previous characterizations, wild-type mice experience an increase in VOR gain with an increase in rotation frequency (Fig. 1B). For stimuli with peak velocity of 8°/s, the recorded gains ranged from 0.146±0.045 at 0.1 Hz to 1.014±0.143 at 3 Hz, and for 16°/s, gains ranged from 0.201±0.079 at 0.1Hz to 1.069±0.243 at 3 Hz. Additionally, phase decreases as a function of frequency (Fig. 1C), such that the VORd initially leads with respect to the head velocity at lower frequencies and has a higher degree of compensation for higher frequencies. Surprisingly, knockout mice experience no attenuation in VORd gain compared to controls under the same peak velocity condition, as their gain values did not significantly differ across any frequencies (Fig. 1B; $p > 0.05$). Furthermore, knockout mice did not significantly differ from controls with respect to phase at all frequencies tested (Fig. 1C; $p > 0.05$).

In order to control for the possibility of the genetic mutation disrupting neural function elsewhere in the processing stream from the vestibular afferents within the VOR pathway, the dynamics of the optokinetic nystagmus (OKN) were assessed. The OKN pathway converges with the VOR pathway at the level of the vestibular nuclei; it is important to discount any alterations in OKN efficacy. Figures 2B, C represent the Bode plots for gain and phase respectively during the OKN condition. Comparing knockout mice to wild-type mice, differences in values for gain and phase across all frequencies in a given peak velocity condition were non-significant (Fig. 2B, c; $p > 0.05$). Both knockout mice and wild-type mice demonstrate the general OKN trend in which gain decreases with increased frequency of the stimulus (Fig. 2B) and initial compensatory eye movement at low frequencies begins to lag with respect to the velocity stimulus at higher frequencies (Fig. 2C).

To further evaluate VOR and OKN efficacy, VOR was quantified in a lit environment (VORl). VORl represents a compound response of both OKN and VOR, where at lower frequencies OKN compensation dominates and at higher frequencies VOR compensates for the head motion. As VORd and OKN were not significantly altered between the two strains, we predicted that there should be no attenuation in VORl response. Figures 3B, C are the Bode plots for gain and phase respectively during the VORl condition. In accordance with our hypothesis, both knockout and wild-type mice show a general trend of near-perfect gain regardless of frequency and no significant difference in gain values across all frequencies in a given peak velocity condition (Fig. 3B; $p > 0.05$). In addition, phase values were non-significant between both mice strains across all frequencies in a given peak velocity condition (Fig. 3C; $p > 0.05$), where the eye movement remained compensatory throughout all frequencies.

Postural Regulation and Balancing Capabilities Preserved in α-9 Knockout Mutants

The vestibular system's projections from the vestibular nuclei to the spinal cord, descending via the vestibulospinal tracts, provide necessary information for both head stabilization (14-16) and balancing through reflexive control of the limbs. (17) Should the α-9 knockout impair normal development of vestibular hair cells, the neurons of the vestibular nuclei would receive distorted signals from the primary vestibular afferents and we would consequently anticipate abnormal motor behaviours when the subject attempts to balance or stabilize their head position. In order to assess the presence of such impairments, we chose to use two paradigms: the balance beam and accelerating rotor rod, both of which are common in evaluating balance and defective motor skill in mice. (18-19)
Both the control wild-type mice and α-9 knockout mutant mice were subject to an initial training period of rotor rod testing, in which the angular velocity remained constant for a given trial but increased with subsequent trials, and a 3-day testing period with accelerating rotations (Fig. 4A). Comparing time to fall averaged across trials conducted on a given day (Fig. 4B), α-9 knockout and control wild-type mice did not significantly differ on any given day (Fig. 4b; $p > 0.05$). The balance beam test was performed on both wild-type and knockout mice, where the time taken to traverse the beam in order to reach a goal platform was measured. The knockout mice did not differ significantly from the wild-type controls in any of the trials (Fig. 4C).

Discussion

In this study, we demonstrated that mice with a deletion of the α-9 gene display normal vestibular function. We hypothesized that the loss of the α-9 subunit used in vestibular efferent signaling would result in deficits in the development of vestibular hair cells and consequently alter the information transmitted by vestibular afferents, disrupting normal vestibular functions. However, characteristic features of the VOR, such as gain and phase, did not significantly differ between α-9 knockout null mutants and α-9 wild type specimens. Additionally, the lack of significant differences in OKN response dynamics confirmed that loss of the α-9 gene has no effect downstream of the afferents within the VOR pathway, specifically on neurons in the vestibular nuclei. Furthermore, the rotor rod and balance beam paradigms suggest that vestibular postural control pathways remain functional despite the absence of the α-9 gene.

The α-9 subunit and EVS function

It is well established that acetylcholine is the main neurotransmitter released by the efferent vestibular system (EVS) onto peripheral vestibular targets. (3, 20-21) Though EVS's pattern of innervation is species-dependent, (3, 22) birds, reptiles, and mammals exhibit efferent innervation onto bouton and calyx vestibular afferents, type II hair cells, and transient interactions with type I hair cells. The α-9 subunit investigated in this study plays a functional role within the α-9/10 nicotinic acetylcholine receptor, which mediates efferent inhibition. (22-23) Despite detailed knowledge of the circuitry, the function of the EVS is still the object of contention. Often, the auditory efferent system is used as a means of comparison when considering the function of the EVS due to the similarities between the systems and the known functional role of the auditory efferent system.

A recent demonstration of such a parallel between these systems is their mutual reliance on the neurotransmitter calcitonin gene-related peptide (CGRP) for efferent signaling. The lateral olivocochlear (LOC) subdivision of the auditory efferent system innervates cochlear afferents and signals using CGRP, ACh, and gamma-aminobutyric acid (GABA). (24) While the loss of the α isoform of the CGRP gene does not affect the function of the medial olivocochlear (MOC) efferent subdivision, which is involved in preventing acoustic trauma from injuring the cochlea, (25) it does result in a large attenuation of nerve activity in the auditory system. Similarly, CGRP is also involved in efferent signaling in the vestibular system. It has been shown that the loss of αCGRP results in a 50% reduction in the gain, but not phase, of VOR. (26)

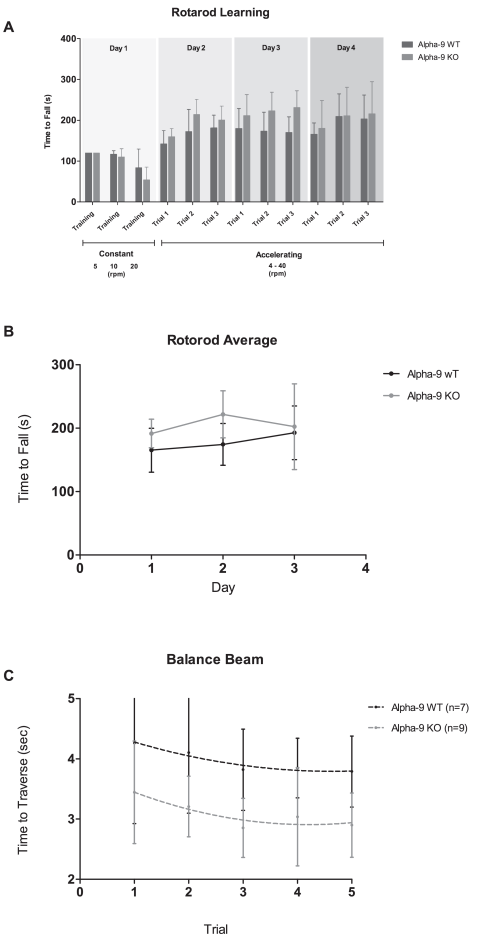

Fig. 4. Balance and postural regulation remain unaffected between strains for rotorod (A, B) and balance beam (C) paradigms. (A) Time to fall measured per trial (mean±SD) for α-9 (+/+) (n=7) and α-9 (-/-) (n=9). (B) Time to fall averaged across trials per day demonstrate non-significant difference between strains. (C) Time to traverse the beam unaffected by α-9 knockout.

Within the auditory system, α-9/10 nAchRs have been shown to potentially influence synaptic strength through transient synapses and prevent deterioration of auditory hair cells by mediating auditory efferent signaling. (23, 27) Specifically, the α-9 subunit appears to play a functional role in auditory hair cell maturation by influencing gene expression during development. (10, 28) These findings suggest a potential role for the α-9 subunit in vestibular end organ development through vestibular efferent signaling. (29-32) Further supporting this proposition, the vestibular efferent system briefly synapses with Type I hair cells, similar to the transient synapses in the auditory system. These transient vestibular synapses are seen in early stages of development in mammals before calyces have formed and thus make direct contact with Type I hair cells. (33) However, our study suggests that the α-9 subunit is not necessary for normal vestibular function, since α-9 knockout mice and α-9 wild-type control mice did not significantly differ in VOR gain or postural regulation.

The result of this study is surprising given that the α-9 subunit acts in cells that are the initial step in the processing of vestibular information and yet the system is unchanged in its absence. It can therefore be asked whether the α-9 subunit has any fundamental relevance to vestibular end organ development. We first consider that stimulation of the vestibular efferents in mammals produce almost exclusively excitation within the semicircular canal afferents. (22, 34-35) This suggests that a majority of the action from efferents is excitatory within mammals. Efferent-mediated excitation occurs through signaling of α4/β2 nAchR for the fast component of the excitation, (34-37) and potentially through muscarinic acetylcholine receptors for the slow component of the excitation. (38-39) As noted, α-9/10 nAchR

signaling mediates the inhibitory effects of efferent stimulation. Given that this study was performed with mice, it is possible that α-9/10 nAchRs play a negligible role within the EVS of this animal model.

Here we found no significant changes in vestibular function, with respect to both the VOR and postural responses in α-9 knockout mice. However, studies in a different α-9 knockout mouse strain have described some changes in the VOR. Notably, a preliminary report has described changes in the VOR time constant and quick phase generation. (40) Further, a second study has recently reported changes in vestibular compensation after unilateral labyrinthectomy in this same strain. (41) One possible explanation for the discrepancy with our results is the different transgenic model used: we used α-9 knockout mice for which a different location on the α-9 gene (exons 1 and 2) was targeted. Additionally, Hübner and colleagues (41) tested VOR at higher frequencies and peak velocities than were tested in our study. Further experiments will be needed to examine this possibility.

Finally, the lack of vestibular impairment demonstrated in this study may also be the result of compensatory mechanisms that occur during development. It has been demonstrated that transgenic mice with a removal of the α-9 subunit experience no difference in auditory behaviors, in both intensity discrimination and the psychophysical threshold for the detection of tone, when compared to a control strain. (42-43) The possibility arises that similar central and/or peripheral systems could potentially be compensating for such a mutation in the vestibular system as in the auditory system. Thus, any conclusions made on lack of deficits in the vestibular system due to the removal of the α-9 subunit must take such compensation into consideration. If future studies demonstrate a lack of abnormalities in the circuitry connecting efferents with vestibular hair cells, it is still possible that compensatory mechanisms are negating developmental effects due to the deletion of the α-9 subunit. The existence of other neurotrophic factors that may be acting as functional mediators in proper efferent targeting could potentially account for such compensation. To further determine whether the α-9/10 nAchR has a functional role in vestibular end organ development, characterizations of possible compensatory mechanisms as well as any morphological and physiological consequences of a deletion of the α-9 subunit on vestibular hair are required.

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