JARO 19: 483–491 (2018)
DOI: 10.1007/s10162-018-00687-y

Research Article

JARO
Journal of the Association for Research in Otolaryngology

Effects of 3,3′-Iminodipropionitrile on Hair Cell Numbers in Cristae of CBA/CaJ and C57BL/6J Mice

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Received: 12 January 2018; Accepted: 25 June 2018; Online publication: 31 August 2018

ABSTRACT

This study examines absolute hair cell numbers in the cristae of C57BL/6J mice and CBA/CaJ mice from weaning to adulthood as well as the dose required for 3,3′-iminodiproprionitrile (IDPN)-injury of the cristae in C57BL/6J mice and CBA/CaJ mice, the two mouse strains most commonly used by inner ear researchers. In cristae of CBA/CaJ and C57BL/6J mice, no loss of hair cells was observed up to 24 weeks. In both strains, dose-dependent loss of hair cells was observed 7 days after IDPN treatment of 2-month-old mice (IC_{50} = 16.1 mmol/kg in C57BL/6J mice vs. 25.21 mmol/kg in CBA/CaJ mice). Four-month-old C57BL/6J mice exposed to IDPN developed dose-dependent vestibular dysfunction as indicated by increased activity and circling behavior in open field tests and by failure to swim 7 days after treatment. IDPN-hair cell injury in C57BL/6J mice and CBA/CaJ mice represents a fast and predictable experimental model for the study of vestibular degeneration and a platform for the testing of vestibular therapies.

Keywords: hair cell, vestibular, IDPN, mouse, toxicity, cristae

INTRODUCTION

Balance disorders and vertigo are estimated to affect 21–30 % overall (Neuhauser et al. 2008; Mendel et al. 2010) and 36–45 % of the elderly (Gopinath et al. 2009; de Moraes et al. 2011). Balance disorders and vertigo are linked to falls—the cause of 2.8 million treatments in emergency departments and 27,000 deaths for adults over 65 in the USA in 2014 alone (Bergen et al. 2016). Balance disorders and vertigo are also linked to increases in sick leave, in medical consultations, in interruptions to daily life, and in levels of anxiety, stress, and depression (Neuhauser et al. 2008; Gopinath et al. 2009; Mendel et al. 2010; Lin and Bhattacharyya 2012). Findings of a survey study suggest that falls increased in adults over 65 from 28.2 % in 1998 to 36.3 % in 2010 (Cigolle et al. 2015), underscoring the need to treat causes underlying balance dysfunction such as peripheral vestibulopathy.

Approximately one third of all balance dysfunction has a peripheral origin in the vestibular system (Kroenke et al. 2000; Uno et al. 2001; Neuhauser et al. 2008; Gopinath et al. 2009; Yin et al. 2009). While many cases of peripheral vestibulopathy involve inflammation or displacement of the otoliths and are treatable, there is currently no effective treatment for approximately 19 % of those with balance dysfunction (Kroenke et al. 2000). In these cases, the cause is either unknown or due to a loss of the sensory hair cells from the vestibular organs. This can result from disorders such as Ménière's disease, drug-related ototoxicity, or age.

The mammalian vestibular system contains 5 sensory organs—the utricle, the saccule, and the 3 cristae

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at the base of each of the semi-circular canals—each containing support cells and the mechanosensitive hair cells. It is these latter sensory organs that we examine in this study. In outbred (CD-1) mice, the vestibular toxin 3,3′-iminodiproprionitrile (IDPN) lesions hair cells of the cochlea, utricle, saccule, and cristae in a dose-dependent manner, causing hearing loss and vestibular dysfunction (Soler-Martin et al. 2007). C57BL/6J and CBA/CaJ mice are particularly suitable for vestibular research because they have highly sensitive vestibular function as indicated by low thresholds for vestibular evoked potentials relative to many other strains (Ohlemiller et al. 2016). We therefore determined absolute hair cell numbers in the cristae of C57BL/6J mice and CBA/CaJ mice from weaning to adulthood as well as the dose required for IDPN injury of the cristae in C57BL/6J mice and CBA/CaJ mice, the two mouse strains most commonly used by inner ear researchers.

METHODS AND MATERIALS

Hair Cell Counts in Cristae

Animal housing and care was provided by the Department of Comparative Medicine at the University of Washington. All procedures were carried out in compliance with the standards and protocols set forth by the University of Washington's Institutional Care and Use Committee. CBA/CaJ mice (Jackson Laboratories stock #000654; Bar Harbor, ME) and C57BL/6J mice (Jackson Laboratories stock #000664) were used for hair cell counts in anterior, posterior, and horizontal cristae (1–6 mice per age group). Multiple cristae were counted in most mice. Hair cells were lesioned in 2-month-old male mice (2–3 mice per dose) by a single intraperitoneal injection of 8–48 mmol/kg IDPN (Acros Organics) in sterile PBS. Seven days after injection, immunolabeling of whole mount anterior and posterior cristae was performed as described previously (Slowik and Bermingham-McDonogh 2013) using the previously characterized guinea pig anti-Gfi1 (Wallis et al. 2003) raised against amino acids 20–256, a region with no homology to Gfi1b, to specifically mark hair cell nuclei (Wallis et al. 2003; Hertzano et al. 2007; Yang et al. 2010). Briefly, temporal bones were bisected to expose the cristae. Cristae were fixed overnight in 4 % paraformaldehyde/PBS (11.9 mM phosphates, 137 mM NaCl, 2.7 mM KCl) at 4 °C, rinsed in PBS, and permeabilized (30 min. in 0.5 % Triton-X-100/PBS at RT). The cristae were then blocked (3 h in 10 % donkey serum/4 % bovine serum albumin/100 mM glycine/0.5 % Triton-X-100/PBS at RT), incubated overnight in 1:1000 guinea pig anti-Gfi1 (a kind gift form Dr. Hugo J. Bellen at the Baylor College of Medicine in Houston, TX) in blocking solution at RT, and washed 1 day in 0.5 % Triton-X-100/PBS at 4 °C with frequent solution changes. The tissue was then incubated in 1:200 Alexa488-conjugated donkey anti-guinea pig secondary antibody (Jackson Immunoresearch; West Grove, PA) in blocking solution overnight at 4 °C, washed 2 days in 0.5 % Triton-X-100/PBS at 4 °C with frequent solution changes, mounted on slides in Fluoromount-G (SouthernBiotech; Birmingham, AL), and imaged in Nikon NIS Elements (0.5 µm step size using a 20× dry CFI Plan Apochromat VC objective on a Nikon A1R TiE inverted laser scanning confocal microscope). Z-projections and manual cell counts (not blinded) were made using ImageJ. To increase signal:noise in the Z-projections of confocal micrographs with minimal luminosity clipping, linear adjustments to the black and white points were made using the "Levels" tool in Adobe Photoshop v19.1.0. Counters were trained on previously counted files until counts were consistent across counters (i.e., ± 50 cells). Counters were instructed to scroll through each z-series and count each $Gfi1^+$ nucleus once, taking care not to double-count nuclei appearing in multiple sections, by marking each nucleus as it was counted. We combined the hair cell counts made in each anterior and posterior crista into one group because anterior and posterior cristae are very similar in that both contain a hair cell-free region (i.e., the crux eminentia) that mouse horizontal cristae do not contain.

Vestibular Functional Testing and Analysis

For direct quantitation of three indicators of vestibular function (i.e., circling, hyperactivity, and swimming), 7 days after a single intraperitoneal injection of 8–48 mmol/kg 3,3'-IDPN in sterile PBS, 4-month-old male C57BL/6J mice (2–3 mice per dose) were recorded in a 2-min open field test in an empty rat cage followed by a 1-min swim test (i.e., in a cage filled with 37 °C water). For the open field test, 1 min of 20 frames/s video was analyzed. For the swim test, 30 s of 20 frames/s video was analyzed. Mouse movement was tracked manually in video files (TrackMate plugin in ImageJ). For each video frame, a point was made between the mouse's ears. Consecutive points were linked to measure the length of the path in pixels and the rotations (i.e., > 90° turn) of the mouse. Pixels were converted to centimeters by measuring the length of the cage in the image in pixels and the length of the actual cage with a ruler. Distance traveled, distance traveled per unit time (i.e., an average velocity), the percentage of time active, the percentage of time circling, and the total number of rotations were calculated as indicators of hyperactivity and circling behavior. The percentages of time active and inactive were calculated using the sum of the number of frames (0.05 s/frame) in which the mouse moved either > 0.2 or < 0.2 cm, respectively, relative to

the previous and subsequent frames, respectively. The percentages of time circling were calculated using the sum of the number of frames (0.05 s/frame) in which the mouse turned at least 90° relative to the previous frames. Numbers of rotations (i.e., turns > 90°) were counted and binned in 90° increments. Quantitative analysis of vestibular function as described requires no surgery and no equipment other than a video camera (cell phone).

Plots and Statistical Analysis

Metrics were calculated using Microsoft Excel (Redmond, WA). Plots were performed in Prism v5.0f (GraphPad Software; La Jolla, CA). To test for crista hair cell number dependence on age, strain, and crista-type, 3-way ANOVA and multiple linear regression analysis were performed in RStudio v1.1.383. Multiple crista were counted in some mice, raising the possibility of pseudoreplication within some groups. IC_{50} and HillSlope values for IDPN were calculated using the nonlinear regression model $Y = 100/(1 + 10\text{^}((\text{LogIC}_{50}\text{-X})*\text{HillSlope}))$ by way of the "log(inhibitor) vs. normalized response—variable slope" least squares fit in Prism v5.0f. To test for differences in behavioral metrics across the treatment groups, one-way ANOVA with Dunnett's multiple comparisons to untreated control levels were performed in RStudio v1.1.383.

RESULTS

No significant loss of hair cells was evident in the cristae of either CBA/CaJ or C57BL/6J mice from weaning to adulthood. To the contrary, linear regression analysis suggested a small increase in hair cell numbers with age (i.e., ~6 %, see Fig. 1b), although this trend did not reach statistical significance (+ 76.10 ± 39.69 hair cells, S.E., $t = 1.918$, $p = 0.059$). However, ~20–30 % more hair cells were observed in cristae of C57BL/6J mice than in cristae of CBA/CaJ mice (Fig. 1b). For example, mean hair cell numbers in anterior and posterior cristae were 1376 ± 103 S.D. ($n = 32$) in 2–24-week-old C57BL/6J mice versus 972 ± 165 S.D. ($n = 27$) in 2–24-week-old CBA/CaJ mice.

In both strains, more hair cells were observed in anterior and posterior cristae than in horizontal cristae. For example, mean hair cell numbers across all ages were 1376 ± 103 S.D. ($n = 32$) in anterior and posterior cristae versus 1152 ± 81 S.D. ($n = 12$) in horizontal cristae in 2–24-week-old C57BL/6J mice. While the density of the hair cells was consistent across the strains, the mean area of the $Gfi1^+$ field size in cristae was ~20–30 % greater in 2–24-week-old C57Bl/6J mice than in 2–24-week-old CBA/CaJ mice (Fig. 1c, d).

In 2-month-old mice of both strains, dose-dependent loss of hair cells in anterior and posterior cristae was observed after IDPN treatment (Fig. 2a–c). Curve fit analysis of the dose response of hair cell loss after IDPN suggested that CBA/CaJ mice showed greater sensitivity than C57BL/6J mice to IDPN as indicated by lower IC_{50}(95 % CI) = 16.1(14.56 to 17.64) mmol/kg vs. 25.21(24.32 to 26.09) mmol/kg, respectively and steeper HillSlope (95 % CI) = − 0.1042(− 0.1471 to − 0.06137) vs. − 0.05392(− 0.05957 to − 0.04826). Hair cell loss after IDPN varied more at lower concentrations (Fig. 2b, c).

Seven days following IDPN treatment of 4-month-old C57BL/6J mice, vestibular dysfunction was evident in some mice after 32 mmol/kg IDPN and in all mice after > 36 mmol/kg IDPN as indicated by increased activity and circling behavior in open field tests (Fig. 3a–f, Tables 1 and 2) and by failure to swim (Fig. 3g–i). In the open field test, whereas control mice explored mainly along the edges of the field (Fig. 1a) and rotated frequently in ~quarter-turns (Table 1), IDPN-treated mice exhibited increased circling (Fig. 3a, c) and hyperactivity (Fig. 3a, b) in a dose-dependent manner. Mice treated with high IDPN doses moved almost constantly (Fig. 3b) and traveled more distance in the field (Fig. 3d). In mice treated with high IDPN doses, circling bouts of > 3 turns (Table 1), head-bobbing and backward scooting behaviors were also observed.

After low IDPN doses, mice showed variable swimming and floating activity as indicated by the percentage of time inactive in the 30-s period (Fig. 3h). After > 32 mmol/kg IDPN, mice required rescue during the swimming test as they underwent barrel-rolling and full submersion. The amount of time before the mice required rescue shortened with increasing IDPN dose (Fig. 3i). Mice receiving intermediate IDPN doses floated briefly before requiring rescue.

DISCUSSION

We report absolute hair cell numbers in the cristae of C57BL/6J mice and CBA/CaJ mice as well as the dose required for IDPN injury of the cristae in C57BL/6J mice and CBA/CaJ mice, the two mouse strains most commonly used by inner ear researchers. We establish that cristae hair cell numbers (Fig. 2) and related vestibular function (Fig. 3) in C57BL/6J mice are sensitive to IDPN injury, consistent with earlier findings (Table 3).

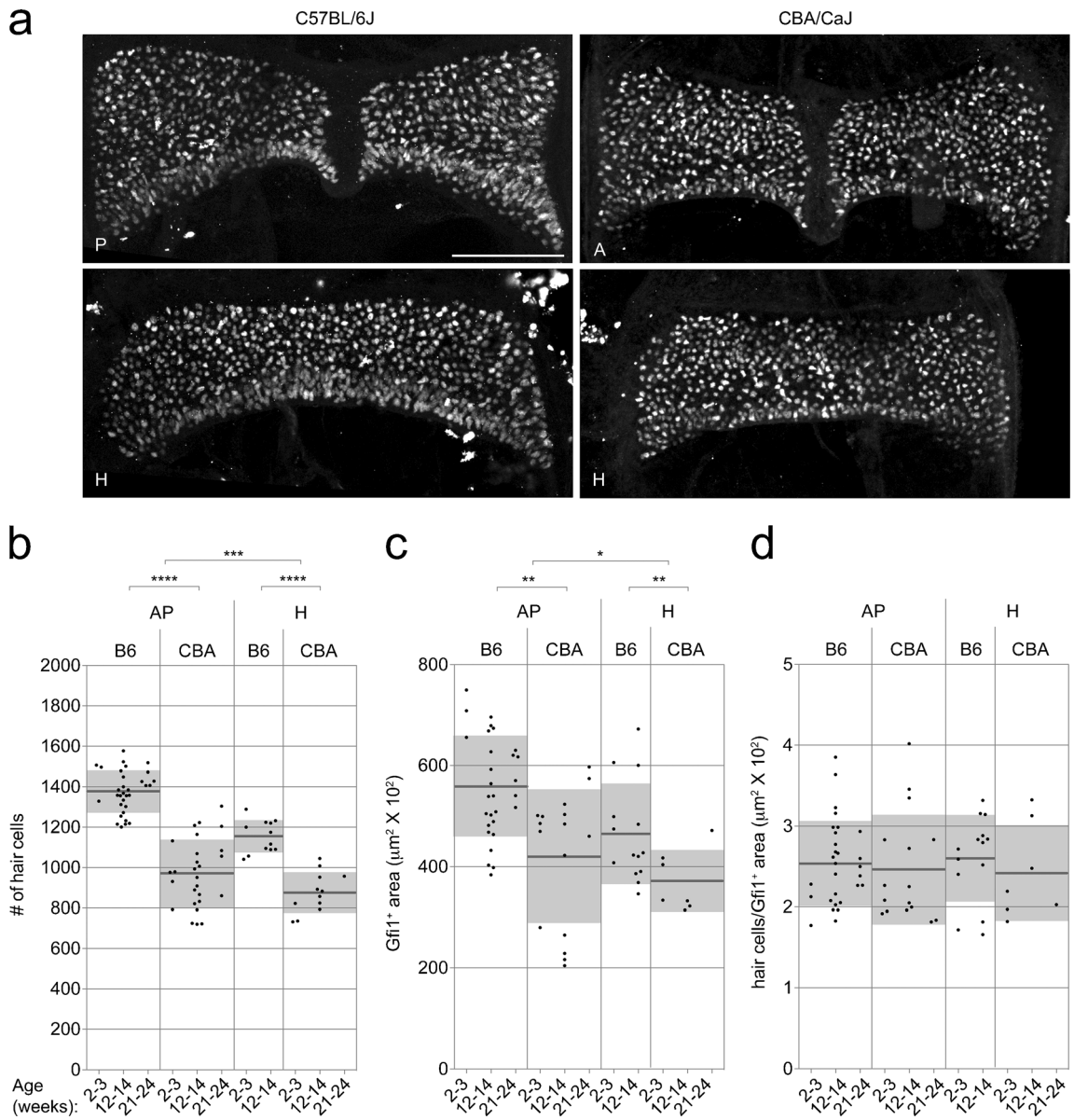

FIG. 1. Hair cells in untreated C57BL/6J and CBA/CaJ mouse cristae. **a** shows Gfi1 immunofluorescence in hair cells of anterior and posterior cristae (AP) and horizontal cristae (H) in 12–14-week-old C57Bl/6J and CBA/CaJ mice. **b** plots hair cell numbers per crista (i.e., 1 dot per crista hair cell count). **c** plots the area of the Gfi1+ field size in cristae. **d** plots crista hair cell density in untreated 0–24-week-old C57BL/6J and CBA/CaJ mice. Bars indicate means and the shaded regions show standard deviation. scale bar = 100 microns; *, + 79.2 $Gfi1^+$ field size in anterior/posterior cristae vs. horizontal cristae $df = 1$, $F = 8.376$, $p = 5.35 \times 10^{-3}$; **, + 123.8 $Gfi1^+$ field size of C57BL/6J cristae vs. CBA/CaJ cristae $df = 1$, $F = 19.9937$, $p = 3.67 \times 10^{-5}$; ***, + 161.5 hair cells in anterior/posterior cristae vs. horizontal cristae $df = 1$, $F = 22.8827$, $p = 8.4 \times 10^{-6}$; ****, + 373 hair cells in C57BL/6J cristae vs. CBA/CaJ cristae $df = 1$, $F = 184.5172$, $p < 2.2 \times 10^{-16}$

Hair cell counts in cristae in the present study are similar to those reported previously in mice (Desai et al. 2005; Fritzsch et al. 2010). Hair cell density measurements in cristae in the present study are similar to those reported previously in mice (Desai et al. 2005). Strain differences in baseline numbers of cristae hair cells are reported: CBA/CaJ mice had ~ 400 fewer hair cells per crista than C57Bl/6J mice (Fig. 1). While the density of the hair cells did not vary between the strains, the mean area of the $Gfi1^+$ field size in cristae was greater in C57Bl/6J mice than in CBA/CaJ mice. Work in the utricle suggests that supporting cell density and the related activity of Yap govern expansion of utricular macula (Gnedeva et al. 2017). Accordingly, we speculate that strain differences in hair cell number could reflect strain differences in the elasticity of surrounding nonsensory tissues constraining the area of the sensory patch.

We were surprised that the hair cell toxicity of IDPN was greater in CBA/CaJ mice than in C57Bl/6J

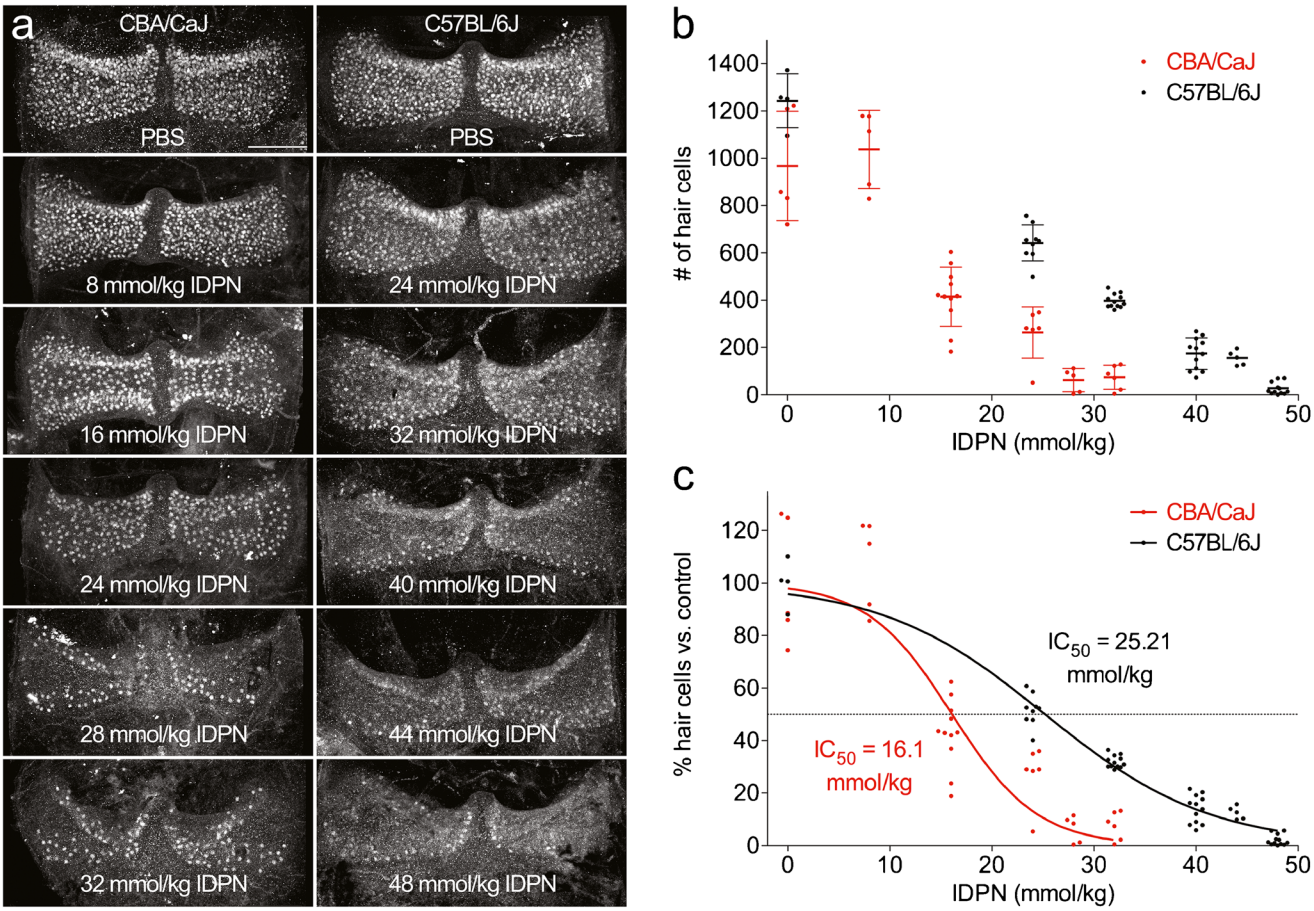

FIG. 2. Hair cell toxicity in C57BL/6J and CBA/CaJ mouse cristae after IDPN. **a** shows Gfi1 immunofluorescence in hair cells of representative anterior and posterior cristae (*AP*) 7 days after the indicated doses of IDPN in 2-month-old male CBA/CaJ and C57BL/6J mice. **b** shows numbers of Gfi1$^+$ hair cells counted in whole mount anterior and posterior cristae. Bars indicate means and error bars indicate standard deviations. **c** replots data in **b** to show the loss of hair cells as a percentage of the mean of hair cell numbers in untreated control mice. Lines show curve fits ($R^2 = 0.8283$ and 0.9650 for CBA/CaJ and C57BL/6J, respectively) of the normalized (i.e., 0–100 %) toxicity responses

mice (Fig. 2). Whether greater IDPN efficacy and lower baseline hair cell numbers per crista in CBA/CaJ mice versus C57Bl/6 mice relates to any of the 7 genes/loci revealed by GWAS that variably influence age-related hearing loss in these strains (Ohlemiller et al. 2016) are open questions. Regardless of the basis for the strain differences, the efficacy of IDPN in CBA/CaJ mice—the standard strain for research of inner ear pathophysiology due to relatively minimal age-related hearing loss (Spongr et al. 1997)—suggests that IDPN could be useful to study vestibular function, and possibly hearing and tinnitus, after peripheral damage. The efficacy of IDPN in C57BL/6J mice suggests that IDPN could be used in widely available transgenic lines such as Sox2-CreER, Glast-CreER, Otof-Cre, and PLP-CreER to study hair cell regeneration in cristae in vivo.

The lack of hair cell loss in the cristae of 24-week-old C57BL/6 mice was surprising given that degeneration of hair cells in the cochlea begins by 1 month of age in C57BL/6J mice (Spongr et al. 1997) related to partial loss of function in *Cadherin23* (*Cdh23*) (Noben-Trauth et al. 2003), a critical component of the tip-link complex (Siemens et al. 2004; Sollner et al. 2004). In contrast to auditory hair cells, however, vestibular hair cells do not degenerate after loss of function in the tip-link complex in mice (Ahmed et al. 2006). Similarly, C57BL/6J mice have no age-related changes in vestibular function as indicated by gain in the vestibulo-ocular reflex (Shiga et al. 2005). Moreover, a recent study controlling for *Cdh23* function in the C57BL/6J background shows that vestibular function as indicated by vestibular sensory evoked potentials is unaffected by either aging or the *Cdh23*^{753A} mutation in C57BL/6J mice (Mock et al. 2016). Differences in central connections and bundle displacement in the vestibular and auditory systems are the proposed basis for the lack of age-related loss of vestibular function in C57BL/6J mice despite hypomorphic *Cdh23* (Mock et al. 2016) and differences in cochlear and utricular

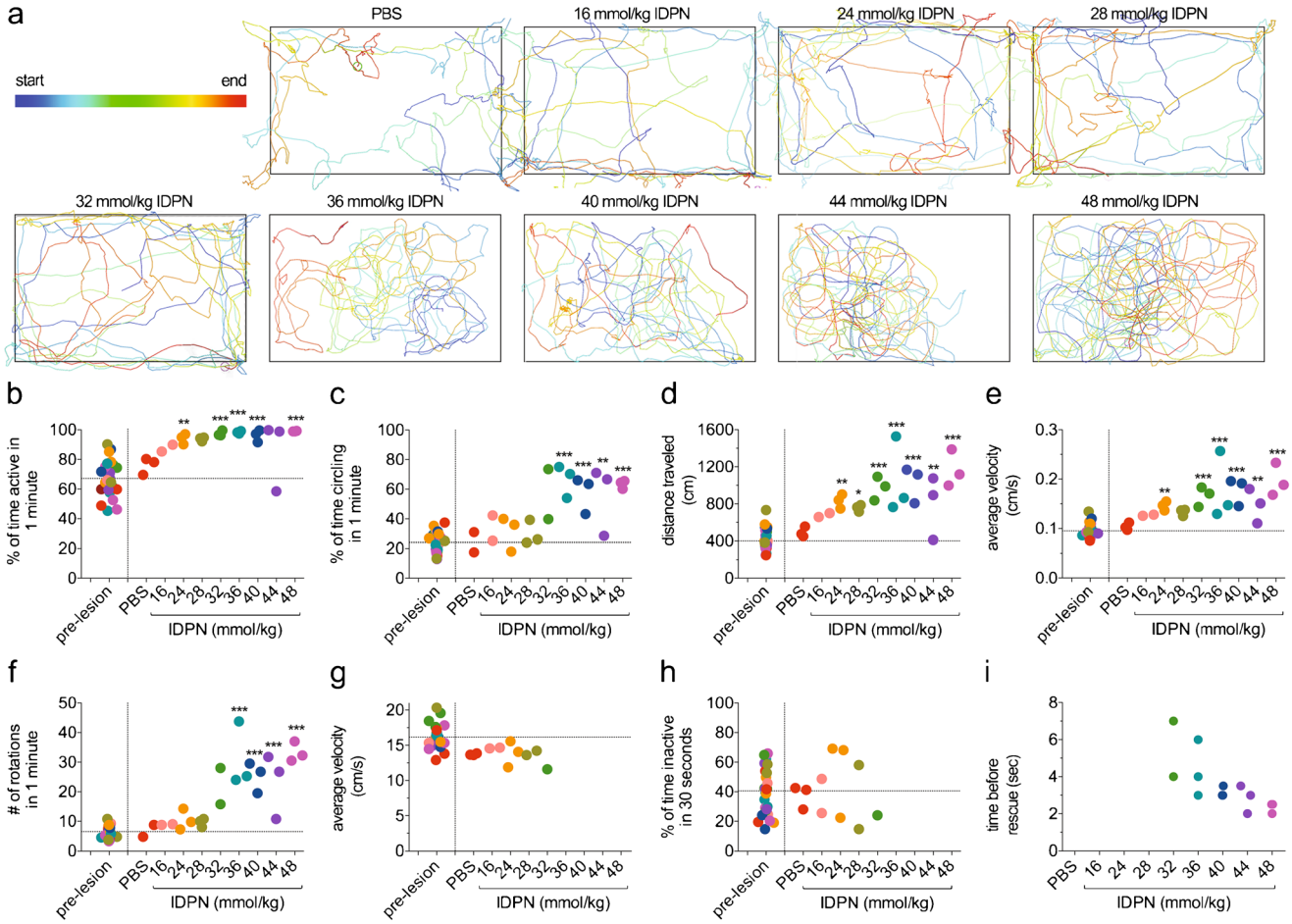

FIG. 3. Vestibular dysfunction after IDPN in C57BL/6J mice. **a–f** show vestibular function as indicated by circling- and hyperactivity-related behaviors in open field tests and **g–i** show behaviors in swimming tests. In **b–i**, each point represents data from an individual mouse, and two points (pre-lesion and post-lesion) are shown for each mouse. Points are color-coded by what dose of IDPN the animal received. Medians of pre-lesion metrics are indicated by horizontal lines. In **a**, examples of paths (*blue* = start, *red* = end) of mice in an open field (indicated by the black box) are shown over a 1-min period 7 days after the indicated treatments. Traces outside of the field indicate the mouse was climbing onto the cage edges. Note that path length and circling increase with IDPN. **b** shows the percentage of time actively moving in 1 min. **c** shows the percentage of time actively circling in 1 min. **d** shows the total distance traveled in 1 min. **e** shows the relationship of the total distance traveled to time. **f** shows the numbers of rotations (i.e., > 90°) observed in 1 min. **g** shows the relationship of the total distance traveled to time in 30-s swimming tests. Missing are data from mice that had to be rescued after treatment with 32 mmol/kg IDPN. **h** shows the percentages of time mice were inactive (i.e., floating) during the 30-s swimming tests. Again, missing are data from mice that had to be rescued. **i** shows the time before rescue of mice that failed the 30-s swimming tests. $^{***}p < 0.001$ versus untreated control; $^{**}p < 0.01$ versus untreated control; $^{*}p < 0.05$ versus untreated control

hair cells are described by transcriptomic studies (Burns et al. 2015) though the cristae hair cells have not been compared in this way. Thus, our findings are consistent with evidence from studies of vestibular function in aging C57BL/6J mice.

The lack of hair cell loss in the cristae of 24-week-old CBA/CaJ mice was surprising given that these mice have gradual age-related decline in vestibular function, i.e., a 2.17 % decline in vestibular sensory evoked potential dynamic range per month, and by 23 months of age, showed an average loss of nearly 50 % in vestibular sensory evoked potential dynamic range (Mock et al. 2011). By 6 months, the oldest mice used in our study there should have been on average a 13 % loss of vestibular function. Moreover, CBA/CaJ mice have age-related hearing loss after 18 months (Li and Borg 1991) associated with loss of hair cells (Spongr et al. 1997). Unpublished findings suggest that age-related decline in vestibular function in CBA/CaJ mice is associated with decreased ribbon synapse density (*personal communication from S. Jones*).

Whereas the present study of IDPN injury focused on hair cell loss in the cristae and related vestibular dysfunction, findings from other studies have established that IDPN affects other systems including the cochlea (Soler-Martin et al. 2007). Moreover, histopathological changes in IDPN-treated rats (Seoane et al. 2001) suggest that IDPN elicits a pattern of progressive degradation of hair cells (i.e., membrane rupture consistent with necrosis in hair cells

TABLE 1

Rotations before and after IDPN versus PBS control

	Pre-lesion	*PBS*	*IDPN (mmol/kg)*							
			16	*24*	*28*	*32*	*36*	*40*	*44*	*48*
90°	21.4 (6.89)	23 (8.49)	27 (4.25)	31.7 (2.31)	32.3 (4.1)	25.5 (23.3)	22.7 (11.6)	19.3 (6.51)	19.3 (4.2)	31.3 (18.2)
180°	2.1 (1.18)	2 (1.42)	3.5 (0.71)	4 (4.59)	3 (1.74)	9.5 (0.71)	15.7 (9.51)	13 (5)	11.7 (7.1)	18.7 (6.51)
270°	0.1 (0.42)	n.o.	0.5 (0.71)	0.7 (1.16)	n.o.	3 (2.83)	6.7 (4.05)	4 (2.65)	6 (5.57)	7.7 (2.31)
360°	0.1 (0.21)	n.o.	n.o.	n.o.	n.o.	1.5 (2.13)	3 (2.65)	2.3 (0.58)	2.3 (1.53)	3 (2)
450°	n.o.	n.o.	n.o.	n.o.	n.o.	2 (2.83)	0.34 (0.58)	1.7 (0.58)	1.3 (1.16)	1 (1)
540°	n.o.	n.o.	n.o.	n.o.	n.o.	0.5 (0.71)	1.7 (0.58)	0.3 (0.58)	0.7 (0.58)	0.3 (0.58)
630°	n.o.	n.o.	n.o.	n.o.	n.o.	0.5 (0.71)	1 (1.74)	1 (1)	0.7 (0.58)	n.o.
720°	n.o.	n.o.	n.o.	n.o.	n.o.	0.5 (0.71)	0.7 (0.58)	n.o.	n.o.	1.3 (0.58)
810°	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0.3 (0.58)	n.o.	n.o.
900°	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	1 (1)	0.3 (0.58)	n.o.
990°	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0.3 (0.58)	0.3 (0.58)
1080°	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0.3 (0.58)	0.3 (0.58)	n.o.	0.7 (0.58)
1170°	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0.3 (0.58)	n.o.	n.o.	n.o.
1260°	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0.3 (0.58)	n.o.	n.o.	n.o.
1350°	n.o.	n.o.	n.o.	n.o.	n.o.	0.5 (0.71)	0.3 (0.58)	n.o.	n.o.	n.o.
1440°	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.
1530°	n.o.	n.o.	n.o.	n.o.	n.o.	n.o.	0.3 (0.58)	n.o.	n.o.	n.o.
n	23	2	2	3	3	2	3	3	3	3

Values are means and standard deviations of counts of rotations of the indicated degrees (i.e., 0.5–8.5 turns) in the indicated numbers of C57BL/6J mice (*n*) during 1 min of observation

n.o. none observed

after 1 day, apoptotic figures and TUNEL-labeling by 4 days, and extrusion of hair cells 5 weeks after treatment), rather than a single period of rapid loss. Limited spontaneous hair cell regeneration and recovery of calyceal junctions are reported after IDPN (Schlecker et al. 2011; Sedó-Cabezón et al. 2015). Thus, a limitation of this study and of the IDPN-vestibular injury model is that IDPN has additional effects that raise the possibility of complex systemic interactions during IDPN exposure. For example, IDPN treatment of rats is reported to increase apoptosis as indicated by Caspase 3 immunolabeling in anterior pituitary cells and in spermatids 4–8 days after treatment (Takahashi et al. 2014). Another study reports histopathological changes in the kidney and liver by day 9 after IDPN treatment in mice (Khan and Ibrahim 2015). Although histopathological changes were not observed in cerebral cortex after IDPN (Khan and Ibrahim 2015), neural function should be evaluated systematically after IDPN to ascertain whether IDPN-toxicity outside the inner ear could contribute to the behavioral changes attributed to vestibular dysfunction. Moreover, a study comparing the onset and extent of vestibular dysfunction after a hair cell-specific lesion [e.g., hair cell-specific lesions induced via Pou4f3-CreER-mediated diphtheria toxin receptor (Buch et al. 2005)] to those after IDPN could ascertain whether IDPN-toxicity outside the inner ear contributes to vestibular dysfunction.

Another recent study reported no significant differences in vestibular dysfunction after IDPN in RjOrl:Swiss/CD-1 mice versus 129S1/SvImJ mice

TABLE 2

Statistical summary of IDPN effects on indicators of vestibular function in open field tests

		% time active in 1 min		*% time circling in 1 min*		*Distance traveled (cm)*		*Average velocity (cm/s)*		*# of rotations in 1 min*	
Tests	*df*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*	*F*	*p*
ANOVA	8	9.757	*1.58E-07*	9.235	*2.72E-07*	16.91	*6.90E-11*	14.92	*4.57E-10*	19.97	*3.62E-12*
Dunnett's	*df*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*	*t*	*p*
16 vs. 0	1	2.472	0.1269	1.293	0.8151	2.084	0.2838	1.732	0.5072	0.862	0.9768
24 vs. 0	2	3.95	*0.0023*	1.268	0.8298	4.047	*0.0017*	3.498	*0.0088*	1.475	0.6951
28 vs. 0	2	3.904	*0.0027*	1.088	0.9166	3.277	*0.0165*	2.551	0.1057	1.219	0.8571
32 vs. 0	1	4.473	*0.0005*	2.044	0.3049	5.486	*< 1e-04*	4.964	*< 1e-04*	2.76	*0.0637*
36 vs. 0	2	4.595	*0.0003*	5.495	*< 1e-04*	6.289	*< 1e-04*	5.849	*< 1e-04*	7.819	*< 1e-05*
40 vs. 0	2	4.266	*0.0009*	4.425	*0.0005*	6.074	*< 1e-04*	5.818	*< 1e-04*	6.047	*< 1e-05*
44 vs. 0	2	2.685	0.0771	4.164	*0.0012*	3.678	*0.0052*	3.577	*0.0070*	5.379	*0.0000*
48 vs. 0	2	4.709	*0.0002*	5.13	*< 1e-04*	7.462	*< 1e-04*	7.223	*< 1e-04*	8.512	*< 1e-05*

TABLE 3

Summary of IDPN effects reported in the mouse vestibular system relative to the present study

	Dose	*Mice*	*IDPN effects*
Present study	8–48 mmol/kg (1 dose)	2–4-month-old C57BL/6J and CBA/CaJ mice	Hair cell loss in cristae by day 7; increased circling and activity in open field, failure to swim by day 7
(Boadas-Vaello et al. 2017)	8–24 mmol/kg (1 dose)	RjOrl:Swiss/CD-1 and 129S1/SvImJ	Diminished hair bundle density in utricle by 22–24 days after treatment; increased circling and activity in open field by 3 days after treatment in both strains
(Khan and Ibrahim 2015)	3.25 mmol/kg (7 daily doses)	30–35 g adult SWR/J mice	Diminished hair bundles in cristae by day 9, increased circling in open field by day 7
(Schlecker et al. 2011)	0.97 mmol/kg (1 dose)	2-month-old C57BL/6 mice	Diminished Myo7a immunofluorescence in crista, saccule, and utricle by day 7; reduced time on rotarod by day 3, urther reductions in rotarod time at days 7 and 11
(Soler-Martin et al. 2007)	8–24 mmol/kg (1 dose)	2-month-old CD-1 mice	Diminished hair bundle density in cristae and saccule by 5–12 weeks; increased activity in open field by 1 day with further increases at 3, 6, and 14 days
(Khan et al. 2004)	6.5 mmol/kg (5 daily doses)	2–8-month-old wildtype littermates of B6C3Fe a/a-Cacng2stg/J mutants	Fell off rotarod, failed to swim, increased circling and activity in open field by 2 weeks after treatment

(Boadas-Vaello et al. 2017), though a faster onset of vestibular dysfunction in female mice of both strains was noted. As we were not aware of these findings, the present study was not designed to examine gender differences in the onset of dysfunction. Gender differences in vestibular function as indicated by vestibular sensory evoked potentials were not found in C57BL/6J (Mock et al. 2016) or CBA/CaJ mice (Mock et al. 2011). Whether gender plays a role in IDPN-vestibular injury in C57BL/6J and CBA/CaJ, however, remains an open question for a future study.

Considering that over half of cristae hair cells were lost after > 32 mmol/kg IDPN in both strains examined (Fig. 2) and that type I and type II hair cells are present in 1.17:1 ratio in mouse cristae (Desai et al. 2005), we infer that IDPN affected both hair cell types. The present study did not examine the relative losses of type I versus type II hair cells. The present study analyzed IDPN efficacy in anterior and posterior cristae and did not compare IDPN efficacy between the cristae. Additionally, whether IDPN affects support cells, transitional epithelial cells or glia of the vestibular organs are open questions.

Currently vestibular prostheses are being developed and there has been limited success using inhibitors of the Notch pathway to regenerate some hair cells (Hori et al. 2007; Lin et al. 2011; Jung et al. 2013; Mizutari et al. 2013; Slowik and Bermingham-McDonogh 2013). Just recently, it has been recognized that there is limited ongoing turnover of hair cells in the utricle: hair cells are replaced by support cells under normal conditions and this replacement is increased in response to hair cell damage (Bucks et al. 2017). While multiple therapeutic strategies to treat hair cell loss are currently under investigation, including protection and repair of hair cells as well as vestibular prostheses, regenerative therapies provide an additional and potentially better approach to replace lost hair cells. IDPN injury in the mouse represents a platform for testing such therapies.

ACKNOWLEDGEMENTS

Special thanks to Dr. Amber Slowik for her contributions to this work, Dr. Lisa Cunningham for commenting on an earlier version, Dr. Thomas Reh for critical comments, and Dr. Sherri Jones for sharing data prior to publication.

Funding information This work was supported by NIDCD grants R21DC010862 (OBM), F32DC016480, and T32DC005361 (BAW).

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