VESTIBULOTOXIC PROPERTIES OF POTENTIAL METABOLITES OF ALLYLNITRILE

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ABSTRACT. This study addressed the hypothesis that epoxidation of the double bound in allylnitrile mediates its vestibular toxicity, directly or after subsequent metabolism by epoxide hydrolases. The potential metabolites 3,4-epoxybutyronitrile and 3,4-dihydroxybutyronitrile were synthesized and characterized. In aqueous solutions containing sodium or potassium ions, 3,4-epoxybutyronitrile rearranged to 4-hydroxybut-2-enenitrile, and this compound was also isolated for study. Male adult Long-Evans rats were exposed to allylnitrile or 3,4-epoxybutyronitrile by bilateral trans-tympanic injection, and vestibular toxicity was assessed using a behavioral test battery and scanning electron microscopy (SEM) observation of the sensory epithelia. Overt vestibular toxicity was caused by 3,4-epoxybutyronitrile at 0.125 mmol/ear and by allylnitrile in some animals at 0.25 mmol/ear. Additional rats were exposed by unilateral trans-tympanic injection. In these studies, behavioral evidences and SEM observations demonstrated unilateral vestibular toxicity after 0.125 mmol of 3,4-epoxybutyronitrile and bilateral vestibular toxicity after 0.50 mmol of allylnitrile. However, 0.25 mmol of allylnitrile did not cause vestibular toxicity. Unilateral administration of 0.50 mmol of 3,4-dihydroxybutyronitrile or 4-hydroxybut-2-enenitrile caused no vestibular toxicity. The four compounds were also evaluated in the mouse utricle explant culture model. In 8h exposure experiments, hair cells completely disappeared after 3,4-epoxybutyronitrile at concentrations of 325 or 450 μM, but not at concentrations of 150 μM or lower. In contrast, no difference from controls was recorded in utricles exposed to 450 μM or 1.5 mM of allylnitrile, 3,4-dihydroxybutyronitrile or 4-hydroxybut-2-enenitrile. Taken together, the present data support the hypothesis that 3,4-epoxybutyronitrile is the active metabolite of allylnitrile for vestibular toxicity.

Keywords: ototoxicity, vestibular toxicity, nitrile, xenobiotic metabolism, 3,4-epoxybutyronitrile, hair cell.

INTRODUCTION

Nitriles are compounds containing cyano (R-CN) groups. They have wide industrial use as solvents and chemical intermediates in the synthesis of plastics, nylons, and elastomers (DeVito, 2007). Many nitriles occur also as natural compounds (Jones, 1998). The four carbon allylnitrile (CAS number 109-75-1) occurs as an industrial product and also as a natural compound (Tanii et al., 2004). Major toxic effects of nitriles are acute lethality, osteolathyrism and neurotoxicity, including sensory toxicity (DeVito, 1996; Crofton and Knight, 1991; Genter et al., 1992; Llorens et al., 1993; Llorens et al., 2011; Selye, 1957; Seoane et al., 1999). In several species, including mammalian and non-mammalian, the vestibular system has been reported to be a major target for 3,3'-iminodipropionitrile (IDPN), allylnitrile, *cis*-crotononitrile, and *cis*-2-pentenenitrile. These nitriles cause degeneration of the vestibular sensory hair cells (HC) and the resulting deficit in vestibular function is recognized by a permanent alteration in the motor behavior of the animals (Llorens et al., 1993; Llorens and Rodríguez-Farré, 1997; Balbuena and Llorens 2001, 2003; Boadas-Vaello et al., 2005; Soler-Martín et al., 2007; Saldaña-Ruíz et al., 2012a,b). Another vestibulotoxic nitrile in the mouse is *trans*-crotononitrile (Saldaña-Ruíz et al., 2012b) although in the rat this nitrile causes neuronal degeneration in discrete brain areas rather than vestibular toxicity (Seoane et al., 2005; Boadas-Vaello et al., 2005).

For several of the vestibulotoxic nitriles there is evidence that metabolic bioactivation is required for vestibular toxicity (Boadas-Vaello et al., 2009; Saldaña-Ruíz et al., 2012a). In the case of IDPN, a hypothesis on metabolic bioactivation has received considerable attention and indirect support (Morandi et al., 1987; Jacobson et al., 1987; Denlinger et al., 1992, 1994; Nace et al., 1997), but no direct evidence has been obtained, and conflicting results are also available (Llorens and Crofton., 1991). So far, the active metabolite(s) and pathways involved in vestibular toxicity have not been identified for any nitrile. In recent years, we have used CYP2E1-null mice to evaluate the hypothesis that CYP2E1-mediated metabolism is responsible for the bioactivation. Available data demonstrate that many low molecular weight nitriles are CYP2E1 substrates, but that in no case is CYP2E1-mediated metabolism associated with vestibular toxicity. Instead, this enzyme appears to frequently be responsible for cyanide release and acute mortality, probably through α-carbon hydroxylation (Boadas-Vaello et al., 2007, 2009; Saldaña-Ruíz et al., 2012b). In the case of allylnitrile, the data obtained led us to hypothesize that epoxidation of the β–γ double bond, perhaps by

CYP2A5, mediates vestibular toxicity either directly, or after subsequent opening of the epoxide by epoxide hydrolase activities (Fig. 1) (Boadas-Vaello et al., 2009). To address this hypothesis, we have now synthesized the hypothesized allylnitrile metabolites, and evaluated their vestibular toxicity *in vivo* and *in vitro* in comparison to that of allylnitrile. These included initially 3,4-epoxybutyronitrile (CAS #: 624-58-8; oxiran-2-ylacetonitrile), and 3,4-dihydroxybutyronitrile (CAS #: 83527-35-9; 3,4-dihydroxybutanenitrile). A third allylnitrile derivative, 4-hydroxybut-2-enenitrile (CAS #: 10479-81-9; (*2E*)-4-hydroxybut-2-enenitrile), was identified as a spontaneous rearrangement product of 3,4-epoxybutyronitrile in aqueous solutions containing alkaline ions. This compound was also isolated and evaluated. The data obtained demonstrate a direct toxic effect of 3,4-epoxybutyronitrile on the vestibular sensory epithelia.

METHODS

Chemicals and reagents

Allylnitrile (>98%) was purchased from Merck-Schuchard (Hohenbrunn bei München, Germany). Potassium fluoride, meta-chloroperbenzoic acid (m-CPBA), glycidol, $LiClO_4$, glutaraldehyde solution (25%), glucose and penicillin-G were from Sigma-Aldrich Química S.A. (Tres Cantos, Spain), and dichloromethane and acetonitrile from Panreac Química (Castellar del Vallès, Spain). The Mowiol 4-88 Reagent was from Calbiochem (Merck KGaA, Darmstadt, Germany). Dulbecco's Modified Eagle Medium – Nutrient Mixture F12 (DMEM-F12) without L-glutamine, L15 (Leibovitz) medium, N2 supplement, GlutaMAX, and 1M HEPES buffer were from Life Technologies (Barcelona, Spain). Mouse monoclonal anti-calmodulin (clone 6D4) was obtained from Sigma, and rabbit polyclonal anti-myosin-VIIa from Proteus Biosciences (Alcobendas, Spain). Alexa-fluor-555-conjugated phalloidin, Alexa-fluor-647 donkey anti mouse IgG, and Alexa-fluor-488 donkey anti-rabbit IgG were obtained from Molecular Probes (Invitrogen S.A., Prat del Llobregat, Spain).

Synthesis of allylnitrile derivatives

Analytical methods

Nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on a Varian Unity 300 MHz machine and a Varian Inova 500 apparatus (^{1}H NMR, 500 MHz; ^{13}C NMR, 125 MHz). Chemical shifts are given in ppm (δ) relative to the $CDCl_3$ signal (7.24

ppm for ^{1}H NMR and 77.23 ppm for ^{13}C NMR), and coupling constants (J) are reported in Hertz (Hz). Multiplicities are reported using the following abbreviations: d, doublet; t, triplet; and m, multiplet. Gas chromatography analyses were carried out on a Carlo Erba MFC 500 chromatograph coupled to a Fisons NPD 800 detector with a GasPRO column (60 m x 0.32 mm internal diameter) or a J&W DB-WAX column (30 m x 0.32 mm).

3,4-Epoxybutyronitrile

3,4-Epoxybutyronitrile was obtained following the procedure described by Fleming et al. (2001) with minor modifications. *m*-Chloroperoxybenzoic acid (20 g, 0.75 eq. each day) was added to a solution of allylnitrile (12 ml, 1 eq.) in dichloromethane (300 ml) for three days. The reaction mixture was stirred at room temperature for five more days (Fig. 2A). Then the organic acids were eliminated by stirring the crude reaction mixture with an excess of KF (13 g, 2 eq.) for 2 h at room temperature, followed by filtration of the insoluble material (Camps et al., 1981). The filtrate was carefully concentrated and purified by vacuum distillation to give 8.0 g of the desired epoxide (62 ºC at 5 mbar) in 64% yield. ^{1}H-NMR ($CDCl_3$): (Supplementary Fig. 1A) 2.75 (d, J= 4.6, 2H); 2.89 (t, J=4.2, 2H); 3.22 (dt, J_1= 4.2, J_2= 8.1, 1H); ^{13}C-NMR: 20.06; 45.43; 46.53; 115.96.

3,4-Dihydroxybutyronitrile

To obtain 3,4-dihydroxybutyronitrile (Jung and Shaw, 1980), NaCN (1.32 g, 1.5 eq.) and $LiClO_4$ (0.97 g, 0.5 eq.) were added to a solution of glycidol (1.2 ml, 1 eq.) in CH_3CN (21 ml) and the mixture was stirred for 3 hours at 55ºC (Fig. 2.B) (Arnone et al., 1995), diluted with a NaCl saturated solution (0.325 ml, 1 eq.) and an excess of *tert*-butyl methyl ether (20 ml), and filtered over Celite (R). The residue obtained from solvent elimination contained the expected 3,4-dihydroxybutyronitrile (as a *R,S* stereoisomer mixture) in 94% yield (1.70 g). ^{1}H-NMR (D_2O): (Supplementary Fig. 1B) 2.75 (d, J= 4.6, 2H); 2.89 (t, J=4.2, 2H); 3.22 (dt, J_1= 4.2, J_2= 8.1, 1H); ^{13}C-NMR: 20.06; 45.43; 46.53; 115.96.

4-Hydroxybut-2-enenitrile

To obtain 4-hydroxybut-2-enenitrile (Nudelman and Keinan, 1982), a solution of 3,4-epoxybutyronitrile (1 ml, 1 eq.) in phosphate buffered saline (PBS, 5 ml) was stirred for 4 h at room temperature (Fig. 3). The crude reaction mixture was extracted with

ethyl acetate and the collected organic fractions were dried over Na_2SO_4 and concentrated to give 0.89 g of the desired compound (89% yield). ^{1}H-NMR (D_2O): (Supplementary Fig. 1C): 4.37 (m, 2H); 5.74 (dt, J_1= 2.2, J_2= 16.2, 2H); 6.83 (dt, J_1= 3.4, J= 16.2, 1H); ^{13}C-NMR: 60.60; 97.03; 117.2; 134.9; 155.2.

Animals

The care and use of animals were in accordance with Acts 5/1995 and 214/1997 of the Regional Government of Catalonia, and approved by the University of Barcelona's Ethics Committee on Animal Experiments. Eight- to nine-week-old male Long-Evans rats (CERJ, Le-Genest-Saint-Isle, France) were used for *in vivo* studies. They were housed two to four per cage in standard Macrolon cages (280 x 520 x 145 mm) with wood shavings as bedding. They were acclimatized for at least seven days before experimentation. For *in vitro* studies, 3- to 6-month-old male and female 129S1/SvImJ mice were used. They were obtained from a local colony established by breeding pairs obtained from the Jackson Laboratory (Bar Harbor, ME, USA). After weaning, mice were housed two to six per cage in standard Macrolon cages (28 x 28 x 15 cm) with wood shavings as bedding. Rats and mice were maintained on a 12:12 L:D cycle (0700:1900 h) at 22 $\pm$ 2ºC and given standard diet pellets (TEKLAD 2014, Harlan Interfauna Ibérica, Sant Feliu de Codines, Spain) *ad libitum*.

Dosing

Animals were dosed by trans-tympanic injection as described below. For complete behavioral assessment, we used bilateral administration of control vehicle (50 µl propylene glycol, n=4) or nitrile solutions as follows: 3,4-epoxybutyronitrile was dosed at 0.0312 mmol/ear (n=2), 0.0625 mmol/ear (n=2), 0.125 mmol/ear (n=3), and 0.250 mmol/ear (n=3). Allylnitrile was dosed at 0.125 mmol/ear (n=5), and 0.250 mmol/ear (n=4). Other animals were administered 3,4-epoxybutyronitrile (0.125 mmol, n=5), allylnitrile (0.25 mmol, n=5; 0.50 mmol, n=4), 4-hydroxybut-2-enenitrile (0.50 mmol, n=4), and 3,4-dihydroxybutyronitrile (0.50 mmol, n=3) on one side only.

Trans-tympanic exposure

The inner ear can be exposed to chemical agents via diffusion from the middle ear after trans-tympanic injection (Parnes et al., 1999). This is a route that is increasingly used for therapeutic drug delivery in humans suffering auditory and

vestibular diseases (Leary Swan et al., 2008). In laboratory animals, trans-tympanic exposure is a well-established model for ototoxicity studies (Horn et al., 1981; Sera et al., 1987; Llorens and Rodríguez-Farré, 1997; Janning et al., 1998).

Rats were anesthetized with isoflurane using a standard vaporizer for small animals (Leica Microsistemas S.L.U., Barcelona, Spain) and they were also administered a dose of analgesia (buprenorphine, 0.05 mg/kg, s.c.). The animals were then placed on their side and, using a surgical microscope, the tympanic membrane was punctured with a 29 G needle to administer 50 µl of nitrile solution into the middle ear cavity. In the case of bilateral exposure, the animals were maintained under anesthesia on their side for 10 min before turning them for administration into the second ear. Once the intratympanic administrations had been completed, the animals were given a dose of meloxicam (0.5 mg/kg, s.c.) and observed for complete recovery from the isoflurane anesthesia. A second dose of the meloxicam analgesia was administered 24 h later.

Behavioral analysis

Disturbance of vestibular function was determined using a battery of behavioral tests well suited to evaluate the bilateral symmetrical loss that occurs following systemic exposure to ototoxic nitriles (Llorens et al., 1993; Llorens and Rodríguez-Farré, 1997; Boadas-Vaello et al., 2005). Briefly, rats were placed for one minute in a 50 x 50 cm glass cube and the experimenter rated the animals from 0 to 4 for circling, retropulsion and abnormal head movements. Circling was defined as stereotypical circling ambulation. Retropulsion consisted of backward displacement of the animal. Head bobbing consisted of intermittent extreme backward extension of the neck. The rats were then rated 0-4 for the tail-hang reflex, contact inhibition of the righting reflex, and air righting reflex tests. When lifted by the tail, normal rats exhibit a "landing" response consisting of forelimb extension. Rats with impaired vestibular function bend ventrally, sometimes "crawling" up towards their tails, thus tending to occipital landing. For the contact inhibition of the righting reflex, rats were placed supine on a horizontal surface and a metal bar grid was lightly placed in contact with the soles of their feet. Healthy rats quickly right themselves, whereas vestibular-deficient rats lie on their back with their feet up and "walk" on the ventral surface. For the air righting reflex, animals were held supine and dropped from a height of 40 cm onto a foam cushion. Normal rats are successful in righting themselves in the air whereas vestibular-deficient rats are not. A summary statistic was obtained by

adding up the scores for all behavior patterns.

In addition, animals were observed for signs of asymmetry in the vestibular damage (Saxon and White, 2006; Vignaux et al., 2011). Unilateral vestibular damage causes animals to tilt their heads to the side. In the tail hang test, unilateral lesions cause body rotation around the tail axis rather than ventral bending.

Corneal opacity

Rats were also observed for presence / absence of corneal opacity. Systemic exposure to vestibulotoxic doses of allylnitrile has been reported to cause dose-dependent opacity of the cornea (Balbuena and Llorens, 2001).

Assessment of vestibular sensory epithelia from *in vivo* studies

We examined surface preparations of the vestibular sensory epithelia using scanning electron microscopy (SEM), following standard procedures as described elsewhere (Llorens et al., 1993; Seoane et al., 2001; Soler-Martín et al., 2007). Briefly, rats were anesthetized with 400 mg/kg chloral hydrate and transcardially perfused with 50 ml heparinized saline followed by 350 ml of 2.5 % glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2). After perfusion, the sensory epithelia in the temporal bones were dissected out in the same fixative and allowed an additional 1.5 h of fixation. The samples were then post-fixed for 1 h in 1% osmium tetroxide in cacodylate buffer and subsequently stored in 70 % ethanol at 4ºC until further processing. The epithelia were then dehydrated with increasing concentrations of ethanol up to 100%, dried in a critical-point dryer using liquid CO_2, coated with 5 nm of gold, stored in a vacuum chamber for 1-3 days, and observed in a Quanta-200 (Fei Company) 360 SEM at an accelerating voltage of 15 kV.

***In vitro* studies**

Mouse utricle cultures were used to evaluate the vestibular toxicity of the allylnitrile derivatives (Cunningham et al., 2002; Cunningham 2006). Mice were anesthetized with 100 mg/kg ketamine and killed by decapitation. The temporal bones were obtained and the utricles dissected out in L15 medium in a tissue culture hood equipped with a stereomicroscope. The utricles were transferred to 12-well tissue culture plates containing 2 ml of DMEM:F12 with 25 mM HEPES, 1% GlutaMAX, 2%

N2, 2 g/L additional glucose, and 1.5 g/L penicillin G. Utricles were incubated free-floating at 37ºC in a 5 % CO2/95% air environment. After 48 h, the utricles were incubated in the same media containing the nitriles to be tested or the vehicle (50 µl propylene glycol). After 8 h of nitrile exposure, the utricles were transferred to clean incubation medium and allowed an overnight washout period. Culture plates included three conditions. The majority consisted of one vehicle control well and two wells exposed to two nitrile concentrations, although some experiments included non-treated controls, vehicle-exposed controls, and one nitrile treatment. One preliminary series of culture analysis explored the effects of 3,4-epoxybutyronitrile at a wide range of concentrations. Then, the toxicities of allylnitrile, 3,4-epoxybutyronitrile, 3,4-dihydroxybutyronitrile, and 4-hydroxybut-2-enenitrile were compared at 150, 325 and 450 µM. Allylnitrile, 3,4-dihydroxybutyronitrile and 4-hydroxybut-2-enenitrile were also assessed at 1.5 mM.

Assessment of utricles from *in vitro* studies

At the end of the incubation protocol, utricles were fixed in 4% freshly depolymerized paraformaldehyde in PBS for 1 h and processed for immunofluorescence analysis following standard protocols (Lysakowski et al., 2011). Briefly, utricles were rinsed with PBS and then incubated with 4% Triton-X-100, 5% donkey serum, and 1% bovine serum albumin (BSA) in PBS for 1 h at room temperature. They were processed to simultaneously label the hair cells, which express calmodulin and myosin VIIa (Cunningham et al., 2002; Hasson et al., 1997; Ogata and Slepecky, 1998; Sahly et al., 1997), and the actin-rich tight junctions and hair-cell stereocilia. Utricles were incubated for 48 h at 4 ºC in 0.3% Triton-X-100 and 5% donkey serum in PBS containing anti-calmodulin (1/150) and anti-myosin-VIIa (1/600) antibodies. The specimens were then incubated overnight at 4º C with Alexa-647 donkey anti-mouse IgG (1/500) and Alexa-488 donkey anti-rabbit IgG (1/500) in 5 % donkey serum and 0.3 % triton-X-100 in PBS containing Alexa-555 phalloidin (1/200) to label actin. After the final washes, the utricles were mounted in mowiol mounting medium (Osborn and Weber, 1982). The specimens were examined in a Nikon E800 fluorescence microscope and photographed with a C3 ProgRes camera (Jenoptik). For cell counts, four to six images of each utricle were obtained with the X100 objective, and the mean numbers of cuticular plates per 6084 μm^2 field shown by phalloidin labeling were obtained with the

help of Image J software. In some experiments, utricles from the *in vitro* experiments were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, and processed for scanning electron microscopy as described above for the utricles from *in vivo* experiments.

Statistics

One-way Kruskal-Wallis ANOVA was used to compare behavioral data, followed by Mann-Withney U-test for two group comparisons. The α level was set at 0.05, and IBM SPSS Statistics 20 for Windows was used for statistical processing.

RESULTS

Synthesis of potential metabolites of allylnitrile

Both 3,4-epoxybutyronitrile and 3,4-dihydroxybutyronitrile were synthesized as racemic mixtures. Although stereospecific synthesis of the two isomers of 3,4-dihydroxybutyronitrile was also carried out (data not shown), their biological activity was not evaluated due to the results obtained with the racemic mixture. The pure compounds were stable for months in tightly sealed vials at -20ºC. However, their stability was low if they were insufficiently purified or exposed to air at room temperature.

When dissolved in saline or phosphate buffered saline (PBS), 3,4-epoxybutyronitrile was observed to undergo spontaneous transformation, as revealed to the naked eye by the appearance of a yellow-brown discoloration of the initial colorless solution. The transformation product was identified as the trans- isomer of 4-hydroxybut-2-enenitrile ((*2E*)-4-hydroxybut-2-enenitrile) (Fleming et al., 2001). The incubation of a 0.4 M solution of 3,4-epoxybutyronitrile in PBS for 6h at room temperature resulted in complete transformation into the rearranged allylic alcohol. This rearrangement was hypothesized to proceed as shown in Fig. 3. In pure water or propylene glycol, 3,4-epoxybutyronitrile was found to be stable, at least for 24 hours. Unlike 3,4-epoxybutyronitrile, 3,4-dihydroxybutyronitrile and 4-hydroxybut-2-enenitrile showed higher stability and no further transformations were observed on standing.

Effects of bilateral trans-tympanic administration of allylnitrile and 3,4-epoxybutyronitrile

Rats given bilateral trans-tympanic injections of allylnitrile or 3,4-epoxybutyronitrile showed a dose-dependent loss of vestibular function (Fig. 4), similar to that observed after intraperitoneal exposure (Balbuena and Llorens, 2001). Kruskal-Wallis analysis of the vehicle, 0.125 and 0.25 mmol/ear groups indicated a significant treatment effect (chi-square, 4 d.f.=10.1, p=0.039). Major vestibular dysfunction was observed at lower doses of 3,4-epoxybutyronitrile (0.125 mmol/ear) than of allylnitrile (0.25 mmol/ear). The animals administered allylnitrile that displayed a noticeable loss of vestibular function also showed corneal opacity. In contrast, corneal opacity was not observed in the 3,4-epoxybutyronitrile animals.

At the SEM level, control animals and animals administered 3,4-

epoxybutyronitrile at doses up to 0.062 mmol/ear, or allylnitrile at doses up to 0.125 mmol/ear, which caused no alterations in behavior, showed a dense presence of hair bundles from the vestibular sensory cells in the crista, utricle and saccule sensory epithelia (Fig. 5A,B), with little or no difference from literature descriptions of control vestibular sensory epithelia (Llorens et al., 1993; Llorens and Demêmes, 1994). Animals exposed bilaterally with high rating scores for loss of vestibular function showed bilateral marked to complete loss of hair bundles (Fig. 5C to 5H). In animals exposed to 0.125 mmol/ear of 3,4-epoxybutyronitrile, a complete loss of hair bundles occurred in the utricles (Fig. 5D), while an extensive but incomplete loss of hair bundles occurred in the crista receptors (Fig. 5C). In contrast, a complete loss of hair cells was evident in the crista receptors (Fig. 5E) of the two animals exposed to 0.25 mmol/ear of allylnitrile that displayed the deepest loss of vestibular function, while some hair bundles were still present in the utricles (Fig. 5F). Complete loss of hair cells in both crista (Fig. 5G) and utricle (Fig. 5H) receptors was recorded in the animals exposed to 0.25 mmol/ear of 3,4-epoxybutyronitrile.

Effects of unilateral trans-tympanic administration of allylnitrile and derivatives

Animals that received unilateral trans-tympanic injections of 0.125 mmol of 3,4-epoxybutyronitrile displayed behavioral evidence of unilateral vestibular damage, including head tilt and body rotation in the tail-hang test. These rats showed no corneal opacity. Histological analysis confirmed that hair cell loss occurred in the injected side only (Fig. 6 A-D). In the injected ears, very extensive loss of hair cells was observed in the crista receptors (Fig. 6A), and complete loss in the utricle receptors (Fig. 6B). The other ear of the same animals showed a control-like density of hair cell bundles in both the crista (Fig. 6C) and utricle (Fig. 6D) receptors.

Unilateral trans-tympanic administration of allylnitrile did not cause vestibular dysfunction at 0.25 mmol. After 0.50 mmol, animals showed behavioral evidence of bilateral, not unilateral, damage. For instance, ventral bending, not body rotation, was observed in the tail-hang test. These animals also showed corneal opacity. Histological analysis confirmed that vestibular damage occurred on both sides after unilateral administration (Fig. 6 E-H). Complete loss of hair cell bundles in the crista receptors and very extensive loss in the utricle receptors occurred in both the injected (Fig. 6 E, F) and the contra-lateral (Fig. 6 G,H) sides.

None of the animals administered 0.50 mmol of 4-hydroxybut-2-enenitrile or

3,4-dihydroxybutyronitrile showed any evidence of unilateral or bilateral vestibular dysfunction. On SEM analysis, the vestibular sensory epithelia of both the injected side (Fig. 7) and the contralateral side (not shown) displayed a control-like density of hair bundles.

In vitro effects of allylnitrile and its derivatives

Utricles maintained in culture with no nitrile treatment showed a high density of HCs as assessed by myosin VIIa immunoreactivity and phalloidin labeling (Fig. 8A). No qualitative difference was observed between non-exposed (n=8) and vehicle (n=44) controls. This was confirmed by quantitative analysis, which resulted in 72.1 $\pm$ 4.5 vs. 79.3 $\pm$ 11.3 HCs per field for non-exposed and vehicle-exposed utricles, respectively (X+SE, n=4/group). At the scanning electron microscope, most utricles displayed evidence of missing or abnormal hair bundles, in comparison to intact utricles from *in vivo* experiments, but a high density of hair bundles was nevertheless present (Fig. 8B). Preliminary observations with 3,4-epoxybutyronitrile indicated that this compound caused HC loss starting at sub-millimolar concentrations. Subsequent comparison of all the compounds under study (Fig. 8) showed that the epoxide derivative of allylnitrile, 3,4-epoxybutyronitrile, caused complete loss of utricule HCs at 325 and 450 μM, while allylnitrile, 3,4-dihydroxybutyronitrile, and 4-hydroxybut-2-enenitrile caused no evidence of toxicity at concentrations of up to 1.5 mM. In the utricles exposed to 325 or 450 μM of 3,4-epoxybutyronitrile, both myosin VIIa immunostaining and phalloidin labeling were completely lost (Fig. 8A). Scanning electron microscopy also showed complete loss of the epithelial surface and hair cells (Fig. 8B).

DISCUSSION

The present study addressed the hypothesis that the metabolism of allylnitrile to 3,4-epoxibutyronitrile, possibly followed by further metabolism to 3,4-dihydroxybutyronitrile, is the bioactivation pathway for its vestibular toxicity. In a previous study (Boadas-Vaello et al., 2009), we concluded that the alternate pathway, hydroxylation of the α-carbon to the nitrile to form a cyanohydrin followed by cyanide release, is unlikely to be the bioactivation pathway for vestibular toxicity but likely to mediate acute lethality. In the present study, the hypothesized allylnitrile metabolites were synthesized, characterized and evaluated for vestibular toxicity in *in vivo* and *in vitro* models.

The toxicity of the candidate compounds was evaluated in rats *in vivo* and in mouse utricules *in vitro*. We used two species for practical and financial reasons. Trans-tympanic administration is easier in rats than in mice due to body size, and the use of mice for utricular explant cultures allowed us to significantly reduce the cost of the studies. We are confident that the use of different species does not alter the value of our conclusions, because similar vestibular toxicity is observed following allylnitrile exposure in rats (Balbuena and Llorens., 2001; Gagnaire et al., 2001) and mice (Boadas-Vaello et al., 2009; Saldaña-Ruíz et al., 2012b), as could be predicted from previously available behavioral data (Tanii et al., 1989, 1991).

Using trans-tympanic exposure we aimed to obtain evidence of the direct ototoxic effect of the compounds *in vivo*. In rats exposed bilaterally, vestibular toxicity could be assessed by means of a well-characterized battery of tests for vestibular dysfunction (Llorens et al., 1993; Llorens and Rodríguez-Farré, 1997; Boadas-Vaello et al., 2005). With this model, we compared the vestibular toxicity of allylnitrile with that of 3,4-epoxibutyronitrile, hypothesized to be its product by CYP-mediated metabolism. The data demonstrated that 3,4-epoxybutyronitrile causes vestibular dysfunction at doses lower than the doses of allylnitrile necessary for a similar effect. The conclusion that the epoxide derivative of allylnitrile is more toxic to the vestibular system than the parent compound was also demonstrated by histological analysis. The pattern of damage differed between the two compounds, with allylnitrile showing a pattern similar to that found after oral or intraperitoneal administration (crista receptors are more affected than utricles) in contrast to the presence of more extensive damage in the utricles than in crista following exposure to 3,4-epoxybutyronitrile. These patterns of damage suggest that 3,4-epoxybutyronitrile caused its effects by direct entrance from

the middle to the inner ear, while allylnitrile could have caused its effects through whole body exposure, as suggested by the presence of corneal clouding in the allylnitrile animals. These hypotheses were confirmed by the unilateral exposure experiments. 3,4-Epoxybutyronitrile caused unilateral vestibular toxicity after unilateral exposure at the same dose/ear previously observed to cause vestibular toxicity after bilateral exposure. In contrast, allylnitrile caused symmetrical bilateral vestibular toxicity after unilateral exposure, and was also associated with corneal toxicity. The unilateral effective dose was the same total dose (i.e., twice the dose/ear) effective after bilateral exposure. Thus, trans-tympanic exposure to allylnitrile did not cause vestibular toxicity by local action, but through absorption and whole body blood distribution.

We also examined *in vivo* the vestibular toxicity of 3,4-dihydroxybutyronitrile and 4-hydroxybut-2-enenitrile. The first compound was the hypothetical result of the action of epoxide hydrolase activities on 3,4-epoxibutyronitrile, with a similarity to the known metabolic pathways of acrylonitrile (Kedderis and Batra, 1993; ElHadri et al., 2005). The second was identified as the spontaneous rearrangement product of 3,4-epoxibutyronitrile in PBS and in potassium phosphate buffer, so it is expected that this rearrangement would actually occur in both the intracellular and extracellular compartments *in vivo*. Due to the chemical instability and expected reactivity of the epoxide, we initially hypothesized that one of the two more stable and probably less reactive compounds would be the circulating ototoxic compound. However, neither of these two compounds caused significant vestibular toxicity by unilateral trans-tympanic exposure at doses four times (0.5 mmol) the effective dose of 3,4-epoxybutyronitrile (0.125 mmol).

The *in vitro* data also supported the conclusion that the epoxide, i.e. 3,4-epoxybutyronitrile, is more toxic to the vestibular system than either the parent allylnitrile or the other candidate derivatives, 3,4-dihydroxybutyronitrile and 4-hydroxybut-2-enenitrile. At concentrations of 325 and 450 μM, the epoxy derivative caused a complete loss of the sensory epithelium, while the other compounds did not show significant vestibular toxicity at these or higher (1.5 mM) concentrations.

Taken together, the data in the present work support the hypothesis that 3,4-epoxybutyronitrile might be the ototoxic metabolite of allylnitrile. Future work may provide further support for the hypothesis, and expand the understanding of nitrile-induced vestibular toxicity. Demonstration that 3,4-epoxybutyronitrile is actually formed by the metabolism of allylnitrile is a pending task. We aimed to indicate the

presence of this epoxide in the blood of allylnitrile exposed animals, or in hepatic microsome preparations incubated with allylnitrile, but our initial attempts were hampered by the lack of an adequate analytical method. Assays based on modifications of our previously developed method for allylnitrile and cyanide analysis in blood by solid-phase microextraction – gas chromatography – nitrogen-phosphorus detection (Boadas-Vaello et al., 2008) were found to be unsuitable for the analysis of the derivatives under study (unpublished results). Another area for future research is the selectivity of ototoxic action. The present *in vitro* experiments showed massive damage to the epithelium by 3,4-epoxybutyronitrile, which is in contrast to the selective degeneration of hair cells with sealing of the scars by supporting cell extension that characterizes ototoxic damage *in vivo* (Llorens and Demêmes, 1994; Meiteles and Raphael, 1994; Hordichok and Steyger, 2007). Thus, more histological data are necessary to demonstrate that 3,4-epoxybutyronitrile has selective toxic action on hair cells, as observed after *in vivo* systemic exposure to ototoxic nitriles. Finally, research on the mechanism of action of nitriles on hair cells is necessary. This goal can be addressed in the future using *in vitro* models.

The question of nitrile bioactivation for toxicity was previously addressed for IDPN. According to Sayre and colleagues, metabolism of that compound would generate N-hydroxy-IDPN and 3-(2-cyanoethylamino)acrylonitrile (Jacobson et al., 1987). In subsequent studies, N-hydroxy-IDPN was shown to be more toxic than IDPN (Morandi et al., 1987; Nace et al., 1997), although the toxic metabolite has not yet been unequivocally identified. By analogy, 3,4-dihydroxybutyronitrile or 4-hydroxybut-2-enenitrile were obvious candidates as the ototoxic metabolites of allylnitrile. Furthermore, since the expected life of 3,4-epoxybutyronitrile was short and its reactivity was likely high, it was not an obvious choice as a circulating ototoxic compound. However, in this study 3,4-epoxybutyronitrile was shown to be directly toxic to the vestibular sensory epithelia, in surprising contrast to the negative data collected regarding the other two compounds. It is thus possible that the chemical properties of the epoxide are responsible for the steepness of the dose response curve of allylnitrile vestibular toxicity (Balbuena and Llorens, 2001) and the small range of doses that are effective to lethal (Balbuena and Llorens, 2001; Saldaña-Ruíz et al., 2012b) in contrast to the larger range that characterize IDPN vestibular toxicity (Llorens et al., 1993). Additional work is required to study whether these properties of IDPN depend on the fact that its toxicity is caused by one of the compounds indicated above

or on the lower reactivity of a yet unidentified epoxide metabolite that mediates the IDPN vestibular effects.

In conclusion, three derivatives of allylnitrile that could mediate the ototoxic properties of the parent nitrile were synthesized and evaluated for vestibular toxicity both *in vivo* and *in vitro*. One of these compounds, 3,4-epoxybutyronitrile, was found to be directly toxic to the vestibular sensory epithelia. Whether epoxide or other functional groups are responsible for the bioactivation of the other ototoxic nitriles, namely IDPN, *cis*-crotononitrile, and *cis*-2-pentenenitrile, remains an open question for future investigations.

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FIGURE LEGENDS

Figure 1. Hypothesized pathways for the CYP-mediated oxidative metabolism of allylnitrile. Hydroxylation at the alpha carbon would generate an unstable cyanohydrin, which would subsequently decompose into 2-propenal (acrolein) and cyanide. The alternate pathway includes the epoxidation of the beta-gamma double bound. The 3,4-epoxybutyronitrile that is formed may be further metabolized to 3,4-dihydroxybutyronitrile by epoxyde hydrolase activity.

Figure 2. Synthesis of allylnitrile metabolites: (A) 3,4-epoxybutyronitrile; (B) 3,4-dihydroxybutyronitrile.

Figure 3. Proposal of a mechanism for the 3,4-epoxybutyronitrile rearrangement into 4-hydroxybut-2-enenitrile. The inset shows the reaction course.

Figure 4. Effects of 3,4-epoxybutyronitrile and of allylnitrile on vestibular function after bilateral trans-tympanic administration. Data are mean $\pm$ SE rating scores for vestibular dysfunction. *: significantly different from control group, $p < 0.05$, Mann-Whitney U-test after significant Kruskal-Wallis ANOVA of the vehicle, 0.125 and 0.25 mmol/ear groups.

Figure 5. Effects of bilateral trans-tympanic administration of allylnitrile (A, B, E, F) or 3,4-epoxybutyronitrile (C, D, G, H) on the vestibular sensory epithelia of the rat, as observed by scanning electron microscopy. (A) Crista and (B) utricle of a rat administered 0.125 mmol/ear of allylnitrile that showed no difference from control epithelia. Each sensory hair cell is identified by a bundle of stereocilia protruding from the epithelial surface (arrows). (C) Crista and (D) utricle of a rat administered 0.125 mmol/ear of 3,4-epoxybutyronitrile; note the scarcity of hair bundles in the crista (arrow in C) and the lack of bundles in the utricle (arrow in D). (E) Almost complete loss of hair bundles in the crista of the worst case example after 0.250 mmol/ear of allylnitrile. (F) Utricle of the same animal shown in E; note that there is extensive loss of hair bundles, but many remain in place (arrow). (G and H) Crista and utricle after 0.250 mmol/ear of 3,4-epoxybutyronitrile; note the complete absence of hair bundles and the large surfaces of the remaining supporting cells, which indicates loss of these cells also. Scale bars: 300 μm A-F, 50 μm in G and H.

Figure 6. Effects of unilateral trans-tympanic administration of 0.125 mmol 3,4-epoxybutyronitrile (A, B, C, D) or 0.5 mmol allylnitrile (E, F, G, H) on the vestibular sensory epithelia of the rat, as observed by scanning electron

microscopy. Nitriles were administered to the right ear only. (A) Right crista and (B) right utricle of a rat administered 3,4-epoxybutyronitrile showing very extensive (crista) and complete (utricle) loss of hair cells. (C and D) Epithelia from the left ear of the same rat as shown in A and B, displaying a control-like density of hair bundles. (E) Right crista and (F) right utricle of a rat administered allylnitrile showing virtually complete (crista) and very extensive (utricle) loss of hair cells. (G and H) Epithelia from the left ear of the same rat shown in E and F, displaying an extensively damaged appearance similar to that of the injected right side. In all panels, arrows point to the surface of the sensory epithelium. Scale bars: 300 μm A, C, E, G, H, 400 μm in D and F, 500 μm in B.

Figure 7. Effects of unilateral trans-tympanic administration of 0.5 mmol 4-hydroxybut-2-enenitrile (A) or 3,4-dihydroxybutyronitrile (B). In both cases, the utricle of the injected (right) ear is shown, and a control-like density of hair bundles (arrows) is observed. Scale bars: 400 μm in A, 500 μm in B.

Figure 8. Effects of 3,4-epoxybutyronitrile (Epoxy), allylnitrile (Allylnitr), 3,4-dihydroxybutyronitrile (Dihydroxy) and 4-hydroxybut-2-enenitrile (Hydroxybut) on the vestibular utricle cultures. (A) Control and treated utricles immunolabeled with anti-myosin VIIa antibodies (green) to label the sensory hair cells, and phalloidin (red) to stain actin fibers. Note the complete loss of labeling in the utricle exposed to 450 μM of 3,4-epoxybutyronitrile, but the control-like appearance of utricles exposed to a lower concentration (150 μM) or to the same concentration of other compounds. The scale bar in top left panel indicates 100 μm and applies to all panels. (B) Scanning electron microscopy views of a vehicle control utricle (left images) or a utricle exposed to 450 μM of 3,4-epoxybutyronitrile (right images). The sensory epithelium is dramatically altered, with no remaining typical surface features, such as hair bundles from the sensory cells or surface microvellosities from the supporting cells. Globular shaped cells are observed attached to the basal lamina (arrow). Scale bars: 300 μm (top panels) and 30 μm (bottom panels). (C) Cell counts based on high magnification of phalloidin stained epithelium. Each point is the mean from two to four utricles, each assessed by counting of 4 to 6 images.

LEGEND FOR SUPPLEMENTARY FIGURES

Supplementary Figure 1. NMR spectra of the synthesized allylnitrile derivatives. (A) 3,4-Epoxybutyronitrile. (B) 3,4-Dihydroxybutyronitrile. (C) 4-Hydroxybut-2-enenitrile.

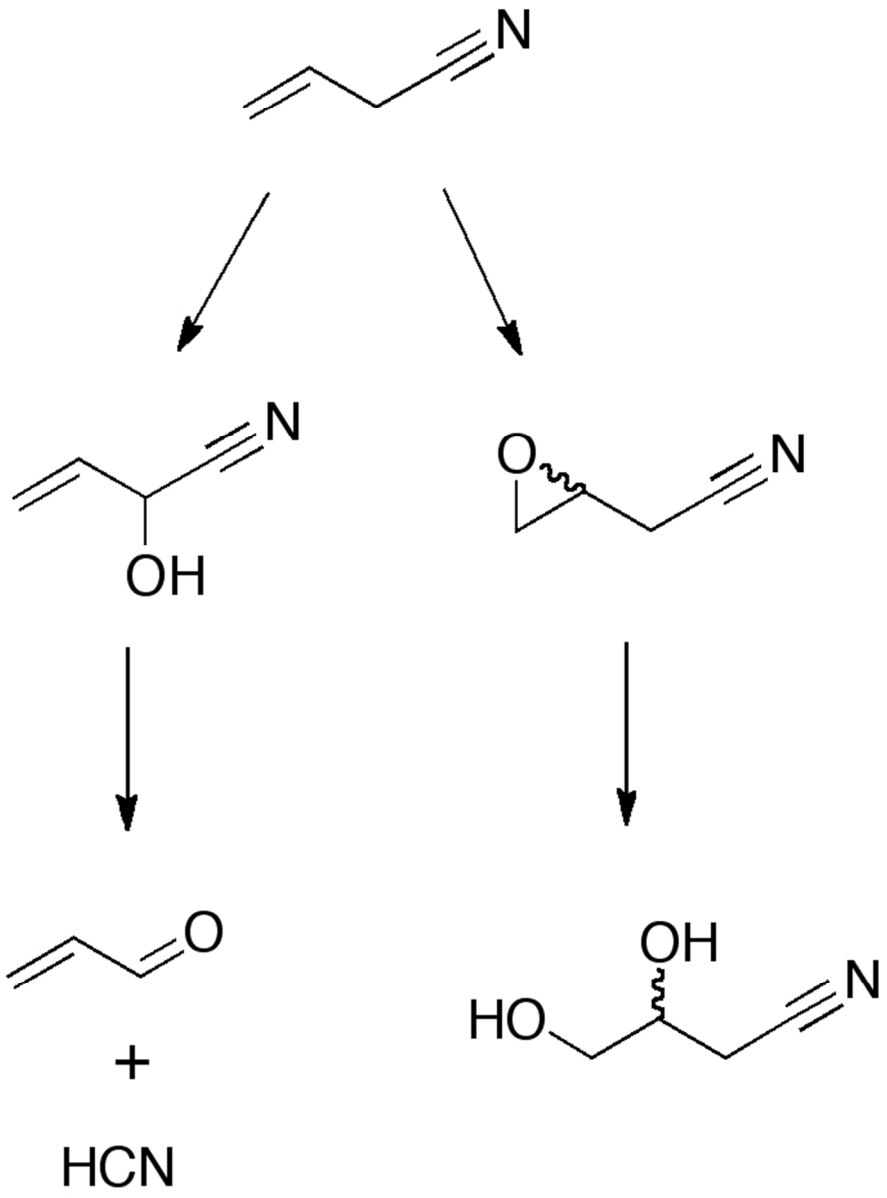

Figure 1. Hypothesized pathways for the CYP-mediated oxidative metabolism of allylnitrile. Hydroxylation at the alpha carbon would generate an unstable cyanohydrin, which would subsequently decompose into 2-propenal (acrolein) and cyanide. The alternate pathway includes the epoxidation of the beta-gamma double bound. The 3,4-epoxybutyronitrile that is formed may be further metabolized to 3,4-dihydroxybutyronitrile by epoxyde hydrolase activity.
59x78mm (300 x 300 DPI)

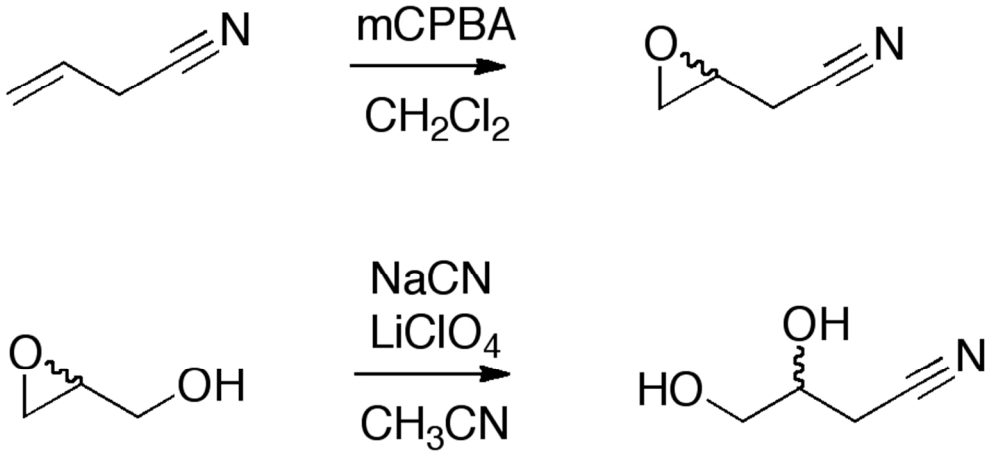

Figure 2. Synthesis of allylnitrile metabolites: (A) 3,4-epoxybutyronitrile; (B) 3,4-dihydroxybutyronitrile.
77x36mm (300 x 300 DPI)

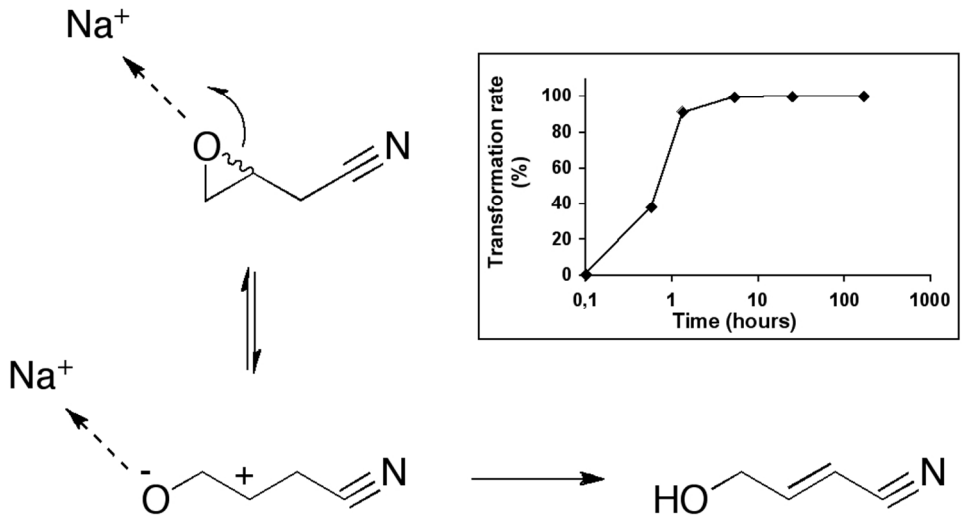

Figure 3. Proposal of a mechanism for the 3,4-epoxybutyronitrile rearrangement into 4-hydroxybut-2-enenitrile. The inset shows the reaction course.
93x49mm (300 x 300 DPI)

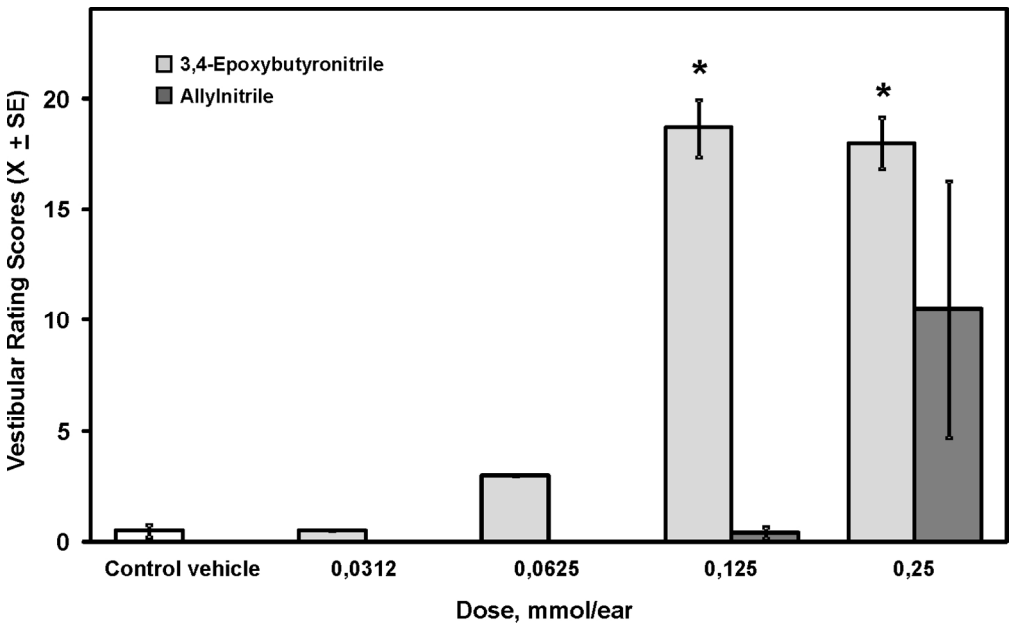

Figure 4. Effects of 3,4-epoxybutyronitrile and of allylnitrile on vestibular function after bilateral trans-tympanic administration. Data are mean + SE rating scores for vestibular dysfunction. *: significantly different from control group, $p < 0.05$, Mann-Whitney U-test after significant Kruskal-Wallis ANOVA of the vehicle, 0.125 and 0.25 mmol/ear groups.
131x80mm (300 x 300 DPI)

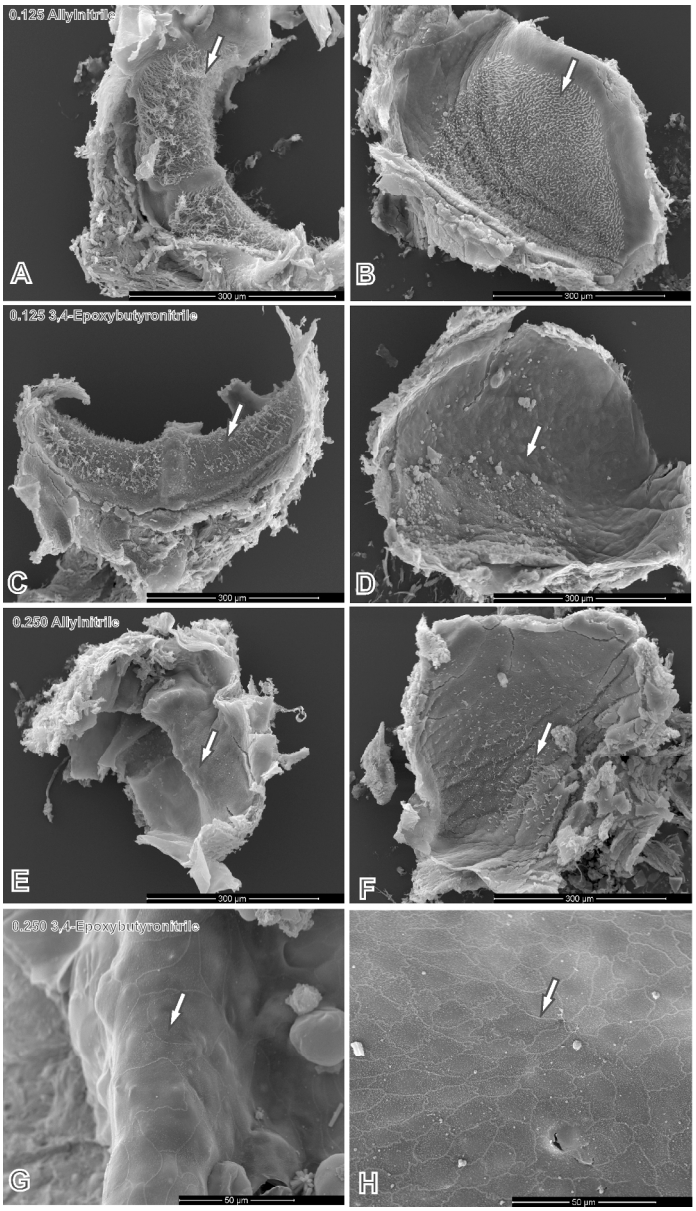

Figure 5. Effects of bilateral trans-tympanic administration of allylnitrile (A, B, E, F) or 3,4-epoxybutyronitrile (C, D, G, H) on the vestibular sensory epithelia of the rat, as observed by scanning electron microscopy. (A) Crista and (B) utricle of a rat administered 0.125 mmol/ear of allylnitrile that showed no difference from control epithelia. Each sensory hair cell is identified by a bundle of stereocilia protruding from the epithelial surface (arrows). (C) Crista and (D) utricle of a rat administered 0.125 mmol/ear of 3,4-epoxybutyronitrile; note the scarcity of hair bundles in the crista (arrow in C) and the lack of bundles in the utricle (arrow in D). (E) Almost complete loss of hair bundles in the crista of the worst case example after 0.250 mmol/ear of allylnitrile. (F) Utricle of the same animal shown in E; note that there is extensive loss of hair bundles, but many remain in place (arrow). (G and H) Crista and utricle after 0.250 mmol/ear of 3,4-epoxybutyronitrile; note the complete absence of hair bundles and the large surfaces of the remaining supporting cells, which indicates loss of these cells also. Scale bars: 300 μμm A-F, 50 μμm in G and H.
138x240mm (300 x 300 DPI)

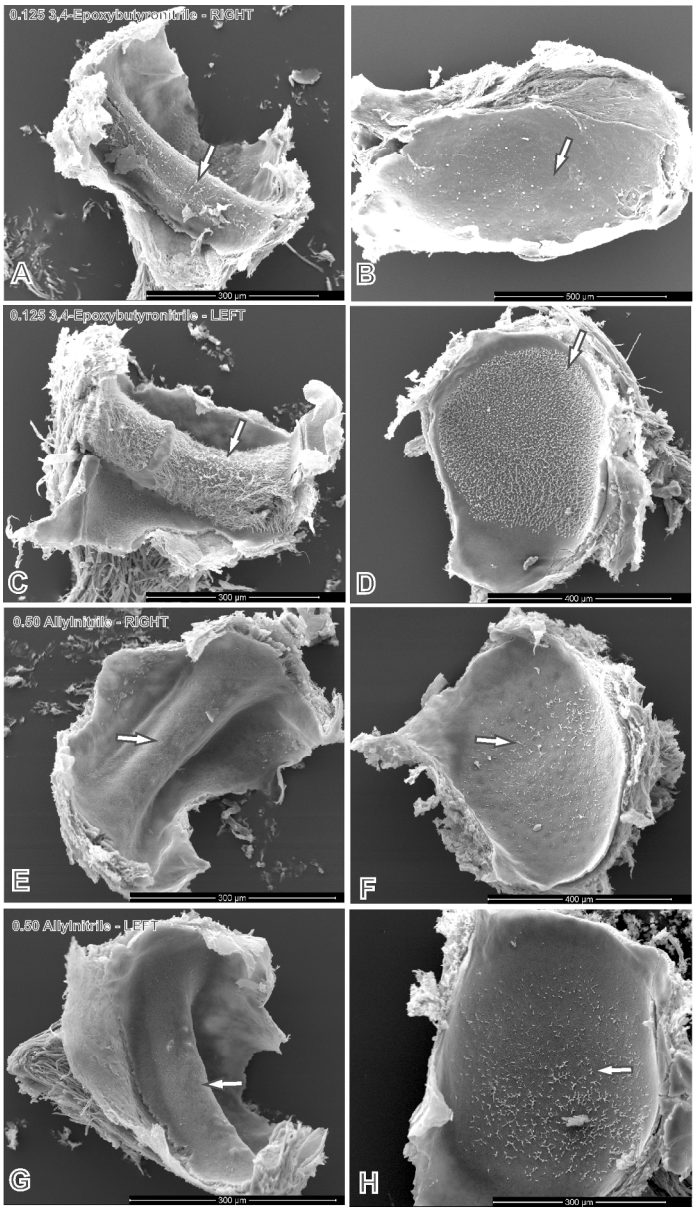

Figure 6. Effects of unilateral trans-tympanic administration of 0.125 mmol 3,4-epoxybutyronitrile (A, B, C, D) or 0.5 mmol allylnitrile (E, F, G, H) on the vestibular sensory epithelia of the rat, as observed by scanning electron microscopy. Nitriles were administered to the right ear only. (A) Right crista and (B) right utricle of a rat administered 3,4-epoxybutyronitrile showing very extensive (crista) and complete (utricle) loss of hair cells. (C and D) Epithelia from the left ear of the same rat as shown in A and B, displaying a control-like density of hair bundles. (E) Right crista and (F) right utricle of a rat administered allylnitrile showing virtually complete (crista) and very extensive (utricle) loss of hair cells. (G and H) Epithelia from the left ear of the same rat shown in E and F, displaying an extensively damaged appearance similar to that of the injected right side. In all panels, arrows point to the surface of the sensory epithelium. Scale bars: 300 μμm A, C, E, G, H, 400 μμm in D and F, 500 μμm in B.
138x240mm (300 x 300 DPI)

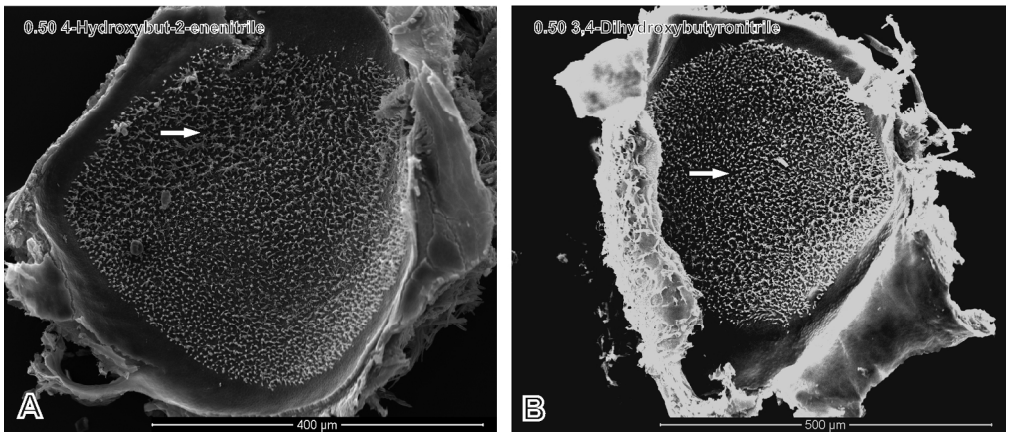

Figure 7. Effects of unilateral trans-tympanic administration of 0.5 mmol 4-hydroxybut-2-enenitrile (A) or 3,4-dihydroxybutyronitrile (B). In both cases, the utricle of the injected (right) ear is shown, and a control-like density of hair bundles (arrows) is observed. Scale bars: 400 µm in A, 500 µm in B.
168x71mm (300 x 300 DPI)

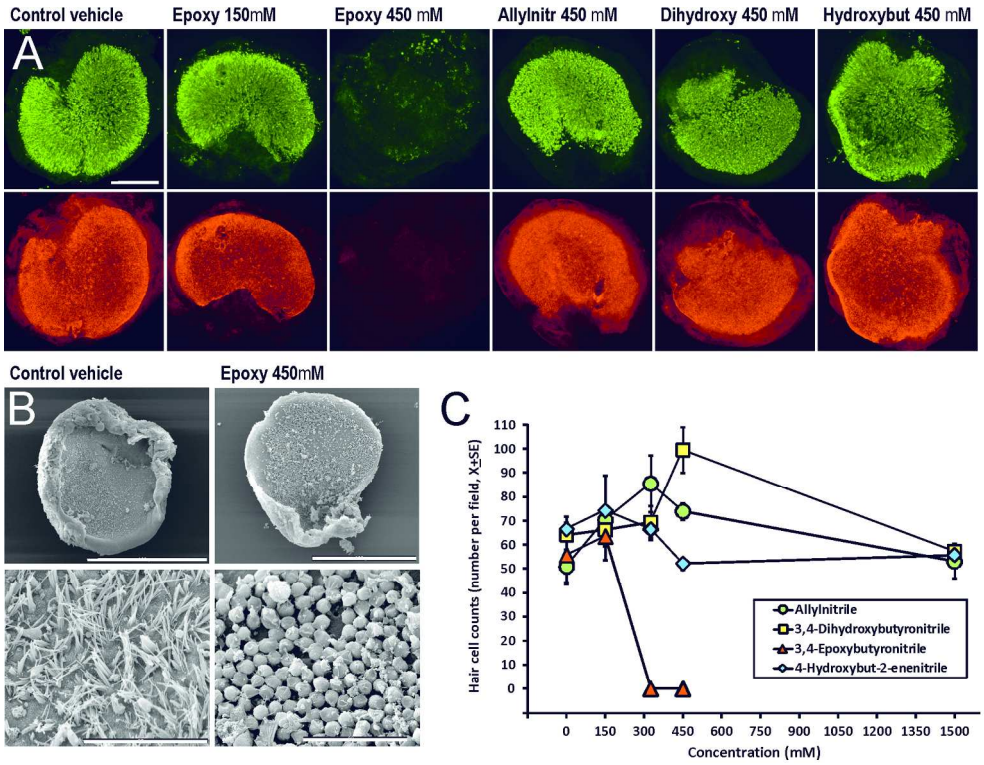

Figure 8. Effects of 3,4-epoxybutyronitrile (Epoxy), allylnitrile (Allylnitr), 3,4-dihydroxybutyronitrile (Dihydroxy) and 4-hydroxybut-2-enenitrile (Hydroxybut) on the vestibular utricle cultures. (A) Control and treated utricles immunolabeled with anti-myosin VIIa antibodies (green) to label the sensory hair cells, and phalloidin (red) to stain actin fibers. Note the complete loss of labeling in the utricle exposed to 450 μμM of 3,4-epoxybutyronitrile, but the control-like appearance of utricles exposed to a lower concentration (150 μμM) or to the same concentration of other compounds. The scale bar in top left panel indicates 100 μμm and applies to all panels. (B) Scanning electron microscopy views of a vehicle control utricle (left images) or a utricle exposed to 450 μμM of 3,4-epoxybutyronitrile (right images). The sensory epithelium is dramatically altered, with no remaining typical surface features, such as hair bundles from the sensory cells or surface microvellosities from the supporting cells. Globular shaped cells are observed attached to the basal lamina (arrow). Scale bars: 300 μμm (top panels) and 30 μμm (bottom panels). (C) Cell counts based on high magnification of phalloidin stained epithelium. Each point is the mean from two to four utricles, each assessed by counting of 4 to 6 images.
185x141mm (300 x 300 DPI)