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>Oral cis-2-pentenenitrile causes loss of vestibular function in rats
>Loss of hair cells in the vestibular sensory epithelia causes the functional deficit
>CYP blocker 1-aminobenzotriazole blocks the vestibular effect of cis-2-pentenenitrile
>Oral cis-2-pentenenitrile does not cause significant neuronal degeneration

VESTIBULAR TOXICITY OF *CIS*-2-PENTENENITRILE IN THE RAT

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ABSTRACT

cis -2-Pentenenitrile, an intermediate in the synthesis of nylon and other products, causes permanent behavioral deficits in rodents. Other low molecular weight nitriles cause degeneration either of the vestibular sensory hair cells or of selected neuronal populations in the brain. Adult male Long-Evans rats were exposed to *cis*-2-pentenenitrile (0, 1.25, 1.50, 1.75, or 2.0 mmol/kg, oral, in corn oil) and assessed for changes in open field activity and rating scores in a test battery for vestibular dysfunction. Surface preparations of the vestibular sensory epithelia were observed for hair cell loss using scanning electron microscopy. A separate experiment examined the impact of pre-treatment with the universal CYP inhibitor,1-aminobenzotriazole, on the effect of *cis*-2-pentenenitrile on vestibular rating scores. The occurrence of degenerating neurons in the central nervous system was assessed by Fluoro-Jade C staining. *cis*-2-Pentenenitrile had a dose-dependent effect on body weight. Rats receiving 1.50 mmol/kg or more of *cis*-2-pentenenitrile displayed reduced rearing activity in the open field and increased rating scores on the vestibular dysfunction test battery. Hair cell loss was observed in the vestibular sensory epithelia and correlated well with the behavioral deficits. Pre-treatment with 1-aminobenzotriazole blocked the behavioral effect. Fluoro-Jade C staining did not reveal significant neuronal degeneration in the central nervous system apart from neurite labeling in the olfactory glomeruli. We conclude that *cis*-2-pentenenitrile causes vestibular toxicity in a similar way to allylnitrile, *cis*-crotononitrile and 3,3'-iminodipropionitrile (IDPN), and also shares other targets such as the olfactory system with these other nitriles. The present data also suggest that CYP-mediated bioactivation is involved in *cis*-2-pentenenitrile toxicity.

Keywords: Ototoxicity, Vestibular toxicity, Nitriles, Hair cells; Neuronal degeneration stain.

1. INTRODUCTION

Nitriles are compounds containing cyano (R-CN) groups; their toxic effects include acute lethality, osteolathyrism and neurotoxicity, including sensory toxicity (DeVito, 1996; Llorens et al., 2011; Saldaña-Ruiz et al., 2012). Among sensory systems, the inner ear is a major target for several nitriles: degeneration of the vestibular and/or auditory sensory hair cells has been reported in rodents exposed to 3,3'-iminodipropionitrile (IDPN) (Llorens et al., 1993; Llorens and Demêmes, 1994; Crofton et al., 1994; Seoane et al., 2001; Soler-Martín et al., 2007), allylnitrile (Balbuena and Llorens, 2001; Gagnaire et al., 2001), racemic crotononitrile (Llorens et al., 1998; Gagnaire et al., 2001), and *cis*-crotononitrile (Balbuena and Llorens, 2003). IDPN has also been shown to cause vestibular toxicity in frogs (Soler-Martin et al., 2007). One nitrile causing vestibular toxicity in mice, *trans*-crotononitrile (Saldaña-Ruiz et al., 2012) has a different profile of neurotoxic effects in the rat, causing selective neuronal degeneration in discrete regions of the brain including the inferior olive and the piriform cortex (Seoane et al., 2005; Boadas-Vaello et al., 2005). Hexadienenitrile shows a similar effect in rat brain (Boadas-Vaello et al., 2005).

Another ototoxic nitrile is *cis*-2-pentenenitrile (CAS no. 25899-50-7). This nitrile has been shown to cause loss of the cochlear hair cells in rodents (Gagnaire et al., 2001) and behavioral disturbances indicative of vestibular toxicity (Tanii et al., 1999; Genter and Crofton, 2000; Lewis et al., 2006; Saldaña-Ruiz et al., 2012), but this vestibular toxicity has not yet been studied in detail. A deeper knowledge of the toxicological properties of *cis*-2-pentenenitrile is desirable, because it is a chemical intermediate associated with the production of nylon monomer and it is also used in a number of chemical synthesis pathways for pesticides, solvents, and other marketed chemicals (Lewis et al., 2006; DeVito, 2007). Therefore, we characterized the acute vestibular toxicity of *cis*-2-pentenenitrile in rats, assessing both its behavioral and pathological effects. Because similar nitriles have been shown to cause CNS toxicity, we also evaluated the CNS for presence of degenerating neurons after *cis*-2-pentenenitrile exposure. Simultaneously, we evaluated whether cytochrome-P450-mediated metabolism is involved in *cis*-2-pentenenitrile vestibular toxicity, as it is in allylnitrile toxicity (Boadas-Vaello et al., 2009).

2. METHODS

2.1. Chemicals and reagents

cis-2-Pentenenitrile (98%) and 1-aminobenzotriazole (>98 %) were purchased from Sigma-Aldrich Química (Madrid, Spain). Hexadienenitrile (>98%) was from Frinton Laboratories (Vineland, NJ, USA). Fluoro-Jade C was from Histo-Chem Inc. (Jefferson, AR, USA).

2.2. Animals

Animal care and use were in accordance with Law 5/1995 and Act 214/1997 of the Government of Catalonia (the Generalitat), and were approved by the Ethics Committee on Animal Experiments of the University of Barcelona. Eight- to 9-week-old male Long-Evans rats were obtained from Janvier (Le-Genest-Saint-Isle, France). They were housed two per cage in standard Macrolon cages (280 x 520 x 145 mm) with wood shavings as bedding at 22 $\pm$ 2ºC. At least seven days were provided for acclimation before experimentation. The rats were maintained on a 12:12 L:D cycle (0700:1900 h) and given standard diet pellets (TEKLAD 2014, Harlan Interfauna Ibérica, Sant Feliu de Codines, Spain) ad libitum. For fluoro-jade B staining, the rats were anesthetized with 400 mg/kg chloral hydrate and transcardially perfused with 50 ml of heparinized saline followed by 350 ml of 4 % paraformaldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.4. For scanning electron microscopy, rats were anesthetized and killed by decapitation.

2.3. Dosing and experimental design

cis-2-Pentenenitrile was administered orally in 1 ml/kg of corn oil. Pilot studies for dose selection were designed based on available data (Genter and Crofton, 2000; Gagnaire et al, 2001; Saldaña-Ruíz et al., 2012). First, two rats were orally administered 1 mmol/kg/day for three consecutive days. Second, two animals were administered 1.5 mmol/kg. Third, one animal was administred 2.0 mmol/kg. In a first experiment, rats were administered with 0 (control vehicle, n=7), 1.25 (n=7), 1.50 (n=7), 1.75 (n=7), or 2.0 (n=4) mmol/kg of *cis*-2- pentenenitrile, and assessed for behavioral evidences of vestibular dysfunction at days 0 (pre-test), 3, 7, and 21 after dosing. The experiment was run in two parts, with three animals per dose (except for the 2.0 mmol/kg dose) in the first part, and four animals per dose in the second part. Selected animals from each group (n=2, 3, 3, 4, and 2, respectively) were used for vestibular histology at 36-42 days after dosing.

In a second experiment, three groups of rats were given control vehicle, 2.0 mmol/kg of *cis*-2- pentenenitrile and saline, or *cis*-2- pentenenitrile and 1-aminobenzotriazole (n=7/group), and were assessed for behavioral evidences of

vestibular dysfunction at days 0 (pre-test), 3 and 7 after dosing. 1-Aminobenzotriazole is an universal P-450 inhibitor (Mico et al., 1988), and was administered in 2 ml/kg of saline, i.p., 1 h before and 24 h after the nitrile administration (Boadas-Vaello et al., 2009). Animals from this experiment were also used for labeling of degenerating neurons with fluoro-jade (Schmued and Hopkins, 2000ab; Schmued et al., 2005) at day 7. As positive control for the degeneration stain, two animals dosed with hexadienenitrile (3.25 mmol/kg/day in 2 ml/kg of corn oil, i.p., for three consecutive days, Boadas-Vaello et al., 2005) were processed in parallel.

2.4. Behavioral Analysis

Vestibular dysfunction was evaluated by observation of spontaneous and reflex motor behaviors as described previously (Llorens et al., 1993; Llorens and Rodríguez-Farré, 1997; Boadas-Vaello et al., 2005). Briefly, rats were placed for 1 min on transparent cage (50 x 50 cm), and the experimenter rated the animals from 0 to 4 for circling, retropulsion, and abnormal head movements. Circling was defined as stereotyped circling movement, retropulsion as backward displacement, and head bobbing as 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 bent ventrally, sometimes "crawling" up toward 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 the animals' feet. Healthy rats quickly right themselves, whereas vestibular-deficient rats lie on their back, with their feet up, and "walk" with respect to the ventral surface. For the air-righting reflex, the 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. The results of all tests were summed to obtain a score of 0 to 24.

2.5. Histology

To identify degenerating neurons in the central nervous system, brain and spinal cord tissues were removed from the perfusion-fixed animals and immersed in the same fixative at 4^{o}C for up to one week. The whole brain and one slice sample from each the cervical and the lumbar regions of the spinal cord were cut in transverse sections (50 µm) using a Leica VT1000M vibrating blade microtome. Every third section was dried onto a microscopy slide for subsequent staining with Fluoro-Jade C (Schmued et al.,

2005). Degenerating neurons were identified by comparison of Fluoro-Jade C stained sections with the appearance of the corresponding structures in the normal brain, according to the atlases by Paxinos et al., 1999ab.

To assess vestibular pathology, we examined surface preparations of the vestibular sensory epithelia by scanning electron microscopy (SEM), as previously done with IDPN (Llorens et al., 1993b; Llorens and Rodríguez-Farré, 1997; Seoane et al., 2001), allylnitrile (Balbuena and Llorens, 2001), and *cis*- and *trans*-crotononitrile (Balbuena and Llorens, 2003). The sensory epithelia were quickly dissected out from the temporal bones in ice-cold 2.5 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.2, using a stereomicroscope in a fume hood. With few exceptions, the complete set of entire vestibular receptors was obtained from one ear. The samples were fixed for 1.5 h in the same solution, post-fixed for 1 h in 1% osmium tetroxide in cacodylate buffer and subsequently stored in 70 % ethanol at 4ºC. For observation, the epithelia were 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, and stored in a vacuum chamber for 1-3 days. The epithelia were then observed in a Quanta-200 SEM (FEI Company, Hillsboro, OR, USA).

2.6. Statistics

Body weight and behavioral data were analyzed using ANOVA or repeated measures MANOVA – Wilks' criterion – with "day" as the within subject factor. Orthogonal contrasts, followed by Duncan's test when applicable, were used for post-hoc analysis. The α level was set at 0.05. The PASW 18.0 for Windows program package was used.

3. RESULTS

3.1. General observations in animals exposed to *cis*-2-pentenenitrile

In the pilot studies for dose selection, the animals administered 1.0 mmol/kg/day of *cis*-2-pentenenitrile for three days showed a 6 % decrease in body weight but neither behavioral evidences of vestibular dysfunction nor other signs of overt toxicity. The two animals dosed with 1.5 mmol/kg showed behavioral evidences of vestibular dysfunction at 24 h after the first dose, and they received no additional doses. The animal dosed with 2.0 mmol/kg showed behavioral evidences of vestibular dysfunction at 24 h after the first dose, received no additional doses, and was killed at 48 h as it reached the criteria of ethical limits of suffering.

After treatment with *cis*-2-pentenenitrile in the dose-response study, one animal in the 1.50 mmol/kg *cis*-2-pentenenitrile group died on day 6, but no mortality was recorded in the other experimental groups. Before administration, mean group body weights were not significantly different, $F(4,26)=2.1$, $p=0.104$, but control rats had a mean value of 308 g compared to 283 g in the group of rats receiving 1.25 mmol/kg of *cis*-2-penteneitrile. To reduce the chances for false positive results, we analyzed body weight data as percent weight respect the mean group weight before administration (Fig. 1). A dose-dependent effect on body weight was recorded. Maximal effects were recorded three days after dosing, when mean body weights were 104%, 99%, 94%, 92%, and 89% of initial means for the control, 1.25, 1.50, 1.75, and 2.0 mmol/kg of *cis*-2-pentenenitrile groups respectively. MANOVA analysis revealed significant effects of day, $F(10, 17)=109$, $p=0.000$, day by treatment, $F(40, 66)=3.55$, $p=0.000$, and treatment, $F(4,26)=7.27$, $p=0.000$. Significant group differences were detected on all days beginning on day 2 after exposure (all $Fs\ (4,26)>3.5$, $ps<0.022$. Compared to control values, body weights were significantly reduced for the two high dose groups at day 2, and for these groups and the 1.50 mmol/kg group on all days from day 3 onwards. Body weights from animals exposed to 1.25 mmol/kg of *cis*-2-pentenenitrile did not differ significantly from control body weights at any time post-exposure.

Other signs of toxicity in the *cis*-2-pentenenitrile animals were changes in spontaneous behavior related to vestibular dysfunction (see below) and reversible corneal opacity. Simple observation revealed a loss of corneal transparency on day 1 after dosing in animals treated with doses of 1.5 mmol/kg or greater. In the 1.5 mmol/kg animals, almost complete recovery was observed on day 2. Corneal opacity was observed up to day 6 in animals exposed to 2.0 mmol/kg.

3.2. Effects of *cis*-2-pentenenitrile on behavior

In the open field, a dose-dependent effect of *cis*-2-pentenenitrile on rearing activity of the rats was observed (Fig. 2A). MANOVA analysis revealed significant effects of day, $F(3,24)=21.5$, $p=0.000$, day by treatment, $F(12,64)=3.5$, $p=0.000$, and treatment, $F(4,26)=4.7$, $p=0.005$. Significant group differences were detected on day 3, $F(4,26)=9.7$, $p=0.000$, day 7, $F=9.2$, $p=0.000$, and day 21, $F=6.0$, $p=0.001$. The groups of animals dosed with 1.75 mmol/kg or 2.0 mmol/kg of *cis*-2-pentenenitrile showed reduced rearing activity at these three time points, while the 1.50 mmol/kg group showed significant differences from the control group at days 3 and 7 but not at day 21.

The effects of *cis*-2-pentenenitrile on horizontal activity in the open field are shown in Fig 2B. MANOVA analysis resulted in a significant effect of day, $F(3,24)=17.6$, $p=0.000$, and day by treatment, $F(12,64)=2.4$, $p=0.014$. However, a high within group variability was present, and day by day analysis showed only marginal effects at day 3, $F(4,26)=2.6$, $p=0.056$, and day 21, $F=2.6$, $p=0.06$. At the latter time point it was evident that some of the rats dosed with 1.5 mmol/kg or more of *cis*-2-pentenenitrile were hyperactive. Thus, while values in the control group ranged from 31 to 147, values in the range of 213 to 492 were recorded from four out of six animals in the 1.50 mmol/kg group, five out of seven animals in the 1.75 mmol/kg group, and two out of four animals in the 2.0 mmol/kg group.

Exposure to *cis*-2-pentenenitrile resulted in a dose-dependent increase in vestibular rating scores, indicating loss of vestibular function (Fig. 3). Analysis of the test battery data indicated significant effects of day, $F(3,24)=21.2$, $p=0.000$, day by treatment, $F(12,64)=4.6$, $p=0.000$, and treatment, $F(4,26)=11$, $p=0.000$. Significant group differences were detected at all the post-dosing experimental times, all F's $(4,26)>8.5$, p's=0.000. The 1.25 mmol/kg of *cis*-2-pentenenitrile group showed no differences from controls, but doses of 1.50 mmol/kg or higher induced a significant loss of vestibular function which was larger in the groups receiving the highest doses.

3.3. Effects of *cis*-2-pentenenitrile on the vestibular sensory epithelia.

SEM assessment of the control vestibular sensory epithelia (Fig. 4A) revealed no pathological alterations. The morphology agreed with that reported for normal adult rat epithelia (Csillag, 2005). The effects of *cis*-2-pentenenitrile are shown in figure 4, and summarized in Table 1. The vestibular epithelia from animals exposed to 1.25 mmol/kg

(Fig. 4B, 4C) showed no difference compared with those from control animals, although minor effects cannot be completely ruled out at the level of observation used. Animals exposed to larger doses of *cis*-2-pentenenitrile showed significant evidence of hair cell loss, up to complete loss of hair bundles in the sensory epithelia (Fig. 4 D, E, F). When comparing animals one by one, animals with higher scores on the behavioral test battery for vestibular dysfunction showed more extensive loss of hair bundles in the epithelia.

3.4. Effects of 1-aminobenzotriazole on the vestibular toxicity of *cis*-2-pentenenitrile.

Co-administration of the animals with the universal cytochrome P450 inhibitor, 1-aminobenzotriazole, blocked the toxicity of *cis*-2-pentenenitrile. In this experiment, animals exposed to *cis*-2-pentenenitrile alone showed a loss of body weight that was maximal at day 2 after dosing, when their mean group weight was 90% of the initial value ($p<0.05$, post-hoc analysis after significant MANOVA day by treatment interaction, $F(10,28)=11.1$, $p=0.000$). Animals co-administered with 1-aminobenzotriazole did not lose weight. Body weights at day 2 were 101 % of the initial body weights in both the control and the aminobenzotriazole + *cis*-2-pentenenitrile groups.

Co-administration with 1-aminobenzotriazole specifically blocked the vestibular toxicity of *cis*-2-pentenenitrile exposure, as assessed by the behavioral test battery at day 7 after exposure. As shown in Fig. 5, animals administered both 1-aminobenzotriazole and *cis*-2-pentenenitrile did not develop the behavioral deficits associated with vestibular damage, in contrast to the effect observed in the animals treated with the nitrile alone. This conclusion was supported by the Duncan's test analysis ($p< 0.05$) after the ANOVA analysis demonstrated a significant group effect ($F(2,18)=10.9$, $p=0.001$).

3.5. Effects of *cis*-2-pentenenitrile in the central nervous system.

Control animals showed no Fluoro-Jade C staining (Fig 6A,D). In the two positive control animals administered with hexadienenitrile, this stain revealed neuronal degeneration in several specific brain regions including the inferior olive and the piriform cortex (Fig 6B), in agreement with previously published results (Boada-Vaello et al., 2005; Seoane et al., 2005).

The rats exposed to 2.0 mmol/kg of *cis*-2-pentenenitrile only did not show

consistent evidence of neuronal degeneration (Fig 6C), although punctuate labeling was noticed in the olfactory glomeruli in two of the three rats examined (Fig 6E). One of these animals also showed a very small focus of degenerating neurons in one side of the deepest layer (6b) of the somatosensory cortex. The third rat showed two small foci of neuronal degeneration: one in the cerebellum, where a Purkinje cell dendritic arborization and several neighboring granule cells were labeled, and a second one in the fimbria of the hippocampus. Both lesions were found in a single section and on one side only. In addition, a small group of axons were apparently labeled in this rat bilaterally in or near the internal capsule.

The rats administered with both 1-aminobenzotriazole and *cis*-2-pentenenitrile did not show Fluoro-Jade C staining (not shown).

4. DISCUSSION

Several low molecular weight nitriles have been shown to have prominent neurotoxic effects, including the induction of degeneration of either the sensory hair cells of the inner ear or selected groups of central nervous system neurons. The nitriles that have been thoroughly assessed for these effects to date, including IDPN, allylnitrile, *cis*- and *trans*-crotononitrile, and hexadienenitrile, are of modest economic importance. In this study, we assessed the vestibular and central nervous system effects in the rat of *cis*-2-pentenenitrile, a compound with a significant industrial occurrence (see Introduction).

Exposure of rats to *cis*-2-pentenenitrile resulted in a dose-dependent effect on body weight; significant differences from control weights were recorded after does of 1.50 mmol/kg or larger. Although not specifically addressed in this study, reversible effects were observed in the cornea, where opacity was evident after administration with doses of 1.50 mmol/kg or larger. Opacification of the cornea is a known effect of IDPN (Selye, 1957; Yamashita, 1973; Seoane et al., 1999), allylnitrile (Balbuena and Llorens, 2001), and *cis*-crotononitrile (Balbuena and Llorens, 2003), but is not observed in rats exposed to *trans*-crotononitrile or hexadienenitrile.

The behavioral effects of *cis*-2-pentenenitrile were dose-dependent and included decreased rearing activity and increased rating scores in a test battery designed to assess vestibular dysfunction. Locomotor activity in the open field showed high variability, but some of the animals dosed with the higher doses of *cis*-2-pentenenitrile were distinctly hyperactive. These effects are similar to those reported in rats exposed to IDPN (Llorens

et al., 1993), allylnitrile (Balbuena and Llorens, 2001), and *cis*-crotononitrile (Balbuena and Llorens, 2003), and are a consequence of the loss of vestibular function (Llorens et al., 1993; Llorens and Rodríguez-Farré, 1997; Balbuena and Llorens, 2003; Boadas-Vaello et al., 2005). Accordingly, our histological data demonstrated loss of the vestibular sensory hair cells and a good correlation between the behavioral and the pathological effects both at group and at individual level. We thus conclude that *cis*-2-pentenenitrile causes a dose-dependent loss of vestibular hair cells in the rat with a lowest effective dose of 1.5 mmol/kg after oral acute exposure. These data confirm and extend the previously available behavioral evidence for this effect (Tanii et al., 1999; Genter and Crofton, 2000; Lewis et al., 2006; Saldaña-Ruiz et al., 2012) and complement the available data on the auditory toxicity of this nitrile (Gagnaire et al., 2001).

The features of the vestibular lesions observed by scanning electron microscopy were remarkably similar to the ones reported previously for the other ototoxic nitriles (Llorens et al., 1993; Llorens and Demêmes, 1994; Balbuena and Llorens 2001, 2003), and were also congruent with the "classic" pattern of vestibular toxicity defined by aminoglycoside antibiotics (Forge and Schacht, 2000). This pattern is characterized by an earlier hair cell loss in the central part of the vestibular sensory epithelia progressing towards the periphery with increasing doses, as well as a greater susceptibility of the crista receptors in comparison to the maculae.

We used co-administration of 1-aminobenzotriazole to assess whether the vestibular toxicity of *cis*-2-pentenenitrile depends on cytochrome P450 (CYP)-mediated bioactivation. Although the results of a pharmacological experiment in vivo should be considered with caution because of the possibility of unspecific effects, 1-aminobenzotriazole is well known as a specific and effective universal CYP inhibitor (Mico et al., 1988). Therefore, the diminished toxicity of *cis*-2-penteninitrile observed after aminobenzotriazole most likely resulted from the reduced formation of the ototoxic metabolite(s) of the nitrile. Evidence that the vestibular toxicity depends on CYP bioactivation is also available for allylnitrile (Boadas-Vaello et al., 2009), but the ototoxic metabolite has not been identified. In the case of IDPN, the data available support the conclusion that flavin monooxygenase-mediated generation of N-hydroxy-IDPN is a bioactivation step (Morandi et al., 1987; Nace et al., 1997), but conclusive identification of the ototoxic metabolite is lacking.

In the central nervous system, vestibulotoxic doses of *cis*-2-pentenenitrile did

not cause overt evidence of specific neuronal degeneration. The only consistent finding in more than one animal, the presence of punctuate fluoro-jade C deposits in the olfactory glomeruli, indicates axonal or dendritic degeneration. Toxicity to the olfactory mucosa has been reported for this nitrile (Genter and Crofton, 2000; Lewis et al., 2006), so degeneration of the incoming axons of the primary olfactory neurons probably accounts for the stain observed, as demonstrated with silver degeneration staining in the olfactory glomeruli of rats treated with IDPN (Genter et al., 1992). Other findings in *cis*-2-pentenenitrile rats were inconsistent; they were found in only one rat, and did not show bilateral symmetry except for a few axons in the internal capsula area. The occasional presence of local foci of degeneration, apparently not related to the specific neuronal degeneration presenting as bilateral lesions in all animals, has also been reported for hexadienenitrile (Boadas-Vaello et al., 2005). The lack of consistent central nervous system toxicity in *cis*-2-pentenenitrile was not modified after aminobenzotriazole co-treatment, which probably increased the circulating concentrations of the parent molecule.

In conclusion, the present study characterized the vestibular toxicity of acute oral exposure to *cis*-2-pentenenitrile in the rat. Vestibular toxicity was found at doses of 1.5 mmol/kg and larger, and with similar characteristics to those previously determined for allylnitrile, *cis*-crotononitrile and IDPN.

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FIGURE CAPTIONS:

Fig. 1. Effects of *cis*-2-pentenenitrile on body weight. Data are mean ± SE body weight values expressed as percentages of initial group mean values.

Fig. 2. Effects of *cis*-2-pentenenitrile on 5-min open field activity. Data are mean ± SE. (A) Rearing activity. a,b,c : groups not sharing a letter are significantly different, $p < 0.05$, Duncan's test after significant repeated-measures MANOVA and post-hoc day by day analysis. (B) Horizontal locomotor activity (square crossings).

Fig. 3. Effects of *cis*-2-pentenenitrile on vestibular function. Data are mean ± SE rating scores for vestibular dysfunction. a,b,c : groups not sharing a letter are significantly different, $p < 0.05$, Duncan's test after significant repeated-measures MANOVA and post-hoc day by day analysis.

Fig 4. Effects of *cis*-2-pentenenitrile on the vestibular sensory epithelia, as assessed by scanning electron microscopy at 36-42 days after exposure. (A) Control crista. A dense covering (arrow) of hair bundles, each one corresponding to one vestibular hair cell, characterizes the sensory epithelium. (B) Crista from a 1.25 mmol/kg of *cis*-2-pentenenitrile rat, showing control-like appearance. This rat had shown no evidence of vestibular dysfunction (rating score of 0 at day 21). Note the hairy appearance of stereocilia bundles (arrow). (C) Utricle from a 1.25 mmol/kg of *cis*-2-pentenenitrile rat, showing control-like appearance. This rat had shown no evidence of vestibular dysfunction (rating score of 0 at day 21). In the utricles, stereocilia bundles (arrow) are shorter than in the cristas. (D) Crista from a 1.75 mmol/kg of *cis*-2-pentenenitrile rat, showing a surface of the sensory epithelium (arrow) almost completely devoid of hair bundles. At day 21 this rat received a vestibular rating score of 16. (E) Utricle from a 1.75 mmol/kg of *cis*-2-pentenenitrile rat, showing a noticeably reduced density of hair bundles (arrow). At day 21 this rat received a vestibular rating score of 11. (F) Crista from a 2.0 mmol/kg of *cis*-2-pentenenitrile rat, representing the worst case example. No normal hair bundles remain in the sensory epithelium (arrow), and the acquired fragility of the tissue favored its distortion during manipulation. At day 21 this rat received a vestibular rating score of 20. Scale bars = 300 μm in A, C,D, E, and F; 100 μm in B.

Fig. 5. Decrease due to 1-aminobenzotriazole co-treatment of the effect of *cis*-2-pentenenitrile on vestibular function. Data are mean ± SE rating scores for vestibular dysfunction at day 7 after exposure to control vehicles, *cis*-2-

pentenenitrile (c2PN) plus vehicle, or 1-aminobenzotriazole (ABT) plus *cis*-2-pentenenitrile. *: significantly different from the other two groups, $p < 0.05$, Duncan's test after significant ANOVA.

Fig 6. Effects of *cis*-2-pentenenitrile on the central nervous system, as assessed by Fluoro-Jade C staining of degenerating neurons at 7 days after exposure. (A) Control brain, piriform cortex region, showing no Fluoro-Jade C staining. (B) Positive control section, piriform cortex region of a rat exposed to hexadienenitrile. Labeled neurons were observed in the whole layer (arrows). The inset shows a higher magnification of the degenerating neurons. (C) The same region in a *cis*-2-pentenenitrile rat. No Fluoro-Jade C labeling was observed. (D) Glomerular layer of the olfactory bulb of a control rat. (E) Glomerular layer of the olfactory bulb of a *cis*-2-pentenenitrile rat. Note the punctuate Fluoro-Jade C deposits (arrows). Scale bars = 100 μm in A, B, and C; 25 μm in D, and E; 10μm in the inset in B.

Table 1. Effects of *cis*-2-pentenenitrile on vestibular sensory epithelia: summary.

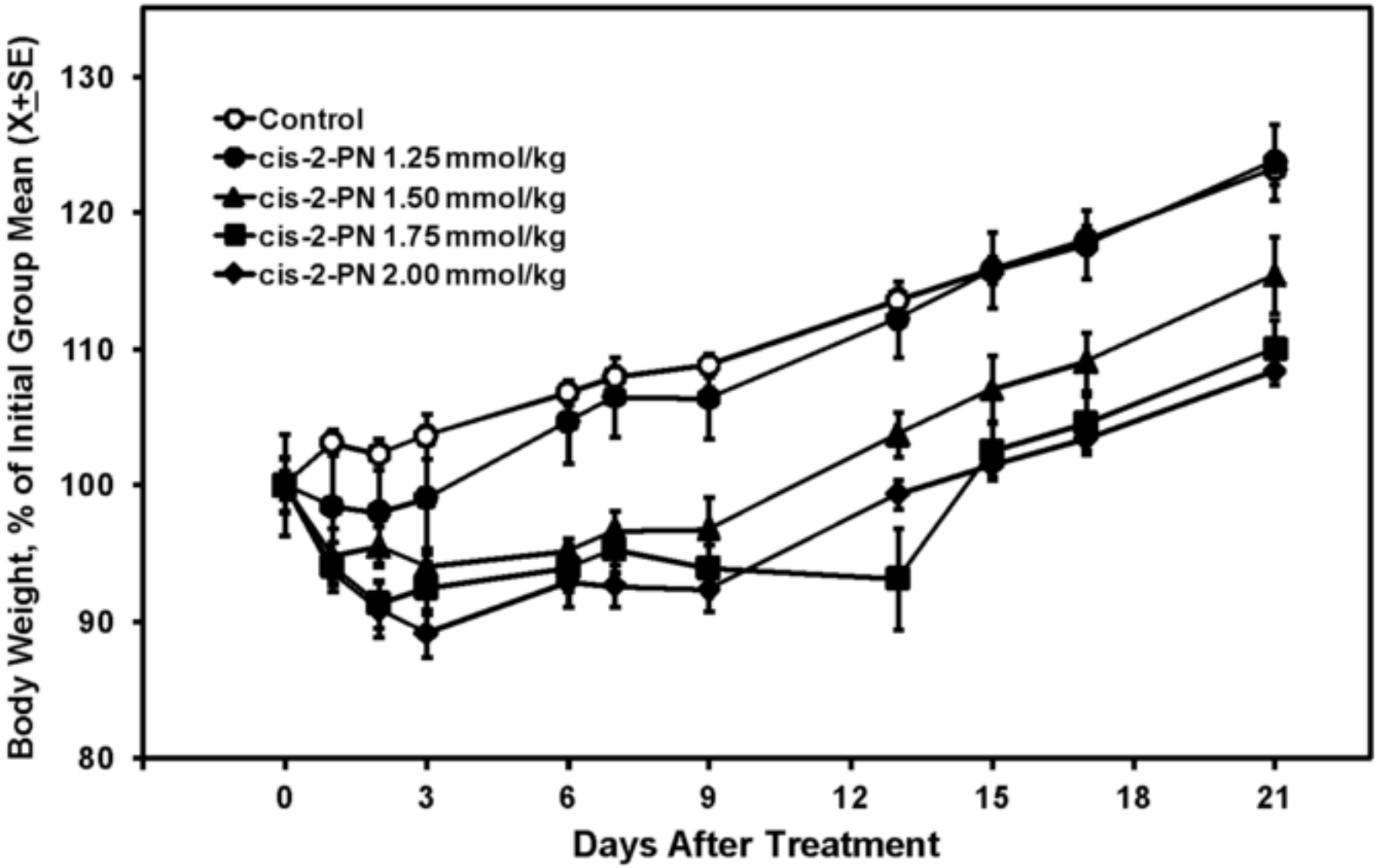
Allylnitrile		Vestibular epithelia		
(mmol/kg)	(n)	Crista	Utricle	Saccule
0	(2)	0-1	0-1	0
1.25	(3)	0-1	0-1	0
1.50	(3)	1-3	1-2	1
1.75	(4)	3-4	2	1-2
2.0	(2)	3-4	3	3-4

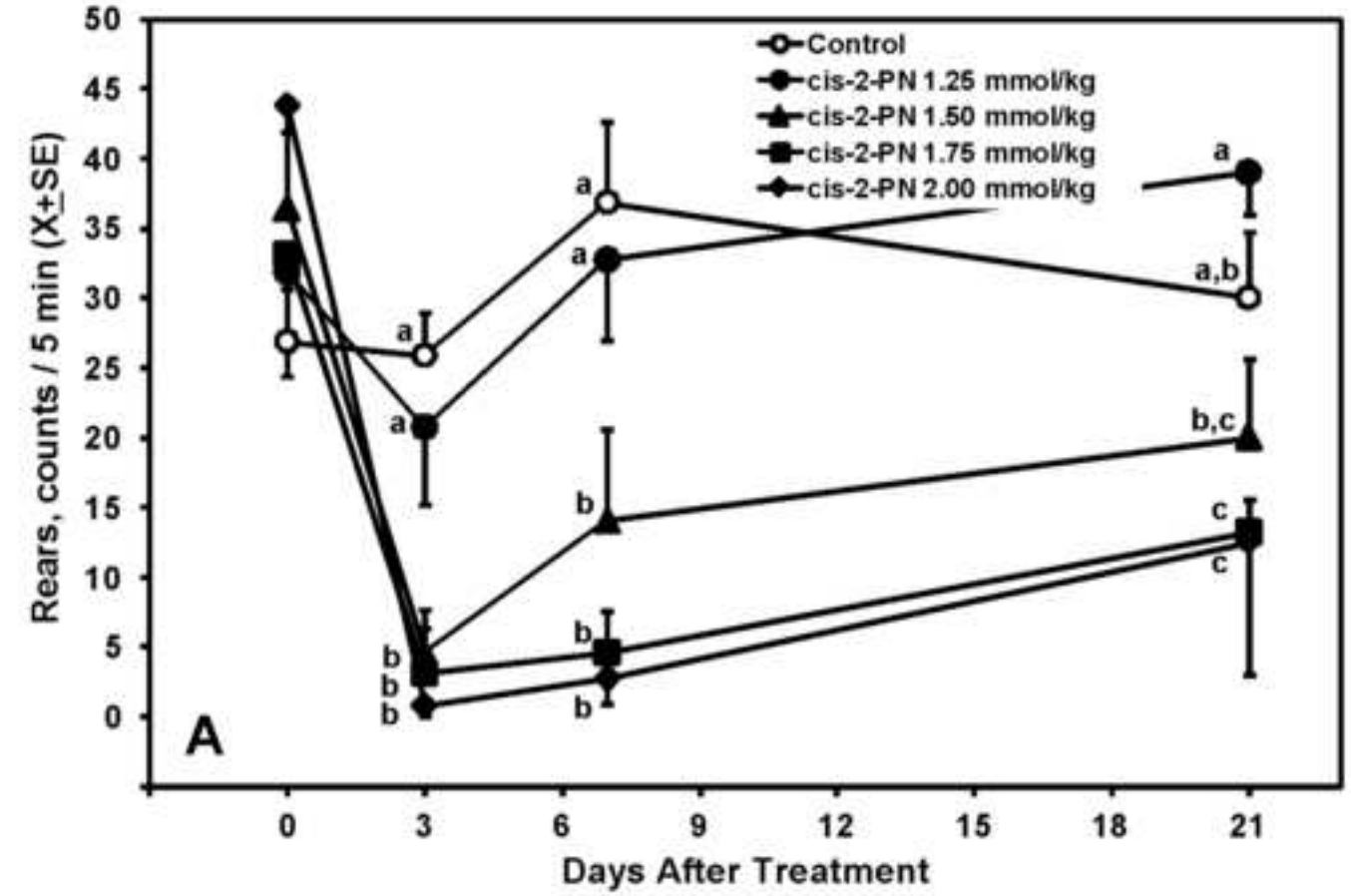
Data are arbitrary ratings after scanning electron microscopy observations of the sensory epithelia. Rats were given 0, 1.25, 1.5, 1.75, or 2.0 mmol/kg of *cis*-2-pentenenitrile and examined at 36-42 days after dosing. (n): Number of animals examined. VESTIBULAR RATINGS: 0, no differences from literature descriptions of control adult tissue; 1, presence of hair bundles with abnormal configuration of stereocilia or lack of a few hair bundles in the central part of the receptor; 2, loss of hair bundles clearly evident at low magnifications but only in the central region of the receptor; 3, widespread loss of hair bundles, usually complete in the central part of the receptor, and evident in more peripheral areas; 4, complete or almost complete loss of hair bundles.

CONFLICT OF INTEREST STATEMENT

Jordi Llorens is a member of the scientific advisory board of Sensorion Pharmaceuticals, a company that generates therapies for vestibular disorders.

Figure 1


Control
cis-2-PN 1.25 mmol/kg
cis-2-PN 1.50 mmol/kg
cis-2-PN 1.75 mmol/kg
cis-2-PN 2.00 mmol/kg
Body Weight, % of Initial Group Mean (X+SE)
130
120
110
100
90
80
0
3
6
9
12
15
18
21
Days After Treatment



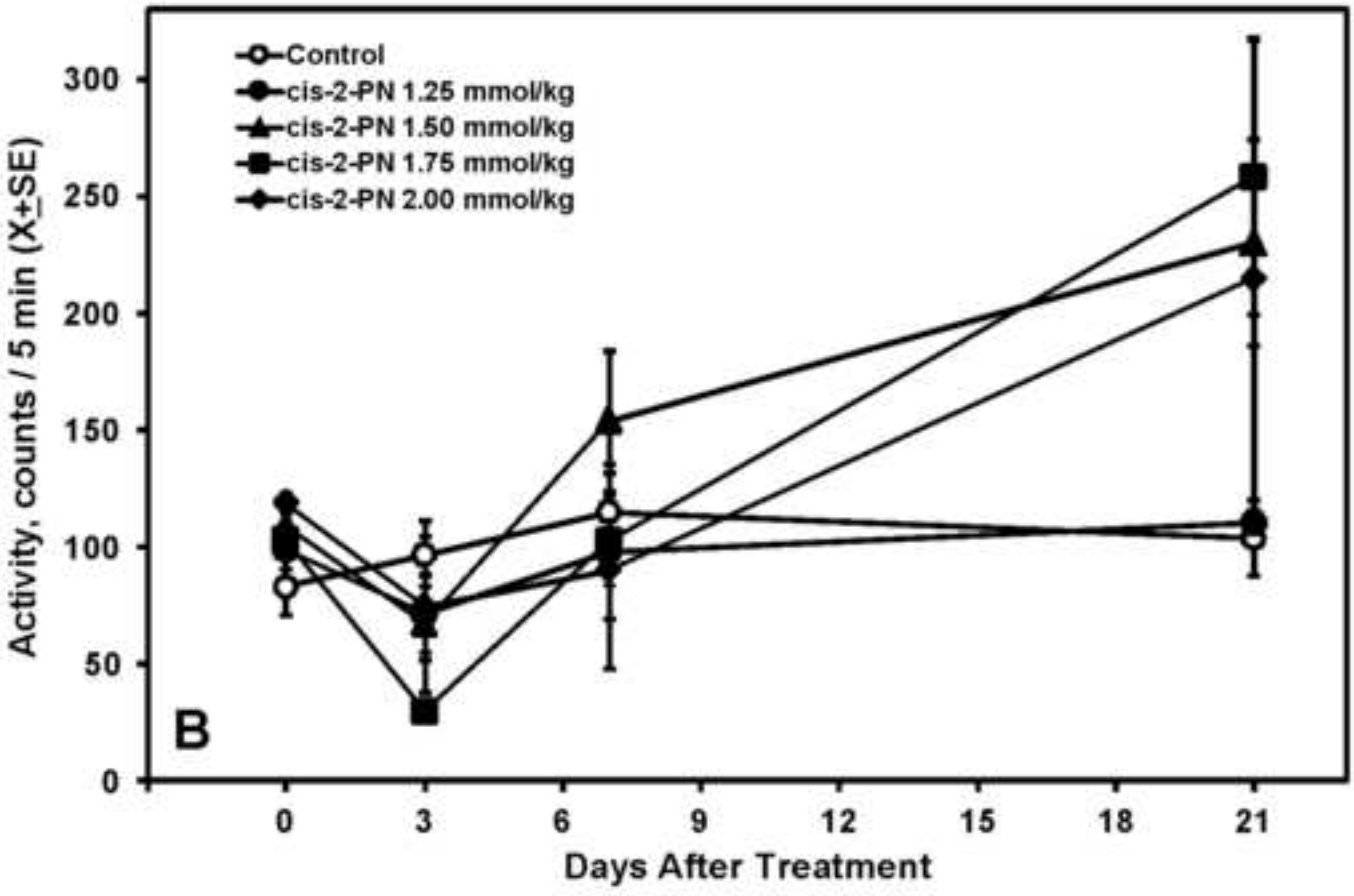
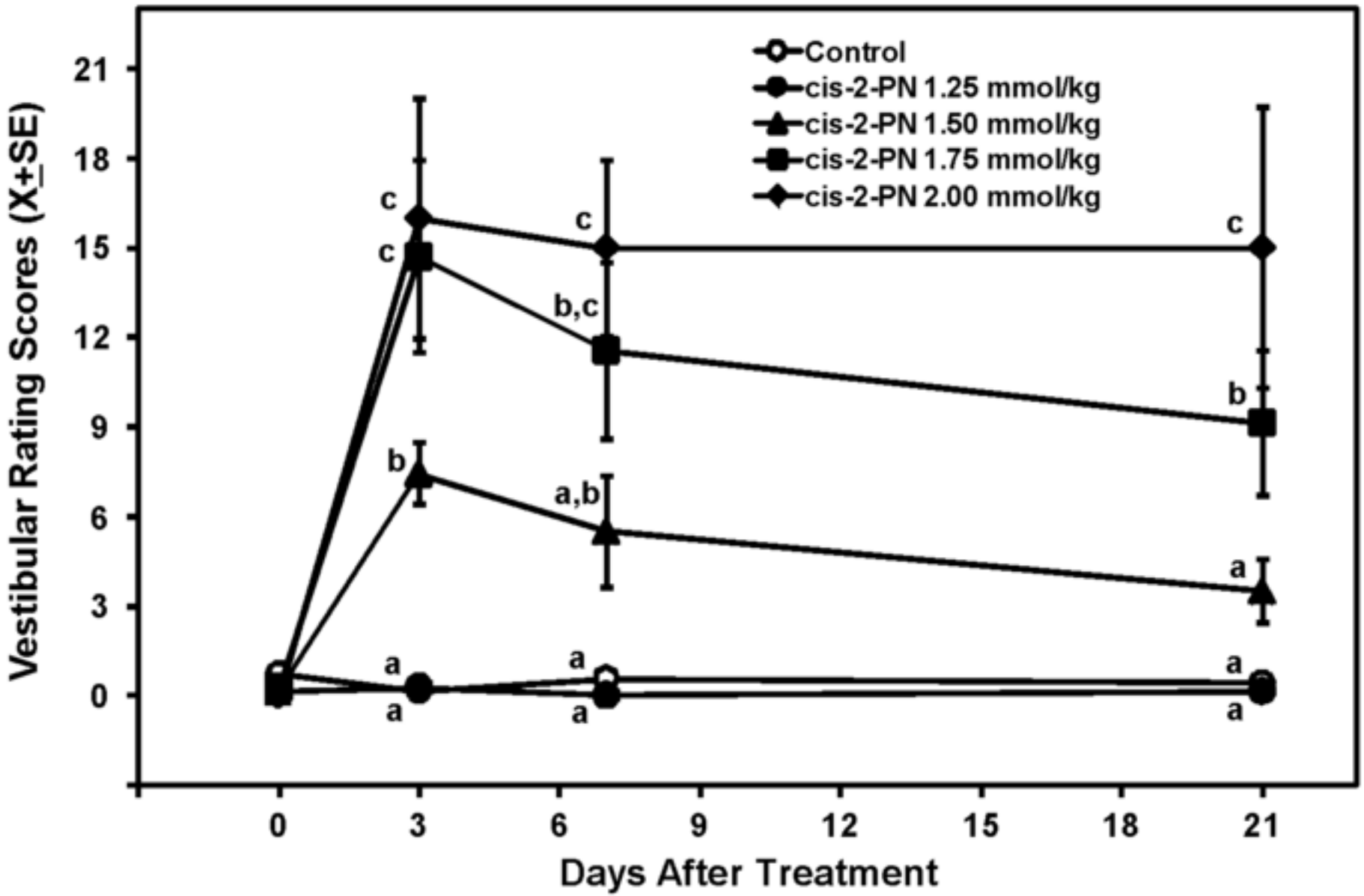
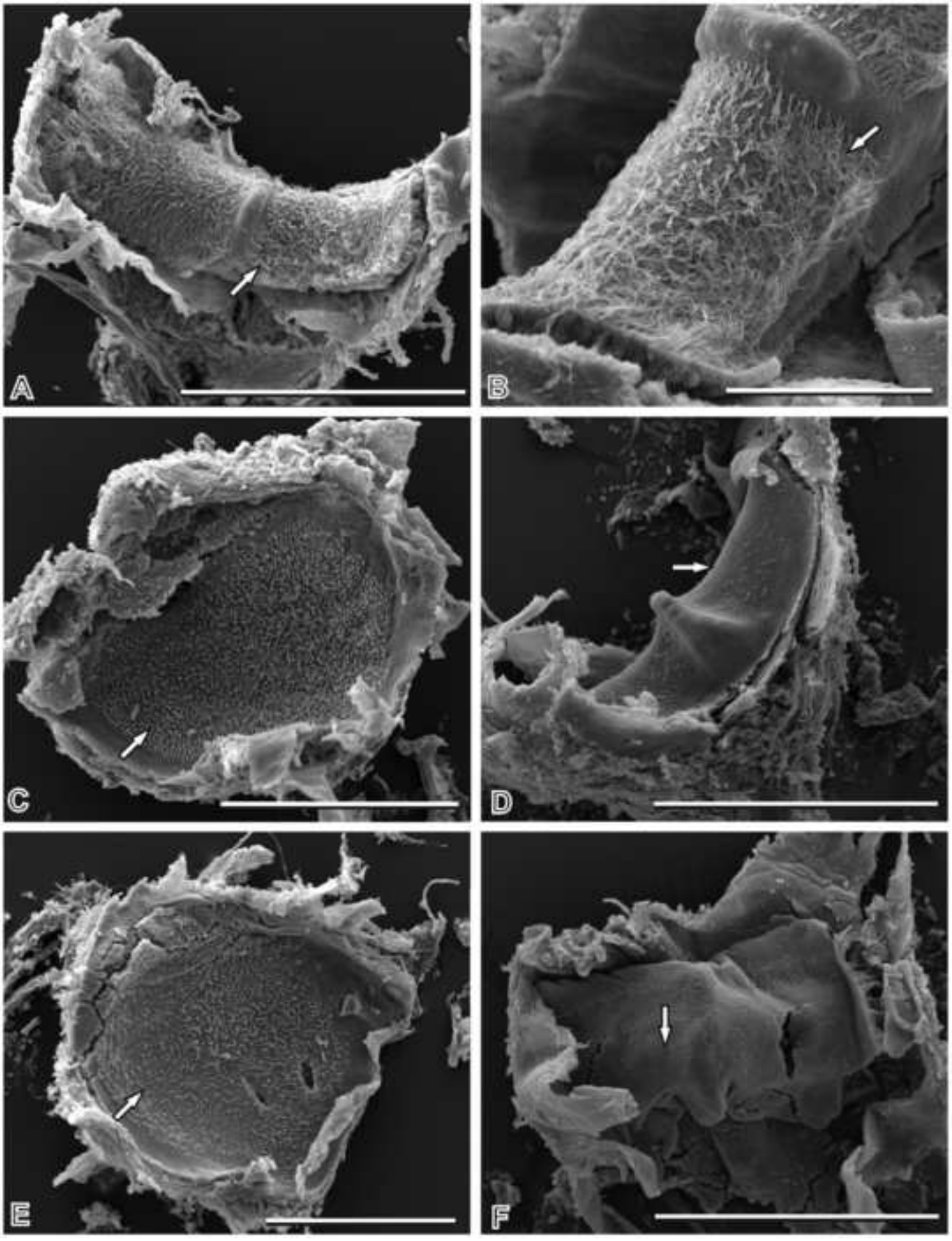


Figure 3


Control
cis-2-PN 1.25 mmol/kg
cis-2-PN 1.50 mmol/kg
cis-2-PN 1.75 mmol/kg
cis-2-PN 2.00 mmol/kg
Vestibular Rating Scores (X+SE)
Days After Treatment
0
3
6
9
12
15
18
21
c
c
c
c
b,c
b
b
a,b
a
a
a
a
a
a



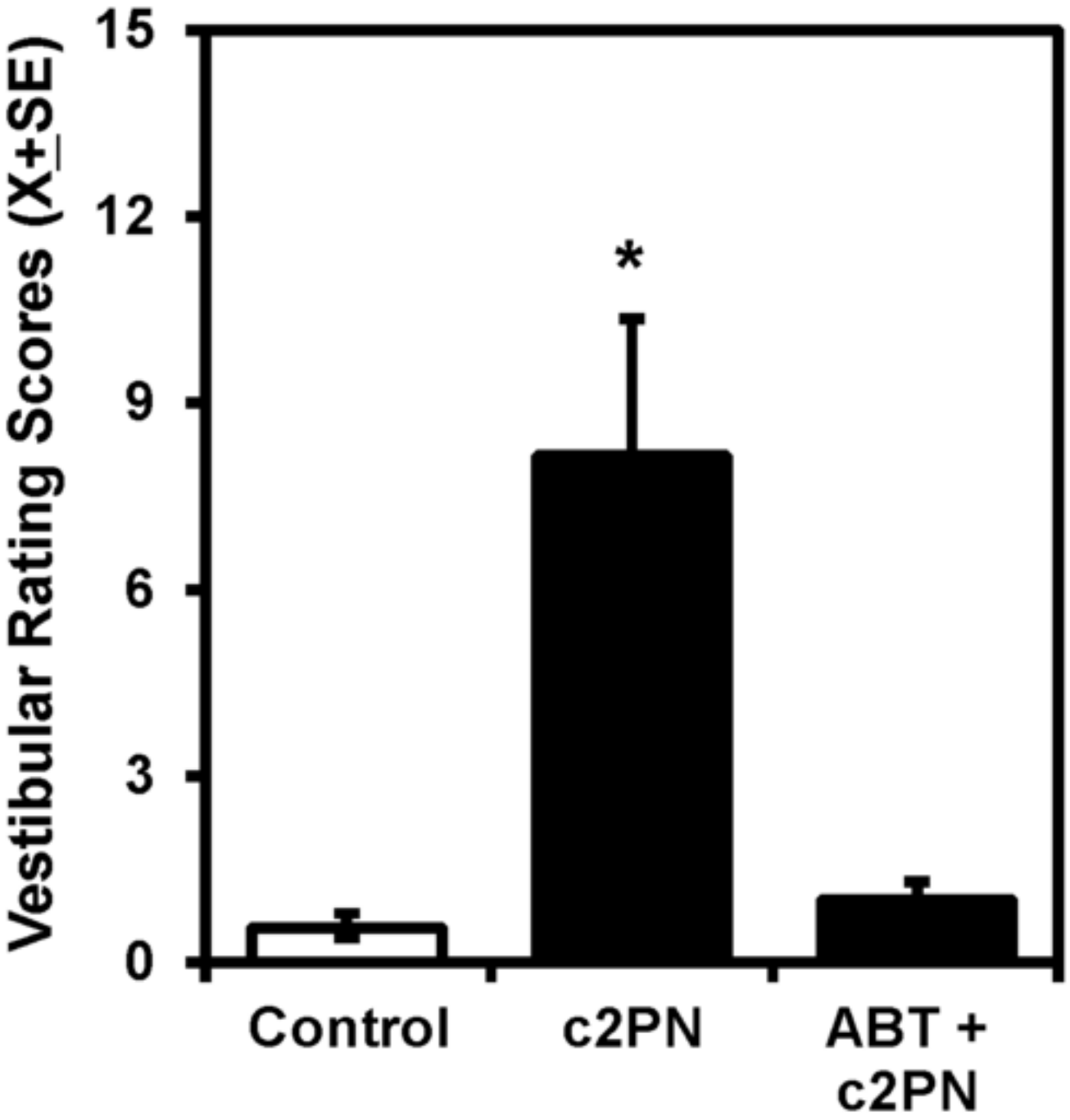


Figure 6