Behavioral Disturbances and Hair Cell Loss in the Inner Ear Following Nitrile Exposure in Mice, Guinea Pigs, and Frogs

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Several nitriles have been demonstrated to cause hair cell loss in the inner ear of the rat, but the susceptibility of other species to this toxic effect has not been investigated. Adult male Swiss mice were administered (po) control vehicle, cis-crotononitrile (2.75 mmol/kg), or 3,3’-iminodipropionitrile (IDPN, at 8, 16, and 24 mmol/kg), and the changes in vestibular function were assessed by behavioral endpoints. In addition, surface preparations of the vestibular sensory epithelia were examined for hair cell loss using scanning electron microscopy (SEM). IDPN, in a dose-dependent manner, and cis-crotononitrile induced both vestibular dysfunction and loss of hair bundles. Male Dunkin Hartley guinea pigs were administered IDPN (0, 1.6, 2.4, or 3.2 mmol/kg, ip), and their vestibular and auditory sensory epithelia were examined by SEM. The guinea pigs developed behavioral abnormalities indicative of vestibular dysfunction, with more overt effects observed in the animals treated with larger doses, and displayed a dose-dependent loss of hair bundles in both the vestibular and the auditory epithelia. Frogs (Rana perezi) were administered IDPN (0, 16, 24, or 32 mmol/kg, ip), and their sensory epithelia in the inner ear were examined by SEM. IDPN caused behavioral abnormalities indicative of vestibular dysfunction and loss of hair bundles. We conclude that some nitriles are thorough ototoxic compounds affecting hair cells in a wide range of species. This conclusion highlights the potential interest of this toxic effect and offers new animal models in which to decipher its basis.

Key Words: iminodipropionitrile; cis-crotononitrile; mechanoreceptors; ototoxicity; mammals; amphibians.

Nitriles are increasingly used in the chemical industry and are also common in crop plants. Their toxic effects include acute lethality, osteolathyrism, and neurotoxicity (DeVito, 1996). This neurotoxic potential was first revealed by the behavioral effects of 3,3’-iminodipropionitrile (IDPN) in rodent species (Delay et al., 1952), termed the ECC syndrome (excitation with choreiform and circling movements; Selye, 1957). The ECC syndrome is also induced by other nitriles, including allylnitrile (3-butenenitrile), crotononitrile (2-butenenitrile), and 2-pentenenitrile (Balbuena and Llorens, 2001, 2003; Llorens et al., 1998). Data from the rat indicate that this syndrome is associated with vestibular sensory hair cell degeneration following exposure to different dosing schedules of IDPN (Llorens and Rodríguez-Farré, 1997; Llorens et al., 1993, 1999), as well as following allylnitrile (Balbuena and Llorens, 2001) and crotononitrile (Llorens et al., 1998). In the case of crotononitrile, only the cis-isomer induces both the ECC syndrome and vestibular hair cell loss; trans-crotononitrile does not induce either of these effects (Balbuena and Llorens, 2003). It has also been demonstrated that the syndrome is similar to the one induced by either surgical or chemical bilateral ablation of the vestibular system (Llorens and Rodríguez-Farré, 1997; Llorens et al., 1993).

Nitriles have other neurotoxic properties. IDPN also causes olfactory deficits associated with degeneration of the olfactory mucosa (Genter et al., 1992, 1996), hearing deficits associated with loss of the auditory hair cells and ganglion neurons (Crofton and Knight, 1991; Crofton et al., 1994), and visual deficits associated with clouding of the cornea and retinal detachment and degeneration (Barone et al., 1995; Herr et al., 1995; Selye, 1957; Seoane et al., 1999). This nitrile is also known to induce a neurofilamentous proximal axonopathy particularly affecting large myelinated neurons (Chou and Hartmann, 1964; Clark et al., 1980; Llorens and Demémés, 1996; Slagel and Hartmann, 1965). Allylnitrile and cis-crotononitrile also cause auditory toxicity (Balbuena and Llorens, 2001, 2003; Gagnaire et al., 2001). Other neurotoxic effects of nitriles include genitourinary toxicity and polynuropathy caused by dimethylaminopropionitrile in humans and rats (Pestrkon, 2000) and the neuronal degeneration occurring in several brain regions, mainly the inferior olive and the piriform cortex, of rats exposed to trans-crotononitrile and hexadienenitrile (Boadas-Vaello et al., 2005; Seoane et al., 2005). Recent data (Boadas-Vaello et al., 2005) indicate that neurotoxic nitriles can be classified in at least two groups: those causing sensory toxicity (IDPN, allylnitrile and cis-crotononitrile) and those causing selective neuronal degeneration in the central nervous system (trans-crotononitrile and hexadienenitrile). Neither the mechanisms of action nor the
ultimate toxic compound are known for any of the neurotoxic effects of nitriles. The hypothesis that IDPN neurotoxicity depends on metabolic bioactivation has been well developed (Jacobson et al., 1987) and has received support for the vestibular and auditory (Nace et al., 1997), the axonopathic (Denlinger et al., 1992), and the olfactory (Genter et al., 1994) effects. However, these evidences are not conclusive and data against this hypothesis are also available (Llorens and Crofton, 1991).

The syndrome of behavioral disturbances has been reported to appear following IDPN exposure in a number of diverse species, including mouse, rat, rabbit, cat, dog, rhesus monkey, and several species of birds (Delay et al., 1952; Hartmann and Stich, 1957; Selye, 1957). However, the vestibular origin of this toxic action has only been established in the rat. Also, no evidence exists for auditory toxicity of nitriles in other species. Thus, the aim of the present study was to demonstrate that the inner ear toxicity of nitriles occurs in species other than the rat. To this end, we first characterized the vestibular toxicity of IDPN and cis-crotononitrile in the mouse by behavioral and histopathological methods. The availability of mutant and transgenic strains make this species a choice for future metabolic and mechanistic studies. Second, we pursued to demonstrate that IDPN causes vestibular and auditory toxicity also in the guinea pig, a species frequently used for inner ear research. Finally, we included in the present series of experiments a small experiment aimed at demonstrating inner ear toxicity of IDPN in an amphibian species.

METHODS

Chemicals and Reagents

IDPN (99%) was purchased from Acros Organics (Geel, Belgium). cis-Crotononitrile was obtained by fractional distillation from a commercially available racemic mixture (99%, cis:trans ratio of approximately 60:40, Aldrich Quimica, Alcobendas, Spain), as described elsewhere (Balbuena and Llorens, 2003); fractions with an isomeric purity greater than 97% (by 1H-NMR, 300 MHz, using CDCl3 as solvent, in a Varian Unity 300 spectrometer) were used in the present series of experiments.

Animals

The care and use of animals were in accordance with the Law 5/1995 and Act 214/1997 of the Autonomous Community (Generalitat) of Catalonia and approved by the Ethics Committee on Animal Experiments of the University of Barcelona. Fifty-three 8- to 10-week-old male Hsd-ICR (CD-1) mice (Harlan Interfauna Ibérica, Sant Feliu de Codines, Spain) were used. They were housed two to four per cage in standard Macrolon cages (28 × 28 × 15 cm) with wood shavings as bedding and given standard diet pellets (Harlan Teklad Global Diet 2014) ad libitum. Ten Dunkin Hartley albino male guinea pigs, 350–400 g on arrival (Charles River Laboratories, Santa Perpètua de Mogoda, Spain) were also used. They were housed two per cage in standard Macrolon cages (28 × 52 × 15 mm) with wood shavings as bedding and given rabbit standard diet pellets (Harlan Teklad Global Diet 2030) ad libitum and ascobic acid in the drinking water (250 mg/l). Mice and guinea pigs were maintained at 22 ± 2°C and on a 12:12 light:dark cycle (0700:1900 h). Seven frogs (Rana perezi, purchased from J.A. Garcia, Mosquerlo, Spain) were also used. Frogs were maintained in Macrolon cages with dechlorinated tap water and stones and given live insects twice a week. Frogs were maintained at 18 ± 2°C. All the animals were provided at least 7 days for acclimation before experimentation.

For histology, mice were anesthetized with 400 mg/kg chloral hydrate, decapitated, and the temporal bones immersed in cold fixative solution for immediate dissection of the sensory epithelia in the inner ear. Guinea pigs were similarly anesthetized, then transcardially perfused with 50 ml of heparinized saline followed by 600 ml of cold fixative solution. Frogs were anesthetized by immersion in 0.18% cold tricaine (ethyl 3-aminobenzoate methane sulphonate, Sigma Chemical, St Louis, MO) before decapitation for inner ear dissection in cold fixative.

Dosing and Experimental Design

Mice. Fourteen mice were used in pilot studies for dose selection. One animal each was successively dosed with 12, 16, 20, 24, 8, 2, 4, and 8 mmol/kg of IDPN (oral, in 10 ml/kg of saline). Overt symptoms of vestibular dysfunction were observed in mice treated with doses of 12 mmol/kg of IDPN or higher, starting 3–7 days after treatment, but the animal dosed with 28 mmol/kg died 4 days after treatment. Other mice were successively dosed with 2 (n = 1), 2.25 (n = 1), 3 (n = 2), and 2.75 (n = 2) mmol/kg of cis-crotononitrile (oral, in 6 ml/kg of corn oil). Starting 1–2 days after treatment, clear symptoms of vestibular dysfunction were observed in mice treated with doses of 2.75 or 3 mmol/kg cis-crotononitrile, but one of the animals receiving the highest dose died the day after treatment.

Thirty-five mice were used for a complete behavioral and pathological study. The behavior of the animals was evaluated in the open field and by a test battery of vestibular dysfunction, as described below, in a pretreatment test session. The animals were then divided in groups of seven and given vehicle solutions as described above (three animals received saline and four received corn oil), IDPN at 8, 16, or 24 mmol/kg or cis-crotononitrile at 2.75 mmol/kg. Open-field activity counts and vestibular rating scores were obtained 1, 3, 7, 14, and 21 days after administration. Three to four animals from each group were used for inner ear histology, as described below, 5–12 weeks after dosing.

Guinea pigs. Two guinea pigs were dosed with 4.8 mmol/kg of IDPN (ip, in 2 ml/kg of saline). These animals displayed symptoms of vestibular dysfunction from day 2 after dosing but died at 7 days. Eight guinea pigs were then dosed with 0, 1.6, 2, or 3.2 mmol/kg (n = 2 per group). These animals were observed for overt alterations in motor behavior and for contact inhibition of the righting reflex at several times after dosing. They were then killed for inner ear histology at 4–6 weeks after treatment. Because the results depicted an unambiguous effect and a definite dose-response relationship, no more animals were added to the study.

Frogs. Five frogs were successively dosed with 8, 16, 24, 32, and 24 mmol/kg of IDPN. These animals were observed for alterations in motor behavior and evaluated for loss of the righting reflex in water at several times after dosing. One frog dosed with 24 mmol/kg died at day 31 after treatment. The other frog receiving this dose was killed 25 days after treatment for inner ear histology, and the one dosed with 32 mmol/kg was killed 17 days after treatment. Two untreated animals were used as controls for histology. Because the results obtained were conclusive in demonstrating the inner ear toxicity of IDPN in this species, no more animals were added to the study.

Behavioral Analysis of Mice

Open field. Open-field behavior was assessed in a white wood 50 × 50-cm arena divided into 10 × 10-cm squares by black lines, enclosed with 40-cm high side walls, and illuminated with a 40 W red light bulb placed 50 cm above the floor. In 5-min sessions, the number of rears and the number of square crossings were counted.

Vestibular rating. The disturbance of vestibular function was determined using a battery of behavioral tests identical to the one developed for the rat (Llorens et al., 1993, including modifications by Llorens and Rodriguez-Farré, 1997). This test battery has been successfully used to assess the loss of vestibular function caused by surgical (Llorens et al., 1993) and chemical
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Llorens and Rodrı́guez-Farré, 1997 bilabyrinthectomies, as well as by IDPN (Llorens et al., 1993), crotononitrile (Llorens et al., 1998), allylnitrile (Balbuena and Llorens, 2001), and cis-crotononitrile (Balbuena and Llorens, 2003) toxicity. The battery includes observation of spontaneous motor behavior (Crofton and Knight, 1991), the tail-hang reflex (Hunt et al., 1987; Selye, 1957), contact inhibition of the righting reflex (Ossenkopp et al., 1990; Shoham et al., 1989), and the air-righting reflex (Ossenkopp et al., 1990). Briefly, mice were placed for 1 min in an open arena (a rat housing cage) and the experimenter rated the animals from 0 to 4 for circling, retropulsion, and abnormal head movements. Circling was defined as stereotyped circling ambulation. Retropulsion consisted of backward displacement of the animal. The head bobbing consisted of intermittent extreme backward extension of the neck. The mice were afterward rated 0–4 by the tail-hang reflex, contact inhibition of the righting reflex, and air-righting reflex tests. When lifted by the tail, normal mice exhibit a “landing” response consisting of forelimb extension. Mice with impaired vestibular function bent ventrally, sometimes “crawling” up toward their tails, thus tending toward occipital landing. For the contact inhibition of the righting reflex, mice were flipped supine on a horizontal surface, and a rigid plastic board was lightly placed in contact with the soles of their feet. Healthy mice quickly right themselves, but the vestibular-deficient mice lie on their back, with their feet up and “walk” with respect to the ventral surface. For the air-righting reflex, the animals were dropped supine from a height of 10 cm onto a foam cushion. Normal mice are able to right themselves in the air, whereas vestibular-deficient mice are not. A summary statistic was obtained by adding up the scores for all behavior patterns.

Histology

To assess inner ear morphology in mice, guinea pigs, and frogs, we examined surface preparations of the vestibular and auditory sensory epithelia with scanning electron microscopy (SEM). Dissection of mouse and guinea pig epithelia followed the method previously used with rats (Balbuena and Llorens, 2003; Llorens and Demémes, 1994; Llorens et al., 1993; Seoane et al., 2001). Frog dissection was performed following literature descriptions of the anuran inner ear (Harada et al., 2001; Jagla and Schneider, 1999; Omura et al., 1989; Smotherman and Narins, 2000). The fixative solution consisted of 2.5% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2). After dissection, the sensory epithelia from the inner ear were allowed an additional 1.5 h of fixation in the same fixative. The samples were then postfixed for 1 h in 1% osmium tetroxide in cacodylate buffer and subsequently stored in 70% ethanol at 4°C. They were then dehydrated with increasing concentrations of ethanol up to 100%, dried in a critical point dryer using liquid CO2, coated with carbon, and stored in a vacuum chamber until observation. The epithelia were then observed in a Hitachi S-4100 Field Emission SEM at an accelerating voltage of 4–5 kV. Some of the samples were also coated with 5 nm of gold, and observed in a LEICA 360 SEM at an accelerating voltage of 7–15 kV.

Statistics

Behavioral data were tested with repeated-measures multivariate analysis of variance—Wilks criterion—with day as the within-subject factor. Orthogonal contrasts, followed by Duncan test when applicable, were used for post hoc analysis. The α level was set at 0.05. The SPSS 12.0.1 for Windows program package was used.

RESULTS

General Observations in Mice Treated with IDPN or cis-Crotononitrile

One mouse in the cis-crotononitrile group died shortly after administration, perhaps, due to an error in the procedure. Another animal in this group died 2 days after treatment, probably from cis-crotononitrile toxicity. One mouse in the IDPN 16 mmol/kg group died on day 10 of unknown causes, not obviously related to IDPN toxicity.

Nitrile treatment significantly affected animals’ body weight. Statistical analysis indicated significant day (F9,19 = 17.7; p = 0.000), day × treatment (F36,72 = 2.91; p = 0.000), and treatment (F4,27 = 5.03; p = 0.004) effects. Significant differences in body weight were recorded from day 3 after treatment until the end of the experiment at day 21 (all F4,27 > 4.0; p > 0.01). On these days, animals treated with 24 mmol/kg of IDPN showed a significant decrease in body weight, with mean values ranging from 86% of control mean at day 3 to 78% of control mean at day 21. The animals treated with 16 mmol/kg of IDPN differed significantly from controls only at days 6–8 after dosing (84% of control mean) and then recovered (91% of controls at day 21). Mean body weights remained above 90% of control means at all times after dosing for both the 8 mmol/kg IDPN and the 2.75 mmol/kg cis-crotononitrile groups.

Effects of IDPN and cis-Crotononitrile on Mouse Behavior

Assessment with a behavioral test battery demonstrated that both IDPN and cis-crotononitrile cause a marked, persistent loss of vestibular function in mice (Fig. 1). The effects of IDPN were dose dependent, and significant effects were recorded by day 3 after treatment with 24 mmol/kg and by day 7 after treatment with 16 mmol/kg, while no significant effects occurred after 8 mmol/kg. All but one of the mice treated with cis-crotononitrile showed a marked loss of vestibular function. Group differences with respect to controls were already obtained at day 1 after treatment and persisted up to day 21. The outlier mouse presented no overt symptoms of vestibular dysfunction at any time in the experimental period.

FIG. 1. Effects of IDPN (8, 16, and 24 mmol/kg) and cis-crotononitrile (2.75 mmol/kg) on tests of vestibular function in mice. Points represent mean ± SE vestibular rating scores of five to seven animals per group. The maximal score is 24. Multivariate analysis of variance analysis indicated significant effects of day (F5,23 = 74.6; p = 0.000), treatment (F4,27 = 29.8; p = 0.000) and day by treatment F20,27 = 14.5, p = 0.000). Significant group differences were detected at all postdosing experimental times (all F4,27 > 18, p = 0.000). *p < 0.05, significantly different from control mean, Duncan test.
In the open field, both IDPN and cis-crotononitrile caused a significant increase in locomotor activity (Fig. 2A). At day 1 after treatment, the animals treated with any of the three IDPN doses, were already hyperactive, but the cis-crotononitrile animals were not, while at day 3 no statistically significant group differences were recorded, owing to the large variability. All the treated groups displayed increased locomotor activity with respect to controls from day 7 posttreatment onward. Nitrile treatment also modified rearing activity (Fig. 2B), but the effects varied according to time and treatment. Increased rearing activity was recorded in the IDPN 8 mmol/kg group at all days after dosing. In contrast, a biphasic effect was observed in the other IDPN groups, with an initial increase in rearing present at day 1 in the 24 mmol/kg group and at days 1 and 3 in the IDPN 16 mmol/kg group, followed by a drop in rearing counts, which were significantly lower than control rearing counts for both groups at day 21 after dosing. Significantly decreased rearing activity was recorded in the cis-crotononitrile group at day 1 after dosing.

Effects of IDPN and cis-Crotononitrile on the Vestibular Sensory Epithelia of the Mouse

We examined the effects of IDPN and cis-crotononitrile on the vestibular sensory epithelia by SEM (Fig. 3; Table 1). Control sensory epithelia showed an even density of hair bundles (Figs. 3A and 3E), although the possibility of the lack of a small number of hair cells could not be ruled out in some of the specimens due to the presence of common preparation artifacts, such as apical blebs emerging from the cuticular plates of the hair cells. IDPN caused a dose-dependent loss of hair cells. Animals dosed with 8 mmol/kg of IDPN showed an overall control-like appearance, but the lack of a small number of hair cells was a consistent finding in the central parts of the crista (Fig. 3B). Animals dosed with IDPN 16 mmol/kg, presented almost–complete loss of hair bundles in the crista (Fig. 3C), with marked loss in the utricle and the saccule as well. The loss of hair bundles was deeper after the highest dose of IDPN (24 mmol/kg), but a significant number of hair bundles remained in utricles and saccules (Fig. 3F). The three animals processed for histology after treatment with cis-crotononitrile included two animals with high vestibular dysfunction scores and an outlier with no overt symptoms of vestibular dysfunction. The first two suffered an almost complete loss of hair bundles in the crista (Fig. 3D) and widespread loss of bundles in the macula receptors, while the outlier showed a density of hair bundles similar to that shown by control mice.

Effects of IDPN on Behavior and Inner Ear Morphology in the Guinea Pig

Guinea pigs treated with IDPN showed a change in both spontaneous and reflex behaviors which indicated a dose-dependent loss of vestibular function. A smaller effect along with significant recovery was observed in guinea pigs dosed with 1.6 mmol/kg, while the deepest and most persistent effect was observed in the animals dosed with 3.2 mmol/kg. In contrast to mice (see above) and rats (Llorens and Rodríguez-Farré, 1997; Llorens et al., 1993), guinea pigs exposed to IDPN did not show overt hyperactivity. However, other abnormalities were similar in all species. When guinea pigs treated with IDPN were held by the hips, they tended to bend ventrally, recalling the change in the tail-hang reflex reported in rats and mice, instead of showing the forelimb extension characteristic of normal animals. The surface-righting reflex was also inhibited in the treated guinea pigs by a bar grid contacting the paws.

Control vestibular sensory epithelia had a normal density of hair cells and supporting cells with no pathological alterations.
Treatment of the guinea pigs with IDPN resulted in a dose-dependent loss of hair bundles (Fig. 4; Table 1). In animals exposed to 1.6 mmol/kg, a marked loss of hair bundles was evident already at low magnifications in the apical parts of the crista (Fig. 4A), with a more limited but significant evidence of hair cell damage seen at higher magnifications in the striola region of the macula receptors (Figs. 4B and 4F). Exposure to 2.4 mmol/kg of IDPN resulted in widespread loss of hair bundles in the crista (Fig. 4C) and obvious loss in the utricle and saccule (Fig. 4G). The most extensive loss of hair bundles was recorded in the guinea pigs exposed to 3.2 mmol/kg (Figs. 4D and 4E). Regenerating hair bundles, characterized by the short size and circular disposition of the hairs, were frequently found in regions lacking mature hair bundles (Figs. 4E, 4F, and 4G).

In the organ of Corti of control guinea pigs, a complete array of inner and outer hair cells was observed in all cochlear regions. In the guinea pigs dosed with 1.6 mmol/kg of IDPN,
The permanent behavioral syndrome caused by acute exposure to IDPN has been reported in several animal species (Delay et al., 1952; Hartmann and Stich, 1957; Selye, 1957), but its association with degeneration of the vestibular hair cells has only been demonstrated in the rat (Llorens and Rodríguez-Farre, 1997; Llorens et al., 1993; Seoane et al., 2001). In this species, the association of the behavioral syndrome and hair cell loss was also established for allylnitrile (Balbuena and Llorens, 2001) and cis-crotononitrile (Balbuena and Llorens, 2003). The present work characterized the association of this syndrome with vestibular hair cell loss in mice exposed to IDPN or cis-crotononitrile. In addition, we obtained limited but conclusive data demonstrating vestibular and auditory toxicity of IDPN in the guinea pig and the frog R. perzii.

The behavioral syndrome shown by mice after nitrile exposure was similar to that of the rat and could be evaluated using the same test battery (Balbuena and Llorens, 2001; Llorens and Rodríguez-Farre, 1997; Llorens et al., 1993). Although the vestibular ratings did not approach maximal scores in the high-dose IDPN and the cis-crotononitrile groups, the fact that significant numbers of hair bundles remained in some utricles and most saccules of these animals offers a reasonable explanation for this observation (compare with data in Balbuena and Llorens, 2001, 2003; Llorens and Rodríguez-Farre, 1997) and does not suggest (as we initially suspected) a difference in sensitivity of the test battery to assessment of mice versus rats.

All the groups of mice exposed to nitriles showed changes in open-field behavior. In previous work in the rat, we concluded that increased locomotor activity and decreased rearing counts are the result of the loss of vestibular function, a conclusion supported by two facts: first, that these behavioral effects correlated with vestibular hair cell loss following exposure to different dosing regimes of IDPN and different nitriles (Balbuena and Llorens, 2001, 2003; Llorens and Rodríguez-Farre, 1997; Llorens et al., 1993); second, they are similarly induced by either surgical (Llorens et al., 1993) or chemical (Llorens and Rodríguez-Farre, 1997) bilateral ablation of the inner ear. The association of increased locomotor activity and decreased rearing with increased vestibular ratings and extensive loss of hair cells is suggested to be due to vestibular dysfunction (Llorens et al., 1993).

### Effects of IDPN on Behavior and Inner Ear Morphology in the Frog

The behavior of the frog dosed with 8 mmol/kg was normal. Frogs administered with 16, 24, or 32 mmol/kg of IDPN showed abnormal jumping and swimming behaviors 7–14 days after treatment. Jumping frequently ended with unsteady, side and even back landing, while swimming included stereotyped circling and sometimes spontaneous loss of the upright position. A loss of the righting reflex was evident when frogs were placed in the water in a supine position. The frog dosed with 16 mmol/kg of IDPN showed a marked recovery in behavior by 2 months after administration.

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Note. Data are arbitrary ratings after SEM observations of the sensory epithelia. Mice were given control vehicle, 8, 16, or 24 mmol/kg of IDPN or 2.75 mmol/kg of cis-crotononitrile (cis-croto), and examined at 5–12 weeks after dosing. Guinea pigs received control vehicle, 1.6, 2.4, or 3.2 mmol/kg of IDPN, and examined at 4–6 weeks 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 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; and 5, complete loss of hair bundles and evident loss of supporting cells.

*Data from one animal displaying little evidence of vestibular dysfunction and two animals displaying an overt behavioral syndrome of vestibular loss; the former presented low ratings and the latter presented high ratings.*
bundles was also found for mice dosed with cis-crotononitrile or medium and high doses of IDPN. However, the mice treated with 8 mmol/kg of IDPN had only limited vestibular damage (Fig. 3B) and showed little evidence of vestibular dysfunction (Fig. 1), yet they displayed a persistent hyperactivity including increased rearing counts. This suggests that IDPN may cause hyperactivity in the mouse for reasons other than vestibular damage, a conclusion supported also by the increased activity, including rearing, displayed by the groups of mice dosed with higher doses of IDPN before vestibular dysfunction and loss of rearing ability begun. A similar early increase in activity after IDPN does not occur in rats (Llorens and Rodríguez-Farré, 1997; Llorens et al., 1993). One possible cause of this hyperactivity effect could be the toxic effects of IDPN on other sensory systems, of which olfactory damage at least appears at doses below the vestibular toxicity (Genter et al., 1992, 1996). Alternatively, the hyperactivity could result from an effect of IDPN on the central nervous system. In the rat, this nitrile does not cause significant neuronal degeneration (Boadas-Vaello et al., 2005), but this possibility has not been examined in the mouse. Also, both the axonopathic effect (Chou and Hartmann, 1964) and modified function in several neurotransmitter

**FIG. 4.** Effects of IDPN on the vestibular sensory epithelia of the guinea pig, as assessed by SEM at 4–6 weeks after exposure. (A) Crista from a guinea pig dosed with 1.6 mmol/kg of IDPN. Note the paucity of hair bundles in the apical region (arrows). (B) Utricle from the same animal whose crista is shown in A. Loss of hair bundles is not evident in this low-magnification view, but some pathological effects were observed at higher magnifications in the central region (striola) of the receptor (arrows). (C) Widespread loss of hair bundles in a crista of a guinea pig dosed with 2.4 mmol kg⁻¹ of IDPN. (D) Complete loss of mature hair bundles in a crista of a guinea pig dosed with 3.2 mmol/kg of IDPN. However, a significant number of immature hair bundles are seen as small dots at this magnification level. (E) Higher magnification of the crista in D, showing immature hair bundles (arrows) in approximately 15–20% of possible locations. (F) Higher magnification of the striola region of the utricle in B. Note the location of missing hair cell (arrow) and the immature hair bundle (arrowhead). (G) Striola region of a saccule of a guinea pig dosed with 2.4 mmol/kg of IDPN. Note the missing hair bundles (arrows), the immature hair bundles (arrowhead) and the degenerating hair bundles (asterisk). Scale bars: A, B, C, and D, 200 μm; E, F, and G, 10 μm.
systems (reviewed by Cadet, 1989) have been described in the central nervous system of rats and mice after IDPN exposure.

Unlike rats and mice, neither guinea pigs nor frogs showed overt spontaneous hyperactivity. However, postural and motor abnormalities indicative of impaired vestibular function were recorded in both species. The most salient deficit was that of the loss of the righting reflex, which can be interpreted as a loss of the sense of gravity. This deficit was obvious in the swimming frog and was highlighted in mice and guinea pigs by tactile stimulus in the paws of the supine animal, as previously shown in rats (Llorens et al., 1993; Ossenkopp et al., 1990; Shoham et al., 1989). The subsequent demonstration of IDPN-induced hair cell loss in these animals allows us to conclude, as predicted, that the syndrome of behavioral abnormalities elicited by IDPN or cis-crotononitrile is associated with vestibular hair cell toxicity in species other than the rat, including not only other rodents but also an amphibian species.

The dose-response relationship for IDPN-induced vestibular toxicity differs among the diverse species studied to date. Thus, the extent of hair bundle loss previously documented in rats after 4.8 mmol/kg (Llorens et al., 1993) was similar to that now recorded in guinea pigs after 3.2 mmol/kg and in mice after 24 mmol/kg. This would suggest that the guinea pig is slightly more sensitive than the rat, while the mouse is significantly more resistant to the IDPN inner ear toxicity. However, one possible factor contributing to this difference is that rats and guinea pigs were administered ip, while mice were administered orally. Oral administration was selected for mice because preliminary observations indicated a high mortality after ip administration of nitriles in this species. Only very few frogs were examined, but the effective doses appeared in the mouse range in spite of ip administration. It is also worth noting that
the death of two out of two guinea pigs dosed with 4.8 mmol/kg of IDPN is in contrast with the survival of rats given up to 16 mmol/kg (e.g., Llorens and Demêmes, 1996).

Previous data in the rat (Balbuena and Llorens, 2001, 2003; Crofton et al., 1994; Llorens and Demêmes, 1994; Llorens et al., 1993; Seoane et al., 2001) indicate that nitriles show a pattern of inner ear toxicity similar to that described for the aminoglycoside antibiotics (Forge and Schacht, 2000). According to the present data, the cristal receptors are more sensitive to nitrile toxicity than the macula receptors in all the three new species examined and base-to-apex and outer hair cell–inner hair cell gradients of sensitivity exist in the guinea pig organ of Corti. Thus, the data confirm and extend the conclusion that nitriles have an ototoxic profile similar to that of aminoglycoside antibiotics, a conclusion also strongly supported by the susceptibility of both mammalian and nonmammalian species. Open questions are the possible existence of gene mutations that confer increased susceptibility to nitrile ototoxicity, as is known in the case of antibiotics (Estivill et al., 1998), or whether different ototoxic nitriles may show differential preference for vestibular or auditory toxicity, a well-known fact in aminoglycoside ototoxicity (Forge and Schacht, 2000).

One clear species difference is the regrowth of hair bundles. In contrast to our previous findings in the rat (Balbuena and Llorens, 2001, 2003; Llorens and Demêmes, 1994; Llorens et al., 1993), many immature hair bundles were observed in the vestibular sensory epithelia of the nitrile-treated guinea pigs. Whether these correspond to new hair cells (Forge et al., 1998) or result from repair of surviving hair cells (Zheng et al., 1999) remains to be determined. In any case, this difference between IDPN-treated rats and guinea pigs emerged in spite of the use of similar experimental paradigms, so it reflects a difference in the physiological response of these animal species to the vestibular damage. Thus, this repair capacity needs to be taken into account when choosing animal species and time points for assessment of vestibular toxicity. In the mouse, a certain repair capacity cannot be completely ruled out by the present observations, but it seems to be rather limited, similar to that in the rat, and much lower than that in the guinea pig. In amphibians, vestibular repair capacity is well documented (for instance, Gale et al., 2002). The fact that we found no evidence of it was probably because the two frogs examined had been treated with supramaximal doses and killed at a time when inner ear damage was still ongoing.

In conclusion, the present data demonstrate that nitriles that are toxic to the vestibular and auditory systems of the rat are also ototoxic to other animal species, both mammals and nonmammals. This suggests that these nitriles are toxic to hair cells by interacting with biological components highly conserved during evolution, so similar effects are to be expected in other species including humans. In addition, the findings open up the possibility of using species other than the rat to study this toxic effect of nitriles and the possibility of using nitriles for inner ear research performed in diverse animal species.

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