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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.

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-Ruíz 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., 2001a,b; 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-Martín et al., 2007). One nitrile causing vestibular toxicity in mice, *trans*-crotononitrile (Saldaña-Ruíz 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., 1989; Genter and Crofton, 2000; Lewis et al., 2006; Saldaña-Ruíz 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

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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 mm × 520 mm × 145 mm) with wood shavings as bedding at 22 ± 2 °C. 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 C 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, 2000a,b; 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 cm × 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 0–24.

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 °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 VT1000 M 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., 1999a,b.

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., 1993; Llorens and Rodríguez-Farré, 1997; Seoane et al., 2001a,b), 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 4 °C. 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).