Abstract 3,3′-iminodipropionitrile (IDPN) is a neurotoxic compound that causes proximal neurofilamentous axonopathy and loss of the vestibular sensory hair cells. During subchronic exposure, the hair cells are eliminated by extrusion of the virtually intact cell from the sensory epithelia towards the luminal cavity. We describe the alterations of the vestibular epithelia before and during hair cell extrusion. Adult male Long-Evans rats were exposed to 0.2% IDPN in the drinking water for 1, 3, 5, 8 or 14 weeks, or to 0.1% IDPN for 14 weeks. Protrusion and subsequent extrusion of hair cells were observed in the cristae and utricular maculae of rats exposed to 0.2% IDPN for at least 5 weeks. At earlier time points and at lower doses, we observed the following pathological signs: blebbing of hair cells, swelling, retraction and fragmentation of the afferent nerve terminals, detachment of hair cells from the surrounding structures and loss of the pre- and post-synaptic membrane thickenings between hair cells and their afferent terminals. Widespread enlargement of the intercellular spaces also preceded and accompanied the extrusion process. The present data challenge the hypothesis that IDPN specifically affects hair cells.

Keywords Nitriles · Axonopathy · Nerve terminals · Cell death · Ototoxicity

Introduction

The experimental neurotoxicity of 3,3′-iminodipropionitrile (IDPN) has been extensively studied given the similarity of the neurofilament-filled swellings in the proximal segments of large calibre axons in IDPN-exposed animals [4] and human diseases, especially amyotrophic lateral sclerosis [9]. Since IDPN impairs the axonal transport of neurofilament proteins [10], it is regarded as a useful model to enlighten both the biology of neurofilament transport and the pathogenesis of diseases involving neurofilament accumulation. However, its mechanism of action on neurofilament transport have not been determined. In addition, IDPN permanently alters motor behaviour [7, 23]. This was first studied under the hypothesis that IDPN damages the extrapyramidal motor system (reviewed by Cadet [3]), but it was finally attributed to the loss of the vestibular sensory hair cells and subsequent loss of vestibular function [15, 17, 18]. The effect of IDPN on hair cells, and whether hair-cell loss and neurofilament effects share a single mechanism, is also unknown.

IDPN neurotoxicity is caused by acute or repeated oral or intraperitoneal administration and by chronic exposure through drinking water administration. The neurofilamentous axonal pathology observed after acute exposure [4] is reproduced by the chronic exposure regimen, which leads to a noticeable accumulation of effects [5, 12, 17]. In the vestibular system, acute IDPN exposure results in necrotic and apoptotic degeneration of hair cells within the sensory epithelia ([15, 18] and unpublished manuscript), while the afferent and efferent nerve endings within the sensory epithelium are apparently well preserved [15]. Thus, hair cells may be the primary target of toxicity. Subchronic IDPN exposure also causes vestibular toxicity, as revealed by behavioural and morphological data [17]. However, this effect has not been studied in detail. We characterised the ultrastructural effects of subchronic IDPN exposure on the vestibular sensory epithelia in rats to elucidate its mechanism(s) of action. Previous data showed that the loss of hair cells occurs by extrusion of intact cells from
the sensory epithelia to the endolymphatic space, in contrast to the necrotic/apoptotic modes of hair cell loss elicited by acute and subacute intoxication (unpublished manuscript). Here, we report other alterations occurring in the epithelium before and during the hair cell extrusion.

Materials and methods

Animals and experimental design

The care and use of animals were in accordance with the European Community Council Directive 86/609/EEC, and approved by the Comité Étique d’Experimentation Animal de la Universitdad de Barcelona. Male Long-Evans rats (CERJ, Le-Genest-Saint-Isle, France), 8–10 weeks old, were used. They were housed two to four per cage in standard macrolon cages (280×520×145 mm) with wood shavings as bedding at 22±2°C. At least 7 days were provided for acclimation before experimentation. The rats were maintained on a 12-h/12-h light/dark cycle (0700:1900 h) and given standard diet pellets (A04, U.A.R., France) ad libitum. They were given tap water containing 0%, 0.1% or 0.2% of IDPN (>99%, Acros, Geel, Belgium) as the sole source of drinking water. Body weights and vestibular function were examined on a regular basis. Animals exposed to 0.2% IDPN were killed at 1, 3, 5, 8 or 14 weeks of exposure (n=3, 3, 4, 3 and 2, respectively). Animals exposed to 0.1% IDPN were killed at 14 weeks of exposure (n=2). One control rat (0% IDPN) was processed at each time point in parallel with the treated rats. The rats were anaesthetised with chloral hydrate (400 mg/kg, i.p.) and transcardially perfused with 50 ml heparinized saline followed by 400 ml of 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2).

Behavioural evaluation of vestibular dysfunction

The tail-hang test [17, 18, 23] was used to monitor the loss of vestibular function. The rat was lifted by the tail and its reflexive behaviour was rated 0 (normal) to 4 (extreme alteration). Normal rats extend their forelimbs towards the earth in a “landing” response. Loss of vestibular function results in the animal’s bending ventrally, sometimes “crawling” up towards its tail, resulting in ocipital landing.

Histology

After perfusion, the vestibular sensory receptors were dissected out in the same fixative, and allowed an additional 1.5 h of fixation. The samples were then postfixed for 1 h in 1% osmium tetroxide in cacodylate buffer, and subsequently dehydrated with increasing concentrations of ethanol up to 100%. For scanning electron microscopy (SEM), samples were dried in a critical-point dryer using liquid CO₂, coated with 5 nm of gold and observed in a LEICA 360 SEM at an accelerating voltage of 7–15 kV. For transmission electron microscopy (TEM), the dehydrated samples were embedded in epoxy resin. Semithin sections (1 μm) were stained with 1% toluidine blue for light microscopy. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined in a Hitachi H600 AB or a Philips 301 transmission electron microscope at 75–80 kV.

Results

IDPN exposure and behavioural effects

IDPN exposure resulted in dose- and time-dependent alterations in vestibular function and morphology. As reported elsewhere [17], rats showed reduced consumption of the intoxicated water, but were nevertheless exposed to larger doses of IDPN when higher concentrations were given. Thus, consumption of water containing 0.1% of IDPN resulted in exposure to 45–75 mg IDPN/kg body weight per day, whereas the rats consuming 0.2% IDPN water received 90–110 mg/kg per day.

Regarding behaviour, 0.1% IDPN animals remained asymptomatic throughout the 14-week exposure period, and the results from the tail-hang test were equivalent to those obtained from control animals (mostly ratings of 0, occasional rating of 1). Animals receiving 0.2% IDPN showed the same behaviour during the first 2 weeks of exposure. At 3 weeks of intoxication, the vestibular function declined, and ratings of 1–2 were recorded in the tail-hang test. From 5 weeks of exposure, a complete syndrome of vestibular dysfunction was observed, and ratings of 3–4 were obtained from the tail-hang test, except for one animal which did not show overt signs of vestibular dysfunction and obtained ratings of 1–2 until sacrifice at 8 weeks of intoxication.

Scanning electron microscopy

The mammalian vestibular system contains three types of neurosensory areas, the cristae ampullaris, the utricular maculae and the saccular maculae. The sensory areas consist of an epithelium containing sensory hair cells and supporting cells. Control specimens showed no evidence of pathological alterations (Fig.1A). Rats exposed to 0.2% IDPN showed progressive blebbing, protrusion and extrusion of hair cells in the crista and utricle. At 1 week of exposure, receptors showed a control-like appearance, except for some blebs behind the hair bundles in the crista. At 3 weeks, large apical blebs were detected in some hair cells in the crista, and in most hair cells in the utricle (Fig.1B). From 5 to 14 weeks of exposure, hair cells in both the crista and utricle showed progressive coalescence of cilia and extrusion towards the endolymphatic cavity (Fig.1C). However, the rat exposed to 0.2% IDPN for 8 weeks, and showing little signs of vestibular dysfunction, showed blebs in the crista, but no evidence of ciliary coalescence or hair cell extrusion. In the epithelia with hair cell extrusion at 5 and 8 weeks, the supporting cells maintained their apical dense lawn of microvilli, although loss of microvilli was apparent after complete loss of hair cells in some areas of the crista after 14 weeks of exposure. Tears near or within the sensory epithelia showed an enlargement of intercellular spaces. Groups of supporting cells assembled in columns extending from the basal membrane to the surface of the epithelia. Cells in the transitional epithelium, the non-sensory epithelium surrounding the sensory area, acquired a spiny columnar shape as they were separated by large intercellular spaces while maintaining discrete focal contacts (Fig.1D).

In contrast with the crista and the utricle, the saccule from rats exposed to 0.2% IDPN was almost normal, with no evident extrusion of hair cells. Nevertheless, apical