Behavioral and Pathological Effects in the Rat Define Two Groups of Neurotoxic Nitriles

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INTRODUCTION

Nitriles are increasingly used in the chemical industry, and they are also common in crop plants. They cause acute lethality and osteolathyrism (DeVito, 1996). Their neurotoxic potential was first revealed by the behavioral effects of 3,3′-iminodipropionitrile (IDPN) (Delay et al., 1952), known as the ECC syndrome (excitation with choreiform and circling movements; Selye, 1957). Histopathological assessment of IDPN-treated animals revealed a neurofilamentous proximal axonopathy particularly affecting large myelinated neurons, and a link between this axonopathy and the behavioral effects was hypothesized (Chou and Hartmann, 1964; Slagel and Hartmann, 1965). However, prominent axonal swelling can be induced without motor alterations by chronic low-dose IDPN exposure (Clark et al., 1980; Llorens and Rodríguez-Farre, 1997). A second hypothesis for the ECC syndrome was that it resulted from altered neurotransmission, mostly within the basal ganglia (reviewed by Cadet, 1989). The permanent nature of the syndrome suggested, however, that it would more likely be associated with permanent histopathological changes. Staining data indicated that sparse axonal degeneration may occur in the cerebral cortex, but only at doses of IDPN above those required to induce the ECC syndrome (Llorens et al., 1993a). We then hypothesized that the motor alterations were the consequence of a toxic effect of IDPN on the vestibular system, and this hypothesis is now supported by the correlation found between the ECC syndrome and vestibular sensory hair cell degeneration after several different dosing schedules of IDPN (Llorens et al., 1993b; Llorens and Rodríguez-Farre, 1997; Seoane et al., 2001). It has also been demonstrated that the same syndrome can be induced by either surgical or chemical bilateral ablation of the vestibular system (Llorens et al., 1993b; Llorens and Rodríguez-Farre, 1997).

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).

Further interest in nitrile neurotoxicity was raised by the finding that allylnitrile (3-butenenitrile), crotononitrile (2-butenenitrile), and 2-pentenenitrile also induce the ECC syndrome (Tanii et al., 1989a,b). The association of motor
disturbances with vestibular toxicity has been demonstrated for allylnitrile (Balbuena and Llorens, 2001) and crotononitrile (Balbuena and Llorens, 2003; Llorens et al., 1998), whereas no axonopathy occurred after administration of crotononitrile (Llorens et al., 1998) or allylnitrile (unpublished results). In the case of crotononitrile, only the cis-isomer induces both the ECC syndrome and vestibular hair cell loss, whereas trans-crotononitrile does not induce either of these effects (Balbuena and Llorens, 2003). We thus concluded that vestibular toxicity fully accounts for the ECC syndrome, and we hypothesized that neuronal degeneration in the central nervous system (CNS) is not involved in its induction. However, several articles report on the deleterious central effects of both IDPN and allylnitrile as an alternative explanation to the vestibular hypothesis for the ECC syndrome. These include studies on apoptosis (Zang et al., 1999), gene expression (Fritschi et al., 2003), neurotransmitter systems (Tani et al., 2000, Wakata et al., 2000), and free radicals (Nomoto, 2004; Wakata et al., 2000). Recent data from our laboratory have demonstrated that trans-crotononitrile, which spares sensory systems (Balbuena and Llorens, 2003), does indeed cause a selective pattern of neuronal degeneration in the CNS in association with behavioral effects that differ from the ECC syndrome (Seoane et al., 2005). There is also evidence suggesting that hexadienenitrile (2,4-hexadiene-1-nitrile) may have neurotoxic effects on the CNS (O’Donoghue, 2000).

In the present study, we compared the behavioral effects of IDPN, allylnitrile, cis-crotononitrile, trans-crotononitrile, and hexadienenitrile in the rat using test methods sensitive to the ECC syndrome or to the hexadienenitrile in the rat using test methods sensitive to the ECC syndrome (Seoane et al., 2005). There is also evidence suggesting that hexadienenitrile (2,4-hexadiene-1-nitrile) may have neurotoxic effects on the CNS (O’Donoghue, 2000).

In the present study, we compared the behavioral effects of IDPN, allylnitrile, cis-crotononitrile, trans-crotononitrile, and hexadienenitrile in the rat using test methods sensitive to the ECC syndrome or to the trans-crotononitrile syndrome. In addition, we assessed the CNS effects of these nitriles with the Fluoro-Jade B stain, which selectively labels degenerating neurons (Schmued and Hopkins, 2000a,b). Finally, the effects of hexadienenitrile on the auditory and vestibular sensory epithelia were examined by scanning electron microscopy (SEM), as these had not been reported previously. Taken together with previous data (Balbuena and Llorens 2001, 2003; Llorens et al., 1993a, 1993b; Seoane et al., 1999, 2005), this study revealed two groups of neurotoxic nitriles: the vestibulotoxic nitriles, IDPN, allylnitrile, and cis-crotononitrile, which cause the ECC syndrome but have no selective neuronal degeneration in the CNS, and the group including trans-crotononitrile and hexadienenitrile, which cause selective neurodegeneration in the CNS and motor deficits resulting from neuronal degeneration in the inferior olivary complex.

METHODS

Chemicals and Reagents

Crotononitrile (99%, cis/trans ratio of approximately 60:40) was obtained from Aldrich Quimica (Alcobendas, Spain), and its isomers were separated by fractionated distillation (Balbuena and Llorens, 2003); fractions with an isomeric purity greater than 95% (by 1H-NMR, 300 MHz, using CDCl3 as solvent, in a Varian Unity 300 spectrometer) were used in the present series of experiments. Sources for other nitriles and reagents were as follows: allylnitrile (>98%), Merck-Schuchardt (Hohenbrunn bei München, Germany); IDPN (99%), Acros Organics (Geel, Belgium); hexadienenitrile (>98%), Fritton Laboratories (Vineland, NJ); Fluoro-Jade B, Chemicon International (Temecula, CA); and 4'-6-diamidino-2-phenylindole dihydrochloride (DAPI), Sigma (St. Louis, MO).

Animals

The care and use of animals were in accordance with Law 5/1995 and Act 214/1997 of the Autonomous Community (Generalitat) of Catalonia, and approved by the Ethics Committee on Animal Experiment of the University of Barcelona. Fifty-eight 8- to 9-week-old male Long-Evans rats (CERJ, Le-Genest-Saint-Isle, France) were used. They were housed two to four per cage in standard Macrolon cages (280 x 520 x 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 12:12 L:D cycle (0700:1900 h) and given standard diet pellets (A04, U.A.R., France) ad libitum.

For histology, rats were anaesthetized with 400 mg kg−1 chloral hydrate and transcardially perfused with 50 ml of heparinized saline followed by 400 ml of fixative solution.

Dosing and Experimental Design

One set of rats was used to compare the effects of the nitriles on behavior. Before testing and dosing, the animals in this set were trained for the vertical ladder task (see below). The rats were then assigned to one of five groups, and dosed i.p. for 3 consecutive days with control vehicle (3 rats received 1 ml kg−1 day−1 of corn oil and 4 rats received 2 ml kg−1 day−1 of olive oil), 110 mg kg−1 day−1 of crotononitrile (in corn oil, n = 10), 250 mg kg−1 day−1 of trans-crotononitrile (in corn oil, n = 7), 50 mg kg−1 day−1 of allylnitrile (in olive oil, n = 10), 300 mg kg−1 day−1 of hexadienenitrile (in olive oil, n = 7), or 400 mg kg−1 day−1 of IDPN (in 2 ml kg−1 of saline, n = 7). The doses were selected on the basis of previous work (Balbuena and Llorens, 2001, 2003; Llorens et al., 1993a; Seoane et al., 1999, 2005) to elicit maximal neurotoxic effects with minimal mortality. A larger number of animals was assigned to the cis-crotononitrile and allylnitrile groups, because these treatments were considered more likely than the other treatments to cause death of some animals. The rats were weighed and examined for vestibular function by surgical (Llorens et al., 1993a, 1993b; including modifications by Llorens and Rodrı´guez-Farré, 1997) bilabyrinthectomies, as well as by IDPN toxicity. The battery includes observation of spontaneous motor behavior

Behavioral Analysis

Vestibular rating. The disturbance of vestibular function was determined by a complete battery of behavioral tests or the tail-hang test alone. The test battery (Llorens et al., 1993b; including modifications by Llorens and Rodrı´guez-Farré, 1997) has been successfully used to assess the loss of vestibular function caused by surgical (Llorens et al., 1993b) and chemical (Llorens and Rodrı´guez-Farré, 1997) bilateral vestibulotomies, as well as by IDPN (Llorens et al., 1993b), 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
and Hopkins, 2000a, 2000b), while the other two sections were cryoprotected with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Brain and spinal segments of the spinal cord were cut in transverse sections (50 μm) using a Leica VT1000M vibrating blade microtome. Every third section was dried with increasing concentrations of ethanol up to 100%, dried in a critical-point dryer using liquid CO2, coated with 5 nm of gold, and stored in a vacuum dryer.

To identify degenerating neurons in the CNS, the fixative solution consisted of 4% paraformaldehyde with affinity for double-stranded DNA that labels cell nuclei; a second series of sections were stained with cresyl violet. Degenerating neurons were identified with affinity for double-stranded DNA that labels cell nuclei; a second series of sections were stained with cresyl violet. Degenerating neurons were identified with affinity for double-stranded DNA that labels cell nuclei. A third series of sections were stained with DAPI, a stain with affinity for DNA. Double-stained sections were examined surface preparations of the vestibular and auditory sensory epithelia in the temporal bones to assess inner ear morphology in rats administered hexadienenitrile (Seoane et al., 2001), allylnitrile (Balbuena and Llorens, 2001), and cis- and trans-crotononitrile (Balbuena and Llorens, 2003). Briefly, the fixative solution consisted 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 dissection procedure usually provides the complete set of vestibular receptors, the entire apical turn of the organ of Corti, and representative fragments of its middle and basilar turn. The samples were then post-fixed for 1 h in 1% osmium tetroxide in cacodylate buffer and subsequently stored for 12–72 h at 70% humidity to improve or confirm the identification of the damaged structures. To assess inner ear morphology in rats administered hexadienenitrile, we examined surface preparations of the vestibular and auditory sensory epithelia by 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). Briefly, the fixative solution consisted 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 dissection procedure usually provides the complete set of vestibular receptors, the entire apical turn of the organ of Corti, and representative fragments of its middle and basilar turn. The samples were then post-fixed for 1 h in 1% osmium tetroxide in cacodylate buffer and subsequently stored for 12–72 h at 70% humidity to improve or confirm the identification of the damaged structures.

RESULTS

General Observations

Allynitrile treatment at 50 mg kg⁻¹ day⁻¹ for 3 consecutive days resulted in 1 death among 10 treated rats, and cis-crotononitrile at 110 mg kg⁻¹ day⁻¹ resulted in 2 deaths among 10 treated rats. No mortality was reported in the groups of 7 animals treated with IDPN (400 mg kg⁻¹ day⁻¹), hexadienenitrile (300 mg kg⁻¹ day⁻¹), or trans-crotononitrile (250 mg kg⁻¹ day⁻¹). All the nitriles significantly decreased the body weight of the animals. The greatest effect was observed for IDPN, with a maximal loss of body weight at day 8 after the last dose, when a 21% loss with respect to pre-dosing values was recorded. Body weight increased thereafter, but it did not recover to control levels, and mean weight for the IDPN group was only 76% of control mean values at day 21. The maximal weight loss caused by the other nitriles was in the 10–15% range and occurred by day 5 after the last dose. As with IDPN, no compensation occurred, and final (day 21) body weights were lower than control body weights in the trans-crotononitrile (90%), cis-crotononitrile (89%), hexadienenitrile (91%), and allylnitrile (87%) groups.

Allylnitrile, cis-crotononitrile, or IDPN showed a clouding of the cornea, largely reversible, as previously described (Balbuena and Llorens, 2001, 2003;
Seoane et al., 1999). In contrast, no changes in corneal transparency were observed in animals treated with hexadiene-nitrile or trans-crotononitrile.

Effects of the Nitriles on Behavior

Simple observation of the animals indicated that nitrile treatment resulted in the development of one of two clearly different syndromes of abnormal motor behavior. Rats treated with IDPN, allylnitrile, or cis-crotononitrile developed the ECC syndrome (Selvy, 1957), characterized by hyperactivity, circling, and head bobbing. This was associated with increased ratings in the tail-hang test. Ratings of 2–3 were recorded on day 1 after the three IDPN doses, and as early as 1 day after the first dose of allylnitrile or cis-crotononitrile. A full ECC syndrome and tail-hang rating scores of 3–4 were observed in all of these animals on day 5 post-dosing. Rats treated with trans-crotononitrile or hexadienonitrile showed a syndrome of marked impairment of limb control with paresis and a gait disturbance characterized by faltering locomotion but with no hyperactivity, circling, or head bobbing. Tail-hang scores for these animals, like those for control animals, were 0 throughout the entire experimental period, although a rating of 1 was occasionally given.

Assessment of vestibular function with the full test battery (Fig. 1) demonstrated a marked and permanent loss in animals treated with IDPN, allylnitrile, or cis-crotononitrile, whereas animals treated with trans-crotononitrile or hexadienonitrile showed no loss of vestibular function.

In the open field animals, the groups of animals treated with any of the nitriles showed a decreased rearing activity at each time post exposure (Fig. 2A), significantly below levels noted in the control group. In contrast, the different nitriles had different effects on locomotor activity (Fig. 2B). At day 1 after treatment, significantly decreased locomotor activity was recorded for IDPN, trans-crotononitrile, and hexadienonitrile animals. The effect of trans-crotononitrile and hexadienonitrile was transient, and no differences from control animals were observed for these groups at later points in time. Rats treated with allylnitrile or cis-crotononitrile were markedly hyperactive on days 6 and 21, and IDPN rats were hyperactive on day 21.

Many rats treated with allylnitrile or cis-crotononitrile on day 1, and most rats treated with these nitriles or IDPN on day 6 were unable to perform the vertical ladder task because of hyperactivity and loss of balance. These groups were thus no longer tested and were excluded from analysis. Rats treated with trans-crotononitrile or hexadienonitrile showed a decreased holding time on the vertical ladder on days 1, 6, and 21 after dosing (Fig. 3). In the gait topography analysis, treatment with different nitriles elicited different effects. Only trans-crotononitrile and hexadienonitrile caused a marked decrease in stride length, whereas rats treated with IDPN, allylnitrile, or cis-crotononitrile showed a stride length that did not significantly differ from control stride length. As expected, similar results were obtained from hindlimb stride length analysis (Fig. 4A) and forelimb stride length analysis (data not shown). In contrast to stride length, stride width did not differ among nitriles. All of the nitriles caused a significant increase in hindlimb stride width (Fig. 4B), whereas no group treatment effect was revealed by ANOVA of the forelimb stride width data (Fig. 4C).

Effects of the Nitriles on the Central Nervous System

Control animals showed no Fluoro-Jade B labeling in any part of the CNS. Similarly, rats treated with IDPN showed no cell labeling with Fluoro-Jade B in any part of the CNS, although punctuate staining was consistently found in many glomeruli of the olfactory bulbs (Fig. 5A, Table 1). Because no bulbar neurons were labeled, this label must correspond to degeneration of the incoming terminals of the olfactory primary neurons, which is in agreement with the known ability of IDPN to damage the olfactory sensory epithelium and the silver staining observed in the olfactory glomeruli in association with this damage (Genter et al., 1992; Llorens et al., 1993a). Like IDPN rats, cis-crotononitrile rats showed no neuronal degeneration, but labeling of the primary terminals was found in a few glomeruli in the olfactory bulbs of only 2 of 3 rats examined. Punctuate staining in a few olfactory glomeruli was also the only finding in brain sections of allylnitrile animals stained with Fluoro-Jade B, while no labeling was evident in any other brain region (Fig. 5B, Table 1).

Rats treated with trans-crotononitrile exhibited Fluoro-Jade B labeling in several brain regions (Fig. 5, Table 1). Extensive staining was found in the inferior olive (Fig. 5C) and piriform cortex. In the olive, intense staining was observed within cell bodies, which were also surrounded by intense punctuate
staining, probably corresponding to olive cell dendrites. In the piriform cortex, labeled cells were found mainly in layer II, although some cells in layers I and III were also labeled; dendritic and axonal staining was also evident. In addition, *trans*-crotononitrile animals exhibited labeling of a small cluster of neurons in the medial part of the lateral entorhinal cortex. Secondary targets of *trans*-crotononitrile found in some but not all of the animals included in a more exhaustive study published elsewhere (Seoane *et al.*, 2005), such as the anterodorsal thalamic nucleus, were not labeled in the two rats examined in the present study (Table 1).

Hexadienenitrile exposure resulted in labeling of neurons by the Fluoro-Jade B stain in several brain regions (Fig. 6, Table 1).

An extensive neuronal degeneration, identical to that recorded for the *trans*-crotononitrile animals, occurred in the inferior olive (Fig. 6A) and the piriform cortex (Fig. 6B). All of the hexadienenitrile animals showed a lesion significantly larger than that of the *trans*-crotononitrile animals in the lateral entorhinal cortex, and this lesion extended to the parasubiculum through a band deep across the medial entorhinal cortex area while sparing the superficial layers of this last area (Fig. 6C). Hexadienenitrile also caused a consistent labeling of neurons in the prelimbic and possibly the anterior cingulate areas of the prefrontal cortex (Fig. 6D), and in a more sparse way in the M2 area of the frontal cortex (Fig. 6E). A few neurons were also labeled in the dorsal transition zone. Only the two animals examined at 2 days after dosing showed labeling of a very few neurons in parietal and temporal regions of the cortex, and also of nerve terminals in the olfactory glomeruli. One of these animals also showed a marked labeling of the anterodorsal thalamic nucleus, as well as two focal areas of neuronal degeneration in the cerebellum. These cerebellar lesions were unilateral and involved both the Purkinje and granular cells (Fig. 6F).

**Effects of Hexadienenitrile on the Vestibular and Auditory Sensory Epithelia**

The ototoxic effects of IDPN, allylnitrile, and *cis*-crotononitrile, as well as the lack of ototoxic effects of *trans*-crotononitrile, are well documented (Balbuena and Llorens, 2001, 2003; Crofton *et al.*, 1994; Llorens *et al.*, 1993b). In the present series of experiments, the ototoxic effects of hexadienenitrile were examined. Scanning electron microscopy observation of surface preparations of vestibular and auditory sensory epithelia from three rats exposed to hexadienenitrile 6 weeks before sacrifice revealed no evidence of hair cell loss (Fig. 7, Table 1).
DISCUSSION

The present study compared the behavioral and pathological effects of five neurotoxic nitriles: IDPN, allylnitrile, cis-crotononitrile, hexadienenitrile, and trans-crotononitrile. IDPN, allylnitrile, and cis-crotononitrile induced high scores in a test battery for vestibular dysfunction and hyperactivity in the open field; in the CNS, they only caused degeneration of nerve terminals in the glomeruli of the olfactory bulbs. Hexadienenitrile and trans-crotononitrile did not increase the vestibular scores or the locomotor activity, but caused a
On the one hand, IDPN, allylnitrile, and cis-crotononitrile induced high scores in the vestibular test battery and hyperactivity in the open field, but did not significantly decrease stride length. The fact that IDPN animals showed reduced activity before the permanent hyperactivity became apparent was in good agreement with previous data (Llorens et al., 1993b; Llorens and Rodríguez-Farré, 1997) and may result from a combination of causes, including the slower progression of the vestibular toxicity of IDPN in comparison with cis-crotononitrile and allylnitrile (Balbuena and Llorens, 2001), the coexistence of other toxic actions, such as the neurofilamentous axonopathy, and a larger degree of body weight loss. The lack of effect of these nitriles on stride length was a remarkable finding, because most aspects of motor control are profoundly affected in the ECC syndrome.

On the other hand, trans-crotononitrile and hexadienenitrile did not modify the vestibular rating scores and did not increase locomotor activity in the open field, but they did cause a marked decrease in stride length. As previously reported for trans-crotononitrile (Seoane et al., 2005), both this nitrile and hexadienenitrile significantly reduced animals’ ability to hold onto a vertical grid, although this test was unsuitable for hyperactive animals treated with the other nitriles. The behavior of the hexadienenitrile and trans-crotononitrile animals on the ladder suggested that they were impaired in their ability to maintain the isometric muscle contraction required by the task. Common effects of all of the nitriles under study were the reduction in rearing activity and the increase in hindlimb stride width. These similarities probably result from the fact that both syndromes include impaired balance through loss of postural control. Neither the lack of effect of these nitriles on stride length nor their ability to cause corneal opacity, whereas cis-crotononitrile and hexadienenitrile caused corneal opacity, whereas trans-crotononitrile and hexadienenitrile did not, which further supports the simple hypothesis that two different mechanisms of toxicity are involved in causing the two behavioral syndromes. The isomeric specificity of the two crotononitrile isomers noticeably illustrates that strict structural requirements critically determine the neurotoxic effect of the nitriles.

### Table 1: Summary of Pathological Effects of Nitriles on the Central Nervous System (CNS) and the Inner Ear

<table>
<thead>
<tr>
<th></th>
<th>IDPN</th>
<th>Allylnitrile</th>
<th>cis-Crotononitrile</th>
<th>trans-Crotononitrile</th>
<th>Hexadienenitrile</th>
</tr>
</thead>
<tbody>
<tr>
<td>CNS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of animals</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2 (9)†</td>
<td>5</td>
</tr>
<tr>
<td>Olfactory bulb</td>
<td>‼</td>
<td>‼</td>
<td>‼</td>
<td>− (−)†</td>
<td>‼</td>
</tr>
<tr>
<td>Prefrontal cortex</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>− (−)†</td>
<td>− (−)†</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>− (−)†</td>
<td>− (−)†</td>
</tr>
<tr>
<td>Temporal/parietal cortex</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>− (−)†</td>
<td>− (−)†</td>
</tr>
<tr>
<td>Piriform cortex</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>− (−)†</td>
<td>− (−)†</td>
</tr>
<tr>
<td>Anterodorsal thalamic nucleus</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>− (−)†</td>
<td>− (−)†</td>
</tr>
<tr>
<td>Lateral entorhinal cortex</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++ (+)†</td>
<td>++ (+)†</td>
</tr>
<tr>
<td>Parasubiculum</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>− (−)†</td>
<td>− (−)†</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>− (−)†</td>
<td>− (−)†</td>
</tr>
<tr>
<td>Inferior olive</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>++ (+)†</td>
<td>++ (+)†</td>
</tr>
<tr>
<td>Inner ear</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vestibular epithelia</td>
<td>Yes²</td>
<td>Yes³</td>
<td>Yes⁵</td>
<td>No⁵</td>
<td>No (n = 3)</td>
</tr>
<tr>
<td>Organ of Corti</td>
<td>Yes⁴</td>
<td>Yes⁵</td>
<td>Yes⁵</td>
<td>No⁵</td>
<td>No (n = 3)</td>
</tr>
</tbody>
</table>

Note. The table indicates extension of neuronal degeneration in the CNS as assessed by Fluoro-Jade B staining, and occurrence of hair cell degeneration as assessed by scanning electron microscopy. n: Number of animals. Multiple ratings result from animal variability. −: lack of Fluoro-Jade B staining. ‼: ‼: ‼: labeling of nerve terminals that is faint, noticeable or extensive, respectively. ++, +++, +++++: cell degeneration in selected areas or neuronal populations that is faint, noticeable, or extensive, respectively. & &: presence of a noticeable focal, unilateral damage. Yes/No: loss of hair cell bundles. Superscript numbers indicate data from published articles, as follows: ¹ Seoane et al., 2005; ² Llorens et al., 1993b; ³ Crofton et al., 1994; ⁴ Balbuena and Llorens, 2001; ⁵ Balbuena and Llorens, 2003. IDPN: 3,3′-iminodipropionitrile.
The Fluoro-Jade B study did not reveal degenerating neurons in the CNS of rats at 7 days after treatment with IDPN, allylnitrile, or cis-crotononitrile, a time at which the ECC was fully developed. The Fluoro-Jade fluorochromes selectively and sensitively label neurons at a late stage in degeneration, with quite a long time window for labeling (Poirier et al., 2000; Schmued and Hopkins, 2000a, 2000b); for instance, they are able to reveal degenerating inferior olive neurons from 1 to at least 12 days after exposure to trans-crotononitrile (Seoane et al., 2005). We thus conclude that no central neuron degeneration was associated with the ECC syndrome. The present Fluoro-Jade B data for IDPN are in good agreement with our previous silver stain data (Llorens et al., 1993a), indicating that the incoming primary olfactory terminals are the only prominent “central” target for IDPN-induced neuronal degeneration, with no evidence for other major central targets that could account for the syndrome. Nevertheless, Fluoro-Jade B is not a reliable marker for terminal degeneration: it failed to reveal in the cerebellum the climbing fibers from the degenerating inferior olive neurons in trans-crotononitrile and hexadienenitrile animals (see also Seoane et al., 2005). Thus, the greater sensitivity of silver stains in revealing degenerating axons may explain why no degenerating axons were observed in brain areas of IDPN animals, where sparse axonal degeneration had been found in the silver stain study (Llorens et al., 1993b). In the olfactory bulbs, the extent of labeling following allylnitrile and cis-crotononitrile was small compared to that following IDPN, which is in agreement with the indirect data suggesting that the olfactory damage caused by the former is smaller than that caused by the latter (Balbuena and Llorens 2001, 2003). As discussed above (see Introduction), axonopathy is not associated with the ECC.

FIG. 6. Neuronal degeneration in the CNS of rats exposed to hexadienenitrile. Fluoro-jade B stain of coronal vibrating microtome sections (50 μm) of brains from rats killed at 7 (A, B, and D) or 2 (C, E and F) days after exposure. A. Extensive degeneration of neurons in the inferior olive complex (IO). B. Piriform cortex region (PirC); note the extensive labeling of layer II neurons, as well as the labeling of some neurons in layers I and III. C. Labeling in the lateral entorhinal cortex (LEnt) extending into the parasubiculum (PaS). Note the sparing of the superficial layers in the medial entorhinal cortex (MEnt). D. Degenerating neurons in the prelimbic area, perhaps extending into the anterior cingulate area at the top, of the prefrontal cortex (PreFC). E. Sparse degenerating neurons (arrows) in the frontal cortex (FC). F. Fluoro-Jade B labeling in the cerebellum (Cer). Note the focal nature of the lesion (arrow). The inset shows a higher magnification of the lesion, to illustrate that it involves both Purkinje (arrow) and granular (arrowhead) neurons. Scale bars = 100 μm.
syndrome either. It thus becomes apparent that the peripheral vestibular toxicity of IDPN, allylnitrile, and cis-crotononitrile fully accounts for the syndrome.

In contrast to the ECC-inducing nitriles, trans-crotononitrile and hexadienenitrile caused selective neuronal degeneration in the CNS. The pattern of neuronal degeneration coincided for both nitriles in its main targets (inferior olive and piriform cortex) and showed a greater involvement of secondary targets (lateral entorhinal/parasubiculum, frontal cortex, prefrontal cortex) in hexadienenitrile-treated rats as compared to trans-crotononitrile-treated rats. Whereas the lack of auditory and vestibular effects has already been reported for trans-crotononitrile (Balbuena and Llorens, 2003), a similar lack of auditory and vestibular toxicity has been determined here for hexadienenitrile. In previous studies, we determined that the motor deficits caused by trans-crotononitrile resemble but are not identical to those induced by 3-acetylpyridine, owing to the fact that both chemicals target the inferior olive but show differences in the extent to which different subnuclei of the olive are affected (Seoane et al., 2005). Hexadienenitrile effects were identical to those of trans-crotononitrile in both stride length, a parameter less severely altered by 3-acetylpyridine, and vertical holding time, a parameter not altered by 3-acetylpyridine (Seoane et al., 2005). Although the functional consequences of some of the toxic effects of trans-crotononitrile and hexadienenitrile, such as the degeneration of the piriform cortex or of the entorhinal/parasubiculum region, remain to be studied, current knowledge about the function of the different brain regions makes it possible to give a satisfactory explanation of their motor effects. The inferior olive is the exclusive source of the climbing fibers to the cerebellum, and its degeneration results in major loss of cerebellar function. As discussed in detail elsewhere (Seoane et al., 2005), the motor effects of trans-crotononitrile are likely to be due mainly—or perhaps only—to the extensive degeneration it causes in the inferior olive complex. In the case of hexadienenitrile, a number of targets were affected to a larger extent than in the case of trans-crotononitrile. However, many of these regions are known to be involved in functions other than the motor function, such as olfaction or memory in the case of the entorhinal cortex. The neurons degenerating in the frontal cortex need to be considered here: these could have a motor function, but they were scarce in number. Taken together with the fact that the motor effects of hexadienenitrile could not be distinguished from those of trans-crotononitrile, this suggests that the motor syndrome caused by hexadienenitrile was also likely to be mainly due to the degeneration of the inferior olive.

Some of the hexadienenitrile animals exhibited terminal degeneration in the olfactory glomeruli indicative of primary olfactory neuron degeneration. Thus, olfactory toxicity could be a property of nitriles that are toxic to either the audiovestibular system or the inferior olive. Available evidence suggests that the olfactory toxicity of IDPN may involve particular metabolic pathways not necessarily identical to those putatively involved in its vestibular toxicity (Genter et al., 1994). Another observation worth discussing is the focal lesion found in one of the 5 hexadienenitrile animals. The focal nature of the lesion would suggest that it was not related to the treatment. However, identical focal lesions had been recorded in the cerebellum in 2 of 3 rats dosed with three daily doses of 60 mg kg$^{-1}$ of allylnitrile from a preliminary study (unpublished data); one of these animals also displayed focal lesions in the striatum. This suggests that these lesions could be related to nitrile treatment. One mechanism causing these lesions could be vascular damage; hemorrhagic spots have been observed in the brains of allylnitrile-treated mice (Tanii et al., 1989b).
In conclusion, the present and previous data indicate that neurotoxic nitriles induce one of two syndromes of abnormal motor behavior: (1) the ECC syndrome caused by IDPN, allylnitrile, and cis-crotononitrile, and resulting from degeneration of the vestibular sensory hair cells with no CNS toxicity role, and (2) the syndrome of faltering movements, caused by trans-crotononitrile and hexadienenitrile, and resulting from degeneration of the inferior olive neurons. In addition, nitriles in either group may eventually cause other neurotoxic effects common to both, such as olfactory mucosa degeneration or neurovascular damage, which are, except for the olfactory toxicity of IDPN, still poorly characterized.

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REFERENCES


Does the documentation discuss the effects of allylnitrile on the vestibular system of rats? If so, could you provide specific details or references related to neurobehavioral changes or cell loss in the vestibular system following allylnitrile exposure?