Ibogaine Produces Neurodegeneration in Rat, but Not Mouse, Cerebellum

Neurohistological Biomarkers of Purkinje Cell Loss

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INTRODUCTION

The compound Ibogaine is being intensively studied as a possible therapeutic aid to interrupt addictive cravings of various sorts (*e.g.*, Ref. 1). Structurally, ibogaine closely resembles serotonin (FIG. 1). It binds, but with only moderate (micromolar) affinity, to a variety of membrane and transporter sites including serotonin, opiate, and NMDA-receptors.[2-5]

Initial clinical reports indicate that ibogaine is a promising therapeutic agent for the difficult, even intractable, downward cycle of drug addiction. However, placebo effects may exert considerable influence on such single-blind trials.[6] Further clinical trials are now being undertaken to evaluate the efficacy of this compound in human cocaine addicts. However, no double-blind clinical studies from which to judge the efficacy of ibogaine have yet been reported. Indeed, the potent psychoactive effects of ibogaine make the design of an effective double-blind study quite challenging. A psychoactive control substance as unfamiliar as ibogaine itself to the patients would be needed.

Preclinical animal studies attempting to establish the mechanism of action and the efficacy of ibogaine against self-administration or withdrawal of such compounds as morphine,[7-14] cocaine,[15-19] and amphetamine[10,22-22] have been conducted. As in the clinical studies, the evidence is promising but remains inconclusive regarding the utility of ibogaine as a therapeutic agent. Ibogaine has also been proposed, but remains unstudied, as a treatment for nicotine and alcohol addiction.

In addition to serotonin, ibogaine also structurally resembles harmaline (FIG. 1), a tremorigenic agent known to produce neurotoxic damage to the cerebellum. This observation lead O'Hearn and colleagues[23,24] to evaluate the neurohistology of rat cerebellum following acute exposure to ibogaine. As with harmaline, they observed necrosis of Purkinje neurons in the cerebellar vermis as indicated by several neurohistological biomarkers: argyrophilic degeneration, loss of calbindin immunoreactivity, astrocytosis, and microgliosis. The dose levels they employed were within the same order of magnitude as those being utilized in human trials. Efforts by other laboratories (unpublished observations) failed to obtain any evidence for the neurotoxicity of ibogaine in nonhuman primates; however, the methods used in those studies were primarily conventional hematoxylin and eosin

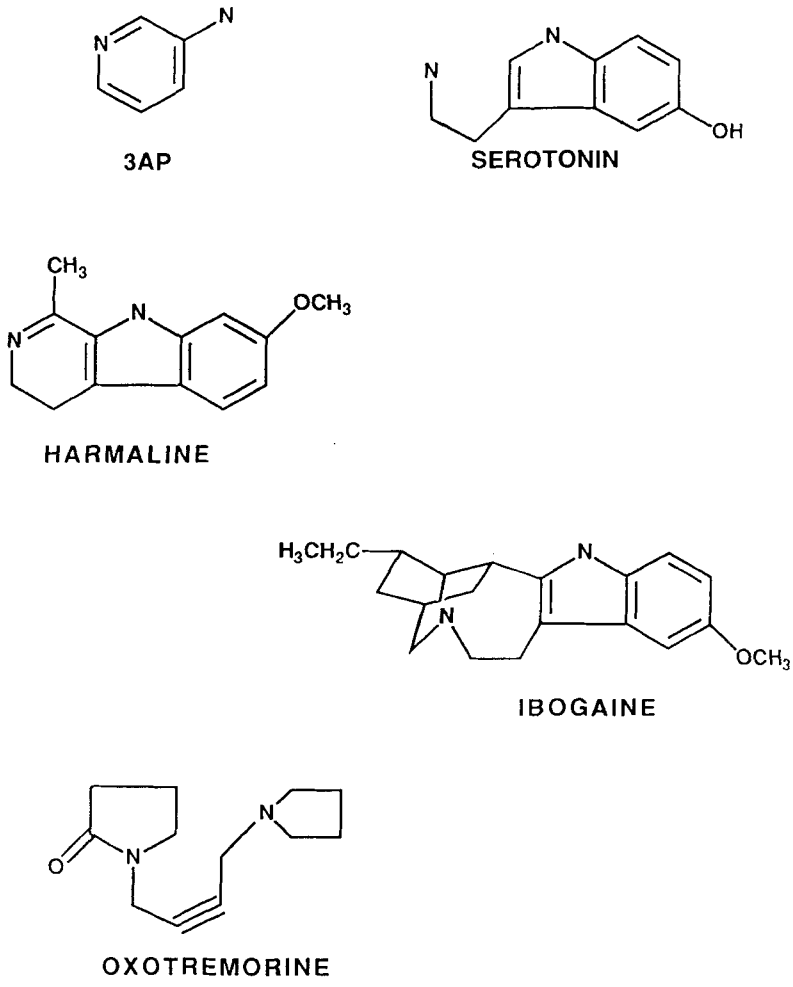

FIGURE 1. The chemical structures of ibogaine and related compounds. Note that all these compounds can have similar positioning of the nitrogens, separated by 5 bond lengths, including a double or triple bond.

(H & E) staining of paraffin sections, rather than the more specialized techniques of O'Hearn *et al.*

In order to provide the necessary information for regulatory decision-making, we must fully understand both the nature and extent of ibogaine neurotoxicity as well as its efficacy, so that the risks and benefits of its therapeutic use in humans can be appropriately balanced. Therefore, the major aim of the present study was to replicate the initial observations of O'Hearn *et al.* using the specialized neurohistological methods, and compare them to the more conventional H & E approach. We also wished to extend the observations to mice treated with comparable doses of ibogaine.

METHODS

Animals

Twenty-three adult male mice (5 months of age) and 23 rats (10 months of age) obtained from the NCTR breeding colony were utilized in these studies. The rats were derived from the Charles River CD · (Crl : CD · (SD)BR) strain (Kingston, New York) and the mice were C57BL6. Rodents were individually housed (rats and mice in separate rooms) under controlled environmental conditions (temperature 22°C, relative humidity 50%, 12 h light : dark cycle with lights out at 1800 h) in plexiglass cages lined with wood chips. Rodent chow (Ralston-Purina, St. Louis, Mo.) was available *ad libitum*.

Drug

Ibogaine hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO) and suspended at a concentration of 10 mg/ml in millipore-filtered, deionized water. It was then warmed to 37°C and sonicated for 2 h in order to maximally solubilize the ibogaine (E. O'Hearn, personal communication). Rodents were injected i.p. with 10 ml/kg body weight of ibogaine, resulting in a dose of 100 mg/kg.

Experiment 1

Ibogaine-treated rats were allowed to survive for either 1 h (n = 3), 1 day (n = 3), or 7 days (n = 3). Two additional saline-dosed control rats were sacrificed at each time-point, resulting in a total of 9 ibogaine and 6 control animals. Sacrifice was by sodium pentabarbital anesthesia (100 mg/kg i.p.), followed by intracardiac perfusion using a peristaltic pump (40 ml/minute) with a saline flush (50 ml) followed by 4% formaldehyde in 0.1 M phosphate buffer (500 ml). The identical experiment was conducted using mice, except that a flow rate of 4 ml/min was used to perfuse 5 ml of saline followed by 50 ml of 4% formaldehyde.

Experiment 2

Rats were dosed i.p. with ibogaine (n = 4) or saline control (n = 4) and sacrificed 7 days after dosing by perfusion, exactly as described above. A parallel set of mice (n = 4) were dosed with ibogaine, together with their controls (n = 4) and they were also sacrificed by perfusion 7 days after dosing.

Histology

Following overnight post-fixation in the perfusate, brains were blocked and sectioned at 50 microns using a Vibratome (Technical Products, Inc., St. Louis, MO). Some blocks were dehydrated and embedded in paraffin. The vibratome sections were stored in the wells of tissue culture plates containing 0.1 M phosphate buffer prior to further histochemical procedures. Paraffin blocks were sectioned with a rotary microtome, and individual ribbons of sections were mounted on slides and stained with hematoxylin and eosin (H & E).

Silver-Degeneration Stain[25,27]

Vibratome sections were utilized in a silver-staining procedure designed to impregnate axon terminals or neuronal cell bodies that are dead or dying. Briefly, sections stored in 2% formaldehyde are washed free of fixative by immersion in three changes of distilled water for 5 min each, using 24-well mesh-bottomed staining baskets as described.[25] Then they are pretreated for 10 min with a dilute alkaline solution of ammonium nitrate, followed by a 10-min impregnation with about 0.25% silver nitrate in alkaline ammonium nitrate. Following three 100-sec washes in a sodium carbonate/ammonium nitrate solution dissolved in 30% ethanol, they are developed for at least 1 min in citric acid in dilute formaldehyde/ethanol. Finally, the sections are mounted on gelatin-subbed glass slides, fixed with acetic acid, dehydrated through an ascending series of ethanol concentrations to xylene, and coverslipped with permount. Sections are then observed under brightfield and/or darkfield optics.

Immunocytochemistry[25,26]

Calbindin. The immunostaining procedure used to localize calbindin was a minor modification of the indirect antibody peroxidase-antiperoxidase method of Sternberger.[26] Briefly, sections stored in 0.1 M phosphate buffer were incubated overnight at 4°C in a 1:500 dilution of a primary antibody that was a mouse-derived monoclonal antibody to calbindin (Sigma Chemical Co., St. Louis, MO). The sections were then rinsed three times for 5 min per rinse in 0.1 M potassium phosphate buffer at room temperature. Then the sections were placed in a 1:200 dilution of goat anti-mouse secondary antibody (Jackson Immunoresearch Labs, West Grove, PA) for 1 h. After three more 5-min rinses in phosphate buffer, sections were placed in a 1:200 dilution of mouse peroxidase-antiperoxidase solution (Jackson Immunoresearch Labs, West Grove, PA) for 60 min. Another three 5-min rinses in phosphate buffer followed, and sections were then placed for 8–10 min in a 0.05% DAB solution (3,3′-diaminobenzidine tetrahydrochloride, Sigma Chemical Co., St. Louis, MO) made 0.1% in hydrogen peroxide just prior to use. After three final 5-min rinses in potassium phosphate buffer, sections were mounted on slides, counterstained with cresyl violet (Eastman Kodak, Rochester, NY), dehydrated and coverslipped.

Glial Fibrillary Acidic Protein (GFAP). Anti-GFAP staining was also performed as described above for calbindin, except a polyclonal rabbit antisera (Incstar, Inc., Stillwater, MN) was utilized, so the secondary antibody was goat anti-rabbit IgG (Gibco, Grand Island, NY), and rabbit peroxidase-antiperoxidase complex (Jackson Immunoresearch Laboratories, West Grove, PA) was utilized.

RESULTS

The silver degeneration-selective stain revealed argyrophilic neuronal damage within the cerebellum of ibogaine-treated rats (FIGS. 2, 3), but not mice (not shown), 1 wk following dosing. Since 1 wk after dosing was the only sacrifice time at which consistent argyrophilic damage could be observed, all the figures and results reported here for silver, calbindin, and GFAP are taken from this

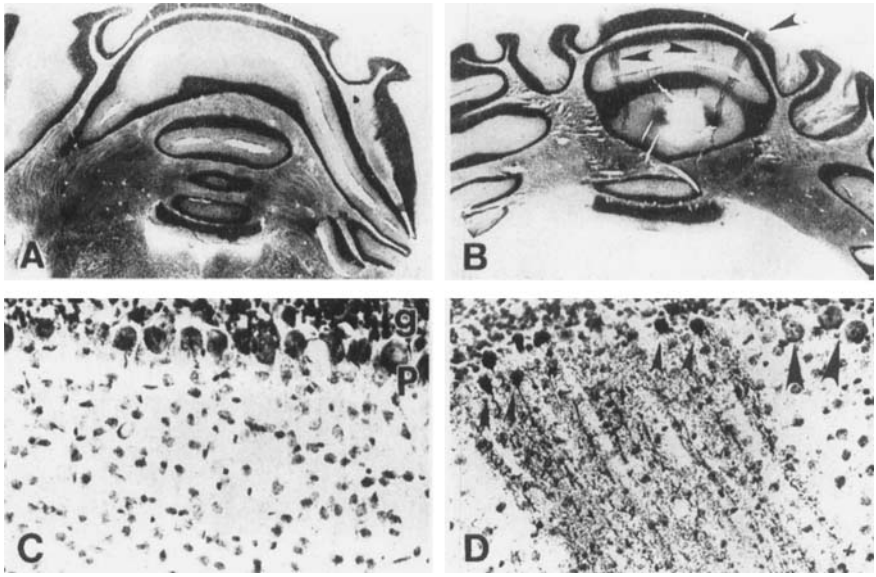

FIGURE 2. All panels are of sections stained with the Nadler-Evenson degeneration-selective silver method, obtained at the 1-wk posttreatment survival time. **(A)** is a low-power micrograph of the cerebellum of a saline-treated control rat, which is absent of any argyrophilia, while **(B)** shows nearly parallel stripes of degenerating axons (*arrowheads*) penetrating down through the layers of the cerebellar vermis toward the deep cerebellar nuclei of an ibogaine-treated rat (both (A) and (B), ×8). **(C)** shows a higher magnification view of the Purkinje cell layer (P) in the vermis of a control animal, as well as the granule cell layer (g) above it. No degeneration is visible. **(D)** shows a portion of one of the stripes of fibers from an ibogaine-treated rat. Note the small, very black degenerating Purkinje neuronal perikarya (*small arrowheads*) compared to the larger, lighter intact neurons (*large arrowheads*). The stripe itself appears to contain both degenerating Purkinje cell dendrites, as well as axons. All the granule cells appear normal (both (D) and (C), ×220). The degeneration in ibogaine-treated rats (B and D) was easily visible to the naked eye in rats, whereas even upon close examination of sections from mice, no argyrophilia was present in the vermis or elsewhere (not shown).

group of animals. The cerebellar damage seemed confined to parasaggital patches or zones of Purkinje cells and their axons, just as described.[23] No argyrophilia was observed in concurrently stained sections containing cortex, corpus striatum, septum, hippocampus, thalamus, hypothalamus, and amygdala (not shown). Those Purkinje cells observed to be degenerating appeared to send degenerating axons to terminal processes ending in the deep nuclei of the cerebellum (FIG. 3D). The degeneration was easily visible at low magnifications (FIGS. 2A, B).

The calbindin stain was quite selective for Purkinje cells and their dendrites in the cerebellum of control rodents (FIGS. 4B, D). In rats, (but not mice) ibogaine treatment resulted in parasaggital blank areas, absent of any of the usual stain (FIGS. 4A, C). The blank areas were easily visible at low magnifications (FIG. 4C).

The glial fibrillary acidic protein (GFAP) immunostain revealed parasaggital patches of reactive astrocytes in ibogaine-treated rats (FIGS. 5B, D), but not

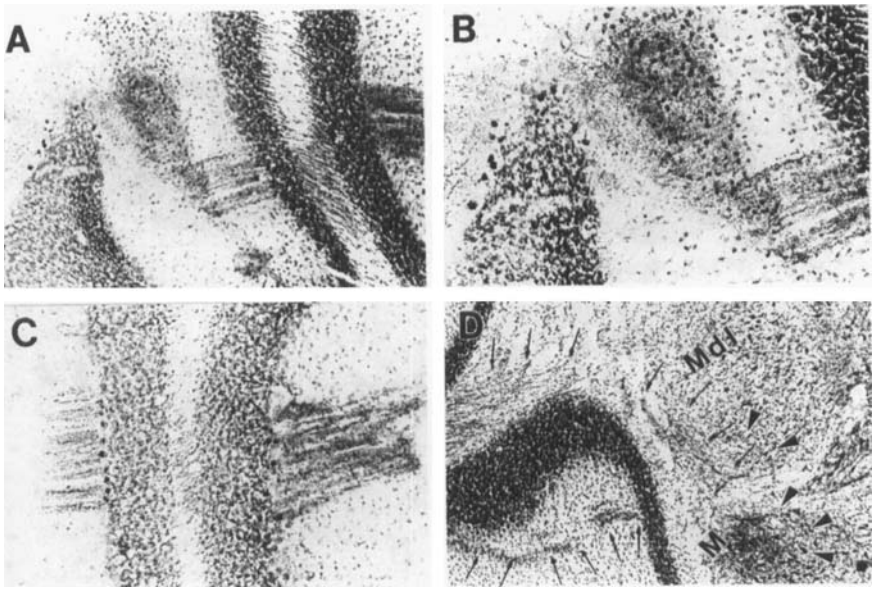

FIGURE 3. Note that dorsal is to the *left* in each of these micrographs, taken of sections stained with the Nadler-Evenson degeneration-selective silver method, also obtained at the 1-wk post-treatment survival time. **(A)** presents an intermediate magnification of another one of the many scenes of degenerating neurons and axons observed in the cerebellar vermi of ibogaine-treated rats (×66). **(B)** is the same scene, at higher magnification (×110). **(C)** further shows how the patches of degeneration in one layer line up with another layer, communicating via axonal fibers penetrating through the intervening layers of granule cells (×85). **(D)** shows that the degenerating, argyrophilic axonal pathways (*small arrows*) can be traced only as far as an apparent termination (*arrowheads*) in the deep cerebellar nuclei (M = medial cerebellar nucleus, Mdl = medial cerebellar nucleus, dorsolateral, ×45). Only degenerating axons and terminals, not perikarya, were observed in the deep cerebellar nuclei.

controls (not shown). The degeneration could be observed with little difficulty, even at low magnifications (FIG. 5B).

Conventional H & E-stained sections revealed several "dark" Purkinje neurons in the cerebellum (FIG. 5C), but these could not be appreciated at low magnification (FIG. 5A).

DISCUSSION

The degeneration-selective silver procedure revealed the very same pattern of ibogaine neurotoxicity in the rat as had previously been described by O'Hearn and colleagues.[23,24] The silver method specific for neurodegeneration made it easy to observe the necrotic Purkinje cells, even at very low magnification (FIG. 2B). However, using the H & E-stained paraffin sections (FIG. 5A), it was very difficult to see the presence of a few dark Purkinje neurons at low magnifications, although they could be seen with careful scrutiny at higher magnification (FIG. 5C). Appar-

ently, this is due to the dark Purkinje neurons being difficult to resolve against the broad band of numerous dark granule cells immediately adjacent to them. It appears that by limiting the evaluation of ibogaine neurotoxicity to simply scanning H & E-stained paraffin sections, one runs a risk of reporting a "false negative" finding simply from overlooking the damage. Thus the conclusions of "negative" studies relying primarily on routine H & E methodologies should be viewed with caution.

Further confirmation of the Purkinje cell necrosis was provided by the calbindin immunohistochemical staining. This marker, present in the cytoplasm of Purkinje neurons and their dendrites, appeared to vanish where the neurons were damaged, revealing such sites as unstained, white patches among the heavily labeled intact Purkinje neurons.

The GFAP immunostaining was useful for defining certain areas of astrocytosis that probably occurred anatomically close to the regions of neuronal damage

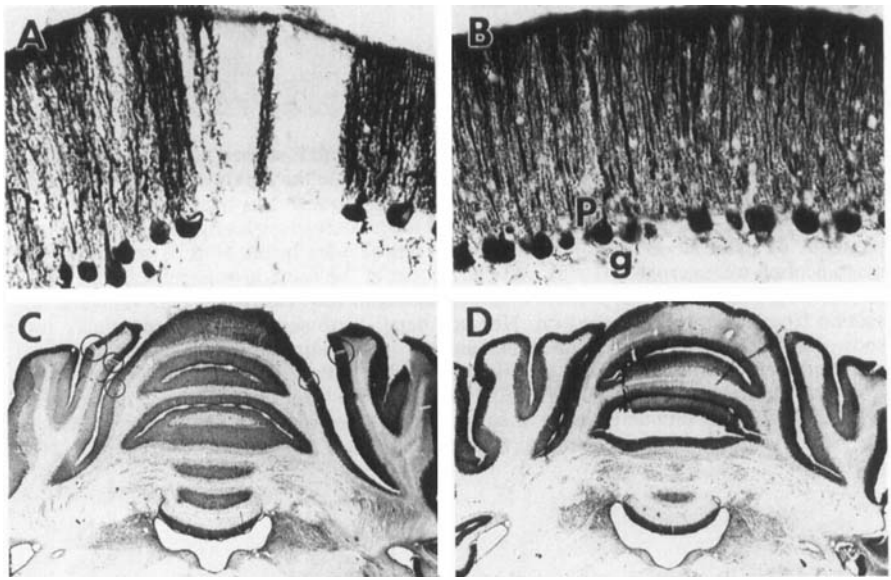

FIGURE 4. All panels are of sections obtained at the 1-wk post-treatment survival time and immunostained with a monoclonal antibody to calbindin. **(A)** shows a portion presumed to correspond to one of the stripes or "patches" of degenerating fibers from an ibogaine-treated rat at high magnification (×220). Note the absence of any immunostaining for calbindin within the patch itself. **(B)** shows the Purkinje cell layer (P) and the Purkinje cell dendrites in the vermis of a control animal, as well as the granule cell layer (g) below it (×220). The Purkinje cell bodies and their dendrites are strongly immunoreactive to the calbindin antibody, with no empty gaps or patches, but the granule cell layer is unstained. **(C)** is a low-power micrograph showing nearly parallel, light-colored patches (*circled*) lined up in stripes through the layers of the cerebellar vermis of an ibogaine-treated rat (×8), while **(D)** is a low-power micrograph of the cerebellum of a saline-treated control rat, which shows continuous bands of dark, calbindin-immunopositive Purkinje neurons and their dendrites (×8).

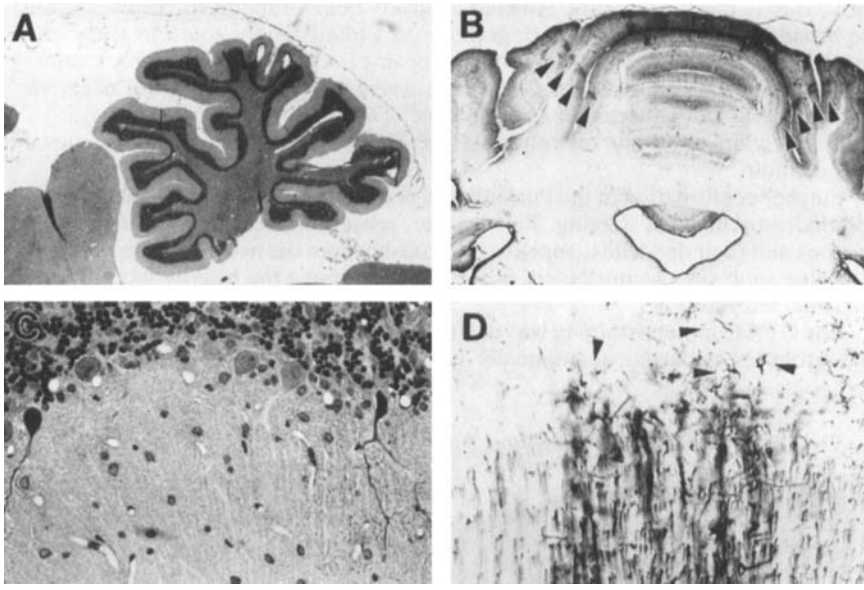

FIGURE 5. (A) is a low-power photomicrograph of an H & E stained paraffin section (24-h survival) of the cerebellum of an ibogaine-treated rat. Note the darkly stained granule cells and the difficulty in resolving individual Purkinje cells against this background, compared to FIGS. 4C and 4D, where the Purkinje cells are selectively stained for their calbindin content. No evidence of neurological damage can be seen in the H & E section at this magnification (all micrographs ×8). **(B)** shows that at the same low magnification (×8) as in (A), dark bands of GFAP-positive immunostaining (*arrowheads*) are easily visible in this section from an ibogaine-treated rat. No such bands were seen in untreated animals (not shown). **(C)** shows two darkly stained, presumably degenerating large Purkinje cells amongst numerous, darkly stained smaller granule cells visible at the *top* of this micrograph, taken from an ibogaine-treated rat (H & E, ×220). **(D)** shows a higher magnification (×220) view of a band of GFAP immunostaining in an ibogaine-treated rat. Note the darker staining of the band and the presence of several reactive astrocytes (*arrowheads*, Alzheimer's type II gliosis).

produced by ibogaine in the cerebellum, based on the appearance of adjacent sections. However, our limited time-course data in these studies do not let us address the question of whether the astrocytosis preceded or followed the onset of the neuronal damage, since both were observed only in the day-7 sections. Further studies will be needed to fill in some of the missing time-course data. Also, the considerable amount of background staining of GFAP in adjacent, but nonreactive, astrocytes (especially strong in cerebellum) makes it sometimes difficult to recognize necrotic areas, particularly at low magnification. The stain is useful for confirming that an area is necrotic as determined from another (*e.g.*, degeneration-specific) method. Since there is a convergence of findings from several different neurohistological procedures, from more than one laboratory, one can be more confident concluding that ibogaine has the potential to produce neurotoxicity.

The damage syndrome described in the previous rat studies[23,24] was completely replicated for all the aspects we examined in our present study, which was performed by a completely separate laboratory. Our efforts to immunostain microglia were unsuccessful (not shown), probably due to overfixation of the surface membrane marker chosen as the antigen (E. O'Hearn, personal communication). Although we do not yet know if the solubility of ibogaine or its potential metabolism is a factor in its neurotoxicity, our study nevertheless shows that the previous observations could be repeated under closely similar circumstances. Further work is planned on the metabolism of this compound and its pharmacokinetics. The relative difficulty of demonstrating the Purkinje cell damage with H & E staining indicates this method may be less useful for evaluating ibogaine neurotoxicity than calbindin immunostaining, GFAP-immunostaining or degeneration-specific methods. Therefore, reports of lack of ibogaine neurotoxicity employing relatively insensitive methods must be considered incomplete until the addition of one or more of the methods shown to be most sensitive.

In our study, mice were resistant to ibogaine neurotoxicity, even at the same high dose shown in concurrent studies to be neurotoxic to rats. The finding of differences in susceptibility even between rats and mice suggests caution in attempting the extrapolation of ibogaine's actions from rodents to primates. Further work including drug and metabolite levels as well as quantitation of the neurotoxic response will aid in predicting the potential costs and benefits of this interesting psychoactive compound in humans.

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