7. SU, T-P. 1993. Delineating biochemical and functional properties of *sigma* receptors: emerging concepts. Crit. Rev. Neurobiol. **7:** 187–203.
8. WALKER, J. M., W. D. BOWEN, F. O. WALKER, R. R. MATSUMOTO, B. DE COSTA & K. C. RICE. 1990. *Sigma* receptors: biology and function. Pharmacol. Rev. **42:** 355–402.

Acute and Chronic Administration of Ibogaine to the Rat Results in Astrogliosis That Is Not Confined to the Cerebellar Vermis[a,e]

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INTRODUCTION

Ibogaine (IBG) is a naturally occurring alkaloid derived from the root of the African shrub, *Tabernanthe iboga*. Recent evidence suggests that IBG interrupts the physiological and psychological aspects of drug addiction, both in experimental animals and in man.[3] Based on these findings, IBG is under consideration for treatment of human drug addictions. In addition to its therapeutic potential, however, IBG also has the potential to induce neurotoxicity; it results in degeneration of Purkinje cells of the cerebellar vermis that is accompanied by reactive gliosis, a generic response of astrocytes to CNS injury.[1,2] Here we evaluated the potential of acute and chronic exposure to IBG to cause neurotoxicity in male and female rats as evidenced by the presence of reactive gliosis in a variety of brain regions. Because enhanced expression of the astrocyte intermediate filament protein, glial fibrillary acidic protein (GFAP) is the hallmark of astrogliosis, we quantified the astroglial response to IBG using a sandwich ELISA for GFAP. In brain areas that showed a positive GFAP response to chronic exposure, other CNS structural proteins were assayed to further characterize neural responses to IBG. The data indicate that exposure to IBG causes astrogliosis and alterations in microtubule and neurofilament proteins that are consistent with IBG-induced brain damage, effects which are not confined to the cerebellar vermis.

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[e] Abbreviations: CNS, central nervous system; ELISA, enzyme-linked immunosorbant assay; SDS, sodium dodecyl sulfate.

METHODS

Materials

IBG (hydrochoride), manufactured by Omnichem, Belgium, was provided by the National Institute on Drug Abuse (Rockville, MD). Chemicals and reagents used in the GFAP assay were as previously described.[4] Antibodies to neurofilament-68 (NF-68) were from Boehringer (Indianapolis, IN) and those directed against β-tubulin were from Amersham (Arlington Heights, IL).

Animals

Sprague-Dawley rats, approximately 6 weeks old, were obtained from Hazleton Research Products, Inc. (Raleigh, NC). Zeigler NIH-07 certified feed and tap water (Birmingham public water supply) were available *ad libitum*. Cage size and animal care conformed to the guidelines of the U.S. Department of Agriculture and those of the U.S. Department of Health and Human Services. Animal weights were recorded on a daily basis as were morbidity and mortality.

Dosing Regimens

Formulations of IBG, calculated as the free base, were administered according to two different regimens. 1) *Acute study:* IBG (dissolved in deionized water) was administered to male and female rats (0, 50, 100, or 150 mg/kg/dose, i.p.), once daily for three days. Rats were sacrificed on days 3, 5, 7, and 14 post the last daily dose. 2) *Chronic study:* IBG (suspended in 0.5% methylcellulose) was administered to male and female rats (0, 25, 75, or 150 mg/kg, p.o.) once daily for 14 days. Rats were sacrificed on days 1 and 17 post the last daily dose.

Clinical Observations

For both dosing regimens, detailed physical examinations for clinical signs of toxicity were conducted for each rat at approximately 1, 3, and 5 hours after dosing on day 1; approximately 3 hours after dosing on days 2 and 3; and daily thereafter.

Histopathology

A variety of tissues from both dosing regimens were weighed, fixed in neutral buffered formalin (10%), embedded in paraffin, sectioned and stained with hematoxylin and eosin for subsequent analysis for gross pathology. These tissues included, but were not limited to brains, lungs, livers, kidneys, testes and ovaries.

Brain Tissue Preparation

Brains were removed and immersed in 70% ethanol/150 mM NaCl fixative. Brains prepared in this manner can be subjected to both immunohistochemistry and immunoassay for GFAP.[5] Brains were bisected in the sagittal plane. One hemi-brain was stored for subsequent morphological analysis in a future study and the other hemi-brain was dissected into the following regions: cerebellum (minus vermis), vermis, hippocampus, striatum, cortex, olfactory bulb and brain stem. Each region was homogenized in 10 volumes of 95°C 1% SDS and stored frozen prior to immunoassay for GFAP.

GFAP Assay

GFAP was assayed by a previously described sandwich ELISA.[4] All samples and standards were processed robotically using a Tecan 5052 system (Tecan US, Research Triangle Park, NC); values were expressed as μg GFAP per mg total homogenate protein. GFAP values are based on a standard purified from a cytoskeletal preparation of rat spinal cord.[4]

PhosphorImager Analysis of Neurofilament-68 and β-Tubulin

Samples of hippocampus and olfactory bulbs obtained from the chronic IBG study (35 μg total protein each) were resolved on SDS-polyacrylamide gels and then electrophoretically transferred to nitrocellulose sheets. The blots were then probed with antibodies directed against NF-68 and β-tubulin followed by sequential incubation with rabbit anti-mouse IgG and ^{125}I protein A.[6] The exact incubation conditions are described in O'Callaghan[4] under "Slot-immunobinding Protocol." Quantification of each protein was achieved by PhosphorImager analysis of the band volume corresponding to each protein using ImageQuaNT software (Molecular Dynamics, Sunnyvale, CA). Arbitrary volume units were normalized to percent of the corresponding control group.

Statistical Analyses

Body weights were evaluated with a one-way ANOVA and Dunnett's Test for multiple comparisons. GFAP data were subjected to statistical analysis using the Statistical Analysis System (SAS Institute, Inc., Cary, NC). All dose groups were compared simultaneously within a brain region across sex and day using the multisample Wilcoxon procedure. NF-68 and β-tubulin were analyzed by Fischer's exact test for mean comparisons.

RESULTS

Mortality, Body Weights and Clinical Observations

In the acute study 19/20 males and 16/19 females in the 150-mg/kg IBG group died by the third day of dosing. In the chronic study there was only one death in

the 150-mg/kg IBG group. Body weight was not affected in the acute study, whereas transient decreases in body weight were observed for both males and females in the 75-mg/kg and 150-mg/kg IBG groups in the chronic study (data not shown); these deficits were resolved by day 31 (data not shown). In the acute study, the primary clinical signs of toxicity in rats dosed with IBG in both males and females included ataxia, tremors, hyperexcitability, hyperactivity, squinting eyes, outstretched forelimbs and splayed hindlimbs (data not shown). Other clinical signs that were considered to be drug related were hypoactivity, hunched posture, tense body, excessive salivation, a clear or red ocular discharge, a red crusty nasal discharge, altered breathing rates, hypothermia and Straub tail (data not shown). Most of these signs were dose-related and were evident within 1–3 hours after the first dose and had resolved by day 7 (data not shown). In the chronic study, a similar constellation of clinical signs were observed for both sexes throughout the dosing period (days 1–14); few signs were noted after day 17 and rats appeared normal by the end of the study (day 31).

Histopathology

No gross necropsy findings, histopathological abnormalities or changes in relative organ weights could be attributed to IBG following either the acute or chronic regimens.

Analysis of GFAP

Acute Study

IBG caused dose-, time-, and region-dependent increases in GFAP. In males (FIG. 1), 100 mg/kg of IBG caused consistently larger increases in GFAP than did 50 mg/kg in the affected areas. Increases were seen in cerebellum (minus vermis), hippocampus, striatum, cortex and olfactory bulbs. The largest (approximately 150% of control) and most consistent effects were observed in cerebellum (minus vermis) and hippocampus. In contrast to males, both the cerebellar vermis and the rest of cerebellum showed large increases in GFAP in females (FIG. 2). Increases were observed as early as day 5 post dosing and these increases persisted through day 14 post dosing (FIG. 2). Alterations in GFAP observed in other brain areas of females (striatum and cortex) were not dose-related and did not correspond to the time course of the increases in GFAP seen in cerebellum (vermis or rest of cerebellum) (FIG. 2) or the increases seen in all affected regions of the males (FIG. 1). Due to the high mortality in the 150-mg/kg IBG group (males and females), GFAP analysis of the remaining animals in this dose group is not presented.

Chronic Study

IBG did not affect the concentration of GFAP in any brain region of male rats on day 1 (FIG. 3) or 17 (data not shown) post dosing. IBG caused large dose-dependent increases in GFAP in hippocampus, brain stem striatum and olfactory bulbs of female rats (FIG. 4). The greatest increase (215% of control) was seen in brain stem whereas the smallest increase was observed in striatum (142% of control). In contrast to the results of the acute study, no alterations were observed in vermis or the rest of cerebellum. The increases in GFAP observed in females at 1 day post dosing had resolved to control levels by 17 days post dosing (data not shown).

Analysis of Neurofilament-68 and β-Tubulin

In the chronic study IBG did not affect NF-68 or β-tubulin in hippocampus or olfactory bulbs in male rats (data not shown). In female rats at day 1 post dosing, however, IBG caused a greater than 2-fold increase in NF-68 and β-tubulin in hippocampus (FIG. 5) without affecting these proteins in olfactory bulbs (data not shown). NF-68 and β-tubulin were not examined in male or female rats at day 17 post dosing.

DISCUSSION

The principal finding of this study is that acute and chronic administration of IBG results in gliosis in a number of regions of the rat brain. Our results are in general agreement with those of O'Hearn and Molliver[1] and O'Hearn *et al.*,[2] who reported that IBG, when administered according to the acute regimen followed in the present study, causes degeneration of cerebellar Purkinje cells and elicits reactive gliosis in the cerebellar vermis. In contrast to these previous reports, however, our acute IBG exposure data suggests that gliosis extends beyond the vermis within the cerebellum and it occurs in other brain regions as well.

The outcomes of neurotoxic exposures often are influenced by a number of variables including, for example, strain,[7] species,[8] temperature,[9] stress,[9,10] gender,[11] and dosing regimen.[12] With respect to the latter two variables, the present data obtained for IBG are no exception. Thus, large acute (i.p.) doses of IBG resulted in a general state of hyperexcitability, high mortality, and gliosis of the cerebellum in both sexes. In contrast, while the chronic (p.o.) exposure to IBG was associated with the same clinical signs, mortality was low, gliosis was restricted to females and it occurred in brain areas other than the cerebellum. Thus, although the data suggest that IBG has the potential to cause gliosis, the brain regions affected are dependent on sex and dosage regimen. In comparison to recent data obtained for mice,[13] our findings also suggest that rats are more sensitive to IBG-induced gliosis. While in aggregate these results may be indicative of complex species-, sex- and regimen-specific mechanisms underlying IBG-induced gliosis, different pharmacokinetic profiles may be the simplest explanation for the different outcomes observed with respect to these variables.[14] Given this possibility, future studies of IBG neurotoxicity would benefit from a regional analysis of IBG and its metabolites.

Reactive gliosis, characterized by an accumulation of glial filaments and, therefore, of GFAP, occurs at any site of damage to the adult CNS.[15,16] Previously we showed that dose-dependent increases in GFAP reflect dose-dependent damage that results from a variety of chemical insults of the CNS and that these effects often occur at exposure levels below those that result in overt histopathology.[16,17]

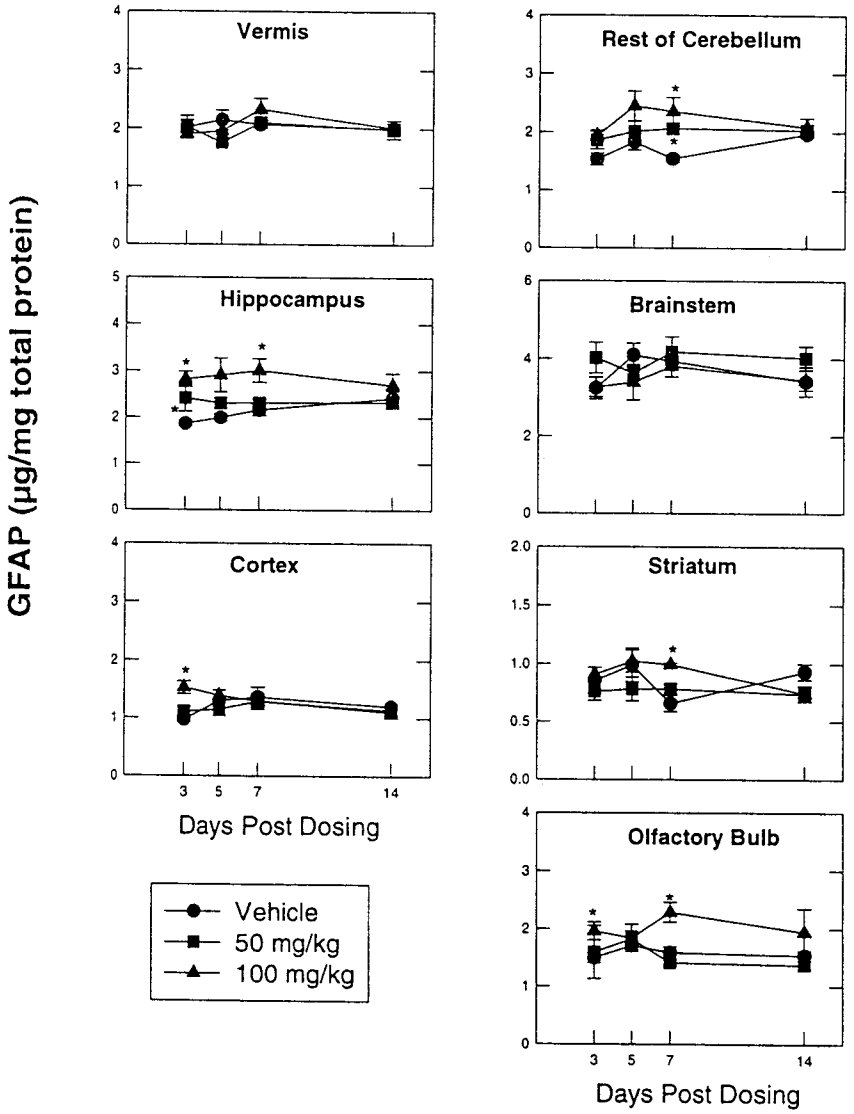

FIGURE 1. Effects of acute IBG on GFAP in male rats. Each value represents the mean ± SEM for 5 rats. *p <0.05 compared to vehicle control. Where error bars are not shown they are within the radius of the data point.

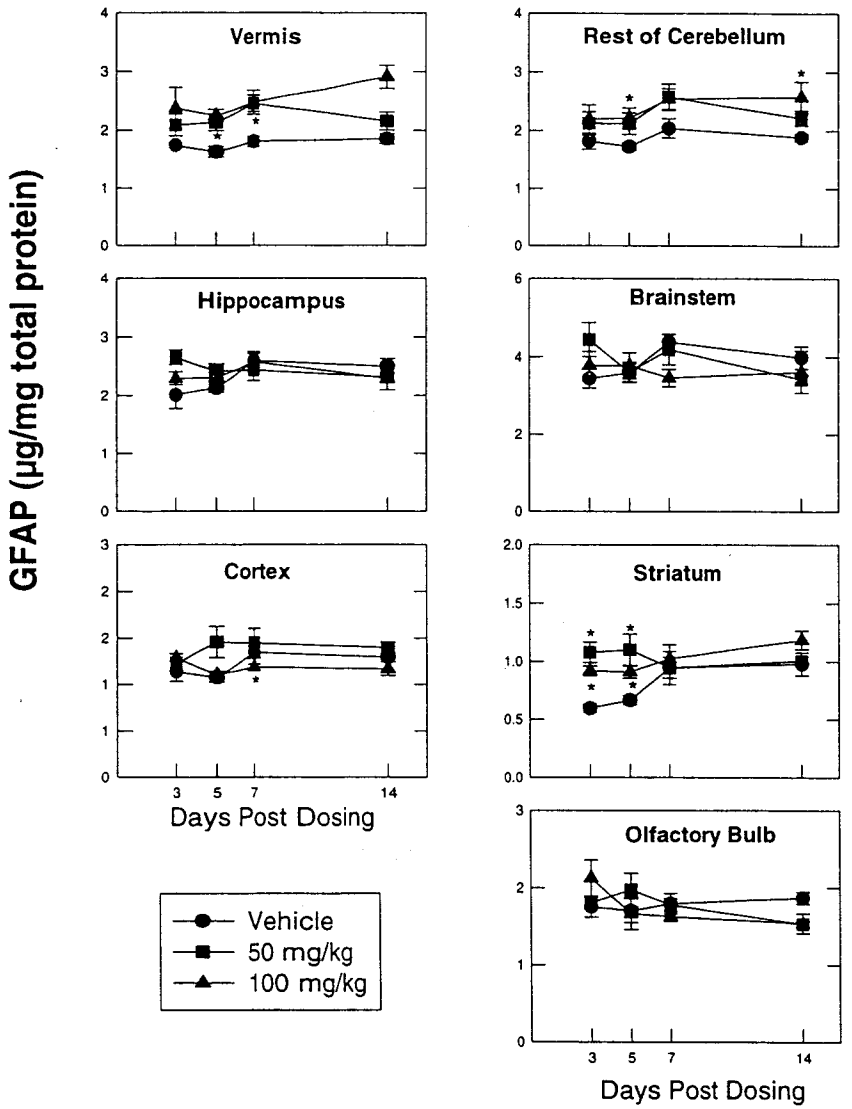

FIGURE 2. Effects of acute IBG on GFAP in female rats. Each value represents the mean ± SEM for 5 rats. *p <0.05 compared to vehicle control. Where error bars are not shown they are within the radius of the data point.

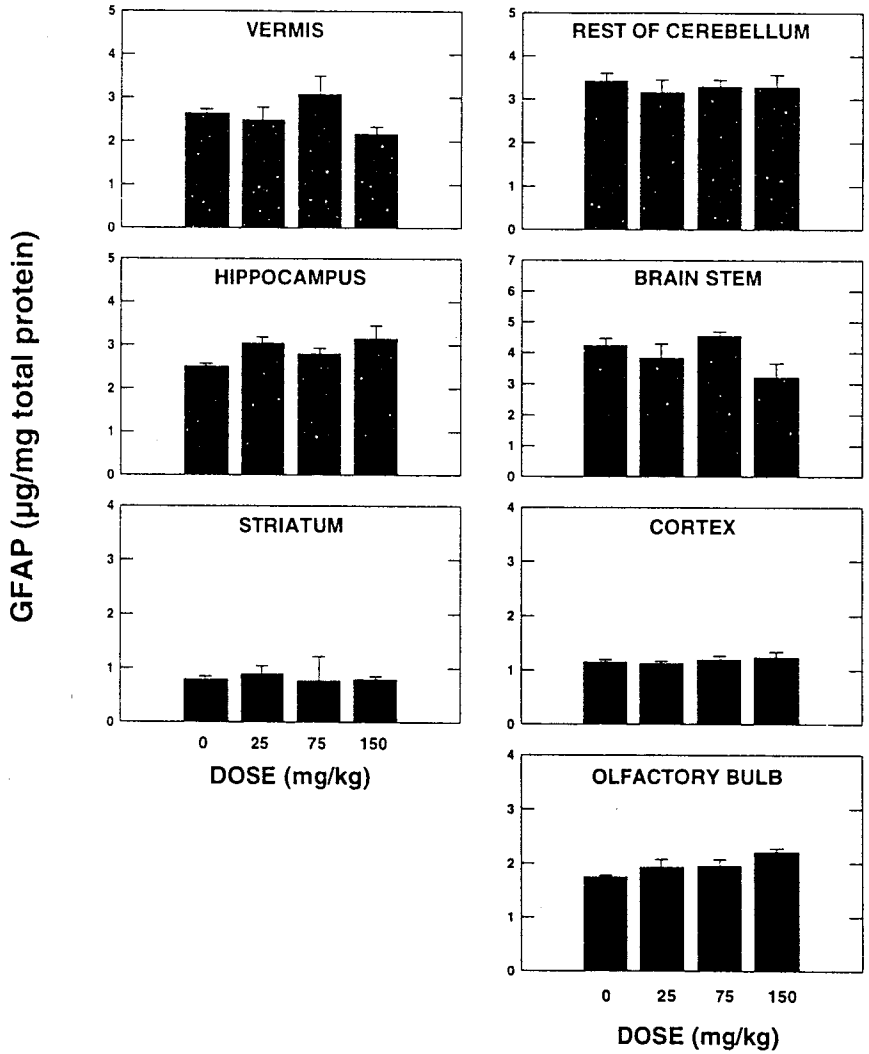

FIGURE 3. Effects of chronic IBG on GFAP in male rats. Each value represents the mean ± SEM for 5 rats. *p <0.05 compared to vehicle control. Rats were sacrificed on day 1 post dosing; all values for rats sacrificed on day 17 post dosing did not differ with respect to treatment (data not shown).

Consistent with these previous observations, most of the effects of IBG on GFAP were dose-related and they occurred in the absence of evidence of histopathology as assessed by light microscopic analysis of conventionally stained tissue. To obtain evidence of underlying neural damage in brain areas showing enhanced expression of GFAP, we assayed the content of the NF-68 and β-tubulin, proteins

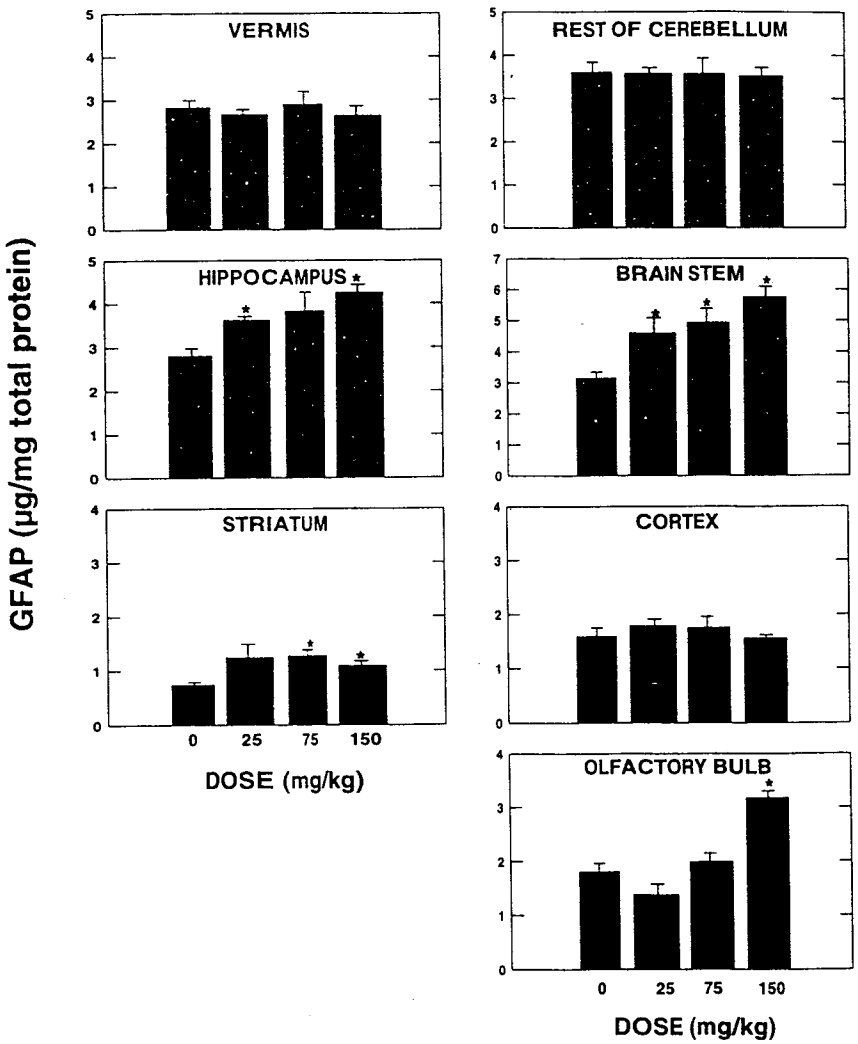

FIGURE 4. Effects of chronic IBG on GFAP in female rats. Each value represents the mean ± SEM for 5 rats. *p <0.05 compared to vehicle control. Rats were sacrificed on day 1 post dosing; all values for rats sacrificed on day 17 post dosing did not differ with respect to treatment (data not shown).

associated with the axonal cytoskeleton and microtubules, respectively,[18] in samples of hippocampus and olfactory bulbs obtained from the chronic IBG study. While IBG did not affect these proteins in the olfactory bulbs, samples of hippocampus from the females showed large increases in both NF-68 and β-tubulin, findings consistent with the sex-dependent increase in GFAP in this area. The hippocampus

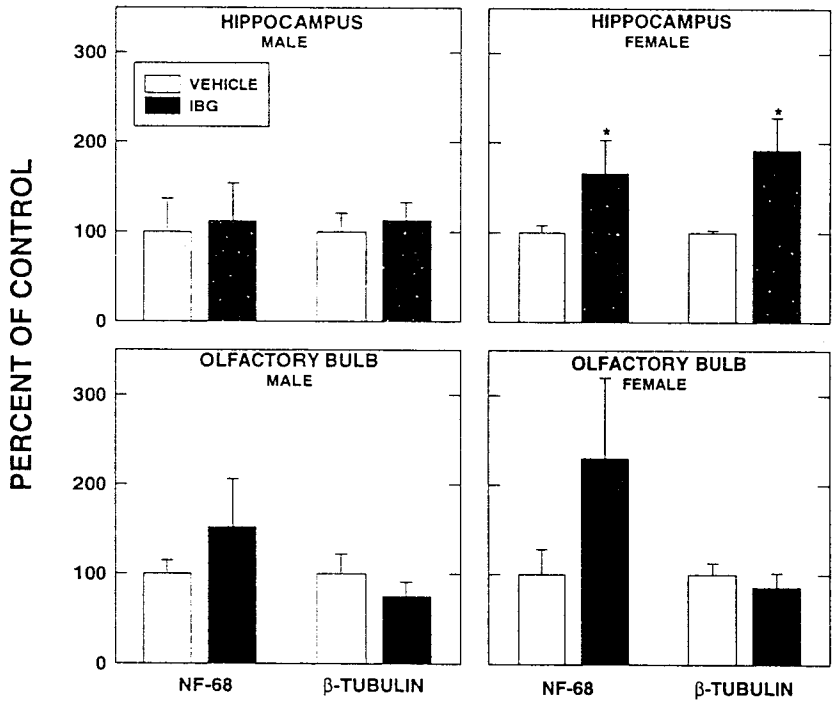

FIGURE 5. Effects of chronic IBG (150 mg/kg) on the concentration of NF-68 and β-tubulin in hippocampus and olfactory bulbs of male and female rats. Each value represents the mean ± SEM for 3 rats. *p <0.1 compared to vehicle control. Rats were sacrificed on day 1 post dosing.

is a "plastic" area of the CNS containing neuronal projections that "sprout" in response to damage.[19] The increase in NF-68 and β-tubulin, proteins associated with structural elements of neurons, is consistent with such a response to injury.[18,20] Alternatively, IBG may have disrupted the normal turnover of these proteins in hippocampus, resulting in their accumulation in this area. An immunohistochemical examination of NF-68 and β-tubulin in hippocampus, along with other proteins associated with spouting, may help resolve this issue.

In conclusion. the observed increases in GFAP following acute and chronic exposure to IBG are suggestive of sites of underlying damage to the CNS. These findings indicate that a more in-depth neuroanatomical evaluation of the affected brain regions is warranted, especially of those areas showing large increases in GFAP and changes in other structural proteins of the CNS.

SUMMARY

Acute administration of high doses of ibogaine (IBG) to the male rat results in degeneration of Purkinje cells and reactive gliosis in the cerebellar vermis.[1,2]

We examined whether acute and chronic administration of IBG to male and female rats results in gliosis as determined by quantification of the astroglial intermediate filament protein, glial fibrillary acidic protein (GFAP). After acute administration of IBG, rats of both sexes showed dose-related increases in GFAP that were not confined to the cerebellar vermis. After chronic administration of IBG, female, but not male rats, showed large (as much as 200% of control), dose-related increases in GFAP in hippocampus, olfactory bulbs, brain stem and striatum, but not cerebellum. In hippocampus, the cytoskeletal proteins, neurofilament 68 (NF-68) and β-tubulin were increased in females treated chronically with IBG, findings consistent with a damage-induced sprouting response. Together, the data indicate that IBG damages areas of the brain outside the cerebellum and that the sites damaged are dependent on sex and dosage regimen.

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REFERENCES

1. O'HEARN, E. & M. E. MOLLIVER. 1993. Degeneration of Purkinje cells in parasagittal zones of the cerebellar vermis after treatment with ibogaine or harmaline. Neuroscience **55:** 303–310.
2. O'HEARN, E., D. B. LONG & M. E. MOLLIVER. 1993. Ibogaine induces glial activation in parasagittal zones of the cerebellum. NeuroReport **4:** 299–302.
3. GLICK, S. D., K. ROSSMAN, N. C. RAO, I. M. MAISONNEUVE & J. N. CARLSON. 1992. Effects of ibogaine on acute signs of morphine withdrawal in rats: independence from tremor. Neuropharmacology **31:** 497–500.
4. MARTIN, P. M. & J. P. O'CALLAGHAN. 1995. A direct comparison of GFAP immunocytochemistry and GFAP concentration in various regions of ethanol-fixed rat and mouse brain. J. Neurosci. Methods **58:** 181–192.
5. O'CALLAGHAN, J. P. 1991. Quantification of glial fibrillary acidic protein: comparison of slot-immunobinding assays with a novel sandwich ELISA. Neurotoxicol. Teratol. **13:** 275–281.
6. BURNETTE, W. N. 1981. "Western blotting:" electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein. Anal. Biochem. **112:** 195–203.
7. SONSALLA, P. K. & R. E. HEIKKILA. 1988. Neurotoxic effects of MPTP and methamphetamine in several strains of mice. Prog. Neurol. Biol. Psychiatry **12:** 345–352.
8. GIOVANNI, A., B-A. SIEBER, R. E. HEIKKILA & P. K. SONSALLA. 1994. Studies on species sensitivity to the dopaminergic neurotoxin MPTP. Part 1: systemic administration. J. Pharmacol. Exp. Ther. **270:** 1000–1007.
9. MILLER, D. B. & J. P. O'CALLAGHAN. 1994. Environment-, drug- and stress-induced alterations in body temperature affect the neurotoxicity of substituted amphetamines in the C57BL/6J mouse. J. Pharmacol. Exp. Ther. **270:** 752–760.
10. MILLER, D. B. & J. P. O'CALLAGHAN. 1996. Neurotoxicity of *d*-amphetamine in the C57BL/6J and CD-1 mouse: interactions with stress and the adrenal system. Ann. N. Y. Acad. Sci. This volume.

11. DLUZEN, D. E., J. L. MCDERMOTT & B. LIU. 1996. Estrogen alters MPTP-induced neurotoxicity in female mice: effects on striatal dopamine concentrations and release. J. Neurochem. **66:** 658–666.
12. SONSALLA, P. K. & R. E. HEIKKILA. 1986. The influence of dose and dosing interval on MPTP-induced dopaminergic neurotoxicity in mice. Eur. J. Pharmacol. **129:** 339–345.
13. ALI, S. F., X. M. MENG & J. P. O'CALLAGHAN. 1994. Neurotoxicity of ibogaine in CD mice. Soc. Neurosci. Abstr. **20** (Part 2): 1650.
14. CAMPBELL, D. B. 1995. The use of toxicokinetics for safety assessment of drugs acting in brain. Mol. Neurobiol. **11:** 193–216.
15. NORTON, W. T., D. A. AQUINO, I. HOZUMI, F-C. CHIU & C. F. BROSNAN. 1992. Quantitative aspects of reactive gliosis: a review. Neurochem. Res. **17:** 877–885.
16. O'CALLAGHAN, J. P. 1993. Quantitative features of reactive gliosis following toxicant-induced damage of the CNS. Ann. N. Y. Acad. Sci. **679:** 195–210.
17. O'CALLAGHAN, J. P., K. F. JENSEN & D. B. MILLER. 1995. Quantitative aspects of drug and toxicant-induced astrogliosis. Neurochem. Int. **26:** 115–124.
18. HOFFMAN, P. N. & D. W. CLEVELAND. 1988. Neurofilament and tubulin expression recapitulates the developmental program during axonal regeneration: induction of a specific β-tubulin isotype. Proc. Natl. Acad. Sci. USA **85:** 4530–4533.
19. WEST, J. R. & S. L. DEWEY. 1984. Mossy fiber sprouting in the fascia dentata after unilateral entorhinal lesions: quantitative analysis using computer-assisted image processing. Neuroscience **13:** 377–384.
20. WONG, J. & M. M. OBLINGER. 1990. A comparison of peripheral and central axotomy effects on neurofilament and tubulin gene expression in rat dorsal root ganglion neurons. J. Neurosci. **10:** 2215–2222.

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