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Neurotoxicology and Teratology 24 (2002) 227–233

NEUROTOXICOLOGY AND TERATOLOGY

www.elsevier.com/locate/neutera

Differential vulnerability of snake species to MPTP: A behavioral and biochemical comparison in ratsnakes (*Elaphe*) and watersnakes (*Nerodia*)

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Received 5 October 2001; accepted 25 January 2002

Abstract

The synthetic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a Parkinsonian-like syndrome in humans and nonhuman primates, and also causes movement disorders in rodents, fish, amphibians and lizards. To date, the effects of MPTP have not been characterized in snakes. In this study, the behavioral and biochemical effects of MPTP were assessed in the black ratsnake *Elaphe o. obsoleta* and the banded watersnake *Nerodia f. fasciata*—species that display contrasting behavioral sensitivities to dopaminergic antagonists and to amphibian toxins. We report that MPTP induces depletion of norepinephrine and serotonin in fore, mid and hindbrain regions and depletion of dopamine in fore and midbrain regions in *E.o. obsoleta*. MPTP also induced a marked reduction in righting ability in *E.o. obsoleta*. In *N.f. fasciata*, norepinephrine and dopamine were depleted by MPTP in all three brain regions and serotonin was only significantly reduced in the forebrain. In contrast to *E.o. obsoleta*, *N.f. fasciata* demonstrated no behavioral disorders. This study demonstrates a behavioral and biochemical sensitivity to MPTP in *E.o. obsoleta* that differs from that in *N.f. fasciata*. The differential sensitivities to monoaminergic modulation may be related to the contrasting diets of these species.

Keywords: MPTP; Dopamine; Serotonin; Reptile; Motor control

1. Introduction

The synthetic neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induces a Parkinsonian-like condition marked by postural abnormalities, muscle rigidity, tremor and bradykinesia in a variety of vertebrates. These effects were first noted in humans who were exposed to MPTP as an impurity in an illicitly manufactured narcotic [9,14]. The behavioral and biochemical effects of MPTP have been studied extensively since then in nonhuman primates and rodents (see Ref. [10], for review). The induction of movement disorders by MPTP has been attributed to the destruction of dopaminergic neurons in the nigrostriatal tract of the brain by the metabolite 1-methyl-4-phenylpyridinium (MPP+), which ultimately results in a reduction of dopamine in the striatum, a condition also noted in patients with Parkinson's disease. The conversion of MPTP to MPP+ is predominantly regulated by monoamine oxidase type B (MAO-B) in human brain tissue.

The effects of MPTP have been characterized previously in nonmammalian vertebrates as well, and the behavioral effects are consistent across all groups tested. MPTP impairs righting ability and causes marked reductions in brain monoamine levels when administered to the frogs, *Rana pipiens* [1] and *R. clamitans clamitans* [2], and also impairs the righting ability of the salamander *Taricha torosa torosa* [2]. When administered to the lizard *Anolis*, MPTP causes damage to striatal terminals in the brain and induces a variety of Parkinsonian-like symptoms such as hypokinesia, rigidity and stereotyped head and neck movements which vary in intensity depending on the dose administered [12]. Parkinsonian-like bradykinesia, reduction of brain monoamines [15,19] and degeneration of dopaminergic neurons [11] also have been documented in the goldfish *Carassius auratus* treated with MPTP. These studies demonstrate

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0892-0362/02/$ – see front matter
PII: S0892-0362(02)00204-0

similar patterns of biochemical and behavioral effects of MPTP across a variety of vertebrate groups.

To date, the behavioral and biochemical effects of MPTP have not been assessed in snakes. The purpose of this study was to assess the acute behavioral effects of dopamine depletion by MPTP in the black ratsnake *Elaphe obsoleta obsoleta* (eastern population) [8] and the banded watersnake *Nerodia fasciata fasciata*. Ratsnakes and watersnakes were of interest due to the fact that they differ considerably in their susceptibility to behavioral effects induced by amphibian skin toxins and drugs that affect monoaminergic pathways. Studies have shown that oral application of skin mucus from the African clawed frog *Xenopus laevis* induces extreme muscle dystonia and often death in *E.o. obsoleta*, but milder stereotyped orofacial dyskinesias in the northern watersnake *N.s. sipedon*, a species closely related to *N.f. fasciata* [4,7]. These contrasting sensitivities are intriguing in light of the fact that the mucus contains dopamine-blocking agents such as caerulein, xenopsin and thyrotropin-releasing hormone and other neuroactive agents including serotonin and bufotenidine (see Ref. [4] for review).

Pharmacological studies have shown that the mucus-induced effects in *N.s. sipedon* are modulated by pretreatment with selective D-1 and D-2 dopamine receptor blockers, implicating the involvement of dopaminergic pathways in the mucus-induced response [3,6]. Further, chronic treatment with the D-2 dopamine receptor blocker haloperidol induces dystonia and movement disorders that are more pronounced in *E.o. obsoleta* than in *N.f. fasciata* [27]. The role of monoaminergic pathways was further implicated by the demonstration that inhibition of monoamine oxidase type B (MAO-B) alters mucus-induced behaviors in *N.s. sipedon* [5]. However, when administered alone, MAO-B inhibitors decrease tongue flicking and increase climbing in *N.s. sipedon* [5] but decrease both tongue flicking and climbing behaviors in *E.o. obsoleta* [26]. These studies suggested fundamental differences in the role that monoaminergic pathways play in the behavior of these two snake species.

The purpose of the present experiment was to (1) assess the dopamine-depleting effect of MPTP, and to (2) determine the acute behavioral effects of MPTP in *E.o. obsoleta* and *N.f. fasciata*. Given the relatively high sensitivity of *E.o. obsoleta* to the movement disorders and dystonia induced by amphibian skin toxins and by D-2 dopamine receptor blockade, the hypothesis that *E.o. obsoleta* would be more susceptible to movement disorders caused by MPTP-induced dopamine depletion as compared to *N.f. fasciata* was tested in this study.

2. Methods

2.1. Animals

Male sibling *E.o. obsoleta* (194–239 g) and *N.f. fasciata* (64–83 g) were born and raised in the laboratory for 26 and 28 months, respectively. *E.o. obsoleta* and *N.f. fasciata* were fed a diet of thawed juvenile mice and live minnows, respectively, supplemented with Terrafauna Vitalife Reptile supplement. The snakes were housed in plastic cages ($41\times28\times15$ cm for *E.o. obsoleta*; $31\times16\times8$ cm for *N.f. fasciata*) which, in turn, were placed in incubators (Lyon Electric) and were maintained on a 14:10 LD cycle at 26 °C. The snakes were provided fresh water but were not fed during the study. The experimental protocol was approved by the Institutional Animal Care and Use Committees of North Carolina State University and the United States Environmental Protection Agency.

2.2. Drugs and chemicals

MPTP hydrochloride was obtained from Aldrich Chemical. Lyphomed 0.9% saline was used as the drug vehicle and the control. Drug delivery of 7.5 mg/kg body weight was achieved by preparing a 1.5 mg/ml solution of MPTP (calculated as the free base) and injecting 0.005 cc/g body weight.

5-hydroxytryptamine creatinine sulfate complex, dopamine hydrochloride salt, norepinephrine hydrochloride, 3,4-dihydroxybenzylamine hydrobromide (DHBA) and heptane sulfonic acid (HSA) were obtained from Sigma (St. Louis, MO). Perchloric acid (70%), HPLC-grade methanol and sodium hydroxide were obtained from Fisher Scientific (Atlanta, GA). Ethylenediaminetetraacetic acid (EDTA) and ortho-phosphoric acid (85%) were obtained from Fluka Chemika-Biochemika. HPLC-grade water (Dracor Water Systems) was used in all preparations for HPLC analysis.

2.3. Experimental procedure

A separate preliminary dose range study (5–40 mg/kg body weight), based upon doses used in previous studies, was conducted to determine an effective dose of MPTP in *Elaphe* and *Nerodia*. The higher doses of 10 and 40 mg/kg were found to be lethal after two to four daily injections. An effective daily dose of 7.5 mg/kg was shown to induce behavioral effects after five daily injections.

Control- (*E.o. obsoleta*, $n=3$; *N.f. fasciata*, $n=4$) and drug-treated (*E.o. obsoleta*, $n=4$; *N.f. fasciata*, $n=4$) snakes were injected intraperitoneally between 10:00–10:30 am daily for 6 days. The righting ability of each snake was assessed during daily observation periods between 4:00–5:00 pm (session A; 6–7 h postinjection) and 8:15–9:15 am (session B; 22–23 h postinjection). The drug was administered daily for 6 days with the two observations per day testing regime in order to ensure maximal neurochemical depletion and to allow for the assessment of recovery from the initial effects of each drug treatment. During each testing session, snakes were placed on their backs in an aquarium ($75\times30\times30$ cm) containing 11 cm of water, released, and their righting time was measured using a foot-operated

stopwatch. The snakes were tested in water to force them to use fine locomotor skills and to exaggerate any motor control deficits. Although *E.o. obsoleta* is a terrestrial species, it is not averse to water and is a capable swimmer. This righting test was used to compare the decrement in righting speed caused by MPTP, relative to the control for each species, and not the absolute righting speeds of each species. This test was repeated ten times per observation period for each snake. A maximum righting time of 15 s was permitted, after which snakes that failed to right themselves were removed from the aquarium.

On day 7, snakes were decapitated between 10:00 am and 2:00 pm, and the brain was quickly excised and dissected on a cold plate (Thermoelectrics Unlimited) into right and left fore, mid and hind regions. The forebrain region included the cerebral cortex, underlying dorsal ventricular ridge and basal ganglia; the midbrain included the optic tectum, thalamus, hypothalamus and pineal body; the hindbrain included the medulla and cerebellum. The tissues were weighed and placed in amber 1.5 ml microfuge tubes, frozen immediately on dry ice and stored at −70 °C until analysis. Samples of the right side were analyzed for norepinephrine, dopamine and serotonin content using liquid chromatography with electrochemical detection.

2.4. Neurochemical analysis

Brain regions were diluted in 0.2 N perchloric acid containing 0.1 μg/ml of DHBA as the internal standard and were sonicated for one burst of 8–10 s in duration, using a Kontes microultrasonic cell disrupter. Samples were then centrifuged for 10 min at 12,200 rpm (12,385×*g*) using a Beckman microfuge 12 centrifuge at 4 °C. The supernatant was removed, centrifuged again and the resulting supernatant was pipetted into TSK-6080 autosampler vials from which 20 μl was injected into the chromatography system.

The mobile phase for liquid chromatography consisted of 3.2 mM HSA, 62 mM ortho-phosphoric acid, 8 μM EDTA and 18% methanol. The mobile phase was filtered through a Millipore type GV 0.22-μm filter and then was degassed under vacuum for 20 min in a Branson 1200 ultrasonic cleaner. The final pH was adjusted with NaOH pellets to 2.8.

Standard stock solutions were prepared in 0.2 N perchloric acid at a concentration of 0.1 mg per ml (calculated as the free base) and aliquots of each were frozen at −70 °C. Working solutions were freshly prepared on the day of the assay by diluting the stock solution with 0.2 N perchloric acid.

The chromatography system hardware consisted of a Waters 6000 A solvent delivery system, operated at a flow rate of 1.2 ml/min. Samples were injected into the system with a Tosoh TSK-6080 automatic injector with an injection loop volume of 20 μl and the monoamines were separated using a Radial-Pak C18 cartridge housed in an RCM 8×10 cartridge holder. A Nova-Pak C-18 Sentry guard column preceded the column cartridge. An ESA Coulochem II Electrochemical detector with a model 5020 guard cell and a model 5011 analytical cell was used for detection of chemicals. The two electrodes in the analytical cell were set at −50 and +450 mV, respectively, and the guard cell was set at +500 mV. Peak heights from the chromatographs were analyzed with Gilson 712 HPLC Controller (version 1.20, Gilson Medical Electronics) software using the internal standard method of analysis for all samples. Using this method, all monoamine peaks were compared to that of an internal standard (DHBA) present in the sample homogenate in order to control for variation in injection volume.

2.5. Statistical analysis

The inverse transformation (righting speed) of the righting times was calculated in order to yield homogeneous variances for righting responses of control- and MPTP-treated snakes. The mean values of the ten righting speeds recorded for each snake during each trial session were used in the statistical analysis. Righting speed data for control and MPTP-treated snakes were analyzed independently for each species (*E.o. obsoleta* and *N.f. fasciata*) and trial session (A or B) using a repeated measures analysis of variance (ANOVA). Least squares mean values were obtained from this analysis.

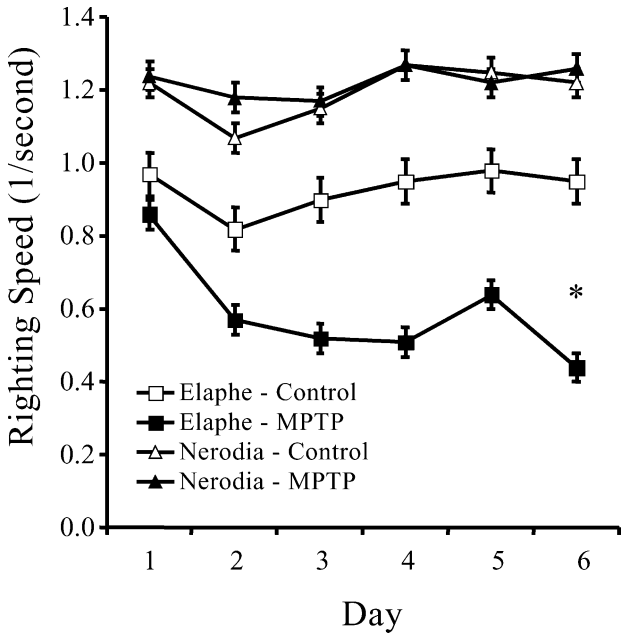

Fig. 1. Mean righting speed of control and MPTP-treated *E.o. obsoleta* ($n=3$ and 4, respectively) and *N.f. fasciata* ($n=4$ per treatment) during session A (6–7 h postinjection) only. Values reported are the least squares means. The error bars represent standard error of the mean. *$P<.05$ vs. control group (repeated measures ANOVA) for *E.o. obsoleta*, but not for *N.f. fasciata*.

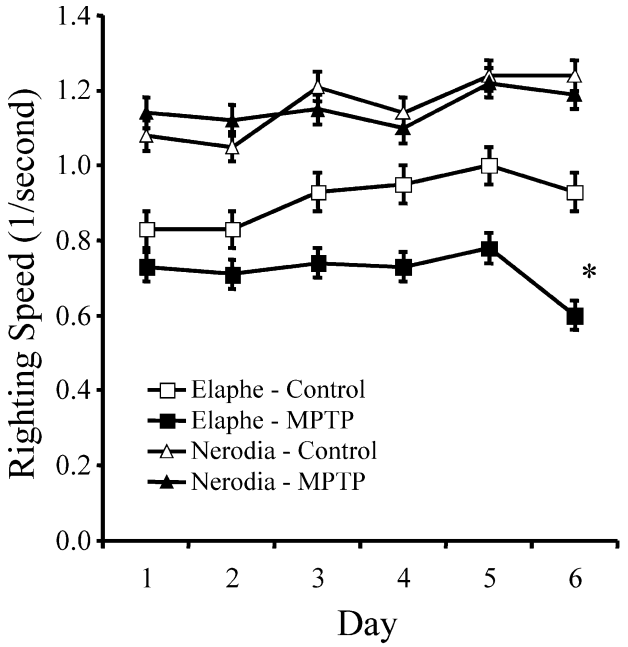

Fig. 2. Mean righting speed of control and MPTP-treated *E.o. obsoleta* ($n=3$ and 4, respectively) and *N.f. fasciata* ($n=4$ per treatment) during session B (22–23 h postinjection) only. Values reported are the least squares means. The error bars represent standard error of the mean. *$P<.05$ vs. control group (repeated measures ANOVA) for *E.o. obsoleta*, but not for *N.f. fasciata*.

The effect of the treatment (control vs. drug) on the level of each neurochemical was assessed for each species and brain region by an analysis of variance followed by Bonferroni's test of inequalities for multiple comparisons. An alpha level of .05 was used in all tests of significance. Statistical analyses were performed using SAS [22] and SPSS [24] software.

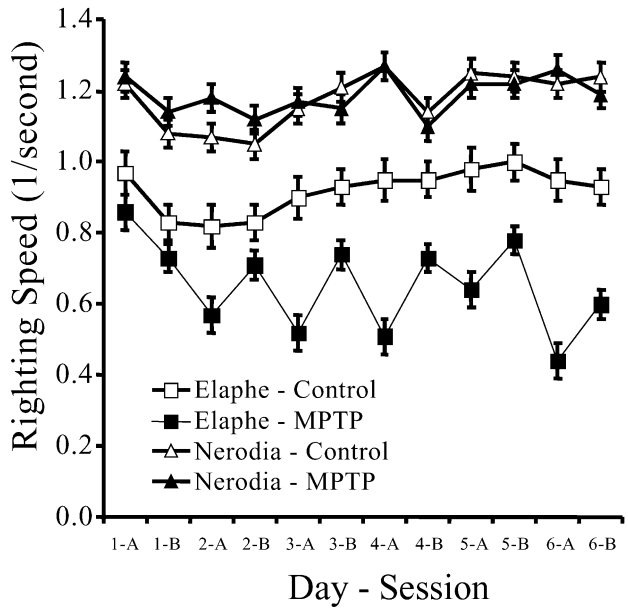

Fig. 3. Mean righting speed of control and MPTP-treated *E.o. obsoleta* ($n=3$ and 4, respectively) and *N.f. fasciata* ($n=4$ per treatment) during sessions A (6–7 h postinjection) and B (22–23 h postinjection). Values reported are the least squares means. The error bars represent standard error of the mean.

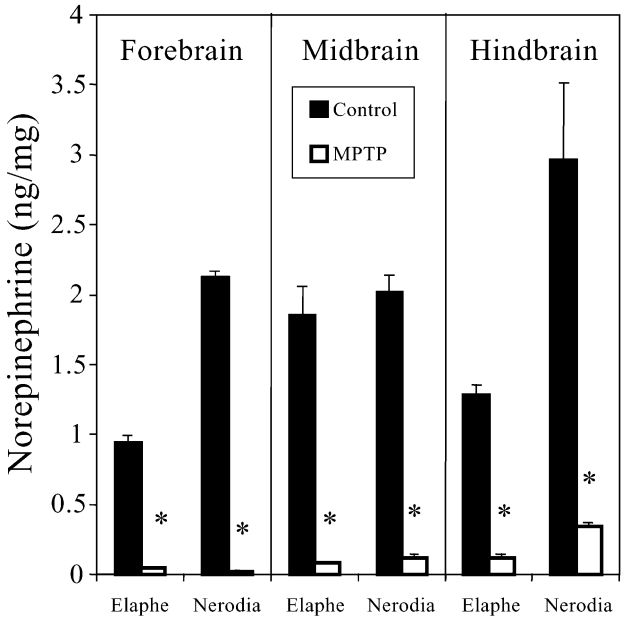

Fig. 4. Mean concentrations (ng/mg) of norepinephrine in fore, mid and hindbrains of *E.o. obsoleta* ($n=3$ and 4, respectively) and *N.f. fasciata* ($n=4$ per treatment) treated with saline (control) or MPTP. The error bars represent standard error of the mean. *$P<.05$ vs. control group (Bonferroni's test of inequalities for multiple comparisons) within each species and brain region.

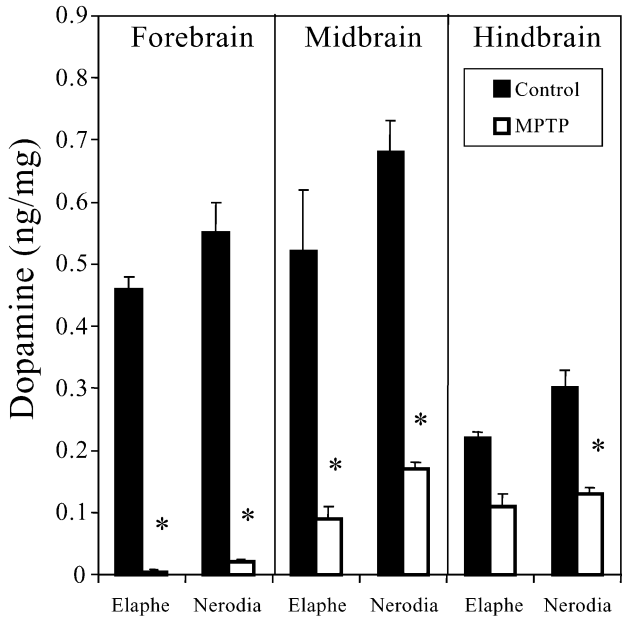

Fig. 5. Mean concentrations (ng/mg) of dopamine in fore, mid and hindbrains of *E.o. obsoleta* ($n=3$ and 4, respectively) and *N.f. fasciata* ($n=4$ per treatment) treated with saline (control) or MPTP. The error bars represent standard error of the mean. *$P<.05$ vs. control group (Bonferroni's test of inequalities for multiple comparisons) within each species and brain region.

3. Results

The mean righting speeds of each species are shown separately for sessions A and B in Figs. 1 and 2, respectively. The administration of MPTP significantly reduced the righting speed of *E.o. obsoleta* as compared to control-treated animals during sessions A (P=.01) and B (P=.02), but did not significantly affect the righting ability of *N.f. fasciata* during either session. The mean righting speeds of control and MPTP-treated snakes of each species during sessions A and B are displayed together in Fig. 3. A notable recovery between sessions A and B was observed for *E.o. obsoleta*. In addition to the impairment of righting ability, spiraling body movements with dorsoflexion of the head referred to as corkscrews, or dorsoflexion alone were noted in two of the four MPTP-treated *E.o. obsoleta*, but not in *N.f. fasciata*. As this study was not designed to quantify such behaviors, no quantitative results are presented.

The biochemical effects of MPTP on norepinephrine, dopamine and serotonin in fore, mid and hindbrain regions are summarized in Figs. 4–6. The MPTP treatment significantly decreased norepinephrine by at least 89% in all three brain regions of both species (Fig. 4). Dopamine was significantly reduced in the fore and midbrain regions of *E.o. obsoleta* and in all three brain regions of *N.f. fasciata* (Fig. 5). Dopamine was depleted by at least 75% in the fore and midbrains, and in the hindbrains it was depleted by 57% for *N.f. fasciata* and 50% for *E.o. obsoleta*, the latter being statistically insignificant. In *E.o. obsoleta*, serotonin was significantly depleted in fore (83%), mid (68%) and hindbrains (51%). In *N.f. fasciata*, serotonin was significantly depleted only in the forebrain (37%), but not in the mid (0%) or hindbrain (16%) regions (Fig. 6).

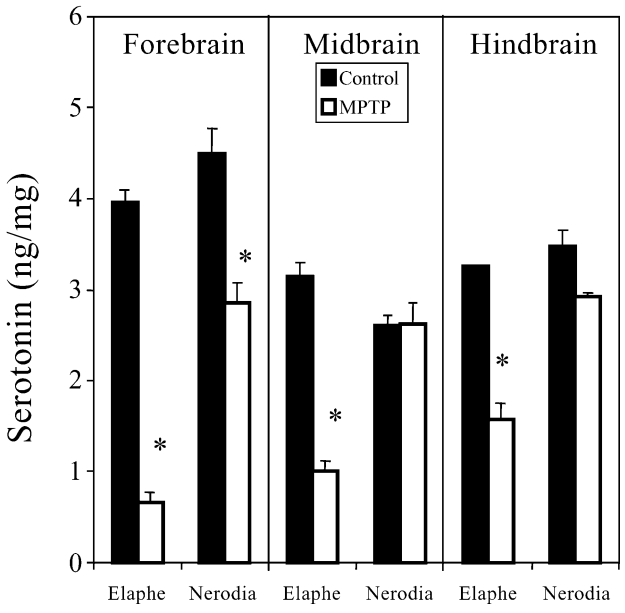

Fig. 6. Mean concentrations (ng/mg) of serotonin in fore, mid and hindbrains of *E.o. obsoleta* (n=3 and 4, respectively) and *N.f. fasciata* (n=4 per treatment) treated with saline (control) or MPTP. The error bars represent standard error of the mean. *P<.05 vs. control group (Bonferroni's test of inequalities for multiple comparisons) within each species and brain region.

4. Discussion

This study provides evidence that the acute behavioral and biochemical effects of MPTP differ between the snake species *E.o. obsoleta* and *N.f. fasciata*. MPTP significantly impaired the righting ability of *E.o. obsoleta* in a fashion consistent with other animal models. However, the righting time of *N.f. fasciata* was not significantly affected. In addition to the impairment of righting ability, corkscrewing body movements and dorsoflexion were also observed in some of the MPTP-treated *E.o. obsoleta*, but not in any of the *N.f. fasciata*. This study also demonstrates that MPTP causes a decrease in brain norepinephrine and dopamine which is similar in *E.o. obsoleta* and *N.f. fasciata*, but induces a more pronounced reduction of brain serotonin in *E.o. obsoleta*. The dose of MPTP used in this study (7.5 mg/kg) is within the range (1–50 mg/kg) of doses known to induce movement disorders in other vertebrates.

The structure and neurochemistry of the reptilian and mammalian basal ganglia are quite similar, suggesting a common role in movement control between these two classes of vertebrates [16,21]. Further, neuroanatomical studies have provided detailed maps of the monoaminergic systems in brains of the snake *Python regius*, and have indicated a concentration of dopaminergic tracts in the striatum and substantia nigra [23], as is seen in mammals. Thus, the reptilian brain serves as an effective vertebrate model for studying the extrapyramidal regulation of movement.

In the present study, the righting ability was tested in water in order to force each snake to use its fine locomotor skills to right itself and to exacerbate any motor deficits. The faster control righting speed of *N.f. fasciata* is believed to be due to their smaller size as compared to *E.o. obsoleta*. The change in righting speed of MPTP-treated snakes of each species relative to their control speeds was used to assess the behavioral effects of MPTP. Thus, righting ability served as an effective index of movement control and coordination. The righting ability of MPTP-treated *E.o. obsoleta* was significantly impaired both 6–7 and 23–24 h following the injections. Those behavioral effects are qualitatively similar to those previously reported for frogs administered MPTP in a similar experimental design [1,2] and indicate a decrease in motor activity and coordination.

The corkscrewing body movements observed in *E.o. obsoleta* were characterized by pronounced dystonia and dorsoflexion of the spine immediately posterior to the head. These effects are qualitatively similar to those induced in the same species by D-2 receptor blockade with haloperidol [27] and also by oral treatment with skin mucus of *X. laevis* [4], which suggests a correlation between the behavioral

disorders and dopamine depletion. The behaviors noted in the present study are also qualitatively similar to those recorded in MPTP-treated lizards (*Anolis*), which also displayed rigidity and head and neck dyskinesias [12]. In contrast to the effects observed in *E.o. obsoleta*, the MPTP-treated *N.f. fasciata* demonstrated neither impairment of righting ability, nor the presence of corkscrewing behaviors, dorsoflexion or muscle dystonia.

The biochemical and behavioral effects of MPTP are known to vary between mammalian species with the effects being more pronounced and longer-lasting in primates than rodents, the latter requiring repeated high doses to achieve a reduction of dopamine and motor control [25]. Further, research has suggested that depletion of central dopamine by MPTP treatment is not necessarily linked to the permanent destruction of striatal dopaminergic neurons [17]. Rather, the reduction of striatal dopamine may be due instead to an acute pharmacological effect of MPTP, which may explain the relatively fast recovery of MPTP-treated rodents in other studies. The partial recovery of righting speed between sessions A and B noted for *E.o. obsoleta* in this study suggests an acute pharmacological effect of MPTP on dopamine levels and not a permanent neurotoxic effect on dopaminergic neurons. This is comparable to the effects seen in rodents.

The analysis of brain monoamine levels indicates that MPTP induced comparable deficits of dopamine in *E.o. obsoleta* and *N.f. fasciata* by the end of the drug treatment period. The depletion of dopamine in the fore and midbrain regions was of particular interest since the nigrostriatal component is located in those regions. Interestingly, the MPTP treatment affected brain dopamine levels similarly in *E.o. obsoleta* and *N.f. fasciata*, causing a substantial reduction ($\geq$75%) in both fore and midbrains. Given the similarities between the basal ganglia in reptiles and mammals, it is not surprising that the reduction of dopamine by MPTP affected movement control in *E.o. obsoleta*. However, it is quite intriguing that no behavioral deficits were observed for *N.f. fasciata*, which also displayed marked reductions of dopamine of the same magnitude as those seen in *E.o. obsoleta*. These data, along with the results of the previously mentioned pharmacological studies involving snakes, suggest that the central dopaminergic pathways of these two species of snakes may be differentially involved in the regulation of movement.

The most obvious species-specific difference regarding the biochemical effects of MPTP in this study was the more pronounced reduction of serotonin in *E.o. obsoleta* than in *N.f. fasciata*. Reduction of serotonin by MPTP treatment has been previously demonstrated in mammals [13,18,20]. Further research is necessary to explore a possible correlation between the behavioral effects in *E.o. obsoleta* and reduction of serotonin and to address the differential sensitivities of serotonergic systems to MPTP in these species.

The contrasting behavioral and biochemical effects of MPTP observed in these two species may be related to the evolution of dietary preferences in each genus. The diet of *Nerodia* includes amphibians, while *Elaphe* preys primarily on small mammals and birds, but not amphibians. Perhaps *Nerodia* has a natural ability to detoxify or tolerate amphibian toxins—a capacity that may provide a higher tolerance to the modulation of dopaminergic and serotonergic systems by exogenous agents.

In summary, this study demonstrates that the behavioral and biochemical effects of MPTP in *E.o. obsoleta* are, indeed, comparable to those reported for other vertebrate classes. Most intriguing are the contrasting behavioral sensitivities of *E.o. obsoleta* and *N.f. fasciata* to MPTP. MPTP induced comparable deficits of dopamine in both snake species, but only affected the behavior of *E.o. obsoleta*. The contrasting effects of dopaminergic modulation shown in this study are consistent with the results of previous pharmacological studies involving these species. This reptilian model provides an interesting opportunity for assessing the central monoaminergic pathways involved in motor control in vertebrates.

Acknowledgments

This work was supported in part by a North Carolina Agricultural Research Service Grant to G. Barthalmus. The authors thank Dr. Marcia Gumpertz, Department of Statistics, North Carolina State University, for statistical advice and Liz Boykin and Lisa Bishop, US EPA, for technical advice. I thank Drs. Harold Heatwole, John Roberts and Babetta Breuhaus, North Carolina State University, and Drs. Deborah O'Dell and Katherine Loesser-Casey, Mary Washington College, for reviewing this manuscript.

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