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Neuronal and Astroglial Responses to the Serotonin and Norepinephrine Neurotoxin: 1-Methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine

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ABSTRACT

1-Methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine (2′-NH_2-MPTP) causes long-term loss of forebrain serotonin (5-HT) and norepinephrine (NE) and consequently, is unlike 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and its other 2′-analogs that primarily deplete striatal dopamine (DA). In the present investigation into the acute effects of 2′-NH_2-MPTP in mice, profound decreases in cortical and hippocampal 5-HT and NE to 10 to 40% of control were observed as early as 30 min post-treatment and lasted throughout the ensuing 21 days. Striatal DA was decreased to 60 to 80% of control during the first 48 h but returned to normal by 72 h. Reactive gliosis, which occurs in response to neurodegeneration was not evident by immunocytochemistry but was detected by enzyme-linked immunosorbent assay, where glial fibrillary acidic protein (GFAP) was increased to 130% of control in cortex, hippocampus, and brain stem 48 to 72 h post-treatment. To explore the possibility that 5-HT modulates the astrocytic response to injury, 2′-NH_2-MPTP was used to damage 5-HT axons 2 weeks before administration of the potent DA neurotoxin 1-methyl-4-(2′-methylphenyl)-1,2,3,6-tetrahydropyridine (2′-CH_3-MPTP). Despite a 90% decrement in striatal DA in 2′-NH_2-MPTP/2′-CH_3-MPTP-treated mice, increases in GFAP were attenuated compared to mice treated with 2′-CH_3-MPTP alone. Thus, 2′-NH_2-MPTP causes severe and immediate decrements in 5-HT and NE in frontal cortex and hippocampus, yet induces a modest GFAP response compared with other MPTP analogs that have their primary effect on DA. These results demonstrate the importance of obtaining quantitative assessments of GFAP to detect astroglial responses associated with selective damage to neurotransmitter systems with low-density innervation and suggest that serotonin may facilitate the astrocytic response to striatal injury.

The irreversible Parkinsonian-like syndrome that rapidly developed in a group of Northern California substance abusers in the early 1980s fueled intense investigation of the causative agent 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as a model of dopaminergic neurodegeneration. MPTP has since been shown to damage DA neurons of the nigrostriatal pathway in humans (Speciale, 2002) and experimental animals (Heikkila et al., 1984a). As such, MPTP has proven to be an important tool for modeling various aspects of DA degeneration occurring in idiopathic Parkinson's disease (Speciale, 2002).

As part of the mechanistic evaluation of MPTP, many analogs were synthesized to elucidate structure-activity relationships (Youngster et al., 1989a; Maret et al., 1990). These studies focused primarily on the ability of MPTP-related compounds to decrease DA in striatum. In fact, a number of analogs were shown to be at least as potent as MPTP in their ability to deplete DA and many of these possess a substituent at the 2′-position (Youngster et al., 1989b). However, a single 2′-substituted analog, 1-methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine (2′-NH_2-MPTP),

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ABBREVIATIONS: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; DA, dopamine; 2′-NH_2-MPTP, 1-methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine; MAO, monoamine oxidase; 5-HT, serotonin; NE, norepinephrine; GFAP, glial fibrillary acidic protein; ELISA, enzyme-linked immunosorbent assay; 2′-CH_3-MPTP, 1-methyl-4-(2′-methylphenyl)-1,2,3,6-tetrahydropyridine; HPLC-ECD, high-performance liquid chromatography-electrochemical detection; TH, tyrosine hydroxylase; 5-HIAA, 5-hydroxyindoleacetic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; PBS, phosphate-buffered saline.

was discovered to deplete forebrain serotonin and norepinephrine in mice (Andrews and Murphy, 1993b) and rats (Unger et al., 2002) while having no long-term effects on striatal DA.

Although the mechanism of 2′-NH_2-MPTP neurotoxicity has not been fully established, those steps that have been investigated have been found to be similar, but not identical to those of MPTP. MPTP is oxidized by monoamine oxidase (MAO), and inhibitors specifically of MAO type-B prevent MPTP-induced DA neurotoxicity (Heikkila et al., 1984b). On the other hand, inhibition of MAO type-A attenuates decreases in 5-HT and NE due to 2′-NH_2-MPTP (Andrews and Murphy, 1993a). Furthermore, the MAO-derived metabolite of MPTP, 1-methyl-4-phenylpyridinium, requires uptake by the dopamine transporter to gain access to dopaminergic neurons. Evidence for this comes from studies using selective dopamine uptake inhibitors (Javitch et al., 1985; Melamed et al., 1985), as well as more recent data showing that MPTP toxicity is prevented in mice genetically engineered to lack the dopamine transporter (Gainetdinov et al., 1997). Similarly, 2′-NH_2-MPTP depletes 5-HT and NE by a mechanism involving the serotonin and norepinephrine transporters and administration of serotonergic or noradrenergic uptake inhibitors before 2′-NH_2-MPTP provides protection from depletions in 5-HT or NE, respectively (Andrews and Murphy, 1993a). Finally, MPTP is thought to cause neurotoxicity by inhibiting mitochondrial complex I, which depletes ATP and subsequently produces toxic oxygen radical species, including superoxide and hydroxyl radicals (Ali et al., 1994; Chiueh et al., 1994). Mice with genetically engineered increases in Cu-Zn superoxide dismutase are protected from either MPTP or 2′-NH_2-MPTP toxicity, implicating oxyradicals in the mechanism of action of both compounds (Przedborski et al., 1992; Andrews et al., 1996).

MPTP is considered a powerful neurotoxin not only because it produces long-lasting decrements in DA neurotransmitter levels (Sedelis et al., 2000) but also because its administration results in neurodegeneration as evidenced by astrogliosis and argyrophilia. Astrocyte hypertrophy seems to be a universal response to a variety of nervous system insults (for review, see Little and O'Callaghan, 2002) and changes in GFAP, the major astrocyte intermediate filament protein, can be used to assess reactive gliosis. Studies with MPTP show a robust time-dependent increase in striatal GFAP that peaks at 48 h post-treatment (Reinhard et al., 1988; O'Callaghan et al., 1990). In addition, silver impregnation (argyrophilia) of nigrostriatal DA cell bodies, dendrites, and axons has been observed in response to MPTP (Jackson-Lewis et al., 1995; Przedborski et al., 1996). Considering the overall similarities between the mechanisms of MPTP and 2′-NH_2-MPTP, it is probable that 2′-NH_2-MPTP causes degenerative damage to serotonergic and noradrenergic neurons and that this underlies the long-term decrements in 5-HT and NE neurotransmitter levels (Andrews and Murphy, 1993a).

To test this hypothesis, the present study first determined the extent of monoamine neurotransmitter depletion during the period immediately after administration of 2′-NH_2-MPTP. The acute time course of decreases in 5-HT, NE, and DA was then correlated with the effects of 2′-NH_2-MPTP on GFAP determined by enzyme-linked immunosorbent assay (ELISA). Maximal changes in neurotransmitter levels and GFAP were present 48 to 72 h after 2′-NH_2-MPTP; therefore, this time point was selected for further investigation of astrogliosis by immunocytochemistry and argyrophilia by silver staining. The present investigation revealed that 2′-NH_2-MPTP causes a rapid and profound depletion in forebrain serotonin and norepinephrine accompanied by an increase in GFAP that only is detectable by ELISA. The latter may be due to the comparatively sparse forebrain innervation associated with the 5-HT and NE systems.

A dual neurotoxin paradigm then was used to explore the alternate hypothesis that 5-HT may modulate the astrocyte response to neuronal damage and loss of 5-HT after 2′-NH_2-MPTP may interfere with the induction of astrogliosis. In this case, 2′-NH_2-MPTP was administered 2 weeks before 1-methyl-4-(2′-methylphenyl)-1,2,3,6-tetrahydropyridine (2′-CH_3-MPTP), a potent DA neurotoxin. Despite significant decreases in striatal DA in 2′-NH_2-MPTP/2′-CH_3-MPTP-treated mice, increases in GFAP in striatum were attenuated compared with mice given saline before 2′-CH_3-MPTP. These results suggest that serotonin may at least partly influence astrogliosis in specific brain regions.

Materials and Methods

Drugs and Chemicals. 2′-NH_2-MPTP was synthesized both at the National Institutes of Mental Health (Bethesda, MD) and The Pennsylvania State University (University Park, PA) and is currently available from Sigma-Aldrich (A7969; St. Louis, MO). The identity of 2′-NH_2-MPTP was verified by ^{1}H NMR and mass spectrometry. The purity was ~99% by gas chromatography. 2′-NH_2-MPTP was stored desiccated at 4°C and checked periodically for stability by HPLC. Antibodies and detection reagents for the GFAP and tyrosine hydroxylase (TH) ELISAs were rabbit anti-GFAP (DAKO, Carpinteria, CA), mouse anti-GFAP (Oncogene Research Products, Boston, MA), mouse anti-TH and rabbit anti-TH (Calbiochem-Novabiochem, San Diego, CA), alkaline phosphatase-conjugated rabbit anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA), *p*-nitrophenol (Bio-Rad, Richmond, CA), peroxidase-conjugated donkey anti-rabbit IgG (Amersham Biosciences Inc., Piscataway, NJ) and Quantablu Substrate (Pierce Endogen, Rockford, IL). All other reagents have been described previously (O'Callaghan, 1999, 2002). Somnifer (sodium pentobarbital, 10 mg/ml) was purchased from Richmond Veterinary Supply Company (Richmond, VA). All other drugs and chemicals were obtained from Sigma-Aldrich or a comparable source and were of analytical grade or higher.

Animal Treatments. Swiss-Webster male mice from Taconic Farms (Germantown, NY), weighing 25 to 35 g, or CD-1 male mice from Charles River Laboratories, Inc. (Wilmington, MA), weighing 30 to 40 g, were housed in facilities approved by the American Association for Accreditation of Laboratory Animal Care. Experimental protocols adhered to National Institutes of Health guidelines and were approved by the National Institute of Mental Health or The Pennsylvania State University Institutional Animal Care and Use Committees. 2′-NH_2-MPTP was administered in four injections of 20 mg/kg at 2-h intervals. 2′-CH_3-MPTP was administered in two injections of 20 mg/kg at a 6-h interval. Doses were calculated as the free base and given in a volume of 0.1 ml. Control animals received similarly timed injections of saline. These dosing regimens were selected because they have been shown to cause long-term depletions in cortical and hippocampal 5-HT and NE or striatal DA (Andrews and Murphy, 1993a,b) and so that comparisons could be made between the effects of 2′-NH_2-MPTP and those of other neurotoxins that have been administered in a similar multidose regimen (O'Callaghan and Miller, 1994).

To determine the acute time course of effects of 2′-NH_2-MPTP on regional brain neurochemistry and GFAP levels, seven cohorts of

mice (n = 12) were treated with 2′-NH_2-MPTP (n = 6) or saline (n = 6) and a single cohort was sacrificed at each of the following time points: 30 min, 2, 24, 48, or 72 h, or 7 or 21 days post-treatment. The mice were killed by cervical dislocation, and their brains were rapidly removed and dissected over ice to obtain frontal cortex, hippocampus, striatum, brain stem, and hypothalamus. Striatum and hippocampus were dissected bilaterally, so that one sample could be analyzed for monoamine neurotransmitters and the other for GFAP. Frontal cortex and brain stem were split on the midline to produce two samples. Hypothalamus was not divided and was analyzed solely for neurochemistry. All samples were stored at −70°C pending analysis.

Mice treated for GFAP immunocytochemistry were randomly assigned to one of three treatment groups (n = 6/group): saline, 2′-NH_2-MPTP or 2′-CH_3-MPTP. Mice were sacrificed by cervical dislocation 48 to 60 h after the final injection. This time point was selected based on the results of the GFAP ELISA experiment, which revealed that maximal increases in GFAP occurred 48 to 72 h after treatment. Brains were rapidly removed and stored at −70°C pending analysis.

Separate groups of mice were treated for silver staining. One group (n = 8) received four injections of 2′-NH_2-MPTP and a second group (n = 8) received similarly timed saline injections. Both groups were killed 48 to 60 h later. After sodium pentobarbital (100 mg/kg i.p.), mice were perfused via the left ventricle with 100 ml of 4% paraformaldehyde, 1.4% sodium cacodylate, and 4% sucrose. Brains were stored at 4°C in 10% sucrose pending analysis.

Finally, the experiment testing the effects of serotonin depletion on astrogliosis included four groups of mice: saline/saline (n = 8), 2′-NH_2-MPTP/saline (n = 9), saline/2′-CH_3-MPTP (n = 10), and 2′-NH_2-MPTP/2′-CH_3-MPTP (n = 11). 2′-CH_3-MPTP or saline was given 14 days after 2′-NH_2-MPTP or saline (Fig. 1). Desipramine (10 mg/kg i.p.) was administered to all mice 30 min before 2′-NH_2-MPTP to prevent depletions in NE (Andrews and Murphy, 1993a). Mice were sacrificed by cervical dislocation 48 to 60 h after the last injection with either 2′-CH_3-MPTP or saline and the brains were rapidly removed and dissected over ice to obtain frontal cortex, hippocampus, striatum, brain stem, and hypothalamus. Striatum and hippocampus were dissected bilaterally so that one sample could be analyzed for monoamine neurotransmitters and the other sample for GFAP and TH ELISA.

Neurotransmitter Analysis. Brain samples were analyzed for monoamine neurotransmitters and their metabolites by high-performance liquid chromatography using electrochemical detection (HPLC-ECD) at +0.3 V using established methods (Andrews and Murphy, 1993b). Briefly, samples were sonicated in 200 to 250 μl of 0.1 M $HClO_4$ and centrifuged at 7,200g (12,000 rpm) for 10 min. Supernatants (50 μl) were injected onto a 10 cm × 4.6 mm Spherisorb 3 μm octadecylsulfate reversed-phase chromatography column (Thomson Instruments, Springfield, VA) in a mobile phase containing 0.14 M monochloroacetic acid, 5 to 9% acetonitrile, 0.9 to 1.8 mM 1-octanesulfonic acid, 0.01% triethylamine, and 0.0025% EDTA. 5-Hydroxy-N^{ω}-methyltryptamine oxalate was used as the internal standard, and sample amounts were calculated by comparing the relative peak areas of sample peaks to external standards. 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), NE, DA, and the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured in a single chromatogram. Protein was determined by the Lowry method and concentrations are expressed as nanograms per milligram of protein (mean ± S.E.M.).

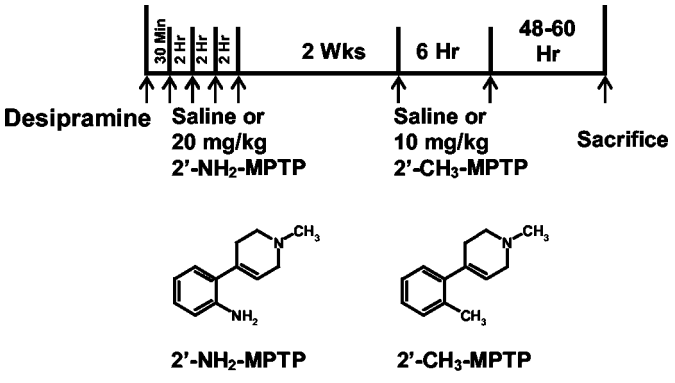

Fig. 1. Timeline depicting the administration schedule for the combined 2′-NH_2-MPTP/2′-CH_3-MPTP dose regimen (chemical structures shown below). Desipramine (to prevent decreases in NE) was administered 30 min before the first of four 2′-NH_2-MPTP or saline injections given at 2-h intervals. After a period of 2 weeks, two injections of 2′-CH_3-MPTP or saline were administered at a 6-h interval. Forty-eight to 60 h after the last injection of 2′-CH_3-MPTP or saline, mice were sacrificed.

GFAP and TH ELISA. GFAP was analyzed by a detergent-based sandwich ELISA, a detailed protocol that has been described previously (O'Callaghan, 1991) and updated (O'Callaghan, 2002). Briefly, homogenates of the dissected brain regions were prepared in hot (>90°C) 1% SDS. Total protein concentration of the SDS homogenates was determined by the bicinchoninic acid method (Smith et al., 1985) using bovine serum albumin as the standard. Aliquots of the SDS homogenates and standards were diluted in phosphate-buffered saline (PBS; pH 7.4) containing 0.5% Triton X-100 and added to microtiter plate wells that had been coated with rabbit antibody to GFAP and blocked with 5% nonfat dry milk in PBS. After washes, a mouse monoclonal antibody to GFAP was added to "sandwich" the GFAP between the two antibodies. An alkaline phosphatase-conjugated antibody directed against mouse IgG then was added, and a colored reaction product was generated by subsequent addition of the enzyme substrate. Quantification was achieved by spectrophotometry of the microplate wells at 405 nm in a microplate reader running Soft Max Pro Plus software (Spectra Max Plus; Molecular Devices, Menlo Park, CA). The amount of GFAP in the samples was expressed as micrograms of GFAP per milligram of total protein.

For measurements of TH, the apoenzyme protein also was assayed by detergent-based ELISA. Essentially, the protocol was the same as for GFAP ELISA except the antibody pairs were mouse monoclonal anti-TH for "capture" of TH onto the wells of the plate and rabbit polyclonal anti-TH to sandwich TH. Subsequent detection was achieved using a peroxidase-labeled antibody directed against rabbit IgG and a fluorogenic substrate. Quantification was achieved by fluorometry of the microplate wells with excitation/emission maxima of 325/420 nm. As for GFAP, TH data were expressed as micrograms per milligram of total protein.

GFAP Immunocytochemical Analysis. Brains were sectioned sagittally (30 μm) with a sliding microtome and stored at −20°C in an ethylene glycol solution containing sucrose and sodium azide. Free-floating sections were incubated overnight (at 4°C with gentle agitation) in antiserum containing monoclonal antibodies against GFAP (Chemicon International, Temecula, CA) diluted 1:400 in PBS. Immunoreactive astrocytes were visualized with the avidin-biotin peroxidase (avidin-biotinylated enzyme complex) method (Vector Laboratories, Burlingame, CA) and stained with 3,3′-diaminobenzidine (Rockland, Gilbertsville, PA) enhanced with nickel sulfate. Sections were mounted on gelatin-coated slides, coverslipped with DPX (Sigma-Aldrich) and examined with bright-field microscopy. Before analysis, the slides were coded to mask the treatment of each animal from the scorer. Analysis of reactive gliosis was performed by examining each brain region and assigning an overall rating (1–5, with 5 representing the greatest amount of gliosis) based on both the number of astrocytes and the degree to which astrocytes were swollen and elongated. Brain regions included CA1, CA3, and dentate gyrus subregions of hippocampus, striatum, cortex, substantia nigra, and hypothalamus.

Silver Degeneration Staining. Brains were freeze-sectioned coronally (30 μm), and adjacent sections were stained with either a cupric silver stain (Carlsen and De Olmos, 1981) to visualize argyrophilic evidence of neuronal degeneration or thionine to identify cell

bodies. The brains of all 16 animals were embedded in a single gelatin matrix block and processed together on 3 × 4-inch slides. Histological analysis was performed at Neuroscience Associates (Knoxville, TN) where the slides were rated by an observer blind to the treatment of individual animals. Additionally, the slides were examined by one of the authors (J. P.O.) who was also blind to the treatment code.

Data Analysis. Data were analyzed by one-way or two-way analysis of variance followed by Duncan's multiple range test using the Statistical Analysis System (SAS Institute, Cary, NC). Probabilities <0.05 were considered statistically significant.

Results

2′-NH_2-MPTP Causes Immediate and Profound Reductions in 5-HT and NE Predominantly in Frontal Cortex and Hippocampus. Seven cohorts of mice were studied, each of which contained equal numbers of animals receiving 2′-NH_2-MPTP or saline. Cohorts were killed 30 min, 2 h, 24 h, 48 h, 72 h, 7 days, or 21 days after treatment to assess the short-term effects of 2′-NH_2-MPTP on 5-HT, NE, DA, and their metabolites. Statistically significant decreases in 5-HT to 10 to 40% of control were observed in frontal cortex and hippocampus at all time points ($p < 0.001$) (Fig. 2, A and B). The lowest levels of serotonin in frontal cortex and hippocampus occurred within the first 2 h after 2′-NH_2-MPTP and 5-HT remained decreased to less than 40% of control as late as 21 days post-treatment. NE was depleted to such a great extent that the levels were not detectable in frontal cortex at all time points and at 30 min and 2 h in hippocampus. Thereafter, NE levels were less than 20% of control in hippocampus ($p < 0.001$) (Fig. 2).

2′-NH_2-MPTP elicited a different pattern of effects on the 5-HT metabolite 5-HIAA. Decreases in 5-HIAA only began to emerge within the first 2 h post-treatment in frontal cortex and hippocampus ($p < 0.05$ in hippocampus) (Fig. 2B) and did not reach statistical significance in frontal cortex until 24 h after treatment. ($p < 0.001$) (Fig. 2A). 5-HIAA levels remained decreased to 30 to 50% of control for the remaining times examined.

The acute effects of 2′-NH_2-MPTP on 5-HT, 5-HIAA, and NE also were determined in other brain regions (Fig. 3). In striatum, 5-HT was decreased to 20% of control ($p < 0.001$) 30 min after the last injection of 2′-NH_2-MPTP but by 72 h postdose, the levels had risen to 60% of control ($p < 0.001$). Unlike frontal cortex and hippocampus, striatal 5-HT levels recovered to 90% of control by 21 days post-treatment ($p < 0.001$) (Fig. 3A). Striatal 5-HIAA rose to 130% of control at 30 min post-treatment, but otherwise exhibited a time-response pattern similar to that observed in frontal cortex and hippocampus by dropping steadily over the course of the first 72 h to ~60% of control ($p < 0.01$) where it stayed for the remainder of the times examined (Fig. 3A).

The same general trend of immediate decreases in 5-HT and NE with a subsequent rise in these levels over the initial 72-h period was similarly noted in brain stem and hypothalamus. In the latter brain regions, 5-HT levels were still decreased to 50% of control at 7 days post-treatment ($p < 0.001$) (Fig. 3, B and C); however, 5-HT levels were not significantly different from control 21 days after 2′-NH_2-MPTP. NE was significantly decreased compared with control at all time points in brain stem and hypothalamus except at 21 days post-treatment when NE levels had completely

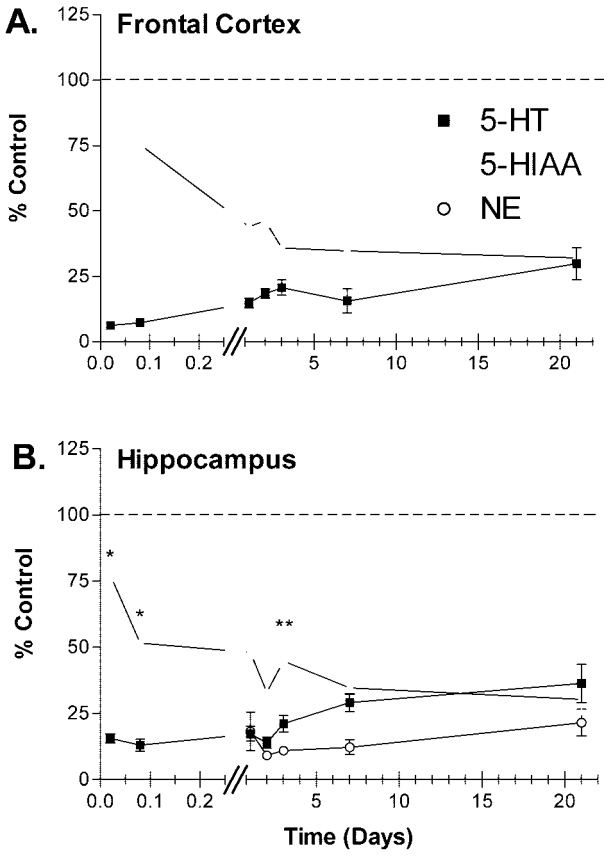

Fig. 2. Acute effects of 2′-NH_2-MPTP on 5-HT, 5-HIAA, and NE levels in frontal cortex and hippocampus. Seven cohorts of mice ($n = 12$) were injected i.p. with either 2′-NH_2-MPTP ($n = 6$) or saline ($n = 6$), and one cohort was sacrificed at each of the following time points: 30 min, 2 h, 24 h, 48 h, 72 h, 7 days, or 21 days post-treatment. 5-HT, 5-HIAA, and NE levels were measured by HPLC-ECD. The results shown are percentages of the respective control group means ± S.E.M. The values for frontal cortex and hippocampus, respectively, at 21 days were 5-HT, 30 ± 6 and 36 ± 7; 5-HIAA, 32 ± 5 and 30 ± 6; and NE, not detectable and 21 ± 5 ng/mg protein. These values are representative of control group means ± S.E.M. for the remaining time points. Probabilities are identified as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for differences from control groups. A, ***, 5-HT and 5-HIAA versus control at all time points, except 5-HIAA was not detectable at 30 min due to technical problems and not significantly different from control at 2 h. B, ***, 5-HT, 5-HIAA, and NE versus control at all time points, except where indicated. NE was not detectable at 30 min and 2 h.

recovered in hypothalamus (Fig. 3, B and C). 5-HIAA was not significantly different from control in brain stem except at 24 h post-treatment ($p < 0.05$). In hypothalamus, 5-HIAA was not detectable at several time points due to an interfering peak in the chromatograms but where measurable, was significantly increased at 2 h post-treatment ($p < 0.05$) and significantly decreased at 21 days post-treatment ($p < 0.01$).

The effects of 2′-NH_2-MPTP on dopaminergic neurochemistry in striatum are pictured in Fig. 4. DA levels were significantly, but transiently decreased to 60 to 70% of control 30 min and 2 h after the last injection of 2′-NH_2-MPTP ($p < 0.001$) and to 90% of control at 48 h post-injection ($p < 0.01$) (Fig. 4). However, striatal DA was unchanged compared with control at 24 h, 72 h, 7 days, and 21 days post-treatment. DOPAC was not significantly different at any time after 2′-NH_2-MPTP with the exception of the 24-h time point where it was increased to 160% of control ($p < 0.01$). HVA was acutely increased after 2′-NH_2-MPTP to 220% of control

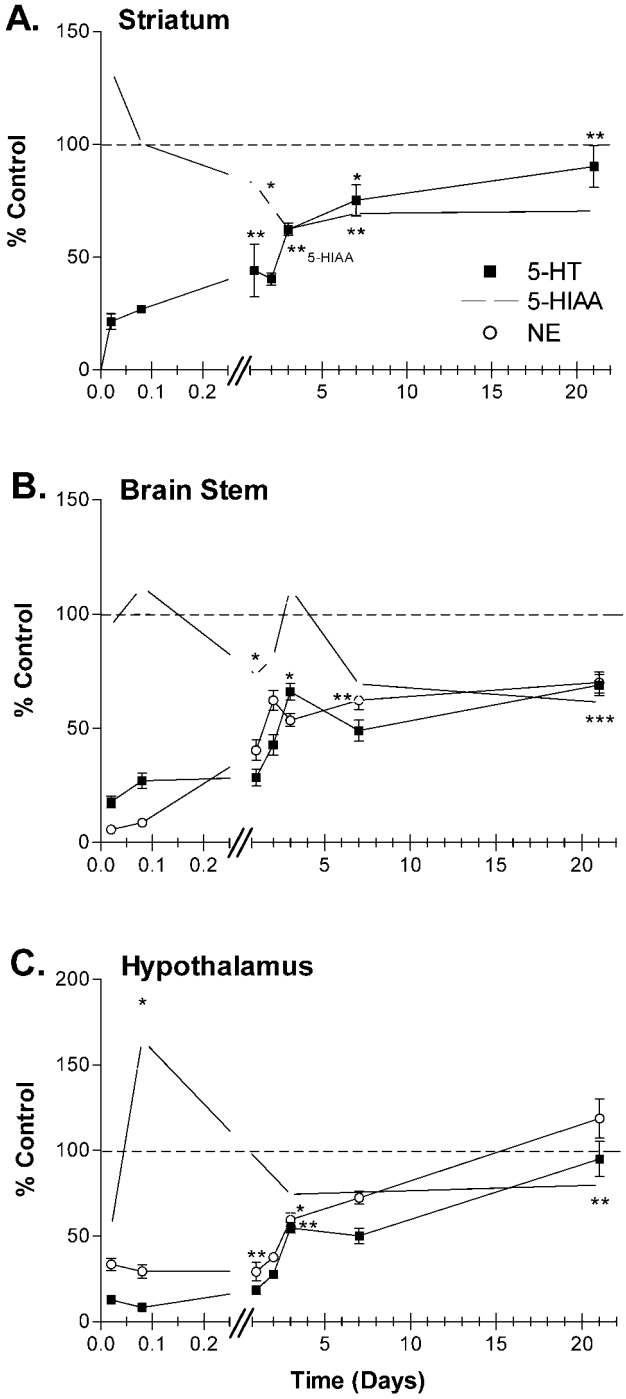

Fig. 3. Acute effects of 2′-NH_2-MPTP on 5-HT, 5-HIAA, and NE levels in striatum, brain stem, and hypothalamus. Treatment and analysis were the same as those described in Fig. 2. The results shown are percentages of the respective control group means ± S.E.M. The values for striatum, brain stem, and hypothalamus, respectively, at 21 days were 5-HT, 90 ± 9, 69 ± 5 and 95 ± 10; 5-HIAA, 70 ± 4, 61 ± 2 and 80 ± 4; and NE, not detectable, 70 ± 5 and 119 ± 11 ng/mg protein. These values are representative of control group means ± S.E.M. for the remaining time points. Probabilities are identified as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for differences from control groups. A, ***, 5-HT and 5-HIAA versus control, except where indicated. 5-HIAA was not significantly different from control at 30 min, 2 h, and 24 h. B, ***, 5-HT and NE versus control, except where indicated. 5-HT was not significantly different from control at 21 days. 5-HIAA is as marked. C, ***, 5-HT and NE versus control, except where indicated. 5-HT and NE were not significantly different from control at 21 days. 5-HIAA is as marked and was not detectable at 24 h, 48 h, and 7 days due to technical problems.

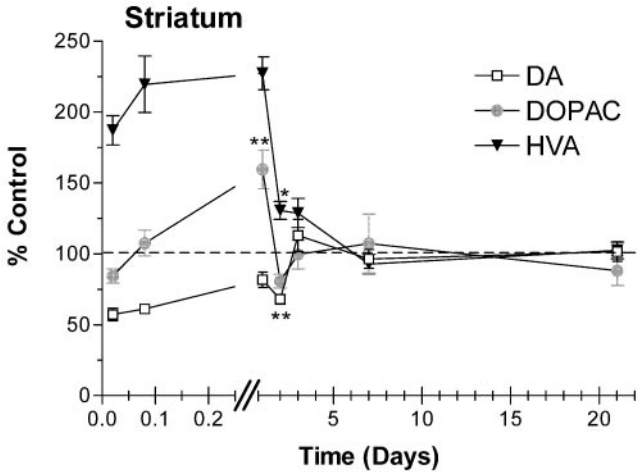

Fig. 4. Acute effects of 2′-NH_2-MPTP on DA, DOPAC, and HVA levels in striatum. Treatment and analysis were the same as those described in Fig. 2. The results shown are percentages of respective control group means ± S.E.M. The values for striatum at 21 days were DA, 102 ± 7; DOPAC, 88 ± 11; and HVA, 102 ± 6 ng/mg protein. These values are representative of the control group means ± S.E.M. for the remaining time points. Probabilities are identified as *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ for differences from control groups. ***, DA and HVA versus control, except where indicated. DA and HVA were not significantly different from control at 24 h (DA only), 72 h, 7 and 21 days. DOPAC is as marked.

during the first 24 h ($p < 0.001$) after which it fell to 130% of control at 48 h post-treatment ($p < 0.05$). By 7 days post-treatment, HVA levels in 2′-NH_2-MPTP-treated animals were not significantly different from those in saline-treated mice.

Overall, 2′-NH_2-MPTP depleted 5-HT and NE to a much greater extent in frontal cortex and hippocampus than in striatum, brain stem, and hypothalamus. 2-NH_2-MPTP also induced long-lasting 5-HT and NE depletions in frontal cortex and hippocampus, whereas 5-HT and NE levels recovered by 21 days in striatum, hypothalamus, and brain stem. Changes in 5-HIAA levels after 2′-NH_2-MPTP treatment followed a different pattern than that of 5-HT and NE. Unlike immediate decreases followed by gradual increases in 5-HT and NE over time, 5-HIAA levels fell more slowly. 2′-NH_2-MPTP also caused a transient decrease in striatal DA and a transient increase in striatal HVA.

2′-NH_2-MPTP Causes a Time-Dependent Increase in Astrogliosis. In frontal cortex, GFAP levels measured by ELISA were significantly increased to 130% of control at 48 h post-treatment ($p < 0.01$) and to 120% of control at 72 h after 2′-NH_2-MPTP ($p < 0.05$) (Fig. 5). In hippocampus and brain stem, GFAP levels were increased to 130% of control at 72 h post-treatment ($p < 0.001$ and 0.01, respectively), whereas in striatum, a nonsignificant increase on the order of 20% was seen 48 h after treatment (Fig. 5).

In contrast to assessment by quantitative ELISA, evidence for increased GFAP in response to 2′-NH_2-MPTP was not detectable by immunocytochemistry (Fig. 6). Reactive astrocytes were observed in hippocampal subregions in all groups of mice; however, there were no significant differences in relative reactivity scores between treatment groups in frontal cortex (Fig. 6, A–C) or hippocampus (Fig. 6, D–F). By contrast, mice administered 2′-CH_3-MPTP demonstrated a significant increase in GFAP immunoreactivity in striatum compared with mice treated with saline ($p < 0.001$) (Fig. 6,

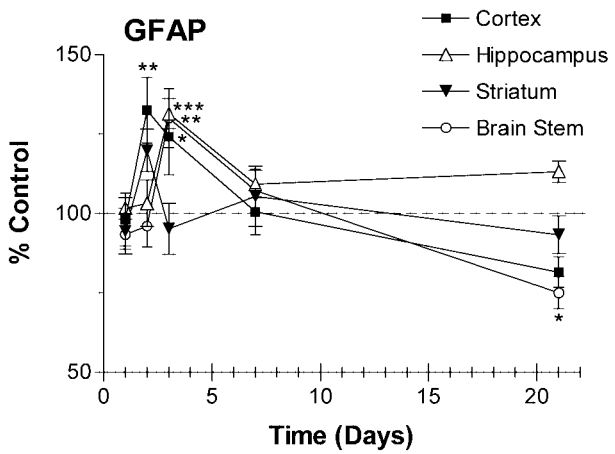

Fig. 5. Acute effects of 2′-NH_2-MPTP on GFAP levels in frontal cortex, hippocampus, striatum, brain stem, and hypothalamus. Five cohorts of mice (n = 12/cohort) were injected i.p. with either 2′-NH_2-MPTP or saline and sacrificed at each of the following time points: 24 h, 48 h, 72 h, 7 days, or 21 days post-treatment. GFAP levels were measured by ELISA. The results shown are percentages of the respective control group means ± S.E.M. The values for GFAP at 21 days were cortex, 0.26 ± 0.03; hippocampus, 0.80 ± 0.07; striatum, 0.29 ± 0.04; and brain stem, 1.4 ± 0.4 μg/mg protein. These values are representative of control group means ± S.E.M. for the remaining time points. Probabilities are identified as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$ for differences from control groups.

G–I). Differences in GFAP were not observed by immunocytochemistry in any of the other brain regions examined (Table 1). Overall, 2′-NH_2-MPTP induced significant but modest increases in GFAP in cortex, hippocampus and brain stem as detected by ELISA; however, visualization of astrogliosis via immunocytochemical methods only revealed an increase in reactive astrocytes in striatum in response to 2′-CH_3-MPTP.

2′-NH_2-MPTP Causes Modest Argyrophilia in Serotonin Cell Body Regions. In seven of eight 2′-NH_2-MPTP-treated mice, limited numbers of degenerating cell bodies were detected in the dorsal raphe as evidenced by silver degeneration staining (Fig. 7). In median raphe, axonal damage was detected in three of eight 2′-NH_2-MPTP-treated mice (data not shown). By contrast, no silver staining of nerve terminals was observed in hippocampus or various cortical regions of 2′-NH_2-MPTP-treated mice 48 to 60 h post-treatment.

Depletion of 5-HT by 2′-NH_2-MPTP Attenuates 2′-CH_3-MPTP-Induced Striatal Astrogliosis. 2′-NH_2-MPTP caused severe short-term depletions in 5-HT yet the GFAP response was modest compared with that induced by MPTP (Reinhard et al., 1988); therefore, we explored the possibility that under certain circumstances, 5-HT may be involved in the induction of the astrocytic response to neuronal damage. To investigate this, 5-HT was depleted via 2′-NH_2-MPTP and dopaminergic damage was induced in striatum by 2′-CH_3-MPTP. Desipramine was administered before 2′-NH_2-MPTP to prevent loss of NE (Fig. 1).

In mice given 2′-NH_2-MPTP 2 weeks before 2′-CH_3-MPTP, 5-HT levels were significantly decreased to 20% of control in frontal cortex and hippocampus ($p < 0.001$), 50% of control in striatum ($p < 0.001$) and 30% of control in brain stem ($p < 0.001$) 48 to 60 h after 2′-CH_3-MPTP treatment (data not shown, except striatum) (Fig. 8). Serotonin was depleted to the same extent in mice treated with 2′-NH_2-MPTP alone. Dopamine levels were severely depleted to 15% of control in striatum in mice administered 2 × 20 mg/kg 2′-CH_3-MPTP 2 weeks after 4 × 20 mg/kg 2′-NH_2-MPTP ($p < 0.001$, Fig. 8). Striatal DA levels were similarly depleted in mice treated with 2′-CH_3-MPTP alone.

Striatal tyrosine hydroxylase levels were determined by ELISA as a second measure of dopamine nerve terminal integrity. TH was significantly reduced to 30% of control 48 to 60 h post-treatment in mice administered 2′-NH_2-MPTP before 2′-CH_3-MPTP ($p < 0.001$), as well as in mice given saline before 2′-CH_3-MPTP ($p < 0.001$) (Fig. 8). In contrast, there was no change in striatal TH levels in mice treated with 2′-NH_2-MPTP alone.

Finally, in the group of mice administered 2′-NH_2-MPTP followed by saline 2 weeks later, no changes in GFAP levels were detected in striatum by ELISA. Consistent with the immunocytochemical data described above, mice treated with saline followed 2 weeks later by 2′-CH_3-MPTP showed an increase in GFAP levels to 180% of control in striatum ($p < 0.01$). However, in mice treated with both MPTP analogs, the 2′-CH_3-MPTP-induced increase in striatal GFAP was completely attenuated (Fig. 8).

Discussion

The present study demonstrates that administration of a multidose regimen of 2′-NH_2-MPTP to mice causes a massive and immediate loss of forebrain serotonin. Unlike the substituted amphetamine *p*-chloroamphetamine that elicits an acute, biphasic effect on serotonin levels (Steranka et al., 1977), 2′-NH_2-MPTP causes a persistent depletion of 5-HT in the acute period in brain regions where it has its greatest effects. Only in brain stem was a biphasic pattern of 5-HT depletion observed (Fig. 3B). This may be indicative of an acute effect of 2′-NH_2-MPTP to release 5-HT followed by a compensatory increase in serotonin synthesis in cell bodies in response to neuronal damage.

The largest depletions in 5-HT occurred in frontal cortex and hippocampus with more moderate losses detected in striatum, hypothalamus, and brain stem. These findings indicate that 2′-NH_2-MPTP induces its most severe effects in the terminal plexus, specifically in frontal cortex and hippocampus. These brain regions are innervated by projections originating from the dorsal and median raphe, respectively (Molliver et al., 1990), and both of these brainstem nuclei showed evidence of degenerative changes by silver staining. The present findings also suggest that the projection areas furthest away from their origin in the brain stem may be most vulnerable to 2′-NH_2-MPTP-induced neurotoxicity, and this may explain the lesser magnitude loss of 5-HT observed in hypothalamus and striatum. 2′-NH_2-MPTP was similarly neurotoxic to noradrenergic nerve terminals with the greatest depletions in NE occurring also in frontal cortex and hippocampus. Immunocytochemical studies using antibodies against tyrosine hydroxylase, tryptophan hydroxylase or 5-HT itself would be useful for further investigating the effects of 2′-NH_2-MPTP on cell bodies and specific terminal fields.

Previous studies have revealed that 2′-NH_2-MPTP causes reductions in 5-HT and NE as opposed to causing long-term striatal DA depletion (Andrews and Murphy, 1993a,b). The present study demonstrates that striatal dopamine levels are affected during the first 48 h after 2′-NH_2-MPTP adminis-

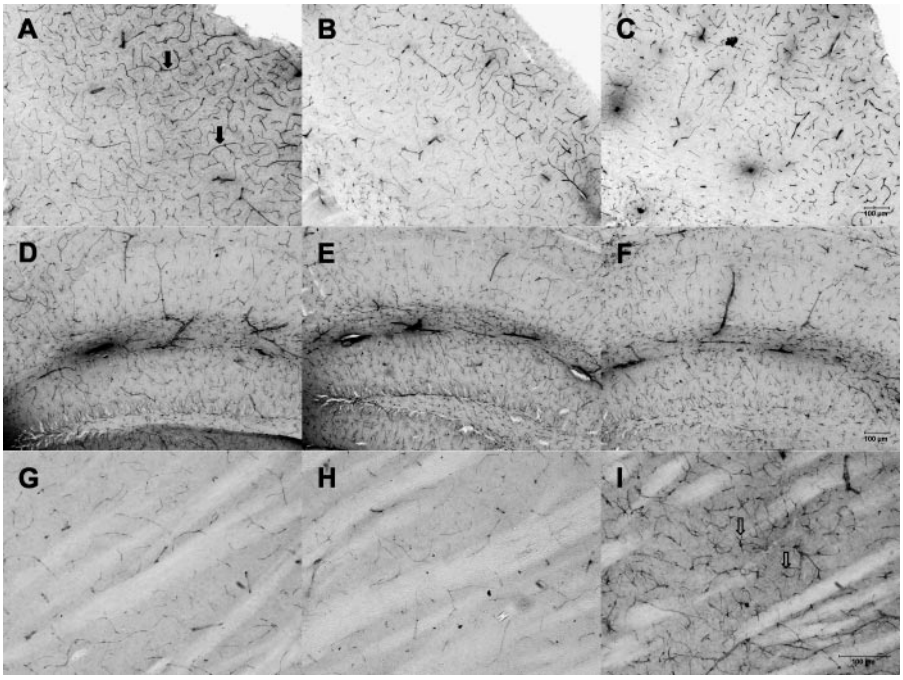

Fig. 6. GFAP Immunocytochemistry. Astrogliosis was detected with a monoclonal antibody against GFAP using the avidin-biotin peroxidase (avidin-biotinylated enzyme complex) visualization method and was photographed with bright-field microscopy. Panels depict representative sections showing GFAP-positive astrocytes in sagittal sections of frontal cortex (A–C), hippocampus (D–F), and striatum (G–I) 48–60 h after treatment with either saline (A, D, and G), 2′-NH_2-MPTP (B, E, and H) or 2′-CH_3-MPTP (C, F, and I) (n = 6/group). No significant differences were observed between groups in frontal cortex or hippocampus; however, mice treated with 2′-CH_3-MPTP showed a significant increase in striatal GFAP ($p < 0.001$) compared with the other treatment groups. Solid arrows (A) indicate blood vessels visualized during the staining process, and open arrows (I) indicate astrocytes.

TABLE 1

Reactive gliosis in 2′-NH_2-MPTP-treated mice

Three groups of mice (n = 6/group) were treated with saline, 2′-NH_2-MPTP, or 2′-CH_3-MPTP and sacrificed 48 to 60 h later. Sagittal sections (30 μm) processed with an antibody against GFAP were blindly rated for astrogliosis whereby examined brain regions in each sample were assigned a number 1 to 5 (1 representing no gliosis and 5 representing the highest amount of gliosis) based on both the number of astrocytes and on the degree to which the astrocytes were swollen and elongated. The results are expressed as the mean of each treatment group ± S.E.M.

Brain Region	Saline	2′-NH_2-MPTP	2′-CH_3-MPTP
Frontal cortex	1.0 ± 0	1.0 ± 0	1.0 ± 0
Parietal cortex	1.0 ± 0	1.0 ± 0	1.0 ± 0
CA1	2.8 ± 0.3	2.8 ± 0.2	3.2 ± 0.3
CA3	4.2 ± 0.5	4.2 ± 0.3	4.5 ± 0.2
Dentate gyrus	4.8 ± 0.3	4.8 ± 0.2	4.8 ± 0.2
Striatum	1.0 ± 0	1.0 ± 0	4.5 ± 0.3[a]
Substantia nigra	3.5 ± 0.4	3.7 ± 0.4	4.0 ± 0.3
Hypothalamus	1.7 ± 0.3	1.0 ± 0	1.7 ± 0.3

[a] $p < 0.001$ for differences from control.

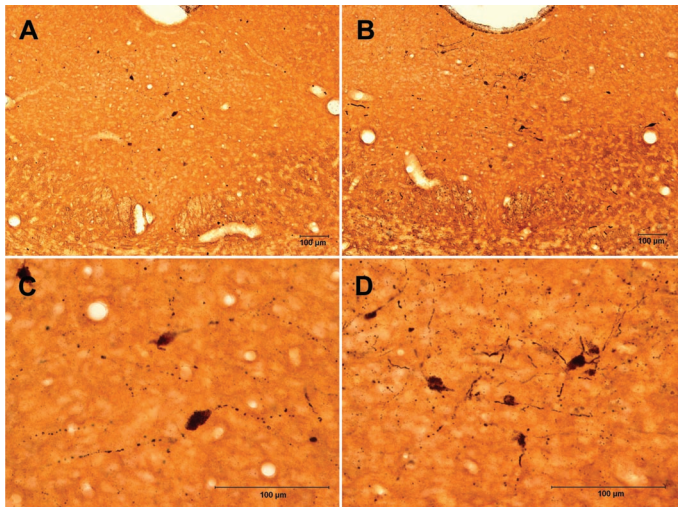

Fig. 7. Silver degeneration staining. Cupric silver staining was used to identify degenerating cell bodies in dorsal raphe. A and B, cell bodies of damaged serotonin neurons in dorsal raphe from two representative animals treated with 2′-NH_2-MPTP and sacrificed 48 to 60 h later (10×). C and D, higher magnification (40×) of same sections in A and B.

tration, but they return to control levels by 72 h post-treatment. Thus, although acute and transient depletions in dopamine occur, 2′-NH_2-MPTP seems to be selective in its lasting toxicity to the 5-HT and NE neurotransmitter systems.

As stated above, 2′-NH_2-MPTP may induce an immediate release of 5-HT, and this is evidenced further by the pattern of 5-HIAA levels observed in this study. A time lag in the reduction of 5-HIAA may be due to the metabolism of large amounts of 5-HT. Only after intracellular stores of 5-HT are significantly depleted would metabolism of 5-HT be reduced, resulting in a loss of 5-HIAA. A similar release and metabolism of DA also is suggested by the acute increases occurring in DOPAC and more prominently, in HVA.

Neurodegeneration is often evidenced by two common markers: reactive gliosis as demonstrated by increases in GFAP and silver staining indicative of degenerating neurons. 2′-NH_2-MPTP causes an astrocytic response in the brain regions most severely affected; however, GFAP levels rose by only 30%, a smaller change in comparison with dopamine neurotoxins such as MPTP (Reinhard et al., 1988; O'Callaghan et al., 1990) or methamphetamine (O'Callaghan and Miller, 1994). Furthermore, 2′-NH_2-MPTP-induced increases in GFAP were only detectable by ELISA. This is in contrast to the readily observable increase in reactive astrocytes in striatum after 2′-CH_3-MPTP evident by immunocytochemistry. GFAP immunocytochemistry is more subjective in its interpretation and most likely less capable of detecting low-level increases in GFAP such that differences in reactive gliosis between saline and 2′-NH_2-MPTP-treated mice were not detected using this technique. Thus, the induction of GFAP after 2′-NH_2-MPTP may be relatively modest, and it can only be detected by specific quantitative analysis.

Several possibilities exist to explain the magnitude of the astrocytic response to 2′-NH_2-MPTP. First, the density of

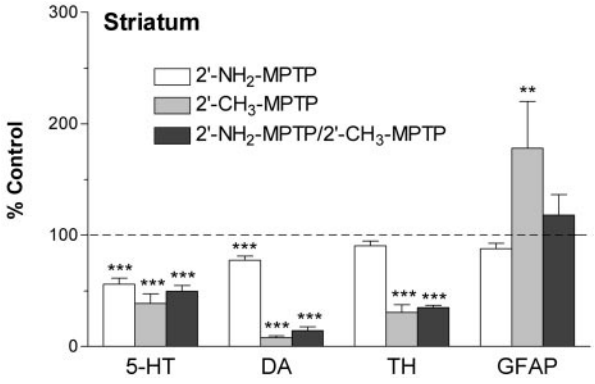

Fig. 8. Effects of 2′-NH_2-MPTP treatment 2 weeks before 2′-CH_3-MPTP on 5-HT, DA, TH, and GFAP in striatum. Mice were injected with either saline/saline (n = 8), 2′-NH_2-MPTP/saline (n = 9), saline/2′-CH_3-MPTP (n = 10), or 2′-NH_2-MPTP/2′-CH_3-MPTP (n = 11) and were sacrificed 48 to 60 h after the last 2′-CH_3-MPTP or saline injection. Although all mice receiving 2′NH_2-MPTP/saline survived, six of 10 mice administered saline/2′CH_3-MPTP died shortly after 2′CH_3-MPTP administration, whereas only one mouse administered 2′NH_2-MPTP/2′CH_3-MPTP died. 5-HT and DA levels were measured by HPLC-ECD, and TH and GFAP levels were measured by ELISA. The results shown are percentages of the respective control group means ± S.E.M. The values for the saline/saline group were 5-HT, 3.0 ± 0.1; DA, 88 ± 2 ng/mg protein and TH, 1.2 ± 0.1; GFAP, 0.15 ± 0.01 μg/mg protein. Probabilities are identified as **, $p <$ 0.01 and ***, $p < 0.001$ for differences from control groups.

serotonergic and noradrenergic innervation in forebrain is low compared with the number of dopamine nerve terminals in striatum. For example, 5-HT and NE neurotransmitter concentrations in hippocampus and cortex are only 10 and 5%, respectively, of DA levels in striatum (Andrews and Murphy, 1993a,b; Andrews et al., 1996). The magnitude of the GFAP response to 2′-NH_2-MPTP is most likely indicative of this difference in relative terminal density. Similarly, 5,7-dihydroxytryptamine, which damages 5-HT terminals and cell bodies, is reported to cause increases in GFAP to 130 to 150% of control (Frankfurt et al., 1991; O'Callaghan and Miller, 2002) and results in only modest argyrophilia in the serotonergic terminal field (O'Callaghan and Miller, 2002). Thus, serotonin neurotoxins in general would not be expected to cause large increases in GFAP or a high degree of silver degeneration staining.

Alternately, it is possible that functionally intact serotonin nerve terminals may modulate the astrocytic response to injury in specific brain regions. Astrocytes express 5-HT transporters and various 5-HT receptors (Azmitia et al., 1996; Hirst et al., 1998), and they may be receptive to serotonin signaling during degenerative conditions, particularly in a trophic capacity (Wu et al., 1999). Administration of the serotonin synthesis inhibitor p-chlorophenylalanine results in a loss of the GFAP response to spinal cord trauma (Sharma et al., 1993; Hadley et al., 1999). We used 2′-NH_2-MPTP to deplete 5-HT before administration of 2′-CH_3-MPTP to further investigate the idea that intact serotonin neurons may facilitate reactive gliosis. Mice treated with 2′-NH_2-MPTP 2 weeks before receiving 2′-CH_3-MPTP demonstrated an attenuated GFAP response in striatum compared with mice given saline before 2′-CH_3-MPTP. These results suggest that the increase in striatal GFAP that normally occurs after 2′-CH_3-MPTP is reduced under conditions of 5-HT depletion. Notably, serotonergic modulation of reactive gliosis may not be important in all brain regions. For instance, neurons devoid of serotonin or serotonergic innervation such as cerebellar Purkinje cells show robust astroglial responses (O'Callaghan and Miller, 1985).

In the present study, cell body degeneration was observed in dorsal raphe and axonal degeneration was present in median raphe as indicated by silver staining in these regions. However, we were not able to detect evidence of terminal degeneration in cortex or hippocampus by the same method. Silver staining carried out at a variety of time points or other histological methods, including electron microscopy, may have to be used to detect serotonergic and noradrenergic nerve terminal degeneration in response to 2′-NH_2-MPTP administration.

Selective neurotoxins are valuable tools for studying the biologic role of specific neurotransmitter systems in central nervous system function and dysfunction. When the effects of neurotoxicants reproduce aspects of human neurodegenerative disorders, they can be used in laboratory animals to produce precisely controlled damage to elucidate molecular mechanisms underlying disease processes and to evaluate potential therapeutic strategies. The results of the present investigation combined with those of previous studies collectively demonstrate that 2′-NH_2-MPTP is a serotonergic and noradrenergic neurotoxin that causes significant short- and long-term depletions in 5-HT and NE in frontal cortex and hippocampus. The current study further shows that 2′-NH_2-MPTP induces astrogliosis, albeit at a modest level, and argyrophilia indicative of degenerative damage to serotonin cell bodies. Disturbances in the serotonergic and noradrenergic systems may occur in various neuropsychiatric illnesses such as obsessive-compulsive disorder, depression, and anorexia (Coccaro and Murphy, 1990) and in neurodegenerative disorders, including Parkinson's and Alzheimer's diseases (Hornykiewicz, 1998; Lyness et al., 2003), yet much remains to be discovered about the role of these neurotransmitters systems in normal and dysfunctional behavior. 2′-NH_2-MPTP is a tool that selectively perturbs the 5-HT and NE systems, and continued investigation using this neurotoxin will benefit research efforts aimed at understanding the roles of serotonin and norepinephrine in mammalian brain function.

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