The Role of Membrane and Vesicular Monoamine Transporters in the Neurotoxic and Hypothermic Effects of 1-Methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine (2′-NH2-MPTP)

Adam L. Numis, Erica L. Unger, Douglas L. Sheridan, Angela C. Chisnell, and Anne M. Andrews

Department of Chemistry, The Pennsylvania State University, University Park, Pennsylvania

This article is available online at http://molpharm.aspetjournals.org

ABSTRACT

The neurotoxin 1-methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine (2′-NH2-MPTP) damages forebrain serotonin (5-HT) and norepinephrine (NE) nerve terminals while sparing striatal dopaminergic innervation. Previous studies suggest that 2′-NH2-MPTP acts by a mechanism that involves uptake by the plasma membrane 5-HT and NE transporters. The present investigation further explores the molecular mechanism of 2′-NH2-MPTP with regard to cellular transport and effects on body temperature. Mice with genetically controlled decreases in serotonin transporter (SERT) expression were studied to corroborate pharmacologic evidence implicating SERT in 2′-NH2-MPTP-induced serotonin neurotoxicity. To investigate whether sequestration by the intracellular vesicular monoamine transporter type 2 (VMAT2) occurs, mice with reduced VMAT2 expression or mice receiving the VMAT2 inhibitor Ro 4-1284 (2-hydroxy-2-ethyl-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydrobenzo[α]chinolizin hydrochloride) were treated with 2′-NH2-MPTP. Body tempera-ture was measured as a function of reduced SERT or VMAT2 expression. 2′-NH2-MPTP caused a 2°C drop in temperature that was attenuated by decreased SERT but not VMAT2. In addition, complete loss of SERT attenuated cortical and hippocampal depletions in 5-HT but not NE. In contrast, mice with a 50% reduction in VMAT2 exhibited similar 5-HT and NE toxicity when compared with wild-type mice at higher doses of 2′-NH2-MPTP, whereas a slight potentiation of toxicity was observed at very low doses of 2′-NH2-MPTP. Pharmacologic inhibition of VMAT2 caused minimal potentiation of neurotransmitter depletions in response to moderate doses of 2′-NH2-MPTP. Thus, 2′-NH2-MPTP seems to be similar to MPTP in its requirement for selective plasma membrane transport and the expression of acute hypothermia; however, unlike MPTP, VMAT2 does not appear to play a major role in the toxic mechanism of 2′-NH2-MPTP.

Modeling neurodegeneration is important for investigating the mechanisms underlying late-onset neurological disorders such as Alzheimer's and Parkinson's diseases, as well as for understanding normal aging processes. Dopamine neurotoxicity and the subsequent Parkinsonian-like symptoms resulting from the ingestion of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) have been well documented in humans, establishing the importance of MPTP as a degenerative model for the dopamine (DA) system (Bloem et al., 1990). Further investigation of the mechanism of action of MPTP in nonhuman primates, rats, mice, and other species has provided significant insight into various facets of DA neuronal degeneration (Przedborski and Vila, 2003).

MPTP neurotoxicity is mediated via oxidative metabolism by monoamine oxidase (MAO) type B to 1-methyl-4-phenylpyridium (MPP+) (Markey et al., 1984), the latter of which is taken up by the DA transporter (DAT) (Melamed et al., 1986; Gainetdinov et al., 1997). Intracellular MPP+ is then concentrated in mitochondria where it interferes with oxidative phosphorylation by inhibiting complex I, thus preventing ATP production. This is thought to induce superoxide and hydroxyl radical formation and, ultimately, to cause neuronal death (Ramsay et al., 1986; Przedborski et al., 1992; Ali et al., 1994). Synaptic vesicles also can take up MPP+ via the vesicular monoamine transporter (VMAT2), and the ex-

ABBREVIATIONS: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; DA, dopamine; MAO, monoamine oxidase; MPP+, 1-methyl-4-phenylpyridium; DAT, dopamine transporter; VMAT, vesicular monoamine transporter; 2′-NH2-MPTP, 1-methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine; 5-HT, serotonin; NE, norepinephrine; SERT, serotonin transporter; NET, norepinephrine transporter; Ro 4-1284, 2-hydroxy-2-ethyl-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydrobenzo[α]chinolizin hydrochloride; 5-HIAA, 5-hydroxyindoleacetic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; HVA, homovanillic acid; ANOVA, analysis of variance.

tent of this sequestration confers species- and cellular-specific resistance to MPTP toxicity (Reinhard et al., 1987, 1988; Liu et al., 1992; Speciale et al., 1998; German et al., 2000; Staal and Sonsalla, 2000; Staal et al., 2000; Uhl et al., 2000).

Many structural analogs of MPTP have been synthesized, and their ability to deplete striatal DA has been investigated (Bradbury et al., 1985; Booth et al., 1989; Maret et al., 1990; Saporito et al., 1992). Most compounds bearing a substituent at the 2′ position are potent dopamine neurotoxins (Youngster et al., 1987; Heikkila et al., 1988; Johnson et al., 1989; Youngster et al., 1989); however, the 2′ amine-substituted analog, 1-methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine (2′-NH₂-MPTP), has been demonstrated to destroy forebrain serotonin (5-HT) and norepinephrine (NE) nerve terminals in mice and rats while sparing striatal DA innervation (Andrews and Murphy, 1993c; Unger et al., 2002; Luellen et al., 2003).

Although the mechanism of 2′-NH₂-MPTP neurotoxicity has not been fully ascertained, the steps that have been investigated are similar but not identical to those of MPTP. 2′-NH₂-MPTP is oxidized by MAO type A (Andrews and Murphy, 1993a). In addition, studies with the 5-HT uptake inhibitors fluoxetine and paroxetine or the NE uptake inhibitor desipramine show that pretreatment with these drugs attenuates 2′-NH₂-MPTP-induced depletions in 5-HT and NE, respectively (Andrews and Murphy, 1993a,b). Finally, mice genetically engineered to express high levels of human Cu-Zn superoxide dismutase exhibit protection from 2′-NH₂-MPTP-induced neurotoxicity, suggesting that oxyradicals are involved in the toxic effects of this compound (Andrews et al., 1996).

The focus of the present study was to further establish the involvement of the serotonin transporter (SERT) and to investigate the role of VMAT2 in the mechanism of action of 2′-NH₂-MPTP. Previous studies have demonstrated that the dopamine transporter is involved in MPTP toxicity using selective dopamine uptake inhibitors, as well as mice genetically engineered to lack the dopamine transporter (Mayer et al., 1986; Gainetdinov et al., 1997). In the current investigation, mice with constitutive reductions in SERT expression were used to further assess whether this transporter is critical for the serotonin neurotoxicity caused by 2′-NH₂-MPTP. The role of VMAT2 in 2′-NH₂-MPTP toxicity was investigated using mice with reduced VMAT2 expression, as well as mice receiving a VMAT2 inhibitor during the course of 2′-NH₂-MPTP treatment. Furthermore, since previous studies have shown that increases in body temperature are associated with long-term decreases in brain serotonin caused by substituted amphetamines (Frey, 1975; Schmidt et al., 1990; Gordon et al., 1991; Stewart et al., 1997), changes in core temperature in response to 2′-NH₂-MPTP were evaluated.

The results of the present investigation indicate that rather than hyperthermia, a hypothermic response occurs during 2′-NH₂-MPTP treatment, similar to what has been observed after MPTP administration (Freyaldenhoven et al., 1995; Moy et al., 1998). Furthermore, the current data indicate that SERT is necessary for 2′-NH₂-MPTP-induced hypothermia, as well as the manifestation of serotonin neurotoxicity. In contrast, VMAT2 does not seem to be appreciably involved in the mechanism of 2′-NH₂-MPTP-induced hypothermia or neurotoxicity, and potentiation of toxicity generally was not observed in either model of decreased VMAT2

activity except at very low doses of 2′-NH₂-MPTP that caused little to no changes in serotonin and norepinephrine levels.

Materials and Methods

Animals. Male mice 2 to 3 months of age weighing 30 to 40 g were used for all experiments. SERT knockout mice lacking one (SERT⁺/⁻) or both (SERT⁻/⁻) functional copies of the SERT gene were generated as described previously (Bengel et al., 1998; Heils et al., 1998) at the National Institute of Mental Health (Bethesda, MD) on a mixed CD-1 × 129S6/SVev background. Animals used in the present study were the products of seven to eight generations of heterozygous brother-sister mating. VMAT2 knockout mice were produced at the National Institute on Drug Abuse (Baltimore, MD) on a mixed C57BL/6J × 129S6/SVev background. Because VMAT⁻/⁻ mice are not viable past postnatal day 12 (Takahashi et al., 1997), only VMAT⁺/⁺ and VMAT⁺/⁻ mice were used in the present experiments. CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA).

All animals were kept in groups of two to four per cage until 1 week before dosing, when they were moved to individual cages. Mice were housed in facilities approved by the American Association for the Accreditation of Laboratory Animal Care at 20–22°C on a 12-h light/dark cycle with food and water ad libitum. Experimental protocols strictly adhered to National Institutes of Health guidelines and were approved by The Pennsylvania State University Institutional Animal Care and Use Committee.

Drugs and Reagents. 2′-NH₂-MPTP was synthesized at The Pennsylvania State University (University Park, PA) and is currently available from Sigma-Aldrich (catalog no. A7969; St. Louis, MO). The VMAT2 inhibitor Ro 4-1284 was a gift from Hoffman-La Roche (Nutley, NJ). All other drugs and chemicals were purchased from Sigma-Aldrich and were of analytical grade.

Neurotoxin Treatments. Mice were weighed 48 h before drug administration, and animals with body weights greater than two standard deviations from the mean were placed in control groups to minimize differences in drug to body mass ratios. SERT⁺/⁺, SERT⁺/⁻, and SERT⁻/⁻ mice were administered 2′-NH₂-MPTP at 4 × 15 mg/kg by the i.p. route in 0.1 ml of sterile water at 2-h intervals. VMAT⁺/⁺ and VMAT⁺/⁻ mice were administered 2′-NH₂-MPTP at 4 × 7.5, 4 × 10, 4 × 12.5, or 4 × 15 mg/kg, i.p. in 0.1 ml of sterile water at 2-h intervals. The highest dose of 2′-NH₂-MPTP was selected on the basis of a previous study demonstrating significant (70–80%) depletions in cortical and hippocampal 5-HT and NE in CD-1 mice (Andrews et al., 1996). Lower doses of 2′-NH₂-MPTP also were investigated in VMAT knockout mice to produce submaximal (<50%) toxicity. These lower doses were necessary to test for possible increases in toxicity in animals with reduced intracellular vesicular monoamine uptake (Gainetdinov et al., 1998). Control animals received similarly timed injections of sterile water in a volume of 0.1 ml.

To further investigate the role of VMAT2 in 2′-NH₂-MPTP neurotoxicity, a pharmacologic model was used in which CD-1 mice were administered Ro 4-1284 at 3 × 10 mg/kg, i.p. in sterile water at 3-h intervals. Beginning 15 min after the first injection of Ro 4-1248, 2′-NH₂-MPTP was administered at 4 × 10 mg/kg, i.p. at 2-h intervals. This moderate dose of 2′-NH₂-MPTP was used to produce submaximal toxicity to ascertain whether potentiation of 5-HT and/or NE depletions occur as a result of inhibition of VMAT2 function. The Ro 4-1284 dose regimen was based on a previous report that demonstrated nearly complete inhibition of VMAT2 for the duration of a multidose neurotoxin exposure (Staal and Sonsalla, 2000).

Temperature Measurements. Body temperature was recorded with a digital thermometer equipped with a rectal temperature probe that was inserted 1.5 cm into the colon (Physitemp model BAT-12/probe RET-3; Physitemp Instruments Inc., Clifton, NJ). Animals were placed in the testing room (22.5 ± 1°C) at least 1 h before baseline temperature measurements and were habituated to the

probe during several exposures the day before each experiment. Temperature was determined twice in the 30 min before drug administration to establish baseline core body temperature. Temperature measurements then were repeated 30 min after each injection of 2′-NH$_2$-MPTP.

Neurochemistry. At the end of each experiment, mice were killed by cervical dislocation, and their brains were removed rapidly and dissected over ice to obtain samples of frontal cortex, hippocampus, brain stem, and striatum, which were stored at −70°C before analysis. Samples were analyzed for monoamine neurotransmitters and their metabolites by high performance liquid chromatography using electrochemical detection at +300 mV as previously reported (Unger et al., 2002). 5-HT, 5-hydroxyindoleacetic acid (5-HIAA), NE, DA, and the DA metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), and homovanillic acid (HVA) were separated and detected in a single chromatogram with 5-hydroxy-$N\omega$-methyltryptamine added as an internal standard. All neurotransmitter levels were quantified by comparing their relative peak areas with external standards. Protein concentrations were determined by the method of Lowry et al. (1951).

Statistics. Neurochemical data were first normalized to the appropriate control groups. For example, each genotype of SERT knockout mice treated with 2′-NH$_2$-MPTP was normalized to the same genotype of mice treated with water. In the case of CD-1 mice treated with combinations of 2′-NH$_2$-MPTP and Ro 4-1284, data were normalized to CD-1 mice receiving water only. Next, data from different treatment groups from a single experiment were analyzed by one-way ANOVA with group as the independent variable using the Statistical Analysis System (SAS Institute, Carey, NC).

For the temperature data, all baseline temperatures were analyzed initially by one-way ANOVA across the three genotypes of SERT knockout mice or Student's t test in the two genotypes of VMAT knockout mice. Individual temperatures at time −0.5 h (baseline) then were subtracted from subsequent temperature measurements for each animal to calculate the change in body temperature from baseline. Temperature data from all treatment groups for each experiment were analyzed by two-way ANOVA with group and time as the independent variables and repeated measures on time. For the neurochemical and temperature data, significant differences among groups are indicated by a priori t test probabilities. All values are expressed as means ± S.E.M. with $p < 0.05$ considered statistically significant.

Results

2′-NH$_2$-MPTP-Induced Serotonin Neurotoxicity Is Attenuated by Genetic Inactivation of SERT. Depletions in 5-HT caused by 2′-NH$_2$-MPTP are reduced by pharmacologic inhibition of SERT (Andrews and Murphy, 1993a,b). To further substantiate the role of SERT in the mechanism of action of 2′-NH$_2$-MPTP, mice with constitutive reductions in SERT expression were administered 2′-NH$_2$-MPTP (4 × 15 mg/kg), and monoamine neurotransmitter levels were determined 1 week later. One-way analysis of variance revealed a significant effect on 5-HT levels in frontal cortex, hippocampus, and brain stem in SERT knockout mice [$F(5,45) = 18$, $p < 0.0001$; $F(5,45) = 28$, $p < 0.0001$; $F(5,43) = 19$, $p < 0.0001$; respectively]. 5-HIAA levels were similarly affected [$F(5,45) = 24$, $p < 0.0001$; $F(5,45) = 22$, $p < 0.0001$; $F(5,43) = 10$, $p < 0.0001$; respectively]. Cortical 5-HT in 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ and SERT$^{+/-}$ mice was depleted to 30 to 40% of 5-HT levels in the corresponding water-treated groups of SERT$^{+/+}$ and SERT$^{+/-}$ mice ($p < 0.001$; Fig. 1A). In contrast, 5-HT in frontal cortex of SERT$^{-/-}$ mice was not affected by 2′-NH$_2$-MPTP treatment, and 5-HT levels in 2′-NH$_2$-MPTP-treated SERT$^{-/-}$ mice

were significantly higher than those in 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ mice ($p < 0.001$).

In hippocampus, a similar pattern of 5-HT depletions was observed. Again, only SERT$^{+/+}$ and SERT$^{+/-}$ mice exhibited significant decreases in 5-HT to 25 to 40% of the levels in water-treated SERT$^{+/+}$ and SERT$^{+/-}$ mice, respectively, in
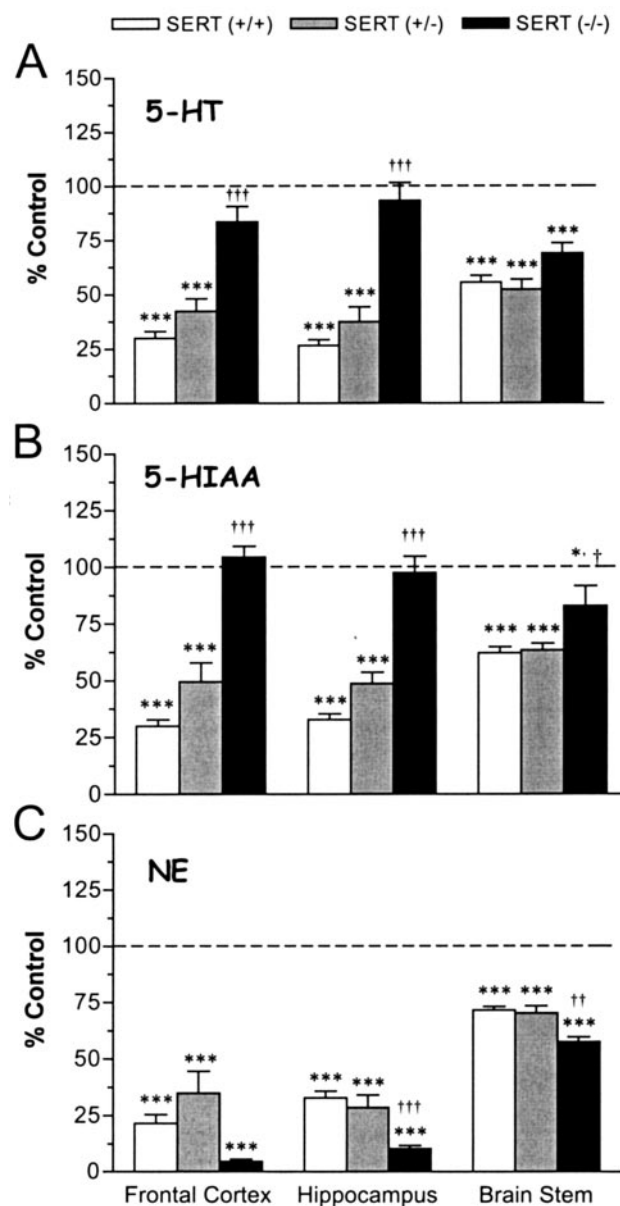

Fig. 1. Changes in 5-HT (A), 5-HIAA (B), and NE (C) levels 1 week after 2′-NH$_2$-MPTP administration in SERT$^{+/+}$, SERT$^{+/-}$, and SERT$^{-/-}$ mice. Animals were injected with 4 × 15 mg/kg 2′-NH$_2$-MPTP, i.p. at 2-h intervals ($n = 9$/genotype) or water using the same schedule ($n = 8$/genotype). Probabilities are $p < 0.05$ (∗) and $p < 0.001$ (∗∗∗) for differences between 2′-NH$_2$-MPTP-treated mice and their genotype-specific control groups and $p < 0.05$ (†), $p < 0.01$ (††), and $p < 0.001$ (†††) between 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ and 2′-NH$_2$-MPTP-treated SERT$^{-/-}$ mice. Results shown are percentages of the following control group means ± S.E.M. in frontal cortex, hippocampus, and brain stem, respectively: A, SERT$^{+/+}$: 7.7 ± 0.4, 4.9 ± 0.2, and 6.8 ± 0.3; SERT$^{+/-}$: 8.5 ± 0.7, 4.1 ± 0.3, and 6.4 ± 0.4; and SERT$^{-/-}$: 3.0 ± 0.4, 0.93 ± 0.08, and 1.7 ± 0.2; B, SERT$^{+/+}$: 2.3 ± 0.2, 3.6 ± 0.3, and 3.7 ± 0.2; SERT$^{+/-}$: 2.1 ± 0.2, 2.7 ± 0.2, and 3.3 ± 0.2; and SERT$^{-/-}$: 0.90 ± 0.05, 1.6 ± 0.1, and 2.4 ± 0.2; C, SERT$^{+/+}$: 5.5 ± 0.8, 4.4 ± 0.2, and 6.1 ± 0.1; SERT$^{+/-}$: 5.0 ± 0.9, 4.9 ± 0.2, and 6.0 ± 0.4; and SERT$^{-/-}$: 5.3 ± 0.8, 4.8 ± 0.3, and 6.1 ± 0.3 ng/mg protein.

response to 2′-NH$_2$-MPTP ($p < 0.001$). Serotonin levels in SERT$^{-/-}$ mice were unchanged with respect to water-treated SERT$^{-/-}$ mice. In both frontal cortex and hippocampus, a minimal trend toward higher 5-HT levels was observed in 2′-NH$_2$-MPTP-treated SERT$^{+/-}$ mice when compared with 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ mice; however, differences between these two groups were not statistically significant.

In brain stem, which contains the serotonergic neuronal perikarya, mice were less vulnerable to the effects of 2′-NH$_2$-MPTP as reflected by 5-HT depletions to 60 to 70% of the respective water-treated groups ($p < 0.001$). Interestingly, SERT$^{-/-}$ mice did not exhibit protection with regard to 2′-NH$_2$-MPTP in this brain region. Overall, similar results were observed for 5-HIAA levels in frontal cortex, hippocampus, and brain stem (Fig. 1B), with the exception that in brain stem, a significant difference between 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ and 2′-NH$_2$-MPTP-treated SERT$^{-/-}$ mice was observed ($p < 0.05$).

In contrast to the lack of 5-HT and 5-HIAA depletions in 2′-NH$_2$-MPTP-treated SERT$^{-/-}$ mice, potentiated reductions in NE levels were observed in these mice in frontal cortex, hippocampus, and brain stem compared with 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ mice (Fig. 1C). One-way analysis of variance revealed overall significant differences between 2′-NH$_2$-MPTP-treated and water-treated groups of mice in frontal cortex [$F_{(5,45)} = 15$, $p < 0.0001$], hippocampus [$F_{(5,45)} = 88$, $p < 0.0001$], and brain stem [$F_{(5,43)} = 28$, $p < 0.0001$]. In frontal cortex, 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ and SERT$^{+/-}$ mice showed decreases in NE to 20 to 35% of NE levels in water-treated SERT$^{+/+}$ and SERT$^{+/-}$ mice ($p < 0.001$), whereas SERT$^{-/-}$ mice exhibited further reductions in NE to 5% of water-treated SERT$^{-/-}$ mice ($p < 0.001$). In hippocampus, NE levels in neurotoxin-treated SERT$^{+/+}$ and SERT$^{+/-}$ mice were reduced to 30% of the respective control levels ($p < 0.001$), whereas SERT$^{-/-}$ mice exhibited NE levels that were 10% of control ($p < 0.001$ versus water-treated SERT$^{-/-}$ mice and $p < 0.001$ versus 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ mice). Locus coeruleus noradrenergic cell bodies were also less sensitive to the effects of 2′-NH$_2$-MPTP, and SERT$^{+/+}$ and SERT$^{+/-}$ mice showed decreases in NE to 70% of the respective water-treated groups in brain stem ($p < 0.001$), whereas SERT$^{-/-}$ mice administered 2′-NH$_2$-MPTP exhibited NE reductions to 60% of water-treated SERT$^{-/-}$ mice ($p < 0.001$). Moreover, NE levels in SERT$^{-/-}$ mice were significantly less than those in 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ mice ($p < 0.01$).

No significant differences with respect to group were observed in striatal DA [$F_{(5,44)} = 1.9$, $p = 0.11$], DOPAC [$F_{(5,45)} = 2.2$, $p = 0.066$], or HVA levels [$F_{(5,45)} = 1.1$, $p = 0.37$] or in cortical DA concentrations [$F_{(5,45)} = 1.4$, $p = 0.25$] in SERT$^{+/+}$, SERT$^{+/-}$, and SERT$^{-/-}$ mice 1 week after treatment with 2′-NH$_2$-MPTP (data not shown). These data, together with previous pharmacologic studies, demonstrate that SERT is necessary for 2′-NH$_2$-MPTP-induced serotonin neurotoxicity in frontal cortex and hippocampus but not for norepinephrine neurotoxicity.

2′-NH$_2$-MPTP Causes SERT-Dependent Hyperthermia in Mice. Substituted amphetamines have been reported to cause depletions in serotonin via a mechanism related to their ability to produce hyperthermia (Frey, 1975; Schmidt et al., 1990; Gordon et al., 1991; Stewart et al., 1997). We assessed the effects of 2′-NH$_2$-MPTP on body temperature

and the modulation of these effects by SERT. SERT$^{+/+}$, SERT$^{+/-}$, and SERT$^{-/-}$ mice were administered 4×15 mg/kg 2′-NH$_2$-MPTP or water, and core body temperature was measured 30 min after each injection. Initial statistical analysis revealed minor differences in baseline temperatures with respect to SERT knockout genotype [$F_{(2,57)} = 3.4$, $p = 0.041$]; therefore, changes in body temperature for individual mice were compared with their own baseline temperatures. Injection of water alone produced no effect on body temperature (data not shown). When 2′-NH$_2$-MPTP-treated and water-treated groups were considered together, two-way analysis of variance revealed a significant interaction between time and group ([$F_{(15,162)} = 7.3$, $p < 0.0001$]; Fig. 2), indicating that the groups responded differentially with respect to time. The simple effect of group was highly significant at each time point ($t = 0.5$ h [$F_{(5,54)} = 11$, $p = 0.0001$]; $t = 2.5$ h [$F_{(5,54)} = 7.4$, $p = 0.0001$]; $t = 4.5$ h [$F_{(5,54)} = 7.3$, $p = 0.0001$]; $t = 6.5$ h [$F_{(5,54)} = 8.5$, $p = 0.0001$]). Specifically, a significant elevation in core temperature 0.5 h after the initial injection of 2′-NH$_2$-MPTP was observed in SERT$^{+/-}$ and SERT$^{-/-}$ mice ($p < 0.01$ and $p < 0.001$ versus water-treated SERT$^{+/+}$ and SERT$^{+/-}$ mice, respectively). This was followed by a robust hypothermia in 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ and SERT$^{+/-}$ mice at the 2.5, 4.5, and 6.5 h time points (see Fig. 2 for significance levels). A hypothermic effect was not observed in 2′-NH$_2$-MPTP-treated SERT$^{-/-}$ mice, and core temperatures in these mice were significantly different from those in 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ and SERT$^{+/-}$ mice at all time points. Thus, administration of
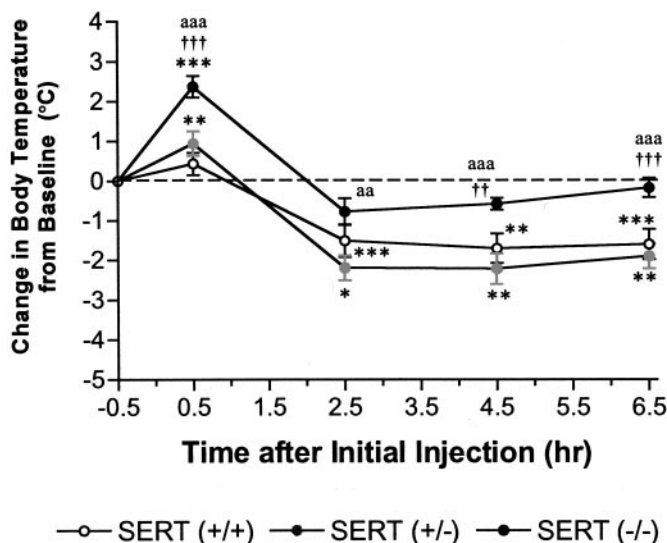

Fig. 2. Time course of effects of 2′-NH$_2$-MPTP (4×15 mg/kg, i.p. at 2-h intervals) on body temperature in SERT$^{+/+}$, SERT$^{+/-}$, and SERT$^{-/-}$ mice. Temperature measurements were taken twice approximately 0.5 h before 2′-NH$_2$-MPTP injections (baseline) and 0.5 h after each injection of 2′-NH$_2$-MPTP. Each value represents the mean change in body temperature of 2′-NH$_2$-MPTP-treated groups ($n = 12$/group) from their individual baseline temperatures $\pm$ S.E.M. Probabilities are $p < 0.05$ (∗), $p < 0.01$ (∗∗) and $p < 0.001$ (∗∗∗) for differences between 2′-NH$_2$-MPTP-treated groups and water-treated groups (data not shown), $p < 0.01$ (††) and $p < 0.001$ (†††) for differences between 2′-NH$_2$-MPTP-treated SERT$^{+/+}$ and 2′-NH$_2$-MPTP-treated SERT$^{-/-}$ mice, and $p < 0.01$ (aa) and $p < 0.001$ (aaa) for differences between 2′-NH$_2$-MPTP-treated SERT$^{+/-}$ and 2′-NH$_2$-MPTP-treated SERT$^{-/-}$ mice. Baseline temperatures were as follows. SERT$^{+/+}$: water = 37.3 $\pm$ 0.2°C, 2′-NH$_2$-MPTP = 37.2 $\pm$ 0.2°C; SERT$^{+/-}$: water = 37.6 $\pm$ 0.2°C, 2′-NH$_2$-MPTP = 36.9 $\pm$ 0.2°C; SERT$^{-/-}$: water = 37.0 $\pm$ 0.3°C, 2′-NH$_2$-MPTP = 36.5 $\pm$ 0.2°C.

$2'$-NH_2-MPTP causes a predominantly hypothermic effect in mice that is suppressed in the absence of SERT.

VMAT2 Is Minimally Involved in $2'$-NH_2-MPTP-Induced Neurotoxicity: Genetic Evidence. Sequestration of MPP^+ by VMAT2 has been shown to be neuroprotective in the case of MPTP-induced striatal DA neurotoxicity (Reinhard et al., 1987, 1988; Speciale et al., 1998; German et al., 2000; Staal and Sonsalla, 2000). To investigate whether uptake of $2'$-NH_2-MPTP or a putative metabolite by VMAT2 similarly occurs, $2'$-NH_2-MPTP was administered to $VMAT^{+/+}$ and $VMAT^{+/-}$ mice over a wide range of doses (4×7.5, 4×10, 4×12.5, and 4×15 mg/kg), and monoamine neurotransmitter levels were assessed 1 week later. When analyzed as a percentage of water-treated mice of the same genotype using one-way ANOVA, neurotransmitter levels revealed consistent VMAT2 gene dose-dependent effects only at the lowest (4×7.5 mg/kg) dose of $2'$-NH_2-MPTP (Figs. 3, 4, and 5).

Depletions in 5-HT were similar in $VMAT^{+/+}$ and $VMAT^{+/-}$ mice administered higher doses of $2'$-NH_2-MPTP (4×12.5 and 4×15 mg/kg). Here, 5-HT was reduced to 20 to 25% of the 5-HT levels in water-treated control $VMAT^{+/+}$ and $VMAT^{+/-}$ mice in frontal cortex ($p < 0.001$; Fig. 3A), 30 to 35% of control in hippocampus ($p < 0.001$; Fig. 3B), and 60 to 70% of control in brain stem ($p < 0.01$ at 4×12.5 mg/kg and $p < 0.05$ at 4×15 mg/kg in $VMAT^{+/-}$ mice; Fig. 3C). Decreases in 5-HT at the 4×10 mg/kg dose of $2'$-NH_2-MPTP were less pronounced as evidenced by 5-HT reductions to 35 to 40% of respective water-treated control groups in frontal cortex ($p < 0.001$; Fig. 3A), 80 to 85% of control in hippocampus ($p < 0.01$ in $VMAT^{+/-}$ mice; Fig. 3B), and 65 to 70% of control in brain stem ($p < 0.05$ in $VMAT^{+/+}$ and $p < 0.01$ in $VMAT^{+/-}$ mice; Fig. 3C).

With respect to differential neurotransmitter depletions in $VMAT^{+/+}$ versus $VMAT^{+/-}$ mice, the most consistent results were small differences in 5-HT and NE levels at the lowest 4×7.5 mg/kg dose of $2'$-NH_2-MPTP. In frontal cortex, 5-HT levels in $2'$-NH_2-MPTP-treated $VMAT^{+/+}$ mice were not significantly different from water-treated $VMAT^{+/+}$ mice, whereas $2'$-NH_2-MPTP-treated $VMAT^{+/-}$ mice showed a significant depletion to 65% of control ($p < 0.001$ versus water-treated $VMAT^{+/-}$ mice and $p < 0.05$ versus $2'$-NH_2-MPTP-treated $VMAT^{+/+}$ mice). In hippocampus and brain stem, 4×7.5 mg/kg $2'$-NH_2-MPTP also failed to cause significant decreases in 5-HT levels in $VMAT^{+/+}$ mice, as well as $VMAT^{+/-}$ mice; however, a significant difference between 5-HT levels across the two genotypes was observed in hippocampus ($p < 0.05$ for $2'$-NH_2-MPTP-treated $VMAT^{+/-}$ versus $2'$-NH_2-MPTP-treated $VMAT^{+/+}$ mice; Fig. 3B). Overall, decreases in 5-HIAA followed the same general trends as those observed for 5-HT with a significant difference between 5-HIAA levels in $2'$-NH_2-MPTP-treated $VMAT^{+/+}$ versus $VMAT^{+/-}$ mice in frontal cortex at the lowest 4×7.5 mg/kg dose ($p < 0.05$; Fig. 4). A difference between 5-HIAA levels in frontal cortex also was noted at the 4×15 mg/kg dose ($p < 0.05$ for $2'$-NH_2-MPTP-treated $VMAT^{+/+}$ mice versus $2'$-NH_2-MPTP-treated $VMAT^{+/-}$ mice).

$2'$-NH_2-MPTP-treated $VMAT^{+/+}$ and $VMAT^{+/-}$ mice showed greater overall decreases in NE at the two higher dose regimens, similar to 5-HT, with reductions in NE to 10 to 15% of NE levels in the respective water-treated control groups in frontal cortex ($p < 0.001$; Fig. 5A), to 20 to 30% of

control in hippocampus ($p < 0.001$; Fig. 5B), and to 70 to 90% of control in brain stem (see Fig. 5C for significance levels). Administration of 4×10 mg/kg $2'$-NH_2-MPTP resulted in
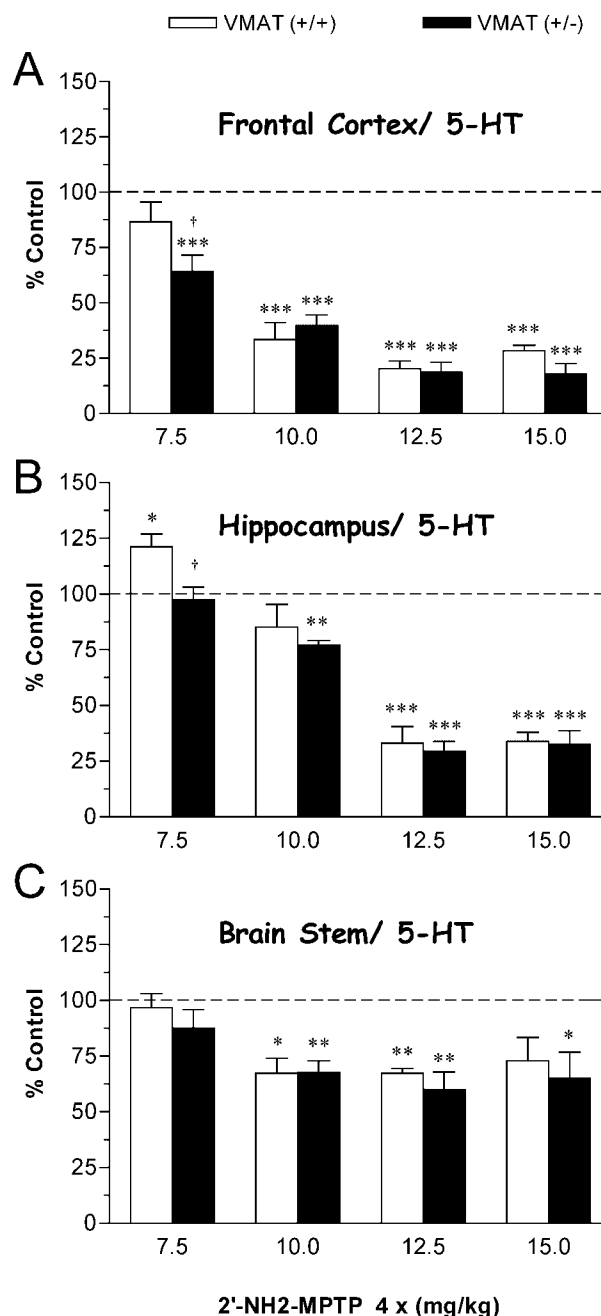

Fig. 3. Changes in 5-HT levels 1 week after $2'$-NH_2-MPTP administration in $VMAT^{+/+}$ and $VMAT^{+/-}$ mice in frontal cortex (A), hippocampus (B), and brain stem (C). Animals were injected with 4×7.5, 4×10, 4×12.5, or 4×15 mg/kg $2'$-NH_2-MPTP, i.p. at 2-h intervals ($VMAT^{+/+}$ mice: $n = 5$, 5, 7, and 4; $VMAT^{+/-}$ mice: $n = 8$, 8, 7, and 4, respectively) or with water using the same schedule. Results are percentages of the following control group means $\pm$ S.E.M. in frontal cortex, hippocampus, and brain stem for $4 \times 7.5/10$ mg/kg and $4 \times 12.5/15$ mg/kg of $2'$-NH_2-MPTP, respectively. A, $VMAT^{+/+}$: 4.9 ± 0.2, 5.4 ± 0.2; $VMAT^{+/-}$: 4.8 ± 0.2, 5.3 ± 0.4; B, $VMAT^{+/+}$: 3.5 ± 0.2, 5.1 ± 0.3; $VMAT^{+/-}$: 4.1 ± 0.2, 4.5 ± 0.4; and C, $VMAT^{+/+}$: 3.5 ± 0.5, 5.2 ± 0.3; $VMAT^{+/-}$: 3.0 ± 0.2, 5.4 ± 0.7 ng/mg protein. Probabilities are $p < 0.05$ ($*$), $p < 0.01$ ($**$), and $p < 0.001$ ($***$) for differences between $2'$-NH_2-MPTP-treated groups and the corresponding water-treated genotypes of VMAT knockout mice and $p < 0.05$ ($\dagger$) between $2'$-NH_2-MPTP-treated $VMAT^{+/+}$ and $2'$-NH_2-MPTP-treated $VMAT^{+/-}$ mice at the same dose of $2'$-NH_2-MPTP.

less severe depletions in NE (Fig. 5). Once again, at the lowest 4 × 7.5 mg/kg dose of 2′-NH₂-MPTP, a VMAT2 gene dose-dependent reduction in NE levels was observed. This

effect also was observed in NE levels in brain stem at the 4 × 10 mg/kg dose ($p < 0.05$ for 2′-NH₂-MPTP-treated VMAT$^{+/+}$ mice versus 2′-NH₂-MPTP -treated VMAT$^{+/-}$ mice; Fig. 5C).
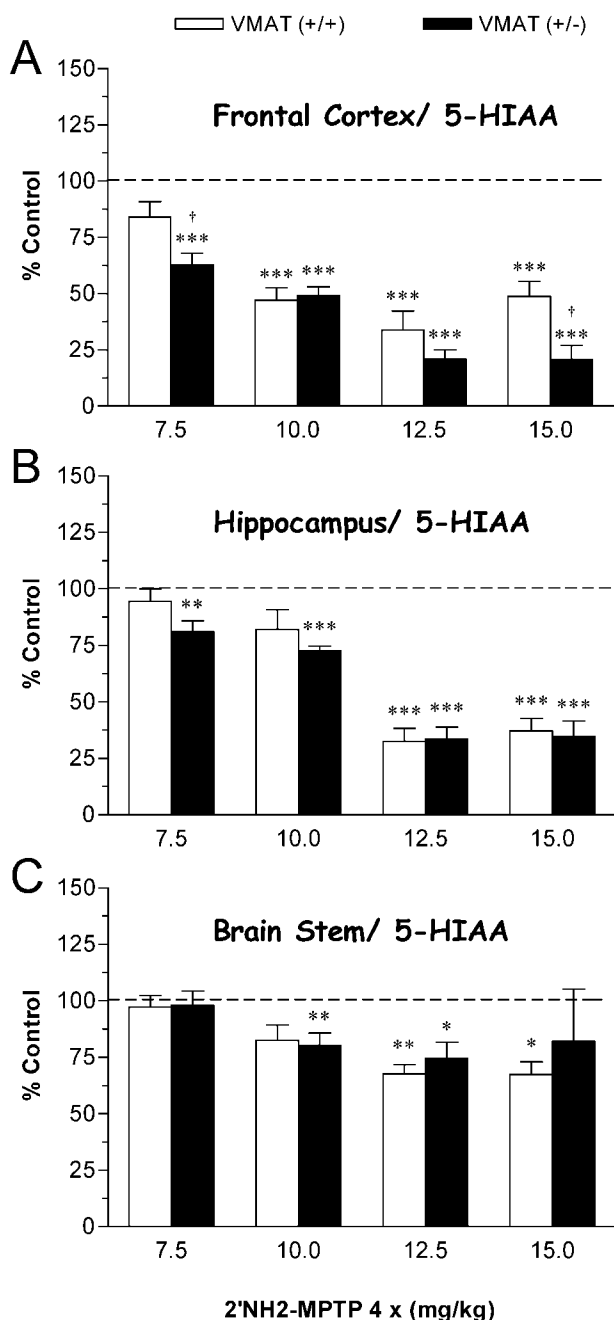
Fig. 4. Changes in 5-HIAA levels 1 week after 2′-NH₂-MPTP administration in VMAT$^{+/+}$ and VMAT$^{+/-}$ mice in frontal cortex (A), hippocampus (B), and brain stem (C). Animals were injected with 4 × 7.5, 4 × 10, 4 × 12.5, or 4 × 15 mg/kg 2′-NH₂-MPTP, i.p. at 2-h intervals (VMAT$^{+/+}$ mice: $n = 5, 5, 7$, and 4; VMAT$^{+/-}$ mice: $n = 8, 8, 7$, and 4, respectively) or with water using the same schedule. Results are percentages of the following water-treated group means ± S.E.M. in frontal cortex, hippocampus, and brain stem for 4 × 7.5/10 mg/kg and 4 × 12.5/15 mg/kg of 2′-NH₂-MPTP, respectively. A, VMAT$^{+/+}$: 4.9 ± 0.2, 5.4 ± 0.2; VMAT$^{+/-}$: 4.8 ± 0.2, 5.3 ± 0.4; B, VMAT$^{+/+}$: 3.5 ± 0.2, 5.1 ± 0.3; VMAT$^{+/-}$: 4.1 ± 0.2, 4.5 ± 0.4; and C, VMAT$^{+/+}$: 3.5 ± 0.5, 5.2 ± 0.3; VMAT$^{+/-}$: 3.0 ± 0.2, 5.4 ± 0.7 ng/mg protein. Probabilities are $p < 0.05$ (∗), $p < 0.01$ (∗∗), and $p < 0.001$ (∗∗∗) for differences between ′-NH₂-MPTP-treated groups and the corresponding water-treated genotypes of VMAT knockout mice and $p < 0.05$ (†) between 2′-NH₂-MPTP-treated VMAT$^{+/+}$ and 2′-NH₂-MPTP-treated VMAT$^{+/-}$ mice at the same dose of 2′-NH₂-MPTP.
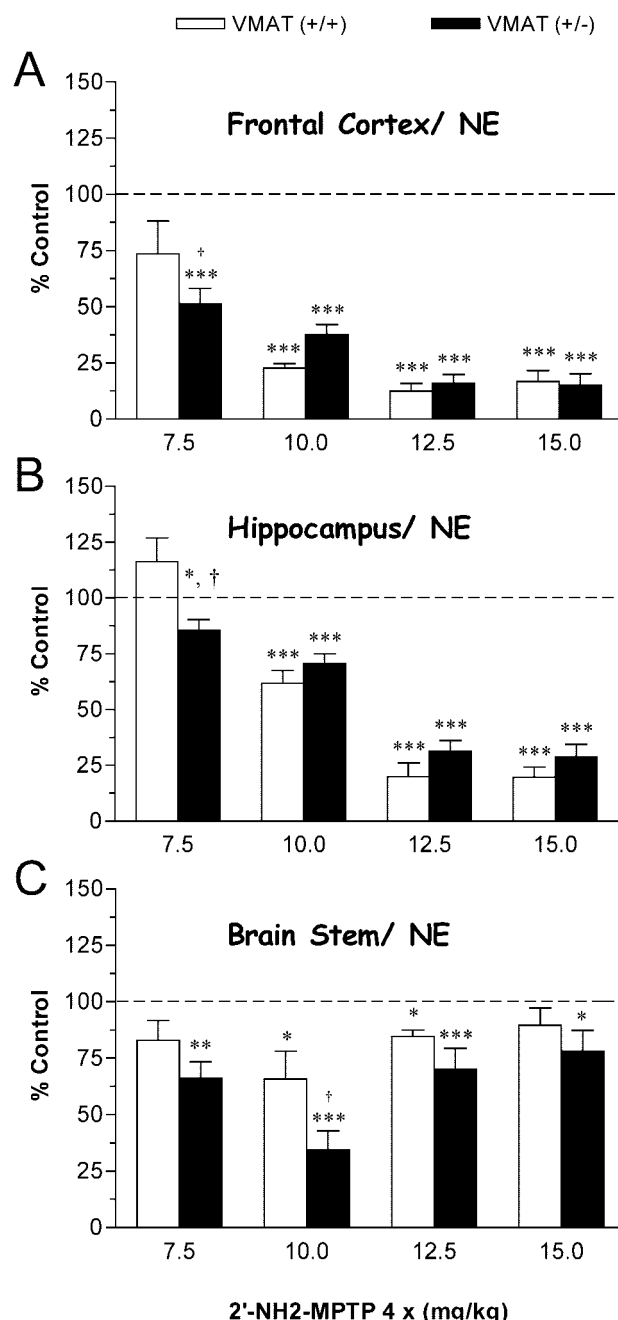
Fig. 5. Changes in NE levels 1 week after 2′-NH₂-MPTP administration in VMAT$^{+/+}$ and VMAT$^{+/-}$ mice in frontal cortex (A), hippocampus (B), and brain stem (C). Animals were injected with 4 × 7.5, 4 × 10, 4 × 12.5, or 4 × 15 mg/kg 2′-NH₂-MPTP, i.p. at 2-h intervals (VMAT$^{+/+}$ mice: $n = 5, 5, 7$, and 4; VMAT$^{+/-}$ mice: $n = 8, 8, 7$, and 4, respectively) or with water using the same schedule. Results are percentages of the following control group means ± S.E.M. in frontal cortex, hippocampus, and brain stem for 4 × 7.5/10 mg/kg and 4 × 12.5/15 mg/kg of 2′-NH₂-MPTP, respectively. A, VMAT$^{+/+}$: 2.7 ± 0.2, 3.1 ± 0.1; VMAT$^{+/-}$: 2.8 ± 0.3, 3.3 ± 0.4; B, VMAT$^{+/+}$: 2.0 ± 0.1, 4.9 ± 0.2; VMAT$^{+/-}$: 2.4 ± 0.1, 4.4 ± 0.3; and C, VMAT$^{+/+}$: 3.2 ± 0.5, 6.1 ± 0.3; VMAT$^{+/-}$: 2.8 ± 0.2, 5.6 ± 0.4 ng/mg protein. Probabilities are $p < 0.05$ (∗), $p < 0.01$ (∗∗), and $p < 0.001$ (∗∗∗) for differences between 2′-NH₂-MPTP-treated groups and the corresponding water-treated genotypes of VMAT knockout mice and $p < 0.05$ (†) between 2′-NH₂-MPTP-treated VMAT$^{+/+}$ and 2′-NH₂-MPTP-treated VMAT$^{+/-}$ mice at the same dose of 2′-NH₂-MPTP.

In frontal cortex, VMAT$^{+/+}$ mice receiving 4×7.5 mg/kg 2'-NH$_2$-MPTP showed no statistically significant reductions in NE, whereas similarly treated VMAT$^{+/-}$ mice had reductions to 50% of control ($p < 0.001$ versus water-treated VMAT$^{+/-}$ mice and $p < 0.05$ versus 2'-NH$_2$-MPTP-treated VMAT$^{+/+}$ mice; Fig. 5A). VMAT2 gene dose-dependent reductions in NE after 4×7.5 mg/kg 2'-NH$_2$-MPTP were also observed in hippocampus ($p < 0.05$ for 2'-NH$_2$-MPTP-treated VMAT$^{+/-}$ versus 2'-NH$_2$-MPTP-treated VMAT$^{+/+}$ mice; Fig. 5B). In brain stem, no reductions in NE were observed in 4×7.5 mg/kg 2'-NH$_2$-MPTP-treated VMAT$^{+/+}$ mice, although similarly treated VMAT$^{+/-}$ mice showed reductions in NE to 65% of control ($p < 0.01$ versus water-treated VMAT$^{+/-}$ mice).

No significant differences with respect to treatment group were detected in DA, DOPAC, or HVA levels in striatum or DA levels in frontal cortex in response to 2'-NH$_2$-MPTP in VMAT knockout mice at all doses (data not shown). In brief, these data demonstrate that only at very low subtoxic doses of 2'-NH$_2$-MPTP where 5-HT and NE depletions were barely discernible was a modest potentiation in neurotoxicity evident in mice with a 50% reduction in VMAT2 expression.

Effects of 2'-NH$_2$-MPTP on Body Temperature in VMAT Knockout Mice. As demonstrated above, 2'-NH$_2$-MPTP causes a hypothermic effect that can be mediated by SERT. We further assessed the effects of 2'-NH$_2$-MPTP on temperature and the possibility of modulation by VMAT2. Core temperature was measured 30 min after each injection of 2'-NH$_2$-MPTP (4×12.5 or 4×15 mg/kg) or water in VMAT$^{+/+}$ and VMAT$^{+/-}$ mice. Initial statistical analysis revealed no significant differences in baseline temperatures between the two genotypes of mice [$t(34) = 1.11$, $p = 0.30$]. Two-way ANOVA with repeated measures on time revealed an overall significant interaction between time and group ([$F(15,90) = 5.6$, $p < 0.0001$; Fig. 6), indicating that the treatment groups responded differentially to 2'-NH$_2$-MPTP administration with respect to time. The simple effect of group was significant at each time point after 2'-NH$_2$-MPTP administration except at 0.5 h ($t = 0.5$ h [$F(5,30) = 2.4$, $p = 0.059$]; $t = 2.5$ h [$F(5,30) = 6.9$, $p = 0.0002$]; $t = 4.5$ h [$F(5,30) = 3.8$, $p = 0.0087$]; $t = 6.5$ h [$F(5,30) = 9.1$, $p = 0.0001$]). Both groups of 2'-NH$_2$-MPTP-treated mice exhibited a hypothermic response at all time points after the second injection of 2'-NH$_2$-MPTP (Fig. 6); however, in contrast to the gene dose-dependent hypothermia exhibited by 2'-NH$_2$-MPTP-treated SERT knockout mice, no significant differences in core body temperature between 2'-NH$_2$-MPTP-treated VMAT$^{+/+}$ and 2'-NH$_2$-MPTP-treated VMAT$^{+/-}$ mice were observed at either dose of 2'-NH$_2$-MPTP. These data further substantiate the finding that 2'-NH$_2$-MPTP induces hypothermia. Additionally, they parallel the neurochemical results in VMAT knockout mice demonstrating no differential effect of 2'-NH$_2$-MPTP between VMAT$^{+/+}$ and VMAT$^{+/-}$ mice at either the 4×12.5 or 4×15 mg/kg doses of 2'-NH$_2$-MPTP with regard to temperature.

VMAT2 Is Minimally Involved in 2'-NH$_2$-MPTP-Induced Neurotoxicity: Pharmacologic Evidence. The ability of reduced VMAT2 activity to potentiate 2'-NH$_2$-MPTP toxicity may not be fully evident in mice with 50% reductions in VMAT2 gene expression. Thus, VMAT2 activity was inhibited to a greater extent during 2'-NH$_2$-MPTP administration using 3×10 mg/kg Ro 4-1284. This dose regimen of Ro 4-1284 has been shown to cause nearly complete

VMAT2 inhibition for 3 h after administration (Staal and Sonsalla, 2000). Four groups of CD-1 mice were administered either water/water, Ro 4-1284/water, water/2'-NH$_2$-MPTP (4×10 mg/kg, i.p.), or Ro 4-1284/2'-NH$_2$-MPTP. One-way analysis of variance revealed significant differences in 5-HT levels among treatment groups in frontal cortex, hippocampus, and brain stem ([$F(3,35) = 8.0$, $p = 0.0003$; $F(3,35) = 5.1$, $p = 0.005$; $F(3,36) = 2.6$, $p = 0.07$], respectively). Likewise, differences in 5-HIAA ([$F(3,35) = 12$, $p = 0.0001$; $F(3,35) = 11$, $p = 0.0001$; $F(3,36) = 2.9$, $p = 0.048$]) and NE levels [$F(3,34) = 24$, $p = 0.0001$; $F(3,35) = 11$, $p = 0.0001$; $F(3,36) = 9.3$, $p = 0.0001$] in frontal cortex, hippocampus, and brain stem, respectively, were detected among treatment groups. Once again, however, 2-NH$_2$-MPTP caused significant depletions in regional 5-HT, 5-HIAA, and NE, but very little potentiation of 2-NH$_2$-MPTP-induced neurotoxicity was observed in the presence of pharmacologic inhibition of VMAT2 (Fig. 7).

Ro 4-1284 alone had no significant effects on 5-HT, 5-HIAA, or NE levels 1 week post-treatment (Fig. 7). 2'-NH$_2$-MPTP alone caused 5-HT reductions to 70% of 5-HT levels in mice treated with water/water in frontal cortex ($p < 0.01$; Fig. 7A) but had no significant effect on hippocampal 5-HT levels. In mice receiving both Ro 4-1284/2'-NH$_2$-MPTP, 5-HT was reduced to 60% in frontal cortex and 65% in hippocampus ($p < 0.001$ and $p < 0.01$ versus water/water-treated mice, respectively). Only in hippocampus was a statistically significant potentiation of 2'-NH$_2$-MPTP serotonin neurotoxicity ob-
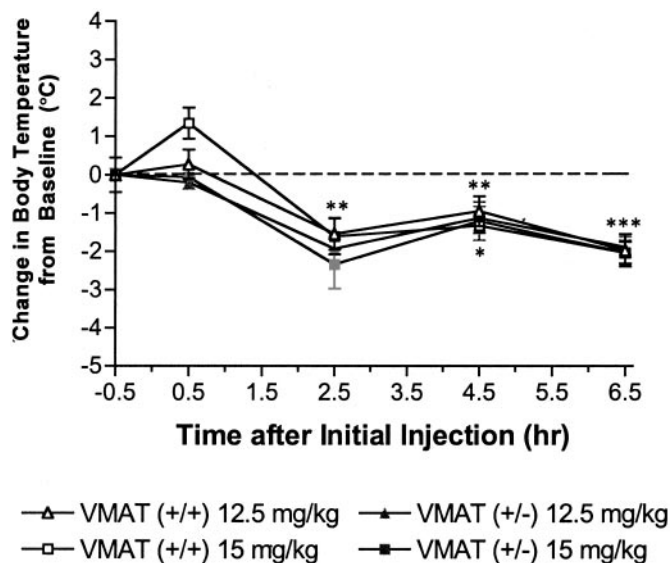

Fig. 6. Time course of effects of 2'-NH$_2$-MPTP (4×12.5 and 4×15 mg/kg, ip at 2-h intervals) on body temperature in VMAT$^{+/+}$ and VMAT$^{+/-}$ mice. Temperature measurements were made twice approximately 0.5 h before 2'-NH$_2$-MPTP injections (baseline) and 0.5 h after each injection of 2'-NH$_2$-MPTP. Each value represents the mean change in body temperature of 4×12.5 mg/kg and 4×15 mg/kg 2'-NH$_2$-MPTP-treated mice ($n = 7$/genotype and $n = 4$/genotype, respectively) from their individual baseline temperatures $\pm$ S.E.M. Baseline temperatures were as follows. VMAT$^{+/+}$: water $= 36.9 \pm 0.1°$C; 4×12.5 mg/kg 2'-NH$_2$-MPTP $= 37.1 \pm 0.1°$C; 4×15 mg/kg 2'-NH$_2$-MPTP $= 36.9 \pm 0.4°$C. VMAT$^{+/-}$: water $= 36.7 \pm 0.1°$C; 4×12.5 mg/kg 2'-NH$_2$-MPTP $= 36.9 \pm 0.1°$C; 4×15 mg/kg 2'-NH$_2$-MPTP $= 36.9 \pm 0.2°$C. $p < 0.01$ (**) for both 2'-NH$_2$-MPTP-treated groups versus genotype-matched water-treated control animals at 2.5 h, $p < 0.01$ (**) for 2'-NH$_2$-MPTP-treated VMAT$^{+/+}$ mice, and $p < 0.05$ (*) for VMAT$^{+/-}$ mice versus genotype-matched water-treated controls at 4.5 h, and $p < 0.001$ (***), for both 2'-NH$_2$-MPTP-groups versus genotype-matched water-treated controls at 6.5 h.

served in the Ro 4-1284/2′-NH₂-MPTP-treated group ($p <$ 0.05 versus water/2′-NH₂-MPTP-treated mice). Overall, decreases in 5-HIAA followed the same general patterns as those observed in 5-HT (Fig. 7B).

As with 5-HT and 5-HIAA, administration of 4×10 mg/kg 2′-NH₂-MPTP to CD-1 mice resulted in modest depletions in
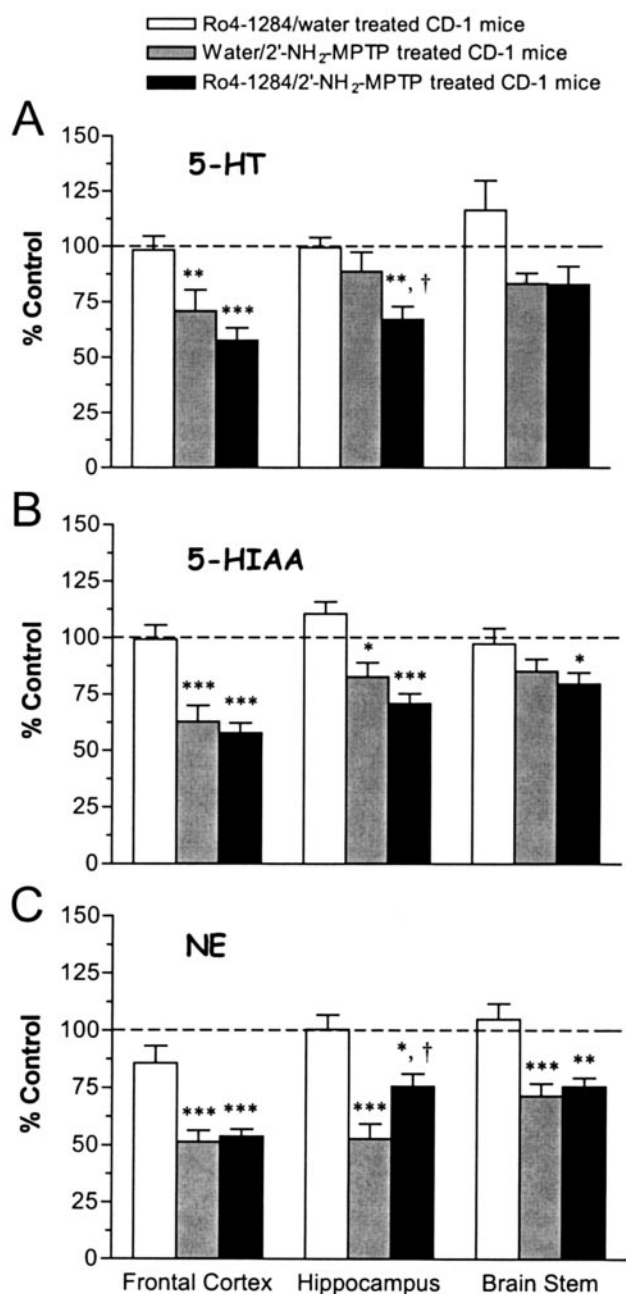
Fig. 7. Changes in 5-HT (A), 5-HIAA (B), and NE (C) levels 1 week after Ro 4-1284/2′-NH₂-MPTP administration in CD-1 mice. Animals were injected with water or Ro 4-1284 (3×10 mg/kg, i.p.) beginning 15 min before the initial administration of water or 2′-NH₂-MPTP (4×10 mg/kg, i.p. water/water $n = 9$; Ro 4-1284/water $n = 8$; water/2′-NH₂-MPTP $n = 10$; Ro 4-1284/2′-NH₂-MPTP $n = 13$). Results are percentages of the following water/water-treated control group means ± S.E.M. in frontal cortex, hippocampus, and brain stem, respectively. A, 3.6 ± 0.3, 4.5 ± 0.4, 3.7 ± 0.5; B, 2.5 ± 0.2, 3.5 ± 0.2, 6.5 ± 0.4; and C, 2.6 ± 0.1, 5.1 ± 0.4, 9.0 ± 0.6 ng/mg protein. Probabilities are $p < 0.05$ (∗), $p < 0.01$ (∗∗), and, $p < 0.001$(∗∗∗) for differences between drug-treated CD-1 mice and the water/water control group and $p < 0.05$ (†) between water/2′-NH₂-MPTP- and Ro 4-1284/2′-NH₂-MPTP-treated CD-1 mice.

NE in frontal cortex, hippocampus, and brain stem (Fig. 7C). No significant potentiation of NE depletions was observed in all brain regions assessed in Ro 4-1284/2′-NH₂-MPTP-treated mice versus water/2′-NH₂-MPTP-treated mice. In mice receiving 2′-NH₂-MPTP alone, NE was reduced to 50% of control in frontal cortex, 55% of control in hippocampus and 70% of control in brain stem ($p < 0.001$ in all cases versus water/water-treated mice). Similarly, in mice receiving the combination of Ro 4-1284 and 2′-NH₂-MPTP, NE was reduced to 55% of control in frontal cortex, 70% of control in hippocampus, and 75% of control in brain stem ($p < 0.001$, $p < 0.05$, and $p < 0.01$ versus water/water-treated mice, respectively). In the case of hippocampus, mice receiving Ro 4-1284 and 2′-NH₂-MPTP had significantly *higher* levels of NE than mice receiving 2′-NH₂-MPTP alone ($p < 0.05$; Fig. 7C). No significant changes in DA, DOPAC, or HVA levels in striatum or DA in frontal cortex were detected in all treatment groups (data not shown). These results, together with the data in VMAT knockout mice, support the conclusion that reductions in VMAT2 activity do not substantially potentiate the neurotoxic effects of 2-NH₂-MPTP.

Discussion

Pharmacological inhibition of SERT or NET results in attenuated serotonergic or noradrenergic 2′-NH₂-MPTP toxicity, respectively (Andrews and Murphy, 1993a,b); however, the molecular specificities of SERT and NET inhibitors are not absolute, and these compounds are known to have varying degrees of affinity for each of the monoamine transporters (Owens et al., 1997). Thus, we assessed the ability of constitutive reductions in SERT expression to modulate 2′-NH₂-MPTP-induced serotonin neurotoxicity. The present findings are consistent with the results of previous pharmacologic experiments whereby 2′-NH₂-MPTP-induced 5-HT neurotoxicity is prevented in mice lacking both copies of the SERT gene, further implicating the serotonin transporter in the mechanism of action of 2′-NH₂-MPTP. The observation that SERT$^{+/-}$ mice tend to display reductions in 5-HT levels similar to those of SERT$^{+/+}$ mice suggests that the serotonin transporter is not saturated during 2′-NH₂-MPTP administration, and animals with 50% reductions in SERT expression appear to take up sufficient 2′-NH₂-MPTP to manifest 5-HT depletions similar but not equal to those in SERT$^{+/+}$ mice. Thus, SERT plays a critical role in transporting 2′-NH₂-MPTP or its MAO-derived metabolite from the extracellular space into the interior of the neuron. In combination with similar studies on MPTP, these results also lend further support to the hypothesis that the plasma membrane monoamine transporters are the mechanism by which the family of MPTP analogs gains access to monoaminergic neurons (Gainetdinov et al., 1997). Furthermore, the affinity of the related pyridinium metabolites for individual monoamine transporters ultimately seems to determine the specific population of neurons affected.

Differential NE levels in 2′-NH₂-MPTP-treated SERT knockout mice point to the fact that SERT$^{-/-}$ mice have an increased susceptibility to 2′-NH₂-MPTP-induced noradrenergic neurotoxicity. 2′-NH₂-MPTP-treated SERT$^{+/+}$ and SERT$^{+/-}$ mice displayed significant reductions in NE levels in frontal cortex, hippocampus, and brain stem; however, 2′-NH₂-MPTP-treated SERT$^{-/-}$ mice showed further NE reductions in these same brain regions. Higher extracellular levels of 2′-NH₂-MPTP or

its metabolite in mice lacking SERT may result in increased transport via NET, resulting in enhanced NE neurotoxicity in these mice. Neuroadaptive changes in NET expression were not detected in hippocampus in SERT$^{-/-}$ mice (Montanez et al., 2003), ruling out the possibility that altered NET expression is responsible for the potentiation of NE depletions in this brain region. Therefore, the results of the present experiments with 2′-NH$_2$-MPTP in SERT knockout mice additionally substantiate the role of NET in the mechanism of 2′-NH$_2$-MPTP-induced noradrenergic neurodegeneration.

Previous studies have shown that MPP$^+$ is a substrate for VMAT2, and its sequestration into synaptic vesicles results in reduced neurotoxicity (Gainetdinov et al., 1998; Staal and Sonsalla, 2000). Our findings with 2′-NH$_2$-MPTP in VMAT knockout mice indicate a weak relationship between 2′-NH$_2$-MPTP and VMAT2. Gene dose-dependent differences in 5-HT depletions in frontal cortex and hippocampus only were evident at very low doses of 2′-NH$_2$-MPTP. Of the four dose regimens investigated in VMAT knockout mice, only the lowest 4×7.5 mg/kg dose of 2-NH$_2$-MPTP led to consistent discernible differences in 5-HT levels between VMAT$^{+/+}$ and VMAT$^{+/-}$ mice. Similar findings with regard to 5-HIAA and NE further support the hypothesis that 2′-NH$_2$-MPTP or its oxidative metabolite are poor substrates for VMAT2.

Because we were unable to investigate the role of VMAT2 in the mechanism of 2′-NH$_2$-MPTP toxicity in genetically altered mice having a complete reduction in VMAT2 expression, a second experiment was conducted in which VMAT2 was pharmacologically inhibited during the course of 2′-NH$_2$-MPTP administration using Ro 4-1284. In this case, only a very modest potentiation of depletion in neurotransmitter levels was observed in the case of hippocampal 5-HT, whereas hippocampal depletions in NE were slightly attenuated in mice treated with Ro 4-1284 and 2′-NH$_2$-MPTP. In both types of experiments involving reduced vesicular monoamine uptake, lower doses of 2-NH$_2$-MPTP were administered to allow for the possibility of further reductions in 5-HT and NE. It seems that even under these conditions, decreases in VMAT2 uptake were not associated with a large magnitude potentiation of 2′-NH$_2$-MPTP toxicity.

With regard to VMAT2, the pharmacologic findings strongly agree with the genetic data; however, because Ro 4-1284 is a reversible VMAT2 inhibitor, the possibility cannot be ruled out that the duration of neuronal exposure to 2-NH$_2$-MPTP exceeds that of Ro 4-1284 inhibition, leading to later onset uptake of 2-NH$_2$-MPTP or its metabolite by VMAT2. This could result in vesicular sequestration of 2-NH$_2$-MPTP in mice treated with Ro 4-1284, which might potentially confound the interpretation of these data. Administration of an irreversible VMAT2 inhibitor would prevent VMAT2 activity for longer periods of time; however, treatment with these compounds by themselves has been shown to significantly alter monoamine levels as long as 28 days post-treatment (Staal and Sonsalla, 2000). In addition, the use of 2′-NH$_2$-MPTP in conjunction with irreversible VMAT2 inhibitors results in high mortality rates (A. M. Andrews, unpublished observations).

Our investigation of body temperature indicates that the mechanism of action by which 2′-NH$_2$-MPTP induces serotonergic and noradrenergic neurodegeneration seems more similar to MPTP than to that of the substituted amphetamines (Miller and O'Callaghan, 1994; O'Callaghan and Miller, 1994). In SERT knockout mice, 2′-NH$_2$-MPTP admin-

istration resulted in a gene dose-dependent pattern of hypothermia that was similar to the pattern of 5-HT depletions observed in these mice. Thus, whereas SERT$^{+/+}$ and SERT$^{+/-}$ mice exhibited hypothermia and had significant depletions in 5-HT and 5-HIAA, SERT$^{-/-}$ mice did not display either of these 2-NH$_2$-MPTP-induced effects. The lack of 2-NH$_2$-MPTP-related hypothermia in SERT$^{-/-}$ mice may be associated with their resistance to serotonergic neurotoxicity, thus implicating depletions in 5-HT but not NE as being critical to decreases in core temperature. Given that 5-HT has been established as a neurotransmitter important to temperature regulation (Lin et al., 1983; Murphy et al., 1991), it follows that acute 5-HT neurotoxicity would have substantial effects on core temperature (Luellen et al., 2003).

Similar to SERT$^{+/+}$ and SERT$^{+/-}$ mice, VMAT$^{+/+}$ and VMAT$^{+/-}$ mice administered higher doses of 2′-NH$_2$-MPTP exhibited hypothermia shortly after 2′-NH$_2$-MPTP administration. Core temperature measurements in these mice also paralleled neurochemical data where both showed no differences with respect to genotype and/or dose regimen. A gene dose-dependent temperature response to 2-NH$_2$-MPTP in VMAT$^{+/+}$ versus VMAT$^{+/-}$ mice might have been present at lower doses of 2′-NH$_2$-MPTP; however, it seems unlikely given that low subtoxic dose regimens result in only very minor changes in 5-HT levels.

Interestingly, a modest hyperthermic effect was observed at the single time point immediately after 2-NH$_2$-MPTP administration in SERT$^{+/-}$ and SERT$^{-/-}$ mice. On the other hand, no statistically significant increases in temperature were observed in SERT$^{+/+}$ mice or in any of the groups of VMAT knockout mice receiving 2-NH$_2$-MPTP. These data suggest that constitutive decreases in SERT expression alter serotonin-mediated temperature regulation (Li et al., 1999). Thus, brief hyperthermia, possibly mediated by NE, seems to predominate in mice with reduced capacity to take up 2-NH$_2$-MPTP or its metabolite into serotonergic neurons.

In summary, the present investigation provides additional evidence for the importance of plasma membrane neurotransmitter transporters, and SERT in particular, to act as "molecular gateways" for compounds causing neurotoxicity to specific populations of neurons (Miller et al., 1999). Furthermore, this study demonstrates that administration of 2-NH$_2$-MPTP is associated with a reduction in body temperature that seems to be mediated by the serotonin system. Thus hyperthermia, such as that occurring in response to serotonin-depleting amphetamines, is not a universal prerequisite for serotonin neurotoxicity. Finally, although many aspects of the mechanism of action of 2-NH$_2$-MPTP are analogous to those of MPTP, the affinity of these neurotoxins for the vesicular monoamine transporter does not seem to be similar. Subcellular sequestration of exogenous (and possibly endogenous) toxins by VMAT2 has been proposed as a general neuroprotective phenomenon (Miller et al., 1999). The current findings suggest that not all neurotoxic substances are subject to modulation by VMAT2.

tional Institute of Mental Health) for providing the SERT knockout mice breeding pairs used to generate the animals for this study, but most importantly, for continued intellectual contributions on many levels regarding the investigation of 2-NH$_2$-MPTP and the serotonin transporter.

References

Ali SF, David SN, Newport GD, Cadet JL, and Slikker W Jr (1994) MPTP-induced oxidative stress and neurotoxicity are age-dependent: evidence from measures of reactive oxygen species and striatal dopamine levels. *Synapse* **18:**27–34.

Andrews AM, Ladenheim B, Epstein CJ, Cadet JL, and Murphy DL (1996) Transgenic mice with high levels of superoxide dismutase activity are protected from the neurotoxic effects of 2′-NH$_2$-MPTP on serotonergic and noradrenergic nerve terminals. *Mol Pharmacol* **50:**1511–1519.

Andrews AM and Murphy DL (1993a) 2′-NH$_2$-MPTP in Swiss Webster mice: evidence for long term (six month) depletions in cortical and hippocampal 5-HT and NE, differential protection by selective uptake inhibitors or clorgyline and functional changes in central 5-HT neurotransmission. *J Pharmacol Exp Ther* **267:**1432–1439.

Andrews AM and Murphy DL (1993b) Fluoxetine and desipramine selectively attenuate 2′-NH2-MPTP-induced depletions in serotonin and norepinephrine. *Eur J Pharmacol* **250:**215–221.

Andrews AM and Murphy DL (1993c) Sustained depletion of cortical and hippocampal serotonin and norepinephrine but not striatal dopamine by 1-methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine (2′-NH$_2$-MPTP): a comparative study with 2′-CH$_3$-MPTP and MPTP. *J Neurochem* **60:**1167–1170.

Bengel D, Murphy DL, Andrews AM, Wichems CH, Feltner D, Heils A, Mossner R, Westphal H, and Lesch KP (1998) Altered brain serotonin homeostasis and locomotor insensitivity to 3,4-methylenedioxymethamphetamine ("Ecstasy") in serotonin transporter-deficient mice. *Mol Pharmacol* **53:**649–655.

Bloem BR, Irwin I, Buruma OJ, Haan J, Roos RA, Tetrud JW, and Langston JW (1990) The MPTP model: versatile contributions to the treatment of idiopathic Parkinson's disease. *J Neurol Sci* **97:**273–293.

Booth RG, Trevor A, Singer TP, and Castagnoli N Jr (1989) Studies on semirigid tricyclic analogues of the nigrostriatal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Med Chem* **32:**473–477.

Bradbury AJ, Costall B, Domeney AM, Testa B, Jenner PG, Marsden CD, and Naylor RJ (1985) The toxic actions of MPTP and its metabolite MPP+ are not mimicked by analogues of MPTP lacking an N-methyl moiety. *Neurosci Lett* **61:**121–126.

Frey HH (1975) Hyperthermia induced by amphetamine, p-chloroamphetamine and fenfluramine in the rat. *Pharmacology* **13:**163–176.

Freyaldenhoven TE, Ali SF, and Hart RW (1995) MPTP- and MPP(+)-induced effects on body temperature exhibit age- and strain-dependence in mice. *Brain Res* **688:**161–170.

Gainetdinov RR, Fumagalli F, Jones SR, and Caron MG (1997) Dopamine transporter is required for in vivo MPTP neurotoxicity: evidence from mice lacking the transporter. *J Neurochem* **69:**1322–1325.

Gainetdinov RR, Fumagalli F, Wang YM, Jones SR, Levey AI, Miller GW, and Caron MG (1998) Increased MPTP neurotoxicity in vesicular monoamine transporter 2 heterozygote knockout mice. *J Neurochem* **70:**1973–1978.

German DC, Liang CL, Manaye KF, Lane K, and Sonsalla PK (2000) Pharmacological inactivation of the vesicular monoamine transporter can enhance 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurodegeneration of midbrain dopaminergic neurons, but not locus coeruleus noradrenergic neurons. *Neuroscience* **101:**1063–1069.

Gordon CJ, Watkinson WP, O'Callaghan JP, and Miller DB (1991) Effects of 3,4-methylenedioxymethamphetamine on autonomic thermoregulatory responses of the rat. *Pharmacol Biochem Behav* **38:**339–344.

Heikkila RE, Youngster SK, Panek DU, Giovanni A, and Sonsalla PK (1988) Studies with the neurotoxicant 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and several of its analogs. *Toxicology* **49:**493–501.

Heils A, Wichems C, Mossner R, Petri S, Glatz K, Bengel D, Murphy DL, and Lesch KP (1998) Functional characterization of the murine serotonin transporter gene promoter in serotonergic raphe neurons. *J Neurochem* **70:**932–939.

Johnson EA, Wu EY, Rollema H, Booth RG, Trevor AJ, and Castagnoli N Jr (1989) 1-methyl-4-phenylpyridinium (MPP+) analogs: in vivo neurotoxicity and inhibition of striatal synaptosomal dopamine uptake. *Eur J Pharmacol* **166:**65–74.

Li Q, Wichems C, Heils A, Van De Kar LD, Lesch KP, and Murphy DL (1999) Reduction of 5-hydroxytryptamine (5-HT)(1A)-mediated temperature and neuroendocrine responses and 5-HT(1A) binding sites in 5-HT transporter knockout mice. *J Pharmacol Exp Ther* **291:**999–1007.

Lin MT, Wu JJ, and Tsay BL (1983) Serotonergic mechanisms in the hypothalamus mediate thermoregulatory responses in rats. *Naunyn Schmiedebergs Arch Pharmacol* **322:**271–278.

Liu Y, Roghani A, and Edwards RH (1992) Gene transfer of a reserpine-sensitive mechanism of resistance to N-methyl-4-phenylpyridinium. *Proc Natl Acad Sci USA* **89:**9074–9078.

Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:**265–275.

Luellen BA, Miller DB, Chisnell AC, Murphy DL, O'Callaghan JP, and Andrews AM (2003) Neuronal and astroglial responses to the serotonin and norepinephrine neurotoxin: 1-methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine. *J Pharmacol Exp Ther* **307:**923–931.

Maret G, el Tayar N, Carrupt PA, Testa B, Jenner P, and Baird M (1990) Toxication of MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) and analogs by monoamine oxidase. A structure-reactivity relationship study. *Biochem Pharmacol* **40:**783–792.

Markey SP, Johannessen JN, Chiueh CC, Burns RS, and Herkenham MA (1984) Intraneuronal generation of a pyridinium metabolite may cause drug-induced parkinsonism. *Nature (Lond)* **311:**464–467.

Mayer RA, Kindt MV, and Heikkila RE (1986) Prevention of the nigrostriatal toxicity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine by inhibitors of 3,4-dihydroxyphenylethylamine transport. *J Neurochem* **47:**1073–1079.

Melamed E, Rosenthal J, Globus M, Cohen O, and Uzzan A (1986) Effect of pharmacological manipulations of dopamine metabolism on the dopaminergic neurotoxicity of MPTP in mice, in *MPTP: A neurotoxin producing a Parkinsonian syndrome* (Markey SP, Castagnoli NP, Trevor AJ, and Kopin IJ eds) pp 473–479, Academic Press, Inc., New York.

Miller DB and O'Callaghan JP (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.

Miller GW, Gainetdinov RR, Levey AI, and Caron MG (1999) Dopamine transporters and neuronal injury. *Trends Pharmacol Sci* **20:**424–429.

Montanez S, Owens WA, Gould GG, Murphy DL, and Daws LC (2003) Exaggerated effect of fluvoxamine in heterozygote serotonin transporter knockout mice. *J Neurochem* **86:**210–219.

Moy LY, Albers DS, and Sonsalla PK (1998) Lowering ambient or core body temperature elevates striatal MPP+ levels and enhances toxicity to dopamine neurons in MPTP-treated mice. *Brain Res* **790:**264–269.

Murphy DL, Lesch KP, Aulakh CS, and Pigott TA (1991) Serotonin-selective arylpiperazines with neuroendocrine, behavioral, temperature and cardiovascular effects in humans. *Pharmacol Rev* **43:**527–552.

O'Callaghan JP and Miller DB (1994) Neurotoxicity profiles of substituted amphetamines in the C57BL/6J mouse. *J Pharmacol Exp Ther* **270:**741–751.

Owens MJ, Morgan WN, Plott SJ, and Nemeroff CB (1997) Neurotransmitter receptor and transporter binding profile of antidepressants and their metabolites. *J Pharmacol Exp Ther* **283:**1305–1322.

Przedborski S, Kostic V, Jackson-Lewis V, Naini AB, Simonetti S, Fahn S, Carlson E, Epstein CJ, and Cadet JL (1992) Transgenic mice with increased Cu/Zn-superoxide dismutase activity are resistant to N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced neurotoxicity. *J Neurosci* **12:**1658–1667.

Przedborski S and Vila M (2003) The 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model: a tool to explore the pathogenesis of Parkinson's disease. *Ann N Y Acad Sci* **991:**189–198.

Ramsay RR, Salach JI, and Singer TP (1986) Uptake of the neurotoxin 1-methyl-4-phenylpyridine (MPP+) by mitochondria and its relation to the inhibition of the mitochondrial oxidation of NAD+-linked substrates by MPP+. *Biochem Biophys Res Commun* **134:**743–748.

Reinhard JF Jr, Daniels AJ, and Viveros OH (1988) Potentiation by reserpine and tetrabenazine of brain catecholamine depletions by MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) in the mouse; evidence for subcellular sequestration as basis for cellular resistance to the toxicant. *Neurosci Lett* **90:**349–353.

Reinhard JF Jr, Diliberto EJ Jr, Viveros OH, and Daniels AJ (1987) Subcellular compartmentalization of 1-methyl-4-phenylpyridinium with catecholamines in adrenal medullary chromaffin vesicles may explain the lack of toxicity to adrenal chromaffin cells. *Proc Natl Acad Sci USA* **84:**8160–8164.

Saporito MS, Heikkila RE, Youngster SK, Nicklas WJ, and Geller HM (1992) Dopaminergic neurotoxicity of 1-methyl-4-phenylpyridinium analogs in cultured neurons: relationship to the dopamine uptake system and inhibition of mitochondrial respiration. *J Pharmacol Exp Ther* **260:**1400–1409.

Schmidt CJ, Black CK, Abbate GM, and Taylor VL (1990) Methylenedioxymethamphetamine-induced hyperthermia and neurotoxicity are independently mediated by 5-HT2 receptors. *Brain Res* **529:**85–90.

Speciale SG, Liang CL, Sonsalla PK, Edwards RH, and German DC (1998) The neurotoxin 1-methyl-4-phenylpyridinium is sequestered within neurons that contain the vesicular monoamine transporter. *Neuroscience* **84:**1177–1185.

Staal RG, Hogan KA, Liang CL, German DC, and Sonsalla PK (2000) In vitro studies of striatal vesicles containing the vesicular monoamine transporter (VMAT2): rat versus mouse differences in sequestration of 1-methyl-4-phenylpyridinium. *J Pharmacol Exp Ther* **293:**329–335.

Staal RG and Sonsalla PK (2000) Inhibition of brain vesicular monoamine transporter (VMAT2) enhances 1-methyl-4-phenylpyridinium neurotoxicity in vivo in rat striata. *J Pharmacol Exp Ther* **293:**336–342.

Stewart CW, Bowyer JF, and Slikker W (1997) Elevated environmental temperatures can induce hyperthermia during d-fenfluramine exposure and enhance 5-hydroxytryptamine (5-HT) depletion in the brain. *J Pharmacol Exp Ther* **283:**1144-1150.

Takahashi N, Miner LL, Sora I, Ujike H, Revay RS, Kostic V, Jackson-Lewis V, Przedborski S, and Uhl GR (1997) VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. *Proc Natl Acad Sci USA* **94:**9938–9943.

Uhl GR, Li S, Takahashi N, Itokawa K, Lin Z, Hazama M, and Sora I (2000) The VMAT2 gene in mice and humans: amphetamine responses, locomotion, cardiac arrhythmias, aging, and vulnerability to dopaminergic toxins. *FASEB J* **14:**2459–2465.

Unger EL, Mazzola-Pomietto P, Murphy DL, and Andrews AM (2002) 2′-NH(2)-MPTP [1-methyl-4-(2′-aminophenyl)-1,2,3,6-tetrahydropyridine] depletes serotonin and norepinephrine in rats: a comparison with 2′-CH(3)-MPTP [1-methyl-4-(2′-methylphenyl)-1,2,3,6-tetrahydropyridine]. *J Pharmacol Exp Ther* **303:**527–533.

Youngster SK, Sonsalla PK, and Heikkila RE (1987) Evaluation of the biological activity of several analogs of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine. *J Neurochem* **48:**929–934.

Youngster SK, Sonsalla PK, Sieber BA, and Heikkila RE (1989) Structure-activity study of the mechanism of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced neurotoxicity. I. Evaluation of the biological activity of MPTP analogs. *J Pharmacol Exp Ther* **249:**820–828.

Address correspondence to: Dr. Anne Milasincic Andrews, The Pennsylvania State University, 104 Chemistry Building, University Park, PA 16802-4615. E-mail: ama11@psu.edu

MOL PHARM

MOLECULAR PHARMACOLOGY