SYNAPSE 30:205–210 (1998)

Reduced MPTP Neurotoxicity in Striatum of the Mutant Mouse *Tottering*

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KEY WORDS vesicular monoamine transporter; monoamine; dopamine

ABSTRACT The effects of MPTP treatment (4 × 10 mg/kg, 2-h intervals) on in vivo striatal binding of (+)-α-[^{3}H]dihydrotetrabenazine ((+)-[^{3}H]DTBZ) to the vesicular monoamine transporter type 2 (VMAT2) were examined in wild type (+,+) and *tottering* (*tg/tg*) mice of the C57BL/6J strain. The *tottering* mutant has been previously characterized as having hyperinnervation of noradrenergic terminals in the brain, with increased concentrations of norepinephrine and increased numbers of VMAT2 binding sites. In wild-type mice, MPTP caused a significant decrease in specific striatal (+)-[^{3}H]DTBZ binding in both males (-71%) and females (-57%), consistent with dopaminergic terminal losses. In the *tottering* mice, the neurotoxic effects of MPTP were diminished, with smaller losses of (+)-[^{3}H]DTBZ binding observed both in males (-45%) and females (-26%). These results are consistent with the hypothesis that vesicular storage (as a result of hyperinnervation) offers neuroprotection toward MPTP toxicity, although the confounding effects of increases in norepinephrine concentrations or changes in calcium ion channel function (both also characteristics of the *tottering* mutant) cannot be ruled out. The *tottering* mutant does, however, offer another animal model to examine the biochemical features responsible for MPTP toxicity. **Synapse 30:205–210, 1998.**

INTRODUCTION

A satisfactory explanation for the species and strain selectivity of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) toxicity to dopaminergic neurons of the brain is still lacking. Although the metabolic enzyme monoamine oxidase B and the neuronal membrane dopamine transporter are required for production and intracellular localization of the neurotoxic species 1-methyl-4-phenylpyridinium (MPP^+), the toxicity of MPTP does not correlate with numbers or activities of the enzyme or transporter (Liu and Edwards, 1997). A good correlation has been reported between brain tissue levels of MPP^+ and losses of striatal dopamine in vivo among mouse strains (Giovanni et al., 1991), although studies within a single strain of mice found a poor correlation between striatal MPP^+ level and dopamine loss (Vaglini et al., 1996). The resistance of rats to MPTP remains difficult to explain, as rats are reported to accumulate MPP^+ to brain tissue levels higher than mice (Giovanni et al., 1991; Nwanze et al., 1995).

Recently, a number of investigators have suggested that differential compartmentalization of MPP^+ might offer a method of neuroprotection; such sequestration of the toxin might be accomplished by storage in vesicles via the actions of the vesicular monoamine transporter type 2 (VMAT2) (Russo et al., 1994; Zuddas et al., 1994). This vesicular transporter is specific for the monoaminergic neurons in the brain and is very capable of moving MPP^+ from the cytosol into the lumen of the vesicle (Liu and Edwards, 1997), thus removing it from potential interactions with mitochondrial complex I sites and the subsequent inhibition of cellular energy metabolism. Evidence for the importance of VMAT2 in MPTP toxicity comes from pharmacological and genetic studies. Pretreatment of animals with inhibitors of the transporter, such as tetrabenazine or reserpine, potentiates the toxicity of MPTP (Reinhard et al., 1988; Russo et al., 1994). Reductions of the numbers of VMAT2 in heterozygous knockout mice (>50% loss of [^{3}H]dihydrotetrabenazine binding to the transporter in the striatum) results in greater than double the toxicity of MPTP, as measured by losses of tissue dopamine (Takahashi et

Contract grant sponsor: National Institutes of Health; Contract grant numbers: MH-47611, NS-16810.

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al., 1997). Both of these experiments support a role of VMAT2 in the dopaminergic neurotoxicity of MPTP.

These pharmacological and transgenic mouse studies have indicated that decreased numbers or availability of VMAT2 results in increased MPTP neurotoxicity. It would be equally interesting to demonstrate that *increased* vesicular storage capability results in *decreased* toxicity of MPTP. This might be done using a pharmacological treatment which upregulates the numbers of VMAT2 and/or the numbers of vesicles, but we and others have shown that the levels of VMAT2 protein cannot be regulated by administration of dopaminergic or cholinergic drugs, even where clear regulation of other functions of the dopaminergic system (e.g., neuronal membrane dopamine transporters and dopamine receptors) can be demonstrated (for review, see Kilbourn et al., 1997). Use of multiple strains of mice with different striatal concentrations of VMAT2 (if such were to be identified) would be complicated by potential differences in peripheral metabolism of the neurotoxin, which may account for some or all of the unusually high brain concentrations of MPP^+ observed in certain mouse strains. Therefore, in this study we examined the effects of MPTP on striatal dopaminergic terminal losses in a genetic model, the *tottering* mouse. *Tottering* is an autosomal recessive mutation, with homozygous animals (*tg*,*tg*) exhibiting ataxia, an intermittent movement disorder resembling focal myoclonus, and seizures with behavioral arrest similar to human absence epilepsy. Histological studies show a hyperinnervation by adrenergic neurons arising from the locus ceruleus. Biochemically, the *tottering* mouse displays a spectrum of altered concentrations of neurotransmitters, enzymes, and receptors (Kostopoulos, 1992), with the most notable a significant increase of tissue norepinephrine concentrations in numerous brain regions (Levitt and Noebels, 1981; Levitt et al., 1987). We recently reported that the *tottering* mouse strain also can be characterized by elevated in vivo binding of radioligands to VMAT2 in many regions of the brain (Kilbourn et al., 1995). Molecular biology studies have identified a gene for an α_{1A} voltage-sensitive calcium channel, which is mutated in *tottering* mice, although the functional link between the mutation and the observed biochemical and cytological abnormalities of the mutant has not been established (Fletcher et al., 1996).

The *tottering* mouse represents an animal model of increased monoaminergic innervation, and potentially increased concentrations of VMAT2. In this study, we examined the effects of MPTP on losses of dopaminergic nerve terminals in the striatum of wild-type and homozygous *tottering* mice; to minimize potential strain differences in peripheral metabolism and neurotoxin distribution, all studies were done in wild-type and *tottering* mice derived from the C57BL/6J strain. As an indicator of terminal loss, we employed measures of the in vivo specific binding of (+)-α-[^{3}H]dihydrotetrabenazine ((+)-[^{3}H]DTBZ), a specific and high affinity (K_d = 1.5 nM) radioligand for VMAT2 (Frey et al., 1997; Kilbourn and Sherman, 1997).

MATERIALS AND METHODS

Materials

All studies were done using (+)-α-[^{3}H]dihydrotetrabenazine ((+)-[^{3}H]DTBZ: 81 Ci/mmol), prepared by custom tritiation as previously described (Kilbourn et al., 1997).

Animals

Wild-type (+/+) and homozygous *tottering* (*tg*/*tg*) C57BL/6J mice were obtained from a breeding colony maintained at Texas A&M University, College Station, Texas. Animals were 26–28 weeks old at the time of the study. All procedures were approved by the University of Michigan University Committee on Use and Care of Laboratory Animals.

In vivo radioligand distribution studies

In vivo studies of radioligand distributions were done by a well-established protocol (Kilbourn and Sherman, 1997) which will be briefly described here. Animals were anesthetized (diethyl ether), injected via the tail vein with 5–10 μCi of (+)-[^{3}H]DTBZ in saline solution, and allowed to awaken. After 10 min, the animals were anesthetized (ether) and killed by decapitation. The brains were then rapidly removed and dissected into regions of interest (striatum, whole cortex, hippocampus, hypothalamic region, and cerebellum). Tissue samples were weighed, digested, and counted for tritium using liquid scintillation counting. Data was calculated as the percent injected dose per gram of tissue (%ID/g). Specific striatal binding of (+)-[^{3}H]DTBZ was calculated as the ratio $[(\%ID/g_{striatum} - \%ID/g_{cerebellum})/\%ID/g_{cerebellum}]$.

Statistics

Comparisons between groups were examined first by ANOVA, with significant interactions further tested using unpaired Student's *t*-tests. A significance level of 0.05 was used throughout the study.

RESULTS

In control animals (no MPTP pretreatment), the in vivo distribution of (+)-[^{3}H]DTBZ in the brains of both wild-type and *tottering* mice showed the expected rank order of VMAT2 binding among brain regions (Vander Borght et al., 1995), with higher concentrations in the striatum and hypothalamic region, and essentially equivalent and lower values in the cortex, hippocampus, and cerebellum (Table I). This rank order was also evident in the MPTP-treated animals, although the differences between regions of high (striatum) and low

TABLE I. Regional distribution of (+)-[^{3}H]DTBZ in the brains of wild-type (+,+) and tottering (tg,tg) C57BL/6J mice, with and without prior treatment with MPTP (4 × 10 mg/kg, 2-h intervals, 14 days prior)

	Controls (no MPTP)			
	(+,+) male	(+,+) female	(tg,tg) male	(tg,tg) female
N =	5	5	4	5
	% Injected dose/g tissue			
Striatum	8.48 ± 1.04	7.72 ± 0.64	8.20 ± 0.42	8.85 ± 1.37
Cortex	3.41 ± 0.28	2.81 ± 0.26	2.81 ± 0.32	3.42 ± 0.64
Hypothalamus	4.86 ± 0.48	4.35 ± 0.34	5.11 ± 0.40	5.18 ± 0.75
Hippocampus	3.38 ± 0.33	2.79 ± 0.19	2.87 ± 0.30	3.52 ± 0.51
Cerebellum	3.4 ± 0.38	2.96 ± 0.14	2.89 ± 0.19	3.39 ± 0.54
	MPTP treated			
	(+,+) male	(+,+) female	(tg,tg) male	(tg,tg) female
N =	6	4	5	5
	% Injected dose/g tissue			
Striatum	3.91 ± 0.30	6.81 ± 0.41	5.24 ± 0.54	8.83 ± 1.37
Cortex	2.69 ± 0.11	3.1 ± 0.31	2.96 ± 0.24	3.53 ± 0.49
Hypothalamus	3.09 ± 0.09	5.47 ± 0.37	3.61 ± 0.37	7.14 ± 1.07
Hippocampus	2.55 ± 0.13	3.48 ± 0.27	2.84 ± 0.25	4.02 ± 0.60
Cerebellum	2.73 ± 0.12	3.55 ± 0.28	2.85 ± 0.21	4.01 ± 0.46

(cerebellum) concentrations of transporters was diminished in most groups.

Treatment of wild-type and *tottering* C57BL/6 mice with MPTP produced variable losses of specific striatal binding of (+)-[^{3}H]DTBZ (Fig. 1). A three-way ANOVA analysis of the combined specific striatal binding data showed significant ($P < 0.01$) effects of strain, sex, and treatment. For the combined dataset, there was a significant interaction between sex plus treatment ($F = 14.4$, $P = 0.0006$), but not between strain and treatment ($F = 1.6$, $P = 0.22$), strain plus sex ($F = 3.3$, $P = 0.078$) or an interaction of all three variables ($F = 0.73$, $P = 0.4$).

The effects of sex, strain, and MPTP treatment were then further examined by comparisons between any two groups of animals. In control groups of both wild-type and *tottering* mice there were no significant gender differences in specific striatal (+)-[^{3}H]DTBZ binding. For female mice, there were no differences between wild-type and *tottering* mice; in males, however, there was an apparently higher specific striatal (+)-[^{3}H]DTBZ binding in the *tottering* mice ($P < 0.001$).

Changes in (+)-[^{3}H]DTBZ binding after MPTP treatment were both sex- and strain-dependent. In the wild-type mice, neurotoxin treatment reduced striatal binding in both females (-57%, $P < 0.001$ vs. +/+ controls) and males (-71%, $P < 0.001$ vs. +/+ controls), and these gender differences in striatal (+)-[^{3}H]DTBZ binding were significant ($P < 0.001$). In *tottering* mice, MPTP also produced smaller but significant decreases of (+)-[^{3}H]DTBZ binding in the males (-45%, $P < 0.001$ vs. *tg/tg* controls) and females (-26%, $P = 0.02$ vs. *tg/tg*

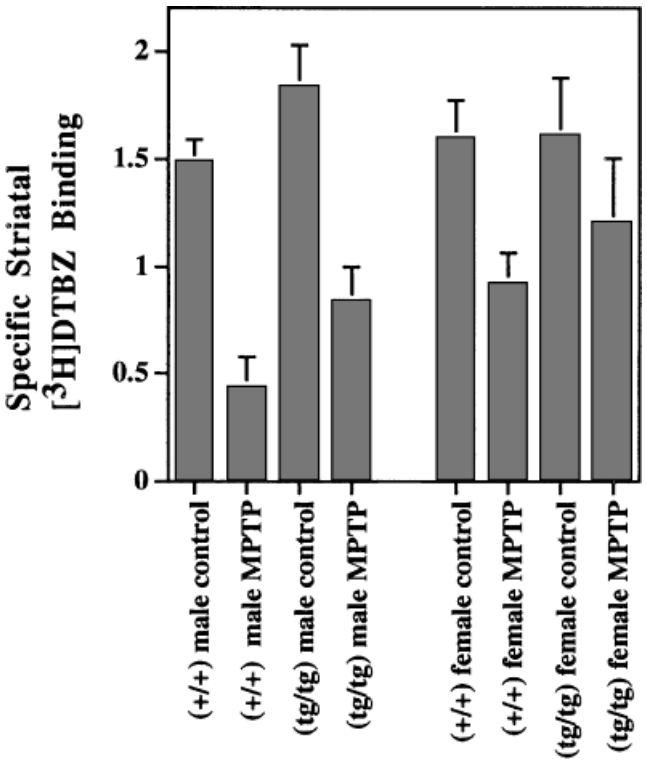

Fig. 1. Specific (+)-[^{3}H]DTBZ binding in striatum of control (no MPTP) and MPTP-trated mice. Specific binding was calculated as the ratio of tissue concentrations ratio, $[(\%ID/g_{striatum} - \%ID/g_{cerebellum})/\%ID/g_{cerebellum}]$. Data are shown as mean ± s.d.

controls). Finally, the MPTP-induced decreases in specific striatal (+)-[^{3}H]DTBZ binding in the *tottering* mice were smaller than the changes observed in the wild-type mice, and this was highly significant for males ($P < 0.001$) but did not quite reach significance for females ($P = 0.067$).

DISCUSSION

The vesicular monoamine transporter type 2 (VMAT2) is a specific marker of presynaptic monoaminergic nerve terminals. We (Frey et al., 1997; Vander Borght et al., 1995) and others (Henry and Sherman, 1989; Staley et al., 1997; Takahashi et al., 1997; Wilson et al., 1996) have utilized radioligand binding to this transporter as a measure of monoaminergic terminal integrity; in the striatum, due to the preponderance of dopaminergic projections, the measures of radioligand binding to VMAT2 reflect mainly the integrity of the dopaminergic system. In this study, we used the binding of the radioligand (+)-α-[^{3}H]DTBZ to this transporter as a measure of losses of terminals in specific brain regions of mice following a sequence of injections of the neurotoxin MPTP. The hypothesis being tested was that the excessive noradrenergic innervation of the tottering mouse brain, which should provide an accompanying increase in tissue concentrations of VMAT2,

would provide a protective effect against the neurotoxicity of MPTP.

In wild-type C57BL/6J mice, MPTP treatment produced a significant loss of in vivo striatal binding sites for (+)-[^{3}H]DTBZ. This loss, ranging from 57% in females to 71% in males, is in reasonable agreement with losses of tissue dopamine (Ali et al., 1993; Dluzen et al., 1996; Marien et al., 1993; Reinhard et al., 1988; Ricuarte et al., 1987a,b) and neuronal membrane dopamine transporters (Cline et al., 1992: Donnan et al., 1987; Kilbourn et al., 1991) measured after similar or identical MPTP treatment regimens. The greater neurotoxicity in male animals is consistent with the observation that female mice are less sensitive to the toxin (Dluzen et al., 1996; Freyaldenhoven et al., 1996; Miller et al., 1997), perhaps due to protective effects of estradiol.

In the *tottering* mice, the toxic effects of MPTP on binding of (+)-[^{3}H]DTBZ to VMAT2 were diminished, producing smaller losses of radioligand binding and, presumably, smaller losses of dopaminergic terminals. This protective effect was clearly significant in the male *tg/tg* mice, with an indication that the same was true for the female mice (although the data did not reach significance). The simplest explanation of our results is that the increased noradrenergic innervation of the tottering mouse brain results in increased numbers of VMAT2, and, by inference, increased numbers of synaptic vesicles and/or increased capacity of MPP^+ storage in the brain results in decreased toxicity of peripherally administered MPTP. This explanation fits the data obtained with the female *tottering* mice, where we consistently found increased concentrations of VMAT2 binding sites in many regions of the brains of both young mature (6–8 weeks: Kilbourn et al., 1995) and older (26–28 weeks, this study) *tottering* mice, as compared to age-matched wild-type C57BL/6 mice. In male mice, however, where the numbers of in vivo VMAT2 binding sites were equivalent or even slightly lower in most regions of the brains of *tottering* mice at 26–28 weeks of age (the higher specific binding ratio for the striatum is due to a lower cerebellar concentration, not increased striatal binding sites), the blunting of the toxicity of MPTP is not as easily explained. Due to the lack of available young male *tottering* mice, we were unable to determine if such animals exhibit increased (+)-[^{3}H]DTBZ binding, as do females of that age. The greater sensitivity of male mice (of either type) is likely not simply due to pharmacokinetics of the toxin, as no gender differences in striatal MPP^+ levels have been reported for single or repeated MPTP administrations to CD-1 mice (Miller et al., 1997).

In this study, we utilized an estimate of the specific binding of the radioligand (+)-[^{3}H]DTBZ in the striatum, calculated as a ratio of radiotracer concentrations in striatum and cerebellum; the latter is a region with a very low concentration of VMAT2 binding sites (Henry and Sherman, 1989; Vander Borght et al., 1995). Although calculations of ratios to areas which represent predominantly nonspecific binding is a common method for in vivo radiotracer studies, and we have shown that such ratios for (+)-[^{3}H]DTBZ reduce the variance in the data and produce good estimates of specific binding of this radioligand (Kilbourn and Sherman, 1997), it should be considered that unexpected changes in the region of the brain used as the denominator (in this instance, the cerebellum) can enhance or obscure changes in the specific binding of radioligand in the striatum. We therefore examined whether the decreases in the specific binding (the STR-CBL/CBL) ratio correlated with changes in striatal or cerebellar radiotracer concentrations. That analysis showed that the specific binding ratio correlated with the striatal ($r^2 = 0.68$) but not at all with the cerebellar concentrations ($r^2 = 0.02$) of radioactivity. Not surprisingly, then, use of simple striatal concentrations of radioligand binding as an alternate in vivo measure also indicates a protective effect of the *tottering* mutation; the striatal radioactivity concentrations represent total radioligand binding, a combination of specific (85%) and nonspecific (15%) binding of (+)-[^{3}H]DTBZ (Kilbourn et al., 1995). Although there is a potential for MPTP toxicity to monoaminergic nerve terminals in other regions of the mammalian brain, including the cerebellum, these treatments of C57BL/6 mice did not produce any clear changes in (+)-[^{3}H]DTBZ binding in any other regions which could be attributed to monoaminergic nerve terminal toxicity, and the use of the ratio method to estimate specific binding in the striatum appears justified.

Although a simple inverse relationship between concentrations of VMAT2 and MPTP toxicity is enticing, other possible explanations for the enhanced resistance of *tottering* mice need to be seriously considered. It has been reported that depletion of brain norepinephrine (NE) results in an enhancement of MPTP neurotoxicity in mice and primates (Bing et al., 1994; Marien et al., 1993), and an enhancement of methamphetamine neurotoxicity in mice and rats (Fornai et al., 1996). Prior lesioning with the neurotoxin DSP-4, which caused severe decreases (77–96%) in tissue norepinephrine levels in cortex, cerebellum, and hippocampus (regions innervated from the locus ceruleus), produced significantly greater MPTP-induced losses of striatal dopamine (~75% loss after DSP-4 + MPTP as compared to ~50% after MPTP alone (Fornai et al., 1997)). A clear explanation of the norepinephrine effect on MPTP toxicity is lacking; the DSP-4 treatment has no effect on the sparse concentration of NE terminals in the striatum, nor does the treatment with DSP-4 affect the pharmacokinetics of MPTP or MPP^+ in the striatum (Fornai et al., 1997). If, however, it is accepted that loss of NE exacerbates MPTP toxicity, then higher regional brain levels of norepinephrine found in the *tottering*

mouse brain (ranging from 125–300% of controls; Levitt and Noebels, 1981) might be responsible for reducing, in a manner yet unknown, the toxicity of MPTP in this mutant strain. The involvement, if any, of norepinephrine might be tested in future studies by treatment of *tottering* mice with the norepinephrine-depleting neurotoxin DSP-4 prior to administration of MPTP.

There may also be an effect due to differential handling of calcium ions in the *tottering* mice. As the genetic defect of the *tottering* mouse has been localized to a region encoding a calcium channel (Fletcher et al., 1996), it could be speculated that in these animals the genetic defect leads to a dysfunction of that calcium channel and perhaps differential handling of calcium by dopaminergic neurons that carry the α_{1A} channel subunit. Alterations of the neuronal calcium balance has also been postulated as part of the toxic cascade of MPP^+ action (Kass et al., 1988), and pretreatment of animals with calcium channel antagonists can diminish neurotoxic effects of MPTP (Gerlach et al., 1993; Kupsch et al., 1995). In the *tottering* mouse, then, it could be postulated that the neurological defect results in a beneficial effect in maintaining intracellular calcium balance. Finally, it must be recognized that the protective effects of the *tottering* mutation may lie in any one of a number of other biochemical abnormalities, known or unknown for this neurological mutant.

Our results showing decreases in MPTP toxicity in mice with monoaminergic hyperinnervation (and with likely higher levels of VMAT2), together with the prior pharmacological and transgenic mice studies showing enhanced MPTP toxicity in animals with fewer available vesicular monoamine transporters, would support the hypothesis that MPTP toxicity is influenced by the storage capacity of the monoaminergic vesicles. If we ignore for now the questions of the roles of norepinephrine and calcium channels in *tottering* mice, can differences in MPTP toxicity between strains, or between species, be so easily explained as simply differences in intracellular compartmentalization? We recently reported studies designed to evaluate the role of vesicular storage in the relative toxicity of MPTP toward different strains of mice. The brains of C57BL/6 and CD-1 mouse strains did not show any significant differences in the striatal concentrations of binding sites for radioligands specific for VMAT2 (Kilbourn and Frey, 1996), but studies have shown that the C57BL/6 strain is markedly sensitive to MPTP but that the CD-1 strain is much less affected (Freyaldenhoven et al., 1996; Giovanni et al., 1991; Sonsalla and Heikkila, 1988). Thus, the greater MPTP sensitivity of the C57BL/6 strain cannot be explained simply on the basis of levels of VMAT2, but may include an inability to handle the higher tissue levels of MPP^+ which, for as yet unexplained reasons, are found in the brains of the C57BL/6 strain of mouse (Giovanni et al., 1991). Similarly, differences in vesicular transporter numbers or function have been suggested as the reason for MPTP resistance of rats (Russo et al., 1994; Staal et al., 1997), with both a higher V_{max} for MPP+ transport and a higher number of (+)-[^{3}H]DTBZ binding sites reported in one study (Staal et al., 1997). In contrast, identical K_m and V_{max} values for synaptosomal uptake of MPP^+ were found in a second study comparing rats and two mouse strains (Sundstrom and Samuelsson, 1997), and we have found essentially identical numbers of in vitro striatal binding sites for (+)-[^{3}H]DTBZ in Sprague-Dawley rat (3.8 fmol/µg protein), C57BL/6 mouse (3.12 fmol/µg protein) and CD-1 mouse (3.22 fmol/µg) (Frey et al., 1997; Kilbourn and Frey, 1996). Although MPTP is not an effective systemic neurotoxin in rats, when injected directly into the striatum at a sufficient concentration MPP+ is clearly neurotoxic (Jenkins et al., 1996), likely through binding to complex I sites (Kilbourn et al., 1997). Thus, it remains unclear if changes in the numbers or functions of VMAT2 are the only factors responsible for species or strain sensitivity to MPTP. Neurological mutants such as the *tottering* offer one more model for the study of factors crucial for MPTP toxicity.

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