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The Role of Endogenous Serotonin in Methamphetamine-Induced Neurotoxicity to Dopamine Nerve Endings of the Striatum

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Abstract

Methamphetamine (METH) is a neurotoxic drug of abuse that damages the dopamine (DA) neuronal system in a highly delimited manner. The brain structure most affected by METH is the striatum where long-term DA depletion and microglial activation are maximal. Endogenous DA has been implicated as a critical participant in METH-induced neurotoxicity, most likely as a substrate for non-enzymatic oxidation by METH-generated reactive oxygen species (ROS). The striatum is also extensively innervated by serotonin (5HT) nerve endings and this neurochemical system is modified by METH in much the same manner as seen in DA nerve endings (i.e., increased release of 5HT, loss of function in tryptophan hydroxylase and the serotonin transporter, long-term depletion of 5HT stores). 5HT can also be modified by ROS to form highly reactive species that damage neurons but its role in METH neurotoxicity has not been assessed. Increases in 5HT levels with 5HTP do not change METH-induced neurotoxicity to the DA nerve endings as revealed by reductions in DA, tyrosine hydroxylase and dopamine transporter levels. Partial reductions in 5HT with p-chlorophenylalanine (PCPA) are without effect on METH toxicity, despite the fact that PCPA largely prevents METH-induced hyperthermia. Mice lacking the gene for brain tryptophan hydroxylase 2 are devoid of brain 5HT and respond to METH in the same manner as wild-type controls, despite showing enhanced drug-induced hyperthermia. Taken together, the present results indicate that endogenous 5HT does not appear to play a role in METH-induced damage to DA nerve endings of the striatum.

Keywords

methamphetamine; serotonin; dopamine; neurotoxicity; tryptophan hydroxylase 2; PCPA; 5HTP

Methamphetamine (METH) is a psychostimulant drug with high abuse potential. METH use continues at high rates and places extreme demands on numerous social, professional and law enforcement networks in response to the consequences associated with its abuse. Perhaps the most dangerous element associated with METH is its ability to cause persistent damage to the central nervous system in humans and in animal models of drug-induced

neuronal damage. The mechanisms that mediate METH-induced neurotoxicity are numerous and include elevated oxidative stress (Yamamoto and Bankson 2005, Cadet *et al.* 2007, Gibb *et al.* 1990, Fleckenstein *et al.* 1997b, Volz *et al.* 2007), mitochondrial dysfunction (Burrows *et al.* 2000, Brown *et al.* 2005, Tian *et al.* 2009) and inflammatory processes associated with microglial activation (Thomas *et al.* 2004b, Bowyer *et al.* 2008, Thomas *et al.* 2004a, LaVoie *et al.* 2004). The neurotoxic effects of METH are remarkably delineated and are most often associated with reductions in function of dopamine (DA) nerve endings of the striatum. However, more extensive damage to include neuronal death can also occur after high-dose METH intoxication (Cadet *et al.* 2007, Davidson *et al.* 2001, Cadet *et al.* 2003, Krasnova and Cadet 2009).

A great deal of effort has been directed at achieving a better understanding of the mechanisms by which METH damages the CNS. In consideration of the fact that DA nerve endings of the striatum are primary targets of METH-induced damage, and realizing the extreme manner by which METH disrupts DA homeostasis, DA itself has become a primary focus of attention. DA nerve endings comprise the majority of monoamine synaptic input to the striatum. METH leads to a release of DA from vesicle stores into the less protective environments of the cytoplasm and synaptic space where it is exposed to rapid non-enzymatic oxidation to several reactive species such as DA quinones (LaVoie and Hastings 1999, Kuhn *et al.* 2006). Changes in local presynaptic DA status can have powerful influences on METH toxicity. It is generally observed that treatments that shift DA from vesicles into the cytoplasm (e.g., reserpine) increase toxicity while reductions in the METH-releasable pool of DA (e.g., α -methyl-p-tyrosine) are neuroprotective (Albers and Sonsalla 1995, Thomas *et al.* 2008, Schmidt *et al.* 1985, Wagner *et al.* 1983, Thomas *et al.* 2009). A role for DA in mediating METH-induced toxicity has been challenged by studies showing that changes in core body temperature (Yuan *et al.* 2001), more than alterations in intracellular DA status, can explain modifications in METH neurotoxicity. It has also been suggested that norepinephrine (NE) input to the striatum can influence METH-toxicity. Reductions in NE levels have been shown to increase dramatically the damaging effects of METH on DA nerve endings (Weinshenker *et al.* 2008, Fornai *et al.* 1995, Fornai *et al.* 1999, Fornai *et al.* 1996), despite the fact that NE content of the striatum is extremely low (e.g., about 3–5% of DA).

The striatum contains a predominant input of serotonin (5HT) containing nerve endings from the midbrain raphe nuclei (Michelsen *et al.* 2007, Steinbusch 1981). Although 5HT synapses represent a significant portion of the monoamine input to the striatum, a role for 5HT in the neurotoxic properties of METH has received relatively little attention. This is surprising because METH is a powerful releaser of 5HT (Berger *et al.* 1992, Kuczenski *et al.* 1995, Wall *et al.* 1995, Cass 2000) and it disrupts presynaptic 5HT status in much the same manner seen for DA. 5HT nerve endings show persistent reductions in function after METH intoxication to include diminished levels of activity for tryptophan hydroxylase and the 5HT transporter (Fleckenstein *et al.* 2000, Hanson *et al.* 1998, Fleckenstein *et al.* 2007, Fleckenstein *et al.* 1997a). 5HT, like DA, can also serve as a substrate for attack by METH-generated reactive oxygen species (ROS) and the downstream products are known to exert damaging effects on neuronal tissue (Wrona and Dryhurst 1988, Wrona *et al.* 1986). For example, oxidation of 5HT by superoxide, nitric oxide or peroxynitrite can form tryptamine-4,5-dione (Wrona and Dryhurst 2001, Wrona *et al.* 1997a), a powerful inhibitor of TPH2 (Wrona and Dryhurst, 2001). Oxidation of 5HT by hydroxyl radical forms 5-hydroxy-3-ethylamino-2-oxindole and 5,6-dihydroxytryptamine (Wrona *et al.* 1995), both of which are potential 5HT neurotoxins, but these byproducts have not yet been detected in brain after high dose METH treatment (Yang *et al.* 1997). 5HT can also bind to and activate nitric oxide synthase and increase production of superoxide radical and hydrogen peroxide (Breard and Grillon 2009, Breard *et al.* 2007). Many of the treatments that profoundly alter

METH-toxicity to DA nerve endings of the striatum (e.g., reserpine) cause near-total depletion of 5HT as well, leaving open the possibility that 5HT could react with METH-generated reactive oxygen and nitrogen species to modulate drug-induced neurotoxicity. In light of the precedence implicating endogenous 5HT as a substrate for attack by ROS in the generation of neurotoxic species, the present studies investigated the possibility that 5HT participates in METH-induced neurotoxicity to DA nerve endings of the striatum.

Materials and methods

Materials

(+) methamphetamine hydrochloride, pentobarbital, 5HTP, carbidopa, Triton X-100, Tween 20, methanol, EDTA, all buffers, and HPLC reagents were purchased from Sigma-Aldrich (St. Louis, MO). Bicinchoninic acid protein assay kits were obtained from Fisher Scientific (Rockford, IL). Antibodies against TH (Kuhn and Billingsley 1987) were produced as previously described. Antibodies against DAT were generously provided by Dr. Roxanne Vaughn (University of North Dakota). HRP-conjugated secondary antibodies were obtained from Amersham (Piscataway, NJ) and Jackson ImmunoResearch Laboratories (West Grove, PA).

Animals

Female C57BL/6 mice (Harlan, Indianapolis, IN) and $TPH2^{+/+}$ and $TPH2^{-/-}$ mice (see below) weighing 20–25 g at the time of experimentation were housed 5 per cage in large shoe-box cages in a light (12 hr light/dark) and temperature controlled room. Mice had free access to food and water. The Institutional Care and Use Committee of Wayne State University approved the animal care and experimental procedures. All procedures were also in compliance with the NIH *Guide for the Care and Use of Laboratory Animals*.

Generation of mice with null-mutations in the gene for TPH2

A targeting vector was designed that deleted 105 bp of exon 1 coding sequence of TPH2, including the ATG initiation codon. This vector contained two negative selection cassettes (thymidine kinase and diphtheria toxin A) and a neomycin positive selection cassette. The vector was linearized and transfected into ES cells using standard protocols. Positive selection was achieved by incubation of transfected cells with G418 and resistant clones were isolated and screened for homologous recombination. Positive ES cell clones were confirmed by 5' and 3' screening (Southern blotting and PCR) and two of these were expanded for injection into C57BL/6J blastocysts. Upon injection, the blastocysts were re-implanted into pseudo-pregnant females and allowed to develop to term. Six highly chimeric males were generated and mated to wild-type C57BL/6J females. Numerous F1 offspring showed transmission of the mutant TPH2 allele to the germline as assessed by agouti coat color. The genotypes of these F1 offspring were confirmed as heterozygous ($TPH2^{+/-}$) and $TPH2^{+/-}$ males and females were set up as mating pairs to generate homozygous ($TPH2^{-/-}$), wild-type ($TPH2^{+/+}$) and $TPH2^{+/-}$ offspring. For further details, see Appendix S1.

Pharmacological and physiological procedures

Mice (wild-type and $TPH2^{-/-}$ mice) were exposed to a neurotoxic regimen of METH comprised of 4 injections of 5 mg/kg i.p. with a 2 hr interval between each injection. This METH regimen is known to cause microglial activation and DA nerve ending damage (Thomas *et al.* 2004b). To assess the effect of increases in the newly synthesized pool of 5HT on METH toxicity, mice were treated with 5HTP, the immediate precursor to 5HT, 1 hr before the first and third METH injections in a dose of 50 mg/kg along with carbidopa (25 mg/kg) to inhibit peripheral decarboxylase enzymes. PCPA, the irreversible inhibitor of

TPH (Koe and Weissman 1966), was used to reduce brain 5HT levels. PCPA was injected in a dose of 300 mg/kg once per day for 3 days prior to the neurotoxic regimen of METH. The last PCPA injection was given 24 hr before the first METH injection. Controls for drug pretreatments and METH received injections of physiological saline on the same schedule used for each respective compound. All injections were given via the i.p. route. Mice were sacrificed 48 hr after the last METH treatment. Body temperature was monitored by telemetry using IPTT-300 implantable temperature transponders from Bio Medic Data Systems, Inc. (Seaford, DE). Temperatures were recorded non-invasively every 20 min starting 60 min before the first METH injection and continuing for 10 hr thereafter using the DAS-5001 console system from Bio Medic.

Determination of striatal monoamine content

The present experiments were restricted to the determination of treatment effects on METH-induced toxicity to the DA system. Thus, striatal tissue was dissected bilaterally from brain 48 hr after treatments and stored at -80°C . Frozen tissues were weighed and sonicated in 10 volumes of 0.16 N perchloric acid at 4°C . Insoluble protein was removed by centrifugation and DA was determined by HPLC with electrochemical detection. In order to characterize the 5HT neurochemical phenotype of TPH2^{-/-} mice, the striatal levels of DA, 5HT and 5-hydroxyindole acetic acid (5HIAA) in TPH2^{-/-} mice and wild-type controls were determined by HPLC (Wolf *et al.* 1991). TPH2 enzyme activity was determined by measuring the formation of 5HTP from tryptophan by HPLC with fluorescence detection in an in vitro reaction as previously described (Kuhn *et al.* 2007). For additional information on the TPH2^{-/-} phenotype, see Appendix S1.

Determination of protein levels by immunoblotting

The effects of the pharmacological treatments on striatal TH and DAT levels were determined by immunoblotting as part of the characterization of toxicity to striatal DA nerve endings. Mice were sacrificed by decapitation 48 hr after treatments and striatum was dissected bilaterally. Tissue was stored at -80°C . Frozen tissue was disrupted by sonication in 1% SDS at 95°C and insoluble material was sedimented by centrifugation. Protein was determined by the bicinchoninic acid method and equal amounts of protein (70 $\mu\text{g}/\text{lane}$) were resolved by SDS-polyacrylamide gel electrophoresis and then electroblotted to nitrocellulose. Blots were blocked in Tris buffered saline containing Tween 20 (0.1% v/v) and 5% non-fat dry milk for 1 hr at room temperature. Primary antibodies were added to blots and allowed to incubate for 16 hr at 4°C . Blots were washed 3X in Tris-buffered saline to remove unreacted antibodies and then incubated with HRP-conjugated anti-IgG secondary antibody (1:4000) for 1 hr at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence and the relative densities of TH- and DAT-reactive bands were determined by scanning films on an Alpha Innotech (San Leandro, CA) AlphaImager HP using automatic spot detection. Immunoblotting results for TH and DAT were normalized to tubulin for quantitation of band densities. For further information on the TPH2^{-/-} phenotype, see Appendix S1.

Data analysis

The effects of drug treatments on striatal DA, TH and DAT content were tested for significance by ANOVA. Individual treatment groups were compared to appropriate controls with a one-way ANOVA followed by Tukey's Multiple Comparison Test in GraphPad Prism 5. Results from studies of METH-induced hyperthermia in wild-type versus TPH2^{-/-} mice were compared at individual times when core temperatures were maximally increased (i.e., 20–30 min after each drug injection) using a Student's T-test. Differences were considered significant if $p < 0.05$.

Results

Effect of increased 5HT content on METH-induced toxicity to striatal DA nerve endings

Mice were treated with 5HTP, the immediate precursor to 5HT, before the first and third injections of METH and animals were sacrificed 2 days later. The results in Fig. 1 show results of these treatments on striatal DA levels. It can be seen that METH lowered DA levels by 60–65%. Treatment of mice with 5HTP alone did not change striatal DA levels. Combined treatment of mice with METH + 5HTP caused the same reduction in striatal DA as seen in mice treated with METH alone. The effects of these same treatments on striatal levels of TH and DAT are presented in Fig. 2. It can be seen that METH caused significant reductions in TH (Fig. 2A) and DAT (Fig. 2B) protein levels (50% reductions for each). 5HTP alone did not change TH or DAT levels by comparison to controls and the combined treatment of 5HTP + METH had effects that were the same as those seen after METH alone. 5HTP alone did not change basal core body temperatures and METH hyperthermia was not altered by 5HTP as shown in Fig. 3.

PCPA-induced reduction in 5HT content and effects on METH-induced toxicity to striatal DA nerve endings

PCPA was injected once daily at a dose of 300 mg/kg for 3 days prior to the neurotoxic METH regimen. This treatment lowered striatal 5HT content by 60–70% at the time of the first METH injection. Results in Fig. 4 show that PCPA alone did not alter striatal DA content 2 days after treatments. Similarly, the METH-induced reduction in striatal DA (60–65%) was not modified by prior treatment with PCPA (55% reduction). The effects of these same treatments on striatal TH and DAT protein levels are presented in Fig. 5. In agreement with Fig. 2, only METH lowered TH (Fig. 5A) and DAT (Fig. 5B) levels by comparison to controls. PCPA had no effect of its own on TH and DAT and was likewise without effect on the METH-induced reductions in these proteins. PCPA alone did not change basal core body temperature but METH-induced hyperthermia was reduced significantly by PCPA (Fig. 6).

Mice lacking brain TPH2 and 5HT show the same METH-induced toxicity to striatal DA nerve endings as wild-type controls

Mice with a null mutation in the gene for TPH2 (Fig. S1) were devoid of brain TPH2, 5HT and 5HIAA and they lacked the ability to hydroxylate tryptophan (Table S1). Immunoblotting (Fig. S2) and immunohistochemical (Fig. S3) analyses confirmed a lack of TPH2 in brains of homozygous mice and established that 5HT neuronal processes remained intact. TPH2^{-/-} and wild-type controls were treated with the standard METH neurotoxic regimen and the response of the striatal DA system was assessed. The results in Fig. 7 show that METH reduced striatal DA levels to same extent in both genotypes, lowering wild-types to 32% of control and TPH2^{-/-} mice to 28% of control. The effect of METH on DA levels in wild-type and TPH2^{-/-} mice was not different ($p > 0.05$). Fig. 8 presents immunoblotting results for TH and DAT and show that METH-induced reductions in these proteins were the same in wild-type controls (Figs. 8A & B) and TPH2^{-/-} mice (Figs. 8C & D), respectively. Heterozygous mice (i.e., TPH2^{+/-}) were not different from wild-type with regard to striatal neurochemical status and their response to METH was likewise the same as wild-type controls (data not shown). Resting core body temperatures were slightly higher in TPH2^{-/-} mice by comparison to wild-type mice prior to initiation of drug treatments. TPH2^{-/-} controls showed a gradual reduction in core temperature (from approximately 38°C to 36°C over the 8 hour period of measurement), an effect that was slightly greater than seen in wild-type controls. METH caused significant hyperthermia in both wild-type and TPH2^{-/-} mice. The overall effect of METH on body temperature did not differ between wild-type and TPH2^{-/-} groups, but TPH2^{-/-} mice did show significant elevations (approximately 1°C

higher) by comparison to controls at the 20, 140 and 380 min time points ($p < 0.05$). These results are presented in Fig. 9.

Discussion

The rationale for testing endogenous striatal 5HT for its influence on METH-induced damage to DA nerve endings of the striatum is compelling. Striatal 5HT homeostasis is disrupted by METH in much the same manner as is seen for DA. An essential role for endogenous DA in METH-induced toxicity to the DA system has been established (Albers and Sonsalla 1995, Thomas *et al.* 2008, Schmidt *et al.* 1985, Wagner *et al.* 1983, Thomas *et al.* 2009) but a similar role for 5HT in this effect has scarcely been studied. The powerful release of 5HT from vesicles into the cytoplasm and the synapse under conditions of heightened oxidative stress caused by METH, would expose the neurotransmitter to a variety of reactants (e.g., superoxide, hydroxyl radical, nitric oxide, peroxynitrite, see Introduction) that could lead to the local production of numerous 5HT-derived neurotoxins (Wrona and Dryhurst 1988, Wrona and Dryhurst 1998, Wrona and Dryhurst 2001, Wrona *et al.* 1995, Wrona *et al.* 1997b). 5HT itself can even bind to nitric oxide synthase and increase its production of oxygen-based radicals (Breard and Grillon 2009, Breard *et al.* 2007). However, despite this strong precedence, the present data do not assign a role for endogenous 5HT in METH-induced toxicity to DA nerve endings of the striatum. Increases in 5HT levels using 5HTP do not modify METH-induced reductions in DA, TH or DAT, and leave METH-induced hyperthermia unchanged. Partial reductions in 5HT using PCPA are likewise without effect on reductions in DA, TH and DAT caused by METH. Mice lacking the gene for TPH2 are devoid of brain 5HT and TPH2 protein, and show the same METH-induced reductions in striatal DA, TH and DAT as seen in wild-type controls.

In light of the fact that METH can increase 5HT release in striatum, several groups have examined if blockade of the SERT or 5HT receptors modulates METH-induced DA neurotoxicity. Results with the SERT blocker fluoxetine show conflicting results with regard to METH-induced toxicity to the DA system. Fluoxetine has been shown to enhance (Ricaurte *et al.* 1983) or have no effect (Matuszewich and Yamamoto 2004) on METH-induced reductions in striatal DA. The fluoxetine-induced enhancement of toxicity was attributed to increased brain concentrations of METH (Ricaurte *et al.* 1983). Fluoxetine and other SERT inhibitors provide substantial protection against MDMA- (Schmidt and Taylor 1987, Li *et al.* 2010, Sanchez *et al.* 2001, Goni-Allo *et al.* 2006) and METH-induced (Rau *et al.* 2006) (Fleckenstein *et al.* 1997a) damage to the 5HT neuronal system. In contrast, fluoxetine does not protect against MDMA-induced deficits to the DA neuronal system (O'Shea *et al.* 2001) but it does partially prevent the malonate potentiation of MDMA reductions in DA (Goni-Allo *et al.* 2006). The possibility that 5HT released into the synapse by METH is contributing to DA neurotoxicity via 5HT receptor mechanisms has been ruled out as well by studies showing that 5HT₂ and 5HT_{3/4} receptor antagonists (Johnson *et al.* 1994, Johnson *et al.* 1988) fail to prevent drug-induced reductions in DA neurochemical function. In sum, it does not appear that the amount of 5HT in nerve endings and in the synaptic space exerts any influence on METH-induced toxicity to the striatal DA system.

A number of investigators have shown that reserpine leads to substantial increases in DA neurotoxicity after METH treatment and this effect has generally been attributed to alterations in DA homeostasis (Albers and Sonsalla 1995, Thomas *et al.* 2008, Thomas *et al.* 2009, Wagner *et al.* 1983, Yuan *et al.* 2001). Similarly, mice lacking the gene for VMAT2 show increased toxicity after METH treatment, effects attributed to reduced storage but increased release of DA (Fumagalli *et al.* 1999, Guillot *et al.* 2008). However, reserpine and null mutations in the gene for VMAT2 also disrupt 5HT homeostasis, making it difficult to attribute alterations in METH toxicity solely to DA. Our present results, and particularly

those in mice lacking TPH2 and brain 5HT, resolve this interpretational difficulty by showing that TPH2^{-/-} mice are equally susceptible to METH-induced toxicity to the DA system as are wild-type mice. Together, these results indicate that endogenous 5HT does not contribute to or influence METH toxicity to DA nerve endings of the striatum.

A role for endogenous NE in the toxic actions of METH on striatal DA nerve endings has also been indicated and derives from the general neuroprotective and antioxidant properties attributed to this monoamine (Heneka *et al.* 2002, Heneka *et al.* 2003, Kalinin *et al.* 2006). With regard to METH, Fornai and colleagues have shown that conditions associated with reduced striatal NE lead to substantial increases in drug-induced toxicity to DA nerve endings. These conditions include treatment of animals with DSP-4, a selective NE neurotoxin (Fornai *et al.* 1995, Fornai *et al.* 1999, Fornai *et al.* 1996), ablation of the gene for dopamine β -hydroxylase and inhibition of NE synthesis with the dopamine β -hydroxylase inhibitor fusaric acid (Weinshenker *et al.* 2008). The interpretational problems that arise from the use of reserpine and VMAT2 knockout mice mentioned previously also apply in the case of NE, because disruption of vesicle storage capacity severely depletes all monoamines including NE. The input of NE to the striatum is very small by comparison to DA and 5HT, comprising levels that are from 2–5% of the other monoamines, and dopamine β -hydroxylase levels in striatum are among the lowest in the CNS (Ross and Reis 1974). DSP-4 lesions of the locus coeruleus reduce striatal NE levels by about 30% (Fornai *et al.* 1999) indicating an extremely powerful influence of NE over the sensitivity of DA nerve endings to METH-induced toxicity. Unfortunately, our present approach cannot resolve the issue of whether NE depletion in reserpinized mice is the mechanism by which METH-toxicity is increased and remains an open question.

Many of the studies that have investigated the role of endogenous 5HT, DA or NE in METH-induced neurotoxicity depended by necessity on the use of drugs or neurotoxins to reduce the levels of the desired neurotransmitter. The non-specific nature of monoamine depletion by reserpine has already been discussed above. PCPA is another such drug and is the classical TPH inhibitor used to lower brain 5HT levels (Koe and Weissman 1966). PCPA is only moderately effective and useful in this regard because larger doses used to cause the greatest losses in 5HT also result in significant reductions in striatal DA [see for example (Dailly *et al.* 2006)]. PCPA is also a powerful inhibitor of liver phenylalanine hydroxylase (Koe and Weissman 1966) and will result in hyper-phenylalaninemia, a condition that could complicate its specificity as elevated levels of phenylalanine in blood could compete with other large neutral amino acids (e.g., tyrosine, tryptophan) for uptake into brain. The depletion of brain 5HT by PCPA is also relatively short-lived and in the doses used in our experiments (see Fig. 4), 5HT returns to untreated control levels in 1–2 days. The use of mice lacking the gene for TPH2 allows a superior approach to these types of experiments. The TPH2^{-/-} mice are shown presently to be devoid of TPH2 protein, tryptophan hydroxylating activity and 5HT using several complementary approaches. Supplementary results establish further that the expression of SERT in the TPH2^{-/-} mice is the same as wild-type controls, and TPH2^{-/-} mice also express NPT-2, the protein product of the neomycin resistance cassette used in the targeting vector for positive selection of homologous recombination events. These data establish that 5HT neurons are intact in TPH2^{-/-} mice but they totally lack their neurotransmitter content. TPH2^{-/-} mice showed similar psychostimulant responses to METH as wild-type controls (data not shown) and METH-induced toxicity within DA nerve endings of the striatum was the same as controls.

The responses of mice to METH neurotoxicity after dramatic shifts in the status of their brain 5HT content is all the more interesting when considered in light of the role of hyperthermia in amphetamine-induced neuronal damage. It is generally observed that the neurotoxicity caused by METH and MDMA is accentuated by elevated ambient or core

temperatures and prevented by low ambient temperature or by treatments that cause hypothermia (Yamamoto *et al.* 2010, Green *et al.* 2004). Nonetheless, it seems clear that this relation does not hold in the present experiments. Increases in 5HT with 5HTP injections do not change either METH-induced hyperthermia or neurotoxicity. The effects of 5HT reductions on METH toxicity are quite paradoxical. PCPA provides near-total protection against METH-induced hyperthermia but does not change its neurotoxicity. TPH2^{-/-} mice actually show significantly enhanced hyperthermia after 3 of the 4 METH injections (1°C above core temperatures measured in wild-type mice given METH) yet toxicity within striatal DA nerve endings was not changed. It is known that genetically modified mice lacking 5HT neurons (Hodges *et al.* 2008) or TPH2 (Alenina *et al.* 2009) have difficulty maintaining core temperature control and show rapid losses of body heat within 1–2 hours of a cold challenge. The present work suggests further that mice lacking brain 5HT also show exaggerated responses to at least drug-induced hyperthermic challenges.

While development and expansion of our TPH2^{-/-} colony was just underway, several other groups have published accounts of a similar mutant mouse line. Gutknecht *et al.* (Gutknecht *et al.* 2008) published a paper on a TPH2 knockout mouse that offered limited phenotype characterization. Savelieva and colleagues created knockout lines for TPH1, TPH2 and a double TPH1/TPH2 knockout, and these mice exhibited a relatively mild phenotype (Savelieva *et al.* 2008). More recently, Bader and colleagues described a TPH2 null mutant with more significant autonomic dysfunction and growth deficiencies (Alenina *et al.* 2009). Finally, Yadav *et al.* reported that brain derived 5HT was important in leptin regulation of bone mass and energy expenditure through the use of TPH2^{-/-} mice (Yadav *et al.* 2009). The fact that at least 5 independent groups are working with mice lacking TPH2 attests to the high level of interest and value in studying the molecular and neurobiological roles played by brain 5HT. Removal of the TPH2 gene generally results in > 95% reduction in brain 5HT content with varying effects on behavior and survival (Alenina *et al.* 2009, Yadav *et al.* 2009, Savelieva *et al.* 2008). Our TPH2^{-/-} mice were totally devoid of 5HT and tryptophan hydroxylating capability and their overall phenotype agrees most with the work presented by Alenina *et al.* (Alenina *et al.* 2009), particularly with regard to survival and growth retardation. In addition, the TPH2^{-/-} mice used presently and in other papers appear to have a fully intact 5HT neuronal system. This sets the TPH2^{-/-} mouse apart from mice with null mutations in the genes for *PET1* (Hendricks *et al.* 2003), *Lmx1b* and *Nkx2.2* (Cheng *et al.* 2003), all of which show severe reductions in CNS 5HT content because of a near-complete absence of central 5HT neurons. Work with the TPH2^{-/-} mouse has revealed some very interesting results and the present approach represents a new extension of this model organism into the study of psychostimulant and neurotoxic drugs of abuse.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used

5HT	serotonin
5HTP	5-hydroxytryptophan

5HIAA	5-hydroxyindole acetic acid
AUC	area under the curve
DA	dopamine
DAT	dopamine transporter
HRP	horseradish peroxidase
METH	methamphetamine
NPT-2	neomycin phosphotransferase-2
NE	norepinephrine
PBS	phosphate-buffered saline
PCPA	para-chlorophenylalanine
ROS	reactive oxygen species
TH	tyrosine hydroxylase
TPH2	tryptophan hydroxylase 2
SERT	serotonin transporter

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Fig. 1.

Effects of increases in 5HT on METH-induced depletion of striatal DA. Mice (N = 5–8/group) were treated with saline (control), 5HTP (50 mg/kg) + carbidopa (25 mg/kg), METH alone (4 injections of 5 mg/kg at 2 hr intervals) or with 5HTP + carbidopa 60 min before the first and third injections of METH and sacrificed 48 hr after the last injection. Striatal DA levels were determined by HPLC with electrochemical detection. Results are presented as mean $\pm$ SEM relative to controls. Differences between treatment groups were tested for significance by one-way ANOVA followed by Tukey's multiple comparison test using Graphpad Prism 5. *** indicates significantly different from control ($p < 0.001$).

Fig. 2.

Effects of increases in 5HT on METH-induced reductions in TH and DAT levels in striatum. Mice ($N = 5-8$ /group) were treated as described in the legend to Fig. 1 and striatal TH (A) and DAT levels (B) were determined by western blot analysis. Representative results from blots are included in each panel and samples for each subject in all groups were scanned and plotted in bar graph format. Results are presented as mean $\pm$ SEM. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparison test. *** indicates significantly different from control ($p < 0.001$).

Fig. 3.

Effects of increases in 5HT on METH-induced hyperthermia. Mice ($N = 5-8/\text{group}$) were treated as described in the legend to Fig. 1 and core body temperatures were recorded by telemetry at 20 min intervals for the indicated time period. Results are presented as group means. SEMs are omitted for the sake of clarity and were always $< 10\%$ of the respective mean values. Areas under the curve (AUC) for all treatment groups were calculated using GraphPad prism with respect to an arbitrary baseline set to 35°C for all groups. AUC values for the SHTP + carbidopa group (996) did not differ from controls (985) whereas groups treated with METH (1560) and SHTP/carbidopa + METH (1433) were not different from one another but differed significantly by comparison to controls ($p < 0.001$ for each).

Fig. 4. Effects of PCPA on METH-induced depletion of striatal DA. Mice (N = 5–8/group) were treated with saline (control), PCPA (300 mg/kg daily for 3d prior to the first METH injection), METH alone (4 × 5 mg/kg at 2 hr intervals) or with PCPA + METH. Mice were sacrificed 48 hr after the last injection of METH and striatal DA levels were determined by HPLC with electrochemical detection. Results are presented as mean ± SEM relative to controls. Differences between treatment groups were tested for significance by one-way ANOVA followed by Tukey's multiple comparison test using Graphpad Prism 5. *** indicates significantly different from control ($p < 0.001$).

Fig. 5.

Effects of PCPA on METH-induced reductions in TH and DAT levels in striatum. Mice ($N = 5-8/\text{group}$) were treated as described in the legend to Fig. 4 and striatal TH (A) and DAT levels (B) were determined by western blot analysis. Representative results from blots are included in each panel and samples for each subject in all groups were scanned and plotted in bar graph format. Results are presented as mean $\pm$ SEM. Significant differences were determined by one-way ANOVA followed by Tukey's multiple comparison test. Symbols (*, $p < 0.05$; ***, $p < 0.001$) indicate significant differences relative to the respective controls.

Fig. 6.

Effects of PCPA on METH-induced hyperthermia. Mice ($N = 5-8/\text{group}$) were treated as described in the legend to Fig. 4 and core body temperatures were recorded by telemetry at 20 min intervals. Results are presented as group means. SEMs are omitted for the sake of clarity and were always $< 10\%$ of the respective mean values. Areas under the curve (AUC) for all treatment groups were calculated using GraphPad Prism 5 with respect to an arbitrary baseline set to 35°C for each group. AUC values for the PCPA (1017) and PCPA + METH (928) groups did not differ from controls (944) whereas METH (1340) treated mice showed significant increases in core temperature by comparison to controls ($p < 0.001$).

Fig. 7.

Effects of METH on striatal DA levels in TPH2^{-/-} mice. Wild-type mice or mice lacking the gene for TPH2 (TPH2^{-/-}) were treated with 4 injections of METH (5 mg/kg at 2 hr intervals). Mice were sacrificed 48 hr after the last METH injection and striatal DA levels were determined by HPLC with electrochemical detection. Results are presented as mean $\pm$ SEM relative to controls. Differences between treatment groups were tested for significance by one-way ANOVA followed by Tukey's multiple comparison test using GraphPad Prism 5. *** indicates significantly different from the respective control ($p < 0.001$).

Fig. 8.

Effects of METH on striatal TH and DAT levels in $TPH2^{-/-}$ mice. Wild-type (A & B) or $TPH2^{-/-}$ (C & D) mice ($N = 5-8/\text{group}$) were treated with METH as described in the legend to Fig. 7 and striatal TH (A & C) and DAT levels (B & D) were determined by western blot analysis. Representative results from blots are included in each panel and samples for each mouse in all groups were scanned and plotted in bar graph format. Results are presented as mean $\pm$ SEM. Differences between treatment groups was tested for significance by one-way ANOVA followed by Tukey's multiple comparison test. Symbols (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$) indicate significant differences relative to the respective controls.

Fig. 9.

Effects of METH on core body temperature in TPH2^{-/-} mice. Wild-type or TPH2^{-/-} mice (N = 5–8/group) were treated as described in the legend to Fig. 7 and core body temperatures were recorded by telemetry at 20 min intervals. Results are presented as group means. SEMs are omitted for the sake of clarity and were always < 10% of the respective mean values. Areas under the curve (AUC) for all treatment groups were calculated using GraphPad Prism 5 with respect to an arbitrary baseline set to 35°C for each group. AUC values for untreated TPH2^{-/-} mice (927) did not differ from wild-type controls (1153) whereas wild-type (1707) and TPH2^{-/-} mice (1731) treated with METH were significantly higher ($p < 0.001$) than their respective controls. The effects of METH on core temperatures were significantly greater in TPH2^{-/-} mice by comparison to wild-type controls only at the 20, 140 and 380 min time points ($p < 0.05$; Student's T-test).