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The newly synthesized pool of dopamine determines the severity of methamphetamine-induced neurotoxicity

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Abstract

The neurotransmitter dopamine (DA) has long been implicated as a participant in the neurotoxicity caused by methamphetamine (METH), yet, its mechanism of action in this regard is not fully understood. Treatment of mice with the tyrosine hydroxylase (TH) inhibitor α -methyl-*p*-tyrosine (AMPT) lowers striatal cytoplasmic DA content by 55% and completely protects against METH-induced damage to DA nerve terminals. Reserpine, by disrupting vesicle amine storage, depletes striatal DA by more than 95% and accentuates METH-induced neurotoxicity. L-DOPA reverses the protective effect of AMPT against METH and enhances neurotoxicity in animals with intact TH. Inhibition of MAO-A by clorgyline increases pre-synaptic DA content and enhances METH striatal neurotoxicity. In all conditions of altered pre-synaptic DA homeostasis, increases or decreases in METH neurotoxicity paralleled changes in striatal microglial activation. Mice treated with AMPT, L-DOPA, or clorgyline + METH developed hyperthermia to the same extent as animals treated with METH alone, whereas mice treated with reserpine + METH were hypothermic, suggesting that the effects of alterations in cytoplasmic DA on METH neurotoxicity were not strictly mediated by changes in core body temperature. Taken together, the present data reinforce the notion that METH-induced release of DA from the newly synthesized pool of transmitter into the extracellular space plays an essential role in drug-induced striatal neurotoxicity and microglial activation. Subtle alterations in intracellular DA content can lead to significant enhancement of METH neurotoxicity. Our results also suggest that reactants derived from METH-induced oxidation of released DA may serve as neuronal signals that lead to microglial activation early in the neurotoxic process associated with METH.

Keywords

dopamine; methamphetamine; microglial activation; neurotoxicity; reserpine

Abuse of designer drugs such as methamphetamine (METH) and 3,4-methylenedioxymethamphetamine (MDMA) continues to increase at an alarming rate. These substituted amphetamine drugs cause persistent damage to dopamine (DA) and serotonin (5HT) nerve terminals in animal models of drug abuse (O'Callaghan and Miller 1994; Lyles and Cadet 2003; Fleckenstein *et al.* 2007), and human abusers of at least METH are now known

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to suffer neuronal damage as well (Thompson *et al.* 2004; Chang *et al.* 2007). The mechanisms by which these drugs cause neurotoxicity are not understood fully but considerable evidence points to oxidative stress and disruptions in mitochondrial function as likely mediators (Yamamoto and Bankson 2005). Emerging data is also implicating microglial activation in the toxic properties of METH (Bowyer *et al.* 1994; LaVoie *et al.* 2004; Thomas *et al.* 2004c; Thomas and Kuhn 2005a) and MDMA (Thomas *et al.* 2004a). Microglia are the primary antigen presenting cells in the CNS and they can serve immune-like functions to protect the brain from injury or invading pathogens (Streit 2002). However, under conditions that are not fully understood, microglia can become activated and release a variety of reactants that damage neurons (Block *et al.* 2007). In fact, activated microglia could be the source of virtually all reactant species that have been implicated in amphetamine-induced neurotoxicity including reactive oxygen (Cadet *et al.* 1994, 2007) and reactive nitrogen species (Imam *et al.* 2001). In light of results implicating microglial activation in the pathogenesis of neurological disorders, such as Parkinson's disease (Klegeris *et al.* 2007), Alzheimer's disease (Streit 2004), and multiple sclerosis (Cassiani-Ingoni *et al.* 2007), as well as in the neurotoxic actions of excitotoxins (Benkovic *et al.* 2004) and MPTP (Kim *et al.* 2007), additional studies on the mechanism by which METH causes microglial activation are clearly needed. A better understanding of how METH intoxication is linked to microglial activation would also help identify early steps in the toxic cascade associated with this drug of abuse.

It may seem paradoxical, but the neurotoxic effects of METH on DA nerve terminals have long been linked to DA itself. Wagner *et al.* (Wagner *et al.* 1983) first showed that depletion of brain DA with the tyrosine hydroxylase (TH) inhibitor α -methyl-*p*-tyrosine (AMPT) provided significant protection against drug-induced neurotoxicity. These early and important studies have been confirmed on numerous occasions (Schmidt *et al.* 1985; Albers and Sonsalla 1995). The mechanisms by which DA damages those neurons in which it serves as a neurotransmitter are not fully understood, but the protective effects of DA depletion do not appear to be linked exclusively to alterations in METH-induced hyperthermia (Albers and Sonsalla 1995), although one study to the contrary has been published (Yuan *et al.* 2001). Several related findings provide important clues for the role of DA in METH-induced neurotoxicity: (i) depletion of vesicle stores of DA with reserpine enhances METH-induced damage to the DA system (Wagner *et al.* 1983), (ii) reserpine causes a marked rise in 5-S-cysteinyl-DA levels in striatum, a marker for elevated production of DA quinones (Fornstedt and Carlsson 1989), (iii) METH results in a significant increase in 5-S-cysteinyl-DA levels (LaVoie and Hastings 1999) and 4) cysteinylcatechol conjugates can damage neurons (Montine *et al.* 1997; Spencer *et al.* 2002) and lead to microglial activation (Le *et al.* 2001; Thomas *et al.* 2006). We have extended this line of research presently by showing that depletion of the newly synthesized DA pool with AMPT protects against METH-induced neurotoxicity and totally prevents drug-induced microglial activation. Reserpine enhances METH-induced damage to DA nerve terminals and results in heightened microglial activation. L-DOPA reverses the protective effects of AMPT on METH and enhances METH-induced neurotoxicity and microglial activation in mice with intact TH. Clorgyline, which inhibits MAO-A in DA neurons and increases cytoplasmic DA levels, also significantly increases METH neurotoxicity and microglial activation. Collectively, these results show that the size of the newly synthesized pool of DA determines the severity of METH-induced neurotoxicity and they link METH-induced release of cytoplasmic DA into the synapse to microglial activation.

Materials and methods

Materials

(+) methamphetamine hydrochloride, pentobarbital, horseradish peroxidase (HRP)-conjugated Isolectin B₄ (from *Griffonia simplicifolia*), 3,3'-diaminobenzidine (DAB), L-DOPA, carbidopa,

clorgy-line, paraformaldehyde, Triton X-100, dopamine, methanol, EDTA, all buffers, and HPLC reagents were purchased from Sigma-Aldrich (St Louis, MO, USA). CitriSolv and Permout were products of Fisher Scientific (Pittsburgh, PA, USA).

Animals

Female C57BL/6 mice (Harlan, Indianapolis, IN, USA) weighing 20-25 g at the time of experimentation were housed five per cage in small shoe-box cages in a light 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*.

Pharmacological and physiological procedures

Mice were exposed to a neurotoxic regimen of METH comprised of four injections of 5 mg/kg i.p. with a 2 h interval between each injection. This METH regimen is known to cause microglial activation and DA nerve ending damage (Thomas *et al.* 2004c). To assess the role of DA in METH toxicity, mice were treated with various drugs that alter the size of the newly synthesized pool of DA. AMPT was injected i.p. in a dose of 100 mg/kg and was given 24, 16, 4, and 1 h before the METH regimen. Reserpine was injected i.p. in a dose of 2.5 mg/kg 24 h before METH. L-DOPA was injected 1 h 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. Clorgyline (10 mg/kg), an inhibitor of MAO-A, was injected 1 h before the METH regimen. Controls for drug pretreatments and METH received i.p. injections of physiological saline on the same schedule used for each respective compound. Mice were killed at various times after the METH regimen to assess the status of striatal DA and microglial activation (specified below for each experiment). Body temperature was monitored by telemetry using IPTT-300 implantable temperature transponders from Bio Medic Data Systems Inc. (Seaford, DE, USA). Temperatures were recorded every 60 min non-invasively using the DAS-5001 console system from Bio Medic.

Lectin histochemical staining of microglia

Microglial activation was assessed by staining fixed brain sections with HRP-conjugated Isolectin B₄ (ILB₄) as developed by Streit (1990) and as previously described in our studies with METH (Thomas *et al.* 2004c). At the time of killing, mice were deeply anesthetized with pentobarbital (120 mg/kg) and perfused transcardially with ice-cold 4% paraformaldehyde. Brains were removed and stored overnight in fixative at 4°C. Sections of 50 µm thickness were cut through the striatum (+1.2 through - 0.1 mm with respect to Bregma). Sections were floated into phosphate-buffered saline (PBS) containing 3% H₂O₂ for 30 min, washed once in PBS + 0.1% Triton X-100, then incubated in fresh PBS + 0.1% Triton X-100 for an additional 30 min. Microglia were labeled with HRP-conjugated ILB₄ (10 µg/mL in 0.1% Triton X-100) overnight at 4°C. Excess ILB₄ was removed by three washes with 0.1% PBS + Triton X-100 (5 min each) followed by a single wash in PBS before exposure to DAB substrate (0.1 mg/mL) in PBS for 25 min. Following a single wash in PBS, sections were transferred to glass slides, then air dried and dehydrated through a series of graded ethanol washes. Sections were incubated in Citrisolv for 5 min then coverslipped under Permout. For all pharmacological studies presented below, brain sections from drug-treated mice were processed simultaneously with controls to normalize staining among treatment groups. Microglial reactivity was viewed under the light microscope and the number of stained cells observed after various treatments was quantified using NIH Image. Cell counts were sampled from a 0.38 mm² area of the striatum by a person blinded to the treatment conditions. Cells were counted from three independent sections from all like-treated mice, bilaterally, generating an average count for each treated subject.

Striatal DA content

Depletion of striatal DA after METH treatment is widely used as an index of METH-induced toxicity to DA nerve endings. DA depletion from striatum faithfully reflects other measures of DA nerve ending damage caused by METH, such as reduced TH immunoreactivity or reduced ligand binding to the dopamine transporter. Striata were dissected from brain at the times listed above and stored frozen at -80°C. Tissues were weighed and sonicated in 10 vol of 0.16 N perchloric acid at 4°C. Insoluble protein was removed by centrifugation at 18 000 g and DA was determined by HPLC with electrochemical detection.

Data analysis

Individual treatment groups were compared to appropriate controls for DA and microglial counts with a one-way ANOVA followed by Tukey's Multiple Comparison Test in GraphPad Prism 5 (San Diego, CA, USA). The effects of drug treatments on core body temperature were analyzed by two-way ANOVA followed by Bonferroni's post-test. Differences were considered significant if $p < 0.05$. Unless otherwise indicated, all references to levels of significance in the results section were determined using ANOVA and Tukey's Multiple Comparison test.

Results

The AMPT treatment protocol used presently reduced striatal DA levels by about 55% at time 0 (i.e., immediately prior to initiation of METH treatment, see below), as shown in Fig. 1(a). Reserpine lowered DA content by 97% within 24 h of treatment. The effects of METH on striatal DA content were determined in mice pre-treated with saline (controls), AMPT, or reserpine. Mice were killed 2 days after the last METH injection, corresponding to the time at which drug-induced microglial activation is maximal (Thomas *et al.* 2004c), and the results are presented in Fig. 1(b). It can be seen that METH lowered DA by about 65% when given alone. The depleting effects of AMPT on striatal DA recover to normal within 2 days in controls as well as in mice subsequently treated with METH, demonstrating the protective effects of AMPT on METH-induced DA nerve terminal damage. In contrast to AMPT, the initial effect of reserpine on striatal DA recovers to approximately 78% of control within 2 days unless mice are treated with METH. In this case, the effect of METH on DA is accentuated in mice pre-treated with reserpine, with DA levels falling to < 5% of controls. When the post-METH survival time is increased from 2 to 7 days, to allow greater repletion of DA after AMPT or reserpine pre-treatment, the effect of METH treatment alone is not changed. Figure 1(c) shows that DA levels are reduced by 71% at 7 days after METH. As expected, striatal DA content in mice treated with AMPT alone or AMPT + METH are no different from controls at this time point. DA levels in mice treated with reserpine remain at about 74% of controls. However, mice treated with reserpine + METH show enhanced neurotoxicity and remain more than 97% depleted of DA at 7 days (Fig. 1c) and 14 days (data not shown) after treatment. The reduction in DA content caused by METH was significant by comparison to controls ($p < 0.01$) and the effect of reserpine + METH was significantly different from both controls ($p < 0.01$) and METH treatment alone ($p < 0.05$).

The effects of AMPT and reserpine on the METH-induced microglial activation at 2 days after METH are presented in Fig. 2. Neither AMPT nor reserpine treatment alone caused microglial activation in striatum at any survival time (data not shown). It can be seen in Fig. 2(b) that METH causes a significant increase in microglial activation 2 days after treatment by comparison to controls (Fig. 2a). Mice treated with AMPT + METH do not display increased microglial activation, in keeping with the ability of AMPT to prevent DA nerve ending alterations (see Fig. 1b and c, above). In contrast, mice treated with reserpine + METH show slightly higher levels of microglial activation (Fig. 1d) than those treated with METH alone. In agreement with previous findings on the time-course of METH-induced microglial

activation (Thomas *et al.* 2004c), it was observed that microglial activation has returned to control levels within 7 days of treatment with METH alone or reserpine + METH (data not shown). Increases in microglial activation in mice treated with METH or reserpine + METH were significant by comparison to controls ($p < 0.01$) and the effect of reserpine + METH was significantly different from METH alone ($p < 0.01$).

Considering the dramatically divergent effects of AMPT and reserpine on METH-induced neurotoxicity, the effects of drug treatments on body temperature were assessed and the data are presented in Fig. 3. METH caused its expected hyperthermic effect, increasing body temperature by 1-2°C within 1 h after the first injection and remaining relatively stable at this level throughout the ensuing 6-7 h. It is also apparent in Fig. 3 that body temperatures rise for about 60 min after METH injections and then fall to near controls over the next 60 min. This pattern repeats itself after each METH injection. Animals treated with AMPT alone did not differ from controls throughout the experiment and mice pre-treated with AMPT had normal body temperatures at the time when METH treatment was started. The initial response to METH in AMPT pre-treated mice was a 1-2°C drop in body temperature for approximately 2 h, after which mice remained slightly less hyperthermic than those treated with METH alone. Reserpine-treated mice showed a profound hypothermia at the time when METH treatment was initiated, with body temperatures 5-6°C below those of controls. The reserpine-only group remained hypothermic throughout the experiment. When METH was injected in reserpine pre-treated mice, body temperatures rebounded toward control within 60 min. Thereafter, body temperatures of mice treated with reserpine + METH were no different from those treated with METH alone. The reserpine effect on body temperature was the only treatment that differed significantly from controls ($p < 0.001$, two-way ANOVA followed by Bonferroni's post-test). To give an overall measure of the effects of drug treatments on body temperature throughout the entire experiment, areas under the curve were calculated (against a reference line at 31°C) for each treatment group. The resulting values were 3114 for controls, 3405 for METH, 3012 for AMPT, 810 for reserpine, 3042 for AMPT + zMETH, and 2946 for reserpine + METH, confirming that METH-treated mice showed an overall hyperthermic effect by comparison to controls whereas mice treated with reserpine + METH showed an overall hypothermic effect.

It was hypothesized that if the AMPT-mediated protection against METH resulted from a depletion of cytoplasmic DA, replacement of DA with L-DOPA treatment should override this effect. Results in Fig. 4(a) confirm the protective effect of AMPT shown in Fig. 1 above and establish further that administration of L-DOPA 1 h before the first and third METH injections overcomes the protective effect of AMPT on METH-induced depletion of DA seen 2 days after the last METH injection. Mice treated with METH alone were depleted of DA by about 65% whereas those treated with AMPT + L-DOPA before METH were depleted of DA by 90% (Fig. 4a). The effect of METH was significantly different from control ($p < 0.01$) and the effect of L-DOPA in mice treated with AMPT + METH was significantly different from controls and METH alone ($p < 0.01$). The effect of L-DOPA on METH toxicity was also tested in mice with normal TH activity (i.e., not treated with AMPT). L-DOPA increased striatal DA content by approximately 50% at the time when METH treatment was initiated (data not shown). The data in Fig. 4(b) show that L-DOPA, while having no effect on DA content at 2 days post-treatment, significantly increased the DA-depleting effects of METH. Mice treated with METH were depleted of DA by 67% whereas those treated with L-DOPA + METH were depleted of DA by 93% 2 days after treatment. Figure 4b also shows that the L-DOPA-induced enhancement of METH neurotoxicity was unabated at 7 and 14 days after treatment. The effect of L-DOPA to increase METH-induced depletion of DA was significantly different from both control ($p < 0.01$) and METH alone ($p < 0.01$) at all times. Mice treated with AMPT + L-DOPA + METH were not different in body temperature responses than those treated with AMPT + METH (data not shown).

Figure 5 shows the effects of L-DOPA on METH-induced microglial activation 2 days after treatment. Because L-DOPA, AMPT, and AMPT + METH did not differ from control with respect to microglial activation, these data are not included in Fig. 5. The profound extent of microglial activation caused by METH is evident in Fig. 5(b) and it is clear from Fig. 5(c) that L-DOPA overrides the protective effect of AMPT, leading to extensive microglial activation in striatum. L-DOPA also enhances microglial activation in mice with normal TH activity (Fig. 5d). Microglial activation was no longer apparent 7 or 14 days after treatments that caused activation at 2 days (data not shown). The effects of L-DOPA on METH-induced microglial activation in animals with inhibited or normal TH were increased significantly by comparison to controls ($p < 0.01$) and the effect of L-DOPA + METH was significantly different from METH alone ($p < 0.01$).

It is clear that alteration of pre-synaptic DA dynamics with AMPT, reserpine, or L-DOPA can lead to dramatic changes in the neurotoxicity caused by METH. The next experiment used a more subtle and specific approach to increase DA content, namely inhibition of MAO-A. Mice were treated with clorgyline (10 mg/kg) 1 h before the standard METH neurotoxic regimen and this increased striatal DA content by 12% at the time when METH treatment was initiated (data not shown). The effects of clorgyline and METH on DA levels 2 days after drug treatment are presented in Fig. 6. DA returned to control levels 2 days after clorgyline but when paired with METH, the depletion of DA (< 2% of control) was much greater than the effect of METH alone on DA (32% of control). It can also be seen in Fig. 6 that the clorgyline-induced enhancement of METH neurotoxicity persisted for 7 and 14 days after treatment. The effect of METH on DA content was significant by comparison to controls ($p < 0.01$) and the effect of clorgyline + METH was significantly different from controls ($p < 0.01$) and METH alone ($p < 0.01$) at all times. The effect of clorgyline on microglial activation paralleled its effects on striatal DA content. It can be seen in Fig. 7 that the MAO-A inhibitor alone did not change microglial activation from control levels but when given before METH, microglial activation was dramatically increased. The extent of microglial activation caused by clorgyline + METH was significantly higher than the effect of METH alone ($p < 0.01$), and both treatments caused significantly greater microglial activation than control or clorgyline alone ($p < 0.01$).

In light of the enhanced effects of L-DOPA and clorgyline on METH-induced DA loss, the effects of these treatments on core body temperature were recorded and the results are presented in Fig. 8. L-DOPA caused a modest drop in body temperature 60 min after the first injection and this effect dissipated to control levels thereafter. METH administration after L-DOPA caused a rapid reversal of the L-DOPA hypothermia, resulting in core temperatures that were about 2°C higher than controls and METH-treated mice. Core temperatures of mice treated with L-DOPA + METH gradually decreased over the entire recording session until they were just slightly below those of mice treated with METH alone at 9 h after treatments started. Clorgyline alone resulted in a mild hypothermia and when paired with METH, core temperatures increased gradually such that these animals were hyperthermic (by comparison to controls) from 4-8 h after the start of treatment. The clorgyline + METH group was the only treatment that was significantly different from control and clorgyline only conditions ($p < 0.001$ for each, two-way ANOVA followed by Bonferroni's post-test). Areas under the curve were calculated (against a reference line at 31°C) for each treatment group as described above for Fig. 3. The resulting values were 3774 for controls, 4027 for METH, 3776 for L-DOPA, 3592 for clorgyline, 4201 for L-DOPA + METH, and 4248 for clorgyline + METH. These results confirm that METH alone and paired with clorgyline or L-DOPA caused an overall hyperthermia by comparison to controls. Clorgyline and L-DOPA alone did not differ from controls over the full extent of the experiment.

Discussion

METH intoxication causes persistent reductions in function of DA nerve terminals. The mechanisms by which METH exerts its highly selective neurotoxic effects are not fully understood, but oxidative stress and mitochondrial dysfunction are likely candidates (Yamamoto and Bankson 2005; Cadet *et al.* 2007; Fleckenstein *et al.* 2007). Emerging research has also linked METH-induced neurotoxicity to microglial activation. Numerous genes whose expression is increased by METH within 2-4 h after treatment suggest the involvement of processes often linked to microglial activation (Thomas *et al.* 2004b). In fact, the time-course, dose-response, and pharmacological profile of microglial activation caused by the substituted amphetamines suggest that microglial activation contributes to METH toxicity and is not merely responding to nerve terminal damage (LaVoie *et al.* 2004; Thomas *et al.* 2004a,c). What is more, drugs that block microglial activation (Thomas and Kuhn 2005b), and tolerance conditions that lead to a blunting of microglial activation (Thomas and Kuhn 2005a) are protective against METH-induced neurotoxicity. DA has been established as an essential component in the neurotoxic effects of METH (Schmidt *et al.* 1985) and this fact may contribute a great deal to the remarkable specificity associated with METH neurotoxicity. In light of the possibility that microglial activation is an early manifestation of the METH toxic cascade, we have attempted to link DA to drug-induced microglial activation through the present experiments.

AMPT blocks the *de novo* synthesis of DA by inhibiting TH and it increases DA transport by the vesicle monoamine transporter (Brown *et al.* 2001) both of which lead to reductions in the newly synthesized (or cytoplasmic) pool of neurotransmitter. The effect of AMPT is reversible and short-lived, but if METH is administered while cytoplasmic DA is maximally depleted (i.e., to approximately 50% of control), its neurotoxic effects are prevented. The recovery of DA to normal levels after AMPT administration is complete at 2 and 7 days, and this repletion of DA is not altered by METH as shown in Fig. 1. Reserpine causes the near-total loss of DA from striatum via its disruption of vesicle storage but it leaves DA synthetic capacity intact. The recovery of DA after reserpine administration is slower than AMPT and reaches 75-80% of control at 2-7 days after treatment. However, we found that the neurotoxic effects of METH are accentuated if it is injected at the time when the DA depleting effects of reserpine are maximal (see Fig. 1). The effects of AMPT and reserpine on METH-induced microglial activation mirrored what was observed at the neurochemical level. The robust activation of striatal microglia normally seen after METH administration was prevented in mice treated with AMPT, reflecting the protective effects of this treatment on METH-induced neurotoxicity. Reserpine caused greater depletions of DA than AMPT and resulted in a greater extent of microglial activation.

Additional attempts to relate disruptions in pre-synaptic DA homeostasis to METH-induced neurotoxicity involved treatments with L-DOPA, the immediate precursor of DA. By itself, L-DOPA causes a transient increase in brain DA, and this effect neither leads to persistent deficits in DA nor does it cause microglial activation. On the other hand, when L-DOPA is administered to AMPT-treated mice to restore cytoplasmic DA levels, the protective effects of AMPT on METH-induced neurotoxicity are reversed. The same dose regimen of L-DOPA given to mice with normal TH activity causes a significant enhancement of METH neurotoxicity and microglial activation. A more subtle and specific approach to increase pre-synaptic DA was undertaken by treating mice with clorgyline, an inhibitor of MAO-A and the form of MAO that is preferentially located in DA neurons. Clorgyline alone did not cause persistent alterations in striatal DA content nor did it provoke microglial activation. However, clorgyline led to a significant increase in METH-induced DA depletion and microglial activation.

The results of the present line of investigation show that pharmacological alterations in the dynamics of the newly synthesized pool of DA can significantly alter the neurotoxicity associated with METH intoxication. Removal of cytoplasmic DA with AMPT, while leaving vesicular stores intact, completely prevents both METH-induced damage to DA nerve endings and activation of microglia. L-DOPA replenishes cytoplasmic DA and overrides the protective effect of AMPT and it also enhances METH toxicity in mice with intact TH. It is generally accepted that the amphetamine drugs, including METH, release DA from vesicles into the cytoplasm via a weak base effect (Sulzer *et al.* 2005), but it must be the case that METH-induced release of DA from vesicles into the cytoplasm in AMPT treated mice does not sufficiently increase DA release into the synapse to cause toxicity. L-DOPA restores cytoplasmic DA content in AMPT-treated mice and increases cytoplasmic DA content in normal mice sufficiently to enhance METH-induced neurotoxicity and microglial activation. Clorgyline, by inhibiting the catabolism of pre-synaptic DA by MAO-A, also increases cytoplasmic DA content and leads to greater METH neurotoxicity and microglial activation. Collectively, these results suggest that METH toxicity is determined by drug-induced release of DA from the newly synthesized cytoplasmic pool into the extracellular space. When DA levels in this pool are very low (i.e., AMPT), toxicity is prevented and when levels are normal or increased (i.e., L-DOPA or clorgyline) toxicity is enhanced. Results from studies showing reserpine-induced enhancement of METH neurotoxicity may seem paradoxical but they are consistent with the rationale that cytoplasmic DA is the most critical source of DA in mediating METH neurotoxicity. The amphetamine-induced release of DA is generally attributed to the newly synthesized pool of transmitter (Sulzer *et al.* 2005) and it is well known that the DA-releasing effects of the amphetamines are prevented by AMPT but not by reserpine (e.g., Butcher *et al.* 1988) or reductions in VMAT2 protein (Fon *et al.* 1997).

Our present findings that changes in pre-synaptic DA synthesis and storage can substantially modify the neurotoxicity associated with METH agree very well with previously published literature. Wagner *et al.* (1983) were the first to show that AMPT prevents METH neurotoxicity and that reserpine accentuated it. These findings were attributed to the ability of METH to mobilize DA release preferentially from the cytoplasmic pool (Wagner *et al.* 1983). Schmidt *et al.* (1985) also showed that AMPT could protect against METH and this protective effect was overcome by L-DOPA (Schmidt *et al.* 1985). Marshall and colleagues have emphasized that the binge method of METH administration as used presently (i.e., four injections with a 2 h interval between injections), causes a marked increase in the release of DA after the 4th injection (500-3600% of basal) and an ensuing neurotoxicity, effects not seen after just three injections (O'Dell *et al.* 1991). These investigators also showed that L-DOPA substantially potentiates METH-induced DA release and enhances long-term reductions in DA caused by a single injection of METH more than sixfold (Weihmuller *et al.* 1993). A limited set of experiments has also established that MAO inhibitors increase the magnitude of METH-induced DA depletion (Wagner and Walsh 1991; Kita *et al.* 1995). Mice lacking the VMAT2 show heightened neurotoxicity in response to METH (Fumagalli *et al.* 1999; Larsen *et al.* 2002), in agreement with findings that reserpine enhances METH toxicity. Taken together, METH-induced release of DA from the newly synthesized pool of transmitter into the synaptic space appears to be essential for subsequent development of striatal neurotoxicity and microglial activation after METH intoxication.

These results notwithstanding, the precise role of DA in METH neurotoxicity remains somewhat controversial. One unresolved issue relates to whether released (i.e., extracellular) or intracellular pools of DA contribute to METH neurotoxicity. For example, LaVoie and Hastings (1999) observed that treatment of rats with METH at a very low ambient temperature (i.e., 5°C) prevented drug-induced toxicity and formation of DA oxidation products. Because METH-induced DA release was not reduced at low ambient temperature, these investigators concluded that extracellular DA did not play a role in METH neurotoxicity. However,

considering that hypothermia is neuroprotective against oxidative stress (Hsu *et al.* 2006) and free radical formation (Zhao *et al.*, 2007), and would likely prevent the formation of DA oxidative products that are thought to play a role in METH neurotoxicity, the conclusions of LaVoie and Hastings (1999) that extracellular DA does not play a role in METH toxicity may be premature.

Another important determinant of the role of DA in METH toxicity is core temperature changes caused by METH and challenge drugs used to modify its toxicity. For instance, Yuan *et al.* (2001) reported that the protective effect of AMPT against METH was prevented if drug administration was carried out at the extremely high ambient temperatures of 33°C to prevent treatment-induced hypothermia, leading these investigators to conclude that endogenous DA does not play any role in METH-induced DA neurotoxicity (Yuan *et al.* 2001). Interpretation of results from studies where the effects of METH and other treatments on core temperature are prevented by increasing ambient temperature is made quite difficult in light of the intense stress associated with these conditions. In fact, exposure of animals to high ambient temperature induces substantial physiological and gene expression changes not seen at normal temperatures (and without drug treatments), provoking a set of symptoms referred to collectively as the heat shock response (Brown 1990; Horowitz and Robinson 2007). The effects of METH on the heat shock response are not known but it is difficult to rule out a role for DA in METH neurotoxicity based primarily on studies carried out at high ambient temperature. An indication of the extreme stress caused by combining multiple drug treatments with elevated ambient temperature is evident in the Yuan *et al.* (2001) study. These investigators reported that lethality resulting from treatment of rats with AMPT, reserpine, and METH increased from 17% to 70% when ambient temperature was increased from 28°C to 33°C (Yuan *et al.* 2001). In the present work, treatment of reserpinized mice with METH led to a significant hypothermia, an effect that would lead to the expectation of neuroprotection if METH toxicity was primarily mediated by hyperthermia. Our findings agree very well with the results of Albers and Sonsalla (1995) who also showed that reserpine enhanced METH neurotoxicity while making rats hypothermic, and they indicate that hyperthermia is not solely responsible for METH toxicity. However, the early changes in body temperature are interesting and somewhat predictive of the ensuing effects of treatments on METH neurotoxicity. For example, AMPT protects against METH neurotoxicity and causes an initial drop in body temperature. Treatments that enhance METH neurotoxicity (i.e., reserpine, L-DOPA, and clorgyline) cause an early increase in body temperature. Other literature precedents regarding the actions of reserpine make its current interactions with METH seem counterintuitive. For example, reserpine prevents malonate-induced striatal neurotoxicity (Moy *et al.* 2000; Xia *et al.* 2001), blocks METH-induced formation of inclusion bodies (Fornai *et al.* 2003), and protects against MDMA neurotoxicity (Yuan *et al.* 2002), effects that are thought to be DA-dependent. Reserpine also significantly reduces brain uptake of METH (Inoue *et al.* 1990). The fact that reserpine enhances METH-induced neurotoxicity emphasizes the central role played by the newly synthesized pool of DA from which METH release occurs to cause neurotoxicity.

It does not seem likely that DA itself, whether in intracellular or extracellular pools, is contributing to METH-induced neurotoxicity. Normal DA neurotransmission and receptor-mediated signal transduction is not neurotoxic. Reserpine dramatically alters intracellular DA homeostasis and storage while leaving synthesis intact, but does not itself cause neurotoxicity. Drugs that increase synaptic DA levels by blocking uptake (e.g., cocaine, nomifensine) are not neurotoxic when given alone and, in fact, prevent METH-induced neurotoxicity (Fleckenstein *et al.* 2007). It is more likely that METH creates conditions in brain that lead to the heightened formation of DA breakdown products and it is these reactants that mediate at least some of the neurotoxic effects of METH. One downstream DA by-product that is a strong candidate for this role is DA quinone. Non-enzymatic degradation of DA can result in the increased

production of reactive oxygen species and 5-S-cysteinyl-DA, a marker for the formation of DA quinones. DA quinone production is increased by METH (LaVoie and Hastings 1999) and it is known that cysteinylcatechol conjugates can cause many effects seen after METH administration to include modification and inhibition of TH (Kuhn *et al.* 1999) and DA transporter function (Whitehead *et al.* 2001; Park *et al.* 2002), and ultimately, damage to neurons (Montine *et al.* 1997; Spencer *et al.* 2002). DA quinones are also powerful activators of microglia (Le *et al.* 2001; Thomas *et al.* 2006).

In summary, it is clear that subtle alterations in the size of the newly synthesized pool of DA can dramatically influence the neurotoxicity and microglial activation associated with METH. With all data considered, it appears that METH preferentially releases DA from the newly synthesized pool of neurotransmitter. As a result of METH-induced inhibition of DA uptake, synaptic levels of DA are increased and the transmitter is exposed to heightened non-enzymatic degradation. Exposure of extracellular DA to reactive species, such as nitric oxide, hydroxyl radical, and peroxynitrite, all have been implicated in METH toxicity (Yamamoto and Bankson 2005; Cadet *et al.* 2007; Fleckenstein *et al.* 2007), will convert DA to the quinone species (Park *et al.* 2003; Kuhn *et al.* 2004). DA quinones then provoke the earliest steps in the cascade leading to nerve ending damage by causing extensive microglial activation and the ensuing production of numerous oxidative reactants and proinflammatory species. It is certainly possible that activated microglia are the source of the reactive oxygen and nitrogen species that have been linked to METH toxicity. In this scheme, activated microglia are not the sole cause of nerve ending damage, but serve as participants in a gradual process of glial-neuronal crosstalk that is initiated by METH-induced disruptions in pre-synaptic DA homeostasis.

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Abbreviations used

5-HT, serotonin; AMPT, α -methyl-p-tyrosine; DA, dopamine; METH, methamphetamine; PBS, phosphate-buffered saline; TH, tyrosine hydroxylase.

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Fig. 1.

Effects of AMPT and reserpine on DA depletion caused by a neurotoxic METH regimen. Mice ($n = 5-8$ per group) were treated with AMPT (4×100 mg/kg; $t = -24, -16, -4$ and -1 h) or reserpine (2.5 mg/kg; $t = -24$ h) alone, and before a neurotoxic METH regimen (4×5 mg/kg; $t = 0, 2, 4$, and 6 h). Striatal DA levels were determined at (a) $t = 0$, (b) $t = 2$ days and (c) $t = 7$ days. Results are presented as mean $\pm$ SEM relative to controls. Significant differences were determined via one-way ANOVA followed by Tukey's multiple comparison test, and are indicated as follows: * $p < 0.01$ relative to control (CON); # $p < 0.01$ relative to METH; ^ $p < 0.05$ relative to METH.

Fig. 2.

Effects of AMPT and reserpine on microglial activation caused by a neurotoxic METH regimen. Mice ($n = 3-5$ per group) were treated as described in Fig. 1 and analyzed for microglial activation in the striatum 2 days after the last METH injection. Microglia counts were obtained as described in the Materials and methods and are presented as means $\pm$ SEM. Treatment conditions and microglia counts for each panel are (a) Control (14 ± 1), (b) METH (142 ± 5), (c) AMPT + METH (18 ± 2) and (d) Reserpine + METH (179 ± 4). Significant differences were determined via oneway $_{ANOVA}$ followed by Tukey's multiple comparison test: $p < 0.01$, METH and Reserpine + METH relative to control; Reserpine + METH relative to METH. Scale bar represents $150 \mu\text{m}$.

Fig. 3.

Effects of a neurotoxic METH regimen on core body temperatures of mice pre-treated with AMPT or reserpine. Mice ($n = 5$ per group) were treated as indicated, and core body temperatures monitored by telemetry every hour for 8 h. Results are presented as mean body temperature ($^{\circ}\text{C}$) for each group at the indicated time points. METH was administered at $t = 0, 120, 240$, and 360 min. SEM bars were omitted for the sake of clarity and were $< 10\%$ of the mean in all groups. The reserpine group is the only statistically significant treatment effect overall ($p < 0.0001$, two-way ANOVA), and was also different from all other conditions ($p < 0.01$, Bonferroni's post-test).

Fig. 4.

Effect of L-DOPA on DA depletion caused by a neurotoxic METH regimen. L-DOPA was administered to (a) AMPT treated and (b) non-AMPT treated mice ($n = 5-8$ per group) to assess its effect on METH-induced DA depletion. Striatal DA levels were determined 2, 7, or 14 days after the METH regimen. Results are presented as mean $\pm$ SEM relative to controls. Significant differences were determined via one-way ANOVA followed by Tukey's multiple comparison test, and are indicated as follows: * $p < 0.01$ relative to control (CON); # $p < 0.01$ relative to METH.

Fig. 5.

Effects of *l*-DOPA on microglial activation caused by a neurotoxic METH regimen. Mice ($n = 3-5$ per group) were treated as described in Fig. 4 and analyzed for microglial activation in the striatum 2 days after the last METH injection. Microglia counts were obtained as described in the Materials and Methods and are presented as means $\pm$ SEM. Treatment conditions and microglia counts for each panel are (a) Control (17 ± 2), (b) METH (155 ± 10), (c) AMPT + *l*-DOPA + METH (171 ± 4) and (d) *l*-DOPA + METH (178 ± 3). Significant differences were determined via one-way ANOVA followed by Tukey's multiple comparison test: $p < 0.01$, all conditions relative to control; $p < 0.05$ *l*-DOPA + METH relative to METH. Scale bar represents 150 μm .

Fig. 6.

Effects of clorgyline on DA depletion caused by a neurotoxic METH regimen. Mice ($n = 5-8$ per group) were treated with clorgyline (10 mg/kg) alone, and 1 h before a neurotoxic METH regimen. Striatal DA levels were determined 2, 7, or 14 days after the METH regimen. Results are presented as mean $\pm$ SEM relative to controls. Significant differences were determined via one-way ANOVA followed by Tukey's multiple comparison test, and are indicated as follows:

* $p < 0.01$ relative to control (CON); # $p < 0.01$ relative to METH.

Fig. 7.

Effects of clorgyline on microglial activation caused by a neurotoxic METH regimen. Mice ($n = 3-5$ per group) were treated as described in Fig. 6 and analyzed for microglial activation in the striatum 2 days after the last METH injection. Microglia counts were obtained as described in the Materials and Methods and are presented as means $\pm$ SEM. Treatment conditions and microglia counts for each panel are (a) control (15 ± 1), (b) METH (149 ± 5), (c) clorgyline (21 ± 2) and (d) clorgyline + METH (186 ± 9). Significant differences were determined via one-way ANOVA followed by Tukey's multiple comparison test: $p < 0.01$, METH and clorgyline + METH relative to control; $p < 0.01$, clorgyline + METH relative to METH. Scale bar represents 150 μm .

Fig. 8.

Effects of a neurotoxic METH regimen on core body temperatures of mice pre-treated with clorgyline or L-DOPA. Mice ($n = 5$ per group) were treated as indicated, and core body temperatures monitored by telemetry every hour for 8 h. Results are presented as mean body temperature ($^{\circ}\text{C}$) for each group at the indicated time points. Clorgyline was administered at $t = 0$; METH was administered at $t = 60, 180, 300$ and 420 min. SEM bars were omitted for the sake of clarity and were $< 10\%$ of the mean in all groups. The clorgyline + METH group is the only statistically significant treatment effect overall ($p < 0.001$, two-way ANOVA), and is also different from control and clorgyline only conditions ($p < 0.01$, Bonferroni's post-test). Note that the ordinate scale used in this figure is expanded by comparison to Fig. 3 to make viewing of all body temperature traces easier.