Enhancement of 3,4-methylenedioxymethamphetamine neurotoxicity by the energy inhibitor malonate

W. L. Nixdorf,* K. B. Burrows,* G. A. Gudelsky† and B. K. Yamamoto*

*Program in Basic and Clinical Neuroscience, Department of Psychiatry, Case Western Reserve University, Cleveland, USA
†College of Pharmacy, University of Cincinnati, Ohio, USA

Abstract
The acute and long-term effects of the local perfusion of 3,4-methylenedioxymethamphetamine (MDMA) and the interaction with the mitochondrial inhibitor malonate (MAL) were examined in the rat striatum. MDMA, MAL or the combination of MAL with MDMA was reverse dialyzed into the striatum for 8 h via a microdialysis probe while extracellular dopamine (DA) and serotonin (5-HT) were measured. One week later, tissue immediately surrounding the probe was assayed for DA and 5-HT tissue content. Local perfusion of MDMA increased DA and 5-HT release but did not produce long-term depletion of DA or 5-HT in tissue. Malonate also increased both DA and 5-HT release but, in contrast to MDMA, produced only long-term depletion of DA. The combined perfusion of MDMA/MAL synergistically increased the release of DA and 5-HT and produced long-term depletion of both DA and 5-HT in tissue. These results support the conclusion that DA, compared with 5-HT, neurons are more susceptible to mitochondrial inhibition. Moreover, MDMA, which does not normally produce DA depletion in the rat, exacerbated MAL-induced DA depletions. The effect of MDMA in combination with MAL to produce 5-HT depletions suggests a role for bio-energetic stress in MDMA-induced toxicity to 5-HT neurons. Overall, these results highlight the importance of energy balance to the function of DA and 5-HT neurons and to the toxic effects of MDMA.

Keywords: malonate, MDMA, mitochondrial inhibition, microdialysis.


3,4-Methylenedioxymethamphetamine (MDMA) is a substituted amphetamine that has recently gained popularity among drug users (Kozel 1997). Although structurally similar to methamphetamine (METH), MDMA produces different pharmacological effects (Seiden and Sabol 1996). MDMA selectively targets serotonin (5-HT) neurons and in high doses, produces selective long-term depletion of rat brain 5-HT (Ricaret et al. 1985; Stone et al. 1986). It also decreases tryptophan hydroxylase (Stone et al. 1986) and 5-HT uptake sites (Battaglia et al. 1987) without causing changes in dopaminergic neuronal markers. However, the exact mechanisms that mediate the toxic effects of MDMA on 5-HT neurons remain to be elucidated.

There are several lines of evidence to suggest that alterations in energy metabolism might be involved in the toxic actions of the amphetamines. Repeated high doses of MDMA acutely decrease mitochondrial cytochrome oxidase activity (Burrows et al. 2000). In addition, the systemic administration of MDMA or METH produces hyperthermia, a critical mediator of the neurotoxicity produced by these drugs (Bowyer et al. 1994; Albers and Sonsalla 1995; Miller and O’Callaghan 1995; Malberg and Seiden 1998). Both hyperthermia and p-amphetamine also enhance energy utilization more than production (Nilsson et al. 1975; Nowak 1988). Moreover, MDMA appears to produce bio-energetic stress by increasing glycogen breakdown in astroglial-rich cell cultures (Poblete and Azmitia 1995). Prolonged glycogen phosphorylase activity may lead to the depletion of synaptic energy stores which eventually promote terminal degeneration. Other substituted amphetamines, such as p-chloroamphetamine, which cause similar degeneration of 5-HT neurons, also acutely deplete glycogen in frontal...
cortex (Huether et al. 1997). In addition, the continued reversal of the dopamine (DA) and 5-HT transporters by MDMA would increase intracellular sodium and consequently activate the ATP-dependent Na/K ATPase, thereby depleting energy stores (Bowyer and Holson 1995; Zeevak and Nicklas 1996).

Although the above studies provide a correlative relationship between changes in energy metabolism and MDMA toxicity, few studies to date have examined directly whether bio-energetic stress differentially affects 5-HT and DA neurons and interacts with the pharmacological action of MDMA to produce long-term depletion of 5-HT. We recently reported that a local perfusion of METH synergizes with mitochondrial inhibition to deplete striatal DA, but not 5-HT, tissue concentrations (Burrows et al. 2000). There are, however, no studies that have directly examined the interaction of mitochondrial inhibition with MDMA and whether bio-energetic stress contributes to MDMA-induced 5-HT depletion.

A local perfusion of MDMA was used in these experiments in order to obviate the potential confounding influence of hyperthermia and altered energy metabolism produced by the systemic administration of MDMA (Schmidt et al. 1990; Farfel and Seiden 1995; Malberg et al. 1996; Taraska and Finnegan 1997; Colado et al. 1998, 1999; Hervias et al. 2000). Malonate (MAL) was used to produce bio-energetic stress because it inhibits succinate dehydrogenase and causes a decrease in striatal ATP (Beal et al. 1993). Although MAL damages DA neurons (Zeevak and Nicklas 1996) and synergizes with METH to deplete striatal DA (Burrows et al. 2000), nothing is known about its interactions with MDMA and its effects on 5-HT terminals. Therefore, the local perfusion of MAL in conjunction with MDMA was used to examine the interaction between the inhibition of electron transport and the acute and long-term effects of MDMA on striatal 5-HT and DA terminals.

Materials and methods

Animals
Male Sprague–Dawley rats (200–290 g, Zivic-Miller, Allison Park, PA, USA) were housed three per cage and provided with food and water ad libitum in a temperature-controlled environment (20–22°C) with a 12/12 h light/dark cycle. After the surgical procedure, rats were housed individually for the duration of the experiments. All experiments were performed in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the local animal care and use committee.

Drugs
The following drugs were used: 3,4-methylenedioxyamphetamine (NIDA) and malonic acid (Sigma Chemical Co., St Louis, MO, USA).

Surgical procedures
Rats were anaesthetized with xylazine/ketamine (6 mg/kg, 70 mg/kg) and placed in a stereotaxic apparatus. The skull was exposed and a stainless steel guide cannula (11 mm) with a stylet obturator was lowered onto the dura directly above the striatum on each side of the brain (AP: +1.2; ML: ±3.2) (Paxinos and Watson 1986). The two cannulae were secured in place using cranioplastic cement, three stainless steel skull screws and cyanoacrylate glue. Rats were allowed to recover for at least 3 days prior to microdialysis.

In vivo microdialysis
On the day of dialysis, the obturators were removed from the guide cannulae and a microdialysis probe inserted slowly through each cannula into the brain of the awake rat. The probe was a concentric flow design and was constructed as described previously (Yamamoto and Pehek 1990). The probe was designed so that the dialysis membrane of 4.0 mm (Spectrapor, 13 0000 mV cut-off, 210 µm O.D.) sampled from the entire dorso-ventral extent of the lateral striatum. The probes were connected via spring-covered PE-50 tubing to a dual channel swivel (Instech, Plymouth Meeting, PA, USA) that allowed for relatively unrestrained movement of the animal. For experiments in which extracellular DA and 5-HT were measured, dialysate was collected into 250-µl tubes clipped to the tether. The probes were perfused with modified Dulbecco’s phosphate-buffered saline (138 mM NaCl, 2.1 mM KCl, 0.5 mM MgCl₂, 1.5 mM KH₂PO₄, 8.1 mM NaHPO₄, 1.2 mM CaCl₂, and 0.5 mM D-glucose, pH 7.4), which was pumped at a flow rate of 2.0 µL/min (Syringe infusion pump, Harvard Apparatus, Holliston, MA, USA) for a 3-h equilibration period prior to the collection of two 60-min baseline samples. The perfusion medium of the probe on one side of each animal was then switched to Dulbecco’s containing either MDMA (100 µM), MAL (100 mM) or the combination of both MDMA/MAL. The perfusion continued for an additional 8 h. Samples were collected every 60 min. In separate groups of rats, no dialysate was collected (i.e. probes were used only for the local perfusion of drugs) but tissue was dissected and assayed for DA and 5-HT content in a similar manner for both groups as described below. The methods in these experiments were identical to those described above except that perfusion of drug occurred immediately following insertion of the probe and continued for 8-h. The dialysate was not collected but flow was monitored to insure that the probes were functional. The ambient temperature was maintained at 22 ± 0.5°C for all experiments. The concentration of MDMA was based on preliminary experiments which indicated that 100 µM MDMA produces an increase in the extracellular concentration of DA equivalent to that observed after the systemic administration of neurotoxic doses of MDMA (Nash and Yamamoto 1992). The dose of MAL used was based on previous studies which demonstrate that the perfusion of 100 mM MAL results in the depletion of DA tissue concentrations (Burrows et al. 2000).

Body temperatures (colonic temperature) were monitored throughout the dialysis experiments using a Thermaalert TH-8 monitor (Physitemp Instruments, Inc., Clinton, NJ, USA). Baseline body temperatures were measured 30 min prior to the perfusion of drug, 30 min after drug perfusion began, and then every 60 min for the next 4 h. A final measurement was recorded 60 min prior to the termination of the perfusion.

Determination of extracellular serotonin and dopamine

Each dialysate sample was divided and assayed for 5-HT or DA using HPLC with electrochemical detection. Samples (20 µL) were injected onto a 3-µm C18 reverse-phase column (100 × 2.0 mm, Phenomenex, Torrance, CA, USA). Dopamine and 5-HT were eluted with a mobile phase consisting of 32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM ethylenediaminetetraacetic acid (Na EDTA), 0.215 mM octyl sodium sulfate and 3% methanol (pH 4.2). Separation of 5-HT and 3-methoxytyramine was confirmed prior to each dialysis experiment. Compounds were detected with an LC-4B amperometric detector (Bioanalytical Systems, West Lafayette, IN, USA) with a glassy carbon working electrode maintained at a potential of +0.7 V relative to an Ag/AgCl reference electrode. Data were recorded using a Hewlett-Packard Integrator.

Striatal dopamine and serotonin content in tissue

MDMA and/or MAL were locally perfused into the striatum. Because the diffusion distance of the drugs from the probe is not known, only tissue directly surrounding the probe was assayed for neurotransmitter content to maximize the detection of local changes in neurotransmitter content. We observed previously that damage produced by the probe itself may affect neurotransmitter content (unpublished observation). Therefore, in all studies we compared tissue surrounding the probe perfused with drug to the contralateral side perfused with vehicle medium.

One week following dialysis, rats were killed by rapid decapitation and the brain quickly removed and frozen with dry ice. Forty-micrometer thick coronal sections were taken until both probe tracts were visualized. A 400-µm thick section was then collected for the dissection of tissue around the probe tracts. The tissue was sonicated in 300 µL of 0.1 M perchloric acid and centrifuged at 14 000 g for 6 min. Dopamine and 5-HT were quantified using HPLC with electrochemical detection as described above. Concentrations were expressed as ng/mg protein. Protein content was determined using a Bradford protein assay.

Statistics

Body temperatures were compared using a two-factor repeated measures ANOVA across time. Dopamine and 5-HT tissue concentrations from striata perfused with drug were compared with those striata perfused with vehicle and were analyzed by one-way ANOVA followed by Newman–Keuls. Extracellular DA and 5-HT were analyzed with a two-factor repeated measures ANOVA across time and Newman–Keuls. For all analyses, significance was set as α = 0.05.

Results

Acute effects

The local perfusion of MDMA, MAL or the combination of MDMA/MAL produced a significant increase in extracellular DA concentrations (two-way repeated measures ANOVA, F27,306 = 342.2, p < 0.001). During the perfusion of MDMA, DA concentrations peaked 1 h after the perfusion began and remained elevated throughout the 8-h period.

Fig. 1 Extracellular dopamine (DA) in the striatum during the local perfusion of vehicle (n = 13), 3,4-methylenedioxymethylamphetamine (MDMA; 100 µM, n = 7), malonate (MAL; 100 mM, n = 7), or MDMA/MAL (n = 9). Local perfusion of MDMA increased extracellular DA concentrations and this increase was maintained throughout the perfusion. Perfusion of MAL alone significantly increased extracellular DA but this increase was not sustained and DA concentrations returned to baseline by the fifth hour of drug perfusion. Error bars represent SEM.

Fig. 2 Extracellular concentrations of serotonin (5-HT) in the striatum during the reverse dialysis of: (a) vehicle (n = 9), 3,4-methylenedioxymethylamphetamine (MDMA; 100 µM, n = 7), malonate (MAL; 100 mM, n = 7), or MDMA/ MAL (n = 9). The local perfusion of MDMA or MAL alone significantly increased extracellular concentrations of 5-HT, whereas MDMA/MAL caused a synergistic increase. Error bars represent SEM.
The peak amount of DA was 77.8 pg and the total for the 8 h of the perfusion was 375.4 pg. Local perfusion of 100 mM MAL also caused an initial significant increase in extracellular DA (peak amount of 310.8 pg; total DA 492.9 pg) but the concentrations returned to baseline levels by the third hour of drug perfusion (Fig. 1). In contrast to MDMA alone, the increase observed with MDMA/MAL (peak amount 463.9 pg; total DA 786.2) was not maintained and by the third hour of the perfusion extracellular concentrations were not different from the Dulbecco’s vehicle group (Fig. 1).

Extracellular 5-HT concentrations increased significantly during the perfusion of MDMA, MAL or MDMA/MAL (two-way repeated measures ANOVA, F_{27,216} = 6.91, p < 0.001). The initial increase caused by MDMA or MAL was similar during the first hour of the perfusion (peak 5-HT amounts of 8.5 and 5.8 pg, respectively) and persisted throughout the perfusion (total 5-HT MDMA 40.5, MAL 25.8; Fig. 2). The increase observed with MDMA/MAL (peak 5-HT 30.82 pg, total 5-HT 62.36 pg) was greater than MDMA or MAL alone.

There were no significant changes in rectal body temperatures during the perfusion of MDMA, MAL or the combination of MDMA/MAL (two-way repeated measures ANOVA, F_{20,80} = 0.942, p ≥ 0.538; Fig. 3).

Tissue concentrations of dopamine and serotonin

Local perfusion of MDMA alone did not change striatal DA tissue content measured 7 days after the perfusion (Fig. 4). Local perfusion of MAL did, however, decrease DA tissue content compared with Dulbecco’s controls (ANOVA, interaction F_{3,66} = 9.497, p < 0.001). Perfusion of MDMA/MAL depleted DA concentrations to a greater extent than that observed with MAL alone (ANOVA, interaction F_{3,70} = 28.837, p < 0.001, Fig. 4). Striatal 5-HT tissue concentrations were unchanged by the local perfusion of MDMA or MAL (Fig. 5). Only the combination of MDMA/MAL caused a significant reduction in 5-HT.
The acute and long-term effects of a local striatal perfusion with MDMA and the interactions with the mitochondrial complex II inhibitor, MAL, were examined to investigate the interaction of MDMA with bio-energetic stress. MDMA increased striatal 5-HT and DA release during local perfusion but did not produce long-term depletion of these transmitters. Malonate also acutely increased 5-HT and DA release, but produced only long-term depletion in DA tissue content. The combined perfusion MDMA/MAL exacerbated the increased release of 5-HT and DA, and produced a long-term depletion of DA content. Moreover, the effect of MAL was synergistic with MDMA on the depletion of 5-HT.

These findings support the hypothesis that a compromised bio-energetic state underlies the long-term depleting effects of MDMA. Because hyperthermia disrupts cellular energetics, inhibits mitochondrial electron transport (Nowak 1988; Huether et al. 1997), and mediates, in part, amphetamine toxicity (Bowyer et al. 1994; Malberg and Seiden 1998), the depleting effects following the systemic administration of MDMA may be produced through a hyperthermia-dependent compromise in bio-energetic state. Consequently, the absence of tissue depletions following the local infusion of MDMA may be due to the lack of effect on body temperature (Fig. 3). Therefore, we combined the local infusion of MDMA with the inhibition of energy metabolism to examine the role of bio-energetic stress in mediating the long-term depletions typically observed following systemic administration. Because MAL inhibits succinate dehydrogenase and decreases striatal ATP (Webb and Enzyme 1966; Beal et al. 1993), local infusion of MAL may reproduce some of the biochemical effects associated with hyperthermia. In fact, the effects of systemic MDMA appear to parallel the consequences of MAL on energy production. MDMA increases glycogen breakdown in astroglial-rich cell cultures (Poblete and Azmitia 1995), and inhibits cytochrome c oxidase activity (Burrows et al. 2000). Thus, the similarities between mitochondrial inhibition and the effects produced by MDMA suggest that MAL would exacerbate the acute and long-term effects of MDMA.

Acute effects
Striatal dopamine release was increased during the perfusion of MDMA and remained elevated throughout the 8-h perfusion. The peak increase was roughly half that observed during the local perfusion of METH (Burrows et al. 2000), which suggests that, compared with MDMA, METH is a more potent releaser of dopamine.

The perfusion of MAL also significantly increased the extracellular concentrations of DA but the pattern of release differed from MDMA. The transient increase, despite the continuous perfusion of the drug alone or in combination with MDMA, suggests that transmitter efflux produced by MAL alone, or in combination with MDMA, is not sustained during mitochondrial inhibition. Although the neurotoxic effects of MAL on striatal neurons have been documented (Green and Greenamyre 1995), the mechanisms that cause DA or 5-HT release are unknown. Further experiments that utilize more frequent sampling periods than the 60-min collection periods used here are needed to examine the pattern and mechanisms underlying the increases in DA and 5-HT release produced by MAL.

The combined perfusion of MDMA/MAL produced a synergistic increase in DA release (Fig. 1) as revealed by an increase greater than the sum of the individual effects of the drugs. Malonate-induced increases in intracellular calcium or the accumulation of intracellular sodium resulting from the inhibition of Na/K ATPase may have augmented the MDMA-induced transporter-mediated DA release.

Perfusion of MDMA increased and sustained 5-HT release throughout the perfusion. Although the peak increase in 5-HT release was similar to that observed with METH (18-fold increase with MDMA and 16-fold with METH), release was not sustained during perfusion with METH (unpublished observation). These results are consistent with previous findings that MDMA is a more potent releaser of 5-HT (Berger et al. 1992).

A synergistic increase in 5-HT release was observed with the combination of MDMA/MAL (Fig. 2). It is possible that MDMA combines with MAL to increase 5-HT release through a mechanism similar to that described above for their effects on DA release.

Long-term effects
Our results confirm previous findings that the central administration of MDMA (Paris and Cunningham 1992) does not produce long-term depletion of 5-HT. This lack of depletion is interesting in light of the findings that DA and 5-HT release have been implicated in mediating the depleting effects of MDMA following systemic administration (Schmidt et al. 1985; Schmidt 1987). Although elevated extracellular concentrations of DA and 5-HT were observed following the local perfusion of MDMA, this route of administration did not produce long-term depletion of striatal 5-HT. These data are nevertheless consistent with the finding that extracellular DA is not associated with the long-term depleting effects of METH (LaVoie and Hastings 1999).

Similar to our previous study, local perfusion of MAL produced a long-term depletion of DA tissue content without affecting 5-HT. Dopamine neurons are particularly vulnerable to the toxic effects of mitochondrial inhibition.
produced by MAL (Beal et al. 1993; Zeevak et al. 1997) and rotenone (Marey-Semper et al. 1995). This increased vulnerability may be mediated largely through an NMDA-receptor-mediated excitotoxicity (Beal et al. 1993; Green and Greenamyre 1995; Marey-Semper et al. 1995). Thus, it appears that increases in glutamate release following MAL (Messam et al. 1995; Burrows et al. 2000) and the subsequent enhanced increase in intracellular calcium produced by the inhibition of mitochondrial oxidative phosphorylation (Khodorov et al. 1999) may lead to the long-term depletion of DA content within the striatum.

An interesting result of this study was that the perfusion of MDMA/MAL produced a synergistic depletion of DA. This result was surprising because systemic administration of MDMA does not typically result in depletion of striatal DA in the rat. In contrast, systemic administration of MDMA to mice depletes DA content only when the mice are exposed to conditions that produce excessive hyperthermia (Miller and O’Callaghan 1994). Although the precise mechanism by which MDMA synergizes with MAL to deplete DA is still unknown, the MAL- and hyperthermia-induced inhibition of energy production and enhancement of glutamate release (Zeevak and Nicklas 1996) may render the DA terminal vulnerable to the effects of MDMA. Alternatively, the synergistic effects of MAL and MDMA on the long-term depletion of DA tissue content may be due to the massive release of 5-HT associated with MDMA/MAL combination. It has been hypothesized that large increases in 5-HT release could result in the production of the toxic metabolite, tryptamine-4,5-dione (Commons et al. 1987; Wrona et al. 1995; Jiang et al. 1999). Regardless, MAL appears to synergize with MDMA to deplete DA content, an effect strikingly similar to that observed after the systemic administration of METH or after the local perfusion of higher concentrations of MAL in combination with METH (Burrows et al. 2000).

Although neither MDMA nor MAL alone produced depletion of tissue 5-HT, the combination of MDMA/MAL markedly depleted the striatal concentration of 5-HT. Similar to the combined effects of MDMA and MAL on DA content, the addition of MAL and the consequent inhibition of energy metabolism may mimic the effects of hyperthermia and contribute to the acute and long-term toxic effects of MDMA on 5-HT terminals. Alternatively, the synergistic enhancement of 5-HT release following the perfusion of MDMA/MAL may underlie these long-term changes in 5-HT content. Stimulation of the 5-HT2A receptor appears to mediate the depleting effects of MDMA (Nash 1990; Schmidt et al. 1990; Poblete and Azmitia 1995) as evidenced by the protective effect of 5-HT2 antagonists. Although these results may be confounded by the hypothermic effects of 5-HT2 antagonists, 5-HT2 antagonism by MDL 11,939 provides complete protection against the long-term depletion of 5-HT in tissue while only partially attenuating the acute hyperthermic responses to MDMA (Schmidt et al. 1990). Nevertheless, stimulation of the 5-HT2A receptor along with the binding of MDMA to the 5-HT transporter activates and translocates protein kinase C (Conn and Sanders-Bush 1986; Wang and Friedman 1990) to increase intracellular calcium (Kramer et al. 1997, 1998; Park and Azmitia 1991) which, in turn, could produce calcium-mediated proteolysis. Therefore, the combined effects of MDMA on second messenger-mediated increases in intracellular calcium, inhibition of mitochondrial function and the diminished sequestration of calcium that ensues (Babcock et al. 1997), could synergize to activate calcium-mediated proteolysis and damage 5-HT terminals.

In contrast to the combined effects of MDMA and MAL on 5-HT content, we have shown that the combined perfusion of METH and MAL did not deplete tissue 5-HT (Burrows et al. 2000). These differences between METH and MDMA in combination with MAL and their effects on 5-HT terminals can also be explained by a differential dependence on calcium-mediated mechanisms. In support of this, Johnson et al. (1992) demonstrated that the calcium channel blocker, flunarizine, attenuated the decrease in tryptophan hydroxylase produced by MDMA but not METH. These data are suggestive of the possibility that calcium-mediated events are more closely linked with the toxic actions of MDMA on the 5-HT system than those produced by METH.

Overall, our results highlight the importance of energy balance to the function of DA and 5-HT neurons and to the long-term effects of MDMA on the depletion of DA and 5-HT. Although the inhibition of mitochondrial complex II by MAL alone only targets DA compared with 5-HT neurons, the combination with MDMA produced depletions of 5-HT and DA. The synergistic interactions between MAL and MDMA on both the acute and long-term changes in dopamine and 5-HT suggest that a compromised energetic state renders these neurons vulnerable to the pharmacological effects of MDMA and produces tissue depletions that resembles the long-term effects produced by the systemic administration of METH. These findings emphasize the importance of interactions of metabolic stress with the pharmacologic and toxic actions of MDMA and highlight the differential vulnerabilities of DA and 5-HT neurons to these insults.

Acknowledgements

This work was supported by DA07606, DA07427, DAMD17-99-1-979, and by a gift from Hitachi America.

References

Albers D. and Sonsalla P. K. (1995) Methamphetamine-induced hyperthermia and dopaminergic neurotoxicity in mice:


