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The neurotoxic effects of methylenedioxymethamphetamine (MDMA) and its metabolites on rat brain spheroids in culture

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Abstract

Rat whole-brain spheroids were used to assess the intrinsic neurotoxicity of methylenedioxymethamphetamine (MDMA, Ecstasy) and two of its metabolites, dihydroxymethamphetamine (DHMA) and 6-hydroxy-MDMA (6-OH MDMA). Exposure of brain spheroids to MDMA or the metabolite 6-OH MDMA (up to 500 μmol/L) for 5 days in culture did not alter intracellular levels of glutathione (GSH), glial fibrillary acidic protein (GFAP) or serotonin (5-HT). In contrast, exposure to the metabolite DHMA, which can deplete intracellular thiols, significantly increased GSH levels (up to 170% of control) following exposure to 50 and 100 μmol/L DHMA. There was also a significant reduction in the levels of glial fibrillary acidic protein (GFAP) and GSH by DHMA at the highest concentration tested (500 μmol/L) but there was no effect on 5HT. This may constitute a sublethal neurotoxic compensatory response to DHMA in an attempt to replenish depleted intraneural GSH levels following metabolite exposure. Rat whole-brain spheroids may thus be a useful *in vitro* model to delineate mechanisms and effects of this class of neurotoxin.

Abbreviations: DHMA, dihydroxymethamphetamine; GFAP, glial fibrillary acidic protein; GSH, glutathione; 5-HT, 5-hydroxytryptamine; LDH, lactate dehydrogenase; MDA, methylenedioxyamphetamine; MDMA, methylenedioxymethamphetamine; 6-OH MDMA, 6-hydroxymethylenedioxymethamphetamine; THMA, trihydroxymethamphetamine

Introduction

Methylenedioxymethamphetamine (MDMA, Ecstasy) is known to selectively target the serotonergic system in rats and humans, resulting in both reversible and irreversible toxicity. Reversible toxicity results from a marked depletion in 5-hydroxytryptamine (5-HT), which may arise from a stimulation of 5-HT release coupled with an inhibition of reuptake mechanisms (Schmidt, 1987). Irreversible toxicity is manifested as a prolonged depletion of 5-HT in various brain regions (Commins et al., 1987). This prolonged depletion of 5-HT is accompanied by a reduction in the number of 5-HT uptake sites, and is interpreted as a loss of serotonergic nerve terminals (Schmidt, 1987).

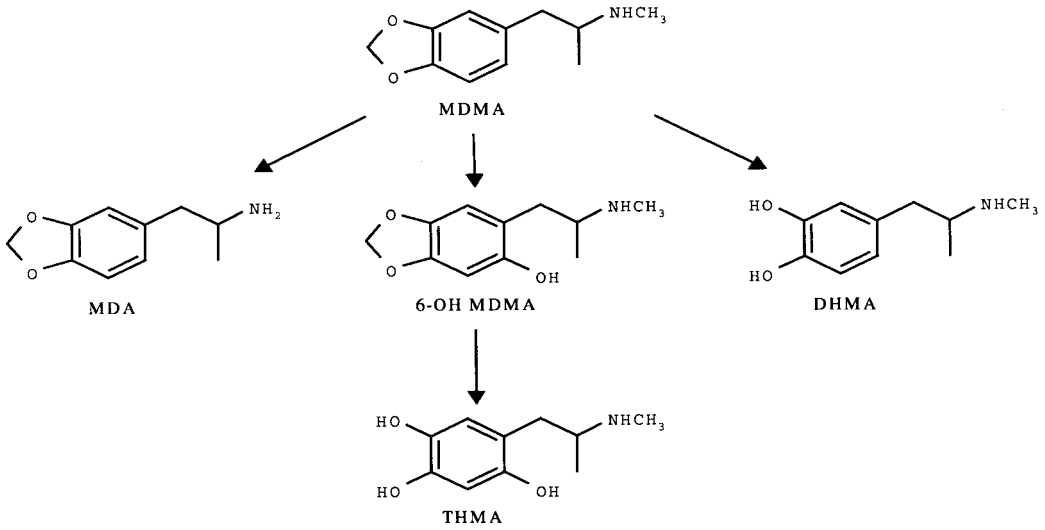

Figure 1. Metabolites of MDMA (Chu et al., 1996).

It was reported that direct intracerebral injection of MDMA to rats did not produce a significant depletion in 5-HT (Schmidt and Taylor, 1988) and, therefore, it was suggested that a metabolite of MDMA may be involved in 5-HT depletion. This was supported by the finding that dark agouti female rats, which are deficient in cytochrome P450 2D, accumulated higher brain levels of MDMA but had a reduced depletion of 5-HT (Chu et al., 1996).

There are a number of metabolites of MDMA (Figure 1). The major metabolite detected in rat brain *in vivo*, following subcutaneous administration, is methylenedioxyamphetamine (MDA). This has been shown to cause 5-HT depletion with a potency comparable to that of MDMA, but levels in the brain are not believed to be sufficient to account for the acute depletion of 5-HT (Chu et al., 1996). However, two other metabolites of MDMA, DHMA (dihydroxymethamphetamine) and 6-OH MDMA (6-hydroxy-MDMA), do have neurotoxic potential, and their formation has been demonstrated *in vitro* with rat brain microsomes (Lin et al., 1992). DHMA can be readily oxidized to the *o*-quinone, which can combine with and deplete intracellular thiols (Tucker et al., 1994). 6-OH MDMA failed to deplete 5-HT levels *in vivo* following either intracerebral or systemic administration, indicating that it is unlikely to play a direct role in neurotoxicity (Zhao et al., 1992). However, it can undergo further metabolism to yield THMA (trihydroxymethamphetamine, Figure 1), which is analogous to 6-hydroxydopamine, a potent neurotoxin (Chu et al., 1996). In addition, direct intrastriatal injection of THMA caused a significant depletion in 5-HT and dopamine levels one week following administration (Zhao et al., 1992).

Freshly isolated cells from fetal tissue can spontaneously reaggregate in rotation-mediated culture to form three-dimensional spherical structures – brain spheroids (Moscona, 1952) – that permit maximal cell-to-cell interactions and development (Honegger and Schilter, 1995). The expression of a highly differentiated phenotype and its longevity in culture make spheroids a useful model for mechanistic toxicological studies in differentiated neural cells (Atterwill and Purcell, 1999).

The aim of the present study was to assess

the neurotoxic potential of MDMA and two of its metabolites, DHMA and 6-OH MDMA, in rat brain spheroids using a variety of cytotoxicity markers. Leakage of a cytosolic enzyme (lactate dehydrogenase, LDH) into the culture medium and levels of intracellular reduced glutathione (GSH) were measured as general cytotoxicity markers. More specific neurological markers were 5-HT and glial fibrillary acidic protein (GFAP) levels. 5-HT levels were measured to determine compound-related depletions in the neurotransmitter, while GFAP has been reported to be a useful marker of neurotoxicity as it accumulates in response to nervous system damage (O'Callaghan, 1991a).

Material and methods

Culture details

Rat brain spheroids were prepared as previously described by Fox et al. (1996). Briefly, whole rat brains were removed from 16-day-old fetuses and a single-cell suspension was obtained by mechanical dissociation. The cells were seeded into Delong conical flasks (3.5×10^7 cells per flask) in DMEM supplemented with 10% FCS and placed on a gyratory shaker at 85 rpm, 37°C, 9% CO_2. On the twelfth day of culture, the spheroids were transferred into 6-well plates and treated with either MDMA, DHMA or 6-OH MDMA (50, 100, 500 μmol/L) or solvent control (1% (v/v) ethanol). Cultures were re-treated on the third day of exposure to MDMA/metabolite and harvested for cytotoxicity assays on the fifth day.

Cytotoxicity assays

LDH leakage into the culture medium was detected by the spectrophotometric method of Korzeniewski and Callewaert (1983). Intracellular GSH was determined by the fluorometric method of Hissin and Hilf (1976). 5-HT content was measured by a fluorometric method, as described by Vanable (1963) and Snyder et al. (1965). GFAP was determined by an ELISA assay, based on the method of O'Callaghan (1991b). Total protein was determined using BioRad protein dye reagent, which is based on the Coomassie blue assay (Bradford. 1976).

Results and discussion

Data from the treatment of 12-day-old rat brain spheroids with either MDMA, DHMA or 6-OH MDMA are illustrated in Figures 2–4 below.

No neurotoxicity was seen following treatment of 12-day-old rat brain spheroids with MDMA for 5 days (Figure 2). This is in agreement with previous studies that suggest that a metabolite of MDMA may be responsible for its toxicity (Zhao et al., 1992; Chu et al., 1996).

DHMA caused concentration-related cytotoxicity in rat brain spheroids (Figure 3). Following treatment with 500 μmol/L DHMA, cytotoxicity was apparent with a reduction in GSH and GFAP, which was accompanied by an increase in extracellular LDH (361% of concurrent control, data not shown). However, a significant, concentration-related increase in intracellular GSH (up to 170% of concurrent control) was observed following exposure to lower concentrations of DHMA (50 and 100 μmol/L) without any parallel changes in 5-HT levels. DHMA, a reactive catechol, may readily be oxidized to an *o*-quinone species that is able to combine with, and deplete, intracellular thiols, including GSH (Hiramatsu et al., 1990). These GSH conjugates may also be involved in the neurotoxicity of DHMA (Miller et al., 1997). Therefore, the increase in intracellular GSH in spheroids may reflect an increase in its synthesis, to compensate for that depleted through conjugate formation.

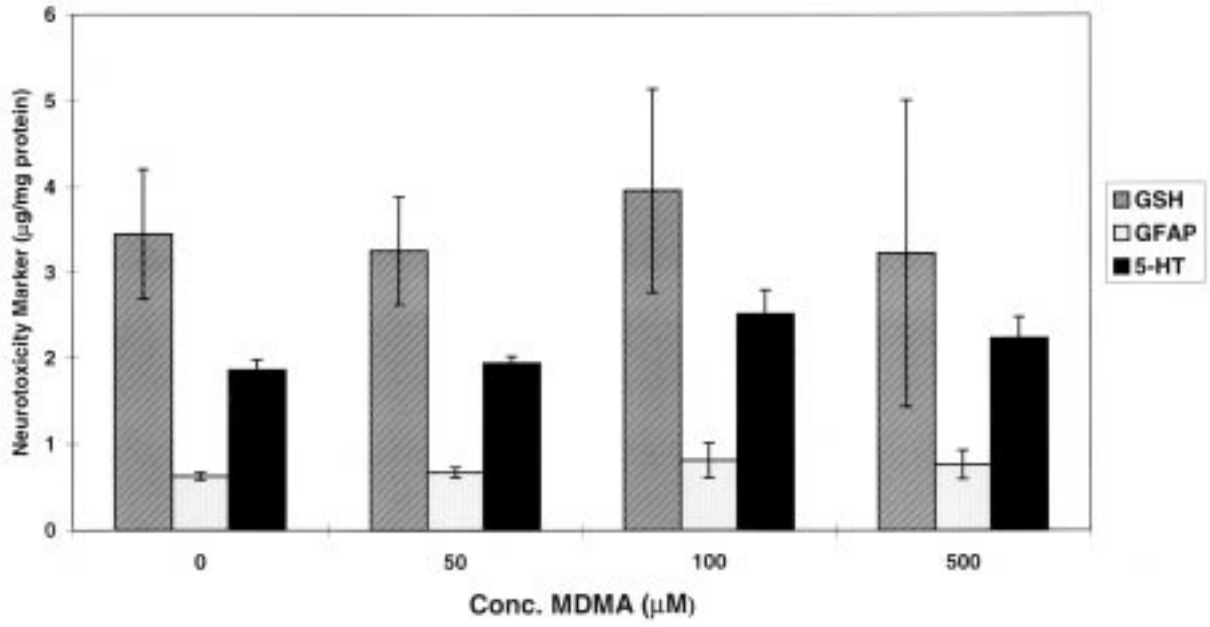

Figure 2. Effect on GSH, GFAP, and 5-HT of exposure of day-12 rat brain spheroids to MDMA, for 5 days. The results are expressed as µg/mg protein and are the mean ± SEM of duplicate samples from three independent experiments.

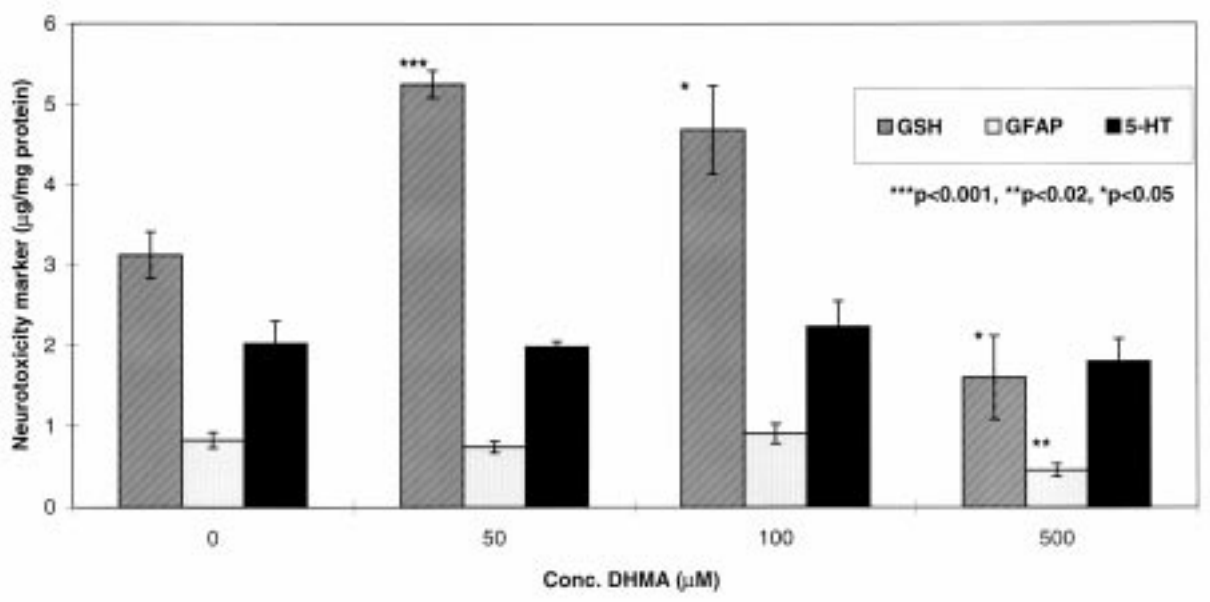

Figure 3. Effect on GSH, GFAP, and 5-HT of exposure of day-12 rat brain spheroids to DHMA for 5 days. The results are expressed as µg/mg protein and are the mean ± SEM of duplicate samples from three independent experiments. (*$p < 0.05$, **$p < 0.02$, ***$p < 0.001$, *t*-test).

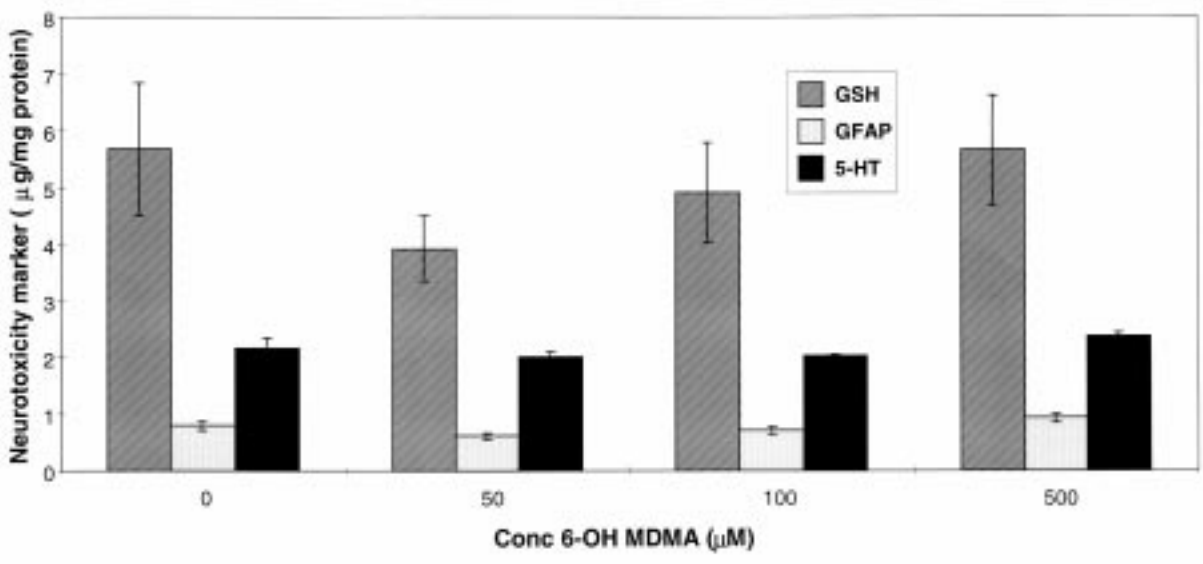

Figure 4. Effect on GSH, GFAP, and 5-HT of exposure of day-12 rat brain spheroids to 6-OH MDMA for 5 days. The results are expressed as µg/mg protein and are the mean ± SEM of duplicate samples from three independent experiments.

Treatment of spheroids with the other metabolite, 6-OH MDMA, did not cause any changes in the cytotoxicity markers measured (Figure 4). As discussed earlier, 6-OH MDMA is not believed to play a direct role in the neurotoxicity of MDMA. It requires further, cytochrome P450 dependent, metabolism to yield the neurotoxic metabolite THMA (Figure 1). Although this metabolic activity has been demonstrated in rat brain microsomes (Lin et al., 1992), no attempt was made to assess this activity in the present study. However, the lack of toxicity of 6-OH MDMA would suggest limited, if any, activity in the cultures.

In the present study no increases in GFAP together with 5-HT depletion were seen with any of the compounds tested, suggesting that they had not resulted in specific serotonergic neurotoxicity and associated reactive astrogliosis. More subtle effects on 5-HT function may perhaps have been detected with measurement of 5-HT turnover or 5-HIAA. Increased GFAP levels should also have been a sensitive marker for neurotoxicity with DHMA, as increases in astroglial GFAP have been reported following intrahypothalamic administration to rats of the serotonergic neurotoxin 5,7-dihydroxytryptamine (Frankfurt et al., 1991). The reduction in GFAP seen following DHMA treatment at the highest concentration tested (500 µmol/L) may reflect a generalized cytotoxic effect at this concentration with astroglial necrosis and concomitant reduction in GSH levels.

In conclusion, exposure of rat whole-brain spheroids to MDMA or two of its metabolites, DHMA and 6-OH MDMA, did not produce changes in specific markers of neurotoxicity. However, lower concentrations of DHMA (50 and 100 µmol/L) caused an increase in the intracellular levels of GSH without evidence of general cytotoxicity (i.e. no change in LDH) or serotonergic neurotoxicity. In contrast, generalized cytotoxicity did result from exposure to higher DHMA concentrations (500 µmol/L). As DHMA is known to conjugate with GSH, the increase in intracellular GSH in spheroids may have been caused by sublethal

neurotoxicity that stimulated GSH synthesis to compensate for diminished levels of endogenous antioxidant through DHMA conjugation. This data indicates that rat brain spheroid cultures *in vitro* (coupled with sensitive neurotoxicity markers) can detect the neurotoxic potential of MDMA-like compounds and their metabolites, by virtue of the compounds' ability to conjugate and deplete cellular GSH. However, this model cannot quantitatively assess the neurotoxicity of MDMA following systemic administration owing to the absence of a liver metabolizing activity in the culture models. Future comparison of the present results with, for example, a coculture of brain and liver spheroids may enable the neurotoxic potential of systemically administered MDMA-like compounds to be assessed.

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