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Research report

Fetal exposure to (±)-methylenedioxymethamphetamine in utero enhances the development and metabolism of serotonergic neurons in three-dimensional reaggregate tissue culture

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Abstract

Methylenedioxymethamphetamine (MDMA, Ecstasy) is a potent psychomotor stimulant with neurotoxic potential which is widely abused by females of childbearing age raising serious public health concerns in terms of exposure of the fetus to the drug. The current study was conducted using the three-dimensional reaggregate tissue culture system as an approach to the assessment of risk to fetal brain cells following exposure to MDMA during early to mid-gestation. In this culture system, the serotonergic and dopaminergic mesencephalic-striatal projections are reconstructed and develop with a time course similar to that observed in vivo. Pregnant C57Bl/6J mice were injected twice daily with 40 mg/kg MDMA or saline from gestational day 6 to 13. On gestational day 14, mesencephalic and striatal cells from MDMA- and saline-exposed embryos were used to prepare reaggregate cultures. Levels of neurotransmitters and their metabolites in the reaggregates and culture medium were assessed at 22 and 36 days of culture. There was a long-term enhancement of serotonergic development and metabolism by fetal exposure to MDMA as evidenced by increased reaggregate serotonin levels as well as the elevated production and release of 5-hydroxyindoleacetic acid in cultures prepared from MDMA-exposed embryos which persisted for up to 36 days of culture. Dopaminergic neurons in such cultures also exhibited increased metabolism as indicated by elevated levels of dihydroxyphenylacetic acid in reaggregate tissue and culture medium. The data obtained suggest that exposure to MDMA in utero during early to mid-gestation may result in more active serotonergic and dopaminergic neurons.

Theme: Neural basis of behavior

Topic: Drugs of abuse: amphetamine and other stimulants

Keywords: Ecstasy; MDMA; Mesostriatal; Prenatal drug; Serotonin; Tissue culture

1. Introduction

Methylenedioxymethamphetamine (MDMA, Ecstasy) is a recreational drug whose use has escalated among young adults including women of childbearing age [4]. There is growing concern about the drug's potential neurotoxicity in humans given that chronic heavy recreational use of MDMA is associated with sleep disorders, memory impairment and psychiatric problems which can persist for months [27]. Memory deficits have been correlated with reductions in serotonergic function [16,25,27]. There are few reports of the effects of MDMA on children prenatally exposed to the drug. Neonates born to MDMA abusing mothers are reported to show an increased incidence of congenital anomalies including cardiovascular and musculoskeletal defects [26], but follow-up studies in exposed children are not available.

MDMA produces long-term neurochemical and neuroanatomical alterations to central serotonergic projections of adult rodents and primates [2,9,15,28], but little is known regarding the effect of this drug on the developing fetus. Gestational exposure to MDMA appears to have no effect on basal forebrain levels of serotonergic markers and only minimal effects on the behavioral development of the offspring [6,32]. Studies on the effects of prenatal exposure to psychomotor stimulants on developing neurons are

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complicated by a number of experimental variables. The effects of prenatal exposure to such agents may be more complex than effects on basal levels of transmitters, as we have recently shown for methamphetamine [12].

Given the experimental complexity of gestational drug exposure, we have used the three-dimensional reaggregate tissue culture system to survey and 'pin-point' times in development at which drug-induced effects occur as well as their nature and duration. This culture system permits the in vitro reconstruction of specific neuronal circuits with a developmental time pattern similar to that observed in the intact brain [14,21,39]. Such cultures can be maintained for periods of 1 year [5] and allow for monitoring of effects of psychostimulants with neurotoxic potential at various developmental stages in the life history of these neurons [13,19,20,38]. Utilizing such cultures, we have demonstrated that there is a long-lasting increased release of monoamine metabolites following in utero exposure to methamphetamine suggesting that the monoaminergic neurons are more active [35]. This finding led us to the demonstration that exposed offspring were more responsive to the drug as adults in terms of release properties and neurotoxicity [12].

Given our findings of enhanced neuronal activity following gestational exposure to methamphetamine, the present investigation, using three-dimensional reaggregate cultures, was undertaken to determine whether similar effects occur on developing monoaminergic neurons following prenatal exposure to MDMA. The results obtained suggest, in fact, that fetal exposure to MDMA also results in more active serotonergic and dopaminergic neurons.

2. Materials and methods

2.1. Subjects

All procedures were approved by the University of Chicago's Institutional Animal Care and Use Committee and conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Adequate measures were taken to minimize pain or discomfort to the animals.

Pregnant female C57Bl/6 mice, approximately 3.5–5 months old, were injected subcutaneously twice daily (08:00 h and 16:00 h) with 40 mg/kg of (±)-MDMA hydrochloride (obtained from the National Institute on Drug Abuse) or saline from gestational day (GD) 6 through 13 (presence of vaginal plug=GD 1). This dose of MDMA was used because it is similar to that used in a study of maternal administration of methamphetamine which produced fetal brain concentrations of the methamphetamine similar to that seen in postmortem human infants of methamphetamine abusing mothers [36]. The dams were housed individually and maintained in an environment with ambient temperature between 22 and 24.5 °C with a 12:12 h light/dark cycle and ad libitum access to food and water. At GD 14, the pregnant dams were killed and their embryos harvested for the preparation of reaggregate cultures. The striatum of the dams was obtained for neurochemical analysis of monoamine and metabolite levels.

2.2. Culture preparation

All of the reaggregate cultures were prepared on the same day from embryos of either MDMA- or saline-treated mice from a single, timed-pregnant mating. The embryonic brains were dissected into the mesencephalic tegmentum (containing developing dopaminergic and serotonergic neurons) and the corpus striatum (a region to which dopaminergic and serotonergic neurons normally project). The tissues were dissociated into single cell suspensions using trypsin and flushing through a fine-bore Pasteur pipette, and then 2.5 million mesencephalic cells and 5 million striatal cells were dispensed into 25-ml Erlenmeyer flasks containing culture media (for culture method details, see Ref. [13]). The culture medium contained sera that had been previously dialyzed to remove serotonin (5-HT) and 5-hydroxyindoleacetic acid (5-HIAA). The flasks were placed into a rotary incubator where reaggregates formed over the course of 24 h. The culture media were changed every 2–3 days. Eleven flasks of reaggregates were initially prepared from MDMA- or saline-exposed embryos. After 18 days in culture, reaggregates from the 11 flasks for a given treatment group (MDMA or saline) were combined and redistributed to seven experimental flasks. The pooling of reaggregates and their subsequent redistribution into an appropriate number of experimental flasks results in a homogeneous population of reaggregates and minimizes the variability in the neurochemical indices of interest between flasks [38].

2.3. Neurochemical analysis

At culture days 22 and 36, samples of culture media and reaggregates were collected from each flask for high performance liquid chromatography (HPLC) analysis of monoamine and metabolite levels. Reaggregates were washed three times with ice-cold Tyrode's solution before the addition of 250 μl of 0.5 N perchloric acid containing 0.2 mM disodium salt, dihydrate (EDTA). The reaggregates were then homogenized by sonication, centrifuged (4000×*g*, 0 °C, 25 min) and the supernatant and pellet separated. Striatal tissue from the dams was processed in a similar manner with omission of the Tyrode's wash. The supernatants from the reaggregate and striatal tissue were used for the analysis of endogenous dopamine (DA), dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), 5-HT and 5-HIAA by HPLC [18] and the pellet used to measure protein content. For analysis of monoamine metabolites in the culture medium, 0.5 ml of medium was mixed with an equal volume of 0.5 N

Table 1
Effect of repeated administration of MDMA on maternal weight gain and litter size

	% Weight gain	Embryos per litter	Resorptions per litter
Saline (n=9)	18.8±1.3	7.2±0.6	1.2±0.5
MDMA (n=9)	11.2±1.8*	6.3±0.8	1.6±0.4

Pregnant dams were treated twice daily with (±)-MDMA (40 mg/kg) or saline from gestational day 6 to 13. Maternal weight gain is expressed as a percentage of the increase in weight from GD 5 to 13 (i.e. [(GD 13−GD 5)/GD 5]×100). The number of embryos and resorbed embryos per litter was assessed at GD 14. The values represent the mean±S.E.M. *Significantly different from saline, $P<0.01$, two-tailed t-test.

perchloric acid and centrifuged as described above for reaggregates. A dilution of the supernatant from the acidified reaggregates or culture medium was directly injected onto the HPLC column. Protein content of the reaggregates was determined spectrophotometrically using bicinchoninic acid [30].

3. Results

3.1. Effect of repeated administration of MDMA on the dams and litter size

Pregnant dams treated with (±)-MDMA for 7 days (GD 6 to 13) gained significantly less weight (−7.6%, $P<0.005$) over this gestational period compared to saline-treated dams (Table 1). Despite the difference in maternal weight gain, there was no significant effect of maternal MDMA administration on average litter size or the number of resorbed embryos at GD 14 when fetal tissue was obtained for the preparation of reaggregate cultures (Table 1). No gross malformations were observed in embryos from either treatment group.

Administration of 40 mg/kg of (±)-MDMA twice daily to pregnant mice during GD 6 to 13 did not have a significant effect on striatal levels of DA, DOPAC, 5-HT or 5-HIAA of the dams 1 day following the last drug injection (Table 2). Striatal HVA was, however, significantly elevated in MDMA-treated dams by 25% ($P<0.01$).

3.2. Effect of maternal MDMA administration on monoaminergic neuronal development as assessed in three-dimensional reaggregate culture

Mesencephalic-striatal reaggregates prepared from embryos of dams treated with (±)-MDMA during GD 6 to 13 showed a significant elevation of tissue 5-HT levels at 22 (+54%) and 36 (+83%) days of culture compared to reaggregates prepared from saline-treated embryos (Fig. 1A). Reaggregate 5-HIAA was also significantly increased (22 days, +78%; 36 days, +97%) at both time points in cultures derived from MDMA-exposed embryos (Fig. 1B). Media 5-HIAA levels from such reaggregates were elevated as well at 22 (+133%) and 29 (+96%) days of culture (Fig. 1C).

Exposure of embryos to MDMA in utero did not have an effect on reaggregate DA levels at either 22 or 36 days of culture (Fig. 2A). Reaggregate DOPAC, however, was significantly elevated at both time points (22 days, +49%; 36 days, +56%) in cultures prepared from MDMA compared to saline-exposed embryos (Fig. 2B). Media DOPAC levels from 22-day-old reaggregates prepared from drug-exposed embryos were also 21.7% greater ($P<0.01$) than from cultures prepared from saline-exposed embryos (Fig. 2C).

4. Discussion

The three-dimensional reaggregate tissue culture system was used to follow the effects of in utero exposure to MDMA during early to mid-gestation on the developing monoaminergic mesostriatal projections. As shown in the current study, there was a long-term enhancement of serotonergic neuronal activity by fetal exposure to MDMA as evidenced by increased reaggregate 5-HT levels as well as the elevated production and release of 5-HIAA in the cultures. This effect persisted for up to 36 days of culture. Although prenatal exposure to MDMA did not have a demonstrable effect on reaggregate DA levels per se, dopaminergic neurons did appear to be more active as indicated by elevated levels of DOPAC in reaggregate tissue and culture medium.

Such increases in monoaminergic neuronal activity observed in the reaggregates are not limited to MDMA. We

Table 2
Effect of (±)-MDMA administration during pregnancy on striatal monoamine and metabolite levels of the dams

	DA	DOPAC	HVA	5-HT	5-HIAA
Saline (n=9)	74.4±1.2	7.7±0.4	6.2±0.3	6.1±0.2	3.2±0.2
MDMA (n=9)	70.2±1.6	8.6±0.3	7.8±0.4*	6.0±0.3	3.4±0.2

Pregnant dams were treated twice daily with (±)-MDMA (40 mg/kg) or saline from gestational day 6 to 13 and killed on gestational day 14, 1 day post-injection. Data represent the mean±S.E.M. striatal monoamine and metabolite levels (ng/mg protein). *$P<0.01$, significantly different from saline, two-tailed t-test.

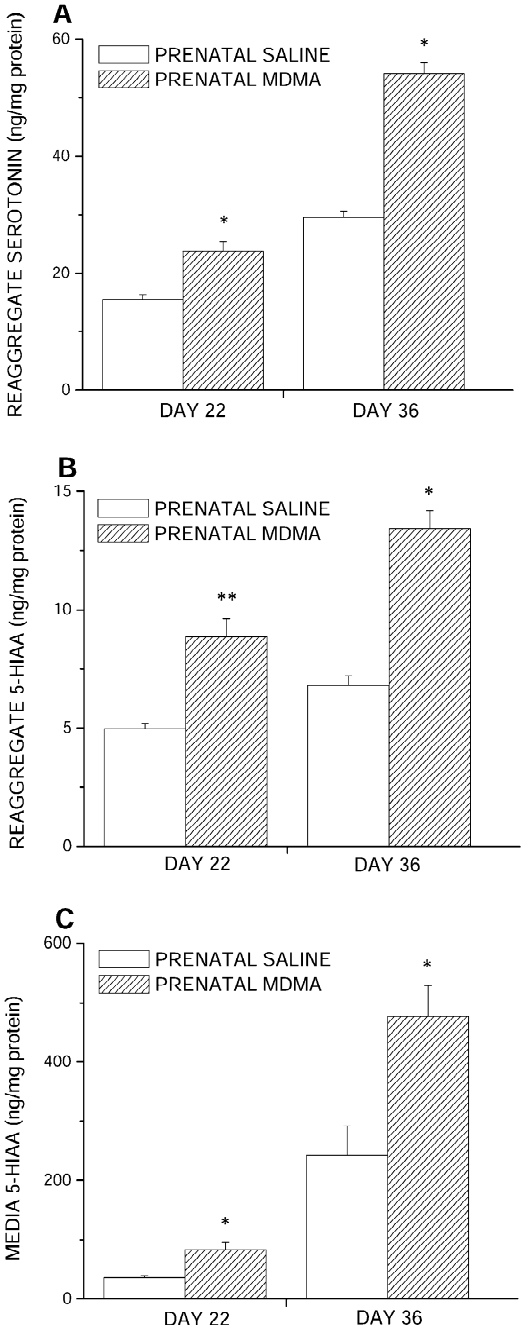

Fig. 1. Mesencephalic-striatal reaggregates were prepared from embryonic day 14 fetuses of pregnant C57Bl/6 mice treated with (±)-methylenedioxymethamphetamine (MDMA) or saline from gestational day 6 through 13. Samples of reaggregates and culture medium from individual flasks of MDMA- ($n=7$ flasks) or saline-exposed cells ($n=7$ flasks) were collected on culture days 22 and 36 for analysis of endogenous tissue content of (A) serotonin and (B) 5-HIAA as well as media levels of 5-HIAA (C). *$P<0.05$, **$P<0.01$, significantly different from reaggregates prepared from saline-exposed embryos at the respective culture age, two-tailed *t*-test.

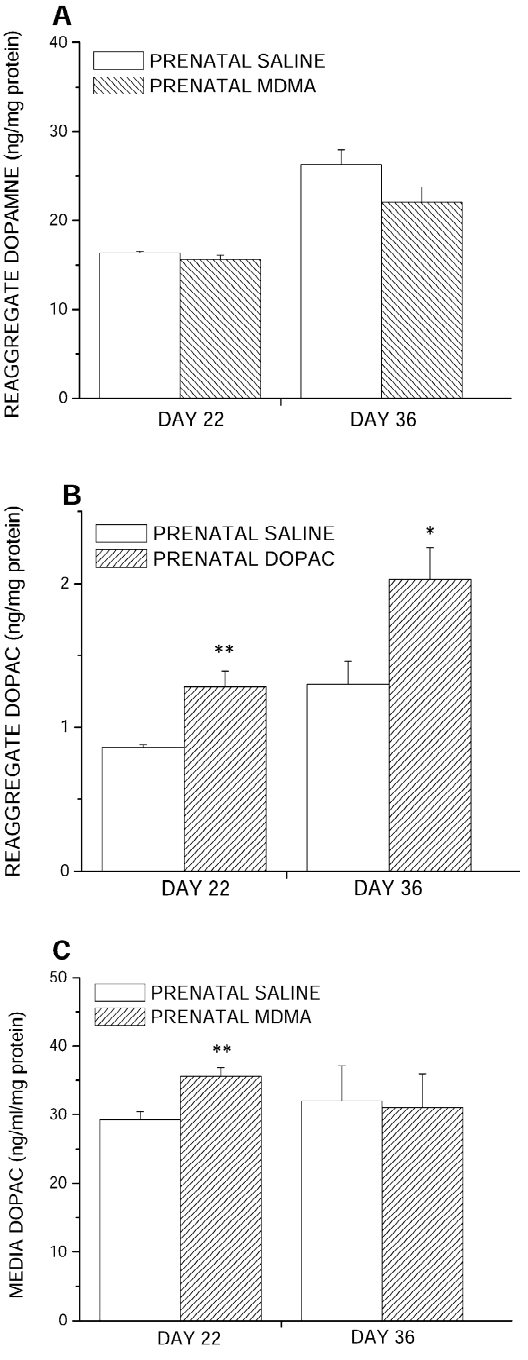

Fig. 2. Mesencephalic-striatal reaggregates were prepared from embryonic day 14 fetuses of pregnant C57Bl/6 mice treated with (±)-methylenedioxymethamphetamine (MDMA) or saline from gestational day 6 through 13. Samples of reaggregates and culture medium from individual flasks of MDMA- ($n=7$ flasks) or saline-exposed cells ($n=7$ flasks) were collected on culture days 22 and 36 for analysis of endogenous tissue content of (A) dopamine and (B) DOPAC as well as media levels of DOPAC (C). *$P<0.05$; **$P<0.01$; significantly different from reaggregates prepared from saline-exposed embryos at the respective culture age, two-tailed *t*-test.

have found that mesencephalic-striatal reaggregates prepared from embryos exposed in utero to nicotine from GD 2 to 13 [37] also show a more rapid neurochemical

development with increased 5-HT levels and greater release of 5-HIAA and DOPAC compared to cultures prepared from saline-treated animals. In addition, serotonergic neurons in reaggregates prepared from fetuses of pregnant mice treated with (+)-methamphetamine from GD 6 to 13 exhibit accelerated development with respect to elevated production and release of 5-HT [35]. The dose of methamphetamine that was administered to these dams results in a peak fetal brain drug concentration of approximately 122 ng/mg protein [36] which is similar to brain drug levels seen in human twin infants of a drug abusing mother [3].

The results from these reaggregate studies suggested that there may be long-term, enhancement of monoaminergic function following in utero exposure to psychostimulants. The effects observed in culture from gestational exposure to such agents provide a clue of possible neural alterations that may not be manifested directly in the intact animal. In that regard, we have found that adult offspring exposed to the methamphetamine in utero have normal brain dopamine and serotonin levels [11] despite the fact that dopamine is increased in fetal rostral mesencephalic tegmentum and corpus striatum at the time of prenatal exposure [10]. The findings of elevated fetal brain dopamine levels following maternal administration of methamphetamine are in agreement with our observation of increased dopaminergic responsivity in adult animals exposed to the drug in utero. Striata from such animals release more dopamine when challenged with methamphetamine [12] and there is a resultant enhanced neurotoxic response of the dopaminergic nigrostriatal projection in the adult male offspring [11]. It would appear that compensatory factors come into play resulting in normal levels of transmitter by adulthood. Given that this is the case, it is not surprising that basal levels of serotonin and 5-HIAA are unaltered in the forebrain of postnatal rat offspring from dams treated with MDMA during pregnancy [6,32].

Repeated maternal administration of MDMA did not affect striatal monoamine levels of the dams in contrast to the effect of this agent on developing monoaminergic neurons in mesencephalic-striatal reaggregates. There was a small, but significant, 26% elevation in striatal HVA in drug-treated dams, 1 day following MDMA administration on GD 14, which may represent increased drug-induced release of dopamine, a well-known action of this agent on mature dopaminergic neurons [17,40]. The lack of an effect of MDMA on striatal serotonergic markers in the dams is consistent with studies on the species-dependent effects of this agent on serotonin depletion [24,33].

MDMA administration does have an effect on the body weight of the dam. In the present study, pregnant mice given 40 mg/kg of MDMA twice a day for 7 days, from GD 6 to 13, gained 7.6% less weight than the saline control group. Similar reductions in maternal weight gain ranging from 6% [32] to 15% [6] have been reported following MDMA treatment of pregnant rats. The decrease in weight gained by MDMA-treated pregnant mice in the current study did not produce detrimental effects with respect to litter size or the number of resorbed embryos and did not result in gross malformations.

MDMA, like other amphetamines, stimulates the release of peripheral catecholamines producing vasoconstrictive effects on the cardiovascular system [7]. Maternal administration of MDMA may be decreasing uterine flow resulting in potential hypoxia-ischemic injury to the developing fetal brain. The possibility that the vasoconstrictive action of MDMA is responsible for the increase in monoaminergic neuronal activity cannot be ruled out in the current study. The effect of fetal hypoxia–ischemia in combination with in utero exposure to MDMA is an issue currently under examination.

It is of interest that the effect of gestational exposure to MDMA seen in the reaggregates is expressed primarily as an increase in the production and release of serotonin with some limited effects on dopaminergic neurons. In the case of serotonin, the enhanced and/or accelerated development of this monoamine in the reaggregates could involve a number of neurochemical or anatomic mechanisms including induction of a serotonergic phenotype in additional mesencephalic neurons, effects on tryptophan hydroxylase, or effects on serotonergic process outgrowth in either the mesencephalic tegmentum or the striatum. The latter possibility has been demonstrated in the case of prenatal exposure to cocaine where serotonergic hyperinnervation of the striatum has been observed [31].

Serotonergic neurons develop early in ontogeny and the transmitter is detectable in raphe neurons by histofluorescence methods as early as GD 13 in the mouse [8]. Fetal levels of serotonin can be pharmacologically manipulated during gestation. Maternal administration of the tryptophan hydroxylase inhibitor, *para*-chloroalanine, to pregnant rats from GD 6 to GD 13–14 decreases the serotonin content of embryonic raphe neurons at GD 13–14 and GD 14–15 [23]. There is in vitro and in vivo evidence that serotonergic neuronal development is under the regulation of the neurotransmitter. Treatment of embryonic raphe cultures with low concentrations of the serotonergic 5-HT1/5-HT2 receptor agonist, 5-methoxytyramine (5-MT), inhibits serotonergic fiber outgrowth while high doses enhance outgrowth [34]. A similar dose-dependent effect on serotonergic terminal density has been observed in the brainstem and forebrain of postnatal day 30 rat offspring following prenatal exposure to 5-MT [29]. MDMA which is known to evoke serotonin release, also stimulates serotonergic neuronal maturation, as evidenced by increased serotonin uptake, in raphe cultures at low drug concentrations [1]. This latter observation is in agreement with the findings of the present study of enhanced serotonin neurochemical development in reaggregates prepared from MDMA-exposed fetuses.

In summary, maternal administration of MDMA to

pregnant C57Bl/6 mice from gestational day 6 through 13 results in persistent effects on the developing monoaminergic striatal projection which can be detected in reaggregate cultures prepared from the embryos of the treated dams. There is an enhancement of serotonergic and dopaminergic markers lasting for at least 36 days of culture. The marked effect on serotonergic development, in particular, is of considerable interest since serotonin may serve as an early differentiation signal in the ontogeny of the nervous system [22]. Accelerated development of serotonergic systems during critical periods of ontogeny could have serious consequences for the neurochemical and behavioral status of the developing and adult animal. The results of the present study raise concerns that the human fetus of an Ecstasy-abusing mother may be at risk for development of alterations in neural function.

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