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MDMA-induced loss of parvalbumin interneurons within the dentate gyrus is mediated by 5HT_{2A} and NMDA receptors

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Abstract

MDMA is a widely abused psychostimulant which causes a rapid and robust release of the monoaminergic neurotransmitters dopamine and serotonin. Recently, it was shown that MDMA increases extracellular glutamate concentrations in the dorsal hippocampus, which is dependent on serotonin release and 5HT_{2A/2C} receptor activation. The increased extracellular glutamate concentration coincides with a loss of parvalbumin-immunoreactive (PV-IR) interneurons of the dentate gyrus region. Given the known susceptibility of PV interneurons to excitotoxicity, we examined whether MDMA-induced increases in extracellular glutamate in the dentate gyrus are necessary for the loss of PV cells in rats. Extracellular glutamate concentrations increased in the dentate gyrus during systemic and local administration of MDMA. Administration of the NMDA receptor antagonist, MK-801, during systemic injections of MDMA, prevented the loss of PV-IR interneurons seen 10 days after MDMA exposure. Local administration of MDL100907, a selective 5HT_{2A} receptor antagonist, prevented the increases in glutamate caused by reverse dialysis of MDMA directly into the dentate gyrus and prevented the reduction of PV-IR. These findings provide evidence that MDMA causes decreases in PV within the dentate gyrus through a 5HT_{2A} receptor-mediated increase in glutamate and subsequent NMDA receptor activation.

Keywords

MDMA; glutamate; parvalbumin; GABA; hippocampus; serotonin

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA, ecstasy) is a psychostimulant popular amongst both teenagers and younger adults. MDMA targets primarily the serotonin (5HT) neurotransmitter system to produce a rapid acute release of 5HT and a long-term depletion

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of 5HT resulting from what can be best described as a distal axotomy (Nichols et al., 1982, Johnson et al., 1986). This persistent decrease in 5HT is observed within the striatum, cortex and hippocampus and may underlie the cognitive deficits evident in human abusers of MDMA (Battaglia et al., 1987, O'Hearn et al., 1988, Parrott et al., 1998).

Several studies support the notion that MDMA-induced neurotoxicity may extend beyond serotonin terminals to include apoptosis within the hippocampus (Tamburini et al., 2006, Wang et al., 2009, Kermanian et al., 2012, Soleimani Asl et al., 2012). Further evidence for neuronal cell loss caused by MDMA originates from recent reports of a loss of parvalbumin (PV) interneurons in the dentate gyrus of MDMA-exposed rodents (Anneken et al., 2013, Abad et al., 2014). These studies concluded that the loss of PV interneurons is associated with MDMA-induced increases in hippocampal glutamate; however, a direct causal role for glutamate receptor activation in mediating this effect remains to be established. Along these lines, PV neurons express mGluR1/5 receptors, GluR2 lacking AMPA receptors as well as NMDA receptors within the hippocampus such that their coordinate or convergent activation may convey susceptibility to excitotoxicity (Kerner et al., 1997, Catania et al., 1998, Le Roux et al., 2013). In fact, decreases in PV immunoreactivity (PV-IR) are evident after neurotoxic insults in which glutamate neurotransmission is a presumed mechanism (Kwon et al., 1999, Gorter et al., 2001, Sanon et al., 2005).

The increases in extracellular glutamate concentrations caused by systemic injections of MDMA appear to be mediated by 5HT efflux and prevented by the systemic injections of the non-specific 5HT₂ receptor antagonist ketanserin (Anneken and Gudelsky, 2012, Anneken et al., 2013). It remains to be determined which 5HT₂ receptor subtype within the hippocampus mediates the increases in extracellular glutamate concentrations. Therefore, the current study determined if 5HT_{2A} and/or NMDA receptor activation within the dentate gyrus of the hippocampus during MDMA exposure is responsible for the increases in extracellular glutamate and decreases in PV-IR.

2. Methods

2.1 Animals

Adult male Sprague-Dawley rats (200–275 g. Harlan Sprague Dawley, In, USA) were used in all experiments. Upon arrival, rats were grouped two rats per cage and allowed one week to acclimate. The rats were kept on a 12/12-hr light dark cycle in a temperature and humidity controlled room with food and water available ad libitum. All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and in approval with Institutional Animal Care and Use Committee at the University of Toledo.

2.2 Drug Treatments

MDMA was obtained from the National Institutes of Drug Abuse (NIDA, Research Triangle). For PV cell count experiments, rats were injected with 0.9% (1 ml/kg) saline or MDMA (7.5 mg/kg, once every 2 hours X 4 injections). MDL100907 (generous gift from Wyeth) and MK801 (Tocris) were dissolved in saline with a pH 3 (adjusted with HCl) and were given via IP injections (0.1mg/kg), 30 minutes before each administration of MDMA. These doses were based on previous studies where this dose was used (Schreiber et al.,

1998, Tutka et al., 2002). Core body temperatures were recorded 1 hour after each injection of saline or MDMA using a rectal probe digital thermometer (Thermalert TH-8, Physitemp Instruments). To control for decreases in MDMA-induced hyperthermia caused by MK801 or MDL100907, treatments of these groups were performed in an elevated ambient temperature (28.7°C).

2.3 Microdialysis

Rats were anesthetized with a xylazine/ketamine hydrochloride mixture (6/70 mg/kg i.p.) and placed in a stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The active membrane (~1mm, 13KDa cutoff) of a microdialysis probe was placed in the dentate gyrus of the dorsal hippocampus (coordinates: 3.9 mm rostral from bregma, 2.2 mm lateral from the midline and 4.2 mm ventral). Three additional holes, with stainless steel screws were placed in the skull to act as anchors for the dental cement.

The morning after surgeries, 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) (Sigma-Aldrich) was perfused at a flow rate of 1.5 µl/min using a model 22 syringe perfusion pump (Harvard Apparatus). After a 1 hour equilibration period, four 30 minute baseline samples were collected. MDMA exposure began immediately following baseline collections and dialysate samples were collected every hour. MDMA exposure consisted of either 4 i.p. injections of 7.5 mg/kg, each injection given every other hour or reverse dialysis of 100 µM MDMA directly into the dentate gyrus. Reverse dialysis of the antagonists (100 nM) MDL100907 was started 30 minutes prior to reverse dialysis of MDMA. This dose was used based on previous findings highlighting selectivity of MDL100907 for 5HT_{2A} receptors as compared to 5HT_{2C} receptors (Kehne et al., 1996). Brains from rats used for microdialysis were sectioned and probe placement was verified.

2.4 High-performance Liquid Chromatography Analysis of Extracellular Glutamate

Dialysate samples (20µl) were injected onto a C18 column (150 x 2mm, 3µm particle filter, Phenomenex) and were eluted using a mobile phase which consisted of 0.1 M Na₂HPO₄, 0.1mM EDTA and 10% methanol (pH 6.4). As previous described, o-phthaldialdehyde was used to derivatize glutamate for electrochemical detection a using a LC-4C amperometric detector (BAS Inc.) (Donzanti and Yamamoto, 1988).

2.5 Immunohistochemistry

Fixation of the brain was performed by transcardially perfusing 0.1M phosphate-buffered saline (PBS) followed by 4% paraformaldehyde on the 10th day after drug or saline exposure. Brains were cryoprotected, flash frozen and hippocampi sectioned into 50 µm thick slices. Sections were treated with 1% H₂O₂ for 30 min at room temperature (RT). The sections were blocked for 2 hr at RT with 10% normal goat serum (Life Technologies) in 0.1M PBS containing 0.5% Triton-X 100 and Avidin block (4 drops/mL; Vector Laboratories). For PV immunostaining, 50µm thick sections were incubated for 36 hr at 4°C with a mouse monoclonal PV antibody (1:2000; Swant, cat# PV235) in 0.1 M PBS containing 0.5% Triton-X 100, 1% NGS and Biotin block (4 drops/ml; Vector Laboratories). Sections were then incubated in goat anti-mouse biotinylated secondary antibodies

(Millipore, cat# AP124b) for 2hr at RT followed by incubation in avidin-biotin-horseradish peroxidase (Vectastain Elite ABC Kit; Vector Laboratories) for 2hr at RT. Sections were then developed in diaminobenzidine (DAB/Metal Concentrate; Pierce) and mounted on glass slides and coverslipped with Eukitt mounting medium (Sigma- Aldrich). All slides were coded and the code was not broken until the end of quantitative analysis.

2.6 Stereology

To assess PV-IR GABA interneurons in the dorsal hippocampus, a modified optical fractionator counting technique was used (West et al., 1991, Gundersen et al., 1999). Neuronal counts were made using a BX51 Olympus microscope equipped with a DVC camera interfaced with StereoInvestigator 8.21 software (MBF Bioscience). During quantification, every fourth section for a total of six sections through the dorsal hippocampus was systematically sampled. A border which contained the granule cell layer and hilus region was drawn with a 10x objective and counts were performed using a 20x objective. Tissue shrinkage due to processing prevented the use of guard zones above and below the dissector. PV counts were performed using 100µm X 100µm grid dimensions. Parameters for stereology experiments were determined in order to obtain a Gundersen coefficient of around 0.1 or below, which allows for an accurate estimate of total PV interneurons within the region.

2.7 Statistical Analyses

Stereological neuronal counts were analyzed using a two-way ANOVA to compare the effects of saline or MDMA and an interaction between these effects and that of the antagonists (MK801 or MDL100907). Extracellular glutamate concentrations were compared between groups using a two-way ANOVA with repeated measures. Post-hoc analysis was performed using Tukey's test. Statistical significance was set at $P < 0.05$

3. Results

3.1 Effects of systemic injections of MDMA on extracellular glutamate concentrations in the dentate gyrus

The effects of systemic injections of MDMA (7.5 mg/kg x 4 injections, each injection every 2 hrs) on extracellular glutamate concentrations within the dentate gyrus were determined via microdialysis. Two-way repeated measures ANOVA revealed a significant effect of MDMA treatment on extracellular glutamate concentrations ($F_{(1,110)}=9.635$; $P < 0.01$). Extracellular glutamate concentrations were significantly increased 1 hour after the second injection of MDMA ($P < 0.05$) and at subsequent sample collection times (Fig. 1).

3.2 NMDA receptor inhibition prevents MDMA-induced PV cell losses

The role of NMDA receptor activation in mediating MDMA-induced PV deficits within the dentate gyrus was examined. Representative images (10x magnification) of the dentate gyrus from treatment groups, 10 days following drug exposure are shown in Fig. 2A. PV cells within the dentate gyrus were localized to the granule cell layer and hilus region. MDMA+vehicle treated rats had significantly fewer (30%, $P < 0.001$) PV interneurons in the dentate gyrus than saline vehicle treated control animals. MDMA+vehicle treated animals

had significantly increased core body temperatures ($P<0.05$) compared to saline+vehicle controls, but were not significantly different than MDMA+MK801 treated animals (mean temperatures were 39.1 °C and 39.2 °C for MDMA+vehicle and MDMA+MK801 respectively (data not shown). As shown in fig. 2B, two-way ANOVA revealed a significant effect of MDMA on PV cells ($F_{(1,27)}=22.7$; $P<0.001$) as well as an interaction between MDMA and MK801 treatment on PV cell counts ($F_{(1,27)}=9.283$; $P<0.01$). PV cell counts in the dentate gyrus of rats treated with MK801 prior to and during MDMA exposure were not significantly different from saline vehicle controls.

3.3 MDL100907 prevents increased glutamate concentrations caused by local perfusion of MDMA

The effects of local administration of the 5HT_{2A} receptor antagonist MDL100907 on extracellular glutamate increases caused by reverse dialysis of 100 µM MDMA into the dentate gyrus was determined. As shown in Fig. 3, reverse dialysis of 100 µM MDMA +vehicle resulted in a significant increase in extracellular glutamate concentrations compared to aCSF+vehicle ($P<0.01$). Two-way repeated measures ANOVA on MDMA +vehicle and aCSF+vehicle groups revealed a significant effect of MDMA treatment ($F_{(1,100)}=6.68$; $P<0.05$) and time ($F_{(6,100)}=2.27$; $P<0.05$). A significant effect of MDL100907 treatment ($F_{(1,107)}=4.95$; $P<0.05$) was revealed when two-way repeated measures ANOVA was performed on MDMA+vehicle and MDMA+MDL10097 groups. Additionally, extracellular glutamate concentrations were not significantly different between MDMA+MDL100907 and aCSF+vehicle groups.

3.4 Effect of MDL100907 on MDMA-induced PV cell losses

The ability of MDL100907 to prevent MDMA-induced decreases in PV cell counts was determined. As shown in Fig. 4, MDMA+vehicle treated animals had significantly fewer PV interneurons in the dentate gyrus compared with saline+vehicle treated rats ($P<0.05$). A two-way ANOVA analysis revealed a main effect of MDMA ($F_{(1,21)}=9.26$; $P<0.01$) as well as an interaction between MDMA and MDL100907 ($F_{(1,21)}=11.5$; $P<0.01$) on the decreases in the number of PV-positive cells. MDL100907 prior to and during MDMA exposure blocked the decrease in PV cell counts and were not significantly different from saline vehicle controls. Core body temperatures of MDMA+MDL100907 treated rats did not differ significantly from those of MDMA+vehicle treated rats (mean temperatures were 39.1 °C and 39 °C. for MDMA+vehicle and MDMA+MDL100907, respectively).

4. Discussion

Our findings support a role for glutamate within the dentate gyrus in mediating the long-term loss of PV interneurons caused by MDMA. It has been reported that systemic injections of MDMA as well as reverse dialysis of 100 µM MDMA increased glutamate concentrations within the hippocampus; however these experiments did not localize this effect to the dentate gyrus where PV cell losses occur (Anneken and Gudelsky, 2012, Anneken et al., 2013). We found that both the systemic injections of 7.5mg/kg MDMA (1 injection given every 2 hr for a total of 4 injections) as well as the direct administration of 100 µM MDMA into the dentate gyrus produced an increase in extracellular glutamate

concentrations within the dentate gyrus. Therefore, these findings support the idea that the loss of PV interneurons in the dentate gyrus is a result of local increases in glutamate during MDMA exposure.

PV interneurons within the hippocampus are known to express NMDA receptors (Le Roux et al., 2013). Antagonism of NMDA receptors during MDMA exposure prevented the deficits in PV interneurons observed 10 days following MDMA exposure (Fig. 2). This effect was not dependent on the ability of MK801 to reduce MDMA-induced hyperthermia (Colado et al., 1998) since the increases in body temperatures between MDMA and MDMA +MK801 groups were similar. Our findings that MK801 pretreatment is able to prevent MDMA-induced decreases in PV cell numbers support the conclusion that increases in glutamate during MDMA exposure play a role in the PV cell losses through increased activation of NMDA receptors. Other studies have reported deficits in PV interneurons within the hippocampus resulting from neurotoxic insults where excitotoxicity is a presumed mechanism (Phillips et al., 1998, Kwon et al., 2000). These studies showed that these PV deficits could be prevented through inactivation of NMDA receptors. Because NMDA receptor activation plays a role in mediating excitotoxicity, it is possible that MDMA-induced glutamate increases are excitotoxic through activation of NMDA receptors on PV interneurons.

Anneken and Gudelsky (2012) concluded that the increases in hippocampal glutamate concentrations were the result of MDMA-induced increases in serotonin and subsequent 5HT₂ receptor activation. In support of this conclusion, we found that MDL100907, an antagonist with high selectivity for 5HT_{2A} receptors, prevented MDMA-induced glutamate increases in the dentate gyrus. Immunoreactivity of 5HT_{2A} receptors is known to be relatively dense in the dentate gyrus, in particular the hilus region bordering the granule cell layer, and is similar to the densities of PV expressing interneurons in the region (Fig. 2A) (Luttgen et al., 2004). The relative density of serotonin innervation follows a similar pattern to that of 5HT_{2A} receptor expression (Moore and Halaris, 1975). Thus, MDMA causes the release of 5HT to presumably increase 5HT_{2A} receptor activation within the dentate gyrus.

5HT_{2A} receptors within the dentate gyrus are known to be expressed by astrocytes and granule cells within the dentate gyrus (Xu and Pandey, 2000). MDMA-induced 5HT and activation of 5HT_{2A} receptors would increase downstream signaling cascades including the activation of phospholipases. 5HT_{2A} receptors in comparison to 5HT_{2C} receptors are known to preferentially activate phospholipase A2 and subsequent arachidonate production (Felder et al., 1990, Berg et al., 1998). Arachidonate production could thus contribute to cyclooxygenase 2 (COX2) mediated synthesis of prostaglandins such as PGE₂, to increase glutamate release from astrocytes and neurons and cause excitotoxicity (Bezzi et al., 1998, Lin et al., 2014). It was previously reported that MDMA-induced increases in glutamate within the hippocampus could be prevented via inhibition of COX2 (Anneken et al., 2013) and supports the interpretation that MDMA-induced increases in glutamate (Figs. 1 and 3) are mediated via activation of 5HT_{2A} receptors (see Fig. 3) with subsequent production of prostaglandins.

As mentioned, the localization of 5HT innervations and 5HT_{2A} receptors is particularly dense within the inner portions of the granule cell layer and hilus region (Luttgen et al., 2004). This corresponds with the pattern of PV interneuron localization within the dentate gyrus and suggests that PV cells in this region have an increased likelihood of being impacted by increased 5HT_{2A} receptor activation and subsequent increases in glutamate caused by MDMA. In support of this idea, we found that the 5HT_{2A} receptor antagonist MDL100907 prevents MDMA-induced decreases in PV cells observed 10 days later (Fig. 4). The PV decreases are not mediated by the reduction in MDMA-induced hyperthermia since the average core body temperatures were similar between MDMA+vehicle and MDMA+MDL100907 treated rats.

The loss of PV interneurons following MDMA exposure may help explain a number of findings that suggest MDMA exposure may impair inhibition within the hippocampus and promote a loss of hippocampal function. PV interneurons within the dentate gyrus are known to synapse directly onto the somatic region of granule cells, which allows them to exert strong inhibitory control of action potential firing (Freund and Buzsaki, 1996, Miles et al., 1996). For this reason, a dysfunction in PV interneuron activity has been implicated in epileptogenesis. Along these lines, MDMA exposure in rodents has been shown to increase susceptibility to kainate induced seizures which correlates with a loss of PV interneurons within the dentate gyrus (Giorgi et al., 2005, Abad et al., 2014). In addition, PV interneurons play a role in feed-forward and feed-back inhibition within the dentate gyrus and thereby are integral components of information processing within the hippocampus (Kneisler and Dingledine, 1995a,b). Thus, a loss of PV interneurons could potentially impair hippocampus-mediated information processing. In fact, several studies have reported spatial memory impairments typically associated with dysfunction of hippocampal based cognitive processes in both adolescent and adult rats treated with MDMA (Robinson et al., 1993, Sprague et al., 2003, Williams et al., 2003, Vorhees et al., 2004, Skelton et al., 2006, Arias-Cavieres et al., 2010). Further studies are needed to determine the extent to which GABAergic function is impaired in the hippocampus after MDMA and whether these impairments underlie changes in hippocampus-mediated cognitive function caused by exposure to the drug.

In conclusion, MDMA-induced decreases in PV interneurons are mediated by the 5HT_{2A} receptor, increases in glutamate neurotransmission and subsequent activation of NMDA receptors within the dentate gyrus during MDMA exposure. These effects of MDMA on PV interneurons within the hippocampus may have a significant impact on hippocampal physiology and explain spatial memory deficits as well as seizure susceptibility in animals treated with MDMA or human MDMA abusers.

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Abbreviations

MDMA	3,4-Methylenedioxymethamphetamine

PV	parvalbumin
PV-IR	parvalbumin immunoreactive
5HT	serotonin
GABA	<i>gamma</i> -Aminobutyric acid
NMDA	N-Methyl-D-aspartate
AMPA	α -Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
RT	room temperature
COX-2	cyclooxygenase 2
PBS	phosphate buffered saline

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Fig. 1.

Effect of systemic MDMA injections on extracellular glutamate levels within the dentate gyrus. Rats received MDMA (7.5mg/kg every 2h X 4 i.p. injections) or saline (1mL/kg, every 2h). n=8–12 per group. Two-way repeated measures ANOVA revealed a significant main effect of MDMA treatment ($P<0.005$) *=statistically significant from saline treated controls ($P<0.05$).

Fig. 2.

Effect of MK801 on MDMA-induced PV-ir interneuron decreases in the dentate gyrus. Rats were treated with MK801 (0.1mg/kg, i.p.) 30 minutes prior to each MDMA (7.5mg/kg every 2h X 4 i.p injections) or saline injection. Ten days after exposure, stereologic assessment of dentate PV-IR interneurons was performed. A) Representative photomicrographs of PV-IR for treatment groups with PV positive cells labeled with black arrows. B) Quantitative assessment of PV-IR (n=5–8 per group). MDMA+vehicle significantly decreased PV-IR neurons in the dentate gyrus compared to saline+vehicle treatments ($P<0.001$). MK801 pretreatment prevented decreases in PV cell counts caused by MDMA ($P<0.005$).

*=statistically significant from saline+vehicle controls ($P<0.05$).

Fig. 3. MDL100907 prevents increases in extracellular glutamate within the dentate gyrus caused by reverse dialysis 100 μ M MDMA. Microdialysis measurements of extracellular glutamate concentrations were performed in the dentate gyrus in which 100 μ M of MDMA was perfused for 7 hours (noted by bar). MDL100907 (100nM) or vehicle was reverse dialysed 30 minutes prior and during MDMA. $n=6-11$ per group. Two-way repeated measures ANOVA revealed a significant main effect of MDMA ($P<0.05$). MDL100907 prevented MDMA-induced increases in glutamate ($P<0.05$). * denotes values statistically significant from aCSF+vehicle controls ($P<0.05$).

Fig. 4.

Effect of MDL100907 on MDMA-induced PV-ir interneuron decreases in the dentate gyrus. Rats were treated with MDL100907 (0.1mg/kg) 30 minutes prior to each MDMA (7.5mg/kg every 2h X 4 injections) or saline injection. Ten days following exposure, stereologic assessment of dentate PV-ir interneurons was performed. PV counts for saline+vehicle and MDMA+vehicle groups are repeated from Fig. 2. $n=6-8$ per group. MDMA+vehicle significantly reduced PV-IR neurons in the dentate gyrus compared to saline+vehicle treatments ($P<0.001$). MDL100907 pretreatment prevented MDMA-induced decreases in PV neurons ($P<0.001$). *=statistically significant from saline+vehicle controls ($P<0.05$).