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Neuroscience Letters 362 (2004) 6–9

Neuroscience Letters

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No effect of MDMA (ecstasy) on cell death and 5-HT$_{2A}$ receptor density in organotypic rat hippocampal cultures

Marie-Louise Sveen, Gitte M. Knudsen, Susana Aznar*

Neurobiology Research Unit, Copenhagen University Hospital, Unit 9201, Blegdamsvej 9, 2100 Copenhagen, Denmark

Received 9 October 2003; received in revised form 7 January 2004; accepted 7 January 2004

Abstract

MDMA (3,4 Methylenedioxy-methamphetamine) binds and blocks the presynaptic serotonin reuptake transporters and postsynaptic serotonin 5-HT$_{2A}$ receptors, with highest affinity for the first. Whether 5-HT$_{2A}$ receptor density decreases due to MDMA's direct effect on postsynaptic serotonin receptors is at present not known. This study analyzes whether direct stimulation of the postsynaptic 5-HT$_{2A}$ receptor by MDMA in organotypic hippocampal cultures results in cell death and downregulation of this receptor. Fifty or 100 μM MDMA was added to 1 week old cultures, made of 11 day old rat pups. Fluorojade and immunostaining for MAP2 and 5-HT$_{2A}$ to determine neurodegeneration, and changes in receptor density, respectively, resulted in no significant differences. MDMA's neurotoxicity and regulation of post-synaptic 5-HT$_{2A}$ receptors thus seems to require the presence of intact serotonergic terminals.

Keywords: 3,4 Methylenedioxy-metamphetamine; In vitro; Organotypic cultures; Neurotoxicity; Serotonin receptor; Hippocampus

MDMA (3,4 Methylenedioxy-metamphetamine) has its psychological and physiological effects through its direct interaction with the serotonergic, and possibly indirectly to a lesser extent dopaminergic system [9]. This is mainly attributed to MDMA's binding to, and reversal of the 5-HT reuptake transporter function, resulting in 5-HT release, and 5-HT reuptake inhibition. Later findings have also suggested the possible role of its 5-HT$_{2A}$ receptor binding in the acute effect of MDMA [16].

To what extent MDMA is neurotoxic is at present not well known. Experimental studies have shown a reduction of serotonergic axons, but not of serotonergic cell bodies, in animals treated with MDMA [11]. In humans decrements in global brain binding of the 5-HT transporter ligand [^{11}C] McN-5652, which correlated to the extent of previous MDMA use have been observed [15].

The idea that there may be a direct effect of MDMA through the post-synaptic 5-HT$_{2A}$ serotonin receptor has been suggested. In humans, ketanserin blockage of the 5-HT$_{2A}$ receptor resulted in attenuation of MDMA induced perceptual changes, emotional excitation, and acute adverse responses, but it had little effect on MDMA induced positive mood, well being, extroversion, and short-term sequelae. These results indicate that the 5-HT$_{2A}$ receptor plays a contributing role in MDMA's psychological effects [7]. Furthermore, recent studies have shown that 5-HT$_{2A}$ receptor densities are decreased both in rats [17] and in humans [13] after chronic exposure to MDMA.

In this study we made use of organotypic hippocampal cultures, which constitute an isolated system devoid of the afferent serotonergic projection and thereby serotonin reuptake sites, to study whether MDMA has a direct neurotoxic effect on the postsynaptic neurons and whether the downregulation of the 5-HT$_{2A}$ receptor observed after MDMA exposure is mediated through a direct effect of MDMA on postsynaptic 5-HT$_{2A}$ receptors. Neurotoxicity was determined by Fluoro-Jade, which stains neurodegenerating cells, and the cytoskeleton marker MAP2. Changes in 5-HT$_{2A}$ receptor density were measured by immunostaining and subsequent densitometric analysis.

Hippocampal cultures were prepared according to the interface culture method of Stoppini et al. [18]. Quickly, 11 day old rat pups were decapitated and their brains taken out under sterile conditions. The hippocampi were dissected out and sectioned transversely at 400 μm with a McIlwain tissue chopper. The resulting slices were placed in chilled Gey's balanced salt solution with glucose (6.5 mg/ml) and

* Corresponding author. Tel.: +45-3545-6706; fax: +45-3545-6713.
E-mail address: saznar@nru.dk (S. Aznar).

0304-3940/03/$ - see front matter © 2004 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2004.01.051

inspected under a microscope. Only intact slices without visible damage and including fimbria, were selected. Six slices were placed on one Millicell CM membrane insert (Millipore). The membrane inserts were transferred to six-well culture trays, each well containing 1 ml medium consisting of 25% inactivated horse serum, 25% Hanks balanced salt solution and 50% Optimem-medium, supplemented with D-glucose (25 mM) and L-glutamine (1 mM; all from Gibco). No antimitotic drugs or antibiotics were used at any stage. The cultures were kept in an incubator at 37 °C and 5% CO_2 and during the 1st week the medium was changed twice.

After the 1st week, MDMA (generously provided by Lundbeck A/S, Denmark) was added to the medium at different concentrations (50 and 100 μM). Controls were exposed to medium alone. The medium was changed every 3rd day for 1 week.

After 1 week of exposure to different concentrations of MDMA, the cultures were fixed in 4% PFA for 1 h and transferred to 20% sucrose solution for cryoprotection for 1 h. The cultures were then embedded in Tissue Tek, frozen and cut in two parallel series at 16 μm in a cryostat. Once cut and thaw-mounted on glass slides the cultures were stored at − 80 °C until processed.

For Fluoro-Jade staining, slices were rehydrated in 99% ethanol (3 min), 70% ethanol (1 min) and distilled H_2O (1 min). The sections were then treated for 15 min with 0.06% potassium permanganate (Sigma), washed in distilled H_2O and stained for 30 min with 0.001% Fluoro-Jade (Histo-Chem). The slices were washed in distilled H_2O, dehydrated in 70 and 99% ethanol, air dried and mounted with a xylene-based medium.

For immunocytochemical staining the sections were first blocked for endogenous peroxidase with a solution of 10% methanol and 10% H_2O_2 in phosphate buffered saline (PBS) for 5 min., washed and blocked for non-specific binding of the secondary antibody for 1 h in PBS containing 2% heat-inactivated goat serum (HINGS), 2% bovine serum albumin (BSA) and 0.2% triton. Parallel sections were then incubated for 3 h at 37 °C with either a monoclonal antibody raised against the 5-HT_{2A} receptor (BD PharMingen, 1:500), or a monoclonal antibody raised against MAP2 (Sigma, 1:1000), this was followed by three washes in PBS containing 2% HINGS, 2% BSA and 0.2% triton and incubation for 1 h at room temperature with either a biotinylated goat anti-rabbit (Dako, 1:600), or a biotinylated goat anti-mouse antibody (Dako, 1:600). Sections were washed again and incubated with streptavidin (Dako, 1:600) for 1h at room temperature. After washing the sections in PBS the immunostaining was visualized by treatment with 3,3-diaminobenzidine (DAB; Dako) for 5 min. The slices were washed in distilled H_2O, dehydrated in 70 and 99% ethanol, air dried and mounted with a xylene-based medium.

Fluoro-Jade stained sections were visualized with a fluorescence microscope using a FITC-filter. Neurons stained for Fluoro-Jade were counted in all sections for each culture and the average of stained neurons was calculated for each group; Control $n = 47$; 50 μM MDMA $n = 56$; 100 μM MDMA $n = 39$.

All sections to be compared with each other were immunostained under exactly the same conditions. The MAP2 and 5-HT_{2A} stained sections were analyzed using a Zeiss axiolab light microscope and the images recorded digitally at a magnification of × 40 with a Sony DSC-S70 camera. The optical density of all the MAP2, and 5-HT_{2A} stained slices was measured using the Scion-Image analysis program. The average density for each culture was calculated from the total of measured slices belonging to each single culture. The number of cultures included in each group was; Control $n = 6$; 50 μM MDMA $n = 6$; 100 μM MDMA $n = 6$.

All densitometric data are expressed as mean ± SEM percentage of control cultures. Statistical significance was assessed using a single factor ANOVA and *t*-tests or Mann–Whitney test on the groups of interest. Differences were considered statistically significant at $P < 0.05$.

Sections stained with Fluoro-Jade (Figs. 1A,B) did not

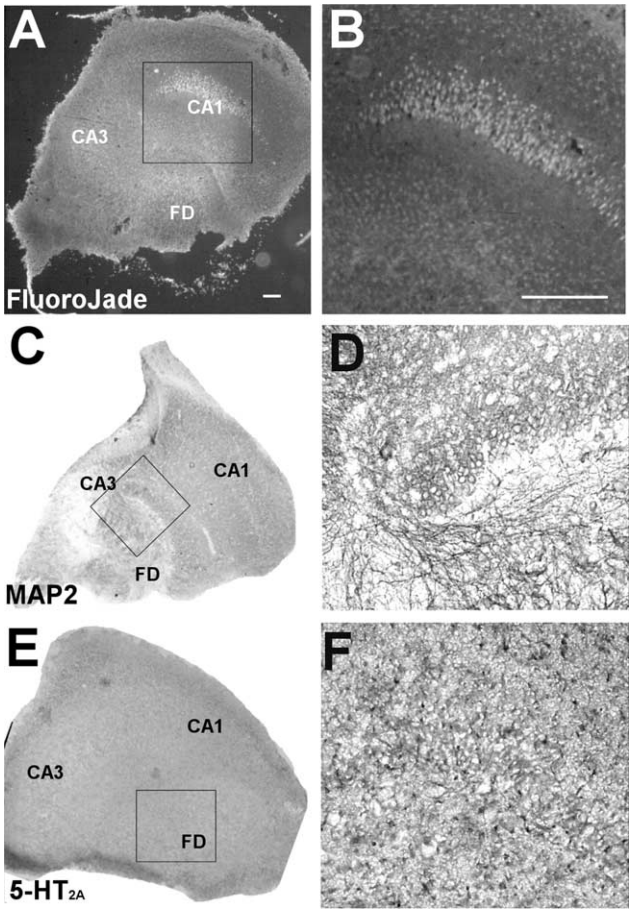

Fig. 1. Sections histochemically stained for Fluoro-Jade (A); immunostained for the MAP2 (B); and for the 5-HT_{2A} receptor (C) of 11 day-old organotypic hippocampal cultures exposed to control medium. B, D and F are details of the framed area in respectively A, C and E. CA1, CA1 pyramidal layer; CA3, CA3 pyramidal layer; FD, fascia dentata. Bar in A, C and E = 500 μM; B, D and F = 200 μM.

display a significantly higher number of stained cells, and thereby neurodegenerating cells in cultures exposed to MDMA in any of the concentrations used in this study (Fig. 2A). Fluoro-Jade stained neurons, when present, were restricted to the CA1 area. The average (± SEM) of Fluoro-Jade stained neurons in each group was as follows: control: 13.9 ± 18.2; 50 μM MDMA: 31 ± 39.2; 100 μM MDMA: 22.4 ± 32.9.

The antibody against MAP2 stained cell bodies and dendrites in all cultures (Figs. 1C,D). Densitometric analysis of the MAP2 immunostaining did not reveal significant differences between the control group and cultures exposed to the two concentrations of MDMA (Fig. 2B).

The antibody against the 5-HT_{2A} receptor stained neurons and processes in all hippocampal layers (Figs. 1E, F). Densitometric analysis of the 5-HT_{2A} immunostaining did not show significant differences between the control groups and cultures exposed to the two concentrations of MDMA (Fig. 2C).

Our results did not show a significant association between MDMA exposure and neurotoxicity in hippocampal organotypic cultures. Likewise, there was no significant change in 5-HT_{2A} receptor density in cultures exposed to MDMA versus control.

The organotypic culture model used in this study offers the advantage that it provides an isolated system devoid of afferents. Serotonin reuptake sites are primarily present on serotonergic terminals [19]. All serotonergic projections to the forebrain originate from the raphe nuclei in the brainstem, and when culturing our hippocampal slices, all serotonergic terminals degenerate after 1 week. Organotypic hippocampal cultures have previously been used for monitoring cell death, using FluoroJade and MAP2 as markers [10] and for studying changes in serotonin receptor density [2].

The lack of neurotoxicity observed in our cultures suggests that there is no direct toxic effect of MDMA on postsynaptic neurons. The removal of presynaptic serotonin

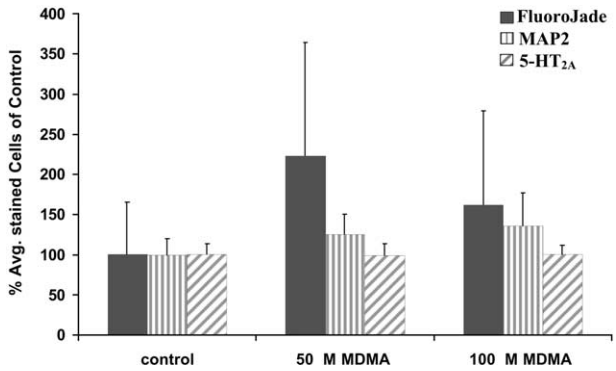

Fig. 2. Number of Fluoro-Jade stained cells, and densities of MAP2 and 5-HT_{2A} immunostaining (mean ± SEM) measured in sections of organotypic hippocampal cultures exposed to control medium and to various concentrations of MDMA for 1 week.

transporters in this system meant that MDMA only could bind to postsynaptic receptors. Homogenate studies have shown that MDMA binds to the 5-HT_{2A} receptor (k_i = 5.1 μM), though with lower affinity than to the pre-synaptic reuptake transporter (k_i = 0.61 μM) [3]. This difference in affinity suggests that the agonist effect of MDMA on the post-synaptic 5-HT_{2A} receptor only takes place in the presence of higher concentrations of the drug [3]. The concentrations of MDMA used in our study (50 and 100 μM) correspond to the local concentrations measured (20–80 μM) when MDMA is directly infused in the brain to produce serotonin release [5]. These concentrations were high enough to expect MDMA to bind to the 5-HT_{2A} receptors. A role of this receptor in the MDMA mediated neurotoxicity has been proposed as blockage of the 5-HT_{2A} receptor prevents the MDMA induced nerve terminal degeneration [12]. According to our data agonist stimulation of the 5-HT_{2A} receptor by MDMA does not lead to cell death.

MDMA's neurotoxicity was first suggested by Ricaurte et al. [14]. Later studies showed that MDMA causes a profound loss of serotonergic axons, in the rat forebrain, while the serotonergic cell bodies in the raphe nucleus, remained immunoreactive and did not show cytopathological changes [11]. Along with the fact that 5-HT persisted in these cell bodies, it indicates that they also remain functionally intact. It thus seems that the term neurotoxicity in the past has been used interchangeably by attenuation of serotonin markers, terminals and axons while no study has directly shown neuronal loss caused by MDMA. Our results support the idea that the effect of MDMA is limited to the depletion or degeneration of the serotonergic and dopaminergic terminals.

There are some in vivo studies reporting that young rats, up to postnatal days 35–40 are resistant to the neurotoxic effects of single injections of MDMA, causing no significant decrease in labelled serotonin levels [1]. Later studies, however, have shown that repeated administration of MDMA (which corresponds with our experimental paradigm) to developing rat pups result in lasting cognitive changes and serotonin reduction in the hippocampus, with the period of greater vulnerability being from postnatal day 11 to postnatal day 20 [4]. In this study we made use of 11 day-old rats, which is the oldest that can be used for organotypic hippocampal cultures according to the approach by Stoppini et al. [18]. These cultures, furthermore, go through a maturation process which corresponds to the in situ maturation and which is exponential to the age of the cultured tissue [8]. A factor we have to take into account when using a culture paradigm is the absence of metabolites. Previous studies have suggested that a MDMA metabolite rather than MDMA itself might be responsible for its neurotoxic effects [20]. Since the most likely is that this MDMA is not metabolized in the cultures, we cannot exclude this as the reason why we did not observe neuronal cell death.

No decrease in 5-HT_{2A} receptor density was detected after MDMA application. We were interested in looking for changes in the levels of this receptor as according to animal studies there is a decrease in the density of post-synaptic 5-HT_{2A} receptors, which persists for at least 1 month after the last MDMA administration [13,17] and SPECT studies in humans have indicated that MDMA users present significant lower 5-HT_{2A} receptor densities in most cortical areas [13]. Whether these changes in receptor density are due to direct stimulation of the postsynaptic 5-HT_{2A} receptors by MDMA or to indirect stimulation caused by the massive release of serotonin that results from the binding of MDMA to presynaptic serotonin reuptake sites is not known. Evidence that MDMA directly binds to 5-HT_{2A} receptor in humans has been deduced from the fact that ketanserin blockage of it results in decreased emotional excitation which is not similarly inhibited by addition of the reuptake site blocker citalopram [6]. Our results suggest that the decrease of 5-HT_{2A} receptor density observed in MDMA abusers is not due to the direct binding of MDMA to this receptor but rather as an indirect effect of the MDMA binding to serotonin reuptake sites.

The principal finding of the present study supports the importance of the presynaptic serotonin reuptake site in MDMA's effects on the brain. The postsynaptic effect cannot be entirely excluded however, but the idea that MDMA's effect on these postsynaptic receptors is indirect and via the blockage of the presynaptic complex resulting in a sharp rise in serotonin, which in turn stimulates the postsynaptic receptors is strengthened. Furthermore, our results support the importance of the presynaptic serotonin reuptake site's initiating role in the cascade of cellular events due to MDMA stimulation, possibly resulting in neuronal damage.

Acknowledgements

Excellent technical assistance was provided by Anja Pedersen. This work was supported by research grants from the 1991 Pharmacy Foundation, Health Insurance Foundation, the Lundbeck Foundation, Danish Medical Research Council, Novo Nordisk Foundation, sawmill owner Jeppe Juhl and Ovita Juhl Memorial Foundation, Augustinus Foundation and Simon Fougner Hartmanns Family Foundation.

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