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NEUROPEPTIDE Y PROTECTS RETINAL NEURAL CELLS AGAINST CELL DEATH INDUCED BY ECSTASY

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Abstract—Ecstasy (3,4-methylenedioxymethamphetamine; MDMA) has potent CNS stimulant effects. Besides the acute effects of MDMA, such as psychomotor activation, euphoria, decreased appetite, and hyperthermia, long-term damage of dopaminergic and serotonergic nerve terminals in multiple brain areas have also been reported. Although some studies have demonstrated that considerable amounts of MDMA reach the vitreous humor of the eye, and that serious visual consequences can result from MDMA consumption, the toxic effect of MDMA on the retina has not been completely elucidated. Neuropeptide Y (NPY) is present in the CNS, including the retina. The aim of the present study was to evaluate the effect of MDMA on rat retinal neural cell viability and investigate the involvement of 5-HT 2A-receptor (5-HT_{2A}) activation. Moreover, the neuroprotective role of NPY on MDMA-induced toxicity was also investigated. MDMA induced necrosis [MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and propidium iodide assays] and apoptosis (immunoreactivity of cleaved caspase-3) in mixed cultures of retinal neural cells (neurons, macroglia and microglia), in a concentration-dependent manner. MDMA-induced toxicity was enhanced at higher temperature (40 °C) and was reduced by the $5HT_{2A}$-receptor antagonist, ketanserin (1 μM). Interestingly, necrotic and apoptotic cell death induced by MDMA was inhibited by NPY (100 nM).

In conclusion, MDMA induces cell death in retinal neural cells, which is potentiated by elevated temperature. The toxic effect of MDMA involves the activation of 5-HT_{2A}-receptor and can be inhibited by exogenous NPY. Thus, NPY or NPY analogues might be useful agents against retinal degeneration induced by drugs or in neurodegenerative eye diseases.

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Abbreviations: BSA, bovine serum albumin; $[Ca^{2+}]_i$, intracellular calcium concentration; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; Hepes, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); MDMA, 3,4-methylenedioxymethamphetamine (ecstasy); MEM, Minimum Essential Medium; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NPY, neuropeptide Y; PBS, phosphate-buffered saline; PI, propidium iodide.

Key words: MDMA, retina, NPY, toxicity, apoptosis, neuroprotection.

The recreational drug 3,4-methylenedioxymethamphetamine (MDMA; "ecstasy", "Adam", "X", "e"), often ingested at dance clubs, is a ring-substituted phenyl-isopropylamine that is related to both amphetamines and hallucinogens (Parrott, 2005; Cadet et al., 2007). The effects of MDMA are mediated, in part, by the increased release of 5-HT and subsequent stimulation of 5-HT receptors (Ricaurte et al., 2000).

MDMA is known to cause degeneration of 5-HT nerve terminals in different animal models (Stone et al., 1986; Schmidt, 1987; Ricaurte et al., 1988b). After MDMA chronic ingestion, a decrease in 5-HT levels and its metabolite, 5-hydroxyindoleacetic acid (5-HIAA), in the number of 5-HT uptake sites, and also in tryptophan hydroxylase (TPH) activity was observed (Commins et al., 1987; Ricaurte et al., 1988a,b, 1992; Green et al., 2003; Xie et al., 2006). Other studies reported that the post-synaptic 5-HT_{2A}-receptor is involved in the mechanism of MDMA-induced toxicity (Schmidt et al., 1990; Johnson et al., 1993; Malberg et al., 1996; Capela et al., 2006, 2007). Other studies showed that MDMA-induced cell death occurs mainly by apoptosis, via caspase-3 activation (Montiel-Duarte et al., 2002; Jimenez et al., 2004; Meyer et al., 2004; Cunha-Oliveira et al., 2006; Tamburini et al., 2006; Warren et al., 2007).

A major feature of clinical cases related to MDMA toxicity is hyperthermia. Body temperatures reaching up to 43 °C have been reported (Henry, 1992; Green et al., 2003). This is of extreme importance, since ecstasy is often consumed at "rave" parties, where dancing takes place in a warm environment that could exacerbate the effect of MDMA on thermoregulation. In animal studies, the increase in core temperature of MDMA-treated animals enhances neurotoxicity (Malberg and Seiden, 1998; Carvalho et al., 2002).

Although MDMA was detected in significant amounts in the eye globe and vitreous humor (Clauwaert et al., 2000; De Letter et al., 2000, 2002), and some serious consequences to vision have been reported in MDMA users (Jacks and Hykin, 1998; Michael et al., 2003; Firth, 2006), the effect of MDMA on the retina has only been examined in one very recent study (Miranda et al., 2007).

Neuropeptide Y (NPY) is a 36 amino acid peptide that belongs to NPY family, and its actions are mediated by six G-protein-coupled receptor subtypes, Y_1, Y_2, Y_3, Y_4, Y_5 and y_6 (Michel et al., 1998; Silva et al., 2002). NPY is one of the most abundant and widely distributed neuropeptides

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in the mammalian CNS, being involved in various physiological functions, including feeding, memory processing and cognition (Wettstein et al., 1995; Silva et al., 2005a). It has also been reported that NPY has a neuroprotective role against excitotoxicity in rat hippocampus (Silva et al., 2003a,b, 2005b). Moreover, it was recently described that NPY has a protective role against methamphetamine-induced neuronal apoptosis in the mouse striatum (Thiriet et al., 2005). NPY and its receptors are present in the retina of different mammalian and non-mammalian species (Bruun et al., 1986; Jen et al., 1994; Hutsler and Chalupa, 1995; Oh et al., 2002; D'Angelo and Brecha, 2004; Álvaro et al., 2007). Recently we showed that primary cell cultures of rat retinal neural cells express NPY and NPY receptors (Álvaro et al., 2007).

The aim of the present study was to evaluate the putative protective role of NPY in MDMA-induced toxicity of rat retinal neural cells. We used primary cell cultures of rat retinal neural cells and tested different MDMA concentrations (100–1600 μM) for 48 h, at normothermic (37 °C) and hyperthermic conditions (40 °C). The type of cell death (necrosis/apoptosis) and changes in morphology of different retinal cell types were also evaluated.

EXPERIMENTAL PROCEDURES

Materials

Minimum Essential Medium (MEM), ketanserin, penicillin, streptomycin, bovine serum albumin (BSA), Hepes, poly-D-lysine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and the anti-glial fibrillary acidic protein (GFAP) antibody were purchased from Sigma Chemical, St. Louis, MO, USA. The fetal bovine serum (FBS) was obtained from Biochrom, Berlin, Germany. Trypsin was purchased from Gibco BRL, Life Technologies, Scotland, UK. NPY was purchased from Novabiochem, Laufelfingen, Switzerland. The rabbit anti-cleaved caspase-3 (Asp 175) antibody was purchased from Cell Signaling, Danvers, MA, USA. The antibody to anti-CD68 (ED1), a marker of activated microglial cells, was obtained from Serotec, Raleigh, NC, USA. The anti-TUJ1 antibody was purchased from Covance Research Products Inc, Berkeley, CA, USA. Hoechst 33342 dye was obtained from Molecular Probes, Eugene, OR, USA. The secondary antibodies, Alexa^{TM} 488 anti-mouse IgG and Alexa^{TM} 488 anti-rabbit IgG were purchased from Invitrogen, Eugene, Oregon, USA.

Rat retina neural cell cultures

All procedures involving animals were in compliance with the Association for Research in Vision and Ophthalmology (ARVO) statement for vision and ophthalmic research. All efforts were made to minimize the number of animals used and their suffering. Wistar rat pups (3–5 days old) were used to prepare mixed primary cultures of retinal cells as previously described (Santiago et al., 2006). Briefly, retinas were dissected under sterile conditions, using a light microscope, in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (in mM: 137 NaCl, 5.4 KCl, 0.45 KH_2PO_4, 0.34 Na_2HPO_4, 4 $NaHCO_3$, 5 glucose, pH 7.4), and digested with 0.1% trypsin (w/v) for 15 min at 37 °C. Cells were diluted in MEM, supplemented with 25 mM Hepes, 26 mM $NaHCO_3$, 10% FBS and penicillin (100 U/mL) –streptomycin (100 μg/mL), and plated on poly-D-lysine (0.1 mg/mL) -coated coverslips or 24-multiwell plates for 9 days, at a density of 2×10^6 cells/cm^2 (37 °C, 5% CO_2).

Cell viability studies

MTT reduction assay. Cell viability was evaluated using the MTT reduction assay that measures the reducing capacity of cells, an general indicator of metabolic function. MTT is taken up by living cells and converted from a yellow to a water-insoluble blue-colored product by cellular dehydrogenases (Mosmann, 1983). Rat retinal cells cultured in 24-well plates were exposed to MDMA (100, 200, 400, 800 and 1600 μM) for 48 h, at 37 °C or 40 °C. After incubation with MDMA, cells were washed twice with Krebs buffer (in mM: 132 NaCl, 4 KCl, 1.4 $MgCl_2$, 1 $CaCl_2$, 6 glucose, 10 Hepes, pH 7.4), and then cell viability was evaluated. Cells were incubated with MTT (0.5 mg/mL) in Krebs buffer for 1 h at 37 °C. After incubation, the medium was removed and the formazan crystals formed were dissolved with 0.04 M HCl in isopropanol. The extent of MTT reduction was measured with a spectrophotometer at 570 nm. All experiments were carried out in triplicate. The cell content of reduced tetrazolium salt was expressed as the percentage of absorbance comparing to control cells.

PI staining. PI [3,8-diamino-5-(3-(diethylmethylamino)propyl)-6-phenyl phetananthridinium diiodide] is a polar substance that is only absorbed by dead or dying cells with disrupted cell membranes due to necrosis or late apoptosis, and binds to DNA emitting a bright red fluorescence (630 nm) when excited by blue–green light (493 nm). Cells plated on coverslips were exposed to MDMA (100, 200 400, 800 and 1600 μM) for 48 h, at 37 °C or 40 °C. In order to test a potential protective role of NPY, cells were also incubated with NPY 100 nM before exposure to MDMA. The protective role of ketanserin (5-HT_{2A}-receptor antagonist) was also evaluated. After drug incubations, the cells were washed twice and incubated with PI (0.04 mg/mL) for 3 min, and then observed with a fluorescence microscope (Zeiss Axioshop 2 Plus) coupled to an Axiocam HRc camera. The number of PI positive cells was counted in five random fields in each coverslip, and the average number of PI positive cells per random field was determined for each condition tested.

Immunocytochemistry

Rat retinal neural cells plated on coverslips were exposed to MDMA (400, 800, and 1600 μM) for 48 h at 37 °C. After incubation, retinal cells were washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 27 mM KCl, 18 mM KH_2PO_4, 100 mM Na_2HPO_4, pH 7.4) and fixed with 4% paraformaldehyde for 20 min at room temperature. Cells were permeabilized with 1% Triton X-100 for 5 min at room temperature, and non-specific binding of the antibodies was prevented by incubation with 3% (w/v) fatty acid-free bovine serum albumin supplemented with 0.2% Tween 20 for 1 h. Cells were then incubated for 90 min at room temperature with the primary antibody: mouse anti-TUJ1 (neuronal marker, 1:500), mouse anti-GFAP (glial cell marker, 1:500), mouse anti CD68 antigen (ED1) (microglial cell marker; 1:200), and rabbit anti-cleaved caspase-3 (Asp175) (cleaved caspase-3 marker, 1:500). After incubation, cells were washed three times with PBS and incubated with the secondary antibody: Alexa^{TM} 488 goat anti-mouse IgG (1:200) or Alexa^{TM} 488 goat anti-rabbit IgG for 1 h at room temperature in the dark. Finally, after 5 min washing, cell nuclei were stained with Hoechst 33342 (1 μg/mL in PBS) for 5 min. Cells were washed twice in PBS and mounted using a Prolong Antifade Kit, Dako Cytomation, DK-2600 Glostrup, Denmark. All antibody solutions were prepared in 3% fatty acid–free BSA solution. Cells were visualized with a Zeiss Axioshop 2 Plus microscope, coupled to an Axiocam HRc camera.

Statistical analysis

All data are presented as mean±S.E.M. Statistical analysis was performed using analysis of variance (ANOVA) followed by Dunnet's or Bonferroni's post-tests, as indicated in the figure legends.