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-HT2A) 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 5HT2A-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-HT2A-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. © 2008 IBRO. Published by Elsevier Ltd. All rights reserved.

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-HT2A-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, Y1, Y2, Y3, Y4, Y5 and Y6 (Michel et al., 1998; Silva et al., 2002). NPY is one of the most abundant and widely distributed neuropeptides...
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 (Thriet 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; Hutslers 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-glia 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-CD68 (ED1), a marker of activated microglial cells, was obtained from Serotec, Raleigh, NC, USA. The antibody to anti-CD68 (ED1) 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 Axioham 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, cells were washed twice with phosphate-buffered saline (PBS) (137 mM NaCl, 27 mM KCl, 18 mM KH2PO4, 100 mM Na2HPO4, 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™ 488 goat anti-mouse IgG and Alexa™ 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 pri-

**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 MgCl2, 1 CaCl2, 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 were formed 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-diamo-no-5-(3-diethylamino)propyl]-6-phenyl phentantridinium 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-HT2A-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 Axioskop 2 Plus) coupled to an Axioham 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.

**Statistical analysis**

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

MDMA-induced cell death in cultured rat retinal neural cells: concentration and temperature dependency

The effect of MDMA on viability of cultured rat retinal neural cells was evaluated by the MTT reduction assay. Cells were exposed to MDMA (100 μM to 1600 μM) during 48 h, under normothermic (37 °C) and hyperthermic (40 °C) conditions (Fig. 1A, B). At 37 °C and 40 °C, there was a significant decrease in cell viability when retinal cells were exposed to 400 μM, 800 μM or 1600 μM MDMA. The highest concentration of MDMA used (1600 μM) decrease cell viability more in hyperthermic conditions (40 °C) than in normothermic conditions (37 °C) (26.8%±5.4% of the control, and 51.2±2.8% of the control, respectively). PI uptake was also used to evaluate the effect of MDMA on cell viability (Fig. 2). MDMA (400 μM, 800 μM and 1600 μM) increased the number of PI+ cells (318.7±67.0%, 348.5±30.7%, 796.5±100.0% compared with control, respectively).

Protective effect of a selective 5-HT2A-receptor antagonist against MDMA-induced toxicity

The protective role of 5-HT2A-receptor blockade against MDMA-induced toxicity (at 37 °C) was evaluated by pre-incubating the cells with ketanserin (1 μM) 1 h before, and during, MDMA incubation. Ketanserin significantly inhibited the increase in the number of PI+ cells induced by MDMA (Fig. 2). Ketanserin alone did not change PI uptake compared with control.

Toxic effect of MDMA on different retinal neural cells: neurons, macroglial and microglial cells

By immunocytochemistry, we also evaluated the toxic effect of MDMA on different retinal neural cells. The toxic effect of MDMA on retinal neurons (TUJ1 positive cells), macroglial cells (GFAP positive cells) and microglial cells (ED1 positive cells) is shown in Fig. 3. In the presence of MDMA (800 and 1600 μM, 48 h, 37 °C) there were fewer cell nuclei compared with controls (Hoechst staining, Fig. 3A, B, C). The number of neurons (β-3-tubulin positive cells) was also less than control, and a retraction on cell processes was evident (Fig. 3D, E, F). Moreover, there were fewer GFAP-positive cells (Müller cells and astrocytes) compared with controls, after highest MDMA concentration used (Fig. 3G, H, I) and the number of microglial cells was also less in MDMA-treated cells compared with controls (Fig. 3J, K, L).

Protective effect of NPY against MDMA-induced cell death

The potential for NPY to protect against MDMA-induced retinal neural cell death was also investigated. NPY (100 nM) inhibited the increase in the number of PI+ cells induced by MDMA (Fig. 4). We also found that when retinal cells were exposed to glutamate (500 μM) for 48 h, the number of PI+ cells increased (279.9±31.0% compared with control), and NPY inhibited this effect (121.1±13.3% compared with control; P<0.001 compared with glutamate, n=6).

NPY alone did not change PI uptake, compared with control (Fig. 4A, B, F). The appearance of cleaved caspase-3 immunoreactivity, used as a marker of apopto-
sis, was also evaluated in retinal cultures exposed to MDMA, in the absence or in the presence of NPY (100 nM). The antibody that was used detects endogenous levels of the large fragment (17/19 kDa) of activated caspase-3 resulting from cleavage adjacent to Asp175, and does not recognize full length caspase-3 or other cleaved caspases. MDMA (400, 800 and 1600 µM) increased the immunoreactivity of cleaved caspase-3 in retinal cells (Fig. 5). In control conditions, as well as in NPY-treated cultures, only a few cells were positive for cleaved caspase-3 (Fig. 5B, F). However, the number of caspase 3-positive cells was significantly increased in cell cultures exposed to 400 µM, 800 µM and 1600 µM MDMA (Fig. 5). Additionally, to determine whether the protective mechanism of NPY was through inhibition of apoptosis, the immunoreactivity of activated caspase-3 was evaluated in retinal cells exposed to MDMA plus NPY (100 nM). The presence of NPY significantly decreased the number of activated caspase 3-positive cells induced by MDMA (Fig. 5).
The injurious effects of MDMA in various brain regions, including cortex, striatum and thalamus, are widely recognized (Commins et al., 1987; Schmued, 2003). The neurotoxicity induced by MDMA was also demonstrated in cultured rat cortical neurons (Capela et al., 2006; Warren et al., 2006), liver cells (Montiel-Duarte et al., 2002), cerebellum granule cells (Jimenez et al., 2004), and PC12 cells (Milhazes et al., 2006). Although some studies showed that considerable amounts of MDMA can reach the vitreous humor and the eye globe (Clauwaert et al., 2000; De Letter et al., 2000, 2002), the toxic effect of MDMA on the retina is not completely elucidated. MDMA causes retinal hemorrhage (Jacks and Hykin, 1998), impairment of retinal function (Firth, 2006) and central serous chorioretinopathy (Michael et al., 2003). In the present study, we showed that MDMA induced toxicity in cultured rat retinal neural cells in a concentration- and temperature-dependent manner. Our findings are correlated with the neurotoxic effect caused by MDMA in the retina, in vivo, reported recently (Miranda et al., 2007). It was shown that MDMA administration induces an increase in the production of oxygen- and nitrogen-derived species, which in turn can cause retinal cell degeneration.

MDMA causes hyperthermia in humans and rats, and MDMA-induced hyperthermia can be fatal (Henry, 1992; Green et al., 2003). Previous studies also showed that hyperthermia plays a role in MDMA-induced neuronal death (Broening et al., 1995; Farfel and Seiden, 1995). In rat retinal cells, the toxic effect of MDMA was potentiated by increasing the incubation temperature to 40 °C. Similar results were obtained by others using cultured rat cortical neurons (Capela et al., 2006). Although neurodegeneration induced by MDMA has been reported to occur in normothermic animals (Broening et al., 1995; Farfel and Seiden, 1995), long-term neurotoxic effects of MDMA have been shown to be related to hyperthermia produced by the drug (Broening et al., 1995). The prevention of MDMA-induced hyperthermia decreases its neurotoxicity, and drugs that protect against MDMA-induced neurotoxicity also decrease body temperature (Farfel and Seiden, 1995; Malberg et al., 1996). The pathologic pathways involved in the aggravation of MDMA-induced toxicity by hyperthermia are not completely understood, though oxidative stress (Carvalho et al., 2001, 2002) activation of inflammatory processes (Roth et al., 2006) may have an important role in this interaction.

Recently, we showed that different cell types, such as neurons, astrocytes, Müller and microglial cells, are...
present in rat retinal cell cultures (Álvaro et al., 2007). In the present work, we show that MDMA is toxic to neurons, astrocytes and Müller cells (GFAP-positive cells), and microglial cells. Furthermore, we found that retinal neurons and microglial cells were less resistant to MDMA exposure than GFAP-positive cells, since the toxic effect of MDMA on both cell types is more evident at the lower concentration used. The toxic effect of MDMA on glial cells has not been reported in previous studies. However, the methods utilized in in vitro studies to analyze the toxic effect of MDMA do not distinguish between cell death of astrocytes and neurons, but glial cells could have also been affected (Stumm et al., 1999; Capela et al., 2006). Retinal glial cell death induced by MDMA might also suggest a higher susceptibility of retinal glial cells compared with brain glial cells. In vivo studies have evaluated the reactivity of brain glial cells, and not glial cell death. It was found that MDMA administration does not change GFAP expression (Wang et al., 2004) or increases GFAP expression and microglial activation (Orio et al., 2004; Baumann et al., 2007) in rat brain. In fact, the in vivo effect of MDMA on glial cells is still a matter of controversy, and further studies are necessary to clarify whether MDMA only induces glial cell reactivity in the brain, or if glial cell death can also occur, in addition to neuronal cell death.

After MDMA incubation we also observed a decrease in the number of neurons and reduction in the length of cytoplasmic processes compared with control conditions. It is well documented that MDMA produces long-term damage to nerve terminals in dopaminergic and serotoninergic neurons (Quinton and Yamamoto, 2006). Several studies have also implicated the 5-HT2A-receptor in the mechanism of MDMA-induced brain toxicity (Schmidt et al., 1990; Johnson et al., 1993; Malberg et al., 1996). Given that these receptors are present in the retina (Pootanakit et al., 1999), we investigated the involvement of 5-HT2A-receptors in MDMA-induced cell toxicity in cultured retinal neural cells. The results indicate that MDMA-induced toxicity in retinal cells is partially dependent on the direct activation of 5-HT2A-receptors, since ketanserin significantly inhibited the cell death induced by MDMA. Similar results were found previously in cultured rat cortical neurons (Capela et al., 2006, 2007). In humans and rats, acute and chronic administration of MDMA decreases cortical 5-HT2A-receptor density (Reneman et al., 2002). In vivo, ketanserin and other 5-HT2A-receptor antagonists showed efficacy in preventing neuronal damage in rats, but this effect was correlated to the hypothermic effect induced by the antagonists alone (Schmidt et al., 1990; Johnson et al., 1993; Malberg et al., 1996).

**Fig. 5.** NPY inhibits the increase in the activation of caspase-3 induced by MDMA. NPY (100 nM) was added to cell cultures 1 h prior to MDMA incubations (400, 800, 1600 µM, 48 h, 37 °C). The results are expressed as percentage of cleaved caspase-3 positive cells (green) in control conditions (no drug, B), and represent the mean±S.E.M. of at least three independent experiments. * P<0.05, ** P<0.01 compared with control, Dunnet’s post hoc test; ++ P<0.05, +++ P<0.01, ++++ P<0.001 compared with MDMA; Bonferroni’s post hoc test.
In the present study, and for the first time, we describe the protective effect of NPY against MDMA toxicity. The neuroprotective role of NPY in the CNS is well documented. NPY protects hippocampal and cortical neurons exposed to excitotoxic conditions (Silva et al., 2003b, 2005a, 2005b; Wu and Li, 2005; Domin et al., 2006; Xapelli et al., 2007) and has a protective role against methamphetamine (METH) -induced neuronal apoptosis in the mouse striatum (Thiriet et al., 2005). Our results show the involvement of 5-HT2A-receptors and a protective role of NPY on MDMA-induced retinal neural cell death. It was described that activation of 5-HT2A-receptors promoted neuronal firing by enhancing the sensitivity of glutamatergic AMPA-receptors and increasing intracellular calcium concentration ([Ca2+]i) (Azmitia, 2001). Moreover, it has been suggested that glutamate might be involved in MDMA toxicity, since the NMDA antagonist, MK-801, protects against MDMA toxicity (Battaglia et al., 2002; Capela et al., 2006). The protective effect of NPY may be linked to its inhibitory effect on glutamate release, as found in rat hippocampus (Silva et al., 2003a, 2005b). Indeed, we show that NPY prevents glutamate-induced retinal neural cell death, measured by PI uptake. Moreover, we also found that NPY inhibits the increase in [Ca2+]i and glutamate release in these cultures (unpublished observations).

Therefore, the putative mechanism of MDMA-induced toxicity may involve an excessive release of glutamate caused by 5-HT2A-receptor activation, and consequently an increase in [Ca2+]i, that could be prevented by the presence of NPY.

Besides necrotic death, several evidences suggest that apoptosis, which is often accompanied by caspase activation, may underlie drug abuse neurotoxicity. Our results show that retinal neural cells exposed to MDMA also die by a caspase-dependent apoptotic pathway, which are in agreement with in vivo and in vitro studies reporting that MDMA stimulates apoptotic cell death by activating caspase-3 in rat cortical neurons (Stumm et al., 1999; Meyer et al., 2004; Capela et al., 2006, 2007; Cunha-Oliveira et al., 2006), PC12 cells (Milhazes et al., 2006), rat primary cerebrocortical neurons (Warren et al., 2006), human retinoreceptive cells (Simantov and Tauber, 1997) and in rat limbic system (Tamburini et al., 2006). In order to dissect the mechanism of the neuroprotection induced by NPY, we investigated the protective effect of this peptide on apoptotic cell death caused by MDMA. Our results show that exogenous NPY decreases cleaved caspase-3 immunoreactivity in rat retinal cells in culture. Similar results were also found for kainate-induced toxicity in primary neocortical and hippocampal neurons in culture (Domin et al., 2006).

**CONCLUSION**

In conclusion, we showed that MDMA causes necrotic and apoptotic cell death in retinal neural cells in a concentration-dependent manner that is potentiated by elevated temperature. The toxic effect of MDMA is partially dependent on 5-HT2A-receptor activation and is significantly reduced by exogenous NPY.

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