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CHRONIC AND ACUTE EFFECTS OF 3,4-METHYLENEDIOXY-*N*-METHYLAMPHETAMINE ('ECSTASY') ADMINISTRATION ON THE DYNORPHINERGIC SYSTEM IN THE RAT BRAIN

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Abstract—The prodynorphin system is implicated in the neurochemical mechanism of psychostimulants. Exposure to different drugs of abuse can induce neuroadaptations in the brain and affect opioid gene expression. The present study aims to examine the possibility of a common neurobiological substrate in drug addiction processes. We studied the effects of single and repeated 3,4-methylenedioxy-*N*-methylamphetamine ('Ecstasy') on the gene expression of the opioid precursor prodynorphin, and on the levels of peptide dynorphin A in the rat brain. Acute (8 mg/kg, intraperitoneally) 3,4-methylenedioxy-*N*-methylamphetamine markedly raised, two hours later, prodynorphin mRNA levels in the prefrontal cortex, and in the caudate putamen, whereas it decreased gene expression in the ventral tegmental area. Chronic (8 mg/kg, intraperitoneally, twice a day for 7 days) 3,4-methylenedioxy-*N*-methylamphetamine increased prodynorphin mRNA in the nucleus accumbens, hypothalamus and caudate putamen and decreased it in the ventral tegmental area. Dynorphin A levels increased after chronic treatment in the ventral tegmental area and decreased after acute treatment in the nucleus accumbens, prefrontal cortex and hypothalamus. These findings confirm the role of the dynorphinergic system in mediating the effects of drugs of abuse, such as 3,4-methylenedioxy-*N*-methylamphetamine, in various regions of the rat brain, which may be important sites for the opioidergic mechanisms activated by addictive drugs. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: opioids, dynorphin A, gene expression, abuse, amphetamine.

3,4-Methylenedioxy-*N*-methylamphetamine (MDMA, 'Ecstasy') is a psychostimulant currently considered to be one of the most popular drugs of abuse in Europe. MDMA has been shown to be a rewarding substance, not only in humans (Camì et al., 2000), but also in experimental animals. Thus, self-administration of MDMA has been reported in rats, rhesus macaques and baboons in the cocaine substitution paradigm (Beardsley et al., 1986; Lamb and Griffiths, 1987; Ratzenboeck et al., 2001), and the establishment of conditioned place preference (CPP) has been demonstrated in rats (Bilsky et al., 1991). In mice, MDMA induces CPP but only within a very narrow range of doses (9–10 mg/kg, i.p.) (Salzmann et al., 2003; Robledo et al., 2004), and it also elicits dramatic increases in locomotion (Callaway et al., 1992).

Ecstasy is thought to produce its behavioral effects on animals primarily via the release of serotonin (5-HT) and dopamine (DA) (Johnson et al., 1986; McKenna et al., 1991; Rudnick and Wall, 1992). MDMA induces marked increases in the synaptic levels of 5-HT (McKenna and Peroutka, 1990; Koch and Galloway, 1997). Treatment with 5-HT re-uptake inhibitors, such as fluoxetine, prevents the effects of MDMA on locomotion and 5-HT release (McKenna and Peroutka, 1990), but is unable to block the effects of amphetamine (Callaway et al., 1992).

Like most drugs of abuse, MDMA increases the release of DA in the nucleus accumbens (NA) (Yamamoto and Spanos, 1988). This effect on the mesolimbic dopaminergic system is thought to be related to the rewarding properties of prototypical drugs of abuse (Koob and Le Moal, 2001) and has been reported to be responsible, at least in part, for MDMA's rewarding properties (Bilsky et al., 1998). MDMA administration has been shown (*in vivo* and *in vitro*) to cause dose-dependent DA release in the striatum, NA, caudate putamen (CP) and hippocampus in rats (Lyles and Cadet, 2003). By releasing DA, MDMA also alters other brain neurotransmitter systems that are regulated by the dopaminergic system.

Pharmacological studies performed in rats have shown that the delta-opioid antagonist naltrindole blocks the enhancement induced by MDMA on reinforcing brain stimulation (Reid et al., 1996), and that naltrexone attenuates MDMA's ability to produce CPP (Bilsky et al., 1991).

More recent data in mice show that naloxone blocks the hyperlocomotion produced by MDMA, demonstrating the involvement of the endogenous opioid system in some of the behavioral effects induced by MDMA (Compan et al., 2003). Like methamphetamine, a single administration of MDMA increases striatal and nigral neurotensin and dynorphin (DYN) A concentrations (Johnson et al., 1991). This increase in the neurotensin and DYN A content is not related to neurotoxic changes induced by amphetamine analogues, since their concentration returns to normal within a week (Hanson et al., 1988).

It has already been shown that various drugs of abuse, acting through different neuronal mechanisms, evoke long-

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Abbreviations: CP, caudate putamen; CPP, conditioned place preference; CREB, c-AMP responsive element binding protein; DA, dopamine; DAT, dopamine transporter; DYN, dynorphin; HPA, hypothalamic–pituitary–adrenal; HYP, hypothalamus; MDMA, 3,4-methylenedioxy-*N*-methylamphetamine; NA, nucleus accumbens; NE, norepinephrine; NET, norepinephrine transporter; PDYN, prodynorphin; PFC, prefrontal cortex; SDS, sodium dodecyl sulfate; SERT, serotonin transporter; VTA, ventral tegmental area; 5-HT, serotonin; 5-PDYN, prodynorphin.

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term changes in the activity of endogenous opioid systems. It has been demonstrated that chronic cocaine administration can result in alterations in the various brain areas in the rat, e.g. alterations in preprodynorphin mRNA levels following different treatment schedules (Hurd and Herkenham, 1992; Daunais et al., 1993; Spangler et al., 1993; Romualdi et al., 1996, 2001; Mathieu-Kia and Besson, 1998; Turchan et al., 1998; Adams et al., 2000). In general, treatment with high doses of indirect DA agonists, such as methamphetamine and cocaine, increases preprodynorphin mRNA levels in the striatum (Hurd and Herkenham, 1992; Steiner and Gerfen, 1993; Smith and McGinty, 1994; Wang and McGinty, 1996a,b) and the prodynorphin (PDYN) mRNA level in the NA was markedly higher after single or repeated amphetamine administration (Turchan et al., 1998). DYN can modulate DA release in addition to directly producing dysphoria and euphoria, respectively, by activating opioid receptors (Reid et al., 1990; Spanagel et al., 1990). Changes in opioid peptide systems may contribute to the psychoactive properties and abuse potential of different drugs of abuse. Finally, Adams et al. (2005) recently conducted *in situ* hybridization studies on neuropeptide mRNA content after one single injection of a high dose of MDMA (10 mg/kg), showing an enhancement in PDYN message in striatum.

Therefore, since the MDMA effects on PDYN gene expression have been poorly explored, the measurement of both the PDYN mRNA and peptide content following chronic and acute treatment with MDMA in different brain areas could represent a useful basis for drawing conclusions about PDYN system activity.

EXPERIMENTAL PROCEDURES

Chemicals

MDMA was obtained from the National Institute on Drug Abuse/National Institutes of Health (Research Triangle Institute, Research Triangle Park, NC, USA) and dissolved in saline (0.9% NaCl).

Drug treatment

Male Sprague–Dawley rats (200–250 g, 7–8 weeks old, Harlan Italy S. Pietro al Natisone, UD, Italy) were maintained on a 12-h light/dark cycle with unrestricted access to rat chow and water. Animals were subdivided into four groups ($n=6$ for each group) and i.p. treated as follows: acute saline (2 ml/kg, first group), acute MDMA (8 mg/kg, second group), chronic saline (2 ml/kg twice daily for seven days, third group) and chronic MDMA (8 mg/kg twice daily for seven days, fourth group). The experiment was repeated four times. Two hours after the acute treatment or one day after the last injection in the chronic treatment, the rats were killed by decapitation, their brains were rapidly removed and the ventral tegmental area (VTA), NA, CP, hypothalamus (HYP) and prefrontal cortex (PFC) were dissected and frozen on dry ice.

All animal experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and National (Ministry of Health) laws and policies (Authorization no.: 19/2002-B). Care was taken to minimize the number of experimental animals and to take measures to minimize their suffering.

Northern analysis

Tissue processing. Total RNA was prepared according to the method of Chomczynski and Sacchi (1987). Briefly, RNA was extracted from single tissue samples by homogenizing in a mixture of acid guanidinium thiocyanate/phenol (2 ml/100 mg tissue), adding 0.2 ml chloroform/2 ml homogenate, and centrifuging the suspension at 12,000×*g* for 15 min at 4 °C. The aqueous phase was transferred to a fresh tube, an equal volume of isopropanol was added, incubated for 15 min at 4 °C and the RNA pellet was isolated by centrifugation at 12,000×*g* for 25 min at 4 °C. The pellet was washed twice with 75% ethanol, dried under a vacuum and then resuspended in 0.5% sodium dodecyl sulfate (SDS). Total RNA content was quantified by measurement of absorbance at 260 nm (1 OD/ml=40 g RNA/ml). The ratio OD260/OD280>1.8 provided an estimate of the purity of the total RNA.

Probes. Blots were hybridized with a probe, fragment of 815 base pairs of the rat genomic DNA complementary to the PDYN mRNA, consisting of the 5′-translated region of the PDYN gene, encoding for all PDYN. The cDNA fragment, inserted into the plasmid vector pUC13, was kindly supplied by Dr. O. Civelli (Civelli et al., 1985). It was released by restriction enzyme *Bam*HI digestion, labeled by random priming methods, using α-[^{32}P]dCTP to a specific activity of 7–9×10^5 cpm/ng. A cDNA fragment recognizing β-actin mRNA (clone pHFA-1, containing the full-length cDNA insert for human cytoplasmic β-actin) was used as an internal standard to hybridize the same blots (Gunning et al., 1983).

Northern blot analysis. Total RNA from each tissue (30 μg) was electrophoresed through a 1% agarose gel, containing 2.2 M formaldehyde at 75 V, using a 0.04 M morpholinopropanesulfonic acid (MOPS, pH 7.0) buffer containing 10 mM sodium acetate and 1 mM ethylenediaminetetracetic acid disodium (EDTA). RNA was transferred by overnight capillary blotting to nylon membranes, and then air-dried, UV cross-linked and hybridized in an oven (Maniatis et al., 1982). After prehybridization for 3–6 h, blots were hybridized for 24 h at 42 °C in a solution of 6× SSC (sodium chloride/sodium citrate) (1× SSC=0.15 M NaCl, 0.015 M sodium citrate), 1× Denhardt's solution (0.02% polyvinylpyrrolidone, Ficoll and bovine serum albumine (BSA)), 100 g/ml denatured salmon sperm DNA, 0.1% SDS, 50% formamide, 10 mM Tris and 10% dextran sulfate, containing the probe at the concentration of 1–2×10^6 cpm/ml. Upon removal of the probe solution, blots were washed three times for 10 min at 42 °C with a solution of 2× SSC/0.1% SDS, followed by three times for 10 min at 55 °C, with a solution of 0.1× SSC/0.1% SDS. X-ray films (Amersham β-max) were exposed to the hybridized blot while backed by an intensifying screen (Dupont Cronex) at −70 °C for 3–6 days. Blots were twice hybridized serially, with the probes directed against PDYN and β-actin mRNA. For β-actin mRNA hybridization, blots were prehybridized and hybridized overnight at 65 °C in a solution of 4× SSC, 50 mM NaH_2PO_4, 5× Denhardt's solution and 10% dextran sulfate. Blots were washed three times for 10 min at 65 °C with a solution of 0.5× SSC, 0.1% SDS on a rocker, and then exposed to X-ray films at −70 °C for 24 h. Total RNA from the treated animals was compared with RNA from control rats. Optical densities for the autoradiographic bands produced by PDYN and β-actin hybridization were determined using a Video Densitometer system (MDL 620). The PDYN mRNA/β-actin mRNA ratios of the hybridization values for treated or control animals were analyzed, and then expressed as percentages of the controls (100%) for each experiment. Data were statistically analyzed by *t*-test. Briefly, background densities from the areas of film away from the lanes were subtracted from the observed values obtained from non-saturated autoradiographic exposures, in which standardizing lanes revealed a linear relationship between the amount of PDYN mRNA and the hybridization signal. Two autoradiograms for each blot were scanned. Multiple exposures to film and the presence of standardizing lanes allowed us to avoid saturation of the X-ray film.