Sex differences in MDMA-induced toxicity in Sprague-Dawley rats

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Summary

Recent evidence demonstrates that female subjects show exaggerated responses to 3,4-methylenedioxymethamphetamine (MDMA) compared with males. The aim of our study was to evaluate sex differences and the role of endogenous gonadal hormones on the effects of MDMA. Fifty-six intact and gonadectomized male and female Sprague-Dawley rats were randomly assigned to either MDMA (5 mg/kg) or saline treatment. Learning and memory were assessed using the Morris water maze (MWM). The expression of Bax and Bcl-2 in the hippocampus was detected by Western blotting.
Behavioral analysis showed that MDMA led to memory impairment in both male and female rats. The female rats showed more sensitivity to impairment than the males, as assessed using all the memory parameters in the MWM. Ovariectomy attenuated the MDMA-induced memory impairment. By contrast, orchiectomized rats showed more impairment than MDMA-treated intact male rats. Bcl-2 and Bax were down-regulated and up-regulated in MDMA-treated male and female rats, respectively.
MDMA treatment in the orchiectomized rats led to up-regulation of Bax and down-regulation of Bcl-2. Ovariectomy attenuated the MDMA-induced up-regulation of Bax and caused more expression of Bcl-2 compared with what was observed in the MDMA-treated intact female rats.
In summary, female rats showed exaggerated responses to the effects of MDMA and this may be explained by endogenous gonadal hormones.

KEY WORDS: Bax, Bcl-2, ecstasy, gonadectomy, spatial memory

Introduction

3,4-methylenedioxymethamphetamine (MDMA), also known as ecstasy, is an illicit drug of abuse that can cause behavioral and neurotoxic damage in humans and animals (Ros-Simó et al., 2012; Watkins et al., 2013). MDMA influences serotonergic, dopaminergic and noradrenergic nerve endings in the brain, showing greater affinity for the serotonergic system. It causes an increase in synaptic serotonin levels and binds to the serotonin transporter. At high doses, MDMA produces an acute depletion in serotonin in serotonergic endings at 4-6 h followed by a recovery by 24 h (Schmidt, 1987). MDMA also affects α-adrenergic receptors, serotonin 5-HT2 receptors, muscarinic M1 receptors and histamine H1 receptors, with more affinity for 5-HT2 receptors (Lyles and Cadet, 2003). The neurotoxic effects of MDMA are relatively well described in humans and animals. For example, it has been shown that MDMA causes feelings of closeness accompanied by enhanced emotion and increased blood pressure, heart rate and body temperature, as well as many other effects (Steinkellner et al., 2011). Exposure to MDMA causes memory impairment and cell death in many regions of the brain (Stephenson et al., 1999; Soleimani Asl et al., 2012). However, little is known about sex differences related to MDMA-induced neurotoxicity. Studies in humans have described that women report greater thought disturbances, perceptual changes, and fear of loss of body control. By contrast, men experience greater increases in blood pressure (Liechti et al., 2001). The other sex differences in the response to ecstasy include increased subjective

effects in females compared with males, and greater susceptibility to the effects of the drug, more pronounced depression following ecstasy use, and a potential increase in serotonergic neurotoxicity in association with long-term use of MDMA in women (Liechti et al., 2001; Verheyden et al., 2002). Another study showed greater reduction in 5-hydroxyindoleacetic acid levels in females than in males (McCann et al., 1994). There are only a few studies that have described the unequal effects of MDMA in male and female primates. Fitzgerald et al. (1989) reported a higher level of MDMA but not metabolic 3,4-methylenedioxyamphetamine (MDA) in the plasma of female rats, whereas Chu et al. (1996) showed a lower brain concentration of MDA, but not MDMA, in female rats after MDMA administration. Babenikova et al. (2005) showed differences between male and female rats in the test of prepulse inhibition of the acoustic startle reaction, while Palenicek et al. (2005) demonstrated high sensitivity of females to the locomotor stimulant effects of MDMA. They observed a dose-dependent locomotion-enhancing effect of MDMA both in male and female rats in an elevated plus-maze; this response was exaggerated in female rats and the authors hypothesized that the increased sensitivity of females to the behavioral effects of MDMA can be explained by increased reactivity of the serotonergic and dopaminergic systems (Palenicek et al., 2005). It is clear that gonadal hormones affect the levels of dopamine and serotonin in the different parts of the brain (McEwen, 2002). It seems that the possible sex differences in the neurotoxic effects of MDMA may be due to gonadal hormones. To our knowledge, there is no evidence on gender differences in MDMA-induced memory impairment and cell death in animals or on relations between gonadal hormones and MDMA effects. In this study, we evaluated the sex differences in the neurotoxic effects of MDMA and the relationship of these effects with gonadal hormones in Sprague-Dawley rats.

Materials and methods

Materials

MDMA was obtained from the Presidency Drug Control Headquarters (Tehran, Iran). Other chemicals were purchased from commercial sources.

Animals

All experiments were carried out on adult male (200-250 g) and female (180-220 g) Sprague-Dawley rats (Razi Institute, Iran). The animals were kept under standard laboratory conditions (12:12 h light-dark cycle, 50% humidity, 22±2°C, and *ad libitum* access to water and food). All animal surgeries, behavioral tests and experimental protocols were approved by the Ethics Committee of Iran University of Medical Sciences.

Fifty-six male and female rats were assigned as follows (n=7 per group)

1- Two saline-treated intact groups (male and female) that received normal saline, 1 ml twice daily, IP for 7 days.
2- Two MDMA-treated intact groups (male and female) that received MDMA, 5 mg/kg twice daily, IP for 7 days.
3- Two saline-treated gonadectomized groups (male and female) that received normal saline, 1 ml twice daily, IP for 7 days.
4- Two MDMA-treated gonadectomized groups (male and female) that received MDMA, 5 mg/kg twice daily, IP for 7 days.

Surgical procedures

One week after their arrival, the saline-treated and MDMA-treated gonadectomized groups underwent bilateral ovariectomy or orchiectomy to remove circulating hormones.

Surgical procedures were performed under ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthesia. The ovaries were typically approached by two separate flank incisions and were gently pulled through the incision with a blunt forceps by grasping the fat pad surrounding the instrument. A hemostat was placed at the boundary between the oviduct and uterus, a ligature was placed just below the hemostat (next to the uterus), and a cut was made just above the hemostat. Once the ovary and oviduct had been removed, the hemostat was released and the uterus was returned to the abdomen. Muscles and skin were closed with suture.

For orchiectomy, the testis was approached by a single midline incision on the scrotal sac. Both testes were pushed down into the scrotum by gentle pressure on the abdomen. A hemostat was placed below the testes and epididymis across the testicular cord (this contains blood vessels and the vas deferens). A ligature was placed below the hemostat and the testes and epididymis were removed with scissors. The hemostat was released and the incision closed in two layers with suture.

Gonadectomy also ensured a constant level of gonadal receptor expression within the brain because receptor levels are regulated by hormones (Suzuki and Handa, 2005). Treatment with MDMA was begun one week after gonadectomy (Donner and Handa, 2009).

Morris water maze

There exist various methods for measuring memory impairments. In animals, memory impairing agents, such as MDMA, generally impair spatial memory (Soleimani Asl et al., 2012). In this study, therefore, we used the Morris water maze (MWM) to evaluate spatial memory.

The MWM tank was 210 cm in diameter, 51 cm in height, painted black, and filled to a depth of 35 cm with water maintained at a temperature of 22±1°C. Around the room, numerous visual cues (e.g. bookcase and tables) were present which remained constant throughout the experiment. The pool was divid-

ed into four quadrants with four starting locations - northern (N), eastern (E), southern (S) and western (W), each located at equal distances along the pool rim. An invisible Plexiglas platform (10 cm diameter) was located 1 cm below the water in the center of the northern quadrant. The position of the escape platform remained the same for all the animals across the training trials. We trained the animals for three days at approximately the same time (10:00-12:00 a.m.) each day. Each training day included two blocks of four trials. The time limit for each trial was 90 s. During training, the animals were allowed to swim until they located the hidden platform or until the 90 s had elapsed. All groups were trained from each of the starting positions (N, S, E and W). A 30 s period was allowed between trials, which was spent on the platform. Rats were also allowed to rest for 5 min between the two blocks. The day after the last learning trial, each rat was given a single 60 s probe trial and a visible trial. The probe trials were conducted without a platform. In the visible trials, the platform was covered with aluminum foil. A video camera (Nikon, Melville, NY, USA) linked to a computer was mounted directly above the MWM pool to record, for each rat, the time taken to reach the hidden platform (escape latency), the length of the swim path (distance traveled), and the percentage of time spent in the target quadrant.

Western blotting analysis

After the spatial memory assessment, for immunoblotting of Bax and Bcl-2 proteins, three rats from each group were killed by cervical dislocation and their hippocampi were removed and immediately frozen in liquid N2. The hippocampi were minced in lysis buffer, containing RIPA buffer with a protease inhibitor cocktail (1:10), homogenized and centrifuged (Eppendorf, Hamburg, Germany) at 12000 g for 20 min at a temperature of 4°C. 100 µg of total proteins were denatured with sample buffer (6.20 mM tris-HCL, 10% glycerol, 2% SDS, 0.01% bromophenol blue and 50 mM 2-ME) at 95°C for 5 min, then separated on a 10% sodium dodecyl sulfate polyacrylamide gel for 90 min at 120 volts, and transferred to nitrocellulose membrane. Membranes were incubated with anti Bax, anti Bcl-2, and B-actin (1:1000; Sigma Aldrich, St. Louis, MO, USA) for 2 h followed by alkaline phosphatase-conjugated secondary antibody (1:10000; Sigma Aldrich, St. Louis, MO, USA) for 1 h. Bands were detected by incubation with chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate in the presence of nitroblue tetrazolium. Each Western blot was performed in triplicate.
Densitometric ratios were obtained using image analysis software (UVIdoc, Houston, TX, USA).

Statistical analysis

All values are expressed as mean ± SEM. The results of all data were statistically evaluated by two-way analysis of variance (ANOVA) with MDMA treatment as one factor and sex as the second factor. Comparisons between treatment groups were conducted using Tukey's post-hoc test where appropriate. Values of $p<0.05$ were considered significant.

Results

Effect of MDMA on escape latency in the Morris water maze

A two-way ANOVA showed a significant effect of treatment [$F(3, 97) = 24.47$, $p<0.001$], but not sex, on escape latency. There was a significant interaction between sex and MDMA treatment [$F(3, 12) = 3.18$, $p<0.05$]. ANOVA of the three training days, showed that the saline-treated groups spent less time in locating the hidden platform (escape latency) than the MDMA-treated groups. Overall, MDMA significantly increased the escape latency, when compared with saline-treated groups, both in male and female rats ($p<0.01$ for males and $p<0.001$ for females, Fig. 1). As shown in figure 1, there was a significant difference between MDMA-treated female and male intact rats in the time taken to reach the hidden platform ($p<0.05$). Gonadectomy in saline-treated rats resulted in a non-significant increase in escape latency compared with the saline-treated intact groups. Escape latency was higher in MDMA-treated orchiectomized rats (mean 60.89± 3.82) than MDMA-treated intact male rats (mean 43.37±4.77). Exposure to MDMA in ovariectomized rats led to a significant reduction in escape latency when compared with MDMA-treated intact females ($p<0.05$, Fig. 1). There was no significant difference in escape latency in

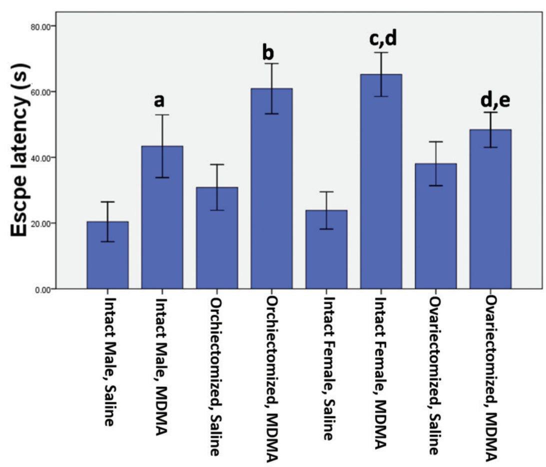

Figure 1 - The effect of MDMA on the escape latency in the MWM in male and female rats.
Data are expressed as mean ± SEM. Exposure to MDMA caused an increase in escape latency in both male and female rats ([a] $p<0.01$, [b] $p<0.001$ vs saline-treated male group, [d] $p<0.001$ vs saline-treated female rats). Escape latency differed significantly between female and male rats ([c] $p<0.05$ vs MDMA-treated male rats). Ovariectomy attenuated the escape latency in MDMA-treated rats ([e] $p<0.05$ vs MDMA-treated intact female rats).

the visible trial session between the treatment and saline groups (data not shown).

Effect of MDMA on distance traveled in the Morris water maze

A two-way ANOVA showed a significant effect of MDMA treatment [F (3, 11) = 11.41, p<0.001], but a non-significant effect of sex on distance traveled in the MWM (swimming distance to the escape platform). There was a near significant interaction between sex and MDMA treatment [F (3, 160) = 2.51, p=0.056]. As shown in figure 2, exposure to MDMA in the intact groups caused a significant increase in distance traveled compared with saline-treated intact rats (p<0.05 for male and p<0.001 for female rats, Fig. 2). In the gonadectomized rats, the MDMA-treated females demonstrated a significant increase in swimming distance to the escape platform compared with the MDMA-treated males (p<0.001). MDMA treatment in the ovariectomized rats caused a significant reduction in distance traveled compared with MDMA-treated intact female rats (p<0.05). There was no significant difference in the distance traveled in the visible trial session between the treatment and saline groups (data not shown).

Effect of MDMA on time spent in the target quadrant in the Morris water maze

Analysis of variance of probe trials showed a significant effect of MDMA treatment [F (3, 11) = 11.925, p<0.001] and an insignificant effect of sex on the time spent in the target quadrant. There was no significant interaction between the factors. Figure 3 shows that MDMA administration significantly decreased the time spent in target quadrant in both male and female intact rats (p<0.001 for males and p<0.01 for females). MDMA-treated intact female rats spent less time in the target quadrant compared with MDMA-treated intact males (p<0.01). Subsequent analysis for each sex showed that the MDMA-treated ovariectomized rats spent more time in the target quadrant (p<0.001) as compared to the MDMA-treated intact female rats, while the MDMA-treated orchiectomized rats spent less time in the target quadrant (mean 30.17±1.60) compared with the MDMA-treated intact male rats (mean 32.70±2.57).

Effect of MDMA on Bax expression in the hippocampus

On Western blot, anti-Bax antibody reacted specifically with 28 KD protein bands (Fig. 4). There was a significant effect of MDMA treatment [F (3, 27) = 114.3, p<0.001] and sex [F (1, 16) = 68.5, p<0.001] on Bax expression in the hippocampus. A significant interaction was observed between the factors sex and MDMA treatment [F (3, 49) = 20.33, p<0.001).The intensity of Bax was significantly enhanced in the MDMA-treated intact rats compared with the saline-treated intact rats (p<0.001 for males and females, Fig. 4). Bax was more expressed in the MDMA-treated female rats (mean 679.47±70) than male rats (mean 653.54±60) but the difference was not significant. Gonadectomy led to an increase in Bax expression in both the MDMA- and saline-treated groups. The MDMA-treated

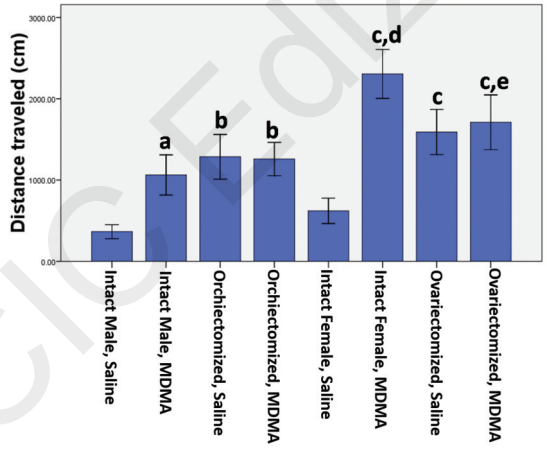

Figure 2 - The effect of MDMA on the distance traveled in the MWM in male and female rats.
Data are expressed as mean ± SEM. MDMA treatment caused an increase in the distance traveled ([a] p<0.05 and [b] p<0.01 vs saline-treated male group, respectively, and [c] p<0.001 vs saline-treated female group). Female rats were more sensitive than male rats ([d] p<0.01 vs MDMA-treated male group). MDMA-treated ovariectomized rats showed a reduction in distance traveled ([e] p<0.05) as compared to MDMA-treated intact female rats.

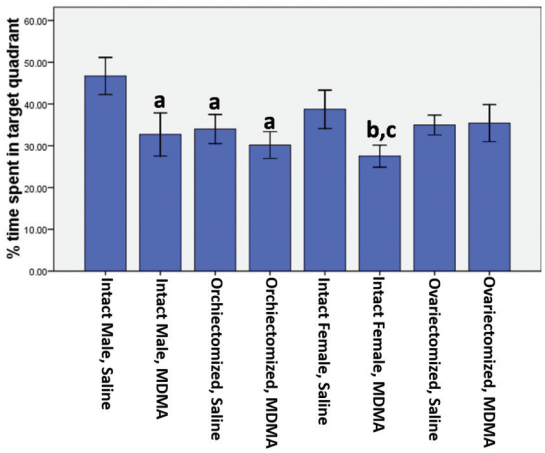

Figure 3 - The effect of MDMA on the time spent in the MWM target quadrant by male and female rats.
Data are expressed as mean ± SEM. MDMA administration caused a significant reduction in the time spent in the target quadrant in both male and female rats ([a] p<0.001 and [b] p<0.01 vs saline-treated male and female rats, respectively). MDMA-treated ovariectomized rats spent significantly more time in the target quadrant ([c] p<0.001) as compared to the MDMA-treated intact female rats.

orchiectomized rats showed greater Bax expression than did the saline-treated intact male rats ($p<0.001$). Exposure to MDMA in the ovariectomized rats resulted in a significant reduction in Bax expression when compared with the MDMA-treated intact rats ($p<0.001$).

Effect of MDMA on Bcl-2 expression in the hippocampus

Anti-Bcl-2 antibody reacted with 26 KD protein bands (Fig. 5). A two-way ANOVA showed a significant effect of MDMA treatment [$F (3, 44) = 227.78$, $p<0.001$] and sex [$F (1, 77) = 39.23$, $p<0.05$] on Bcl-2 expression in the hippocampus. A significant interaction was found between the factors sex and MDMA treatment [$F (3, 10) = 5.11$, $p<0.01$]. Bcl-2 was expressed more in the saline-treated rats. In both the male and female intact groups, MDMA treatment caused a significant reduction in Bcl-2 expression compared with the saline-treated groups ($p<0.001$, Fig. 5). Bcl-2 was less expressed in the MDMA-treated intact females (mean 291.96±58.63) than male rats (mean 314.54±60.10). Gonadectomy caused a significant reduction in Bcl-2 expression compared with the values recorded in the saline-treated intact male and female rats ($p<0.001$). Bcl-2 was up-regulated in the saline-treated and MDMA-treated ovariectomized rats compared with the MDMA-treated intact female rats ($p<0.05$ for both).

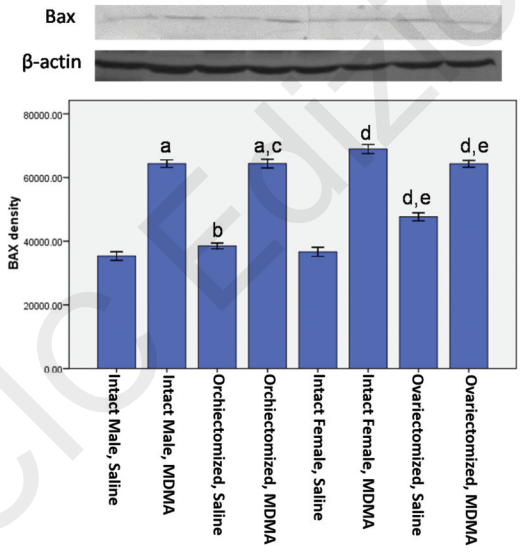

Figure 4 - The effect of MDMA on Bax expression in the Western blotting.
Data are expressed as mean ± SEM. Exposure to MDMA increased Bax expression in the hippocampi of male and female rats ([e] and [d] $p<0.001$ vs male and female saline-treated groups, respectively, [b] $p<0.001$ vs intact male MDMA group). MDMA treatment in the orchiectomized rats caused an increase in Bax expression ([c] $p<0.05$ vs saline-treated orchiectomized rats). Saline- and MDMA-treated ovariectomized rats showed less Bax expression ([e] $p<0.001$ vs MDMA-treated intact female rats).

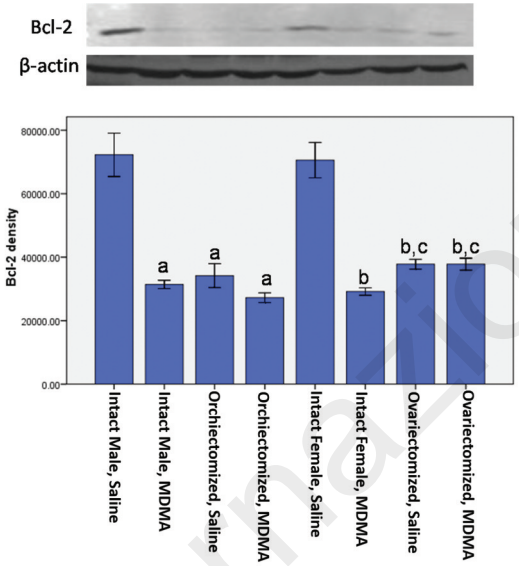

Figure 5 - The effect of MDMA on Bcl-2 expression in the Western blotting.
Data are expressed as mean ± SEM. Exposure to MDMA decreased Bcl-2 expression in the hippocampi of male and female rats ([a] $p<0.001$ and [b] $p<0.001$ vs saline-treated male and female rats, respectively). MDMA treatment in ovariectomized rats upregulated Bcl-2 expression ([c] $p<0.05$ vs MDMA-treated intact female rats).

Discussion

In the present study, we found that the female rats, compared with the male rats, were more sensitive to MDMA toxicity and demonstrated an exaggerated response to MDMA administration. Furthermore, MDMA induced greater responses in orchiectomized rats compared with non-orchiectomized rats, whereas ovariectomy attenuated MDMA-induced neurotoxicity compared with what was observed in non-ovariectomized rats.
Consistent with our study, Walker et al. (2007) showed that MDMA elicits substantially greater locomotor activation in female rats than in male rats. Other sex differences in the response to MDMA have previously been reported and include increased subjective effects in women compared with men (Liechti et al., 2001), and more pronounced depression following ecstasy use in women (Verheyden et al., 2002).
There is growing evidence that females are more susceptible to the effects of drugs of abuse (Carroll et al., 2004). It has been reported that pharmacological sensitivity to ecstasy is dependent on gonadal hormone changes during the estrous cycle in female rats (Bubenikova et al., 2005). The estrous cycle comprises the physiological changes that are induced by reproductive hormones in females. Variations in endogenous estrogen levels across the estrous cycle modulate some aspects of memory (Warren and Juraska, 1997).
Ovarian steroids are associated with changes in the expression of serotonin receptors and transporters and in serotonin metabolism; these changes result in a higher level of serotonin available for release (Ramírez

and Carrer, 1982; McEwen, 2002). It has been established that estrogen influences synaptic plasticity in the hippocampus and that gonadectomy results in a loss of post-synaptic dendritic spines of hippocampal neurons of the CA1 region (Vedder et al., 2014). Spine density varies with the estrous cycle, peaking at the proestrous stage, shortly before ovulation, when serum estradiol concentrations are highest (Woolley and McEwen, 1992). It is clear that serum levels of estradiol control release of gonadotropin-releasing hormone (GnRH) from the hypothalamus, which in turn stimulates the follicle-stimulating hormone/luteinizing hormone secretion that enhances the synthesis of estradiol in ovarian granulose cells. It has been reported that GnRH receptors are abundant in the hippocampus (Prange-Kiel et al., 2008) and GnRH can also directly stimulate estradiol synthesis in the hippocampus, similar to its effect on granulose cells (Brandt et al., 2013).

The mechanism by which MDMA induces greater neurotoxicity in females is likely due to gonadal steroid effects on the serotonergic mechanism through which MDMA acts. A potential increase in serotonergic neurotoxicity in association with long-term use of MDMA has been reported in women (Reneman et al., 2000). Fedotova and Ordyan (2010) suggested that chronic administration of NAN-190 as a 5-HT1A receptor antagonist in adult female rats in the proestrous and estrous phase resulted in the appearance of passive avoidance performance, as distinct from the control animals. By contrast, chronic treatment with 8-OH-DPAT as a 5-HT1A receptor agonist impaired non-spatial and spatial learning in females during all phases of the ovarian cycle. The results of this study point to a modulatory role of 5-HT1A receptors in learning/memory processing during the ovarian cycle in adult female rats.

Both stimulatory and inhibitory effects of MDMA on 5-HT1B and 5-HT2 receptors have been reported (Kehne et al., 1996; Bankson and Cunningham, 2002). The enhanced response in females may reflect a sex-specific receptor up-regulation mediated by estrogen-facilitated 5-HT2 receptor synthesis (Kehne et al., 1996; Reneman et al., 2000; Bankson and Cunningham, 2002).

Females exhibit increased sensitivity to the anxiolytic effects of 5-HT1A agonists (Simerly et al., 1985), and increased levels of basal 5-H2 function in the hippocampus (Haleem et al., 1990). Also, hippocampal levels of acetylcholine and 5-HT are consistently higher in female rats than in age-matched males (Hörtnagl et al., 1993) and estradiol increases 5-HT2A receptor density in the dorsal raphe and frontal cortex in female rats (Sumner and Fink, 1993). It has been found that ovarian steroids in different parts of the rat brain affect dopamine and serotonin levels (McEwen, 2002).

In the present study, removal of the source of gonadal hormones resulted in an increase in neurotoxicity in male rats, whereas in female rats it attenuated MDMA-induced spatial learning impairment and cell death. Taken together, these findings seem to show that gonadal hormones affect MDMA-associated neurotoxicity.

Gonadectomy results in an increase in cellular 5-HT mRNA content in the cortex, hippocampus, hypothalamus and amygdala, where the androgen receptors are abundant (Simerly et al., 1990), as well as in the dorsal raphe nucleus. Testosterone inhibits the serotonergic function that is responsible for the enhanced serotonergic responsivity seen in female rats (Fischette et al., 1984). Zhang et al. (1999) reported modulatory effects of testosterone on serotonergic receptors at the level of transcription or transcript processing. Studies have shown that estradiol, a metabolite of testosterone, modulates neuronal 5-HT content and receptor binding as well as receptor mRNA levels (Sumner and Fink, 1993). In female rats, estradiol has been shown to increase 5-HT2A (but not 5-HT1A) receptor mRNA density in multiple brain regions (Cyr et al., 2002). Developmentally, 5-HT appears early and exerts widespread effects on brain morphogenesis and synaptogenesis (Kobe et al., 2012). Gonadal steroids may therefore alter brain morphology and function by regulating the development of the 5-HT system, as well as by regulating neural cell death or apoptosis (Dodson and Gorski, 1993). Sexual dimorphism in brain circuitry might mediate the gender-related differences in physiology or behavior, depending upon the brain region involved. The serotonergic system plays a role in the regulation of affective state, learning and memory, all of which have been demonstrated to differ according to gender, and disturbances of which characterize affective disorders in humans (Price et al., 2013).

Overall, we observed impairment in spatial memory and apoptosis following MDMA treatment both in male and female rats. The female rats were more sensitive to the MDMA effects than the males. The increased sensitivity of the females can be explained by an increased reactivity of the serotonin system due to the effect of ovarian hormones. Understanding how gonadal hormone-MDMA interaction affects gender differences in the brain is essential to develop effective pharmacotherapy for both women and men. Finally, further investigation of the sex steroids involved in these effects appears to be necessary.

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