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The Recreational Drug Ecstasy Disrupts the Hypothalamic-Pituitary-Gonadal Reproductive Axis in Adult Male Rats

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Abstract

Reproductive function involves an interaction of three regulatory levels: hypothalamus, pituitary, and gonad. The primary drive upon this system comes from hypothalamic gonadotropin-releasing hormone (GnRH) neurosecretory cells, which receive afferent inputs from other neurotransmitter systems in the central nervous system to result in the proper coordination of reproduction and the environment. Here, we hypothesized that the recreational drug ±-3,4-Methylenedioxymethamphetamine (MDMA; "ecstasy"), which acts through several of the neurotransmitter systems that affect GnRH neurons, suppresses the hypothalamic-pituitary-gonadal (HPG) reproductive axis of male rats. Adult male Sprague-Dawley rats self-administered saline or MDMA or saline either once (acute) or for 20 days (chronic), and were euthanized 7 days following last administration. We quantified hypothalamic GnRH mRNA, serum luteinizing hormone (LH) concentrations, and serum testosterone levels, as indices of hypothalamic, pituitary, and gonadal functions, respectively. The results indicate that the hypothalamic and gonadal levels of the HPG axis are significantly altered by MDMA, with GnRH mRNA and serum testosterone levels suppressed in rats administered MDMA compared to saline. Furthermore, our finding that hypothalamic GnRH mRNA levels are suppressed in the context of low testosterone concentrations suggests that the central GnRH neurosecretory system may be a primary target of inhibitory regulation by MDMA usage.

Keywords

MDMA; ecstasy; neuroendocrinology; GnRH; testosterone; endocrine disruption; male reproduction

Recreational use of 3,4-Methylenedioxymethamphetamine (MDMA, "ecstasy") is particularly popular among college students and those involved in the dance culture [1,2]. MDMA abusers often consume the drug at all-night dance parties because it induces a state of euphoria, increased energy, insomnia, and enhanced sensory perception. MDMA causes neurocognitive deficits in attention, verbal and non-verbal learning and memory, psychomotor speed and executive systems functioning [reviewed in 3]. Other central nervous system effects of MDMA

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have been reported for cognitive function [4,5], circadian rhythms [6,7], and thermoregulation [8,9].

The widespread and diverse actions of MDMA on brain and behavior are explained at least in part by its numerous target neurotransmitter systems [reviewed in 10]. The serotonergic and dopaminergic systems are most strongly implicated as primary targets of MDMA action, as shown by *in vivo* microdialysis studies [11–14] and by *in vitro* and *ex vivo* studies [15,16]. In addition, MDMA stimulates norepinephrine [17–20], acetylcholine [21–23], and GABA release [24]. Thus, MDMA has potential to alter disparate nervous system functions through its multiple targets.

The impact of MDMA on reproductive neuroendocrine function and the reproductive axis has not, to our knowledge, been rigorously explored. Nevertheless, this question is biologically relevant, because the same neurotransmitter systems that control these aforementioned central nervous system actions of MDMA, including (but not limited to) serotonergic and dopaminergic pathways, also regulate reproduction [25–27; for review see 28]. Reproductive function in mammals is driven by about 1000 gonadatropin-releasing hormone (GnRH) neurons, localized in the preoptic area and hypothalamus of rodents [28], which in turn are regulated by afferent inputs from other brain regions. This neural circuitry enables the hypothalamic GnRH cells to coordinate reproduction with other environmental and homeostatic cues. Thus, GnRH neurons, through these central afferent inputs, are a potential target of MDMA.

The objective of the present study was to assess the neuroendocrine disrupting effects of MDMA in adult male Sprague-Dawley rats as a model for reproductive effects of ecstasy usage in humans. Although our primary interest was actions of MDMA on hypothalamic GnRH neurons, we also assessed actions of MDMA on the other two levels of the hypothalamic-pituitary-gonadal (HPG) axis: serum luteinizing hormone (LH) concentrations as an index of pituitary output, and sex steroid hormones as an indicator of testicular function.

METHODS

MDMA

(±)3,4-Methylenedioxymethamphetamine HCl (MDMA) was obtained through NIDA Drug Inventory Supply and Control, and was dissolved in 0.9% saline.

Animals and Procedures

All experimental procedures were carried out in accordance with the National Institute of Health Guide for Care and Use of Laboratory Animals, and performed following protocols approved by the Institutional Animal Care and Use Committee at the University of Texas at Austin. Adult male (250 – 300 g) Sprague-Dawley rats Charles River Laboratories, Inc. Wilmington, MA) were used in this study. The experimental model used herein was to train rats to level press for 45 mg sugar pellets (Bio-Serv, Frenchtown, NJ) on a fixed ratio 1 schedule for a minimum of 8 days, as part of a separate study seeking to enable rats to self-administer either MDMA or vehicle [published in 8]. Additional details on animals, surgeries, and procedures, are reported in that paper. In brief, rats self-administered saline or MDMA either once (acute) or for 20 days (chronic), and were euthanized 7 days following last administration, for a total of 4 treatment groups (Figure 1). All administration occurred intravenously, via a chronically implanted jugular catheter (see [8] for details). The acute MDMA experimental group administered a single 3 mg/kg dose of MDMA, chosen to mimic a recreational MDMA “binging” experience with the drug, when a large dose of the drug is ingested in one session. This dose was selected upon results of preliminary microdialysis experiments in one of our

labs (C.L.D.; data not shown), which demonstrated significant increases in serotonin and dopamine in the nucleus accumbens at this dose. The chronic MDMA experimental group was designed to mimic long-term heavy use of MDMA among young people at dance clubs or "raves," where repeated doses are taken intermittently in the same evening. Male rats administered unit doses of MDMA (or saline control) *ad libitum* for five consecutive days for a period of 4 weeks (2 hour sessions, 5 days on, 2 days off each week for the 4 week period). During Days 1–10 the unit dose was 1 mg/kg per injection, and during Days 11–20 the unit dose was 0.5 mg/kg per injection [8] to optimize self-administration behavior [29]. Average daily MDMA intake for chronic MDMA animals was found to be 4 mg/kg body weight. The number of animals per experimental group were as follows: acute saline: 5, chronic saline: 6, acute MDMA: 5, chronic MDMA: 9. In the results section, any deviation from the number of animals per group noted here represents a lost data point due to experimental error or tissue loss.

Blood & Tissue Collection

Acute- and chronic-treated MDMA and saline rats were euthanized by carbon dioxide asphyxiation 7 days following the final treatment. Brains were removed and one half of the preoptic area - anterior hypothalamus (POA-AH), which contains the majority of GnRH cell bodies, was dissected out on wet ice, and snap frozen within 2 minutes of removal on dry ice and stored at −80°C until RNA extraction. Terminal blood samples were taken, and serum was separated by centrifugation at 6000 g and stored at −80°C until further processing.

Serum Hormone Assays

The LH level in serum samples was measured in the laboratory of Dr. Michael Woller, University of Wisconsin-Whitewater, by double antibody competitive binding RIA as previously described [30,31], using reagents provided by Dr. A. F. Parlow of the National Hormone and Pituitary Program at NIDDK. The reference standard used was rat LH-RP-3. Duplicate volumes of 100 µl serum were used for each sample, and three separate assays were performed. The assay sensitivity was 0.2 ng/ml, the intra-assay coefficient of variability (CV) based on duplicate samples for each assay was 2.36%, 4.39%, and 4.35%, respectively, and the inter-assay CV was 7.23%.

Total serum testosterone was determined in two assays using the Active® Testosterone coated well EIA kit (Catalog # DSL-10-4000, Lot # 08035-B, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), according to the manufacture instructions. Duplicate volumes of 50 µl serum were used for each sample. The assay limit of detection was 0.04 ng/ml, and the intra-asay CV based on duplicate samples for each assay was 3.11% and 5.76%, and the inter-assay CV was 8.07%.

Progesterone concentrations were determined in a single assay using the ACTIVE®. Progesterone Coated-Tube Radioimmunoassay Kit (Catalog # DSL-3900, Lot # 07076, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), according to the manufacturer's instructions. Duplicate volumes of 25 µl serum were used for each sample. The sensitivity of the assay was 0.12 ng/ml, and the intra-assay CV was 2.3%. For all assays, samples for which the CV between duplicates was 10% or greater were excluded from analysis.

Estradiol concentrations were determined in a single assay using an ultrasensitive double-antibody RIA kit (Catalog # DSL-4800, Lot # 07076, Diagnostic Systems Laboratories, Inc., Webster, TX, USA), according to the manufacture instructions. Duplicate volumes of 200µL serum were used for each sample. The assay limit of detection was 2.2 pg/mL, and the intra-assay coefficient of variability based on duplicate samples for each assay was 2.83%.

GnRH and Cyclophilin Gene Expression Analysis by Real-time PCR

GnRH and cyclophilin (internal control: [32,33]) gene expression were measured using real-time PCR with the Stratagene Brilliant® qPCR kit and the Stratagene MX3000 detection system. Messenger RNA from frozen POA-AH dissections of individual rats was extracted using a double-detergent lysis buffer system [32]. In brief, frozen tissues were homogenized in cold lysis buffer through a 22-gauge needle, and cytoplasmic RNA was separated from nuclear RNA using a sucrose gradient. Samples were treated with proteinase K to remove proteins, and cytoplasmic RNA was extracted with chloroform and isopropanol, followed by precipitation at −20°C. The RNA was pelleted by centrifugation, washed, and resuspended in 10 μl nuclease-free water (Catalog # AM9937, Applied Biosystems, Foster City, CA, USA). Genomic contamination was removed using the DNA-free™ Kit (Catalog #AM1906, Applied Biosystems) according to the manufacture instructions. RNA concentration was quantified using a Nanodrop ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and its RNA purity was assessed using the 260:280 and 260:230 nm ratios. All samples had 260:280 ratios between 1.8 and 2.1, and 260:230 ratios above 1.7. RNA integrity was assessed by examining representative samples loaded onto a 1.5% agarose gel stained with ethidium bromide. Two μg of RNA were then converted to cDNA via a reverse-transcriptase (RT) PCR reaction using the SuperScript™ First-Strand Synthesis System for RT-PCR (Catalog # 11904-018, Invitrogen Corporation, Carlsbad, CA) according to the manufacturer's instructions. All RT reactions contained a negative control, which consisted of nuclease-free water instead of reverse transcriptase enzyme, to confirm absence of genomic contamination in each sample. cDNA reactions were diluted 1:5 and a 2 μl aliquot of cDNA was used as template for amplification in PCR. Primers used were 5'-TGTGCCAGGGTGGTGACTT-3' (sense) and 5'-TCAAATTTCTCTCCGTAGATGGACTT-3' (anti-sense) and probe 5'-CCACCAGTGCCATTATGGCGTGT-3' for cyclophilin [34] and 5'-CCCTTTGGCTTTCACATCCA-3' (sense) and 5'-AACAGCGGCCATCAGTTTG-3' (anti-sense) and probe 5'-ACAGAATGGAAACGATCC-3' for GnRH. PCR conditions were 95°C for 10 min, followed by 50 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 60 sec, and extension at 72°C for 30 sec. For each sample, GnRH gene expression was normalized to cyclophilin and relative expression was determined using the Comparative Ct Method [35]. RNA extracted from the POA-AH of an intact untreated male rat was used as a positive control, and was included as a standard on each assay plate. Samples were run in triplicate and analyzed in duplicate to account for pipetting errors. The mean positive control value was used as a calibrator for inter-plate variability. The interassay CV was 6%.

Statistical Analysis

The effects of MDMA on serum hormone levels and GnRH gene expression were analyzed using two-way ANOVAs [variables: drug (saline, MDMA) X duration (acute, chronic)] with Statview 5.0 software for Macintosh computer. Significant interactions and main effects were probed further with Fisher's PLSD *post-hoc* tests. Differences were considered significant at $p < 0.05$. All data are reported as mean ± S.E.M. unless otherwise noted.

RESULTS

Effects of MDMA on Hypothalamic GnRH Gene Expression

GnRH gene expression was measured using real-time PCR in POA dissections. Two-way ANOVA revealed a significant main effect of drug treatment upon GnRH mRNA levels, with overall lower levels of GnRH mRNA expressed in MDMA compared to saline rats ($F = 8.584$, $p < 0.01$; Figure 2). No significant main effect was detected for duration of treatment (chronic vs. acute), nor were any significant interactions between the two variables observed.

Effects of MDMA on Serum LH Levels

Serum LH levels varied considerably among rats, probably due to the pulsatile nature of LH release [31]. Two-way ANOVA showed no significant main effect of drug ($p = 0.11$) or duration ($p = 0.91$) upon LH levels, nor were any significant interactions between the two variables observed ($p = 0.71$; Figure 3).

Effects of MDMA on Serum Testosterone Levels

A significant main effect of drug treatment was found for serum testosterone concentrations, with decreased levels in the MDMA compared to the saline group ($F = 33.23$, $p < 0.0001$; Figure 4). A significant main effect of duration ($F = 5.15$, $p < 0.05$) on serum testosterone levels was also found, with chronic rats having higher testosterone levels than acute (Figure 4). There was a non-significant trend for an interaction of treatment with duration ($p = 0.054$), and results suggest that effect of duration is largely due to differences among the saline treated animals. Nevertheless, MDMA significantly suppressed serum testosterone levels at both durations compared to respective saline controls.

Effects of MDMA on Serum Progesterone and Estradiol Levels

Serum estradiol and progesterone concentrations were measured to further assess the effect of systemic MDMA on steroid hormones. For progesterone, two-way ANOVA revealed no significant main effects of drug ($p = 0.62$) or duration ($p = 0.11$), nor were any interactions between drug and duration observed ($p = 0.29$; Figure 5). For the estradiol assay, a high percentage of samples had a large assay CV, and a lack of serum for repeating the assay resulted in a very small sample size that did not provide adequate statistical power for analysis. For those animals with detectable estradiol concentrations, preliminary results suggested that there were no differences among any of the groups. Estradiol concentrations (pg/ml) for the Acute-Saline, Chronic-Saline, Acute-MDMA and Chronic-MDMA groups were 10.0 ± 4.1, 7.1 ± 4.5, 9.3 ± 1.9 and 10.3 ± 4.1, respectively (data are presented as mean ± standard deviation due to small N's).

Effects of MDMA on Paired Testes Weights

Paired testes were removed and weighed, with Acute-Saline, Chronic-Saline, Acute-MDMA and Chronic-MDMA groups having weights (g) of 2.66 ± 0.72, 3.37 ± 0.25, 3.79 ± 0.96 and 3.25 ± 0.46, respectively (mean ± SEM). Although there was no effect of treatment or duration, there was a significant interaction of these two variables ($p < 0.05$), attributable to differences between Acute-MDMA and Acute-Saline.

DISCUSSION

The present study demonstrates that MDMA administered at dosages relevant to human intake [36–38] causes a significant disruption of hypothalamic and gonadal function. Specifically, and as discussed in more detail below, rats taking MDMA had significantly lower GnRH gene expression and lower serum testosterone concentrations compared to their saline control counterparts. Because rats were euthanized seven days after the last MDMA administration, these findings further suggest that both acute and chronic MDMA use has lasting endocrine disrupting actions on the HPG axis. Although we recognize that metabolism and secretion of MDMA varies between species [10], these results showing that MDMA disrupts reproductive neuroendocrine function in healthy adult male rats has potential relevance to humans who use MDMA even once.

The results show that MDMA administered either acutely or chronically resulted in a significant, approximately 50% decrease in GnRH mRNA levels seven days later compared

to saline administered rats. Pituitary LH levels were slightly lower in these same MDMA animals but this latter result did not attain significance [e.g. 31]. Because we do not know when during the LH pulse a rat was euthanized, it is possible that more careful analyses of effects of MDMA on LH pulsatility would reveal differences between the treatment groups. In addition, our current observation that serum testosterone was profoundly suppressed in MDMA rats is consistent with decreased drive upon the testes by the serum gonadotropins. Therefore, our results showing significant decreases in GnRH mRNA and serum testosterone concentrations, together with the non-significant decrease in serum LH, is consistent with diminished drive from hypothalamic GnRH neurons upon the rest of the HPG axis. Moreover, we speculate that the GnRH neurosecretory system is the primary target for HPG axis disruption by MDMA. If the gonad were the primary target of the MDMA suppression, thereby resulting in decreased testosterone concentrations, we would predict that negative feedback upon the hypothalamus would be reduced, resulting in an *increase* in GnRH gene expression, as reported in other experimental models [39]. As this was not the case, the suppressed testosterone levels are likely due to decreased feed-forward input from the hypothalamus, and subsequently the pituitary, upon the testes.

In addition to androgens, the male adrenal and testes also produce substantial levels of progesterone [40,41], a hormone that plays a role in the sexual behavior of male rodents [42]. This hormone was assayed in the current study, with no effects of drug treatment found. We also assayed serum estradiol, and although our sample sizes were too small to perform statistics, the preliminary results suggest that there was no apparent effect of any MDMA treatment. Therefore, effects of MDMA on steroid hormones measured thus far appear to be specific to testosterone.

Although it was beyond the scope of the current study to determine which neurotransmitter systems mediate the effects of MDMA on the hypothalamic GnRH system, these are likely to be similar to those that underlie MDMA's other central nervous system actions. The two most plausible candidates are serotonin and dopamine, as they have been reported to be the primary targets of MDMA in other regions of the brain [8; reviewed in 10]. Serotonin and dopamine neurons innervate the hypothalamus, and receptors for both these neurotransmitters are expressed in the preoptic area, the location of the GnRH cell bodies [43,44; reviewed in 28]. Serotonin agonists have been reported to be either stimulatory or inhibitory to GnRH neurons depending upon the experimental model [26,45,46]. A similar finding has been made for dopaminergic actions on GnRH cells [47,25,48]. Although it is difficult to reconcile both stimulatory and inhibitory effects of these neurotransmitter systems, they are probably best explained by there being a permissive window of normal functional activity of serotonin or dopamine, manipulations above or below which cause disruption of GnRH function. It is possible that through alterations in hypothalamic monoaminergic receptors, MDMA can influence these inputs into the GnRH neurosecretory system, either directly or indirectly.

This study was conducted to evaluate the impact of MDMA on the HPG axis of rats. However, we would like to note that the model of MDMA self-administration in rats is a topic of considerable controversy [49]. Several laboratories [50,29,51] including one of ours (C.L.D., [8]), but not others [reviewed in 49], have shown that rats and mice self-administer MDMA. Although acquisition occurs at a slower rate and supports fewer lever responses compared to other psychostimulants [49,50;52–55], the salient point is that our rats in the current study administered MDMA at levels comparable to voluntary human intake. Thus, we were able to make comparisons between animals given MDMA versus saline to determine specific effects on the reproductive axis.

The major novel finding of this study is that, to our knowledge, it provides the first evidence that MDMA disrupts the HPG axis of adult male rats, and even more specifically, that the

mechanism for this effect involves the targeting of the hypothalamic-preoptic GnRH system. Our results are consistent with observations that environmental or pharmaceutical substances that disrupt monoaminergic neurotransmitter functions in the hypothalamus, including pesticides [56,57] and PCBs [58,59], affect GnRH and LH release. The popularity of MDMA among humans has increased in recent years [1], leading to concern over the potential health hazards associated with recreational drug use. The results of this study suggest that dosages approximating the recreational use of MDMA may impact the male reproductive axis.

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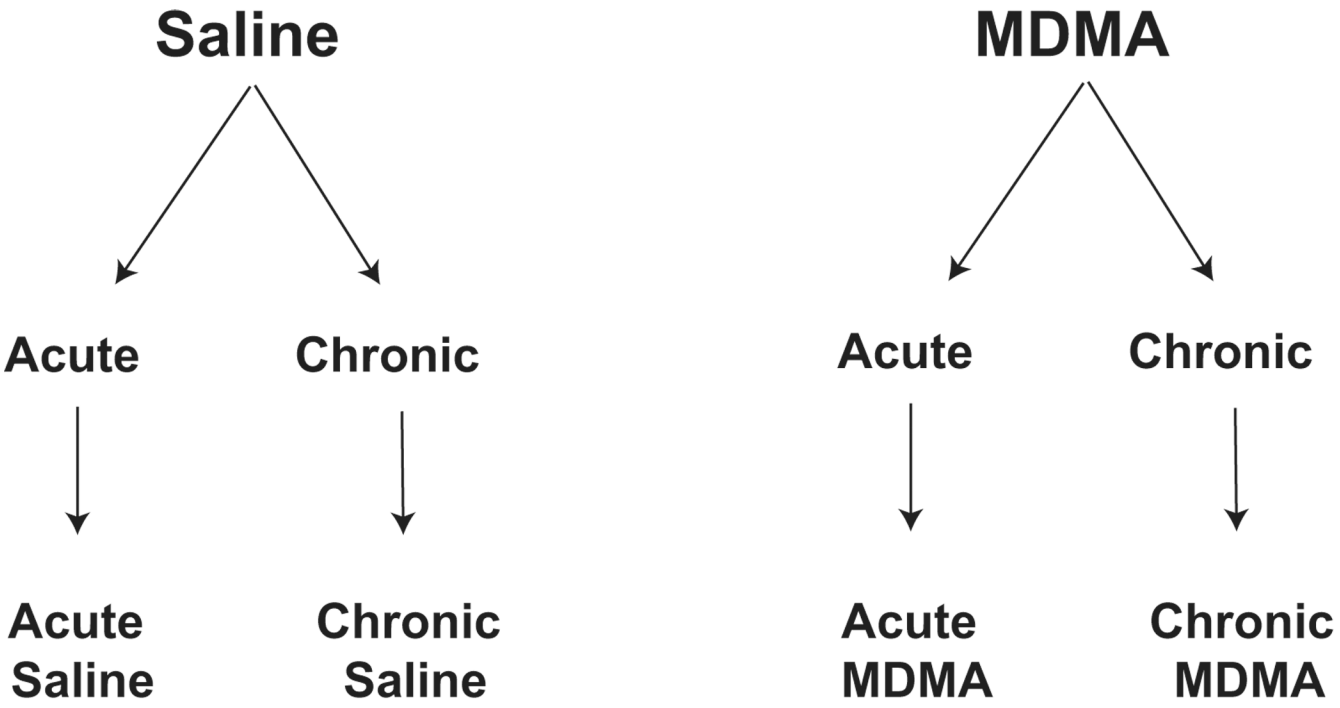

Figure 1.
Experimental model. Adult male Sprague-Dawley rats self-administered saline or MDMA either once (acute) or *ad libidum* for a period of 5 days over a 4-week time period, resulting in a total of 20 days (chronic). They were then euthanized 7 days following last administration, for a total of 4 treatment groups.

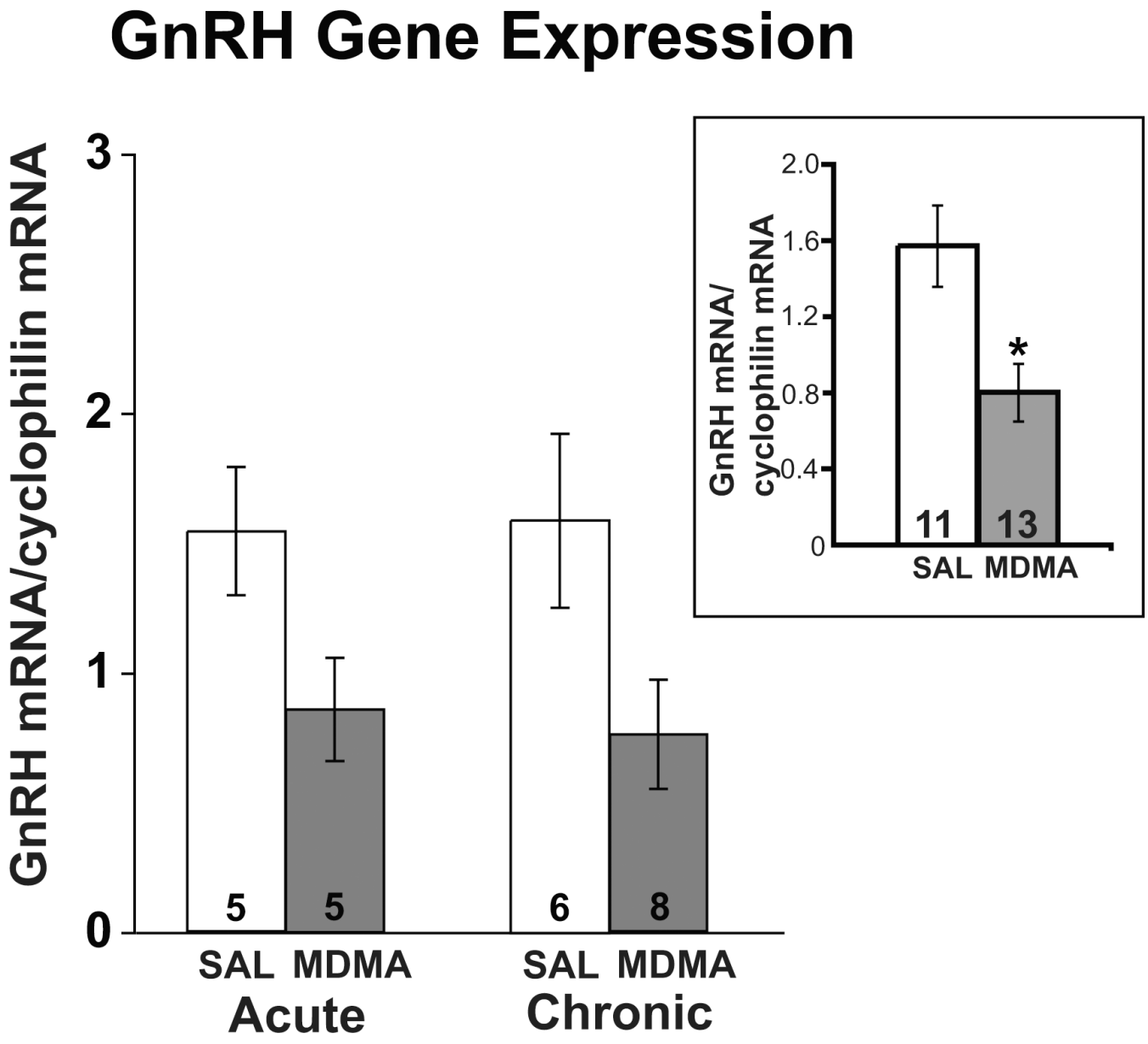

Figure 2.

Effects of acute and chronic MDMA or saline (SAL) on POA-AH GnRH mRNA levels, normalized relative to cyclophilin mRNA levels. Group data (mean ± SEM) are shown here and in subsequent figures. A significant main effect of drug was detected, with MDMA rats having decreased GnRH mRNA levels compared to saline rats. Because there were no differences in results between the acute and chronic groups, the inset shows the comparison between all MDMA and SAL rats combined for both durations of treatment. Numbers of rats per group are shown within each bar in this and all other figures. *$p < 0.01$ compared to SAL.

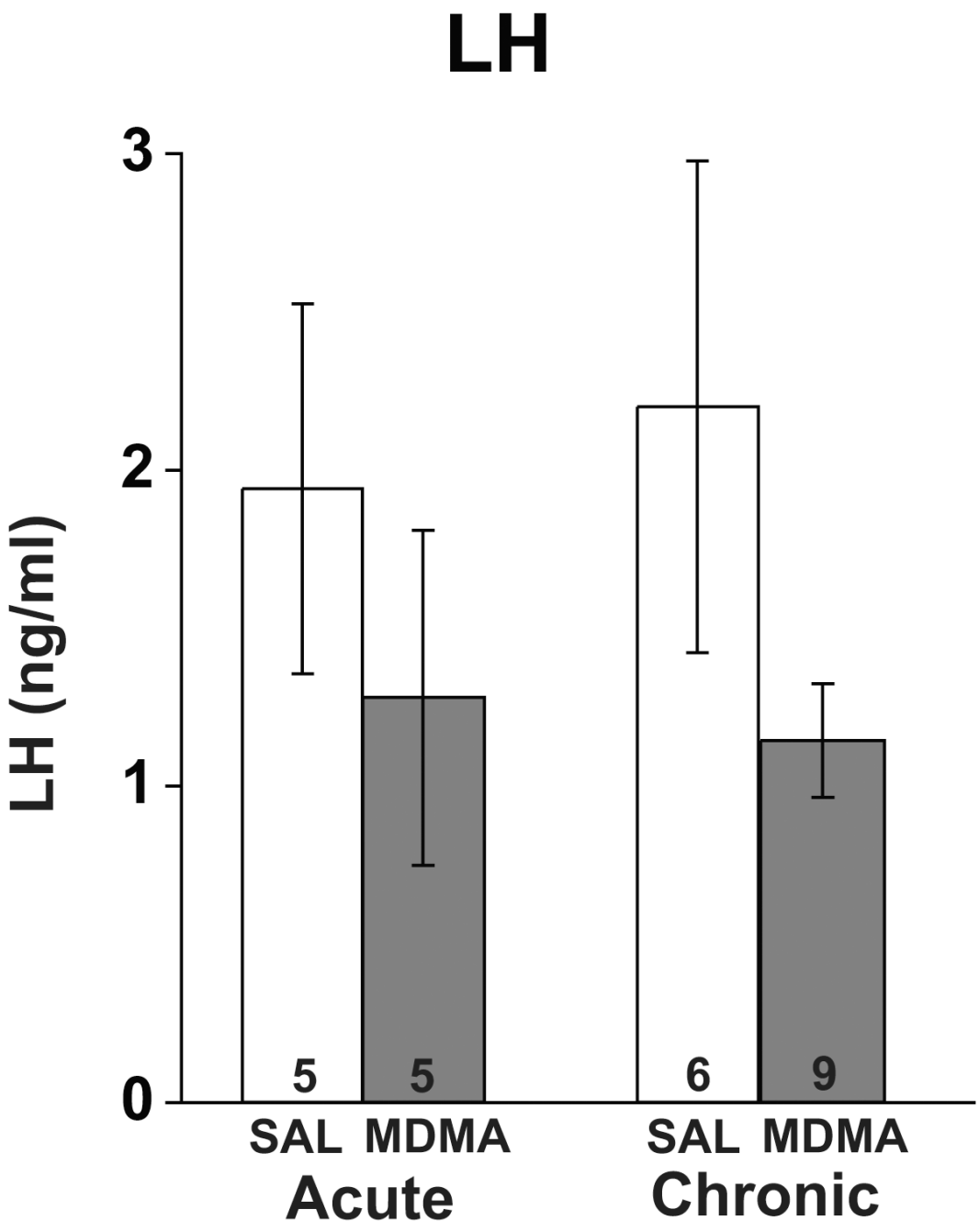

Figure 3.
Effects of acute and chronic MDMA or saline (SAL) on serum luteinizing hormone (LH) concentrations. No significant differences in serum LH were detected for treatment or duration, with no significant interactions of the two variables.

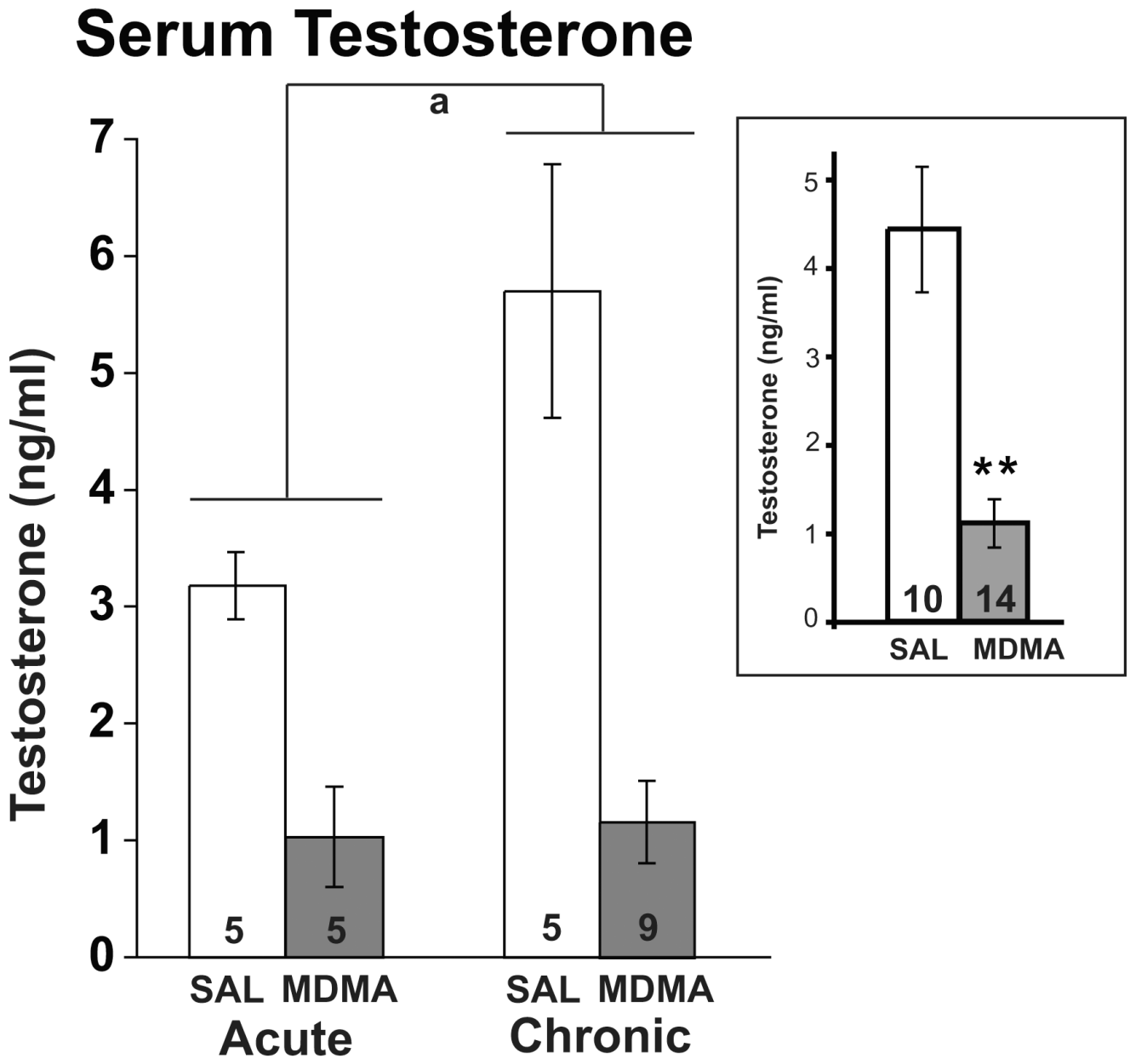

Figure 4.

Effects of acute and chronic MDMA or saline (SAL) on serum testosterone concentrations. Testosterone concentrations were significantly lower in the MDMA compared to the saline group, as indicated in the inset ($p < 0.0001$). Chronic rats also had higher testosterone levels than acute rats ($p < 0.05$). **, $p < 0.0001$ compared to SAL; a, $p < 0.05$ for acute vs. chronic treatments.

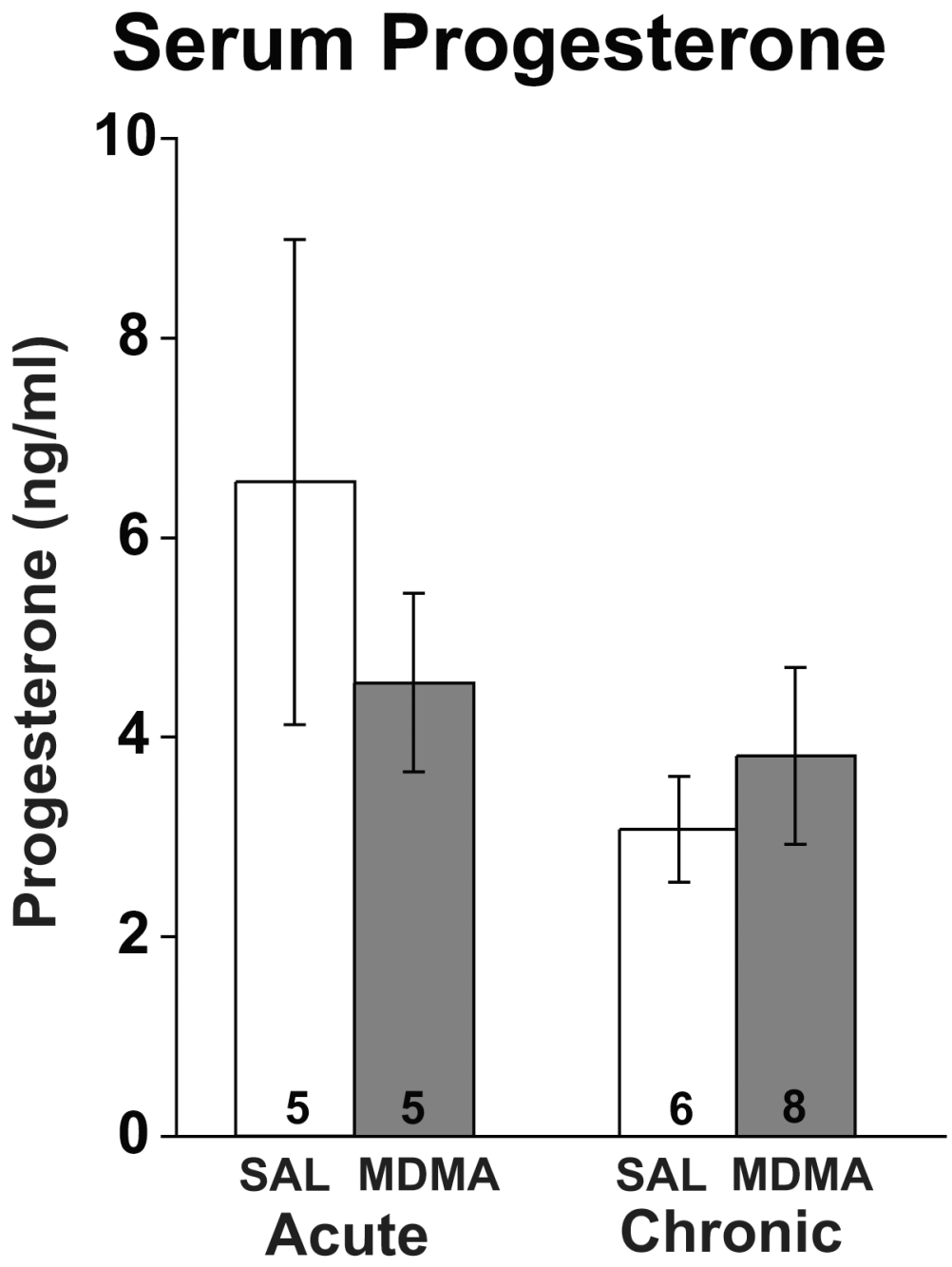

Figure 5.
Effects of acute and chronic MDMA or saline (SAL) on serum progesterone concentrations. No significant differences in serum progesterone were found for drug or duration of treatment.