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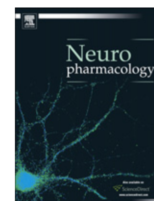

An estrogenic effect of 5α-androstane-3β, 17β-diol on the behavioral response to stress and on CRH regulation

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A B S T R A C T

The gender difference in behavioral and hormonal response to stress is well known, but the underlying mechanism remains elusive. Arginine-vasopressin (AVP) and corticotrophin-releasing hormone (CRH) are two major regulatory peptides in the brain involved in stress regulation. Their response to stress has been shown to be modulated by sex hormones. The androgen metabolite, 5α-androstane-3β, 17β-diol (3β-diol), has been identified as an estrogenic hormone. It binds to estrogen receptors (ERs) and modulates estrogen response element mediated promoter activities via the ER pathway. The present study involved in vitro transfection assays to examine whether 3β-diol can directly modulate CRH and AVP promoter activity. Our results demonstrate that in CHO-K1 cell lines, when ERs were over-expressed, 3β-diol could significantly stimulate CRH and AVP promoter activity through an ER pathway. The effect of 3β-diol on the behavioral, the CRH and the AVP response to stress in the rat was also investigated. We found that chronic, but not acute administration of 3β-diol significantly decreased the immobile duration in the forced swim test. In rats exposed to the forced swim test, CRH mRNA expression in the hypothalamus was enhanced by chronic 3β-diol administration, while the AVP mRNA expression was not affected. These results suggest that 3β-diol may play an anti-depressive role in affective behavior and may have a direct effect on CRH expression.

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1. Introduction

The hypothalamic–pituitary–adrenal (HPA) axis plays an important role in the stress response in vertebrates. Previous studies demonstrated that the HPA activity is related with behavioral outcomes when exposed to a stressor. For example, passive coping behavior, like immobility, was suggested to be associated with a higher plasma glucocorticoid level (Bohus et al., 1987; Liebsch et al., 1998). However, some other experimental evidence denied such an association (Abel, 1991; Brush, 1991). Arginine-vasopressin (AVP) and corticotrophin-releasing hormone (CRH) are two major regulatory peptides in the HPA system. They co-localize in the parvocellular part of the paraventricular nucleus of the hypothalamus (PVN), which is highly responsive to stressors (Mouri et al., 1993; Tramu et al., 1983) and they may act synergistically to stimulate the secretion of adrenocorticotropin (ACTH) at the adenohypophysis (Antoni, 1993; Plotsky, 1991; Whitnall, 1993). It has been reported that psychological stresses, such as restraint (Harbuz et al., 1994; Ma et al., 1997) and forced swimming (Jiang et al., 2004), as well as physical stress, such as intraperitoneal hypertonic saline injection (Harbuz et al., 1994), may result in increased levels of CRH and AVP mRNA in the PVN.

Many studies revealed an involvement of sex hormones in HPA axis regulation and stress response. Estrogen responsive elements (EREs) have been identified on the CRH and AVP promoter region, and promoter activity may be enhanced by estradiol (E2) (Shapiro et al., 2000; Vamvakopoulos and Chrousos, 1993). A physiological E2 regimen to ovariectomized (ovx) rats decreases anxiety or depression behavior, while very low or very high E2 concentrations have no such effect (Walf and Frye, 2005). The same is true for E2's modification of the HPA response. When subjected to stress, E2 administration to ovx rats either enhances or reduces the plasma corticosterone level, depending on the dosage and the duration of exposure (Burgess and Handa, 1992; Walf and Frye, 2005; Young et al., 2001).

Depending on the presence of different enzymes, testosterone (T) may be aromatized to E2 or converted to 5α-dihydrotestosterone (DHT). DHT may be further metabolized to 5α-androstane-3β, 17β-diol (3β-diol) by 3β-hydroxysteroid oxidoreductase (3β-HSD), 3α-HSD and 17β-HSD (Gangloff et al., 2003; Steckelbroeck et al.,

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2004; Torn et al., 2003). Despite its androgenic origin, 3β-diol does not play an androgenic role. While other androgens (testosterone, DHT) or androgen metabolites (3α-diol, androsterone) show little binding affinity to estrogen receptors (ERs) (Kuiper et al., 1997), 3β-diol is able to bind to estrogen receptor (ER) with relative high affinity, and it is considered as an endogenous estrogen in the prostate (Weihua et al., 2002, 2001). Also, 3β-diol stimulated promoter activity mediated by ER binding to an ERE, while its stereoisomer 3α-diol had no effect (Pak et al., 2005). Recently, the biological function of 3β-diol in the central nervous system was revealed: it decreases ACTH and corticosterone release after restraint stress (Lund et al., 2006, 2004). Pak et al. (2007) found that in a neuronal cell line, 3β-diol stimulates AVP promoter activity via ERβ pathway. These results indicate that, like other steroid hormones, 3β-diol plays an important role in stress response and in the regulation of HPA activity.

Since 3β-diol directly stimulates AVP promoter activity in vitro, it is of interest to investigate whether it can also directly influence CRH promoter activity. Also, given the influence of 3β-diol in the ACTH and corticosterone response to stress, it is of interest whether it may influence the affective behavior and the CRH and AVP response to stress. The present study was carried out according to the following hypotheses: first, 3β-diol directly stimulates the CRH promoter activity via an ER pathway, just as it did the AVP promoter; second, as a steroid hormone, 3β-diol plays a role in affective behavior, for example, it influences the immobility in the forced swim test (FST); third, 3β-diol also influences the CRH and AVP expression in basal conditions or in stress response.

2. Methods

2.1. Plasmid and cell culture

CHO-K1 cells were cultured in phenol red-free Dulbecco's Modified Eagle Medium/F12 medium (1:1) supplemented with 10% (v/v) charcoal-treated new born calf serum, in a humidified atmosphere of 5% (v/v) CO_2 at 37 °C. The CRH luciferase plasmid containing a 2.2 kb CRH promoter has been described before as pDFF146 (Bao et al., 2006). The human AVP promoter from −796 to −1 was amplified from human genomic DNA and inserted into the pGL3-basic plasmid. The human estrogen receptor expression vectors pSG5-hERα and pSG5-hERβ was generously provided by Dr. J.A. Gustafsson (Karolinska Institute, Sweden). The pRL-CMV plasmid was kindly provided by Dr. M. Wu (University of Science and Technology of China, China).

2.2. Transfection and luciferase reporter assay

Cells were plated at 1×10^5 cells per well in 24-well plates 24 h before transfection to reach a final confluency of 80–90%. Transfection was carried out using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Briefly, cells were transfected with either a pSG5 empty plasmid or ERα or ERβ expression vector and with a CRH luciferase reporter or an AVP luciferase reporter plasmid. Cells were incubated in transfection media for 6 h and then the media was replaced with complete medium containing 10% charcoal-treated new born calf serum and different hormones were added. 17β-Estradiol (E2, purchased from Sigma, USA), 3β-diol (purchased from Sigma, USA) and tamoxifen (tam, purchased from Sigma, USA) were first dissolved in ethanol and used at a final concentration of 100 nM in 0.01% ethanol. Cells were harvested 24 h after hormone treatment and luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, USA). Luciferase activity was normalized for transfection efficiency by dividing the measured firefly luciferase activity by the measured renilla luciferase activity. All transfections were carried out in at least three independent experiments.

2.3. Animals

Adult male Sprague–Dawley rats (8–10 weeks) were housed three to four to a cage in a room under controlled 12 h light/dark schedule (light on at 7:00 A.M.) and temperature (23 ± 2 °C) conditions with ad libitum access to food and water. Rats were gonadectomized (GDX) under anesthetization with chloral hydrate (7%, 1 ml/100 g body weight). Rats were allowed 1-week recover before the experiment. All animal handling procedures were carried out in accordance with all relevant local guidelines and legislation to minimize the animals' suffering.

2.4. Hormone treatment

In the following experiments, 3β-diol was administrated subcutaneously in a dose of 1 mg/kg body weight. Tamoxifen was administrated subcutaneously in a dose of 1 mg/kg body weight in combination with 3β-diol. The vehicle was sesame oil containing 8% ethanol. The dosage was chosen based on previous studies by Lund et al. (2004, 2006) that had demonstrated its effect.

2.4.1. Experiment 1: effect of chronic 3β-diol administration on behavior and on the expression of CRH and AVP

Rats were divided into three groups and received a daily injection of vehicle ($n = 8$), 3β-diol ($n = 8$) or 3β-diol in combination with tamoxifen ($n = 6$) for 9 days. On the eighth day, the FST paradigm was carried out as described below. The last injection was performed 1 h before the FST. To determine the effect of 3β-diol in basal condition, six rats of each group went through the same treatment without being subjected to the FST.

2.4.2. Experiment 2: effect of acute 3β-diol administration on behavior and on the expression of CRH and AVP

Rats were divided into three groups. One hour before the FST, they received acute injection of vehicle, 3β-diol or 3β-diol in combination with tamoxifen ($n = 6$ for each group). To determine the effect of acute 3β-diol treatment in basal condition, five rats of each group went through the same treatment without being subjected to the FST.

2.5. Forced swim test (FST)

The FST is a well-established behavioral paradigm used to test the efficacy of antidepressants. Many antidepressants, such as fluoxetine, imipramine and reboxetine significantly decrease the immobility time in this test (Cryan et al., 2002; Porsolt et al., 1978, 1977). Two active behaviors in the test, climbing and swimming, also predict the efficacy of antidepressant treatment. In our study, the FST was conducted according to the method of Porsolt et al. (1977, 1978) with some modifications. The behavioral apparatus was a stainless steel cylinder 60 cm high and 25 cm in diameter filled with 30 cm of water maintained at 25 °C. On day 1, rats were forced to swim in water for 15 min. Twenty-four hours later they were replaced in the cylinder for a 5-min test. The movements of the rats were recorded by a video camera and then counted by an observer. Rats were considered to be immobile when they did not make any active movements. They were considered to be climbing when they made active movements with their forepaws in and out of the water along the side of the swim chamber. They were considered to be swimming when they made active swimming or circular movements.

2.6. Tissue preparation

Rats were anesthetized and then decapitated 2 h after the test and their brains were removed quickly. Hypothalamic parts were dissected according to Yasin et al. (1993) with the following limits: anterior border of the optic chiasm, anterior border of the mamillary bodies, and lateral hypothalamic sulci. The depth of dissection was approximately 3 mm. The hypothalamus was then quickly frozen in liquid nitrogen and preserved at −80 °C.

2.7. Quantitative RT-PCR

Each frozen hypothalamus was homogenized, and total RNA was isolated with TRIzol Reagent (Invitrogen, USA). cDNA was synthesized using reverse transcriptase (Promega, USA). Q-PCR was performed using SYBR Green PCR Kit (Applied Biosystems, USA) and an ABI Prism 7000 Sequence Detector system in 25 μl volume for 40 cycles (15 s at 95 °C and 60 s at 64 °C). Rat AVP cDNA was amplified using primers 5′-CAGATGCTCGGCCCGAAG-3′ and 5′-TTCCAGAACTGCCCAAGAG -3′(100 bp, intron spanned). Rat CRH was amplified using primers 5′-CAGAACAA CAGTGCGGGCTCA-3′ and 5′-AAGGCAGACAGGGCGACAGAG-3′ (121 bp, intron spanned). Primers for rat beta-actin were 5′-TTGCTGACAGGATGCAGAA-3′ and 5′-ACCAATCCACACAGAG TACTT-3′ (100 bp, intron spanned). The relative amount of target gene was calculated using the $2^{-\Delta\Delta Ct}$ method. The relative amplification efficiencies of the primers were tested and shown to be similar.

2.8. Statistical analysis

Statistical analyses were carried out using Kruskal–Wallis multiple comparison tests for differences among groups. If a significant difference was detected, differences between two groups were evaluated by the Mann–Whitney test. Statistical significance was considered at the $p < 0.05$. Data are expressed as mean ± S.E.M.

3. Results

3.1. Effect of 3β-diol on ER mediated CRH and AVP promoter activity in vitro

To determine whether 3β-diol, like E2, regulates CRH (or AVP) promoter activity through an ER pathway, we cotransfected CHO-K1 cells with CRH (or AVP) promoter-luciferase reporter plasmid and a vector that expressed either ERα, ERβ or pSG5 empty plasmid. Our results show that when either ERα or ERβ was presented, treatment with E2 significantly stimulated CRH and AVP promoter activity compared with the vehicle treated group. Co-treatment with ER antagonist tamoxifen completely abolishes this effect (Fig. 1B, C; Fig. 2B, C). 3β-Diol was similar to E2 in its effect on CRH and AVP promoter activity. Compared to vehicle treatment, the promoter activities were significantly increased by administration of 3β-diol when either ERα or ERβ presented. Also, this phenomenon was reversed when tamoxifen was co-administered (Fig. 1B, C; Fig. 2B, C). Without expression of ERs (pSG5 empty expression plasmid), none of these hormones influence the expression of CRH or AVP (Fig. 1A; Fig. 2A). These results show that 3β-diol had a direct stimulatory effect on CRH and AVP promoter activity mediated by ERs.

3.2. Effects of 3β-diol administration on the behaviors of rats in the FST

The present study shows that 3β-diol influenced the affective behavior of rats. Chronic administration of 3β-diol significantly decreased the duration of immobility in the FST compared to the vehicle treated group ($p = 0.020$, Fig. 3A). This effect may be completely abolished by co-injection with the tamoxifen ($p = 0.002$ versus the single 3β-diol injection group, Fig. 3A). However, when administrated acutely, 3β-diol did not influence the immobile duration (Fig. 3B).

For the two positive behaviors in the FST, swimming and climbing, neither chronic nor acute administration of 3β-diol had an influence on the duration (Table 1).

3.3. Effect of 3β-diol on CRH and AVP mRNA expression in the hypothalamus

After exposure to FST, CRH mRNA expression in the hypothalamus was significantly higher in the chronic 3β-diol treated group compared to the vehicle treated group ($p = 0.008$, Fig. 4A). This effect of 3β-diol could be inhibited by co-injection with tamoxifen. There seemed to be a trend that chronic 3β-diol treatment increased AVP mRNA expression but this trend did not reach significant difference. We did see a significant difference between single 3β-diol treatment and 3β-diol in combination with tamoxifen treatment groups ($p = 0.001$, Fig. 3). For the basal levels of CRH or AVP mRNA in the hypothalamus, in rats that were not subjected to FST, chronic 3β-diol administration had no effect (data not shown).

In the present study, we found that acute 3β-diol administration did not influence the basal or FST induced CRH and AVP expression (data not shown).

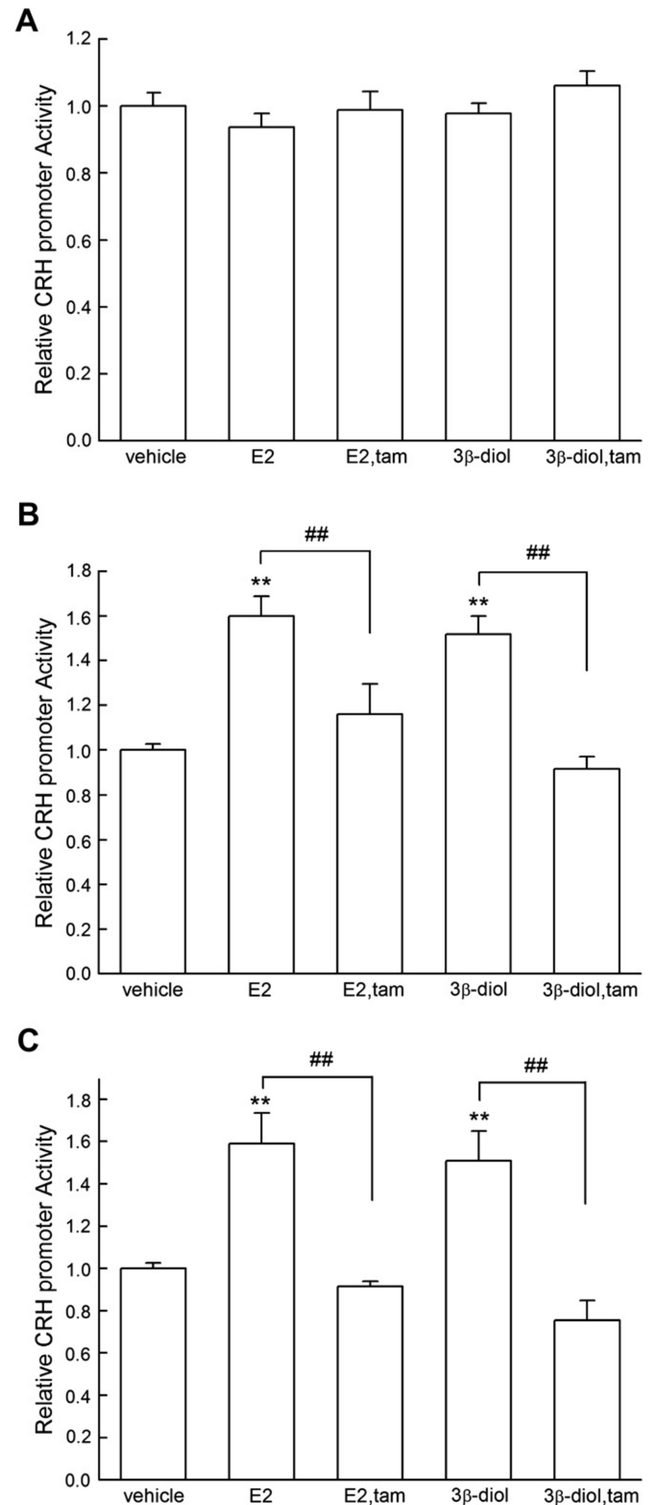

Fig. 1. Effect of 3β-diol on pSG5 (A), pSG5-ERα (B) or pSG5-ERβ (C) mediated CRH promoter activity in vitro. Data represent mean ± S.E.M., $^{**}p < 0.01$ versus vehicle group, $^{\#\#}p < 0.01$.

4. Discussion

The present study provides experimental evidence that 3β-diol could directly stimulate AVP and CRH promoter activity via ERα and ERβ receptors in vitro. In vivo, chronic 3β-diol administration significantly decreased the immobile duration of GDX rats in the FST, while acute treatment had no effect. Quantitative analysis of CRH and AVP mRNA expression shows that chronic 3β-diol administration enhanced CRH mRNA expression in the hypothalamus of those rats subjected to the FST.

Many studies have shown that sex hormones are involved in the regulation of CRH and AVP expression; for example, estrogen may stimulate CRH and AVP transcription via ERs binding to ERE on the gene promoter region (Shapiro et al., 2000; Vamvakopoulos and

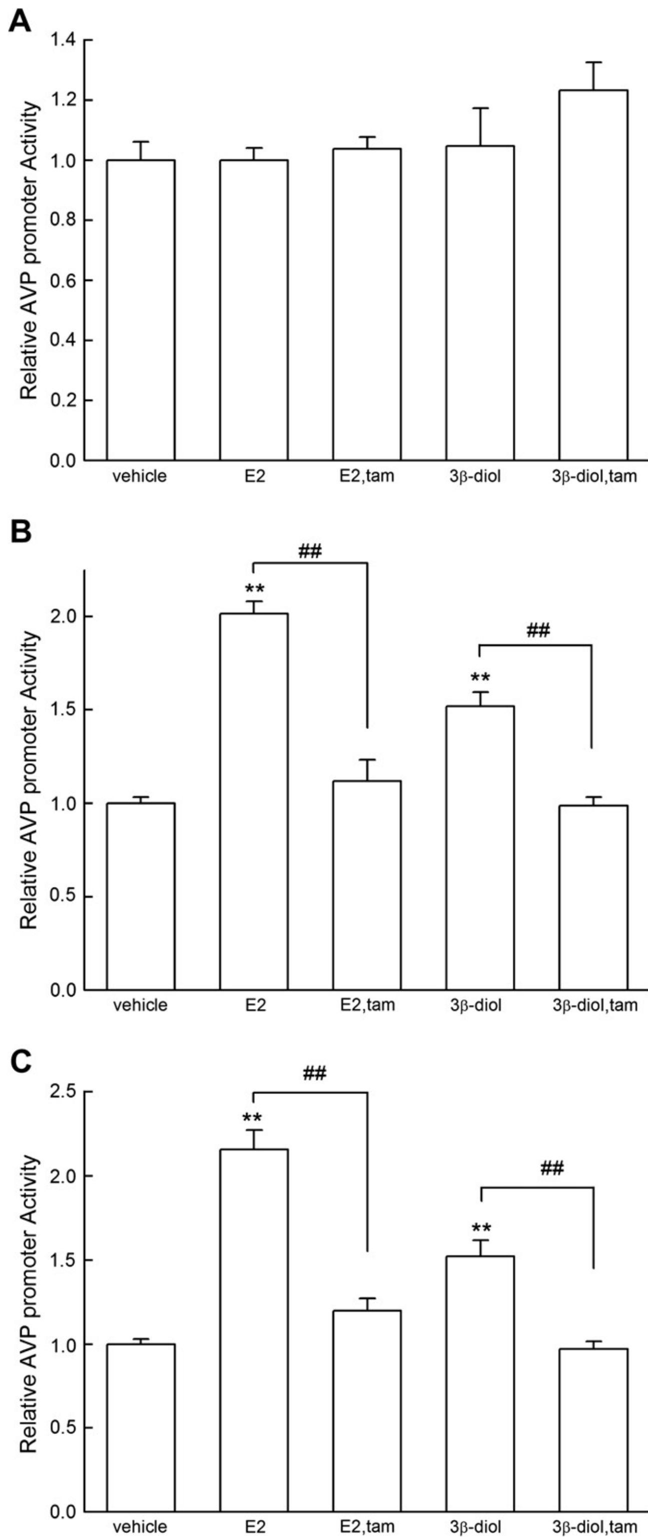

Fig. 2. Effect of 3β-diol on pSG5 (A), pSG5-ERα (B) or pSG5-ERβ (C) mediated AVP promoter activity in vitro. Data represent mean ± S.E.M., $^{**}p < 0.01$ versus vehicle group, $^{\#\#}p < 0.01$.

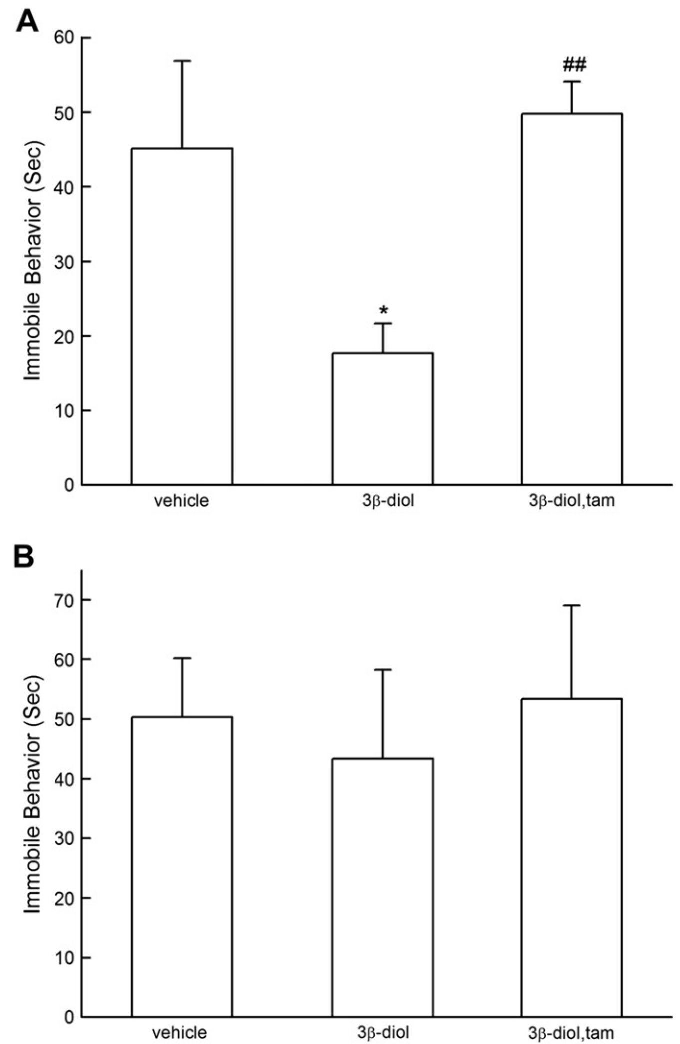

Fig. 3. Effect of chronic (A) or acute (B) 3β-diol, or 3β-diol in combination with tamoxifen administration on immobility behavior in the forced swim test. Data represent mean ± S.E.M., $^{*}p < 0.05$ versus vehicle group, $^{\#\#}p < 0.01$ versus 3β-diol group.

Chrousos, 1993). 3β-Diol has been shown to play an estrogenic role in the prostate (Weihua et al., 2002, 2001). In the present study, we therefore investigated the hypothesis that it also plays an estrogenic role in CRH and AVP transcription. As expected, our results show that in vitro 3β-diol had a direct stimulatory effect on CRH and AVP promoter activity mediated by ERα or ERβ. This effect is similar to that of E2, and, like E2, 3β-diol could thus play a role in affective behavior and in stress response.

3β-Diol is a metabolite of DHT. Some other metabolites of androgen, 3α-diol and androsterone, have also been reported to exert an anti-anxiety effect and alter the affective behavior in the FST or burying behavior test (Fernandez-Guasti and Martinez-Mota, 2005; Frye et al., 2007). Although these are all androgen metabolites, they have different biological function. 3α-Diol and androsterone are weak androgens, while 3β-diol has been proved to be a potent estrogenic hormone. In adult male rats, the plasma concentration of 3β-diol is about 200 pg/ml, which is about 20 times higher than that of E2 (Weihua et al., 2003). This suggests an important role in the

Table 1
The effect of 3β-diol administration on two positive behaviors in the FST

Groups	Chronic administration		Acute administration	
	Swimming (s)	Climbing (s)	Swimming (s)	Climbing (s)
Vehicle	184.1 ± 16.3	70.8 ± 9.9	159.2 ± 21.5	90.5 ± 17.6
3β-Diol	182.6 ± 18.8	99.6 ± 20.2	156.5 ± 14.2	100.2 ± 27.0
3β-Diol + tamoxifen	164.0 ± 18.4	86.2 ± 16.9	148.5 ± 31.1	98.2 ± 40.7

Data expressed as mean ± S.E.M.

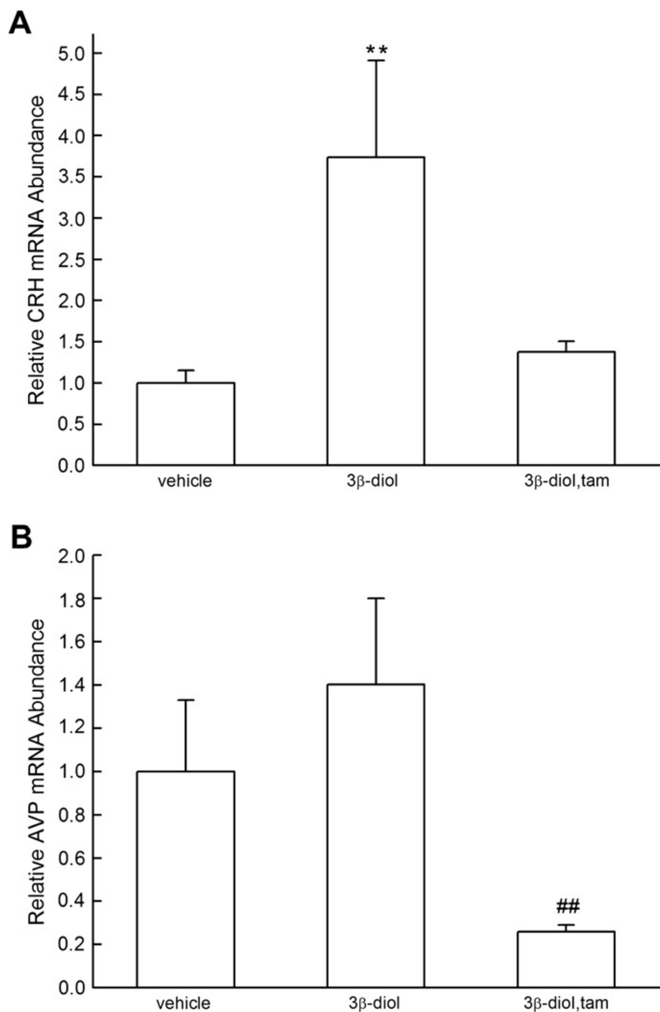

Fig. 4. Effect of chronic 3β-diol administration on CRH (A) and AVP (B) gene transcription in hypothalamus after the FST, detected by Q-PCR. Data represent mean ± S.E.M., $^{**}p < 0.01$ versus vehicle group, $^{\#\#}p < 0.01$ versus 3β-diol group.

organism. In the present study, we adopted a dose of 1 mg/kg body weight of 3β-diol. Although higher doses of 3β-diol (e.g. 3 mg/kg body weight) were used in other studies related to prostate growth (Weihua et al., 2001), the dose we chose has been proved to be effective in the ACTH and corticosterone response to stress (Lund et al., 2006, 2004). As expected, we found that 3β-diol played a role in the affective behavior. Chronic administration of 3β-diol significantly decreased the immobile duration in the FST, which indicates its anti-depressive effect. However, acute administration of 3β-diol had no effect on the duration of immobility. This discrepancy may be due to the different hormone regimen. Indeed, the regimen of hormone administration has a great influence on affective behavior. For example, the hormone E2 may or may not have an anti-anxiety or anti-depressive effect, that depends on its dosage and the duration of the administration (Walf and Frye, 2005).

Previous studies found that chronic administration of 3β-diol into the PVN or peripheral 3β-diol injections suppressed ACTH and corticosterone responses to restraint stress, without any influence on basal ACTH and corticosterone levels (Lund et al., 2006, 2004). However, so far there are no studies dealing with the direct effect of 3β-diol on CRH or AVP expression. In the present study, we examine the effect of 3β-diol on CRH and AVP mRNA expression in basal conditions and after FST. Our results show that in basal condition neither chronic nor acute 3β-diol administration had an effect on the expression of CRH and AVP. Acute injection of 3β-diol did not influence the CRH or AVP expression. Since chronic 3β-diol administration exerts antidepressant effects in the FST in our assay, and suppressed ACTH and corticosterone responses to restraint stress, we expected that it could reduce the CRH and AVP response to the FST. However, to our surprise, we found that chronic 3β-diol administration significantly stimulated CRH mRNA expression levels during FST. Also, there is a tendency towards 3β-diol enhancing AVP mRNA expression. Again, the discrepancy between our results and the results of Lund et al. (2004, 2006) may have resulted from different hormone regimens. The HPA response to stress was proven to be influenced by the hormone regimen, as was the affective behavior. E2 administration to ovx rats may reduce or increase stress-induced corticosterone response, according to the dose or administration regimen (Walf and Frye, 2005). Of course there may be other factors involved in the discrepancy. For example, we adopted a 2-day FST to investigate the antidepressant effect of 3β-diol, so the rats may have become habituated to the stressor.

The PVN, a highly responsive brain area in stress process, contains many ERα and ERβ expressing neurons (Alves et al., 1998; Bao et al., 2005; Hrabovszky et al., 1998), and it may thus well be that estrogenic hormones are involved in the stress response. In rats, ERβ is the main ER isoform in this brain region (Laflamme et al., 1998). Furthermore, this receptor has been found to be differentially co-localized with CRH and AVP. Experimental evidence showed that there were only few PVN neurons that expressed both AVP and ERβ. Meanwhile, a high percentage of the ERβ expressing neurons was co-localized with CRH (Alves et al., 1998; Laflamme et al., 1998). This different co-localization of ERβ with CRH or AVP may explain why in our study, chronic 3β-diol administration influenced the CRH expression after the FST but had no significant effect on AVP expression. Another explanation for this result is the limitation of the RT-PCR approach to determine AVP expression. In fact, both magnocellular and parvocellular neurons in the PVN express AVP, but only the latter are responsible for the stress response (Herman and Sherman, 1993). It is therefore possible that the alteration of AVP expression in the parvocellular neurons could not be detected with this method because the results might be diluted by the AVP mRNA from the magnocellular neurons.

3β-Diol can be converted from DHT by 3β-hydroxysteroid oxidoreductase (3β-HSD), 3α-HSD and 17β-HSD (Gangloff et al., 2003; Steckelbroeck et al., 2004; Torn et al., 2003), and these enzymes have been found in the hypothalamus (Lund et al., 2006). Previous studies have shown that depression is correlated with an increased activity of 17β-HSD (Raven and Taylor, 1998). Administration of antidepressant mirtazapine altered 3α-HSD activity in patients suffering from a major depressive episode (Schule et al., 2006). These results indicate that these two enzymes play a role in the affective process, possibly partly due to their effect as local modulators of sex hormones. As we know, the CRH level is significantly increased during depression and stress (Bao et al., 2005, 2007). Since our results demonstrate that 3β-diol stimulates CRH expression in the hypothalamus, it will be of interest to determine the expression and activity of these two enzymes in this brain region of depressed subjects.

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