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# Androstenedione Effects on the Vasopressin Innervation of the Rat Brain

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## ABSTRACT

The steroid hormone androstenedione profoundly influences the development and expression of sexual and aggressive behavior. The neural basis of these effects are, however, poorly understood. In this study we evaluated androstenedione's ability to maintain vasopressin peptide levels in the gonadal steroid-responsive vasopressin cells of the bed nucleus of the stria terminalis and the centromedial amygdala, and their projections. Adult male rats were castrated and given testosterone, androstenedione or no hormonal treatment for five weeks. Their brains were then processed for vasopressin immunoreactivity. Androstenedione and testosterone treatment were equally effective in preventing the reduction of vasopressin immunoreactivity associated with castration. Androstenedione may therefore be able to mimic the effects of testosterone on testosterone-responsive neural systems.

Despite its low affinity for the androgen receptor and weak peripheral androgenic effects, the androgen androstenedione (1,2) has profound masculinizing effects on the development and expression of sexual (3,4,5) and aggressive behavior (6). Although the behavioral effects of androstenedione suggest neural actions, no studies have demonstrated its ability to act at the neuronal level.

The vasopressin (AVP) projections of the bed nucleus of the stria terminalis (BST) and the centromedial amygdala (CMA) form an attractive system to test whether androstenedione acts at the neuronal level. Levels of AVP mRNA expression and AVP immunoreactivity in this system depend entirely on the presence of gonadal steroids (7,8,9,10). Furthermore, the AVP projections of the BST and CMA have been implicated in the neural control of aggressive behavior (11, 12), suggesting that steroid hormones, including androstenedione, act at the level of the AVP system to alter aggressive behavior. To test whether androstenedione is able to stimulate AVP expression in the BST and CMA and its projections, we have compared AVP immunoreactivity in the brains of male rats castrated in adulthood and treated with testosterone, androstenedione, or no hormone.

## Materials and Methods

*Hormone treatment and tissue preparation*

Nineteen male Sprague-Dawley (Taconic, Germantown, NY) rats between the ages of sixty to seventy days old were castrated under Ketamine Cocktail anesthesia (150 mg ketamine, 30 mg xylazine, 5 mg acepromazine in 3.5 ml sterile water; given at a dose of 0.9 cc/kg I.P.) and given either two empty subcutaneous silicon tubing implants (n=6; 2.5 cm long, 1.5 mm inner diameter, 2.4 mm outer diameter), two silicon tubing implants containing crystalline 4-androstene3,17dione (Sigma, St Louis, MO; n=7), or one silicon tubing implant containing crystalline testosterone (Sigma; n=6). One testosterone-filled implant of these dimensions yields serum testosterone levels that fall within the physiological range of 2.5 - 3.6 ng/ml for adult male rats (8). Two androstenedione-filled capsules of these dimensions yielded levels of $2.9 \pm 0.4$ ng/ml in this experiment (see below). Five weeks later, the rats were anesthetized, and blood samples were taken through cardiac puncture. Blood samples were allowed to clot overnight at $4^0$C and then centrifuged for 15 minutes at 2,500 RPM. Serum concentrations of androstenedione were determined using the ICN (Costa Mesa, CA) Androstenedione Tritium Kit.

Immediately after blood sample collection, the rats were perfused transcardially with physiological saline followed by 5% acrolein in 0.1M sodium phosphate buffer (pH 7.4) for ten minutes. The brains were removed and immersed in 30% sucrose in phosphate buffer (pH 7.4) overnight. Transverse, frozen sections (50 μm thick) were cut on a freezing microtome.

*Immunocytochemistry*

Every fourth section was processed for AVP

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immunocytochemistry at room temperature unless stated otherwise. After a 10-minute pretreatment with 0.01% sodium borohydride, the sections were rinsed in 0.05 M tris buffered saline (pH 7.6; TBS) and incubated in: a) TBS with 0.03% Triton X100 (Tris-triton) and 20% normal goat serum for 20 min; b) rabbit anti-AVP serum (ICN Laboratories, Costa Mesa, CA), 1:4,000 in Tris-triton with 2% normal goat serum (Tritigo) for 90 min at 37°C; c) Tritigo three times, for five min each at 37°C; d) biotinylated goat anti-rabbit serum (Vector Laboratories, Burlington, CA) 1:200 in Tritigo for 60 min; e) Tritigo three times, for five min each; f) TBS, five min; g) ABC complex (Vector *Elite* Kit, Vector Laboratories) in TBS for 60 min; h) TBS three times, for five min each; i) 0.05% 3-3'-diaminobenzidine, 0.04% ammonium chloride, 1.5% nickel ammonium sulfate, 0.02% glucose oxidase (Sigma; 46,000 I.U./g solid) and 0.15% B -D-glucose in TBS for 35 min. The sections were rinsed four times in TBS, mounted, air-dried, and cover-slipped. Preadsorption of the AVP antiserum with 50 μM AVP or omission of the AVP antiserum from the procedure eliminated all immunoreactivity.

*Analysis*

AVP-immunoreactive (-ir) cell bodies were counted under bright-field illumination at 40X magnification in three consecutive sections at the level of Plate 21 and Plate 27 of the rat brain atlas of Paxinos and Watson (13) for the BST and CMA, respectively. In order to determine that cell size did not vary by group, randomly selected AVP-ir cells from all groups were measured and compared. Cell size was determined by measuring the area covered by the cell in a digitized image captured under 100X magnification. AVP-ir fiber density in the lateral septum (LS) was estimated bilaterally under bright-field illumination at 40X magnification in the section at the level of Plate 19 of Paxinos and Watson (13). Fiber density was analyzed by computerized gray-level thresholding using the NIH Image program developed by Dr. Rasbaud at NIH. Light intensity and camera settings were kept constant across the sections. The density was expressed as the number of pixels covered by AVP-ir fibers in a 30,000 $\mu m^2$ sampling area bordering the ventricular wall. Differences between groups were tested with a one-way ANOVA.

## Results and Discussion

Castration without hormonal treatment dramatically diminished AVP-ir fiber density in the LS (ANOVA, $p<0.001$; $F(2,16)= 26.41$; Figures 1 and 2). Hormonal treatment did not affect AVP-ir cell size in the BST, and CMA. However, castration virtually eliminated

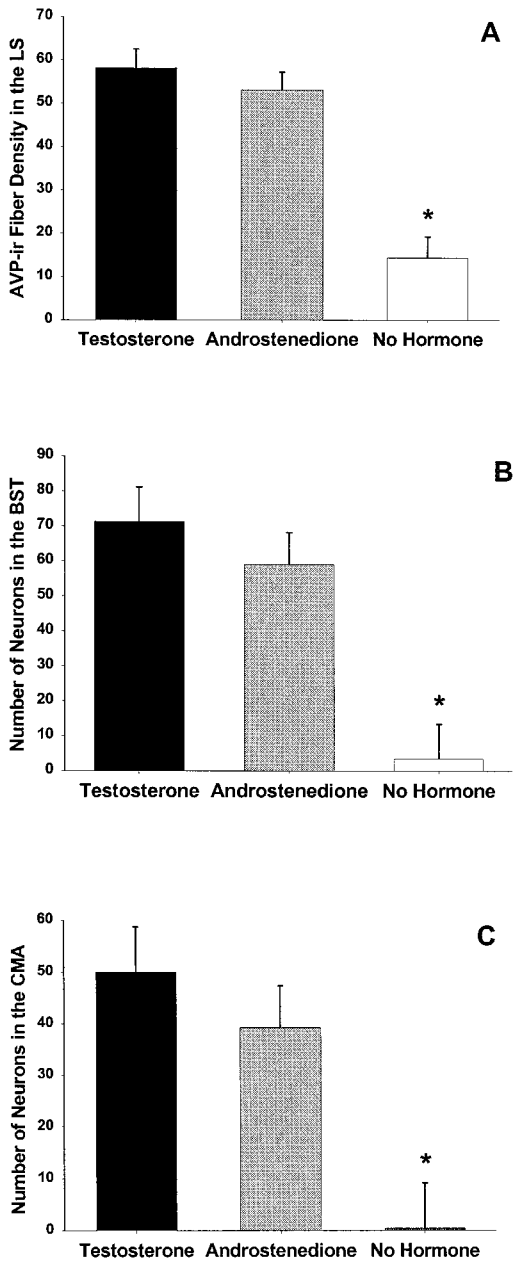


Figure 1. Density of AVP-ir fibers in the LS (A), and number of AVP-ir cells in the BST (B) and CMA (C) of rats treated with testosterone, androstenedione or no hormone.

AVP-ir cells in the BST (ANOVA $p<0.001$; $F(2, 16)= 13.75$; Figures 1 and 2) and the CMA (ANOVA, $p<0.001$; $F(2,16) = 8.98$; Figure 1). Androstenedione treatment was as effective as testosterone treatment in preventing the effects of castration on this system. Hormonal treatment did not notably affect AVP-ir structures in other brain areas such as the paraventricular nucleus of the hypothalamus or the suprachiasmatic nucleus.

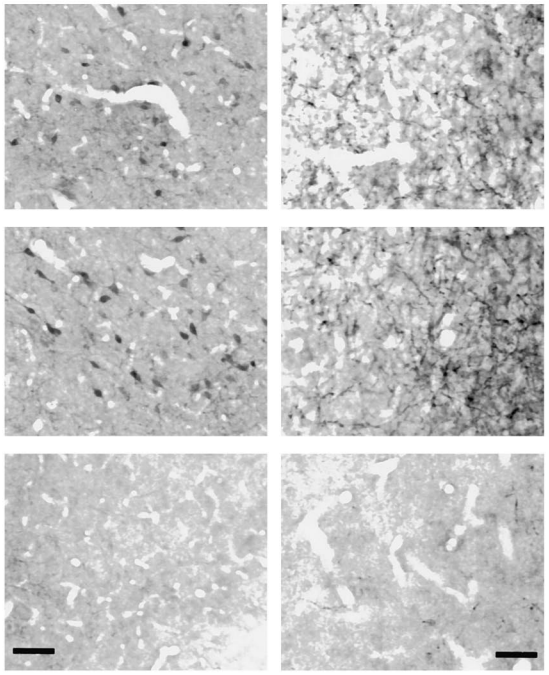

Figure 2. AVP-ir cells in the BST (left; bar 100 μm) and AVP-ir fibers in the lateral septum (right; bar = 50μm) of rats treated with testosterone (top), androstenedione (middle) or no hormone (bottom).

Animals that received testosterone, androstenedione, or no hormonal treatment had serum androstenedione concentrations averaging 0.05 ± 0.43 ng/ml, 2.9 ± 0.4 ng/ml, and 0.02 ± 0.43 ng/ml, respectively. The circulating level of androstenedione in androstenedione-treated rats fell just above the physiological range of androstenedione for healthy women (0.8-2.7 ng/ml; 14) and well within the physiological range of androstenedione for female spotted hyenas (15, 16). This level of androstenedione is, however, pharmacological for rats, which normally have circulating androstenedione levels of about 0.9 ng/ml (17). Because there are species differences in the levels and sensitivities to steroid hormones (18), it is unclear whether androstenedione would be as effective in stimulating vasopressin expression in animals with naturally high levels of androstenedione.

The analogous effects of androstenedione and testosterone treatment on levels of AVP immunoreactivity in the LS, BST, and CMA suggest that androstenedione, which is traditionally referred to as a weak androgen because of its inability to maintain normal genital function (2), actually has potent androgenic effects in the brain.

The mechanism by which androstenedione affects limbic AVP expression is unclear. Androstenedione treatment increases testosterone and estradiol serum levels (19, 20). Furthermore, androstenedione can be converted to other steroids such as, estrone and dihydrotestosterone (DHT) in vitro in rat brain tissue (21, 22). Therefore, like testosterone it may affect AVP expression by activating androgen and estrogen receptors, after it has been metabolized. The testosterone metabolite estradiol partly restores AVP peptide and messenger RNA levels in cells and projections of the BST and CMA of castrated male rats (7,8,10), while a combination of the testosterone metabolite DHT and estradiol fully restores AVP peptide and messenger RNA levels (7,8,10). Whether androstenedione's effects on AVP expression depend on conversion to active metabolites could be elucidated, for example, by using metabolizing enzyme blockers (*e.g.*, aromatase inhibitors), and specific estrogen or androgen receptor antagonists.

The central effects of androstenedione demonstrated in the present study may have functional importance in animal and clinical models in which androstenedione levels are endogenously or pharmacologically elevated. Androstenedione treatment may stimulate aggression (6), for example, through its actions on the AVP system. Application of AVP in projection areas of the BST and CMA prevent the reduction of aggression normally seen with castration (11, 12). Similarly, the high levels of aggression exhibited by female spotted hyenas (15, 16) may result from stimulation of AVP expression through endogenously produced androstenedione. Furthermore, the ability of androstenedione to influence neural systems suggests that humans that have high levels of androstenedione because of clinical conditions such as congenital adrenal hyperplasia (23) or polycystic ovarian syndrome (24), or because of androstenedione self-administration, may undergo changes in neural function.

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