

# Androgens Alter Corticotropin Releasing Hormone and Arginine Vasopressin mRNA Within Forebrain Sites Known to Regulate Activity in the Hypothalamic-Pituitary-Adrenal Axis

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Key words: corticotropin releasing hormone (CRH), arginine vasopressin (AVP), paraventricular nucleus, bed nuclei of the stria terminalis (BNST), amygdala, testosterone.

## Abstract

To reveal direct effects of androgens, independent of glucocorticoids, we studied the effects of gonadectomy (GDX) in adrenalectomized (ADX) rats with or without androgen replacement on corticotropin releasing hormone (CRH) and arginine vasopressin (AVP) mRNA expression within various forebrain sites known to regulate the hypothalamic-pituitary-adrenal axis. These included the medial parvocellular portion of the paraventricular nucleus of the hypothalamus (mp PVN), the central and medial nuclei of the amygdala and bed nuclei of the stria terminalis (BNST). In the mp PVN, ADX stimulated both CRH and AVP mRNA expression. Combined ADX + GDX inhibited only AVP, and testosterone and dihydrotestosterone (DHT) restored AVP mRNA. In the central nucleus of the amygdala, ADX decreased CRH mRNA expression, and this response was unaffected by GDX ± testosterone or DHT replacement. In the medial amygdala, AVP mRNA expression was decreased by ADX, abolished by ADX + GDX, and restored by androgen replacement. ADX had no effect on CRH and AVP mRNA expression in the BNST. GDX + ADX, however, reduced CRH mRNA expression only within the fusiform nuclei of the BNST and reduced the number of AVP-expressing neurones in the posterior BNST. Androgen replacement reversed both responses. In summary, in ADX rats, AVP, but not CRH mRNA expression in the amygdala and mp PVN, is sensitive to GDX ± androgen replacement. Both CRH- and AVP-expressing neurones in the BNST respond to GDX and androgen replacement, but not to ADX alone. Because androgen receptors are not expressed by hypophysiotropic PVN neurones, we conclude that glucocorticoid-independent, androgenic influences on medial parvocellular AVP mRNA expression are mediated upstream from the PVN, and may involve AVP-related pathways in the medial amygdala, relayed to and through CRH- and AVP-expressing neurones of the BNST.

Hypothalamic-pituitary-adrenal (HPA) responses to homeostatic insults are initiated by stimulatory inputs conveying stress-related information to medial parvocellular (mp) neurosecretory neurones of the paraventricular nucleus of the hypothalamus (PVN). During stress, mp neurones release several peptides from their terminal stores into the portal capillary zone of the median eminence and these subsequently stimulate the release of adrenocorticotropin (ACTH) from anterior pituitary corticotropes (1). Foremost among these peptides are corticotropin releasing hormone (CRH) and arginine vasopressin (AVP), which potentiates the stimulatory effects of CRH on ACTH secretion. While some

CRH-producing neurones coexpress AVP, the amount of AVP synthesized under basal conditions is normally low, and is dependent on the state of the organism (2). AVP synthesis is upregulated in CRH-expressing neurones following glucocorticoid removal by adrenalectomy and in response to chronic stress. Thus, increased AVP synthesis and release are thought to sustain ACTH release, which is otherwise inhibited by the negative feedback actions of elevated glucocorticoid levels.

Gonadal influences on basal and stress function in the HPA axis appear to be particular to AVP-expressing parvocellular neurosecretory neurones in the PVN. Thus, the dose-related inhibitory effect of testosterone on the magnitude of ACTH

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responses to restraint stress is strongly correlated with resting-state AVP content, but not CRH content in the median eminence (3). In models of social stress, subordinates secrete lower levels of testosterone compared to dominant males, and show elevations in AVP-, but not CRH-immunoreactivity in the external (hypophysiotropic) zone of the median eminence (4). Confronted with a dominant male, subordinates hypersecrete ACTH and show a selective depletion of AVP (versus CRH) from the median eminence (5). Gonadectomy reduces the stimulatory effects of adrenalectomy on AVP, but not CRH mRNA expression within mp PVN neurones (6). Gonadectomy also blocks the stimulatory effects of ADX on ACTH secretion (AVP-dependent), but not pituitary ACTH synthesis (CRH-dependent).

Androgen receptors are not expressed by mp neurosecretory neurones (7), suggesting that the actions of testosterone on mp AVP expression are indirect, and mediated by neurones that are upstream from the PVN. We have provided evidence identifying the medial preoptic area as an important site of the inhibitory effects of testosterone on hypophysiotropic AVP and stress-induced ACTH release (3). Regulation of HPA function by testosterone probably occurs at multiple target sites, given the distribution of androgen receptors within several hypothalamic and extrahypothalamic cell groups known to regulate ACTH release (7, 8). Androgen receptors are expressed by anatomically and functionally connected cell groups of the bed nuclei of the stria terminalis (BNST) and the medial and central nuclei of the amygdala, all of which send multimodal information to the PVN, express CRH and AVP, and regulate basal and stress-induced HPA function (9, 10).

Building on our previous findings with respect to the glucocorticoid-independent effects of testosterone on AVP mRNA expression in the mp PVN (6), we aimed to reveal additional sites in the brain where testosterone could act to regulate the HPA axis. CRH and AVP are strongly expressed in PVN-projecting neurones of the BNST and amygdala. Thus, we examined the manner in which CRH- and AVP-expressing neurones in the BNST, amygdala and PVN respond to gonadectomy and androgen replacement in ADX rats.

## Materials and methods

### Animals

Adult male Sprague-Dawley rats (Bantin-Kingman, CA, USA) weighing  $256.5 \pm 2.6$  g at the time of surgery (see Treatment and Table 1) were used in all experiments. The rats were group housed (three per cage) under controlled temperature and lighting conditions (12:12 h light:dark cycle; lights on at 06.30 h), with food and water (0.9% NaCl) available *ad libitum*. Seven days after surgery, rats were removed from their home cages and immediately anaesthetized for perfusion using 35% chloral hydrate (500 mg/kg, i.p.). This was performed during the light phase of the cycle between 10.00 h and 12.00 h. All experimental protocols were approved by the UCSF Committee on Animal Research.

### Treatment

To reveal direct effects of androgens on CRH and AVP mRNA expression in the brain, independently of glucocorticoids, male rats were divided into six groups (three rats per group) containing Sham endocrinectomy (control, ctl); gonadal-intact, adrenalectomized rats (ADX); and gonadectomized + adrenalectomized (GDX-ADX) rats. The latter group was subdivided into four groups: no androgen replacement (+0); rats with low (+t) or high (+T) testosterone, or replaced with dihydrotestosterone (+DHT), the nonaromatizable form of testosterone.

Bilateral gonadectomy, gonadectomy + adrenalectomy, and Sham surgeries were performed using a rodent anaesthetic cocktail of ketamine, xylazine, and acepromazine (77:1.5:1.5 mg/ml, respectively, 1 ml/kg, i.p.). Testosterone replacement was performed using either 1 (+t) or 2 (+T) subcutaneous SILASTIC capsules (2.5 cm length; 0.062 i.d., 0.125 o.d.) filled with crystalline testosterone designed to provide low and high plasma concentrations of testosterone, respectively. The attained concentrations of testosterone were within the physiological range seen in ADX and gonadal-adrenal intact rats, respectively (3, 6) (Fig. 1). DHT-replaced rats received two SILASTIC capsules (2.5 cm length; 0.062 i.d., 0.125 o.d.) of crystalline steroid. Rats with Sham endocrinectomy surgeries, in which the glands were not removed, were implanted with subcutaneous cholesterol-filled capsules.

Seven days after surgery, the rats were removed from their home cages and immediately anaesthetized for perfusion. As verified by corneal and tail pinch

TABLE 1. Treatment and Body Weight (BW).

Treatment	BWi (g)	BWf (g)	Delta BW (% BWi)
CTL	245.6 ± 6.5	273.6 ± 7.6	11.4 ± 2.6**
ADX	243.3 ± 9.4	261.3 ± 9.2	7.4 ± 3.9**
GDX + ADX + 0	271.3 ± 0.8	263.3 ± 9.2	-2.9 ± 5.7
GDX + ADX + t	264.3 ± 4.1	249.0 ± 3.6	-5.8 ± 2.7
GDX + ADX + T	260.3 ± 2.3	241.7 ± 11.7	-5.5 ± 9.9
GDX + ADX + DHT	254.0 ± 1.0	234.3 ± 5.8	-6.4 ± 1.5

BWi, day 1 body weight; BWf, day 7 body weight. \*\* $P < 0.01$  versus gonadectomized (GDX) ± androgen-replaced, adrenalectomized (ADX) rats. t, low testosterone; T, high testosterone.

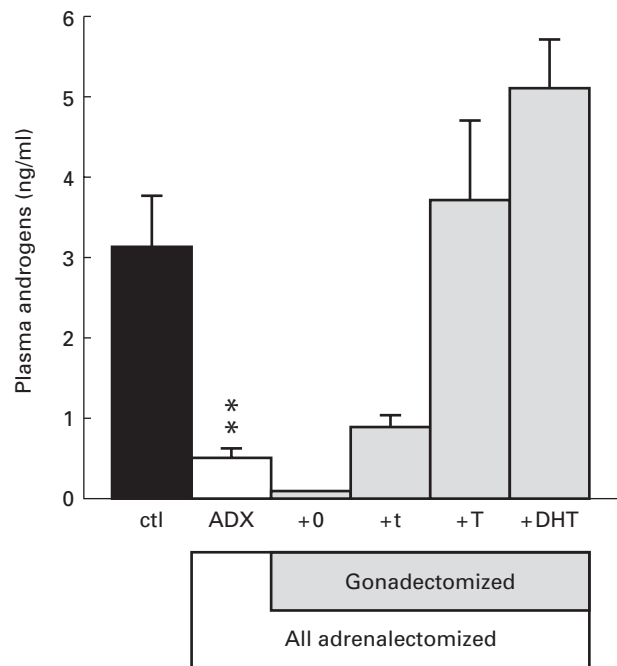


FIG. 1. Plasma testosterone response to adrenalectomized (ADX) (mean ± SEM), and androgen replacement strategy in gonadectomized (GDX) + ADX rats. Note the significant drop in the plasma concentration of testosterone in ADX rats. This prompted us to examine the effects of low versus high testosterone replacement (GDX + t versus GDX + T, respectively), in addition to the nonaromatizable dihydrotestosterone (DHT);  $n = 3$  per group. \*\* $P < 0.01$  versus control (ctl) (i.e. Sham GDX-ADX). For the DHT replacement group, plasma androgen levels represent the concentration of plasma DHT.

reflexes, deep anaesthesia was reliably achieved within 45 s of chloral hydrate administration. At this time, blood samples obtained from the right atrium of the heart were collected into ice-chilled EDTA-treated tubes, centrifuged at 3000 g for 10 min, and stored at  $-20^{\circ}\text{C}$  until assayed for corticosterone to validate adrenalectomy, androgen replacement levels and plasma ACTH concentrations. Rats were perfused via the ascending aorta with 0.9% saline and then 4% paraformaldehyde and 0.1 M borate buffer (pH 9.5). Brains were then postfixed for 5 h, and cryoprotected overnight with 10% sucrose in 0.1 M phosphate buffer. Six series of coronal sections (20  $\mu\text{m}$ ) through the entire length of the hypothalamus (0.5 to  $-4.0$  mm Bregma) were collected and stored at  $-20^{\circ}\text{C}$  in cryoprotectant (30% ethylene glycol and 20% glycerol in 0.5 M phosphate buffer) until hybridization processing.

#### Radioimmunoassays

Plasma testosterone concentrations were measured using the RIA kit of ICN Biomedicals (Costa Mesa, CA, USA) with [ $^{125}\text{I}$ ]testosterone as tracer. The antibody (liquid phase) cross-reacts 100% with testosterone, slightly with 5 $\alpha$ -DHT (3.40%), 5 $\alpha$ -androstane-3 $\beta$ , 17 $\beta$ -diol (2.2%) and 11-oxotestosterone (2%), but does not cross-react with progesterone, oestrogen or the glucocorticoids (all  $<0.01\%$ ). The detection limit of the assay was 0.1 ng/ml.

Plasma dihydrotestosterone (DHT; 17 $\beta$ -Hydroxy-5 $\alpha$ -androstane-3 $\beta$ -1) was extracted with n-hexane and measured using the RIA kit of Diagnostic Systems Laboratories (Webster, TX, USA) with [ $^{125}\text{I}$ ]DHT as tracer and anti-DHT-coated tubes. The antibody cross-reacts 100% with DHT, 1.9% with androstenedione, 1.4% with oestradiol, 0.25% with androstanediol, 0.02% with testosterone, but does not cross-react with progesterone, or the glucocorticoids (all  $<0.01\%$ ). The detection limit of the assay was 25 pg/ml.

Plasma corticosterone concentrations were measured using the RIA kit of ICN Biomedicals with [ $^{125}\text{I}$ ]B as tracer. The antibody cross-reacts 100% with corticosterone, slightly with desoxycorticosterone (0.34%), testosterone and cortisol (0.10%), but does not cross-react with the progestins or oestrogens ( $<0.01\%$ ). The detection limit of the assay was 0.2  $\mu\text{g}/\text{dl}$ .

Plasma ACTH concentrations were determined by RIA as previously described (11, 12). Briefly, plasma (intact = 50  $\mu\text{l}$ ; ADX = 12.5  $\mu\text{l}$ ) was first incubated overnight at  $4^{\circ}\text{C}$  with a specific ACTH antiserum (Dr W. C. England, University of Minnesota, Minneapolis, MN, USA) at a final dilution of 1:120 000. The ACTH antibody cross-reacts 100% with ACTH1-39, ACTH 1-18 and ACTH 1-24, but not with ACTH1-16,  $\beta$ -endorphin,  $\alpha$ - and  $\beta$ -melanocyte-stimulating hormone, or  $\alpha$ - and  $\beta$ -lipotropin (all  $<1\%$ ). After an additional 24 h incubation with [ $^{125}\text{I}$ ]ACTH trace (5000 c.p.m./tube; Instar, Stillwater, MN, USA), precipitation serum (Peninsula Laboratories, Belmont, CA, USA) was added, and bound peptide was obtained by centrifugation at 5000 g for 45 min. The detection limit of the assay was 10 pg/ml.

#### In situ hybridization

Hybridization histochemical localization was performed using a  $^{33}\text{P}$ -labelled antisense cRNA probe transcribed from a full length (1.2 kb) cDNA encoding CRH mRNA (Dr K Mayo, North-western University, Evanston, IL, USA), and a  $^{33}\text{P}$ -labelled antisense cRNA probe transcribed from a 230-bp cDNA fragment encoding the vasopressin-specific 3' end (exon C) of AVP (Dr D. Richter, University of Hamburg, Germany). Techniques for riboprobe synthesis, hybridization and autoradiographic localization of mRNA signal are described in greater detail elsewhere (13, 14). Briefly, free-floating sections were first rinsed in 0.1 M phosphate buffer (pH 7.4) to remove cryoprotectant, then mounted and vacuum dried on glass slides overnight. After postfixation with 10% formaldehyde for 30 min at room temperature, sections were digested in proteinase K (10 mg/ml,  $37^{\circ}\text{C}$ ) for 30 min, acetylated for 10 min (2.5 mM acetic anhydride, 0.1 M triethanolamine, pH 8.0), rapidly dehydrated in ascending ethanol concentrations (50–100%), and then vacuum dried. Radionucleotide cRNA probes were used at concentrations approximating  $10^7$  c.p.m./ml in a solution of 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 0.05% tRNA, and 10 mM dithiothreitol, 1  $\times$  Denhardt's solution, and 10% dextran sulphate, and applied to individual slides containing sections through the rostro-caudal extent of the BNST, PVN and amygdala, with sampling intervals of 120  $\mu\text{m}$ . Slides were coverslipped, and then incubated overnight at  $55^{\circ}\text{C}$ , after which the coverslips were removed, and the sections washed three times in  $2 \times \text{SSC}$  (SSC; 0.15 M NaCl, 15 mM citric acid, pH 7.0) at room temperature, treated with ribonuclease A (20  $\mu\text{g}/\text{ml}$ ) for 30 min at  $37^{\circ}\text{C}$ , desalted in descending SSC concentrations ( $2-0.1 \times \text{SSC}$ ), washed in  $0.1 \times \text{SSC}$  for 30 min at  $60^{\circ}\text{C}$ , and dehydrated in ascending ethanol concentrations. Hybridized sections were then exposed to X-ray film ( $\beta$ -max,

Amersham, Arlington Heights, IL, USA), defatted in xylenes, and subsequently coated with Kodak NTB2 liquid autoradiographic emulsion, and exposed at  $4^{\circ}\text{C}$  in the dark with desiccant, the duration determined by the strength of signal on X-ray film. Slides were developed with Kodak D-19 for 3.5 min at  $14^{\circ}\text{C}$ , briefly rinsed in distilled water ( $14^{\circ}\text{C}$ ) for 15 s, fixed in Kodak fixer for 6.5 min at  $14^{\circ}\text{C}$ , and then washed in running water for 45 min at room temperature. Adjacent series of sections were stained for reference purposes.

Semiquantitative densitometric analysis of the relative levels of CRH and AVP mRNAs in the PVN, and CRH mRNA in the central nucleus of the amygdala (CeA) and in the anterior division of the BNST was performed using Macintosh-driven NIH Image software (Rasband, v 1.61; <http://rsb.info.nih.gov/nih-image/>). This was assisted by redirected sampling of dark-field autoradiographic images aligned to corresponding Nissl stained sections. Optical density readings through any given cell group, corrected for background, were taken at regularly spaced (120  $\mu\text{m}$ ) intervals. Mean group values were determined from averaged readings of multiple sections from individual rats. The number of slices analysed for each rat varied depending on the region and distribution of the transcript (3, 3, and 4 sections per rat for the PVN, CeA and BNST, respectively). In all cases, alternate and adjacent series of sections, spaced 20  $\mu\text{m}$  apart, were used for CRH and AVP hybridizations.

Given the widely dispersed distribution of AVP-expressing neurones in the medial amygdala (MeA) and posterior BNST, analysis of AVP mRNA expression in these cell groups required an individual cell and grain-counting procedure, as previously described (15, 16). Briefly, individual cell and grain counts were obtained by first sampling the optical density of identified clusters of hybridized cells using a 33- $\mu\text{m}$  diameter circular field. Only those individual clusters with an optical density  $\geq 5 \times$  background were deemed to be positive and counted. Grains per cell were generated from a standard curve which best fit the relationship between the optical density of emulsion-dipped C-14 standards and the amount of silver grains per unit area using a 33- $\mu\text{m}$  diameter circular field. This revealed a linear relationship where optical density (OD) =  $[1.77 \times \text{grain number} + 0.198]$ ;  $r^2 = 0.86$ . The total numbers of AVP-expressing neurones were counted bilaterally throughout the extent of the posterior BNST and medial amygdala (four and seven sequential sections, respectively).

#### Statistical analysis

The data were analysed by one-way analyses of variance (ANOVA). Post-hoc analysis was performed, when appropriate, using Newman-Keuls for multiple pairwise comparisons.

## Results

### Body weight responses to ADX and androgen replacement

In rats with testes, no significant effect of ADX on body weight was seen 1 week after surgery, although weight gain tended to be lower in ADX rats compared to controls (Table 1). In contrast, GDX + ADX caused a loss in body weight. This decrease in body weight in GDX + ADX rats was not reversible with any form of androgen replacement, including low testosterone replacement (Fig. 1). Lower replacement doses may be required to elicit positive effects of testosterone on body weight gain, particularly in ADX rats. While reduced weight gain and hypophagia produced by elevated testosterone doses have been explained by its aromatization to oestrogen (17), this may be applicable only to rats with adrenals. Thus, comparable reductions in body weight occurred in GDX + ADX rats replaced with nonaromatizable DHT.

### Hormone responses to ADX and androgen replacement

Plasma corticosterone concentrations in Sham endocrinectomized controls (ctl) were  $4.8 \pm 1$   $\mu\text{g}/\text{dl}$ , and below detection ( $<0.2$   $\mu\text{g}/\text{dl}$ ) in all ADX rats. Note that ADX caused a

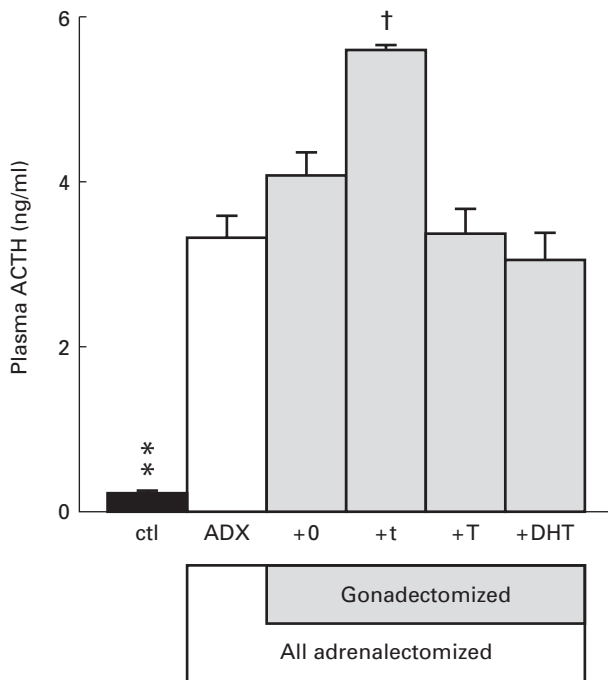


FIG. 2. Plasma adrenocorticotropin (ACTH) levels (mean  $\pm$  SEM) vary as a function of androgen replacement in adrenalectomized (ADX) rats. \*\* $P < 0.01$ ; † $P < 0.05$  versus all others ( $n = 3$  per group).

significant decrease in plasma concentrations of testosterone:  $ctl = 3.1 \pm 0.7$ ;  $adx = 0.5 \pm 0.1$  ng/ml (Fig. 1). Therefore, GDX + ADX rats were replaced with two doses of testosterone, providing plasma testosterone concentrations comparable to those of intact controls and ADX rats: testosterone replacement =  $3.6 \pm 1.0$  and  $0.9 \pm 0.1$  ng/ml in + high testosterone and + low testosterone rats, respectively.

The loss of glucocorticoid negative feedback on ACTH release was reflected in all ADX rats. Thus, relative to intact controls, all ADX rats hypersecreted ACTH ( $P < 0.0001$ ). Amongst androgen-replaced, GDX + ADX rats, ACTH responses to ADX varied as a function of androgen replacement (Fig. 2). Thus, compared to ADX and GDX + ADX rats, GDX + ADX rats with low testosterone replacement showed significantly higher ACTH concentrations. This was reversed with either high testosterone or DHT replacement.

*CRH and AVP mRNA in the PVN*

In rats with intact gonads, ADX stimulated both CRH and AVP mRNA expression within the PVN. This stimulatory effect of ADX was restricted to the mp PVN (Fig. 3). As revealed by quantitative densitometric analysis, CRH mRNA expression in the mp PVN was uniformly higher in all ADX groups compared to intact controls (Fig. 4, top). This CRH response was not affected by GDX with or without androgen

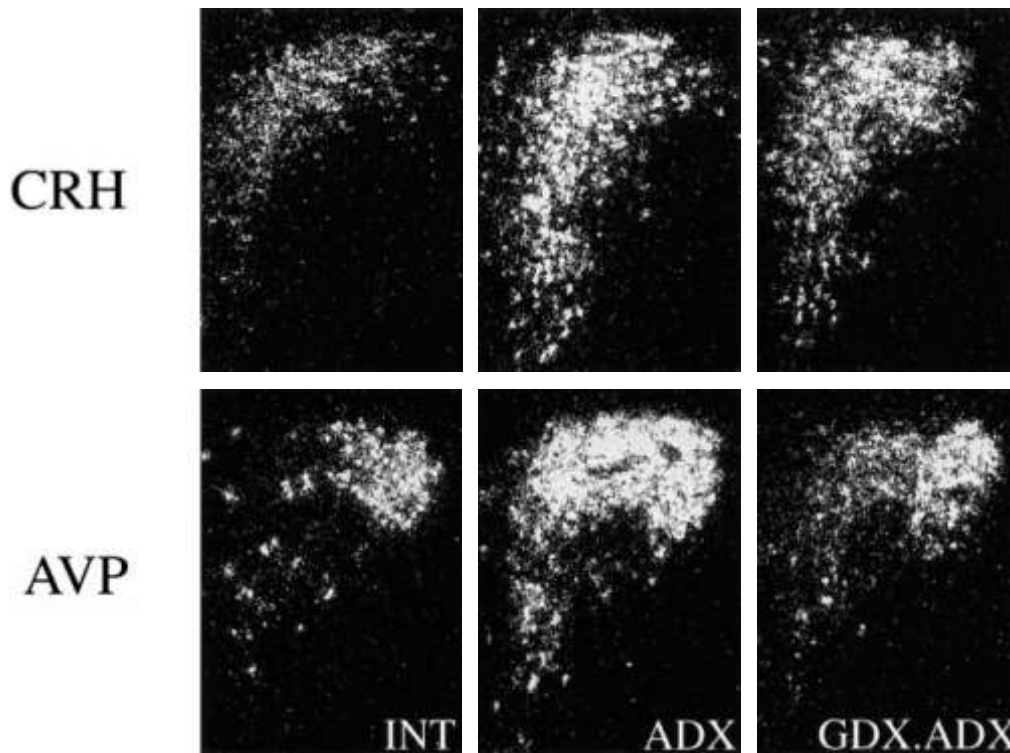


FIG. 3. Arginine vasopressin (AVP), but not corticotropin releasing hormone (CRH) mRNA responses to adrenalectomized (ADX) in the medial parvocellular paraventricular nucleus (PVN) are sensitive to gonadal status. Darkfield photomicrographs of CRH (top) and AVP (bottom) mRNA in the PVN in control, ADX, and gonadectomized (GDX) + ADX rats. Note the selective inhibitory effects of GDX + ADX on AVP mRNA, restricted to the medial parvocellular PVN. Photomicrographs, magnification  $\times 50$ .

replacement. By contrast, there was a clear androgenic influence on AVP mRNA expression in ADX rats (Fig. 4, bottom). AVP responses to ADX were significantly blunted in GDX rats, and this response was effectively restored with either low testosterone, or high testosterone replacement. DHT replacement in GDX+ADX rats reversed, but did not fully restore AVP mRNA expression to ADX levels: AVP mRNA levels in the mp PVN were significantly lower in DHT-replaced rats than in ADX rats with testes ( $P < 0.05$ ). Finally, no effects of ADX or GDX ± androgen replacement on AVP mRNA expression were seen within magnocellular neurons of the PVN (Fig. 3, bottom panels).

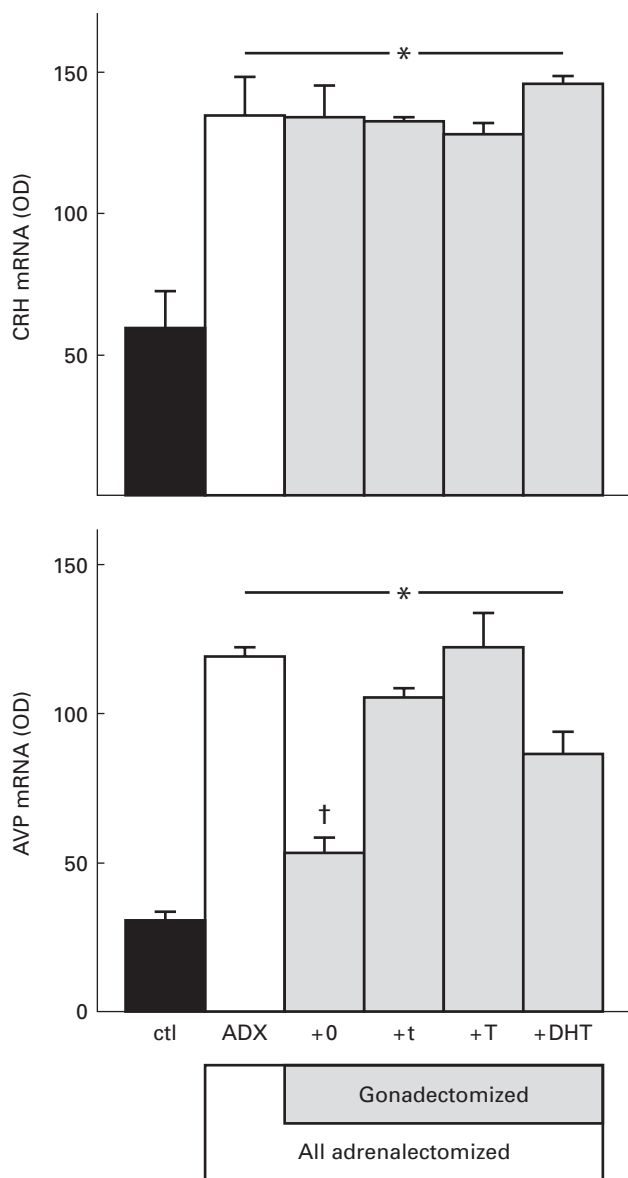


FIG. 4. Mean  $\pm$  SEM medial parvocellular corticotropin releasing hormone (CRH) (top) and arginine vasopressin (AVP) mRNA (bottom) responses to adrenalectomized (ADX) and gonadectomized (GDX) + ADX with or without androgen replacement (see replacement strategy in Fig. 1). \* $P < 0.05$  versus control (ctl); † $P < 0.05$  versus ADX ( $n = 3$  per group).

#### CRH and AVP mRNA in the amygdala

Unlike the CRH response in the mp PVN, ADX was associated with decreased CRH mRNA expression in the central nucleus (CeA) of the amygdala (Fig. 5, top). This inhibitory effect of ADX on CRH mRNA expression was not

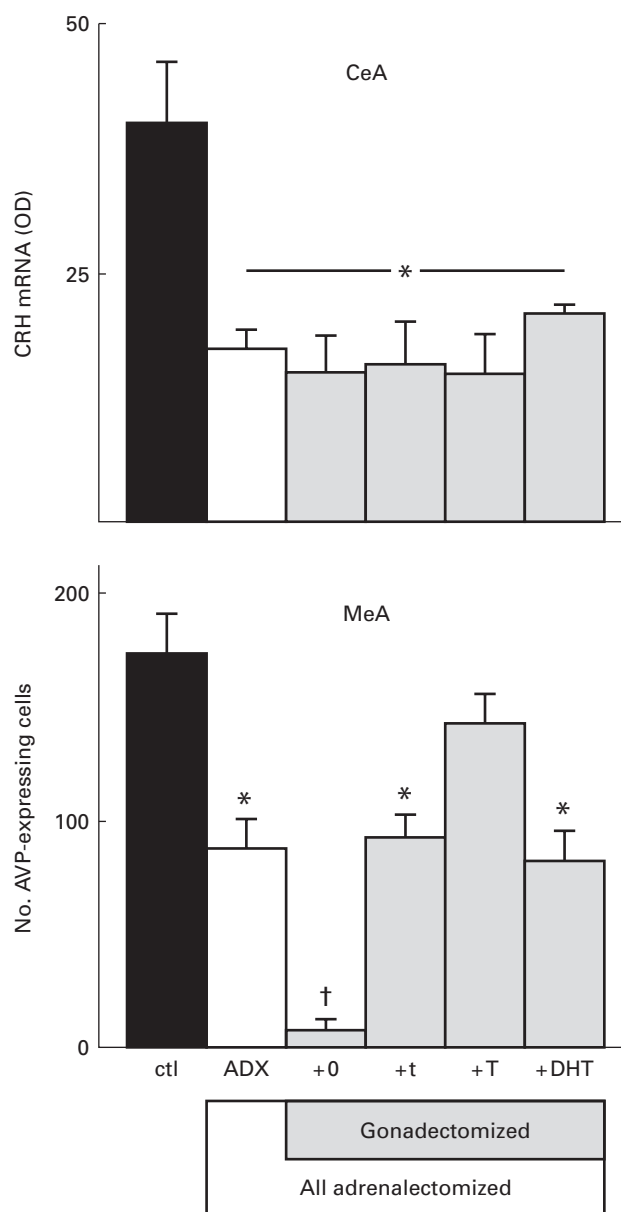


FIG. 5. Corticotropin releasing hormone (CRH) (top) and arginine vasopressin (AVP) (bottom) mRNA expression (mean  $\pm$  SEM) in central (CeA) and medial (MeA) nuclei of the amygdala in response to adrenalectomized (ADX) and gonadectomized (GDX) + ADX with or without androgen replacement. \* $P < 0.05$  versus control (ctl); † $P < 0.05$  versus ADX ( $n = 3$  per group). Because of the dispersed nature of AVP-expressing neurons in the MeA (unlike CRH in the CeA), AVP expression in this region was amenable to single-cell analysis (See Methods and Figs 6 and 9). In this case, a 30- $\mu$ m circular field was used to count individual AVP mRNA-expressing neurons, sampled over a 200-mm<sup>2</sup> surface area. Note that decrements in the number of AVP-expressing neurons in the MeA produced by ADX fail to occur in high testosterone-replaced rats.

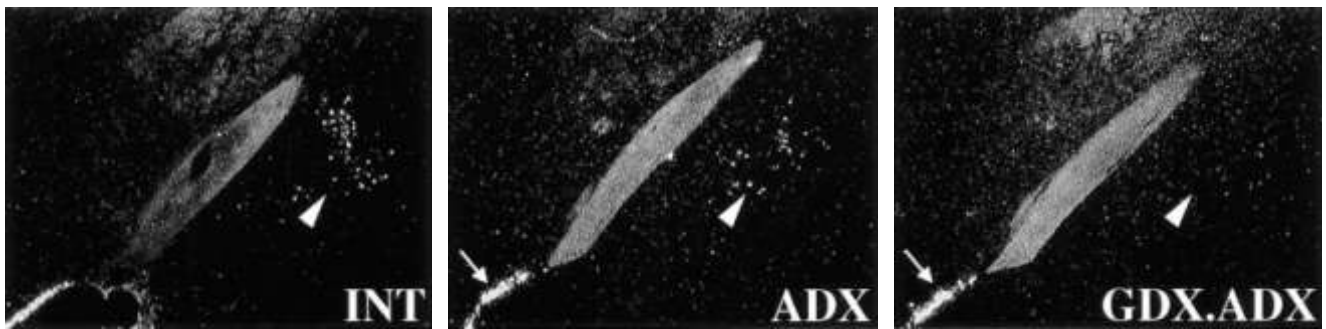


FIG. 6. Arginine vasopressin (AVP) expression in the medial amygdala. Darkfield photomicrographs showing that the number of AVP mRNA-expressing neurones in the medial amygdala is reduced by adrenalectomized (ADX), and abolished by gonadectomized (GDX)+ADX. Note that AVP expression within the retrochiasmatic part of supraoptic nucleus (SOR) is unaffected by ADX or GDX+ADX. Arrowheads and arrows identify cells expressing AVP mRNA in the medial amygdala and SOR, respectively. Photomicrographs, magnification  $\times 16$ .

influenced by GDX with or without androgen replacement. Androgenic influences on AVP mRNA expression in the medial nucleus (MeA) of the amygdala were clear (Fig. 5, bottom), and most apparent within the posterodorsal part of the MeA (Fig. 6). In rats with intact testes, the number of AVP mRNA-expressing neurones in the MeA was decreased in ADX, and nearly obliterated in GDX+ADX rats. Replacement with either low concentrations of testosterone or with DHT reversed this effect of GDX. High testosterone replacement restored the number of AVP mRNA-expressing neurones further to the range of intact-control rats ( $P > 0.05$ ). As shown in Fig. 6, despite the major effects of ADX and GDX+ADX on the number of AVP mRNA-expressing neurones in the MeA, expression levels of the transcript remained relatively unaffected within the AVP-rich, retrochiasmatic magnocellular neurones of the supraoptic nucleus.

#### CRH and AVP mRNA in the BNST

Androgenic effects on CRH mRNA expression within the BNST were studied at two rostro-caudal levels, within four cell groups (Fig. 7). At the most anterior extent of the BNST (0.0 mm Bregma), dense clusters of CRH mRNA-expressing neurones were observed within the anterolateral and anteroventral divisions of the BNST (Fig. 7, top). At, or just beyond the decussation of the anterior commissure ( $-0.4$  mm Bregma), CRH mRNA-expressing neurones were consistently found within the oval and fusiform BNST nuclei (Fig. 7, bottom) (18, 19). More posteriorly, scattered complements of CRH mRNA-expressing neurones were only occasionally detected, just ventral to the anterior commissure and medial to the anterodorsal preoptic nucleus. Due to the small sample size and large sampling interval ( $120 \mu\text{m}$ ), CRH mRNA expression within these posterior, scattered groups could not be reliably studied, and was not analysed.

Densitometric analysis revealed no significant effect of ADX on CRH mRNA expression within the BNST. CRH mRNA expression was reduced in GDX+ADX rats, but the reduction was significant only within the fusiform nucleus of the BNST (Fig. 8, top). Similar, but nonsignificant, trends were observed within the other BNST divisions ( $P = 0.06, 0.09$ , and  $0.1$ ; anterolateral, oval and anteroventral, respectively). Low testosterone, high testosterone, and DHT replacements

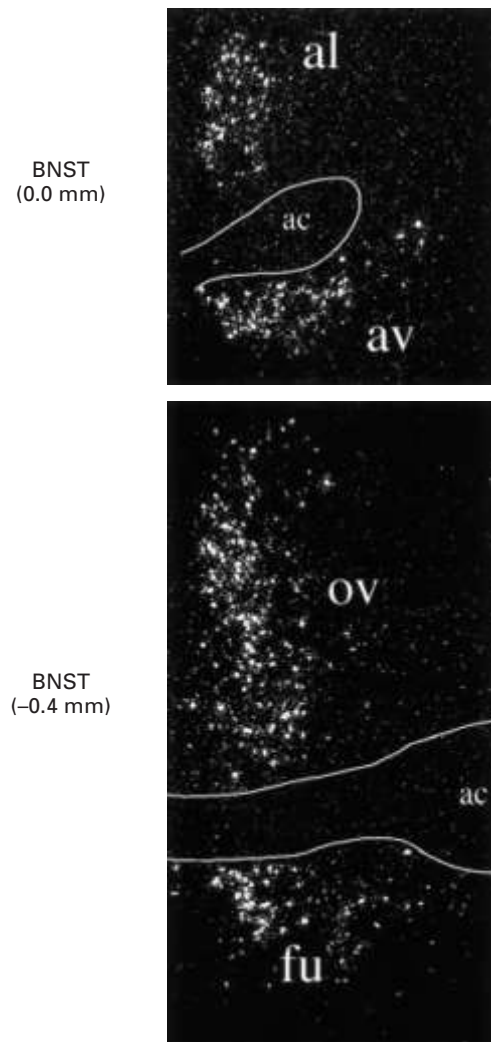


FIG. 7. Corticotropin releasing hormone (CRH) expression within bed nuclei of the stria terminalis (BNST). Taken from control rats, darkfield photomicrographs illustrating the distribution of CRH mRNA within the anterior division of the BNST: Top: anterolateral (al) and anteroventral (av) nuclei. Bottom: oval (ov) and fusiform (fu) nuclei. ac; anterior commissure. (18, 19, 53). Photomicrographs, magnification  $\times 25$ .

were equally effective in reversing the inhibitory effect of GDX+ADX on CRH mRNA expression in the fusiform nucleus.

Scattered complements of AVP-mRNA expressing cells were readily detected within the posterior extent of the BNST. While spanning approximately 480 μm of the posterior division BNST (four sections), a clear majority of these AVP-positive neurones was consistently found in the same transverse plane as the rostral portion of the suprachiasmatic nucleus (-0.8 to -1.0 mm Bregma) (16). In rats with intact gonads, ADX alone had no effect on AVP mRNA expression

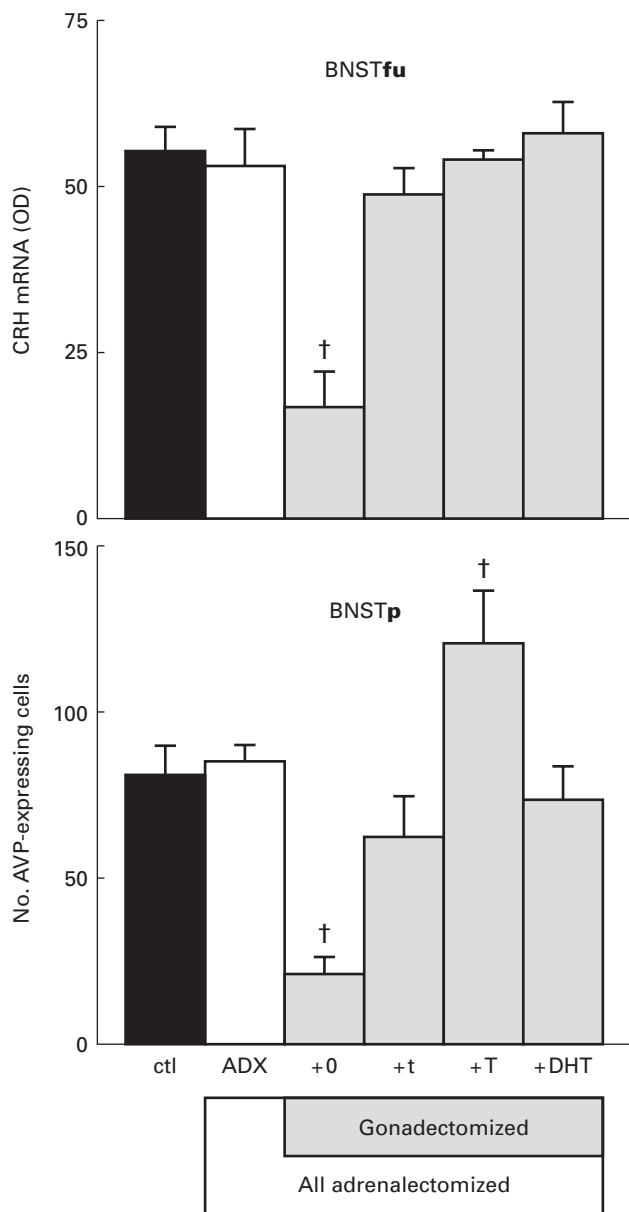


FIG. 8. Corticotropin releasing hormone (CRH) (top) and arginine vasopressin (AVP) (bottom) mRNA expression (mean ± SEM) within the anterior and posterior divisions of the bed nuclei of the stria terminalis (BNST), respectively, in response to adrenalectomized (ADX) and gonadectomized (GDX)+ADX with or without androgen replacement. †P < 0.05 versus all others (n = 3 per group).

(Fig. 8, bottom). GDX+ADX decreased the number of AVP mRNA-expressing neurones in the posterior BNST. Replacement with either low levels of testosterone (t) or with DHT reversed the effect of GDX. Higher testosterone replacement significantly increased the number of AVP-expressing neurones to levels surpassing those of control and ADX rats. Finally, suprachiasmatic neurones displayed no changes in AVP mRNA expression (data not shown).

Analysis of the numbers of grains per cell revealed similarities in the frequency distribution of grains in AVP mRNA-expressing cells amongst intact-controls, ADX, and high T-replaced ADX rats in the MeA and BNST (Fig. 9). Thus, independently of the way the numbers of AVP-expressing

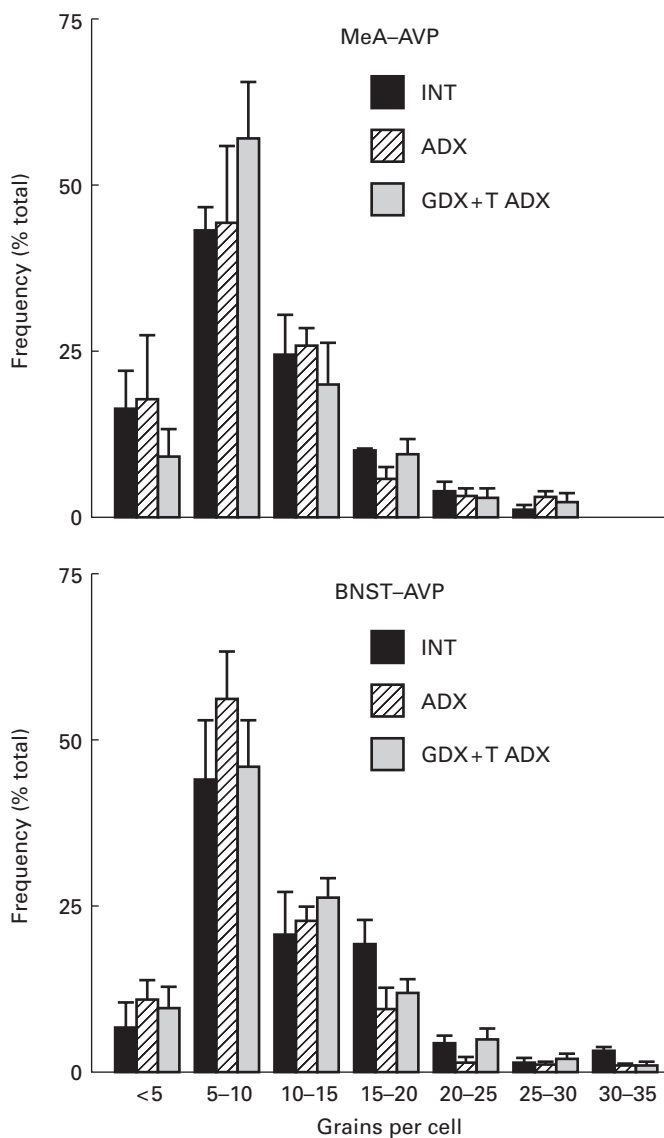


FIG. 9. Frequency distribution (mean ± SEM) of arginine vasopressin (AVP) expressing bed nuclei of the stria terminalis (MeA) (top) and medial (BNST) nuclei of the amygdala (bottom) as a function of grains per cell in intact, adrenalectomized (ADX), and gonadectomized (GDX) + high testosterone (T). ADX rats (n = 3 per group). Note that gonadal regulation of AVP expression is not reflected in grain density, but by the number of AVP mRNA-expressing neurones (Figs 5 and 8).

neurones change in response to ADX (decreased) and high testosterone replacement (increased), in the MeA and BNST, respectively, the amount of AVP mRNA expressed per cell remained stable. Taken together, these analyses suggest that androgen-dependent alterations in AVP mRNA expression in these cell populations are produced by changes in the number of neurones expressing the transcript, and not by the amount of transcript expressed in each cell.

## Discussion

We studied six groups of rats. Sham endocrinectomies, adrenalectomy, and gonadectomy+adrenalectomy with or without androgen replacement (Fig. 1). This design revealed glucocorticoid-independent effects of testosterone on AVP and CRH mRNA expression within the amygdala and bed nuclei of the stria terminalis. Given their functional and anatomical connectivity with the PVN, the results suggest a role for these cell groups in mediating the central effects of circulating testosterone on HPA function.

Consistent with our previous studies (3, 6), gonadal influences on peptide expression within the hypophysiotropic (ACTH-regulating) zone of the PVN were restricted to AVP (Figs 3 and 4). In rats with testes, CRH and AVP mRNA levels were significantly increased by ADX. However, GDX attenuated the stimulatory effect of ADX selectively on AVP mRNA expression within mp PVN neurones; CRH mRNA responses to ADX did not vary with gonadal status. Both testosterone replacement doses and nonaromatizable DHT reversed the inhibitory effect of GDX on AVP mRNA expression. This indicates that testosterone regulates mp AVP expression in PVN through androgen, not oestrogen receptors.

ADX resulted in decreased plasma testosterone concentrations in rats with testes (Fig. 1). This normal decrease may be important in mediating AVP transcriptional and secretory responses to ADX. This is suggested, in part, by the inhibitory effects of GDX on mp AVP mRNA, and the restorative and facilitatory effects of low testosterone replacement on AVP mRNA expression and the ACTH secretory response to ADX, respectively (Figs 2,4). A role for decreased testosterone in mediating ADX-induced HPA drive is further indicated by the relative reduction in the ACTH response in high versus low testosterone-replaced ADX+GDX rats. This could also reflect a dose-related inhibitory effect of high testosterone replacement on the AVP secretory response to ADX (3, 6). With respect to GDX+ADX without androgen replacement, we studied rats 1 week following surgery here, and deficits in AVP peptide secretion may take longer to emerge; at 2 weeks postsurgically GDX+ADX rats do not hypersecrete ACTH (6).

Glucocorticoid-independent effects of testosterone on HPA function cannot be explained by direct actions at the level of the anterior pituitary, or within mp PVN neurones. Androgen receptors are not expressed by pituitary corticotropes (20, 21), and are restricted to autonomic (non-neurosecretory) neurones in the PVN (7). Because of the high concentrations of androgen receptors in the amygdala and its role in regulating PVN function, both in response to stress and ADX, we measured CRH and AVP mRNA expression in two nuclei of

the amygdala. ADX significantly decreased CRH expression within the CeA (Fig. 5). This response to ADX was not altered by GDX±androgen replacement. Interestingly, however, in rats with adrenals, CRH mRNA levels in the CeA vary as a function of plasma testosterone, with a negative correlation (22). Taken together, these findings suggest that testosterone can operate on CRH expression in the CeA, but only in the presence of corticosterone. This interaction is consistent with the distribution of both glucocorticoid and androgen receptors in the lateral part of the CeA (8, 23), where CRH-expressing neurones are concentrated.

The number of AVP-expressing neurones in the MeA showed a graded response to increasing testosterone replacement in ADX groups (Fig. 5). Unlike the posterior BNST, discussed below, androgen taken up by cells in the MeA is not converted to oestrogen (24), indicating that, if direct, the dose-related effect of testosterone on AVP neurones is via androgen receptors. Discrepancies between DHT and high testosterone replacement in inducing numbers of AVP-expressing neurones in the MeA may reflect a relative impediment to brain uptake of DHT (25), since delivery of DHT centrally stimulates AVP expression far more effectively than peripheral implants (26). Interestingly, in rats with testes, ADX decreased the number of AVP-expressing neurones in the MeA (Figs 5 and 6). This effect of ADX may be secondary to the reduction in circulating testosterone concentrations, as over-replacement with high testosterone restored the number of AVP expressing neurones to, but not higher than controls. With respect to androgen-dependent influences on AVP mRNA expression in the MeA and BNST, these findings are in agreement with previous studies (16, 27). Wistar rats show neither changes in AVP mRNA in the MeA, nor decreased plasma testosterone concentrations following ADX (28). Thus, the extent to which MeA neurones respond to ADX is strain-dependent, possibly through sensitivity of the gonadal system of the strain to adrenal manipulation.

In the anterior BNST, ADX had no effect on CRH mRNA expression within any region examined, including the anterolateral and anteroventral areas, and the oval and fusiform nuclei (Fig. 7). CRH mRNA levels were significantly reduced by GDX+ADX only within the fusiform nucleus (Fig. 8). This effect was reversed to the same extent by all forms of androgen replacement, indicating that regulation of CRH expression by testosterone in the fusiform nucleus is mediated by androgen receptors. This is compatible with the demonstration of androgen, but not oestrogen receptor mRNA in the fusiform nucleus (8), and the sparsity of aromatase activity within the anterior division of the BNST (29, 30). Moreover, GDX in the absence of ADX can effectively decrease CRH mRNA levels within the fusiform BNST, and this is reversed by testosterone replacement (22). These findings indicate that gonadal influences on CRH expression in the BNST occur independently of glucocorticoids.

In GDX+ADX rats, both low testosterone and DHT replacement restored the number of AVP-expressing neurones in the posterior BNST to that in controls (Fig. 8), supporting an androgenic mode of action over the lower physiological range of circulating testosterone levels. However, in contrast to the MeA, high testosterone replacement increased the number of cells expressing AVP further, to values exceeding

those in intact controls. Because plasma testosterone concentration in the ADX, GDX + testosterone group was comparable to intact controls (Fig. 1), this suggests an interactive stimulatory effect of testosterone and ADX on the number of AVP-expressing cells in the BNST. This may be explained by aromatization of testosterone to estradiol. Oestrogen receptors, together with androgen receptors, are localized in AVP-expressing cells of the BNST (7, 31), and aromatase activity is relatively prominent in the posterior BNST (29, 30). Finally, the number of AVP-expressing neurones in the BNST was not decreased by ADX. Thus, AVP neurones in the BNST appear insensitive to the reduced testosterone levels associated with ADX.

The known anatomical connections between the amygdala, BNST and the PVN, taken together with our current results, form the basis for postulating a steroid-sensitive circuit mediating indirect effects of testosterone on mp AVP expression in the PVN. Direct projections from the medial and central cell groups of the amygdala to the hypophysiotropic (ACTH-regulating) PVN are few and limited to the periventricular and autonomic-related cell groups (32–35). Thus, amygdaloid regulation of HPA function is probably both indirect and multisynaptic. Functional and connective studies have shown that different portions of the BNST act as an obligatory relay between the amygdala and PVN (36–39). A large proportion of MeA afferents have been shown to contact neurones of the posterior BNST that, in turn, project directly to the PVN (34, 40). These contacts are formed within the medial aspect of the posterior BNST, where AVP-expressing neurones are concentrated. Although the phenotypic nature of this pathway remains to be defined, AVP released within the PVN inhibits ACTH secretion (41). Moreover, posterior BNST lesions tend to increase AVP expression within mp PVN neurones (42). These findings suggest an inhibitory role for the posterior BNST that may be conveyed by AVP-containing afferents to the PVN (43). The MeA is also a major recipient of input arising from the olfactory bulbs. Interestingly, lesions of the olfactory bulb increase synthesis of hypophysiotropic AVP, but not CRH in the PVN (44). The importance of scent marking in social recognition, androgen-dependent effects of social hierarchy on stress-reactivity (4, 45,46) and, as shown here, the sensitivity of AVP neurones in the MeA and posterior BNST to androgens, implicate these sites as important integrators of social behaviour and HPA function (47).

Central amygdaloid connections to the PVN are more sporadic than medial amygdaloid afferents, involving multiple relays within several interconnected subnuclei of the anterior BNST (48, 49). Disynaptic contacts between the lateral CeA and mp PVN have been observed, notably within the oval and fusiform nuclei of the BNST (34). A clear majority of PVN-projecting, fusiform neurones also express CRH (50, 51). CRH mRNA in the fusiform nucleus is increased in rats exposed to elevated corticosterone levels (52, 53). Moreover, CRH type 1 receptor expression in the parvocellular PVN is upregulated by CRH and restraint stress (54), and this response to restraint stress is enhanced by GDX (Viau and Sawchenko, unpublished observation). Taken together with our current findings, upstream androgenic influences on PVN

function may likely involve these CRH-expressing, PVN-projecting fusiform neurones.

The proposed routes by which androgens regulate HPA function represent a small fraction of larger circuits in the brain regulating stress reactivity. Several subnuclei of the BNST, including those examined here, receive and reciprocate input from a variety of forebrain, limbic, and brainstem nuclei supplying homeostatic input to the PVN (55–58). Some of these cell groups overlap with part of a large network processing gustatory and metabolic information, including extended circuitries of the CeA and the oval and fusiform nuclei of the BNST (59). Moreover, anterior, but not posterior, BNST lesions alter body weight (42). Thus, androgen-dependent alterations in fusiform CRH may play a pivotal role in mediating the inhibitory effects of androgen replacement on body weight gain in ADX rats (Table 1).

In models of social stress, dominant and subordinate males show reciprocal patterns of androgen and glucocorticoid release. The shared inhibitory characteristics of androgens and glucocorticoids on HPA function, suggesting an overlap in their signalling pathways, have made it difficult to define the central substrate mediating the effects of social hierarchy on stress responsiveness. We have recently provided a solution to the problem by manipulating both the adrenal and gonadal endocrine systems simultaneously, revealing glucocorticoid-independent effects of testosterone on HPA function that are mediated upstream from the PVN (6). Building on these findings, our current design provides a framework with which to begin constructing and segregating androgen- from glucocorticoid-sensitive pathways in the brain.

#### Acknowledgement

This work was supported by DK 28172.

Accepted 15 January 2001

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