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REGION-SPECIFIC MECHANISMS FOR TESTOSTERONE-INDUCED FOS IN HAMSTER BRAIN

Anita Nagypál¹ and Ruth I. Wood*

Neuroscience Program, Department of Cell and Neurobiology, Keck School of Medicine of the University of Southern California, Los Angeles, CA 90033

Abstract

Hamsters self-administer androgens. Previously, we determined that testosterone (T) activates select steroid- and opiate-sensitive brain regions. Is T-stimulated neuronal activation androgenic? 35 castrated males with physiologic T replacement (n=7/group) were pre-treated with the androgen antagonist flutamide (15 mg/kg sc) or ethanol (0.25 ml), and infused into the lateral ventricle (ICV) for 4h with 40 µg T (TF and TE, respectively) or 40 µl vehicle (VF and VE). To determine if androgens and opiates activate overlapping brain areas, 7 additional males received 20 µg morphine sulfate ICV following ethanol injection (ME). Immediately after ICV infusion, animals were perfused. 60 µm coronal brain slices were stained for Fos. Fos-positive neurons were counted in a 0.3 mm² area from 5 regions previously shown to express T-induced Fos: the posteromedial bed nucleus of the stria terminalis (BSTPM), posteromedial amygdala (MeP), lateral habenula (LHb), ventral tegmental area, and lateral pontine nucleus. T induced Fos in all areas reported previously (TE vs. VE, p<0.05), except LHb (p>0.05). Morphine induced Fos in all 5 brain regions (ME vs. VE, p<0.05), indicating that androgens and opiates activate overlapping brain regions. Flutamide alone did not induce Fos (VF vs. VE, p>0.05). Moreover, flutamide treatment blocked T-induced Fos expression only in the steroid-sensitive BSTPM, suggesting that androgens mediate neuronal activation in this area (mean ±SEM: TF: 68.4±13.2 vs. TE: 137.9±17.6, p<0.05). The absence of flutamide effects on T-induced Fos in the steroid-sensitive MeP (TE: 210.6±50.0 vs. TF: 215.3±28.2, p>0.05) suggests that distinct mechanisms activate Fos in individual androgen-responsive nuclei.

Keywords

hamsters; testosterone; anabolic agents; immediate-early gene products; reinforcement; bed nucleus of the stria terminalis

1. Introduction

Androgenic-anabolic steroids (AAS) are drugs of abuse (Brower, 2002; Yesalis et al, 1993). Animal studies have demonstrated that androgens are reinforcing via actions in the brain, suggesting the potential for dependence (Packard et al, 1997; Wood et al, 2004). In particular, a recent study from our laboratory using hamsters indicated that testosterone induces neuronal

¹ Address all reprint requests and correspondence to Ruth I. Wood, Department of Cell and Neurobiology, Keck School of Medicine of the University of Southern California, 1333 San Pablo St., BMT 401, Los Angeles, CA 90033. Telephone (323) 442-1980, Fax: (323) 442-3466, e-mail: riw@usc.edu..

¹Present address: Department of Gerontology, Leonard Davis School of Gerontology, University of Southern California, Los Angeles, 90033

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activation in brain areas activated by other commonly-abused drugs, including the ventral tegmental area (VTA, DiMeo and Wood, 2006). In addition, testosterone activates select steroid- and opiate-sensitive brain regions. The present study investigated the role of androgen receptors in brain activation, and the potential overlap in neuronal activation after morphine and testosterone exposure.

In another study from our laboratory, the competitive non-steroidal androgen antagonist, flutamide, blocked testosterone self-administration, indicating that androgens *per se* are essential for testosterone reinforcement (Peters and Wood, 2004). This is significant because many effects of testosterone on brain are mediated by aromatization to estrogen. In the present study, we applied a similar approach to determine if flutamide can block testosterone-induced Fos activation. The aim was to identify brain regions that respond to the androgenic effects of acute intracerebroventricular (ICV) testosterone infusion, thereby linking form and function in the neural control of androgen reinforcement. Fos-immunoreactive neurons were counted in brain regions previously shown to express testosterone-induced Fos (DiMeo and Wood, 2006): the posteromedial bed nucleus of the stria terminalis (BSTPM), posteromedial amygdala (MeP), lateral habenula (LHb), VTA, and lateral pontine nucleus (Pn). Interestingly, all of these areas have been shown to be responsive to opiate agonists. In particular, the BSTPM plays an important role in opiate reinforcement (Walker et al., 2000). Furthermore, we previously showed that the opiate antagonist naltrexone also blocks testosterone self-administration (Peters and Wood, 2004). This suggests that opioids may also play role in testosterone reinforcement. Thus, to determine if androgens activate opiate-responsive brain regions, we compared morphine- and testosterone-induced neuronal activation.

2. Results

2.1. Flutamide pretreatment and testosterone-induced neuronal activation

Hamsters ($n=7/\text{group}$) were pretreated with flutamide or ethanol vehicle sc 30 min prior to ICV infusion of 40 μg testosterone (TF and TE, respectively) or 40 μl aqueous vehicle (VF and VE). Photomicrographs in Figure 1 illustrate Fos expression in the MeP from representative males after TE, TF, VE, and VF treatment. As in our previous study (DiMeo and Wood, 2006), a 4h infusion of testosterone ICV induced Fos in MeP. Cell counts are presented as the mean $\pm$ SEM of Fos-positive neurons in a 0.3 mm² region of brain. The number of Fos neurons in the MeP of TE males was nearly twice that of VE controls (TE: 210.6 $\pm$ 50.0 vs. VE: 113.8 $\pm$ 23.1, $p<0.05$). Flutamide treatment alone did not induce Fos expression above control (VF: 118.6 $\pm$ 17.3 vs. VE: 113.8 $\pm$ 23.1, $p>0.05$). Furthermore, flutamide pre-treatment failed to block testosterone-induced neuronal activation (TE: 210.6 $\pm$ 50.0 vs. TF: 215.3 $\pm$ 28.2, $p>0.05$). A similar pattern of Fos expression was observed in the VTA and lateral Pn.

Photomicrographs in Figure 2 illustrate Fos expression in the BSTPM from representative males after TE, TF, VE, and VF treatment. As for MeP, testosterone infusion in BSTPM nearly doubled Fos expression above vehicle controls (TE: 137.9 $\pm$ 17.6 vs. VE: 77.5 $\pm$ 16.2, $p<0.05$), and flutamide alone did not induce Fos (VF: 66.1 $\pm$ 10.7 vs. VE: 77.5 $\pm$ 16.2, $p>0.05$). However, flutamide pre-treatment abolished testosterone-induced neuronal activation (TF: 68.4 $\pm$ 13.2 vs. TE: 137.9 $\pm$ 17.6, $p<0.05$).

Figure 3 summarizes the number of Fos-immunoreactive neurons in BSTPM, MeP, VTA, lateral PN, and LHb after TE, TF, VE, and VF treatment in male hamsters ($n=7/\text{group}$). In the BSTPM, MeP, VTA and lateral PN, the number of Fos-positive neurons was similar to that in our previous report (DiMeo and Wood, 2006). However, in contrast to our previous study, testosterone infusion did not stimulate Fos in the LHb (TE: 134.4 $\pm$ 26.0 vs. VE: 77.9 $\pm$ 18.0, $p>0.05$). As in MeP, flutamide pre-treatment failed to abolish neuronal activation in the in the VTA (TE: 92.8 $\pm$ 19.7 vs. TF: 90.5 $\pm$ 17.0, $p>0.05$). Likewise, in the lateral Pn testosterone-

induced Fos expression was similar in TE and TF males (TE: 304.1 ± 27.1 vs. TF: 257.9 ± 26.0 , $p > 0.05$).

2.2. Morphine- vs. testosterone-induced neuronal activation

Another 7 males received ethanol 30 min prior to ICV infusion of morphine sulfate (30 nmol, ME). The photomicrograph in Figure 4 illustrates Fos expression in the MeP from a representative male after ME treatment. As in our study of testosterone-induced Fos (DiMeo and Wood, 2006), a 4h infusion of morphine ICV induced Fos in BSTPM, MeP, LHb, VTA and lateral Pn. The number of Fos neurons in the MeP after ME treatment was over twice that of VE controls (ME: 235.0 ± 28.4 vs. VE: 113.8 ± 23.1 , $p < 0.05$). After ME infusion, a similar pattern of Fos expression was observed in the BSTPM (ME: 161.2 ± 23.6 vs. VE: 77.5 ± 16.2 , $p < 0.05$), LHb (ME: 162.9 ± 20.7 vs. VE: 77.8 ± 18.0 , $p < 0.05$), VTA (ME: 71.3 ± 11.4 vs. VE: 30.7 ± 10.4 , $p < 0.05$), and lateral Pn (ME: 358.9 ± 38.5 vs. VE: 185.7 ± 34.5 , $p < 0.05$). In addition, areas previously shown to respond to opiates, including the dorsomedial caudate putamen, ventrolateral periaqueductal grey, and paraventricular nucleus of the hypothalamus (Bontempi and Sharp, 1997; D'Este et al, 2002; Grabus et al, 2004), were also extensively stained for Fos following ME infusion.

3. Discussion

3.1. Flutamide selectively blocks testosterone-induced neuronal activation in BSTPM

The present study provides neuroanatomical evidence that mechanisms for testosterone-induced neuronal activation are region-specific. In BSTPM, Fos expression is dependent on androgen action, but neural activation in MeP, VTA, and lateral Pn is not. In our previous study, we surveyed the entire brain for testosterone-induced neuronal activation including brain areas involved in drug reinforcement, particularly opioid responses, and those with steroid-responsive neurons. Fos was induced by testosterone in the BSTPM, MeP, VTA, LHb, and lateral Pn (DiMeo and Wood, 2006). In the present study, testosterone infusion elevated Fos expression in the BSTPM, MeP, VTA and lateral Pn, but not in LHb. Moreover, the anti-androgen flutamide blocked testosterone-induced neuronal activation only in the BSTPM, and not in the MeP, VTA or lateral Pn. Because flutamide blocks both testosterone self-administration (Peters and Wood, 2004) and Fos expression in the BSTPM, this suggests that the BSTPM may play a key role in androgen reinforcement.

In the present study, testosterone delivery ICV focuses on the central effects of androgens. Considering the range of testosterone's systemic androgenic effects, ICV infusion is used here not so much to mimic human use as to probe brain sites of action independent of testosterone's systemic effects. One limitation of the present study is that Fos expression does not necessarily indicate testosterone reinforcement. Rather it reveals the neural substrates responsive to acute testosterone. In this regard, testosterone has wide-ranging effects in brain, including aggression or mating. Fos is thought to reflect neural activation, but the cellular and behavioral consequences of Fos expression will require further study. A further caveat is that although the present study focused on 5 brain regions previously shown to express Fos in response to testosterone, the absence of Fos protein does not preclude the involvement of other brain regions in testosterone reinforcement.

For each brain region expressing Fos in the present study, testosterone may act through 4 principal mechanisms: 1) aromatization to estradiol and binding to estrogen receptors, 2) binding to classical genomic androgen receptors, 3) actions on non-genomic androgen receptors, or 4) indirect stimulation via afferents from steroid-sensitive brain regions. The brain has both androgen and estrogen receptors, as well as the aromatase enzyme to synthesize estradiol from testosterone (Lephart et al, 2001). Classical genomic actions of steroids involve

binding to intracellular steroid receptors and translocation into the nucleus to induce RNA transcription and protein synthesis (McEwen, 1991). The time-course of these effects is slow: hours-days. By contrast, non-genomic effects of steroids are observed within seconds to minutes after hormonal stimulation, too rapid for protein synthesis (Heinlein and Chang, 2002). Moreover, these rapid actions of steroids may occur in brain regions that do not express classical steroid receptors (Day and Good, 2005; Frye and McCormick, 2000). The non-genomic effects of steroids may be transduced either at the cell surface or via interaction with intracellular receptors (Heinlein and Chang, 2002). Ultimately, induction of Fos protein requires transcriptional activation. Thus, in some sense, the presence of Fos represents a genomic response to testosterone stimulation. The issue is whether classical genomic androgen receptors are essential for Fos induction.

There is precedent that androgen reinforcement may involve both genomic and non-genomic actions of steroids. Chronic AAS treatment up-regulates classical androgen receptors in the brain (Menard and Harlan, 1993), implying possible genomic mechanisms. On the other hand, conditioned place preference is induced within 30 minutes after injection of testosterone into the shell of nucleus accumbens (Packard et al, 1997), a brain region with few classical steroid receptors (Kritzer, 1997). This supports the possibility of non-genomic actions. In a similar vein, testicular feminized (Tfm) rats self-administer DHT despite a mutation in the genomic androgen receptor that interferes with androgen binding (Sato et al, 2006).

Flutamide is a competitive non-steroidal anti-androgen (Yin et al, 2003) which has the potential to block testosterone's action on either classical or non-genomic androgen receptors (Gorczyńska and Handelsman, 1995; Farla et al, 2005). It is also a useful approach to investigate the role of aromatization in testosterone reinforcement. Previously, we determined that male hamsters will self-administer either estradiol or DHT (DiMeo and Wood, 2006b). This demonstrates that either androgens or estrogens are reinforcing. This finding brings up the question: when male hamsters self-administer testosterone, are the reinforcing effects mediated via androgen receptors, or by actions on estrogen receptors following aromatization? Recently, we determined that flutamide pretreatment blocks testosterone self-administration (Peters and Wood, 2004). This result suggests that aromatization to estrogen cannot account for the reinforcing effects of icv testosterone self-administration. In the present study, flutamide blocked testosterone-induced Fos in BSTPM, suggesting that BSTPM may be important for androgen reinforcement. Whether this effect involves classical and/or non-genomic androgen receptors is unknown. At high concentrations, flutamide has also been reported to act as an androgen receptor agonist (Maness et al, 1998). However, the absence of Fos in the present study after flutamide injection alone (VF vs. VE) suggests that flutamide did not act as an agonist in the BSTPM, MeP, VTA, and lateral Pn.

BSTPM and MeP each have abundant classical androgen and estrogen receptors (Simerly et al, 1990), and these nuclei are interconnected as part of the extended amygdala (Wood and Newman, 1999). Given these similarities, it was surprising that Fos expression is blocked by flutamide only in BSTPM, and not in MeP. In MeP, Fos activation may be due to estrogenic effects via aromatization of testosterone. By contrast, Fos activation in BSTPM is androgenic. Nonetheless, despite dense interconnections between BSTPM and MeP, testosterone in MeP failed to activate neurons in BSTPM, suggesting that indirect effects of testosterone are insufficient to stimulate BSTPM.

In VTA and lateral Pn, it is somewhat more difficult to interpret the failure of flutamide to block testosterone-induced Fos. Both VTA and lateral Pn are implicated in reinforcement and reward processes (Arvanitogiannis et al, 2000; Erb and Stewart, 1999; Macy et al, 2003). However, VTA and lateral Pn have few classical steroid receptors and little aromatase. Nonetheless, testosterone and progesterone have been shown to act in VTA, presumably via

non-genomic steroid receptors (Frye and Rhodes, 2005; Sipos and Nyby, 1996). In addition, BST sends projections to VTA, and stimulation of NMDA in BST receptors increases firing rates of VTA dopamine neurons (Georges and Aston-Jones, 2002). MeP does not have a substantial direct connection to the VTA (Petrovich et al, 1996; Swanson and Petrovich, 1998). Taken together, it would appear that testosterone-induced Fos in VTA should reflect the combination of activation via non-genomic androgen receptors and indirect stimulation via BST. If so, we would expect flutamide to block Fos in VTA, as it does in BSTPM. This is not the case. Instead, there remains the possibility that the androgenic metabolite 3 α -diol may act on non-genomic estrogen receptors in VTA to facilitate androgen reinforcement (Frye et al, 2001; Pak et al, 2005; Rosellini et al, 2001).

Similar mechanisms may contribute to testosterone-induced Fos in lateral Pn. This region does not connect to the VTA, BSTPM, MeP (Giolli et al, 2001), or other brain regions with abundant classical androgen receptors. In addition, lateral Pn has no discernable classical androgen receptors. Thus, there is no opportunity for testosterone to activate Fos via classical androgen receptors, either directly or indirectly. The lack of connection with steroid-sensitive brain regions, and the absence of genomic steroid receptors suggest that non-genomic mechanisms may be responsible for neuronal activation in lateral Pn.

3.2. Morphine-induced neuronal activation resembles that of testosterone

The present study provides neuroanatomical evidence that testosterone-induced brain activation overlaps with opiate-responsive regions. All brain regions that were responsive to testosterone in our present and previous studies (DiMeo and Wood, 2006) were also activated by morphine. The BSTPM, MeP, VTA, and lateral Pn have dense mu-type opioid receptors (Daunais et al, 2001; Sharif and Hughes, 1989), which are associated with the reinforcing effects of opioids (Contet et al, 2004). In particular, the BST plays a role in opiate reinforcement (Walker et al, 2000) and opiate withdrawal (Aston-Jones and Harris, 2004; Delfs et al, 2002). It may be that testosterone, directly or indirectly via metabolites, activates opiate-responsive neurons to facilitate reinforcement. In accordance with these observations, pre-treatment with the opioid antagonist naltrexone blocks testosterone self-administration in male hamsters (Peters and Wood, 2004), similar to the effects of flutamide.

Steroid hormones and opioid interactions in the brain have been extensively described (Piva et al, 1995). Following morphine administration, 5 α -reductase activity is increased (Amini and Ahmadiani, 2005). Similarly, testosterone increased enkephalin-degrading aminopeptidase activity in the hypothalamus-pituitary-adrenal axis, suggesting an androgenic influence on opiate regulation (Garcia-Lopez et al, 2003). Nandrolone, a commonly abused AAS, down-regulates delta opioid receptor mRNA in vitro (Pasquariello et al, 2000). In addition to androgens, the estrogenic metabolites of testosterone may also interact with the opiate system. Estradiol and progesterone activate kappa-opiate receptors to induce analgesia (Dawson-Basoa and Gintzler, 1996). Estrogen induces mu-opioid receptor internalization via estrogen α receptors (Micevych et al, 2003), while it also increases opioid receptor binding in selective brain regions (Brown et al, 1996). Furthermore, estrogen increases the abuse potential of heroin (Roth et al, 2002), while androgens may increase sensitivity to heroin overdose (Thiblin et al, 1999). Together, these observations indicate that androgens may act in concert with the opioid system to produce reinforcement.

3.3. Summary

Our results suggest that non-genomic androgen receptors may play a significant role in testosterone-induced neural activation. The mechanisms underlying Fos expression are tissue-specific: different brain regions respond to testosterone via distinct mechanisms. In particular, Fos expression in BSTPM appears to be androgenic. Conversely, in the MeP, VTA, and lateral

Pn, Fos expression is not dependent on androgens. Together these observations support the hypothesis that androgen reinforcement may be mediated by rapid non-genomic androgenic actions and, to a lesser extent, by slower genomic androgen receptors. Our results also provide evidence for the possibility that androgens may interact with other neurochemical systems such as opiates to induce reinforcement. Ultimately, neural activation of androgen reinforcement may reflect the combined actions of steroids on several brain regions according to site-specific mechanisms.

4. Methods

All efforts were made to minimize animal suffering, and to reduce the number of animals used. All experimental procedures were approved by the USC Institutional Animal Care and Use Committee and were conducted in accordance with the “NIH Guide for the Care and Use of Laboratory Animals” (NIH Publications No. 80-23, revised 1978).

4.1. Animals and housing

35 adult male Syrian hamsters (122–172 g BW) were obtained from Charles River Laboratories (Kingston, NY). Males were singly-housed with food and water available *ad libitum*. Hamsters were maintained under a long-day photoperiod (14:10 LD).

4.2. Surgeries and apparatus

All males were orchidectomized and received a 10-mm Silastic implant (id: 1.98 mm, od: 3.18mm; Dow Corning, MI) subcutaneously to maintain physiologic levels of testosterone (Powers et al, 1985). At the time of orchidectomy, hamsters were implanted with a 22-ga stainless steel guide cannula (Plastics One, Roanoke, VA) into the lateral ventricle (AP: +1.0 mm, ML: +1.0 mm, DV: –3.0–5.0 mm relative to bregma). Except during ICV infusions, the guide cannula was protected by a dummy cannula with dust cap. All surgeries were performed under anesthesia with sodium pentobarbital (80 mg/kg, Abbott Laboratories, North Chicago, IL).

ICV infusions were conducted in an operant chamber (Med Associates, St. Albans, VT) enclosed in a sound-attenuating cubicle with a fan for ventilation. Chambers were equipped with a house light and a computer-controlled syringe pump (Med Associates) with balance arm and fluid swivel (Instech, Plymouth Meeting, PA). Solutions from a 100 µl glass syringe (Hamilton, Reno, NY) were delivered to the swivel through Teflon tubing. The tubing connecting the swivel to the cannula was protected by a metal spring.

4.3. Experimental design

Methods followed closely those of DiMeo and Wood (2006). One week after surgery, 28 hamsters received a single 4h ICV infusion of 40 µg testosterone (1.0 µg/µl [Steraloids, Newport, RI]) or 40 µl vehicle (13% 2-hydroxypropyl-β-cyclodextrin in H₂O, [RBI, Natick, MA]) via a 28-ga internal cannula inserted into the guide cannula immediately before testing. This 4h ICV T infusion induces Fos expression in male hamsters (DiMeo and Wood, 2006). Moreover, the 1.0 µg/µl concentration produces maximal rates of operant behavior during ICV testosterone self-administration (DiMeo and Wood, 2004; Triemstra and Wood, 2004; Wood et al, 2004). 40 µg is a substantial, but non-lethal, dose of testosterone, as described in Peters and Wood (2004). ICV Infusions were not continuous. Instead, males received a 1.0 µl injection every 6 minutes for 4h (40 µl total). This mode of intermittent ICV infusion has been used previously to demonstrate depressive effects of testosterone (Peters and Wood, 2004), and parallels our previous studies using testosterone infusion to stimulate Fos expression (DiMeo and Wood, 2006). Half of the males in each group (n=7 each) received 15 mg/kg flutamide in 0.25 ml 95% ethanol or 0.25 ml ethanol vehicle sc 30 min prior to ICV infusion of testosterone

(TF and TE, respectively) or vehicle (VF and VE). In parallel with testosterone treatment, 7 additional males received 0.25 ml ethanol vehicle sc 30 min prior to ICV infusion of 30 nmol (20 µg) morphine sulfate (Sigma Chemical Co, St. Louis, MO, 0.5 µg/µl). Based on studies using ICV morphine sulfate injection in various rodents, including guinea-pigs (Bot and Chahl, 1996), rats (Easterling and Hotlzman, 2001; Ito et al, 2001), and mice (Kest and Hopkins, 2001), 30 nmol morphine should be sufficient to induce Fos expression in hamster brain.

4.4. Perfusion

Immediately after ICV infusion, hamsters were deeply anesthetized with sodium pentobarbital (150 mg/kg), and perfused through the heart with 150 ml 0.1 M sodium phosphate buffer (NaPB, pH 7.4) containing 0.9% NaCl and 0.1% NaNO₃ for vasodilation, followed by 250 ml of 4% paraformaldehyde in NaPB. The brains were removed, post-fixed for 1 h at room temperature in the perfusion fixative, and then cryoprotected overnight in NaPB containing 20% sucrose at 4°C. The brains were rapidly frozen, then sectioned coronally at 60 µm on a freezing microtome. Brain slices were stored at 4°C in NaPB with 0.01% sodium azide as a preservative until Fos immunostaining.

4.5. Immunocytochemistry

Every 4th section of each brain was immunostained for Fos. Brains from each experimental group were stained at the same time. The sections were extensively rinsed in NaPB between steps. To reduce excess aldehydes, sections were washed for 10 min in 0.2% sodium borohydrate, followed by 30 min in 0.1M glycine. Next, sections were incubated overnight at room temperature in anti-Fos antiserum (sc-253, 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA) with 4% normal donkey serum and 0.3% Triton X-100 in NaPB. Sections then were incubated in biotinylated donkey anti-rabbit IgG (1:200, Jackson ImmunoResearch Laboratories, West Grove, PA), followed by the avidin-biotin horseradish peroxidase complex (1:50, Vectastain ABC Elite kit, Vector Laboratories, Burlingame, CA), each in Triton-NaPB for 1h at room temperature. The horseradish peroxidase complex was visualized using NiCl-enhanced 3,3'-diaminobenzidine (DAB, Sigma), with 0.08 % hydrogen peroxidase (Sigma). Sections were mounted onto gelatin-coated slides, dehydrated in alcohols, cleared in xylenes, and cover-slipped with Permount.

4.6. Cell counting and image analysis

Brain regions and nomenclature are according to the hamster brain atlas of Morin and Wood (Morin and Wood, 2001). Fos-immunoreactive nuclei were counted in 5 brain regions (BSTPM, MeP, LHb, VTA and lateral Pn). Previously, these brain regions expressed Fos after 4h testosterone infusion (DiMeo and Wood, 2006). Images of the BSTPM were collected at the level of the foramen of Monroe (Atlas plate 21). Photomicrographs of the LHb and MeP were collected at the rostral limit of the arcuate nucleus (Arc [Atlas plate 28]), while the VTA was analyzed at the caudalmost extent of Arc (Atlas plate 36). In the lateral Pn, images were collected at the decussation of the superior cerebellar peduncle (Atlas plate 41).

Digital images of select brain regions at a single focal plane were obtained at 10x by an investigator blind to the treatment. The images were converted to grayscale in Adobe Photoshop 7.0 (Adobe Systems Inc., San Jose, CA). To correct for uneven illumination, a white reference image from a blank cover-slipped slide was subtracted from each digital image. At the same time, a small (70x70 pixels at 72 dpi) image from an unstained region of cortex was collected from each brain slice to aid in thresholding. Fos neurons were counted in a 0.3 mm² area (375x375 pixels at 72 dpi) except in BSTPM. In the BSTPM, a 0.205 mm² (275x350 pixels) area was selected at a 45° angle to accommodate the orientation of this small, oval subnucleus. For the BSTPM, cell counts were normalized to 0.3 mm².

Neurons expressing Fos protein were counted bilaterally in thresholded images using NIH Image, version 1.63. The threshold was determined as the highest value at which no pixels were detected in the unstained cortical image from the same brain slice. To eliminate stray pixels and tissue artifacts, only objects between 5–45 pixels were counted as Fos-positive nuclei. Cell counts from the right and left hemispheres were averaged for each male.

4.7. Statistical analysis

The number of Fos neurons (mean±SEM) in males exposed to vehicle or testosterone after ethanol or flutamide pre-treatment were compared by factorial analysis of variance (ANOVA) with post-hoc analysis by Fisher's PLSD. Statistical analyses were completed using Statview 5.0 (SAS Institute). For all comparisons, $p < 0.05$ was considered significant.

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Figure 1.

Photomicrographs of the posterial medial amygdala showing Fos protein expression in representative male hamsters after 4h of 40 μ g ICV testosterone (1.0 μ g/ μ l, top) or 40 μ l vehicle (bottom) with pre-treatment of ethanol (left) or flutamide (right). Abbreviations: TE: Testosterone+Ethanol; TF: Testosterone+Flutamide; VE: Vehicle+Ethanol; VF: Vehicle +Flutamide. Scale bar =200 μ m.

Figure 2.

Photomicrographs of the bed nucleus of stria terminalis showing Fos protein expression in representative male hamsters after 4h of 40 μ g ICV testosterone (1.0 μ g/ μ l, top) or 40 μ l vehicle (bottom) with pre-treatment of ethanol (left) or flutamide (right). Abbreviations: TE: Testosterone+Ethanol; TF: Testosterone+Flutamide; VE: Vehicle+Ethanol; VF: Vehicle +Flutamide. Scale bar =200 μ m.

Figure 3. Mean±SEM Fos-immunoreactive neurons after 4h ICV testosterone (black) or vehicle (white) infusion in male hamsters (n=7/group). 30 min prior to infusions, males received either ethanol or flutamide (shaded). Abbreviations: BSTPM: posteromedial bed nucleus of the stria terminalis; LHb: lateral habenula; MeP: posterior medial amygdala; Pn: lateral pontine nucleus; TE: Testosterone+Ethanol; TF: Testosterone+Flutamide; VTA: ventral tegmental area; VE: Vehicle+Ethanol; VF: Vehicle+Flutamide. Bars with common letter subscripts are not significantly different.

Figure 4. Photomicrograph of the posterolateral medial amygdala showing Fos protein expression in a representative male hamster after 4 h of 20 μ g ICV morphine sulfate (0.5.0 μ g/ μ l). Males received a subcutaneous ethanol injection 30 min prior to morphine infusion. Scale bar =200 μ m.