

Corticotropin-Releasing Factor Modulation of Forebrain GABAergic Transmission has a Pivotal Role in the Expression of Anabolic Steroid-Induced Anxiety in the Female Mouse

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Increased anxiety is commonly observed in individuals who illicitly administer anabolic androgenic steroids (AAS). Behavioral effects of steroid abuse have become an increasing concern in adults and adolescents of both sexes. The dorsolateral bed nucleus of the stria terminalis (dlBnST) has a critical role in the expression of diffuse anxiety and is a key site of action for the anxiogenic neuromodulator, corticotropin releasing factor (CRF). Here we demonstrate that chronic, but not acute, exposure of female mice during adolescence to AAS augments anxiety-like behaviors; effects that were blocked by central infusion of the CRF receptor type 1 antagonist, antalarmin. AAS treatment selectively increased action potential (AP) firing in neurons of the central amygdala (CeA) that project to the dlBnST, increased the frequency of GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) in dlBnST target neurons, and decreased both c-FOS immunoreactivity (IR) and AP frequency in these postsynaptic cells. Acute application of antalarmin abrogated the enhancement of GABAergic inhibition induced by chronic AAS exposure whereas application of CRF to brain slices of naïve mice mimicked the actions of this treatment. These results, in concert with previous data demonstrating that chronic AAS treatment results in enhanced levels of CRF mRNA in the CeA and increased CRF-IR in the dlBnST neuropil, are consistent with a mechanism in which the enhanced anxiety elicited by chronic AAS exposure involves augmented inhibitory activity of CeA afferents to the dlBnST and CRF-dependent enhancement of GABAergic inhibition in this brain region.

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INTRODUCTION

Anabolic androgenic steroids (AAS) comprise a large class of synthetic androgens designed for therapeutic purposes, but whose present use is predominantly illicit self-administration of supratherapeutic doses for image and performance enhancement (for review, Basaria *et al*, 2001; Bahrke and Yesalis, 2004; Trenton and Currier, 2005). Elevated levels of anxiety have been reported in AAS users (Su *et al*, 1993; Cooper *et al*, 1996; Galligani *et al*, 1996; Hall and Chapman, 2005), and AAS-dependent anxiety may contribute to other noted behavioral effects of AAS, including increased impulsivity, hostility, and aggression (Annitto and Layman, 1980; Pope and Katz, 1988; Burnett and Kleiman, 1994; Cooper *et al*, 1996; Hall and Chapman, 2005; Pagonis *et al*, 2006). Consistent with reports in human subjects, chronic AAS exposure increases anxiety-like behavior in adult male rodents, as assessed on the elevated plus maze

(EPM), in open field paradigms, and in risk-assessment behaviors (Rocha *et al*, 2007; Ambar and Chiavegatto, 2009). Although the public focus of steroid abuse and the preponderance of animal studies have been on adult males, estimated use among adolescents indicates that several hundred thousand current AAS users are girls and young women (Elliot *et al*, 2007; Johnston *et al*, 2009). The risks associated with AAS use in this population may be compounded by the high degree of hormone sensitivity present during adolescence (O'Connor and Cicero, 1993; Sato *et al*, 2008; Schulz *et al*, 2009) and by sex-specific differences in susceptibility to behavioral disorders, such as anxiety, which is more prevalent in women (Zender and Olshansky, 2009).

The extended amygdala is a corridor of interrelated forebrain structures including the central amygdala (CeA) and adjoining structures of the bed nucleus of the stria terminalis (BnST) that are fundamental to the expression of anxious states (for review, Alheid, 2003; Davis *et al*, 2010). The CeA provides the major source of GABAergic input to the BnST (Krettek and Price, 1978; Sun and Cassell, 1993; Schmued, 1994; Petrovich and Swanson, 1997; Veinante and Freund-Mercier, 1998; Pitkanen, 2000), and GABAergic transmission in circuits of the extended amygdala has been shown to be critical in the expression of anxious states

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(Möhler, 2012). Prior studies in rodents suggest that the CeA is paramount in the acquisition and expression of phasic fear to specific threats whereas the BnST is key in sustained fear/diffuse anxiety (for review, Shekhar *et al*, 2005; Davis *et al*, 2010; although see Resstel *et al*, 2008). Changes to both specific threats and generalized anxiety are noted in steroid abusers, but the latter may be particularly important with respect to the expression of inappropriate reactions and increased negative perception of self and others (Pagonis *et al*, 2006).

The corticotropin releasing factor (CRF) family of peptides acts to integrate sensory, endocrine and autonomic information and formulate an appropriate response to stress (for review, Korosi and Baram, 2008). Elevated levels of central CRF are implicated in anxiety and stress disorders in humans. The BnST is believed to be the primary site of anxiogenic action of CRF, and in rodents CRF stimulates anxiety-like behaviors, as assessed by the acoustic startle response (ASR), the EPM, open field tests, conditioned place aversion, and social interaction tests (for review, Bale and Vale, 2004; Davis *et al*, 2010). The lateral portion of the CeA (CeA_L), which provides strong GABAergic input to the dorsolateral BnST (dlBnST), is also the major extrahypothalamic site of CRF mRNA expression (Swanson *et al*, 1983; Day *et al*, 1999; Asan *et al*, 2005). Corticotropin-releasing factor receptor type 1 (CRF-R1) mRNA is expressed throughout the BnST (van Pett *et al*, 2000; Justice *et al*, 2008) and has been shown to mediate CRF-dependent enhancement of the ASR (Swerdlow *et al*, 1986, 1989; Liang *et al*, 1992; Risbrough *et al*, 2003, 2004; Risbrough and Geyer, 2005; Walker *et al*, 2009a,b).

Prolonged exposure of adolescent female mice to three AAS commonly self-administered in the human population results in enhanced anxiety-like behaviors, as assessed by the ASR (Costine *et al*, 2010). CRF signaling is likely to have an important role in this elevated level of anxiety, as treatment is also associated with increased CRF mRNA and CRF-immunoreactivity (IR) in the somata of CeA neurons and CRF-IR in the neuropil of their target region, the dlBnST (Costine *et al*, 2010). CRF has been shown to augment inhibitory transmission mediated by GABA_A receptors in other regions of the extended amygdala (Kash and Winder, 2006; Kirby *et al*, 2008; Nie *et al*, 2004, 2009). These studies suggest that AAS-dependent increases in anxiety may involve enhanced GABAergic inhibition to the dlBnST and that CRF signaling may have a significant role in both the enhancement of this inhibition and in the expression of AAS-induced anxiety. The aim of the current study was to test this hypothesis.

MATERIALS AND METHODS

Animal Care and Use

Female C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were group-housed (4/cage) with food and water *ad libitum* in a temperature-controlled and 12 h light cycle facility with lights on starting at 0700 h. Care was taken to minimize the discomfort and the number of animals used, and all procedures were approved by the Dartmouth College Institutional Animal Care and Use Committee and conducted in accordance with guidelines from the National Institutes of Health. Adolescent female mice were injected i.p. at post-natal day (PN) 24 for 6 days per week for 4 weeks with equal

concentrations of testosterone cypionate (Sigma; St Louis, MO), nandrolone decanoate (Sigma) and methandrostenolone (Sternaloids; Newport, RI) dissolved in sesame oil at a final concentration of 7.5 mg/kg per day. Age-matched control subjects were administered the same volume (20–30 μ l based on body weight) of sesame oil alone. Behavioral testing commenced at \sim PN55, and treatment with either AAS or oil was continued during testing. AAS treatment imposes an anestrus state characterized by diestras-like profiles (Blasberg and Clark, 1997; Penatti *et al*, 2009a, 2011), and control mice were assessed in diestras (Cooper *et al*, 1993). In some experiments, PN55 diestras female mice that had not received any injections (naïve) were used.

Behavioral Testing

ASR testing was performed using the MED-ASR-PRO1 apparatus (Med Associates; St Albans, VT) (Costine *et al*, 2010). The day following acclimation, ASR testing consisted of 5 min of acclimation followed by exposure to the startle stimulus of a 50-ms/100 dB burst of white noise (1 ms rise/fall; 10 s intra-trial interval) for 60 trials. In one experiment, 55-day old mice received single injections of oil on day 1 and day 3 of testing followed by a single injection of either the AAS mixture or oil prior to testing on day 5. The EPM (Columbus Instruments, Columbus, OH) was 45 cm high and arms were 30 cm long. Walls of the closed arms were 15.25 cm high. EPM testing was done according to standard procedures (Lister, 1987; Walf and Frye, 2007). The luminous intensity in the center of the maze and at both ends of the open arms was 3.8 cd, equivalent to the intensity of the home cage during non-testing periods. The mouse was placed in the center of the EPM facing an open arm, and the time spent in the open and closed arms, and the number of entries was scored (5 min). Testing occurred between 4 and 6 h after lights on, and the experimenter was blind to treatment conditions.

Intracerebroventricular (i.c.v.) Infusions

Infusions of the CRF-R1 antagonist, antalarmin (10 μ g/kg), or saline (vehicle) were made into the lateral ventricle (Briscoe *et al*, 2000; Pelleymounter *et al*, 2000; Risbrough *et al*, 2004). Infusate (5.0 μ l) was pressure-ejected (30 s) in isoflurane-anesthetized mice through a 27-gauge 2.0 mm guide cannula placed lateral to the midline of the skull halfway between bregma and lambda. The incision was then sealed with cyanoacrylate. Mice were ambulatory within 10 min and were placed in the ASR chamber after 5 additional minutes. An additional group of AAS- and oil-injected mice received a sham surgical procedure that included exposure to anesthesia, skull exposure (but no injection), and cyanoacrylate wound closure. Following testing and euthanasia, brains were dissected and fixed overnight in 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB). Coronal brain sections were Nissl-stained with 0.25% cresyl violet and cover-slipped following serial dehydration. Placement of the guide cannula was verified under 200 \times magnification and classified as either ventricular or extra-ventricular. Sera were collected and assayed for corticosterone in singlet by radioimmunoassay at the

University of Virginia Ligand Assay Core Laboratory. Detection limits for the assay were 6.7–739.0 ng/ml.

c-FOS Immunolabeling

Mice were ASR-tested as described above and compared to mice that were injected with AAS or oil, but not tested on the ASR. At 1 h and 45 min after placement of a mouse into the chamber (or time matched for not-tested groups), animals received an overdose of ketamine/xylazine (9:1) and 0.05 ml heparin (1000 U/ml; APP Pharmaceuticals, Schaumburg, IL) and were intracardially perfused with 4% PFA in PB. Following overnight fixation and cryoprotection at 4 °C, coronal free-floating sections were labeled for c-FOS (Martel and Baum, 2009). Sections were reacted with 10% H₂O₂ solution, washed three times for 10 min in PB and incubated in blocking buffer: 10% normal goat serum in PBT (PB containing 0.3% Triton X-100; Thermo Fisher) for 3 h at room temperature. The sections were reacted overnight in primary antibody (rabbit anti-c-FOS, Santa Cruz sc-52; 1: 1000), followed by a 90-min incubation with a biotinylated goat anti-rabbit antibody (Vector Labs, Burlingame, CA; 1:2000 in PBT) and visualized with ABC Vectastain/Nickel-enhanced 3,3' diaminobenzidine (Vector). Sections were washed in PB three times for 15 min between primary and secondary antibodies, and before and after ABC Vectastain. Bilateral images from two sections were obtained for the ventrolateral BnST (vlBnST) and the dlBnST (also called the anterolateral portion of the BnST_{ALG}; Dong and Swanson, 2005; Hammack *et al*, 2007) (Bregma 0.14 mm); the CeA_L (Bregma −1.46 mm); the basolateral nucleus of the amygdala (BLA; Bregma −1.46 mm); and the auditory cortex (AuC; Bregma −2.06) (Franklin and Paxinos, 1997). The numbers of c-FOS-IR cells in each image were counted and averaged for the four images per mouse using ImageJ and with the investigator blind to the experimental conditions.

Physiological Recordings

Coronal brain slices (250–300 μm) were prepared as described (Penatti *et al*, 2010, 2011). Sections corresponding the dlBnST (Bregma 0.14 mm) or the CeA (Bregma −1.46 mm) were superfused in artificial cerebrospinal fluid (aCSF: 95% O₂/5% CO₂ saturated aCSF, containing in mM: 125 NaCl, 1.2 CaCl₂, 10 glucose, 4 KCl, 1 MgCl₂, 26 NaHCO₃, 1.25 NaH₂PO₄, and 1 ascorbic acid at pH 7.35) for 20 min (1–2 ml aCSF per min) at 22 or 35 °C. Spontaneous action potentials (APs) were recorded from neurons using the 'loose-patch' on-cell conformation with electrodes (3–5 MΩ) filled with aCSF. In some experiments, either 100 μM picrotoxin or 1 μM strychnine (Sigma) were added to the recording bath to block GABA_A or glycine receptors, respectively. All physiological data was acquired and stored using a HEKA-EPC9 amplifier (HEKA Instruments, Bellmore, NY, USA) (Penatti *et al*, 2005, 2009a, b, 2010, 2011).

Fluorescent latex microspheres conjugated to Alexa-488 (Retrobeads, Lumafuor) were stereotactically pressure injected in the dlBnST (Lammel *et al*, 2011; Carrillo *et al*, 2011). A 4.4-mm guide cannula was inserted to target the dlBnST, and 300 nl of retrobeads were delivered over 1 min through a Hamilton syringe, with the needle left in place for an

additional minute. 4–7 days after Retrobead infusion, APs were recorded from the ipsilateral CeA from both labeled and neighboring unlabeled cells. The site of the Retrobead injection was verified in the dlBnST following recording; only those animals showing correct injection of the Retrobeads were used for analysis.

GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) were recorded in the whole-cell configuration ($V_{\text{hold}} = -70$ mV) in aCSF containing 2.0 mM kynurenic acid to block excitatory ion channels. Pipettes were filled with (in mM): 153 CsCl, 1 MgCl₂, 5 EGTA, and 10 HEPPES, to which 2 MgATP was added just before each experiment. Biocytin (0.5% w/v) or Lucifer yellow (0.5% w/v) was added in some experiments to confirm the neuron's location within the BnST (Campbell *et al*, 2005). The identity of synaptic currents as GABAergic was confirmed in some experiments by antagonism by bicuculline (20 μM). To record miniature IPSCs (mIPSCs), 1 μM tetrodotoxin (TTX) was added to the kynurenic acid-containing aCSF. Data were acquired for >5 min and accepted for analysis only if the seal resistance (>1 GΩ), access resistance (<25 MΩ), and the holding current did not change more than $\pm \sim 10\%$ during the recording. In some experiments, antalarmin (40 or 4 μM), CRF (100 nM) (Anaspec, Fremont, CA), or the mixture of the three AAS (1 or 10 μM in 0.01% DMSO) was added to the aCSF.

Frequency analysis of APs was derived from direct assessment of inter-spike intervals. Patterning was determined using autocorrelation analysis and classified as regular, irregular, or 'bursty' (Penatti *et al*, 2009a, b, 2010, 2011). Autocorrelational profiles correlated with the coefficient of variation (CV) of AP firing: the bursty pattern with high CVs (≥ 4), the irregular pattern with CVs between 0.5 and 2.5, and the regular pattern with CVs <0.5. Cells with fewer 10 APs over the 5 min of recordings were classified as quiet and not used in frequency comparisons. Averaged IPSCs were analyzed for peak current amplitude (I_{peak}), frequency, and decay kinetics (biphasic and fitted with two time constants, τ_1 and τ_2) or with a single weighted time constant (τ_w) (Penatti *et al*, 2010, 2011).

Statistical Analysis

Values are means \pm SE of the mean. Significance was determined by one-way, two-way or repeated measures analysis of variance (ANOVA) followed by *post hoc* analyses using the Student's *t*-test with Bonferroni correction. The Kolmogorov-Smirnov test was used for cumulative probability distributions. Non-normally distributed data were log-transformed prior to statistical assessment. For all data, the α level was set at $p < 0.05$.

RESULTS

Chronic, but not Acute, Exposure to AAS Significantly Increases Anxiety-like Behavior in Female Mice

Female C57BL/6J mice were treated throughout a period that spans adolescence (Laviola *et al*, 2003) with three AAS that are commonly self-administered by human subjects and at a dosage that reflects a high human abuse regime (for discussion, Costine *et al*, 2010). Our previous study

demonstrated that this same regime promotes anxiety-like behaviors in C57BL/6J female mice, as assessed by the ASR (Costine *et al*, 2010); results that were confirmed in the current study (Figure 1a). Anxiogenic effects of AAS were also indicated by a significant ($F_{1,10} = 22.05$, $p < 0.001$) decrease in the percent time spent in the open arms on the EPM (3.90 ± 0.96 for AAS vs 10.30 ± 0.96 for oil; $n = 6$, each group), a behavioral test that, like the ASR, is an assessment of sustained fear and heightened anxiety (Davis *et al*, 2010). No significant ($F_{1,10} = 0.16$, $p = 0.70$) differences were evident in the total number of arm entries (oil: 22.67 ± 1.87 ; AAS: 23.67 ± 1.67), indicating that animals were not stationary in the closed arms and that the increased time spent in the closed arms was not attributable to AAS-dependent differences in locomotion.

AAS have been reported to promote short-term effects on anxiety-like behaviors (Ågren *et al*, 1999; Aikey *et al*, 2002), as well as behavioral actions that necessitate prolonged exposure. AAS can act as acute allosteric modulators of GABA_A receptors (Henderson and Jorge, 2004; Henderson, 2007; Oberlander *et al*, 2012), and such acute action may give rise to immediate behavioral effects. To determine whether a single exposure to the AAS altered anxiety-like behaviors, female mice that had not been treated during adolescence were tested on the ASR and the EPM beginning on PN55 (the age when behavioral testing was initiated for chronically treated mice) after a single injection of AAS or

oil. No significant differences were observed on either the ASR or the EPM tests between animals receiving an acute injection of AAS vs oil (ASR: $F_{1,590} = 0.18$, $p = 0.68$; EPM: $F_{1,10} = 2.23$, $p = 0.17$). On the EPM, the percentage of time spent in the open arm was significantly higher for mice receiving a single AAS injection vs those that were chronically treated (18.50 ± 2.81 vs 3.90 ± 0.96 , respectively; $p < 0.001$), and those receiving a single AAS injection were not different than the chronic oil-injected group ($p = 0.16$). These results suggest that the anxiogenic effect of AAS require long-term exposure to the AAS.

Antagonism of the CRF-R1 Abrogated AAS-Dependent Enhancement of the ASR

Sustained fear and heightened anxiety-like behaviors are believed to arise from changes in neural circuitry of the extended amygdala. In particular, CRF-containing afferents from the CeA_L that project to the dlBnST are believed to be key mediators in the expression of diffuse anxiety and sustained fear. CRF, acting primarily through CRF-R1, augments the ASR, and the BnST is the critical site for CRF-dependent enhancement of the ASR (Swerdlow *et al*, 1986, 1989; Liang *et al*, 1992; Risbrough *et al*, 2003, 2004; Risbrough and Geyer, 2005; Walker *et al*, 2009a, b). To test whether CRF-R1-mediated signaling is also required for AAS-induced increases in anxiety-like behaviors, chronically treated female mice were given i.c.v. infusions of the CRF-R1 antagonist, antalarmin (Briscoe *et al*, 2000; Pellemounter *et al*, 2000), or saline prior to ASR testing. Antagonism of CRF-R1 has been shown to block the ASR (Risbrough *et al*, 2003), and antalarmin, a non-peptide antagonist of the CRF-R1 receptor (Webster *et al*, 1996) reduces anxiety-like behavior on the EPM (Zorrilla *et al*, 2002) without affecting the stress response to restraint (Wong *et al*, 1999; however, see Deak *et al*, 1998). Both AAS- and oil-injected mice habituated to the 100 dB white noise burst during the ASR testing at similar rates ($F_{11,418} = 10.48$, $p < 0.01$). The effects of antalarmin were, therefore, assessed for the first 20 trials of the ASR. As expected, AAS-injected groups exhibited significantly higher ASR amplitudes than oil-injected groups, following sham surgery procedures ($F_{1,4} = 4.58$, $p < 0.05$; Figure 1b) or i.c.v. infusion of saline ($F_{1,6} = 24.83$, $p < 0.001$) (Figure 1b). However, the effect of the AAS treatment was abrogated by infusion of antalarmin into the lateral ventricle ($F_{1,8} = 0.40$, $p = 0.70$) (Figure 1b). Significant AAS-induced potentiation of the ASR was evident in animals where infusions of antalarmin were made off-target in either the corpus callosum or the cerebellum ($F_{1,13} = 16.69$, $p < 0.0001$). Although these data do not localize the actions of antalarmin solely to the dlBnST, they do suggest that the ability of the AAS to augment anxiety-like behaviors requires signaling mediated by CRF-R1 in brain regions in the extended amygdala, including the dlBnST, that are likely to be the predominant targets of antalarmin during this i.c.v. infusion procedure.

An alternative explanation for the anxiogenic actions of AAS is that they could arise from changes in the activation of the HPA axis, as CRF is an important central regulator of the stress response via its actions in the paraventricular nucleus (PVN) of the hypothalamus (Baram *et al*, 1997; Gallagher *et al*, 2008; for review, Gray and Bingaman, 1996;

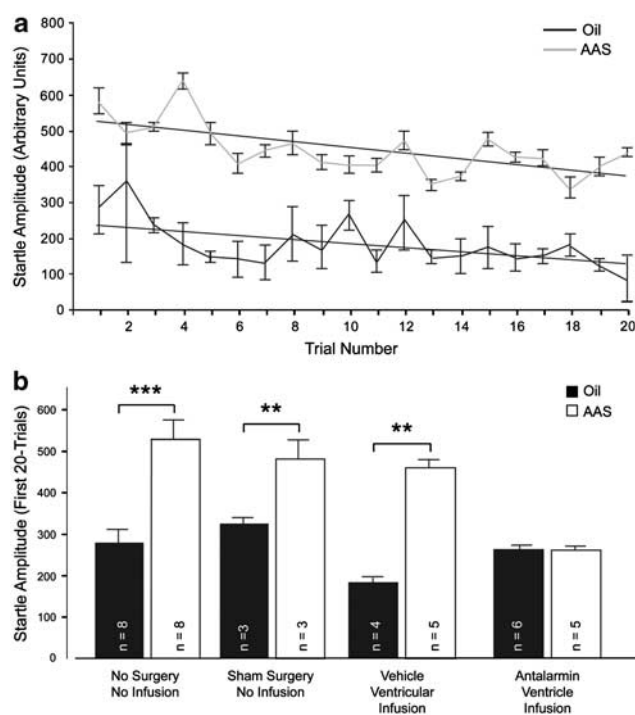


Figure 1 Acoustic startle response (ASR) testing in mice that had received oil- or anabolic androgenic steroids (AAS)-injections. (a) Average amplitudes on the first 20 individual trials to the 50 ms 100 dB burst of white noise were significantly greater on all trials in the intact AAS-injected animals vs oil-injected animals ($n = 8$ each group). Both cohorts exhibit comparable habituation over the testing period. (b) Average startle amplitudes over the first 20 trials for oil-injected and AAS-treated animals that received no infusions or surgery, mock surgery, ventricular infusion of saline, or ventricular infusion of antalarmin. n values represent the number of animals per group/condition. ** $p < 0.01$; *** $p < 0.001$.

Herman *et al*, 1996). Our previous studies argue against this mechanism as, although AAS treatment enhanced CRF expression in the extended amygdala, exposure to these steroids did not alter CRF expression in the median eminence or the PVN and did not increase basal levels of serum corticosterone in animals not subjected to behavioral testing (Costine *et al*, 2010). Interestingly, central treatment of adult male rats with the androgen, dihydrotestosterone, also failed to augment CRF expression in the PVN (Bingham *et al*, 2011). Here, we extended this analysis by assessing corticosterone levels following i.c.v. infusions and ASR testing.

Corticosterone levels in animals subjected to sham surgery procedures (no infusion), saline infusions, or extra-ventricular administration of antalarmin and subsequently tested on the ASR were significantly higher in the AAS-injected mice than in oil-injected mice (sham surgery: $F_{1,4} = 4.16$, $p < 0.05$; saline: $F_{1,7} = 5.65$, $p < 0.001$; extra-ventricular antalarmin: $F_{1,14} = 2.99$, $p < 0.01$). Mice that had received i.c.v. antalarmin showed no differences in corticosterone levels between AAS- and oil-injected groups ($F_{1,10} = 0.40$, $p = 0.70$), consistent with the lack of differences in ASR amplitudes between these two conditions. As previously reported (Costine *et al*, 2010), corticosterone levels were indistinguishable between AAS- and oil-injected mice that had received no infusions or testing ($F_{1,7} = 0.41$, $p = 0.69$). Moreover, mice who had not been subjected to ASR testing had significantly lower levels of corticosterone than any group of behaviorally tested mice ($p < 0.0001$), irrespective of the permutations of manipulations associated with infusions to which they had been subjected. Taken together with the prior results (Costine *et al*, 2010), these data suggest that although the basal state of the HPA axis is not altered by AAS exposure, AAS-treatment results in an enhanced response to a stressor, in this case the startle noise, that is CRF-R1-mediated and results in elevated peripheral levels of corticosterone.

Neuronal Excitability is Increased in the CeA_L by AAS Exposure

CRF action on neural activity in the BnST is believed to be critical for the expression of diffuse anxiety (for review, Davis *et al*, 1997; Shekhar *et al*, 2005; Davis *et al*, 2010). Our previous work demonstrated that AAS treatment increased the levels of CRF mRNA and somal-associated CRF-IR in the CeA_L and enhanced CRF-IR in the neuropil of the dlBnST (Costine *et al*, 2010). The CeA provides a robust projection to the dlBnST from neurons that express both CRF and the classical neurotransmitter, GABA (Sun *et al*, 1991; Sun and Cassell, 1993; Arluison *et al*, 1994; Cassell *et al*, 1999). We hypothesized that AAS treatment would increase the activity of these CeA_L projection neurons, and thus also promote an increase in CRF release and CRF-R1-mediated signaling in the dlBnST. On-cell recordings of neurons in the rostral extent of the CeA_L support this assertion. The frequency of APs was found to be significantly higher ($F_{1,69} = 8.40$, $p < 0.005$; 35 °C) in CeA_L neurons from AAS-treated than oil-injected animals (Figures 2a–c). The percentage of bursty cells in this region was greater in AAS-treated animals (31.6% in oil-injected vs 42.9 % AAS-treated), but the difference was not significant.

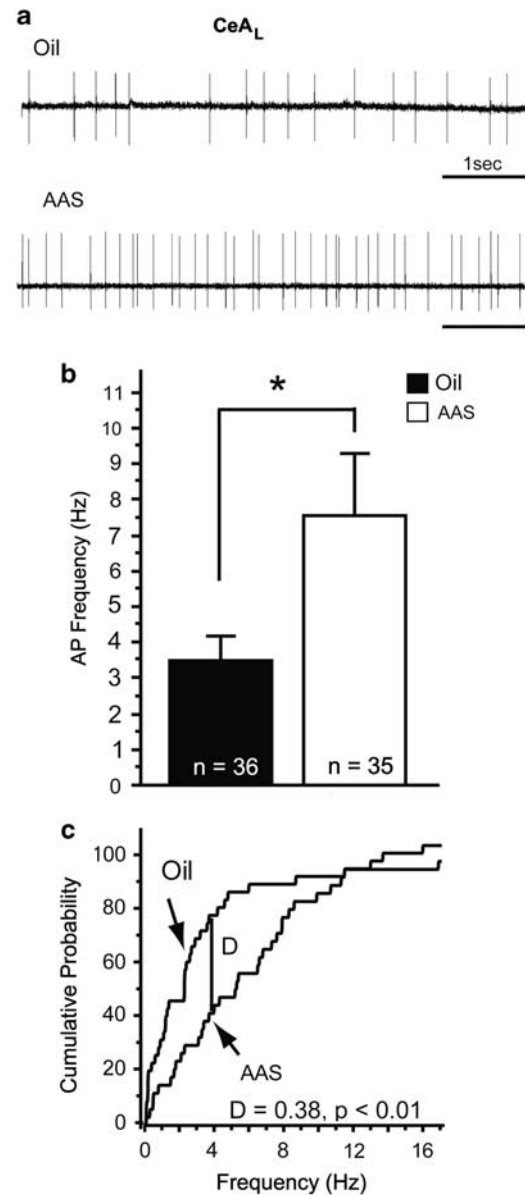


Figure 2 (a) Representative on-cell recordings from the lateral part of the central amygdala (CeA_L) from oil- and anabolic androgenic steroids (AAS)-injected mice in recordings made at 35 °C. (b) Action potential (AP) frequency; * $p < 0.05$. (c) Cumulative probability distributions of AP frequencies. Inset: Kolmogorov-Smirnov 'D' values.

Immunoreactivity for the immediate early gene, c-FOS (Hoffman *et al*, 1993; Sharp *et al*, 1993; Clayton, 2000) can provide an assessment of concurrent neural activity in inter-related brain regions involved in the expression of fear and anxiety (Scicli *et al*, 2004; Veening *et al*, 2009; Skórzewska *et al*, 2008). Surprisingly, whereas electrophysiological recordings demonstrated a significant increase in AP frequency in CeA_L neurons with AAS treatment, c-FOS-IR did not reveal a significant effect of treatment in this area (Figure 3). Such a discrepancy between these two approaches might arise if the AAS promote an increase in electrical activity that occurs within a select subset of CeA_L neurons, so that the differences in activity in this more restricted population are lost in the broader assessment of

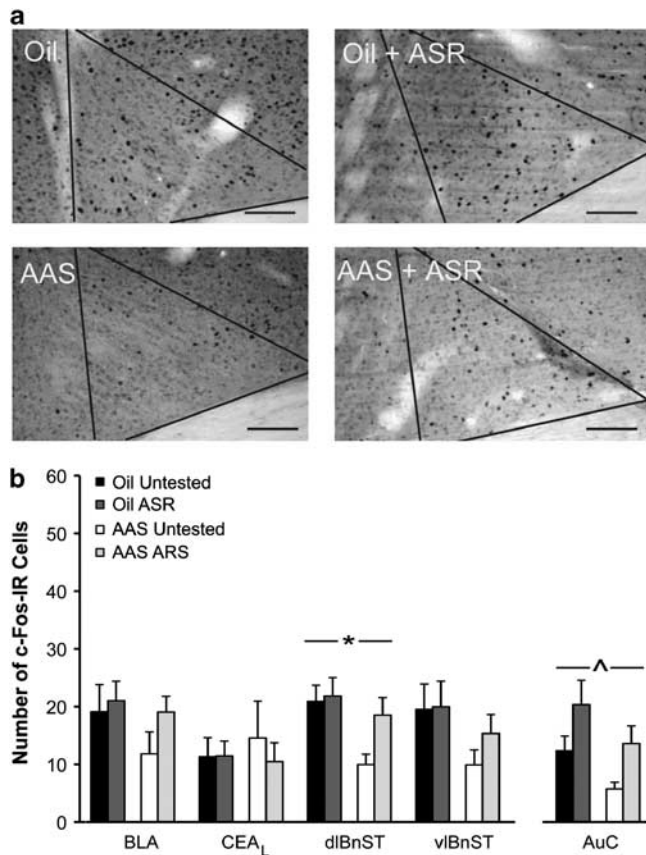


Figure 3 (a) Representative photomicrographs of the dorsolateral bed nucleus of the stria terminalis (dlBnST) (as indicated by the triangular lines) demonstrating c-FOS-immunoreactivity. Scale bar = 50 μ m. (b) Mean number of c-FOS-immunoreactive cells in regions of the extended amygdala and the hypothalamus that have a role in the expression of anxiety and stress as well as the auditory cortex (AuC) ($n=5$ for oil/untreated; $n=8$ for oil/acoustic startle response (ASR); $n=5$ for AAS/untreated; $n=7$ for anabolic androgenic steroids (AAS)/ASR). *Two-way analysis of variance (ANOVA) indicated a significant effect of treatment ($p<0.027$) with the number significantly lower for AAS-treated than oil-injected animals not subjected to testing (untreated; $p<0.01$); for untreated vs ASR-treated animals in the AAS-group ($p<0.04$) and for untreated/AAS-treated vs oil/ASR ($p<0.01$). ^ Two-way ANOVA indicated a significant effect of testing ($p<0.035$).

c-FOS expression. To test whether or not this restricted population may correspond to the neurons within the CeA_L that project to the dlBnST, fluorescent latex microspheres (Retrobeads) were stereotactically injected into the dlBnST, and recordings were then made from retrogradely labeled neurons in the CeA_L (Figure 4a). Labeled neurons localized in the CeA_L were significantly more active in the AAS-treated mice than either non-labeled neurons in AAS-injected mice or either population of neurons in the oil-injected mice ($F_{1,39} = 12.98$, $p<0.001$) (Figure 4b). These data are consistent with the hypothesis that AAS selectively enhance the activity of CeA neurons that project to the dlBnST, and are likely to provide both GABAergic inhibition and CRF input in this region. Interestingly, AAS treatment also promoted a significant shift in the distribution of bursty vs non-bursty cells in the CeA_L projection neurons (Figure 4c). The mechanism underlying this shift is not yet known, but it suggests that the AAS impose changes that may alter the network interactions in this key region either through direct

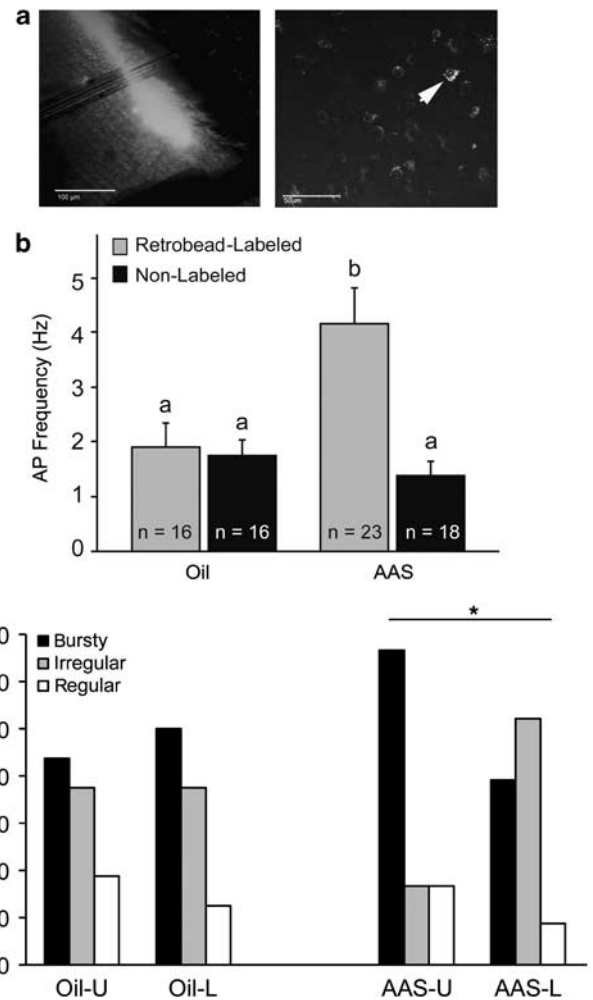


Figure 4 (a) Representative photomicrographs of the Retrobead injection site in the dorsolateral bed nucleus of the stria terminalis (dlBnST) (left), the transverse mesh wire is holding down the coronal slice, and of retrogradely labeled neurons in the lateral part of the central amygdala (CeA_L) (right) as captured by the DAGE-MTI VE-1000 camera on the recording set-up. (b) Average data indicating the mean action potential (AP) frequency in labeled (gray bars) and unlabeled (black bars) neurons from oil-injected and anabolic androgenic steroids (AAS)-injected mice. The 'b' indicates that the value for labeled neurons in AAS-treated mice was significantly different from the other three states ('a'); n values represent the number of cells. (c) The distribution of action potential (AP) patterning amongst labeled (L) and unlabeled (U) neurons from AAS- and oil-injected mice. *The distribution between labeled and unlabeled neurons in the AAS-injected mice was significantly different ($p<0.001$).

effects on the electrical excitability of these neurons or on the synaptic inputs to them.

Neuronal Excitability is Decreased in the dlBnST by AAS Exposure

The BnST is the key site of action of CRF in eliciting diffuse anxiety (Davis *et al*, 1997; Shekhar *et al*, 2005; Davis *et al*, 2010). To determine whether the AAS-dependent increases in anxiety-like behavior is correlated with significant changes in neuronal activity within the dlBnST, on-cell recordings of AP activity were made from neurons within this region. These recordings revealed that the

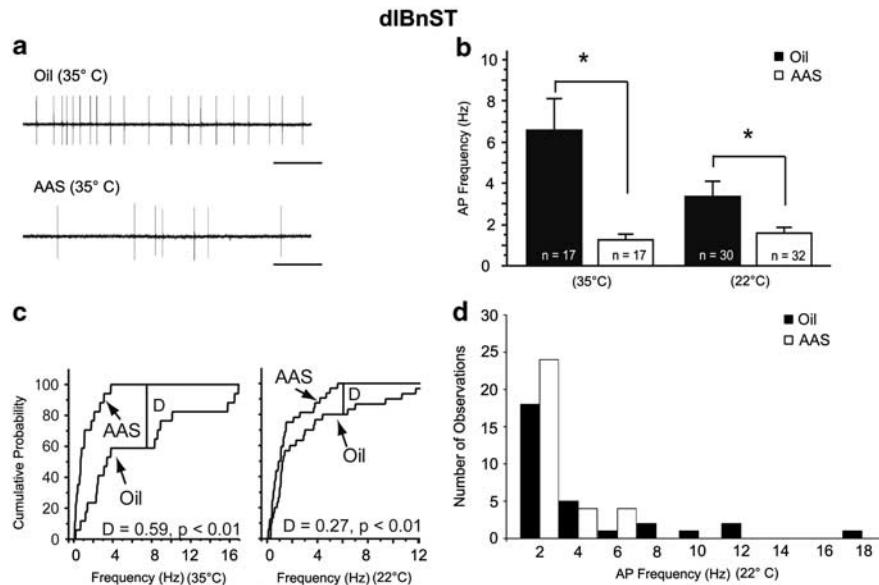


Figure 5 (a) Representative on-cell recordings of action potentials (AP) from the dorsolateral bed nucleus of the stria terminalis (dlBnST) from oil- and anabolic androgenic steroids (AAS)-injected mice in recordings made at 35 °C. (b) AP frequency in recordings from dlBnST neurons at 22 and 35 °C; * $p < 0.05$. (c) Cumulative probability distributions of AP frequencies at 22 and 35 °C. Inset: Kolmogorov-Smirnov 'D' values. (d) Distribution of AP frequencies (22 °C).

frequency of APs in dlBnST neurons was significantly lower in AAS-injected than in oil-injected animals ($F_{1,32} = 16.70$, $p < 0.0003$) (Figures 5a–c). Autocorrelational analysis of firing patterns revealed that irregularly firing cells accounted for the majority of recordings under both treatment conditions, and although the percentage of irregularly firing cells was lower in AAS-treated than oil-injected animals (35.3% vs 52.9%, with a concomitant increase in the percentage of cells with regular firing patterns), the difference was not significant.

Consistent with the electrophysiological assessment in the dlBnST, two-way ANOVA of c-FOS-IR revealed a significant effect of treatment in this region ($F_{1,21} = 5.37$, $p < 0.030$). *Post-hoc* analysis indicated that the number of c-FOS-IR cells was significantly lower in AAS-treated vs oil-injected animals not subjected to behavioral testing (untested; $p < 0.01$), and also lower ($p < 0.04$) for untested mice than those subjected to the startle stimulus in the AAS-treated group (Figures 3a and b). No significant effects of either testing or treatment were observed in c-FOS expression in other regions of the extended amygdala (the BLA and the vlBnST), although, as expected for an auditory stimulus, startle testing elicited a significant increase in c-FOS in the AuC ($F_{1,21} = 5.26$, $p < 0.034$) (Figure 3b). These electrophysiological and immunocytochemical data suggested that AAS exposure might impose a lower basal level of activity in dlBnST neurons, and thus accentuate the response to the startle itself. Such an action of the AAS may arise if there is an enhanced inhibition of dlBnST neurons in the AAS-treated mice.

AAS Exposure Augments GABA_A Receptor-Mediated Inhibition in the dlBnST

We next used whole-cell recording to determine whether the lower level of activity in the dlBnST of AAS-treated mice arose from increased GABAergic inhibition in these neurons.

To allow prolonged recordings of GABA_A receptor-mediated sIPSCs in the dlBnST, these experiments were performed at 22 °C. As a prelude to these whole-cell recordings, we first determined in on-cell recordings that the AAS-dependent decrease in AP frequency in dlBnST neurons was still significant at this lower temperature, and indeed it was (Figures 5a–c). As with recordings made at 35 °C, the mean AP frequency was significantly lower in AAS-treated vs oil-injected animals for recordings made from the dlBnST at 22 °C ($F_{1,60} = 4.88$, $p < 0.031$) (Figures 5a–c). We next assessed whether blockade of GABA_A receptor-mediated transmission abrogated the effects of AAS treatment on AP frequency in the dlBnST by recording from oil-injected and AAS-treated animals in the presence of the non-competitive GABA_A receptor antagonist, picrotoxin (100 μM). In the presence of picrotoxin, the AP frequencies were not different between the two groups (oil: 2.19 ± 0.69 Hz; AAS: 2.89 ± 1.08 Hz; $F_{1,49} = 0.51$, $p = 0.48$). Addition of the glycine receptor antagonist strychnine had no effect on AP frequency (data not shown), further implicating GABA_A receptors as the mediators of AAS-dependent enhanced inhibition in the dlBnST.

Whole-cell recordings revealed that the frequency of GABA_A receptor-mediated sIPSCs was significantly ($F_{1,77} = 24.13$, $p < 0.0001$) higher in recordings from AAS-injected mice than from oil-injected mice (Figures 6a and b). AAS treatment had no effect on sIPSC amplitudes or the time constants of synaptic decay (Figure 5c; legend). Analysis of mIPSCs recorded in the presence of 1 μM TTX revealed no significant differences in the frequency, amplitude, or decay kinetics of these currents (frequency: 1.82 ± 0.27 Hz vs 1.97 ± 0.32 Hz; I_{peak} : 27.2 ± 4.0 pA vs 26.6 ± 3.9 pA; τ_w : 27.7 ± 3.6 ms vs 35.2 ± 3.5 ms in dlBnST neurons from oil-injected vs AAS-treated animals, respectively). These data suggest that AAS treatment augments AP-dependent pre-synaptic release of GABA onto dlBnST neurons, but does not alter postsynaptic sensitivity to GABA.

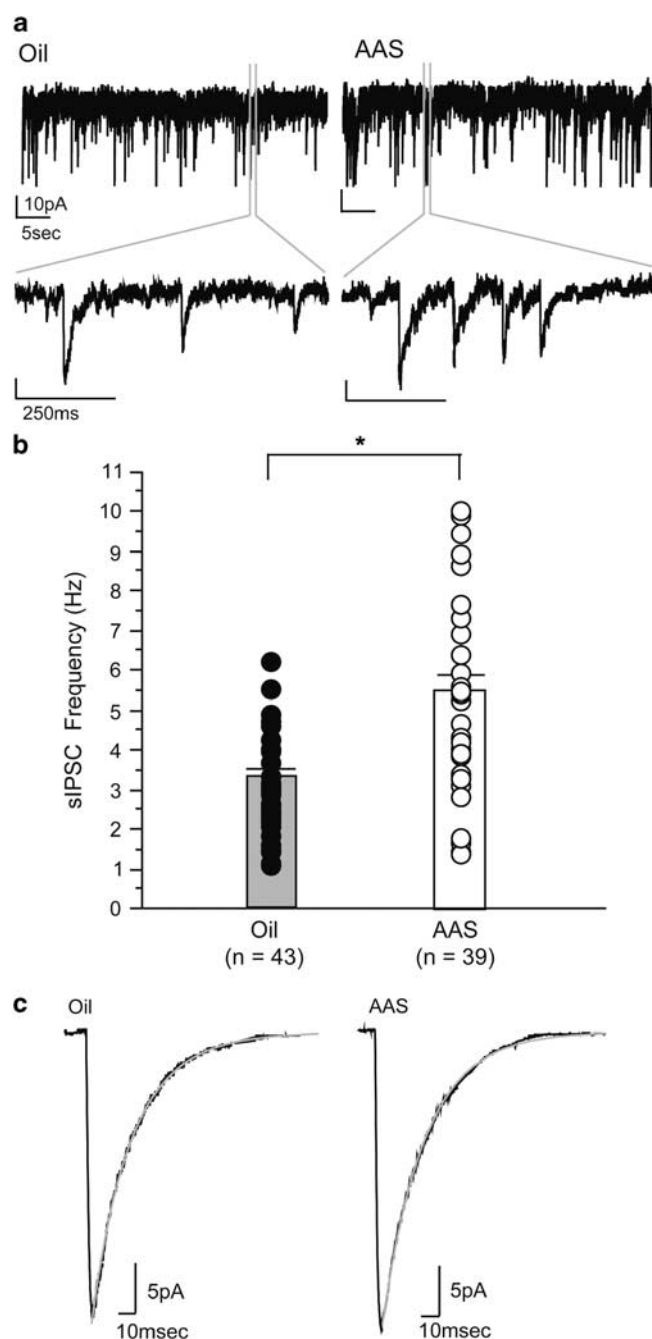


Figure 6 (a) Representative whole-cell recordings of GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) in dorsolateral bed nucleus of the stria terminalis (dlBnST) neurons from oil- and anabolic androgenic steroids (AAS)-injected mice. (b) sIPSC frequencies from individual dlBnST neurons from oil-injected (black circles) and AAS-treated (white circles) mice. Histograms display the same data as means with error bars (SEM); *n* values represent the number of cells. **p* < 0.0001. (c) Averaged sIPSCs from dlBnST neurons under the two treatment conditions. No differences between oil-injected and AAS-treated mice in *I*_{peak} (36.8 ± 2.5 vs 42.0 ± 3.3 pA) or decay kinetics (τ_1 : 18.1 ± 1.1 vs 21.2 ± 1.3 ms), (τ_2 : 76.3 ± 10.0 vs 86.0 ± 11.5) or (τ_w : 33.2 ± 2.9 vs 37.1 ± 2.3 ms) were evident. Fitted line to data reflects τ_w .

We have shown that AAS treatment augments AP firing selectively in CeA_L neurons that project to the dlBnST and separately that AAS augments GABA_A receptor-mediated

responses in dlBnST cells, together suggesting that the enhanced GABAergic drive arises from these CeA afferents. A recognized caveat to this interpretation, however, is that in the slice configuration used here, the cell bodies of the CeA neurons would not have been retained in the BnST slice. CeA neurons may continue to generate spontaneous APs even in the absence of cell bodies if the sites of AP initiation are distal to the somata (see Peng *et al*, 2007; Sheffield *et al*, 2011). Conversely, AAS may augment GABA release from the population of intrinsic interneurons within the BnST. If AAS enhanced the activity of interneurons within the dlBnST itself, we might expect to see a bimodal distribution of AP frequencies: local interneurons whose activity was enhanced by AAS treatment and target dlBnST neurons who received the corresponding increase in inhibitory input. Assessment of the distribution of AP frequencies in dlBnST neurons did not reveal such an effect. At both recording temperatures (Figure 5d; 22 °C and 35 °C; data not shown), the effect of AAS was to shift the distribution to lower frequencies by the loss of cells with the highest firing rates. Moreover, there was no correlation between AP patterning and firing frequency at either temperature. Thus, the enhanced GABAergic drive to dlBnST neurons is likely to either reflect an increase of release from the desomatized CeA afferents and/or afferents arising from other regions of the extended amygdala, including neighboring subregions of the BnST.

The Ability of AAS to Enhance GABAergic Inhibition in the dlBnST is Mediated via a CRF-R1-Dependent Mechanism

AAS treatment augments CRF expression (Costine *et al*, 2010) and activity in CeA_L projection neurons to the dlBnST; neurons that provide not only GABA, but also CRF signaling to the dlBnST (Sun *et al*, 1991; Sun and Cassell, 1993; Arluison *et al*, 1994; Cassell *et al*, 1999). Moreover, our data demonstrate that AAS enhance CRF expression in the CeA_L and that the increase in anxiety-like behaviors observed in ASR testing in AAS-treated mice is abrogated by infusion of the CRF-R1 antagonist, antalarmin (Costine *et al*, 2010; present study). Because CRF has been shown to increase the frequency of GABA_A receptor-mediated sIPSCs in other forebrain regions (Kirby *et al*, 2008; Nie *et al*, 2004, 2009), we hypothesized that enhanced CRF release from CeA_L afferents into the dlBnST might also mediate, via a CRF-R1-dependent mechanism, the AAS-dependent increase in sIPSCs observed in dlBnST target neurons. To test this hypothesis, antalarmin was added to the bath during recordings from dlBnST neurons in acutely isolated brains slices of oil-injected or AAS-treated animals. As previously observed, the frequency of sIPSCs was significantly higher in dlBnST neurons from the AAS-treated than oil-treated groups prior to addition of antalarmin ($F_{1,35} = 15.08$, *p* < 0.001). However, in the presence of 40 μM antalarmin, no significant differences ($F_{1,19} = 0.019$, *p* = 0.89) were evident in sIPSC frequency between oil-injected and AAS-treated mice (Figures 7a and b). Moreover, the effect of antalarmin was found to be due to a significant decrease in sIPSC frequency only in recordings from the AAS-injected group (*p* < 0.001). The effects of antalarmin were reversible upon washout, and addition of this antagonist did not alter either the peak

current or the decay kinetics of the sIPSCs (data not shown). Acute application of 4 μ M antalarmin had an intermediate effect, lowering sIPSC frequency in AAS-treated animals to a value partway between that observed in control solutions and that observed in the presence of 40 μ M antalarmin (Figure 7c). As with the 40 μ M concentration, 4 μ M antalarmin had no effect on the frequency of sIPSCs in dlBnST neurons of oil-injected mice (Figure 7c).

AAS given to mice during the treatment period are likely washed out or substantially diluted during slice preparation. To assess whether direct exposure to CRF (100 nM) altered GABAergic inhibition in the dlBnST and whether the action of CRF was different if AAS were present, recordings were made from dlBnST neurons recordings were made from dlBnST neurons either in aCSF alone or with co-application of AAS from slices of age-matched naïve mice that had not received injections of any type. Real-time PCR analysis indicates that BnST neurons express high levels of GABA_A receptor subunits, including α_1 , α_2 , γ_2 , and γ_1 (data not shown), that we have shown form receptors that are sensitive to acute positive allosteric modulation by the AAS (Yang *et al*, 2002, 2005; Clark *et al*, 2004). Thus, not surprisingly, we

found that acute exposure to the AAS mixture at a concentration (1 μ M) that approximates the concentration measured in the CSF of adult volunteers administered AAS on a moderate, short-term basis (Daly *et al*, 2001), had a modest, but significant effect on the peak amplitude of sIPSCs in dlBnST neurons (22.2 ± 1.4 pA in aCSF; 26.3 ± 1.8 pA in the presence of 1 μ M AAS; $n = 13$) (Figure 8a). Acute AAS application had no significant effect on sIPSC decay kinetics or frequency. Acute exposure of brain slices to 100 nM CRF significantly increased the frequency of sIPSCs in dlBnST neurons in recordings made in aCSF alone (47%; $p < 0.016$) and in neurons in which CRF was applied following exposure to 1 μ M AAS (39%; $p < 0.023$) (Figures 8b and c). CRF was without effect on either the peak amplitudes of the sIPSCs or the decay kinetics of sIPSCs. A similar, albeit somewhat smaller, increase in sIPSC frequency (21%) was also observed in slices exposed to 10 μ M AAS; a concentration that may be present in the CNS in individuals who administer high doses for a prolonged period of time. The percentage of neurons that showed an increase in sIPSC frequency when exposed to CRF that was $>10\%$ was comparable in both recording conditions (70% for saline alone; 69% for saline + 1 μ M AAS). As with sIPSCs, CRF had no effect on the amplitude or frequency of mIPSCs recorded in the presence of 1 μ M TTX. In contrast to sIPSCs, CRF had no significant effect on mIPSC frequency (0.91 ± 0.1 Hz in saline alone vs 0.99 ± 0.15 in saline + CRF, $n = 10$ cells each condition). These results suggest that the actions of CRF in the dlBnST are presynaptic and require AP-dependent release.

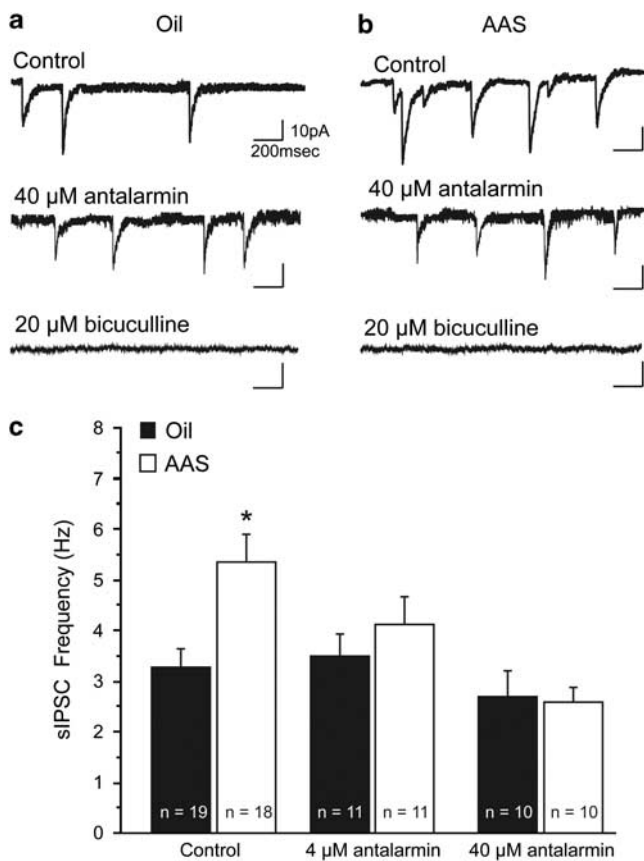


Figure 7 Representative whole-cell recordings of GABA_A receptor-mediated sIPSCs from dorsolateral bed nucleus of the stria terminalis (dlBnST) neurons of (a) oil- or (b) anabolic androgenic steroids (AAS)-injected mice made in artificial cerebrospinal fluid (aCSF) alone (control, top), following the addition of 40 μ M antalarmin (middle) or in the presence of 20 μ M bicuculline (bottom). (c) Average spontaneous inhibitory postsynaptic currents (sIPSC) frequency in the dlBnST in aCSF alone (control) or in the presence of 4 or 40 μ M antalarmin. *Frequency for AAS (control) value significantly ($p < 0.05$) different from oil-injected (control); n values represent the number of cells.

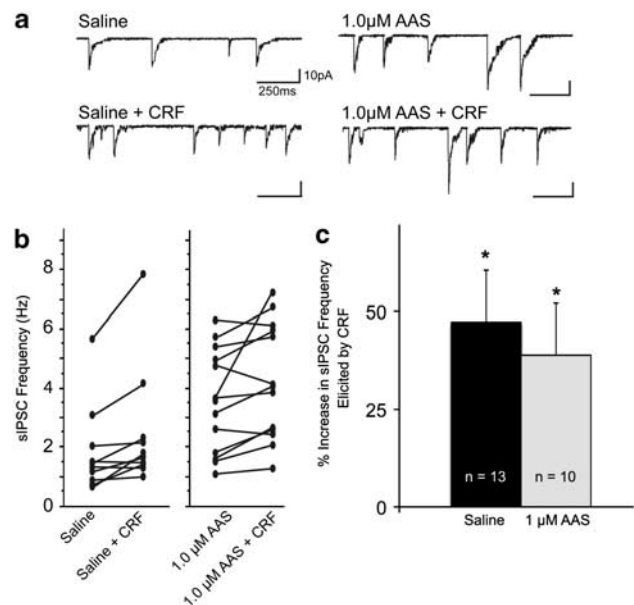


Figure 8 (a) Representative recordings illustrating effects of acute application of corticotropin releasing factor (CRF) on GABA_A receptor-mediated spontaneous inhibitory postsynaptic currents (sIPSCs) from dorsolateral bed nucleus of the stria terminalis (dlBnST) neurons of naïve mice in recordings made in saline alone or saline supplemented with 1 μ M anabolic androgenic steroids (AAS). (b) Data points indicate the change in sIPSC frequency in individual dlBnST neurons exposed to CRF alone (100 nM) or CRF following exposure to 1 μ M AAS. (c) Averaged data indicating the percent increase (\pm SEM) in sIPSC frequency elicited by acute exposure to CRF for dlBnST neurons in saline alone or saline supplemented by 1 μ M AAS. * $p < 0.016$ (saline); $p < 0.023$ (1 μ M AAS). n values represent the number of cells.

DISCUSSION

The prevalence of illicit self-administration of supratherapeutic doses of AAS has been estimated to be approximately 0.5% of adolescent girls and 2% of adolescent boys (Bahrke *et al*, 2000; Miller *et al*, 2005; Johnston *et al*, 2009). In both adults and adolescents, AAS use has been associated with changes in behavior, including anxiety, irritability, poor impulse control, and mood fluctuations, in addition to their actions on physique and performance (Annitto and Layman, 1980; Pope and Katz, 1988; Burnett and Kleiman, 1994; Cooper *et al*, 1996; Franke and Berendonk, 1997; Rashid, 2000; Pagonis *et al*, 2006). Interestingly, human subjects have reported both transient euphoria and hypomania early in the course of AAS administration (Pope and Katz, 1994; Thiblin and Petersson, 2005; Basaria, 2010), and higher levels of anxiety with prolonged, chronic use (Cooper *et al*, 1996; Galligani *et al*, 1996; Hall and Chapman, 2005; Pagonis *et al*, 2006). Consistent with the multiplicity of effects on anxiety observed in humans who self-administer these drugs, treatment of rodents with AAS has also been reported to elicit anxiolytic/diminished fear responses (Bitran *et al*, 1993; Ågren *et al*, 1999; Barreto-Estrada *et al*, 2004; Steensland *et al*, 2005; Frye *et al*, 2008b; Kouvelas *et al*, 2008), anxiogenic/enhanced fear responses (Rocha *et al*, 2007; Agis-Balboa *et al*, 2009; Ambar and Chiavegatto, 2009; Costine *et al*, 2010), or minimal observable effects on anxiety-like behaviors (Rojas-Ortiz *et al*, 2005). The plethora of effects on anxiety-like behaviors is likely to reflect not only the temporal chronology in the exposure to AAS (transient *vs* prolonged exposure), but also a complex matrix of interactions that include, age, sex, the genetic and the social background of the subjects, and the interface of these factors with the types of AAS taken and the paradigms of their administration (Clark and Henderson, 2003; Clark *et al*, 2006; Pinna *et al*, 2008).

Here, we report that chronic exposure of C57BL/6J female mice during adolescence to a mixture of three commonly abused AAS at a dose that reflects a high human abuse concentration augments anxiety-like behaviors. These results are consistent with our prior study in female mice (Costine *et al*, 2010). In contrast, a single acute injection of this mixture of three AAS promoted neither anxiolytic nor anxiogenic effects, suggesting that effects on anxiety of this combination of AAS in these mice require long-term, likely genomic actions. The increase in anxiety in mice chronically treated with AAS was paralleled by a concomitant increase in the expression of CRF and CRF-dependent changes in neural signaling within the extended amygdala, as both the behavioral and the physiological effects of AAS treatment were shown to require signaling through the CRF-R1 (Costine *et al*, 2010; current study). Our findings provide a mechanistic link between a large body of literature highlighting the importance of CRF signaling in the BnST to the production of diffuse anxiety/sustained fear (for review, Reul and Holsboer, 2002; Holsboer and Ising, 2008; Davis *et al*, 2010) and the demonstrated anxiogenic actions of prolonged AAS use (Cooper *et al*, 1996; Galligani *et al*, 1996; Hall and Chapman, 2005; Pagonis *et al*, 2006; Rocha *et al*, 2007; Kanayama *et al*, 2008; Agis-Balboa *et al*, 2009; Ambar and Chiavegatto, 2009; Costine *et al*, 2010). Specifically, our data demonstrate that CRF signaling is required for the

AAS-dependent increase in anxiety-like behaviors and for changes in neural circuits of the extended amygdala that underlie their generation.

Although we cannot rule out the possibility that AAS-dependent changes in CRF expression act to alter synaptic inputs onto neurons in the amygdala itself, previous studies argue against this being the critical site with respect to the AAS-elicited increase in diffuse anxiety and the enhanced startle response. First, our previous study (Costine *et al*, 2010) demonstrated that CRF mRNA is enhanced in CeA neurons, as is somal-associated CRF-IR. However, the overall IR in the CeA is not affected by AAS treatment. These data suggest that the AAS increase CRF expression within CeA neurons, but not in neurons that provide the afferent CRF innervation to the CeA neurons themselves. Second, a substantial body of work from Davis and colleagues supports the postulate that although the CeA is an integral part of the startle circuitry and that afferent disinhibition of CeA neurons is involved in the startle response, the action of CRF in enhancing the startle is localized to the BnST (Liang *et al*, 1992; Davis *et al*, 1997; Walker *et al*, 2009a,b; for review Davis *et al*, 2010). Finally, we note that AAS treatment enhances generalized anxiety as measured in the ASR, but not fear-potentiated startle (Costine *et al*, 2010), again, suggesting that the BnST is the critical site of AAS actions.

Our data also suggest that CRF-R1-dependent changes in GABA_A receptor-mediated transmission between the CeA_L and its major projection site, the dlBnST, is a critical intermediary in the AAS-dependent expression of anxiety. Specifically, AAS treatment was shown to promote increased AP frequency selectively in those CeA_L neurons that project to the dlBnST and to increase presynaptic release of GABA in this region. We note that while AAS do promote this selective increase in firing of CeA projection neurons, CeA somata are unlikely to be present in the slice preparations used for dlBnST recordings. Although we do not know the site of AP initiation in the CeA afferents, and initiation sites quite distal from cell somata have been described in other preparations (Peng *et al*, 2007; Sheffield *et al*, 2011), CRF effects on local GABAergic interneurons must also be considered as the source of the increase in GABAergic sIPSCs recorded in the dlBnST. Irrespective of the source, the enhanced GABAergic tone in the dlBnST is, in turn, associated with a lower level of neural activity in this region, as indicated by both c-FOS expression and the frequency of spontaneous APs. The role of GABA in imposing AAS-dependent effects in the dlBnST was further reinforced by experiments demonstrating that the effects of the AAS on AP activity in the dlBnST could be abrogated by the GABA_A receptor antagonist, picrotoxin, and that CRF-R1 antagonism could negate the AAS-induced increase in sIPSC frequency in dlBnST neurons. This proposed mechanism (Figure 9), by which AAS act to enhance transmission from GABA- and CRF-containing CeA_L afferents, resulting in enhanced GABAergic inhibition imposed on target dlBnST neurons, is consistent with several separate, but interrelated, findings from previous studies. First, CRF-positive terminals originating in the CeA co-express GABA (Veinante *et al*, 1997), and CRF has been shown to augment GABAergic transmission via CRF-R1-mediated signaling in other brain regions (Nie *et al*, 2004, 2009; Kash and Winder, 2006; Bajo *et al*, 2008; Kirby *et al*, 2008; Roberto *et al*, 2010).

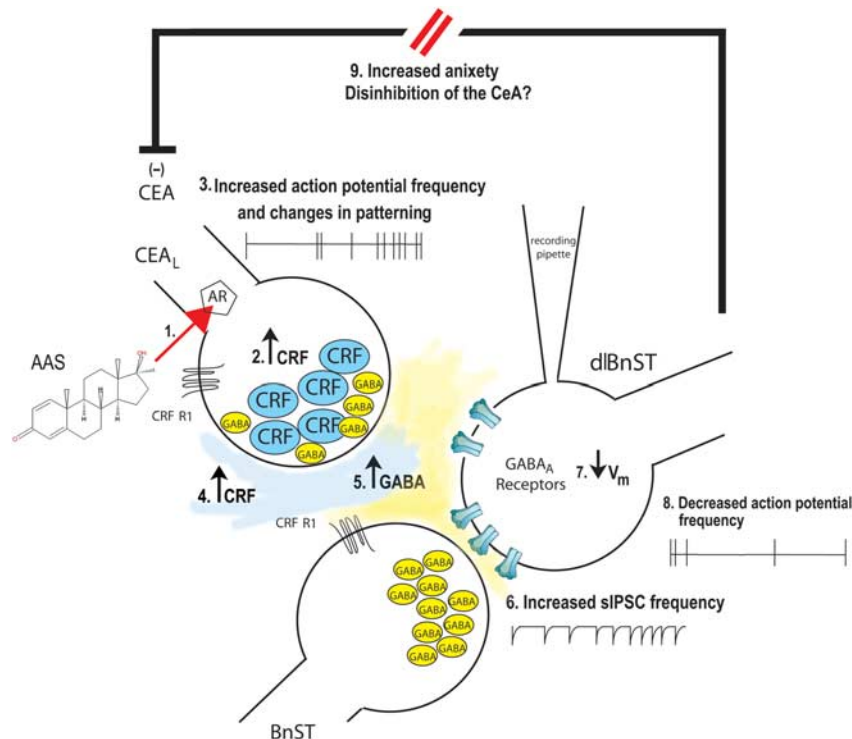


Figure 9 Putative model for anabolic androgenic steroids (AAS) actions in the extended amygdala: Chronic AAS exposure acting through classical androgen receptors (1) increases CRF expression (2) and increases action potential firing/bursting of GABAergic/CRF projection neurons from the lateral central amygdala (CeA_L) (3) that promotes both a direct increase in GABA release and increased release of CRF. CRF acting through the CRF type 1 receptor (CRF R1) (4) on CeA_L or other bed nucleus of the stria terminalis (BnST) terminals further enhances the release of GABA (5). The increased release of GABA is reflected in an increased frequency of sIPSCs (6), hyperpolarization (7), and decreased action potential activity in postsynaptic dorsolateral BnST (dlBnST) neurons (8). The diminished output from the dlBnST gives rise to increased anxiety as measured by the acoustic startle response and on the elevated plus maze, potentially through disinhibition (double red line) of the CeA itself through reciprocal connections (9).

Second, it has been well established that the GABA_A receptor is one of the key molecular targets for drugs that alter the expression of anxiety (for review, Reddy, 2010; Möhler, 2012). Third, steroid-dependent changes in GABA_A receptor-mediated transmission significantly modify anxiety-like behaviors (Shen *et al*, 2007; Frye *et al*, 2008a,b; Smith *et al*, 2009; Maguire and Mody, 2009).

Although our data are consistent with previous studies demonstrating that CRF enhances neurotransmitter release from GABAergic neurons in regions of the extended amygdala (Kirby *et al*, 2008; Nie *et al*, 2004, 2009), our results do diverge from these previous studies in that we found that CRF in the dlBnST enhanced the frequency of sIPSCs, but not mIPSCs. A growing body of literature suggests that the processes leading to mIPSCs, sIPSCs, and evoked IPSCs are not always coordinately regulated (Sara *et al*, 2005; Wasser *et al*, 2007; Wasser and Kavalali, 2009; Fredj and Burrone, 2009; Chung *et al*, 2010), and region-specific effects of CRF may exist between the CeA and the dlBnST. In addition, we note that CRF has been shown to enhance evoked IPSCs in the vlBnST through a postsynaptic mechanism (Kash and Winder, 2006). Taken together, these data raise the possibility that this neuropeptide may have separate synapse-specific mechanisms of augmenting GABAergic inhibition within the different regions of the extended amygdala.

Prior studies have suggested that activity within the BnST as a whole is correlated with enhanced anxiety (for review, Shekhar *et al*, 2005; Hammack *et al*, 2009; Davis *et al*, 2010). Thus, at first blush increased GABAergic inhibition to the dlBnST may seem paradoxical with this proposed role. However, it must be considered that the BnST is a complex and heterogeneous structure in which the neurons make abundant and often reciprocal connections with other structures, as well as with a rich network of local inhibitory circuits (Dong and Swanson, 2005). The majority of GABAergic neurons in the BnST are localized to the dlBnST (Sun and Cassell, 1993). Thus, inhibition of these dlBnST GABAergic neurons may, through local circuit interactions, result in disinhibition of BnST efferents. The CeA not only sends a dense projection to the dlBnST, but receives an equivalently robust one from this region as well (Dong *et al*, 2001; Dong and Swanson, 2005). This reciprocal innervation may promote disinhibition in the CeA, which has been suggested to be an important component of the startle response (Davis *et al*, 1997). Conversely, the AAS may alter glutamatergic excitatory synaptic drive onto CeA neurons either directly or indirectly, or may affect synaptic inputs to these cells by altering the expression of key peptides, such as oxytocin, Neuropeptide Y, arginine vasopressin, and PACAP that have important effects on the expression of anxiety. The AAS are also known to significantly alter the

expression of receptors for serotonin in other brain regions (Grimes and Melloni, 2005; Ricci *et al*, 2006; Schwartz *et al*, 2009). As regulation of 5-HT transmission is the cornerstone of current treatments for anxiety, and changes in serotonergic modulation of transmission within the BnST have been proposed to have an important role in the etiology of pathological anxiety (for review, Hammack *et al*, 2009), steroid-dependent changes in serotonergic signaling within the extended amygdala and its targets may also have an important role in AAS-dependent increases in anxiety.

Androgens are also known to have widespread and significant effects on the expression of voltage-gated channels that regulate electrical excitability (for review, Penatti and Henderson, 2009). Preliminary microarray data from our lab suggests that AAS treatment may alter the expression of several different potassium channel genes (including GIRK3, $K_v1.3$, $K_v3.4$, and HCN4) that are known to regulate repetitive firing behavior. Interestingly, some of these channel genes are selectively expressed in the populations of neurons that exhibit distinctly different firing patterns as assessed in neighboring subregions of the BnST (Egli and Winder, 2003; Hammack *et al*, 2007; Hazra *et al*, 2011). These findings raise the possibility that AAS may also affect the output of the BnST by altering the intrinsic membrane properties of these neurons, although care must be made in extrapolating from studies that have been made in adult male rats to properties of BnST neurons in adolescent female mice, especially as the BnST displays a marked sexual dimorphism (Polston *et al*, 2004; Forger *et al*, 2004).

The signaling mechanisms by which AAS increase CRF in the extended amygdala have yet to be determined. Costine *et al* (2010) demonstrated that AAS treatment results in increases in CRF mRNA in the CeA and CRF peptide in the BnST. Although previous studies have underscored the importance of the androgen receptor in mediating the chronic effects of some of the AAS used in this study in both males and females (Penatti *et al*, 2009a,b) and in regulating the HPA axis response to stress in males (Handa *et al*, 1994), estrogens have also been shown to alter CRF expression *in vivo* and *in vitro* (Paulmyer-Lacroix *et al*, 1996; Lunga and Herbert, 2004; Lalmanasingh and Uht, 2010; for review, Kageyama and Suda, 2009), raising the possibility that estrogen receptors may also have a pivotal role in AAS-dependent changes in CRF in the extended amygdala. The demonstrated ability of androgens to interact directly with estrogen receptor β to affect the stress response (Handa *et al*, 2009) further highlights the complexity in the potential hormone pathways that may mediate the effects of the AAS. In addition, it is possible that AAS may regulate the levels of CRF within the extended amygdala by altering the expression or binding interactions of the CRF-binding protein (Potter *et al*, 1992; Westphal and Seasholtz, 2006). Finally, data demonstrating that down-stream coupling of CRF receptors with different G proteins varies with strain (Blank *et al*, 2003), and that there are sex-specific differences in CRF receptor trafficking (Bangasser *et al*, 2010) highlight that variables such as sex and genetic background are likely to be significant parameters in determining the effects of AAS on the neural circuits that underlie the expression of anxiety.

An interesting facet to emerge from this study is the concept that AAS may impose their effects on the production of

anxious behaviors by lowering the basal level of activity in the dlBnST, as was indicated by both c-FOS and AP frequency. This would be expected to extend the dynamic range of the circuits in this system that regulate the expression of behaviors to an anxiety-producing stimulus. In the hippocampus, it has been proposed that stress associated with a novel environment may impair learning and memory retrieval by saturating the mechanisms that generate glutamate receptor-mediated long-term potentiation (LTP) such that they are limited in their ability to subsequently respond to other stimuli that would normally promote learning (for review, Diamond *et al*, 2005; Kim *et al*, 2006; Howland and Wang, 2008; Collingridge *et al*, 2010). Consistent with this model, CRF has been shown to prevent LTP in the hippocampus (Rebaudo *et al*, 2001). It is provocative to consider a mirror mechanism by which AAS-dependent increases in CRF at the synapse may 'de-saturate' transmission between the CeA and dlBnST by enhancing GABAergic inhibition, extending the response range in this critical circuit in the extended amygdala and thus allowing the more pronounced expression of sustained fear/anxiety to stressful stimuli that was observed in these animals. It will be of great interest to ascertain whether or not chronic AAS exposure also alters glutamate receptor-mediated synaptic plasticity at the CeA to dlBnST synapse as changes in anxiety-like behaviors in animals exposed to social isolation conditions and to ethanol result in blunted LTP in the dlBnST (Conrad and Winder, 2011).

Our results also suggest important and intriguing parallels in the effects of the AAS and the actions of ethanol. Human studies have documented covariability in use of AAS and alcohol (Brower *et al*, 1991; Perry *et al*, 2005; Dodge and Hoagland, 2011), and data from animal models suggest a convergence in behavioral and neurophysiological effects of these two classes of drugs on CRF-dependent modulation of GABAergic transmission. In rodents, increased anxiety and augmented ASR are associated with ethanol withdrawal (Rassnick *et al*, 1992), and the anxiety elicited by ethanol withdrawal is associated with increased levels of CRF release in both the CeA (Merlo Pich *et al*, 1995) and the BnST (Olive *et al*, 2002). Furthermore, the increase in CRF with withdrawal is counteracted by subsequent ethanol intake (Olive *et al*, 2002). Both CRF mRNA and the sensitivity of GABA release to CRF modulation are increased in ethanol-dependent rats (Roberto *et al*, 2004, 2010; Sommer *et al*, 2008), and CRF/CRF-R1-dependent changes in transmission mediated by GABA_A receptors have been implicated as having a major role in anxiety associated with ethanol dependence and in ethanol consumption (for discussion, see Roberto *et al*, 2010). The BnST has been identified as the critical region in CRF-dependent reinstatement of drug-seeking behavior (Erb and Stewart, 1999; Erb *et al*, 2001), and acute injections of ethanol have been shown to elicit increases in c-FOS expression in the lateral BnST in GABAergic neurons (Leriche *et al*, 2008). Finally, injections of GABA_A receptor antagonists into the BnST reduce ethanol responding in rats (Hyytiä and Koob, 1995). These studies, as well as preliminary data from our laboratory indicating enhanced ethanol consumption and preference on the two-bottle test in AAS-treated mice (M. Onakomaiya, unpublished data) strongly support the assumption that there is a common neural basis for the anxiety associated

with ethanol withdrawal and with AAS use. This convergence further suggests that this shared neural mechanism may underlie the covariance in AAS and alcohol use. Future experiments designed to test the interactions of AAS and ethanol on CRF-dependent modulation of GABAergic transmission in the extended amygdala will provide important information to alert AAS users, especially adolescents, that they could be at an increased risk for alcohol abuse.

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DISCLOSURE

The authors declare that, except for income received from our primary employer, no financial support or compensation has been received from any individual or corporate entity over the past three years for research or professional services (with the exception of honoraria for invited talks at other universities and for service to the NSF and the NIH for LPH) and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest.

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