Behavioral/Systems/Cognitive

Altered GABA$_A$ Receptor-Mediated Synaptic Transmission Disrupts the Firing of Gonadotropin-Releasing Hormone Neurons in Male Mice under Conditions That Mimic Steroid Abuse

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Gonadotropin-releasing hormone (GnRH) neurons are the central regulators of reproduction. GABAergic transmission plays a critical role in pubertal activation of pulsatile GnRH secretion. Self-administration of excessive doses of anabolic androgenic steroids (AAS) disrupts reproductive function and may have critical repercussions for pubertal onset in adolescent users. Here, we demonstrate that chronic treatment of adolescent male mice with the AAS 17α-methyltestosterone significantly decreased action potential frequency in GnRH neurons, reduced the serum gonadotropin levels, and decreased testes mass. AAS treatment did not induce significant changes in GABA$_A$ receptor subunit mRNA levels or alter the amplitude or decay kinetics of GABA$_A$ receptor-mediated spontaneous postsynaptic currents (sPSCs) or tonic currents in GnRH neurons. However, AAS treatment significantly increased action potential frequency in neighboring medial preoptic area (mPOA) neurons and GABA$_A$ receptor-mediated sPSC frequency in GnRH neurons. In addition, physical isolation of the more lateral aspects of the mPOA from the medially localized GnRH neurons abrogated the AAS-induced increase in GABA$_A$ receptor-mediated sPSC frequency and the decrease in action potential firing in the GnRH cells. Our results indicate that AAS act predominantly on steroid-sensitive presynaptic neurons within the mPOA to impart significant increases in GABA$_A$ receptor-mediated inhibitory tone onto downstream GnRH neurons, resulting in diminished activity of these pivotal mediators of reproductive function. These AAS-induced changes in central GABAergic circuits of the forebrain may significantly contribute to the disruptive actions of these drugs on pubertal maturation and the development of reproductive competence in male steroid abusers.

Introduction

The anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone whose therapeutic use has been overshadowed by illicit self-administration of excessive doses of these drugs, resulting in adverse effects on reproduction and sexual behaviors (Kam and Yarrow, 2005). AAS abuse by teenagers (Irving et al., 2002; Kanayama et al., 2008) may present even greater risks than for adults, given the greater hormone sensitivity of the brain during adolescence (Sato et al., 2008). Administration of supraphysiological levels of AAS in rodents alters pubertal onset and reproductive behaviors and is associated with suppression of gonadotropin secretion and disruption of the hypothalamic–pituitary–gonadal (HPG) axis (Clark and Henderson, 2003). Activity of gonadotropin-releasing hormone (GnRH) neurons, which constitute the epicenter of HPG control, is highly sensitive to changes in physiological gonadal steroids (Kelly and Wagner, 2002; Moenter et al., 2003; Pielecka and Moenter, 2006; Clarkson and Herbison, 2009), and AAS-dependent changes in their activity may disrupt reproductive function in adolescence.

The AAS comprise a chemically diverse group of steroids that can signal via androgen receptors (ARs) and, after aromatization, via estrogen receptor (ER) α and β. Although ERβ mRNA and protein has been measured in GnRH neurons of male rodents (Hrabovszky et al., 2001), the consensus is that neither ERαs (cf. Hu et al., 2008) nor ARs are expressed. In contrast, neighboring non-GnRH neurons of the medial preoptic area (mPOA), the majority of which are GABAergic (Gao and Moore, 1996; Sagrillo and Selmanoff, 1997), express robust levels of ARs and ERα and moderate levels of ERβ (Lu et al., 1998; Mitra et al., 2003; Nomura et al., 2003; Kudwa et al., 2004; Shah et al., 2004). GnRH neuronal somata in male mice are nestled within a dense surround of GABAergic neurons (Turi et al., 2003; Chen and Moenter, 2009), and neurons within the mPOA and neighboring bed nucleus of the stria terminalis (BnST) are some of the likely sources of these GABAergic afferents in male rodents (Simerly and Swanson, 1988; Hutton et al., 1998). These previous studies have led to the consensus that the effects of gonadal steroids on GnRH function are predominantly indirect (Scott et al., 2000; Grattan et al., 2007; Herbison, 2008), with GABAergic neurons

that supply afferents to the GnRH neurons being among the prime candidates as the targets for steroid action. Although important links between gonadal steroids, GABAergic tone in the mPOA, GnRH neuronal activity, and HPG function have been established in adult male rodents (Grattan et al., 1996; Mitsushima et al., 1999, 2003; Tin-Tin-Win-Shwe et al., 2002, 2004; Pielecka and Moenter, 2006; Chen and Moenter, 2009), the impact of exposure to supraphysiological levels of synthetic androgens during adolescence is unknown. Here we demonstrate that chronic treatment of adolescent male mice with AAS imparts significant effects on GABAergic inputs to GnRH neurons and on the activity of these final effector cells. Such changes are likely to play an important role in the ability of the AAS to disrupt pubertal onset and reproductive maturation during adolescent male development.

Materials and Methods

Drugs and reagents. 17α-Methyltestosterone (17αMT) was purchased from Steraloids. All other drugs and reagents were from Sigma. Salts used for making biological buffers and solutions were mainly from Thermo Fisher Scientific and Sigma.

Animal care and use. Male transgenic mice in which the green fluorescent protein (GFP) is driven from the gonadotropin releasing hormone promoter (GFP–GnRH mice) (Suter et al., 2000) were obtained from an in-house breeding colony at Dartmouth Medical School. GFP expression in this transgenic line allows accurate identification of GnRH neurons in the living tissue by fluorescence microscopy. In particular, 84–94% of GFP fluorescent-GnRH neurons have been shown to be immunopositive for GnRH labeling and are predominantly found within the medial and lateral portions of the mPOA (Suter et al., 2000). All animal care procedures were approved by Institutional Animal Care and Use Committee at Dartmouth, in agreement with the guidelines and recommendations of the National Institutes of Health and American Veterinary Medical Association. All animals were housed in a temperature-controlled and 12 h light/dark cycle facility with lights on starting at 7:00 AM.

Drug treatment paradigm. The median age of steroid initiation in the human population is reported to be 15 years old, with appreciable initiation noted as early as 12 years of age (Bahrke et al., 2000), adolescent ages that precede completion of reproductive maturation. Assessments of the development of the HPG axis in male mice of comparable strain background to the GFP–GnRH animals used in this study suggest that changes associated with reproductive maturation occur as early as weeks 3–4 of postnatal development. Specifically, luteinizing hormone (LH) levels increase from ~10 ng/ml at birth to ~90 ng/ml by postnatal day 24 (P24) (Selmanoff et al., 1977), and balanopreputial separation occurs by P27–P28 (Keene et al., 2002). To determine the effects of AAS exposure during adolescence on reproductive maturation, male GFP–GnRH mice in this study were administered $7.5 \, \text{mg} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ 17αMT in sesame oil 6 d per week beginning on P25–P28 for a period of 4 weeks. This dosage reflects a high human abuse regimen, alters the onset of puberty and inhibits reproductive behaviors in both male and female rats (Clark et al., 2006), and significantly alters testes and seminal vesicle weights in adolescent male mice (McIntyre et al., 2002). Control subjects were administered the same volume (10–30 μl based on body weight) of sesame oil alone. 17αMT was chosen because it is predominantly an androgenic AAS (AAS that are α-alkylated at C_{17} cannot be aromatized to 17β-estradiol) (Winters, 1990; Kochakian and Yesalis, 2000), and these compounds may partially inhibit aromatase *in vitro* (de Gooyer et al., 2003) and *in vivo* (Penatti et al., 2009b).

LH and follicle-stimulating hormone measurements. Quantification of serum levels of LH and follicle-stimulating hormone (FSH) was made according to protocols described previously (Gay et al., 1970; Fallest et al., 1995). Sera from three different cohorts of control and AAS-treated animals were collected from animals also used for electrophysiological recording and were assayed in singlet via radioimmunoassay by the University of Virginia Ligand Assay Core Laboratory (http://www.healthsystem. virginia.edu/internet/crr/). Lower concentration limits for the assays were 20 pg/ml for LH and 0.8 ng/ml for FSH, and the intra-assay coefficient of variation (CV) was 4.9%.

Slice preparation. Coronal sections were prepared using an Electron Microscopy Sciences OTS-4000 vibroslicer as described previously (Penatti et al., 2005, 2009a,b). Briefly, whole brains were quickly removed and placed in ice-cold oxygenated low-sodium solution consisting of the following (in mM): 250 sucrose, 1.2 $CaCl_2$, 10 glucose, 4 KCl, 7 $MgSO_4$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 1 ascorbic acid, pH 7.35. Sections (300 μm) were cut that included the mPOA as defined by Franklin and Paxinos (1997). This area encompasses nearly all of the population of GFP–GnRH neurons, which are mainly localized in its anterior and medial limits. Single identified GFP–GnRH neurons within the mPOA from individual subjects were also harvested and pooled for reverse transcription coupled with quantitative real-time PCR (qRT-PCR).

Electrophysiological recordings. Patch-clamp recordings were performed as described previously according to Penatti et al. (2005, 2009a,b). All recordings were made between 2:00 P.M. and 6:00 P.M. at room temperature. Recordings of spontaneous action potential currents (APs) were made in the loose-patch on-cell configuration (R_{seal} of ~50– 100 MΩ). Slices were superfused with 95%O_2/5%CO_2-saturated artificial CSF (aCSF) (in mM): 125 NaCl, 1.2 $CaCl_2$, 10 glucose, 4 KCl, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 NaH_2PO_4, and 1 of the antioxidant ascorbic acid, pH 7.35 (20–22°C); aCSF was also present in the pipette. APs were recorded for a minimum of 3 min for each experimental condition, resulting in an average total time of recording of 12–20 min for GnRH neurons. Data acquisition was started only after baseline parameters (I_{hold}, R_{seal}) were stable. Data were recorded to tape and subsequently digitized using Acquire 4.0 software. AP frequency and patterning were analyzed using software written locally by Brian L. Jones (Oregon Health and Science University, Portland, OR) in MATLAB 6.5 R13 (MathWorks). Frequency analysis was derived from direct assessment of interspike interval, and patterning was determined using autocorrelation analysis (Penatti et al., 2009a,b) and classified according to Bar-Gad et al. (2001) as regular, irregular, or "bursty." Assignment of autocorrelational profiles to these three groups was found to be correlated with the coefficient of variation of AP firing: the bursty pattern was characterized by high CVs (≥4), the irregular pattern with CVs between 0.5 and 2.5, and the regular pattern with CVs of ≤0.5. The designations of patterning based on the autocorrelogram classifications were found to be in universal agreement of classifications of firing patterns as regular, irregular, or bursty that were independently made from the raw data by an independent observer.

For recordings of GABA$_A$ receptor-mediated spontaneous postsynaptic currents (sPSCs) and tonic currents, recordings were made in aCSF supplemented with 2 mM kynurenic acid to block receptors for excitatory transmission in the whole-cell configuration at a holding potential (V_{hold}) of −70 mV (20–22°C) (Yang et al., 2002; Penatti et al., 2005, 2009a,b). The pipette solution consisted of the following (in mM): 153 CsCl, 1 $MgCl_2$, 5 EGTA, and 10 HEPES, to which 2 MgATP was added just before each experiment. The identity of synaptic currents as GABAergic was confirmed in some experiments by demonstrating blockade of events by the selective GABA$_A$ receptor competitive antagonist bicuculline (20 μM). To record miniature PSCs (mPSCs), 1 μM tetrodotoxin was added to the kynurenic acid-containing aCSF bath solution to block AP-dependent GABA release (Nusser et al., 1997; Hájos et al., 2000). Digitized data were analyzed using MiniAnalysis software (Synaptosoft). Averaged PSCs 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) as described previously (Yang et al., 2002; Penatti et al., 2005, 2009a,b). Average membrane capacitance was 20 pF, average holding current (I_{hold}) was 50 pA, and the average series resistance (R_{series}) was 19 mΩ. Recordings were only accepted for analysis if the seal resistance (>1 GΩ), access resistance (<25 MΩ), and I_{hold} (in the absence of drug application) did not change more than approximately ±10% during the recording. The magnitude of tonic GABA$_A$ receptor-mediated currents (I_{tonic}) was estimated from the difference in the amplitude of the baseline holding current before and after addition of a saturating concentration of the competitive GABA$_A$ receptor antagonist picrotoxin (PTX) (100 μM) (Farrant and Nusser, 2005; Jones et al., 2006). Acquisition of data in the presence of 100 μM PTX was initiated ~1 min after the perfusion of this drug into the bath, and data were acquired for 3–4 min.

For physiological recordings during which we physically isolated GnRH neurons from neurons in the more lateral aspects of the mPOA neurons, a unilateral linear and longitudinal cut was made under fluorescence optics using a stainless steel fine-point microscalpel with a 4 mm edge (Fine Science Tools). The cut was made parallel to the third ventricle border, ran throughout the mPOA lateral to the pool of the GnRH neurons, and ended dorsally at the limits of the anterior commissure (this area is called the "disrupted mPOA" throughout text). A minimum distance of ~50 μm between the lateralmost GnRH neurons and the cut was maintained to preserve the viability/health of the GnRH neurons within the more medial aspect of the slice. Recordings from non-GnRH neurons in the mPOA were made from the central region of the medial preoptic nucleus (MPN) corresponding to the dorsal aspect of the MPN-medial and encompassing the MPN-central, as defined by Franklin and Paxinos (1997, their Figs. 29–32) (see also Penatti et al., 2005). For assessment of the effects of lateral isolation on AP firing frequency, recordings were made from the disrupted mPOA and the intact, contralateral mPOA of the same slice for both control (oil-injected) and AAS-treated animals. Thus, the contralateral intact side served as an internal control for the effects of the cut regardless of treatment condition. Data were analyzed only when recordings were successfully made from GnRH neurons from both the disrupted and the corresponding intact sides from a given mPOA slice. The low probability of meeting acceptable criteria inherent in this procedure resulted in one "pair" of successful recordings (i.e., one GnRH neuron on the cut side and one on the uncut side) per animal per condition. For experiments in which we assessed the effects of PTX on AP activity, independent recordings were made on a disrupted mPOA in one slice and an intact mPOA in a separate slice to avoid potential artifacts arising from the prolonged duration of these recordings or inefficient washout of PTX.

Single-cell qRT-PCR of GnRH neurons. GFP–GnRH neurons were harvested between 2:00 P.M. and 6:00 P.M. from coronal brain slices using borosilicate glass patch pipettes (Sutter Instruments) with tip diameters of ~7–10 μm. The pipettes were filled with aCSF containing 0.5 U/μl RNase inhibitor (Ambion). The GnRH neurons were identified based on their fluorescence in the brain slice under a BX50 fluorescence microscope. Cell debris and tissue contaminants were avoided by aiming for superficial and healthy GnRH neurons (i.e., cells for which fluorescence was stable until the pipette approach and from which processes were partially visible as emerging from the soma). The GnRH neuron harvest was done for matched pairs of control and AAS-treated animals harvested on the same day. For each PCR sample, five GnRH neurons from one animal (typically from the same slice, although in some instances two slices per animal were required) were collected into a 0.5 ml microcentrifuge tube containing 49.5 μl of cell lysis buffer plus 0.5 μl of DNaseI provided in the Ambion TaqManPreAmp Cells-to-CT kit, followed by cDNA reverse transcription, cDNA preamplification (PreAmp), and quantitative real-time PCR for the GABA$_A$ receptor subunit mRNAs. cDNA synthesis and qRT-PCR were performed using minor modifications of procedures described by Li et al. (2008) using the Ambion TaqManPreAmp Cells-to-CT kit. Briefly, the collected cells were lysed at room temperature for 5 min, the reaction was stopped by the addition of 5 μl of Stop Solution, and the mixture was incubated at room temperature for 2 min. RNA (22.5 μl) was reverse transcribed for 60 min at 37°C in a total reaction volume of 50 μl that contained 1× RT buffer and 1× RT Enzyme Mix, followed by enzyme inactivation for 5 min at 95°C. In a 50 μl reaction volume, 12.5 μl of cDNA from five single cells was preamplified in a reaction solution containing 25 μl of TaqMan PreAmp mix, 0.2× pooled primer/probe sets (TaqMan Gene Expression Assays) commercially available for the GABA$_A$ receptor subunits α1 (Mm00439040_m1), α2 (Mm00433435_m1), α5 (Mm00621092_m1), β1 (Mm00433461_m1), β2 (Mm00549788_s1), β3 (Mm00433473_m1), δ (Mm00433476_m1), ε (Mm00489932_m1), γ1 (Mm00439047_m1) and γ2 (Mm00433489_m1), GnRH (Mm01315605_m1), and β-actin as the endogenous control (Mm00607939_s1) (Applied Biosystems). The reaction was preamplified with an initial denaturation step for 10 min at 95°C, followed by 10 cycles of PCR at 95°C for 15 s, 60°C for 4 min. Preamplified product was diluted 1:5 with 1× Tris/EDTA buffer, and qRT-PCR was performed for each TaqMan Gene Expression Assay indi-

vidually. Reaction (20 μl) containing 1× TaqMan Gene Expression Master Mix, 1× TaqMan Gene Expression Assay, and 5 μl of diluted PreAmp product was run on an Applied Biosystems 7500 real-time instrument with an initial denaturation step for 10 min at 95°C, followed by 40 cycles at 92°C for 15 s, 60°C for 1 min. The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001; Peirson et al., 2003) was used for quantification of subunit mRNA. All primers used were demonstrated according to protocols in the TaqManPreAmp Cells-to-CT kit to amplify with equal efficiencies. Samples with reverse transcriptase omitted were used to control for genomic DNA contamination and a no-template control to control for any reagent contamination. GnRH transcripts were always detected in samples of GnRH neurons, and these transcripts were not detected in single-cell harvests made from non-GnRH neurons. Similarly, GnRH transcripts were not amplified from mock harvests in which the pipette was placed on the surface of the GnRH neuron or in samples of aCSF solution superfusing the slices.

Statistical analyses. Values are presented as means ± SE. To test for normality, Shapiro–Wilks or Kolmogorov–Smirnov tests were applied on the raw data. For serum LH and FSH assays, normally distributed values were accepted that fell within the first and third quartile of all analyzed samples from each group. For qRT-PCR analysis, C_T values were defined as outliers when they lay outside ±3 SDs from the mean. Results were qualitatively the same whether or not outliers were included in the final analysis. Differences in the relative abundance of each mRNA between control and AAS-treated subjects were assessed using pairwise fixed reallocation randomization t test using REST (Excel-based Relative Expression Software Tool) (Pfaffl, 2001; Pfaffl et al., 2002). For mRNA analysis, only positive error bars are depicted in the results; positive and negative error bars differed by ≤20%. For electrophysiological experiments, non-normally distributed data were log transformed before statistical assessment. Significance for electrophysiological data and gonadotropin (LH/FSH) measurements was determined by one-way ANOVA, followed by *post hoc* analyses using either Tukey's or Fisher's tests for means comparison (Origin8Pro; OriginLab). The same statistical tests were directly applied on all normally distributed data as well. For all data, the α level was set at $p < 0.05$. Except when indicated to the contrary, n values indicate the number of neurons per condition.

Results

AAS treatment decreases electrical activity in GnRH neurons and lowers serum LH and FSH of male mice

On-cell recordings from GnRH neurons within the mPOA of male mice were performed to assess spontaneous AP currents. After AAS treatment, the average AP frequency in GnRH neurons was significantly decreased ($p = 2.03 \times 10^{-4}$) from 1.15 ± 0.25 Hz in control animals to 0.36 ± 0.06 Hz in AAS-treated mice (Fig. 1). Autocorrelational analysis demonstrated that bursty firing patterns were evident in ~61% and irregular firing patterns in 39% of the GnRH neurons from control subjects. AAS treatment did not significantly alter the relative proportion of cells with bursty versus irregular firing (73 and 27%, respectively). Whereas the average frequency of AP firing was decreased with AAS treatment for cells with both patterns of firing, the decrease attained significance only for cells with bursty patterning ($p = 4.19 \times 10^{-5}$). The AAS-dependent decrease in firing frequency in bursty GnRH neurons correlated with a diminution in the number of APs per burst from 10.4 ± 3.0 in GnRH neurons from control subjects to 6.6 ± 0.8 in GnRH neurons from AAS-treated subjects. Consistent with previous reports, intraburst and interburst intervals were variable (Kelly and Wagner, 2002), and there was no effect of AAS treatment on these parameters.

Activity of GnRH neurons has been shown to be required for GnRH secretion and control of the peripheral signaling that regulates reproduction (Roseweir et al., 2009). Concomitant with the decrease in AP frequency in GnRH neurons, we observed lower levels of LH and FSH levels in AAS-treated versus control

mice (Table 1). The observed decrease in FSH attained significance ($p = 0.005$). Although the mean value of LH in AAS-treated animals was ~33% that observed in control animals (Table 1), these differences were not significant. The absence of statistical significance for serum LH levels arises from a large degree of interanimal variability in both treated and control groups. This variability likely reflects the fact that blood was collected as a single point assay, but LH release in male rodents is pulsatile. Continuous serum sampling indicates that LH peaks occur with a frequency of tens of minutes to a few hours and that both peak amplitude and pulse frequency depend on the level of serum testosterone (Bartke et al., 1973; Steiner et al., 1982; Pierroz et al., 1999). Sampling at a single point from individual subjects in different phases of this pulsatile pattern will therefore introduce variability in the determination of mean LH levels. AAS treatment did result in a significant decrease in testes mass (Table 1) ($p = 7.506 \times 10^{-4}$), consistent with an AAS-dependent diminution in the levels of both gonadotropins.

AAS treatment alters presynaptic GABAergic inputs but not postsynaptic GABA_A receptors in GnRH neurons

GnRH neurons are ubiquitously sensitive to GABA and express a broad repertoire of GABA_A receptor subunit genes (Clarkson and Herbison, 2006). The most prevalent GABA_A receptor subunit mRNAs expressed by GnRH neurons are reported to be α_1, α_2, α_5, β_1, β_2, β_3, γ_1, and γ_2 (Sim et al., 2000; Pape et al., 2001, Todman et al., 2005). In addition to these transcripts, we also assessed levels of ε and δ subunit mRNAs in identified GnRH neurons. Although levels of these ε and δ subunit mRNAs have not been assessed previously in GnRH neurons, immunocytochemical studies have indicated preferential expression of ε subunits in GnRH neurons and a limited number of other peptidergic cells (Moragues et al., 2003). Moreover, both ε- and δ-containing receptors may play important roles in receptors that mediated extrasynaptic tonic currents (Henderson, 2007). All of the GABA_A receptor subunit mRNAs assayed were detected in identified GnRH neurons in both control and AAS-treated male mice. AAS treatment did not significantly alter the levels of any of the individual subunit mRNAs (Fig. 2). A parallel cohort of control and AAS-treated animals revealed that GnRH mRNA was robustly expressed in the GFP-positive cells, but, as with the GABA_A receptor subunit mRNAs, AAS treatment did not significantly alter the levels of GnRH transcripts (Fig. 2).

AAS treatment may promote posttranslational modifications or alter the association of receptors with other postsynaptic components that may impose significant differences in receptor function independent of any effects on subunit composition. To further assess the effects of AAS treatment on GABAergic transmission to GnRH neurons, recordings were made of GABA_A receptor-mediated sPSCs and tonic currents in identified GnRH cells. These recordings indicated that
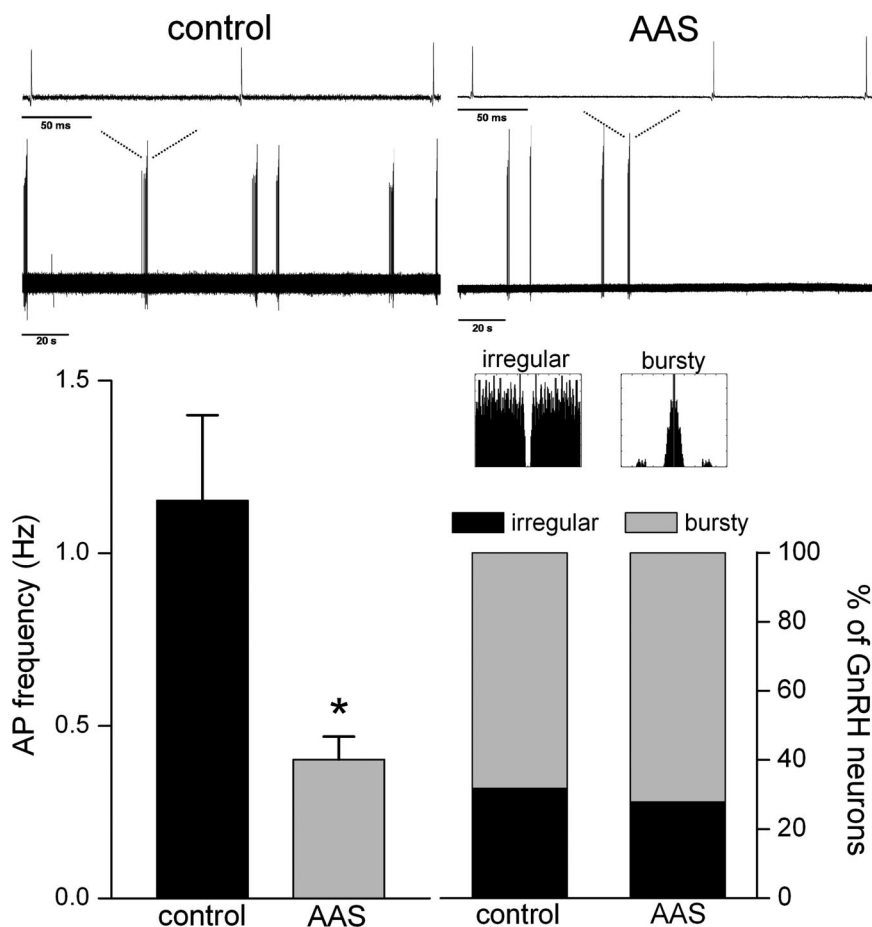

Figure 1. AAS-dependent effects on AP firing in GnRH neurons from control and AAS-treated mice. Top, Three continuous minutes of recording in the loose-patch on-cell configuration demonstrating bursty AP firing from control and AAS-treated subjects from the same cohort depicting the differences of AP frequency; insets show individual APs within the burst. Display of responses on the left was scaled to match amplitudes of those on the right. Bottom left, AP frequency is decreased (*$p = 2.03 \times 10^{-4}$) in GnRH neurons from AAS-treated (gray; $n = 18$ cells) versus control (black; $n = 22$ cells) male mice. Bottom right, AAS treatment did not alter AP patterning in GnRH neurons. Inset, Representative autocorrelograms corresponding to irregular and bursty firing patterns.

Table 1. Serum gonadotropins and testes mass in control and AAS-treated male mice

	LH (pg/ml)	FSH (ng/ml)	Testes mass (mg)
Control	222.8 ± 12.1 ($n = 9$)	17.3 ± 1.2 ($n = 9$)	172.4 ± 2.7 ($n = 8$)
AAS	71.9 ± 4.1 ($n = 10$)	12.4 ± 1.0 ($n = 10$)	150.3 ± 4.1 ($n = 8$)

Means $\pm$ SEs of the levels of serum LH and FSH and testes mass from control and AAS-injected mice. n values indicate numbers of animals used for each assay per treatment condition.

AAS treatment was without effect on the amplitude (I_{peak} of 23.8 ± 1.5 pA for control and 24.3 ± 1.7 pA for AAS treated) or the decay kinetics (τ_w of 38.9 ± 4.4 ms for control and 39.4 ± 2.9 ms for AAS treated) of sPSCs (Fig. 3A). Assessment of mPSCs also indicated that AAS treatment was without effect on either I_{peak} (23.8 ± 1.7 pA for control and 24.9 ± 2.9 pA for AAS treated) or τ_w (39.0 ± 4.1 ms for control and 40.2 ± 4.2 ms for AAS treated). Finally, the magnitude of GABA_A receptor-mediated tonic currents was also unchanged by AAS treatment: 3.83 ± 0.97 pA for control ($n = 6$ cells) versus 3.64 ± 0.63 pA for AAS treated ($n = 8$ cells), with no differences in holding currents in either group. Together, these data indicate that AAS treatment was without an appreciable effect on the complement or function of postsynaptic GABA_A receptors expressed in GnRH neurons.

AAS treatment did, however, result in a significant ($p = 0.014$) increase in the frequency of sPSCs (0.10 ± 0.01 Hz) compared with control (0.062 ± 0.02 Hz) (Fig. 3B). The increase in
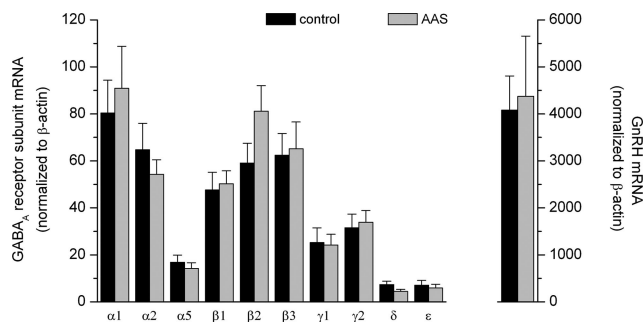
Figure 2. AAS-dependent effects on GABA_A receptor subunit and GnRH mRNA levels in GnRH neurons from control and AAS-treated mice. Data are presented as the $2^{-\Delta C_T}$, which indicate the average levels (relative to the housekeeping gene β-actin) of subunit mRNAs in GnRH neurons isolated from control (black; $n = 10$ mice) and AAS-treated (gray; $n = 10$ mice) for analysis of GABA_A receptor subunit mRNA levels and from a separate cohort of seven control and seven AAS-treated mice for analysis of GnRH mRNA levels.
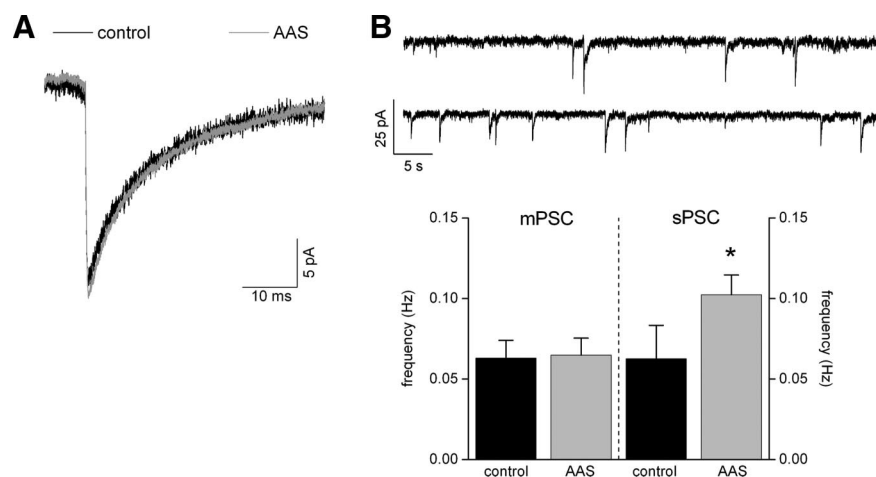

Figure 3. AAS-dependent effects on GABA_A receptor-mediated miniature and spontaneous PSCs in GnRH neurons. **A**, Averaged sPSCs recorded from GnRH neurons in slices isolated from control (black line) and AAS-treated (gray line) subjects ($n = 12$ neurons for control and $n = 13$ neurons for AAS-treated). **B**, Top, Representative sPSCs recorded from a GnRH neuron in a control and an AAS-treated mouse. Bottom, Average frequencies of sPSCs and mPSCs in GnRH neurons from control (black) and AAS-treated (gray) mice (data are from the same cells as shown in **A**). Asterisk indicates the sPSC frequency is greater in AAS condition ($p = 0.014$).

the number of GABA_A receptor-mediated synaptic events was evident only for sPSCs; AAS treatment did not alter the frequency of mPSCs (~0.06 Hz) (Fig. 3B), suggesting that AAS treatment may alter the activity, but not the number, of presynaptic GABAergic inputs to these GnRH targets.

AAS alters AP firing of non-GnRH mPOA neurons

The data presented above are consistent with the prevailing models that indicate that the effects of physiological steroids on GnRH neurons are likely to occur via the steroid-sensitive neurons within the basal forebrain/anterior hypothalamus that make synaptic inputs on these cells (Scott et al., 2000; Herbison, 2008). In this coronal slice orientation, neuronal networks encompassing steroid-sensitive mPOA neurons are likely candidates for the source of GABAergic afferents to GnRH neurons in the male rodent (Simerly and Swanson, 1988; Gao and Moore, 1996; Hutton et al., 1998). These neurons are also likely, because of their high level of expression of ARs (Lu et al., 1998; Shah et al., 2004), to be primary targets for the direct action of 17αMT, resulting in the observed changes in GABAergic tone to postsynaptic GnRH cells. Recordings were therefore also made to determine the effects of chronic exposure to 17αMT on non-GnRH neurons within the MPN of the mPOA.

Treatment of adolescent male mice with 17αMT significantly ($p = 0.015$) increased AP firing by these mPOA neurons from 2.6 ± 0.33 Hz for control to 3.4 ± 0.35 Hz for AAS-treated mice (Fig. 4). Autocorrelational analysis indicated that AAS treatment also decreased the percentage of cells displaying irregular firing patterns and concomitantly increased the percentage displaying regular firing in mPOA neurons (Fig. 4), although this trend did not attain significance ($p = 0.090$ for the decrease in irregular firing).

Disruption of inputs from mPOA neurons abrogates AAS-induced changes in presynaptic GABAergic tone and AP frequency in GnRH neurons

Data presented above suggest that long-term treatment with 17αMT increased the activity of presynaptic GABAergic mPOA neurons and thus their inhibitory drive onto GnRH neurons. To confirm that the AAS-mediated increase in sPSC frequency in GnRH neurons indeed arose from mPOA neuron afferents, whole-cell recordings were made from GnRH neurons that were isolated from their putative lateral presynaptic partners (e.g., ipsilateral mPOA projections) within a disrupted mPOA (see Materials and Methods) (Fig. 5A). AP recordings from the uncut (unmanipulated) mPOA on the contralateral side of the slice were made in tandem with recordings from the disrupted side to be able to compare the effects of this surgical deafferentation in a single control or single AAS-treated animal.

Acute interruption of inputs from the more lateral regions of the mPOA (inclusive of the MPN) completely abrogated the AAS-induced increase in GABA_A receptor-mediated sPSC frequency from a value of 0.10 ± 0.01 Hz in the intact side to 0.05 ± 0.01 Hz in the disrupted mPOA ($p = 0.023$) (Fig. 5B), a value that was not significantly different from that observed in the intact mPOA of control subjects (0.063 ± 0.02 Hz; $p = 0.718$). As important, disruption of the more lateral mPOA elicited mirrored changes in AP frequency in GnRH neurons: the AP frequency within the disrupted mPOA of AAS-treated mice (0.88 ± 0.15 Hz) was significantly higher ($p = 0.020$) than that observed in the intact mPOA (0.33 ± 0.05 Hz). Thus, as with sPSCs, acute interruption of lateral mPOA afferents restored the average AP frequency to a level not significantly different from that observed in GnRH neurons in the intact mPOA of control subjects (1.11 ± 0.17 Hz; $p = 0.834$) (Fig. 5C). Interruption of inputs from the lateral mPOA had no effect on either peak amplitude or decay kinetics of PSCs, corroborating the conclusion noted above that the effects of AAS treatment are predominantly presynaptic. Although interruption of connections from the more lateral regions of the mPOA elicited changes of comparable direction in control subjects (i.e., a decrease in sPSC frequency and a concomitant increase in AP frequency), in neither case was the effect significant (Fig. 5B,C).

GABAergic projections from the mPOA are the critical source of increased inhibition for GnRH neuron activity in male mice under chronic AAS abuse conditions

GnRH neurons belong to a complex network comprising not only local GABAergic afferents from the mPOA but also neurochemically heterogeneous inputs from other steroid-sensitive hy-
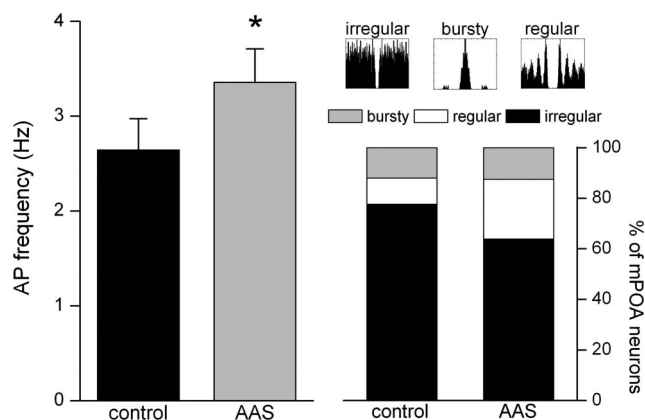
Figure 4. AAS-dependent effects on AP firing in non-GnRH mPOA neurons. Left, AP frequency in mPOA neurons from control (black; $n = 72$ neurons) and AAS-treated (gray; $n = 58$ neurons) mice. Right, Representative autocorrelograms corresponding to bursty, regular, and irregular firing patterns and the percentages of neurons characterized by each firing pattern in control and AAS-treated mice. Asterisk indicates the AP frequency is greater in AAS condition ($p = 0.015$).

pothalamic/forebrain nuclei that are likely to be retained in this coronal slice, including the anteroventral periventricular area (AVPV) (Hahn and Coen, 2006; Herbison, 2008), BnST (Simerly et al., 1990; Pompolo et al., 2002) and the suprachiasmatic nucleus (Gu and Simerly, 1997; Van der Beek et al., 1997). Therefore, to examine the importance of GABAergic projections in mediating the actions of AAS on GnRH neuron activity, we next assessed the impact of pharmacological blockade of GABAergic transmission on AP frequency in GnRH neurons and its dependence on the physical integrity of mPOA.

On-cell recordings from GnRH neurons were made in the presence of the GABA$_A$ receptor antagonist PTX (100 μM). Consistent with data discussed above indicating that interruption of GABAergic afferents from the more lateral mPOA did not significantly alter sPSC or AP frequency in control animals, pharmacological inhibition of GABA$_A$ receptors did not significantly alter AP frequency in either the intact or the disrupted mPOA of control subjects (Fig. 6A). Conversely, in AAS-treated subjects, blockade of GABA$_A$ receptors by PTX significantly increased AP frequency in GnRH neurons in the intact mPOA ($p = 0.019$) (Fig. 6B) but was without effect in the disrupted mPOA. Moreover, as predicted if the action of the AAS is to enhance inhibitory GABAergic inputs from more lateral mPOA afferents, pharmacological blockade of GABA$_A$ receptors resulted in AP firing rates in treated animals that were not statistically different in the intact versus the disrupted sides (Fig. 6B).

Discussion

Self-administration of high doses of AAS in men and boys has been associated with changes in libido and a hypogonadal state characterized by diminished levels of serum LH and FSH, testicular atrophy, and decreased sperm production (Kam and Yarrow, 2005). Effects of exposure to AAS on pubertal onset in human subjects has not been assessed, but exposure to 17αMT decreased testes weight in adolescent male mice (McIntyre et al., 2002) and advanced vaginal opening, delayed the day of first estrus, and suppressed estrous cyclicity in adolescent female rats (Clark et al., 2006).

The mechanisms by which chronic AAS exposure disrupts the HPG axis are unknown and may reflect both peripheral effects on gonadal tissues and central effects on neuronal circuits in the forebrain. The mPOA is a key site for the regulation of behaviors
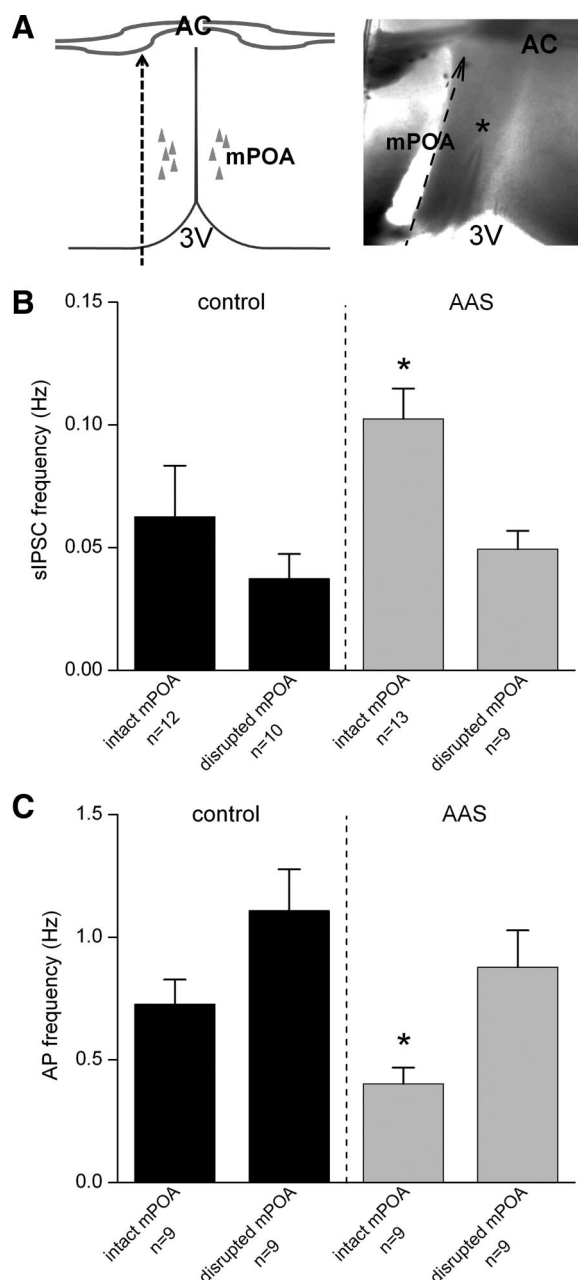

Figure 5. Effects of acute interruption of inputs from the more lateral regions of the mPOA on GABA$_A$ receptor-mediated sPSC and AP firing frequencies in GnRH neurons in control and AAS-treated mice. *A*, Illustration depicting the bilateral distribution of GnRH neurons (gray triangles) and the location of longitudinal cuts (as indicated by the dotted line and arrow) made to physically isolate these neurons from their upstream lateral afferents in the mPOA (right) and representative slice that shows the intact and disrupted mPOA (right). Asterisk indicates electrode tip poised above the GnRH neurons in disrupted mPOA. 3V, Third ventricle; AC, anterior commissure. *B*, Averaged data demonstrating sPSC frequency in GnRH neurons from control (black) and AAS-treated (gray) mice in recordings from intact and disrupted mPOAs. *n* values indicate numbers of neurons for each condition. Asterisk indicates that sPSC frequency was significantly greater ($p = 0.023$) in the intact mPOA than in the disrupted mPOA of AAS-treated mice. *C*, Averaged data demonstrating AP frequency in GnRH neurons from control (black) and AAS-treated (gray) mice in recordings from intact and disrupted mPOAs. *n* values indicate numbers of neurons for each condition. Asterisk indicates that sPSC frequency was significantly lower ($p = 0.020$) in the intact mPOA than in the disrupted mPOA of AAS-treated mice.

in male rodents (Hull and Dominguez, 2007), and GABAergic activity from the mPOA exerts critical control over reproductive function in males (Fernández-Guasti et al., 1985; Seltzer and Donoso, 1992; Ojeda et al., 2006). Here we show that chronic
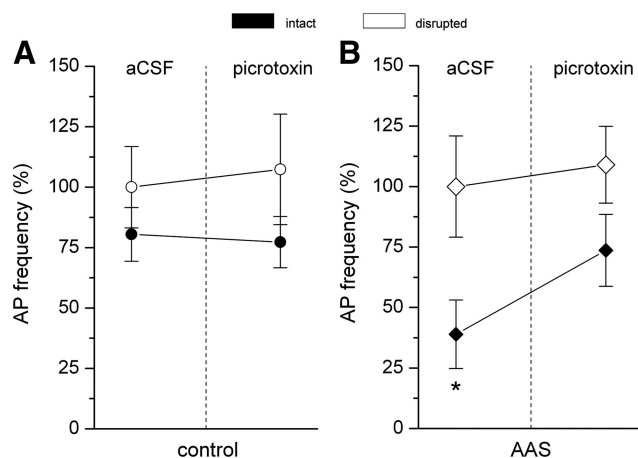
Figure 6. Blocking GABA$_A$ receptor function enhances GnRH neuron AP firing rate only in the intact mPOA from AAS-treated male mice. Recordings of APs were made from GnRH neurons under the same conditions as described in Figure 5C before and after the addition of 100 μM PTX. Data from each group (control and AAS-treated) were normalized to their respective average AP frequency from disrupted mPOA in aCSF (100%). *A*, PTX did not alter AP firing in control subjects from either intact (n = 10; filled circles) or disrupted (n = 10; open circles) mPOA. *B*, Conversely, in AAS-treated subjects, AP frequency was significantly (*p = 0.019) increased with PTX application in GnRH neurons from the intact mPOA (n = 10; filled diamonds) but was without significant effect in the disrupted mPOA (n = 10; open diamonds). For AAS-treated animals, AP firing rates were comparable in slices in which GABAergic inputs were physically interrupted (disrupted POA) and in those in which the mPOA was intact, but GABA$_A$ receptor-mediated transmission was blocked.

exposure of adolescent male mice to high doses of 17αMT significantly enhanced AP firing frequency of non-GnRH neurons within the mPOA. The increase in the activity of mPOA neurons occurred in tandem with enhanced GABA$_A$ receptor-mediated sPSC frequency in putatively downstream GnRH neurons, diminished AP firing in these neuroendocrine effectors, and decreased the concentration of gonadotropins in the serum of treated mice.

Previous studies have shown that the anterorostral portion of the mPOA is composed of a dense population of GABAergic neurons (Gao and Moore, 1996; Sagrillo and Selmanoff, 1997). These GABAergic neurons within the mPOA, unlike GnRH neurons themselves, are markedly steroid sensitive and express high levels of ARs (Lu et al., 1998; Shah et al., 2004). The presence of the C_{17} methyl group precludes aromatization of 17αMT to 17β-estradiol (Winters, 1990; Kochakian and Yesalis, 2000), suggesting that signaling by this AAS is heavily weighted to AR versus ER. Our data indicate that the most likely mechanism by which 17αMT exerts its action on the HPG axis is via enhancement of the activity of presynaptic GABAergic neurons within the mPOA, which, in turn, promote decreased activity of downstream GnRH neurons. The mechanism by which this AAS alters presynaptic activity is not known but may involve changes in the expression and/or function of voltage-gated conductances. An attractive candidate for such regulation may be small-conductance calcium-activated potassium (SK) channels, because inhibition of these channels decreases afterhyperpolarization (AHP) and increases AP frequency in GABAergic neurons within the mPOA (Wagner et al., 2000, 2001). SK channel activity is also of critical importance in regulating the AHP and AP firing in GnRH neurons themselves (Liu and Herbison, 2008). Our data, however, demonstrate only a modest (and not significant) effect of AAS effect on AP patterning in GnRH neurons in the intact slice and abrogation of AAS-mediated effects on GnRH firing by disrup-

tion of lateral afferents. These results are most consistent with a presynaptic effect of this AAS on GABAergic afferents rather than a direct action on the GnRH neurons themselves.

Effects of chronic steroid exposure on AP firing are likely to involve AR-mediated signaling because comparable effects were also observed in wild-type, but not AR-deficient, male mice treated with a mixture of AAS (Penatti et al., 2009b). However, alternative actions are also possible. Specifically, some of the AAS, including 17αMT, can inhibit aromatase activity (de Gooyer et al., 2003; Penatti et al., 2009b) and thus may interfere with endogenous estrogenic signaling in the mPOA, signaling that provides negative feedback on GnRH neuronal activity (Pielecka and Moenter, 2006). AAS may also allosterically modulate GABA$_A$ receptors in these neurons (Henderson, 2007) and may have the potential, as yet untested, to signal through membrane ARs. Estrogens have been shown to have important nongenomic actions on GnRH neuron function (Abrahám et al., 2004; Abe and Terasawa, 2005; Abrahám and Herbison, 2005; Abe et al., 2008; Chu et al., 2009). The nongenomic actions of estrogens in GnRH neurons are mediated via cell signaling pathways that are used by membrane ARs in other cell systems (Nguyen et al., 2005), suggesting a point of potential convergence for nongenomic androgen and estrogen actions. Of particular note, estrogens have been shown to act via a nongenomic mechanism to regulate AP-independent release of GABA from presynaptic afferents to GnRH neurons (Romanò et al., 2008).

The critical role played by GABAergic neurons within the mPOA in mediating AAS effects on GnRH neuronal function is supported by experiments demonstrating that the AAS-dependent suppression of GnRH neuronal activity and AAS-dependent enhancement of GABA$_A$ receptor-mediated sPSC frequency was evident only when the mPOA circuitry lateral to the GnRH neurons was intact. Disruption of these lateral afferents restored both sPSC frequency and AP firing frequency in GnRH neurons of treated animals to levels observed in control subjects. Similarly, pharmacological antagonism of GABA$_A$ receptors in AAS-treated subjects significantly increased AP frequency in the intact mPOA but not the disrupted mPOA. Thus, our data are consistent with the hypothesis that AAS treatment increases AP firing in GABAergic mPOA neurons, which in turn provides enhanced inhibitory tone that suppresses the firing rate of GnRH neurons. Although this is the most parsimonious interpretation of our data, mPOA GABAergic afferents may also diminish GnRH neuronal firing by providing inhibitory inputs to excitatory glutamatergic or kisspeptin neurons in the AVPV or arcuate nucleus.

GnRH neurons are developmentally atypical in that GABA depolarizes these neurons well beyond early postnatal development. Whether GnRH neurons continue to be excited by GABA after puberty has prompted a lively scholarly debate (DeFazio and Moenter, 2002; Han et al., 2002, 2004; Moenter and DeFazio, 2005; Christian and Moenter, 2007; Yin et al., 2008; Chen and Moenter, 2009), and data from dissociated adult male rat neurons suggest that there may be heterogeneity within the GnRH population with respect to the action of GABA (Watanabe et al., 2009). It is also interesting to consider complexities arising from the fact that dendrites are the primary site for AP generation in GnRH neurons (Roberts et al., 2008, 2009). Thus, GABA$_A$ receptor-mediated conductance changes, although depolarizing, may nonetheless shunt more distally generated active responses and result in diminished firing frequency recorded at the soma. Although additional studies are required to determine whether GABA depolarizes or hyperpolarizes GnRH neurons in AAS-treated subjects, the results presented here contrast with previous

studies in gonadectomized male mice indicating coordinate enhancement of GABA$_A$ receptor-mediated PSC frequency (Chen and Moenter, 2009) and AP firing (Pielecka and Moenter, 2006) in GnRH neurons. These data further underscore that actions of supraphysiological levels of synthetic steroids may significantly diverge from effects imposed by castration or by replacement in castrates with physiological androgens or estrogens.

Results presented here indicate that chronic exposure to 17αMT did not elicit significant effects on the expression of GABA$_A$ receptor subunit mRNAs, on GABA$_A$ receptor-mediated sPSC amplitude or kinetics of current decay, on tonic GABA$_A$ receptor-mediated currents, or on the level of GnRH mRNA in GnRH neurons themselves. The lack of changes in either subunit mRNAs or in properties of GABA$_A$ receptor-mediated currents suggests that the AAS do not alter the complement of GABA$_A$ receptors expressed in these cells or posttranslational modifications that would alter their function. The absence of AAS-dependent effects on these postsynaptic molecules is consistent with data demonstrating an absence of AR and a paucity of ER expression in these cells. Data demonstrating a lack of effect on GnRH mRNA levels and yet a decrease in serum gonadotropin levels are not irreconcilable because previous studies indicate a lack of correlation between these levels and GnRH message in male rodents (Gore et al., 2000; Richardson et al., 2002, 2004), except under conditions of persistent absence of steroids after gonadectomy (Spratt and Herbison, 1997; Thanky et al., 2003).

Currently, ~2% of high school-age boys are estimated to have used anabolic steroids (Johnston et al., 2009). Wesson and McGinnis (2006) have shown that treatment of adolescent male rats at the onset of puberty until adulthood with a high dose (5 mg/kg) of the 17α-alkylated AAS stanozolol decreased testes weight, ejaculation frequency, scent marking, and vocalizations. Treatment of male Syrian hamsters with a mixture of three AAS (none 17α-alkylated) augmented sexual behavior in adolescents but suppressed it in adults (Salas-Ramirez et al., 2008), underscoring a pivotal distinction in neural templates between youth and adulthood. Gonadal steroids have been shown to have significant organizational actions on the neural circuits that give rise to male social behaviors not only during early perinatal development but also during adolescence (Sisk et al., 2003). Although our data point to central substrates through which the AAS may impair reproduction in adolescence, a key question that remains to be addressed is whether AAS exposure during this developmental period imparts permanent organizational changes on hypothalamic physiology and reproductive behaviors. Such data are particularly pertinent because studies of men (Kanayama et al., 2008) suggest that AAS users may be subject to long-term effects arising from steroid use after cessation of exposure.

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