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ANABOLIC STEROIDS ALTER THE PHYSIOLOGICAL ACTIVITY OF AGGRESSION CIRCUITS IN THE LATERAL ANTERIOR HYPOTHALAMUS

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Abstract

Syrian hamsters exposed to anabolic/androgenic steroids (AAS) during adolescence consistently show increased aggressive behavior across studies. Although the behavioral and anatomical profiles of AAS-induced alterations have been well characterized, there is a lack of data describing physiological changes that accompany these alterations. For instance, behavioral pharmacology and neuroanatomical studies show that AAS-induced changes in the vasopressin (AVP) neural system within the latero-anterior hypothalamus (LAH) interact with the serotonin (5HT) and dopamine (DA) systems to modulate aggression. To characterize the electrophysiological profile of the AAS aggression circuit, we recorded LAH neurons in adolescent male hamsters *in vivo* and microiontophoretically applied agonists and antagonists of aggressive behavior. The interspike interval (ISI) of neurons from AAS-treated animals correlated positively with aggressive behaviors, and adolescent AAS exposure altered parameters of activity in regular firing neurons while also changing the proportion of neuron types (i.e., bursting, regular, irregular). AAS treated animals had more responsive neurons that were excited by AVP application, while cells from control animals showed the opposite effect and were predominantly inhibited by AVP. Both DA D2 antagonists and 5HT increased the firing frequency of AVP responsive cells from AAS animals and dual application of AVP and D2 antagonists doubled the excitatory effect of AVP or D2 antagonist administration alone. These data suggest that multiple DA circuits in the LAH modulate AAS-induced aggressive responding. More broadly, these data show that multiple neurochemical interactions at the neurophysiological level are altered by adolescent AAS exposure.

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Keywords

Vasopressin; serotonin; dopamine; aggression; development; hypothalamus

Introduction

In male Syrian hamsters (*Mesocricetus auratus*), exposure to anabolic/androgenic steroids (AAS) during adolescent development produces a mature and escalated aggressive phenotype (DeLeon, Grimes, & Melloni, 2002; Grimes, Ricci, & Melloni, 2007; Harrison, Connor, Nowak, Nash, & Melloni, 2000; Melloni & Ricci, 2010). In a series of studies, we have shown that adolescent AAS exposure alters several neurotransmitter systems implicated in the control of aggression within the latero-anterior subregion of the hypothalamus (LAH) that include the vasopressin (AVP) (Carrillo et al., 2011a; Grimes, Ricci, & Melloni, 2006; Grimes et al., 2007; Harrison et al., 2000; Melloni & Ricci, 2010), serotonin (5HT) (Grimes & Melloni, 2002, 2005; Ricci, Rasakham, Grimes, & Melloni, 2006), and dopamine (DA) (Morrison, Ricci, & Melloni, 2015b; Schwartz, Ricci, & Melloni, 2009) neural systems.

The source of AVP and DA to the LAH region are neurons located within the medial supra-optic nucleus (mSON) as well as those in a small cluster centered directly within the anterior hypothalamus (AH) proper within the nucleus circularis (Ferris, Axelsson, Martin, & Roberge, 1989; Ferris, Irvin, Potegal, & Axelsson, 1990; Mahoney, Koh, Irvin, & Ferris, 1990; Ricci, Schwartz, & Melloni, 2009), while 5HT is supplied to the region by afferents extending from both the dorsal and median raphe nuclei (Delville, De Vries, & Ferris, 2000). All three neurotransmitter systems within the LAH undergo anatomical alterations that correlate with changes in aggressive behavior after adolescent exposure to AAS. For example, there is a greater density of AH AVP afferent fibers and peptide content in aggressive AAS-treated hamsters than non-aggressive vehicle-treated controls (Grimes et al., 2006; Harrison et al., 2000). Increases in AVP afferent density correlate with the onset of a highly aggressive phenotype over the course of adolescent AAS exposure and coincides with decreases in 5HT afferent fibers (Grimes et al., 2007). Similarly, AAS exposure affects 5HT signaling in the LAH by reducing the number of excitatory 5HT₃ receptors (Morrison, Ricci, & Melloni, 2015a), and by also reducing the number of 5HT_{1A} and 5HT_{1B} receptors localized to AVP neurons (Grimes & Melloni, 2005; Melloni & Ricci, 2010; Ricci et al., 2006).

Modified anatomical interactions between the 5HT and AVP systems in the LAH as a function of AAS exposure has been suggested in part to account for 5HT's role in AAS-induced aggressive behavior, whereby an AAS-reduced 5HT system disinhibits AVP release into the LAH and escalates the frequency of aggressive displays (Melloni & Ricci, 2010). Behavioral data support the presence of an AVP-activated aggression circuit partially gated by 5HT signaling. For instance, pretreatment with systemic fluoxetine (i.e., a selective serotonin reuptake inhibitor), and co-microinjection of 8-OH-DPAT (i.e., a 5HT_{1A} receptor agonist) blocks the aggression enhancing effects of AVP microinjection in the AH (Ferris et

al., 1997; Ferris, Stolberg, & Delville, 1999), and both fluoxetine and 8-OH-DPAT pretreatment inhibit AAS-induced aggression (Grimes & Melloni, 2002; Ricci et al., 2006).

Besides 5HT, AVP-mediated circuits that facilitate aggressive behavior after AAS-exposure are also known to interact with the DA system in the LAH. Anabolic androgenic steroids enhance the presence of D2 receptors within the LAH (Ricci et al., 2009; Schwartz et al., 2009), and local injection of Eticlopride (i.e., a D2-receptor antagonist) dose-dependently inhibits AAS-induced aggression (Schwartz & Melloni, 2010a). Recently, we showed that co-injection of AVP with Eticlopride into the LAH rescues the AAS-aggressive phenotype from the inhibitory effects of D2 antagonism, suggesting that DA mechanisms that regulate aggressive behavior in the LAH lie upstream of AVP-sensitive cells (Morrison et al., 2015b).

The neuroanatomical and behavioral characteristics of the adolescent AAS-exposed hamster have been well documented, however little is known about the physiological properties that underlie the aggressive behavioral phenotype. Chronic AAS exposure during adolescence alters the firing rate of neurons (*in vitro*) within the medial preoptic area of female and male mice, and differentially alters spontaneous synaptic potentials between males and females without altering cell populations (Penatti, Costine, Porter, & Henderson, 2009; Penatti, Davis, Porter, & Henderson, 2010; Penatti, Oberlander, Davis, Porter, & Henderson, 2011; Penatti, Porter, Jones, & Henderson, 2005). In male rats, nandrolone decanoate exposure throughout adolescence decreases firing rates of raphe 5HT neurons while enhancing the firing frequency of noradrenergic cells within the locus coeruleus (Rainer et al., 2014). Conversely, intracerebroventricular injections of testosterone increase the firing rates of raphe 5HT neurons in both castrated male and intact female rats (Robichaud & Debonnel, 2005). Although seemingly contradictory, together these data indicate that the activity of cells within the 5HT neural system are developmentally sensitive to AAS exposure.

Previously, single unit recordings from behaving mice show that the firing frequency of ventromedial hypothalamic neurons increase during agonistic encounters with conspecifics (Lin et al., 2011). Interestingly, microdialysis studies from our own lab show that AH-AVP release is increased in resident AAS treated hamsters during aggression testing when confronted with an intruder animal (Melloni & Ricci, 2010). Given the stimulatory nature of AH-AVP on aggressive display (Caldwell & Albers, 2004; Ferris et al., 1997; Gobrogge, Liu, Young, & Wang, 2009), these studies suggest the sensitivity of hypothalamic neurons to AVP is altered following chronic adolescent AAS exposure.

Although neural models of the aggression circuit within the anterior hypothalamic region have been proposed (Ferris et al., 1997), and updated to include AAS-induced anatomical modifications specifically within the LAH (Melloni & Ricci, 2010; Morrison & Melloni, 2014; Morrison, Ricci, & Melloni, 2014a; Schwartz & Melloni, 2010b), none incorporate physiological evidence that is necessary to understand how various neurochemical systems interact to affect aggressive behaviors. Using electrophysiological recording techniques, the data presented here describe the neurophysiological profile of LAH neurons in the aggressive adolescent AAS-treated hamster. Further, to understand how AVP/5HT and AVP/DA interactions are altered at the cellular level in the LAH to facilitate AAS-induced

aggressive behavior, we microiontophoretically applied AVP, 5HT, and the D2 receptor antagonist Eticlopride into the LAH and recorded the neuronal response to these aggression altering substances.

Methods

Animals

Male Syrian hamsters (N=40) were obtained from Charles River (Wilmington, MA) and individually housed in polycarbonate cages, and maintained at ambient room temperature (22–24 °C, with 55% relative humidity) on a reverse light–dark cycle (14L:10 D; lights off at 08:00) as previously described (Grimes & Melloni, 2002). Food (Prolab RMH 3000, Land O’Lakes, Inc. Arden Hills, MN) and water were provided ad libitum. For aggression testing, stimulus (intruder) males of equal size and weight to the experimental animals were obtained from Charles River one week prior to the behavioral test, group-housed (five animals per cage) in large polycarbonate cages, and maintained as above to acclimate to the animal facility. All studies were preapproved by the Northeastern University Institutional Animal Care and Use Committee and all methods employed are consistent with guidelines provided by the National Institutes of Health for the scientific treatment of animals.

Drugs

Testosterone cypionate, nandrolone decanoate, and boldenone undecylenate were purchased from Steraloids Inc (Newport, RI) and prepared in sesame oil. Eticlopride hydrochloride (ETIC), serotonin hydrochloride (5HT) and arginine vasopressin (AVP) were purchased from Sigma Aldrich (St. Louis, MO). The ETIC and AVP were dissolved in 0.9% (wt/vol) normal saline, and the 5HT was dissolved in ddH₂O.

Experimental Treatment and Behavioral Testing

On Postnatal day (P) 27, hamsters (n = 20) received daily subcutaneous (SC) injections (0.1–0.2 ml) of an AAS mixture consisting of 2 mg/kg testosterone cypionate, 2 mg/kg nandrolone decanoate, and 1 mg/kg boldenone undecylenate dissolved in sesame oil, for 30 consecutive days during adolescent development (P27–P56). This treatment regimen has been shown repeatedly to produce highly aggressive animals in greater than 85% of the treatment pool (DeLeon et al., 2002; Grimes & Melloni, 2005). As a nonaggressive control, a separate set of animals received SC injections of vehicle (sesame oil [VEH]; n = 20).

One day following the last injection of AAS or VEH (P57), hamsters were tested for offensive aggression using the resident-intruder paradigm, i.e., a well-characterized and ethologically valid model of offensive aggression in Syrian hamsters (Floody & Pfaff, 1974; Lerwill & Makings, 1971). Briefly, a novel intruder of similar size and weight was introduced into the home cage of the experimental animal (resident) and the resident was scored for specific and targeted aggressive responses including upright offensive postures, lateral attacks, and flank/rump bites, as previously described (Grimes, Ricci, & Melloni, 2003; Ricci et al., 2006). An “attack” was scored each time the resident animal would pursue and then either lunge toward and/or confine the intruder by upright and sideways threat; each generally followed by a direct attempt to bite the intruder’s dorsal rump and/or

flank target area(s). The composite aggression score, used as a general measure of offensive aggression, was defined as the total number of attacks (i.e., upright offensives and lateral attacks) and bites (i.e., flank/rump bites) during the behavioral test period. Attack and bite latencies were also recorded with the former defined as the period of time between the beginning of the behavioral test and the first attack the residents made toward an intruder. In the case of no attacks, attack and bite latencies were assigned the maximum latency (i.e., 600 seconds). Each aggression test lasted for 10 min and was videotaped and scored manually by two observers blind to experimental treatment. Inter-rater reliability was set at 95%. No intruder was used for more than one behavioral test, and all subjects were tested during the first 4 hours of the dark cycle under dim red illumination to control for circadian influences on behavioral responding. In addition to aggressive behaviors, residents were measured for social interest toward intruders defined as the period of time during which the resident deliberately initiated contact with the intruder (i.e., total contact time between the intruder and resident).

Electrophysiology

One day following behavioral testing (P58), hamsters were anesthetized with a ketamine/xylazine cocktail (80 mg/kg:12 mg/kg, i.p.) and prepared for extracellular single cell recording. Anesthesia was maintained through booster doses administered every 45 minutes (ketamine 10 mg/kg: xylazine 0.5 mg/kg, i.p.). Hamsters were placed into a stereotaxic instrument (David Kopf Instruments, Tujunga, CA) equipped with a micromanipulator, and secured with ear bars. The snout was positioned at -3.3 mm for the purpose of consistency in head angle across animals. The skull was exposed and a burr hole was drilled over the lateral region of the anterior hypothalamus at 0.6 – 1.2 mm lateral, and 0 – 0.7 mm anterior to bregma in accordance with coordinates identified previously (i.e., Schwartz & Melloni, 2010b).

Carbon fiber recording electrodes (impedance 5 – 7 $\mu\Omega$, 6 – 7 μm in diameter), surrounded by 6 micropipettes (impedances 3 – 7 $\mu\Omega$; Carbestar-7S, Kation Scientific, St. Paul, MN) were filled with the following substances for each experiment: AVP (0.2M in 2 barrels), 5HT (30 mM), ETIC (0.01 M), 1 M NaCl, and 2% pontamine sky blue (in 0.5 M sodium acetate) for current balancing and tissue marking. Electrodes were lowered to a depth between 6.9 and 8.0 mm ventral to dura to record spontaneous action potentials from the LAH region. Any cell encountered within the region of interest was monitored for at least 10 min before data collection to ensure stability throughout the duration of the experiment.

Activity from units were obtained through a plexon recording system (Plexon, Dallas, TX), the signals amplified, filtered (200 – 2000 Hz), and sampled at 15 kHz. All data were then stored on a server for offline analysis. Data collection was followed by ejection of pontamine sky blue at the last site of neuronal recording via a -20 μA current for 25 – 30 minutes, consistent with marking protocols reported elsewhere (e.g., Heidenreich, Mailman, Nichols, & Napier, 1995). After dye ejection, hamsters were euthanized with ketamine and transcardially perfused with 0.9% NaCl, and then 4% paraformaldehyde solution in 0.1 M phosphate buffer. The brains were removed and fixed in paraformaldehyde for 24 hours, and then stored in 20% sucrose solution until histological processing. After at least 72 hours in

sucrose, brains were sectioned into 30 μm thick sections beginning at the anterior portion of the hypothalamus. Sections were mounted onto gelatin-coated slides and dried. Slides with brain sections were then re-hydrated, stained using cresyl violet or neutral red, defatted with ethanol and Hemo-D, and coverslipped. Light microscopy and the atlas of Morin and Wood (2001) were used to confirm proper localization of the dye marker in the LAH in the brain slices. Physiological data from any animals with marker placement outside the LAH were removed from the data set before data analysis.

Experimental design

All recorded cells underwent a drug microinfusion protocol that measured their response to individually applied drugs (Figure 1, see Single Drug Protocol). To evaluate the physiological relevance of AVP sensitive cells' interactions with either 5HT or D2 receptor targets within the LAH, stable cells exposed to the Single Drug Protocol were also exposed to one of two variations of a second protocol (Figure 1, see Dual Drug Protocol) that measured the neuronal response to dual application of AVP along with either ETIC (ETIC variation of Dual Drug Protocol) or 5HT (5HT variation of Dual Drug Protocol).

The Single Drug Protocol consisted of a counterbalanced microiontophoretic drug application regimen where AVP (45 nA), 5HT (60 nA), or ETIC (50 nA) were each individually microinfused into the LAH for 30 s once over the course of the protocol. The order in which drugs were individually microinfused was counterbalanced across experiments to control for order effects of drugs on neuronal activity. The Single Drug Protocol regimen started with a 5 min baseline recording period, followed by a 30 s period when the first drug (Drug 1; AVP, 5HT, or ETIC) was individually applied in the LAH followed by 5 min period of washout plus another 5 min period of baseline recording (10 min total). Following the 10 min baseline and washout period the second drug was infused for 30 s (Drug 2, i.e., depending on the counterbalanced design, Drug 2 was either AVP, 5HT, or ETIC, but not the same drug as Drug 1, thus for example, if Drug 1 was AVP, Drug 2 would have been ETIC or 5HT). The infusion of Drug 2 was followed by another 5 min period of washout plus a 5 min period of baseline recording. Following this 10 min baseline and washout period the third drug was applied for 30 s (Drug 3; i.e., depending on the counterbalanced design, Drug 3 was either AVP, 5HT, or ETIC, thus for example if Drug 1 was AVP and Drug 2 was 5HT, Drug 3 would have been ETIC). The infusion of Drug 3 was followed by a washout period that lasted 10 min. All drug concentrations and ejection currents were selected based on previous studies that detailed their efficacy in altering neuronal firing rates (Sesack & Bunney, 1989; Shen, Asdourian, & Chiodo, 1992; Yang, Bourquet, & Renaud, 1991). In some cases, not all cells were exposed to all three drugs due to clogged micropipettes.

After completion of the Single Drug Protocol, cells underwent a 20 min monitoring period. During this time, if cells varied ($\pm 15\%$) from their initial baseline firing frequency or their firing pattern was altered, their data were not included in the final analyses. Stable cells that completed the Single Drug Protocol were exposed to one of the two variations (i.e., ETIC variation or 5HT variation, see Figure 1) of the Dual Drug Protocol where baseline activity was recorded for 5 min followed by AVP application within the LAH for 30 s and then a 5

min washout period. Following washout and another 5 min period of baseline recording (washout + baseline = 10 min total), the second drug (i.e., either 5HT or ETIC) was applied for 30 s followed by a 5 min washout plus a 5 min period of baseline recording. Following this baseline recording period, the second drug was again constantly applied for a 1 min time period during which, after 30 seconds, AVP was co-applied for the remaining 30 s of the 1 min period. All data for any cell that did not complete all three microinfusions for the Dual Drug Regimen was removed from analyses.

Data analysis

Offline spike sorting and analysis were completed through the use of Offline Sorter and NeuroExplorer (Plexon; Dallas, TX). The carbon fiber electrodes simultaneously recorded 1–3 waveforms at each site. These waveforms were sorted into spike trains from individual neurons using principal component cluster analysis followed by template matching, as described previously (see Sikes, Vogt, & Vogt, 2008). Graphpad Prism (La Jolla, CA), and custom algorithms implemented using *Mathematica* software (Wolfram Research; Champaign, IL) were used for handling spike train and all statistical analyses. Interspike interval histograms (ISIH) and autocorrelograms were computed using a bin width of 1 ms and a sample of baseline unit activity ranging between 120 and 300 s. ISIHs, autocorrelograms, and raster plots as well as ISI coefficients of variation (CV) were used to identify cell types as “regular”, “irregular”, or “bursting” based on guidelines as described (Bar-Gad, Ritov, & Bergman, 2001; Oberlander & Henderson, 2012; Penatti et al., 2010). Comparisons between baseline neuronal activity and drug exposure periods were normalized as the percentage change of mean firing rates. Any unit showing a change $\pm 15\%$ was considered responsive and classified according to the direction of its response relative to baseline (i.e., “excitatory”, “inhibitory”, “non-responsive”).

In the case of baseline data comparisons between treatment groups (i.e., AAS vs VEH), raw mean firing rates were compared. The proportions of neuron type (e.g., regular, irregular) and response classes (i.e., excitatory, inhibitory, or non-responsive) were compared between treatment groups using the Chi-square test followed in some cases by post-hoc Binomial exact test planned comparisons. All baseline firing and behavioral parameters were compared between treatment groups using independent t-tests. The association between behavior (i.e., attacks, bites, attack latency, bite latency) and unit activity (i.e., firing frequency and interspike intervals) was assessed through correlation analyses. For the Single Drug Protocol, changes in firing frequency were compared using within-subjects t-tests. For the Dual Drug Protocol, neurons were grouped according to their responsiveness to AVP from the Single Drug Protocol and data were analyzed using a mixed-design 2-way ANOVA with microinfusion as the repeated measure and, given a significant interaction, followed by post hoc planned comparison t-tests. The α level for all tests was set at 0.05.

Results

Resident-intruder testing

Of the 40 animals tested, there was a total of 15 AAS and 15 VEH animals with correctly placed recording electrodes (see Figure 2). Resident-intruder behavioral testing showed that

adolescent exposure to AAS significantly increased the number of attacks and bites towards intruders ($t(28)=3.7, p < 0.001, t(28)=2.5, p < 0.02$, respectively), and reduced attack latencies ($t(28)=2.3, p = 0.02$), but only marginally reduced bite latency times ($t(28) = 2.0, p = 0.06$; see Figure 3). Although AAS altered offensive aggressive behaviors, it did not alter the amount of contact time between animals during the aggression testing period ($t(28) = 1.41, p > 0.05$).

Spontaneous activity of lateral anterior hypothalamic neurons in aggressive AAS hamsters and controls

From all animals with correctly placed recording electrodes, for the Single Drug Protocol, 69 LAH neurons were recorded from AAS animals and 38 were recorded from VEH treated animals. There was a total of 34 and 23 successful recording sites (typically 2 sites from each animal were analyzed, but no more than 3) in AAS and VEH (respectively) treated animals with 1–3 waveforms collected per recording site in each animal. Table 1 shows a summary of baseline unit parameters of cells from the LAH region between AAS and VEH treated animals and Figure 4 shows ISI histograms, autocorrelograms, and raster plots of representative neurons for the three classes of neurons that were encountered. AAS significantly increased the mean ISI ($t(38)=2.7, p < 0.01$), and the median ISI ($t(38) = 2.3, p < 0.05$) for regular firing cells, and marginally increased the mode ISI for bursting cells ($t(11), p = 0.06$), while all other parameters for all cell types were not found to be significantly different from VEH treated controls ($p > 0.05$ for all).

Anabolic-androgenic steroid exposure during adolescence also significantly altered the proportion of cell types within the LAH ($\chi^2(2) = 7.95, p < 0.05$, Figure 5). Irregular firing neurons accounted for the majority of all neurons in both AAS (53%) and VEH (45%) treated animals, with the proportion of regular firing neurons being nearly equal between the treatment groups (39% for AAS and 34% for VEH). In contrast, bursting neurons were the only neuron type that was proportionally greater in VEH treated animals (21%) compared to AAS (7%).

Unit activity and behavior correlations

The relationship between aggressive behavior and LAH unit activity was assessed in all but one outlier cell that was in an AAS-treated animal and showed an extremely fast firing cell frequency (i.e., $> 50\times$ the mean). In AAS treated animals, there was a significant positive correlation between the interspike interval of LAH neurons and bite number ($R = 0.24, p < 0.05$), however, no such relationship was found for vehicle treated animals ($p > 0.05$, see Figure 6). Similarly, no other significant correlation was found between unit activity and attack frequency, attack latency, or bite latency for either treatment group ($p > 0.1$ for all).

Vasopressin microinfusion

Results from the Single Drug Protocol show that AAS exposure differentially alters the proportion of neurons that are sensitive to AVP, 5HT, and ETIC. Overall, AVP microinfusion significantly altered the proportion of responses of cells between AAS and VEH treated animals ($\chi^2(2) = 33.35, p < 0.0001$). Post-hoc Binomial exact tests revealed that AAS marginally significantly decreased the number of AVP responsive cells (49%)

within the LAH compared to VEH treated animals (61%; $p = 0.06$, Figure 7). When the proportion of response types were examined, however, of the cells that responded to AVP, AAS-treated animals had 50% more cells that showed excitatory activity than VEH-treated animals, while VEH animals had 50% more cells that showed an inhibitory response than AAS-treated animals; this difference was significant ($p < 0.0001$ see Figure 7 inset).

Figure 8 (top left) shows changes in activity after drug infusion in the majority of cells from each response class (i.e., excitatory or inhibitory) in both VEH and AAS treated animals. Compared to baseline unit activity, in AAS treated animals AVP significantly increased the firing rate of LAH neurons from the majority of responsive neurons (2.6 ± 0.3 to 3.2 ± 0.4 Hz; $t(27) = 3.9$, $p < 0.001$) while the activity of the majority of responsive cells in VEH animals after AVP exposure was significantly decreased (7.5 ± 3.5 Hz to 6.5 ± 3.3 Hz; $t(14) = 3.0$, $p < 0.01$). Neurons in VEH treated animals from the minority of responsive cells showed a significant increase in activity after AVP exposure (5.2 ± 1.3 to 6.0 ± 1.4 Hz; $t(7) = 4.3$, $p < 0.005$) while cells from the inhibitory response class in AAS treated animals were not significantly decreased by AVP exposure ($p > 0.05$).

Serotonin microinfusion

Like AVP responsive cells, AAS exposure also significantly altered the overall proportion of neuronal responses to 5HT microinfusion ($\chi^2(2) = 10.81$, $p < 0.005$). Post hoc analyses showed that there was no significant difference in the proportion of cells that were responsive to 5HT between AAS (59%) and VEH (55%) treated animals ($p = 0.54$ Figure 7); however, of the cells that responded to 5HT, in contrast to AVP responsive cells there was a significant difference in the proportion of response classes between AAS and VEH treated animals with 66% of VEH responsive cells showing excitatory activity (vs 44% for AAS), and AAS cells (56%) showing a greater proportion of cells with inhibited activity (vs 33% for VEH). These differences were significant ($p < 0.01$; Figure 7 inset).

Serotonin microinfusion significantly increased the activity in the majority of cells in VEH treated animals (4.6 ± 1.3 Hz to 6.1 ± 1.6 Hz; $t(13) = 3.34$, $p < 0.005$; Figure 8 -- middle left), but did not affect the firing rate of the minority of responsive neurons from this treatment group ($p > 0.05$). In AAS treated animals, neurons primarily were inhibited by 5HT microinfusion and this change in firing rate was significant (8.8 ± 2.2 Hz to 7.2 ± 1.8 Hz; $t(22) = 3.9$, $p < 0.005$; Figure 8 middle). The minority of 5HT sensitive neurons in AAS animals also showed a significant change with increased firing after 5HT microinfusion (3.3 ± 0.7 Hz to 4.1 ± 0.8 Hz; $t(17) = 5.391$, $p < 0.0001$).

Eticlopride microinfusion

Overall, ETIC microinfusion significantly altered the proportion of responses of neurons from AAS-treated animals compared to vehicle treated controls ($\chi^2(2) = 7.42$, $p < 0.05$). Post hoc planned comparisons showed that similar to AVP microinfusion, AAS significantly reduced the proportion of ETIC responsive cells in the LAH region compared to VEH cells (AAS: 47%; VEH: 63%; $p < 0.01$, Figure 7). In contrast to both 5HT and AVP microinfusions, of the cells that responded to ETIC, there were no significant differences in the proportion of cells showing an excitatory (AAS: 39%; VEH: 46%) or inhibitory response

(AAS: 65%; VEH: 54%) between cells from AAS and VEH treated animals ($p > 0.5$ Figure 7).

Figure 8 (bottom left) shows changes in firing rates from the majority of neurons that were responsive to ETIC in both AAS and VEH treated animals. ETIC significantly decreased the firing frequency in the majority of cells from AAS (6.2 ± 2.0 Hz to 4.9 ± 1.6 Hz; $t(16) = 2.9$, $p < 0.01$) and VEH (5.4 ± 1.6 Hz to 4.3 ± 1.5 Hz; $t(12) = 3.7$, $p < 0.005$) treated animals. ETIC also significantly increased activity in the minority of responsive cells in both AAS (2.7 ± 0.6 Hz to 3.2 ± 0.6 Hz; $t(10) = 5.02$, $p < 0.0005$) and VEH treated animals (5.0 ± 2.5 Hz to 5.8 ± 2.7 Hz; $t(10) = 3.8$, $p < 0.005$).

Vasopressin and serotonin

Of the 107 cells used in the Single Drug Protocol, 46 (AAS $n = 27$ cells from 6 animals; VEH $n = 19$ cells from 7 animals) were used to in the Dual Drug Protocol to measure the response of AVP and 5HT. For all analyses, the frequency changes from baseline for response types were pooled using the results of the Single Drug Protocol and identified as responsive to AVP (AAS $n = 15$ cells from 3 animals; VEH $n = 10$ cells from 4 animals) or non-responsive to AVP (AAS $n = 12$ cells from 3 animals; VEH $n = 9$ from 3 animals). For AVP-responsive cells, there was a significant interaction effect of drug pretreatment (i.e., AAS vs VEH) and microinfusion (e.g., AVP, 5HT, and AVP+5HT; $F[3, 69] = 3.45$, $p < 0.05$; see Figure 9 -- top left). Post hoc tests showed that in addition to altering the proportion of neurons, the action potential firing frequency of cells from AAS (but not VEH) treated animals was significantly increased in response to AVP ($t(14) = 7.15$, $p < 0.0005$), 5HT ($t(14) = 2.25$, $p < 0.05$), and dual application of AVP+5HT ($t(14) = 2.86$, $p < 0.02$) when compared to baseline. There was also a significant difference for AAS treated animals between the change from baseline activity between 5HT and dual microinfusion to AVP +5HT ($t(14) = 2.52$, $p < 0.02$, but not between AVP and dual infusion ($p > 0.1$). There were no significant differences within the results of cell activity changes from VEH treated animals ($p > 0.1$). Between subjects effects showed that the change from baseline activity after AVP ($t(23) = 2.07$, $p < 0.05$), 5HT ($t(23) = 2.22$, $p < 0.05$), and AVP+5HT ($t(23) = 3.92$, $p < 0.0005$) microinfusions was significantly greater in cells from AAS compared to VEH treated controls.

For AVP non-responsive cells (Figure 9 – top right), there was a main effect of microinfusion $F(3, 57) = 3.39$, $p < 0.05$), however the interaction between pretreatment and microinfusion was insignificant ($p > 0.1$).

Vasopressin and Eticlopride

Of the 107 cells used in the Single Drug Protocol, 45 (AAS $n = 26$ cells from 9 animals; VEH $n = 19$ cells from 8 animals) were exposed to the Dual Drug Protocol using AVP and ETIC. For AVP responsive cells (AAS $n = 16$ cells from 5 animals; VEH $n = 10$ cells from 4 animals), similar to AVP and 5HT, there was a significant interaction of pretreatment type and microinfusion regimen ($F[3, 72] = 4.85$, $p < 0.005$; see Figure 9 – bottom left). Post hoc testing showed that in cells from AAS exposed animals, AVP and dual application of AVP +ETIC (but not ETIC; $p > 0.05$) significantly increased the action potential firing frequency

from baseline ($t(15) = 6.04$, $p < 0.0001$ and $t(15) = 3.62$, $p < 0.005$, respectively). When compared to dual application of AVP+ETIC, the alteration from baseline was significantly higher than either AVP ($t(15) = 2.45$, $p < 0.02$) or ETIC ($t(15) = 4.00$, $p < 0.001$) microinjections. In VEH treated animals, the pooled response of AVP responsive cells was not significantly altered from baseline after AVP, ETIC, or AVP+ETIC microinjections ($p > 0.1$ for all). Between subjects effects showed that the firing frequencies of neurons from AAS animals in response to AVP ($t(24) = 1.98$, $p < 0.05$), ETIC ($t(24) = 2.35$, $p < 0.05$), and AVP+ETIC ($t(24) = 4.6$, $p < 0.0001$) were significantly greater than from neurons in VEH animals.

For AVP-non responsive cells (AAS $n = 10$ cells from 4 animals; VEH $n = 9$ cells from 4 animals) there was a significant interaction between pretreatment and microinfusion ($F[3, 51] = 6.51$, $p < 0.001$; see Figure 9 – bottom right). Post hoc testing revealed that cells from AAS (but not VEH) treated animals were significantly altered by ETIC exposure ($t(9) = 4.28$, $p < 0.005$) and dual (AVP+ETIC) application elevated activity levels above that observed during baseline ($t(9) = 4.37$, $p < 0.005$) and after AVP exposure ($t(9) = 3.11$, $p < 0.02$). For both of these effects, changes from baseline were significantly greater in neurons from AAS compared to VEH treated animals (ETIC: $t(17) = 4.71$, Dual: $t(17) = 4.91$; $p < 0.0001$ for both). In contrast, dual application in AAS and VEH animals did not raise activity levels beyond that of ETIC, nor did AVP alter activity beyond baseline levels for cells within or between pretreatment groups ($p > 0.1$ for all).

Discussion

Exposure to AAS throughout adolescence alters the activity patterns, responsivity, and proportions of cell types within the lateral anterior hypothalamus (LAH). Behavioral data from our laboratory's past studies suggest that AAS alters at least two AVP-sensitive circuits within the LAH region to alter aggressive behaviors (see Melloni & Ricci, 2010 for review). One such circuit is modulated by 5HT receptors while another involves the D2 dopamine receptor. Our results support the existence of these circuits along with a additional DA circuit, and show that substances that alter aggressive behavior in the LAH also alter the physiological activity of neurons in this region.

Anabolic steroids alter the activity of neurons associated with aggression

Anabolic/androgenic steroid exposure extended the time interval between action potentials in both regularly firing and bursting (marginally) neurons while also reducing the proportion of bursting cells within the LAH. These effects of AAS in our animal model are similar to those in adolescent female mice showing that AAS exposure alters the distribution of bursting cells in the amygdala that control neurons in the bed nucleus of the stria terminalis (BNST) (Oberlander & Henderson, 2012), as well as in the medial preoptic area (mPOA) in adult male mice (Penatti, Porter, & Henderson, 2009). Moreover, similar to the effects we observed in regular firing neurons, AAS differently alters the firing parameters of neurons in aggression-associated regions between male and female mice (Oberlander & Henderson, 2012; Penatti, Costine, et al., 2009; Penatti, Porter, et al., 2009). Interestingly, the LAH of the male hamster is essential to the AAS-induced aggression circuit and connects with

various aggression and anxiety regions including the mPOA, amygdala, and BNST (Morrison & Melloni, 2014). Changes to the proportion of bursting cells and overall firing activity in these brain regions across sexes suggests that AAS-exposure offsets the cellular interaction between androgen-sensitive regions of the brain that contain portions of the cellular network controlling aggression. More generally, the effects of AAS we observed in comparison to previous studies highlight the importance of bursting cells in AAS-induced behavioral alterations.

AAS-altered proportions of specific cell types in the LAH suggests that AAS exposure changes the communication patterns between cells that mediate aggressive behavior and (combined with anatomical data) offers clues as to which neurons are most important in controlling AAS-induced behavior changes. For example, AAS dramatically increases the density of GABA containing neurons in the LAH after adolescent exposure (Schwartz et al., 2009). Interestingly, blockade of GABA receptor signaling increases bursting activity in neurons within various brain regions (Urbain, Rentéro, Gervasoni, Renaud, & Chouvet, 2002), and together with our results, this report suggests that increased GABA signaling can tonically silence bursting neuron activity in a given brain region. Along with previous behavioral pharmacology studies from our lab showing that the GABA system, in part, modulates AAS-induced aggressive behavior (Morrison, Ricci, & Melloni, 2014b), our electrophysiological data may help characterize novel characteristics of GABA circuits and identify unique drug targets that can be used to reverse maladaptive AAS alterations to behavior.

Correlational analyses showed that AAS alterations to the firing frequency of hypothalamic neurons are associated with bite frequency and suggest that LAH cells with longer interspike intervals are present in animals that produce more bites during aggressive encounters. This finding is modestly similar to data from Lin et al. (2011) that show that ventromedial hypothalamic neurons are excited during social and aggressive encounters between male mice. It is interesting to note that bites are androgen-dependent in rodents (Wagner, Beuving, & Hutchinson, 1979) and are a highly aggressive form of attack behavior. The behavioral phenotype of our animal model relies on AAS-induced alterations within the LAH that may result in the activation of androgen-sensitive attack pathways that are similar to those involved in Lin et al.'s results. The behaviorally active component of this proposed pathway, however, is likely downstream (e.g., perhaps in the BNST and/or amygdala) of hypothalamic circuits since, unlike our animal model (see Carrillo, Ricci, & Melloni, 2009), Lin et al.'s mice show no evidence of neuronal activation specifically in the anterior hypothalamus during attack behavior. Together, these data suggest that the activity of neurons in the LAH are sensitive to androgen receptor activation and modulate a highly aggressive form of attack behavior that is unique to the AAS-aggression phenotype, further highlighting the LAH as being critically important to the AAS aggression circuit.

Microinfusion of vasopressin, serotonin, and Eticlopride

When LAH neurons were analyzed in terms of their responsiveness to substances known to alter AAS-induced aggressive displays, AAS exposure reduced the proportion of cells sensitive to both ETIC and AVP, but did not affect the number of cells sensitive to 5HT.

These differences may in part be due to alterations to specific cell types that occur after AAS exposure. For example, the density of GABA cell bodies within the LAH of the hamster is increased after AAS treatment (Schwartzter et al., 2009). Although a portion of these GABA neurons is known to possess DA D2 receptors (Schwartzter et al., 2009), there is no evidence that they possess AVP receptors. There is also no indication that AVP receptors are reduced in the LAH after adolescent AAS exposure (DeLeon et al., 2002). Together these data indicate that although AAS does not likely reduce AVP-responsive cells, *proportionally* there are fewer AVP-responsive cells in AAS animals due to the increased presence of non-AVP responsive GABA-containing cells.

Opposite to the effects observed in 5HT-sensitive neurons, AAS increased the presence of AVP-sensitive cells that were excited by AVP, while reducing those showing an inhibitory response. These alterations are presumably not a direct function of AVP-receptor containing neurons since AAS exposure does not increase AVP receptors in the AH (DeLeon et al., 2002), but more likely reflects increased activity of cells that, in the absence of AAS exposure, are silent. These putative AAS-activated cells have previously been hypothesized to be glutamate projection cells to the BNST that are responsible for activating AAS-induced aggressive responding (Carrillo et al., 2011a; Carrillo, Ricci, & Melloni, 2011b), and are regulated by both 5HT and AVP receptor signaling. In terms of behavior, increased AVP signaling in the LAH is associated with enhanced aggressive displays that can be blocked by increasing central 5HT or by activating 5HT1A receptors (Altemus, Cizza, & Gold, 1992; Ferris et al., 1997; Ferris, Stolberg, & Delville, 1996; Ferris et al., 1999; Gobrogge et al., 2009; Melloni & Ricci, 2010; Veenema, Beiderbeck, Lukas, & Neumann, 2010). More specific to our findings, reduced 5HT innervation in the LAH after AAS exposure coincides with both heightened aggressive responding (Grimes & Melloni, 2006) and increased AVP afferents and peptide content in the LAH region (Grimes et al., 2006, 2007; Harrison et al., 2000). Taken together, these data suggest a greater proportion of excitatory AVP-sensitive cells exist in aggressive AAS animals at least in part due to the loss of inhibitory 5HT afferents fibers. This notion is supported by our finding that, across all experimental conditions, AVP increased the activity of LAH neurons from aggressive AAS treated animals. Moreover, in general 5HT-sensitive cells from AAS animals primarily showed an inhibitory response, further aligning the activity of AVP/5HT sensitive putative GLU cells with AVP-increased aggression and 5HT-induced aggression inhibition.

In contrast to AVP-sensitive cells, our finding of a lack of D2 antagonist responsive cells in AAS-treated animals seemingly conflicts with previous data from our lab showing AAS-exposure increases D2-receptors on GABA and other types of cells within the LAH (Ricci et al., 2009; Schwartzter et al., 2009). Our previous behavioral pharmacological experiments exploit these changes by showing that D2 antagonist injection into the LAH inhibits AAS-induced aggressive behavior (Morrison et al., 2015b; Schwartzter & Melloni, 2010a, 2010b). A putative neural model circuit (Model 1 in Figure 10) that explains ETIC's effect on aggression assumes that AAS-increased DA neurons within the AH tonically inhibit GABA neurons (that synapse onto AVP terminals) via activation of inhibitory D2 receptors (Schwartzter et al., 2009). Tonic inhibition of GABA release effectively disinhibits AVP release into the LAH, thus facilitating AAS-induced aggression. Infusion of D2 antagonists

into this circuit allows for GABA release that subsequently inhibits AVP release and aggression. This model is supported by data that show that AAS increases: LAH AVP release, the density of both DA cells and D2 receptors in the LAH, and the density of LAH GABA neurons (Melloni & Ricci, 2010; Schwartz et al., 2009). Behavioral data align with this model by showing that exogenous AVP in the LAH rescues AAS-induced aggression that is inhibited by D2 antagonists (Morrison et al., 2015b). In support of this model, it is likely that we recorded fewer ETIC sensitive cells within the LAH of AAS animals because we only recorded spontaneous action potentials, and thus although there exists more D2 containing neurons in AAS animals than vehicle controls, the majority of these cells are (presumably) tonically silenced GABA neurons. Future electrophysiological experiments using D2 agonists in silent regions of the LAH may provide further insight into the density and function of D2 containing GABA neurons in this region.

Anabolic steroids and vasopressin/dopamine system interactions

Examination of AVP interactions with the DA systems in aggressive, AAS-treated animals showed that AVP responsive cells were generally excited by AVP and D2 antagonists. The response of these neurons to dual AVP and ETIC infusion induced a level of excitation that was twice as large as the response elicited by AVP or ETIC alone. In terms of activity alteration, our finding conflicts with a previous report showing DA and D2 agonists increase spontaneous hypothalamic neuronal firing in tissue from adult rats (Yang et al., 1991). Differences in the effect of D2 receptor stimulation between studies may underscore the significance of alterations to the DA system in the LAH that occur specifically after adolescent AAS exposure.

Increased firing frequency of AVP sensitive cells after ETIC along with an even greater excitatory response of these neurons to dual application of ETIC and AVP suggests that some GABA neurons in the LAH, after AAS exposure, are directly or indirectly regulated by both AVP and D2 receptor activation. Specifically, the additive excitatory activity occurring after dual application may be a function of a separate set of GABA neurons that possess both D2 and excitatory AVP receptors (Model 2 in Figure 10), and exist in addition to those described in Model 1. The existence of two distinct classes of D2-regulated GABA neurons is supported by our data from non-AVP responsive neurons in AAS-treated animals that show similar levels of elevated firing activity in response to both ETIC and dual ETIC +AVP application. AVP receptors on GABA neurons in AAS-treated animals may be components of a feedback mechanism that limit AVP release modulating the intensity of aggressive display. Evidence for this model exists in the literature describing the dose-dependent effects of AH AVP microinjections on aggressive behaviors. High concentration AVP microinjection into the AH increases flank marking behavior (i.e., an indication of social dominance [Ferris, Axelsson, Shinto, & Albers, 1987; Irvin, Szot, Dorsa, Potegal, & Ferris, 1990]) without altering aggressive attacks (Ferris, Albers, Wesolowski, Goldman, & Luman, 1984; Ferris & Potegal, 1988). Conversely, at low concentrations, AVP increases the frequency of attacks directed at conspecifics (Ferris et al., 1997). Since AAS exposure does not alter the density of AVP receptors in the AH region (DeLeon et al., 2002), AVP receptor-containing GABA neurons likely exist prior to AAS exposure, but co-express D2 receptors only *after* adolescent treatment. Regardless of existing localization data and

altered physiological responses to AVP and ETIC, future triple label co-localization experiments examining the presence of GABA neurons with both D2 receptors and AVP (V1A) receptors in the LAH of AAS treated animals are necessary to rule out this mechanism.

Anabolic steroids and vasopressin/serotonin system interactions

Unlike ETIC and AVP microinjections, AAS did not change the number of 5HT responsive cells between AAS and VEH treated animals. However, in terms of specific response, AAS treated animals showed greater numbers of cells inhibited by 5HT, while VEH animals showed the opposite with a greater number of cells that were excited by 5HT. The effects of 5HT in VEH treated animals likely highlights the greater presence of excitatory 5HT₃ receptors in the AH region compared to AAS treated animals (Morrison et al., 2015a). AAS reduces 5HT₃ receptors in many aggression regions and increases inhibitory somatic 5HT_{1B} receptors in the LAH (Grimes & Melloni, 2005) providing a plausible mechanism for 5HT's inhibitory effect we recorded from LAH cells in AAS treated animals. This mechanism correlates with behavioral data that show that 5HT_{1B} agonists are effective in reducing adolescent AAS-induced aggression (Grimes & Melloni, 2005) while 5HT₃ agonists enhance aggressive behavior (Ricci, Knyshevski, & Melloni, 2005).

Similar to the effects of D2 antagonists, AVP sensitive cells from AAS treated animals were excited by 5HT exposure. Dual application of AVP and 5HT elicited an excitatory effect that was greater than the response of 5HT application alone. The effects of 5HT and dual AVP/5HT on AVP-sensitive cells is surprising since, proportionally we found a greater density of LAH cells that were inhibited by 5HT in AAS-treated animals. These contradictory results suggest multiple 5HT-modulated circuits underlying neuronal activity in the LAH, and are possibly related to the variability of 5HT-induced alterations to aggressive behaviors that exist between studies. For example, chronic exposure to fluoxetine increases the release of 5HT in various locations throughout the brain, including the hypothalamus (Dremencov, Gur, Lerer, & Newman, 2002), but has been shown to both increase and inhibit aggression-associated behaviors across experimental conditions (McDonald, Gonzalez, & Sloman, 2011; Mongillo, Kosyachkova, Nguyen, & Holmes, 2014; Perreault, Semsar, & Godwin, 2003; Ricci, Morrison, & Melloni, 2012; Ten Eyck & Regen, 2014).

Conclusions

To our knowledge, this is the first investigation of the effects of adolescent AAS exposure on the physiological response of LAH neurons to substances that are known to alter aggressive behavior. Anatomical and behavioral alterations brought on by AAS exposure have been extensively characterized (see Melloni & Ricci, 2010 and Morrison & Melloni, 2014 for review), but have previously only provided clues as to how interacting neurochemical systems in the LAH work to affect aggressive behavior. Our results carry the anatomical model of AAS-induced aggression forward by revealing how neural substrates interact in response to substances that affect aggression and offer translational value by showing that the activity patterns of neurons that mediate maladaptive behavior can be disrupted.

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Highlights

- * Adolescent AAS exposure alters neuronal firing parameters in the hypothalamus
- * LAH neuronal activity and aggressive behavior are correlated in AAS animals
- * AAS increases the proportion of responsive cells that are excited by AVP
- * AAS lowers the proportion of cells that respond to DA D2 receptor antagonists

Figure 1.

For the Single Drug Protocol, each drug was individually applied (AVP@45 nA, 5HT@60 nA, and ETIC@50 nA) into the LAH for 30 s after baseline data collection or baseline data collection + washout in a counterbalanced manner. No effect of drug administration order was detected. For the Dual Drug Protocol, after an initial monitoring period and baseline period (25 min total), AVP was individually applied, followed by individual application of either 5HT or ETIC. After a washout period (5 min) plus baseline (5 min), 5HT or ETIC was

constantly applied for 1 min along with AVP that was co-infused for 30 seconds beginning 30 seconds after the start of the 1 min 5HT or ETIC injection period.

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Figure 2.

Left: diagram and micrograph of sample electrode placement. Arrow points to pontamine blue ejection showing the recording location within the LAH. **Right:** schematic diagram of LAH region with the recording sites from all animals with hits 0.6 caudal from Bregma. This caudal-rostral location contains the majority of recording sites (AAS: 25/34; VEH: 17/23, 15 animals per treatment group) from all animals tested; ✕ = Hits; ○ = Misses. (AH: Anterior Hypothalamus, F: fornix, NC: Nucleus Circularis, LH: Lateral Hypothalamus, LAH: Lateral Anterior Hypothalamus, son: supraoptic nucleus, 3RD Ven: Third Ventricle). Adapted from A Stereotaxic Atlas of The Golden Hamster Brain (p. 24), by L. P. Morin and R. I. Wood, Copyright Elsevier (2001).

Figure 3.

Adolescent exposure to anabolic/androgenic steroids (AAS) significantly alters the parameters of aggressive behavior compared to vehicle (VEH) treated animals. AAS-treated animals ($n = 15$) showed significantly more attack behaviors (i.e., upright offensives, lateral attacks, and bites) towards intruders while also showing greater levels of impulsivity as evidenced by their significantly short attack and bite (marginal) latencies compared control animals ($n = 15$). Independent t-tests, + $p = 0.06$; * $p < 0.05$; $p < 0.01$; $p < 0.001$.

Figure 4.

Typical baseline firing activity patterns from neurons within the lateral anterior hypothalamus (LAH) of the adolescent hamster. Interspike interval (ISI) histograms (**Top**) and their respective autocorrelograms (**Middle**) with raster plots (Second from bottom), and units from each class (**Bottom**) show the three firing patterns from three characteristic cells that were encountered using single-cell recording within the LAH. The regular pattern was characterized by uniformly firing cells evident in their autocorrelograms that showed multiple (> 3) uniform peaks. Irregular firing cells exhibited less predictable firing patterns and had greater levels of variability in the spiking patterns exhibited by a wider histogram and a flat autocorrelogram. Bursting cells typically showed tightly clustered action potentials separated by a pause in activity. This pattern is most evident in the raster plots,

though also within the autocorrelograms that show a less evident initial peak followed by a flattened rate of activity. Bin size = 1 ms.

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Figure 5.

Anabolic/androgenic steroid (AAS) treatment throughout adolescence significantly altered the proportion of cell firing patterns in the lateral anterior hypothalamus compared to cells from vehicle controls (VEH). Bursting cells accounted for 7% ($n = 5$ cells from 3 animals) of all cells in AAS-treated animals, while this firing pattern was found in more than 20% ($n = 8$ cells from 6 animals) of cells from VEH controls. Cells with a regular firing pattern accounted for 39% ($n = 27$ cells from 14 animals) and 34% ($n = 13$ cells from 9 animals) of neurons in AAS vs VEH treated animals, respectively; and irregular cells accounted for 53% ($n = 37$ cells from 14 animals) in AAS and 45% ($n = 17$ cells in 11 animals) in VEH-treated animals of all cells in each treatment group. * $p < 0.05$.

Figure 6.

Correlational analysis revealed that the interspike interval in AAS-treated animals was significantly positively correlated with bite frequency (**Left**), suggesting that slower firing cells are involved in a highly aggressive form of offensive attack behavior after adolescent AAS exposure. This relationship was not found in vehicle-treated controls (**Right**). * $p < 0.05$. AAS: 68 cells (1 outlier removed) from 15 animals; VEH: 38 cells from 15 animals.

Figure 7.

Anabolic/androgenic steroid (AAS) treatment throughout adolescence significantly differentially alters the proportion of action potential firing frequency responses to AVP, 5HT, and ETIC microinfusions into the lateral anterior hypothalamus of the hamster brain. Bar graphs (**Left** column) show the proportion of cells that responded to AVP, 5HT, and ETIC microiontophoretic applications. Overall, there were proportionally fewer AVP responsive cells in AAS treated animals than in VEH treated animals. When these responses were separated based on whether their action potential frequency was excited or inhibited,

AAS-treated animals had more excitatory responses than VEH-treated animals, while VEH animals had more cells showing an inhibitory response than AAS animals. This effect was reversed for cells that responded to 5HT application. Alternatively, although more VEH cells were responsive to ETIC than AAS cells, there was no difference in the proportion of response types between the treatment groups. Post hoc Binomial exact test: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$. AAS: 69 cells from 15 animals; VEH: 38 cells from 15 animals.

Figure 8.

The bar graphs in the left column show the primary response changes in firing frequency (Hz) from LAH neurons 30 s before and 30 s after microiontophoretic application of vasopressin (AVP), serotonin (5HT), or Etiplopride (ETIC). The line graphs on the right detail how these changes occurred over the entire 1 min interval. Action potential firing frequency was primarily increased after AVP application in AAS animals, while it primarily decreased the activity of cells from VEH-treated animals (**Top**). This effect was the opposite of that observed in response to 5HT where, AAS cells primarily showed decreased activity

and VEH cells showed increased activity (**Middle**). ETIC exposure primarily decreased the activity of responsive cells from both AAS and VEH treated animals (**Bottom**). Within-subjects t-test between basal neuronal activity and activity during drug application: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. AAS: 69 cells from 15 animals; VEH: 38 cells from 15 animals.

Figure 9.

Line graphs show the change in action potential firing frequency from baseline of AVP responsive cells (Top row) and non-AVP responsive cells (**Bottom** row) after AVP exposure (45 nA for 30 s) followed by 5 min washout, 5 min baseline, and then 5HT (60 nA for 30 s) or Eticlopride (ETIC) exposure (50 nA for 30 s), 5 min washout, 5 min baseline, and finally dual application of either AVP and 5HT or AVP and ETIC (30 s). Post hoc results from 2-way mixed design ANOVA with microinfusion as the repeated measure: **a**: Sig. different from baseline, **b**: Sig different from AVP, **c**: Sig different 5HT, **d**: Sig different from ETIC, **#**: Sig different from VEH control. AVP+5HT regimen: AAS: n=27 cells, VEH: n=19 cells. AVP+ETIC regimen: AAS: n = 27 cells, VEH: n = 19 cells.

Figure 10.

Previously we have shown that DA D2 receptors, DA neurons, as well as GABA neurons that co-localize with and without D2 receptors are increased within the lateral anterior hypothalamus (LAH) after AAS exposure throughout adolescence. The **left panel of Model 1 (Top)** shows that increased AVP release is made possible (after AAS exposure) through tonic inhibition of putative GABA neurons that possess D2 receptors. In the presence of ETIC (**Top Right**), D2 receptors are blocked leading to the disinhibition of GABA release, and subsequent tonic inhibition of AVP release in the LAH, thereby inhibiting the activation

of aggression enhancing glutamate (GLU) cells. Behavioral data from our lab (Melloni & Ricci, 2010, Morrison et al., 2015a, Carrillo et al., 2011a, Carrillo et al., 2011b) and elsewhere (Ferris et al., 1997) suggests that AVP release and activation of AVP V1A receptors on GLU cells are necessary for the aggressive phenotype that emerges after adolescent AAS exposure. **Model 2** illustrates our hypothesis that two types of GABA neurons within the LAH exist to modulate aggressive behavior. The first type is comprised of GABA neurons that possess D2 receptors and emerge after AAS exposure (see Model 1). The second type of GABA neuron may exist prior to AAS exposure and co-express both AVP and D2 receptors. AVP receptors on these neurons may serve as a homeostatic mechanism that limits the intensity of aggressive display through blockade of AVP release and activation of downstream LAH aggression stimulating neurons (GLU). In Model 2, in the presence of ETIC (**Bottom Left**), inhibitory D2 receptors on GABA neurons are blocked leading to an increase in both the firing frequency of these neurons and the release of GABA. Increased GABA release inhibits AVP release via activation of terminal GABA receptors on AVP neurons. In the presence of microinjected ETIC and AVP (**Bottom Right**), the activity of GABA neurons is enhanced by the simultaneous blockade of inhibitory D2 receptors *and* the activation of excitatory AVP V1A receptors by exogenous AVP. The exogenous AVP also enhances aggression by activating AVP V1A receptors on downstream GLU cells.

Table 1

Baseline electrophysiological properties of specific LAH neuron types between drug treatment groups

Parameter	Neuron Type		
	Regular	Irregular	Bursting
<i>No. of neurons</i>			
VEH	13	17	8
AAS	27	37	5
<i>Mean AP Firing (Hz)</i>			
VEH	14.0 ± 2.6	11.5 ± 3.4	3.6 ± 1.3
AAS	9.3 ± 2.3	12.7 ± 3.5	1.6 ± 0.2
<i>Mean ISI (ms)</i>			
VEH	139.1 ± 41.3	226.1 ± 77	733.2 ± 176.7
AAS	391.9 ± 61.3 *	292.5 ± 49.1	1058 ± 239.1
<i>Median ISI (ms)</i>			
VEH	110.1 ± 29.2	189.8 ± 65.4	680.3 ± 126.7
AAS	282.9 ± 48.9 *	241.6 ± 38.3	808.2 ± 283.1
<i>Mode ISI (ms)</i>			
VEH	41.5 ± 6.5	105.5 ± 28.7	330 ± 177.3
AAS	227.1 ± 74.9	220.9 ± 72.03	1167 ± 416.8 +
<i>ISI CV</i>			
VEH	0.68 ± 0.06	0.75 ± 0.03	0.86 ± 0.03
AAS	0.70 ± 0.04	0.76 ± 0.02	0.79 ± 0.09

Data represent Mean ± SEM, + p < 0.06;

*
p • 0.05;**
p • 0.01;***
p • 0.001

Significant difference vs. vehicle. n=15 animals/treatment group