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Vasopressin Differentially Modulates Aggression and Anxiety in Adolescent Hamsters Administered Anabolic Steroids

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Abstract

Adolescent Syrian hamsters (*Mesocricetus auratus*) treated with anabolic/androgenic steroids display increased offensive aggression and decreased anxiety correlated with an increase in vasopressin afferent development, synthesis, and neural signaling within the anterior hypothalamus. Upon withdrawal from anabolic/androgenic steroids, this neurobehavioral relationship shifts as hamsters display decreased offensive aggression and increased anxiety correlated with a decrease in anterior hypothalamic vasopressin. This study investigated the hypothesis that alterations in anterior hypothalamic vasopressin neural signaling modulate behavioral shifting between adolescent anabolic/androgenic steroid-induced offensive aggression and anxiety. To test this, adolescent male hamsters were administered anabolic/androgenic steroids and tested for offensive aggression or anxiety following direct pharmacological manipulation of vasopressin V1A receptor signaling within the anterior hypothalamus. Blockade of anterior hypothalamic vasopressin V1A receptor signaling suppressed offensive aggression and enhanced general and social anxiety in hamsters administered anabolic/androgenic steroids during adolescence, effectively reversing the pattern of behavioral response pattern normally observed during the adolescent exposure period. Conversely, activation of anterior hypothalamic vasopressin V1A receptor signaling enhanced offensive aggression in hamsters exposed to anabolic/androgenic steroids during adolescence. Together, these findings suggest that the state of vasopressin neural development and signaling in the anterior hypothalamus plays an important role in behavioral shifting between aggression and anxiety following adolescent exposure to anabolic/androgenic steroids.

INTRODUCTION

In the United States (U.S.) and worldwide, the use of anabolic-androgenic steroids (AAS) by teenagers has been a concern for decades, yet AAS use has risen in this population in recent years (Eaton et al., 2012; Harmer, 2010). In the U.S., an estimated 1.3 million youth report

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AAS use, with the incidence of use ranging between 2.5% to 8% depending upon the reporting region (Eaton et al., 2012). These findings are similar to those from Europe and Australia where AAS use by adolescent males often exceeds 6% (Palleson et al., 2006; Williamson, 1993a, 1993b). Early onset AAS use is concerning given the abundance of clinical evidence showing its link to serious negative acute and long-term psychological consequences. For instance, while the most common negative psychiatric side effect of AAS use is increased aggression in adults (Kouri et al., 1995; Pope and Katz, 1994; Pope et al., 2000; Su et al., 1993) and teens (Beaver et al., 2008; Dabbs et al., 1991; Johnson, 1990; Johnson et al., 1989; Mattsson et al., 1980; Olweus et al., 1980), an increasing number of AAS users are being diagnosed with anxiety-related disorders (Bahrke et al., 1990; Johnson, 1990; Pagonis et al., 2006a, 2006b; Pope and Katz, 1994), which tend to emerge more frequently after discontinuing AAS (Bahrke et al., 1990; Brower, 2002; Corrigan, 1996; Lindqvist et al., 2007; Malone et al., 1995; Malone and Dimeff, 1992). In fact, both novice and seasoned AAS users report increased aggression and anxiety over time (Pope et al., 2000; Su et al., 1993; Hall and Chapman, 2005; Pagonis et al., 2006a), indicating that AAS use can promote the development of both behaviors in the same individual. Importantly, the range and extent of aggression and anxiety in users correlate with the severity of AAS use, with earlier and longer use times correlating with higher levels of both of these pathologic behaviors (Pagonis et al., 2006a, 2006b). Yet, while numerous studies have consistently shown that AAS produces escalated aggression across animal models (Lumia et al., 1994; McGinnis, 2004; McGinnis et al., 2002a, 2002b; Melloni and Ricci, 2010), there are far fewer studies that examine the effects of AAS on anxiety. Those that are available report varying outcomes between animal species, exposure periods, AAS, doses, and experimental paradigms. For instance, in adult and adolescent male and female rats and mice, AAS have been reported to be both anxiolytic (Aikey et al., 2002; Bing et al., 1998; Bitran et al., 1993; Fernández-Guasti and Martínez-Mota, 2005; Kouvelas et al., 2008; Ovsukova et al., 2003) and anxiogenic (Agis-Balboa et al., 2009; Ambar and Chiavegatto, 2009; Barreto-Estrada et al., 2004; Minkin et al., 1993; Parrilla-Carrero et al., 2009; Rainer et al., 2014; Rocha et al., 2007; Rojas-Ortiz et al., 2006). Although these findings support the notion that AAS exposure alters aggression and anxiety, they fail to provide specific insight into how these two behaviors are related.

For over two decades we have used pubertal male Syrian hamsters (*Mesocricetus auratus*) as an animal model to study the effects of adolescent AAS exposure on the neurobiology of aggression. Our data show that adolescent hamsters repeatedly administered AAS show high levels of aggressive responding during AAS exposure (Grimes and Melloni, 2002; Harrison et al., 2000; Melloni and Ricci, 2010) that subsequently falls to non-aggressive, control levels during AAS withdrawal (Carrillo et al., 2011; Grimes and Melloni, 2006; Grimes et al., 2006). More recent studies from our lab show that AAS exposure during adolescence increases aggression while also reducing anxiety (Ricci et al., 2013). The relationship between these two behaviors is reversed during AAS withdrawal at which point anxiety-like behaviors increase while aggressive behavior returns to control levels (Ricci et al., 2013; Morrison et al., 2015; Ricci et al., 2012). Moreover, changes in aggressive and anxiety-like behaviors between AAS exposure and withdrawal are linearly related suggesting that these two phenotypes are linked (Ricci et al., 2013), perhaps due to alterations in the signaling of

shared neural circuits and/or neurochemical systems that mediate both aggression and anxiety.

The arginine vasopressin (AVP) system in the hypothalamus has repeatedly been implicated in the facilitation of aggressive behavior in a number of animal models including hamsters administered AAS during adolescence (Melloni and Ricci, 2010; Morrison and Melloni, 2014). Conversely, while AVP neural signaling has been shown to affect anxiety (Appenrodt and Schwarzborg, 2000; Bhattacharya et al., 1998; Bielsky et al., 2005; Everts and Koolhaas, 1999; Landgraf and Wigger, 2002; Liebsch et al., 1996; McCarthy et al., 1996; Wigger et al., 2004; Williamson and Edwards, 1982), the available studies have primarily focused on *extrahypothalamic* AVP neural signaling, with little or no research examining the role of *hypothalamic* AVP in the modulation of anxiety.

Adolescent AAS-treated hamsters show high levels of aggression modulated by increased AVP afferent development, AVP production, and AVP release within the latero-anterior hypothalamic (LAH) subdivision of the anterior hypothalamus (AH) (Harrison et al., 2000; Melloni et al., 1997; Melloni and Ricci, 2010). In contrast, reduced aggression in these animals correlates with reduced afferent development of the AVP neural system within the LAH during AAS withdrawal (Grimes et al., 2006). It is during this withdrawal period that adolescent AAS-treated animals show increased anxiety-like responding (Ricci et al., 2012). Given that AAS-mediated alterations in LAH AVP neural circuitry are reduced from AAS exposure to AAS withdrawal during the behavioral shift from increased aggression to increased anxiety, we questioned whether altering the functioning of this neural circuit could affect the behavioral shift from aggression to anxiety as observed in our previous studies (Morrison et al., 2015; Ricci et al., 2012; Ricci et al., 2013).

Here we present the first set of preclinical studies that investigate the effects of adolescent AAS exposure on AVP neural signaling in the LAH as it pertains to the neurobehavioral relationship between aggression and anxiety. We hypothesized that during adolescent AAS exposure (i.e., when animals express heightened offensive aggression and diminished anxiety-like responding alongside increased afferent development, production, and release of AVP in the LAH), inhibition of LAH AVP neural signaling would facilitate a behavioral shift from heightened offensive aggression to a heightened anxiety state. To examine this question, animals were treated with AAS throughout adolescent development and tested for aggressive behavior or anxiety following direct pharmacological manipulation of LAH AVP receptor activity to determine whether alterations in AVP signaling in the LAH modulate a behavioral shift between adolescent AAS-induced offensive aggression and anxiety.

METHODS

Animals

Intact male Syrian hamsters (*Mesocricetus auratus*) postnatal day 21 (P21; pubertal hamsters) were obtained from Charles River Laboratories (Wilmington, MA), 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:10D; lights off at 7:00). For aggression testing, stimulus (intruder) males of equal size and weight to the experimental

animals were obtained one week prior to the behavioral test, group housed at 3–4 animals/cage in large polycarbonate cages, and maintained as above to acclimate to the animal facility. All experimental treatments and behavioral tests described below were administered during the first four hours of the dark cycle under dim-red illumination to control for circadian influences.

Drugs

Testosterone cypionate, nandrolone decanoate, and boldenone undecylenate were purchased from Steraloids Inc (Newport, RI) and prepared in sesame oil (SO). The neuropeptide arginine vasopressin (AVP) and the linear AVP V1A receptor antagonist (Manning Compound) [OH-Phaa-D-Tyr(Me)-Phe-Gln-Asn-Arg-Pro-Arg-NH₂] were purchased from Sigma Aldrich (St. Louis, MO). Manning compound was dissolved in 0.9% (wt/vol) normal saline and AVP was dissolved in ddH₂O.

Experimental Treatment

Postnatal day 27 Syrian hamsters (n=72) were weighed and received daily subcutaneous (SC) injections (0.1 ml – 0.2 ml) for 30 consecutive days (P27 – P56) of a mixture of 3 commonly used AAS (Hall et al., 2005) in doses consistent with repeated, moderate use in humans as described (see Melloni and Ricci, 2010 for a review). The AAS cocktail was composed of 2 mg/kg testosterone cypionate, 2 mg/kg nortestosterone and 1 mg/kg dihydrotestosterone undecylate dissolved in SO. As a control, a separate set of hamsters (n=16) received daily SC injections (0.15 ml) of SO alone.

Experimental Design

The day following the last AAS or SO injection (P57), AAS- and SO- treated animals were randomly split across four Experiments (n = 25–35 animals/Experiment). In Experiment 1, AAS-treated animals (n=18) and SO-treated controls (n=17) were randomly assigned to one of two groups and tested for either offensive aggression using the Resident/Intruder (RI) test (AAS; n=8, SO; n=8) or anxiety-like responding using the Elevated Plus Maze (EPM) (AAS; n=10, SO; n=9). In Experiment 2, a separate cohort of AAS-treated animals (n=25) were randomly assigned to one of three groups (n=8–9/group) and tested for offensive aggression after microinjection of one of three drugs into the LAH. The drugs were as follows: (1) saline (NaCl; 0.9%; n = 8), (2) Manning Compound, i.e., a AVP V1A receptor antagonist (9.0 uM; n = 8), or (3) AVP (0.09 uM; n = 9). In Experiment 3, a third cohort of AAS-treated animals (n = 28) were randomly assigned to one of three counterbalanced groups (n = 5–12/group) and tested for generalized anxiety-like responding using the Elevated Plus Maze (EPM) test after receiving one of three microinjections into the LAH. The drugs were as follows: (1) saline (NaCl; 0.9%; n=5), (2) Manning Compound (9.0 uM; n=11), or (3) AVP (0.09 uM; n=12). In Experiment 4, a fourth cohort of AAS-treated animals (n=25) were randomly assigned to one of three counterbalanced groups (n=8–9/group) and tested for social anxiety using the Social Interaction Test after receiving one of three microinjections into the LAH. The drugs were as follows: (1) saline (NaCl; 0.9%; n=8), (2) Manning Compound (9.0 uM; n=8), or (3) AVP (0.09 uM; n=9). During the Social Interaction Test, animals in Experiment 4 were also scored for motor behavior and grooming as described. The doses of Manning compound and AVP were selected based on previous

reports of efficacy in modulating aggression in hamsters (Ferris et al., 1997; Harrison et al., 2000).

Surgical Procedure

One week before aggression testing (P50), animals used in Experiments 2–4 were anesthetized with Ketamine/Xylazine (80 mg/kg:12mg/kg) and placed into a stereotaxic device for unilateral implantation of a 26 gauge guide cannula aimed at the LAH. An incision was made to expose the dorsal surface of the skull. The skull surface was then wiped clean to reveal the position of lambda and bregma landmarks. A small hole was drilled into the skull at the coordinate position necessary to gain access to the LAH located at 0.7mm anterior to bregma, 0.6mm lateral to the midsagittal suture, 6.8mm ventral from dura. The cannula was lowered into the brain angled at 8 degrees and anchored to the skull using dental screws and acrylic. The head-wound was then sutured closed and topical antibiotic ointment applied to the wound area. To verify cannula placement, animals were anesthetized with carbon-dioxide one day following behavioral testing and transcardially perfused with 4% paraformaldehyde. Brains were removed, postfixed for 90 min in perfusion fixative, and cryoprotected overnight in 30% sucrose at 4 °C. Brains were cut at 40 µm on a freezing microtome in serial coronal sections and mounted on gelatin-coated slides. Sections were stained with cresyl violet, dehydrated through a series of alcohols, cleared with xylene, and coverslipped with Permount (Fischer Scientific, Pittsburgh, PA). Only animals with correctly placed cannula tips into the LAH were included in the statistical analysis (Figure 1).

Microinjections

A 2-µl Hamilton syringe was connected to a 33-gauge stainless injection needle via polyethylene tubing. Infusion of drugs into the LAH was performed by lowering the injection needle through the guide cannula, delivering a final volume of 0.5µl of drug over 3 minutes and left in position for an additional minute to allow for drug diffusion away from the injector tip. The internal-injector cannula protruded 1 mm beyond the guide cannula toward the LAH (Figure 1). Based on the volume selected and the diffusion rate of microinjected substances (Routtenberg, 1972), spread of drug beyond the LAH of animals with correctly verified cannula placements is limited. Importantly, while it is difficult to restrict the spread of drug to the LAH and limit diffusion dorsal of the injection site (i.e. to the medial dorsal AH), the markedly lower distribution of AVP receptors in the dorsal medial regions of the AH (DeLeon et al., 2002) would suggest that receptor antagonism effects were produced in the LAH.

Aggression Testing

Hamsters were tested for offensive aggression using the Resident/Intruder (RI) test (Floody and Pfaff, 1977; Lerwill and Makings, 1971). For this, a novel intruder of similar size and weight was introduced into the home cage of the experimental animal (resident) and the resident scored for specific and targeted aggressive responses observed as described (Grimes et al., 2003; Ricci et al., 2006). An attack was scored each time the resident animal would pursue and either [1] lunge toward and/or [2] confine the intruder by upright and sideways threat; each generally followed by a direct attempt to bite the intruder's dorsal rump and/or

belly target area(s). The latency to attack was defined as the period of time between the beginning of the test and the first attack of the resident on an intruder. In the case of no attacks, latencies were assigned the maximum time (ie, 600s). Each test lasted for 10 minutes and was videotaped and scored manually by two observers unaware of the hamsters' experimental treatment. Inter-rater reliability was set at 95%. No intruder was used for more than one behavioral test.

Anxiety Testing

Elevated Plus Maze—Hamsters were tested for generalized anxiety behavior using the Elevated Plus Maze (EPM) test as in our prior studies (Morrison et al., 2015; Ricci et al., 2013, 2012). The EPM has been used extensively in rodents as an animal model of anxiety, with particular use as a sensitive behavioral test to screen for anxiolytic drug effects (Pellow et al., 1985; Pellow and File, 1986), including Syrian hamsters Gannon et al., 2011; Morrison et al., 2015; Ricci et al., 2013, 2012; Ricci et al., 2013). The EPM apparatus consisted of two open arms and two closed arms (30 × 5 cm) elevated to a height of 38.5 cm and intersecting in a central platform (5 × 5 cm). The closed arms had black Plexiglas walls (15 cm high) and Plexiglas lid. The apparatus was arranged such that the open arms were opposite to each other. Animals were individually placed in the center of the apparatus facing one of the closed arms. The number of entries into- and the duration of time (sec) spent beyond one complete body length within the open arms was calculated for each animal over a 5-min period. An increase in the percentage of entries and time spent within the open arms of the EPM was used as an index of anxiolytic behavior as described (Lister, 1987; Pellow et al., 1985). Each anxiety test was videotaped and coded by two observers unaware of experimental treatment.

Social Interaction Test—The Social Interaction Test consisted of the introduction of an experimental animal to a novel conspecific in an open field. The open field consisted of a large (*L* 42cm; *W* 42cm; *H* 36cm) opaque Plexiglas box with black tape on the underside of the floor of the box dividing the box into 4 separate quadrants. Animals (experimental and novel) were placed in opposite quadrants to start the beginning of the 10 min test. Social interest in conspecifics was determined by measuring physical contact time and social investigation; defined as the duration of time during which the AAS-treated animal initiated and maintained contact with the conspecific through olfactory investigation during the test period. In addition to social interaction, animals were also scored for motor and comfort behaviors. Animals were scored for open field matrix crossings (i.e., locomotion) and extent of self-grooming (i.e., comfort) throughout the 10 min social interaction test. A 'line-cross' was scored when the animal showed four paws over any given line thus belonging within a quadrant. Animals were tested during the first four hours of the dark cycle under dim-red illumination to control for circadian influences in behavioral responding.

Statistical Analyses

Behavioral results from aggression (i.e., flank/belly attacks, bites and latency to attack) and anxiety (i.e., number and % of entries and duration of time spent within the open arms of the EPM, contact time with conspecifics) during AAS exposure were compared between AAS and SO treatment groups using paired Student's *t*-test (two-tailed). Behavioral data from all

microinjection studies were compared using one-way, analysis of variance (ANOVAs) followed by Fisher's protected least significance difference post-hoc test (two-tailed) when appropriate. The α level for all statistical analyses was set at 0.05.

RESULTS

Adolescent AAS Exposure, Aggression and Anxiety

As previously reported (Ricci et al., 2013), during adolescent exposure, AAS-treated hamsters were significantly more aggressive and less anxious than SO-treated littermates (Figure 2). Adolescent AAS-treated animals directed significantly more rump/belly attacks ($t(14)=7.56$, $p<0.001$), bites ($t(14)=7.66$, $p<0.001$) and were faster to attack ($t(14)=5.14$, $p<0.001$) intruders than SO-treated controls. In fact, AAS animals exhibited a greater than tenfold increase in the number of rump/belly attacks, nearly thirtyfold increase in the number of bites, and were eight times faster to attack intruders compared to SO-treated controls. In a separate set of animals, AAS reduced anxiety-like behavior during adolescent AAS exposure (i.e., on P57) compared to SO-treated control animals as measured by the duration of time spent within the open arms of the EPM ($t(17)=6.32$, $p<0.001$). During adolescent AAS exposure, AAS-treated animals spent nearly twice as much time in the open arms of the EPM as SO-treated control animals. Despite this finding, there was no difference in the number of entries into the open arms of the EPM between AAS and SO treatment groups ($t(17)=0.82$, $p>0.05$).

Adolescent AAS Exposure, LAH AVP and Aggression

As reported previously (Carrillo et al., 2011b; Harrison et al., 2000), during adolescent AAS exposure the local infusion of AVP V1A receptor antagonists and agonists in the LAH produced an overall effect on offensive aggression as measured by flank/belly attacks ($F_{(2, 22)}=28.91$, $p<0.001$), bites ($F_{(2, 22)}=9.33$, $p<0.01$), and latency to attack ($F_{(2, 22)}=17.15$, $p<0.001$) (Figure 3). Post hoc analyses revealed that AAS-treated hamsters infused with AVP V1A receptor antagonists in the LAH showed a significant decrease in the number of rump/belly attacks ($t(14)=6.37$, $p<0.001$) and bites ($t(14)=3.59$, $p<0.01$) compared to saline infused controls and hamsters infused with AVP (rump/belly attacks $t(15)=7.56$, $p<0.001$; and bites $t(15)=4.95$, $p<0.001$). AAS-treated hamsters infused with AVP V1A receptor antagonists in the LAH showed on average fourfold fewer attacks and bites onto intruders during the behavioral test period than saline infused controls and animals infused with AVP. Interestingly, AAS-treated hamsters infused with AVP in the LAH also showed a trend towards an increase in the number of Rump/Belly Attacks ($t(15)=1.79$, $p=0.09$), but not bites ($t(15)=0.30$, $p>0.1$), compared to saline controls.

The local infusion of AVP antagonists into the LAH also significantly lengthened the latency to first attack of AAS-treated residents. Hamsters infused with AVP V1A receptor antagonists showed a significant increase in the time to attack intruders compared to both saline infused controls ($t(14)=3.98$, $p<0.01$) and animals infused with AVP ($t(15)=4.44$, $p<0.001$); nearly quadrupling the time to first attack onto intruders compared to these treatment groups. No significant difference in latency to attack was observed between AAS-

treated hamsters infused with AVP in the LAH and saline infused controls ($t(15)=0.72$, $p>0.1$).

Adolescent AAS Exposure, LAH AVP and Anxiety

During adolescent AAS exposure, the local infusion of AVP V1A receptor antagonists and agonists directly into the LAH produced an overall effect on generalized anxiety as measured by entries into- ($F_{(2, 25)}=8.79$, $p<0.01$) and time spent within- ($F_{(2, 25)}=10.57$, $p<0.001$) the open arm of the EPM in hamsters (Figure 4A). Post hoc analysis revealed AAS-treated hamsters infused with AVP V1A receptor antagonists in the LAH showed a significant decrease in the entries into- ($t(14)=2.83$, $p<0.01$) and time spent within- ($t(14)=2.49$, $p<0.05$) the open arms of the EPM compared to saline infused controls and hamsters infused with AVP (Entries, $t(21)=3.97$, $p<0.001$; Time, ($t(21)=4.54$, $p<0.001$). On average, AAS-treated hamsters in saline infused control and AVP treatment groups spent practically 80% of the test period in the open arms of the EPM, however, AAS-treated hamsters infused with AVP V1A receptor antagonists in the LAH spent and spent nearly 30% less time in the open arms than did animals infused with saline or AVP. Conversely, AAS-treated hamsters infused with AVP in the LAH showed no difference in entries into- ($t(15)=0.24$, $p>0.05$) and time spent within- ($t(15)=1.04$, $p>0.05$) the open arms of the EPM compared to saline infused controls. The local infusion of AVP antagonists and agonists into the LAH had no effect on the entries ($F_{(2, 25)}=1.76$, $p>0.05$) or time spent ($F_{(2, 25)}=1.96$, $p>0.05$) in all arms of the EPM (i.e., an indication that drug treatment had no significant effect on locomotor activity).

During adolescent AAS exposure, the local infusion of AVP V1A receptor antagonists and agonists directly into the LAH also produced an overall effect on social anxiety as measured by total contact time between conspecifics in the social interaction test ($F_{(2, 22)}=4.53$, $p<0.05$) (Figure 4B). Post hoc analysis revealed that AAS-treated hamsters infused with AVP V1A receptor antagonists in the LAH showed a significant decrease in the total duration of time spent smelling and attending to conspecifics compared to saline-infused controls ($t(14)=3.05$, $p<0.01$) and hamsters infused with AVP ($t(15)=2.40$, $p<0.05$). On average, AAS-treated hamsters in each treatment group spent greater than 6 min of the 10 min test period smelling and exploring conspecifics, however, AAS-treated hamsters infused with AVP V1A receptor antagonists in the LAH spent 1–2 min less investigating conspecifics during the social interaction test period than did animals infused with saline or AVP. AAS-treated hamsters infused with AVP in the LAH showed no difference in contact time with conspecifics compared to saline-infused controls ($t(15)=0.56$, $p>0.1$).

Adolescent AAS Exposure, LAH AVP, and General Behavioral Activation

During adolescent AAS exposure, the local infusion of AVP V1A receptor antagonists and agonists directly into the LAH produced an overall trend towards an effect on locomotion as measured by total number of line crosses ($F_{(2, 22)}=2.97$, $p=0.07$), while no effect of AVP V1A antagonists or agonists were observed on comfort behavior as measured by self-grooming ($F_{(2, 22)}=0.57$, $p>0.1$) (Table 1). Post hoc analysis revealed that while AAS-treated hamsters infused with AVP V1A receptor antagonists into the LAH showed no significant difference in total line crosses compared to saline infused controls ($t(14)=1.65$, $p>0.1$),

hamsters infused with AVP V1A receptor antagonists displayed significantly fewer total line crosses when compared to hamsters infused with AVP ($t(15)=2.37$, $p<0.05$). Resident AAS-treated hamsters infused with AVP in the LAH showed no difference in line crosses compared to saline infused controls ($t(15)=0.76$, $p>0.1$).

DISCUSSION

In recent studies, we investigated the influence of AAS exposure on the relationship between aggression and anxiety as these behaviors present during AAS exposure and withdrawal (Morrison et al., 2015; Ricci et al., 2012; Ricci et al., 2013). We found that repeated administration of moderate doses of AAS during adolescent development simultaneously increased aggression and decreased anxiety during the AAS exposure period, then decreased aggression and increased anxiety during AAS withdrawal. Linear regression analysis revealed that the difference in aggressive responding between the AAS exposure and withdrawal periods was a significant predictor of differences in anxiety within individuals, specifically showing that from adolescent AAS exposure to withdrawal, decreased aggression predicts increased anxiety (Ricci et al., 2013). These findings support the idea that during withdrawal, adolescent AAS-treated animals no longer responded aggressively because they are anxious (Carrillo et al., 2011; Grimes and Melloni, 2006; Grimes et al., 2006), suggesting that these behavioral phenotypes are linked – perhaps due to alterations in the signaling of neural systems that influence aggression and anxiety. Evidence of a relationship between aggression and anxiety exists across several animal models. For instance, in animals bred for high (HAB) or low (LAB) anxiety behavior, aggression and anxiety negatively correlate, such that LAB mice display higher aggression than HAB mice while rats bred for high levels of anxiety show lower levels of aggression (Veenema et al., 2007; Beiderbeck et al., 2012; Kantor, Anheuer, & Bagdy, 2000). This relationship is not absolute since mice selected for extremely high aggression have also been shown to exhibit high anxiety compared to those with low aggression, as do aggressive mice from other inbred strains (Apter et al., 1990; Fehon et al., 2001; Guillot and Chapouthier, 1996; Veenema and Neumann, 2007; Veenema et al., 2007). Taken together, these studies outline a behavioral relationship between aggression and anxiety, yet comparatively little is known about the neurobiological nature of this interaction, particularly as it pertains to AAS use. Close examination of the neural circuits that modulate these behaviors reveal overlapping neural loci and neurochemical systems, supporting the hypothesis that aggression and anxiety share a common neurobiology (Delville et al., 2000; Ernst and Fudge, 2009; Ferris, 2000; Newman et al., 1999; Pratt, 1992). For instance, the anterior hypothalamus (AH) appears positioned at the center of overlap for neural inputs that influence both aggression and anxiety (Delville et al., 2000; Nelson and Trainor, 2007; Pratt, 1992). Within the AH, the development and activity of the arginine vasopressin (AVP) neural system has been shown to play a critical role in the control of offensive aggression in a number of animal species (Morrison and Melloni, 2014). However, while AH neural circuits exist at the center of aggression research, few studies have focused on the role that this brain region and its local neural circuitry play in the modulation of anxiety – particularly as it pertains to the relationship between aggression and anxiety resulting from drug abuse.

Here we present the first set of preclinical studies to date that investigate the effects of adolescent AAS exposure on the neurobehavioral relationship between anterior hypothalamic AVP neural signaling, offensive aggression, and anxiety-like behavior. In these studies, pubertal male hamsters were treated with AAS and tested for offensive aggression or anxiety-like responding following direct pharmacological manipulations of AH AVP neural signaling to determine whether local stimulation or blockade of AVP V1A receptors within the AH modulate behavioral shifting between adolescent AAS-induced aggression and anxiety. We found that AAS administration has potent aggression-stimulating and anxiety-suppressing effects during the adolescent AAS exposure period that were modulated by the state of AVP neural signaling within the lateral aspects of the AH, i.e., the latero-anterior hypothalamus (LAH). Specifically, we found that AVP V1A receptor blockade within the LAH during adolescent AAS exposure suppressed offensive aggression and enhanced anxiety-like responding, effectively reversing the pattern of behavioral responding normally observed during the adolescent AAS exposure period. Adolescent AAS-treated animals administered AVP V1A receptor antagonists directly into the LAH elicited fewer attacks and bites and were slower to attack, while during anxiety testing AAS treated animals ventured less into- and spent less time within- the open arms of the EPM after AVP V1A blockade; indicative of increases in generalized anxiety. In addition, AAS animals administered AVP V1A receptor antagonists also spent less time in contact with conspecifics during the social interaction test than saline-infused controls, indicating that a reduction of AVP activity within the LAH also enhances social anxiety and avoidance in adolescent AAS-treated animals. This notion is supported by studies showing that although infusions of AVP V1A receptor antagonists to the AH also reduce odor-induced flank marking (i.e., a stereotypic motor behavior that serves as a measure of social communication in hamsters), they fail to increase (and in several instances decrease) social interaction between conspecifics (Ferris et al., 1985; Ferris and Potegal, 1988; Ferris et al., 1988). Further, more recent studies show that that juvenile rats infused with AVP V1A receptor antagonists into the lateral ventricle display significantly reduced social play; a characteristic feature observed in rats bred for juvenile high anxiety (i.e., HAB rats) (Lukas and Wöhr, 2015). Together, these findings not only confirm our earlier observations that a reduction in AVP neural signaling within the LAH during adolescent AAS exposure suppresses offensive aggression (Harrison et al., 2000), they report the novel observation that a similar reduction in AVP V1A receptor activity within the LAH enhances both generalized and social anxiety in adolescent AAS-treated animals.

In contrast to AVP V1A blockade, AVP V1A receptor activation within the LAH during adolescent AAS exposure marginally enhanced aggression above that of animals administered saline or AVP V1A receptor antagonists. Relatedly, animals administered AVP showed the same degree of anxiolytic behavior as saline-treated animals across all measures in both the EPM and the Social Interaction tests. The negligible effects of exogenous AVP on aggression and anxiety are likely due the fact that adolescent AAS-treated animals already have significantly more AVP in the LAH. Animals exposed to AAS during adolescence possess modest, approximately 3 fold increases in AVP afferent innervation to the LAH and 2–3 fold increases in AVP peptide content within the LAH (Grimes et al., 2003; Grimes et al., 2007; Harrison et al., 2000), and release nearly 3 fold more AVP into

the LAH during aggressive encounters with conspecifics (Melloni and Ricci, 2010). Functional studies show that low-moderate elevations in AVP within the AH are *necessary* to facilitate offensive aggression in hamsters (Ferris et al., 1997), while overstimulation of AVP signaling in this brain region by markedly increasing AVP (>10 times that required to stimulate aggressive responding) leads to an overwhelming tendency for the animal to flank mark, i.e., a stereotypic motor behavior that is part of the ethogram of offensive aggression in hamsters (Albers et al., 1986; Ferris et al., 1984).

Data from these studies further suggest that the state of AVP neural development and signaling within the LAH may play an important role in the behavioral shifting between aggression and anxiety that we have shown to occur in hamsters between the adolescent AAS exposure and withdrawal periods (Ricci et al., 2013; Ricci et al., 2012). In particular, these findings indicate that during periods of high AVP activity within the LAH, animals are likely to respond aggressively, while during periods of low AVP activity within the LAH, animals respond with high levels of anxiety. Support for this interpretation comes from our prior studies that examined the development of the LAH AVP system during AAS exposure and withdrawal. During AAS exposure (when AAS animals are aggressive, not anxious) we found that the density of AVP afferent development to the LAH are positively correlated with the temporal onset- and continued expression- of the aggressive behavioral phenotype (Grimes et al., 2003; Grimes et al., 2007; Harrison et al., 2000), while during AAS withdrawal (when AAS animals are anxious, not aggressive), the non-aggressive behavioral phenotype appeared alongside a significant reduction in AVP afferent innervation to the LAH (Grimes et al., 2006). Considering that the timeframe over which AAS-mediated alterations in LAH AVP neural circuitry occurs mirrors the timeframe over which the behavioral change from aggression to anxiety takes place, our findings suggest that altered AVP neural signaling within the LAH may modulate the behavioral shift from aggression to anxiety observed in our previous studies (Morrison et al., 2015; Ricci et al., 2012; Ricci et al., 2013).

Other studies that have examined the role of extrahypothalamic and hypothalamic AVP neural signaling in aggression and anxiety support our findings and the hypothesis that altered AVP activity corresponds to changes in aggressive and anxiety-like behavior. For instance, direct infusions of AVP into the AH increase the frequency of aggressive behaviors (Ferris et al., 1997; Gobrogge et al., 2009) while AVP infusions delivered to the cerebral ventricles or the lateral septum (LS) decrease anxiety (Appenrodt et al., 1998; Appenrodt and Schwarzberg, 2000). Similarly, when AVP receptors are blocked in the septum, anxiety-related behaviors are increased (Everts and Koolhaas, 1999). Conversely, there is also evidence that AVP receptor activation increases- (Andrews and File, 1992; Beiderbeck et al., 2007; Mak et al., 2012; Mitchell et al., 1991) or has no influence on- (Crisp and Kerchner, 2006; Liebsch et al., 1996; Mikics et al., 2009) anxiety suggesting that the effects of AVP signaling on behavior are highly subregion-dependent. For instance, local infusion of AVP into the medial-dorsal portion of the LS reduces anxiety (Appenrodt et al., 1998), while more lateral and ventral LS microinjections have no effect (Liebsch et al., 1996). Along similar lines, in inbred mice, a sensitized AVP system facilitates anxiety while in outbred mouse strains, AVP infusion to the cerebral ventricles facilitates anxiety (Bhattacharya et al., 1998; McCarthy et al., 1996). Furthermore, over-expression of AVP V1A receptors increases

anxiety while V1A knock out mice and unilateral blockade of AVP V1A receptors in the septum reduce anxiety (Appenrodt and Schwarzberg, 2000; Bielsky et al., 2005; Liebsch et al., 1996). Although seemingly convoluted, together with our findings, these data generally suggest that AVP neural signaling in brain regions implicated in aggression also play a role in the modulation of anxiety.

From a mechanistic standpoint, adolescent AAS may modulate behavioral shifting from aggression during exposure to anxiety during withdrawal by altering the balance of function of AVP-sensitive neurons in regions of the brain that modulate both these behaviors. For instance, the AH, medial amygdala (MeA), and the bed nucleus of the stria terminalis (BNST) appear to be sites of overlap for AVP neural inputs that influence both aggression and anxiety (Davis and Shi, 1999; Delville et al., 2000; Nelson and Trainor, 2007; Pratt, 1992). Previously, we reported that the onset and maintenance of adolescent AAS-induced aggression correlates with an increase in the development of both the AH AVP and *glutamate* neural systems (Grimes et al, 2006; Carrillo et al., 2009a,b; Carrillo et al., 2010b; Fischer et al., 2007). During adolescent AAS exposure, AAS-treated hamsters have more AH-glutamate cells innervating the BNST and fewer AH-glutamate cells innervating the MeA (Carrillo et al., 2009; Carrillo et al., 2010a; Carrillo et al., 2011c). In subsequent studies we showed that increased glutamate-mediated stimulation of the BNST was dependent on AH-AVP signaling and that this neural pathway modulated offensive aggression during adolescent AAS exposure (Carrillo et al., 2011b). Thus, it appears that the increased excitatory glutamate output from the AH to the BNST is responsible for the expression of aggression, not anxiety, during adolescent AAS exposure. We hypothesize that during AAS withdrawal, AVP-mediated neuroplastic alterations in AH-glutamate neural signaling occur between the BNST and the MeA, and these alterations modulate the behavioral shift from aggression during AAS exposure to anxiety during withdrawal. This hypothesis is currently being investigated in the laboratory.

In summary, here we present the first set of preclinical studies that investigate the consequence of adolescent AAS exposure on AVP neural signaling in the AH brain region as it pertains to the neurobehavioral relationship between the expression of aggression and anxiety. Blockade of AVP V1A receptor signaling within the LAH both suppressed offensive aggression and enhanced general and social anxiety in hamsters, effectively reversing the pattern of behavioral responding normally observed during the adolescent AAS exposure period. Conversely, activation of AVP V1A receptor signaling within the LAH during adolescent AAS exposure enhanced offensive aggression and suppressed anxiety. These findings are novel and significant in that they are the first to illustrate that the state of AVP neural development and signaling within the LAH brain region plays a role in behavioral shifting between aggression and anxiety.

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HIGHLIGHTS

- 1.** Blockade of anterior hypothalamic vasopressin V1A receptor signaling suppresses offensive aggression and enhances general and social anxiety in hamsters administered anabolic/androgenic steroids during adolescence
- 2.** Activation of anterior hypothalamic vasopressin V1A receptor signaling enhances offensive aggression in hamsters exposed to anabolic/androgenic steroids during adolescence
- 3.** Vasopressin neural signaling in the anterior hypothalamus modulates behavioral shifting between aggression and anxiety in hamsters exposed to anabolic/androgenic steroids during adolescence

Figure 1. LAH microinjection

Micrograph of sample microinfusion with a properly placed guide cannula. Top arrow indicates guide cannula location; bottom arrow indicates site of drug microinfusion within the latero-anterior subregion of the hypothalamus (LAH). AH: Anterior Hypothalamus, F: fornix, NC: Nucleus Circularis, LH: Lateral Hypothalamus, LAH: Latero-Anterior Hypothalamus, sox: supraoptic decussation, ot: optic tract, MeA: Medial Amygdala, son: supraoptic nucleus. Adapted from *A Stereotaxic Atlas of The Golden Hamster Brain* (p. 24), by L. P. Morin and R. I. Wood, Copyright Elsevier (2001).

Figure 2. Adolescent AAS, Aggression and Anxiety

AAS exposure facilitates offensive aggression and suppresses anxiety in adolescent hamsters. On P57, adolescent hamsters exposed to AAS were tested for either aggression using the Resident/Intruder paradigm, or anxiety using the Elevated Plus Maze. Measures of aggressive behavior and impulsivity were significantly enhanced in AAS exposed animals while anxiety-related behavior was reduced. *** $p < 0.001$.

Figure 3. Adolescent AAS, LAH AVP and Aggression

AVP V1A receptor blockade in the latero-anterior hypothalamus (LAH) reduces offensive aggression in hamsters administered AAS during adolescence. Aggressive AAS pretreated animals were given one of three treatments and tested for offensive aggression using the Resident/Intruder test. The treatments were (1) no microinjection, (2) microinjection of NaCl (vehicle) into the LAH, or (3) microinjection of 9.0uM AVP V1A receptor antagonist (ie., Manning Compound) into the LAH. As seen previously (Harrison et al., 2000), LAH AVP V1A receptor blockade reduces the number of attacks and bites targeted towards the rump/belly region of intruders and increases the latency to first attack in adolescent AAS-treated animals. ($0.05 < ^+p < 0.1$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$).

Figure 4. Adolescent AAS, LAH AVP and Anxiety

AVP V1A receptor blockade in the latero-anterior hypothalamus (LAH) enhances anxiety-like responding in hamsters administered AAS during adolescence. AAS pretreated animals were given one of three treatments and tested for anxiety-like responding using the (A) Elevated Plus Maze (EPM) or (B) Social Interaction Test. The treatments were (1) microinjection of NaCl (vehicle) into the LAH, (2) microinjection of 9.0uM AVP V1A receptor antagonist (ie., Manning Compound) into the LAH, or (3) microinjection of 0.9uM AVP into the LAH. (A) LAH AVP V1A receptor blockade reduces the % of Entries into- and % of Time within- the Open Arms of the EPM compared to animals microinjected with vehicle (NaCl) or AVP into the LAH. (B) LAH AVP V1A receptor blockade reduces Contact Time between conspecifics in the neutral arena compared to animals microinjected with vehicle (NaCl) or AVP into the LAH. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Table 1**Ancillary Behaviors**

Effect(s) of AVP V1A receptor antagonists (i.e., Manning Compound) or agonists (i.e., AVP) on general measures of behavioral activation.

BEHAVIOR	NaCl	AVP V1A Receptor Antagonist (9.0uM)	AVP (0.09uM)
<i>Locomotion ($\pm$SEM)</i>			
Line Crosses	44.2 $\pm$ 3.77	35.3 $\pm$ 3.6	48.5 $\pm$ 3.3 * (<i>a</i>)
<i>Comfort Behavior ($\pm$SEM)</i>			
Grooming	7.25 $\pm$ 1.7	5.4 $\pm$ 0.9	6.78 $\pm$ 1.1

Data represents mean values plus standard error of the mean (SEM).

(*a*) compared to AVP V1A Receptor Antagonist

*
p<0.05