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Plasticity in Anterior Hypothalamic Vasopressin Correlates With Aggression During Anabolic–Androgenic Steroid Withdrawal in Hamsters

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In hamsters, adolescent anabolic–androgenic steroid (AAS) exposure facilitates offensive aggression, in part by altering the development and activity of anterior hypothalamic arginine vasopressin (AH–AVP). This study assessed whether these effects were lasting by examining aggression and AH–AVP during AAS withdrawal. Adolescent hamsters administered AAS were tested as adults for aggression at 1, 4, 11, 18, or 25 days of withdrawal, sacrificed the following day, and examined for AH–AVP afferent innervation using immunohistochemistry. Through Day 12 of withdrawal, aggression and AVP were significantly higher in AAS-treated hamsters than in controls. These differences were no longer observable by Day 19 of withdrawal, at which point the behavior and neurobiology of AAS-treated hamsters reverted to that observed in controls. These data indicate that adolescent AAS exposure has short-term, reversible effects on both aggression and AH–AVP, correlating AH–AVP with the aggressive/nonaggressive behavioral phenotype during AAS withdrawal.

Keywords: adolescence, anabolic–androgenic steroids, arginine vasopressin (AVP), anterior hypothalamus, aggression

Studies from the National Institute on Drug Abuse estimate that nearly half million 8th and 10th grade students are using anabolic–androgenic steroids (AASs) in the United States each year (NIDACapsules, 2005). Reports indicate that AAS use has remained relatively stable in this population with 1.3% of male 8th graders, 2.3% of male 10th graders, and 3.3% of male 12th graders reporting use in 2004 (NIDACapsules, 2005). This pattern of abuse is important, because the onset of AAS use during adolescence is correlated with more frequent and heavier use later in life despite the associated physical and psychological ramifications (Buckley et al., 1988; Yesalis et al., 1988). Thus, this population of users may constitute a significant portion of the stable, long-term AAS-abusing population, increasing risk for the numerous behavioral and psychiatric side effects associated with AAS abuse, including increased aggressive behavior and hostility (Pope & Katz, 1994; Strauss, Wright, & Finerman, 1987; Su et al., 1993).

In a number of previous studies, we have used subadult male Syrian hamsters as an adolescent animal model to examine the link between developmental AAS exposure and the behavioral neurobiology of offensive aggression (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002, 2005; Grimes, Ricci, & Melloni, 2003; Harrison, Connor, Nowak, Nash, & Melloni, 2000; Melloni, Connor, Hang, Harrison, & Ferris, 1997; Melloni & Ferris, 1996). Behavioral data from these studies indicated that animals repeatedly exposed to high doses (5.0 mg/kg/day) of AAS during adolescent development display highly elevated levels of offensive aggression 1 day following the suspension of drug treatment. This escalated behavioral phenotype is characterized by intense bouts of biting and attacking, primarily directed toward the flanks and rump of the intruder, as well as high amounts of lateral offensive movements (i.e., lateral attacks) toward the intruder. The finding that adolescent AAS-treated hamsters demonstrated escalated, offensive aggression in the absence of prior social interactions and dominance cues suggested that adolescent exposure to AAS stimulated aggression directly, likely by affecting the development and/or activity of neural circuits that regulate this behavior.

In hamsters, the neural circuit controlling offensive aggression has been uncovered at the level of the anterior hypothalamus (AH). In this brain region, arginine vasopressin (AVP) activity facilitates offensive aggression through an interaction with the AVP $V1_A$ receptor subtype (Caldwell & Albers, 2004; Delville, De Vries, & Ferris, 2000; Ferris, 1992; Ferris, Axelson, Martin, & Roberge, 1989; Ferris et al., 1997; Ferris & Potegal, 1988; Jackson et al., 2005; Potegal & Ferris, 1989). Previous work from our laboratory has shown that adolescent AAS-induced offensive aggression correlates with developmental alterations in the AH-AVP neural system and is modulated by AH–AVP activity (DeLeon, Grimes, & Melloni, 2002; Harrison et al., 2000). For instance, AH-AVP neurons contain more AVP and display a significantly more developed pattern of AVP afferent innervation to the AH in aggres-

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sive, adolescent AAS-treated animals than in nonaggressive, vehicle-treated littermates, implicating an enhancement of AH-AVP neural signaling in aggressive, AAS-treated animals (Harrison et al., 2000). Thus, it appears that the development of the AH–AVP neural system is sensitive to the biological effects of long-term, high dose AAS exposure during adolescent development. Behavioral pharmacology studies have shown that the aggressive phenotype displayed by adolescent AAS-exposed animals could be inhibited by microinjections of selective AVP $V1_A$ receptor antagonists directly into the AH (Harrison et al., 2000), supporting the notion that AH–AVP activation plays an important role in adolescent, AAS-induced offensive aggression. Together, these data indicate that adolescent AAS exposure enhances the development and activity of the AH–AVP neural system, increasing AH–AVP tone and producing neurodevelopmental changes consistent with the generation of the aggressive behavioral phenotype. Given these data, one question that remains is whether the adolescent AAS-induced changes in aggression and AH–AVP are static or plastic, continuously reshaping to alter the expression of the aggressive phenotype.

To date, there has been little investigation of whether adolescent AAS effects are short-lived and reversible or persist well into adulthood, and those studies that have addressed this important issue have focused entirely on the behavioral effects of AAS during withdrawal. For instance, long-term increases in aggression (i.e., through 17 weeks of withdrawal) were observed in rats treated with AAS during puberty when compared with gonadally intact controls; however, this behavioral phenotype was only displayed when aggression was experimentally provoked through administration of a tail pinch prior to behavioral testing (Farrell & McGinnis, 2004). No differences in aggression were observed between rats exposed to AAS during puberty and control animals when the tail-pinch provocation was not administered prior to the aggression test. Although significant, these studies are limiting in that they did not include analyses correlating short- or long-term maintenance of the behavioral abnormalities (i.e., increased aggression) with neurobiological alterations. Thus, it remains unknown whether AAS exposure during adolescence has any lasting effects on the maintenance and/or activity of neural circuits important for aggression regulation. Does the exposure to AAS during adolescent development have lasting effects on the maintenance–activity of the AH–AVP neural system implicated in the control of aggression, predisposing adolescent users to prolonged periods of increased aggression?

The present study was conducted to examine whether repeated exposure to high-dose AAS during adolescent development has long-term effects on offensive aggression and the AH–AVP neural system—that is, the neural circuit important for aggression control in hamsters. First, to determine whether adolescent AAS effects on aggression were lasting, we measured offensive aggression in adolescent AAS-treated animals and oil-treated controls at 1, 4, 11, 18, or 25 days of withdrawal. Then, to determine whether adolescent AAS effects on AH–AVP were lasting, we examined the density of AVP afferent innervation to the AH in these same animals 1 day later in efforts to correlate the behavioral response patterns to AH–AVP neural circuitry.

Method

Animals

In Syrian hamsters, the adolescent period of development can be identified as the time between postnatal Days 25 and 56 (P25–P56). Weaning generally occurs around P25, with the onset of puberty beginning around P40 (Miller, Whitsett, Vandenbergh, & Colby, 1977). During this developmental time period, hamsters wean from their dams, leave the home nest, establish new solitary nest sites, and learn to defend their territory and participate in social dominance hierarchies (Schoenfeld & Leonard, 1985; Whitsett, 1975)

For the experimental treatment paradigm, intact preadolescent male hamsters (P21) were obtained from Charles River Laboratories (Wilmington, Massachusetts), individually housed in Plexiglas cages, and maintained at ambient room temperature on a reverse 14:10-hr light–dark cycle (lights on at 1900). Food and water were provided ad libitum. For aggression testing, stimulus (intruder) male hamsters of equal size and weight to the experimental animals were obtained from Charles River 1 week prior to the behavioral test, group housed at 5 animals per cage in large polycarbonate cages, and maintained as above to acclimate to the animal facility. All intruders were prescreened for low aggression (i.e., disengage and evade) and submission (i.e., tail-up freeze, flee, and fly away) 1 day prior to the aggression test to control for behavioral differences between stimulus animals, as previously described (Ferris et al., 1997; Melloni et al., 1997; Ricci, Grimes, & Melloni, 2004; Ricci, Knyshevski, & Melloni, 2005). Intruders displaying significantly low aggression and/or submissive postures (<5%) were excluded from use in the behavioral assay. All methods and procedures described below were preapproved by the Northeastern University Institutional Animal Care and Use Committee.

Experimental Treatment

On P28, hamsters ($N = 76$) were weighed and randomly assigned into five groups (Groups 1–5) corresponding to the time at which animals would be tested for offensive aggression and sacrificed for immunohistochemistry (see Figure 1). Each group was divided into two drug treatment groups: those administered a high-dose mixture of AAS suspended in sesame oil and those administered sesame oil alone (vehicle control). Hamsters ($n = 8$–10 animals/drug treatment/group) received daily subcutaneous injections (0.1 μl–0.2 μl) of an AAS mixture consisting of 2 mg/kg testosterone cypionate, 2 mg/kg nortestosterone, and 1 mg/kg dihydroxytestosterone undecylate (Steraloids Inc., Newport, Rhode Island) or sesame oil vehicle for 30 consecutive days (P28–P58), as previously described (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002, 2005; Grimes et al., 2003; Harrison et al., 2000). This daily treatment of AAS was designed to mimic a chronic "heavy use" regimen (Pope & Katz, 1988, 1994). Following the last injection on P58, animals were tested for offensive aggression (as detailed below) and sacrificed for immunohistochemistry at varied time points of AAS withdrawal. Group 1 (P59[60]) animals were tested for offensive aggression 1 day following cessation of drug treatment and then sacrificed 24 hr later (i.e., 1[2] days; see Figure 1), whereas animals in Groups 2 (P62[63]), 3 (P69[70]), 4 (P76[77]), and 5 (P83[84]) were tested and sacrificed at 4[5], 11[12], 18[19], and 25[26] days of AAS withdrawal, respectively. AAS- and oil-treated hamsters in each group were sacrificed via transcardial perfusion, and brains were removed and processed for AVP immunohistochemistry as detailed below.

Aggression Testing

Experimental animals were tested for offensive aggression using the resident/intruder paradigm, a well-characterized and ethologically valid model of offensive aggression in Syrian hamsters (Floody & Pfaff, 1977; Lerwill & Makaings, 1971). For this measure, an intruder of similar size

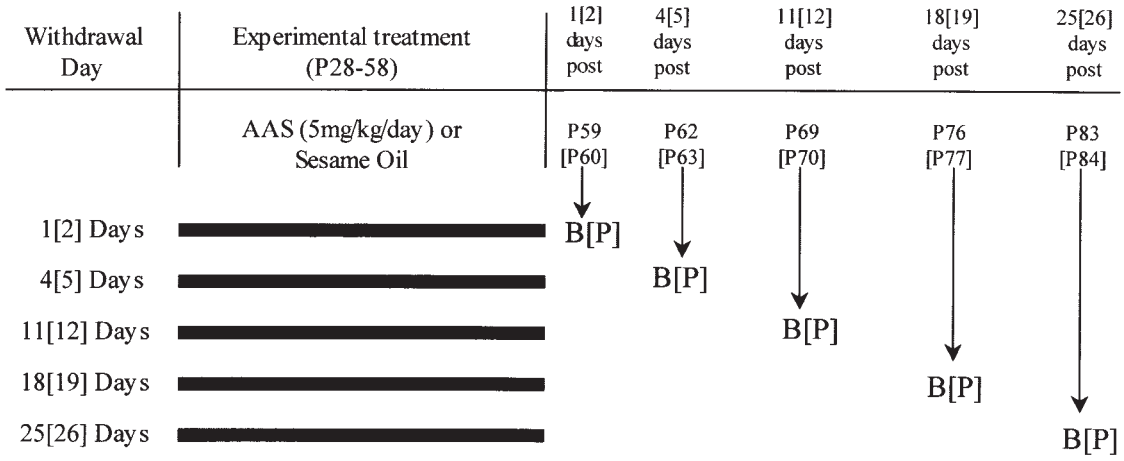

Figure 1. Diagram showing the experimental treatment paradigm. Hamsters were administered anabolic–androgenic steroid (AAS) or sesame oil throughout adolescent development, then tested aggression (i.e., behavior testing [B]) and processed for arginine vasopressin immunohistochemistry (i.e., physiology [P]) at 1[2], 4[5], 11[12], 18[19], or 25[26] days of withdrawal.

and weight was introduced into the home cage of experimental animals, and the resident was scored for offensive aggression (i.e., number of lateral attacks, upright offensive attacks, head/neck bites, flank/rump bites, and chases). Briefly, an attack was scored each time the resident animal would chase and then either (a) lunge toward and/or (b) confine the intruder by upright and sideways threat; each attack was generally followed by a direct attempt to bite the intruder's flank and/or rump. A composite aggression score, used as general measure of offensive aggression, was defined as the total number of attacks (i.e., upright offensives and lateral attacks) and bites (i.e., head/neck and flank/rump bites) during the behavioral test period. Each aggression test lasted for 10 min and was scored by an observer unaware of the hamsters' experimental treatment. No intruder was used for more than one behavioral test, and all tests were performed during the first 4 hours of the dark phase under dim red illumination and videotaped for behavioral verification of the findings.

Immunohistochemistry

One day following the behavioral test for aggression, AAS and sesame oil-treated hamsters were anesthetized with 80 mg/kg Ketamine and 12 mg/kg Xylazine and the brains fixed by transcardial perfusion with 4% paraformaldehyde. Brains were then cryoprotected by incubating in 30% sucrose in phosphate-buffered saline (PBS; 0.001M KH_2PO_4, 0.01M Na_2HPO_4, 0.137M NaCl, 0.003M KCl, pH 7.4) overnight at 4 °C. A consecutive series of 35-μm coronal sections were cut on a sliding microtome, collected as free-floating sections in 1X PBS, and labeled for AVP by single-label immunohistochemistry using a modification of an existing protocol (Ricci et al., 2004). For AVP immunohistochemistry, free-floating sections were pretreated with 4.5% H_2O_2 (30% stock solution) followed by preincubation in 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) with 0.6% Triton X-100. Sections were incubated in primary antiserum (1:10,000) for AVP antirabbit (DiaSorin, Saluggia, Italy) with 10% NGS, 1% BSA, and 0.6% Triton X-100 for 24 hr at 33 °C. After primary incubation, sections were incubated in secondary goat anti-rabbit followed by tertiary antisera (Vectastain ABC Elite Kit–rabbit, Vector Laboratories, Burlingame, California) for 90 min at room temperature and then labeled with diaminobenzidine (Vector Laboratories, Burlingame, California). Sections were mounted on gelatin-coated slides, allowed to air dry, and dehydrated through a series of ethanol and xylene solutions. Then, slides were coverslipped using Cytoseal-60 mounting medium (VWR Scientific, West Chester, Pennsylvania).

Image Analysis

The area covered by AVP-ir fibers was determined within the AH using the BIOQUANT NOVA 5.0 computer-assisted microscopic image analysis software package, as previously described (DeLeon, Grimes, Connor, & Melloni, 2002; Grimes & Melloni, 2002, 2005; Grimes et al., 2003). Slides from each animal were coded by an experimenter unaware of the experimental conditions, and BIOQUANT NOVA 5.0 image-analysis software running on a Pentium III CSI Open PC computer (R & M Biometrics, Nashville, Tennessee) was used to identify the AH at the level of the nucleus circularis at low power (4×) using a Nikon (Melville, New York) E600 microscope. At this magnification, a standard computer-generated box was drawn to fit within the particular region of interest. Then, under this same magnification, images were thresholded at a standard Red Green Blue–scale level empirically determined by observers blinded to treatment conditions to allow detection of stained AVP-ir fibers with moderate-to-high intensity while suppressing lightly stained elements. This threshold value was then applied across subjects to control for changes in background staining and differences in foreground staining intensity between animals. The illumination was kept constant for all measurements. AH–AVP-ir fibers were identified in each field using a mouse-driven cursor, and then AVP-ir measurements were performed automatically by the BIOQUANT software. One to two independent measurements of AVP-ir were taken from consecutive sections of each animal per treatment group depending on (a) identification of the exact position of the nucleus circularis within the region of interest and/or (b) the size of the nucleus in the rostral-caudal plane. Then, the density of AVP-ir fibers were determined for the entire AH (area density) and used for statistical analysis.

Statistics

Behavioral studies. Nonparametric aggression data (i.e., composite aggression scores, number of upright offensive and lateral attacks, head/neck and flank/rump bites and chases) were compared using planned comparison Mann–Whitney *U* tests (two-tailed) for AAS versus vehicle groups for each time point and Kruskal–Wallis analyses of variance

(ANOVAs) followed by Mann–Whitney *U* tests (two-tailed) for post hoc comparisons within treatment groups (i.e., AAS or vehicle) across the 5 separate withdrawal days for AAS-treated animals or age for vehicle-treated animals.

AH–AVP immunohistochemistry. The area density of AVP-ir fibers within the AH were compared between treatment groups for each period of withdrawal by one-way ANOVAs followed by Fisher's protected least significant difference tests post hoc.

Correlational analysis: Behavior and AH–AVP. Composite aggression scores and AH–AVP levels were collapsed across treatment (i.e., AAS and sesame oil) and withdrawal times and correlated using a Pearson's *r* correlation analysis.

Results

Offensive Aggression

As characterized extensively in our previous studies (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002, 2005; Grimes et al., 2003; Harrison et al., 2000), hamsters treated with AAS during adolescent development and tested for offensive aggression 1 day following the cessation of treatment (P59) displayed high levels of aggression compared with sesame-oil-treated controls (see Figure 2). For instance, AAS-treated animals displayed higher composite aggression scores (i.e., total number of attacks and bites) during the test period than sesame-oil-treated controls ($Z = 2.66$, $p < .01$). When examined more precisely, AAS-treated hamsters showed a significant increase in select components of the aggressive response pattern—namely, the numbers of lateral attacks ($Z = 2.20$), flank/rump bites ($Z = 2.19$), and chases ($Z = 2.37$) during the behavioral test compared with sesame-oil controls (all ps $< .05$).

In addition to the behavioral differences observed 1 day following the cessation of AAS treatment, a significant overall effect of time of AAS withdrawal on composite aggression, $\chi^2(4, N = 5) = 19.08$, $p < .001$, was observed. At P62 and P69 (i.e., 4 and 11 days of AAS withdrawal, respectively), composite aggression scores were higher in AAS-treated animals than in controls ($Z = 2.41$ and $Z = 2.16$, respectively, both ps $< .05$; see Figure 3). However, the behavioral differences observed between treatment groups were no longer present by 18 (i.e., P76; $Z = 0.53$) and 25 (i.e., P83; $Z = 0.82$) days of AAS withdrawal (both ps $> .10$). At these time points, composite aggression scores from AAS-treated animals were reduced to those of the nonaggressive behavioral phenotype observed in sesame-oil controls. In addition, within-AAS-group comparisons of composite aggression scores showed that at 1, 4, and 11 days of AAS withdrawal, AAS-treated hamsters were significantly more aggressive than AAS-treated hamsters tested at 18 and 25 days of AAS withdrawal (1 day vs. 18 days, $Z = 2.93$, $p < .01$; 1 day vs. 25 days, $Z = 2.41$, $p < .05$; 4 days vs. 18 days, $Z = 3.13$, $p < .01$; 4 days vs. 25 days, $Z = 2.87$, $p < .01$; 11 days vs. 18 days, $Z = 2.87$, $p < .01$; 11 days vs. 25 days, $Z = 2.43$, $p < .05$).

When examined in more accurate detail, a significant overall effect was observed for length of AAS withdrawal on select components of the aggressive response—namely, the numbers of lateral attacks, $\chi^2(4, N = 5) = 16.75$, $p < .01$; flank/rump bites, $\chi^2(4, N = 5) = 12.36$, $p < .05$; and chases, $\chi^2(4, N = 5) = 21.94$, $p < .001$. No differences in these behaviors were observed at any experimental point in oil-treated control animals, χ^2s$(4, N = 5) = 0.82$, 2.09, and 1.87, respectively, all ps $> .10$. At 4 and 11 days of AAS withdrawal, AAS-treated animals showed a significant increase in the numbers of lateral attacks ($Z = 2.82$, $p < .01$, and $Z = 2.45$, $p < .05$, respectively); flank/rump bites ($Z = 2.04$ and $Z = 2.16$, respectively, both ps $< .05$); and chases ($Z = 3.06$, $p < .01$, and $Z = 2.44$, $p < .05$, respectively) over vehicle-treated littermates (see Figure 4). As with composite aggression scores, however, the behavioral differences observed between treatment groups were no longer present by Days 18 (lateral attacks, $Z = 0.75$; flank/rump bites, $Z = 0.93$; and chases, $Z = 0.49$) and 25 (lateral attacks, $Z = 0.77$; flank/rump bites, $Z = 0.67$; and chases, $Z = 1.35$) of AAS withdrawal (all ps $> .10$). Within-AAS-group comparisons indicated that AAS-treated hamsters tested at 1, 4, and 11 days of AAS withdrawal displayed significant increases in these measures of aggression intensity compared with AAS-treated hamsters tested at 18 and 25 days. For instance, hamsters with 1,

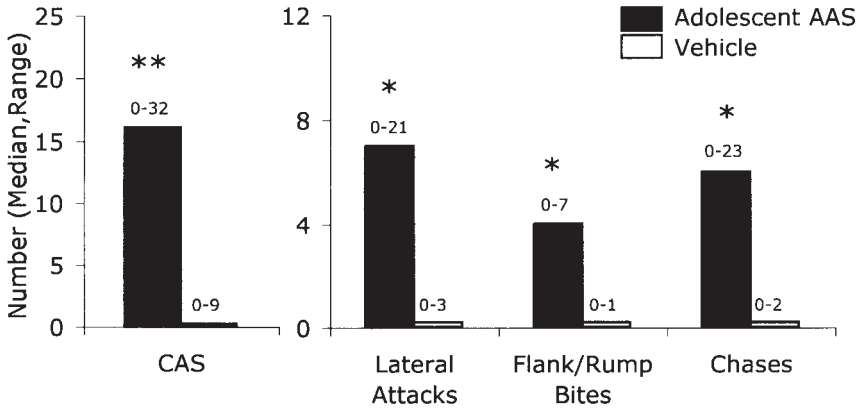

Figure 2. Adolescent anabolic–androgenic steroid (AAS) treatment and offensive aggression immediately following cessation of drug treatment. Composite aggression scores (CASs; total attacks and bites) and select measures of aggression intensity (lateral attacks, flank/rump bites, and chases) in adolescent AAS- and vehicle-treated residents. *$p < .05$; **$p < .01$, Mann–Whitney *U* tests (two-tailed).

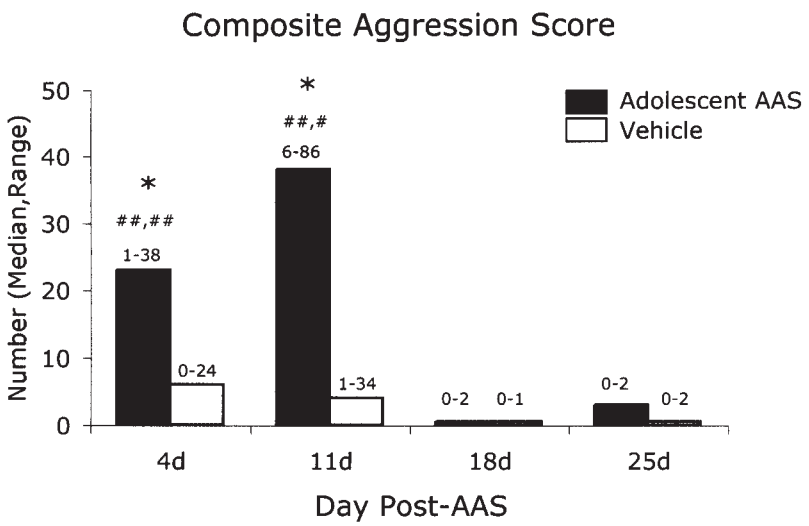

Figure 3. Comparison of composite aggression scores during withdrawal from adolescent anabolic–androgenic steroid (AAS) treatment. Comparisons with controls: $^{*}p < .05$. Within-group comparisons of adolescent AAS-treated animals at 4 and 11 days versus 18 and 25 days of withdrawal, respectively: $^{\#}p < .05$; $^{\#\#}p < .01$; Mann–Whitney *U* tests (two-tailed).

4, and 11 days of withdrawal from AAS showed significant increases in numbers of lateral attacks (1 day vs. 18 days, $Z = 2.41$, $p < .05$; 4 days vs. 18 days, $Z = 2.96$, $p < .01$; 4 days vs. 25 days, $Z = 2.43$, $p < .05$; 11 days vs. 18 days, $Z = 3.09$, $p < .01$; 11 days vs. 25 days, $Z = 2.52$, $p < .05$); flank/rump bites (1 day vs. 18 days, $Z = 2.43$, $p < .05$; 1 day vs. 25 days, $Z = 2.30$, $p < .05$; 4 days vs. 18 days, $Z = 2.03$, $p < .05$; 4 days vs. 25 days, $Z = 1.90$, $p < .05$; 11 days vs. 18 days, $Z = 2.56$, $p < .01$; 11 days vs. 25 days, $Z = 2.47$, $p < .05$); and chases (1 day vs. 18 days, $Z = 3.13$, $p < .01$; 1 day vs. 25 days, $Z = 2.07$, $p < .05$; 4 days vs. 18 days, $Z = 3.66$, $p < .001$; 4 days vs. 25 days, $Z = 2.69$, $p < .01$; 11 days vs. 18 days, $Z = 3.26$, $p < .001$; 11 days vs. 25 days, $Z = 2.47$, $p < .05$) when compared with AAS-treated hamsters with 18 and 25 days of AAS withdrawal.

AH–AVP Immunohistochemistry

A high density of AVP-ir was found in the AH of both AAS- and vehicle-treated hamsters. A dense staining pattern was distributed in the neuronal somata region located in the medial supraoptic nucleus and appeared consistent along the length of fibers from these neurons into the AH (see Figure 5A). As previously observed (Harrison et al., 2000), adolescent AAS treatment had an effect on AH–AVP-ir when examined 1 day following the treatment period. Specifically, at P60 (2 days of AAS withdrawal) animals treated with high-dose AAS throughout adolescence display a significant (nearly twofold) increase in the density of AVP-ir fibers in the AH when compared with vehicle-treated controls, $t(12) = 2.58$, $p < .05$ (see Figure 5B). In addition to these differences observed immediately following the cessation of AAS treatment, a significant overall effect of length of AAS withdrawal was observed on AH–AVP-ir, $F(9, 58) = 6.20$, $p < .0001$. Indeed, differences in AH–AVP-ir between AAS- and oil- treatment groups seen 2 days after the cessation of AAS treatment (i.e., P60 hamsters) were also observed at both 5 and 12 days of withdrawal (see Figure 6). Specifically, at 5 and 12 days of withdrawal, hamsters exposed to AAS during adolescence displayed significant increases in the mean area covered by AVP-ir fibers in the AH when compared with age-matched, oil-treated controls, $t(15) = 4.83$, $p < .001$, and $t(14) = 2.22$, $p < .05$, respectively. However, at 19 days and 26 days of AAS withdrawal, no differences were observed in AH–AVP-ir, $t(8) = 0.76$ and $t(9) = -0.32$, respectively, both $ps > .10$, between AAS-treated hamsters and vehicle-treated littermates. Within-AAS-group comparisons indicated a significant overall effect of length of AAS withdrawal on AH–AVP-ir, $F(4, 29) = 9.68$, $p < .0001$; however, no effect of time (age) on AH–AVP-ir was observed in oil-treated controls, $F(4, 28) = 1.44$, $p > .10$. Specifically, in AAS-treated hamsters, AH–AVP-ir at 2, 5, and 12 days of AAS withdrawal was significantly higher compared with hamsters examined on Days 19 and 26 of AAS withdrawal: 1 day versus 19 days, $t(11) = 7.22$, $p < .0001$; 1 day versus 26 days, $t(11) = 2.55$, $p < .05$; 5 days versus 19 days, $t(12) = 10.58$, $p < .0001$; 5 days versus 26 days, $t(12) = 2.74$, $p < .05$; 12 days versus 19 days, $t(11) = 10.17$, $p < .0001$; 12 days versus 25 days, $t(11) = 2.83$, $p < .05$.

Correlational Analysis: Offensive Aggression and AH–AVP Immunohistochemistry

When composite aggression scores and levels of AH–AVP-ir for each time point were collapsed across treatment (i.e., AAS and sesame oil), a direct linear correlation ($r = .441$, $p < .01$; see Table 1) was observed, with increases in AVP afferent innervation to the AH correlating with higher levels of offensive aggression.

Discussion

In a number of previous studies, we have shown that high-dose AAS exposure during adolescent development increases several

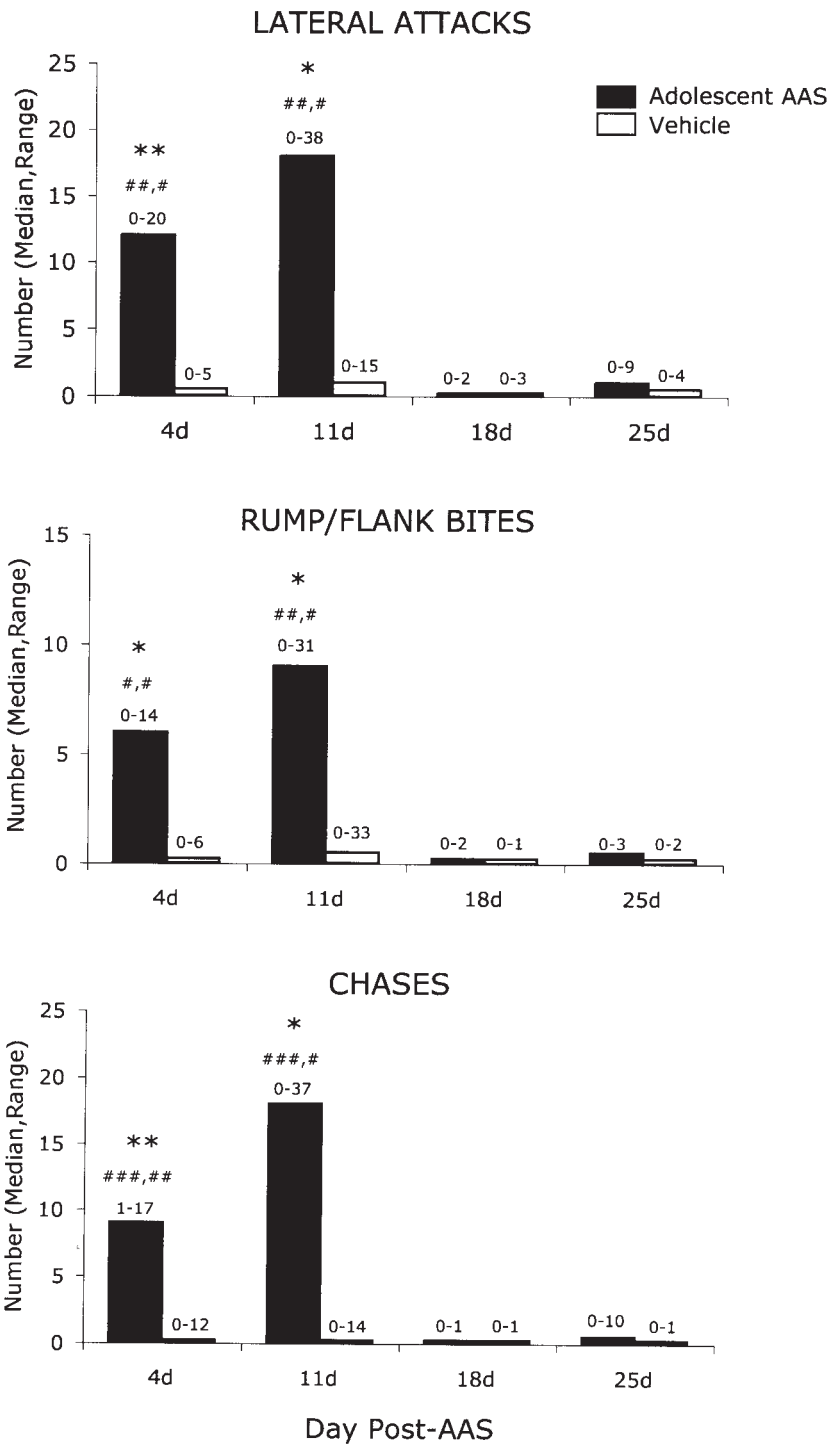

Figure 4. Comparison of specific and targeted measures of aggression intensity (lateral attacks, flank/rump bites, and chases) during withdrawal from adolescent anabolic–androgenic steroid (AAS) treatment. d = day. Comparisons with controls: $^{*}p < .05$; $^{**}p < .01$. Within-group comparisons of adolescent AAS-treated animals at 4 and 11 days versus 18 and 25 days of withdrawal, respectively: $^{\#}p < .05$; $^{\#\#}p < .01$; $^{\#\#\#}p < .001$; Mann–Whitney *U* tests (two-tailed).

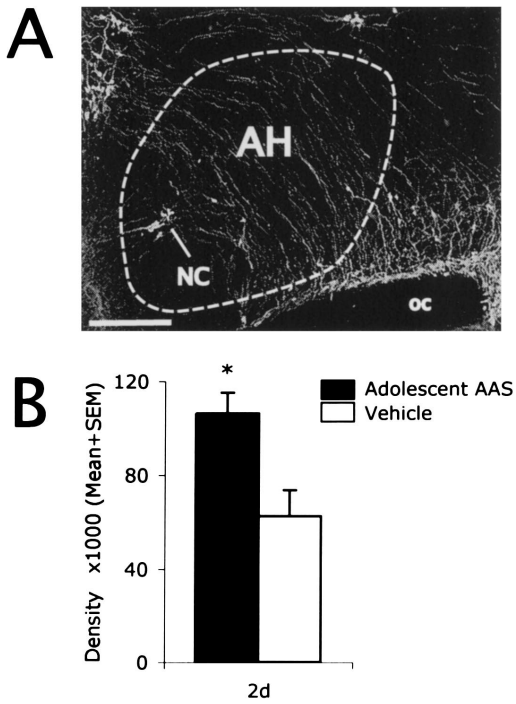

Figure 5. A: Darkfield photomicrograph showing immunoreactive labeling for arginine vasopressin (AVP) in the anterior hypothalamus (AH; seen as white neuronal somata and afferent fiber streams). NC = nucleus circularis; OC = optic chiasm; bar = 250 μm. B: Comparison of the density of AVP afferent fibers in the AH immediately following cessation of drug treatment. Fibers were quantified within standardized surfaces between groups for comparisons. d = day. *$p < .05$; Student's t test (two-tailed).

separate and distinct measures of offensive aggression—including lateral attacks, flank/rump bites, and chases—when behavior was examined immediately following the drug treatment period in young-adult Syrian hamsters (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002, 2005; Grimes et al., 2003; Harrison et al., 2000). To determine whether adolescent AAS exposure had lasting behavioral effects on aggression following the suspension of drug treatment, we examined these measures of offensive aggression after AAS treatment had been suspended for 1, 4, 11, 18 or 25 days. For these studies, gonadally intact, adolescent male Syrian hamsters were treated with high doses of AAS throughout adolescence and tested for offensive aggression following varying periods of AAS withdrawal. Replicating previous results from our laboratory (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002, 2005; Grimes et al., 2003; Harrison et al., 2000; Melloni et al., 1997) 1 day following the suspension of AAS treatment, AAS-treated hamsters were significantly more aggressive than age-matched, vehicle-treated controls as indicated by both composite aggression scores and increased numbers of lateral attacks, flank/rump bites, and chases directed toward the intruder. When examined at longer withdrawal times, adolescent AAS-treated hamsters displayed lasting increases in many indices of offensive aggression when compared with age-matched, vehicle-treated littermates; however, these behavioral effects were not permanent as the reemmergence of the control nonaggressive phenotype appeared by approximately 3 weeks of withdrawal. Animals maintained this nonaggressive phenotype through 25 days of withdrawal (i.e., the study endpoint). Within-AAS-group comparisons supported this movement toward the reemmergence of the nonaggressive phenotype by 3 weeks of withdrawal. Specifically, significant increases in composite aggression scores, lateral attacks, flank/rump bites, and chases were observed in AAS-treated hamsters following 1, 4, and 11 days of withdrawal as compared with littermates tested for aggression following 18 and 25 days of AAS withdrawal. These data are novel and significant; they indicate that adolescent AAS exposure has short-term, reversible effects on offensive aggression in hamsters and that the intensity of each component of the aggressive response heightened by adolescent AAS treatment diminishes back to control (nonaggressive) levels within a 1-week time span during short-term AAS withdrawal.

The finding that adolescent AAS treatment induces lasting but not permanent effects on offensive aggression in hamsters is to some extent similar to data from previous studies examining the behavioral effects of AAS withdrawal on aggression following pubertal and adult exposure in male Long–Evans rats (Farrell & McGinnis, 2004; McGinnis, Lumia, & Possidente, 2002). In these studies, either intact pubertal or adult rats were treated with AAS for more than 9 weeks, followed by a series of behavioral tests to examine aggressive behavior during prewithdrawal periods and/or following short-term (i.e., 3–5 weeks) or long-term (i.e., 9–12 and/or 15–17 weeks) withdrawal (Farrell & McGinnis, 2004; McGinnis et al., 2002). In both studies, lasting increases in aggression were noted for variable periods after the cessation of AAS treatment. In studies examining the effects of withdrawal from pubertal AAS (Farrell & McGinnis, 2004), AAS-treated animals showed heightened aggression compared with controls during the course of short- and long-term withdrawal. However, aggression was only observed in animals provoked to fight by tail pinch prior to testing. No effects of AAS were observed on unprovoked aggression (i.e., aggression test without prior tail-pinch provocation) following either short-term or long-term withdrawal. It is interesting to note that AAS-treated animals provoked by tail pinch failed to show increases in aggression compared with tail-pinched controls during prewithdrawal tests. The lack of an effect of AAS on aggression immediately following the pubertal exposure period and in the absence of tail-pinch provocation is puzzling given the large body of data from our laboratory indicating dramatic increases in aggressive responding in AAS-treated animals tested immediately following the cessation of adolescent AAS exposure using standard resident/intruder procedures (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002, 2005; Grimes et al., 2003; Harrison et al., 2000; Melloni et al., 1997; Melloni & Ferris, 1996). However, various methodological differences exist between these two studies that make a direct comparison difficult, including treatment regimen (i.e., administration of a single AAS vs. a mixture of AASs), dose of individual component AAS (5 mg/kg/day each vs. 1–2 mg/kg/day), duration of treatment (9 weeks vs. 4 weeks), and animal species used (rat vs. hamster). These differences notwithstanding, in similar studies examining the effects of withdrawal from adult AAS, increases in aggression were observed using standard resident/intruder procedures following short-term withdrawal, but only in animals administered prolonged

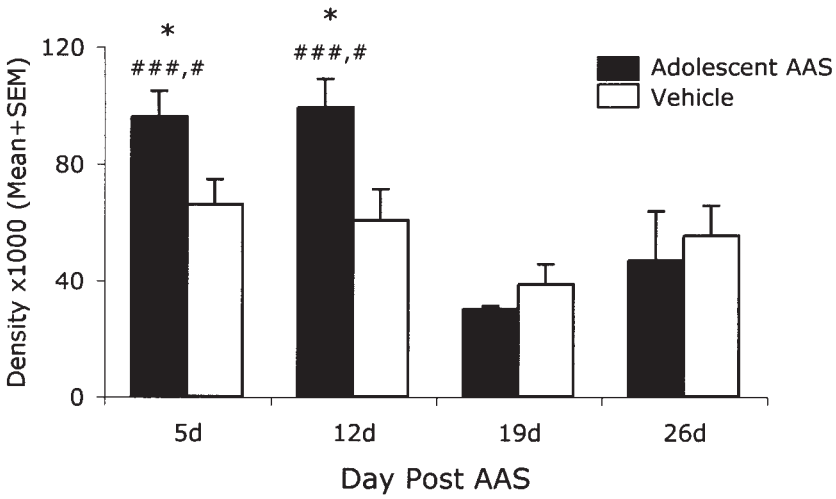

Figure 6. Comparison of the density of arginine vasopressin afferent fibers in the anterior hypothalamus during withdrawal from adolescent anabolic–androgenic steroid (AAS) treatment. d = day. Comparisons with controls: $^{*}p < .05$. Within-group comparisons of adolescent AAS-treated animals at 4 and 11 days versus 18 and 25 days of withdrawal, respectively: $^{\#}p < .05$; $^{\#\#\#}p < .01$; Student's *t* test (two-tailed).

high-dose AAS (5 mg/kg/day × 12 weeks; McGinnis et al., 2002). Together with data from this report, these studies illustrate that AAS may have differential lasting effects on behavior, dependent on age, length, and dose of drug exposure; animal species; and the behavioral testing paradigm used.

Adolescent AAS exposure may facilitate and maintain heightened levels of offensive aggression by producing sustained increases in the activity of neurochemical signals involved in this activation of this response. Previous work from our laboratory and the laboratories of others indicate that AH–AVP is a potent activator of offensive aggression in Syrian hamsters (Caldwell & Albers, 2004; Delville et al., 2000; Ferris, 1992; Ferris et al., 1989, 1997; Ferris & Potegal, 1988; Jackson et al., 2005; Potegal & Ferris, 1989). Aggressive, adolescent AAS-treated animals have an altered AH–AVP neural system; specifically, AH–AVP neurons contain more AVP in aggressive, adolescent AAS-treated animals and display increased AVP afferent development to the AH brain region implicated in the control of aggression, suggesting that increased AH–AVP tone underlies the development of the adolescent AAS-induced aggressive phenotype (Harrison et al., 2000). This notion was supported by behavioral pharmacology studies using select AVP-receptor antagonists (Harrison et al., 2000), strengthening the assertion that AH–AVP activity plays an important role in adolescent, AAS-induced offensive aggression. Together, these data suggested that adolescent AAS exposure increased offensive aggression by enhancing AH–AVP production, afferent development, and activity, providing direct evidence for a causal role of AH–AVP expression and function in early-onset AAS-induced aggression. Given these data, we questioned whether the exposure to AAS during adolescent development had lasting effects on the maintenance–activity of the AH–AVP neural system implicated in the control of aggression, predisposing adolescent users to prolonged periods of increased offensive aggression. To address this question, we examined whether adolescent AAS-induced increases in AH–AVP afferent innervation would persist during AAS withdrawal or the observed increases in AH–AVP afferent development would undergo neuroplastic reductions after the cessation of AAS treatment, correlating with the reemergence of the nonaggressive phenotype during withdrawal. As expected, in this study, the density of AH–AVP afferent fibers was greater in aggressive, adolescent AAS-treated animals than in nonaggressive, sesame-oil-treated controls when examined immediately following the treatment period. These increases persisted through 12 days of withdrawal in adolescent AAS-treated hamsters—that is, withdrawal times during which AAS-treated animals still showed heightened aggressive responding. However, the differences in AH–AVP between AAS- and vehicle-treated hamsters were no longer observed at later times of withdrawal—that is, at times when AAS-treated animals no longer showed heightened aggressive responding. When aggression levels and extent of AH–AVP afferent innervation for each time point were collapsed across treatment, a strong, positive correlation between AH–AVP fiber density and levels of offensive aggression was observed, indicating that at times of increased AH–AVP tone, animals respond more aggressively than they do when levels of AH–AVP are low. This correlation between aggression and AH–AVP across short-term withdrawal further strengthens the notion that the interactions between AAS and the AH–AVP neural system directly underlie adolescent AAS-induced alterations in offensive aggres-

Table 1
Pearson's r Correlational Analysis of Offensive Aggression and Anterior Hypothalamic Arginine Vasopressin (AH–AVP)

Aggression measure	AH–AVP
Composite aggression score	$r = .441^{**}$

$^{**}p < .01$.

sion. Together, these data indicate that adolescent AAS exposure has short-term, reversible effects on AH–AVP in hamsters and that changes in the AH–AVP neural system are linked to the display of the aggressive or nonaggressive behavioral phenotype in hamsters during AAS withdrawal. These data are novel and significant in that no studies to date have linked changes in the maintenance of defined neural systems implicated in aggression control with the display of the aggressive/nonaggressive behavioral phenotype during AAS withdrawal.

The finding here that lower levels of AH–AVP correlate with the nonaggressive phenotype is not surprising given studies examining the relationship between social status and AH–AVP. For instance, in recent studies using adult bluehead wrasse, pharmacologic manipulations that reduce aggression (Perreault, Semsar, & Godwin, 2003) correlated with lower arginine vasotocin (the teleost homologue of AVP) in the preoptic area (i.e., the AH equivalent in fish) relative to controls (Semsar, Perreault, & Godwin, 2004). Thus, marked neuroplastic reductions in AH–AVP are associated with a pharmacologic stimulus that predisposes hamsters to behave in a nonaggressive manner. In earlier studies examining AH–AVP during the establishment of dominant/subordinate relationships between adult hamsters, castrated hamsters repeatedly defeated by larger, more dominant conspecifics displayed little offensive aggression (Ferris et al., 1989). This decline in aggressive behavior in subordinated animals is correlated with a decrease in AH–AVP-ir neurons and levels compared with dominant partners (Ferris et al., 1989). Hence, marked neuroplastic reductions in AH–AVP afferent innervation are also associated with a nonpharmacologic stimulus that predisposes hamsters to behave in a nonaggressive manner. Together with findings from the present study, these data provide compelling evidence that neuroplastic alterations in AH–AVP occur in response to a diverse array of stimuli and that these changes can have dramatic impact on the behavioral phenotype presented by animal species.

In summary, this study provides the first examination of the effects of withdrawal from adolescent AAS exposure on aggression and the basic neurobiological mechanisms by which these agents exert their aggression-modulating effects. These findings show that offensive aggression and AH–AVP innervation were significantly higher in AAS-treated hamsters than in controls through nearly the first 2 weeks of AAS withdrawal. These differences were no longer observable at later withdrawal times, when the behavior and neurobiology of AAS-treated hamsters reverted to those observed in nonaggressive controls. These data suggest that adolescent AAS exposure has short-term, reversible effects on both offensive aggression and AH–AVP and correlate levels of AH–AVP with the aggressive/nonaggressive behavioral phenotype during AAS withdrawal, providing further evidence for a causal role of AH–AVP activity–function in adolescent AAS-induced offensive aggression.

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