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Prolonged Alterations in the Serotonin Neural System Following the Cessation of Adolescent Anabolic–Androgenic Steroid Exposure in Hamsters (*Mesocricetus auratus*)

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In hamsters (*Mesocricetus auratus*), anabolic–androgenic steroid (AAS) exposure during adolescence facilitates offensive aggression that is modulated, in part, by serotonin (5-HT) signaling and development and by signaling and expression of 5-HT_{1B} receptors. To examine whether these effects are persistent or reversible, the authors administered AAS to hamsters, then examined them for aggression at 1, 4, 11, 18, or 25 days following cessation of AAS treatment. Then, 1 day later, hamsters were killed by transcardial perfusion and examined for 5-HT afferents to and 5-HT_{1B} receptor-containing neuronal puncta and somata in areas of the brain altered by AAS, namely, the anterior hypothalamus, ventrolateral hypothalamus, and medial amygdala. Although aggression resulting from AAS exposure returned to control, nonaggressive levels by 18 days following cessation of AAS treatment, alterations in 5-HT afferent innervation and 5-HT_{1B} receptor localization were observed throughout the extended time period examined. These data suggest that adolescent AAS exposure may have long-term, irreversible effects on 5-HT neural systems and that return to nonaggressive behavioral phenotypes following adolescent AAS exposure may not be a function of plasticity in central 5-HT systems.

Keywords: adolescence, anabolic–androgenic steroids, serotonin, anterior hypothalamus, aggression

The serotonin (5-HT) system has been implicated in the control of aggression in humans (Brown et al., 1982; Coccaro, Bergeman, Kavoussi, & Seroczynski, 1997; Kruesi et al., 1990; Linnoila et al., 1983) and in a number of animal models of aggression (Higley et al., 1996; Kyes, Botchin, Kaplan, Manuck, & Mann, 1995; Sijbesma, Schipper, & De Kloet, 1990; Vergnes, Depaulis, Boehrer, & Kempf, 1988). In Syrian hamsters (*Mesocricetus auratus*), 5-HT activity in the anterior hypothalamus (AH) and the ventrolateral hypothalamus (VLH) has been shown to regulate offensive aggression (Delville, Mansour, & Ferris, 1996a; Ferris, 1996; Ferris et al., 1997; Ferris, Stolberg, & Delville, 1999) where 5-HT acts to suppress aggressive responding. The inhibitory nature of 5-HT on aggression has been predominately attributed to 5-HT action at a subset of 5-HT receptors, including (but not limited to) the 5-HT_1 subtype (i.e., 5-HT_{1A} and 5-HT_{1B}) receptors (Bell, Donaldson, & Gracey, 1995; de Almeida & Miczek, 2002; Miczek, Hussain, & Faccidomo, 1998; Muehlenkamp, Lucion, & Vogel, 1995; Rilke, Will, Jahkel, & Oehler, 2001; Sanchez, Arnt, Hyttel, & Moltzen, 1993).

In previous studies, we used pubertal Syrian hamsters as an animal model to examine the link among adolescent anabolic–androgenic steroid (AAS) exposure, 5-HT, and offensive aggression (Grimes & Melloni, 2002, 2005; Ricci, Rasakham, Grimes, & Melloni, 2006). Behavioral data from these studies showed that hamsters repeatedly exposed to AAS during development display high levels of offensive aggression. Neuroanatomical studies revealed that aggressive, adolescent AAS-treated hamsters had significant deficits in 5-HT afferent innervation in select areas of the brain implicated in aggression control (i.e., namely the AH, the VLH, and the medial amygdala [MeA]) when the hamsters were compared with nonaggressive, vehicle-treated littermates, implicating marked 5-HT hypofunctioning in these brain areas in AAS-treated hamsters (Grimes & Melloni, 2002). Subsequent studies showed that aggressive, adolescent AAS-treated hamsters had altered expression of 5-HT_{1B} receptors in each of these same brain regions (Grimes & Melloni, 2005). Specifically, aggressive, adolescent AAS-treated hamsters displayed significant decreases in 5-HT_{1B}-containing neuronal puncta and increases in the number of 5-HT_{1B}-containing neuronal somata in these select brain regions. Similarly, altered expression of 5-HT_{1A} receptors was detected in a subset of these areas (i.e., the AH) in aggressive, adolescent AAS-treated hamsters (Ricci et al., 2006). Together, these data suggest that changes in 5-HT neural signaling through 5-HT_1 receptors in select areas of the brain may underlie the aggressive phenotype observed in AAS-treated hamsters. Behavioral pharmacology studies showed that the aggressive response pattern displayed by adolescent AAS-exposed hamsters could be blocked by

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enhancing 5-HT neural signaling (Grimes & Melloni, 2002) through 5-HT_{1A} (Ricci et al., 2006) and 5-HT_{1B} receptors (Grimes & Melloni, 2005), supporting the notion that 5-HT hypofunctioning via 5-HT_1 receptors in select hypothalamic (and amygdaloid) brain regions may play an important role in adolescent, AAS-induced offensive aggression. Together, these data indicate that adolescent AAS exposure alters the development and activity of the 5-HT neural system, altering 5-HT tone and producing changes consistent with the generation of the aggressive behavioral phenotype. Given these data, the question that remains is whether the adolescent AAS-induced changes in 5-HT afferent innervation and 5-HT_{1B} receptor expression in the AH, VLH, and MeA are static or plastic—continuously reshaping to modulate the aggressive phenotype.

Recently, we have shown that exposure to AAS during adolescent development has lasting but not permanent effects on both the display of offensive aggression and on one basic neurobiological mechanism by which these agents exert their aggression-stimulating effects (i.e., the anterior hypothalamic arginine vasopressin [AH AVP] neural system; Grimes, Ricci, & Melloni, 2006). These findings showed that offensive aggression and the AH AVP were significantly higher in AAS-treated hamsters than in control hamsters from 1 or 2 days to 11 or 12 days post-AAS, and these differences were no longer observable from 18 or 19 days to 25 or 26 days following the cessation of AAS exposure. These data indicate that adolescent AAS exposure has short-term, reversible effects on both offensive aggression and the AH AVP neural system and that reductions in the AH AVP correlate with the return to the nonaggressive behavioral phenotype following AAS exposure. The present study was conducted to examine whether repeated exposure to high-dose AAS during adolescent development has long-term effects on the 5-HT neural system, that is, a neural system that acts with AVP to control aggression in hamsters. First, in a repeat of a previous study done in our laboratory (Grimes et al., 2006), we measured offensive aggression in adolescent AAS-treated hamsters at 1, 4, 11, 18, and 25 days post-AAS exposure to verify and extend findings that AAS induced increases in offensive aggression nearly 3 weeks after the cessation of AAS exposure. Second, to determine whether the observed effects of adolescent AAS on the 5-HT neural system were lasting, we examined the density of 5-HT afferent fibers and varicosities and 5-HT_{1B} receptor-containing puncta and neuronal somata in the AH, VLH, and MeA in these same hamsters 1 day later at 2, 5, 12, 19, or 26 days post-AAS exposure, in an effort to correlate the aggressive behavioral response pattern to 5-HT neural circuitry.

Method

Subjects

In Syrian hamsters (*Mesocricetus auratus*), the adolescent period of development can be identified as the time between Postnatal Days 25 and 56 (PDs 25 & 56). Weaning generally occurs around PD 25, with the onset of puberty beginning around PD 40 (Miller, Whitsett, Vandenbergh, & Colby, 1977). During this developmental time period, hamsters wean from their dams, leave their home nest, establish new solitary nest sites, and learn to defend their territory and participate in social dominance hierarchies (Schoenfeld & Leonard, 1985; Whitsett & Vanderbergh, 1975).

For the experimental treatment paradigm, intact preadolescent male Syrian hamsters (aged PD 21) were obtained from Charles River Laboratories (Wilmington, MA), individually housed in Plexiglas cages, and maintained at ambient room temperature on a reverse light–dark cycle of (14:10-hr light–dark; lights on at 1900). Food and water were provided ad libitum. For aggression testing, stimulus (intruder) male hamsters of a size and weight equal to that of the experimental hamsters were obtained from Charles River 1 week prior to the behavioral test, group housed with 5 hamsters per cage in large polycarbonate cages, and maintained as above to acclimate them to the hamster 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 with experimental hamsters, as a control for behavioral differences between stimulus hamsters, as previously described in other articles (Ferris et al., 1997; Melloni, Connor, Hang, Harrison, & Ferris, 1997; Ricci, Grimes, & Melloni, 2004; Ricci, Knyshevski, & Melloni, 2005). Intruders displaying significantly low aggression and/or displaying 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 PD 28, hamsters ($n = 71$) were weighed and randomly assigned to five groups (Groups 1–5) corresponding to the time at which hamsters would be tested for offensive aggression and killed by transcardial perfusion for immunohistochemistry (see Figure 1 for schematic representation of the experimental paradigm). Each group was divided into two drug treatment groups: those administered a high-dose mixture of AAS suspended in sesame oil (SO) and those administered SO alone (vehicle control). Hamsters ($n = 6–8$ hamsters per drug treatment per group) received daily subcutaneous injections (0.1 ml–0.2 ml) of an AAS mixture consisting of 2 mg/kg testosterone cypionate, 2 mg/kg nortestosterone, and 1 mg/kg dihydroxytestosterone undecylate (Steraloids Inc., Newport, RI) or an SO vehicle for 30 consecutive days (PDs 28–58), as previously described in other articles (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002, 2005; Grimes, Ricci, & Melloni, 2003; Grimes et al., 2006; Harrison, Connor, Nowak, Nash, & Melloni, 2000; Ricci et al., 2006). This daily treatment of AAS was designed to mimic a chronic heavy-use regimen (Pope & Katz, 1988, 1994). After the last injection on PD 58, hamsters ($n = 6–8$ per drug treatment per group) were tested for offensive aggression (as detailed below) and killed by transcardial perfusion for immunohistochemistry at varied time points following the cessation of AAS treatment. Group 1 hamsters (PD 59 or 60) were tested for offensive aggression 1 day following cessation of drug treatment and then killed by transcardial perfusion 24 hr later (i.e., 1 or 2 days later; see Figure 1), whereas hamsters in Groups 2 (PD 62 or 63), 3 (PD 69 or 70), 4 (PD 76 or 77) and 5 (PD 83 or 84) were tested and killed via transcardial perfusion at 4 or 5, 11 or 12, 18 or 19, and 25 or 26 days post-AAS exposure, respectively. AAS- and SO-treated hamsters in each group were killed via transcardial perfusion, and brains were removed and processed for 5-HT or 5-HT_{1B} immunohistochemistry as detailed below.

Aggression Testing

Experimental hamsters were tested for offensive aggression with 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 and weight was introduced into the home cage of experimental hamsters and the resident was scored for offensive aggression (i.e., number of lateral attacks, upright offensive attacks, head and neck bites, flank and rump bites, and chases). Briefly, an attack was scored each time the resident hamster chased and then lunged toward and/or confined 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. Composite Aggression

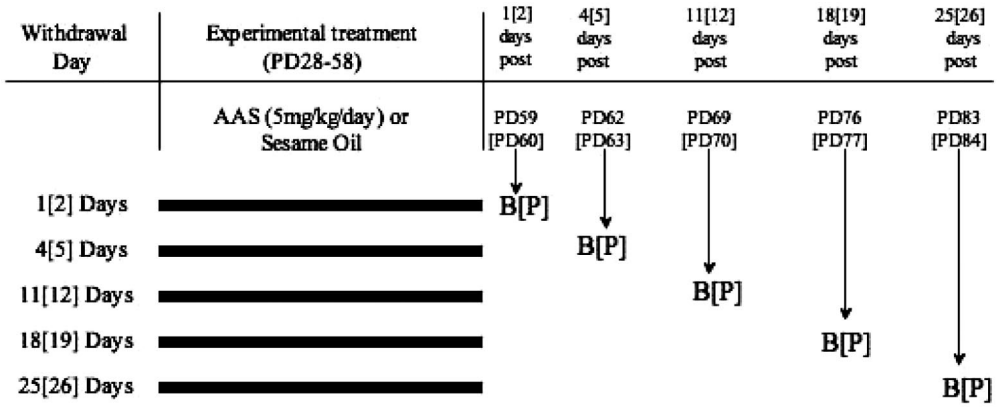

Figure 1. The diagram shows the experimental treatment paradigm. Hamsters were administered anabolic–androgenic steroid (AAS; 5 mg/kg per day) or sesame oil throughout adolescent development. We then tested aggression (behavior testing [B]) at 1, 4, 11, 18, or 25 days posttreatment and processed for serotonin (5-HT) or 5-HT_{1B} receptor immunohistochemistry (physiology [P]) at 2, 5, 12, 19, or 26 days posttreatment. Solid horizontal bars indicate AAS treatment from PD 28 to PD 58. Arrows identify the time frame of behavioral and physiological testing. Withdrawal Day indicates the number of days of post-AAS exposure. Numbers outside the brackets indicate the behavioral testing day, and numbers inside the brackets indicate the day of physiological examination. PD = postnatal day.

Score (Grimes & Melloni, 2005), 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 and neck bites and flank and 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 hr of the dark phase under dim red illumination and videotaped for behavioral verification of the findings.

Immunohistochemistry

At 2 days following the last injection, AAS- and SO-treated hamsters were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine, and the brains were fixed by transcardial perfusion with a fixative containing either 4.0% paraformaldehyde for 5-HT or 4.0% paraformaldehyde with 0.2% gluteraldehyde and 0.2% picric acid for 5-HT_{1B}. Brains were then cryogenically protected by incubation in 30.0% sucrose in a phosphate buffered saline (PBS; 0.001 M KH_2PO_4, 0.01 M Na_2HPO_4, 0.137 M NaCl, 0.003 M 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 1× PBS and labeled for 5-HT or 5-HT_{1B} by single-label immunohistochemistry, as previously described in other articles (Grimes & Melloni, 2002, 2005).

Immunohistochemistry of 5-HT. Free-floating sections were pretreated with 3.0% H_2O_2 (30.0% stock solution) followed by preincubation in 20.0% normal goat serum with 0.6% Triton X-100 (Sigma Chemical Co., St. Louis, MO). Sections were incubated in primary antiserum (1:1,000) for 5-HT antirabbit (Protos Biotech, New York, NY) with 20.0% normal goat serum and 0.6% Triton X-100 for 24 hr at 37 °C. After primary incubation, sections were incubated in secondary goat antirabbit followed by tertiary antisera (Vectastain ABC Elite Kit—rabbit, Vector Labs, Burlingame, CA) for 60 min each at room temperature and then labeled with diaminobenzidine (Vector Labs, Burlingame, CA).

Immunohistochemistry of 5-HT_{1B}. Free-floating sections were washed in PBS for 3–5 min and pretreated with 3.0% H_2O_2 (30.0% stock solution) followed by preincubation in 3.0% bovine serum albumin in PBS with 0.1% Triton X-100. Sections were incubated in primary antiserum for 5-HT_{1B} (i.e., goat anti-5-HT_{1B} receptor polyclonal antibody; Santa Cruz Biotech, Santa Cruz, CA) at a final dilution of 1:2,000 with 3.0% bovine serum albumin and 0.1% Triton X-100 for 24 hr at 37 °C. After primary incubation, sections were incubated in biotinylated secondary antigoat immunoglobulin in PBS and 1.0% bovine serum albumin for 60 min at room temperature, rinsed again followed by tertiary antisera (Vectastain ABC Elite Kit—rabbit) for 60 min each at room temperature, and then labeled with diaminobenzidine. Sections from both immunohistochemistries were mounted on gelatin-coated slides, allowed to air dry, and dehydrated through a series of ethanol and xylene solutions. Then, a coverslip was placed on the slides with Cytoseal-60 mounting medium (VWR Scientific, West Chester, PA).

Image Analysis

The density of 5-HT-immunoreactive or 5-HT_{1B}-immunoreactive (5-HT-ir or 5-HT_{1B}-ir) elements was determined within specific brain areas with the BIOQUANT NOVA 5.0 computer-assisted microscopic image analysis software package (BIOQUANT Image Analysis Corp., Nashville, TN), as previously described elsewhere (DeLeon, Grimes, Connor, & Melloni, 2002). The areas analyzed were selected on the basis of previous data that implicated these regions as part of the circuit important for aggressive behavior or social communication in numerous species and models of aggression (Bunnell, Sodetz, & Shalloway, 1970; Delville, De Vries, & Ferris, 2000; Delville et al., 1996a; Delville, Mansour, & Ferris, 1996b; Ferris et al., 1997; Hammond & Rowe, 1976; Kollack-Walker & Newman, 1995; Potegal, Blau, & Glusman, 1981a, 1981b; Sodetz & Bunnell, 1970). These areas (see Figure 2) include the intermediate part of the lateral septal nucleus, the medial division of the bed nucleus of the stria terminalis, the MeA, the central amygdala, the AH at the level of the nucleus circularis, and the VLH, which includes the medial aspects of the medial tuberal nucleus and the ventrolateral part of the ventromedial hypothalamic nucleus. Elements of 5-HT-ir or 5-HT_{1B}-ir were also quantified in several brain regions not implicated in the aggressive response (i.e., the paraventricular hypothalamic nucleus and the caudate putamen) as controls. Slides from each hamster 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

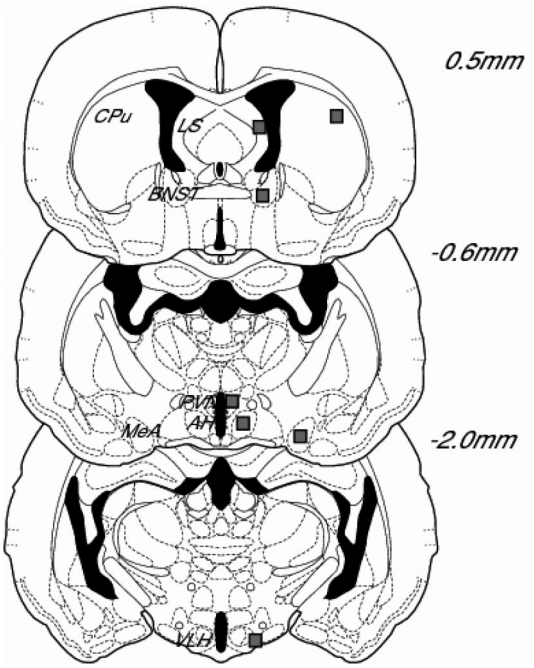

Figure 2. Diagram shows the location of the areas selected to quantify serotonin (5-HT) immunoreactive (ir) fibers and varicosities and 5-HT_{1B}-ir neuronal puncta and somata (gray shaded areas). Dark shaded areas in the figure represent ventricular structures. Plates from pages 50, 54, and 59 of *A Stereotaxic Atlas of the Golden Hamster Brain* (Morin & Wood, 2001) were modified and reflect specific positions in the rostral-caudal plane (i.e., distance in millimeters from bregma to the plane of section at the skull surface). CPu = caudate putamen; LS = intermediate part of the lateral septal nucleus; BNST = medial division of the bed nucleus of the stria terminalis; PVN = paraventricular hypothalamic nucleus; AH = anterior hypothalamus; MeA = medial amygdala; and VLH = ventrolateral hypothalamus. From *A Stereotaxic Atlas of the Golden Hamster Brain* (pp. 50, 54, and 59), by L. P. Morin and R. I. Wood, 2001, London: Academic Press. Copyright 2001 by Elsevier. Reprinted with permission.

Biometrics, Nashville, TN) was used to identify the brain region of interest at low power (4×) with a Nikon E600 microscope. At this magnification, a standard computer-generated box was drawn to fit within the particular region of interest. Then, under 20× (5-HT) or 40× (5-HT_{1B}) magnification, images reached threshold at a standard RGB-scale level empirically determined by observers who were blind to treatment conditions. This allowed observers to detect stained 5-HT-ir or 5-HT_{1B}-ir elements with moderate to high intensity and suppressed 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 hamsters. The illumination was kept constant for all measurements. Then, 5-HT-ir varicosities and fibers or 5-HT_{1B}-ir neuronal puncta and somata were identified in each field with a mouse driven cursor, and counts were performed automatically by the BIOQUANT software. Measurements at 20× or 40× magnification continued until 5-HT-ir or 5-HT_{1B}-ir elements throughout the entire region of interest were quantified. Using the same region of interest used to quantify the area density of 5-HT-ir varicosities and fibers and 5-HT_{1B}-ir puncta, we performed 5-HT_{1B}-ir cell counts manually with a mouse-driven cursor by identifying stained elements and marking each cell until all cells within the region of interest were marked and counted. One to two independent measurements of 5-HT-ir or 5-HT_{1B}-ir elements were taken from several consecutive sections ($n \geq 2$) of each hamster per treatment group, depending on the identification of the exact position of the nucleus within the region of interest and the size of the nucleus in the rostral-caudal plane. Then, the density of 5-HT-ir varicosities and fibers, the density of 5-HT_{1B}-ir neuronal puncta, or the number of 5-HT_{1B}-ir neuronal somata was determined for each region of interest, standardized per 100 μm × 100 μm parcel for regional comparison purposes, and used for statistical analysis.

Statistics

Behavioral studies. The results from the aggression tests were compared between AAS- and SO-treatment groups and within treatment group for length of time following AAS exposure or age (for controls). Nonparametric data (number of attacks and bites) were compared with a Kruskal–Wallis analysis of variance for main effects tests and with Mann–Whitney *U* tests (two-tailed test) for post hoc comparisons.

Immunohistochemistry. The area densities of 5-HT-ir varicosities and fibers, 5-HT_{1B}-ir puncta, and 5-HT_{1B}-ir cells within each brain region were compared between AAS- and SO-treatment groups and within treatment groups for length of time after AAS exposure or age (for controls) with Student's *t* test (two-tailed test).

Results

Offensive Aggression

As characterized extensively in a recent study (Grimes et al., 2006), a significant overall effect of time following the cessation of AAS exposure on aggression was observed for AAS-treated hamsters, $\chi^2(4, N = 5) = 21.44$, $p < .001$, but not for SO-treated controls, $\chi^2(4, N = 5) = 4.61$, $p = .329$. Specifically, within-AAS-group comparisons of Composite Aggression Scores showed that AAS-treated hamsters tested at 1, 4, and 11 days post-AAS administration were significantly more aggressive than AAS-treated hamsters tested 18 and 25 days following the cessation of drug exposure (1 day vs. 18 days, $Z = 3.26$; 1 day vs. 25 days, $Z = 3.13$; 4 days vs. 18 days, $Z = 2.89$; 4 days vs. 25 days, $Z = 2.87$; 11 days vs. 18 days, $Z = 3.07$; 11 days vs. 25 days, $Z = 2.78$, $p < .01$ for each comparison; Figure 3). In addition, adolescent AAS-treated hamsters displayed high levels of composite aggression when compared with SO-treated controls for several weeks following the end of AAS treatment. Specifically, from PD 59 to PD 69, at 1, 4, and 11 days post-AAS, Composite Aggression Scores were higher ($Z = 3.05$; $Z = 2.78$, $Z = 3.13$, respectively, $p < .01$ for each comparison) in AAS-treated hamsters compared with controls (Figure 3). However, the behavioral differences observed between treatment groups were no longer present by 18 days (i.e., PD 76, $Z = 1.09$, $p > .1$) and 25 days (i.e., PD 83, $Z = 0.58$, $p > .1$). At these time points, Composite Aggression Scores from AAS-treated hamsters were reduced to that of the nonaggressive behavioral phenotype observed in SO controls.

Immunohistochemistry

In previous studies, we and others have used immunohistochemical staining of 5-HT fibers or puncta as a sensitive marker of the development of 5-HT afferent projections into brain areas implicated in aggression control (DeLeon, Grimes, Connor, et al., 2002; Delville, Melloni, & Ferris, 1998; Grimes & Melloni, 2002; Taravosh-Lahn, Bastida, & Delville, 2006). Similar to subjects in one prior report (Grimes & Melloni, 2002), aggressive, adolescent

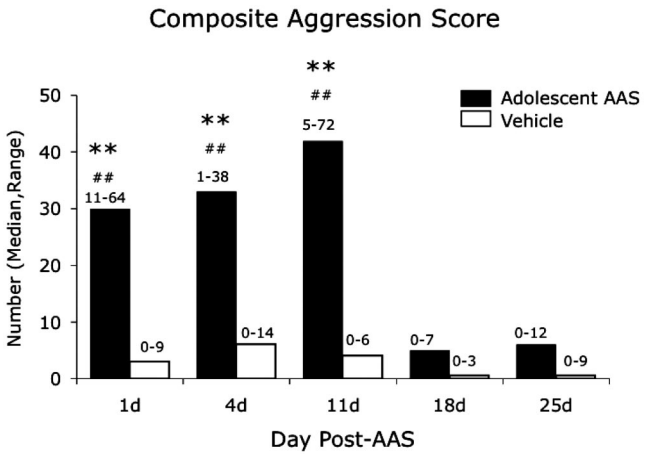

Figure 3. Comparisons of Composite Aggression Scores during withdrawal from adolescent anabolic–androgenic steroid (AAS) treatment are shown. The range of numbers at the top of each column indicates the *y*-axis range. Mann–Whitney *U* tests (two-tailed) were performed. d = day. $^{**}p < .01$ (in comparisons with controls). $^{\#\#}p < .01$ (for within-group comparisons of adolescent AAS-treated hamsters at 1, 4, and 11 days versus 18 and 25 days post-AAS exposure).

AAS-treated hamsters displayed a significant decrease in the density of 5-HT-ir varicosities and fibers (indicative of 5-HT afferent innervation; Grimes & Melloni, 2002) in the AH, VLH, and MeA immediately following the cessation of AAS treatment (i.e., PD 60) compared with nonaggressive, SO-treated controls: AH, $t(11) = -6.3$, $p < .0001$; VLH, $t(9) = -3.1$, $p < .05$; and MeA, $t(10) = -2.9$, $p < .05$ (Figure 4). As also previously observed (Grimes & Melloni, 2005), at PD 60, aggressive, adolescent AAS-treated hamsters displayed a significant decrease in the density of 5-HT_{1B}-ir-containing puncta (indicative of pre-synaptic 5-HT_{1B} receptors on 5-HT-containing varicosities; Grimes & Melloni, 2005) in the AH, $t(8) = -5.0$, $p < .01$; the MeA, $t(3) = -5.3$, $p < .05$; and the VLH, $t(5) = -2.8$, $p < .05$, and a significant increase in number of 5-HT_{1B}-ir neuronal somata in the AH, $t(3) = 5.8$, $p < .05$; the MeA, $t(3) = 3.4$, $p < .05$; and the VLH, $t(4) = 6.6$, $p < .01$, when compared with SO-treated controls (Figure 4).

In addition to these differences observed immediately following the cessation of AAS treatment, differences in 5-HT-ir between AAS- and SO-treatment groups were also observed across the extended time period following AAS exposure. Specifically, aggressive, adolescent AAS-treated hamsters displayed significant decreases in the density of 5-HT-ir fibers and varicosities in the AH when compared with age-matched, nonaggressive, SO-treated controls at all time points examined: PD 63, $t(13) = -7.8$, $p < .0001$; PD 70, $t(15) = -6.0$, $p < .0001$; PD 77, $t(9) = -6.4$, $p < .0001$; PD 84, $t(9) = -3.0$, $p < .05$ (Figure 4). Similar decreases in the density of 5-HT-ir fibers and varicosities were observed in the MeA on PD 63, $t(15) = -3.3$, $p < .01$; on PD 70, $t(16) = -4.0$, $p < .01$; on PD 77, $t(14) = -3.9$, $p < .01$; and on PD 84, $t(11) = -4.4$, $p < .001$; and in the VLH on PD 63, $t(9) = -2.9$, $p < .05$; on PD 70, $t(13) = -2.3$, $p < .05$, and on PD 84, $t(15) = -2.6$, $p < .05$, of AAS-treated hamsters across the same time period (Figure 4). In brain regions in which no significant differences in 5-HT-ir were observed between AAS- and SO-treated hamsters at PD 60 (i.e., medial division of the bed nucleus of the stria terminalis, lateral septal nucleus, and paraventricular hypothalamic nucleus), no differences were observed at any other time point ($p > .1$, each comparison). Also, no within-AAS- or within–SO-group differences in 5-HT-ir were observed across the time period examined ($p > .1$, each comparison).

In addition to the differences in 5-HT-ir observed following AAS treatment, differences in 5-HT_{1B}-ir puncta and cells between AAS- and SO-treatment groups were also observed across the entire time period sampled here. Specifically, hamsters exposed to AAS during adolescence displayed significant decreases in the density of 5-HT_{1B}-ir neuronal puncta in the AH on PD 63, $t(9) = -4.2$, $p < .01$; on PD 70, $t(8) = -3.1$, $p < .05$; on PD 77, $t(7) = -3.1$, $p < .05$; and on PD 84, $t(8) = -3.5$, $p < .01$, as well as significant increases in the number of 5-HT_{1B}-ir neuronal somata in the AH on PD 63, $t(7) = 15.8$, $p < .0001$; on PD 70, $t(4) = 10.7$, $p < .001$; on PD 77, $t(4) = 6.2$, $p < .01$; and on PD 84, $t(5) = 3.9$, $p < .05$ when compared with age-matched, SO-treated controls at all time points examined following the cessation of AAS treatment. Similar decreases in 5-HT_{1B}-ir puncta in AAS-treated hamsters were observed in the MeA on PD 63, $t(9) = -4.2$, $p < .01$; on PD 70, $t(6) = -5.4$, $p < .01$; on PD 77, $t(9) = -3.5$, $p < .05$; and on PD 84, $t(10) = -2.8$, $p < .05$; and in the VLH on PD 63, $t(6) = -4.8$, $p < .05$; on PD 70, $t(8) = -5.7$, $p < .01$; on PD 77, $t(9) = -3.6$, $p < .05$; and on PD 84, $t(8) = -5.5$, $p < .01$, across the same time period. Similarly, increases in 5-HT_{1B}-ir neuronal somata in AAS-treated hamsters were also observed in the MeA on PD 63, $t(3) = 4.3$, $p < .05$; on PD 70, $t(4) = 3.4$, $p < .05$; on PD 77, $t(3) = 4.9$, $p < .05$; and on PD 84, $t(5) = 8.5$, $p < .01$; and in the VLH on PD 63, $t(3) = 5.6$, $p < .05$; on PD 70, $t(4) = 3.5$, $p < .05$; on PD 77, $t(3) = 5.7$, $p < .05$; and on PD 84, $t(6) = 29.6$, $p < .0001$, across the entire time period sampled. In brain regions in which no differences in 5-HT_{1B}-ir (neuronal puncta and/or somata) were observed between AAS- and SO-treated hamsters at PD 60 (i.e., medial division of the bed nucleus of the stria terminalis, lateral septal nucleus, CeA, and caudate putamen—either here or in our previous studies; Grimes & Melloni, 2005), no differences were observed at any other time point following AAS treatment ($p > .1$, each comparison). Also, no within-AAS-group differences or within-SO-group differences in 5-HT_{1B}-ir were observed across the time period examined ($p > .1$, each comparison).

Discussion

In previous studies, we have shown that adolescent AAS exposure significantly increases offensive aggression when hamsters are tested immediately following the drug treatment period (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002, 2005; Grimes et al., 2003, 2006; Harrison et al., 2000; Ricci et al., 2006). In one study, examination of the persistence of this heightened behavioral response pattern indicated that hamsters treated with AAS during adolescent development displayed the highly aggressive phenotype for nearly 2 weeks following the cessation of drug treatment, with the reemergence of the nonaggressive phenotype occurring by the 3rd week (Grimes et al., 2006). These data indicate that adolescent AAS exposure produces lasting but not permanent increases in offensive aggression in hamsters. In a replication of this prior study, we show here that adolescent AAS-treated hamsters display both acute and lasting increases in

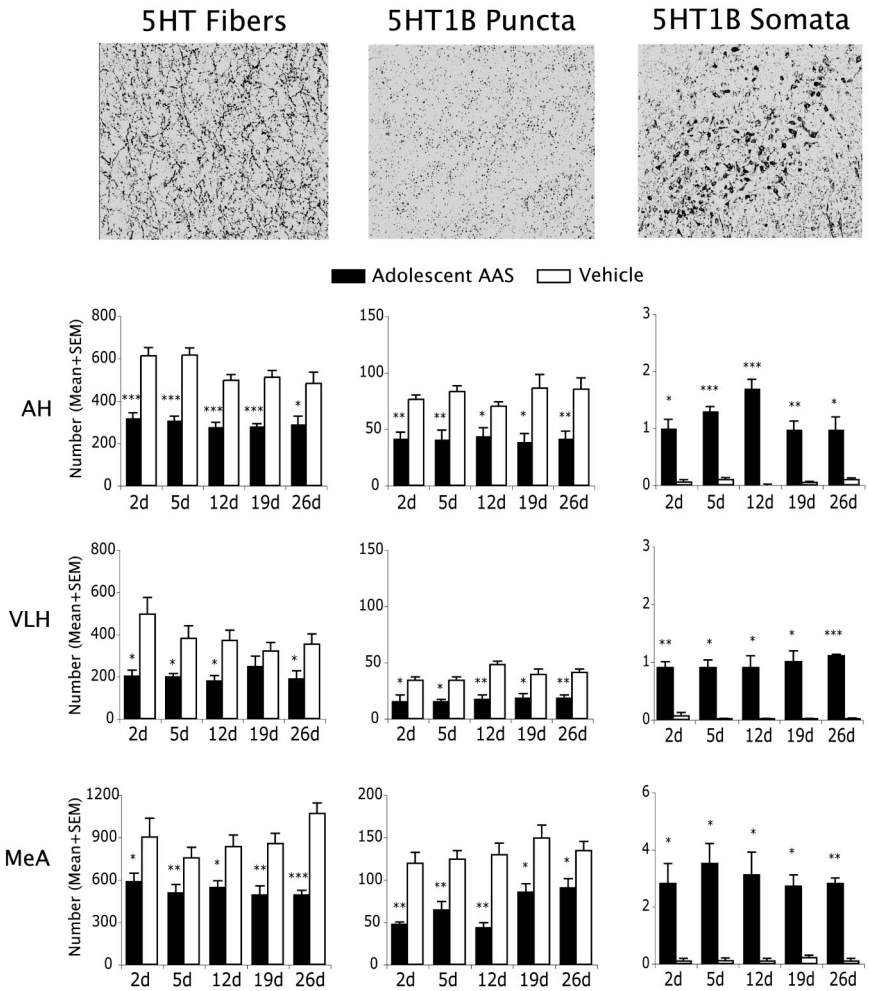

Figure 4. The top of the figure shows bright-field photomicrographs illustrating representative immunoreactive labeling for serotonin (5-HT) afferent fibers and 5-HT_{1B} receptor-containing neuronal puncta and somata. Graphs depict comparison of the density of 5-HT-containing afferent fibers (left column) and 5-HT_{1B} receptor-containing neuronal puncta (middle column) and the number of 5-HT_{1B} receptor-containing neuronal somata (right column) in the anterior hypothalamus (AH), ventrolateral hypothalamus (VLH), and medial amygdala (MeA) immediately following cessation of drug treatment (2 days post-anabolic–androgenic [AAS] treatment) and after more extended periods following the cessation of adolescent AAS treatment (5–26 days post-AAS). Fibers were quantified within standardized surfaces between groups for comparisons. A two-tailed Student's *t* test was performed. Error bars indicate the standard error of the mean. d = day. In comparisons with controls: $*p < .05$; $**p < .01$; and $***p < .001$.

offensive aggression when compared with age-matched, vehicle-treated littermates. And, as previously described, these behavioral alterations are not permanent, as the control, nonaggressive phenotype reemerged by approximately 3 weeks (18 days) following the end of AAS administration. Hamsters maintained this nonaggressive phenotype through the study end point, that is, 25 days post-AAS exposure. The finding that adolescent AAS treatment produces lasting but not permanent effects on offensive aggression in hamsters is similar to data from studies in which researchers examined the effects of adult AAS exposure on aggression during short-term (i.e., 3–5 weeks) withdrawal in male Long Evans rats (Farrell & McGinnis, 2004; McGinnis, Lumia, & Possidente, 2002). In these studies, researchers used the resident–intruder paradigm to observe increases in aggression following short-term AAS withdrawal, but only in animals administered prolonged high-dose AAS (5 mg/kg per day × 12 weeks; McGinnis et al., 2002). In other studies in which researchers examined the effects of withdrawal from pubertal AAS, AAS-treated Long Evans rats showed heightened aggression compared with controls during the course of both short- and long-term withdrawal periods (Farrell & McGinnis, 2004). Together with a replicate data set from this report, these studies illustrate that AAS may have lasting, yet not always permanent, effects on aggressive behavior.

Adolescent AAS exposure may facilitate and maintain heightened levels of offensive aggression by producing sustained alterations in the activity of neurochemical signals known to modulate

this behavioral response. Previous work from our laboratory and the laboratories of others indicate that 5-HT is an important inhibitor of offensive aggression in Syrian hamsters. In particular, 5-HT activity in the AH and VLH has been shown to suppress offensive aggression in hamsters (Delville et al., 1996a; Ferris, 1996; Ferris et al., 1997, 1999). It is interesting that aggressive hamsters with a history of adolescent AAS exposure have altered development and activity of the 5-HT neural system in these and other select brain regions that are important for the control of offensive aggression in hamsters. Specifically, aggressive, AAS-treated hamsters possess decreased 5-HT-containing afferent fibers (Grimes & Melloni, 2002), decreased pre-synaptic 5-HT_{1B} receptor positive neuronal puncta (Grimes & Melloni, 2005), and increased numbers of postsynaptic 5-HT_{1B} receptor expressing neuronal somata (Grimes & Melloni, 2005) in the AH, the VLH, and the MeA, that is, the three brain sites implicated in the neural control of aggression. These findings suggest that decreased 5-HT development and 5-HT_{1B} receptor activity underlies the development of the adolescent AAS-induced aggressive phenotype. This viewpoint was supported by behavioral pharmacology studies that used selective 5-HT reuptake inhibitors and 5-HT_{1B} receptor agonists (Grimes & Melloni, 2002, 2005). These studies showed that activation of the 5-HT neural system via 5-HT_{1B} receptors could suppress the adolescent AAS-induced aggressive response. Together, these data suggested that adolescent AAS exposure increased offensive aggression by suppressing the development and activity of the 5-HT neural system, providing strong evidence of a role of this neural system in AAS-induced aggression. Given these data, we questioned whether the exposure to AAS during adolescent development had lasting effects on the maintenance and activity of the 5-HT neural system implicated in the control of aggression, predisposing those exposed adolescents to prolonged periods of increased aggression. To address this question, we examined whether adolescent AAS-induced reductions in 5-HT afferent innervation and alterations in 5-HT_{1B} receptor localization and expression would persist for an extended period following the cessation of AAS administration or whether the observed changes in the 5-HT neural system would undergo neuroplastic adaptations after the AAS exposure, correlating with the reemergence of the nonaggressive phenotype over time.

Given previous data from our laboratory (Grimes & Melloni, 2002, 2005), we hypothesized a strong, negative correlation between the density of 5-HT afferent fiber and 5-HT_{1B} neuronal puncta and the levels of offensive aggression (i.e., low 5-HT-ir elements with high levels of offensive aggression and higher levels of 5-HT-ir elements with nonaggressive, control level behavior). Contrary to our hypothesis, there was no correlation between the density of 5-HT-containing fibers and/or 5-HT_{1B} receptor-containing neuronal puncta and somata and offensive aggression. Immunoreactive staining of 5-HT and 5-HT_{1B} followed patterns in the AH, VLH, and MeA that were similar to those seen previously (Grimes & Melloni, 2005), that is, immediately following the treatment period, the density of 5-HT-containing afferent fibers and varicosities and 5-HT_{1B} receptor-containing neuronal puncta was significantly decreased in the AH, VLH, and MeA, whereas the number of 5-HT_{1B}-containing neuronal somata were significantly increased in these same brain regions in adolescent AAS-treated hamsters compared with nonaggressive, SO-treated control hamsters. However, these alterations persisted throughout the entire time period examined (through 26 days post-AAS exposure) despite the return of the nonaggressive phenotype in these hamsters by 18 days. Specifically, at 2, 5, and 12 days after adolescent AAS exposure, aggressive, AAS-treated hamsters displayed significant decreases in the density of 5-HT-containing afferent fibers and 5HT_{1B} receptor-containing neuronal puncta and significant increases in 5HT_{1B} receptor-containing neuronal somata in the AH, VLH, and MeA when compared with nonaggressive, age-matched, vehicle-treated littermates. Although differences in levels of offensive aggression between AAS- and SO-treated hamsters disappeared by 18 days after adolescent AAS treatment (i.e., behavioral recovery), the 5-HT neural system did not recover back to control levels at correlate time frames or at any time during the extended period examined. Together with the behavioral results above, these data indicate that although adolescent AAS exposure has short-term, reversible effects on aggression in hamsters, changes in the 5-HT neural system alone do not explain the behavioral recovery that occurs following adolescent AAS exposure. These data are novel as they dissociate alterations in the 5-HT neural system from the reemergence of the nonaggressive phenotype that occurs following cessation of adolescent AAS exposure.

Perhaps the reemergence of the nonaggressive phenotype following adolescent AAS exposure can be explained by paralleled alterations in other neurochemical systems in the hypothalamus and/or amygdala that modulate offensive aggression. For instance, a number of studies have demonstrated an anatomical and functional relationship between the 5-HT and AVP neural systems in the AH and VLH and the control of aggression (Delville et al., 1996a; Ferris, 1996; Ferris et al., 1997, 1999). Anatomical studies reveal a dense 5-HT afferent innervation onto AVP neurons in the AH (Delville et al., 2000; Ferris et al., 1997, 1999), and the VLH contains both AVP and 5-HT_{1B} receptors (Delville et al., 1996a). Functionally, treatment of hamsters with fluoxetine increases AH 5-HT release (Pergola, Sved, Voogt, & Alper, 1993), decreases AH AVP release (Altemus, Cizza, & Gold, 1992; Ferris, 1996), and blocks aggression resulting from application of AVP directly onto the AH (Ferris, 1996; Ferris et al., 1997) or VLH (Delville et al., 1996a). Together, these data suggest that 5-HT inhibits aggression by suppressing AVP activity within the AH and/or the VLH. Previously, we have shown that aggressive, adolescent AAS-treated hamsters display increased AVP afferent development and AVP levels within the AH (Harrison et al., 2000) and AVP V_{1A} receptor binding within the VLH (DeLeon, Grimes, & Melloni, 2002), suggesting that increased AVP tone in these brain sites underlies the development of the AAS-induced aggressive phenotype. This notion was supported by behavioral pharmacology studies that used AVP receptor antagonists (Harrison et al., 2000), strengthening the assertion that enhanced AVP activity within the AH (at a minimum) plays an important role in adolescent, AAS-induced offensive aggression. Recently, we have shown that the AAS-induced augmentation of AH AVP afferent development is plastic and returns to control levels during discrete time frames after the cessation of AAS treatment (Grimes et al., 2006). In this study, a positive correlation between AH AVP fiber density and offensive aggression was observed following adolescent AAS exposure, indicating that at times of increased AH AVP tone, hamsters respond more aggressively than at times when levels of AH AVP are low. This correlation between aggression and AH AVP strengthens the notion that the interactions between AAS and AH

AVP directly underlie adolescent AAS-induced alterations in aggressive behavior and that other changes in aggression circuitry may play a more modulatory role in AAS-induced aggression. Accordingly then, although 5-HT is an important modulator of AH AVP activity and aggression, in the presence of a changing AH AVP neural system, alterations in the 5-HT neural system (e.g., increases in 5-HT afferent development) may not be necessary for the behavioral recovery to occur.

What other neurobehavioral implications might long term alterations in the hypothalamic and/or amygdaloid 5-HT neural systems (i.e., reductions in 5-HT afferent development and alterations in 5-HT_{1B} receptor localization and expression) then have for adolescent AAS exposed individuals? Central 5-HT systems play a critical role in the regulation of normal and abnormal behavior. Evidence suggests that dysfunction of the central 5-HT neural system contributes to various pathological conditions, including mood disorders (see Malhi, Parker, & Greenwood, 2005 for review). Among these, there is an emerging data set indicating that 5-HT function is blunted in the psychopathology of clinical depression in humans (Asberg, Thoren, Traskman, Bertilsson, & Ringberger, 1976; Elliott, 1991; Maes & Meltzer, 1995; Meltzer, 1990; Owens & Nemeroff, 1994) and in stress-induced learned helplessness (i.e., an animal model of depression) in hamsters (Dwivedi, Mondal, Payappagoudar, & Rizavi, 2005; Wu et al., 1999). It is interesting that several studies have shown that discontinuation of high-dose, long-term AAS use may lead to the development of withdrawal symptoms that include severe mood disorders, including depression (Allnutt & Chaimowitz, 1994; Malone & Dimeff, 1992; Malone, Dimeff, Lombardo, & Sample, 1995; Pope & Katz, 1994; Pope, Kouri, & Hudson, 2000; Thiblin, Runeson, & Rajs, 1999). Symptomatic relief for this condition, termed anabolic steroid withdrawal depression, includes antidepressants (most notably the selective serotonin reuptake inhibitor class of medications) and endocrine medications that are targeted to improve hypothalamic–pituitary–gonadal as well as hypothalamic–pituitary–adrenal function (Malone & Dimeff, 1992; and see Medras & Tworowska, 2001 for review). This treatment strategy indicates a role for both 5-HT and the hypothalamus in this clinical condition. Given the long-term reductions in 5-HT development in hypothalamic and amygdaloid nuclei observed in this study following adolescent exposure to AAS and the link between 5-HT underactivity and depression, it is conceivable that AAS-induced behavioral depression is modulated, in part, by the underdevelopment and activity of the 5-HT neural system in these important brain regions. This hypothesis is currently under investigation in the laboratory.

In summary, the study presented in this article provides an examination of the residual effects of adolescent AAS exposure on offensive aggression and of select components of one of the neurobiological systems modulating this behavioral phenotype. These findings show that offensive aggression was significantly higher in AAS-treated hamsters than in controls from 1 through 11 days following cessation of AAS administration, which is a replication of previous results from our laboratory. Novel results from these studies show that decreases in 5-HT afferent innervation into the AH, VLH, and MeA observed 2 days post-AAS treatment persist into adulthood through 26 days. In addition to the persistent decreases in 5-HT innervation during this time period, lasting changes in 5-HT_{1B} receptor expression also occur as indicated by the decreases in 5-HT_{1B}-ir puncta and increases in 5-HT_{1B}-ir neuronal somata. These data suggest that adolescent AAS exposure has short-term, reversible effects on the display of offensive aggression but has long-lasting, perhaps permanent, effects on the 5-HT neural system. These results also indicate that the 5-HT neural system may play a more modulatory role in the control of offensive aggression, as there does not appear to be a direct correlation between the persistence of the adolescent AAS-induced aggressive phenotype and the activity of this neural system.

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