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Serotonin-1B Receptor Activity and Expression Modulate the Aggression-Stimulating Effects of Adolescent Anabolic Steroid Exposure in Hamsters

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Repeated high dose (5.0 mg/kg) anabolic–androgenic steroid (AAS) exposure during adolescence stimulates offensive aggression in male Syrian hamsters. These studies examined whether AAS-induced aggression was regulated by the activity of serotonin (5HT) type-1B receptors and correlated with altered 5HT1B expression. AAS-treated hamsters were tested for offensive aggression following the administration of the 5HT1B agonist anpirtoline (0.125–0.5 mg/kg). Anpirtoline dose-dependently reduced select components of the AAS-induced aggressive response, with significant reductions observed at 0.25 mg/kg. Aggressive, AAS-treated hamsters showed significant decreases in the area covered by 5HT1B-containing neuronal puncta and increases in the number of 5HT1B-containing neuronal somata in select brain regions implicated in aggression control. Together, these data support a role for site-specific alterations in 5HT1B signaling and expression in adolescent AAS-induced aggression.

Keywords: adolescence, anabolic–androgenic steroids, serotonin-1B receptor, development, aggression

Previously, we have used adolescent Syrian hamsters as an animal model to examine the link between adolescent anabolic–androgenic steroid (AAS) exposure and the behavioral neurobiology of offensive aggression (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002; Grimes, Ricci, & Melloni, 2003; Harrison, Connor, Nowak, Nash, & Melloni, 2000; Melloni, Connor, Hang, Harrison, & Ferris, 1997; Melloni & Ferris, 1996). Behavioral data from these studies showed that hamsters repeatedly exposed to high doses of AAS (5.0 mg/kg per day) during adolescent development display high, adult forms of offensive aggression. 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 upright offensive postures and lateral movements (i.e., lateral attacks) toward the intruder. The finding that adolescent AAS-treated hamsters demonstrated high, mature forms of offensive aggression in the absence of prior social interactions and dominance cues suggested that adolescent exposure to AAS stimulated aggression directly, perhaps by affecting the development and/or activity of neural circuits that regulate this behavior.

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Serotonin (5HT) has been implicated in the control of aggression in adolescent and adult humans (Brown et al., 1982; Coccaro, Kavoussi, Trestman, et al., 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, 5HT activity in the anterior hypothalamus (AH) and 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 5HT acts to inhibit aggression. Recently, we showed that aggressive, adolescent AAS-treated hamsters had significant deficits in 5HT afferent innervation to these two brain areas plus the medial amygdaloid (MeA) and central amygdaloid (CeA) nuclei when compared with nonaggressive, vehicle-treated littermates, implicating a reduction in 5HT neural signaling in discrete hypothalamic and amygdaloid nuclei in aggressive, AAS-treated hamsters (Grimes & Melloni, 2002). Behavioral pharmacology studies have shown that the aggressive phenotype displayed by adolescent AAS-exposed hamsters could be blocked by enhancing 5HT neural signaling (Grimes & Melloni, 2002), supporting the notion that 5HT hypofunctioning plays an important role in adolescent, AAS-induced offensive aggression.

The inhibitory nature of the 5HT neural system on aggression has been predominately attributed to the action of 5HT at specific 5HT receptors, namely the 5HT Type I (i.e., 5HT1A and 5HT1B) and 5HT Type II 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). For instance, selective 5HT1B receptor agonists have potent antiaggres-

sive properties across a variety of aggression paradigms, including alcohol-heightened (Fish, Faccidomo, & Miczek, 1999; Miczek & de Almeida, 2001), social-instigation (de Almeida & Miczek, 2002; Fish et al., 1999), frustration-induced (de Almeida & Miczek, 2002), intermale (Bell et al., 1995), and isolation-induced (Rilke et al., 2001) aggression in mice and rats, and territorial (i.e., social) aggression in hamsters (Joppa, Rowe, & Meisel, 1997). Furthermore, mice lacking the 5HT1B receptor display significantly decreased offensive aggression (Ramboz et al., 1996; Saudou et al., 1994). From a neuroanatomical standpoint, 5HT1B receptors have been localized to several regions of the brain that are important for aggression control, such as the hypothalamus and amygdala in a number of species, including the rat (Makarenko, Meguid, & Ugrumov, 2002; Riad et al., 2000; Sari et al., 1997, 1999), mouse (Maroteaux et al., 1992), and guinea pig (Bonaventure, Langlois, & Leysen, 1998; Bonaventure, Voorn, et al., 1998). 5HT1B receptor expression has been characterized as punctate and diffuse (Riad et al., 2000; Sari et al., 1999), consistent with its localization to both 5HT-containing (i.e., 5HT1B autoreceptors) and non-5HT-containing (i.e., 5HT1B heteroreceptors) nerve terminals located throughout the neuropil (for a review, see Barnes & Sharp, 1999). 5HT1B receptors have also been localized to neuronal somata distributed throughout the hypothalamus of the rat (Makarenko et al., 2002), consistent with reports of 5HT1B mRNA expression and in situ binding sites in neurons in the guinea pig hypothalamus (Bonaventure, Langlois, & Leysen, 1998; Bonaventure, Voorn, et al., 1998). These studies illustrate that 5HT1B receptors are present in areas of the brain that are important for aggression control and, more significantly, that demonstrate reduced 5HT afferent innervation following adolescent AAS exposure, that is, the hypothalamus and amygdala. Perhaps exposure to AAS during adolescence stimulates aggression in hamsters by disrupting the activity, localization, and/or expression of 5HT1B receptors in these brain sites, thus altering 5HT activity and, subsequently, 5HT's inhibitory influence on aggression. Sex differences in the ability of 5HT1B agonists to decrease offensive aggression have been observed (Cologer-Clifford, Simon, Lu, & Smoluk, 1997; Joppa et al., 1997) and may be due to site-specific differences in 5HT1B receptor activity/expression patterns. To date, however, it is unknown whether AAS exposure during adolescence has any effects on the development or function of the 5HT1B receptor system and/or whether 5HT1B signaling plays a significant role in adolescent AAS-induced aggression.

We conducted these studies to establish a direct link between adolescent AAS exposure, 5HT1B receptor signaling and expression, and offensive aggression using the adolescent Syrian hamster as an animal model. First, to determine whether 5HT1B receptor signaling played a role in adolescent AAS-induced aggression, we tested whether the mature aggressive phenotype could be inhibited by activation of these receptors using anpirtoline hydrochloride, that is, a selective 5HT1B receptor agonist. Then, to determine whether adolescent AAS exposure altered 5HT1B receptor expression in areas of the hamster brain implicated in aggressive behavior, we used immunohistochemistry to visualize and quantify 5HT1B receptor distribution/localization patterns in these brain regions.

Method

Hamsters

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 hamsters were obtained from Charles River 1 week prior to the behavioral test; these hamsters were group housed at 5 hamsters per cage in large polycarbonate cages and maintained as above to acclimate 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 to control for behavioral differences between stimulus hamsters, 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 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 Procedures

Aggression testing. We tested experimental hamsters for offensive aggression using the resident/intruder paradigm, a well-characterized and ethologically valid model of offensive aggression in golden 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/neck bites, flank/rump bites, chases, and latency to attack and bite toward intruders), as previously described (Grimes et al., 2003; Grimes & Melloni, 2002; Ricci et al., 2004; Ricci et al., 2005). Briefly, an attack was scored each time the resident hamster 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. Composite aggression scores, used as general measures of offensive aggression, were 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. The latency to attack and bite was defined as the period of time between the beginning of the behavioral test and the first attack and bite of the residents toward an intruder. In the case of no attacks, latency to attack was assigned the maximum latency (i.e., 600 s). Also, in Experiment 1, residents were measured for social interest toward intruders (i.e., contact time between resident and intruder), and the frequency of grooming bouts were counted to control for nonspecific effects of 5HT1B agonists on hamster behavior. Contact time was defined as the period of time during which the resident initiated contact with the intruder either through olfactory investigation (i.e., sniffing) or aggression. Each aggression test lasted for 10 min and was scored by an observer unaware of the hamster's 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.

Anpirtoline effects on adolescent AAS-induced offensive aggression. In Experiment 1, P27 hamsters ($n = 60$) received daily subcutaneous injec-

tions (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) for 30 consecutive days (P27–P56), as previously described (DeLeon, Grimes, & Melloni, 2002; Grimes & Melloni, 2002; Grimes et al., 2003; Harrison et al., 2000; Melloni et al., 1997; Melloni & Ferris, 1996). This treatment regime was designed to mimic a chronic "heavy use" regimen (Pope & Katz, 1994; Pope, Katz, & Champoux, 1988). The day following the last injection, adolescent AAS-treated hamsters were tested for offensive aggression after an intraperitoneal injection of anpirtoline hydrochloride (0.125, 0.25, or 0.5 mg/kg in 0.9% normal saline; Tocris; n = 15–16/dose) or saline vehicle (n = 14) in a volume of 1 μl/kg. All injections were performed on unanesthetized hamsters and took no longer than 10 s. Administration of anpirtoline in this manner and at these doses has been shown previously to be selective for its antiaggressive properties, with no generalized effects observed on social or motor behavior patterns (de Almeida & Miczek, 2002; Miczek & de Almeida, 2001). After injection, hamsters were returned to their home cage. Hamsters were tested for offensive aggression 15 min later, as described below.

Adolescent AAS-induced offensive aggression and 5HT1B receptor localization. In Experiment 2 (as previously reported in Grimes et al.'s, 2003, study), P27 hamsters were weighed and randomly distributed into two groups. One group of hamsters (n = 24) received subcutaneous injections of the AAS mixture as described above, whereas a second group (n = 14) was injected with an equal volume of sesame oil vehicle alone. Following the 30-day treatment period, hamsters in both AAS and sesame oil groups were tested for offensive aggression as described, sacrificed 1 day later (P58), and the brains from a subset of these hamsters selected at random (n = 5 hamsters per group) were removed and processed for the immunohistochemical localization of 5HT1B receptors as detailed below.

Immunohistochemistry. One day following the behavioral test for aggression (P58), AAS and sesame oil-treated hamsters from Experiment 2 were anesthetized with 80 mg/kg Ketamine and 12 mg/kg Xylazine and the brains fixed by transcardial perfusion with a fixative solution containing 4% paraformaldehyde, 0.2% glutaraldehyde, and 0.2% picric acid. Brains were removed, postfixed in perfusion fixative, and then cryoprotected by incubation 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. We processed a subset of the collected brains (n = 5 per experimental treatment group) for the immunohistochemical localization of 5HT1B receptors using a modification of an existing protocol (Grimes et al., 2003; Grimes & Melloni, 2002). Briefly, a consecutive series of 35-μm coronal sections were cut on a freezing microtome and collected as free floating sections in PBS. Every third brain section was washed in PBS for 3–5 min, pretreated with 2% H_2O_2 in distilled water for 10 min, and rinsed thoroughly with PBS. Sections were then pretreated in 3% bovine serum albumin (BSA) in PBS for 60 min at 4 °C, then incubated in primary antiserum for 5HT1B (i.e., goat anti-5HT1B receptor polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, California) at a final dilution of 1:2,000 in 3% BSA and 0.1% Triton X-100 for 24 hr at 4 °C. Sections were then rinsed with PBS for 3–10 min, incubated in biotinylated secondary antigoat Immunoglobulin G (Vector Laboratories, Burlingame, California) in PBS and 1% BSA for 60 min at room temperature, rinsed again in PBS for 3–10 minutes, and then incubated in Avidin-biotin-complex (Vectastain ABC kit; Vector Laboratories, Burlingame, California) with 1% BSA in PBS for 60 min at room temperature. We revealed the peroxidase reaction using 0.5% 3,3′-diaminobenzidine in distilled water as per manufacturer's recommendations (DAB Kit; Vectastain; Vector Laboratories, Burlingame, California). The sections were mounted on gel-coated slides, air dried, dehydrated through a series of alcohols, cleared with xylene, and coverslipped with Cytoseal (Stephens Scientific, Kalamazoo, Michigan). Omission of the primary and secondary antibodies were run as controls during the procedure.

Image analysis. We determined the area density of 5HT1B-immunoreactive (5HT1B-ir) puncta and the number of 5HT1B-ir cells within specific brain areas using the BIOQUANT NOVA 5.5 computer-assisted microscopic image analysis software package as previously described (DeLeon, Grimes, Connor, & Melloni, 2002; Grimes et al., 2003; Grimes & Melloni, 2002). The areas analyzed were selected on the basis of previous data implicating these regions as part of the circuit important for aggressive behavior in numerous species and models of aggression, with the notable exception of the caudate putamen, that is, a nonaggression area used as a control region (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 1) included the intermediate part of the lateral septal nucleus (LS), the medial division of the bed nucleus of the stria terminalis (BNST), the MeA nucleus, the AH, the CeA nucleus, and the VLH, which included the medial aspects of the medial tuberal nucleus and the ventrolateral part of the ventromedial hypothalamic nucleus. Slides from each hamster were coded by an experimenter unaware of the experimental treatment, and BIOQUANT NOVA 5.5 image analysis software running on a Pentium III CSI Open PC computer (R & M Biometrics, Nashville, Tennessee) was used to identify the brain region of interest at low power (4×) with a Nikon (Melville, New York) E600

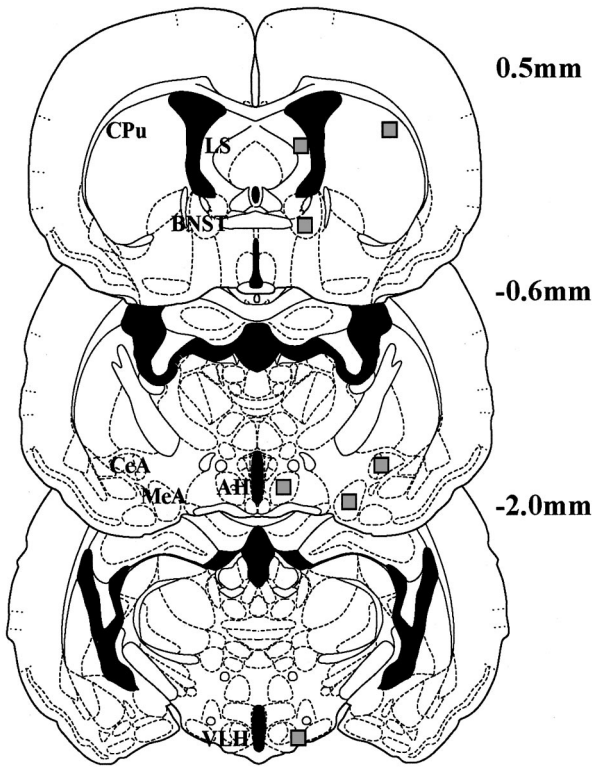

Figure 1. Diagram showing the location of the areas selected to quantify 5HT1B-immunoreactive puncta and cells (shaded areas). Plates were reprinted from *Stereotaxic Atlas of the Golden Hamster Brain*, by L. P. Morin and R. I. Wood, 2001, with permission from Elsevier. The plates reflect specific positions 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; CeA = central amygdala; MeA = medial amygdala; AH = anterior hypothalamus; VLH = ventrolateral hypothalamus.

microscope. At this magnification, a standard computer-generated box was drawn to fit within the particular region of interest. Then, under 40× magnification, images were thresholded at a standard Red Green Blue-scale level empirically determined by observers blinded to treatment conditions, such as to allow detection of stained 5HT1B-ir puncta 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 hamsters. The illumination was kept constant for all measurements. 5HT1B-ir puncta were identified in each field with a mouse-driven cursor, and then 5HT1B-ir counts were performed automatically by the BIOQUANT software. Measurements at 40× continued until 5HT1B-ir puncta throughout the entire region of interest were quantified. Using the same region of interest used to quantify the area density of 5HT1B-ir puncta, we performed 5HT1B-ir cell counts manually, using a mouse-driven cursor, by identifying stained elements and marking each cell until all cells within the region of interest were marked and counted. Two to three independent measurements of 5HT1B-ir elements were taken from several consecutive sections ($n \geq 3$) of each hamster per treatment group depending on the following: (a) identification of the exact position of the nucleus within the region of interest and (b) the size of the nucleus in the rostral-caudal plane. Then, the number of 5HT1B-ir puncta and cells were determined for each region of interest, standardized per 100 μm × 100 μm parcel for regional comparison purposes, and then used for statistical analysis.

Statistical Analysis

Behavioral studies. For Experiment 1, the results from the aggression tests were compared between (a) anpirtoline- and saline-treatment groups and (b) doses of anpirtoline. For Experiment 2, results from the aggression tests were compared between AAS and sesame oil treatment groups. In both experiments, nonparametric data (number of lateral attacks, upright offensive attacks, total attacks, flank/rump bites, chases, and grooming) were compared with Mann–Whitney *U* tests (two-tailed) and Kruskal–Wallis analysis of variance, whereas parametric data (attack and bite latency and contact time) were compared with Student's *t* test (two-tailed) and analysis of variance.

5HT1B immunoreactivity. The area covered by 5HT1B-ir puncta and the number of 5HT1B-ir cells were compared between treatment groups with Student's *t* test (two-tailed) for each area analyzed.

Results

Anpirtoline Effects on Adolescent AAS-Induced Offensive Aggression

Systemic administration of the 5HT1B agonist, anpirtoline, produced an overall effect on aggression intensity as measured by composite scores of offensive aggression (i.e., total number of attacks and bites, $\chi^2(3, N = 4) = 12.29$, $p < .01$, with the antiaggressive effects to be significant at the 0.25 mg/kg and 0.5 mg/kg doses. At these doses, anpirtoline treatment significantly reduced composite aggression (0.25 mg/kg, $Z = 2.61$, $p < .01$; 0.5 mg/kg, $Z = 2.50$, $p < .01$) of AAS-treated hamsters toward intruders when compared with AAS-treated hamsters that received saline prior to behavioral testing (see Figure 2). Similarly, at the effective doses (i.e., 0.25 mg/kg and 0.5 mg/kg) of anpirtoline, there were significant trends toward decreases in offensive aggression of AAS-treated hamsters toward intruders when compared with the lower dose (i.e., 0.125 mg/kg) of anpirtoline (0.125 vs. 0.25 [$Z = 2.40$, $p < .05$]; 0.125 vs. 0.5 [$Z = 2.33$, $p < .05$]). Conversely, 5HT1B receptor activation with anpirtoline failed to produce an overall effect on the initiation of offensive aggression as measured by the latency to first attack, $F(3, 53) = 1.91$, $p = 0.14$, and/or first bite, $F(3, 53) = 1.56$, $p = .21$, in AAS-treated hamsters. Further analysis showed that systemic administration of anpirtoline did not produce a significant effect on total contact time, $F(3, 30) = 0.72$, $p = .54$ (i.e., a measure of social interest), and there was not an effect observed on the number of grooming bouts, $\chi^2(3, N = 4) = 3.67$, $p = .3$ (i.e., a comfort measure), during the behavioral test.

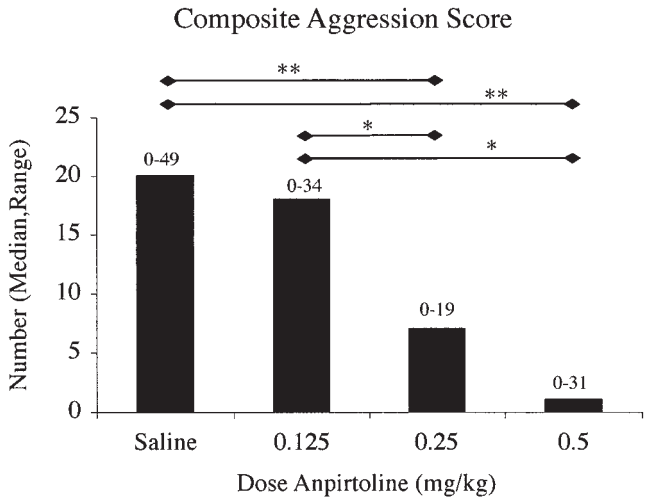

Figure 2. Antiaggressive effects of anpirtoline on composite aggression scores (i.e., total number of attacks and bites) of adolescent, anabolic–androgenic steroid (AAS)-treated hamsters. Agonist treatment (0.25–0.50 mg/kg, intraperitoneal) decreases composite aggression in aggressive adolescent AAS-treated residents. *$p < .05$; **$p < .01$; Mann–Whitney *U* tests (two-tailed) and Kruskal–Wallis analysis of variance.

When examined more precisely, 5HT1B receptor activation with anpirtoline produced an overall effect on several specific and targeted offensive responses. In particular, systemic administration of anpirtoline produced an overall effect on the number of lateral attacks, $\chi^2(3, N = 4) = 13.59$, $p < .01$, and flank/rump bites, $\chi^2(3, N = 4) = 9.11$, $p < .05$, with significant effects observed at the 0.25 and 0.5 mg/kg doses. At these doses, anpirtoline significantly reduced the number of lateral attacks (0.25 mg/kg, $Z = 2.75$, $p < .01$; 0.5 mg/kg, $Z = 2.78$, $p < .01$) and flank/rump bites (0.25 mg/kg, $Z = 2.00$, $p < .05$; 0.5 mg/kg, $Z = 2.27$, $p < .05$) of AAS-treated hamsters toward intruders when compared with AAS-treated hamsters treated with saline prior to aggression testing (see Figure 3). Similarly, at the effective doses of anpirtoline (i.e., 0.25 mg/kg and 0.5 mg/kg), there were significant decreases in the number of lateral attacks of AAS-treated hamsters toward intruders when compared with the lowest dose (i.e., 0.125 mg/kg) of anpirtoline (0.125 vs. 0.25 [$Z = 2.34$, $p < .01$]; 0.125 vs. 0.5 [$Z = 2.49$, $p < .01$]). The same was true for the lowest dose (i.e., 0.125 mg/kg) of anpirtoline when compared with the effective doses (i.e., 0.25 mg/kg and 0.5 mg/kg) with respect to flank/rump bites (0.125 vs. 0.25 [$Z = 2.05$, $p < .05$]; 0.125 vs. 0.5 [$Z = 2.11$, $p < .05$]). Conversely, the systemic administration of anpirtoline at any dose did not produce significant overall

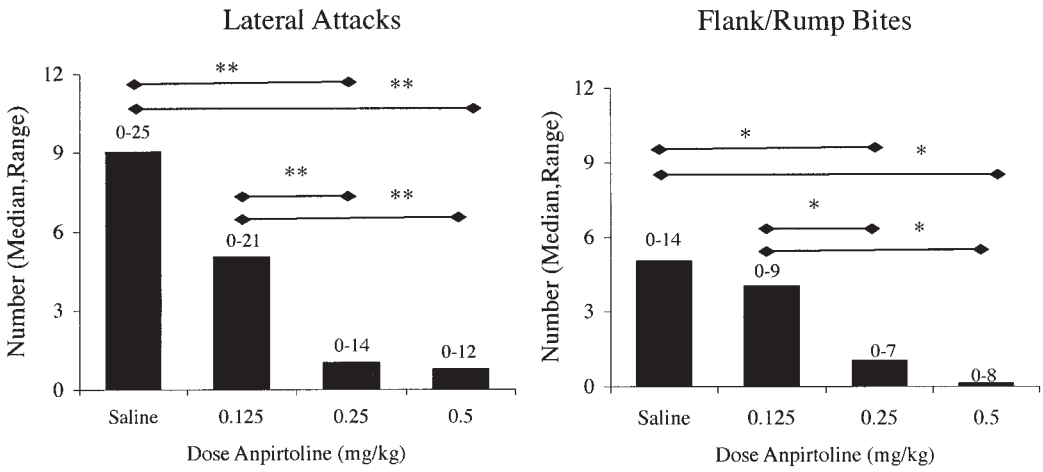

Figure 3. Antiaggressive effects of low-to-moderate doses of anpirtoline on the offensive response pattern of adolescent, anabolic–androgenic steroid (AAS)-treated hamsters. Agonist treatment (0.25–0.50 mg/kg, intraperitoneal) selectively decreases specific and targeted measures of aggression intensity (i.e., number of lateral attacks and flank/rump bites) in aggressive adolescent AAS-treated residents. $*p < .05$; $**p < .01$; Mann–Whitney *U* tests (two-tailed) and Kruskal–Wallis analysis of variance.

effects on the number of upright offensive attacks, $\chi^2(3, N = 4) = 5.34$, $p = .15$, and/or chases, $\chi^2(3, N = 4) = 4.09$, $p = .25$, in AAS-treated hamsters compared with AAS-treated hamsters treated with saline prior to aggression testing.

5HT1B Receptor Distribution in Brains of Aggressive, Adolescent AAS-Treated Hamsters

As reported previously (DeLeon, Grimes, & Melloni, 2002; Grimes et al., 2003; Grimes & Melloni, 2002; Harrison et al., 2000; Melloni et al., 1997; Melloni & Ferris, 1996), hamsters treated with high-dose AAS throughout adolescent development display high levels of offensive aggression when compared with oil-treated controls. This behavioral alteration was present in the AAS-treated hamsters selected at random for this study to examine the expression of 5HT1B receptors. Specifically, the subset of AAS-treated hamsters selected for 5HT1B immunohistochemistry showed a significant increase in the number of lateral attacks ($Z = 2.69$, $p < .01$), and flank/rump bites ($Z = 2.80$, $p < .01$) over vehicle-treated littermates (see Figure 4). In addition, AAS-treated hamsters displayed significantly decreased attack and bite latencies toward intruders—latency to attack, $t(4) = 3.06$, $p < .05$; latency to bite, $t(4) = -2.4$, $p < .01$—than vehicle-treated control hamsters (see Figure 4).

In aggressive AAS-treated hamsters, the immunohistochemical staining pattern for 5HT1B receptors was altered in several areas of the hamster brain implicated in offensive aggression, including those in the hypothalamus. For example, in oil-treated controls, the

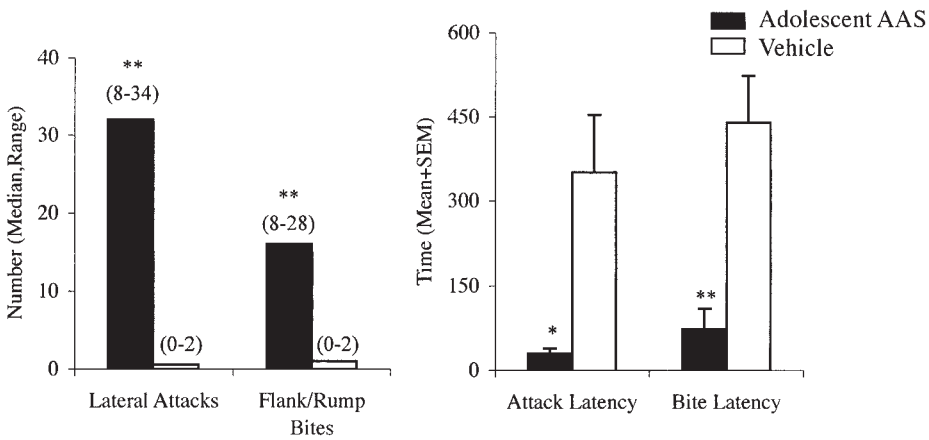

Figure 4. Adolescent anabolic–androgenic steroid (AAS) treatment increases offensive aggression. Aggression intensity (total lateral attacks and flank/rump bites) and aggression initiation (latency to first attack and bite) measures in AAS- and vehicle-treated residents. Bars denote standard error of the mean (*SEM*). $*p < .05$; $**p < .01$; Mann–Whitney, two-tailed (intensity measures) and Student's *t* test, two-tailed (initiation measures).

staining of 5HT1B-containing puncta in the AH displayed a dense pattern of 5HT1B receptor immunoreactive puncta with very few 5HT1B receptor-containing somata indicative of the normal distribution of receptor localization in this brain region (see Figure 5). By comparison, aggressive, AAS-treated hamsters displayed a less dense pattern of 5HT1B-ir puncta in this brain region. Quantitative analysis of the density of 5HT1B receptor immunoreactive puncta in the AH showed that aggressive, AAS-treated hamsters had approximately 30% of the 5HT1B receptor-containing puncta of nonaggressive, oil-treated littermates (see Figure 7A). This reduction was statistically significant, $t(8) = 5.07$, $p < .01$, between treatment groups. An interesting finding is that aggressive, AAS-treated hamsters also displayed an increase in the density of 5HT1B-ir neuronal somata in the AH (see Figure 6, Panels A and C) compared with nonaggressive, oil-treated littermates (see Figure 6, Panels B and C). Analysis of the density of 5HT1B receptor-containing somata in the AH showed that aggressive, AAS-treated hamsters had approximately 11-fold more 5HT1B immunopositive cell bodies when compared with nonaggressive, oil-treated littermates (see Figure 7B). This difference was also statistically significant, $t(8) = 5.19$, $p < .01$. Similar results were found in the VLH, in which aggressive, AAS-treated hamsters had significantly less, $t(8) = -4.79$, $p < .01$ (i.e., less than half), 5HT1B receptor-containing puncta than nonaggressive, oil-treated littermates (see Figure 7A). However, only a trend toward an increase in the density of 5HT1B receptor immunoreactive neuronal somata were found in the VLH of aggressive, AAS-treated hamsters compared with nonaggressive, oil-treated littermates, $t(8) = 2.03$, $p = .09$ (see Figure 7B). These findings were not restricted to the hypothalamus, however, as other regions of the hamster brain important for aggression regulation showed similar decreases/increases in the density of 5HT1B receptor-containing puncta/somata following adolescent AAS exposure. For instance, the density of 5HT1B-ir puncta in the MeA of aggressive, AAS-treated hamsters was approximately 60% of that found in nonaggressive, oil-treated littermates, a statistically significant, $t(9) = -2.38$, $p < .05$, difference between treatment groups (see Figure 7A). As was seen in the AH and VLH, the MeA of AAS-treated hamsters also displayed a significant increase in the density of 5HT1B receptor-containing somata when compared with oil-treated controls, $t(9) = 5.45$, $p < .01$ (see Figure 7B). Finally, although the CeA of AAS-treated hamsters expressed a significant decrease in 5HT1B-ir puncta when compared with oil-treated controls, $t(8) = -4.68$, $p < .01$, there were no 5HT1B-ir cells observed in either AAS- or oil-treated hamsters (see Figure 7, Panels A and B).

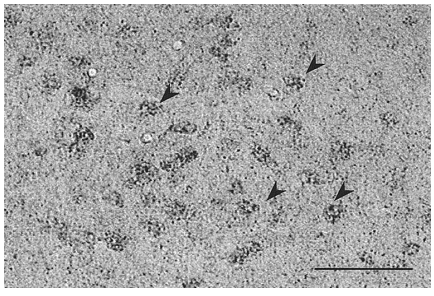

Figure 5. Brightfield photomicrograph showing immunoreactive labeling for 5HT1B-immunoreactive puncta in the anterior hypothalamus. Bar = 200 μm.

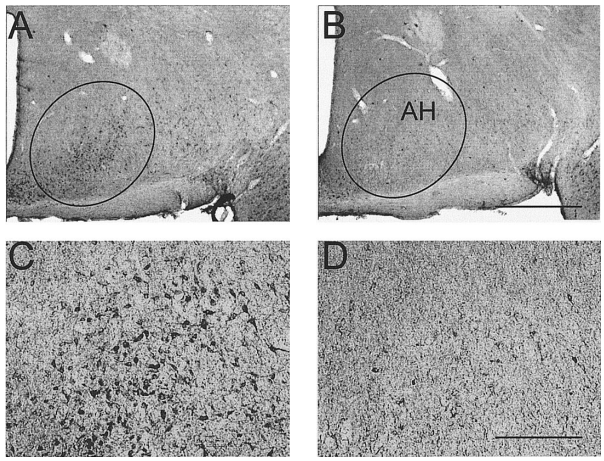

Figure 6. Brightfield photomicrographs showing 5HT1B-immunoreactive cells in the anterior hypothalamus (AH) of anabolic–androgenic-steroid-treated (Panels A and C) and vehicle-treated (Panels B and D) hamsters. Bar = 600 μm (Panels A and B) and 300 μm (Panels C and D).

Not all brain regions implicated in the aggressive response showed significant alterations in the density of 5HT1B-ir puncta/somata following adolescent AAS exposure (see Figure 7, Panels A and B). For instance, similar densities of 5HT1B receptor-containing puncta and somata were found in the medial division of the BNST and in the intermediate part of the LS of both AAS- and oil-treated hamsters. These counts were not significantly different between treatment groups ($p > .1$ each comparison). Similarly, no significant differences were found in the density of 5HT1B-ir puncta in the caudate putamen, a brain area not involved in aggressive behavior in the hamster ($p > .1$).

Discussion

5HT has been observed to play an inhibitory role in aggression in humans (Brown et al., 1982; Coccaro, Kavoussi, & Hauger, 1997; Kruesi et al., 1990; Linnoila et al., 1983) and in many animal models and species (Higley et al., 1992; Kyes et al., 1995; Sijbesma et al., 1990; Vergnes et al., 1988), including hamsters (Delville et al., 1996a; Ferris et al., 1997, 1999). We have shown that intact male hamsters repeatedly treated with high-dose AAS throughout adolescence display significantly high levels of offensive aggression (DeLeon, Grimes, & Melloni, 2002; Grimes et al., 2003; Grimes & Melloni, 2002; Harrison et al., 2000; Melloni et al., 1997; Melloni & Ferris, 1996) that can be attenuated by pharmacologically increasing extracellular 5HT (Grimes & Melloni, 2002). Hamsters that responded aggressively following adolescent AAS exposure showed significant decreases in 5HT afferent innervation to many areas of the hamster brain important for aggression control (Grimes & Melloni, 2002). Together, these data suggest that adolescent AAS exposure stimulated aggression by suppressing the activity and development of the 5HT neural system implicated in the inhibition of offensive aggression. Such alterations in 5HT development and function might have drastic

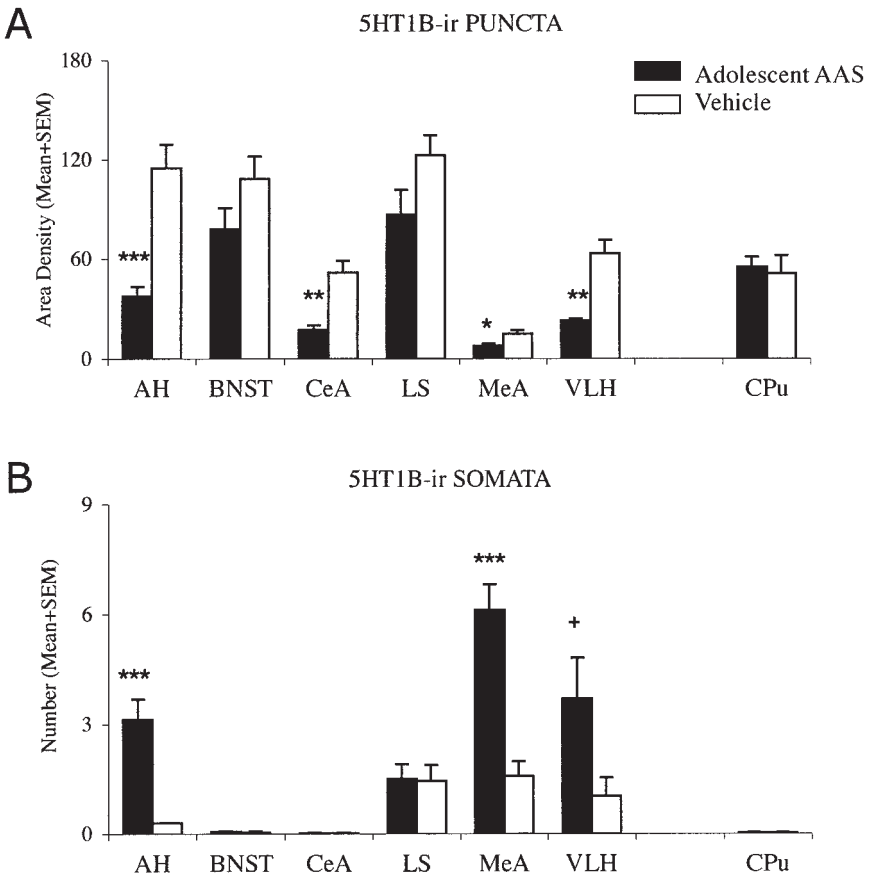

Figure 7. 5HT1B in brains of anabolic–androgenic steroid (AAS)- versus vehicle-treated hamsters. (A) Density of 5HT1B-immunoreactive (5HT1B-ir) neuronal puncta and (B) number of 5HT1B-ir neuronal somata in AAS- and vehicle-treated hamsters. Numbers were normalized to a standard area (100 μm $\times$ 100 μm) for regional comparisons. *$p < .05$; **$p < .01$; ***$p < .001$; Student's t test, two-tailed. SEM = standard error of the mean; AH = anterior hypothalamus; BNST = medial division of the bed nucleus of the stria terminalis; CeA = central amygdala; LS = intermediate part of the lateral septal nucleus; MeA = medial amygdala; VLH = ventrolateral hypothalamus; CPu = caudate putamen.

consequences on the activity and/or expression of the 5HT receptor populations that subserve the antiaggressive properties of 5HT.

The inhibitory nature of 5HT on aggression has been predominately attributed to the 5HT Type I (i.e., 5HT1A and 1B; Miczek et al., 1998; Muehlenkamp et al., 1995; Sanchez et al., 1993) and Type II (Bell et al., 1995; de Almeida & Miczek, 2002; Rilke et al., 2001) receptors. AAS exposure has been previously shown to decrease 5HT1B receptor binding in various regions of the adult rat brain (Kindlundh, Lindblom, Bergstrom, & Nyberg, 2003). Perhaps exposure to AAS during adolescent development stimulates aggression by decreasing 5HT signaling through 5HT1B receptor pools in brain regions implicated in the control of offensive aggression in hamsters. To address this question, we performed experimental manipulations using a selective 5HT1B agonist to test the hypothesis that 5HT1B receptor activation would reduce AAS-induced aggression. The behavioral data presented here support our hypothesis that 5HT1B receptors play an inhibitory role in adolescent AAS-induced offensive aggression in Syrian hamsters. For instance, aggressive, AAS-treated hamsters administered saline prior to behavioral testing displayed high levels of offensive aggression, analogous to that observed in our previous studies (DeLeon, Grimes, & Melloni, 2002; Grimes et al., 2003; Grimes & Melloni, 2002; Harrison et al., 2000; Melloni et al., 1997; Melloni & Ferris, 1996) and presented here (see Figures 2–4). Nearly all saline-treated (12 of 14) hamsters showed a high intensity of aggression (as defined by composite aggression measures and targeted attack and bite scores) and a quick onset (initiation) of the aggressive response (defined by the latency to the first attack and bite). The response pattern and high level of aggression seen in aggressive, AAS-treated hamsters administered saline is similar to that of experienced adult "fighters" (i.e., hamsters trained to respond aggressively by repeated exposure to conspecifics) stimulated to respond hyper-aggressively by activation of the neural circuits stimulating the aggressive response (Ferris et al., 1997). Conversely, administration of the 5HT1B receptor agonist anpirtoline to aggressive, adolescent AAS-treated hamsters prior to behavioral testing dose-dependently reduced adolescent AAS-induced aggression. Anpirtoline-treated hamsters showed a greater than 65% decrease in composite aggression at 0.25 mg/kg, with a maximal effect seen at a dose of 0.5 mg/kg in

which nearly 95% of composite measures of aggression were eliminated when compared with saline-treated counterparts. The antiaggressive effects of these low doses of anpirtoline are in agreement with a set of studies in which anpirtoline decreased instigation induced- and frustration- and ethanol-heightened aggression in male mice (de Almeida & Miczek, 2002; Miczek & de Almeida, 2001). Because acute 5HT1B receptor stimulation with anpirtoline dose-dependently reduced only certain aspects of the adolescent AAS-induced aggressive response (i.e., aggression intensity but not initiation), it was possible that anpirtoline was acting in a highly discriminating antiaggressive fashion, having 5HT1B-selective effects on specific and targeted measures of the aggressive response. To address this issue, we investigated the antiaggressive properties of anpirtoline by examining several more specific and targeted determinates of offensive aggression. Specifically, along with a nearly 95% decrease in the total number of attack and bites (i.e., composite aggression scores), anpirtoline-treated hamsters showed a greater than 90% decrease in the number of lateral attacks and flank/rump bites compared with saline-treated controls. No effect of anpirtoline was noted on upright offensive attacks or chases, or on latency to first attack and/or bite. This finding is interesting given that bites targeted toward either the flank or hind quarter (i.e., rump) region of the intruder have been shown to be intense and highly organized adult forms of aggressive behavior (Delville, David, Taravosh-Lahn, & Wommack, 2003; Wommack & Delville, 2003). These data suggest that 5HT1B activity may play an important role in modulating (i.e., attenuating) the intensity of mature forms of offensive aggression (i.e., lateral attacks and flank/rump-directed bites) in adolescent AAS-treated hamsters but not aggression initiation (i.e., attack and bite latency). Furthermore, the highly selective nature of anpirtoline's antiaggressive properties combined with the lack of any effect on contact time and/or grooming (i.e., a comfort behavior) indicated that activation of 5HT1B receptors did not attenuate adolescent AAS-induced offensive aggression through general nonspecific behavioral inhibition. These behavioral data are important and novel in that they indicate that 5HT activity via the 5HT1B receptors plays a significant role in attenuating the intense, adult-aggressive phenotype that arises in response to adolescent-AAS exposure.

The finding that 5HT1B agonism (via anpirtoline) produced a dose-dependent reduction of select components of the AAS-induced offensive response suggested there may be a specific mechanism of action for this receptor in regulating the adult offensive phenotype. Indeed, the effective doses tested in this study are the same as those shown to be effective for reducing various types of aggression in studies that have used adult animal models (de Almeida & Miczek, 2002; Miczek & de Almeida, 2001). A plausible explanation for the ability of anpirtoline to attenuate intense, adult forms of offensive aggression is that 5HT1B receptors play a direct role in aggression control, acting within discrete subregions of the brain involved in aggressive behavior. In Syrian hamsters, neurons located in the AH, VLH, LS, CeA, MeA, and BNST have been implicated in offensive aggression (Bunnell et al., 1970; Delville et al., 1996a, 2000; Ferris et al., 1997, 1999; Kollack-Walker & Newman, 1995). In the AH and VLH, 5HT has been shown to suppress offensive aggression (Delville et al., 1996a; Ferris, 1996; Ferris & Delville, 1994; Ferris et al., 1997) by acting through 5HT1 receptors (Ferris et al., 1999). Adolescent AAS exposure may alter 5HT neural signaling through 5HT1B receptors in these brain regions by decreasing the extent to which neurons in these areas express this receptor subtype, functionally activating the neural circuits stimulating offensive aggression. To determine this, we quantified the distribution/localization of 5HT1B receptor-containing neuronal elements in brains of aggressive, AAS-treated hamsters and nonaggressive, oil-treated littermates. A number of these brain regions showed significant decreases in 5HT1B receptor-containing neuronal puncta. Specifically, aggressive, adolescent AAS-treated hamsters had significantly less 5HT1B-ir neuronal puncta within the AH, VLH, MeA, and CeA. Because 5HT1B receptors have been localized as autoreceptors on 5HT-containing presynaptic terminals (Boschert, Amara, Segu, & Hen, 1994; Riad et al., 2000; Sari et al., 1999; see Barnes & Sharp, 1999, for a review), this reduction likely represents a loss of 5HT afferents into these brain regions. In support of this assertion, we have previously shown significant reductions in the number of 5HT-containing neuronal puncta in these very same brain sites (i.e., the AH, VLH, MeA, and CeA) following adolescent AAS exposure (Grimes & Melloni, 2002). In further support, no differences in the density of 5HT1B-ir puncta were found in aggression areas that failed to show decreases in 5HT afferent innervation following adolescent AAS treatment (i.e., the LS and BNST; Grimes & Melloni, 2002). Together, these data provide compelling evidence that the reduction in punctate 5HT1B-ir neural elements localized throughout the AH, VLH, MeA, and CeA of aggressive, adolescent AAS-treated hamsters are due to AAS-induced losses in 5HT afferent innervation to these brain regions.

Adolescent AAS-induced reductions in 5HT trophic influence to the AH, VLH, MeA, and CeA may drive postsynaptic neurons in these regions to up-regulate the expression/activity of 5HT1B heteroreceptors. Indeed, pharmacodynamic up-regulation of 5HT1B receptors in response to pharmacologic influence and/or decreased 5HT afferent input has been documented. For instance, up-regulation of 5HT1B binding sites has been detected after exposure to (+) 3,4-methylenedioxymethamphetamine (Sexton, McEvoy, & Neumaier, 1999) and during cocaine withdrawal (Przegalinski, Czepiel, Nowak, Dlaboga, & Filip, 2003), the latter of which has been previously shown to be characterized by significant losses of 5HT afferents to brain areas important for aggression control in hamsters, including the AH (DeLeon, Grimes, Connor, & Melloni, 2002). Along those same lines, increases in postsynaptic 5HT1B receptors have been reported following 5HT denervation with 5,7-DHT (Compan, Segu, Buhot, & Daszuta, 1998). In the study reported here, aggressive, adolescent AAS-treated hamsters had more 5HT1B-ir neuronal somata in areas noted to contain reduced densities of 5, 7-dihydroxytriptamine-ir puncta and 5HT-containing afferents (namely, the AH and MeA, as well as a trend toward an increase in the VLH) as compared with nonaggressive, oil-treated littermates. However, no differences in the number of 5HT1B-ir neurons were found in aggression areas that failed to show decreases in 5HT1B-ir puncta and 5HT afferent innervation (Grimes & Melloni, 2002) following adolescent AAS treatment (i.e., the LS and BNST). From a mechanistic standpoint, these findings are consistent with the hypothesis that the loss of 5HT trophic influence to areas of the hamster brain important for aggression control activates pharmacodynamic mechanisms that stimulate the de novo synthesis and/or altered posttranscriptional regulation of postsynaptic 5HT1B receptors in

neurons occupying these zones of afferent denervation. One inconsistency with this hypothesis, however, arises from results of 5HT1B neuronal immunostaining in the CeA. In our previous study, we found a significant reduction in 5HT afferent innervation and 5HT1B-containing presynaptic terminals in the CeA in aggressive, AAS-treated hamsters compared with nonaggressive, vehicle-treated littermates. By our model, we would predict an increase in the number of 5HT1B-ir neurons in the CeA of aggressive, AAS-treated hamsters compared with controls. However, in these analyses, no ostensible differences were observed in neuronal CeA-5HT1B immunostaining between treatment groups. One possible explanation for this disparity may lie in the fact that little, if any, 5HT1B cellular staining was observed in either treatment group, indicating a lack of 5HT1B receptor expression in postsynaptic neurons of this brain region in hamsters. Further study is necessary to identify and characterize the neuronal cell types expressing these receptors in areas of the brain important for aggression control and the molecular mechanisms regulating their expression in efforts to fully understand how these receptors are exerting their aggression-inhibiting effects.

In summary, the studies presented in this article provide data examining the neurobehavioral effects of chronic high-dose AAS exposure during adolescent development on aggression and the basic neurobiological mechanisms by which 5HT psychopharmacological agents may exert their aggression-inhibiting effects. These findings indicate that increases in offensive aggression resulting from adolescent AAS treatment can be attenuated, at least in part, by 5HT via the 5HT1B receptor and that AAS exposure during this developmental period produces marked reductions in 5HT1B-ir neuronal puncta and concurrent increases in 5HT1B-ir neuronal somata in several, but not all, areas of the hamster brain implicated in aggressive behavior. These findings provide a link among adolescent AAS, 5HT1B, and the inhibition of aggression, indicating a role of altered 5HT1B receptor expression and function in AAS-induced aggression in adolescent hamsters.

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