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Serotonin-1A receptor activity and expression modulate adolescent anabolic/androgenic steroid-induced aggression in hamsters

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Abstract

Repeated high dose (5.0 mg/kg) anabolic/androgenic steroid exposure during adolescence stimulates offensive aggression in male Syrian hamsters. These studies examined whether anabolic/androgenic steroid-induced aggression was regulated by the activity and expression of serotonin (5HT) type-1A receptors. In a first experiment, adolescent male hamsters were treated with a mixture of anabolic/androgenic steroids and then scored for offensive aggression in the absence or presence of the selective 5HT1A receptor agonist *R*(+)-8-OH-DPAT (0.1–0.6 mg/kg). Adolescent anabolic/androgenic steroid-treated hamsters displayed high levels of offensive aggression that could be reversed by enhancing the activity of 5HT1A receptors. The agonist *R*(+)-8-OH-DPAT dose-dependently reduced the steroid-induced aggressive response, with significant reductions in aggression observed at 0.1–0.3 mg/kg. In a second set of experiments, adolescent hamsters were administered anabolic/androgenic steroids or vehicle and then examined for 5HT1A receptor localization and expression in regions of the brain important for aggression control. Hamsters treated with anabolic/androgenic steroids showed significant decreases in 5HT1A receptor-immunoreactive staining and protein levels in the anterior hypothalamus (i.e., a brain region central to the control of offensive aggression in hamsters) with no concomitant decrease in the number of 5HT1A receptor-expressing neurons. Together, these data support a role for site-specific down-regulation of 5HT1A receptor activity in adolescent anabolic/androgenic steroid-induced aggression.

Keywords: Adolescence; Anabolic–androgenic steroids; Serotonin; Serotonin 1A receptor; Development; Aggression; Anterior hypothalamus

1. Introduction

The serotonin (5HT) neural system has been implicated in the control of aggression in adolescent and adult humans (Brown et al., 1982; Linnoila et al., 1983; Kruesi et al., 1990; Coccaro et al., 1997) and in a number of animal models of aggression (Vergnes et al., 1988; Sijbesma et al., 1990a; Kyes et al., 1995; Higley et al., 1996). In Syrian hamsters, 5HT activity in the anterior hypothalamus (AH) and ventrolateral hypothalamus (VLH) has been shown to regulate offensive aggression (Delville et al., 1996a; Ferris, 1996; Ferris et al., 1997, 1999), where 5HT acts to suppress aggressive responding. The inhibitory nature of 5HT on aggression has been predominately attributed to it's action at a subset of 5HT receptors, including (but not limited to) the 5HT1 subtype (i.e., 5HT1A and 5HT1B) receptors (Sanchez et al., 1993; Bell et al., 1995; Muehlenkamp et al., 1995; Miczek et al., 1998; Rilke et al., 2001; de Almeida and Miczek, 2002). For instance, rats treated with eltoprazine, a mixed 5HT1A/B-receptor agonist, show a dose-dependent decrease in offensive aggression (Sijbesma et al., 1990b). The 5HT1A receptor agonist 8-hydroxy-2-(di-*n*-propylamino) tetralin (i.e., 8-OH-DPAT) effectively decreases aggression in a number of species and models of aggression, including isolation-induced- (White et al., 1991; Sanchez et al., 1993; Bell and Hobson, 1994; Sanchez and Hyttel, 1994), alcohol heightened- (Miczek et al., 1998), and testosterone-dependent (Simon et al., 1998; Cologer-Clifford et al., 1999) aggression in mice and offensive aggression in rats (de Boer et al., 1999) and hamsters (Joppa et al., 1997; Knyshevski et al., 2005). Similarly, 5HT1B receptor agonists have potent anti-aggressive properties across a wide variety of aggression paradigms, including alcohol-heightened (Fish et al., 1999; Miczek and de Almeida, 2001), social instigation (Fish et al., 1999; de Almeida

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and Miczek, 2002), frustration-induced (de Almeida and Miczek, 2002), intermale (Bell et al., 1995) and isolation-induced (Rilke et al., 2001) aggression in mice and rats, and territorial aggression in hamsters (Joppa et al., 1997; Grimes and Melloni, 2005).

Previously, we have used developmentally immature Syrian hamsters (*Mesocricetus auratus*) as an animal model to examine the link between adolescent anabolic/androgenic steroid (AAS) exposure, 5HT and the neurobiology of aggression (Grimes and Melloni, 2002, 2005). Behavioral data from these studies showed that adolescent hamsters repeatedly exposed to AAS (5.0 mg/kg/day) display significantly high levels of offensive aggression. Neuroanatomical studies revealed that aggressive, adolescent AAS-treated hamsters had significant deficits in 5HT afferent innervation to select areas of the brain implicated in aggression control (i.e., namely the hypothalamus and amygdala) when compared to non-aggressive, vehicle-treated littermates, implicating marked 5HT hypofunctioning in these brain areas in AAS-treated animals (Grimes and Melloni, 2002). Subsequent studies showed that aggressive, adolescent AAS-treated hamsters also had altered expression of 5HT1B receptors in these same brain regions (Grimes and Melloni, 2005), further implying that changes in 5HT neural signaling through 5HT1 subtype receptors in select areas of the brain may underlie the aggressive phenotype observed in AAS-treated animals. Behavioral pharmacology studies showed that the aggressive response pattern displayed by adolescent AAS exposed animals could be blocked by enhancing 5HT neural signaling (Grimes and Melloni, 2002) through 5HT1B receptors (Grimes and Melloni, 2005), supporting the notion that 5HT hypofunctioning via 5HT1 receptors in hypothalamic and amygdaloid brain regions may play an important role in adolescent, AAS-induced offensive aggression. To date however, it is unknown whether 5HT signaling through 5HT1A receptors plays a significant role in adolescent AAS-induced aggression and/or whether AAS exposure during puberty has any effects on the localization and/or expression of 5HT1A receptors in these brain regions. Sex differences have been reported in the expression of 5HT1A receptor mRNA, with increases in 5HT1A mRNA seen in the AH area of male rats (Zhang et al., 1999). This expression appears to be down-regulated by androgens however, as castration-induced increases hypothalamic 5HT1A mRNA expression can be reversed by testosterone (Zhang et al., 1999). Perhaps pubertal AAS exposure stimulates aggressive behavior in hamsters by reducing 5HT signaling by down-regulating 5HT1A receptor expression in areas of the brain important for aggression control, e.g., the hypothalamus.

These studies were conducted to establish a direct link between adolescent AAS exposure, 5HT1A receptor signaling and expression, and offensive aggression using the adolescent Syrian hamster as an animal model. First, to determine whether 5HT1A receptor signaling played a role in adolescent AAS-induced aggression, we tested whether the aggressive phenotype could be attenuated by activating 5HT1A receptor signaling using *R*(+)-8-OH-DPAT, i.e., a selective 5HT1A receptor agonist. Then, to determine whether adolescent AAS exposure altered 5HT1A receptor expression in areas of the hamster brain implicated in aggressive behavior, we utilized immunohistochemistry and western blot analysis to visualize and quantify 5HT1A receptor distribution/localization patterns and levels in these brain regions.

2. Experimental procedures

2.1. Animals

In Syrian hamsters, the adolescent period of development can be identified as the time between postnatal days 25 and 65 (P25–P65). Weaning generally occurs around P25 with the onset of puberty beginning around P40 (Miller et al., 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 (Whitsett and Vanderbergh, 1975; Schoenfeld and Leonard, 1985).

For the experimental treatment paradigm, intact pre-adolescent male hamsters (P21) 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 (14L/10D; lights on at 19:00). Food and water were provided ad libitum. For aggression testing, stimulus (intruder) males of equal size and weight to the experimental animals were obtained from Charles River 1 week prior to the behavioral test, group housed at 5 animals/cage in large polycarbonate cages, and maintained as above to acclimate to the animal facility. All intruders were prescreened for low aggression (i.e., Disengage and Evade) and submission (i.e., Tail-up Freeze, Flee, and Fly-away) 1 day prior to the aggression test to control for behavioral differences between stimulus animals, as previously described (Ricci et al., 2004, 2005a). Intruders displaying significantly low aggression and/or submissive postures were excluded from use in the behavioral assay. All methods and procedures described below were pre-approved by the Northeastern University Institutional Animal Care and Use Committee (NU-IACUC).

2.2. Experimental treatment

In the first experiment, P27 hamsters ($n=29$) received daily subcutaneous (s.c.) 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), for 30 consecutive days (P27–P56) as previously described (Harrison et al., 2000). This treatment regime, designed to mimic a chronic use regimen (Pope et al., 1988; Pope and Katz, 1994), has been shown repeatedly to produce highly aggressive animals in greater than 90% of the treatment pool (Harrison et al., 2000; DeLeon et al., 2002b; Grimes and Melloni, 2002, 2005; Grimes et al., 2003). The day following the last AAS injection, adolescent AAS-treated animals were tested for offensive aggression 15–20 min after a single intraperitoneal (i.p.) injection of the selective 5HT1A receptor agonist *R*(+)-8-OH-DPAT (0.1, 0.3

or 0.6 mg/kg in 0.9% normal saline) (Tocris) (n=7–8 animals/dose) or saline vehicle (n=6) in a volume of 1 ml/kg. All injections were performed on unanesthetized animals and took no longer than 10 s. Administration of 8-OH-DPAT in this manner and at these doses has been shown previously to be selective for its anti-aggressive properties, with no generalized effects observed on social or motor behavior patterns (White et al., 1991; Sanchez et al., 1993; Bell and Hobson, 1994; Sanchez and Hyttel, 1994; Joppa et al., 1997; Miczek et al., 1998; de Boer et al., 1999). After injection, animals were returned to their home cage. As a baseline behavioral control, two separate groups of hamsters (n=6–7 each) were treated with either AAS or sesame oil throughout adolescence and tested for aggression.

In a second experiment, P27 hamsters were weighed and randomly distributed into 2 groups. One group of hamsters (n=11) received the AAS mixture as described above, while a second group (n=11) was injected with sesame oil vehicle alone. Injections of AAS or vehicle continued throughout adolescent development (i.e., P27–P56). One day following the 30-day treatment period (P57), animals in both AAS and sesame oil groups were sacrificed and the brains removed and processed for immunohistochemistry and Western blot analysis as detailed below.

2.3. Aggression testing

Experimental animals were tested for offensive aggression using the resident/intruder paradigm, a well-characterized and ethologically valid model of offensive aggression in golden hamsters (Lerwill and Makaings, 1971; Floody and Pfaff, 1977). For this measure, an intruder of similar size and weight was introduced into the home cage of experimental animals and the resident was scored for offensive aggression (i.e., number of lateral attacks, upright offensive attacks, head/neck bites, flank/rump bites, chases, and latency to attack and bite towards intruders), as previously described (Grimes and Melloni, 2002; Grimes et al., 2003; Ricci et al., 2004, 2005a). Briefly, an attack was scored each time the resident animal would chase and then either: (1) lunge towards, and/or (2) confine the intruder by upright and sideways threat; each generally followed by a direct attempt to bite the intruder's flank and/or rump. Composite Aggression Scores (CAS), used as a general measure of aggression intensity, 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 first bite, used as a general measure of aggression initiation, was defined as the period of time between the beginning of the behavioral test and the first bite of the residents onto an intruder. In the case of no bites, latency to bite was assigned the maximum latency (i.e., 600 s). Also, residents were measured for social interest towards intruders (i.e., contact time between resident and intruder) to control for nonspecific effects of 5HT1A agonists on animal 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.

2.4. Immunohistochemistry

For immunohistochemistry a subset of AAS and sesame oil-treated hamsters from Experiment 2 (n=7 animals/group) 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, post-fixed in perfusion fixative, and then cryoprotected by incubation in 30% sucrose in 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. The collected brains were then processed for the immunohistochemical localization of 5HT1A receptors using a modification of an existing protocol (Grimes and Melloni, 2002, 2005; Grimes et al., 2003). Briefly, a consecutive series of 35 μm coronal sections were cut on a freezing microtome and collected as free floating sections in PBS. Every 3rd brain section was washed in PBS for 3×5 min, pretreated with 3% H_2O_2 in distilled water for 10 min, and rinsed thoroughly with PBS. Sections were pretreated with 10% normal goat serum (NGS) and 1% bovine serum albumin (BSA) in PBS containing 0.25% Triton X-100 for 60 min at room temperature (RT), then incubated in primary antiserum for 5HT1A (i.e., goat anti-serotonin-1A receptor polyclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA) at a final dilution of 1:500 in 10% NGS, 1% BSA and 0.25% Triton X-100 for 48 h at 4 °C. Sections were then rinsed with PBS for 3×10 min, incubated in biotinylated secondary anti-goat IgG (Vector Laboratories, Burlingame, CA) in PBS and 10% NGS, 1% BSA, and 0.25% Triton X-100 for 60 min at room temperature, rinsed again in PBS for 3×10 min, and then incubated in avidin–biotin-complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) with 1% BSA in PBS for 60 min at room temperature. The peroxidase reaction was revealed using 0.5% 3,3′-diaminobenzidine in distilled water as per manufacture's recommendations (DAB Kit. Vectastain; Vector Laboratories, Burlingame, CA). The sections were mounted on gelatin-coated slides, air-dried, dehydrated through a series of alcohols, cleared with xylene and coverslipped with Cytoseal-60 (Stephens Scientific, Kalamazoo, MI). Omission of the primary and secondary antibodies were run as controls during the procedure.

2.5. Western blot analysis

To more accurately measure 5HT1A levels, a second subset of AAS and sesame oil-treated hamsters from Experiment 2 (n=4 animals/group) were live decapitated and brains rapidly removed and snap-frozen in liquid nitrogen and stored at −80 °C. The frozen brains were then placed at −20 °C overnight and a consecutive series of 250 μm coronal sections

were cut at −9 °C using an HM 505 E micron cryostat and placed on pre-cooled, gelatin-coated slides. A 0.3 mm stainless steel punch (Palkovitz Punch Kit, Vibratome, Inc.) was used to sample micropunches of the anterior hypothalamus (AH) bilaterally using the hamster atlas (Morin and Wood, 2001) as a guide to locate specific brain regions. Brain punches from control and AAS-treated hamsters were pooled (n=4 punches per brain region per animal), and total protein was extracted using Trizol reagent (Invitrogen) with slight modifications to manufacturer's protocol. Briefly, punches of each brain region from each brain region were collected in 500 µl of ice-cold Trizol reagent. Protein was extracted from the phenol–ethanol phase by precipitation with 750 µl of isopropyl alcohol at 4 °C for 15 min followed by centrifugation at 12,000×g at 4 °C for 10 min. Protein pellets were then washed 2 times with 0.3 M guanidine hydrochloride dissolved in 95% ethanol followed by centrifugations of 10,000×g at 4 °C for 10 min between washes. After the second wash, protein pellets were washed with 100% ethanol. The protein pellets were then vacuum-dried for 10 min using a vacufuge (Eppendorf), re-dissolved in 1% sodium dodecyl sulfate (SDS) in PBS and incubated at 50 °C for 10 min. Protein concentration was determined using the Bradford assay (Bio-Rad).

Trizol extracted proteins were boiled at 95 °C for 10 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% tris-glycine gel (Cambrex) for 1 h at 100 Vs. Following gel electrophoresis proteins were transferred onto Immobillon-P PVDF membranes (Millipore) at 200 mA for 2 h at RT. To block nonspecific binding, membranes were incubated with 5% milk/PBS containing 0.001% Tween-20 (PBST) for 2 h at RT. Membranes were rinsed with PBST for 15 min and probed with primary antibody 5HT1A receptor (Santa Cruz) at 1:500 in 1% BSA/PBST for 2 nights at 4 °C. Following primary antibody incubation membranes were washed 3 times for 20 min with PBST at RT. Membranes were then incubated in goat anti-rabbit horse radish peroxidase-conjugated secondary antibody (Amersham) at 1:5000 in 1% BSA/PBST for 1 h at RT. Membranes were washed 3 times 20 min in PBST followed a last wash in PBS for 15 min. Color development was carried out using Supersignal western blot chemiluminescent reagent (Pierce, Rockford, IL) followed by exposure to Hyper Performance Chemiluminescent films (Amersham Pharmacia; Pascataway, New Jersey). Densitometry analysis was performed using NIH ImageJ software (NIH).

2.6. Image analysis

For immunohistochemistry, the area density of 5HT1A immunoreactive staining (5HT1A-ir) and the number of 5HT1A-ir cells were determined within specific brain areas using the BIOQUANT NOVA 5.5 computer-assisted microscopic image analysis software package as previously described (DeLeon et al., 2002a; Grimes and Melloni, 2002, 2005; Grimes et al., 2003). The areas analyzed were selected based on previous data implicating these regions as part of the circuit important for aggressive behavior in numerous species and models of aggression (Bunnell et al., 1970; Sodetz and Bunnell, 1970; Potegal et al., 1981; Kollack-Walker and Newman, 1995; Delville et al., 1996a,b, 2000; Ferris et al., 1997). These areas (see Fig. 1) included the anterior hypothalamus (AH), the medial division of the bed nucleus of the stria terminalis (BNST), the central amygdala (CeA), the anterior cortico-amygdala (CoMeA), the intermediate part of the lateral septal nucleus (LS), the medial amygdaloid nucleus (MeA), and the ventrolateral hypothalamus (VLH) which included the medial aspects of the medial tuberal nucleus and the ventrolateral part of the ventromedial hypothalamic nucleus. Additionally, we analyzed the S1 neocortex (S1 CTX), a brain region not associated with aggression. Slides from each animal 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, TN, USA) was utilized to identify the brain region of interest at low power (4×) using a Nikon E600 microscope. At this magnification, a standard computer-generated box was drawn to fit within the particular region of interest. Then, under 10× magnification images were assigned a threshold value at a standard RGB-scale level empirically determined by observers blinded to treatment conditions, such as to allow detection of

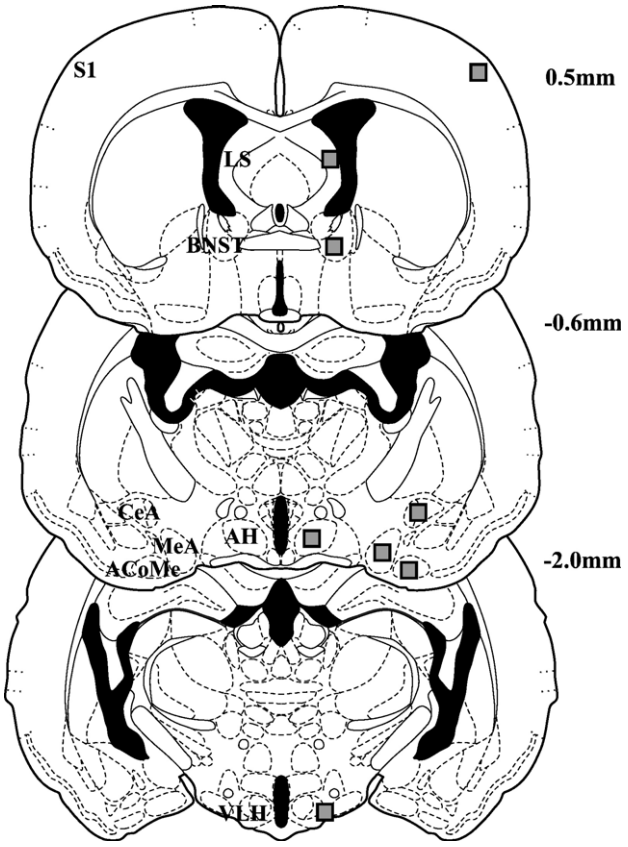

Fig. 1. Diagram showing the location of the areas selected to quantify 5HT1A immunoreactive labeling (shaded areas). Plates were modified from The Hamster Atlas of Morin and Wood (2001) and reflect specific positions in the rostral–caudal plane (i.e., distance in mm from bregma to the plane of section at the skull surface). Abbreviations: ACoMe, anterior corticomedial amygdala; 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; S1, somatosensory cortex; VLH, ventrolateral hypothalamus.

stained 5HT1A-ir neurons with moderate to high intensity, while suppressing lightly stained elements. This threshold value was then applied across subjects to control for changes in background staining and differences in foreground staining intensity between animals. The illumination was kept constant for all measurements. Two types of quantitative measurements were assessed: (1) total area density covered by 5HT1A immunoreactivity and (2) total cell numbers expressing positive 5HT1A immunoreactivity. Immunoreactive staining of 5HT1A receptors was identified in each field using a mouse driven cursor and then the total area density of 5HT1A-ir was calculated automatically by the BIOQUANT software. Using the same region of interest used to quantify the area density of 5HT1A-ir, 5HT1A-ir cell counts were performed 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. A neuron was counted as 5HT1A positive when processes or part of its nucleus was visible. Four to six independent measurements of 5HT1A-ir elements were taken from several consecutive sections ($n>3$) of each animal per treatment group depending upon: (1) identification of the exact position of the nucleus within the region of interest, and (2) the size of the nucleus in the rostral-caudal plane. Then, 5HT1A-ir area density and number of 5HT1A-ir cells were determined for each region of interest and standardized per 100 μm × 100 μm parcel for regional comparison purposes. The average for each brain region was calculated for each individual animal. The collective averages within treatment conditions were then compared and analyzed between treatments.

Western blot films were analyzed using the NIH software program ImageJ. To quantify 5HT1A receptor expression, 5HT1A-ir bands on film autoradiograms were outlined within a region of interest (ROI) box and the signal density was captured in NIH ImageJ software running on a MacIntosh G3 interfaced with a B95 Northern Light Illuminator (Imaging Research, Ontario, Canada), which provided uniform light distribution and intensity across the film autoradiogram. Densitometric analysis using the NIH ImageJ analysis software measures the density of whiteness within the selected ROI on a scale of 0–255 (black to white). Since 5HT1A-ir bands are represented as black bands on a white-gray background on film autoradiograms, the degree of whiteness is inversely related to the amount of protein expression. To standardize, background intensity measurements were taken from the film and subtracted from the 5HT1A-specific signal intensity.

2.7. Statistical analysis

Behavioral studies. The results from the aggression tests were compared between (1) *R*(+)-8-OH-DPAT-and saline-treatment groups and (2) doses of *R*(+)-8-OH-DPAT. Nonparametric data (composite aggression scores, number of lateral attacks, upright offensive attacks, flank/rump bites, chases) were compared by Mann–Whitney *U*-tests (two-tailed) and Kruskal–Wallis ANOVA, while parametric data (bite latency and total contact time) were compared by Student's *t*-test (two-tailed) and ANOVA.

5HT1A immunoreactivity and expression. The area covered by 5HT1B-ir neurons, the number of 5HT1A-ir cells, and the levels of 5HT1A protein were compared between treatment groups by Student's *t*-test (two-tailed).

3. Results

3.1. Experiment 1 — R(+)-8-OH-DPAT effects on adolescent AAS-induced offensive aggression

Peripheral administration of the 5HT1A agonist, *R*(+)-8-OH-DPAT diminished the aggressive response of adolescent AAS-treated animals. As shown in Fig. 2, hamsters treated with high-dose AAS throughout adolescence showed high levels of aggression intensity (as measured by composite scores of offensive aggression) when administered saline prior to the aggression test. Indeed, nearly all of the AAS-treated animals administered saline tested (5 out of 6) scored greater than 30 attacks and bites on intruders during the test period. The

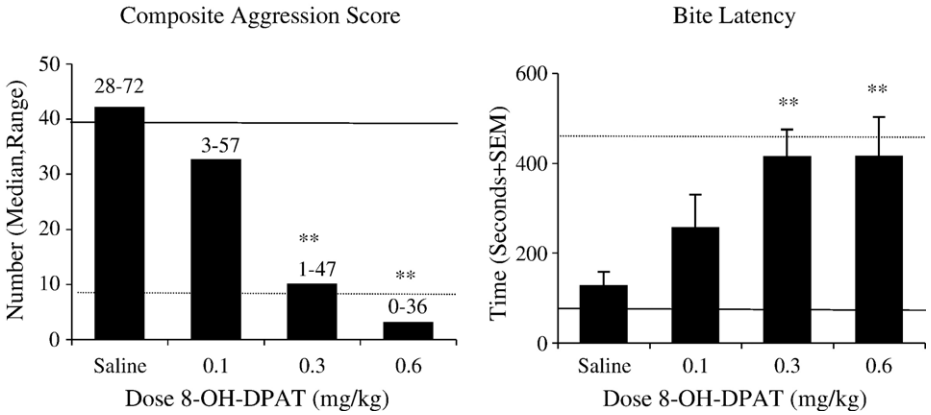

Fig. 2. Anti-aggressive effects of *R*(+)-8-OH-DPAT on Composite Aggression Scores (i.e., total number of attacks and bites) and Latency to Bite of adolescent, AAS-treated hamsters. Agonist treatment (0.1–0.6 mg/kg, i.p.) decreases Composite Aggression and Bite Latency in aggressive adolescent AAS-treated residents. Solid and dashed lines represent the baseline behavioral response of AAS- (solid) or sesame oil- (dashed) treated residents in the absence of *R*(+)-8-OH-DPAT administration. $^{**}p<0.01$, Mann–Whitney *U*-tests (two-tailed) and Kruskal–Wallis ANOVA (Composite Aggression Scores) and Student's *t*-test (two-tailed) and ANOVA (Bite Latency).

remainder of the animals (1 out of 6) scored moderate-to-high levels of offensive attack, directing 28 attacks and bites towards intruders. Similarly, hamsters treated with high-dose AAS throughout adolescence displayed a rapid initiation of the aggressive response (as measured by the latency to first bite) when administered saline prior to the aggression test. As above, in nearly all of the AAS-treated animals administered saline (5 out of 6), the bite latency was under 2.5 min out of a 10 min test duration.

Conversely, the systemic administration of the 5HT1A agonist, *R*(+)-8-OH-DPAT, produced an overall effect on composite scores of offensive aggression ($\chi^2=11.65$, $p<0.01$) with the anti-aggressive effects significant at the 0.3 mg/kg and 0.6 mg/kg doses (Fig. 2). At these doses, *R*(+)-8-OH-DPAT treatment significantly reduced composite aggression (0.3 mg/kg, $Z=2.84$; 0.6 mg/kg, $Z=2.78$; $p<0.01$ each comparison) of AAS-treated animals towards intruders when compared with AAS-treated hamsters that received saline prior to behavioral testing (Fig. 2A). Similarly, at the high effective dose (i.e., 0.6 mg/kg) of *R*(+)-8-OH-DPAT, there was a significant decrease in composite aggression of AAS-treated animals towards intruders when compared with the lowest dose (i.e., 0.1 mg/kg) of *R*(+)-8-OH-DPAT ($Z=2.08$, $p<0.05$). In addition, 5HT1A receptor activation with 0.3- and 0.6-mg/kg *R*(+)-8-OH-DPAT produced an overall effect on the latency to first bite ($F(3,25)=3.82$, $p<0.05$) in AAS-treated hamsters (Fig. 2). At these doses, *R*(+)-8-OH-DPAT treatment significantly increased latency to bite intruders [0.3 mg/kg, $t(8)=2.98$; 0.6 mg/kg, $t(8)=2.88$; $p<0.01$ each comparison] in AAS-treated animals compared with AAS-treated hamsters that received saline prior to behavioral testing. Further analysis showed that systemic administration of *R*(+)-8-OH-DPAT did not produce a significant effect on total contact time (i.e., a measure of social interest) between residents and intruders during the behavioral test ($F(3,25)=2.01$, $p=0.14$).

When examined more precisely, 5HT1A receptor activation with *R*(+)-8-OH-DPAT produced an overall effect on several targeted offensive responses. In particular, systemic administration of *R*(+)-8-OH-DPAT produced an overall effect on the number of upright offensive attacks ($\chi^2=9.78$, $p<0.05$), lateral attacks ($\chi^2=9.78$, $p<0.05$), flank/rump bites ($\chi^2=12.32$, $p<0.01$), and chases ($\chi^2=11.14$, $p<0.05$) with significant effects observed at each dose of *R*(+)-8-OH-DPAT administered dependent upon the specific behavior examined. For instance, at all doses examined *R*(+)-8-OH-DPAT significantly reduced the number of upright offensive attacks (0.1 mg/kg, $Z=2.52$, $p<0.05$; 0.3 mg/kg, $Z=2.78$, $p<0.01$, and 0.6 mg/kg, $Z=2.5$, $p<0.05$) and flank/rump bites (0.1 mg/kg, $Z=2.06$, $p<0.05$; 0.3 mg/kg, $Z=2.96$, $p<0.01$, and 0.6 mg/kg, $Z=2.85$, $p<0.01$) of AAS-treated animals onto intruders when compared with

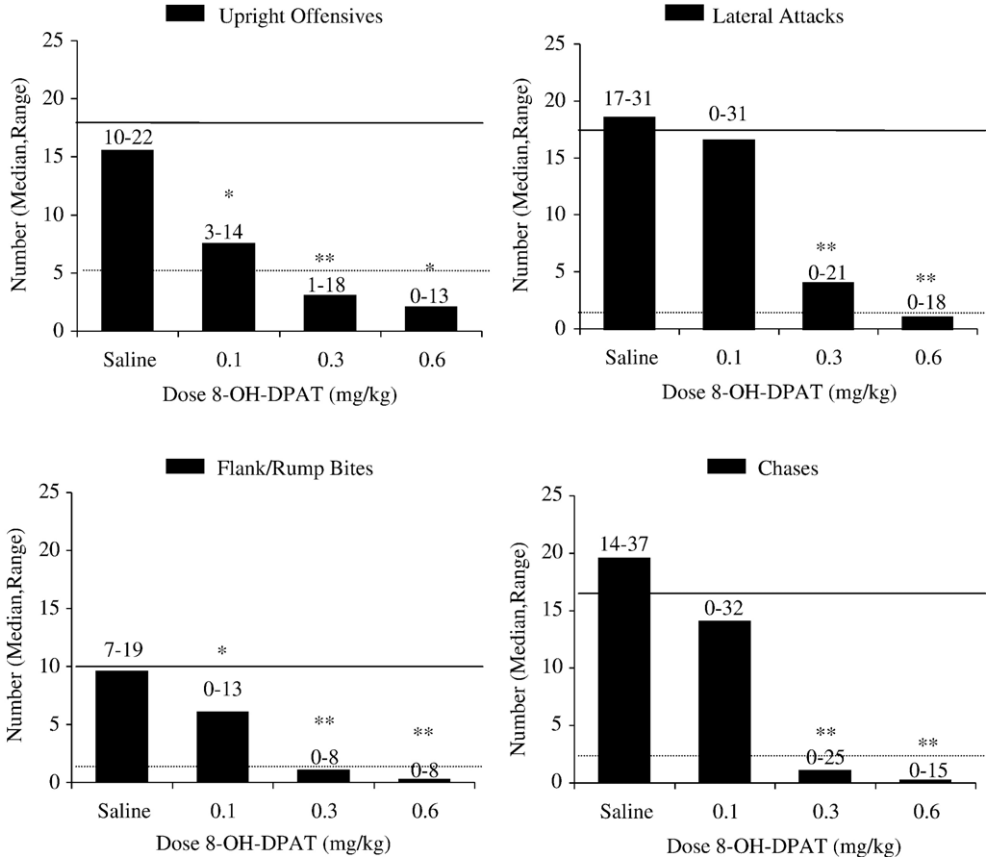

Fig. 3. Anti-aggressive effects of *R*(+)-8-OH-DPAT on the offensive response pattern of adolescent, AAS-treated hamsters. Agonist treatment (0.1–0.6 mg/kg, i.p.) decreases general measures of Aggression Intensity (i.e., number of upright offensive and lateral attacks, flank/rump bites, and chases) in aggressive adolescent AAS-treated residents. Lines represent the baseline behavioral response of AAS- (solid lines) or sesame oil- (dashed lines) treated residents in the absence of *R*(+)-8-OH-DPAT administration. *$p<0.05$; **$p<0.01$, Mann–Whitney *U*-tests (two-tailed) and Kruskal–Wallis ANOVA.

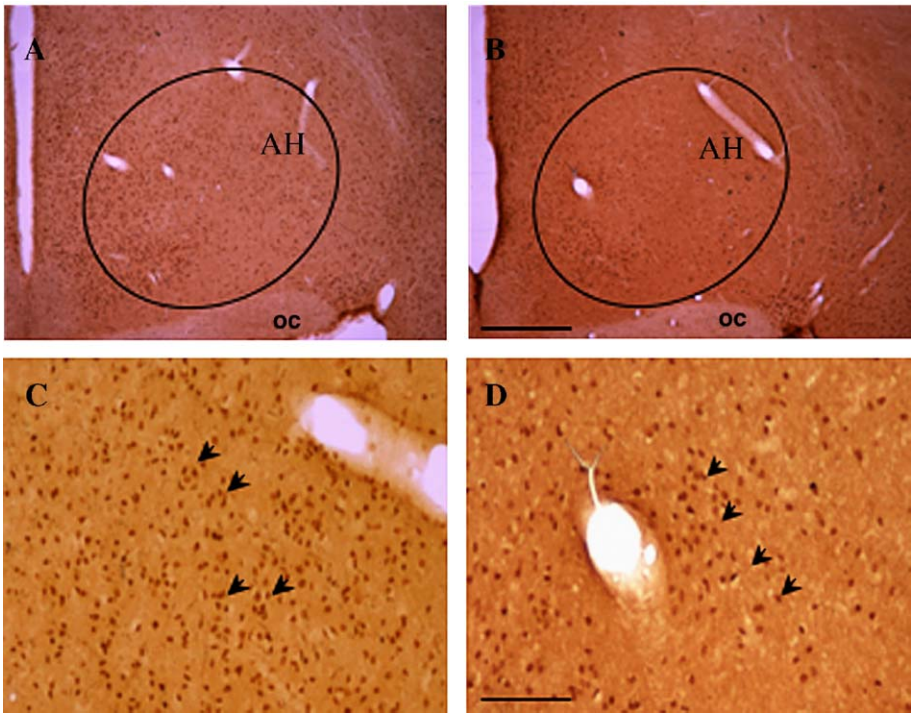

Fig. 4. Brightfield photomicrographs showing 5HT1A receptor-ir cells (arrows) in the anterior hypothalamus (AH) of (A, C) Sesame oil- and (B, D) AAS-treated hamsters. Bar, 600 μm (A, B) and 300 μm (C, D). oc, optic chiasm.

AAS-treated hamsters treated with saline prior to aggression testing (Fig. 3). However, only at higher doses (i.e., 0.3 mg/kg and 0.6 mg/kg) did *R*(+)-8-OH-DPAT significantly reduce the number of lateral attacks (0.3 mg/kg, $Z=2.71$, $p<0.01$; 0.6 mg/kg, $Z=2.78$, $p<0.01$) and chases (0.3 mg/kg, $Z=2.71$, $p<0.01$; 0.6 mg/kg, $Z=3.00$, $p<0.01$) of AAS-treated animals towards intruders as compared with AAS-treated hamsters treated with saline (Fig. 3).

3.2. Experiment 2 — adolescent AAS exposure and 5HT1A receptor localization and expression

5HT1A immunohistochemical analysis revealed distinct neuronal somata staining across the hamster neuraxis that was not present in negative control sections, i.e. omission of primary or secondary antibodies (data not shown). In AAS-treated hamsters, the immunohistochemical staining pattern for 5HT1A receptors was altered only in select areas of the hamster brain implicated in offensive aggression, namely the anterior hypothalamic brain region. For example, in sesame oil-treated controls, the staining of 5HT1A-containing neuronal somata in the AH displayed a moderate-to-dense pattern of 5HT1A receptor immunoreactivity indicative of the normal distribution of receptor localization in this brain region (Fig. 4A,C). By comparison, AAS-treated hamsters displayed a less dense pattern of immunostaining for 5HT1A-containing neuronal somata in this brain region (Fig. 4B,D). Quantitative analysis of the density of 5HT1A receptor immunoreactive staining in the AH showed that AAS-treated hamsters had less than 50% of the 5HT1A receptor signal of sesame oil-treated littermates (Fig. 5). This reduction was statistically significant [$t(12)=4.71$, $p<0.001$] between treatment groups. These findings were restricted to the AH, as other hypothalamic and extrahypothalamic regions of the hamster brain important for aggression

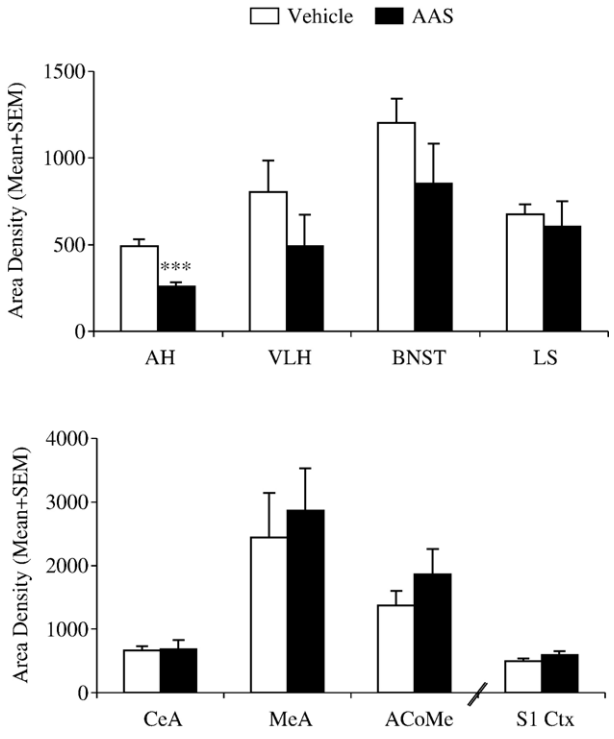

Fig. 5. Density of 5HT1A receptor-ir neuronal somata in AAS- and vehicle-treated hamsters. Numbers were normalized to a standard area (100 μm × 100 μm) for regional comparisons. ***$p<0.001$; Student's *t*-test, two-tailed.

regulation showed no concomitant changes in the density of 5HT1A immunostaining following adolescent AAS exposure. For instance, similar densities of 5HT1A receptor-containing neuronal somata were found in the VLH ($t(12)=1.36$), medial division of the BNST ($t(11)=1.74$), the intermediate part of the LS ($t(12)=0.43$), and several divisions of the amygdala, including the central ($t(12)=0.1$), medial ($t(12)=1.62$) and corticomedial ($t(12)=1.06$) divisions of both AAS- and oil-treated hamsters (Fig. 5). These data were not significantly different between treatment groups [$p>0.1$ each comparison]. Similarly, no significant differences were found in the density of 5HT1A immunoreactive staining in the S1 cortex ($t(12)=1.2$, $p>0.1$), i.e., a brain area not involved in aggressive behavior in the hamster.

To determine whether the changing patterns of 5HT1A-ir observed in the AH by immunohistochemistry are indicative of a decrease in the number of neurons expressing 5HT1A receptors in the brain region, 5HT1A-immunopositive neurons were counted to determine if the number of 5HT1A receptor expressing neurons differ between AAS-treated hamsters and controls. Interestingly, the numbers of 5HT1A-immunoreactive somata in the AH were unchanged between adolescent, AAS-treated animals and sesame-oil controls. In AAS-treated animals, the number of 5HT1A receptor-containing cells averaged 333+78 in the AH, while the number of 5HT1A receptor-containing cells averaged 399+79 in the AH in sesame oil-treated animals. These data were not significantly different between treatment groups ($t(12)=1.54$, $p>0.1$). To determine quantitatively whether the changing patterns of 5HT1A-ir observed in the AH by immunohistochemistry are indicative of a decrease in protein content, Western blot analysis was performed on protein extracts isolated from the AH of animals administered AAS during adolescence and sesame oil-treated littermates (Fig. 6). In all extracts measured, the levels of 5HT1A protein were approximately 50% lower in the AH of animals chronically exposed to AAS as compared to sesame oil-treated littermates. These differences were statistically significant ($t(6)=2.7$, $p<0.05$).

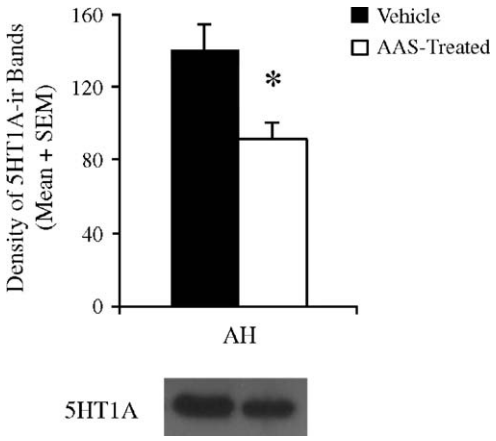

Fig. 6. Quantitative measure of 5HT1A receptor levels in the anterior hypothalamus (AH) of AAS- and vehicle-treated hamsters by Western blot analysis. (Top row) Quantification of the relative amounts of 5HT1A receptor in AH neurons from AAS- (white bars) and vehicle- (black bars) treated animals. Western blots, generated as in (bottom row), were scanned by densitometry. (Bottom row) Western blot autoradiogram of 5HT1A receptor protein in AH neurons of AAS- and vehicle-treated animals. *$p<0.05$; Student's t-test, two-tailed.

4. Discussion

In previous studies, we have shown that intact male hamsters repeatedly exposed to AAS throughout adolescent development display significantly high levels of offensive aggression (Harrison et al., 2000; DeLeon et al., 2002b; Grimes et al., 2003, 2006) that could be attenuated by pharmacologically increasing extracellular 5HT (Grimes and Melloni, 2002) or enhancing the activity of select 5HT type 1 receptors (namely, the 5HT1B subtype receptor) (Grimes and Melloni, 2005). Animals that respond aggressively following adolescent AAS exposure showed significant decreases in 5HT afferent innervation (Grimes and Melloni, 2002) and changes in 5HT1B receptor localization/expression in many areas of hamster brain important for aggression control (Grimes and Melloni, 2005). Together, these data suggested that adolescent AAS exposure stimulated aggression by altering the activity and development of the 5HT and 5HT type-1 receptor-expressing system implicated in the control of aggressive behavior. Since the inhibitory nature of 5HT on aggression has been attributed to activity at the full spectrum of the 5HT type 1 receptors (i.e., 5HT1A and 1B receptors) (Sanchez et al., 1993; Muehlenkamp et al., 1995; Miczek et al., 1998), we hypothesized that 5HT1A receptor activity/expression would also modulate AAS-induced aggression. Perhaps exposure to AAS during adolescent development stimulates aggression by decreasing 5HT signaling through 5HT1A receptor pools in brain regions implicated in the control of offensive aggression in hamsters. To address this question, we performed experimental manipulations employing a selective 5HT1A agonist to test the hypothesis that 5HT1A receptor activation would reduce AAS-induced aggression.

The behavioral data presented here support our hypothesis that 5HT1A receptors play an inhibitory role in adolescent AAS-induced offensive aggression in Syrian hamsters. For instance, the administration of saline to AAS-treated hamsters had no effect on aggression, as animals showed high levels of offensive aggression analogous to that observed in our previous studies (Harrison et al., 2000, Grimes and Melloni, 2002, 2005; Grimes et al., 2003, 2006) and presented here (see solid lines in Figs. 2 and 3). All saline-treated animals (6 out of 6) 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 bite). Conversely, treatment with 5HT1A receptor agonists resulted in a dose-dependent reduction of adolescent AAS-induced aggression, as $R(+)$-8-OH-DPAT-treated hamsters showed a greater than 40–50% decrease in a number of the aggression measures at the 0.1 mg/kg dose, with a maximal effect seen at a dose of 0.3 mg/kg. At this dose of agonist nearly 75–85% of aggression measures were eliminated when compared to saline-treated counterparts. Conversely, there was no difference in contact time between $R(+)$-8-OH-DPAT- and vehicle-treated animals, indicating that animals in both groups

were equally interested in intruders. The anti-aggressive effects of these low doses (0.1–0.3 mg/kg) of *R*(+)-8-OH-DPAT are in agreement with a set of studies in which 8-OH-DPAT decreased resident/intruder aggression in male rats (de Boer et al., 1999) and isolation- and ethanol-heightened-aggression in male mice (Sanchez and Hyttel, 1994; Miczek et al., 1998). To determine whether *R*(+)-8-OH-DPAT had more selective effects on the aggressive response, the aggression-suppressing properties of *R*(+)-8-OH-DPAT were investigated examining more targeted determinates of offensive aggression. In particular, in hamsters, attacks and bites targeted towards either the flank or hind quarter (i.e., rump) region of the intruder have been shown to be highly organized and adult determinants of aggressive behavior, while those targeted to the head and neck region depict more juvenile forms of the behavior (Delville et al., 2003; Wommack and Delville, 2003). Interestingly, adolescent AAS-treated animals administered *R*(+)-8-OH-DPAT showed a greater than 40 and 85% decrease in the number of lateral attacks and flank/rump bites compared to saline-treated controls at the effective doses of 0.1 and 0.3 mg/kg, respectively. No effect of *R*(+)-8-OH-DPAT was observed on latency to first attack or head/neck bites. Together, these behavioral data indicate that 5HT1A receptor activation via *R*(+)-8-OH-DPAT possesses a strong anti-aggressive capability on adolescent AAS-induced offensive aggression. Further, these findings are important and novel in that they indicate 5HT1A activity may play an important role in modulating mature elements of the offensive response in adolescent AAS-treated animals and that enhanced 5HT1A activity does not block AAS-facilitated offensive aggression through a nonspecific behavioral inhibition.

The finding that 5HT1A agonism (via *R*(+)-8-OH-DPAT) produced a dose-dependent reduction of the AAS-induced offensive response suggested that 5HT1A receptors play a direct role in aggression, perhaps acting within discrete brain regions involved in aggressive behavior in Syrian hamsters. In Syrian hamsters, 5HT activity in the AH and VLH has been shown to regulate offensive aggression (Delville et al., 1996a; Ferris, 1996; Ferris et al., 1997, 1999), where 5HT acts to inhibit aggression by acting through 5HT1A receptors (Ferris et al., 1999). Adolescent AAS exposure may alter 5HT neural signaling through post-synaptic 5HT1A 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 localized and quantified the expression of 5HT1A receptor-containing neuronal elements in brains of AAS-treated hamsters and oil-treated littermates. Immunohistochemical and western blotting assays revealed that adolescent AAS exposure significantly reduced 5HT1A receptor protein expression in only one of the number of brain areas implicated in the control of offensive aggression in hamsters, namely the AH. These data show that repeated exposure to AAS during adolescent development reduces 5HT1A localization/expression by nearly 50% in the AH without altering the number of cells expressing this receptor subtype. The reduced level of 5HT1A protein without a concomitant reduction in 5HT1A-receptor expressing cell numbers in the AH suggests that adolescent exposure to AAS does not completely deactivate the synthesis of 5HT1A receptors but instead down-regulates the expression of 5HT1A receptors in AH neurons producing this receptor subtype. There is support for this notion from studies in rats. For instance, sex differences have been reported in the expression of 5HT1A receptors within the AH brain area, with increases in 5HT1A mRNA seen in hypothalamic neurons in male rats (Zhang et al., 1999). This expression appears to be down-regulated by testosterone however, since castration-induced increases hypothalamic 5HT1A mRNA can be reduced by testosterone treatment (Zhang et al., 1999). Therefore, from a molecular standpoint, the reduced levels of 5HT1A receptor protein detected following adolescent AAS treatment may be the result of a decreased synthesis of 5HT1A receptor mRNA in this important brain region.

From a systems standpoint, the consistent pattern of alterations observed in AH-5HT development (Grimes and Melloni, 2002) and-5HT type 1 receptor localization/expression ((Grimes and Melloni, 2005) and those shown here) suggest that this brain region in particular is sensitive to the neurodevelopmental effects of AAS, and that these alterations may have significant functional influences on the aggressive phenotype. Indeed, the AH brain region has been identified as a critical brain region for the consummation of the aggressive response in hamsters (Delville et al., 2000). Neurobiological and behavioral studies have demonstrated an intimate anatomical and functional relationship between the AH 5-HT neural system and the control of offensive aggression (Ferris, 1996, Ferris et al., 1997, 1999). Anatomical studies reveal a dense 5HT innervation originating from neurons in the dorsal and median raphe nucleus to neurons in the AH (Ferris et al., 1997, 1999; Delville et al., 2000), and both the 5HT1A and 1B receptors are expressed in the AH brain region in hamsters (Grimes and Melloni, 2005; Ricci et al., 2005b). Functionally, treatment of animals with fluoxetine (i.e., a selective serotonin reuptake inhibitor) increases AH 5HT release (Pergola et al., 1993) and blocks aggression (Ferris, 1996; Ferris et al., 1997). Similarly, AH microinjections of 5HT1A agonists block offensive aggression in hamsters (Ferris et al., 1999). Together, these data suggest that 5HT activity in the AH can have specific aggression-suppressing effects, and that this inhibition can be mediated through 5HT1A receptor activity. Reductions in 5HT1A receptor expression in this critical brain region (as observed in this study) may reflect a loss in the ability of 5HT1A receptors to suppress the activity of AH neurons facilitating aggression, e.g., AH arginine vasopression (AVP) neurons. Indeed, in support of this notion, we recently co-localized 5HT1A receptors onto AVP neurons in the AH (Ricci et al., 2005b). In conjunction with reductions in the density of aggression-suppressing 5HT afferent fibers (Grimes and Melloni, 2002) and pre-synaptic 5HT1B receptor pools (Grimes and Melloni, 2005) in the AH of AAS-treated animals, these alterations may reflect a loss in the overall inhibitory tone of the AH 5HT neural system, subsequently facilitating the activity of aggression stimulating AVP neurons in the AH, predisposing animals to display the aggressive phenotype.

Finally, although these behavioral pharmacology and neurobiology studies are highly suggestive of 5HT1A receptor

mediated effect in the AH, it cannot be ruled out that the ability of *R*(+)-8-OH-DPAT to selectively block the adolescent AAS-induced aggressive phenotype results from the action of *R*(+)-8-OH-DPAT on receptors other than 5HT1A. Recent studies have shown that 8-OH-DPAT may also activate 5HT7 receptors (Wood et al., 2000; Hedlund et al., 2004) that are positively coupled to adenyl cyclase (Bard et al., 1993). One possible mechanism by which 5HT7 receptor activity may suppress adolescent AAS-induced offensive aggression is by augmenting γ-aminobutyric acid (GABA) inhibition of aggression-facilitating neurons in the AH, e.g., AH AVP neurons. In support of this view, 5HT7 receptors have been identified on GABA terminals in the hypothalamus (Belenky and Pickard, 2001) and we have reported marked increases in GABAergic terminal density in the AH of aggressive, AAS-treated animals compared to non-aggressive, oil-treated littermates (Grimes et al., 2003). Perhaps the combined action of *R*(+)-8-OH-DPAT on inhibitory 5HT1A receptors located on aggression-facilitating AH AVP neurons and excitatory 5HT7 receptors located on aggression-suppressing AH GABA terminals result in the highly anti-aggressive properties of *R*(+)-8-OH-DPAT observed in this study. Speculation aside, further study is needed to determine the site- and receptor-specific action of the anti-aggressive properties of *R*(+)-8-OH-DPAT in adolescent AAS-induced aggression.

In summary, the studies presented in this paper 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 5HT1A receptor and that AAS exposure during this developmental period produces marked reductions in 5HT1A expression in the AH, a critical brain region for the control of aggression in hamsters. These findings provide a link between adolescent AAS, AH 5HT1A and the inhibition of aggression, indicating a role of reduced AH 5HT1A receptor expression and function in AAS-induced aggression in adolescent hamsters.

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