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Serotonin modulates anxiety-like behaviors during withdrawal from adolescent anabolic–androgenic steroid exposure in Syrian hamsters

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Abstract

From the U.S. to Europe and Australia anabolic steroid abuse remains high in the adolescent population. This is concerning given that anabolic steroid use is associated with a higher incidence of pathological anxiety that often appears during withdrawal from use. This study uses pubertal Syrian hamsters (*Mesocricetus auratus*) to investigate the hypothesis that adolescent anabolic/androgenic steroid (AAS) exposure predisposes hamsters to heightened levels of anxiety during AAS withdrawal that is modulated by serotonin (5HT) neural signaling. In the first two sets of experiments, adolescent AAS-treated hamsters were tested for anxiety 21 days after the cessation of AAS administration (i.e., during AAS withdrawal) using the elevated plus maze (EPM), dark/light (DL), and seed finding (SF) tests and then examined for differences in 5HT afferent innervation to select areas of the brain important for anxiety. In the EPM and DL tests, adolescent AAS exposure leads to significant increases in anxiety-like response during AAS withdrawal. AAS-treated hamsters showed long-term reductions in 5HT innervation within several areas of the hamster brain implicated in anxiety, most notably the anterior hypothalamus and the central and medial amygdala. However, no differences in 5HT were found in other anxiety areas, e.g., frontal cortex and lateral septum. In the last experiment, adolescent AAS-treated hamsters were scored for anxiety on the 21st day of AAS withdrawal following the systemic administration of saline or one of three doses of fluoxetine, a selective serotonin reuptake inhibitor. Saline-treated hamsters showed high levels of AAS withdrawal-induced anxiety, while treatment with fluoxetine reduced AAS withdrawal-induced anxiety. These findings indicate that early AAS exposure has potent anxiogenic effects during AAS withdrawal that are modulated, in part, by 5HT signaling.

Keywords

Adolescence; Anabolic–androgenic steroids; Serotonin; Fluoxetine; Anxiety

Introduction

Despite educational programs and legal prohibitions, the illicit use of androgenic–anabolic steroids (AAS) has remained constant in the adolescent population over the past decade in the U.S., with more than a half million 8th–10th graders reporting use each year and a lifetime use of nearly 2% in males (NIDACapsules, 2007). This pattern is eclipsed however by use in European countries and Australia where AAS use among adolescents can well exceed 6% (Palleson et al., 2006; Williamson, 1993a; Williamson, 1993b). This trend is

alarming given that AAS use has been shown to be associated with an increased frequency of an array of adverse psychiatric side effects, including impulsivity, irritability, aggression and mood disorders (Pope and Katz, 1994; Pope et al., 1988). This is significant as much of the clinical data on the psychiatric side effects of AAS stem from the study of users in the 1960s–70s, i.e., a time when few adolescents had access to AAS and the doses used were generally much lower (Kanayama et al., 2008), therefore young AAS users of today may be more vulnerable to a wider range of more severe psychiatric side effects that may persist long after cessation of AAS use.

A common psychological side effect of AAS abuse in adult users is pathological anxiety (Bahrke et al., 1990; Daly et al., 2003; Johnson, 1990; Pagonis et al., 2006a, 2006b; Pope and Katz, 1994; Pope et al., 1988) that often appears during withdrawal from AAS use, either at the end of a use cycle or long after discontinuing AAS use (Bahrke et al., 1990; Brower, 1992; Brower, 2002; Corrigan, 1996; Kashkin and Kleber, 1989; Lindqvist et al., 2007; Malone and Dimeff, 1992; Malone et al., 1995; Perry and Hughes, 1992; Perry et al., 1990; Pope et al., 1996; Su et al., 1993). Often, the range and extent of the anxiogenic effects of AAS correlate with the severity of abuse (Pagonis et al., 2006a, 2006b). In contrast, preclinical studies investigating the relationship between adult AAS exposure and anxiety have produced mixed results indicating that AAS administration has both *anxiolytic* and *anxiogenic* effects (Agis-Balboa et al., 2009; Aikey et al., 2002; Ambar and Chiavegatto, 2009; Bing et al., 1998; Bitran et al., 1993; Fernandez-Guasti and Martinez-Mota, 2005; Koukoulas et al., 1999; Minkin et al., 1993; Ovsukova et al., 2003; Parrilla-Carrero et al., 2009; Rocha et al., 2007; Rojas-Ortiz et al., 2006). Given the incidence of AAS use among that adolescent population, it is surprising that relatively little, if any, preclinical research has focused on elucidating the bio-behavioral effects of adolescent AAS exposure on anxiety-like behaviors, and *none* that have focused on the long-term consequences of withdrawal from repeated, early-onset AAS exposure.

For over a decade we have used pubertal male Syrian hamsters (*Mesocricetus auratus*) as an adolescent animal model to investigate the effects of adolescent AAS exposure on the neurobiology of aggressive behavior (see Melloni and Ricci, 2010 for a comprehensive review). Interestingly, recent reports have shown a neurobiological relationship between aggressive behavior and anxiety (Apter et al., 1990; Fehon et al., 2001; Guillot and Chapouthier, 1996; Veenema and Neumann, 2007; Veenema et al., 2007). Closer examination of the neural networks regulating these behaviors reveal overlapping neural loci, supporting the notion that aggression and anxiety share a common neuroanatomy (Delville et al., 2000; Ernst and Fudge, 2009; Ferris, 2000; Newman et al., 1999; Pratt, 1992) (see Fig. 1). In particular, the anterior hypothalamus (AH), central amygdala (CeA), frontal cortex (FC), lateral septum (LS), and medial amygdala (MeA) appear to be sites of overlap for neural inputs that influence *both* aggressive behavior (Delville et al., 2000; Nelson and Trainor, 2007) and anxiety (Pratt, 1992). Findings from a number of our previous studies identified the serotonin (5HT) neural system in several of these brain sites as important points of convergence for developmental and neuroplastic alterations modulating adolescent AAS-induced aggression. In brief, reductions in 5HT afferent development were observed in two of these sites, i.e., the AH and MeA, in highly-aggressive, adolescent AAS-treated animals (Grimes and Melloni, 2002; Grimes et al., 2007). However, at 21 days of AAS withdrawal, AAS-treated animals were no longer aggressive (Carrillo et al., 2011; Grimes and Melloni, 2006; Grimes et al., 2006), yet reductions in 5HT afferent development to these regions remained (Grimes and Melloni, 2006), an indication that adolescent AAS exposure produced lasting reductions in 5HT signaling that did not correlate with offensive aggression. From these data we hypothesized that perhaps behaviors other than aggression that are influenced by 5HT would emerge during withdrawal from adolescent AAS exposure as a result of this persistent reduction in

5HT signaling. For instance, in humans abnormalities in the 5HT neural system have also been implicated in generalized anxiety disorder, panic disorder, post traumatic stress disorder, depression and obsessive compulsive disorder (Deakin, 1998a, 1998b; File, 2001; Graeff et al., 1996; Kennett et al., 1987; Lesch, 1991; Murphy et al., 1989; Southwick et al., 1999). Likewise in animals, reductions in 5HT signaling have been implicated in the development of anxiety (Collinson and Dawson, 1997; Cowen, 1991; Holmes et al., 2003). Given our previous findings that adolescent AAS exposure reduced the development of the 5HT neural system in several brain areas serving both anxiety and offensive aggression (i.e. most notably the AH and MeA) (Grimes and Melloni, 2002; Grimes et al., 2007), we questioned that reduced 5HT signaling may also modulate anxiety-like behaviors during AAS withdrawal. To date, however, it is unknown whether adolescent AAS exposure has any effects on anxiety-like response during AAS withdrawal. Moreover, there is no clear evidence as to whether 5HT signaling plays a significant role in any anxiety-provoking effects of adolescent AAS exposure.

To investigate the relationship between withdrawal from adolescent AAS exposure, 5HT neural development/signaling and anxiety-like behaviors, we used pubertal male hamsters as an adolescent animal model. First, to determine whether adolescent AAS exposure produced alterations in anxiety-like response during AAS withdrawal, AAS-treated hamsters were tested for anxiety-like behaviors 21 days following the cessation of adolescent AAS administration (i.e., the 21st day of withdrawal — when AAS-treated animals no longer display the aggressive phenotype (Carrillo et al., 2011; Grimes and Melloni, 2006; Grimes et al., 2006)). Then, to determine whether adolescent AAS exposure altered the development of the 5HT neural systems in brain sites implicated in *both* anxiety and aggression, immunohistochemistry was utilized to visualize and quantify 5HT afferent fibers and varicosities in AAS-treated hamsters. Finally, using an experimental design similar to the one we employed previously to highlight the role of 5HT signaling in adolescent AAS-induced offensive aggression, we tested whether the selective serotonin reuptake inhibitor (SSRI), fluoxetine, was capable of inhibiting the anxiety-like behavioral responses that are present during withdrawal from adolescent AAS exposure.

Methods

Animals

Intact pubertal male Syrian hamsters (*M. auratus*) postnatal day (P) 21 were obtained from Charles River Laboratories (Wilmington, MA), individually housed in polycarbonate cages, and maintained at ambient room temperature (22–24 °C with 55% relative humidity) on a reverse light/dark cycle (14L:10D; lights off at 07:00). Food and water were provided ad libitum. All studies using live animals were performed with pre-approval by the Northeastern University Institutional Animal Care and Use Committee (NU-IACUC) and all methods used were consistent with guidelines provided by the National Institute of Health for the scientific treatment of animals.

Experimental treatment

Effects on adolescent AAS on anxiety and aggression during AAS withdrawal

—In the first experiment, P27 Syrian hamsters (n=16) were weighed and randomly distributed into two groups (n=8 animals/group). One group of animals received daily subcutaneous (SC) injections (0.1 ml–0.2 ml) for 30 consecutive days (P27–P56) of a mixture (or “stack”) of 3 of the 10 most commonly abused AAS (Hall et al., 2005) in doses consistent with repeated, moderate use in humans as described (see Melloni and Ricci, 2010 for a comprehensive review). The AAS cocktail was composed of 2 mg/kg testosterone cypionate, 2 mg/kg nortestosterone and 1 mg/kg dihydrotestosterone undecylate

(Steraloids, Newport, RI) dissolved in sesame oil (SO). The second group of hamsters was injected with matched volume of SO as a vehicle control. Experimental and control animals were tested for anxiety using the EPM, DL, and SF tests at 21 days of AAS withdrawal, i.e., on P77 — 21 days following the last AAS/SO injection.

To confirm the absence of an aggressive phenotype at 21 days of AAS withdrawal (as reported previously (Grimes and Melloni, 2006; Grimes et al., 2006)), experimental and control animals were subsequently tested for offensive aggression on P77 using the resident–intruder test.

Effects of adolescent AAS on the 5HT neural system during AAS withdrawal—

In a second experiment, P27 hamsters were weighed and randomly distributed into 2 groups. One group of hamsters (n=6) received SC injections of the AAS mixture while a second group (n=5) was injected with an equal volume of SO vehicle alone for 30 consecutive days (P27–P56) as described above. Animals in each treatment group were then sacrificed 21 days following the cessation of AAS treatment and the brains were removed and processed for immunohistochemistry as detailed below.

Effects of fluoxetine on adolescent AAS withdrawal-induced anxiety—In a third experiment, P27 hamsters (n=31) received daily SC injections of the AAS mixture for 30 consecutive days (P27–P56) as described above. Then, 21 days following the last injection, AAS-treated animals were tested for anxiety-like behavior after an intraperitoneal (IP) injection of fluoxetine (5, 10, or 20 mg/kg in 0.9% saline) (Sigma Chemical Co., St. Louis, MO) (n=7–8/fluoxetine dose) or vehicle (n=8) in a volume of 0.2 ml each. All injections were performed on unanesthetized animals and took no longer than 10 s. Using this regimen, administration of fluoxetine at this dose and manner has been shown previously to have no generalized effects on social or sex behaviors (Delville et al., 1996; Ferris and Delville, 1994; Ferris et al., 1997; Grimes and Melloni, 2002). After injection, animals were returned to their home cage. One-hour later, animals were tested for anxiety-like behavior using the DL test.

Behavioral testing

Elevated plus maze test—The elevated plus maze (EPM) test has been used extensively in rats as an animal model of anxiety, with particular use as a sensitive behavioral test to screen for anxiolytic drug effects (Pellow and File, 1986; Pellow et al., 1985). The apparatus consisted of two open arms and two closed arms (30×5 cm) elevated to a height of 38.5 cm and intersecting in a central platform (5×5 cm). The closed arms had black Plexiglas walls (15 cm high) covered with a black Plexiglas lid on the roof. The apparatus was arranged such that the open arms were opposite to each other. Animals were individually placed in the center of the apparatus facing one of the closed arms. The number of entries to the open and closed arms and the time spent in each arm were recorded for a period of 5 min. The percentage of entries to the open arms and percentage of time spent in the open arms were calculated for each animal. Increased percentage of entries and percentage of time spent in the open arms was used as an index of anxiolytic behavior (Lister, 1987; Pellow et al., 1985). Each anxiety test was videotaped and coded by two observers unaware of the experimental treatment. Animals were tested during the first 4 h of the dark cycle under dim-red illumination to control for circadian influences in behavioral response.

Dark/light test—The dark/light (DL) test has been used extensively in mice as an animal model of anxiety and to screen for anxiolytic or anxiogenic properties of drugs (see Bourin and Hascoet, 2003 for a review). The apparatus consisted of a large Plexiglas box (L: 91.4 cm, W: 61 cm, H: 30.5 cm) separated into two compartments, each measuring L: 45.7 cm,

W: 61 cm, H: 30.5 cm. One compartment (i.e., the dark compartment) was made of black Plexiglas and covered with a black Plexiglas lid, while the other compartment (i.e., the white compartment) was made of clear Plexiglas, had an open roof and illuminated with a 40 W light bulb. One permanent dividing wall separated both compartments and had an opening in the center (W: 5 cm, L: 5 cm) that allowed animals to freely move from one compartment to the other. Animals were individually placed in one of the corners of the dark compartment. The number of entries into the white and dark compartment, the time spent in each compartment, and the latency to enter the white compartment were scored for a period of 10 min. The percentage of entries to the white compartment and percentage of time spent in the white compartment were calculated for each animal. Given the natural preference rodents have to enclosed/dark environments and the fear of open/illuminated spaces, shorter latency to first enter the white compartment, increased percentage of entries to the light compartment, and percentage of time spent in the light compartment were used as an index of anxiolytic response (Crawley and Goodwin, 1980; Hascoet et al., 2001). Each anxiety test was videotaped and coded by two observers unaware of experimental treatment. Animals were tested during the first 4 h of the dark cycle.

Seed finding test—The seed finding (SF) test has been used previously in hamsters as an animal model of anxiety, with particular focus on its use as a behavioral test to screen potential anxiolytic drugs (King et al., 2002). Two minutes prior to the beginning of the test, animals were taken from their home cage and placed in a clean novel cage. During this time, six sunflower seeds (without the shell) were randomly buried in one of the corners of the animal's home cage. Then, the animals were placed back in their home cage in the opposite corner to where the seeds were buried. The latency to find the seeds and pouch or chew at least one of the seeds was scored during a 10 minute period. When animals did not find and pouch or chew the seeds, the maximum latency was assigned (600 s). A shorter latency to find and pouch or chew the seeds was considered to be indicative of anxiolytic behavior (King et al., 2002). Animals were tested during the first 4 h of the dark cycle under dim-red illumination to control for circadian influences in behavioral response.

Resident–intruder test—Experimental animals and control animals were subsequently tested for offensive aggression on P77 using the resident–intruder test, a well-characterized and ethologically valid model of offensive aggression in golden hamsters (Floody and Pfaff, 1977; Lerwill and Makings, 1971). For this measure, an intruder of similar size and weight was introduced into the home cage of experimental animals and the behavior of the resident was examined for a focal analysis of the aggressive response including number of lateral attacks, upright offensive attacks, and latency to attack. Briefly, an attack was scored each time the resident animal would wildly pursue and then lunge towards and confine the intruder by sideway or upright threat; each generally followed by a direct attempt to bite the intruder's ventrum and/or flank. The latency to attack was defined as the period of time between the beginning of the behavioral test and the first attack and bite of the residents towards an intruder. In the case of no attacks or bites, latency was assigned the maximum time of the behavioral test (i.e., 600 s). Each aggression test lasted for 10 min and was scored by an observer unaware of the hamsters' experimental treatment. No intruder was used for more than one behavioral test and all tests were performed during the first 4 h of the dark phase under dim red illumination and videotaped for behavioral verification of the findings.

Immunohistochemistry

One day following the behavioral testing, AAS- (n=6) and SO-(n=5) treated hamsters were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine and the brains fixed by transcardial perfusion with 4% paraformaldehyde. Brains were then cryoprotected by

incubating in 30% sucrose in phosphate buffered saline (PBS; 0.001 M KH_2PO_4 , 0.01 MNa_2HPO_4 , 0.137 MNaCl , 0.003 MKCl , pH 7.4) overnight at 4 °C. A consecutive series of 35 μm coronal sections were cut on a sliding microtome, collected as free floating sections in 1× PBS and labeled for 5HT by single-label immunohistochemistry that we have previously described (DeLeon et al., 2002; Grimes and Melloni, 2002, 2006).

Image analysis

The number of 5HT immunoreactive (5HT-ir) varicosities and fibers was determined within specific brain areas using the BIOQUANT NOVA 5.5 computer-assisted microscopic image analysis software package as previously described in a number of our studies (DeLeon et al., 2002; Grimes and Melloni, 2002, 2006). The areas analyzed were selected based on previous data implicating these regions as part of an overlapping circuit important for both anxiety and aggressive behavior (Fig. 1). These areas included the anterior hypothalamus proper (AH), the central amygdaloid nucleus (CeA), the frontal cortex (FC), the intermediate part of the lateral septal nucleus (LS), and the medial amygdaloid nucleus (MeA). Slides from each animal were coded by an experimenter unaware of the experimental conditions 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 (ROI). 5HT-ir varicosities and fibers were identified in each ROI using a mouse driven cursor and then 5HT-ir counts were performed automatically by the BIOQUANT software as previously described (DeLeon et al., 2002; Grimes and Melloni, 2002, 2006). Measurements continued until 5HT-ir elements throughout the entire region of interest were quantified. Two to three independent measurements of 5HT-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, the number of 5HT-ir varicosities and fibers was determined for each region of interest, standardized per 100 $\mu\text{m} \times 100 \mu\text{m}$ parcel for regional comparison purposes, and then used for statistical analysis.

Statistical analysis

Behavioral results from the anxiety tests as well as the number of 5HT-ir varicosities and fibers were compared between AAS- and SO-treated groups using Student's *t*-test (two-tailed). In addition, the results from behavioral and fluoxetine dose response groups were compared using one-way analysis of variance (ANOVAs) followed by Fisher's protected least significance difference test post hoc. The α level for all comparisons was set at 0.05.

Results

Effects of adolescent AAS on anxiety-like behaviors during AAS withdrawal

Elevated plus maze test—At 21 days of AAS withdrawal, adolescent AAS-treated animals exhibited an increase in anxiety-like behavioral response in the EPM (Fig. 2). Specifically, adolescent AAS-exposed hamsters displayed a significant reduction in the percentage of entries to the open arms of the EPM compared to SO control animals [$t(14)=3.28$, $p<0.01$]. Here, AAS-treated animals displayed over a 30% reduction in the percentage of entries into the open arms of the EPM compared to SO-treated control animals. No significant differences, however, were observed in the percentage of time spent in the open arms of the EPM between AAS treatment and SO treatment groups [$t(14)=0.74$, $p>0.1$]. Similarly, no significant differences between the total number of entries into the open and closed arms were observed between groups [$t(14)=0.91$, $p>0.1$].

Dark/light test—At 21 days of AAS withdrawal, adolescent AAS-treated animals demonstrated a significant increase in anxiety-like behavioral response in the DL test (Fig.

2). Specifically, adolescent AAS-exposed hamsters displayed a significant reduction in the percentage of entries to the white compartment of the DL box compared to SO-treated control animals [$t(14)=3.24$, $p<0.01$]. In fact, AAS-treated animals nearly completely avoided entering the white compartment of the DL box during the test period. As a consequence, adolescent AAS-treated animals spent less than half the amount of time in the white compartment than their vehicle-treated counterparts. In addition, adolescent AAS-treated animals spent a significantly lower percentage of test period in the white compartment of the DL box when compared with SO-treated controls [$t(14)=2.99$, $p<0.05$]. On the occasion that AAS-treated animals did enter the white compartment, they spent less than half the time in the white compartment than vehicle-treated controls. When examined for other variables such as latency to first enter the light compartment no differences between adolescent AAS- and SO-treated animals were observed [$t(14)=0.74$, $p>0.05$].

Seed finding test—At 21 days of AAS withdrawal, no significant difference between adolescent AAS- and SO- treated animals were observed on measures of anxiety-like behavior using the SF test. Specifically, the latency to find seeds in AAS treated animals (174.25 ± 81 sd) was not significantly different from the latency to find seeds of SO-treated controls (269 ± 112 sd) [$t(12)=1.2$, $p>0.1$].

Effects of adolescent AAS on aggressive behavior during AAS withdrawal

As observed previously (Carrillo et al., 2011; Grimes and Melloni, 2006; Grimes et al., 2006), no significant difference between adolescent AAS- and SO-treated animals were observed on targeted measures of aggressive behavior at 21 days of AAS withdrawal (Fig. 2 — inset). Specifically, the number of lateral attacks and upright offensive attacks as well as the latency to first attack was not significantly different between adolescent AAS- and SO-treated control animals [$t(16)=0.97$, $t(16)=0.39$, $t(16)=0.012$ respectively, $p>0.1$ each comparison].

Effects of adolescent AAS exposure on 5HT neural development

In a number of previous studies, we have used immunohistochemical staining of 5HT fibers and varicosities as a sensitive marker of the development of 5HT afferent projections to brain areas implicated in aggressive behavior (DeLeon et al., 2002; Delville et al., 1998; Grimes and Melloni, 2002, 2006). Similar to the findings observed in the studies of Grimes and Melloni (2002, 2006), at 21 days of AAS withdrawal, the immunohistochemical staining pattern for 5HT was altered in select regions of the hamster brain, this time however in areas notably important for both aggression and anxiety. Specifically, adolescent AAS-treated animals displayed significant decreases in the density of 5HT-ir fibers and varicosities in the AH, CeA and MeA compared to SO-treated controls (AH, $t(10)=6.6$, $p<0.0001$; CeA, $t(10)=3.2$, $p<0.01$; MeA, $t(10)=4.2$, $p<0.01$) (see arrows in the photomicrographs in Fig. 3). Quantitative analysis of 5HT innervations showed that AAS-treated animals still had approximately half of the 5HT-ir varicosities and fibers in the AH and MeA of oil-treated littermates while the CeA of AAS-treated animals had just over 40% of the 5HT-ir varicosities and fibers of oil-treated littermates. However, not every brain region that has been implicated in the overlap between both aggressive- and anxiety-behaviors showed significant changes in 5HT afferent innervation following adolescent AAS withdrawal. For instance, similar numbers of 5HT-ir varicosities and fibers were found in the FC and the intermediate part of the LS of both AAS-treated and control animals. These counts were not significantly different between treatment groups [$p>0.1$ each comparison]. Similarly, no significant differences were found in several brain areas not involved in either aggressive or anxiety-like behaviors in the hamster. For example, the number of 5HT-ir varicosities and fibers was not significantly different between treatment groups in the S1 cortex or the caudate-putamen [$p>0.1$ each comparison].

5HT signaling modulates adolescent AAS withdrawal-induced anxiety

When tested for anxiety-like behavior at 21 days of AAS withdrawal, the peripheral administration of fluoxetine produced an overall decrease in anxiety-like response as measured by the percentage of time spent in the white compartment [$F_{(3, 27)}=12.1$, $p<0.001$] and the latency to enter the white compartment [$F_{(3, 27)}=14.62$, $p<0.001$] (Fig. 4). Post hoc analysis revealed that the low (5 mg/kg), moderate (10 mg/kg), and high (20 mg/kg) doses of fluoxetine each reduced the percentage of time that AAS-treated animals spent in the white compartment compared to those administered saline on test day [5 mg/kg, $t(13)=6.8$; 10 mg/kg, $t(14)=4.6$; 20 mg/kg, $t(14)=5.7$, $p<0.001$ each comparison]. Compared to AAS hamsters administered saline prior to the behavioral test, those administered fluoxetine (at any dose administered) spent nearly twice as much time in the white compartment. Specifically, adolescent hamsters treated with AAS spent very little time in the white compartment of the DL box when administered saline prior to the anxiety test. In fact, 100% of the animals administered saline (8 out of 8) spent between 13 and 15 s within the white compartment during the 10 minute behavioral test. By comparison, at the 5 mg/kg, 10 mg/kg, and 20 mg/kg doses of fluoxetine, the majority (nearly 75%) of the animals (17 out of 23 across all treatment groups) spent 30 s or more within the white compartment.

Similarly, in AAS-treated animals, low (5 mg/kg), moderate (10 mg/kg), and high (20 mg/kg) doses of fluoxetine each reduced the latency to enter the white compartment compared to those administered saline on test day [5 mg/kg, $t(13)=6.5$, $p<0.001$; 10 mg/kg, $t(14)=9.1$, $p<0.001$; 20 mg/kg, $t(14)=3.3$, $p<0.01$], with the greatest behavioral effect observed at the 10 mg/kg dose. Specifically, adolescent hamsters treated with AAS showed a moderate latency to enter the white compartment of the DL box when administered saline prior to the anxiety test, with 100% (8 out of 8) of the animals entering the light compartment well after the first 2 min of the behavioral test. By comparison, of the AAS-treated animals administered the 10 mg/kg dose of fluoxetine, nearly 90% (7 out of 8) of the animals entered the light compartment within the first 30 s of the test period, with half of the animals (4 out of 8) entering the light compartment within the first 15 s of the test. Similarly, the majority (60–70%) of AAS-treated animals administered the 5 mg/kg or 20 mg/kg dose of fluoxetine entered the light compartment within the first 60 s of the test period.

Lastly, no significant difference was observed in the percentage of entries into the white compartment between treatment groups at any fluoxetine dose administered [$F_{(3, 27)}=0.59$, $p>0.1$].

Discussion

A common and relatively unexplored psychological side effect of AAS abuse is pathological anxiety (Bahrke et al., 1990; Daly et al., 2003; Johnson, 1990; Pagonis et al., 2006a, 2006b; Pope and Katz, 1994; Pope et al., 1988) that often appears during withdrawal from AAS use (Bahrke et al., 1990; Brower, 1992, 2002; Corrigan, 1996; Kashkin and Kleber, 1989; Lindqvist et al., 2007; Malone and Dimeff, 1992; Malone et al., 1995; Perry and Hughes, 1992; Perry et al., 1990; Pope et al., 1996; Su et al., 1993). Interestingly, the range and extent of anxiety have been shown to correlate with the severity of AAS abuse, with longer abuse times correlating with greater levels of anxiety (Pagonis et al., 2006a; Pagonis et al., 2006b). Given this, it is surprising that no preclinical research has investigated whether AAS withdrawal influences anxiety. Those studies that have investigated the relationship between AAS use and anxiety have produced mixed results that vary with the animal species, AAS, dose, duration, and the experimental paradigm used. In adult mice and rats for example, *anxiolytic-like* actions of AAS have been observed directly following both acute (Aikey et al., 2002; Bing et al., 1998; Ovsukova et al., 2003) and chronic (Bing et al., 1998; Bitran et al., 1993; Fernandez-Guasti and Martinez-Mota, 2005; Koukoulas et al., 1999) exposure to

AAS using the EPM, DL, modified open field, burying behavior, holeboard and social interaction tests as measures of exploratory, anticipatory, and social anxiety. Conversely, *anxiogenic-like* actions of AAS have also been observed directly following acute (Parrilla-Carrero et al., 2009) and chronic (Agis-Balboa et al., 2009; Ambar and Chiavegatto, 2009; Minkin et al., 1993; Rocha et al., 2007; Rojas-Ortiz et al., 2006) exposure to AAS using identical animal models and experimental measures. While informative, these studies fail to provide consistent answers regarding the influence of AAS exposure on anxiety-like behavior. One possible reason for the discrepancies between these studies is that these preclinical investigations fail to consider several significant issues regarding the link between societal AAS abuse and anxiety. First, these studies do not use experimental treatment paradigms that are relevant to the naturalistic patterns of human AAS abuse. Field studies show that ordinary AAS abusers commonly administer two or more types of AAS simultaneously (a practice known as “stacking”) for extended periods of time (Kanayama et al., 2008). Yet of the preclinical studies to date, all have focused on the effects of a select few AAS compounds administered alone, and in many instances acutely. For instance, most studies examine the influence of nandrolone (Ambar and Chiavegatto, 2009; Koukoulas et al., 1999; Minkin et al., 1993; Parrilla-Carrero et al., 2009; Rocha et al., 2007) or testosterone propionate or cypionate (Agis-Balboa et al., 2009; Aikey et al., 2002; Bitran et al., 1993; Fernandez-Guasti and Martinez-Mota, 2005; Frye and Seliga, 2001) at doses and for durations which are varied, ranging from physiologic replacement to 30 mg/kg/day and for periods ranging from 1 day to 8 weeks. Preclinical studies employing a more consistent repeated-dosing and “stacking” regimen using combinations of AAS compounds commonly taken together may produce more consistent results regarding the relationship between AAS use and anxiety. Next, all the preclinical studies investigating the relationship between AAS use and anxiety have focused on the influence of adult AAS use on anxiety-like response during the AAS exposure period despite the documented prevalence and risk of adolescent AAS use and reports indicating that AAS use can increase the incidence of anxiety during AAS withdrawal (Brower, 1992, 2002; Corrigan, 1996; Kashkin and Kleber, 1989; Lindqvist et al., 2007; Malone and Dimeff, 1992; Malone et al., 1995; Perry and Hughes, 1992; Perry et al., 1990; Pope et al., 1996; Su et al., 1993). These variables are important given that much of the clinical data on the psychiatric side effects of AAS stem from the study of competitive users at a time when fewer novice youth had access to AAS and the doses of AAS used were generally much lower (Kanayama et al., 2008). Therefore young, novice AAS users of today may be more vulnerable to a wider range of more severe psychiatric side effects that may emerge long after the cessation of AAS use.

Here we present the first set of preclinical studies to date that has taken each of these important factors into consideration in one experimental design. In these studies we used pubertal male hamsters as an adolescent animal model to investigate the consequences of withdrawal from repeated, early-onset AAS exposure on anxiety-like behavior. Previously, we have used a similar experimental design and adolescent model to investigate the effects of withdrawal from adolescent AAS exposure on the behavioral neurobiology and pharmacology of aggressive behavior (Carrillo et al., 2011; Grimes and Melloni, 2006; Grimes et al., 2006). In these studies, we found that adolescent AAS administration had potent aggression-stimulating effects (Grimes and Melloni, 2002; Harrison et al., 2000) that subsided at extended periods of withdrawal (i.e., 21 days after the cessation of AAS administration) (Carrillo et al., 2011; Grimes and Melloni, 2006; Grimes et al., 2006). At this time adolescent AAS-treated animals were no longer aggressive and displayed a non-aggressive behavioral phenotype representative of vehicle-treated control animals. Further study indicated that the return of AAS-treated animals to the non-aggressive phenotype during AAS withdrawal correlated only with neuroplastic alterations in the arginine vasopressin (AVP) neural system, but not the 5HT neural system, indicating that AVP activity contributed more significantly to AAS-induced aggression than 5HT (Grimes and

Melloni, 2006; Grimes et al., 2006). From these data we questioned whether other adverse behaviors (besides aggression) that are influenced by 5HT would emerge from adolescent AAS exposure as a result of a persistent reduction in 5HT signaling during withdrawal. Given that a reduction in the 5HT signaling has also been implicated in the development of anxiety (Collinson and Dawson, 1997; Cowen, 1991; Holmes et al., 2003), we hypothesized that adolescent AAS exposure would also facilitate the generation of a highly anxious animals during AAS withdrawal and that this behavioral phenotype would be modulated by 5HT activity. In accord with this hypothesis, we found that adolescent AAS exposure had significant anxiogenic effects during AAS withdrawal. The findings are important and may explain, at least in part, why adolescent AAS-treated animals no longer respond in an aggressive manner at extended periods of withdrawal (Carrillo et al., 2011; Grimes and Melloni, 2006; Grimes et al., 2006). Indeed, it has been hypothesized that the aggressive response of an animal is closely related to the animal's response to anxiety provoking stimuli in the environment (Apter et al., 1990). In particular, the aggressive response reflects the coping strategy that each animal adopts when facing environmental challenges. Previous findings indicate that highly aggressive animals adopt an active, non-fearful strategy when they face and explore anxiety provoking environments (i.e., spend more time in species-specific aversive environments) while non-aggressive animals adopt a more passive strategy by avoiding these anxiogenic environments (i.e., spend more time avoiding noxious environments) (Nyberg et al., 2003). Interestingly, there is evidence supporting an intimate (albeit complicated) bio-behavioral relationship between anxiety-like behavior and aggression (Apter et al., 1990; Fehon et al., 2001; Guillot and Chapouthier, 1996; Veenema and Neumann, 2007; Veenema et al., 2007). For example, in animals selectively bred for high and low anxiety-like behavior (HAB/LAB), anxiety and aggressive behavior are negatively correlated, such that LAB animals reliably display higher levels of aggressive behavior when compared with HAB animals (Veenema et al., 2007). These data support the notion of a specific "gating" mechanism (perhaps neuroanatomical and/or neurochemical) whereby the expression of the "aggressive" brain state precludes the expression of the "anxious" brain state and vice versa, an investigation currently ongoing in our laboratory.

In brain, the AH, CeA, FC, LS, and MeA appear to be sites that influence *both* aggressive behavior (Delville et al., 2000; Nelson and Trainor, 2007) and anxiety (Pratt, 1992). Our finding that adolescent AAS exposure reduces the development the 5HT neural system in several of these brain areas leads us to speculate that perhaps persistent reductions in 5HT signaling in these brain sites and/or others may modulate the expression of anxiety-like behavior during AAS withdrawal. This concept is not novel, as abnormalities in the 5HT neural system (particularly reductions in 5HT tone) have been implicated in the development of anxiety (Collinson and Dawson, 1997; Cowen, 1991; Holmes et al., 2003). Together this body of evidence suggests that reductions in 5HT signaling, perhaps in select areas of the brain that modulate aggression (e.g., the hypothalamus and amygdala), also may modulate the onset of anxiety that occurs during withdrawal from adolescent AAS exposure. Therefore, in the final set of studies, we investigated the behavioral effects of increasing extracellular 5HT in adolescent AAS-treated hamsters 21 days following the final AAS administration to determine whether adolescent AAS withdrawal-induced anxiety was modulated by 5HT activity. Fluoxetine administration resulted in a significant reduction of adolescent AAS withdrawal-induced anxiety in almost all measures of anxiety tested. In fact AAS-treated animals administered fluoxetine displayed a behavioral phenotype nearly identical to vehicle-control animals administered SO in Experiment 1, attesting to the capacity of elevated 5HT to completely block the adolescent AAS withdrawal-induced anxious phenotype. These behavioral pharmacology data together with neuroanatomical data showing a significant decrease in 5HT afferent development to areas of the brain implicated in anxiety support the notion that reductions in 5HT signaling may contribute to increases in anxiety-like behavioral response observed during AAS withdrawal. In accord with this view,

a reduction in 5HT tone acting through select 5HT receptors has been implicated in anxiety (Cowen, 1991). Several studies have shown that reductions in 5HT signaling through 5HT1A receptors can increase anxiety-like response (Collinson and Dawson, 1997; Holmes et al., 2003). In contrast, 5HT1A receptor activation as well as overexpression has been shown to have anxiolytic-like properties (Carli et al., 1989; Collinson and Dawson, 1997; Kusserow et al., 2004). Evidence suggests that the expression of 5HT1A receptors during postnatal development is fundamental for normal anxiety-like behaviors as adults, while changes in 5HT1A receptor expression during adulthood do not have a long term impact, indicating that 5HT1A receptor function in anxiety differs in different stages of development (Gross et al., 2002; Kusserow et al., 2004). Along this line, it is noteworthy that we have previously shown that adolescent hamsters treated with AAS express significantly fewer 5HT1A receptors in areas of the brain implicated in anxiety and aggressive behavior and compromised 5HT signaling through 5HT1A receptors (Ricci et al., 2006), supporting the notion that reduced 5HT signaling through 5HT1A receptors in these brain sites may play a role in adolescent AAS withdrawal-induced anxiety. The ability of acute fluoxetine administration to suppress the AAS withdrawal-induced anxiogenic response may be due to its ability to increase extracellular 5HT to levels sufficient to activate postsynaptic 5HT1A receptors in forebrain regions implicated in anxiety (Sprouse et al., 2001). Together, these findings support data from other laboratories implicating a role for 5HT signaling (perhaps through 5HT1A receptors) in anxiety (Clark and Neumaier, 2001; Critchley and Handley, 1987; File, 1986).

From a translational standpoint, there is considerable evidence supporting the efficacy of several classes of 5HT pharmacotherapeutics for the treatment of anxiety (see White, 2009 for review). In particular, SSRIs such as fluoxetine (Prozac), sertraline (Zoloft), paroxetine (Paxil) and escitalopram (Lexapro) are often recommended as first line agents for the long-term clinical treatment of a range of anxiety disorders, including generalized anxiety, panic, and social anxiety disorders (Ballenger et al., 1998; Blomhoff et al., 2001; Davidson et al., 2004; Dunlop and Davis, 2008; Goodman et al., 2005; Kasper et al., 2005; McIntosh et al., 2004; Michelson et al., 2001; Pohl et al., 1998; Pollack et al., 1998, 2001; Rapaport et al., 2001; Stein et al., 1998). The effectiveness of fluoxetine to suppress AAS withdrawal-induced anxiety in the present study lends preclinical support to the medical use of SSRI-like agents for the clinical management of AAS withdrawal-induced anxiety in the human AAS abusing population.

In summary, these studies provide data that describe the neurobehavioral effects of withdrawal from adolescent AAS exposure on anxiety-like behaviors and the basic neurobiological mechanisms by which these drugs may exert their anxiety-inducing effects. The findings show clearly that adolescent AAS exposure leads to significant increases in anxiety during extended periods of withdrawal. Further, the data show that the increase in anxiety-like response that emerges during withdrawal is modulated by 5HT signaling, perhaps due, in part, to persistent reductions in 5HT afferent development that exist across several areas of the hamster brain implicated in the control of anxiety in adolescent AAS exposed animals. These findings provide a link between withdrawal from early onset AAS exposure, 5HT, and anxiety in hamsters indicating a role for reduced 5HT activity in AAS withdrawal-induced anxiety.

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Fig. 1.

Conceptual model of the overlap in neural circuits regulating both offensive aggression and anxiety, identifying the anterior hypothalamus, central amygdala, frontal cortex, medial amygdala and lateral septum as brain regions modulating both aggression and anxiety. Adapted from Delville et al. (2000) and Nelson and Trainor (2007).

Fig. 2.

Adolescent AAS exposure alters anxiety in the elevated plus maze and dark/light box at 21 days of AAS withdrawal. Elevated plus maze graph — percentage (%) of entries into and time within the open arms of the EPM (top left) and number of entries into the open and closed arms of the EPM (top right) of sesame oil- (white bars) and AAS- (black bars) treated residents. Dark/light box graph — percentage (%) of entries into and time within the white compartment of the DL box (middle left) and latency to enter the white compartments of the DL box (middle right) of sesame oil- (white bars) and AAS- (black bars) treated residents. Inset: resident–intruder graph — number of lateral and upright offensive attacks and latency

to attack of vehicle- (white bars) and AAS- (black bars) treated residents. Bars denote SEM.
** $p < 0.01$.

Fig. 3.

Graph: Density of 5HT-ir varicosities and fibers in brains of sesame oil- (white bars) and AAS- (black bars) treated hamsters at 21 days of AAS withdrawal. Numbers were normalized to a standard area (100 $\mu\text{m} \times 100 \mu\text{m}$) for regional comparisons.

Photomicrographs: Brightfield photomicrographs showing 5HT-ir varicosities and fibers (arrows) in the hamster anterior hypothalamus (AH), central amygdala (CeA) and medial amygdala (MeA) of sesame oil- and AAS-treated hamsters at 21 days of AAS withdrawal. Bars denote SEM. *** $p < 0.001$, ** $p < 0.01$.

%Time (White)

Latency to Enter (White)

Fig. 4.

Fluoxetine treatment decreases anxiety at 21 days of AAS withdrawal in the dark/light test. Percentage (%) of time within the white compartment of the DL box (top) and latency to enter the white compartments of the DL box (bottom) of AAS-treated animals administered saline or fluoxetine (5 mg/kg, 10 mg/kg, or 20 mg/kg) prior to the behavioral test. Bars denote SEM. *** $p < 0.001$, ** $p < 0.01$.