

Research report

# Lasting changes in neuronal activation patterns in select forebrain regions of aggressive, adolescent anabolic/androgenic steroid-treated hamsters

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## Abstract

Repeated exposure to anabolic/androgenic steroids (AAS) during adolescence stimulates high levels of offensive aggression in Syrian hamsters. The current study investigated whether adolescent AAS exposure activated neurons in areas of hamster forebrain implicated in aggressive behavior by examining the expression of FOS, i.e., the protein product of the immediate early gene *c-fos* shown to be a reliably sensitive marker of neuronal activation. Adolescent AAS-treated hamsters and sesame oil-treated littermates were scored for offensive aggression and then sacrificed 1 day later and examined for the number of FOS immunoreactive (FOS-ir) cells in regions of the hamster forebrain important for aggression control. When compared with non-aggressive, oil-treated controls, aggressive AAS-treated hamsters showed persistent increases in the number of FOS-ir cells in select aggression regions, namely the anterior hypothalamus and lateral septum. However, no differences in FOS-ir cells were found in other areas implicated in aggression such as the ventrolateral hypothalamus, bed nucleus of the stria terminalis, central and/or medial amygdala or in non-aggression areas, such as the somatosensory cortex and the suprachiasmatic nucleus. These results suggest that adolescent AAS exposure may constitutively activate neurons in select forebrain areas critical for the regulation of aggression in hamsters. A model for how persistent activation of neurons in one of these brain regions (i.e., the anterior hypothalamus) may facilitate the development of the aggressive phenotype in adolescent-AAS exposed animals is presented.

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**Keywords:** Aggression; Anabolic/androgenic steroids; Adolescence; FOS; Neural activation

## 1. Introduction

Previously, we have used developmentally immature Syrian hamsters (*Mesocricetus auratus*) as an animal model to examine the link between repeated adolescent anabolic/androgenic steroid (AAS) exposure and the behavioral neurobiology of offensive aggression [12,25–28,33,67,68]. 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 characterized by intense bouts of biting and attacking primarily directed towards the flanks, rump and ventrum of the intruder, as well as high amounts of upright offensive postures and lateral attacks toward the intruder. The finding that adolescent AAS-treated hamsters demonstrated highly esca-

lated and mature forms of offensive aggression in the absence of prior social interactions and established dominance cues suggested that adolescent AAS exposure stimulated aggression directly, perhaps by impacting the development and/or activity of select brain regions that regulate this behavior.

In hamsters, the anterior hypothalamus (AH) appears to be at the center of a neural network of reciprocal connections between the lateral septum (LS), medial amygdala (MeA) and ventrolateral hypothalamus (VLH) that regulates offensive aggression [13]. The activity of the entire network is regulated (at least partially) by the activity of centrally released arginine vasopressin (AVP) and serotonin (5HT) within the AH. In this instance, AH AVP activity has been shown to facilitate offensive aggression that is normally inhibited by AH 5HT [19]. The AH appears also to be an important point of convergence for AAS-induced developmental changes in the AVP and 5HT neural systems that correlate with the emergence of the aggressive phenotype. For example, recently we have shown that aggressive,

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adolescent AAS-treated hamsters display significant elevations in AVP afferent fiber density and peptide content in the AH commensurate with deficits in 5HT afferent innervation and alterations in 5HT<sub>1A</sub> and 1B receptor expression in this same brain site as compared to non-aggressive, sesame oil-treated littermates [25,26,33,67]. Further, in mice and hamsters, increased aggressive behavior correlates with higher  $\gamma$ -aminobutyric acid (GABA) activity, levels and density of synaptic terminals containing glutamic acid decarboxylase-65 (i.e., the rate-limiting enzyme in the synthesis of GABA) [28,34,64,70]. Aggressive, adolescent AAS-treated hamsters display significant increases in GAD<sub>65</sub>-containing afferent terminals in several aggression regions, including the anterior hypothalamus [28]. It is possible that, either together or alone, these adolescent AAS-induced neurodevelopmental changes stimulate offensive aggression by permanently altering the activation patterns of neurons in these discrete forebrain regions. To date, however, it is unknown whether adolescent AAS exposure activates neurons in these brain areas important for aggression control.

In the nervous system, activated neurons can be identified by the expression of FOS, the protein product of the proto-oncogene *c-fos* [31,35]. The immunohistochemical localization of FOS expression has proven to be a reliably useful tool for mapping cellular activation resulting from a diverse array of physiologic and behavioral stimuli, including agonistic interactions [7–10,13,23,49,50,69,73,77]. For instance, directly following an aggressive interaction, a significant increase in the number of FOS-containing neurons has been reported in several forebrain areas important for aggression control, including the AH, LS, and MeA [13,24,29,43,62,63]. Although FOS had been originally thought to have been expressed for only short periods (i.e., 15–90 min) following stimulus exposure [53,54], instances of persistent FOS expression representative of constitutive cellular activation have been observed following chronic physiologic and behavioral stimulation [15,50,52]. Given these findings, the present studies were conducted to investigate the hypothesis that repeated adolescent AAS exposure would produce *lasting* increases in neuronal activity in forebrain areas important for aggression control. From a functional standpoint, increases in neuronal activation for extended periods of time in these areas may represent constitutive activation of the neural network implicated in the aggressive response, heightening the aggressive response patterns of these animals. To determine whether adolescent AAS exposed animals possessed *persistent* alterations in neuronal activation patterns in areas of the hamster forebrain important for aggression control we quantified FOS-expressing cells in these brain sites 1 day following the behavioral test to assess aggressive responding.

## 2. Methods

### 2.1. Animals

Pre-pubertal (P21–23) male hamsters were obtained from Harlan Sprague-Dawley Labs (Indianapolis, IN), individually housed in polycarbonate 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 Harlan Sprague-Dawley 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-level social interest aggression (i.e., Disengage and Evade) and environmental fear responses (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 [32,65,66]. Intruders displaying significantly low aggression and/or submissive postures (<5%) were excluded from use in the behavioral assay. All methods and procedures described below were pre-approved by the Northeastern University Institutional Animal Care and Use Committee (NU-IACUC).

### 2.2. Experimental treatment

Adolescent Syrian hamsters (P27) were weighed and randomly distributed into two groups ( $n > 5$  animals/group). One group of animals ( $n = 6$ ) received daily subcutaneous (SC) injections (0.1–0.2 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 [33]. This treatment regime, designed to mimic a chronic use regimen [58,59], has been shown repeatedly to produce highly aggressive animals in greater than 90% of the treatment pool [12,25,26,28,33]. The second group of hamsters ( $n = 5$ ) received SC injections of sesame oil alone. The day following the last injection (P58), animals were tested for offensive aggression using the resident-intruder paradigm, sacrificed 24 h later (i.e., on P59), and the brains removed and processed for FOS immunohistochemistry as detailed below.

### 2.3. Aggression testing

Animals were tested for aggressive behavior using the resident/intruder paradigm, a well-characterized and ethologically valid model of offensive aggression in golden hamsters [22,46]. For this measure, a stimulus (intruder) male of similar size and weight was introduced into the home cage of experimental animals and the resident was scored for general measures of offensive aggression (i.e., number of attacks and latency to first attack towards an intruder) as previously described [65,66]. Briefly, an attack was scored each time the resident animal would wildly pursue 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 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 of the residents towards an intruder. In the case of no attacks, 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 independent observer uninformed as to the experimental treatment. No stimulus animal 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.

### 2.4. Immunohistochemistry

One day following the behavioral test for aggression, AAS and sesame oil-treated hamsters ( $n = 6$  and 5, respectively) were anesthetized with 80 mg/kg ketamine and 12 mg/kg xylazine and the brains fixed by transcardial perfusion with 4% paraformaldehyde (50 wt/50 wt). Brains were then cryoprotected in 30% sucrose in phosphate buffered saline (1 × PBS; 0.001 M KH<sub>2</sub>PO<sub>4</sub>, 0.01 M Na<sub>2</sub>HPO<sub>4</sub>, 0.137 M NaCl, 0.003 M KCl, pH 7.4) overnight at 4 °C. A consecutive series of 35  $\mu$ m coronal brain sections from experimental and control animals were cut on a freezing rotary microtome, collected as free floating sections in 1 × PBS, and then every third section was labeled for FOS in one standardized immunohistochemical run using a modification of an existing protocol [11]. Briefly, sections were pretreated with 0.3% hydrogen peroxide for 30 min, washed in PBSTx (1 × PBS/0.5% Triton X-100), and pre-incubated in 5% normal goat serum (NGS) in PBSTx for 90 min. Sections were then incubated in rabbit antiserum (1:2000) generated against FOS (Oncogene Sciences) in 5% NGS/PBSTx for two nights at 4 °C. After primary incubation, sections were incubated in secondary anti-rabbit followed by avidin–biotin

complex (Vectastain ABC Elite Kit-rabbit, Burlingame, CA) and then labeled with diaminobenzidine (DAB, Vector Labs, Burlingame, CA). Sections were mounted on gelatin-coated slides, allowed to air dry, and dehydrated through a series of ethanol and xylene solutions. Then, slides were coverslipped using cyto seal-60 mounting medium (VWR Scientific, West Chester, PA, USA). Representative slides with omission of primary and/or secondary antibodies were processed as above for control purposes.

## 2.5. Image analysis

The number of FOS immunoreactive (FOS-ir) cells was determined within specific brain areas using the BIOQUANT NOVA 5.0 computer-assisted microscopic image analysis software package as previously described [11,26,65]. The areas analyzed were selected based on data from previous studies implicating these regions in aggressive responding across numerous species and models of aggression, with the notable exception of the S1 somatosensory neocortex (S1) and suprachiasmatic nucleus (SCN) included in the assay as non-aggression areas used for control purposes. The specific aggression areas examined included the anterior hypothalamus (AH), the medial and lateral divisions of the bed nucleus of the stria terminalis (BNST), the central amygdaloid nucleus (CeA), the dorsal, intermediate, and ventral parts of the lateral septal nucleus (LS), the medial amygdaloid nucleus (MeA), the medial supraoptic nucleus (mSON), and the ventrolateral hypothalamus (VLH) (Fig. 1). Slides from each animal were coded by an experimenter unaware of the experimental conditions and BIOQUANT NOVA 5.0 image analysis software running on a Pentium III CSI Open PC computer (R&M Biometrics, Nashville, TN, USA) was utilized to identify the brain Region Of Interest (ROI). Specifically, with the aid of The Hamster Atlas [55], a standard computer-generated parcel was drawn to outline the entire ROI at low power (4 $\times$ ) using a Nikon E600 microscope. Each brain region was assigned a separate and distinct ROI parcel, formatted in size specifically for that brain area, with the notable exception of the S1 cortex control region where placement of a size appropriate parcel was not feasible. In cases where the delin-

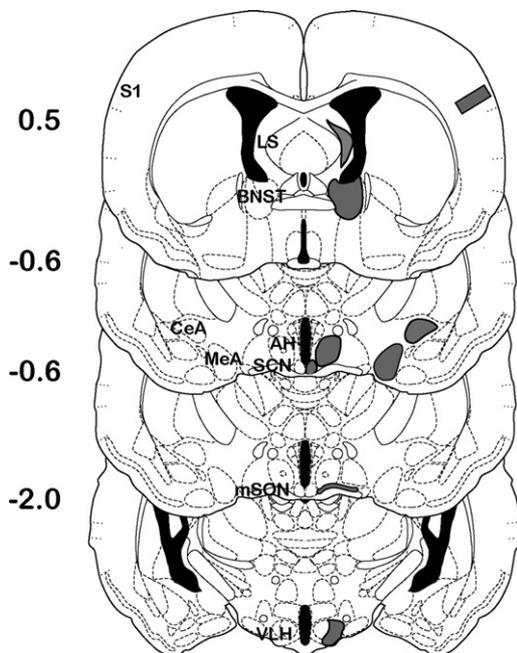


Fig. 1. Diagram showing the location of the areas selected to quantify FOS-containing cells (shaded areas). Plates were modified from hamster atlas of Morin and Wood [55] and reflect specific positions in the rostral-caudal plane (i.e., distance in millimeters from bregma to the plane of section at the skull surface). Abbreviations: 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; mSON, medial division of the supraoptic nucleus; VLH, ventrolateral hypothalamus; S1, somatosensory neocortex; SCN, suprachiasmatic nucleus.

ation of ROI boundaries was questionable, extreme care was taken to localize nuclear compartments by measuring distances to standard neuronal landmarks including white matter fiber tracts, ventricular compartments and cortical borders. Then, under 10 $\times$  magnification images were assigned a threshold value at a standard RGB-scale level empirically determined by observers unaware of the treatment conditions, such as to allow detection of stained FOS-ir cells 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. FOS-ir cells were identified in each field using a mouse driven cursor and then FOS-ir counts were performed automatically by the BIOQUANT software. Measurements at 10 $\times$  continued until FOS-ir elements throughout the entire ROI were quantified. Two to six independent measurements were taken from several consecutive sections ( $n=2-4$ ) of each animal ( $n=5-6$ ) per treatment group. Then, the number of FOS-ir cells was determined for each ROI, averaged for each brain region per animal and used for statistical analysis.

## 2.6. Statistics

### 2.6.1. Behavioral studies

The results from the aggression tests were compared between AAS- and sesame oil-treatment groups. Nonparametric data (total number of attacks) was compared by Mann-Whitney  $U$ -tests (two-tailed). Parametric data (latency to first attack) was compared by Student's  $t$ -test (two-tailed).

### 2.6.2. FOS immunoreactivity

The number of FOS-ir cells was compared between treatment groups by Student's  $t$ -test (two-tailed) for each area analyzed.

## 3. Results

### 3.1. Aggressive behavior

As characterized extensively in our previous studies [12,25,26,28,33], adolescent AAS-treated animals showed significantly elevated offensive aggression (Fig. 2). Here, we show that hamsters treated with AAS showed a significant increase in the number of attacks ( $Z=2.39$ ,  $p<0.05$ ) directed toward the intruder. The majority of the AAS-treated animals (five out of six) scored greater than 10 attacks during the aggression test.

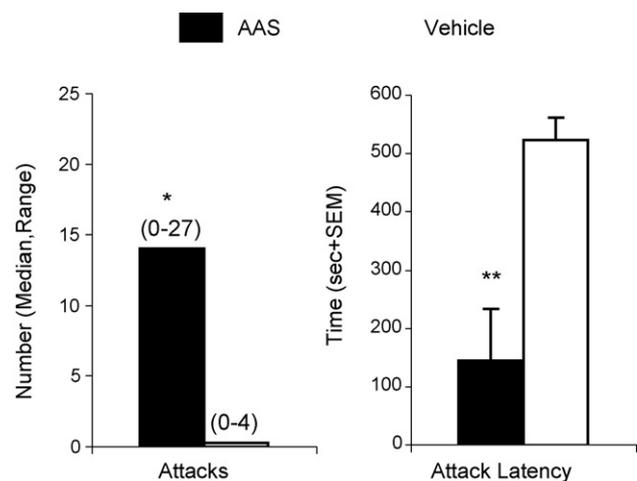


Fig. 2. Adolescent AAS treatment increases offensive aggression. Number of offensive attacks and latency to first attack in AAS- and sesame oil-treated residents. Bars denote S.E.M., \* $p<0.05$ ; \*\* $p<0.01$ ; Mann-Whitney, two-tailed (number of attacks), Student's  $t$ -test, two-tailed (attack latency).

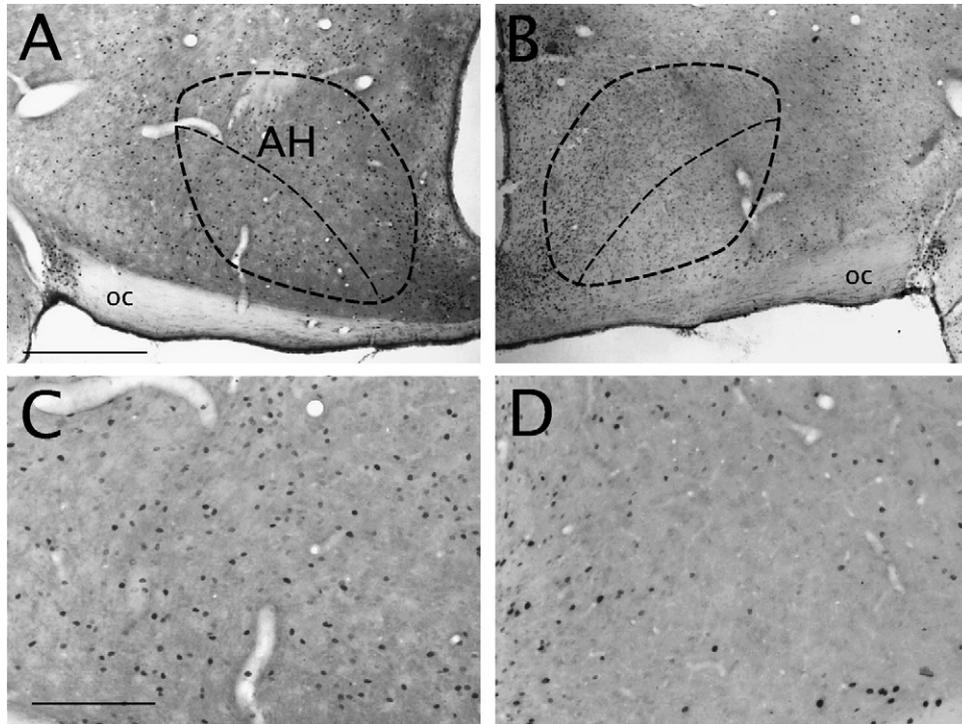


Fig. 3. Brightfield photomicrographs of a coronal section through the Syrian hamster hypothalamus. Shown are FOS-containing cells (arrows) within the anterior hypothalamus (encircled) of (A and C) AAS-treated and (B and D) sesame oil-treated hamsters. High power photomicrographs in (C) and (D) represent FOS immunostaining in the ventrolateral aspects of the anterior hypothalamus delineated below the center dashed line in (A) and (B), i.e., the latero-anterior hypothalamic brain region. oc, optic chiasm; 3V, 3rd ventricle.

By comparison most of sesame oil-treated hamsters (four out of five) scored a single attack or less on opponents. In addition, AAS-treated hamsters displayed significantly decreased attack latencies toward intruders [ $t(10)=3.4$ ,  $p<0.01$ ], than vehicle-treated control animals. All but one AAS-treated animal attacked within the first 2 min of the 10 min test in comparison to sesame oil-treated control animals whose first recorded attack averaged nearly 5 min later, toward the end of the test period.

### 3.2. FOS immunohistochemistry

Adolescent AAS treatment altered the immunohistochemical expression of FOS-containing cells in the areas of hamster brain involved in aggression control when compared with oil-treated controls. Specifically, in aggressive, AAS-treated hamsters, the immunohistochemical staining pattern of FOS-containing cells was altered in two brain areas important for aggressive behavior, the hypothalamus and the lateral septum. AAS-treated hamsters exhibited an increased number of FOS positive cells in the AH proper when compared to sesame oil-treated controls (Fig. 3), which displayed a less dense pattern of staining indicative of the normal distribution of FOS positive cells in this brain region. Quantitative analysis of FOS-ir cells in the AH showed that steroid-treated animals had a two-fold increase in FOS positive cells when compared to oil-treated littermates (Fig. 4). This difference was statistically significant [AH,  $t(9)=3.59$ ,  $p<0.01$ ]. Similar results were observed in the LS where aggressive, adolescent AAS-treated animals had nearly three times the number of FOS positive cells than

oil-treated littermates (Fig. 4). This difference was also statistically significant [LS,  $t(8)=4.96$ ,  $p<0.01$ ]. Interestingly, aggressive, AAS-treated animals did not show an increase in FOS positive cells compared to non-aggressive, oil-treated littermates in several other brain regions implicated in the control of offensive aggression in hamster, most notably the, BNST, CeA, MeA, mSON, and VLH [BNST,  $t(9)=0.1073$ ,  $p>0.05$ ; CeA,  $t(9)=1.23$ ,  $p>0.05$ ; MeA,  $t(9)=0.4437$ ,  $p>0.05$ ; mSON,  $t(8)=0.7915$ ; VLH,  $t(9)=0.7664$ ,  $p>0.05$ ]. Further, no FOS positive cells were found in the NC (i.e., a small neuropeptide-containing nucleus at the center of the AH implicated in the regulation of offensive aggression in hamsters) in either AAS- or sesame oil-treatment groups. In addition, no significant differences were found between treatment groups in the S1

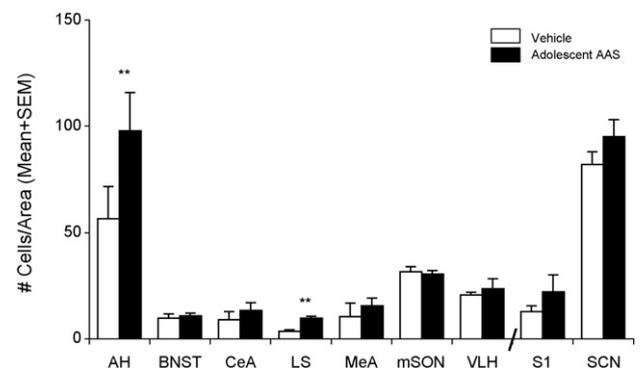


Fig. 4. Number of FOS-immunoreactive cells in select brain regions of AAS- vs. sesame oil-treated hamsters, \*\*  $p<0.01$ ; Student's  $t$ -test, two-tailed.

cortex ( $t(9) = 1.309$ ,  $p > 0.05$ ) or SCN ( $t(7) = 1.666$ ,  $p > 0.05$ ) (i.e., brain areas not involved in aggressive behavior in the hamster).

#### 4. Discussion

As we have previously reported in a number of studies, hamsters treated with AAS during adolescence develop high levels of offensive responding when compared to sesame oil-treated littermates (see Fig. 2 and Refs. [12,25–28,33,67,68]). Compared to non-aggressive, sesame oil-treated controls, aggressive, adolescent AAS-treated residents showed significant increases in the number of FOS-containing neurons in two brain regions implicated in aggression control, namely the AH and LS. These data indicate that a significant number of neurons in these brain sites display *lasting* increases in the expression of FOS following adolescent AAS exposure. Relatively recent studies have shown that persistent FOS expression is representative of constitutive cellular activation that occurs in response to a variety of factors, including chronic physiologic [15,52] and behavioral [15,50] stimuli. Thus, from a functional standpoint, the lasting increase in neuronal activation observed in areas of the hamster brain important for aggressive control (as indicated by an increased number of FOS-containing cells in these regions) in adolescent AAS-treated animals may represent constitutive stimulation of the neural circuit(s) implicated in the aggressive response. The finding that FOS-containing neurons are increased in select regions of the brain following AAS exposure is, in general, not unique. For instance, both acute and chronic exposure to a variety of AAS have been shown to increase FOS expres-

sion in nuclei of the basal forebrain, hypothalamus, amygdala, and brainstem of adult rats [72], guinea pigs [37], and hamsters [14]. However, the finding that *lasting* increases in the number of FOS-expressing neurons occur in very select regions of the hypothalamus and forebrain in *adolescent AAS-treated hamsters* is novel, and suggest a direct activational mechanism underlying the escalated levels of offensive aggression displayed in these animals during the behavioral test. Recently, we have found similar results in another adolescent model linking developmental drug abuse and the neurobiology of aggression [41]. In this study, aggressive adolescent cocaine-treated hamsters showed persistent increases in the number of FOS-ir cells in a number of aggression regions, including the anterior hypothalamus, nucleus circularis, lateral hypothalamus (i.e., the hypothalamic attack area), lateral septum, and medial and corticomedial amygdaloid nuclei. While not the same results, these data sets do overlap in that persistent neuronal activation is observed in the AH and LS in both models of adolescent drug abuse and aggression.

As in the above model, we hypothesize that the sustained activation of neurons in the AH of adolescent AAS-treated animals may function to activate aggressive responding. Indeed, the AH is believed to be at the center of a neural network regulating offensive aggression in hamsters [13]. In this brain region two distinct neural systems, i.e., arginine vasopressin (AVP) and serotonin (5HT), have been identified as important neurochemical signals regulating the state of the aggressive phenotype [17,19,20]. AH AVP activity has been implicated in the facilitation of aggression, while AH 5HT activity inhibits aggressive behavior (Fig. 5, first panel for model) [17,19]. In

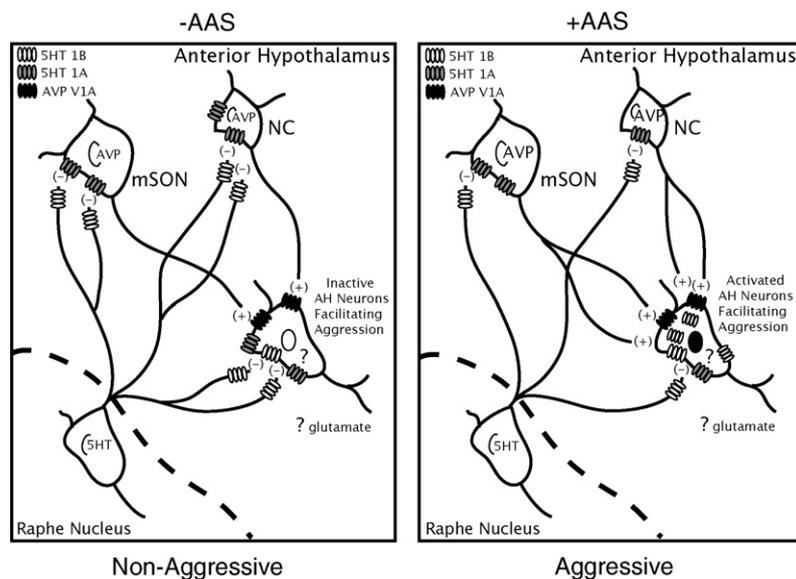


Fig. 5. A model showing the hypothetical relationship between adolescent AAS-induced neuronal activation and the serotonin (5HT) with the arginine vasopressin (AVP) systems in the AH (adapted from Ferris et al. [19]). In the first panel (-AAS) a dense plexus of 5HT afferent fibers originating from neurons in the raphe nucleus innervate AVP neurons localized to the medial supraoptic nucleus (mSON) and nucleus circularis (NC), i.e., vasopressinergic neurons identified as potential sources of AVP innervation to the AH involved in agonistic behavior. These AVP neurons together with 5HT neurons from the raphe nucleus regulate the activity of AH neurons involved in the facilitation of aggression. The identity (?) of these post-synaptic AH neurons is unknown but two potential candidates are glutamate and norepinephrine. 5HT is inhibitory (-), working through a 5HT1A/B receptors, whereas AVP is excitatory (+), working through a V1A receptor. In the second panel (+AAS), adolescent AAS exposure markedly enhances AVP and reduces 5HT afferent innervation to the AH and alters the expression of 5HT1A and 1B receptors throughout this brain region (see Refs. [25,26,33,67]), constitutively dis-inhibiting (i.e., activating) non-AVP neurons (? glutaminergic—see Ref. [21]) in the AH facilitating aggression. Neuronal activation in this model is represented as dark nuclei indicating persistent FOS expression.

previous studies, we have shown that adolescent AAS-induced offensive aggression is modulated by AH AVP activity and correlated with enhanced AVP afferent fiber development – and AVP peptide levels – in the AH than non-aggressive, sesame-oil treated controls [27,33], prompting our hypothesis that heightened AH AVP activity is a critical element for adolescent AAS-induced aggression. The primary source of AVP afferent innervation to the AH appears to be magnocellular neurons located in the nucleus circularis (NC) and the medial supraoptic nucleus (mSON) [16,18,47]. A persistent increase in the activation of neurons in these brain sites following adolescent AAS exposure could have explained the augmented aggressive response patterns observed in these animals, yet neither of these brain regions show any appreciable alterations in neuronal activation following AAS treatment. Rather, increases in FOS expressing neurons were found only in non-AVP containing neural sites in the AH of aggressive, adolescent AAS-treated animals. Therefore, it is possible that constitutive activation of AVPergic neurons is not required for the enhanced aggressive phenotype observed in adolescent AAS-treated animals.

One putative mechanism that might explain the increase in number of FOS-containing cells in non-AVP containing regions of the AH in aggressive, adolescent AAS-treated animals can be gleaned from findings regarding the 5HTergic regulation of AH neurons controlling the aggressive response. A growing body of neurobiological and behavioral data indicates that 5HT inhibits aggression by suppressing the activity of both AVP- and non-AVP-containing neurons in the AH that facilitate aggression. Anatomical studies reveal a dense 5HT afferent innervation originating from neurons in the raphe nucleus onto AVP- and non-AVP neurons in the AH [19,20,26]. Functional studies reveal pharmacologic treatments that increase AH 5HT or activate 5HT1A and 1B receptors reverse aggression resulting from adolescent AAS exposure [25,26,67]. Might a loss of 5HT inhibition onto non-AVP containing neurons in the AH normally facilitate aggression explain the persistent activation of cells in these brain regions? Recently, we have shown a dramatic reduction in 5HT-containing afferents to the AH of aggressive, adolescent AAS-treated hamsters [26], indicating a reduced 5HT tone in this brain region in AAS-exposed animals. Aggressive, adolescent AAS-treated hamsters also express fewer post-synaptic 5HT1A-receptor [67] and pre-synaptic 5HT1B-receptor [25] in the AH, while post-synaptic 5HT1B receptor expression appears to be up-regulated in neurons located in a select subregion of AH [25], namely the hamster equivalent of the latero-anterior hypothalamic nucleus—the ventrolateral portion of the AH located between the two AVP containing compartments of the AH (i.e., the mSON and NC). Interestingly, we recently found a dramatic increase in the expression of phosphate-activated glutaminase (PAG), i.e., a key enzyme in the production of glutamate, in neurons located in this very same brain region in aggressive, adolescent AAS-treated animals [21]. PAG expression has been used in a number of previous studies to visualize and quantify glutamate-transmitting neurons [1,38–40,48,75]. Glutamate has been firmly established as the predominant excitatory transmitter in the hypothalamus

[5,51,74], and it is activity has been extensively linked to aggression in a range of animal models, including rats, mice, cats, and fighting bulls [6,30,36,56,57,71,76], where it appears to be positively associated with the aggressive behavioral phenotype. In preliminary studies, we have shown that PAG containing neurons in the AH express 5HT1B receptors (data not shown). Thus, in the case of aggressive, adolescent AAS-treated animals, the blunted 5HT development/tone that exists in the AH as a result of AAS exposure might chronically activate downstream glutamate-containing neurons in this brain region that facilitate the aggressive response (Fig. 5, second panel). This hypothesis aside, there are other neural systems previously shown to be affected by AAS exposure that may serve the same aggression-facilitating effect. For instance, rats administered 10- to 100-fold the therapeutic dose of AAS show significantly increased levels of norepinephrine (NE) and it is metabolite in the hypothalamus [72]. These animals also showed higher numbers of FOS-ir neurons in this brain region. Since, NE has also been linked to the facilitation of aggression, it is also possible that the blunted 5HT development/tone that exists in the AH as a result of AAS exposure might chronically activate downstream NE-containing neurons in this brain region that facilitate the aggressive response. This hypothesis is currently under investigation in the laboratory.

These data notwithstanding, there are findings from the current study that are inconsistent with this general hypothesis. For example, in this study we show lasting activation of neurons in the LS in aggressive, adolescent AAS-treated animals. However, in this brain region, no significant changes in 5HT afferent innervation and/or 5HT1A or 1B receptor expression have been observed in aggressive, AAS-treated hamsters [25,26,67], nor are there any appreciable changes in the expression of PAG in the LS of these animals [21]. Further, aggressive responding has been shown to be inhibited by LS activity in hamsters [60,61], suggesting that activation of neurons in this brain site may serve to suppress aggressive behavior. While it is true that the LS has previously been reported to be active following bouts of fighting in hamsters [43,45], this brain region has not been specifically characterized as one critical for the regulation of offensive aggression in hamsters. For example, in hamsters, activation of the LS has been implicated in defensive aggression, mating and scent marking [2,3,42–44]. Therefore, activity in these brain regions may reflect gross alterations in social behavior and responsiveness and have less of an effect on *offensive aggression*, remaining consistent with the central hypothesis regarding the relationship between site specific neural activation and the AH and the development of the offensive aggressive phenotype. Taken together, these data above are novel and significant in that they show that repeated exposure to AAS during adolescent development can have *lasting* effects on neuronal activation in the AH, i.e., the area of the brain that has been implicated in aggression facilitation in hamsters. From a neurobiological standpoint, these data support increased neural signaling in this key area as a potential neural substrate for adolescent AAS-induced offensive aggression. Further, the stable nature of neural activation across several other “aggression” and “non-aggression” regions suggest that there is a non-uniform effect

of adolescent AAS-treatment on neuronal activation across the neuraxis.

In summary, the neural mechanisms underlying the elicitation of AAS-induced offensive aggression appears to be complicated and likely involves a number of neurotransmitter systems. That notwithstanding, the studies presented in this paper provide data regarding the neurobiological effects of repeated AAS exposure during adolescent development and propose a basic neurobiological mechanism by which these agents may exert their aggression-stimulating effects. These findings indicate that repeated AAS exposure during adolescent development increases the activation of neurons in few select areas of hamster brain implicated in the control of offensive aggression, with specific emphasis placed on changes observed in the AH. These findings, together with those from our laboratory indicating that the 5HT, AVP and glutamate neural systems in the AH are important in adolescent AAS-induced offensive aggression in hamsters, are synthesized to provide a putative neural mechanism explaining how adolescent AAS exposure might facilitate aggressive responding. Further studies are needed to elucidate whether the aggression-stimulating effects of AAS exposure do indeed occur as a direct result of AAS influence on the signaling between these systems or whether the changes in neural activation observed here were representative of activational changes in other endogenous neural systems.

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