REPEATED ANABOLIC/ANDROGENIC STEROID EXPOSURE DURING ADOLESCENCE ALTERS PHOSPHATE-ACTIVATED GLUTAMINASE AND GLUTAMATE RECEPTOR 1 SUBUNIT IMMUNOREACTIVITY IN HAMSTER BRAIN: CORRELATION WITH OFFENSIVE AGGRESSION

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Abstract

Male Syrian hamsters (*Mesocricetus auratus*) treated with moderately high doses (5.0mg/kg/day) of anabolic/androgenic steroids (AAS) during adolescence (P27–P56) display highly escalated offensive aggression. The current study examined whether adolescent AAS-exposure influenced the immunohistochemical localization of phosphate-activated glutaminase (PAG), the rate-limiting enzyme in the synthesis of glutamate, a fast-acting neurotransmitter implicated in the modulation of aggression in various species and models of aggression, as well as glutamate receptor 1 subunit (GluR1). Hamsters were administered AAS during adolescence, scored for offensive aggression using the resident-intruder paradigm, and then examined for changes in PAG and GluR1 immunoreactivity in areas of the brain implicated in aggression control. When compared with sesame oil-treated control animals, aggressive AAS-treated hamsters displayed a significant increase in the number of PAG- and area density of GluR1- containing neurons in several notable aggression regions, although the differential pattern of expression did not appear to overlap across brain regions. Together, these results suggest that altered glutamate synthesis and GluR1 receptor expression in specific aggression areas may be involved in adolescent AAS-induced offensive aggression.

INTRODUCTION

In a number of previous studies, we have used developmentally immature Syrian hamsters (*Mesocricetus auratus*) as an adolescent animal model to examine the link between developmental anabolic/androgenic steroid (AAS) exposure and the behavioral neurobiology of offensive aggression. Behavioral data from these studies indicated that animals repeatedly exposed AAS during adolescent development display highly elevated levels of offensive aggression when tested immediately following the exposure period on the first behavioral interaction [11,20–23,25,31,46,47,59,60]. The finding that adolescent AAS-treated hamsters demonstrated highly escalated offensive aggression in the absence of prior social interactions

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and dominance cues suggested that adolescent exposure to AAS stimulated aggression directly, perhaps by affecting the development and/or activity of neural circuits that regulate this behavior.

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 [12]. In a number of prior studies, we have shown that adolescent AAS exposure produces dramatic alterations in the development and activity of several neurochemical systems in these brain regions, suggesting that hypothalamic and amygdaloid nuclei may be an important point of convergence for developmental changes that underlie the generation of the AAS-induced aggressive phenotype [11,20–23,25,31,46,47,59,60]. While compelling, this data set is limited in that it only considers the role of very few and select neurotransmitter systems implicated in the aggressive response in adolescent AAS-induced aggression. It is probable that other neurotransmitter systems implicated in this behavioral response are influenced by chronic adolescent AAS exposure and contribute to the AAS-induced aggressive phenotype.

Glutamate has been firmly established as the predominant excitatory transmitter in the hypothalamus [4,45,70], however as expected glutamate is widely distributed through the CNS [7]; for review see [75]. Neuronal glutamate is produced in brain by the enzyme phosphate-activated glutaminase (PAG) [29] and the expression of this enzyme has been used in a number of previous studies to visualize and quantify glutamate-transmitting neurons in the CNS [1, 33–35,44,71]. Glutamate activity has been localized to various brain regions in rat, including the hypothalamus, lateral septum, bed nucleus of the stria terminalis, and medial amygdala [8,15,28,32,36,68,69], i.e., areas of the brain implicated in aggressive behavior in hamsters [6,12–14,16,30,38,56,57,65]. Indeed, glutamate activity has been extensively linked to aggression in a range of animal models, where it appears to be positively associated with the aggressive behavioral phenotype. For instance, in rats, the "hypothalamic attack area" (HAA) has been shown to possess dense glutamatergic activity [32], and infusion of L-glutamate itself into a similar region of the cat hypothalamus activates an aggressive response [5]. Interestingly, genetically aggressive species, such as fighting bulls also show increased ratios of the excitatory amino acids (including glutamate and aspartate) to their inhibitory counterparts, such as GABA and glycine, in the fastigial nucleus of the cerebellum, the anterior and posterior collicui, and the pons, as compared to non-aggressive breeds [52,53].

The excitatory nature of the glutamate neural system on aggression has been linked to the action of glutamate at receptors representing each of the fast-acting ionotropic glutamate receptor families, i.e., N-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA), and kainate (KA) glutamate receptors. For instance, in rats co-infusion of a glutamate KA receptor agonist and a GABA A subtype receptor antagonist into the HAA has been shown to elicit attack behavior in experienced animals [27], while peripheral administration of NMDA receptor antagonists blocks morphine withdrawal-induced aggression in mice [66,67]. Of the AMPA family of glutamate receptors, knock-out mice lacking the glutamate receptor 1 subunit (GluR1) show vastly reduced aggressive behavior in a variety of agonistic paradigms, including the resident-intruder paradigm [72], indicating that signaling through glutamate receptors expressing this subunit may play an important role in aggression facilitation. GluR1 mRNA expression has been localized throughout hypothalamic and limbic brain areas, including the anterior, medial, and ventrolateral hypothalamic nuclei, lateral septum, bed nucleus of the terminal stria, and the amygdaloid nuclei [62,69], consistent with a putative role in aggressive behavior. In addition, GluR1-containing receptors co-localize within androgen receptor-expressing neurons in the hypothalamus and limbic system, and hypothalamic GluR1 protein levels are reduced castrated rats compared to intact and testosterone treated castrated rats [15]. Thus, of all the ionotropic glutamate receptors, those

expressing the GluR1 subunit present as an ideal initial candidates for the investigation of the relationship between glutamate receptor expression and adolescent AAS-induced aggression. It is possible that AAS exposure during adolescent development escalates aggressive responding by altering glutamate and/or GluR1 activity in brain areas important for aggression control. To date however, it is unknown whether adolescent AAS exposure has <u>any</u> effects on the development and/or activity of the glutamate neural system.

Given this documented association between the glutaminergic neural system, aggression and androgens, the present study was conducted to establish a link between the expression of Glu- and GluR1- containing neurons and adolescent AAS-induced offensive aggression using the sub-adult Syrian hamster as an animal model. In Syrian hamsters the adolescent period of development can be identified as the time between postnatal days 27 and 60 (P27–P60). Weaning generally occurs between P23–P25 with the onset of puberty beginning around one week later [48]. During this developmental time period, hamsters wean from their dams, leave the nest, establish new solitary nest sites, participate in social relationships, and learn to defend their territory using offensive aggression [63,76]. To establish whether adolescent AAS exposure altered glutaminergic activity in various brain regions implicated in the control of offensive aggression, we employed immunohistochemistry utilizing an antibody specific against PAG and the GluR1 receptor subunit to visualize and quantify Glu- and GluR1- containing neurons.

METHODS

Animals

Prepubertal male Syrian hamsters (P21–P23) 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 of (14L: 10D; lights off at 07:00). Food and water were provided *ad libitium.* For aggression testing, stimulus (intruder) males of equal size and weight to the experimental animals were obtained from Charles River one week prior to the behavioral test, group housed at five animals per cage in large polycarbonate cages, and maintained as above to acclimate to the animal facility. All intruders were prescreened for low-level social interest and avoidance (i.e., Disengage and Evade) and submission (i.e., Tail-up Freeze, Flee, and Fly-away) one day prior to the aggression test to control for behavioral differences between stimulus animals. Stimulus animals displaying no social interest and/or submissive postures were excluded from use in the behavioral assay. All studies using live animals were produced with approval by The Animal Care and Use Committee at Northeastern University and all methods used were consistent with guidelines provided by the National Institute of Health for the scientific treatment of animals.

Experimental Treatment

Adolescent Syrian hamsters (P27) were weighed and randomly distributed into two groups (n = 10 animals/group). One group of animals received daily subcutaneous (SC) injections (0.1ml – 0.2ml) of an AAS mixture consisting of 2mg/kg testosterone cypionate, 2mg/kg nortestosterone, and 1mg/kg dihydroxytestosterone undecylate (Steraloids Inc., Newport, R.I.), for 30 consecutive days (P27–P56) as previously described [31]. This treatment regime, designed to mimic a chronic use regimen [54,55], has been shown repeatedly to produce highly aggressive animals in greater than 90% of the treatment pool [11,20,21,25,31]. The second group of hamsters 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 hours later (i.e., on P59), and the brains removed and processed for immunohistochemistry for PAG and GluR1 receptors as detailed below.

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 Syrian hamsters [18,43]. Briefly, an intruder of similar size and weight was introduced into the home cage of the experimental animal (resident) and the resident was scored for specific and targeted aggressive responses including lateral attacks and flank bites as described [25]. An attack was scored each time the resident animal would pursue and then either: (1) lunge toward and/or (2) confine the intruder by upright and sideways threat; each generally followed by a direct attempt to bite the intruder's dorsal and/or flank target area(s). The latency to attack and bite was defined as the period of time between the beginning of the behavioral test and the first attack and bite of the residents toward an intruder. In the case of no attacks and/or bites, latencies to attack and bite were assigned the maximum latency (i.e., 600s). In addition, residents were measured for social interest toward intruders (i.e., contact time between resident and intruder) to control for nonspecific effects of AAS treatment. Contact time was defined as the period of time during which the resident deliberately initiated contact with the intruder either through olfactory investigation (i.e., sniffing) or aggression. Each aggression test lasted for 10 min and was videotaped and coded by two observers unaware of the hamsters' experimental treatment. No intruder was used for more than one behavioral test to control for the effects of repeated exposure to conspecifics on the behavior of intruders, and all animals were tested during the first four hours of the dark cycle under dim-red illumination to control for circadian influences on behavioral responding.

Immunohistochemistry

One day following the behavioral test for aggression, AAS and sesame oil-treated hamsters were anesthetized with 80mg/kg Ketamine and 12mg/kg Xylazine and the brains fixed by transcardial perfusion with 4% paraformaldehyde. Brains were removed, post-fixed for 90 mins in perfusion fixative, then cryoprotected in 30% sucrose in phosphate buffered saline (1xPBS; 0.001M KH_2PO_4, 0.01M Na_2HPO_4, 0.137M NaCl, 0.003M KCl, pH 7.4) overnight at 4°C. A consecutive series of 35 μm coronal brain sections from experimental and control animals were cut on a freezing rotary microtome, collected as free floating sections in 1x PBS, and then alternating sections were labeled for PAG or GluR1 in one standardized immunohistochemical run specific modifications of an existing protocol [10]. For PAG immunohistochemistry, sections were washed 3×5 mins in 0.1M PBS, then incubated in antibody buffer comprised of 10% normal goat serum (NGS) in 0.3% PBSTx (1xPBS/0.5% Triton X-100) for 90 mins. Primary antibody (a monoclonal mouse anti-PAG serum, courtesy of T. Kaneko, Kyoto University, Kyoto, Japan) was prepared in antibody buffer diluted to a final concentration of 1:5000 and incubation with free-floating brain sections was carried out overnight at 33°C. The specificity of this antibody has been well established [34] and the antiserum has been used to identify and study neuronal glutamate-containing neurons in the CNS in a number of studies by other laboratories [32,43,70]. Sections were then rinsed 3×5 minutes with 0.3% PBSTx, incubated for 90 minutes in biotinylated secondary horse anti-mouse IGg (Vector Laboratories, Burlingame, CA.) in 0.3% PBSTx then rinsed again 3×5 minutes in 0.3% PBSTx and incubated for 90 mins in Avidin-biotin-complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA.) in 0.3% PBSTx. 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.). Omissions of primary and secondary antibodies were performed as specificity controls during the procedure. GluR1 immunohistochemistry was largely similar to that used for PAG staining. Briefly, sections were washed 3×5 minutes in 0.1M PBS followed by 5% hydrogen peroxide for 10 mins, rinsed 3×10 mins with 0.3% PBSTx, and then incubated in antibody buffer comprised of 10% NGS in PBS for 1 hr. Primary GluR1 antibody (Chemicon, Temecula, CA) was prepared in antibody buffer diluted to final concentration of 1:1000 and free-floating brain sections were incubated

overnight at 21°C on a rotation wheel. Subsequently, sections were incubated for 2 hrs in biotinylated secondary goat anti-rabbit IGg (Vector Laboratories, Burlingame, CA.) in PBS with 10% NGS, rinsed again 3 × 5 minutes in PBS and incubated for 1 hr in Avidin-biotin-complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA.) in PBS. The peroxidase reaction was revealed as above. As with PAG immunochemistry, omissions of primary and secondary antibodies were performed during the procedure as controls.

Image Analysis

The number of PAG immunoreactive cell bodies was determined within specific brain areas using the BIOQUANT NOVA 5.0 computer-assisted microscopic image analysis software package as previously described [10,21,58]. The areas analyzed were selected based on data from previous studies implicating these regions as important for the regulation of offensive aggression, with the notable exception of the S1 somatosensory neocortex (S1) included in the assay as a non-aggression area used for control purposes. The specific aggression areas examined included the anterior hypothalamus (AH) with specific focus on a distinct cellular compartment within the AH, namely the latero-anterior hypothalamic (LAH) brain region (i.e., the brain area in the AH just ventro-lateral to the nucleus circularis and dorso-medial to the medial supraoptic nucleus), the medial and lateral divisions of the bed nucleus of the stria terminalis (BNST), the dorsal, intermediate, and ventral parts of the lateral septal nucleus (LS), the medial amygdaloid nucleus (MeA), and the ventrolateral hypothalamus (VLH). 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 [51], a standard computer-generated parcel was drawn to outline the entire ROI at low power (4X) 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. Then, under 10X 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 PAG-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. PAG-ir cells were identified in each field using a mouse driven cursor and then quantification of PAG-immunopositive cells was performed manually by the experimenter using the BIOQUANT software. Measurements at 10X continued until all PAG-positive cells throughout the entire ROI were quantified. Two to six independent measurements were taken from several consecutive sections of each animal (n=6) per treatment group depending upon the identification of the exact position of the nucleus within the region of interest, and the size of the nucleus in the rostral-caudal plane. Then, the number of PAG-ir cells was determined for each ROI, averaged for each brain region per animal and used for statistical analysis. The density of GluR1-ir was similarly determined. Briefly, under 40X magnification, images were assigned a threshold value as above such as to allow detection of stained GluR1-ir elements (neuropil and somata) showing moderate to high intensity, while suppressing lightly stained elements. and this threshold value was then applied across subjects. GluR1-ir elements were identified in each field using a mouse driven cursor and then GluR1-ir density measurements were performed automatically by the BIOQUANT software. Two to three independent measurements of GluR1 elements were taken from several consecutive sections of each animal per treatment group, and then the quantity (density) of GluR1-ir signal was determined for each region of interested and used for statistical analysis.

Statistical Analysis

Behavioral Studies—The results from the aggression tests were compared between AAS- and sesame oil-treatment groups. Data for all behavioral measures were compared using Student's t-test (two-tailed).

PAG/GluR1 Immunoreactivity—The number of PAG-ir cells and the density of GluR1-ir was compared between treatment groups by Student's t-Test (two-tailed) for each area analyzed. The α level for all comparisons was set at 0.05.

RESULTS

Aggressive Behavior

As seen previously in a number of published studies [11,20–23,25,31,59,60], hamsters treated with anabolic steroids during the majority of adolescent development showed significantly elevated levels of offensive aggression. Specifically, AAS animals demonstrated a greater than two-fold increase in the number of lateral attacks (t (16) = 2.97, p < 0.01) and more than three times the number of flank bites (t (16) = 3.32, p < 0.01) compared to their oil-treated counterparts (Fig. 1). Similarly, although the latency to first attack was not significantly different as a function of treatment (t (16) = −0.99, p > 0.05), latency to first bite was decreased in AAS-treated hamsters versus their controls (t (16) = −2.42, p < 0.05). Social contact time did not differ between groups, t (16) = 0.08, p > 0.05.

PAG Immunohistochemistry

Adolescent AAS treatment altered the immunohistochemical expression of PAG-containing cells in three brain areas important for aggressive behavior, namely the hypothalamus, amygdala and the lateral septum, when compared with oil-treated controls. For example, in oil-treated controls, PAG immunostaining in the AH revealed relatively few PAG-containing neuronal somata indicative of the normal distribution of glutamate neurons in this brain region in hamsters ([17] and see Fig. 2A,C) and rats [35]. By comparison, aggressive, AAS-treated hamsters displayed an increased number of PAG immunopositive somata in the AH (Fig. 2B,D). Quantitative analysis of PAG-ir neurons in the AH showed that AAS-treated animals had a 2 fold increase in PAG immunopositive somata when compared to oil-treated controls (Fig. 3). This difference was statistically significant [AH, t(13) = 2.35, p < 0.05]. However, closer examination of this phenomenon revealed a visually detectable increase in the number of PAG-ir neurons in a select subregion of AH, namely the latero-anterior hypothalamic nucleus (LAH) – the ventro-lateral portion of the AH (Fig. 2C and B). Similar increases in the number of PAG-containing neurons were detected in subregions of the amygdala and septum. For instance, aggressive, AAS-treated hamsters exhibited a 2–3 fold increase in the number of PAG immunopositive somata in the MeA (t (11)=3.06, p < 0.01) and LS (t (12) = 3.49, p < 0.01) compared to sesame oil-treated controls (Fig. 3). Interestingly, aggressive, AAS-treated animals did not show an increase in PAG immunopositive somata compared to non-aggressive, oil-treated controls in several other brain regions implicated in the control of offensive aggression in hamster, most notably the BNST (t (10) = 0.82) and VLH (t (13) = 1.25) (p > 0.05 each comparison). Further, no significant differences were found between treatment groups in the S1 cortex (t (13) = 1.25, p > 0.05), i.e., a brain area not involved in regulation of aggressive behavior in the hamster.

GluR1Immunohistochemistry

In aggressive, AAS-treated hamsters, the immunohistochemical staining pattern for GluR1 receptors was also altered in areas of the hamster brain implicated in offensive aggression, including those in the hypothalamus. For example, in oil-treated controls, the staining of GluR1

receptors displayed a moderately dense pattern of GluR1 receptor immunoreactive fibers and puncta in the VLH with very few GluR1 receptor-containing somata (astericks) indicative of the normal distribution of receptor localization in this brain region (Fig. 4A). By comparison, aggressive, AAS-treated hamsters displayed an increase in the density of GluR1 immunoreactive staining (fibers and neuronal somata (somata identified by astericks) in the VLH (Fig. 4B) compared to non-aggressive, oil-treated controls (Fig. 4A,B). Analysis of the density of GluR1-ir staining in the VLH showed that aggressive, AAS-treated hamsters had approximately 3-fold more GluR1 immunopositive signal (fibrous and somata staining) when compared to non-aggressive, oil-treated controls (Fig. 4, see Graph). This difference was statistically significant [t (10) = 3.68, p < 0.01]. Similar results were found in the BNST where aggressive, AAS-treated animals displayed significantly more GluR1-ir staining [t (12) = 3.10, p < 0.01] (i.e., approximately 2-fold) than non-aggressive, oil-treated controls (Fig. 4, see Graph). However, not all brain regions implicated in the aggressive response showed significant alterations in the density of GluR1 immunoreactive puncta/somata following adolescent AAS exposure (Fig. 4, see Graph). For instance, similar densities of GluR1 receptor-containing puncta and somata were found in the AH (t (11) = 1.05), MeA (t (11) = 0.34), and LS (t (10) = 1.21) (p > 0.05 each comparison) of both AAS- and oil-treated hamsters. Similarly, no significant differences were found in the density of GluR1 immunoreactive puncta/somata in the S1 cortex (t (13) = 0.08, p > 0.05), a brain area not involved in aggressive behavior in the hamster.

DISCUSSION

Aggressive, adolescent AAS treated animals had significant increases in the number and density of glutamate and GluR1-expressing neurons compared to non-aggressive, vehicle-treated controls, although the patterns of change observed across the neuraxis did not parallel one another. For instance, in the AAS-treated hamsters, there were greater than two-fold more PAG-ir neurons in the AH, MeA, and LS. Meanwhile, two- to three- fold increases in the density of GluR1-ir were observed in the BNST and VLH of aggressive, adolescent AAS treated animals. This disparate pattern of expression between transmitter and receptor across different brain sites implicated in aggression control is reminiscent, at least in part, of that observed for arginine vasopressin (AVP), i.e., another neurochemical signal shown previously to stimulate offensive aggression in a number of animal models and species. For instance, aggressive, adolescent AAS-treated hamsters show increased AVP in the AH, but not other brain sites associated with and/or implicated in aggression control, e.g., the BNST, LS, MeA, VLH [23,24,31]. Conversely, AVP V1A subtype receptor binding activity is increased in the BNST, LS, MeA, and VLH of aggressive, AAS-treated animals, while levels of receptor activity remain unchanged in the AH [11]. While most assuredly indicative of different molecular mechanisms, that aside, these data combined indicate that adolescent AAS exposure increases AVP tone across a number of brain regions implicated in aggression control – likely facilitating the development of the aggressive phenotype in drug treated animals. The findings presented in the current study are suggestive of a similar trend. Here aggressive, adolescent AAS-treated hamsters show increased PAG expression (indicative of increased glutamate production/activity) in the AH, MeA, and LS but not other brain sites associated with and/or implicated in aggression control, e.g., the BNST and VLH. However, increases in GluR1 glutamate receptors are noted in these latter brain sites, but not in the aforementioned brain regions. Together, these data strongly suggest that adolescent AAS exposure also increases glutaminergic tone across a number of the same brain regions implicated in aggression control – similarly suggestive of a facilitation the development of the aggressive phenotype in drug treated animals. Of particular relevance are the findings of an increase in the production/activity of neurotransmitters that facilitate aggression in the AH brain region of aggressive, AAS-treated animals, i.e., AVP and glutamate. Given this patterns of overlap, these data suggest that AVP and glutamate may interact in the AH to control adolescent, AAS-induced aggression in

hamsters. Indeed, there is precedent that AVP and glutamate interact in the AH to control flank marking in hamsters, i.e., a stereotypic motor behavior that is part of the ethogram of offensive aggression in this species [3]. In these studies, the activation of flank marking by AVP delivered directly into the AH requires the activity of glutamate – linking the activity of these two neural systems in this particular brain region to the regulation of agonistic behavior.

Overlap between the altered production and/or activity of the aggression-stimulating neurotransmitter glutamate and that of other neural components implicated in aggression control is not limited to the AVP neural system in the AH. Indeed, in recent studies we have shown a dramatic reduction in afferent fibers containing serotonin (5HT – i.e., a neurochemical signal previously shown to inhibit aggression) to the AH of aggressive, adolescent AAS-treated hamsters [21], indicating a reduced 5HT tone in this brain region in AAS-exposed animals. Aggressive, adolescent AAS-treated hamsters also express fewer post-synaptic 5HT1A- [60] and pre-synaptic 5HT1B- [20] receptors in the AH, while the expression of post-synaptic 5HT1B receptors appears to be up-regulated in neurons located in a select subregion of AH [20] namely the hamster equivalent of the latero-anterior hypothalamic nucleus – the ventro-lateral portion of the AH located between the two AVP containing compartments of the AH (i.e., the mSON and NC). Interestingly, more detailed analysis of the data presented in this report indicates that the observed increases in PAG appear to be localized to a great extent within the LAH, suggesting that perhaps the 5HT1B-containing neurons in this brain region are glutaminergic – and that both PAG and 5HT1B receptors are up-regulated in these neurons in aggressive, adolescent AAS-treated hamsters. Preliminary studies support the former assertion as we have shown that PAG containing neurons in the LAH express 5HT1B receptors (data not shown). Interestingly, in a separate set of studies we also showed that aggressive, adolescent AAS-treated animals showed persistent activation of neurons within this same subregion of the AH [59], suggesting that 5HT1B receptor-containing glutamate neurons in the LAH brain region may become constitutively active following adolescent AAS exposure. Thus, together, in the case of aggressive, adolescent AAS-treated animals, the enhanced stimulatory AVP tone combined with the loss of inhibitory 5HT tone that exists in the LAH as a result of AAS exposure might result in the chronic activation of downstream glutamate neurons in this brain region, facilitating the development of a heightened aggressive response pattern. This hypothesis is currently under investigation in the laboratory.

As mentioned above, however, there is increased PAG and GluR1 receptor expression (indicative of increased glutaminergic tone) in several other parts of the forebrain implicated in the control of aggression that should not be overlooked. For instance, neurons in the MeA and BNST regulate aggressive response patterns in rats, mice, prairie voles, and hamsters [6, 12,19,38,41,42,64,73,74], and increased neuronal activation has been observed in these brain regions in aggressive, experienced fighter hamsters after an aggressive encounter with other hamsters [12,38]. In aggressive, adolescent AAS treated animals increases in excitatory AVP V1a receptor binding [11], decreases in inhibitory 5HT afferent innervation [21,22], and alterations in 5HT1B receptor expression [22] have all been observed in the MeA and BNST. The enhanced tone of stimulatory AVP combined with the loss of inhibitory 5HT tone that occur in the MeA and BNST as a result of AAS exposure, along with the increase in PAG- and GluR1- expression (or glutaminergic tone) observed in these brain regions, reflect a neurobiologic state consistent with facilitating the development of a heightened aggressive response pattern in drug exposed animals. Similarly, the VLH has been implicated in the modulation of offensive aggression in hamsters [12–14], and projects extensively into other relevant regions, including the AH and BNST [61]. Alterations in the AVP and 5HT neural systems that are consistent with the development of the aggressive phenotype have been observed in the VLH of aggressive, adolescent AAS treated hamsters [11,21,22]. Similarly here the increased GluR1 expression in the VLH may reflect an increase in excitatory glutamate tone in this brain region. Combined with the aforementioned neural changes that also occur

here, increased glutaminergic tone may function to activate a feed-forward circuit to facilitate the development of excessive offensive responding in animals exposed to AAS during adolescence.

These data notwithstanding, there are findings from the current study that are inconsistent with this general hypothesis. For example, in this study we show increases in PAG-ir 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 [20,21,60], nor are there any alterations in neuronal activation in this brain site [20,21,60]. Further, aggressive responding has been shown to be inhibited by LS activity in hamsters [56,57], suggesting that an increase in the excitatory glutaminergic tone 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 [38,40], 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 [2,3,37–39]. Therefore, activity in these brain regions may reflect gross alterations in social behavior and responsiveness and have less of an affect on *offensive aggression*, remaining consistent with the central hypothesis regarding the relationship between site specific neural activation and the development of the offensive aggressive phenotype.

Speculation aside, currently the mechanisms by which adolescent exposure to AAS would up-regulate PAG expression is unclear. Certainly, there is strong evidence that glutamate affects steroid hormones – glutamatergic activity is critical for the onset of puberty, and appears to underlie secretion of lutenizing hormone, testosterone, and follicle stimulating hormone [4,9]. In fact the development of the glutamatergic system continues through the pubertal years, not reaching full maturation until adulthood [26,49,50]. Glutamate concentrations begin to rise during postnatal development and peak at or very shortly after, the onset of puberty [50]. Such ongoing pubertal development might leave glutamatergic systems particularly vulnerable to pharmacological insult by AAS. However, evidence is considerably sparse regarding an association in this direction – i.e., whereby glutamate levels are influenced by sex steroids. Our data clearly suggest such a developmental sensitivity, and are bolstered by previous studies that have reported a degree of hypothalamic and limbic GluR1 and GluR2/3 receptor dependence upon sex steroid manipulations [15]. Further research is necessary to determine the specific mechanisms underlying the increased expression in hypothalamic and limbic PAG and GluR1 observed in this study.

In summary, existing literature strongly implicate a role for the excitatory glutamatergic systems in the mediation of offensive aggression. In the current report, we explored the possibility that alterations in the expression of PAG and the ionotropic AMPA GluR1 subunit might be affected by chronic, high-dose steroid treatment in adolescent hamsters. Elevations in expression of both PAG and GluR1 were observed in key brain areas previously associated with offensive behavior, including the AH, VLH, MeA, BNST, and LS. The patterns of expression appeared to complement, rather than parallel one another, and overall, the hypothalamic-limbic aggression areas demonstrated an increase in general glutamatergic tone and activity in aggressive, AAS-treated animals.

Acknowledgements

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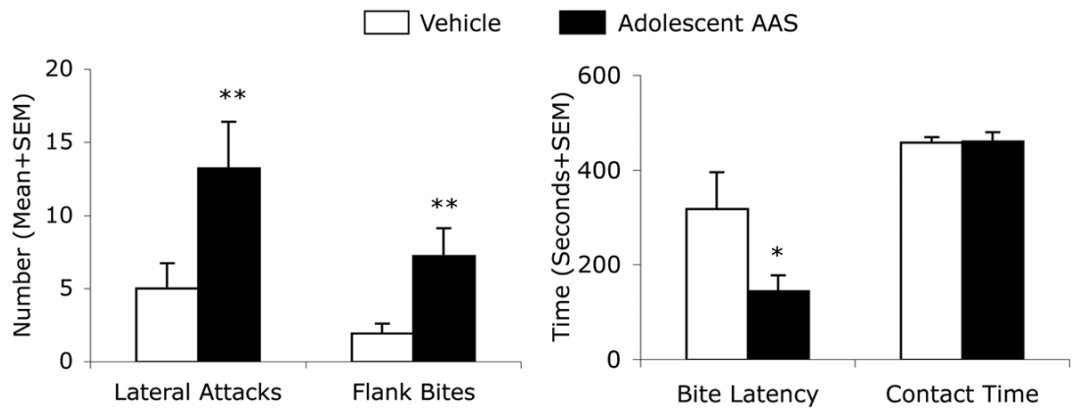
Figure 1.
Adolescent AAS treatment increases offensive aggression. Number of Lateral Attacks and Flank Bites, as well as Latency to First Attack and Total Contact Time in Vehicle- (White Bars) and AAS- (Black Bars) treated residents. Bars denote SEM. *p<0.05; **p<0.01; Mann-Whitney, two-tailed (number of attacks and bites), Student's *t*-test, two-tailed (attack latency and contact time).
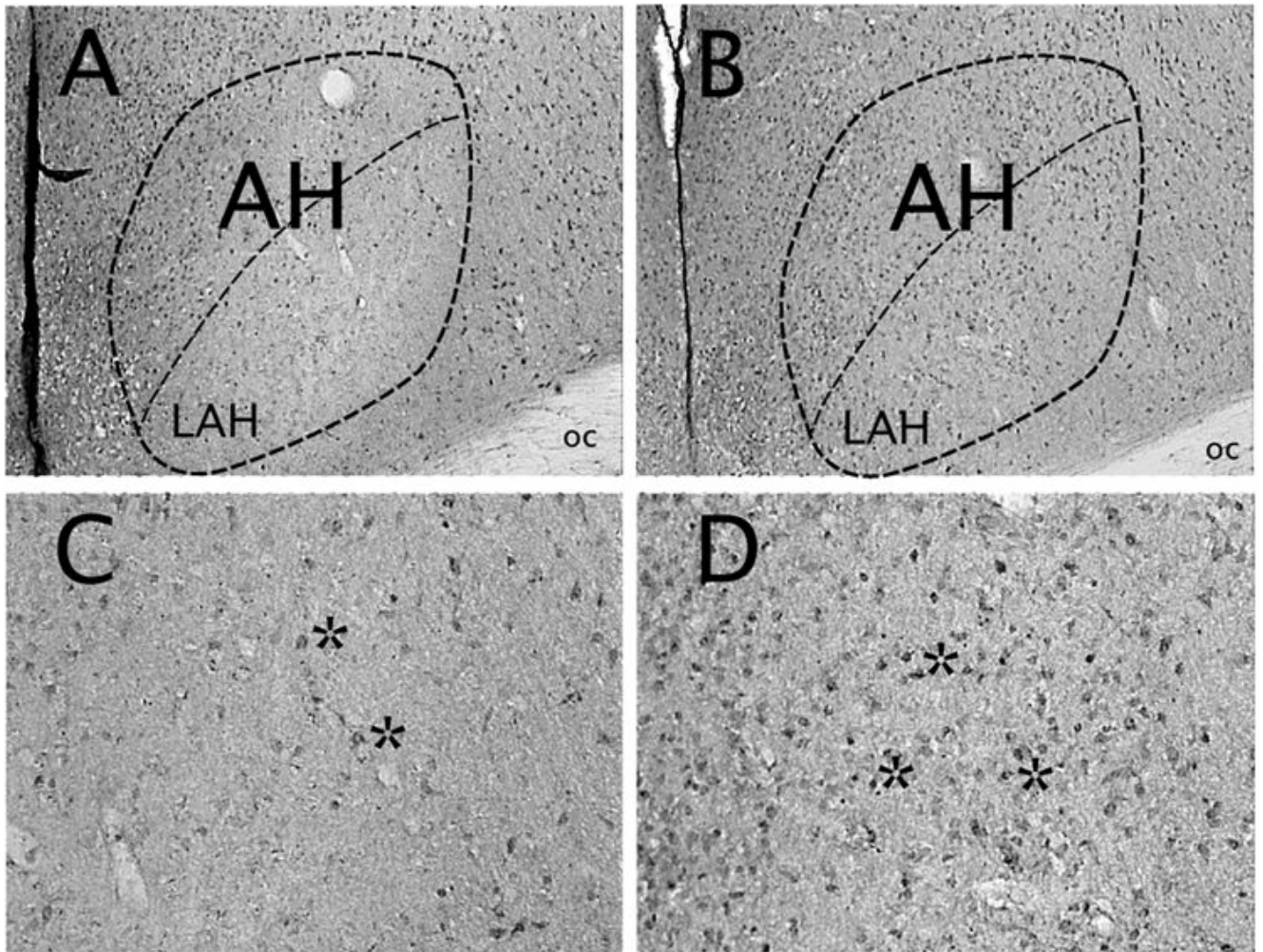
Figure 2.
Brightfield photomicrographs of a coronal section through the Syrian hamster hypothalamus. Shown are PAG-containing cells (astericks) within the anterior hypothalamus (encircled) of Vehicle- (A, C) and AAS- (B, D) treated hamsters. High power photomicrographs in C and D represent PAG immunostaining in the ventro-lateral aspects of the anterior hypothalamus delineated below the center dashed line in A and B, i.e., the latero-anterior hypothalamic (LAH) brain region. oc, optic chiasm.
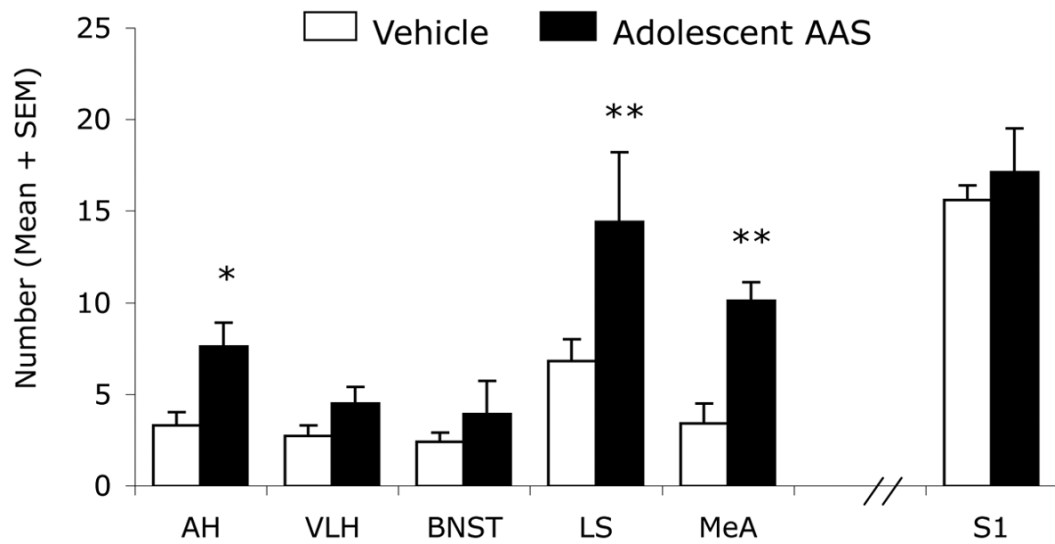
Figure 3.
Number of PAG-immunoreactive cells in select brain regions of Vehicle (White Bars) and AAS (Black Bars) treated hamsters. * $p<0.05$, ** $p< 0.01$; Student's t-test, two-tailed.
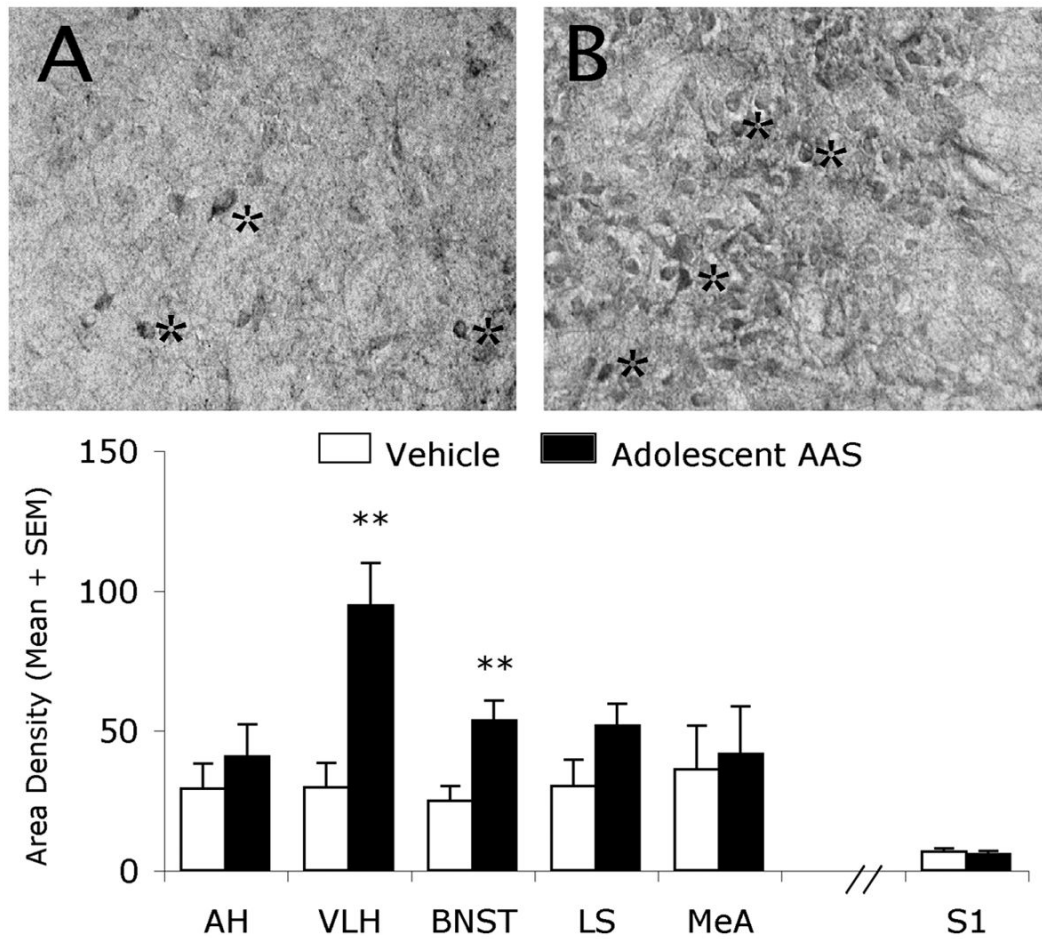
Figure 4.
Brightfield photomicrographs of a coronal section through the Syrian hamster hypothalamus. Shown are GluR1-containing cells (astericks) within the ventrolateral hypothalamus of Vehicle- (A) and AAS- (B) treated hamsters. Graph represents density of GluR1-immunostaining (neuropil and somata) in select brain regions of Vehicle (White Bars) and AAS (Black Bars) treated hamsters. ** $p < 0.01$; Student's t-test, two-tailed.