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Glutamic acid decarboxylase (GAD_{65}) immunoreactivity in brains of aggressive, adolescent anabolic steroid-treated hamsters

Jill M. Grimes, Lesley A. Ricci, and Richard H. Melloni Jr.*

Behavioral Neuroscience Program, Department of Psychology, 125 Nightingale Hall, Northeastern University, 360 Huntington Avenue, Boston, MA 02115, USA

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Abstract

Chronic anabolic–androgenic steroid (AAS) treatment during adolescence facilitates offensive aggression in male Syrian hamsters (*Mesocricetus auratus*). The current study assessed whether adolescent AAS exposure influenced the immunohistochemical localization of glutamic acid decarboxylase (GAD_{65}), the rate-limiting enzyme in the synthesis of γ-aminobutyric acid (GABA), in areas of hamster brain implicated in aggressive behavior. Hamsters were administered high dose AAS throughout adolescence, scored for offensive aggression, and then examined for differences in GAD_{65} puncta to regions of the hamster brain important for aggression. When compared with control animals, aggressive AAS-treated hamsters showed significant increases in the area covered by GAD_{65} immunoreactive puncta in several of these aggression regions, including the anterior hypothalamus, ventrolateral hypothalamus, and medial amygdala. Conversely, aggressive AAS-treated hamsters showed a significant decrease in GAD_{65}-ir puncta in the lateral septum when compared with oil-treated controls. However, no differences in GAD_{65} puncta were found in other aggression areas, such as the bed nucleus of the stria terminalis and central amygdala. Together, these results support a role for altered GAD_{65} synthesis and function in adolescent AAS-facilitated offensive aggression.

Keywords: Adolescence; Anabolic–androgenic steroids; γ-Aminobutyric acid; GABA; GAD_{65}; Development; Aggression

Introduction

The naturally occurring hormone testosterone and its synthetic derivatives (collectively termed androgenic–anabolic steroids (AAS)) have been used by professional and amateur athletes and bodybuilders for over two decades to enhance athletic performance and overall physical appearance (Yesalis et al., 1988, 1993). Over the past decade, the illicit use of AAS among the adolescent population appears to be rising and is reaching near epidemic proportions (Yesalis et al., 1997; NIDACapsules, 2001). Studies from the National Institute on Drug Abuse estimate that more than one-half million 8th- and 10th-grade students are using AAS in the United States each year (NIDACapsules, 2001). Reports indicate that AAS use has risen significantly in this population with 3.6% of male 10th-graders reporting use in 2000, up from 2.8% in 1999, 2.0% in 1997, and 1.8% in 1996 (NIDACapsules, 2001). This pattern of abuse is of particular interest since the onset of AAS use during adolescence is correlated with more frequent and heavier use later in life despite the associated physical and psychological ramifications (Buckley et al., 1988; Yesalis et al., 1988). The illicit use of AAS during adolescence is a significant mental health risk since AAS abuse has been associated with many adverse psychiatric and behavioral effects, including increased aggressive behavior.

Behavioral data from our laboratory indicate that Syrian hamsters treated with chronic, high-dose AAS throughout adolescent development show elevated offensive aggression on the first behavioral interaction with conspecifics (Melloni et al., 1997; Harrison et al., 2000; Grimes and Melloni, 2002; DeLeon et al., 2002b). The finding that AAS-treated hamsters demonstrated heightened offensive aggression in

* Corresponding author. Fax: +1-617-373-8714.
E-mail address: melloni@research.neu.edu (R.H. Melloni Jr.).

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the absence of established social interactions and cues suggested that adolescent exposure to AAS stimulated aggression directly, perhaps by affecting the activity of neural circuits that regulate this behavior. γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter system in the central nervous system (CNS) and is produced in the brain by the two isoforms of the enzyme glutamic acid decarboxylase (GAD_{65} and GAD_{67}). While both GAD_{65} and GAD_{67} have been shown to synthesize GABA in the CNS, it appears that the two GAD isoforms serve different functions and have different cellular distributions (Erlander and Tobin, 1991; Kaufman et al., 1991; for review, see Martin and Rimvall, 1993). In contrast to GAD_{67}, which is almost saturated with cofactor in brain, GAD_{65} exists within GABA neurons as an inactive apoenzyme (about 50% of GAD_{65}) and as an active holoenzyme when bound to its cofactor pyridoxal-P (Kaufman et al., 1991; for review, see Martin and Rimvall, 1993). GAD_{65} has been localized mainly to synaptic terminals and, therefore, may serve to respond to demands for GABA with changes in neuronal activity (Kaufman et al., 1991). Both GAD_{65} mRNA expression and immunohistochemical labeling of GAD_{65} in axon terminals have been has been localized to various brain regions (Tappaz et al., 1977a, 1977b; Feldblum et al., 1993; Fenelon et al., 1995; Bowers et al., 1998; Chen et al., 1998; Sur et al., 1999) including those implicated in aggressive behavior in hamsters such as the hypothalamus, lateral septum (LS), bed nucleus of the stria terminalis (BNST), and the central and medial amygdaloid nuclei (CeA and MeA) (Bunnell et al., 1970; Sodetz and Bunnell, 1970; Hammond and Rowe, 1976; Potegal et al., 1981a, 1981b; Ferris and Potegal, 1988; Ferris and Delville, 1994; Kollack-Walker and Newman, 1995; Delville et al., 1996a, 1996b, 2000; Ferris et al., 1997, 1999).

Several studies have suggested that GABAergic activity may be involved in the modulation of aggression in various species and models of aggression. For example, in mice and hamsters, increased levels of aggressive behavior correlates with significantly higher levels of GABA in various limbic structures such as the hypothalamus, olfactory bulbs, and amygdala (Potegal et al., 1982; Haug et al., 1984). Mice lacking the gene for GAD_{65}, resulting in a GABA deficit, show decreased aggressive behavior when confronted with a conspecific (Stork et al., 2000). In addition, androgens appear to influence the activity of GABA neurons. Indeed, sex differences in GABA neuronal activity and levels of GAD_{65} mRNA have been reported, with increases in activity of GABA neurons and in GAD_{65} mRNA in the hypothalamus and the medial amygdala observed in male rats (Grattan and Selmanoff, 1997; Stefanova, 1998; Searles et al., 2000; Perrot-Sinal et al., 2001). Also, castration- and androgen receptor antagonist-induced decreases in GABA neuronal activity have been observed in male rats (Grattan and Selmanoff, 1993, 1994a; Grattan et al., 1996a; Yoo et al., 2000) and testosterone replacement prevents such castration-induced decreases (Grattan and Selmanoff, 1994a, 1994b).

Given this documented association between the GABAergic neural system, aggression, and testosterone, the present study was conducted to establish a link between the expression of GAD_{65}-ir puncta and AAS-induced offensive aggression using the subadult Syrian hamster as an animal model. To establish whether adolescent AAS exposure altered GABAergic activity in various brain regions implicated in the control of offensive aggression, we employed immunohistochemistry utilizing an antibody specific against the 65-kDa isoform of glutamate decarboxylase to visualize and quantify GAD_{65} puncta.

Methods

Animals

For the experimental treatment paradigm, intact preadolescent male hamsters (P23–25) were obtained from Harlan Sprague–Dawley Labs (Indianapolis, IN), individually housed in Plexiglas cages, and maintained at ambient room temperature on a reverse light:dark cycle of (14L:10D; lights on at 19:00). Food and water were provided ad libitum. For aggression testing, stimulus (intruder) males of size and weight equal to those of the experimental animals were obtained from Harlan Spraque–Dawley one week prior to the behavioral test, group-housed at five animals/cage in large Plexiglas cages, and maintained as above to acclimate to the animal facility. All intruders were prescreened for low aggression (i.e., Disengage and Evade) and submission (i.e., Tail-up Freeze, Flee, and Fly-away) one day prior to the aggression test to control for behavioral differences between stimulus animals, as previously described (Ferris et al., 1997; Melloni et al., 1997). Animals displaying significantly low aggression and/or submissive postures were excluded from use in the behavioral assay. All methods and procedures described below were preapproved by the Northeastern University Institutional Animal Care and Use Committee (NU-IACUC).

Experimental treatment

Adolescent (P27) hamsters ($n = 38$ total) received daily subcutaneous (SC) injections (0.1–0.2 ml) of an AAS mixture ($n = 24$) consisting of 2 mg/kg testosterone cypionate, 2 mg/kg nortestosterone, and 1 mg/kg dihydroxytestosterone undecylate (Steraloids, Inc., Newport, RI), or with an equal volume of sesame oil vehicle alone ($n = 14$) (Sigma, St. Louis, MO) for 30 consecutive days (P27–P56). This daily treatment of AAS was designed to mimic a chronic "heavy use" regimen (Pope and Katz, 1988, 1994). Animals were weighed daily (P27–P57) to determine total body weight gain for between group comparisons. Following the treatment period, animals in both AAS and sesame oil

groups were tested for offensive aggression and sacrificed, and the brains were removed and processed for immunohistochemistry ($n = 12$ total; $n = 6$ animals/group) as detailed below. Testes of animals in both AAS and sesame oil groups ($N = 11$/group) were removed, weighed, and measured for comparisons between the two groups.

Aggression testing

Experimental animals were tested for offensive aggression using the resident/intruder paradigm, a well-characterized and ethologically valid model of offensive aggression in golden hamsters (Lerwill and Makaings, 1971; Floody and Pfaff, 1977). For this measure, an intruder of similar size and weight was introduced into the home cage of experimental animals and the resident was scored for offensive aggression (i.e., number of attacks and bites and latency to attack and bite intruder). The behavior of a subset of experimental and control animals was examined for a more focal analysis of this aggressive response including number of lateral attacks, upright offensive attacks, and chases directed toward an intruder. Briefly, an attack was scored each time the resident animal would wildly pursue and then (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 ventrum and/or flank. 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 or bites, latency was assigned the maximum time of the behavioral test (i.e., 600 s). In addition, residents were also measured for social interest toward intruders (i.e., total contact time between resident and intruder). 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 test for aggression, AAS- and sesame oil-treated hamsters were anesthetized with 80 mg/kg Ketamine and 12 mg/kg Xylazine and the brains fixed by transcardial perfusion with 4% paraformaldehyde, 0.2% picric acid, 0.2% gluteraldehyde (50/50 wt). Brains were then cryoprotected by incubating in 30% sucrose in phosphate-buffered saline (PBS) (0.001 M KH_2PO_4, 0.01 M Na_2HPO_4, 0.137 M NaCl, 0.003 M KCl, pH 7.4) overnight at 4°C. Consecutive series of 35-μm coronal sections were cut on a sliding microtome, collected as free-floating sections in 1× PBS, and labeled for GAD_{65} by single-label immunohistochemistry using a modification of an existing protocol (Grimes and Melloni, 2002). Briefly, free-floating sections were pretreated with 0.03% H_2O_2 followed by preincubation in 10% normal donkey serum with 0.5% Triton X-100. Sections were incubated in primary antiserum (1:4,000) for GAD_{65} anti-mouse (Boehringer Mannheim, Indianapolis, IN) with 5% NDS and 0.5% Triton X-100 for 48 h at 4°C. After primary incubation, sections were incubated in secondary donkey anti-mouse followed by tertiary antisera (Vectastain ABC Elite Kit—mouse, Vector Labs, Burlingame, CA) for 1 h each at room temperature 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 Cytoseal-60 mounting medium (VWR Scientific, West Chester, PA).

Image analysis

The area covered by GAD_{65} immunoreactive (GAD_{65}-ir) puncta was determined within specific brain areas using the BIOQUANT NOVA 5.0 computer-assisted microscopic image analysis software package as previously described (DeLeon et al., 2002a). The areas analyzed were selected based on previous data implicating these regions as part of the circuit important for aggressive behavior in numerous species and models of aggression, with the notable exception of the S1 neocortex (S1), i.e., a nonaggression area used as a control region. These areas (see Fig. 1) included the intermediate part of the LS, the medial division of the BNST, the MeA, the anterior hypothalamus (AH), the CeA, and the ventrolateral hypothalamus (VLH) which included the medial aspects of the medial tuberal nucleus and the ventrolateral part of the ventromedial hypothalamic nucleus. 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 at low power (4×) using a Nikon E600 microscope. At this magnification, a standard computer-generated box was drawn to fit within the particular region of interest. Then, under 20× magnification, images were thresholded at a standard RGB-scale level empirically determined by observers blinded to treatment conditions to allow detection of stained GAD_{65}-ir elements 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. GAD_{65}-ir puncta were identified in each field using a mouse driven cursor and then GAD_{65}-ir counts were performed automatically by the BIOQUANT software. Measurements at 20× continued until GAD_{65}-ir elements throughout the entire region of interest were quantified. Two to three independent measurements of GAD_{65}-ir elements were taken from several consecutive sections ($n \geq 2$) of each animal per treatment group

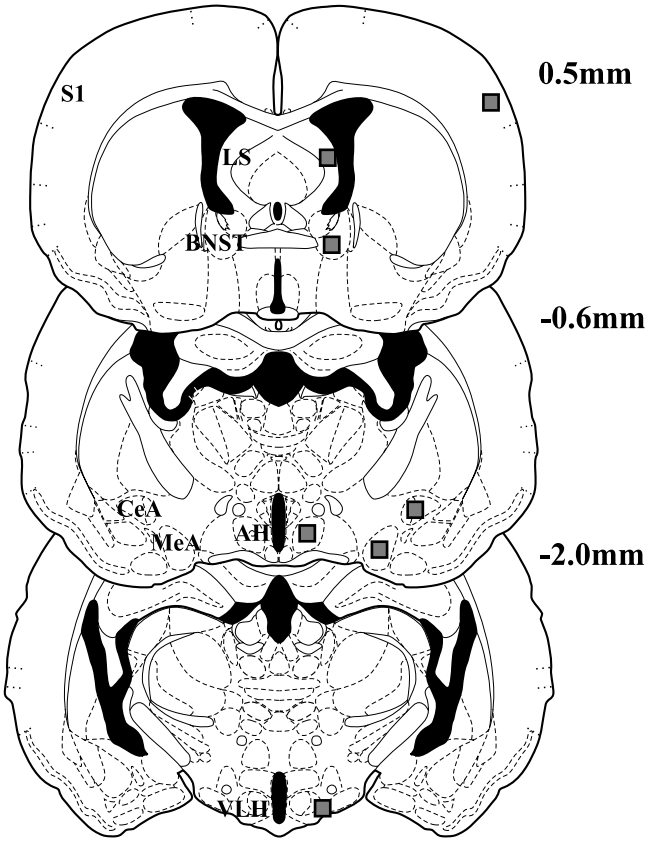

Fig. 1. Diagram showing the location of the areas selected to quantify GAD_{65}-ir puncta (shaded areas). Plates were modified from hamster atlas of Morin and Wood (2001) and reflect specific positions in the rostral–caudal plane (i.e., distance in mm from bregma to the plane of section at the skull surface). Abbreviations: AH, anterior hypothalamus; BNST, medial division of the bed nucleus of the stria terminalis; CeA, central amygdala; LS, intermediate part of the lateral septal nucleus; MeA, medial amygdala; S1, S1 neocortex; VLH, ventrolateral hypothalamus.

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 GAD_{65}-ir puncta was determined for each region of interest, standardized per 100 × 100-μm parcel for regional comparison purposes, and then used for statistical analysis.

Statistics

Behavioral studies

Results from the aggression tests were compared between AAS and sesame oil treatment groups. Nonparametric data (number of total attacks, lateral attacks, upright offensive attacks, bites, and chases) were compared by Mann–Whitney *U* tests (two-tailed), while parametric data (attack and bite latencies and contact times) were compared by Student's *t* test (two-tailed).

Body weight/testicular weight and size

The results of body weight and testicular weight and size were compared between AAS-treated and sesame oil-treated groups. Student *t* tests (two-tailed) were used for the analysis of total body weight at P27 and P57 for both groups, testicular weight, length, and width at P57.

GAD_{65} puncta

The numbers of GAD_{65}-ir puncta were compared between treatment groups by Student's *t* test (two-tailed) for each area analyzed.

Results

As characterized extensively in our previous studies (Melloni and Ferris, 1996; Melloni et al., 1997; Harrison et al., 2000; Grimes and Melloni, 2002; DeLeon et al., 2002b), animals treated with AAS during adolescent development showed significantly heightened measures of offensive aggression (Fig. 2). Specifically, hamsters treated with high-dose AAS showed a significant increase in the number of total attacks ($Z = 3.15$, $P < 0.01$) and bites ($Z = 3.02$, $P < 0.01$) over vehicle-treated littermates (Fig. 2). Indeed, the half of the AAS-treated animals (12 of 24) scored greater than 20 total attacks during the aggression test. By comparison, half of the oil-treated hamsters (7 of 14) scored less than 5 attacks on opponents. The behavior of a subset of animals was rescored to fully characterize the behavioral response to include lateral attacks, upright offensive attacks, and chases to the analysis. AAS-treated hamsters showed significant increases in the number of lateral attacks ($Z = 3.05$, $P < 0.01$), upright offensive attacks ($Z = 3.02$, $P < 0.01$), and chases ($Z = 3.04$, $P < 0.01$) which usually preceded an attack (Fig. 2; see inset). In addition, AAS-treated hamsters displayed significantly decreased attack and bite latencies toward intruders ((latency to attack, $t(36) = -2.36$, $P < 0.05$) (latency to bite, $t(36) = -2.4$, $P < 0.05$)) than vehicle-treated control animals (Fig. 2; see inset). The majority of AAS-treated animals (16 of 24) attacked within the first 100 s of the 10-min test, in comparison to saline-treated control animals, whose first recorded attack averaged nearly 4 min later, toward the middle and end of the test period. Finally, although AAS-treated hamsters showed significant increases in offensive aggression, the duration of physical contact was similar in AAS-treated (395.75 ± 60.17) and oil-treated (408.42 ± 90.87) residents ($t(38) = -0.52$, $P > 0.5$).

Prior to treatment, at P27, initial body weights were not significantly different (mean ± SD) between AAS-treated and control hamsters ($t(18) = -1.85$, $P > 0.05$). Following 30 days of treatment, at P57, AAS-treated animals showed a significant decrease in body weight ($t(18) = -2.759$, $P < 0.05$) compared to controls (Table 1). At P57, AAS-treated hamsters showed significant decreases in testicular weight ($t(18) = -7.92$, $P < 0.001$), length ($t(18) = -7.49$, $P < 0.001$), and width ($t(18) = -6.82$, $P < 0.001$) when compared to oil controls (Table 1).

In aggressive AAS-treated hamsters, the immunohisto-

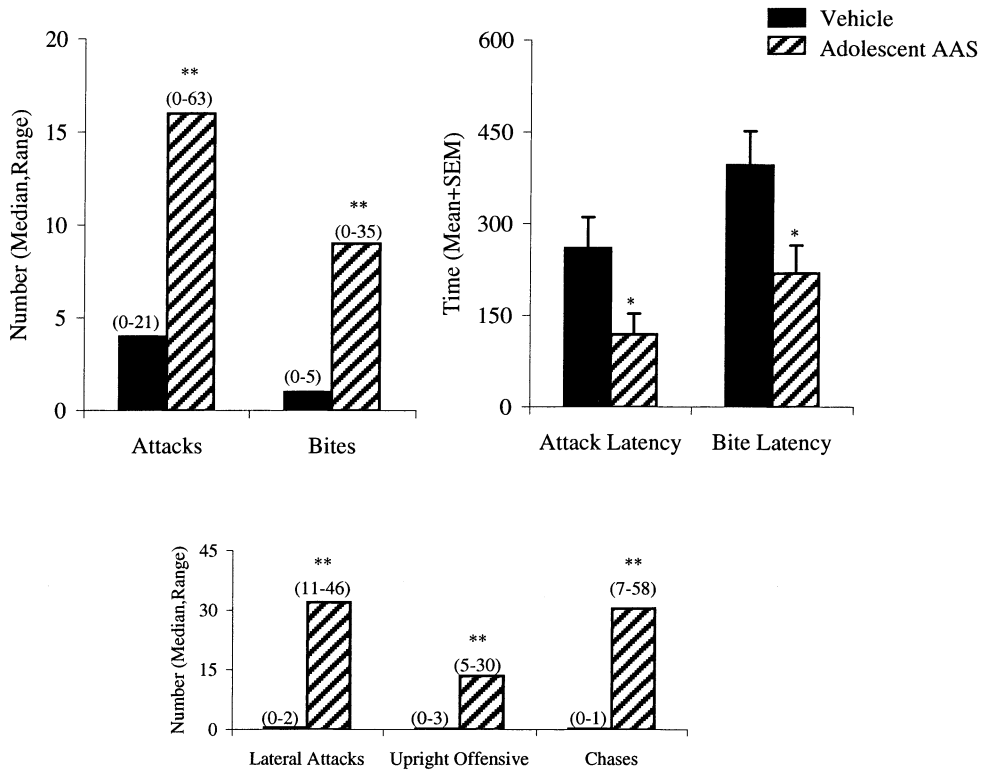

Fig. 2. Adolescent AAS treatment increases offensive aggression. Number of total attacks and bites and latency to first attack and bite as well as lateral attacks, upright offensive attacks, and chases (inset) in AAS- and vehicle-treated residents. Bars denote SEM. *$P < 0.05$, **$P < 0.01$, Mann–Whitney, two-tailed (number measures), Student's t test, two-tailed (latency measures).

chemical staining pattern for GAD_{65} was altered in several areas of hamster brain important for aggressive behavior, including those in the hypothalamus. For example, AAS-treated hamsters exhibited a dense staining pattern of GAD_{65}-ir puncta in the VLH (Fig. 3A) compared to sesame oil-treated controls (Fig. 3B), which displayed less dense pattern of staining, indicative of the normal distribution and density of GABAergic synaptic terminals observed in naïve animals (data not shown). Quantitative analysis of GAD_{65}-ir puncta in the AH showed that AAS-treated animals had nearly a 30% increase in GAD_{65}-ir puncta compared to oil-treated littermates (Fig. 4). This difference was statistically significant (AH, $t(24) = 2.16$, $P < 0.05$). Similar results were observed in the VLH where AAS-treated animals had greater than two times the GAD_{65}-ir puncta of oil-treated littermates (Fig. 4). This difference was statistically significant (VLH, $t(19) = 4.69$, $P < 0.001$). These findings were not restricted to the hypothalamus, however, as other brain regions implicated in the hamster aggression circuit showed changes in GAD_{65}-ir puncta following adolescent AAS exposure (Fig. 4). For instance, the number of GAD_{65}-ir puncta in the MeA of AAS-treated animals was nearly three times that of sesame oil controls and this difference was statistically significant (MeA, $t(16) = 6.02$, $P < 0.001$) (Fig. 4). However, AAS-treated animals did not show an increase in all brain regions implicated in the control of offensive aggression in hamster. For example, the density of GAD65-ir puncta in the LS was significantly decreased in AAS-treated hamsters compared to oil-treated controls (LS, $t(22) = -3.46$, $P < 0.01$) (Fig. 4).

Not every brain region implicated in aggressive behavior in hamsters showed significant changes in GAD_{65}-ir puncta following adolescent AAS exposure. For instance, similar numbers of GAD_{65}-ir puncta were found in the medial division of the BNST and the CeA of both AAS-treated and control animals (Fig. 4). These counts were not significantly difference between treatment groups ($P > 0.2$ each com-

Table 1
Adolescent AAS exposure decreases body weight, testicular weight, and testicular size (length and width)

	AAS (mean + SD)	SO (mean + SD)
Animal weight (g)		
P(27)	43.64 + 12.43	51.9 + 8
P(57)	105 + 12.89*	122 + 13.4
Testes weight (mg)	942.78 + 182.52**	1578 + 156.69
Testes size (mm)		
Length	15.84 + 1.24**	19.43 + 0.73
Width	11.48 + 0.74**	13.75 + 0.68

Note. Mean body (g) and testicular (mg) weights and testicular size (mm) + SD in AAS- and vehicle-treated hamsters.

* $P < 0.05$, Student t-test (two-tailed).

** $P < 0.001$, Student t-test (two-tailed).

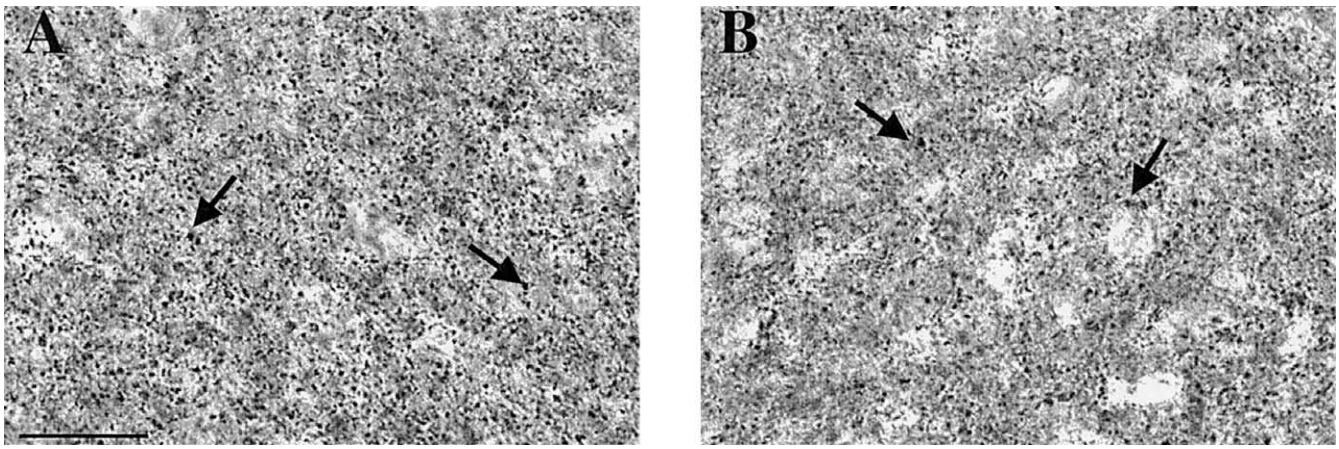

Fig. 3. Brightfield photomicrographs of a coronal section through the Syrian hamster hypothalamus. Shown are glutamic acid decarboxylase (GAD_{65}) immunoreactive puncta (arrows) within the ventrolateral hypothalamus (VLH) of (A) AAS-treated and (B) oil-treated hamsters. Bar, 20 μm.

parison). Similarly, no significant differences were found in the S1 cortex (Fig. 4), a brain area not involved in aggressive behavior in the hamster, between treatment groups ($P > 0.2$).

Discussion

In previous studies, we have shown that repeated high-dose AAS treatment throughout adolescence specifically increased offensive aggression in male Syrian hamsters (Melloni and Ferris, 1996; Melloni et al., 1997; Harrison et al., 2000; Grimes and Melloni, 2002; DeLeon et al., 2002b). One mechanism by which chronic adolescent AAS exposure may facilitate aggression in hamsters is by altering the activity of neurotransmitter systems implicated in this behavioral response. In various species, including hamsters, increases in GAD_{65} enzymatic activity, GABA levels, and $GABA_A$ receptor activity have been correlated with heightened aggressive responding. For instance, highly aggressive rodents display elevated GABA levels (Potegal et al., 1982; Haug et al., 1984), and pharmacological manipulations employing $GABA_A$ receptor agonists facilitate attack behavior in rats confronted with a conspecific in a neutral area compared to controls (Depaulis and Vergnes, 1985). Conversely, using the resident–intruder paradigm, $GABA_A$ receptor antagonists decrease the number of attacks of resident rats toward intruders (Depaulis and Vergnes, 1985). Similarly, benzodiazepine antagonists and partial agonists which block the binding of GABA to its receptor also have been shown to dose-dependently decrease resident–intruder offensive aggression in rats and squirrel monkeys (Weerts et al., 1993). Together, these findings suggest a facilitative role for GABA activity in offensive aggression. In accord with this notion, GAD_{65}-ir puncta and mRNA and $GABA_A$ receptors have been localized to various brain regions implicated in the facilitation (and inhibition) of aggression (Tappaz et al., 1977a, 1977b; Feldblum et al., 1993; Fenelon et al., 1995; Sur et al., 1999; Bowers et al., 1998; Chen et al., 1998), including the hypothalamus, septum, amygdala, and bed nucleus of stria terminalis (Bunnell et al., 1970; Sodetz and Bunnell, 1970; Hammond and Rowe, 1976; Potegal et al., 1981a, 1981b; Ferris and Potegal, 1988; Ferris and Delville, 1994; Kollack-Walker and Newman, 1995;

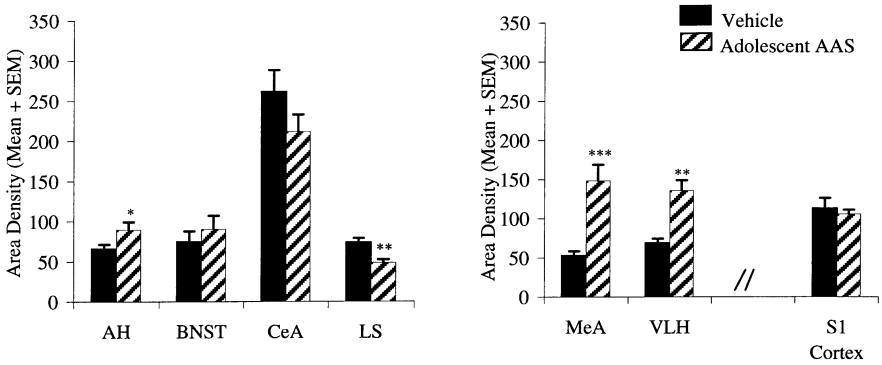

Fig. 4. Density of glutamic acid decarboxylase (GAD_{65}) immunoreactive puncta in brains of AAS- vs vehicle-treated hamsters. Numbers were normalized to a standard area (100 × 100 μm) for regional comparisons. ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, Student's t test, two-tailed.

Delville et al., 1996a, 1996b, 2000; Ferris et al., 1997, 1999), and their levels/activity has been shown to be sensitive to sex steroids in several of these areas (Grattan and Selmanoff, 1993, 1994a, 1994b; Grattan et al., 1996a, 1996b; Perrot-Sinal et al., 2001; Yoo et al., 2000). In the current study, we hypothesized that exposure to AAS during adolescence facilitated offensive aggression in hamsters by influencing GABA function in areas of the hamster brain important for aggression control. For instance, adolescent AAS exposure may enhance GABA function in aggression areas by increasing the density/number of GABA-containing terminals in these regions, functionally activating the neural circuits stimulating offensive aggression. To determine this, we quantified the density of GAD_{65}-ir puncta in the brains of aggressive, adolescent AAS-treated hamsters and nonaggressive vehicle-treated littermates.

Aggressive, adolescent AAS-treated animals had significant increases in GAD_{65}-ir puncta in several areas of the hamster brain that regulate offensive aggression, namely the AH, VLH, and MeA (Delville et al., 1996b, 2000; Ferris et al., 1997), suggesting an increase in the expression of GAD_{65} protein and/or the density of GABAergic terminals in these brain areas. One potential mechanism by which increases in GAD_{65}-ir in these sites may facilitate the development of the aggressive phenotype is by augmenting GABA inhibition of neural components suppressing aggression (e.g., serotonin (5-HT) afferent fibers). There is support for this notion from studies in hamsters showing that 5-HT release from afferents and activity in the AH and VLH inhibits offensive aggression (Ferris and Delville, 1994; Delville et al., 1996b; Ferris et al., 1997, 1999) and others in rats indicating a functional relationship between the 5-HT and GABA neural systems and the regulation of aggression (Soderpalm and Svensson, 1999). While the site(s) of 5-HT/GABA interactions is not completely elucidated, studies have localized GABA receptors to 5-HT afferent terminals (Gale, 1982), so it is possible that changes in signaling between 5-HT and GABA terminals in the AH, VLH, and MeA may facilitate adolescent AAS-induced offensive aggression. Further support for this hypothesis comes from work from our laboratory showing dramatic reductions in 5-HT afferent innervation to these very same brain sites (plus the MeA) in aggressive, adolescent AAS-treated hamsters compared to nonaggressive, vehicle-treated littermates (Grimes and Melloni, 2002). From a mechanistic standpoint, the increased density of GAD_{65}-ir puncta in the AH, VLH, and MeA observed in the present study may represent a neural plastic response to the loss of GABA receptor-containing 5-HT afferent terminals in these regions of the hamster brain important for offensive aggression. Together, these results would support the notion that signaling between 5-HT and GABA inhibitory control systems is linked and suggest a mechanism whereby enhanced GABA activity could cause the disinhibition of a behavioral state (i.e., offensive aggression) that is regulated by 5-HT. This hypothesis is currently under investigation in the laboratory.

One inconsistency with this hypothesis, however, arises from results of GAD_{65} immunostaining in the CeA. In our previous study we found a significant reduction in 5-HT afferent innervation to the CeA in aggressive, AAS-treated hamsters compared to nonaggressive, vehicle-treated littermates. By our model we would predict an increase in GAD_{65}-ir puncta in the CeA of aggressive, AAS-treated animals compared to controls. However, in these analyses, no ostensible differences were observed in CeA-GAD_{65} immunostaining between treatment groups. One possible explanation for this disparity may lie in the fact that the CeA has not been firmly characterized as part of the network of nuclei regulating "offensive aggression." Indeed, while the CeA has been implicated in defensive aggression in cats (Zagrodzka et al., 1998), physical aggression in rats (Bedard and Persinger, 1995), and scent marking in hamsters (Bamshad et al., 1997), no reports have shown CeA activity to be directly involved in the regulation of offensive aggression in hamsters. Therefore, no change in GABA activity in this brain region may have little effect (if any) on offensive responding, remaining consistent with our central hypothesis regarding the site-specific nature of 5-HT/GABA interactions and the development of the aggressive phenotype. Interestingly, aggressive, AAS-treated hamsters had a significant decrease in GAD_{65}-ir puncta in the LS when compared with controls. Decreased GABA levels have been observed in the septum of isolation-induced aggressive mice but these effects appear to be dependent upon the housing condition rather than the aggressive phenotype (Simler et al., 1982). Since offensive aggression has been shown to be inhibited by electrical stimulation of the LS (Potegal et al., 1981a, 1981b), adolescent AAS-induced decreases GAD_{65}-ir here could potentially cause a disinhibition of an inhibitory neural system (e.g., 5-HT), subsequently increasing an inhibitory influence on LS neurons and facilitating offensive aggression. Taken together, the data above are novel and significant in that they show that exposure to high-dose AAS during adolescent development can alter patterns of GAD_{65}-containing afferent terminals to areas of the brain which have been implicated in offensive aggression in hamsters. From a neurobiological standpoint, these data implicate increased GABA neural signaling in several key areas as potential neural substrates for adolescent AAS-facilitated aggression. Further, the different patterns of GAD_{65}-ir staining in several brain regions suggest that there is a nonuniform effect of adolescent AAS treatment on GABA neurons across the neuraxis.

Lastly, in addition to reports which implicate a functional relationship between AAS and the GABA and 5-HT neural systems, AAS themselves, as well as several steroid derivatives (e.g., 3α-androsterone and 3α-androstanediol), have been shown to directly modulate GABA activity by acting as agonists and antagonists at the GABA, receptor (Majewska et al., 1986; Majewska, 1992; Masonis and McCarthy, 1995, 1996; Bitran et al., 1996; Frye et al., 1996a, 1996b, Wilson and Biscardi, 1997; Jorge-Rivera et al., 2000; Yang

et al., 2002). Specifically, 17α-alkylated AAS and nandrolone (a 19-nortestosterone) both positively and negatively modulate the GABA, receptor and these divergent effects seem dependent upon whether the cells predominantly expressed the $\alpha_1\beta_3\gamma_1$ or the $\alpha_1\beta_3\gamma_{2(L)}$ subtype (Jorge-Rivera et al., 2000; Yang et al., 2002). So, consistent with our hypothesis, GABA, receptors present on 5-HT afferents in the AH, VLH, and MeA may also be activated through a nongenomic interaction between AAS and/or testosterone metabolites and subunits of the GABA, receptor complex, further enhancing GABA activity on aggression-suppressing terminals in these brain areas, and leading to the development of the aggressive phenotype in adolescent AAS-treated animals.

In summary, the role of GABAergic mechanisms in the elicitation of offensive aggression remains elusive and is further complicated by the modulatory effects of testosterone on the activity of GABA, receptors and our previous work indicating that adolescent AAS animals also have a compromised 5-HT neural system. That notwithstanding, the studies presented in this article provide data regarding the neurobiological effects of chronic high-dose AAS exposure during adolescent development and propose basic neurobiological mechanisms by which these agents may exert their aggression-stimulating effects. These findings indicate that AAS exposure during adolescent development produces both increases and decreases in GAD_{65}-ir in several areas of hamster brain important for aggressive behavior. These and previous findings from our laboratory provide evidence that multiple neural systems including GABA are important in linking adolescent AAS exposure and offensive aggression in hamsters. Further studies are needed to more precisely elucidate the role of AAS on the GABA system, particularly how treatment during critical periods of development, such as adolescence, alters GABA, receptor expression and function, subsequently leading to the development of aggressive behavior. It will also be of interest to determine what other neural systems may be involved in the control of AAS-induced offensive aggression and how these systems interact with one another to establish the aggressive behavioral phenotype.

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