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ADOLESCENT ANABOLIC STEROID EXPOSURE: EFFECTS ON SOCIAL BEHAVIORS AND NEURAL PLASTICITY

By

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Anabolic-androgenic steroids (AAS) are synthetic derivatives of testosterone used by over half a million adolescents in the US for their tissue-building potency and performance-enhancing effects. Recent studies show AAS use is increasing among teenagers. AAS have behavioral effects such as heightened aggression and changes in sexual libido in humans. The maturation of adult behavior during adolescence involves complex interactions among hormones, experience, and the developing brain. Our research using Syrian hamsters demonstrates that the absence of gonadal hormones during adolescence irreversibly impairs male reproductive and social communication behaviors in adulthood, indicating that adolescence is a particularly sensitive period for steroid-dependent remodeling of neural circuits underlying complex social behaviors. The overall goal of my work was to determine how AAS interact with the adolescent brain to influence social behaviors during adolescence and in adulthood. Results from my studies show that a cocktail of AAS containing testosterone cypionate, nandrolone decanoate, and boldenone undecylenate increase aggressive and reproductive behaviors in male Syrian hamsters treated for two weeks and tested during adolescence. Nevertheless, the same cocktail has different effects on adult males treated for the same length of time. Both adolescent and adult males suffered from long term effects due to AAS exposure, but only for particular aspects of sexual and agonistic behaviors.

Finally, adolescent AAS exposure affects amygdaloid neural plasticity. These data indicate that the adolescent brain responds differently to exogenous hormones than the adult brain, suggesting that the still developing adolescent brain is vulnerable to perturbations in steroid milieu.

Dedication:

I dedicate this body of work to my parents, Dr. Salvador Salas-Quintana and Dr. Doris Ramírez, for being my role models, inspiration and motivation to obtain the highest degree I could in science. To my sister, Ing. Karilin Salas and her children, Kayra Yimar and Ramón Esteban, who have been my pride and joy since the day they were born. And finally, to my husband Dr. Ken Hoyte, for always reminding me to challenge myself intellectually and that one day, we are going to change the world. Thank you, for being my support network.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEY TO AND ABBREVIATIONS SYMBOLS

Anabolic-Androgenic Steroids ..... AAS

Hypothalamic-Pituitary-Gonadal Axis ..... HPG axis

Drug Enforcement Agency ..... DEA

Testosterone ..... T

Testosterone Propionate ..... TP

Testosterone Cypionate ..... TC

Nandrolone Decanoate ..... ND

Boldenone Undecylenate ..... BU

17α-methyltestosterone 17α-MT

Dihydrotestosterone ..... DHT

Estrogen ..... E

Estradiol Benzoate ..... EB

Progesterone ..... P

Luteinizing Hormone ..... LH

Follicle Stimulating Hormone ..... FSH

Gonadotropin Releasing Hormone ..... GnRH

Androgen Receptor ..... AR

Estrogen Receptor ..... ER

Gonadectomy ..... gdx

Overiectomy ..... OVX

Postnatal day ..... P#

Medial Amygdala ..... Me

Anterodorsal Medial Amygdala ..... MeAD

Anteroventral Medial Amygdala ..... MeAV

Posterodorsal Medial Amygdala ..... MePD

Posteroventral Medial Amygdala ..... MePV

Central Amygdala ..... CeA

Medial Preoptic Area ..... MPOA

Lateral Septum ..... LS

Bed Nucleus of the Stria Terminalis ..... BNST

Anterior Hypothalamus ..... AH

Female Hamster Vaginal Secretions ..... FHVS

Dopamine ..... DA

Serotonin 5-HT

γ-aminobutyric acid ..... GABA

Argenine Vasopressin ..... AVP

Neural Stem Cells ..... NSC

Bromodeoxyuridine ..... BrdU

Light:dark ..... L:D

Treatment ..... TMT

p-value ..... P < #

Analysis of Variance ..... ANOVA

Coefficient of Variation CV

CHAPTER 1: ADOLESCENT ANDROGENIC ANABOLIC STEROID EXPOSURE (AAS): POTENTIAL EFFECTS ON SOCIAL BEHAVIORS AND NEURAL PLASTICITY

AAS are synthetic compounds structurally related to testosterone (T) that promote muscle growth (*anabolic*) and have masculinizing (*androgenic*) effects. They were originally used during World War II to treat soldiers suffering from malnutrition due to their anabolic potency. Later, in the 1940's high doses of AAS were used to treat hypogonadal men that suffer from a reduced or absent secretion of T. Clinically, high dose regimens are used to promote muscle deposition after burns, surgery, radiation therapy and, more recently, to treat HIV+ patients (Kuhn, 2002). AAS have also been used by psychiatrists to treat mood disorders in order to improve mental alertness, mood elevation, and concentration (Uzych, 1992). However, the advantages that anabolic steroids introduce for treating physiological and psychological disorders may become disadvantages when they are used inappropriately (Blue and Lombardo, 1999; Corcoran and Longo, 1992).

Athletes and non-athletes (Bahrke and Yesalis, 2004), males and females, of different ages use AAS for cosmetic and anabolic purposes in doses of 10-100 times higher than clinically prescribed. These high doses may cause acne, cardiovascular disease, headaches, and liver disease. In males it may cause baldness, prostate changes and a decreased libido. In females they cause breast shrinkage, clitoral enlargement and menstrual irregularities (Yesalis et al., 1990). In addition to these pathological changes in peripheral tissues, AAS may

create psychological dependency. Recently, a two stage model for AAS dependency was described (Brower, 2002). The first stage is defined when AAS are used solely for aesthetic purposes for their anabolic effects. The second stage begins after users discontinue administration, but remain psychologically dependent on the drug. Users can become depressed and suffer from withdrawal, similar to what someone suffering from cocaine or heroin withdrawal would, to the extent that suicide attempts may occur (Thiblin et al., 1999b; Yesalis and Bahrke, 2000; Yesalis et al., 1997). Therefore, the user must re-administer AAS in order to avoid these consequences. Some of the psychological adverse effects that are increased by inappropriate use of AAS are: aggression, mania, emotional instability, irritability, hostility, and ultimately violence, all describe under the "roid rage" phenomenon in humans. Within the last decade the Drug Enforcement Agency (DEA) has classified androgenic-anabolic steroids as illegal substances because of their potency and addictive characteristics.

Within the adolescent community there are social pressures to be attractive, which in turn, encourages the use of AAS among teenagers for cosmetic purposes (Bahrke et al., 1998). Furthermore, some adolescents claim to use AAS to become intoxicated and braver (Kindlundh et al., 1999). The onset of AAS use has been documented to be as early as 10 years of age. Over 700,000 adolescents have used AAS (Terney and McLain, 1990), two thirds of these do not use AAS for athletic purposes. In addition, among teenagers the

perception of risk of anabolic steroid use is decreasing over time (Monitoring the Future Study 2004). Data from the Youth Surveillance Survey from Center of Disease Control (1993) shows the prevalence of lifetime illegal steroid use ranged from 3.2% to 7.1% across state surveys (median: 4.8%) and from 2.3% to 7.4% across local surveys (median: 3.1%). Surprisingly, a range of 4 – 12% of high school males have admitted to using AAS (Yesalis and Bahrke, 2000). Similar studies indicate that high school students have little understanding of the adverse effects AAS can have when abused (Tanner et al., 1995), NIDA Steroid Report, 2000; Monitoring the Future, 2004). As a result of steroid misuse human adolescents become defiant, aggressive, and are described to have antisocial personalities, along with increased or decreased sexual libido (Thiblin and Petersson, 2005). They may also suffer from physiological adverse effects like stunted growth and acne.

AAS have traditionally been taken in "cycles" which last 6 to 12 wks or more. By staggering and combining these drugs users attempt to avoid developing a tolerance to a particular AAS, this is called "stacking". Other kinds of drugs are used to prevent the adverse effects caused by the abuse of AAS, like antiduiretics, antiestrogens and human chorionic gonadotrophins are used to counteract the adverse effects cause by AAS abuse (Karila et al., 2004; Martikainen et al., 1986). Nevertheless, studies in humans show that even when a single compound is actively abused severe side effects on the body and psyche still occur (Daly et al., 2001; Kouri et al., 1995; Pope et al., 2000).

AAS and Adolescent Development

A stage of development that is under appreciated as a time of neural change is puberty (Romeo, 2003; Sisk et al., 2003). In the last decade, studies on pubertal and adolescent development provide evidence that the adolescent brain undergoes neural changes that result in the expression of adult-typical behaviors. During this developmental period the emergence of sex-biased psychopathologies such as depression and schizophrenia occurs, along with an increase in risk-taking behaviors, such as sexual exploration, drug use and aggression. Puberty and adolescence are sometimes used interchangeably, however, there are distinct definitions for each: (1) *puberty*: the activation of the hypothalamic-pituitary-gonadal axis (HPG-axis) that results in gonadal maturation and (2) *adolescence*: the maturation of adult social and cognitive behaviors (Sisk and Foster, 2004). Thus, adolescence encompasses both the hormonal changes of puberty and the neural changes associated with behavioral maturation.

Gonadal hormones are known to have both activational and organizational effects on brain and behavior. A*ctivational* effects are defined as the occurrence of a given behavioral response only in the presence of a given hormone. In the absence of hormone, the behavior is not expressed. The classical definition of *organizational* effects is permanent and long-lasting effects of steroid hormones on structural features of neural circuits that occur during a maximally sensitive period of perinatal development and that program behavioral responses to

steroid hormones in adulthood. Therefore, since adolescence is characterized by rapid neural development, steroid hormones may exert organizational or activational effects, or both, during this time.

The HPG-axis is governed by a small population of cells in the hypothalamus that secrete the gonadotropin releasing hormone (GnRH). Activation of GnRH neurons at the onset of puberty causes increased released of GnRH hormone pulses into the median eminence of the hypothalamus, where GnRH enters the pituitary portal system. GnRH acts on gonadotropes in the anterior pituitary, to induce pulsatile release of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH). In males, these hormones act on target cells to stimulate the release of testosterone (T) from the testes. As T levels rise, a neuroendocrine negative feedback loop is activated and it shuts down GnRH neurosecretion and the release of T in male rodents. An additional effect of T in the brain is to facilitate male social behaviors in specific environmental contexts. Exogenous androgens, such as anabolic androgenic steroids (AAS), can also result in the negative feedback inhibition of the HPG-axis and facilitation of behavior (McGinnis, 2004). Because puberty is a time of rapid hormonal and behavioral development, this dissertation focuses on understanding the effects of pubertal exposure to AAS on social behaviors and adolescent neural development.

The use of AAS is increasing significantly among adolescents. There are several gaps in our current understanding that this work addresses: (1) a comparison between the adolescent and adult exposure to AAS on brain and behavior, (2) a comparison of short and long term effects of AAS on the adolescent and adult brain and behavior, and (3) the effects of AAS on neural changes in the brain during puberty. Work from our laboratory using the male Syrian hamster has established that the absence of gonadal hormones during adolescence impairs steroid-dependent social behaviors in adulthood, suggesting that during adolescence, steroid hormones organize neural circuits to program adult behavioral responses to hormone; however, how the administration of supraphysiological levels of steroid hormones (which would occur with AAS use) affect brain and behavior has not been well established.

Adolescence is the transition from childhood to adulthood and encompasses neural, hormonal and behavioral maturation by both steroid dependent and independent events. Gonadal steroid hormones play a critical role during adolescent maturation in two important ways (Sisk et al., 2003). First, they alter brain development and organization by influencing fundamental neural processes such as neurogenesis, cell death, and synapse formation. Second, they influence social experience both by activation of neural circuits underlying social behaviors.

Recent studies in male animal models show that pubertal gonadal hormones also organize the adolescent nervous system, resulting in an adult that will more readily display male-typical behaviors (Sisk and Foster, 2004; Sisk et al., 2003). Our laboratory has therefore proposed a two-stage model for behavioral maturation of male social behaviors. During perinatal development steroid hormones act on the central nervous system to sexually differentiate neural circuits, which later are activated to result in the expression of adult development. In addition to the perinatal period, puberty is a second time when gonadal hormones further organize neural circuits to result in the full expression of adult-typical behaviors (Romeo, 2003; Sisk et al., 2003).

Studies in our lab using the male Syrian hamster have repeatedly supported the organizational hypothesis during puberty. Prepubertal males, even when treated with adult physiological levels of T, do not express the full repertoire of reproductive behaviors, suggesting that the prepubertal brain is not prepared for the activation of these behaviors by T (Meek et al., 1997; Romeo et al., 2001). In several studies, an experimental paradigm tested the hypothesis that the presence of gonadal hormones during puberty organizes neural circuits to allow the activation of reproductive behavior in adulthood. In this paradigm, sexually naïve males were gonadectomized (gdx) before or after puberty. Six weeks after castration, in adulthood, a subcutaneous T pellet was implanted for one week and animals were tested with a receptive female for reproductive behaviors. Animals that were castrated before puberty expressed significantly

lower mounts, intromissions and ejaculations (Schulz et al., 2004). Similar results were observed in flank marking behavior when using the same hormonal manipulation and a resident intruder paradigm: animals that were gdx before puberty displayed fewer flank marks (Romeo et al., 2003; Sisk et al., 2003). These results suggest that developmental events occurring during puberty affect the responsiveness of the nervous system to the activational effects of gonadal steroids in reproductive and agonistic behaviors.

Using a similar experimental paradigm, we found that the impairments in reproductive behavior resulting from the absence of gonadal hormones during puberty were long-lasting and not easily reversed. Neither prolonged T treatment (17 days) nor sexual experience (3 exposures to a receptive female) abolishes the behavioral differences between males undergoing adolescent development in the presence versus absence of hormones. Taken together, we conclude that steroid hormones in adolescence program the expression of social behaviors in adulthood (Schulz et al., 2004). These results also suggest that adolescent exposure to pharmacological levels of steroids, such as those resulting from AAS use, may have more severe and permanent consequences than AAS exposure in adulthood.

The adolescent brain undergoes profound change that results in both synaptic reorganization and modifications in gross morphological features. Imaging techniques show dramatic differences in grey matter and white matter

during this developmental period in humans. The brain's grey matter volume increases during early adolescence and then decreases during late adolescence in both males and females. These changes are particularly robust in the frontal cortex and cerebellum (Giedd et al., 1999). Interestingly, the average age at peak gray matter volume occurs earlier in girls than in boys, and corresponds to the earlier age at onset of puberty in girls, suggesting a potential role for gonadal hormones in this process. Biphasic changes are also observed in striatal dopamine receptors in adolescent male rats. There is a large increase of D2 receptors in male rats between postnatal day (P) 25 and 40 (early puberty), and then later there is a "pruning" process when the receptor density decreases almost 55% by P80 (Andersen et al., 2002; Andersen et al., 2000). Interestingly, this phenomenon is still observed in animals that are gonadectomized prepubertally, indicating that this process is steroid-independent.

Pharmacology of Anabolic Androgenic Steroids:

There are three different classes (Figure 1.3) of AAS that can be administered in a variety of ways, a transdermal patch, skin cream, injectable or an oral preparation. Like endogenous androgens, AAS are four-ringed structures with nineteen carbon atoms. Modifications to these carbons were made in order to prolong the metabolic half-life and the efficacy of the synthetic AAS (Clark and Henderson, 2003). Class I AAS, like testosterone cypionate (TC) and testosterone propionate (TP), are injectable and are derived from an esterification of the 17β-hydroxyl group of testosterone. Class II AAS are also

injectable androgen esters; however, they are defined as the 19-nor-testosterone derivatives because of the substitution of a hydrogen bond for methyl group at C19. This substitution extends the half-life of the compound that cannot be attributed to esterification alone. Among steroid users some popular class II AAS are nandrolone decanoate (ND) and boldenone undecylenate (BU), which has slow clearance from the body up to 12 wks (Ballard and Wood, 2005). Most class I and class II AAS are aromatizable and can have significant effects in the central nervous system (CNS) not only via the androgen receptor (AR) but also by actions on the estrogen receptor. Class III AAS are mostly non-aromatizable and cannot be converted to either dihydrotestosterone (DHT) or 17β-estradiol (E_2) although other androgenic and estrogenic metabolites may be created by the degradation of these compounds. They are known as the 17α-alkyl AAS because they are alkylated in the C17 of the four-ring structure. Since the alkylation slows down the metabolism by the liver, these are orally active. Some class III AAS are 17α-methyltestosterone (17α-MT), oxymethalone and stanozolol (ST) (Clark and Henderson, 2003).

Characterization of Social Behaviors in Male Syrian hamsters:

We use the male Syrian hamster to investigate the neural mechanisms that are responsible for pubertal change in male social behaviors, and how these are disrupted. In our lab we have characterized and defined aggressive and reproductive behaviors in male Syrian hamsters through development. We also

have a good understanding of their chemosensory processing neural pathway (Wood, 1998; Wood and Coolen, 1997), along with the neural circuits that mediate social behaviors in both pubertal and adult males. We have demonstrated that behavioral responses to T change as a function of pubertal development in this species (Meek et al., 1997) and the endocrinological profile has been established as the male progresses through puberty (Miller et al., 1977). All these are important aspects for the use of this animal model and understanding the effects of AAS on male social behaviors during adolescence and adulthood.

The amygdala: Social behaviors and Neural Plasticity

Pheromonal communication and hormonal signals are critical for the expression of male social behaviors in male rodents. The neural circuits that underlie male reproductive behaviors (which includes the bed nucleus of the stria terminalis (BNST) and the medial preoptic area (MPOA)) and inter-male aggressive behaviors (which includes the anterior hypothalamus (AH), the BNST, and the lateral septum (LS)) share efferent and afferent connections to and from the medial amygdala (Me) (Ferris et al., 1987; Kollack-Walker et al., 1999; Kollack-Walker and Newman, 1995; Potegal et al., 1996; Romeo and Sisk, 2001) The connections of the amygdala in rats, cats and monkeys can be divided into three systems (1) sensory processing through connections with the olfactory cortex, posterior thalamus, and sensory association cortex, (2) visceral function in relation to the emotional stimuli through connections with the hypothalamus,

and (3) emotional behavior and mood through connections that compose the forebrain circuit (Price, 2003). The Me is a complex structure that has been subdivided based on cytoarchitectonic, synaptic and functional criteria, into four main nuclei: anterodorsal (MeAD), anteroventral (MeAV), posterodorsal (MePD) and posteroventral (MePV) (Alheid, 2003; Cooke and Simerly, 2005). The Me subnuclei contribute to the interpretation of sensory information (Wood and Coolen, 1997), for the regulation of intermale aggression and reproductive behaviors, (Kollack-Walker and Newman, 1995) and to the interpretation of emotionally loaded stimuli and memory modulation (Rasia-Filho et al., 2004) . In addition, it is considered part of the "extended amygdala", composed of parallel rings of cells, extending through the medial and central amygdaloid nuclei, the bed nucleus of the stria terminalis (BNST) and the substantia innominata, along with structures in the basal forebrain, which are related by the shared characteristics in cell morphology, reciprocal neuronal connections and neurochemical/neurostransmitter identity (Alheid, 2003; Canteras et al., 1995; Newman, 1999).

The results of several studies suggest that the subdivisions of the Me play different roles in the mediation of male social behavior (Wood and Newman, 1995b). The MeA is primarily a relay for chemosensory information, while the MeP relays mostly steroidal information (Meredith and Fewell, 2001). However, their functions within circuits underlying social behaviors do overlap. The medial amygdala may be involved in learning and memory for socially significant cues

(Kollack-Walker & Newman, 1995). Both the MeA and MeP nuclei express androgen and estrogen receptors (Wood & Newman, 1995), and both show a significant increase found in Fos-immunostaining, a marker of neuronal activity, in response to pheromonal cues in estrous female vaginal secretions (in both pubertal and adult males) (Romeo et al., 1998), and in response to the presence of a stimulus male (Johansson-Steensland et al., 2002; Kollack-Walker and Newman, 1995). Lesions in the medial amygdala, both the MeA and the MeP, disrupt a male's ability to engage in copulation (Lehman et al., 1980; Newman and Bachevalier, 1997) . Reduced aggressive behavior is observed in lesioned animals after experience with four aggressive interactions, suggesting that these lesions interfere with social learning processes (Vochteloo and Koolhaas, 1987).

Gonadal hormones play a role in neural plasticity during puberty (Romeo & Sisk, 2001). The MeA and MeP are steroid sensitive, and morphological changes in these nuclei may reflect changes in the expression of male social behaviors. The removal of gonadal hormones in adult males results in a decrease in somal size of individual cells and/or overall volume of the Me. Interestingly, the MeA is significantly larger in prepubertal male Syrian hamsters when compared to adults; however the inverse in true about the MePD. This may be explained by function of the amygdala and its sensitivity to gonadal hormones. The MeA receives substantial projections from the accessory olfactory bulb, and lesions to the MeA abolish chemoinvestigatory behavior, suggesting the pubertal development may involve changes that allow appropriate

processing and integration of these cues (Gomez and Newman, 1992; Wood and Newman, 1995b). However, the MeP contains more androgen (AR) and estrogen receptors (ER) than the MeA (Cooke et al., 2003; Wood and Newman, 1995a), suggesting that it may retain the capacity for plasticity throughout life so that the expression of male social behaviors is possible under specific steroidal and/or environmental conditions (Romeo and Sisk, 2001).

When male rodents are gonadectomized and lack gonadal hormones the MePD volume decreases. The MePD contains both androgen (AR) and estrogen receptors (Cooke et al., 2003; Cooke et al., 2001) that may be regulating the plasticity of this neural structure. When adult male rats are castrated and given silastic capsules containing DHT and/or E_2 or blanks, all groups were compared to control shams. Steroid, alone or in combination, maintained ejaculatory behavior and vocalizations patterns. Interestingly, when the volume measures were taken of the amygdala, regional volume and cell soma size of the MePD following castration was lateralized (the left hemisphere neurons were larger than the right). Both DHT and E_2 each maintained neuronal soma size in both hemispheres. This data suggests that steroid hormones that participate in the maintenance of male sexual behavior mediated by both receptors in the MePD contribute to the steroid-regulated structural plasticity in the brain region.

Estrogen increases the number of AR and the duration of the ligand occupancy in brain regions involved in the control of mating, and androgens downregulate the expression of ER (Brown et al., 1994) suggesting an interaction

between both steroids. Brains from adult male rats were used to assess whether AR and ER receptors were co-localized in several structures in the brain that mediate male reproductive behavior, one of which is the MeA, after being exposed to several sexual interactions. Specifically, MeA contains both receptors in male rodents (Greco et al., 1999). In the MePD of male Syrian hamsters, 57.5% of neurons contain AR and 13% of neurons contain ER (Wood & Newman, 1995). The differential distribution of AR and ER in the Me neurons provide information for a potential synergistic actions of androgens and estrogens on their receptors. T bound to AR is converted by aromatase to E_2, and within the same cells activation of ER facilitates androgenic actions by increasing the duration of occupation of AR and the amount of AR. The behavioral effects of androgens could be further enhanced by the binding of T to AR in cells not containing ER.

Androgen-dependent plasticity in the MePD has also been by using Golgi-staining in male Syrian hamsters. In castrated males, neurons in the posterior, but not in the anterior, region of the Me undergo structural changes (Gomez and Newman, 1992). This was indicated by a decrease in the mean highest dendritic branch level and mean somal area when compared to intact hamsters. Animals with implants of a testosterone (T), dihydrotestosterone (DHT) and/or 17β-estradiol (E_2) were also compared to intacts and castrates; all of these were subjected to a behavior test with a receptive female. Castration and treatment with DHT resulted in a decrease in mean somal area, the mean highest dendritic

branch and the percentage of neurons with tertiary branch segments. DHT treatment also resulted in a reduction of total dendritic length and spine density. These results suggest that T or E is sufficient to maintain normal morphology of the neurons in the MePD. Recently, Zehr et al. (2005) demonstrated that that MePD neurons go through dramatic changes and structural remodeling during puberty such as changes in terminal spine densities, axons and dendritic arborization. Prepubertal animals have a greater number of dendrites and higher spine density than midpubertal and postpubertal animals. During puberty, pruning of dendrites and spines, along with axonal changes occur with increasing steroid levels.

Gonadal steroid hormones play an important role in the proliferation (Ormerod and Galea, 2001), survival (Leranth et al., 2000) and activation (Insel, 1990) of neurons. Adult male voles were gdx and treated with TP, EB or DHT. Animals also received injections of a proliferation marker, 5-bromo-2'deoxyuridine (BrdU), which gets incorporated in the DNA of a mitotic cell during the S phase of mitosis. Treatment with TP and EB exerted similar effects in increasing the density of BrdU labeled cells in the Me (44% newly born neurons as identified by a double labeled cells with TuJ1. TuJ1 is considered the earliest marker for cells that have begun to differentiate into neurons), whereas DHT was ineffective. Taken together, these data suggests that gonadal steroid hormones influence the number of newly born cells in the amygdala (Fowler et al., 2002).

A study in adult male Syrian hamsters observed newly proliferated cells in the medial amygdala in adulthood (Huang and Bittman, 2002). Animals were exposed were exposed to an estrous female, an aggressive male or a cotton swab containing FHVS. Tissue sections were stained for BrdU and c-Fos in the olfactory bulb, MPOA, Me and BNST. The activated cells were seen in the olfactory bulbs after the males were exposed to the estrous female. A comparable pattern of activation was seen in the amygdaloid complex when compared to the other nuclei quantified in this study. BrdU labeled cells were observed 3 wks after the last BrdU injection in the amygdala. However, no double labeled cells were seen in any of the hypothalamic nuclei. This data supports previous studies seen in male voles suggesting a proliferation of newly born cells in the Me, however, their function is still not defined.

AAS and the Amygdala:

The medial amygdala plays a role in the mediation of social behaviors that are also affected by the use of AAS during adolescence and adulthood. However, few studies have been performed to determine the effects of AAS on androgen and estrogen receptors in the mammalian brain (Clark and Henderson, 2003; Lynch and Story, 2000). As stated previously, Class I and Class II AAS can be aromatized to bind to AR and ER in the CNS. For changes to occur, AAS must bind to AR and ER and affect cellular gene expression. Treatment of a cocktail of AAS (2 mg/kg testosterone cypionate, 2 mg/kg nandrolone decanoate and 1 mg/kg boldenone undecylanate) to intact male rats for 2 wks increase the

expression of AR in the classical androgen target sites like the MePD, MPOA and BNST (Menard and Harlan, 1993); all of these structures are involved in the mediation of male social behaviors. High levels of AR-ir in non-classical structures that usually express little AR (ie., caudate putamen, ventral tegmental area, central gray, hippocampus, and locus coeruleus) as well as classical AR dense structures were also observed. This provides evidence that AAS can produce significant changes in both "steroid-sensitive" and "steroid-insensitive" brain regions, which in turn can affect the expression of behavior. These results identify an up-regulation of AR in the male rat brain by AAS.

Another study compared the effects on AR-ir by the same AAS cocktail, dihydrostestosterone (DHT), and E_2 for 14 days in intact and castrated rats. The male rats administered the cocktail and DHT fully upregulated AR-ir in the ventromedial hypothalamus (VMH), MPOA and MeP. Estradiol caused partially up-regulated AR-ir in the VMH and MeP, but not in the MPOA in castrated rats when compared to intact males (Lynch and Story, 2000). Taken together, these studies show that androgens can have profound effects on brain metabolism and function via AR and ER. These measurements can be used to elucidate how AAS can modify central nervous system mechanisms.

Studies examining neurochemical changes in the sexually maturing, adolescent brain exposed to supraphysiological levels of AAS have determined that AAS cause changes in the neurochemical communication pathways

associated social behaviors. It is well established that serotonin (5HT) and arginine vasopressin (AVP) mediate aggression in male Syrian hamsters. Reduced serotonin levels and increased vasopressin levels have been associated with increased levels of aggressiveness. Adult male rats were treated with nandrolone decanoate (ND) for 2 wks. Changes in 5HT receptors due to AAS administration were visualized by using autoradiography. A decrease in $5HT_2$ R was observed in the medial amygdala (Kindlundh et al., 2003a). Significant changes are also seen in other hypothalamic regions. A cocktail of AAS causes neuroanatomical changes after 30 days of consecutive administration from 27-53 days of age in male Syrian hamsters. After administration they were tested for aggressive behaviors. AAS treated males had increased levels of offensive aggression. When examining serotonin fibers (5-HT) in the MeA, there was a significant decrease in animals that were treated with the AAS (DeLeon et al., 2002; Grimes and Melloni, 2002). Using the same AAS regimen and autoradiography, a significant increase was seen in the expression of AVP V_{1A} receptors in the cortical nucleus of the Me and other regions that regulate aggressive behavior, like the anterior hypothalamus.

When same cocktail of AAS is used to look at GAD_{65}, the rate limiting enzyme in the synthesis of γ-aminobutyric acid (GABA), in adolescent brains of aggressive male hamsters, a significant increase was observed in the immunoreactive puncta in the medial amygdala of these animals (Grimes et al., 2003). Increased levels of GABA result in increased levels of aggression

(Potegal et al., 1982). Studies of neurohormonal receptors in the adolescent male rodent brain show that a high dose of AAS during puberty can cause changes in the GABA receptor subunits. No significant changes can be observed in adulthood. Taken together, these data demonstrate that the effects of AAS exposure affect the adolescent developing brain and result in modifications to male social behaviors.

Use of AAS can affect brain function leading to behavioral changes. A study using rat neural stem cells (NSC) in culture, and the dentate gyrus in the rat hippocampus was conducted to test the effects nandrolone decanoate has on neurogenesis and cell proliferation. *In vitro,* NSC were stimulated by epidermal growth factor, nandrolone reduced cell proliferation. Nandrolone also decreased neurogenesis (measured by bromodeoxyuridine (Brd-U) labeling) in the dentate gyrus of the rat hippocampus in both adult males and females after 5 days of administration (Brannvall et al., 2005).

Overall, AAS can affect neural organization and circuitry, which in turn results in changes in behavior.

Preliminary Experiments

In the first preliminary experiment we examined the effects of pubertal exposoure to 17-α-methyltestosterone (17α-MT), a class III non-aromatizable AAS, on male aggressive behaviors. 17α-MT (0 mg/kg, 1 mg/kg, 3 mg/kg, and 5

mg/kg) was administered subcutaneously (sc) for three weeks beginning at puberty onset (P21 – P41). One day after the last day of administration, mid-pubertal (P42) male hamsters were then exposed to a weight and age matched stimulus male in a neutral arena (with a 5 minute acclimation period before testing); all tests were videotaped and quantified. Not all aggressive behaviors were increased in AAS-treated males at all doses, but the results were suggestive of an increase in aggression with the highest dose of 17α-MT. We observed a significant decrease in attack latency in a 5 mg/kg dose (Fig. 4; p-value = 0.008), and a trend towards an increase in flank marking (p-value = 0.07) and overall aggression score (Fig. 1.4; attacks + bites + flank marks – submissive behaviors; p-value = 0.07). In addition, when behavior of the stimulus (untreated) males was compared with the experimental treated males, the treated males expressed most, if not all, of the dominant behaviors within the social interaction. It shows that a non-aromatizable AAS can affect some aspects of aggressive behaviors when administered over three weeks during puberty; nevertheless, a combination of aromatizable and non-aromatizable AAS may be needed to activate the full repertoire of aggressive behaviors. Physiological measures such as body weight from the beginning of administration to the end, testes weight and T plasma levels where taken, however there was no difference among any of the treatment groups.

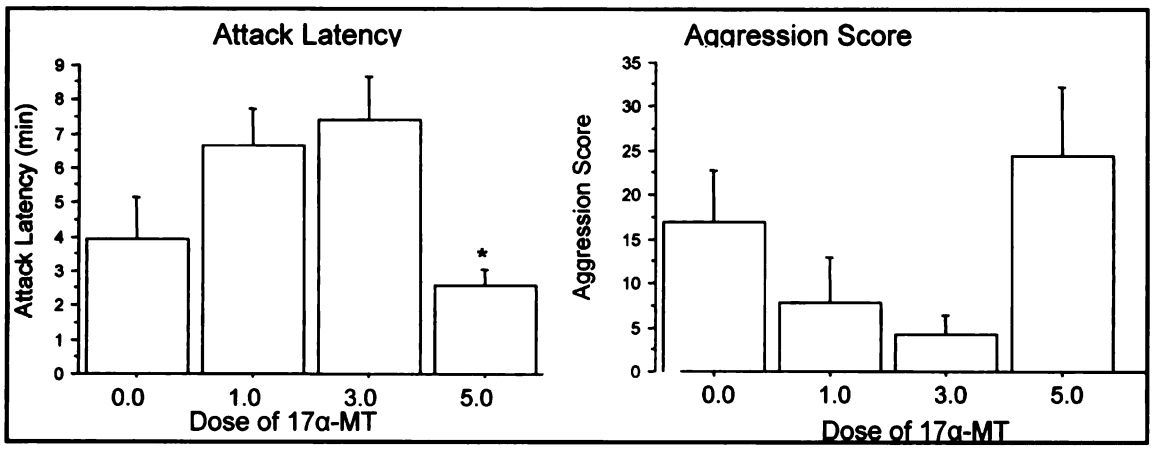


Figure 1.1 Effects of 17α-methyltestosterone on attack latency and aggression in pubertal male Syrian hamsters.

The second experiment compared several doses of 17α-MT (0 mg/kg, 2.5 mg/kg, 5 mg/kg, and 10 mg/kg) with a cocktail of AAS. The cocktail included a compound from all three classes of AAS: class I = testosterone cypionate (TC; 2 mg/kg), class II = nandrolone decanoate (ND; 2 mg/kg), and class III = 17α-MT (5mg/kg). Aggression tests used a neutral arena behavioral paradigm without an acclimation period; therefore, neither animal had the advantage of investigating their surroundings. Behaviorally we wanted to assess social dominance. Stimulus males received saline injections to decrease handling differences among animals. AAS administration was for a total of 4 weeks (P21-P49). After matching the experimental males with age and weight matched partners, a 10 min behavior test was given and videotaped, without any acclimation period. Aggressive behaviors were quantified; however there were no significant differences in any of the treatment groups. Physiological measures were also taken including, seminal vesicle weight, testes weight, body weight and testosterone (T) plasma levels. There was no significant change in seminal

vesicle weight. However, the animals that were injected with the cocktail had overall significantly lower testes weight (p –value < .001) and body weight (p – value <.01) when compared to all of the animals that received 17α-MT. Also, their T plasma levels were significantly lower (p – value <.02) than animals treated with the vehicle. These data suggest that AAS effect reproductive target tissues, and interferes in the normal function of the HPG axis by decreasing T levels in pubertal males; this may result in deficits of the expression of social behaviors in adult males.

Taken together, these experiments suggest several things that affect the expression of aggressive behaviors. First, social context is important when testing for aggressive behaviors. In the first experiment we used a neutral arena paradigm with a five minute acclimation period for the treated male, which resulted in a slight increase in the aggression score. However, there were no behavioral effects of AAS in the second experiment, where there was no acclimation period. Experiments in male rats treated with AAS used a tail-pinch to provoke an aggressive response before a social encounter with another male (McGinnis et al., 2002), suggesting that social context is important for the expression of aggressive behavior. Second, a single compound may not be sufficient to increase aggressive behaviors relative to vehicle treatment. 17α-MT is a class III AAS that is not aromatized, suggesting that AAS that act on AR and ER are more like to induce aggressive behaviors. Finally, a cocktail of class I and II AAS affects the reproductive physiology of male hamsters, especially after a

long period of administration. These data suggest that AAS may induce a negative feedback loop on the HPG axis, resulting in decreased testes weights and decreased plasma levels.

A third experiment focused on comparing the cocktail used in the previous experiment with one that has been documented in the literature to increase aggressive behaviors in pubertal male Syrian hamsters and to upregulate AR in classical and non-classical AR-dense structures in the brain (Lynch and Story, 2000; Menard and Harlan, 1993). Aggressive behaviors were assessed using a resident-intruder paradigm, creating a context where the treated animal must protect his "home" (Romeo et al., 2003) Animals arrived on P18 and were group housed until P25. On P27 animals were divided in three groups in order to compare two cocktails of AAS to a vehicle group. One cocktail (A) was composed of all three classes: 2 mg/kg TC, 2 mg/kg ND and 5mg/kg 17α-MT. The second (B) was made up of class I and class II AAS: 2 mg/kg TC, 2 mg/kg ND and 1 mg/kg boldenone undecylenate. After two weeks of daily injections (P27-P41), one day after the last injection (P42) a behavior test was administered with an age and weight matched gonad-intact male intruder. Aggressive behaviors were significantly increased (Figure 1.5; attacks, attack duration, bites, and contact time; p – value <0.003) and defensive posturing was significantly decreased ($p < 0.06$) by cocktail B. Cocktail A produced levels of aggression intermediate to those seen groups treated with vehicle and cocktail B. Physiological measures taken reflected that cocktail B also significantly

decreased body weight in those two weeks (p - value < 0.03). Testes weight was significantly decreased by both cocktails (p - value < 0.01). There were no significant differences in T blood plasma levels. These results suggest that a cocktail of aromatizable AAS has greater effects on the expression of aggression during puberty that a cocktail with a non-aromatizable AAS and a single non-aromatizable compound.

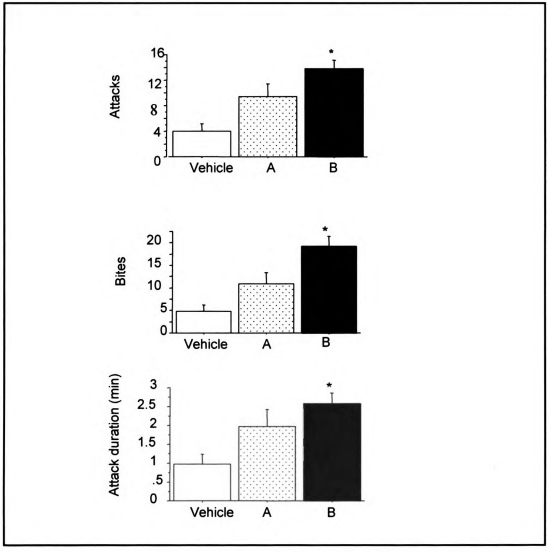


Figure 1.2 Attack number, bites and attack duration were significantly increased with an AAS cocktail containing TC, ND, and BU (B) compared to a cocktail containing TC, ND and 17α-MT (A) and vehicle.

These three experiments determined the optimal behavioral context for assessing aggressive behaviors in intact male Syrian hamsters, along with identifying that a cocktail of AAS results in an increase of aggressive behaviors when compared with single compounds and vehicle controls. The following experiments addressed (1) whether this effect is also seen in adult male hamsters and in reproductive behaviors, (2) whether the AAS exposure have long term effects on social behaviors in pubertal and adult animals and (3) whether AAS affect neural plasticity specifically in the amygdala.

Chapter 2: ANABOLIC ANDROGENIC STEROIDS DIFFERENTIALLY AFFECT SOCIAL BEHAVIORS IN ADOLESCENT AND ADULT MALE SYRIAN HAMSTERS

Introduction

Adolescence is the developmental transition from childhood to adulthood and it encompasses reproductive, neural, and behavioral maturation (Andersen, 2003; Casey et al., 2000; Lenroot et al., 2007; Sisk and Foster, 2004; Sisk and Zehr, 2005; Spear, 2000; Yurgelun-Todd, 2007). The onset of puberty marks the onset of adolescence, and gonadal steroid hormones, which become elevated during puberty, influence subsequent adolescent brain development by both organizing and activating neural circuits underlying social behaviors (Delville et al., 1998; Primus and Kellogg, 1989; Primus and Kellogg, 1990; Romeo, 2005; Schulz and Sisk, 2006a; Schulz and Sisk, 2006b; Sisk and Foster, 2004; Spear, 2004). Importantly, the organizational effects of pubertal hormones program their activational effects in adulthood. For example, studies in the Syrian hamster show that the presence of testicular hormones during adolescence results in adults that more readily display male-typical social behaviors relative to adults that matured in the absence of testosterone (T) (Schulz et al., 2006; Schulz et al., 2004; Schulz and Sisk, 2006a; Sisk et al., 2003). Thus, the adolescent brain is exquisitely sensitive to endogenous testicular steroids, and the action of testicular hormones during adolescence has both short-term and long-term consequences on social behaviors.

Given the significance of endogenous T in normal development of the adolescent male brain, it is important to consider how anabolic androgenic steroids (AAS) influence the adolescent brain and behavior. AAS are synthetic compounds structurally related to T that promote muscle growth (*anabolic*) and have masculinizing (*androgenic*) effects. AAS are clinically used to treat malnutrition and mood disorders (Kopera, 1993; Perry et al., 2002); however, the benefits they have for treating physiological and psychological disorders may become detriments when they are used inappropriately (Blue and Lombardo, 1999; Corcoran and Longo, 1992). Adolescents that use AAS are often described by their peers, teachers, and psychologists as defiant, aggressive, and antisocial. In addition, teenagers may display either increased or decreased sexual libido after AAS misuse (Thiblin and Petersson, 2005). In the United States, the onset of AAS use can be as early as 10 years of age, and use among high school males has been as high as 12% in the last decade (Ahima and Harlan, 1992; Bahrke and Yesalis, 2004; Bahrke et al., 1990; Bahrke et al., 1998; Buckley et al., 1988; Terney and McLain, 1990). Although there is evidence that severe consequences occur after misuse of AAS by adolescents, there has been relatively little systematic investigation of how these exogenous compounds affect the adolescent brain and behavior.

AAS affect the expression of social behaviors in most rodent models. The behavioral responses to AAS depend on the chemical structure of the steroid administered, whether steroid treatment consists of a single compound or as a

cocktail, and the age and duration of treatment. Intact adult male rats treated with T and testosterone cypionate (TC) do not display altered reproductive behaviors when compared to controls (Clark et al., 1997; Farrell and McGinnis, 2003). However, T administered to intact *adolescent* male rats increased sexual behaviors in adulthood (Wesson and McGinnis, 2006). Also, long-term (12 wk) treatment of intact male rats with oxymethelone, stanozolol, nandrolone or 17α-methyltestosterone during adolescence and continuing to adulthood, results in a decrease in sexual behaviors (Clark et al., 1997; Farrell and McGinnis, 2003; Feinberg et al., 1997). AAS also affect aggressive behaviors in rodents. Single compounds such as T and testosterone propionate (TP) increase aggression in intact adult male rats and mice (Albert et al., 1989; Clark and Barber, 1994; Farrell and McGinnis, 2003; Lumia et al., 1994; Martinez-Sanchis et al., 1998). However, adolescent exposure to single compounds such as nandrolone, 17α-methyltestosterone, or stanozolol, has little or no effect on aggression in rats or hamsters (Breuer et al., 2001; Farrell and McGinnis, 2003; McGinnis, 2004; Wesson and McGinnis, 2006). Adolescent male Syrian hamsters exposed to a cocktail of AAS for two or four weeks show an increase in aggressive behaviors (DeLeon et al., 2002; Grimes and Melloni, 2002; Grimes et al., 2003; Melloni and Ferris, 1996; Ricci et al., 2004; Salas-Ramirez and Sisk, 2005). It is clear that a number of variables determine the overall effects of AAS on sexual and aggressive behaviors, and age has not been systematically studied.

The use of anabolic steroids by adolescent males, and growing evidence that the adolescent brain is particularly sensitive to hormones and experience call for a better understanding of how age at the time of exposure to AAS influence behavioral responses to AAS. The objective of this study was to directly compare the immediate effects of AAS on sexual and aggressive behaviors in adolescent and adult intact male Syrian hamsters. Because adolescent male hamsters are more responsive than adults to the organizational effects of endogenous testicular hormones on male social behaviors (Schulz and Sisk, 2006), we hypothesized that adolescents would also be more responsive to activational effects of exogenous AAS, leading to the prediction that AAS will elicit greater behavioral responses in adolescent compared to adult males.

Materials and Methods

Animals

Eighteen-day-old male Syrian hamsters (*Mesocricetus auratus*) were obtained from Harlan Sprague–Dawley Laboratories (Madison, WI). Upon arrival males were housed in groups of eight animals per cage (polycarbonate, 33 x 38 x 17 cm) with ad libitum access to food (Telkad Rodent Diet No. 8640, Harlan) and water. Animal colony temperature was maintained at 22 ± 2°C, and animals were kept on a light-dark cycle of 14:10 L:D (lights off at 1600h EST). At 25 days of age, animals were singly housed in polycarbonate cages (30.5 x 10 x 20 cm, for Experiment 1; 33 x 38 x 17 cm, for Experiment 2). All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and

all protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

AAS Treatment

A cocktail of AAS containing 0.8 mg/ml of testosterone cypionate (TC; Sigma Aldrich, Inc., St. Louis, MO), 0.8 mg/ml of nandrolone decanoate (ND; Sigma Aldrich, Inc.), and 0.4 mg/ml of boldenone undecylenate (BU; Steraloids, New Port, Rhode Island) was dissolved in 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, Inc.). The AAS cocktail was prepared the day before treatment began for each age group in each experiment, and was stored at 4˚C.

Experimental design

Two separate experiments were conducted. The experimental designs were identical except that the final behavioral test in Experiment 1 assessed sexual behavior with a receptive female whereas in Experiment 2 it assessed agonistic behaviors with an intruder male. At 27 days of age, gonad-intact male hamsters were randomly divided into AAS-treated or vehicle-treated groups. These males received a daily subcutaneous (*sc*) injection of either the AAS cocktail (2 mg/kg TC, 2 mg/kg ND, 1 mg/kg BU) or vehicle (2-hydroxypropyl-β-cyclodextrin), either during adolescence (from 27-41 days of age) or during adulthood (from 63 -77 days of age). Group sizes were originally n=10/age and treatment group in Experiment 1 (assessment of sexual behavior) and n=15/age and treatment group in Experiment 2 (assessment of aggressive behavior). Body

weight for each animal was obtained daily in order to calculate the injection volume required to administer the desired dose. Volume ranged from 0.1 ml to 0.25 ml over the two-week course of administration across age groups. Tests for either sexual or aggressive behaviors were conducted one day after the last injection, at 42 days of age for males treated during adolescence and at 78 days of age for males treated as adults. All behavioral interactions were videotaped with a low-light color video camera and were scored blind using a computer program that tags each behavioral code with an elapsed time (software generously provided by Dr. Kim Wallen, Emory University, Atlanta, GA). This system allows the calculation of counts, duration, and latency for each behavior.

Sexual Behavior Tests (Experiment 1)

Males were sexually inexperienced prior to a standardized behavior test with a receptive female. Tests were conducted between 1700 and 2030 h EST under red-light illumination, as described in previous studies (Heise et al., 2003; Romeo et al., 2002; Romeo and Sisk, 2001; Schulz et al., 2004). Following a 5 min acclimation to the test chamber (a 51 x 26 x 31.5 cm glass aquarium with a mirror underneath), males interacted with a stimulus female for 15 min. Ovariectomized stimulus females were hormonally primed to induce receptivity with an estradiol benzoate injection (10 μg in 0.05 ml sesame oil) 48 h before, and a progesterone injection (500 μg in 0.1 ml sesame oil) 4 h before behavior testing. Before the 15 min behavior test with the subject male, stimulus females were tested for receptivity with a sexually experienced stud male. Anogenital

investigation of the female, mounts, intromissions, and ejaculations were scored and quantified as previously described (Schulz et al., 2004). Data from two adolescent and two adult subjects were excluded because females did not remain receptive throughout the 15 minute test. Data from three other adolescent and adult males were excluded because of technical difficulties during videotaping of the behavioral tests. Two adult AAS-treated animals died during treatment. Thus, final sample sizes for Experiment 1 were: adolescent vehicle-treated, n=7; adolescent AAS-treated, n=8; adult vehicle-treated, n=9; adult AAS-treated, n=7.

Agonistic behavior tests (Experiment 2)

Animals were tested for agonistic behaviors using a resident-intruder paradigm. The socially naïve males were tested between 1700 and 2030 h EST in a red-light illuminated room. A plexiglass extension was placed in the home cage of the subject (AAS- or vehicle-treated) in order to make the walls of the cage taller. After a 5 min acclimation period, an age- and weight-matched intruder male was placed in the treated male's home cage. Intruder males were purchased from the same vendor and arrived at the same time as the subject males. Ten-min behavior tests were videotaped and scored for aggressive and submissive behaviors.

The aggressive/dominant behaviors quantified during each behavior test were defined as follows:

(1) Flank marks: the subject rubbed its pigmented sebaceous flank gland against the wall of his home cage. In male-male social encounters, flank marking serves to communicate dominance status (Drickamer et al., 1973; Ferris et al., 1987; Johnston, 1975).

(2) Total contact time: the length of time the subject male approached the intruder male and showed interest by sniffing or remaining in close proximity.

(3) Attacks: the subject moved quickly toward the intruder male to bite or to attempt to bite. The pair either tumbled around or the treated male chased the intruder male.

(4) Bites: The subject male bit the intruder.

(5) Offensive posturing: the subject stood upright and faced the intruder with his front paws raised.

Defensive/Submissive behaviors were defined as follows:

(1) Defensive posturing: the two males approached each other and the subject male twisted sideways with his paw or paws stretched toward the other male to ward off an attack.

(2) Tail-up walking: the subject walked around the home cage with his tail raised and back arched. This behavior was usually exhibited in response to the intruder male sniffing or investigating the hindquarters and communicated submissive status.

(3) Escape dashes: a rapid movement away from the intruder male, which occurred only after some form of social interaction, indicating that the subject was fearful of the intruder male.

To assess overall aggressiveness of each subject, a composite aggression/dominance score was obtained by adding the number of flank marks, attacks and bites, and subtracting tail-up walking.

Two adult males were excluded from the statistical analyses of aggressive behaviors because of extremely low levels of social interaction during the test; they were determined to be outliers by the Dixon outlier test. In addition, two males assigned to the adult vehicle-treated group were not used because of poor health prior to the treatment period. Data from six other males were excluded because of technical difficulties during videotaping of the behavioral tests. Thus, final sample sizes for Experiment 2 were: adolescent vehicle-treated, n = 13; adolescent AAS-treated, n = 14; adult vehicle-treated, n = 11; adult AAS-treated, n = 12.

Physiological Measures

Immediately following the behavior test, animals were administered an overdose of sodium pentobarbital (130 mg/kg i.p.) and a terminal blood sample was taken via cardiac puncture. Testes and seminal vesicles were removed and weighed. At the time of sacrifice, flank gland diameters were assessed by shaving the hindquarters and measuring the gland with a caliper.
Plasma testosterone concentrations were measured in duplicate using a commercially available radioimmunoassay kit (Coat-a-Count Total Testosterone, Diagnostic Products, Los Angeles, CA). This assay has been previously

validated for measuring endogenous plasma testosterone levels in hamsters in our laboratory (Parfitt et al., 1999). The intra-assay coefficient of variation (CV) was 5.9%, and the limit of detectability was 0.1 ng/ml. We determined whether the assay detected exogenously administered AAS by using the Coat-a-Count Total Testosterone kit to assay known amounts (100 µl, 10 µl and 1µl) of the cocktail. The assay detected only 0.15% of the anabolic steroids added to the assay tube.

Plasma luteinizing hormone (LH) levels were determined in duplicate samples by a double antibody RIA using reference preparation RP-3. Reagents in the rat LH kit were obtained from the National Institute of Diabetes and Digestive and Kidney Diseases (courtesy of Dr. A.F. Parlow). This assay has been validated for measurement of LH in the Syrian hamster (Richardson et al., 2002). The intra-assay CV was 8.8% and the lower limit of detectability was 0.8 ng/ml.

Statistics

2-by-2 ANOVAs were used to determine main effects and interactions between age at treatment (adolescent or adult) and treatment (vehicle or AAS treated) on aggressive and reproductive behaviors, hormone levels, testes weights, seminal vesicle weights, and flank gland diameters. To probe significant interactions, simple main effects analyses were performed to compare vehicle- and AAS-treated groups within each age. The Dixon outlier test was used to determine

any behavioral outliers, and Student's t-tests were used to compare treatment groups within adolescent and adult males. Chi-square analyses were performed to determine whether there were differences in the proportion of vehicle- and AAS-treated males that showed defensive/submissive behaviors. All differences were considered significant if they resulted in a $p \leq 0.05$. Data in all figures are reported as group means +/- SEM.

Results

Experiment 1: The effects of AAS on sexual behaviors in adolescent and adult male Syrian hamsters

AAS did not affect the amount of time that either adolescents or adults spent investigating the receptive female (data not shown). There was a significant interaction between treatment and age on the number of mounts [$F(1, 27) = 17.172$; $P < 0.0002$], intromissions [$F(1, 27) = 7.449$; $P < 0.01$], and ejaculations [$F(1, 27) = 15.005$; $P < 0.0006$]. In general, AAS facilitated reproductive behavior in adolescent hamsters and reduced reproductive behavior in adults (Fig 2.1). Post-hoc analyses showed a trend toward increased number of mounts and a significant increase in the number of intromissions [$F(1, 13) = 7.482$; $P = 0.01$] and ejaculations [$F(1, 13) = 7.316$; $P < 0.02$] in AAS-treated adolescents relative to vehicle-treated adolescents. In contrast, AAS-treated adults showed significantly fewer mounts [$F(1, 14) = 17.256$; $P < 0.001$], intromissions [$F(1, 14) = 5.716$; $P < 0.05$] and ejaculations [$F(1, 14)$; $P < 0.015$] compared to vehicle-treated adults. The same pattern of results was found for latencies to show sexual behavior in that AAS treatment reduced latencies in

adolescents and lengthened latencies in adults. Thus, there were significant interactions between treatment and age on latency to mount [F (1, 27) = 10.773; P < 0.003], intromit [F (1, 27) = 14.332; P < 0.001], and ejaculate [F (1, 27) = 8.646; P <0.007] (data not shown).

Experiment 2: The effects of AAS on agonistic behaviors in adolescent and adult male Syrian hamsters

Aggressive/Dominant Behaviors

ANOVAs revealed a significant interaction between age and treatment on overall aggression scores [F (1, 46) = 7.417; P < 0.009]. Post-hoc analyses showed that AAS treatment significantly increased the overall aggression score in adolescents [F (1, 25) = 26.809; P < 0.001], but not in adults [F (1, 21) = 2.781; P < 0.1] (Fig 2.2). Significant interactions between age and treatment were also observed when individual aggressive behaviors were analyzed [number of attacks [F (1, 46) = 5.573; P < 0.03], number of bites [F (1, 46); P < 0.0001], and attack duration [F (1, 45) = 6.510; P < 0.02]. Post-hoc tests determined that AAS treatment significantly increased the number of attacks [F (1, 25) = 31.750; P < 0.0001)], number of bites [F (1, 25) = 27.629; P < 0.0001], and attack duration [F (1, 25) = 17.240; P < 0.0003] in adolescents. In contrast, none of these measures were significantly increased by AAS treatment in adulthood (Fig 2.3).

A two-way ANOVA showed a significant interaction between age and treatment [F (1, 46) = 7.089; P < 0.01] on flank marking (Fig 2.4). Although both

AAS-treated adolescents [F (1, 25) = 1.25; P , 0.5] and adults [F (1, 20); P < 0.05] displayed significantly more flank marks compared to vehicle-treated males, the significant interaction was due to an apparently greater response by AAS-treated adolescent males (Fig 2.4). Two-way ANOVA revealed a main effect of age on offensive posturing [F (1, 46) = 4.528; P < 0.05]. This main effect appeared to be driven by the greater display of offensive postures in AAS-treated adults (Fig 2.5).

Defensive/Submissive Behaviors:

Two-way ANOVAs revealed no effects of treatment or age nor any interactions on defensive behaviors. This was primarily because only occasionally did AAS-treated adolescent males even show these behaviors, and in addition, within group variability of the other treatment groups was high. None of the AAS-treated adolescent males displayed any escape dashes or tail-up walking and only two AAS-treated adolescents displayed defensive posturing (Table 2.1). Therefore, chi-square analyses were performed to determine whether the proportion of males exhibiting defensive behaviors was different in AAS-treated adolescents compared with the other three groups. The proportion of vehicle-treated adolescents and vehicle-treated adults displaying tail-up walking (P < 0.05) and escape dashes (P < 0.05) was significantly greater than the proportion of AAS-treated adolescents (Table 2.1). Similarly, the proportion of AAS-treated adults exhibiting tail-up walking (P < 0.05) and defensive postures (P < 0.05) was greater than that of AAS-treated adolescents. Only two AAS-

treated adults displayed escape dashes, and therefore this group was not significantly different from AAS-treated adolescents on this measure.

Physiological Measures in Experiments 1 and 2

In Experiment 1 (sexual behavior test immediately before terminal blood sample collection), we found that there was neither an effect of treatment nor age on plasma LH levels (Fig 2.6A). In contrast, there was a main effect of treatment on plasma T levels [$F(1, 29) = 10.393$; $P < 0.003$], where AAS treatment significantly reduced endogenous T levels in both adolescents and adults (Fig 2.6A). In Experiment 2 (aggression test immediately before terminal blood sample collection), there were no effects of treatment or age on either plasma LH or T concentrations (Fig. 2.6B).

Main effects of both age and treatment were found on testes weight, seminal vesicle weight and flank gland diameter (Table 2.2). As expected, in both experiments, testes and seminal vesicle weights and flank gland diameters were all larger in adults compared with adolescents. In males tested for sexual behaviors (Exp. 1), testes [$F(1, 29) = 22.053$; $P < 0.0001$] and seminal vesicle [$F(1, 29) = 20.155$; $P < 0.0001$] weights were less in AAS-treated than in vehicle-treated males. In contrast, AAS treatment increased flank gland diameter [$F(1, 29) = 5.530$; $P < 0.03$]. A similar pattern was seen in males tested for agonistic behaviors (Exp 2), where AAS-treatment reduced testes [$F(1, 47) = 11.147$; $P < 0,002$], seminal vesicle [$F(1, 47) = 3.805$; $P < 0.05$] weights, and led to a trend

towards increased flank gland diameter [$F(1, 47) = 2.893$; $P < 0.1$). Males were randomly assigned to treatment groups and there were no statistical differences in their body weights at the beginning of treatment. At the end of the treatment period in Experiment 2, however, mean body weight of AAS-treated adolescent and adults males was less than the corresponding vehicle-treated males at each age ($P < 0.005$; Table 2.2).

Discussion

These experiments demonstrate that adolescent males treated with a cocktail of testosterone cypionate, boldenone undecylenate and nandrolone decanoate show increased levels of aggressive and reproductive behaviors when compared to both vehicle-treated adolescents and AAS-treated adults. This is the first direct comparison of the behavioral effects of anabolic steroid exposure at two different developmental stages, adolescence and adulthood, and in two different social contexts, sexual interactions with a receptive female or agonistic interactions with a male of similar age and weight. The different behavioral responses to anabolic steroids in adolescence and adulthood most likely reflect structural and functional differences in the neural circuits underlying social behaviors at these two stages of life.

Normally, the postnatal maturation of reproductive behaviors in male Syrian hamsters is a function of the increase in circulating testicular hormones that occurs with the onset of puberty. Previous work from this laboratory has

demonstrated that male reproductive behavior cannot be activated by testosterone, dihydrotestosterone or estrogen in prepubertal animals (Meek et al., 1997; Romeo et al., 2002) and that the pubertal rise in testosterone organizes (further masculinizes and defeminizes) neural circuits to enhance or maximize the activation of reproductive behavior in adulthood (Schulz et al., 2004). In the present experiment, adolescent exposure to AAS resulted in levels of sexual behavior comparable to those typically observed in gonad-intact adult hamsters, demonstrating that behavioral responsiveness of adolescent males is greater than that of prepubertal males. Adolescent males may also be more responsive than adults to the activational effects of supraphysiological levels of androgens, since in this experiment AAS treatment increased sexual behavior in adolescents, but not adults. In addition, because endogenous testosterone further masculinizes the nervous system during puberty (Schulz and Sisk, 2006), it is possible that AAS "hypermasculinize" the adolescent brain, resulting in enhanced activational responses in adolescent males compared with adults. Organizational effects of AAS that enhance activational responses would not be expected to occur in AAS-treated adults, since previous work has shown that the potential for organization of sexual behavior is greatly diminished after puberty and adolescence (Schulz and Sisk, 2006). In fact, we found that AAS in adulthood *decreases* sexual behavior. Similar reductions in sexual behavior in response to exposure to supraphysiological levels of androgens have been reported in rats (Farrell and McGinnis, 2003) and hamsters (Meek et al., 1997). The decrement in sexual behavior induced by high levels of exogenous androgen

in adulthood may be secondary to a general suppression of endogenous hypothalamic-pituitary-gonadal axis function. If so, then the current experiments indicate that the adolescent brain is either less responsive to these inhibitory effects, or that there are other mechanisms that override them to result in enhancement of sexual behavior by AAS in adolescence.

Aggressive behavior in Syrian hamsters is not under strong activational control by endogenous testosterone. In spite of this fact, adolescent animals treated with AAS in the present study showed higher levels of aggressive behaviors relative to controls, and spent at least one-third of the 10 minute test attacking their opponent, similar to previous reports of effects of AAS on aggression in adolescent hamsters (Grimes et al., 2006; Melloni et al., 1997; Melloni and Ferris, 1996). In addition, AAS-treated adolescent males in the present study did not express submissive behaviors such as defensive posturing, escape dashes, or tail-up walking. In contrast to the heightened aggression observed in AAS-treated adolescent males, AAS treatment in adulthood did not result in increased levels of overt aggression, consistent with our previous findings that aggressive behavior is not facilitated by testosterone in adulthood (Romeo et al., 2003). Testicular hormones organize flank marking behavior during adolescence in male Syrian hamsters (Schulz et al., 2006). Interestingly, both adolescent and adult males showed increased levels of flank marking behavior after AAS-treatment suggesting that this behavior may be organized and activated during early to mid adolescence. In general, AAS-treated adults

communicated dominance by expressing threatening behaviors (e.g., offensive posturing) and flank marking behavior, rather than by overt aggression. Rats treated with an anabolic steroid throughout adolescence and tested for aggressive behaviors during adulthood also display an increase in threatening behaviors (Farrell and McGinnis, 2003). Thus, the neural mechanisms that in adults favor display of communicative agonistic behaviors (offensive posturing, flank marking) over overt aggression (bites, attacks) appear to be either not engaged or overridden in adolescent males, which in the presence of AAS show heightened overt aggression.

The heightened sexual and aggressive behaviors observed with adolescent exposure to AAS may be indicative of either hyper-responsivity to steroids or to a relative lack of inhibition of the underlying neural circuits at this stage of development (Forbes and Dahl, 2005; Yurgelun-Todd, 2007). The neural circuitries underlying sexual and agonistic behaviors in the male hamster have both overlapping and distinctive components (Kollack-Walker and Newman, 1995). The medial amygdala, lateral septum, and bed nucleus of the stria terminalis (BNST) are common elements of the two circuits, while the medial preoptic area and anterior hypothalamus are areas selectively linked to sexual or agonistic behaviors, respectively (Ferris and Delville, 1994; Swann, 1997; Wood and Coolen, 1997; Wood and Swann, 2005). Most of these structures have direct or indirect connections with cognitive control and reward areas, e.g. nucleus accumbens and prefrontal cortex (Cunningham et al., 2002; Ernst et al.,

2006; Yurgelun-Todd, 2007). This study suggests that adolescent males may not be able to regulate the expression of their social behaviors and/or have atypical expression of behavior after AAS exposure when compared to adult males. Our results fit with previously reported relationships between high levels of endogenous testosterone and heightened aggression and the importance of context in the display of social behaviors in human adolescents (Davey et al., 2007; Rowe et al., 2004).

Endogenous testosterone exerts negative feedback on GnRH and gonadotropin release (Sisk and Foster, 2004). Therefore, AAS would also be expected to exert negative feedback effects on LH secretion, and in fact a previous report found that 8 weeks of testosterone cypionate treatment reduced LH and testosterone levels in adult male rats (Feinberg et al., 1997; McGinnis, 2004). Therefore, it was surprising that AAS cocktail used in the present studies, which contained testosterone cypionate, nandrolone decanoate and boldenone undecylenate, did not result in a general suppression of hormone levels. Instead, the type of social interaction that occurred immediately before blood sample collection appeared to influence hormone levels more robustly than either AAS or age. Males tested with a receptive female had LH levels that were on average 3-4x higher and T levels that were 2-3x higher than those of males tested with an intruder male. This finding suggests that the social interaction acutely overrides direct effects of AAS on the h-p-g axis. In general, LH secretion increases in adult male hamsters after exposure to female pheromones, so these findings

were not too surprising (Richardson et al., 2004). The fact that AAS treatment was associated with reduced T levels in males tested with a female does provide some evidence for negative feedback influences of AAS. This is further supported by the reduction in weight of the testes and seminal vesicles in AAS-treated males at both ages in both experiments. Thus, unlike their behavioral responses, peripheral tissues appear to be similarly responsive to AAS in adolescent and adult males.

Hormonal facilitation of reproductive and aggressive behaviors includes intracellular actions of both androgens and estrogens (Bodo and Rissman, 2006; Nelson and Trainor, 2007; Romeo et al., 2002; Vagell and McGinnis, 1998). Therefore, the mechanisms by which AAS influence neurotransmitter systems and behavior are likely to include regulation and activation of nuclear androgen (AR) and estrogen receptors (ER). All three androgens in the cocktail used in these experiments are aromatizable to estrogenic metabolites that are known to have effects on both the central nervous system and the periphery (Clark and Henderson, 2003). When given to adult male rats, this cocktail upregulates AR immunoreactivity widely throughout the brain (Menard and Harlan, 1993). Self-administration of testosterone induces AR and ER immunoreactivity in steroid-sensitive regions such as the medial amygdala and preoptic area in adult male Syrian hamsters (Dimeo and Wood, 2005). Given that these areas are receptor rich and that there are differential effects of AAS on adolescent and adult males, one can hypothesize that AR and ER expression may be differentially influenced

by anabolic steroids in adolescent and adult males. Further studies are needed to determine the mechanisms by which these compounds affect social behaviors.

In the last decade, increased understanding that pubertal gonadal hormones organize the adolescent brain has heightened interest in discovering how AAS affect the adolescent brain and behavioral maturation. This study was the first to directly compare males treated with anabolic steroids during adolescence and adulthood, and we found significant differences in the direction and magnitude of AAS effects on sexual and aggressive behavior. The particular cocktail used in this study is comparable to what a heavy or chronic anabolic steroid user would use to enhance muscle growth and athletic performance (Menard and Harlan, 1993). An increase in body image concerns during adolescence, along with a decrease in the perceived risk of taking AAS has resulted in increased use of AAS among US teenagers. Importantly, through neuroimaging and behavioral studies early adolescence has been shown to be a sensitive period of neural, cognitive and emotional development. Our data underscore the need for further research on age-specific the effects of AAS on brain circuitry and organization throughout development and whether AAS use during adolescence has enduring consequences on social behaviors.

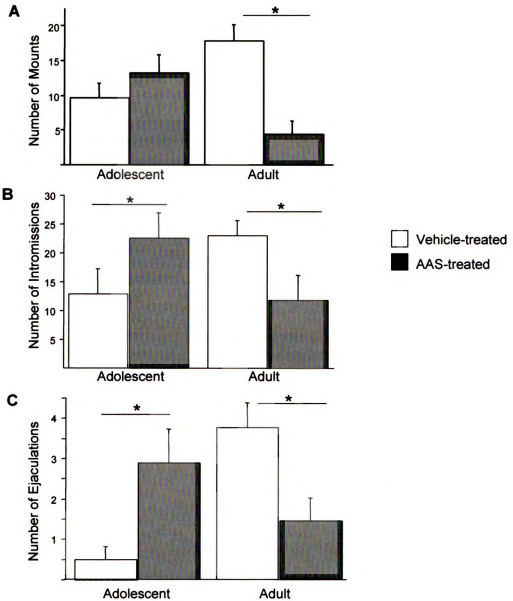


Figure 2.1 AAS affect male sexual behaviors differently in adolescent and adult hamsters. Two-way ANOVA revealed age by treatment interactions in which, relative to age-matched vehicle-treated adolescents (n=8) and vehicle-treated adults (n=9), AAS-treated adolescents (n=7) displayed more (A) mounts, (B) intromissions, and (C) ejaculations, whereas these behaviors were decreased in AAS-treated adults (n=7). Asterisk indicates significant difference ($p<0.05$) between AAS- and vehicle treatment within an age as determined by post hoc analysis.

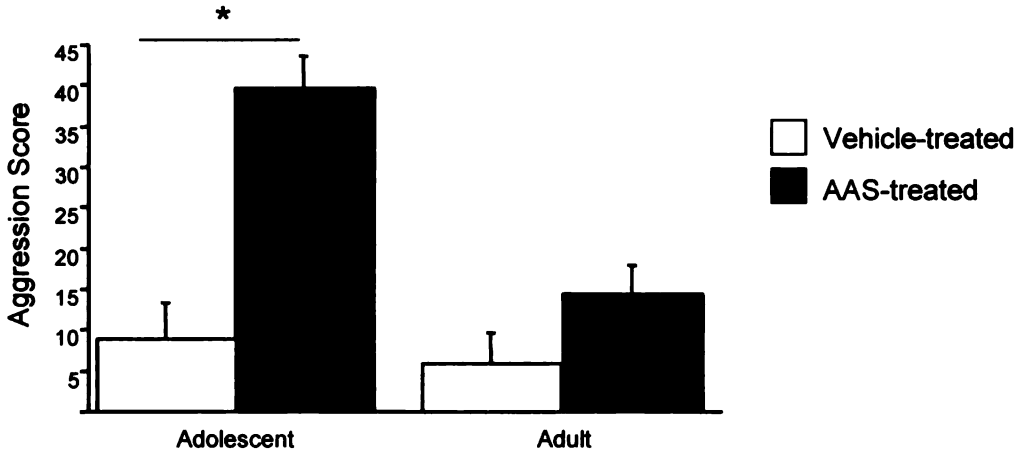


Figure 2.2 AAS treatment increases aggression in adolescents but not in adults. Two-way ANOVA revealed an age by treatment interaction on a global aggression score [(flank marks + attacks + bites) – (tail-up walking)]. Sample sizes: vehicle-treated adolescent, n=13; AAS-treated adolescent, n=14; vehicle-treated adult, n=11; AAS-treated adult, n=13. Asterisk indicates significant difference ($p<0.05$) between AAS- and vehicle treatment within an age as determined by post hoc analysis.

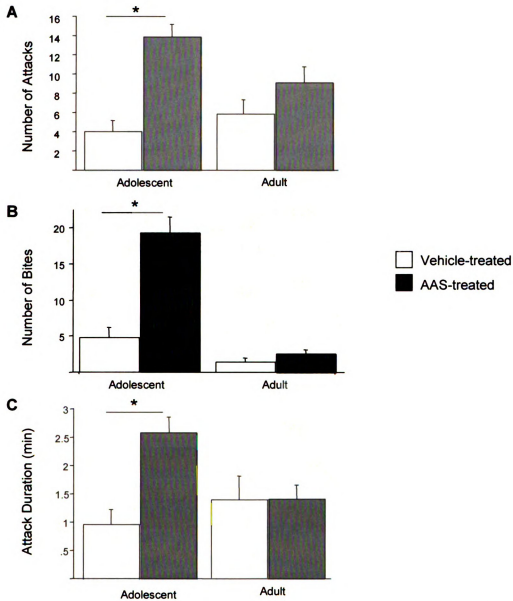


Figure 2.3 AAS affect male aggressive behaviors differently in adolescent and adult hamsters. Two-way ANOVA revealed age by treatment interactions on (A) attacks, (B) bites, and (C) attack durations (min). Asterisk indicates significant difference ($p<0.05$) between AAS- and vehicle treatment within an age as determined by post hoc analysis.

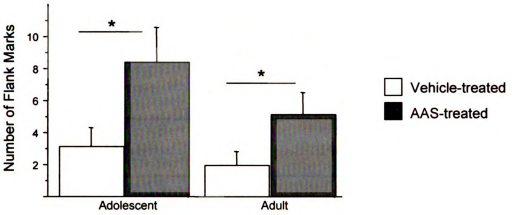


Figure 2.4 AAS increase flank marking in both adolescent and adult males. Two-way ANOVA revealed a significant effect of treatment. Asterisk indicates significant difference ($p<0.05$) between AAS- and vehicle treatment within an age as determined by post hoc analysis.

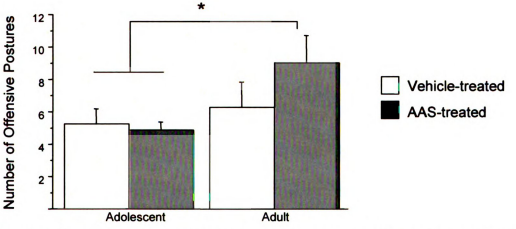


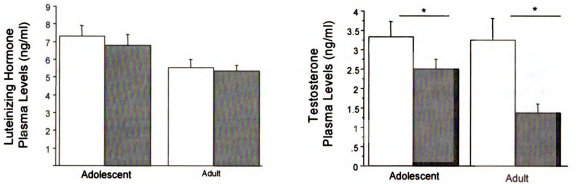
Figure 2.5 Adults engage in more offensive posturing than adolescents during a male-male social interaction. Two-way ANOVA revealed a significant main effect of age (asterisk).

Table 2.1 AAS-treated adolescent males were less likely to display submissive behaviors than vehicle controls and adult males.

Submissive Behavior	***Adolescent – Vehicle (n=13)***	***Adolescent – AAS treated (n=14)***	***Adult – Vehicle (n=11)***	***Adult – AAS treated (n=12)***
Tail Up Walking	3 ± 1.5 (5/13) *	0 (0/14)	3.4 ± 1.9 (3/11) *	1.8 ± 1.2 (3/12) *
Escape Dashes	3.5 ± 1.8 (4/13) *	0 (0/14)	6 ± 3.2 (4/11) *	4.3 ± 3.1 (2/12)
Defensive posturing	4.3 ± 1.6 (7/13) *	0.3 ± 0.2 (2/14)	7 ± 2 (5/11)	5.2 ± 1.5 (6/12)*

** Chi-square analysis, $p < 0.05$

A – Males tested for Reproductive Behaviors



B – Males tested for Aggressive Behavior

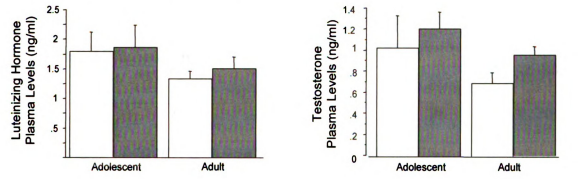


Figure 2.6 Plasma luteinizing hormone (LH) and testosterone (T) concentrations immediately after each behavior test. (A) In males tested with a receptive female (A), there were no effects of age or treatment on LH concentrations, but AAS decreased T concentrations in both AAS-treated adolescent and adult males (main effect of treatment, $p < 0.05$). (B) In males tested with another male for aggressive behaviors, there were no effects of age or treatment on either LH or T concentrations. Asterisk indicates significant difference ($p<0.05$) between AAS- and vehicle treatment within an age as determined by post hoc analysis.

Table 2. Peripheral measures in vehicle- and AAS-treated adolescent and adult males.

Experiment	Adolescent Males		Adult Males	
	Vehicle treated (Mean +/- SEM)	AAS-treated (Mean +/- SEM)	Vehicle Treated (Mean +/- SEM)	AAS-treated (Mean +/- SEM)
Testes Weight (g)				
1	2.31 ± .04	2.0 ± .04*	3.85 ± .13	3.29 ± .10*
2	2.06 ± .07	1.70 ± .07*	3.86 ± .09	3.67 ± .07*
Seminal Vesicle Weight (g)				
1	0.167 ± .01	0.114 ± .02*	0.344 ± .02	0.273 ± .02*
2	0.21 ± .01	0.16 ± .01*	0.36 ± .03	0.32 ± .03*
Flank Gland Diameter (mm)				
1	6.77 ± .33	8.24 ± .34*	7.6 ± .40	7.75 ± .19*
2	7 ± .3	7.9 ± .3	8.5 ± .26	8.5 ± .25
Body Weight – beginning of treatment period (g)				
1	56.41 ± 1.12	59.49 ± 2.0	106.14 ± 4.17	107 ± 2.03
2	49.67 ± 1.0	47.23 ± 1.6	107.78 ± 2.6	108.61 ± 1.9
Body Weight – end of treatment period (g)				
1	93.36 ± 2.33	90.10 ± 1.81	113.32 ± 4.83	111.39 ± 2.36
2	86.9 ± 1.8	81.8 ± 2.1*	120.53 ± 2.9	116.0 ± 2.1*

* main effect of treatment in two-way ANOVA, $p<0.05$

CHAPTER 3: ADOLESCENT ANDROGENIC ANABOLIC STEROID EXPOSURE ENHANCES AGGRESSIVE BEHAVIORS AND REDUCES THE POTENTIAL TO REACH SATIETY AFTER DISCONTINUATION OF TREATMENT

Introduction

The transition from childhood to adulthood during puberty and adolescence comprises reproductive, neural, and behavioral maturation (Lenroot et al., 2007; Sisk and Foster, 2004). The brain undergoes dramatic remodeling during adolescence, and endogenous steroid hormones influence this developmental process (Nelson et al., 2005; Sisk and Zehr, 2005). Thus, the adolescent brain may be particularly vulnerable to the adverse consequences of anabolic steroid use. Anabolic androgenic steroids (AAS) are synthetic derivatives of testosterone used to enhance athletic performance and physical appearance; increased use has been reported among adolescents. AAS influence mood and behavior, including heightened aggression and increased or decreased libido in humans (Bahrke and Yesalis, 2004; Yesalis and Bahrke, 2000; Yesalis and Bahrke, 2002; Yesalis et al., 2000). Although some AAS can be detected up to 12-wks after withdrawal, few studies have focused on understanding the long-term effects of AAS on the adolescent brain and behavior (Ballard and Wood, 2005).

Androgens play a critical role in the pubertal maturation of social behaviors. The absence of gonadal hormones during puberty results in long-lasting impairments in steroid-dependent social behaviors in adulthood (Schulz et

al., 2004), suggesting that steroid hormones in adolescence program the expression of sexual behaviors in adulthood. In addition, sexual experience in adulthood does not compensate for a lack of testosterone during adolescence to bring forth adult-typical levels of behavior (Schulz et al., 2004). These findings also generalize to the expression of flank marking behavior in the male Syrian hamster (Schulz, et al 2007), suggesting that androgens during adolescence are critical for the expression of social behaviors in adulthood. While these studies show that the absence of testosterone during adolescence impairs social behaviors in adulthood, relatively little is known about the long term effects of exposure to pharmacological levels of testosterone or other androgens on adult male social behaviors.

Exposure of male hamsters to a cocktail of AAS during adolescence increases the expression of sexual and aggressive behaviors immediately after treatment (Farrell and McGinnis, 2003; Melloni et al., 1997). In addition, AAS affect aggression and sexual behaviors in other rodent models, such as rats and mice, with specific effects depending on the particular compound and dose used and age at treatment (Clark and Henderson, 2003; McGinnis, 2004). Collectively, these studies show that the adolescent brain responds differently than the adult brain to exogenous hormones, suggesting that the adolescent brain is still developing and vulnerable to perturbations in steroid milieu.

A direct comparison between adolescent and adult exposure of the long-term consequences of AAS has yet to be done. Single AAS compounds, like testosterone, nandrolone, and stanozolol, affect aggressive behaviors up to 17 wks after discontinued treatment and reproductive behaviors up to 9 wks in intact male rats exposed throughout adolescence (Farrell and McGinnis, 2004). However, 19 days after adolescent exposure with a cocktail, the effects of treatment on offensive behaviors did not persist in intact male Syrian hamsters (Grimes et al., 2006). These results suggest that at least some of the alterations in androgen-dependent behaviors by adolescent AAS exposure can persist after drug exposure.

The goal of these experiments was to directly compare the long-term behavioral consequences of AAS exposure during adolescence or adulthood. Therefore, sexual and aggressive behavior was assessed either two or four weeks after discontinuation of treatment with AAS or vehicle, where treatment occurred either during adolescence or in adulthood. Because the adolescent brain is more responsive to organizational effects of testosterone than the adult brain (Schulz, et al 2007), the tested hypothesis was that adolescent, but not adult AAS exposure exerts long-lasting changes in the nervous system, leading to the prediction that AAS exposure during adolescence, but not in adulthood, will result in effects on behavior that persist beyond the period of exposure to AAS.

Materials and Methods

Animals

Eighteen-day-old male Syrian hamsters (*Mesocricetus auratus*) were obtained from Harlan Sprague–Dawley Laboratories (Madison, WI). Upon arrival males were housed in groups of eight animals per cage (polycarbonate, 33 x 38 x 17 cm) with ad libitum access to food (Telkad Rodent Diet No. 8640, Harlan) and water. Animal colony temperature was maintained at 22 ± 2°C, and animals were kept on a light-dark cycle of 14:10 L:D (lights off at 1600h EST). At 25 days of age, animals were singly housed in polycarbonate cages (30.5 x 10 x 20 cm, for Experiment 1; 33 x 38 x 17 cm, for Experiment 2). All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

AAS Treatment

A cocktail of AAS containing 0.8 mg/ml of testosterone cypionate (TC; Sigma Aldrich, Inc., St. Louis, MO), 0.8 mg/ml of nandrolone decanoate (ND; Sigma Aldrich, Inc.), and 0.4 mg/ml of boldenone undecylenate (BU; Steraloids, New Port, Rhode Island) was dissolved in 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, Inc.). The AAS cocktail was prepared the day before treatment began for each age group in each experiment, and was stored at 4˚C.

Experimental design

Two separate experiments were conducted to understand the long-term effects of AAS on males treated during adolescence or in adulthood. Experiment 1 tested treated males with a receptive female to assess behavioral changes in sexual behavior; whereas in Experiment 2 treated males were tested with an intruder male to test for agonistic behavior.

On 25 days of age males were singly housed. At 27 days of age, gonad-intact male hamsters were randomly divided into AAS-treated or vehicle-treated groups. These males received a daily subcutaneous (*sc*) injection of either the AAS cocktail (2 mg/kg TC, 2 mg/kg ND, 1 mg/kg BU) or vehicle (2-hydroxypropyl-β-cyclodextrin), either during adolescence (from 27-41 days of age) or during adulthood (from 63 -77 days of age). Group sizes were originally n= 8-10/age and treatment group in Experiment 1 (assessment of sexual behavior) and n=13-15/age and treatment group in Experiment 2 (assessment of agonistic behavior).

The volume of each daily injection was determined according to the male's body weight. The volume of the injections ranged from 0.1 ml to 0.25 ml over the two-week course of administration across age groups. Tests for either sexual or aggressive behaviors were conducted either two or four weeks after the last injection, at 56 (two-wks) or 70 days (four-wks) of age for males treated during adolescence and at 92 (two-wks) or 106 (four-wks) days of age for males treated as adults. Separate groups of animals were tested 2 and 4 weeks after

discontinuation of AAS or vehicle treatment. A repeated measures design was not used in order to avoid a potential confound and additional source of variability from prior social experience. All behavioral interactions were videotaped with a low-light color video camera and were scored blind using a computer program that tags each behavioral code with an elapsed time (software generously provided by Dr. Kim Wallen, Emory University, Atlanta, GA). This system allows the calculation of counts, duration, and latency for each behavior.

Experiment 1: Sexual Behavior

Males were sexually inexperienced prior to a standardized behavior test with a receptive female. Tests were conducted between 1700 and 2030 h EST, one-hour after "lights-out", under red-light illumination, as described in previous studies (Heise et al., 2003; Romeo et al., 2002; Romeo and Sisk, 2001; Schulz et al., 2004). Following a 5 min acclimation to the test chamber (a 51 x 26 x 31.5 cm glass aquarium with a mirror underneath), males interacted with a stimulus female for 15 min. Males were tested either two- or four- wks after AAS or vehicle treatment during adolescence (P56 or P70) or adulthood (P92 or P106).

Ovariectomized stimulus females were hormonally primed to induce receptivity with an estradiol benzoate injection (10 μg in 0.05 ml sesame oil) 48 h before, and a progesterone injection (500 μg in 0.1 ml sesame oil) 4 h before behavior testing. Prior to the 15 min behavior test with the subject male, stimulus females were tested for receptivity with a sexually experienced stud male.

Anogenital investigation of the female, mounts, intromissions, and ejaculations were scored and quantified as previously described (Schulz et al., 2004). Satiety and latency to reach satiety were also assessed by quantifying long intromissions. These are defined as multiple rhythmic thrusts, about 30 seconds long, performed by subject males after five to eight ejaculations and followed by genital grooming as they approach satiety (Arteaga and Morali, 1997; Arteaga et al., 2000). The efficiency rate was also determined for each male by dividing the number of ejaculations by the sum of mounts and intromissions. Data from three males were excluded because no seminal vesicles were found the day of behavior testing which would skew the data gathered from peripheral tissues. Data from two adolescent and three adult subjects were excluded because females did not remain receptive throughout the 15 minute test, which resulted in atypical behavior of both male and female hamsters. Data from four other adolescent and adult males were excluded because of technical difficulties during videotaping of the behavioral tests (e.g. the tape ran out during testing). Thus, a total of 68 males were tested, and the final sample sizes in Experiment 1 were: adolescent vehicle-treated tested on P56, n=8, and tested on P70, n = 10; adolescent AAS-treated tested on P56, n=7, and tested on P70, n=9; adult vehicle-treated tested on P92. n=8, and tested on P106, n=9; adult AAS-treated tested on P92, n=9, and tested on P106. n=8.

Experiment 2: Agonistic Behavior

Animals were tested for agonistic behaviors using a resident-intruder paradigm. The socially naïve males were tested between 1700 and 2030 h EST in a red-light illuminated room. A riser made by cutting out the bottom part of the polycarbonate cages was placed in the home cage of the subject (AAS- or vehicle-treated) to make the walls of the home cage taller. After a 5 min acclimation period, an age- and weight-matched intruder male was placed in the treated male's home cage. Intruder males were purchased from the same vendor and arrived at the same time as the subject males. Males were tested two- or four- wks after AAS or vehicle treatment during adolescence (P56 or P70) or adulthood (P92 or P106). Ten-min behavior tests were videotaped and scored for aggressive and submissive behaviors, as previously reported (Salas-Ramirez, et al. 2007). Agonistic behaviors included in the analysis were: (1) aggressive/dominant behaviors (flank marking behavior, total contact time, attacks, bites, and offensive posturing) and (2) defensive/submissive behaviors (defensive posturing, tail-up walking, and escape dashes). In this experiment a distinction was made between sniffing and a risk-assessment measure called stretch-attend. It was defined and scored as an approach made by the subject in which the body is stretched and lowered, the hind legs stay planted and the front legs stretch forward, head is out, eyes are on the opponent and the subject is sniffing in the opponent's direction (Albers, et al 2002).

To assess overall aggressiveness of each subject, a composite aggression/dominance score was obtained by adding the number of flank marks, attacks and bites, and subtracting tail-up walking.

A total of 106 males were tested for agonistic behaviors in this experiment. Two males treated during adolescence and four males treated during adulthood were excluded from the statistical analyses of aggressive behaviors because of extremely low levels of social interaction during the test; they were determined to be outliers by the Dixon outlier test. In addition, two males assigned to the adult vehicle-treated group were not used because their body weights were too low to test with a weight- and age- matched intruder. Data from six other males were excluded because of technical difficulties during videotaping of the behavioral tests (e.g. tape ran out, cameras were moved during a test). Thus, final sample sizes for Experiment 2 were: adolescent vehicle-treated tested on P56, n =12, and tested on P70, n=10; adolescent AAS-treated tested on P56, n = 14, and tested on P70, n=10; adult vehicle-treated tested on P92, n = 11, and tested on P106, n=13; adult AAS-treated tested on P92, n = 12, and tested on P105, n=10.

Physiological Measures

Immediately following the behavior test, animals were administered an overdose of sodium pentobarbital (130 mg/kg i.p.). Fifteen to twenty min after the injection, a terminal blood sample was taken from each male tested. Testes and seminal vesicles were removed and weighed. At the time of sacrifice, flank gland

diameters were assessed by shaving the hindquarters and measuring the gland with a caliper.

Plasma testosterone concentrations were measured in duplicate using a commercially available radioimmunoassay kit (Coat-a-Count Total Testosterone, Diagnostic Products, Los Angeles, CA). This assay has been previously validated for measuring endogenous plasma testosterone levels in hamsters in our laboratory (Parfitt et al., 1999). The intra-assay CV was 4.72 % in Experiment 1 and 5.81% in Experiment 2, and the limit of detectability was 0.1 ng/ml for both experiments.

Statistics

Three-way ANOVAs were used to determine main effects and interactions between age of exposure (adolescent or adult), time of testing post-treatment (two or four weeks after discontinuation of treatment) and treatment (vehicle or AAS treated) on aggressive and reproductive behaviors, testes weights, seminal vesicle weights, and flank gland diameters. In this two experiments time of testing post-treatment did not affect the expression of behaviors, therefore groups were collapsed to understand how age of AAS exposure and treatment affected each behavior. Simple main effects analyses were performed to compare vehicle- and AAS-treated groups within each age. The Dixon outlier test was used to determine any behavioral outliers, which only occurred when a male was tested with a non-receptive female or displayed little behavior when

tested with an age-and weight-matched male. In addition, one-way ANOVAs were used to determine any behavioral differences between vehicle treated subjects throughout development.

Results

Experiment 1: The long term effects of AAS on sexual behaviors after exposure during adolescence or adulthood

A. Sexual behavior:

a. General Development of Adult Behavior:

An ANOVA determined that there were no significant differences in the expression of mounts and intromissions in vehicle-treated males tested with a receptive female on P56, P70, P92 and P 106 (Figure 3.1).

b. Treatment effects:

AAS did not affect the amount of time that males spent in anogenital investigation of the receptive female, the number of mounts, intromissions, or ejaculations (data not shown). Interestingly, there was an overall main effect of AAS on satiety measures. AAS treatment significantly reduced the number of long intromissions [$F(1, 60) = 5.975$; $P < 0.02$) and increased the latency to display a long intromission [$F(1, 60) = 5.922$; $P < 0.02$) (Figure 3.2). Across the other two variables (age of exposure, time of testing), subjects treated with AAS took longer to reach satiety and performed fewer long intromissions.

B. Physiological Measures

There were no effects of AAS on testes weight, seminal vesicle weight or flank gland diameter. However, as expected, there was a main effect of age of exposure on the weight and size of the peripheral tissues ($p < 0.05$) (Table 3.1). There were no effects of AAS treatment on plasma testosterone levels.

Experiment 2: The long-term effects of AAS on agonistic behaviors after exposure during adolescence or adulthood

A. *General Development of Agonistic behaviors*

Agonistic behaviors can be classified into aggressive and submissive behaviors. Unlike sexual behaviors, aggressive behaviors decrease significantly with age. A one-way ANOVA on vehicle-treated subjects showed that the number of attacks is significantly reduced over development [Figure 3.3A; $F(3, 42) = 6.09$; $P < 0.002$]. Interestingly, when comparing vehicle treated males, tail-up walking, a typical submissive behavior, increases significantly with age [Figure 3.3B; $F(3,42) = 7.26$; $P < 0.0005$].

B. *Aggressive/Dominant Behaviors*

Adolescent AAS exposure increases the number of attacks and bites even 4-wks after discontinuation of treatment. A three-way ANOVA revealed a main effect of AAS treatment by increasing the number of attacks [Figure 3.4A; $F(1, 84) = 4.579$; $P < 0.05$] and number of bites [Figure 3.4B; $F(1, 84) = 4.496$; $P <$

0.05) on adult male Syrian hamsters. Additionally, a main affect of age of exposure was also found on number of attacks [$F(1, 84) = 31.998$; $P < 0.0001$] and bites [$F(1, 84) = 36.798$; $P < 0.0001$), showing that males that were treated during adolescence display an increase in aggressive behavior. Since time of testing post-treatment did not affect the expression of aggressive behaviors, behavioral data were collapsed across this variable, and a 2-way ANOVA was performed in order to probe the effects of age of exposure and treatment. AAS treatment resulted in increased aggression over vehicle controls regardless of age of treatment. Since behavioral testing in this experiment was performed two weeks or four weeks after AAS exposure, these results show persistent effects of AAS on aggression that last beyond the period of drug exposure.

A critical behavior for establishing dominance in male hamsters is flank marking behavior. Again, an increase in flank marking behavior was observed after AAS exposure. Main effects of age of exposure [$F(1, 84) = 10.53$; $P < 0.002$] and treatment [$F(1, 84) = 6.13$; $P < 0.02$] were found by a three-way ANOVA (Figure 3.5A). Again, since time of testing post-treatment did not affect flank marking behavior, data were collapsed across this variable and a two way ANOVA was performed. As with the three-way ANOVA, there was a significant main effect of age of exposure, in which males treated with vehicle or AAS during adolescence showed more aggression that males treated during adulthood. those groups were collapsed to analyze effects of age of exposure and treatment. AAS treatment resulted in increased flank marking behavior over vehicle controls independent of age of AAS exposure. Since behavioral testing

in this experiment was performed two weeks or four weeks after AAS exposure, these results show persistent effects of AAS on flank marking behavior.

Overall, males that were treated with AAS exposure adolescence had a higher aggression score than males that were treated in adulthood (Figure 3.5B). A main effect of age of exposure was observed [$F(1, 84) = 33.970$; $P < 0.0001$] in this experiment, along with a main effect of treatment [$F(1, 84) = 6.16$; $P < 0.02$]. Time of testing post-treatment did not have a main effect on the expression of aggression, therefore, a 2-way ANOVA was run collapsing the groups by age of exposure and treatment. The same main effects of age and treatment persisted. Again, AAS treatment resulted in increased the composite aggression score over vehicle controls independent of age of AAS exposure. This significant increase persisted four-weeks after exposure.

C. Defensive/Submissive Behaviors:

Tail-up walking, escape dashes and stretch-attend are all behaviors categorized as defensive or submissive behaviors. Interestingly, males exposed to AAS during adolescence did not express tail-up walking, escapes dashes or stretch-attend (Figure 3.6). A main effect of age of exposure was found on the number of tail-up walking [$F(1, 84) = 23.312$; $P < 0.0001$], escape dashes [$F(1, 84) = 9.980$; $P < 0.002$] and stretch-attend [$F(1, 84) = 14.142$; $P < 0.0005$] showing that males treated as adults displayed a significantly higher number of submissive behaviors.

C. Physiological Measures

In this experiment a main effect of age of AAS exposure was observed on testes weight [$F(1, 84) = 24.294$; $P < 0.0001$), seminal vesicle weight [$F(1, 84) = 59.856$; $P < 0.0001$] and flank gland diameter [$F(1, 84) = 19.470$; $P < 0.0001$], as expected and also shown in experiment 1 (Table 3.1). Males that were treated during adolescence had significantly smaller peripheral tissue measurements. However, in this experiment, a main effect of treatment [$F(1, 84) = 22.030$; $P < 0.0001$] and of testing post-treatment [$F(1, 84) = 14.003$; $P < 0.0003$] was found on testes weight. AAS treated males and males that were tested 4 wks after treatment had significantly smaller testes weights. A significant interaction between age of exposure and testing-post treatment was also found on flank gland diameter [$F(1, 84) = 12.165$; $P < 0.0008$], showing that males that were treated during adolescence and tested 4 wks has reduced flank gland measurements. No significant effects of treatment were found on testosterone levels in this experiment, but a main effect of testing post-treatment was determined by a 3-way ANOVA [$F(1, 84) = 5.410$; $P < 0.02$].

Discussion

The purpose of these experiments was to determine if AAS exposure during adolescence or adulthood had long term consequences on social behaviors in an animal model. Using a cocktail comparable to what a heavy or chronic anabolic steroid user would administer, these experiments show long-lasting consequences due to AAS exposure after adolescent and adult treatment

on both sexual and aggressive behaviors. Sexual satiety is significantly decreased after anabolic steroid exposure, independent of age of treatment. Also, aggressive behaviors remained enhanced and submissive behaviors suppressed only after adolescent AAS exposure, while AAS had mild effects on the expression of aggressive behaviors after adult exposure. This is the first study to compare directly the long-term effects of AAS exposure during development on adult social behaviors. Data from these experiments show that anabolic steroid exposure affects specific behavioral characteristics in a social interaction with male and female counterparts.

Androgens play a critical role in socio-behavioral changes that occur during adolescence. Gonadal hormones during puberty exert long-lasting changes in neural circuits responsible for the programming of activational responses to steroids later in adulthood. A two-stage model for maturation of male social behaviors is proposed: a perinatal critical period for sexual differentiation of neural circuits, followed by the pubertal period, during which gonadal steroids further organize the circuits to enhance behavioral responsiveness to hormones in adulthood (Sisk et al., 2003). In support of this hypothesis, the lack of gonadal hormones during adolescence leads to detrimental effects on social behaviors in adulthood (Schulz et al., 2004). Normative behaviors were quantified in the vehicle-treated groups. Like in other studies, sexual maturation did not change throughout adult development (Meek et al., 1997). On the other hand, aggressive behaviors were reduced throughout

development. Previous studies from this laboratory describe developmental changes in agonistic behaviors. Prepubertal males show increased aggression when compared to adult males (Romeo et al., 2003) and flank marking behavior is increases after puberty (Schulz et al., 2006). In this study aggressive and submissive behaviors were quantified. After puberty adult aggressive behavior also goes through developmental changes where in it decreases with age and submissive behaviors increase. Adult male uses less overt aggression to establish dominance hierarchies; however, expression of flank marking behavior and submissive behaviors remain consistent throughout adulthood.

In all treated groups AAS exposure affected the potential to reach satiety. AAS exposure decreased the expression of long intromissions, which are indicative of the onset of sexual satiety (Arteaga, 2000), in hamsters that were exposed to AAS in either adolescence or adulthood. Pubertal AAS exposure resulted in decreases in the expression of sexual behaviors up to 9 wks after withdrawal in intact male rats (Farrell and McGinnis, 2004). These findings are not easy to compare with the current study since there was a longer treatment period (8 wks, throughout adolescence) of single synthetic compounds and repeated testing within subjects. However, potential effects of anabolic steroids on the dopaminergic systems could result in the inability to reach satiety. AAS exposure in adulthood results in an increase of dopamine synthesis and an increase in dopamine receptors in the nucleus accumbens, which plays a critical role in the interpretation of rewarding stimuli and sexual satiety (Birgner et al.,

2007; Kindlundh et al., 2001; Kindlundh et al., 2003b; Kindlundh et al., 2004). However, dopamine regulation in the nucleus accumbens is related to anticipatory sexual behavior, whereas in the MPOA it's related to copulatory rate and sexual motivation (Dominguez and Hull, 2005; Pfaus and Phillips, 1991). Dopamine agonists can reduce or facilitate rodent male sexual behaviors depending on the dose (Bazzett et al., 1991; Mas et al., 1995b; Moses et al., 1995). The inhibition of dopaminergic neurons within the mating circuit (Me, BNST and MPOA) results in the disappearance of long intromissions (Arteaga et al., 2002; Parfitt and Newman, 1998). In addition, copulation leading to sexual exhaustion is associated with elevated levels of dopaminergic activity in the MPOA (Mas et al., 1995a). Unfortunately, the effects of AAS exposure on dopaminergic systems related to the mating circuit has not been explored; however, AAS may regulate dopamine release and the expression of dopamine receptors differentially in neural circuits in the mating and reward circuits.

A link between anabolic steroid use and increased aggression has been well documented in both human subjects and animal models (Clark and Henderson, 2003; Fischer et al., 2007; McGinnis, 2004; Rowe et al., 2004). Intact male rats exposed to AAS during adolescence and adulthood have increased composite aggression scores and intact adolescent male Syrian hamsters show an increase in attacks, bites and attack duration immediately after exposure (Melloni et al., 1997). Follow-up studies focusing on the long-term effects of AAS show that adolescent AAS treatment in male rats can lead to

enhanced expression of aggressive behaviors up 17 wks post-treatment (Farrell and McGinnis, 2004); however, these long term effects were not observed in adolescent male Syrian hamsters, where aggressive behaviors were the same as in control males 19 days after adolescent AAS exposure (Grimes et al., 2006; Ricci et al., 2007). Housing conditions for the intruder males were not controlled in the aforementioned studies. Unpublished observations from this laboratory indicate that it's optimal when resident and intruder males are singly-housed, especially since male Syrian hamsters prefer to be socially isolated. Preliminary studies presented in this dissertation also indicate that social context is a variable that may increase or decrease the expression of aggressive behaviors.

This experiment found long term changes on agonistic behaviors due to AAS exposure during adolescence. A significant increase in number of bites, attacks, flank marking behavior and a composite aggression score in adolescents and not in adults even after four weeks of AAS exposure provides new evidence that the adolescent brain is more vulnerable to exogenous androgens. Interestingly, the display of submissive and/or defensive behaviors is also affected by adolescent AAS exposure. In a previous study, AAS-treated adolescent males showed few or no submissive behaviors when compared to vehicle control and adult-treated groups. Similarly, adolescent males exposed to AAS did not show any escape dashes, tail-up walking or stretch attend, resulting in a main effect of age exposure for all three behaviors. These results suggest

that AAS exposure during adolescence results in a lack of regulation of risk assessment and attenuation of expression of submissive behaviors.

Many studies focused on the regulation of aggression have identified serotonin as key neurotransmitter that modulates this social behavior (Delville et al., 2000; Ferris and Delville, 1994; Ferris et al., 1999; Insel et al., 1990; Nelson and Chiavegatto, 2001). Importantly, a decrease in serotonergic innervations in the amygdala and anterior hypothalamus (Grimes and Melloni, 2002), in serotonin receptors (Grimes and Melloni, 2006), and a decrease in serotonin release in the hypothalamus (Keleta et al., 2007) have been observed after adolescent anabolic steroid exposure. These neurochemical changes are long-lasting (Grimes and Melloni, 2006) and can explain the enhanced overt aggression after AAS exposure, even well after treatment.

In this experiment a significant decrease in testes weight was observed, suggesting cohort differences in the effects of AAS on peripheral tissues (Table 3.1). These results are consistent with previous studies showing a significant decrease in testes weight after AAS-treatment. However, since no other peripheral measure was affected by treatment, it implies that the effects of AAS on behavior are due to their effects on the central nervous system since the periphery is able to recover from adverse effects on seminal vesicles and a decrease in hormone levels exhibited immediately after AAS exposure (Feinberg et al., 1997).

Adolescence is a time marked by impulsivity, risk-taking behaviors and a social re-orientation (Nelson et al., 2005). Areas like the prefrontal cortex, the nucleus accumbens and the amygdala are undergoing dramatic neural changes, all of which may be affected by the anabolic steroid exposure (Ernst et al., 2006). These studies contribute significantly to the understanding that AAS exposure during adolescence may cause permanent changes to adult-typical social behaviors by increasing aspects of impulsivity and inhibiting neural systems involved in the regulation of behavior, such as dopaminergic and serotonergic circuitry. Further understanding of how anabolic steroids may cause organizational neural changes in the brain is still needed; however these profound changes in adult behavior after adolescent anabolic steroid exposure require further investigation.

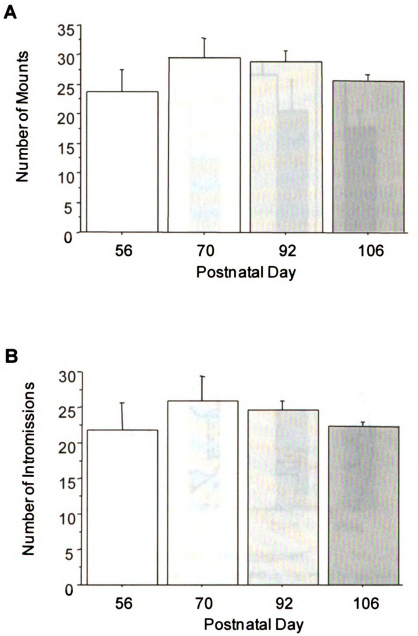


Figure 3.1 Adult sexual behavior remains consistent from P 56 to P106. A one-way ANOVA revealed no significant differences in the number of mounts and intromissions displayed by vehicle-treated males tested on P56, P70, P72 and P106.

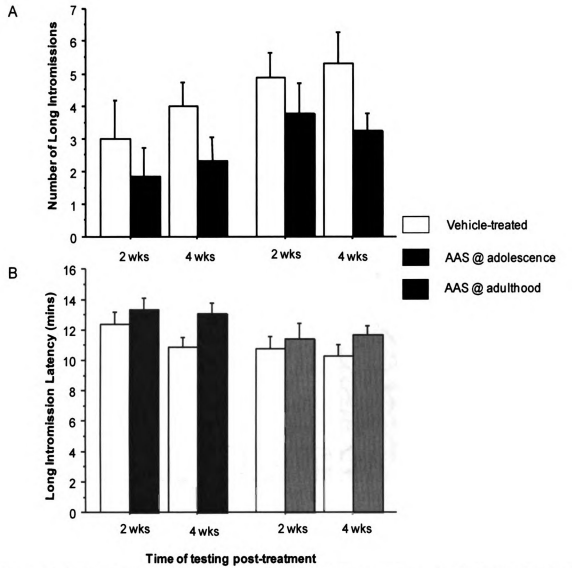


Figure 3.2. AAS exposure reduces the potential to reach satiety independently of age of AAS exposure and time of post-treatment behavioral test. A 3-way ANOVA was used to determine that AAS exposure during adolescence and adulthood significantly ($p < 0.05$) (A) decreases the number of long intromissions and (b) increases long intromission latency.

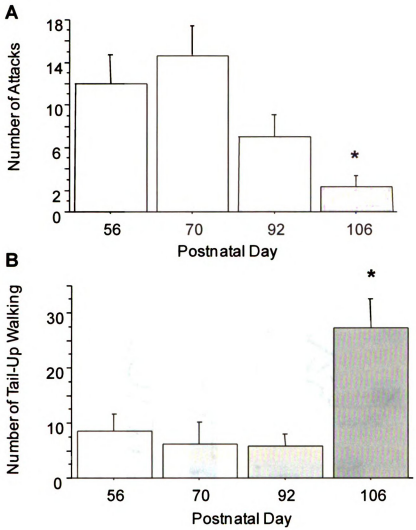


Figure 3.3 Developmental changes were found in the expression of agonistic behaviors. A one-way ANOVA revealed that the older the vehicle-treated males were when tested, the fewer (A) attacks (*, $p < .002$) and the more (B) tail-up walking (*, $p < 0.005$) they displayed.

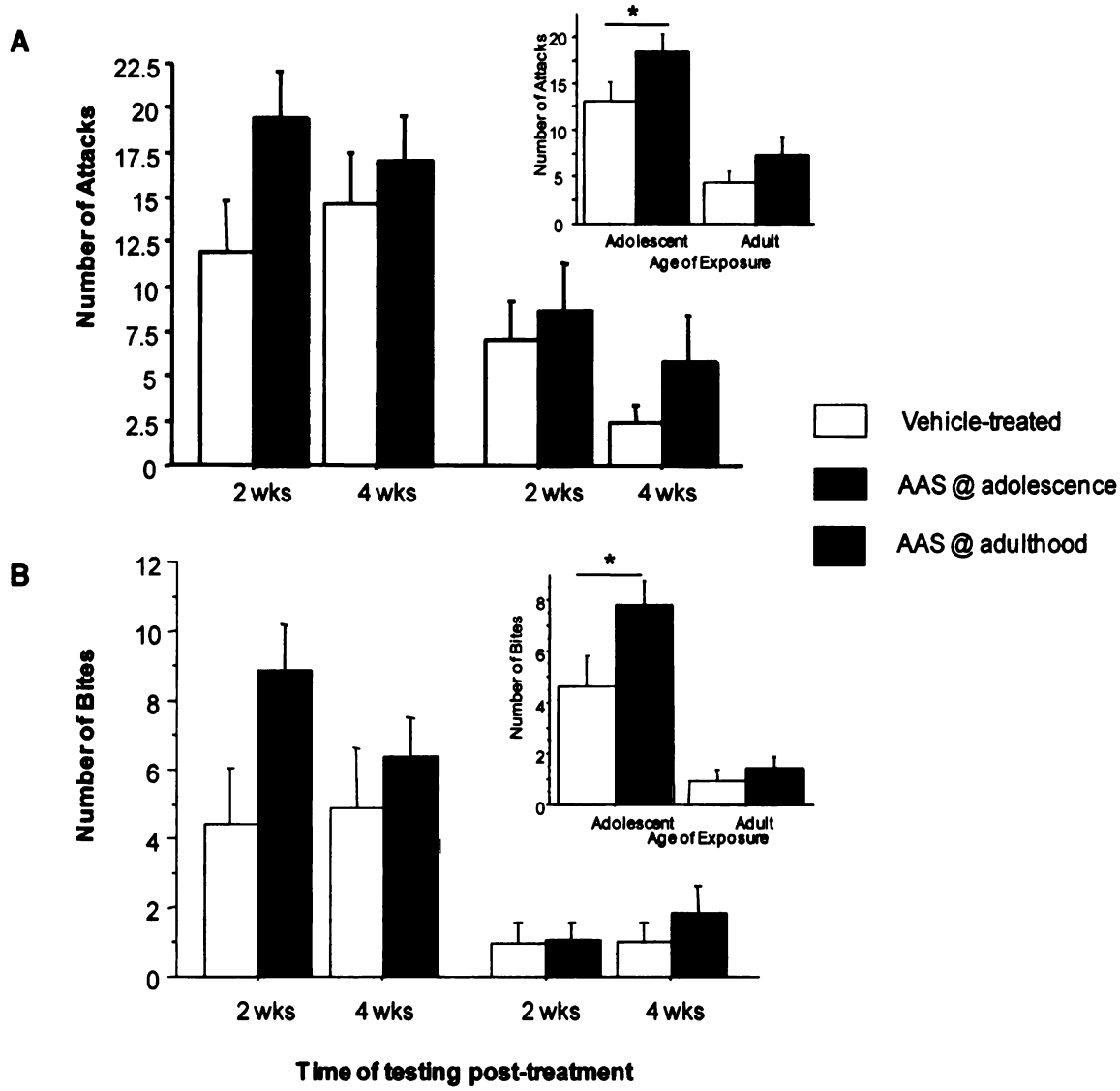


Figure 3.4 A main effect of treatment was found on (A) attacks and (B) bites ($p < 0.05$). A three-way ANOVA also revealed a main effect of age of exposure on the expression of both behaviors ($p < 0.0001$). No significant interactions were found. The insets are ANOVAs comparing age of exposure and treatment. A 2-way ANOVA comparing age of exposure and treatment determined that adolescent AAS exposure significantly increased (A) attacks (*, $p < 0.05$) and (B) bites (*, $p < 0.05$).

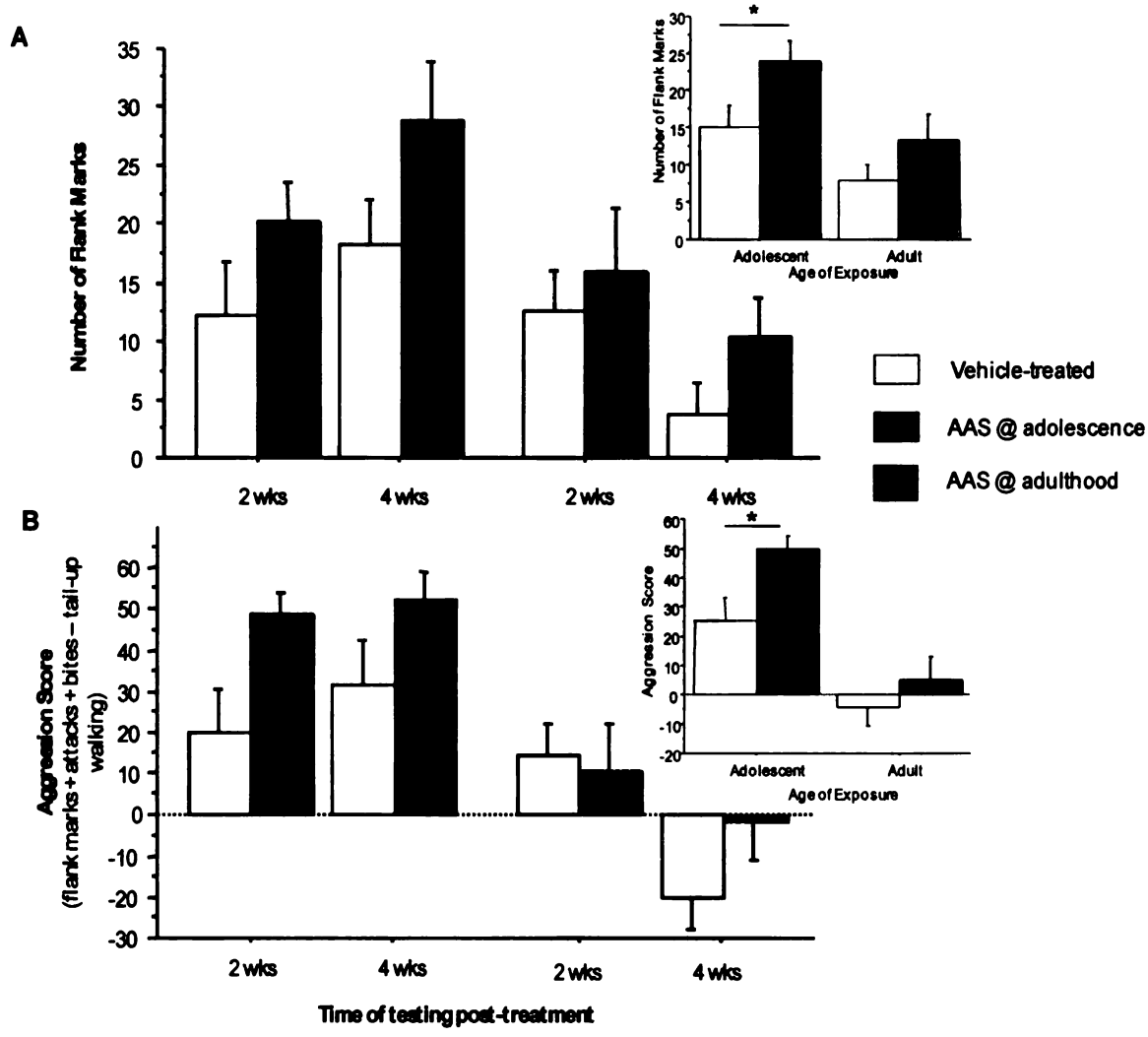


Figure 3.5 AAS exposure during adolescence significantly increased flank marking behaviors and overall aggression. (A) A 3-way ANOVA showed that flank marking behavior was increased after AAS exposure during adolescence and adulthood ($p < 0.02$). A main effect of age of AAS-exposure was observed ($p < 0.002$). The inset is a 2-way ANOVA revealed that adolescent AAS exposure significantly increases flank marking behavior when compared to vehicle- and adult-treated males (*, $p < 0.05$). (B) A 3-way ANOVA showed that AAS treatment increases aggression in males treated during adolescence and adulthood ($p < 0.02$). A main effect of age was also observed ($p < 0.001$). The inset is a 2-way ANOVA determined males exposed to AAS treatment during adolescence had significantly higher composite aggression scores than vehicle- and adult-treated adult (*, $p < 0.005$).

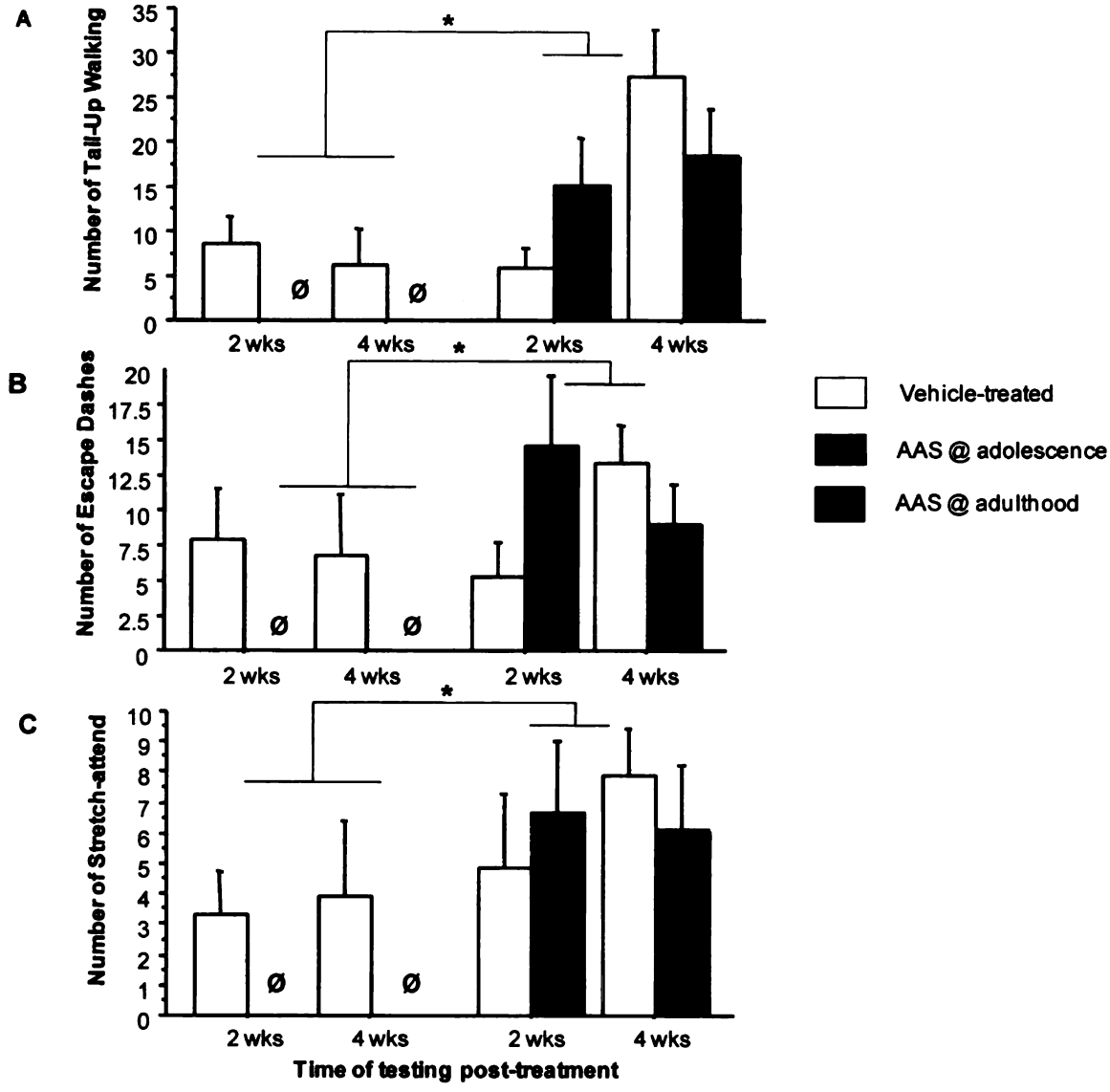


Figure 3.6 Males exposed to AAS during adolescence did not express tail-up walking, escape dashes or stretch-attend. A three-way ANOVA determined a main effect of age of exposure for (A) number of tail-up walking ($p < 0.001$), (b) escape dashes ($p < 0.02$) and (c) stretch attend ($p < 0.0005$).

Table 3.1 Peripheral measures in males exposed to AAS during adolescence or adulthood.

	Adolescent Males				**Adult Males**			
	Vehicle treated (Mean +/- SEM)		AAS-treated (Mean +/- SEM)		Vehicle Treated (Mean +/- SEM)		AAS-treated (Mean +/- SEM)	
Age of testing	P56	P70	P56	P70	P92	P106	P92	P106
Testing post-treatment	2 wks	4 wks	2 wks	4 wks	2 wks	4 wks	2 wks	4 wks
Testes Weight (g)								
Experiment 1	3.43 ± 0.1	3.41 ± 0.7	3.11 ± 0.3	3.81 ± 0.6	3.93 ± 0.4	4.04 ± 0.3	3.75 ± 0.5	3.77 ± 0.8
Experiment 2 *	3.10 ± 0.2	3.53 ± 0.2	2.87 ± 0.3	3.39 ± 0.3	3.87 ± 0.2	3.75 ± 0.2	3.21 ± 0.6	3.45 ± 0.4
Seminal Vesicle Weight (g)								
Experiment 1	0.19 ± 0.07	0. 22 ± 0.04	0.26 ± 0.03	0.24 ± 0.05	0.31 ± 0.04	0.33 ± 0.06	0.30 ± 0.05	0.35 ± 0.07
Experiment 2	0.21 ± 0.07	0.27 ± 0.04	0.24 ± 0.06	0.25 ± 0.06	0.34 ± 0.10	0.35 ± 0.06	0.37 ± 0.08	0.37 ± 0.10
Flank Gland Diameter (mm)								
Experiment 1	8.48 ± 0.6	8.35 ± 1.2	7.77 ± 2.1	8.59 ± 1.6	8.88 ± 0.9	8.63 ± 0.8	9.48 ± 1.6	8.74 ± 0.6
Experiment 2	7.04 ± 1.11	7.87 ± 0.62	7.02 ± 1.32	7.58 ± 0.60	8.73 ± 1.07	7.54 ± 1.07	8.70 ± 1.09	8.30 ± 0.80
Testosterone plasma levels (ng/ml)								
Experiment 1	2.43 ± 0.52	1.71 ± 0.18	2.38 ± 0.49	2.30 ± 0.34	2.03 ± 0.29	1.77 ± 0.25	2.21 ± 0.20	1.74 ± 0.21
Experiment 2 #	1.26 ± 0.36	0.67 ± 0.21	0.82 ± 0.22	0.47 ± 0.09	0.77 ± 0.13	0.47 ± 0.10	0.61 ± 0.08	0.54 ± 0.13

A main effect of age of exposure was found on all measures ($p < 0.05$).
* A main effect of treatment was found on testes weight in Experiment 2 ($p < 0.001$).
# A main effect of testing post-treatment was found on testosterone plasma levels in Experiment 2 ($p < 0.02$).

CHAPTER 4: ADOLESCENT ANABOLIC STEROID EXPOSURE AFFECTS MEDIAL AMYGDALA VOLUME AND CELL ADDITION IN THE MALE SYRIAN HAMSTER

Introduction:

Pheromonal communication and hormonal signals are critical for the expression of male social behaviors in rodents. The neural circuits that underlie male reproductive behaviors [which includes the bed nucleus of the stria terminalis (BNST) and the medial preoptic area (MPOA)] and inter-male aggressive behaviors [which includes the anterior hypothalamus (AH), the BNST, and the lateral septum (LS)] share efferent and afferent connections to and from the medial amygdala (Me) (Ferris et al., 1987; Kollack-Walker et al., 1999; Kollack-Walker and Newman, 1995; Potegal et al., 1996; Romeo and Sisk, 2001). The connections of the amygdala can be divided into three systems (1) sensory processing through connections with the olfactory cortex, posterior thalamus, and sensory association cortex, (2) visceral function in relation to the emotional stimuli through connections with the hypothalamus, and (3) emotional behavior and mood through connections that comprise the forebrain circuit (Price, 2003). The Me is a complex structure that has been subdivided based on cytoarchitectonic, synaptic and functional criteria, into four main nuclei: anterodorsal (MeAD), anteroventral (MeAV), posterodorsal (MePD) and posteroventral (MePV) (Alheid, 2003; Cooke and Simerly, 2005). The Me subnuclei contribute to the interpretation of sensory information (Wood and Coolen, 1997), for the regulation of intermale aggression and reproductive

behaviors, (Kollack-Walker and Newman, 1995) and to the interpretation of emotionally loaded stimuli and memory modulation (Rasia-Filho et al., 2004).

Gonadal hormones play a role in neural plasticity during puberty in the amygdala (Romeo and Sisk, 2001). The anterior medial amygdala (MeA) and posterior medial amygdala (MeP) are steroid sensitive, and morphological changes in these nuclei may reflect changes in the expression of male social behaviors. The removal of gonadal hormones in adult males results in a decrease in somal size of individual cells and/or overall volume of the Me. Interestingly, the MeA is significantly larger in prepubertal male Syrian hamsters when compared to adults; however the inverse in true about the MePD (Romeo and Sisk, 2001). Androgen-dependent plasticity in the MePD has also been observed by using Golgi-staining in male Syrian hamsters. In castrated males, neuronal structure in the posterior, but not in the anterior, region of the Me undergo structural changes such as increase dendritic pruning during adolescent development and due to a rise in gonadal hormones (Gomez and Newman, 1992; Zehr et al., 2006). The MeA receives substantial projections from the accessory olfactory bulb, and lesions to the MeA abolish chemoinvestigatory behavior, suggesting the pubertal development may involve changes that allow appropriate processing and integration of these cues (Gomez and Newman, 1992; Wood and Newman, 1995b). However, the MeP contains more androgen (AR) and estrogen receptors (ER) than the MeA (Cooke et al., 2003; Wood and Newman, 1995a), suggesting that it may retain the capacity for plasticity

throughout life so that the expression of male social behaviors is possible under specific steroidal and/or environmental conditions (Romeo and Sisk, 2001).

There is a major gap in understanding how AAS affect the adolescent brain, specifically the Me; however several studies have been focused on the effects of AAS on the adult brain. In adult male rats androgen receptors are upregulated in the Me after being treated for two weeks with a cocktail of AAS (Lynch and Story, 2000; Menard and Harlan, 1993). A central injection of testosterone in adult male Syrian hamsters increases Fos, expression, a marker for neuronal activity, in the MePD after one day of exposure (Dimeo and Wood, 2005). These findings suggest that Me neurons are sensitive to acute and immediate effects of AAS. Recently, AAS exposure during adolescence was also shown to increase spine density in the MeA and MeP immediately after treatment, however these changes in spine density did not persist into adulthood (Cunningham et al., 2007).

Another amygdaloid nucleus involved in social communication and cognition that may be significantly affected by AAS exposure is the central amygdala (CeA). CeA has been implicated in fear related behaviors (Wilensky et al., 2006), physical aggression in rodents (Bedard and Persinger, 1995) and scent marking in the hamsters (Bamshad et al., 1997). Adolescent AAS exposure can alter developmental patterns of serotonin innervations to other the lateral hypothalamus and the anterior hypothalamus, that are implicated in

aggression and social communication (Grimes and Melloni, 2002). However, little is known about neural changes that occur during adolescence in the CeA.

Gonadal steroid hormones, like testosterone and estrogen, also play an important role in the proliferation (Ormerod and Galea, 2001), survival (Leranth et al., 2000) and activation (Insel, 1990) of neurons. Studies in male rodents show that gonadal steroid hormones influence the number of newly born cells in the amygdala (Fowler et al., 2003; Huang and Bittman, 2002).

In order to understand how androgenic-anabolic steroids (AAS) affect neurogenesis, a study examined the effect of nandrolone decanoate on rat neural stem cells (NSC) in culture on the dentate gyrus in the rat hippocampus. *In vitro,* NSC were stimulated by epidermal growth factor and nandrolone reduced cell proliferation. Nandrolone also decreased neurogenesis (measured by bromodeoxyuridine (BrdU) labeling) in the dentate gyrus of the rat hippocampus in both adult males and females after 5 days of administration (Brannvall et al., 2005). Other drugs of abuse, such as nicotine, cocaine, and alcohol, cause a disruption in neurogenesis, inhibit cell survival, and generally affect neural plasticity (Abrous et al., 2002; He et al., 2005; Kerns et al., 2005; Yamaguchi et al., 2004).

The goal of this experiment was to determine if adolescent AAS exposure affects the number of new cells added to amygdaloid nuclei after treating male

Syrian hamsters with a cocktail of AAS and BrdU throughout two-wks in early adolescence. Evidence from previous studies predicts that AAS will decrease cell proliferation in the adolescent brain. In addition, evidence from other studies predicts that the MePD would be particularly sensitive to the effects of adolescent AAS-exposure.

Materials and Methods

Animals

Thirty-two male Syrian hamsters (Harlan, Madison WI) arrived on postnatal day 18 (P18) without their dams. Upon arrival subjects were housed in groups of eight animals per cage (polycarbonate, 33 x 38 x 17 cm) with ad libitum access to food (Telkad Rodent Diet No. 8640, Harlan) and water. They were singly housed on postnatal day 25 in clear polycarbonate cages and randomly selected for assignment to one of four treatment groups. Animal colony temperature was maintained at 22 ± 2°C, and animals were kept on a light-dark cycle of 14:10 L:D (lights off at 1600h EST). All animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals, and all protocols were approved by the Michigan State University All-University Committee for Animal Use and Care.

AAS Treatment

A cocktail of AAS containing 0.8 mg/ml of testosterone cypionate (TC; Sigma Aldrich, Inc., St. Louis, MO), 0.8 mg/ml of nandrolone decanoate (ND;

Sigma Aldrich, Inc.), and 0.4 mg/ml of boldenone undecylenate (BU; Steraloids, New Port, Rhode Island) was dissolved in 2-hydroxypropyl-β-cyclodextrin (Sigma-Aldrich, Inc.). The AAS cocktail was prepared the day before treatment began, and was stored at 4°C.

Experimental Design

In order to examine the effects of AAS during adolescence on cell proliferation and cell survival, gonad-intact male hamsters were randomly divided into four groups: (1 and 2) vehicle-treated or AAS-treated and perfused during late adolescence on P42, and (3 and 4) vehicle-treated or AAS-treated and perfused in adulthood on P70. Subjects received a daily subcutaneous (*sc*) injection of either the AAS cocktail (2 mg/kg TC, 2 mg/kg ND, 1 mg/kg BU) or vehicle (2-hydroxypropyl-β-cyclodextrin) during adolescence (from 27-41 days of age). Injection volume ranged from 0.1 ml to 0.25 ml throughout the two-week course of administration, depending on subject weight.

The cell birth marker, 5'-bromo-2'-deoxyuridine (BrdU; cat. No. B-9285, Sigma: St. Louis, MO) was used to identify proliferating cells during adolescence. BrdU injections were given intraperitoneally (i.p.; 300 mg/Kg BW, 10mg /ml BrdU in 0.9% NaCl) to all subjects every other day from P28-42 for a total of 8 injections per subject. BrdU was made fresh every day of injection. Injection volume ranged from 1.6 ml to 2.6 ml throughout the two-week course of administration, due to the increase in body weight during development.

Groups treated with either vehicle or AAS were perfused on postnatal day 42, 24 hours after the last AAS-injection and 4 hours after their last BrdU injection to assess for cell proliferation immediately after AAS exposure. The two remaining groups were perfused in adulthood on postnatal day 70, 4 wks after the last AAS and BrdU injections to assess cell survival after adolescent AAS exposure.

Tissue Collection and Physiological Measures

On the day of sacrifice, males were administered an overdose of sodium pentobarbital (130 mg/kg i.p.) and a terminal blood sample was taken via cardiac puncture. Testes and seminal vesicles were removed and weighed. Flank gland diameters were assessed by shaving the subject's side and measuring the left gland with a caliper. Perfusions were performed intracardially with heparinized phosphate-buffered saline rinse (~200 ml) followed by 4% paraformaldehyde (~150 ml). The brains were removed, post-fixed in 4% paraformaldehyde overnight, and then stored in a 30% sucrose solution. All brains were sectioned on a cryostat in four series at 40μm per section and stored in glycerol-based cryoprotectant at –20°C, except for one series that was mounted fresh frozen while sectioning. This set of sections was Nissl-stained and used to determine the boundaries of cell groups within the medial and central amygdala.

Plasma testosterone concentrations were measured in duplicate using a commercially available radioimmunoassay kit (Coat-a-Count Total Testosterone,

Diagnostic Products, Los Angeles, CA). This assay has been previously validated for measuring endogenous plasma testosterone levels in hamsters in our laboratory (Parfitt et al., 1999). The intra-assay CV was 4.7%, and the limit of detectability was 0.1 ng/ml.

BrdU Immunohistochemisty

A set of sections was used to perform a single-label BrdU immunohistochemistry according to previously established protocols (Ahmed, et al.). In brief, free-floating sections were incubated in 50% formamide at 65°C for 2 h, rinsed in 2X standard sodium citrate (SSC), incubated in 2 N HCl for 30 min at 37°C, and rinsed in 0.1 M borate buffer pH 8.5 for 10 min. After three rinses in Tris-buffered saline (TBS), sections were blocked in TBS containing 0.1% Triton X-100 and 3% donkey serum (Jackson ImmunoResearch Lab., Inc, West Grove, PA) for 30 min. Sections were incubated overnight at 4°C with monoclonal rat anti-BrdU (1:1000; MCA2060, Serotec, Ltd, Oxford, UK) and incubated for 2 hours at room temperature with biotinylated donkey anti-rat (1:250; 712-065-150, Jackson ImmunoResearch Lab.,Inc, West Grove, PA). Finally, sections were incubated for 60 min at room temperature with ABC reagent (ABC Elite kit; Vector Laboratories, Burlingame, CA) and reacted for 6 min in diaminobenzedine (0.25 µg/ml in TBS with 0.01% H_2O_2; Sigma-Aldrich, St. Louis, MO). All solutions used in this experiment were made fresh the day before the immunohistochemistry was performed.

To generate positive control tissue, pregnant female rats were injected with BrdU (50 mg/kg body weight) on gestational days 13-15 and allowed to deliver litters naturally. When litters were 21 days old, brains of pups exposed to BrdU on embryonic days 13-15 were collected as described for the experimental groups. Tissue from these animals showed substantial BrdU labeling throughout the brain. To assess nonspecific immunoreactivity, sections from BrdU-injected animals were processed in the absence of either primary or secondary antibody. Omission of primary or secondary antibodies eliminated BrdU-labeling. These histological controls were included in the immunohistochemical processing of the experimental tissue.

Microscopic Analysis

Subregions of the medial amygdala (MeAD, MeAV, MePD, MePV) and the central amygdala (CeA) were traced bilaterally in Nissl-stained sections (Figure 1). Regional boundaries were traced at 4X magnification using Neurolucida (Version number 6.50.1, Microbrightfield, Inc., Williston, VT). Cytoarchitectural characteristics (e.i. cells in the ventral nuclei are darker and densely packed, whereas cells in the dorsal nuclei are organized in a columnar, cone shape) and structural landmarks (e.i. the shape of the optic tract, the location and shape of the basolateral amygdala and the intercalated nuclei of the amygdala) for each nuclei were used to determine contour boundaries and tracings. Total cross-sectional area for each subregion was calculated by summing subregion areas across sections. The volume for each amygdaloid nucleus was calculated by

summing the traced cross-sectional areas and multiplying by the distance between sections (120 μm). Tracings from Nissl sections were aligned with adjacent BrdU-ir sections using the optic tract and section outline, along with a notch on the left hemisphere to determine laterality. All BrdU-ir cells located within the tracing boundaries were counted at 40X magnification (Figure 2). Because the size of medial amygdala subregions varies with age and sex (Cooke et al., 1997; Romeo & Sisk, 2001; Morris et al., 2006), the total number of BrdU-ir cells was divided by the total cross-sectional area for each subregion.

Statistics

Two-way ANOVAs were used to determine the main effects of treatment (AAS or vehicle), time since the last BrdU injection (4-hrs or 4-wks after), and the interactions between these two factors on the mean number of BrdU-labeled cells per unit area in the subdivisions of the medial amygdala and in the central amygdala. Post-hoc Student's T-tests were performed to determine differences between vehicle and AAS-treated males within each age group if an age by treatment interaction was determined.

Results

Number of BrdU-labeled cells and cell group volume

A two-way ANOVA revealed a main effect of age of perfusion (P42, 4-hrs after treatment vs. P70, 4-wks after treatment) on the number of BrdU-labeled cells per mm^2 in the CeA [Fig. 1A; $F(1, 23) = 48.4$; $P < 0.0001$], MeAD [Fig. 2A;

F (1, 23) = 18.6; P < 0.003], MeAV [Fig. 3A; F (1, 23) = 48.1; P < 0.0001], MePD [Fig.4A; F (1, 23) = 64.5; P < 0.0001] and MePV [Fig. 5A; F (1, 23) = 33.6; P < 0.0001]. Subjects that were perfused on P42 had significantly more BrdU-labeled calls in all amygdaloid nuclei than subjects perfused on P70. This result indicates that not all cells born between P28-P42 survive until P70.

A two-way ANOVA revealed a significant interaction between age and treatment in BrdU-labeled cells per mm^2 in MePD [Fig. 4A; F (1, 23) = 6.2; P < 0.02]. Specifically, the number of BrdU-labeled cells was significantly less in the vehicle-treated males perfused 4-hrs after treatment than in AAS-treated males [Fig.4A; F (1, 12) = 9.2; P < 0.01]. However, by 4-wks after treatment, there were no differences in the number of BrdU-labeled cells between vehicle- and AAS-treated males.

No effects of age or treatment were found on the volume of the CeA (Fig 1B), MeAD (Fig 2B), MePD (Fig 4B) or MePV (Fig 5B). However, the volume of the MeAV was significantly increased by AAS exposure during adolescence [Fig. 3B; F (1, 23) = 4.2; P < 0.05). This increase in volume in AAS-treated males persisted even 4-wks after discontinuation of AAS treatment.

Physiological measures:

AAS exposure during adolescence had profound effects on plasma testosterone (T) levels and testes weight. Main effects of age [Fig. 6; F (1, 23) =

7.7; $P < 0.01$] and treatment [Fig. 6; $F(1, 23) = 7.3$; $P < 0.01$] were found on T plasma levels; however no significant interactions were found. T plasma levels were reduced by adolescent AAS treatment, and T samples taken on P42 were higher than samples taken on P70. A significant interaction between age and treatment was found on testes weight [Fig.6; $F(1, 23) = 4.9$; $P < 0.05$], where AAS-treated males perfused 4-hrs after treatment had significantly reduced testes weight than vehicle-treated males [Fig.6; $F(1, 12) = 17$; $P < 0.001$], but no significant reduction in testes weights was found in males perfused 4-wks after treatment. Seminal vesicle weight and flank gland diameter were not affected by adolescent AAS-exposure.

Discussion

This study provides the first evidence that new cells are added to the hamster medial amygdala during adolescence and that approximately one-third of them survive to adulthood. In general, this study found no effect of AAS treatment on the addition of new cells during adolescence, with the exception of a decrease in cell proliferation in the MePD. This finding is similar to that of a previous study showing nandrolone decanoate decreases cell proliferation in the dentate gyrus of adult rodents (Brannvall et al., 2005) . Adolescence AAS treatment did result in an increase MeAV volume that persisted into adulthood. Increased cell proliferation in intact animals has been reported in other brain regions such as hippocampus (He and Crews, 2007).

AAS exposure during adolescence significantly decreased the number of BrdU-labeled cells in the MePD suggesting that this subdivision of the amygdala is vulnerable to supraphysiological levels of synthetic androgens. The MePD has been studied extensively because of its role in sensory-hormonal integration and its reciprocal interconnections with the limbic system in order to control social behaviors (Newman, 1999; Skuse et al., 2003). In male Syrian hamsters, the MePD is involved in regulating the expression of sexual and agonistic behaviors (Kollack-Walker and Newman, 1995). Through studies assessing neurogenesis, spine density, dendritic arborizations, soma size and volume, the medial amygdala has been shown to be very plastic, especially during adolescence and after manipulation of gonadal hormones (Cooke, 2006; Fowler et al., 2003; Rasia-Filho et al., 2004; Romeo and Sisk, 2001; Zehr et al., 2006). The MePD is steroid receptor rich and an increase in androgen receptor immunoreactivity is observed after testosterone treatment in the prepubertal medial amygdala when compared to adults (Meek et al., 1997; Romeo et al., 2000; Wood et al., 1992). Therefore MePD cells may be particularly sensitive to the central actions of AAS exposure during adolescence. In addition, a recent study showed that spine density was increased by AAS immediately after treatment in the MeP, however, these changes did not persist 4-wks after withdrawal (Cunningham et al., 2007). Taken together, adolescent AAS exposure affects neural plasticity in the MePD, however, these particular changes do not persist into adulthood.

AAS treatment increased the volume of the MeAV immediately and 4-wks after exposure, suggesting a permanent change in this particular subdivision of the Me (Fig.2). The MeAD and MeAV are activated after general arousal, especially in relationship to sexual behavior (Gomez and Newman, 1992; Kollack-Walker and Newman, 1997). The MeA is also involved in the interpretation of a social interaction resulting in a defensive behavior (Choi et al., 2005). Studies from this laboratory have shown that adolescent AAS exposure significantly reduced or eliminated the expression of submissive and defensive behaviors, even 4-wks after discontinuation of AAS treatment (see Chapters 2 and 3). These data suggest the AAS-induced changes in volume in the MeAV may be a result of organizational changes at the cellular or molecular level that in turn affect behavior.

The amygdaloid nuclei CeA, MeAD and MePV were not affected by adolescent AAS exposure, however, they are all involved in the interpretation of environmental cues and mediate social behaviors. The central amygdala has been implicated in the coordinating actions of fear and anxiety like behaviors (Jasnow et al., 2004). All subdivisions of the medial amygdala are activated after sexual and agonistic behaviors (Kollack-Walker and Newman, 1995). Male treated with this cocktail of AAS have long lasting changes in serotonergic circuitry in the central and medial amygdala (Grimes and Melloni, 2002), suggesting cell proliferation is not the only neural change responsible for the effects on social behaviors induced by adolescent AAS exposure.

This experiment used BrdU to label cells added to the amygdaloid nuclei. In the last decade several studies have been conducted to assess the BrdU labels newly born cells. Different methods of delivery have been performed and peripheral injections have consistently labeled cells that have undergone DNA synthesis (Kokoeva et al., 2005; Rakic, 2002). Most studies of neurogenesis used a commonly used dose of BrdU injection at a concentration of 50mg/kg. In the present study BrdU was injected at a concentration of 300mg/kg because of a recent report suggests that a higher dose of BrdU (300 mg/kg) is a better quantitative marker of proliferating cells of rats, as a lower dosages only label a fraction of the S-phase cells (Cameron and McKay, 2001). In addition to single label BrdU, fluorescent immunocytochemical techniques have been used to identify mature and immature neurons, astrocytes and whether these new born cells are functional (Huang and Bittman, 2002; Kokoeva et al., 2005; Kokoeva et al., 2007). A natural follow-up to this study would be to assess whether the cells that are affected in the MePD are neurons or glia. In this particular study, many more BrdU-labeled cells were observed in the adolescent brain (immediately after treatment) that the adult brain (cell survival). Previous findings demonstrate a relationship between steroid hormones and neurogenesis in the medial amygdala during in adulthood (Bernier et al., 2002; Fowler et al., 2007) .

Thus far, few studies have focused on organizational changes caused by adolescent AAS exposure and the underlying mechanisms for cell addition to neural structure during adolescence and adulthood. The views on neural

plasticity and organizational changes during adolescence are being modified due to recent imaging evidence (Giedd et al., 1999; Giedd et al., 2006; Lenroot et al., 2007). It is important to continue to investigate the underlying mechanisms that make the adolescent brain vulnerable to exogenous compounds and the consequences of these. This study provides further evidence on how AAS affect the adolescent brain and the effects of supraphysiological androgen levels on neural plasticity.

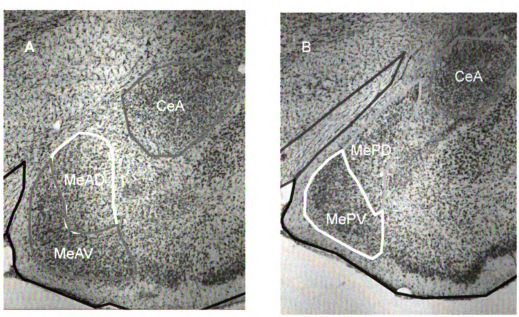


Figure 4.1 Photomicrographs of Nissl-stained tissue at 4X of the amygdaloid nuclei. Neurolucida tracings outline (A) central amygdala (CeA), anterodorsal medial amygdala (MeAD), anteroventral medial amygdala (MeAV); (B) CeA, posterodorsal medial amygdala (MePD), posteroventral medial amygdala (MePV)].

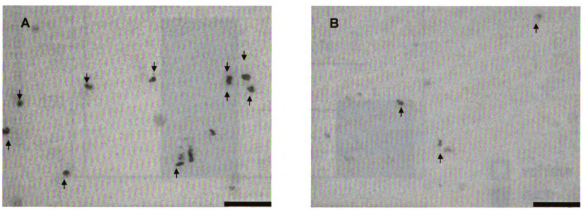


Figure 4.2 Photomicrographs at 40X of BrdU-labeled cells (arrows) in the amygdala (scale bar = 500 μm). In general, a significantly higher number of BrdU-labeled cells was identified in males perfused 4-hrs after the last BrdU injection (A) than in males perfused 4 wk after the last BrdU injection (B). Approximately one third of the cells born during adolescence survive to adulthood.

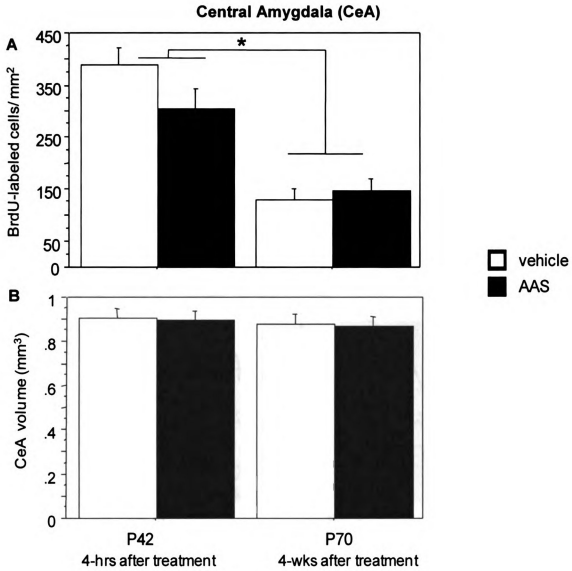


Figure 4.3 AAS exposure during adolescence does not affect cell proliferation or cell survival in the CeA. (A) A 2-way ANOVA revealed a main effect of age on the number of BrdU-labeled cells/mm^2 (*$p < 0.0001$). No effects of treatment were found in BrdU-ir cells/mm^2 or volume (mm^3) (B).

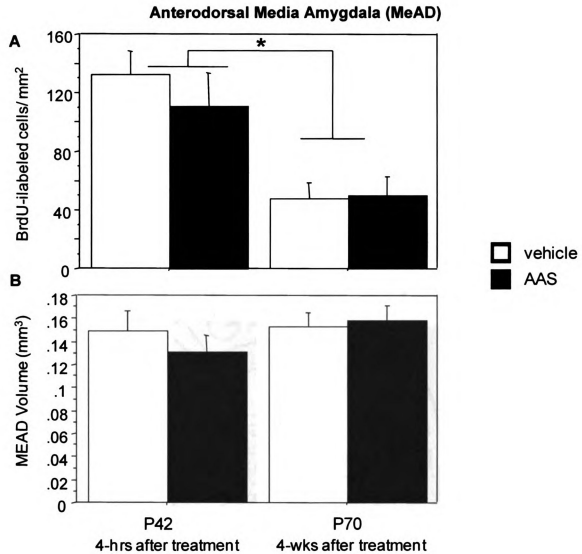


Figure 4.4 AAS exposure during adolescence does not affect cell proliferation or cell survival in the MeAD. (A) A 2-way ANOVA revealed a main effect of age on BrdU-labeled cells/mm^2 (*p< 0.001). No effects of treatment were found on BrdU-labeled cells/mm^2 or volume (mm^3) (B).

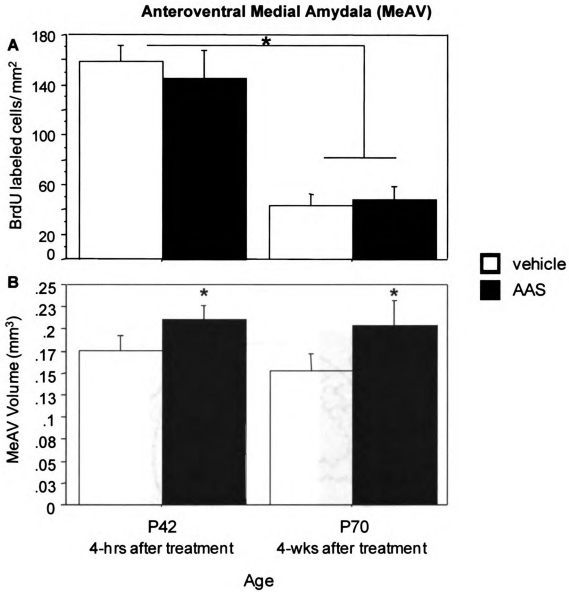


Figure 4.5. AAS exposure during adolescence does not affect cell proliferation or cell survival in the MeAV, however AAS exposure significantly increases MeAV volume even 4 wks after treatment. (A) A 2-way ANOVA revealed a main effect of age on BrdU-ir cells/mm^2 (* $p<0.0001$). (B) AAS-treatment increased MeAV volume (mm^3) in males perfused immediately after treatment and 4 wks after treatment (* $p < 0.05$).

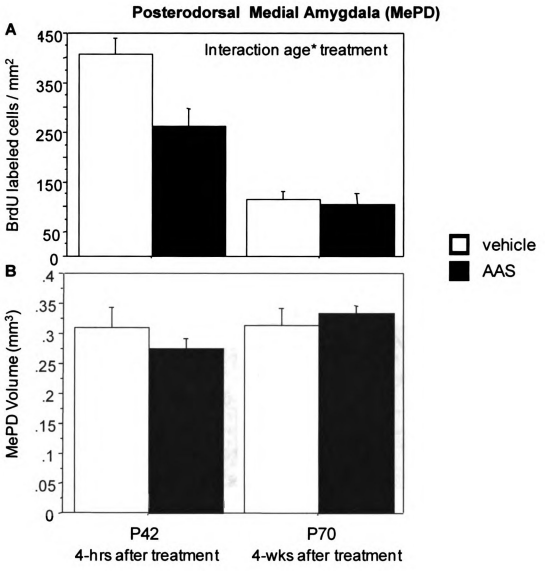


Figure 4.6 AAS exposure during adolescence decreases the number of cells added to the MePD. (A) A significant interaction between age and AAS-treatment was found on BrdU-ir cells/mm^2 (* $p < 0.05$). AAS treated males had significantly less cells added to the MePD than vehicle-treated animals when perfused 4 hr after treatment but not when perfused 4 wk after treatment. (B) No effects of age or treatment were found on MePD volume (mm^3).

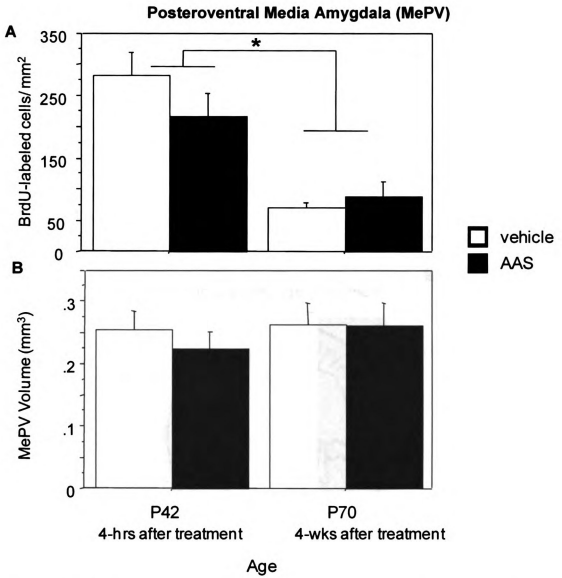


Figure 4.7 AAS exposure during adolescence does not affect cell proliferation or cell survival in the MePV. (A) A 2-way ANOVA revealed a main effect of age on BrdU-ir cells/mm^2 ($p < 0.0001$). No significant of treatment was found on BrdU-ir cells/mm^2 or volume (mm^3) (B).

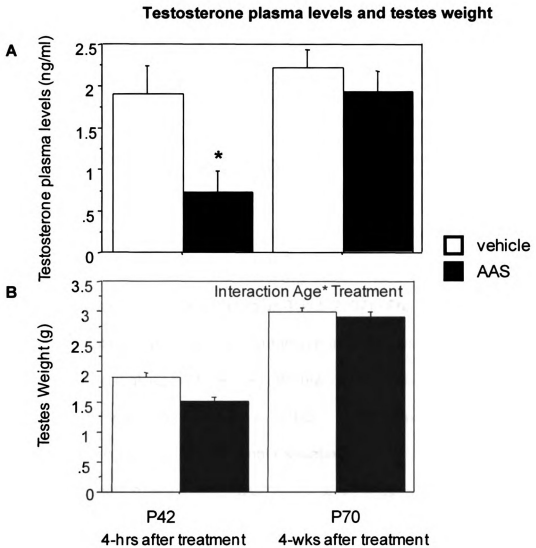


Figure 4.8 AAS exposure during adolescence decreases plasma testosterone levels and testes weight immediately after treatment. (A) A 2-way ANOVA revealed a significant main effect of treatment (*$p < 0.01$) and age ($p < 0.01$) on plasma testosterone levels. (B) A 2-way ANOVA revealed an interaction between age and treatment ($p < 0.05$) on testes weight.

CHAPTER 5: IMPLICATIONS AND CONTRIBUTIONS TO UNDERSTANDING THE DEVELOPMENT OF SOCIAL BEHAVIORS AND THE EFFECT OF ANABOLIC STEROIDS ON THE ADOLESCENT BRAIN

In the last decade, adolescent maturation of brain, behavior and cognition has received more attention than ever before. Most people understand that adolescence is a time when secondary sexual characteristics develop; for example, a male's voice gets deeper. However, there is now an increasing appreciation for the substantial neuroanatomical, emotional, and behavioral changes that also occur during this developmental stage (Lenroot and Giedd, 2006; Nelson et al., 2005; Yurgelun-Todd, 2007). For example, cortical and subcortical regions change in volume in human adolescents, and areas important for decision making such as the prefrontal cortex develop late in adolescence and young adulthood (Giedd et al., 2006). These developmental changes likely underlie the changes in emotional regulation and risk taking behavior exhibited by adolescent males and females (Ernst et al., 2006; Gardner and Steinberg, 2005; Steinberg, 2004). The environment of an adolescent is also in constant flux, and the socio-emotional behavioral changes occurring during adolescence in turn influence their environment. For example, the behavior of teens varies dramatically depending on the presence or absence of peers (Steinberg and Monahan, 2007). Given the dramatic neurodevelopmental and socio-emotional changes occurring during adolescence, it is not surprising that many psychopathologies such as depression, schizophrenia and body image disorders (e.g. anorexia, bulimia) first emerge during adolescence (Choi et al., 2002; Kanayama et al., 2006; Klump et al., 2007; Thompson et al., 2004; Zehr et al.,

2007). Furthermore, both human and animal research clearly demonstrate an increased propensity for risk taking behavior and novelty seeking during adolescence (Laviola et al., 2003; Spear, 2000), which undoubtedly contributes to increased drug use during adolescence. Thus, in many ways, adolescence is a period of developmental vulnerability (Crews et al., 2007).

Although drug use increases during the adolescent period, our understanding of the neurodevelopmental and behavioral consequences of many drugs are poorly understood. Adolescent anabolic steroid use, for example, is rapidly becoming a health problem in the United States (Kanayama et al., 2006; Wood, 2006). At least one million Americans use AAS to gain muscle mass and/or lose body fat, and, unfortunately, the prevalence in AAS use among adolescents has increased significantly in the last 10-yrs. In general, AAS use causes significant medical problems such as suppression of neuroendocrine function, hepatoxicity, myocardial dysfunction and adverse effects on lipoproteins. In addition, AAS use also promotes psychiatric effects such as major mood disorders (Pope and Katz, 1994), dependence syndrome (Brower, 2002), violent behavior (Daly et al., 2003; Midgley et al., 2001; Perry et al., 2003) and possibly an increase in progressing to opioid abuse (Celerier et al., 2006; Celerier et al., 2003; Peters and Wood, 2005). The experiments within this dissertation sought to elucidate the some of the neurobiological and behavioral consequences of AAS use during adolescence by 1) comparing the effects of AAS treatment during adolescence and adulthood, and 2) determining both the short and long-term effects of AAS use on the development of social behaviors

(mating and aggression), and the key brain region regulating these behaviors, the medial amygdala. The results of these experiments clearly demonstrate that the adolescent brain is particularly sensitive to the effects of anabolic steroid exposure.

Chapter 2 Summary.

Experiments in Chapter 2 were designed to determine whether behavioral responses to AAS, immediately following the cessation of treatment, differ between adolescent and adult animals. Interestingly, AAS significantly increased sexual behavior in adolescent males, but significantly decreased sexual behavior in adult males. Aggressive behaviors were also significantly increased in adolescent males, but were relatively unaffected in adult males. Thus, clear differences in behavioral responses to AAS were observed between adolescent and adult animals.

The enhanced behavioral responses to AAS in adolescents could be due to age-related differences in neurotransmitter release, receptors, or connectivity within and among the brain regions comprising these behavioral circuits. For example, several neurotransmitter systems within these areas mature during adolescent brain development. These include dopamine (DA), serotonin (5-HT), argenine vasopressin (AVP), and GABA (Clark and Henderson, 2003). Not only are these systems different pre- and post-pubertally, but they are also in flux during adolescence and are steroid-sensitive. In other experimental paradigms, AAS have been shown to increase 5HT1b receptor expression in the adolescent

male Syrian hamster (Ferris et al., 1997; Grimes and Melloni, 2002; Grimes and Melloni, 2005; Grimes and Melloni, 2006; Ricci et al., 2005b; Ricci et al., 2006), to increase DA receptor expression in the adult male rat (Kindlundh et al., 2002; Kindlundh et al., 2001; Kindlundh et al., 2003b; Kindlundh et al., 2004), to decrease vasopressin immunoreactivity in varicosities and terminals (DeLeon et al., 2002; Ferris et al., 1996; Ferris et al., 1997; Grimes et al., 2006; Harrison et al., 2000), to decrease GAD65 (Grimes et al., 2003; Ricci et al., 2005a), and to alter $GABA_A$ subunit composition in pubertal mice (Clark et al., 2006; Henderson et al., 2006; McIntyre et al., 2002; Penatti et al., 2005) . Furthermore, serotonin decreases and dopamine increases in the rat hypothalamus, striatum and amygdala after AAS-exposure (Keleta et al., 2007; Thiblin et al., 1999a). These previous studies examined neurochemical responses in adulthood after AAS treatment of various durations and times (during adolescence and adulthood), and therefore are not directly comparable to the AAS treatments and age groups in the present experiments. Nevertheless, they suggest that the dissimilar behavioral responses observed here in adolescents and adults could be the result of actions of AAS unique to particular stages of brain development.

Chapter 3 Summary

The experiments within chapter 3 were aimed at determining whether the immediate effects on social behavior observed in Chapter 2 are long-lasting. Therefore, behavior was assessed two- and four- weeks after the discontinuation of AAS treatment. In general there were no long term effects on the display of

sexual behaviors, regardless of age of exposure. However, AAS exposure reduced the likelihood of reaching sexual satiety in treated males, measured by the display and latency of long intromissions. This is a finding unique since Farrell, et al. (2004) found there was an inhibition of sexual behaviors in rats treated with anabolic steroids during adolescence. Satiety is defined as a state of feeling satisfied, and is mostly mediated by the reward circuit composed of the nucleus accumbens and prefrontal cortex. Since satiety was the only measure decreased when males were tested with a receptive female, the data presented in this dissertation suggest that anabolic steroids may alter brain areas that reward-related associative behaviors. Studies done in rats and mice have shown an increase is dopamine synthesis immediately after exposure and D_2 receptors are down-regulated in the key structures in the reward circuit (e.g. nucleus accumbens) (Kindlundh et al., 2001; Kindlundh et al., 2003b; Kurling et al., 2005; Thiblin et al., 1999a). All these changes will alter the potential to reach satiety, therefore delaying the display of long intromissions within a 15-minute interaction of a male and female.

Adolescent AAS exposure increases aggressive behaviors throughout development. Males tested with an age and weight matched opponent displayed significantly more attacks, bites and flank marks immediately after treatment and long after. Interestingly, males exposed to AAS during adolescence did not display any submissive behaviors. This was not the case in males treated in adulthood. In general there was more variability in an intermale interaction between adults. After AAS exposure, adults tended to display more overt

aggression when compared to vehicle-treated adults. Over development a significant increase in the expression of submissive behaviors was observed; however, AAS treated adults displayed less submissive behaviors than vehicle-treated adults. These findings are comparable to human and rodent studies done to assess the long-term effects of AAS on aggression when aggressive behaviors remain elevated up to 17 wks after discontinuation of use (Farrell and McGinnis, 2004; McGinnis, 2004). In addition, even male rats that have been subjected to a subordinate situation tended to display less submissive behaviors after anabolic steroid treatment (Steensland et al., 2005).

Table 5.1 **Short Term vs. Long Term Effects**: *Summary of the Effects of AAS on Social Behaviors Compared to Adult-Typical Social Behaviors*

	Vehicle - treated adolescents	AAS-treated adolescents	Vehicle-treated adults	AAS treated adults
Immediate effects				
Sexual behavior	Significantly less than adults	Significantly increased	Adult-typical	Significantly reduced
Aggressive behavior	High expression of aggression	Significantly increased (dominant)	Adult-typical	Mild effects; increase in flank marking behavior
Submissive behavior	Adult-typical	N/A	Adult-typical	Adult-typical
Long-term Effects				
Sexual behavior	Adult-typical	Reduced satiety	Adult-typical	Reduced satiety
Aggressive behavior	High expression of aggression	Significantly increased (dominant)	Adult-typical	Mild effects; Increase in flank marking behavior
Submissive behavior	Adult-typical	N/A	Adult-typical	Reduced compared to vehicle treated adults

Chapter 4 Summary

The aim of the experiment in Chapter 4 was to determine whether AAS treatment alters cell proliferation within the medial amygdala, a key brain region mediating social behavior. Although a great deal of immunocytochemical work has been conducted investigating changes in serotonin and vasopressin immunoreactivity following AAS exposure, no work has previously investigated whether changes in amygdala cell proliferation occur following AAS exposure. The amygdala contains many androgen and estrogen sensitive neurons that are responsible for processing sensory cues into behaviors and neuroendocrine events which result in the display of social behaviors. The amygdala also exhibits hormone-dependent and hormone-independent changes during adolescence. In male Syrian hamsters, for example, the MePD volume increases significantly during puberty, but this is not due to having more neurons because neuron number does not change. Instead, these morphological changes in the MePD are due to an increased dendritic length and an increase in cell soma size (Cooke et al., 2007). In contrast, the MeAV decreases during adolescence, but this decrease does not appear to be dependent on circulating gonadal hormones (Romeo and Sisk, 2001). Interestingly, the experiments in chapter 4 found that changes in cell proliferation following AAS exposure were localized to the MePD. Adolescent but not adult AAS exposure significantly decreased cell proliferation in the MePD, whereas the volume of this region was unaffected. This suggests that although testicular secretions of testosterone

normally increase the MePD volume, AAS affects cell proliferation independently of regional volume.

Surprisingly, volume changes within the medial amygdala were limited to the MeAV, a region that normally does not exhibit steroid-dependent plasticity. Thus, during the course of normal adolescence, this region usually decreases in size, but AAS exposure caused this region to increase in volume and remained larger than normal. The MeAD and MeAV are activated after general arousal, especially in relationship to sexual behavior (Gomez and Newman, 1992; Kollack-Walker and Newman, 1997). The MeA is also involved in the interpretation of a social interaction that results in a defensive behavior (Choi et al., 2005). Studies from this laboratory have shown that adolescent AAS exposure significantly reduced or eliminated the expression of submissive and defensive behaviors, even 4-wks after discontinuation of AAS treatment (see Chapters 2 and 3). These data suggest the AAS-induced changes in volume in the MeAV may be a result of organizational changes at the cellular or molecular level that in turn affect behavior.

All of the AAS used in the cocktail are aromatizable and can be enzymatically converted to androgenic and estrogenic metabolites, in addition to upregulating androgen receptors and estrogen receptors in the brain (Bartsch, 1993; Clark and Henderson, 2003; Menard and Harlan, 1993; Roselli, 1998). Androgens and estrogens mediate cell proliferation and cell survival in voles and rats in the hippocampus and amygdala in adult animals (Fowler et al., 2003; Fowler et al., 2005; Spritzer and Galea, 2007). In turn, circulating hormones can

also affect neurotrophic factors and growth factors like nerve growth factor (NGF), brain derived neurotrophic factor (BDNF) and insulin growth factor (IGF-1), which have also been shown to mediate neurogenesis in the hippocampus, amygdala and hypothalamus in rodent models throughout development (Fiore et al., 2003; Fowler et al., 2007; He and Crews, 2007). However, further studies are needed to elucidate how AAS affect the nerve growth factors in order to understand how AAS can affect cell proliferation. In addition, androgens, as well as AAS, have also been reported to have affects on different neurotransmitters systems, such as serotonin (Grimes and Melloni, 2002; Grimes and Melloni, 2005; Keleta et al., 2007; Ricci et al., 2005b; Ricci et al., 2006; Thiblin et al., 1999a). A decrease in serotonergic activity results in a decrease in cell proliferation (Fowler et al., 2007). These data indicate that specific neuronal subtypes may be affected by AAS. Because cell proliferation and survival rely on these neural support networks and environmental cues, it suggests that the adolescent brain can compensate for the inhibitory effects of AAS on cell proliferation after discontinuation of treatment.

Normal Development of Social Behaviors:

Although the overarching purpose of the studies described here was to elucidate the effects of AAS treatment on adolescent brain and behavioral development, many interesting developmental changes were also found in the vehicle-treated controls. We found that the trajectory of the maturation of social behaviors is completely different for sexual behaviors compared with aggressive

and submissive behaviors. In these studies males were tested with a receptive female or an age, weight-matched opponent in order to assess sexual and agonistic behaviors. As described previously, the display of sexual behaviors, such as mounts, intromissions and ejaculations was higher in males tested as adults than males tested during adolescence. When males reach adulthood, the display of sexual behaviors remains consistent. Developmental analyses of both aggressive and submissive behaviors across adolescence and adulthood has not previously been conducted. When males were tested during adolescence with an opponent, overt aggression remained high, however, the older the male was, the less overt aggression was displayed. For the most part, adult males used flank marking behavior to establish dominance and displayed submissive behaviors readily. Submissive behaviors increased with age suggesting that an adult male can regulate their aggressive behaviors and will only display the behavior needed to communicate with their opponent to establish a dominance hierarchy, where an adolescent animal may "fight" to establish dominance.

Chapters two and three of this dissertation focused on understanding the effects of anabolic steroids on behavior and how it compared to adult AAS exposure. In addition, insights were gained into the normal development of these behaviors from adolescence to adulthood. In summary, adolescents treated with anabolic steroids show an increase in sexual behaviors immediately after testing; however these changes do not persist. They also show a significant increase in overt aggression, and do not display submissive behaviors. The behavioral

changes on agonistic behaviors persist after adolescent AAS exposure. Adults display differential behavioral responses after AAS exposure. Immediately after treatment sexual behaviors is significantly reduced, and these changes are not permanent. However, the same effects on satiety are observed after adult exposure. With respect to agonistic behaviors, there is a mild increase in aggressive behaviors throughout development; however there is more variability within adult social interactions with another male. These results suggest that the adolescent brain is particularly vulnerable to the adverse effects of AAS and the regulation of aggressive behaviors may be affected permanently.

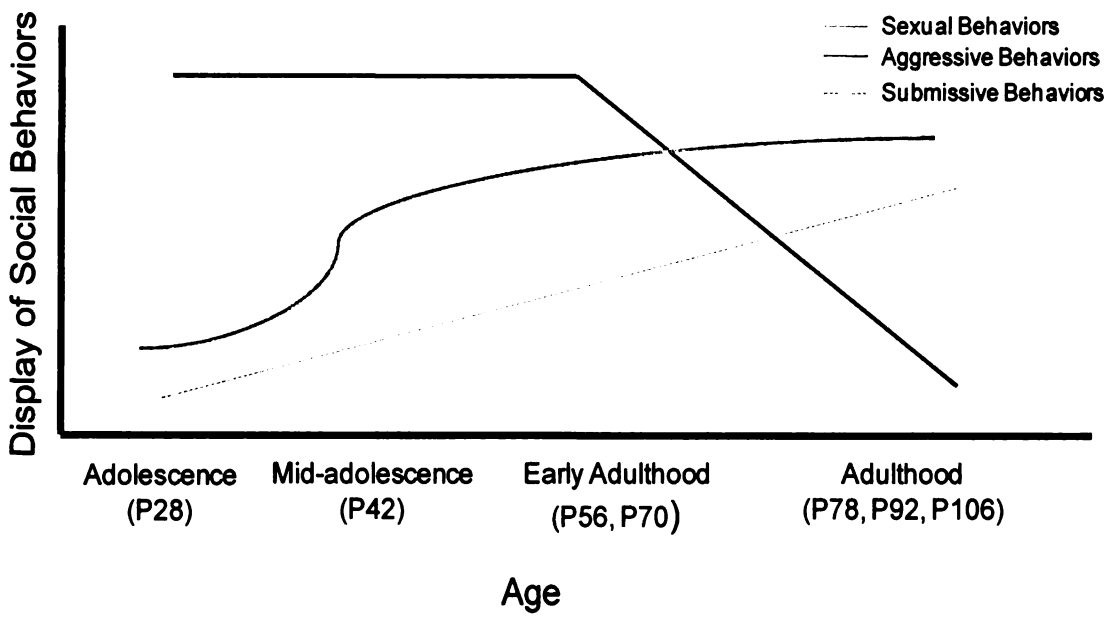


Figure 1. Depiction of the development of male social behavior from adolescence to adulthood.

Are the changes caused by AAS activational, organizational or both?

Definitions for activational and organizational changes due to gonadal hormones were introduced in the first chapter. These studies, which were focused on the effects of AAS on social behaviors and amygdaloid neural plasticity, do not directly address activational affects of gonadal hormones since the males were intact and were administered an exogenous steroid. However, these studies do suggest that AAS may cause organizational changes in sexual behaviors, by their affects on long intromissions, and on aggressive behaviors on adolescents, since they maintain high levels of aggressive behaviors well after discontinuation of treatment. These data also provide some evidence that adolescent AAS exposure causes organizational changes on the brain by increasing MeAV volume.

Implications and Contributions to Understanding Human Anabolic Steroid Use

Interest in understanding how gonadal hormones organize the adolescent brain has increased and has caused a heightened concern in exploring how drug use can affect the adolescent brain and behavioral maturation. In this dissertation the effects of exogenous synthetic androgens are explored. AAS are used to treat malnutrition and mood disorders (Kopera, 1993; Perry et al., 2002); however, the advantages they have for treating physiological and psychological disorders may become disadvantages when they are used inappropriately (Blue

and Lombardo, 1999; Corcoran and Longo, 1992). These studies are the first to directly compare males treated with anabolic steroids during adolescence and adulthood in order to explore immediate and long term effects on social behaviors. Most studies exploring human anabolic steroid use are epidemiological or limited to focusing on adult use. The particular cocktail of anabolic steroids used, which included testosterone cypionate, nandrolone decanoate and boldenone undecylenate, is comparable to what a heavy or chronic anabolic steroid user would combine for effective muscle and performance enhancing. Athletes and non-athletes of different ages use doses of AAS 10-100 times higher than doses clinically prescribed for cosmetic and anabolic purposes (Bahrke and Yesalis, 2004; Thiblin and Petersson, 2005). Unfortunately, an increase in body image issues during adolescence, along with a decrease in the perceived risk of taking AAS, have resulted in increased use of AAS among this age group. First- time AAS use has been reported as early as age 10, and use increases with age. Anecdotally, and through interviews with school counselors and teachers, adolescent AAS users are described as impulsive, spontaneous, and engaging in more risk-taking behaviors when compared to a non-user their age. Interestingly, the enhanced social behaviors displayed by AAS treated adolescent hamsters suggest higher levels of impulsivity and lack of regulation of certain behavioral responses to a stimulus.

In human adult males, especially after long-term use, they are described as explosive, aggressive and reckless; however many times the expression of this behavior is context dependent. Moreover, if they cease to use these

compounds, subjects can experience withdrawal symptoms, just as if they were consuming opiates or other chronic drugs. Additionally, users can also experience symptoms of clinical depression and have suicidal tendencies, which enhance the concern of mental health in AAS users, at any age. Physically, AAS can cause liver problems, heart disease and body acne after long term use.

The male Syrian hamster has been a great animal model for studying how different hormone treatments affect the expression and aspects of complex social behaviors throughout development. The advantage of using this animal model and this treatment regimen is that the basic knowledge on its neuroendocrine profile and patterns of social behaviors along with having some evidence of the neurochemical effects of AAS on offensive behaviors (McGinnis, 2004; Schulz and Sisk, 2006b) . There are three main points that this work can address: (1) adolescent anabolic steroid exposure increases the expression of social behaviors, (2) there are permanent effects on enhanced aggressive and suppressed submissive behaviors after adolescent exposure and (3) the adolescent amygdala is vulnerable to morphological changes after drug exposure.

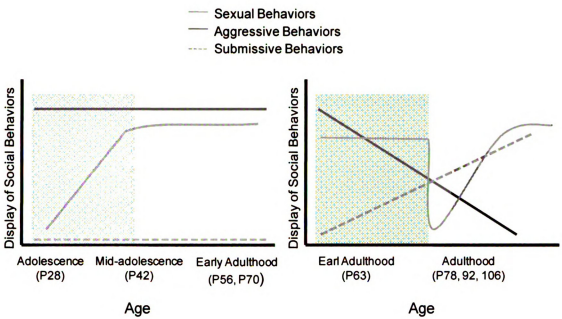


Figure 5.2 Depiction of the display of social behaviors after adolescent and adult AAS exposure.

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