IMPACT OF PREPUBERTAL SOCIAL SUBJUGATION AND ANABOLIC ANDROGENIC STEROID EXPOSURE ON BRAIN MORPHOLOGY IN MALE RATS.

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DEDICATION

To my family

ACKNOWLEDGEMENTS

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

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August 2007

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Abused children have a higher propensity to abuse drugs, including anabolic androgenic steroids (AAS). This research examined the impact of social subjugation (SS), as animal model of childhood abuse, and anabolic androgenic steroids (AAS), on the morphology of the anteroventral periventricular nucleus of the hypothalamus (AVPV) and the medial forebrain bundle (MFB) in pubertal Long-Evans male rats. This design was used to determine if the early experience of social subjugation or pubertal AAS exposure would alter the volume and areal cell density in the AVPV independently or in

combination. The AVPV was selected because it is extremely sensitive to alterations by gonadal steroids and the MFB was selected as a control brain area. Four groups of rats received the following treatments: AAS, SS, AAS + SS, and vehicle control. On postnatal day 26 (P26) prepubertal gonadally intact male rats received subjugation, while controls remained in their cage undisturbed. SS consisted of daily exposures to an aggressive adult male conspecific for 10 minutes a day, 5 days a wk for 2 wks. At the onset of puberty (P40) half of the subjugated and non-subjugated male rats received the AAS testosterone propionate at a dose of 5mg/kg body weight. The AAS treatment continued for 5 days a week for 5 weeks. At the conclusion of testing, animals were sacrificed and perfused. Brains were sectioned at 60 μm and Nissl stained. The volume and cross-sectional cell density was measured unilaterally in anatomically matched sections. All images were collected using an Olympus camera mounted on a microscope. The AVPV and MFB were analyzed with the NIH program Image J version 2.0. The volume in the AVPV was significantly reduced following SS, AAS, and AAS+SS compared to controls. There was no significant difference in MFB volume. There was also no significant difference observed for AVPV and MFB areal cell density. The results suggest that exposing the adolescent brain to either abusive early experiences or high levels of androgens, alone or in combination, can alter certain components of brain morphology. This animal model may provide insight into the impact of early experiences and AAS exposure on altered brain morphology in adolescence.

CHAPTER I

INTRODUCTION

According to the United States Census Bureau, there were 3, 423, 347 cases of suspected child abuse reported in 2004. Research has shown that childhood abuse that occurs within the first 5 years of life has consequences that persist for at least 12 years (Lansford, Dodge, Pettit, Bates, Crozier & Kaplow, 2002). These consequences include heightened levels of aggression, lower grades, and delinquent behavior as compared to their non-abused counterparts (Lansford et al., 2002).

Research on humans has shown that child abuse has a direct impact on brain morphology. For example, Teicher, Andersen, Polcari, Anderson, & Navalta (2002) observed reduced activity in the cerebellum vermis. Tiecher, Andersen, Polcaro, Anderson, Navalta, & Kim (2003) also found that child abuse reduced the size of the mid-portions of the corpus callosum. Both sets of findings indicate quite clearly that the developing brain is susceptible to early traumatic events.

Early child abuse takes its toll physically by altering brain morphology but also in the development of a variety of profound psychological effects. Child abuse can have clear and wide spread neurological repercussions associated with subsequent maladaptive behavior patterns such as high risk activities and a higher likelihood of substance abuse. These may result from an individual adapting an environment that is unhealthy (Teicher

et al., 2003), but in the future these changes make it hard to succeed in the world. For instance, victims of child abuse have a greater likelihood of suffering from mental disorders such as depression, post-traumatic stress disorder (Teicher, et al., 2002), and a higher incidence of dysmorphic disorders (Didie, Tortolani, Pope, Menard, Fay & Phillips, 2006). Abused children are also more likely to display higher levels of aggression than non-abused children (Lansford, et al., 2002; Connor, Doerfler, Volungis, Steingard, & Melloni, 2003). These disparities between abused children versus non-abuse children signify that abuse has a whole host of behavioral and psychiatric consequences stemming from alterations in the brain during development.

These behaviors, particularly the elevated level of displayed aggression, have been observed in animals using the social subjugation paradigm. Social subjugation was first proposed by Delville, Melloni, & Ferris in 1998. Delville et al. (1998) found that early subjugation by a larger more dominant male in golden hamsters resulted in higher levels of aggression exhibited towards younger and weaker intruder. Social subjugation has been proposed as an animal model for human child abuse due to the similar experiences and comparable outcomes. Overall, social subjugation consistently results in significantly higher levels of aggression that are increased in adulthood due to subjugation in puberty (Ferris, Messenger, & Sullivan, 2005; Cunningham & McGinnis, 2007).

Abused children also have been shown to have a greater tendency than non-abused children to use illegal substances (Kindlundh, Isacson, Berglund, & Nyberg, 1999; Bahrke, Yesalis, Kopstein, & Stephens, 2000; Teicher, et al., 2002). One type of drug in particular that is used by abused children is testosterone, an anabolic androgenic

steroid (AAS) (Driessen, M, Mussigbrodt, Dilling, & Driessen, B, 1996; Wisdom, Marmorstein, & White, 2006).

Anabolic androgenic steroids are typically administered 10-100 times the physiological amount (Haupt & Rovere, 1984) in cycles. These cycles consist of six to twelve weeks or more of consecutive abuse (Rogol, & Yesalis, 1992b). The excessive amount and the duration of the abuse heightens the cause for concern. The reinforcing and anabolic qualities of AAS may explain its continued use and addiction potential in children (Johnson & Wood, 2001).

Along with the potential rewarding and reinforcing effects of AAS abuse, the current view of the long-term effects is not shared by all who use it. For instance, a survey conducted by Faigenbaum, Zaichkowsky, Gardner, & Micheli (1998) found that only 54% of AAS abusers believed that they were "bad". The denial of serious consequences may explain why most abused children have a higher incidence of AAS abuse (Driessen et al., 1996).

Anabolic androgenic steroid abuse has been shown to produce aversive behavioral outcomes. Human AAS abusers report ingesting more cocaine and alcohol than non-AAS abusers (DuRant, Escobedo & Heath, 1995). Animal research has shown that AAS, when combined with cocaine, sensitize the mechanisms that are responsible for the expression of aggression (Le Greves, Zhou, Huang, & Nyberg, 2002). AAS abuse has also been consistently found to increase the level of aggression displayed in animals and humans (Lumia, Thorner, & McGinnis, 1994; Melloni & Ferris, 1996; Melloni, Connor, Hang, Harrison, & Ferris, 1997; Breuer, McGinnis, Lumia, & Possidente, 2001; McGinnis, Lumia, & Possidente, 2002; Toot, Dunphy, Turner & Ely, 2004). In addition,

it has been found in animals that AAS exposure appears to lower the threshold for exhibiting aggression when physically provoked (Breuer et al., 2001).

There are profound behavioral differences exhibited in animals that have been subjugated in early life, or administered AAS (Delville, Melloni, & Ferris, 1998; Cunningham & McGinnis, 2006). Specifically, Delville et al. (1998) established that social subjugation altered the behavior in adulthood of aggression. Concerning AAS abuse, Cunningham & McGinnis (2006) found that male rats were physically aggressive towards females when provoked. The behavioral differences found by Delville et al. (1998) and Cunningham & McGinnis (2007) indicate that there may be morphological alterations in brain structures from either of these conditions alone or in combination. The anteroventral periventricular nucleus of the hypothalamus (AVPV), in particular, has been shown to have morphological differences due to early experiences. For example, Rhees, Al-Saleh, Kinghorn, Fleming & Lephart (1999) found that prenatally stressed rats showed a reduction in volume in the AVPV. This morphological alteration may also be due to traumatic early experience (Teicher et al., 2002; Teicher et al., 2003). Therefore, using the Social Subjugation (SS) paradigm as an animal model for child abuse, this study will determine the impact of early experience on the morphology of the AVPV.

Another brain area, the sexually dimorphic nucleus of the preoptic area (SDN-POA), is related to sexually activity in males (Anderson, Fleming, Rhees, & Kinghorn, 1986). The SDN-POA is similar to the AVPV; both are sensitive to androgens and the SDN-POA has been show to have a reduction in volume due to steroids (Bloch & Gorski, 1988). This morphological effect on the volume of the SDN-POA may be present in the AVPV from AAS exposure.

The impact of abuse in humans or social subjugation in young animals seems to have a marked and lasting alteration on behavior during the period of rapid brain development in puberty (Spear, 2004). Puberty is a period of neural maturation that is mediated by steroid-dependent and steroid-independent events which further organize and shape the behavioral potential of the human adult (Romeo, 2003). In rats, puberty occurs on postnatal day 40 (Korenbrot, Huhtaniemi, & Weiner, 1977). It has been shown that the presence of excess steroids in puberty will induce the maturation of the reproductive system (Kraulis, Traikov, Sharpe, Ruf, & Naftolin, 1978). Since many abused children report using AAS in puberty (Driessen et al., 1996), it is important to further understand the impact these two variables may have. Puberty is a critical period to study because it provides us a better understanding of the morphological impact, if any, that AAS may have when administered during puberty either alone or in combination with early subjugation.

Therefore, based on the findings reported, it is hypothesized that the impact of social subjugation and exposure to anabolic androgenic steroids during puberty will have a morphological impact on the AVPV volume. It is further hypothesized that the greatest impact on morphology will be in the socially subjugated animals. This hypothesis is based on the findings by Rhees et al. (1999) that demonstrate the impact of early experience on the morphological development of the AVPV. These results will provide further insight on the impact of early experiences in combination with AAS abuse on brain morphology.

CHAPTER II

IMPACT OF SOCIAL SUBJUGATION AND ANABOLIC ANDROGENIC STEROIDS

Hypothalamic-Pituitary-Gonadal Axis

The hypothamic-pituitary-gonadal axis (HPG), see figure 1, is a neuroendocrine system that integrates the brain and the body through a process of negative feedback. The HPG axis originates in the hypothalamus where gonadotropin-releasing hormone (GnRH) is produced. GnRH then is transported to the pituitary gland where it stimulates the synthesis and release of two gonadotropic hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These two hormones are synthesized in the pituitary gland and cause the testes to release testosterone. For males, LH stimulates testosterone secretion and FSH is important in the initiation and maintenance of spermatogenesis. When there is an environmental, physical, or physiological challenge, especially in early development, the consequences can be detrimental to normal reproductive and sexual expression during adulthood.

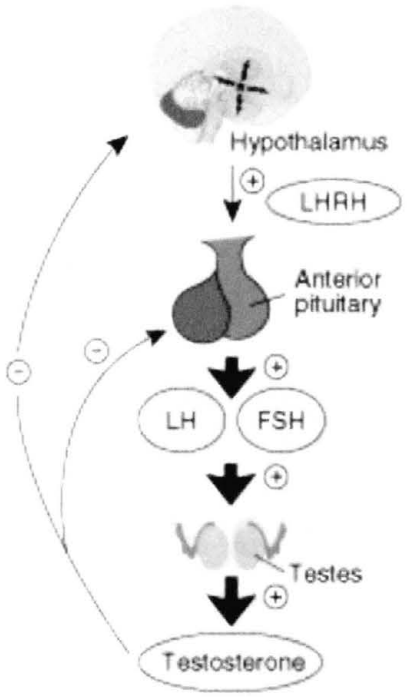

Figure 1. Hypothalamic-Pituitary-Gonadal Axis. LHRH, luteinizing hormone-releasing hormone; LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone. [Obtained from pubs.niaaa.nih.gov]

Exposure to AAS can alter the function of the HPG axis. This occurs because normally, LH interacts with receptors on the Leydig cells within the testes to produce testosterone. Testosterone is then transported to target tissues such as the testes, seminal vesicles, and prostate gland to regulate growth and maintenance of these tissues. The circulating testosterone regulates GnRH release by acting on the hypothalamus. When an individual abuses AAS, high levels of T cause LH from the pituitary gland decreases, and a reduction in testosterone production occurs. These changes can lead to alterations in the AVPV during puberty.

CHAPTER III

SOCIAL DEFEAT VERSUS SOCIAL SUBJUGATION

The use of animal models affords a clear methodological opportunity to circumvent the ethical limitations imposed in conducting human research and affords systematic control over variables associated with early traumatic experience. Several paradigms have been developed and tested to assess the outcome for many conditions, such as the effects of experiences on an organism. Two paradigms in particular, are referred to as social defeat and social subjugation, and they provide insight into the direct impact of specific conditions on brain morphology and changes in behavior.

Social Defeat Paradigm

In 1990, Koolhaas, Hermann, Kemperman, Bohus, Van den Hoofdakker, & Beersma tested the long lasting effects of social defeat on male rats. The results of this experiment show that social defeat has a gradual and enduring impact on behavior resembling depression in humans. This paradigm consists of experimental males being placed in the resident cage of a larger stimulus male. The social defeat paradigm is also characterized by the experimental male exhibiting a subordinate position (Selton & Cantor-Graae, 2005). This intraspecific confrontation occurs in a cage with Plexiglas unequally dividing the experimental from the resident, stimulus male, who is located in

the larger portion. For 10 minutes the experimental and the stimulus resident male are allowed to smell each other, but without contact. Then the Plexiglas is removed and the animals are allowed to interact for ten minutes and their behaviors recorded. These behaviors include the resident male attacking the experimental intruder who consistenly will display submissive behavior (Gardner, Thrivikraman, Lightman, Plotsky, & Lowry, 2005). Overall, the experiences that arise from the social defeat paradigm produce submissive responses when encountering a conspecific. The ability to relate the social defeat paradigm to social conflict in humans makes it a valuable procedure for researchers. Unfortunately, it does not provide insight into the long-term effects of abusive early experiences on brain development and behavior.

Social Subjugation Paradigm

The social subjugation paradigm, which is currently used as an animal model for childhood abuse, consists of placing a prepubertal male in the home cage of an aggressive, larger adult male daily until puberty (Delville et al., 1998; Wommack & Delville, 2004; Cunningham & McGinnis, 2007). There are some differences in experimental duration. For example, Delville et al. allowed the subjugation to occur for thirty minutes, Wommack and Delville used twenty minutes, while Cunningham & McGinnis used a ten minute subjugation period. It is important to note that the duration of the social subjugation does appear to influence behavioral outcome. Repeated social subjugation early in life accelerates the expression of adult aggressive behavior and enhances the intensity of aggression when presented with a smaller opponent (Wommack & Delville, 2003).

Some interesting findings within this paradigm are that subjugated animals as compared to non subjugated animals were more prone to attacking younger, weaker intruders (Delville et al. 1998). Also, a distinct outcome of this paradigm is that subjugation in adolescence produces aggression in adulthood. Paradoxically, adult subjugation promotes submissive behaviors similar to those found in the social defeat paradigm. These submissive behaviors include little or no aggression towards intruders of comparable size, and an increased latency in the experimental male to bite intruders (Ferris, Messenger & Sullivan, 2005).

There are similarities that exist between the social defeat and social subjugation paradigms. For example, social defeat and social subjugation both involve direct contact between the stimulus male and the experimental male. Also, comparing social defeat and social subjugation, both use similar methods but the primary difference is the age at which the abusive experiences occur.

There is also a distinct difference between the social defeat and the social subjugation paradigms, in particular, the amount of physical contact between the experimental and stimulus males. For example, the social defeat paradigm involves minimal contact; therefore, social defeat is not useful in studying the impact of child abuse on the morphology of the brain.

Since the social subjugation paradigm consists only of direct contact between the stimulus male and a young experimental male, it is a useful paradigm to compare to the negative experiences endured by children of abuse. The similarities of this paradigm to child abuse experienced in humans allows an opportunity for further understanding the effects that early physical experiences have on the morphology of the brain.

CHAPTER IV

ANABOLIC ANDROGENIC STEROIDS

As previously described, during puberty the HPG axis is developing and causes a cascading effect resulting in the secretion of testosterone from the testis. Testosterone, see figure 2, can be synthetically derived to form an exogenous male hormone categorized as an anabolic androgenic steroid (AAS). Anabolic refers to the ability of the chemical to build tissue, while androgenic explains that the chemical has a masculinizing effect (Strauss, 1991). These belong to a class of steroid hormones classified as androgens. Androgens are produced in the Leydig cells in the testes and carried by Sertoli cells. Overall, AAS increases protein synthesis and the development of male secondary sex characteristics such as a broadening of the shoulders and a deepening of the voice (Vandenberg, Neumark-Sztainer, Cafri & Wall, 2007).

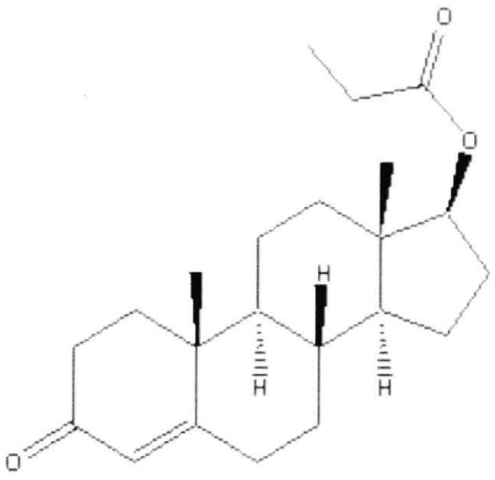

Figure 2. Chemical Structure of Testosterone Propionate. Testosterone propionate has both anabolic (muscle building) and androgenic (masculinizing) effects. No AAS is purely anabolic in nature. [Obtained from *http://www.testosteronepropionate.com/*]

In addition to altering the reproductive system, AAS abuse involving testosterone has directly been linked to aggression (Toot et al., 2004). In fact, the most consistently viewed behavioral difference between AAS abusers as compare to non AAS abusers is the elevated level of displayed aggression (Farrell and McGinnis, 2004). Animal studies have shown that with provocation, testosterone-treated males exhibit significantly more aggression than controls (Farrell & McGinnis, 2004). Also Cunningham and McGinnis (2006) found that AAS male rats were aggressive toward female rats when provoked. Male rats are normally not aggressive toward female rats, indicating that an increase in testosterone, a result from AAS abuse, can lead to an atypical incidence of aggression when combined with external cues.

Testosterone, according to the aromatization hypothesis, influences male sexual behavior in the brain by being aromatized to estradiol by cytochrome P450 aromatase in several brain areas (Naftolin, 1994). The role testosterone plays on male sexual behavior is observable following castration. Castrated rats show a decrease in male sexual behaviors that is returned to that of a gonadally intact male following testosterone treatment (Hull, Meisel, & Sachs, 2002). Other animal research has shown that AAS abuse involving testosterone significantly alters sexual behavior. For example, Feinberg, Lumia, and McGinnis (1997) found that there was a significantly higher incidence of ejaculation in male rats due to an elevated level of testosterone.

The results reported for both aggression and sexual behavior tests indicate that the two behaviors are both androgen sensitive and affected by exposure to AAS. This finding also suggests that the behavioral differences may be due to the impact of AAS on brain morphology.

CHAPTER V

ANTEROVENTRAL PERIVENTRICULAR NUCLEUS OF THE HYPOTHALAMUS

The Anteroventral Periventricular Nucleus of the Hypothalamus (AVPV) is a sexually dimorphic area that is dense with cell clusters (Lund, Salyer, Fleming, & Lephart, 2000). The AVPV is located in the brain, see figure 3, immediately caudal to the vascular organ of the lamina terminalis at the rostal tip of the periventricular zone (Gu & Simerly, 1997; Lund et al., 2000). There have been some inconsistencies in the reference of this area. In the past it has been referred to by many different names such as the anteromedial preoptic nucleus or the medial preoptic nucleus (Rhees et al., 1999). But this led to confusion; it is now referred to as the anteroventral periventricular nucleus of the hypothalamus (Simerly, Swanson, Handa, & Gorksi, 1985)

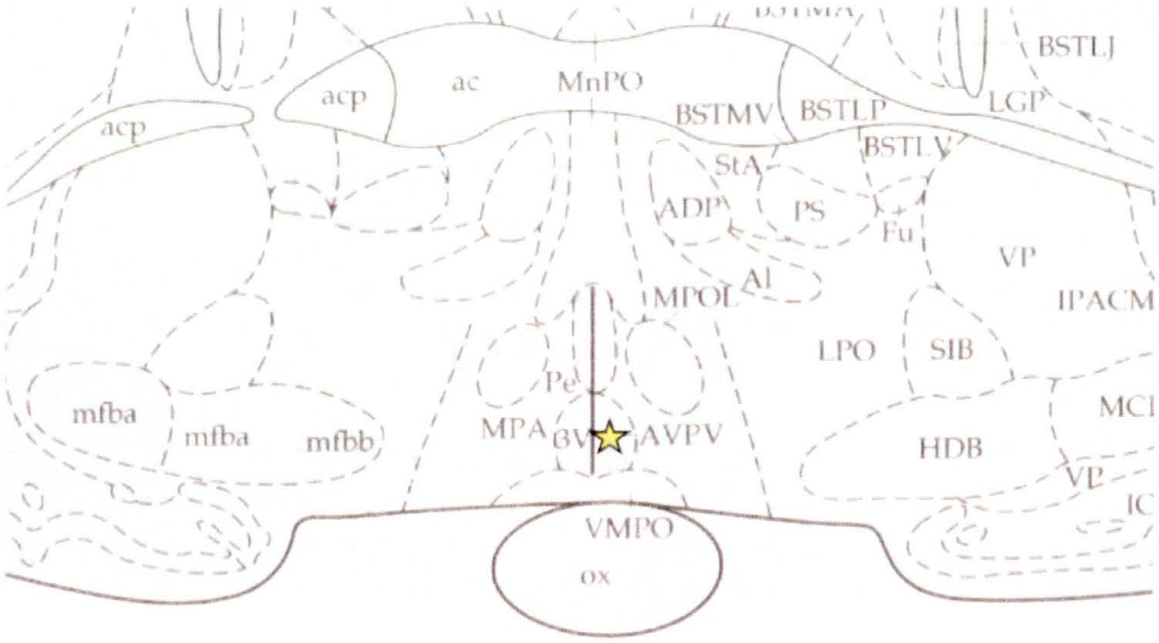

Figure 3. Anteroventral Periventricular Nucleus of the Hypothalamus. ☆ Indicates the Anteroventral Periventricular Nucleus of the Hypothalamus, AVPV; Medial Preoptic Area, MPA; Medial preoptic nucleus, lateral, MPOL; Periventricular Hypothalamic Nucleus, PE; Medial Forebrain Bundle, MFBA; Anterior Commissure, AC; Median Preoptic Nucleus, MnPO; Optic Chiasm, OX; Ventral Medial Preoptic Area, VMPO. [Adapted from Paxinos & Watson, 2002].

Anteroventral Periventricular Nucleus Function

The AVPV receives inputs from regions of the brain that convey olfactory information and neuroendocrine regulation (Simerly, 1997). These afferent connections are what allow the AVPV to play an important role in mediating positive feedback on gonadotropin secretion (Gu & Simerly, 1997; Lephart, Lund, & Horvath, 2001).

Rhees et al. (1999) found that early experiences can impact the morphological development in the AVPV and this change can been seen in an alteration in sexual

behavior. The AVPV volume was significantly increased and this resulted in a decrease in sexual activity, indicating that the AVPV plays a role in sexual behavior.

The AVPV is sensitive to testosterone (Simerly et al., 1985) and this brain area has been shown to have a reduction in volume due to an increase level of this circulating androgen. This indicates that increasing the level of testosterone, such as during AAS abuse, can have a morphological impact on the AVPV.

Anteroventral Periventricular Nucleus Volume

The volume of the AVPV is influenced by hormonal changes that occur during early prenatal and postnatal period of brain development (Rhees et al., 1999). Some evidence indicates that testosterone, when administered during postnatal development, may cause a decrease in AVPV volume (Lund et al., 2000). This may occur by androgens prolonging the active period of cell death and this appears to contribute to the decrease in volume (Murakami & Arai, 1989; Lund et al).

Studies have shown that the volume of the AVPV can be altered. Males that are gonadectomized and receive estrogen and progesterone treatment have a larger AVPV, see figure 4, (Bloch & Gorski, 1988). Yet, gonadectomy later in life does not influence its size or length (Davis, Shryne, & Gorski, 1996), suggesting that there is a vulnerability to morphological changes in early development.

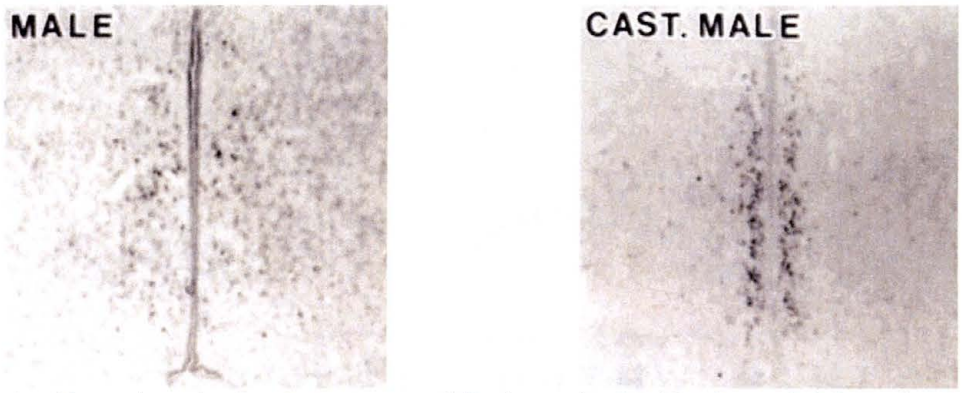

Figure 4. Alterations in the Anteroventral Periventricular Nucleus. Adult male and Adult neonatally castrated male. (Scale = 100 μm.) [Adapted from Orikasa, Kondo, Hayashi, McEwen, & Sakuma (2002)]

Anteroventral Periventricular Nucleus Afferent Connections

The AVPV receives many key sensory inputs, see figure 5, which may have a direct impact on the activity of gonadotropin releasing hormone neurons. The gonadotropin releasing hormone neurons control the secretion of luteinizing hormone from the pituitary gland (Gu & Simerly, 1997; Polston, Gu & Simerly, 2004). The AVPV receives dense projections from the bed nuclei of the stria terminalis (BST) providing a pathway of olfactory information to the hypothalamus, which creates a hormone-sensitive border between the telencephalon and diencephalon (Polston et al., 2004). The AVPV receives input from many brain regions that convey sensory and autonomic signals relevant to reproduction because it integrates hormonal and environmental signals to GnRH neurons (Ottem et al., 2004). This means that alterations in the AVPV can have an impact on many other regions of the brain.

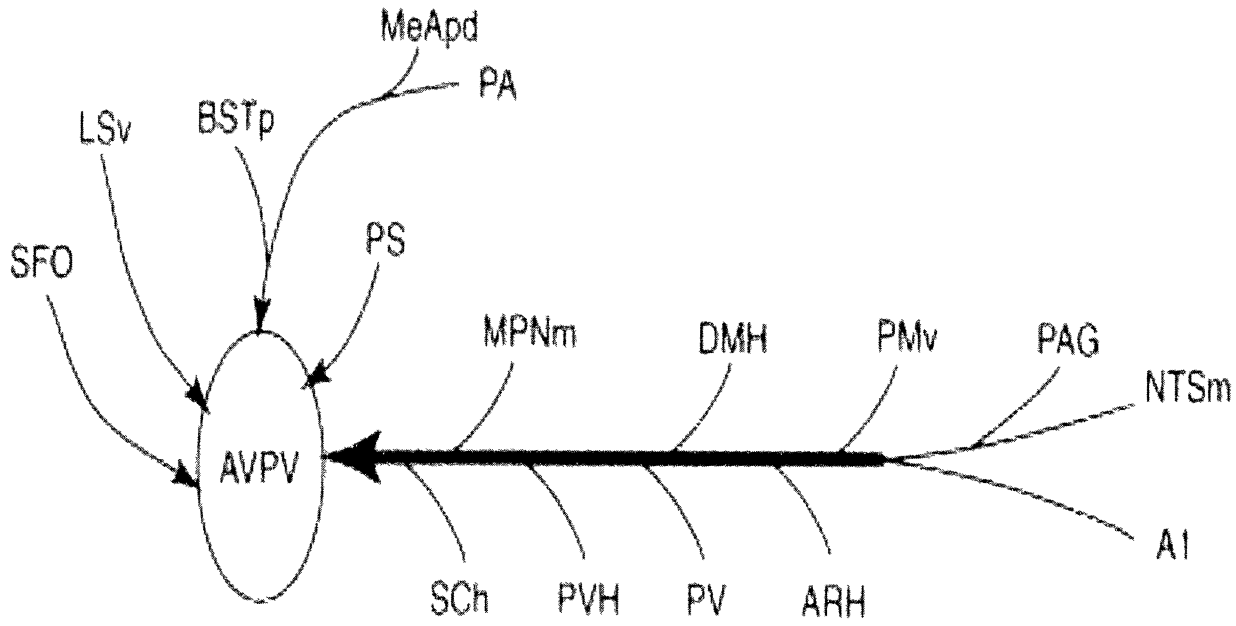

Figure 5. Afferent Projections to the Anteroventral Periventricular Nucleus. Schematic illustration to summarize the major inputs to the AVPV. A1, noradrenergic cell group; ARH, arcuate nucleus of the hypothalamus; AVPV, anteroventral periventricular nucleus.; BSTp, principal nucleus of the bed nuclei of the strict terminalis; DMH, dorsomedial nucleus of the hypothalamus; LSv, ventral part of the lateral septal nucleus.; MPNm, medial part of the MPN; MeApd, medial nucleus of the amygdala (posterodorsal part); NTSm, medial part of the nucleus of the solitary tract; PA, posterior nucleus of the amygdala; PAG, periaqueductal grey; PMv, ventral pre-mammillary nucleus; PS, parastrial nucleus of the preoptic region; PV, periventricular nucleus of hypothalamus (preoptic, anterior, intermediate, posterior parts); PVH, paraventricular nucleus of the hypothalamus; Sch, suprachiasmatic n.; SFO, sub-fornical organ. [Adapted from]

Anteroventral Periventricular Nucleus Efferent Connections

The AVPV projects to many other brain regions, see figure 6. But its strongest projections are to the periventricular zone of the hypothalamus (Gu & Simerly, 1997). This area then projects back to the AVPV, this bidirectional connection may result in the AVPV playing a role in regulation of gonadotropin secretion.

The cells within the AVPV have also been shown to project to GnRH neurons (Bodo, Kudwa & Rissman, 2005). This in turn means that the AVPV can control the

secretion of LH & FSH, which are both important for reproductive function. There are many projections from the AVPV, but many are still unclear and need to be further researched in the future.

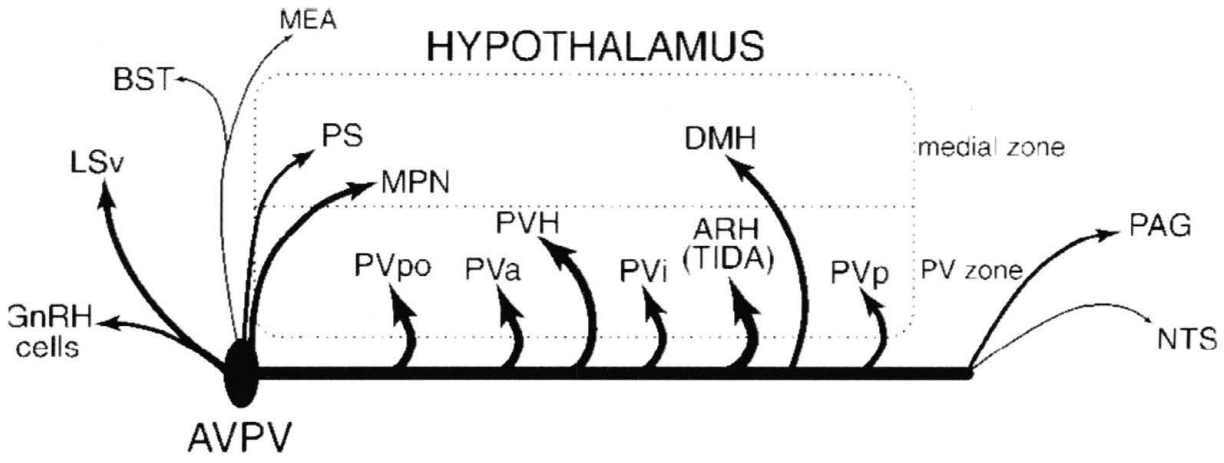

Figure 6. Efferent Projections from the Anteroventral Periventricular Nucleus. The relative size of each pathway is roughly proportional to the thickness of the lines associated with it. ARH, arcuate nucleus of the hypothalamus; BST, bed nuclei of the stria terminalis; DMH, dorsomedial nucleus of the hypothalamus; GnRH, gonadotropin releasing hormone; MEA, medial nucleus of the amygdala; LSv, ventral part of lateral septal nucleus; MPN, medial preoptic nucleus; NTS, nucleus of solitary tract; PAG, periaqueductal gray; PS, parastrial nucleus; PV, periventricular; PVa, anterior periventricular nucleus; PVi, intermediate periventricular nucleus; PVp, posterior periventricular nucleus; PVpo, preoptic periventricular nucleus; PVH, paraventricular nucleus; TIDA, tuberoinfundibular dopamine. [Adapted from Gu & Simerly (1997)].

CHAPTER VI

HYPOTHESIS

This study's purpose was to determine the morphological impact of prepubertal social subjugation (SS) and exposure to anabolic androgenic steroids (AAS) during puberty. It is hypothesized that SS will have a morphological impact on the volume and areal cell density in the anteroventral periventricular nucleus of the hypothalamus (AVPV). It is also hypothesized that AAS will induce similar morphological changes in the volume and areal cell density in the AVPV. It is also expected that there will be morphological changes in combining SS and AAS on the AVPV.

CHAPTER VII

METHODS

Setting

All experiments were performed at the University of Texas at San Antonio and the University of Texas Health Science Center at San Antonio under the supervision of Dr. Marilyn Y. McGinnis.

Subjects

Twenty-four gonadally intact male Long-Evans rats were received from Charles River Laboratories (Wilmington, MA). Subjects were individually housed in Plexiglas cages (25 x 20 x 18 cm) in a temperature controlled room (23°C). Food and water were available ad libitum. The room in which the animals were housed was maintained at a 12 hour light-dark cycle with the lights turned off at noon. Cages were cleaned weekly and the bedding was changed in each cage. All procedures followed guidelines established for the care and use of laboratory animals by the National Institute of Health.

History of Animals

These twenty four Long Evans male rats were divided into four groups. One group served as a control. The second group received the AAS testosterone propionate at a dose of 5mg/kg body weight for five days a week for five weeks. The third group received social subjugation, which consisted of daily exposure to an aggressive adult male conspecific (300-350 g) for ten minutes a day, five days a week, for two weeks beginning on postnatal day 26. Subjugation experiences were counterbalanced to prevent any experimental male being paired with the same stimulus male within the same week. The final group received prepubertal social subjugation and AAS during puberty.

Table 1. Experiment Timeline. Social Subjugation, SS; Anabolic Androgenic Steroids, AAS.

Tissue Samples

Animals were sacrificed on postnatal day 159, flushed with phosphate buffer saline (PBS), and then perfused with 4% paraformaldyhyde. Brains were removed and sectioned using a vibrotome. The brains were then sectioned in to 60 μm coronal sections and stored in PBS. Nine animals were excluded from the analysis due to damaged tissue; two from the SS group, two from the T & SS group; three from the control group, and two from the T group.

Stain Preparation

The sections were removed and placed on gelatin coated microscope slides and allowed to adhere to the slides for two days. Then these sections were stained with a Cresyl Violet Nissl stain, allowed to dry for two days, and labeled. Finally, the slides were coverslipped with coverglass (Fisher Scientific), and Permount mounting medium (Fisher Scientific), and then allowed to dry for two days. After this time period, excess Permount on the outside of the slide was removed from the slides using a razor blade and acetone and cleaned for image collection with glass cleaner.

Image Collection

The AVPV and MFB were analyzed for volume and areal cell density and the anterior commissure (AC), and the Optic Chiasm (OC) were used as landmark brain areas. Slides were then examined under a microscope. Images were taken on an Olympus camera mounted on the microscope. To make accurate measurements, a measurement bar was added when taking the images. Each slide was focused on the microscope and then a picture was taken creating an image for analysis.

To measure the volume in the different brain areas, images were collected under a 4x magnification level. Images of the left and right side of the AVPV, the corpus callosum (CC), the Medial Forebrain Bundle, the AC, and the OC the were taken for analysis.

To determine the areal cell density in the AVPV and the MFB, images were collected under a 10x magnification level. The area determined to be the AVPV or MFB under 4x magnification was magnified to 10x and the image was taken for areal cell density analysis.

Image Analysis

Images from the camera were then uploaded onto a PC for image analysis. Image J version 2.0, a program available on the National Institute of Health Website was used to analyze the volume and the areal cell density of the areas. Due to missing sections and damaged tissues, sections were anatomically matched across animals (Romeo & Sisk, 2001). Only one section containing a crossing anterior commissure and one section containing an approaching anterior commissure were used for each animal. Only the right side of each section was used for analysis. Figure 7 shows a crossing anterior commissure, and Figure 8 shows an approaching anterior commissure.

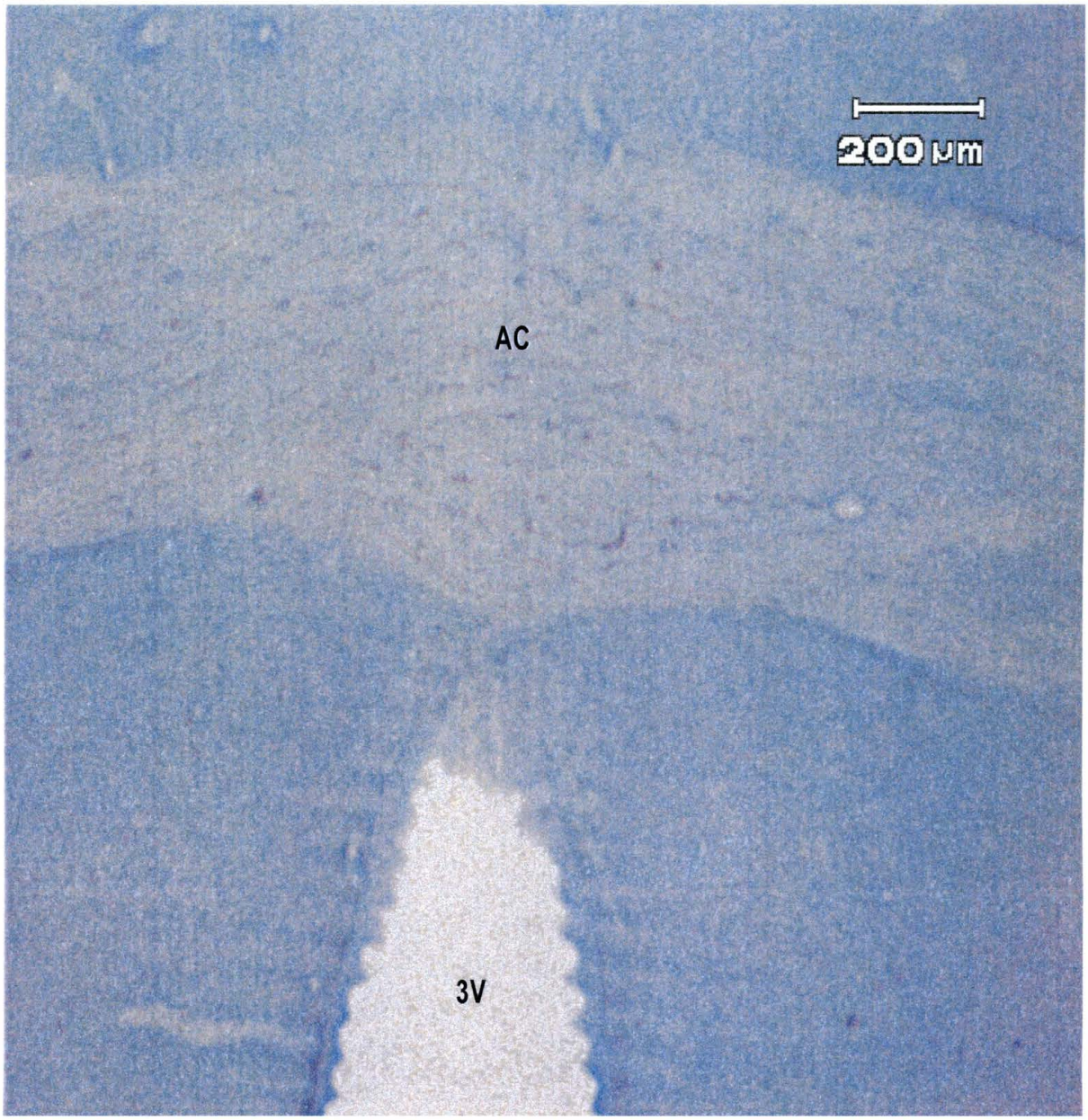

Figure 7. Image of Crossing Anterior Commissure. Also present is the Third Ventricle (3V). This is a 60µm coronal section viewable at 4x magnification.

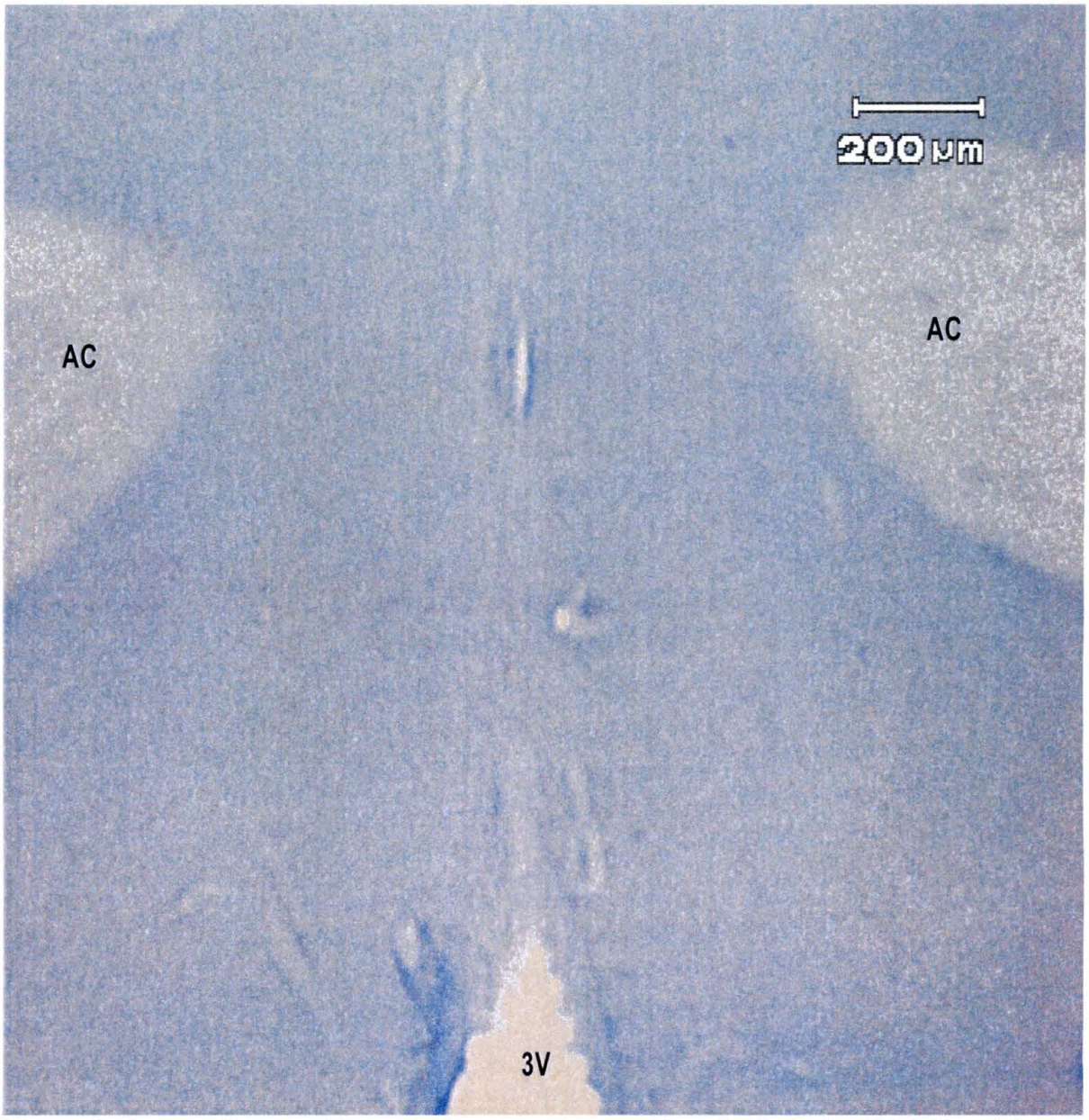

Figure 8. Image of approaching anterior commissure. Also visible is the Third Ventricle (3V). This is a 60µm coronal section viewable at 4x magnification.

Control Brain Area

The medial forebrain bundle (MFB), see figure 3, which served as a control brain area, has many rewarding effects when stimulated (Simmons, Ackermann, & Gallistel, 1998). For example, Hernandez, Hamdani, Rajabi, Conover, Stewart, Arvanitogiannis et al. (2006) found that MFB stimulation produces a substantial elevation of dopamine levels. It appears that long, myelinated fibers crossing between the forebrain and the midbrain appear to mediate the rewarding effects of the medial forebrain bundle (Gallistel, Gomita, Yadin & Campbell, 1985; Simmons, et al., 1998). Many areas, such as the substantia nigra and the ventral tegmental ascend in the MFB to innervate a variety of other brain areas located in the forebrain such as the accumbens and caudate (Gallistel et al., 1995).

Volume Analysis

Volume for the AVPV and the MFB was determined by setting the scale from the available 200 um measurement bar on the 4x magnified images. Then, the contrast and brightness for each image was also adjusted. Using the Image J threshold option, the area became distinct. With the freehand option, the area for the AVPV and the MFB was traced by including only the stained cells that appeared within the area being analyzed. Then the Image J program calculated the volume for each section. The total for each animal was added to its appropriate experimental group for analysis.

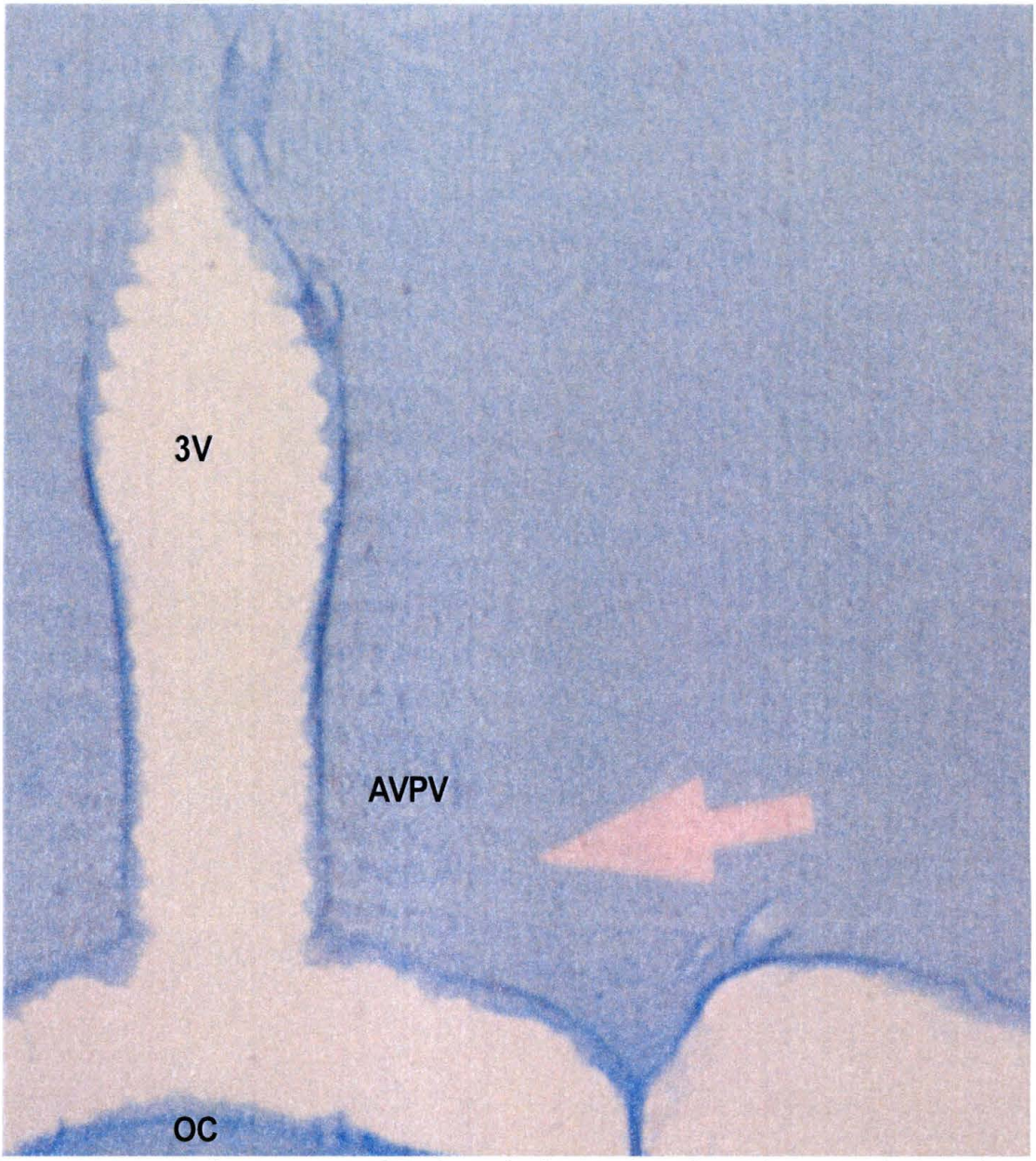

Figure 9. Image of Anteroventral Periventricular Nucleus for Volume Analysis. Also visible are the Optic Chiasm (OC), and the Third Ventricle (3V). This is a 60 μm coronal section viewable under 4x magnification.

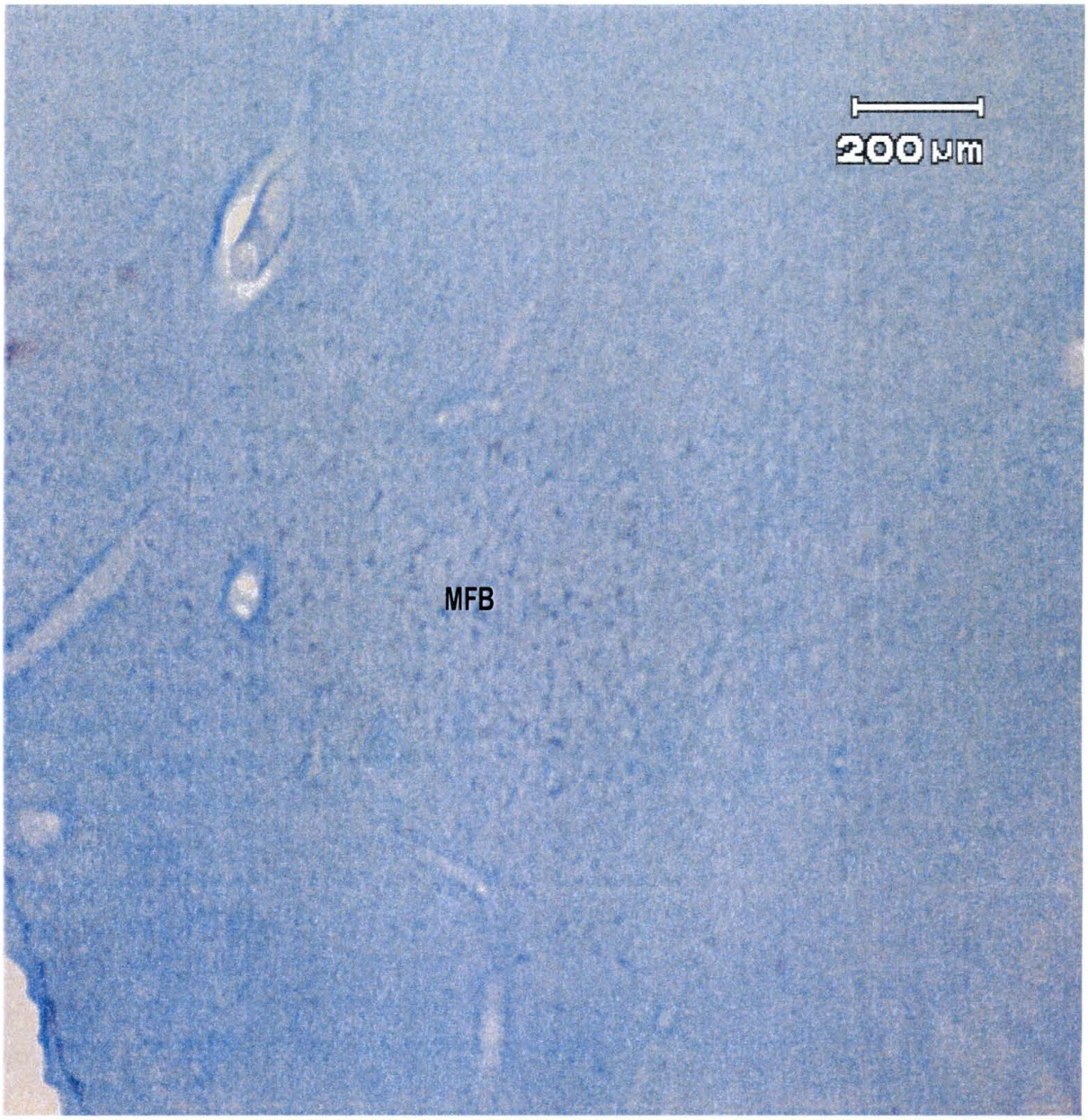

Figure 10. Image of Medial Forebrain Bundle for Volume Analysis. Coronal section 4x magnification right side. This is a 60µm coronal section viewed at 4x magnification,

Areal Cell Density Analysis

In order to calculate the areal cell density, the scale was set from the available 60um measurement bar on the 10x magnified images, see figures 15 and 16. Then a 125μm x 125μm area was taken from the volume of each area. The contrast and brightness was adjusted for each image. The threshold option allowed each cell to become viewable and definitive. Using the cell counter plug-in, each cell was marked on the image and the total number was calculated by the Image J program. The total number for cells present for each section was added to the appropriate experimental group for analysis.

Statistical Analysis

Comparisons of the groups by analysis of variance (ANOVA) was performed using the software package, Statview, detected the main effects. Fisher's PLSD post hoc analysis was used for comparisons between groups for each brain area. The significance level was set at $p<.05$.

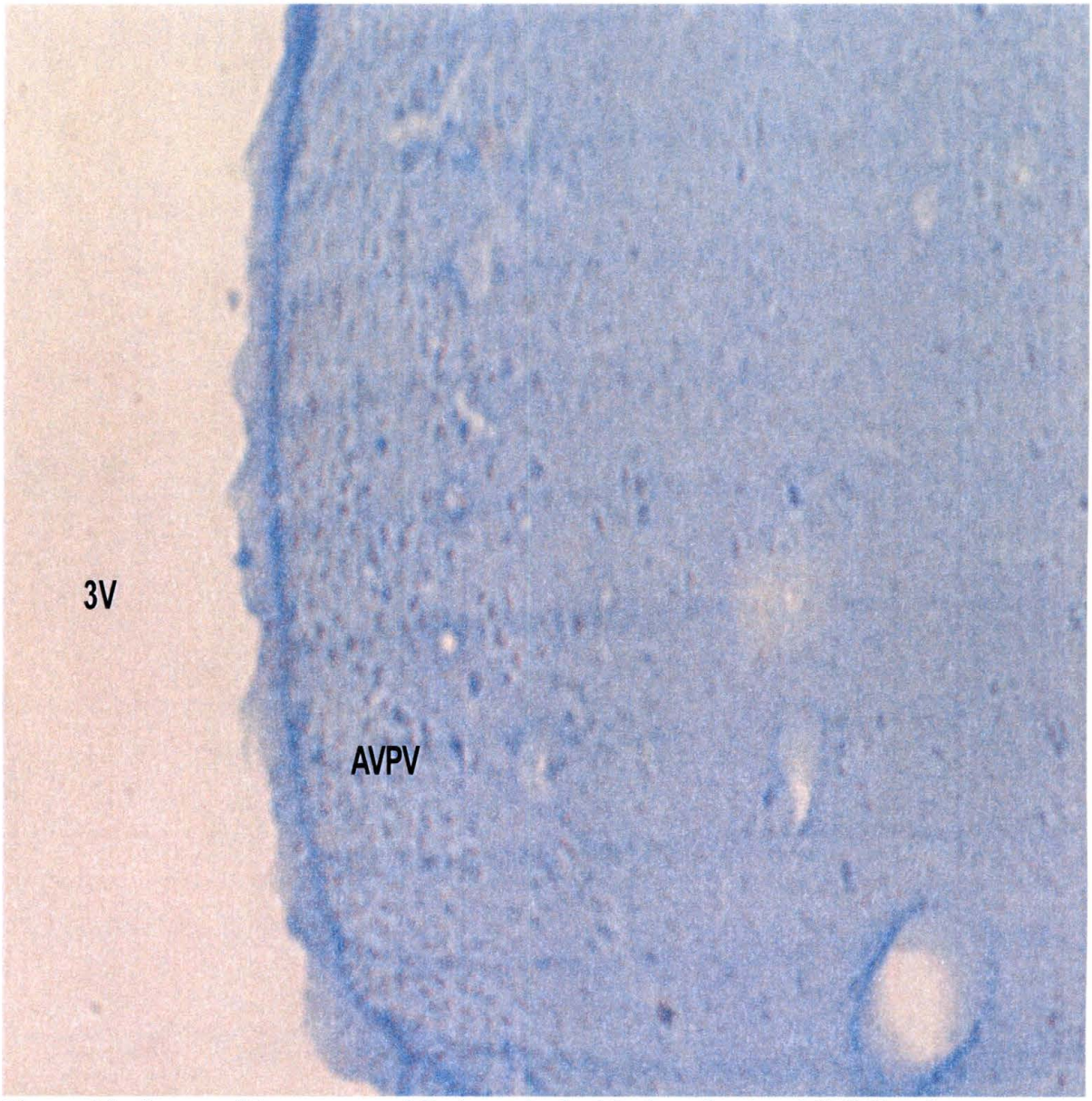

Figure 11. Image of Anteroventral Periventricular Nucleus for Areal Cell Density Analysis. Also visible is the Third Ventricle (3V). This is a 60 μm coronal section viewable at 10x magnification.

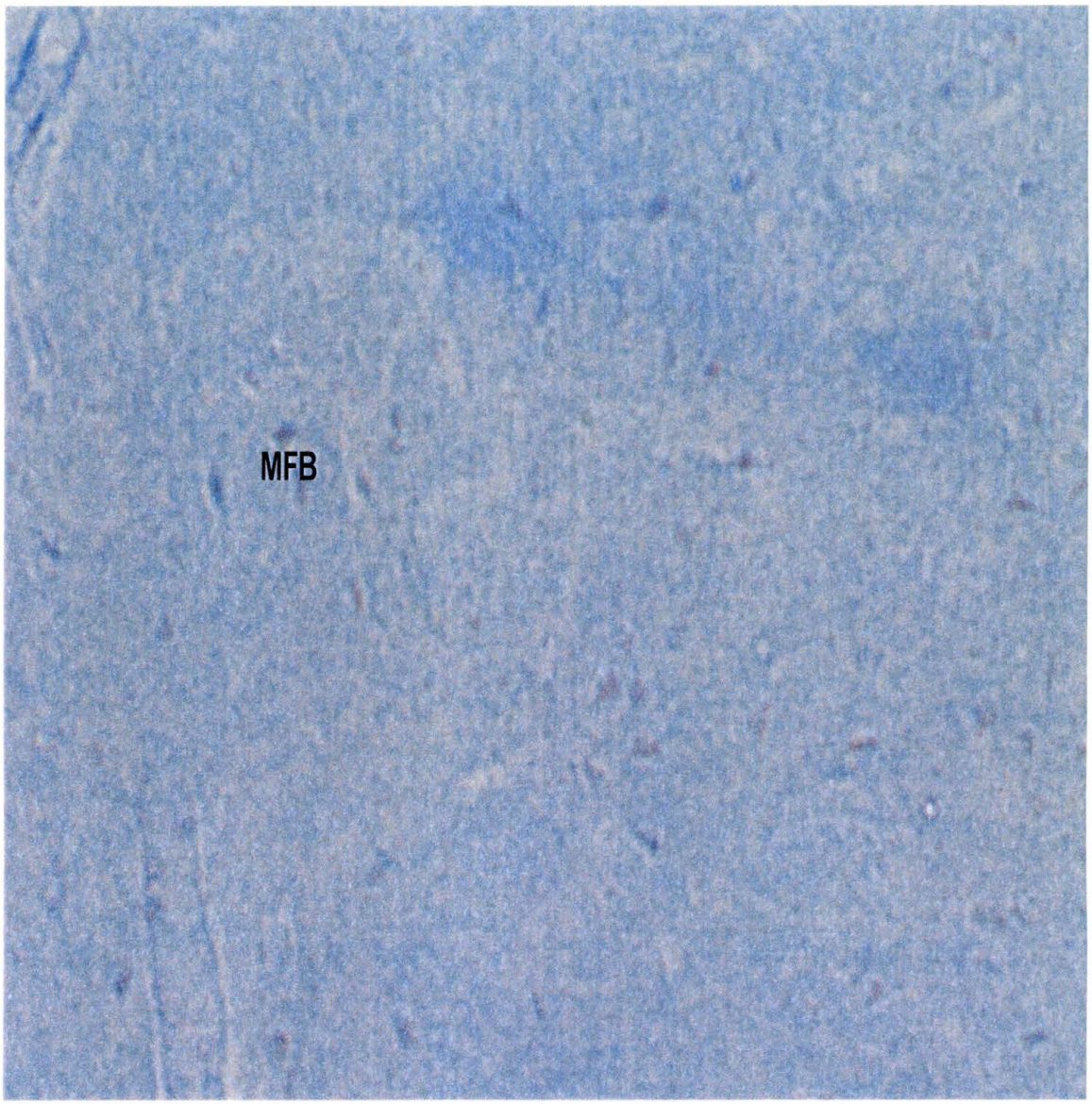

Figure 12. Image of Medial Forebrain Bundle for Areal Cell Density Analysis. This is a 60 μm coronal section under 10x magnification.

CHAPTER VIII

RESULTS

Anteroventral Periventricular Nucleus Volume

Figure 13 shows the total volume of the AVPV for each experimental group. There was a significant overall morphological effect on the AVPV volume $F(3,14)=10.698$ $p<.0001$. SS had a morphological impact on the AVPV by reducing the volume of that area as compared to controls ($p<.05$). AAS also significantly reduced the volume as compared to controls ($p<.05$), indicating this area is susceptible to morphological changes demonstrated in adulthood. In addition, the combination of SS and AAS was sufficient enough to induce morphological changes in the volume of the AVPV in comparison to controls ($p<.05$).

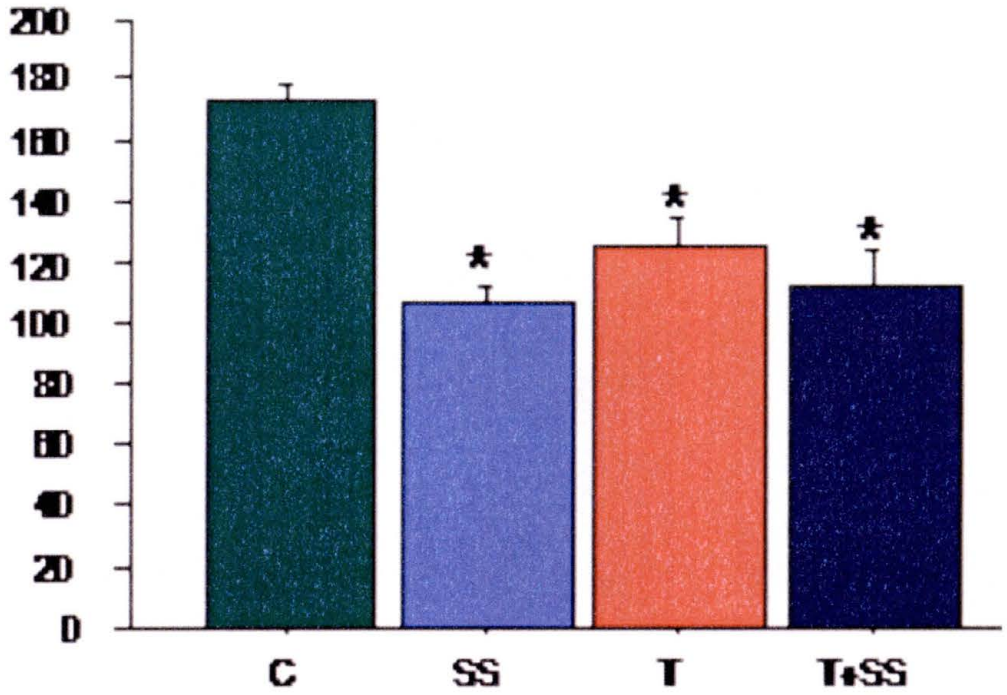

Figure 13. Anteroventral Periventricular Nucleus Volume. Mean (± SEM) AVPV volume *1000 (μ^2). There is a significant difference in the AVPV volume between each group as compared to controls. Control (C), (n=3); Social Subjugation (SS), (n=4); Testosterone, (T), (n=4); Testosterone plus Social Subjugation (T + SS), (n=4)
* Indicates a significant ($p<.05$) difference from controls.

Anteroventral Periventricular Nucleus Areal Cell Density

This analysis compared the impact on the areal cell density in the AVPV due to SS and ASS alone and in combination. Figure 14 shows the total areal cell density of the AVPV for each experimental group in micrometers. There was no significant difference in the number of cells present in the AVPV for the SS, AAS, or SS plus AAS groups $F(3, 14)=2.210$, $p<.1108$. These results indicate that the cell density of the AVPV is not susceptible to morphological changes due to SS or AAS.

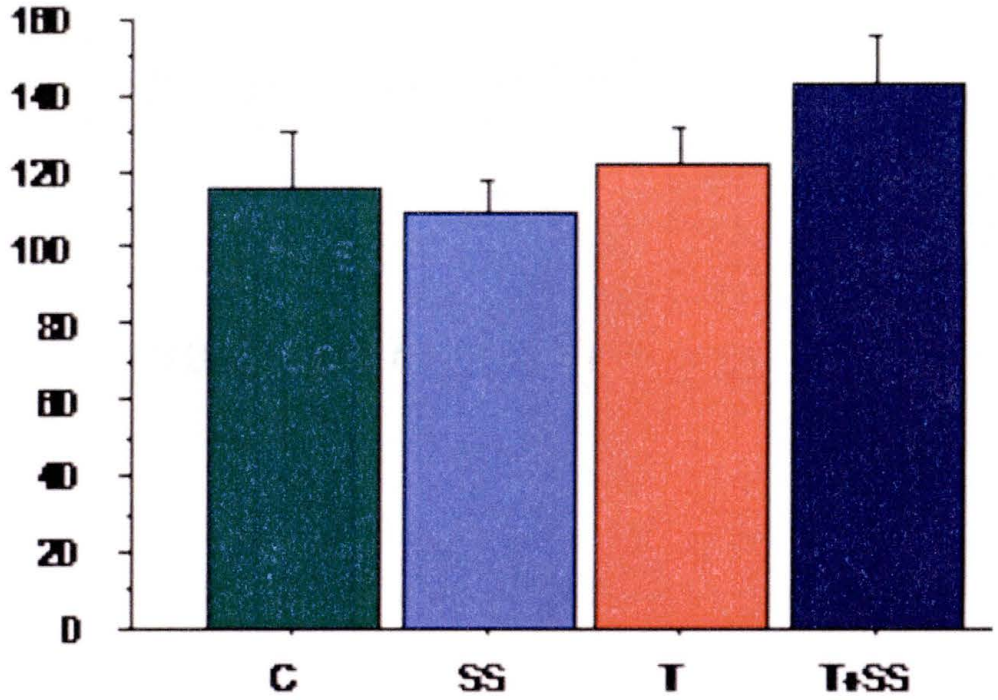

Figure 14. Anteroventral Periventricular Nucleus Areal Cell Density. Mean (± SEM) AVPV areal cell density (cells/μm^2). Area cell density for the AVPV in number of cells combined for each experimental group. Control (C), (n=3); Social Subjugation (SS), (n=4); Testosterone, (T), (n=4); Testosterone plus Social Subjugation (T + SS), (n=4)
* Indicates a significant ($p<.05$) difference relative to controls.

Medial Forebrain Bundle Volume

In order to determine if the MFB may be susceptible to morphological changes, the volume was analyzed. Figure 15 depicts the mean volume of the MFB for each experimental group in μm. The MFB did not exhibit a clear difference in volume $F(3, 14)=1.556$, $p<.2240$ from SS or AAS as compared to controls. This indicates that SS or AAS does not have a morphological impact on the MFB that persist into adulthood.

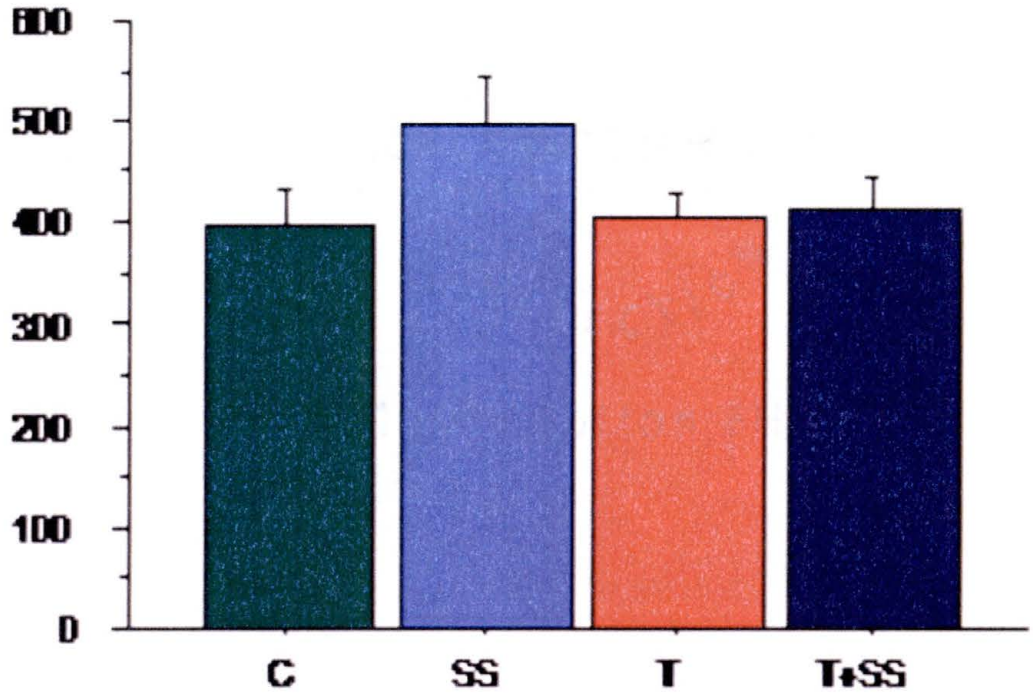

Figure 15. Medial Forebrain Bundle Volume. Mean (± SEM) MFB Volume *1000(μ^2). . There was no significance between any of the groups. Control (C), (n=3); Social Subjugation (SS), (n=4); Testosterone (T), (n=4); Testosterone plus Social Subjugation (T + SS), (n=4)
* Indicates a significant ($p<.05$) difference relative to controls.

Medial Forebrain Bundle Areal Cell Density

Figure 16 shows the total areal cell density of the MFB for each experimental group. There was no significant difference in the number of cells present between any of the groups $F(3, 14)=2.827$, $p<.0582$. This indicates that the MFB is not as vulnerable as the AVPV to morphological changes from SS or AAS that persist into adulthood.

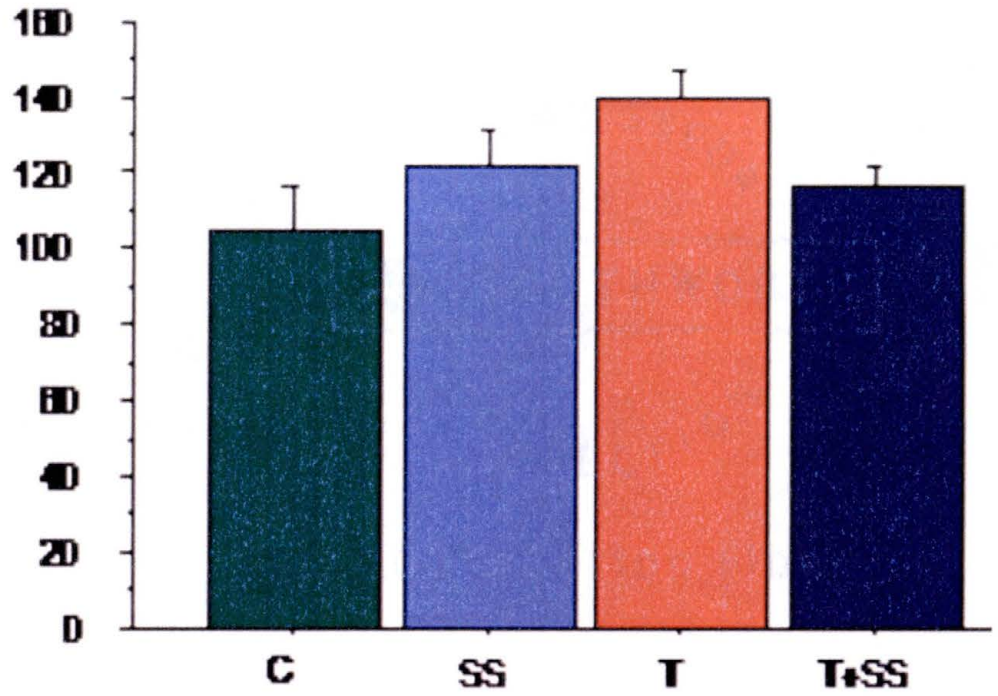

Figure. 16. Medial Forebrain Bundle Areal Cell Density. Mean (± SEM) MFB areal cell density (cells/μm^2). Number of cells present in the MFB combined into the appropriate experimental group. Control (C); (n=3), Social Subjugation (SS), (n=4); Testosterone (T), (n=4); Testosterone plus Social Subjugation (T + SS), (n=4)
* Indicates a significant ($p<.05$) difference relative to controls.

CHAPTER IX

DISCUSSION

This study found that social subjugation is sufficient to alter the morphology of the anteroventral periventricular nucleus of the hypothalamus in adolescent male rats. The volume in the AVPV was significantly reduced due to social subjugation as compared to controls. This morphological alteration supports the findings by Rhees et al. (1999) who found a decrease in AVPV volume as a result of early experience. Specifically, they reported that the mean AVPV volume was significantly less for prenatally stressed sexually active males than for nonstressed controls.

Another finding of this study was that anabolic androgenic steroid exposure also was sufficient to induce morphological changes in adulthood. The volume of the AVPV was significantly reduced due to AAS administration as compared to controls. The present finding that AAS alters brain morphology is supported by the findings of Cunningham (2006).

In addition to each treatment having a distinct morphological effect, the combination of SS plus AAS produced a significant volume reduction in the AVPV as compared to controls. Since independently, both SS and AAS had a significant impact on

brain morphology of the AVPV, it is not surprising that the combination of SS plus AAS produced a similar reduction in volume as compared to controls.

There were no significant morphological changes present in areal cell density in the AVPV due to SS, AAS, or SS plus AAS. This is contrary to previous research by Murakami and Arai (1989) who found a reduced number of neurons in the AVPV from neonatal androgen treatment in female rats. The lack of effect of effect on areal cell density found in this study as compared to the decreased cell number found by Murakami and Arai (1989) may be due to the differences in gender, age of treatment (neonatal versus puberty), or the level of androgen treatment.

Also, there was no significant difference in MFB volume for any of the experimental groups as compared to controls. This outcome is desirable since the MFB was analyzed as a control brain area. There was also no significant difference observed for MFB areal cell density, indicating that the MFB is not susceptible to morphological changes due to SS or AAS exposure. The nonsignificant findings for the MFB volume and areal cell density indicate that the methods employed in this study were sensitive and specific to detect reliable differences on the impact of SS and AAS on a particular brain area, the AVPV.

In light of these findings, it is suggested that exposing the adolescent brain to either early subjugation or high levels of androgens, alone or in combination, can have a morphological impact on brain development. This study provides further insight into the effects of prepubertal SS and AAS exposure in puberty. The use of animal models may provide insight into the impact of early experience and adolescent AAS abuse on future behaviors in humans.

A factor that may have influenced the results of this study was the total number of animals analyzed for morphological changes. The loss of nine animals, two from the SS group, two from the T group, two from the SS plus T groups, and three from the control group resulted in relatively small n's. Larger experimental groups may have resulted in a significant morphological effect in the areal cell density of the AVPV. The areal cell density in the AVPV was approaching significance; therefore, the use of more animals may have resulted in a significant effect. To replicate this study, it should include larger numbers within each group.

Also, the Image J program could have allowed human error to alter the outcome in AVPV and MFB results. Any dark, round area that was present when the landscape option was used was considered to be a cell. This in turn determined the volume and areal cell density for that particular brain region. There could have been other ways that a dark, round area appeared on the image. This may include excess Paramount or something other than a cell being viewable when the threshold was adjusted. This could have slightly altered the areal cell count or volume within the groups. However, since the areal cell counts and volumes were all calculated by one individual, it is likely that the error, if any, was consistent across groups. Therefore, it may not have affected the statistical outcome.

Research and the current body of knowledge abut AAS abuse should be more accessible to the general public (Haupt & Rovere, 1984). The media needs to focus more on the harmful than on the beneficial aspects of AAS abuse and aid in shifting the way society as a whole views the use of this illegal substance (Faigenbaum et al., 1998). Also

there should be more involvement by parents. Instilling appropriate views and having more involvement may reduce the number of adolescents who abuse AAS.

In summary, this research demonstrates the need for specific fields, such as Health Psychology, to primarily focus on preventative and early detection methods to reduce the incidence of child abuse and AAS abuse in the future. The results of this study indicate that there are morphological changes that take place in the brain. These appear to be life-long and therefore, more brain areas should be researched in order to comprehend the true impact of child abuse and AAS abuse on the individual.

Future research should include analyzing the effects of SS and AAS on the female brain. Though AAS abuse is not thought to be as prevalent in females as it is in males, Faigenbaum et al. (1998) found that currently an equal percentage of young females are now abusing AAS. Therefore, an experimental design that includes females should be undertaken to determine if similar morphological changes occur. The AVPV is much larger in females in comparison to males (Hutton, Gu, & Simerly, 1998; Lund, Salyer, Fleming, & Lephart, 2000; Lephart et al. 2001, Lephart, Rhees, Setchell, Bu & Lund, 2003; Bodo et al., 2005), and the impact of SS and AAS on the morphology of the female brain needs to be recognized.

In addition, other brain areas should be researched in the future for morphological changes in volume or areal cell density due to SS or AAS, alone or in combination. For instance, the sexually dimorphic area of the hypothalamus may show similar results as those seen in the AVPV because it is also a steroid sensitive area. Other areas of interest should include the cerebellum and other limbic structures, since marked differences have been observed by Teicher et al. (2002) and Teicher et al. (2003). There have been some

advances in understanding the impact child abuse and AAS abuse has had on the morphology of the brain, but additional consequences need to be understood. Also, once these maladies have occurred, if it is possible to reduce the deleterious consequences.

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