INTEGRATING THE NEURAL SYSTEMS OF THE LATERAL ANTERIOR HYPOTHALAMUS IN ADOLESCENT AAS-INDUCED OFFENSIVE AGGRESSION

A dissertation presented

by

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to
The Department of Psychology

In partial fulfillment of the requirements for the degree of
Doctor of Philosophy

in the field of

Psychology

Northeastern University
Boston, Massachusetts
April 2011
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ABSTRACT OF DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the field of Psychology in the Graduate School of Arts and Sciences of Northeastern University, April 2011
Abstract

Adolescence is a developmental period where neurobiological mechanisms regulating complex behaviors, such as aggression, are particularly sensitive to circulating androgens. For example, an increased incidence of aggressive behavior correlates with elevated levels of endogenous testosterone in mid-to-late adolescent males (Dabbs et al. 1987; Dabbs et al. 1991; Mattsson et al. 1980; Scerbo and Kolko 1994) but not in prepubertal males (Constantino et al. 1993; Schaal et al. 1996; Susman et al. 1987), suggesting a link between circulating androgens and the development of the aggressive phenotype. This link between testosterone and aggression is particularly concerning for adolescent populations who abuse synthetic testosterone and its derivatives, collectively termed anabolic androgenic steroids (AAS), for their performance enhancing effects (NIDACapsules 2008). Abuse of AAS remains high in the adolescent population, with more than a half million 8th–10th graders in the U.S. reporting AAS use each year (NIDACapsules 2008). This is particularly concerning given the high incidence of psychological and physiological ramifications associated with taking AAS, including increased violence, rage, and aggression (Dukarm et al. 1996; Loeber and Hay 1997).

For over a decade, the Syrian hamster has been used as a valid animal model to investigate the neurobiological consequences of adolescent AAS exposure (Melloni et al. 1997a). These studies have revealed that AAS-treatment produces activational and organizational
alterations in distinct neurochemical systems across key aggression loci of the brain (Melloni and Ricci 2010). Of particular interest are the neurochemical systems altered in the anterior hypothalamus (AH), a brain region essential to the control of aggressive behavior. For example, dopamine has been localized to the AH, though its sensitivity to adolescent AAS-exposure and ability to modulate aggression is not well understood.

To investigate the effects of AAS-exposure on the dopamine neural system, male Syrian hamsters were treated with AAS throughout adolescence and their brains were processed for immunohistochemistry of tyrosine hydroxylase (TH: the rate limiting enzyme for dopamine synthesis), as well as dopamine D$_2$ and D$_5$ receptors. Aggressive adolescent AAS-treated hamsters were found to have a significant increase in the number of dopaminergic cell bodies in two subnuclei of the AH, the nucleus circularis (NC) and medial supraoptic nucleus (mSON), i.e., the two principal nuclei responsible for innervating the lateral subdivision of the AH, referred to as the LAH. Increases in LAH TH correlate with aggression intensity in AAS-treated animals, demonstrating a direct link between AAS-induced aggression and hypothalamic dopamine production. Interestingly, both D$_2$ and D$_5$ receptors were localized to cell bodies within the LAH, suggesting that AAS-induced increases in dopamine results in increased activation to these local receptor pools, eliciting the elevated aggressive responses.

To investigate this hypothesis, aggressive AAS-treated hamsters were microinjected with a selective D$_2$ or D$_5$ antagonist into the LAH and measured for changes in aggressive responding. The selective D$_2$ receptor antagonist eticlopride dose-dependently decreased aggression while leaving all other non-aggressive related behaviors intact. Conversely, only high doses of the D$_5$ receptor antagonist SCH-23390 suppressed AAS-induced aggression and these reductions in aggressive responding were met with concomitant reductions in general arousal and motor
activity. While these findings suggest that dopamine’s action at D₅ receptors in the LAH may regulate other hypothalamic-mediated behaviors non-specific to aggression, it is possible that their function in the control of aggression may still be important, albeit difficult to discern. Therefore it was necessary to investigate what neural systems express these D₂ and D₅ receptors in the LAH and how they may be altered by AAS-exposure throughout adolescent development.

Double-label immunofluorescence studies revealed that D₂, but not D₅, receptors are expressed on a subpopulation of GABAergic interneurons in the LAH, suggesting that AAS-induced alterations in dopamine synthesis increases aggression through inhibition of GABAergic neurons resulting in disinhibition of hypothalamic activity. The finding that only a subpopulation of GABA neurons in the LAH is modulated by dopamine led to the investigation of the serotonin neural system and how it modulates GABA through excitatory 5-HT₂ₐ receptors. Aggressive AAS-treated hamsters showed increased expression of 5-HT₂ₐ receptors in the LAH. These increases in receptor levels correlated with the elevated aggressive response produced by AAS-treatment. 5-HT₂ₐ receptors were found to colocalize with a subpopulation of GABAergic neurons indicative of multiple GABAergic pathways in the LAH. Together, serotonin and dopamine are postulated to differentially modulate multiple GABAergic populations in the LAH resulting in differential regulation of inhibitory and excitatory neural inputs. Perturbation of these neural systems as a result of AAS-use during adolescence results in increased hypothalamic activity and exaggerated aggressive responding. Given that pharmacology used to treat aggression in clinical youth target D₂ and 5-HT₂ₐ receptors, findings from this dissertation elucidates a plausible brain locus where these drugs may work to directly modulate aggressive behaviors and provide a putative neural mechanism whereby adolescent AAS-abuse alters brain neurochemistry resulting in increased offensive aggression.
ACKNOWLEDGEMENTS

I am very fortunate to be surrounded by the best; learning and growing from the many great people that I am lucky enough to have in my life. To my wife, Mari, thank you for always being what I need, when I need it. For sharing your life with me so we can take on the world together, and live it to the fullest.

Thank you to my mother, Karyn, for always telling me I can achieve anything I want in life, and believing it to be true. To my father, David, for instilling in me a strong work ethic. Through his own perseverance, he encouraged me to work hard no matter how challenging the project. To both Judy and Melissa, thank you for bringing out the best in my parents so they can be their best for me. Because of you, they have found their paths to true love and happiness.

To Noah, thank you for never letting me forget to laugh, and continuing to be the big brother I look up to. And to my younger brother, Rami, thank you for teaching me that my actions in life impact the lives of those around me.

Last but not least, I would like to extend my appreciation to my advisor, Dr. Rich Melloni, for providing me with an environment in which I was encouraged to make mistakes, grow as a person, and develop as a student, colleague, and scientist. Thank you for taking the risk and giving me a chance. And to Dr. Lesley Ricci, greatest thanks for your tutelage and for always being confident in my ability to succeed. You have been eternally helpful through your hands-on assistance and your careful words of encouragement.
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<tr>
<td>5-HT\textsubscript{1A}</td>
<td>Serotonin type-1A receptors</td>
</tr>
<tr>
<td>5-HT\textsubscript{1B}</td>
<td>Serotonin type-1B receptors</td>
</tr>
<tr>
<td>5-HT\textsubscript{2A}</td>
<td>Serotonin type-2A receptors</td>
</tr>
<tr>
<td>AAS</td>
<td>Anabolic Androgenic Steroids</td>
</tr>
<tr>
<td>AH</td>
<td>Anterior Hypothalamus</td>
</tr>
<tr>
<td>AVP</td>
<td>Arginine Vasopressin</td>
</tr>
<tr>
<td>BNST</td>
<td>Bed Nucleus Stria Terminalis</td>
</tr>
<tr>
<td>CeA</td>
<td>Central Amygdala</td>
</tr>
<tr>
<td>D\textsubscript{2}</td>
<td>Dopamine D\textsubscript{2} receptors</td>
</tr>
<tr>
<td>D\textsubscript{5}</td>
<td>Dopamine D\textsubscript{5} receptors</td>
</tr>
<tr>
<td>GABA</td>
<td>(\gamma)-Aminobutyric Acid</td>
</tr>
<tr>
<td>GAD</td>
<td>Glutamic Acid Decarboxylase</td>
</tr>
<tr>
<td>ir</td>
<td>immunoreactive</td>
</tr>
<tr>
<td>LAH</td>
<td>Lateral Anterior Hypothalamus</td>
</tr>
<tr>
<td>LS</td>
<td>Lateral Septum</td>
</tr>
<tr>
<td>MeA</td>
<td>Medial Amygdala</td>
</tr>
<tr>
<td>mSON</td>
<td>medial Supraoptic Nucleus</td>
</tr>
<tr>
<td>NC</td>
<td>Nucleus Circularis</td>
</tr>
<tr>
<td>oc</td>
<td>Optic Chiasm</td>
</tr>
<tr>
<td>P</td>
<td>Postnatal</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine Hydroxylase</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of Interest</td>
</tr>
<tr>
<td>S1</td>
<td>Somatosensory Cortex 1</td>
</tr>
<tr>
<td>SC</td>
<td>subcutaneous</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic Nucleus</td>
</tr>
<tr>
<td>VLH</td>
<td>Ventrolateral Hypothalamus</td>
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Chapter 1

Background

1.1 Adolescent Anabolic Steroid Abuse

Adolescence is a developmental period where neurobiological mechanisms regulating complex behaviors, such as aggression, are particularly sensitive to circulating androgens. For example, an increased incidence of aggressive behavior correlates with elevated levels of endogenous testosterone in mid-to-late adolescent males (Dabbs et al. 1987; Dabbs et al. 1991; Mattsson et al. 1980; Scerbo and Kolko 1994) but not in prepubertal males (Constantino et al. 1993; Schaal et al. 1996; Susman et al. 1987), suggesting a link between circulating androgens and the development of the aggressive phenotype. In fact, changes in serum testosterone levels are associated with aggressive responses to agonistic encounters (Kudryavtseva et al. 2004; Oyegbile and Marler 2005; 2006; Trainor et al. 2004). For example, transient increases in testosterone levels are observed after a single winning experience in birds (Oliveira et al. 2001; Wingfield et al. 1987), primates (Cavigelli and Pereira 2000) and even humans (Mazur and Booth 1998). The increase in testosterone is hypothesized to be causally associated with the elevated aggressive response observed in agonistic encounters (Oyegbile and Marler 2005). This link between testosterone and aggression is particularly concerning for adolescent populations.
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who abuse synthetic testosterone and its derivatives, collectively termed anabolic androgenic steroids (AAS), for their performance enhancing effects (NIDACapsules 2008).

Studies from the National Institute on Drug Abuse report that abuse remains high in adolescent populations with 1.5% of 8th graders and 1.8% of 10th graders reporting AAS use (NIDACapsules 2008). Furthermore, high abuse trends also exist for high school seniors with an alarming 2.2% of 12th graders reported abusing AAS (NIDACapsules 2008). This pattern of abuse is concerning for several reasons. First, the onset of AAS use during adolescence is correlated with more frequent and heavier use later in life (Buckley et al. 1988), despite physical and psychological side effects. Second, since the frequency of adolescent AAS use is associated with frequency of multiple drug use, including injectable drugs, AAS use during adolescence may represent an increased level of commitment to drug use in general (DuRant et al. 1993). Indeed a study of 227 men admitted to a drug treatment center for heroin or other opioid dependence reported that 9.3% had used AAS before trying any other illicit drug. Additionally, of these 9.3%, 86.7% reported first using opioids to counteract the unwanted side-effects of AAS abuse (i.e. insomnia, irritability, and aggression) (NIDACapsules 2008). This pattern of early use has been associated with continued and frequent abuse later in life, despite potential psychiatric, physiological, and behavioral risks, including heightened offensive aggression (Buckley et al. 1988). To date there is an incomplete understanding of the neurobiological mechanisms modulating AAS-induced aggression limiting the availability and development of appropriate pharmacotherapeutics for treating early onset AAS-induced aggressive individuals and for those youth predisposed to aggression-related disorders across a spectrum.
1. Background

1.2 Animal Models of Aggression: The Syrian Hamster

Violent behavior in humans is classified as one of two subtypes of aggression. Reactive aggression, often referred to as non-instrumental, is regarded as explosive and uncontrolled aggression accompanied by high levels of arousal, including anger and fear (Vitiello and Stoff 1997). Conversely, proactive, or instrumental, aggression refers to non-impulsive aggression exhibited as goal-oriented behavior and marked by an absence of arousal (Vitiello and Stoff 1997). The identification of aggression subtypes is important because the brain mechanisms that subserve different subtypes of aggression are distinct in animals (Eichelman 1992) and in human beings (Blair 2004). In AAS-induced aggression, abusers are reported to exhibit higher instances of reactive, not proactive, aggression (Chantal et al. 2009) suggesting that AAS abuse alters neural systems linked to reactive aggression. Ethical and technological limitation in experimental methods limits our ability to investigate the neurobiological links to AAS-induced reactive aggression in humans. However, the use of preclinical animal models affords the possibility of examining the neurobiological aspects to specific types of aggression, including their probable etiologies. In animal models of aggression, numerous attempts have been made to categorize the aggressive response into a bimodal classification that parallels the human reactive non-instrumental and proactive instrumental categories. Although not an exact fit, offensive aggression in animals possesses many of the characteristics of reactive aggression in human beings (Bambauer and Connor 2005; Blanchard and Blanchard 2003; Moyer 1977), including impulsive responding and intense aggression focused on a specific object identified as threatening. Specifically for AAS-induced aggression, the Syrian hamster has been used as a valid animal model to investigate the neurobiological and behavioral consequences of adolescent AAS-abuse for more than a decade (Melloni et al. 1997a). This research has led to a plausible
1. Background

model underscoring the major neurochemical alterations produced in various brain loci that contribute to the AAS-induced aggressive phenotype (for review see Melloni and Ricci 2010).

Unlike other rodent models (i.e. rat and mouse), the Syrian hamster in the wild establishes a solitary nest site after weaning from the dam (Schoenfeld and Leonard 1985). Once leaving the home nest, the hamster learns to defend its newly established territory and participates in social dominance hierarchies (Schoenfeld and Leonard 1985; Whitsett 1975). Therefore, single housing or isolation of the Syrian hamster is consistent with its natural environment thus providing an ethologically valid model to study fighting experience in the laboratory setting. The aggression ethogram in the Syrian hamster has been well established and qualitatively defined with discrete behavioral patterns and distinguished postures that can be used to measure changes in aggressive motivation and fighting ability (Floody and Pfaff 1977; Johnston 1985). Most social interactions begin with nose-to-nose investigation followed by mutual investigation of the opponent’s face and head region. At these early stages of agonistic interaction, the two hamsters often engage in upright postures. During this upright position, the hamsters begin to jockey for dominance through the display of either offensive or defensive upright postures as defined by body angle, extension of forelegs and paws, and their orientation to the opponent hamster (Grant and Mackintosh 1963; Johnston 1976; 1985). If a dominant status is not reached by one of the hamsters, the animals may move to a later phase incorporating more direct physical contact. At this time, there is a qualitative change in the behavioral sequences in which one hamster initiates a sideways attack on the opponent and the two engage in a rolling fight. During this phase the Syrian hamster will attempt to bite the fur and skin of the opponent’s flank or rump regions. This phase of fighting is often terminated when the subordinate hamster exhibits an explosive movement, known as fly away, which allows the
1. Background

individual to disengage from its opponent (Floody and Pfaff 1977; Johnston 1985). The display of these aggressive behaviors begins near the onset of puberty and are further developed and refined during a time period in hamsters that is comparable to human adolescence.

Hamsters wean around postnatal day 25 (P25), leave the home nest, forage on their own, establish new nest sites, and defend their territory (Schoenfeld and Leonard 1985). They begin to fight and establish dominance hierarchies as early as P35 (Whitsett 1975) and puberty occurs between P35-P70 with a minimal breeding age of 42 days in males (Miller et al. 1977; Vomachka and Greenwald 1979; Wommack and Delville 2007). Between P25 and P56, hamsters achieve independence from the maternal nest, double their body weight and size, reach full sexual maturity and reproductive competence, and establish social relationships. It is during the establishment of these social relationships that aggressive behaviors develop naturally in the wild through repeated interactions in agonistic encounters. In the laboratory settings, animals treated with AAS throughout the developmentally sensitive period of adolescence display this same elevated aggressive phenotype when compared to vehicle treated littermates. (Melloni et al. 1997a). Given that these AAS-treated animals display a high frequency of attacking and biting behavior in the absence of social learning suggests that this treatment regimen circumvents normal adolescent development to produce the exaggerated aggressive phenotype. In fact, adolescent AAS-abuse results in activational changes in neurochemistry across various brain loci to produce elevated offensive aggressive responses.

1.3 The Aggression Circuit

Aggression and other social behaviors are regulated by a discrete network of nuclei that are conserved across species and taxa (Goodson 2005; Newman 1999). These brain regions
1. Background

include three hypothalamic nuclei (the anterior hypothalamus, medial preoptic area, and ventromedial hypothalamus), the lateral septum, medial extended amygdala (including the bed nucleus of the stria terminalis), and various midbrain nuclei (i.e. periaqueductal grey and adjacent tegmentum). Different activation patterns across these nuclei are proposed to elicit different behavioral responses based on contextual inputs (e.g. sexual behavior, aggression) (Newman 1999). Importantly, these nuclei richly express androgen receptors (Simerly et al. 1990) further linking testosterone to the control of aggressive behaviors. While each of these nuclei provide a unique input to the control of aggression, one nucleus appears to be at the center of aggression control, namely the anterior hypothalamus (AH). In the Syrian hamster, the AH is reciprocally connected to all other aggression-related nuclei (Delville et al. 2000) and is the key regulator of aggressive responding (Nelson and Trainor 2007). Pharmacological activation and electrical stimulation of the AH elicits robust increases in aggression intensity while suppression of AH activity abolishes aggressive behaviors (Bermond et al. 1982; Ferris et al. 1984; Ferris and Potegal 1988; Hammond and Rowe 1976; Sweidan et al. 1991). AH activity is highly dependent on androgen levels and many of the neurochemicals modulating aggression are sensitive to the effects of AAS-exposure (Melloni and Ricci 2010). Therefore, it is important to consider the various neuropeptide and neurotransmitter signals implicated in aggression control with an emphasis on their potential influences on modulating AH activity.

1.4 Neurochemistry of Aggression

Arginine Vasopressin

Control of aggression is modulated, in part, by the release of arginine vasopressin (AVP) into the AH. Specifically, microinjection of AVP into the AH triggers the aggressive response in
1. Background

Syrian hamsters (Ferris et al. 1984) while blockade of AVP V$_{1A}$ receptors reduces aggressive responding (Ferris and Potegal 1988). Moreover, treatment with AAS throughout adolescence produces an elevated aggressive response as a result of increased AVP innervation to the AH (Harrison et al. 2000). The source of the aggression stimulating AVP afferents are localized to two distinct nuclei within the AH, specifically the nucleus circularis (NC) and the medial supraoptic nucleus (mSON). Magnocellular AVP neurons within the NC and mSON innervate a lateral subdivision of the anterior hypothalamus (LAH) implicating this subregion of the AH as the key regulator of aggression control. Indeed, many of the AAS-induced alterations in neurochemistry are localized to this subregion of the AH (Melloni and Ricci 2010). Magnocellular AVP neurons in the AH have previously been shown to co-express the catecholamine neurotransmitter dopamine (Panayotacopoulou et al. 1994) suggesting a role for other neurotransmitters in aggression control. Indeed, monoaminergic activity, specifically dopamine and serotonin, as well as excitatory and inhibitory inputs from glutamate and GABA, play integral roles in the control of aggression and are localized to the AH. Studies linking these neurotransmitter systems to aggression control and AAS-abuse have often examined their functions independent of one another. Therefore, it is necessary to investigate how these neural systems may be integrated in the AH to modulate AAS-induced aggression.

Serotonin

1. Background

1995; Sijbesma et al. 1990). Low levels of serotonin are associated with increased impulsivity and aggression (Higley et al. 1996; King et al. 2003; Nelson and Trainor 2007) while pharmacological enhancement of the serotonin system attenuates the aggressive response (Grimes and Melloni 2002; Miczek and Fish 2006). Specifically, treatment with serotonin reuptake inhibitors or L-tryptophan (the precursor for serotonin synthesis) significantly reduces aggressive responding in various animal models (Grimes and Melloni 2002; Winberg et al. 2001). In the Syrian hamster, serotonin activity in the hypothalamus has been shown to regulate offensive aggression (Delville et al. 1996; Ferris 1996; Ferris et al. 1997; Ferris et al. 1999). Indeed, activation of the serotonin system through receptor agonism of serotonin type-1A (5-HT1A) and serotonin type-1B (5-HT1B) receptors markedly reduces impulsivity and aggression in various species (Bell et al. 1995; de Almeida and Miczek 2002; Delville et al. 1996; Miczek et al. 1998; Muehlenkamp et al. 1995; Rilke et al. 2001; Sanchez et al. 1993). In AAS-induced aggression, neurobiological examination of 5-HT1A receptors revealed significant reductions in protein content and immunopositive staining in the AH (Ricci et al. 2006). Treatment with R(+)-8-OH-DPAT, a selective 5-HT1A agonist, abolishes the AAS-induced increase in aggression (Ricci et al. 2006). Similarly, AAS exposure alters site specific 5-HT1B expression by reducing postsynaptic signal and increasing autoreceptor expression in various aggression nuclei (Grimes and Melloni 2005). Interestingly, these alterations in 5-HT1B receptor expression occur exclusively in the LAH. Taken together, this data supports an inhibitory role of AH serotonin in the control of AAS-induced aggression. In addition to 5-HT1A and 5-HT1B receptors, which have an inhibitory influence on neural activity, the excitatory serotonin type-2A (5-HT2A) receptor is also localized to the AH and previously implicated in aggression control. While the role of 5-HT1A and 5-HT1B receptors are hypothesized to suppress AVP release through their inhibitory
1. Background

effects, the role of excitatory 5-HT$_{2A}$ receptors appears more complex.

5-HT$_{2A}$ Receptors

The 5-HT$_{2A}$ receptor is an important receptor governing various psychological and psychiatric processes. Specifically, alterations in the expression of 5-HT$_{2A}$ have been linked to schizophrenia, mood disorders, anxiety disorders, obsessive-compulsive disorder, eating disorders and Alzheimer’s disease (Abbas and Roth 2008; Roth et al. 1998; Roth and Xia 2004). Additionally, 5-HT$_{2A}$ over activation results in psychological and perceptual alterations as this receptor subtype is the site of action for many hallucinogenic agents (Aghajanian 1994; Glennon 1990; Nichols 2004; Roth et al. 2002). Given that 5-HT$_{2A}$ receptors mediate perception, cognition, and emotion, this receptor has become a target of interest for the development of therapeutics for multiple psychiatric disorders. For example, antagonism of 5-HT$_{2A}$ receptors show therapeutic efficacy in antidepressant, anxiolytic, and antipsychotic drugs (Gonzalez-Maeso et al. 2007; Gray and Roth 2007; Kroeze and Roth 1998). Interestingly, the use of atypical antipsychotics, which have high affinity for the 5-HT$_{2A}$ receptor subtype, has been shown to reduce aggressive outbursts in psychiatrically referred youth (Findling et al. 2005; Schur et al. 2003). In preclinical studies, blockade of 5-HT$_{2A}$ receptors using the selective antagonist ritanserin suppresses isolation-induced aggression (Sakaue et al. 2002; White et al. 1991). These studies suggest a plausible excitatory role for serotonin activity in the control of aggression in addition to the already well-established inhibitory mechanisms. Therefore, it is important to investigate the role of 5-HT$_{2A}$ receptors in the AH of AAS-induced aggression and examine what neural systems may express these excitatory receptors to produce the heightened
1. Background

aggressive response. Evidence supporting a role for 5-HT$_{2A}$ receptors in modulating aggression stems from preclinical studies of exaggerated aggressive responding in the Syrian hamster. Administration of antipsychotic medications with high affinity for 5-HT$_{2A}$ receptors dose-dependently decreased cocaine-induced aggression without concomitant motor dysfunction (Ricci et al. 2007a; Schwartzer et al. 2008; Schwartzer et al. 2009a). These receptor antagonists also have high affinity for dopamine receptors suggesting that both serotonin and dopamine may interact to elicit aggressive behaviors. Therefore, dopamine and its receptor pools must also be considered in the neural circuits governing aggression.

Dopamine

Dopamine is a catecholamine neurotransmitter that has been associated with aggressive behavior. Dopamine terminals have been localized to numerous brain areas, including aggression-associated areas such as the hypothalamus, septum, bed nucleus of the stria terminalis and different amygdaloid nuclei (Adams and Moghaddam 2000; Pirnik and Kiss 2005; Wommack and Delville 2002). Indeed, dopamine activity has been shown to play a critical role in the modulation of aggressive behavior across a number of species and behavioral paradigms. For example, male rats have increased limbic dopamine concentration in anticipation and at the beginning of an aggressive encounter (Ferrari et al. 2003; Louilot et al. 1986). Similarly, dominant mice have increased tyrosine hydroxylase (TH; the rate-limiting enzyme for dopamine production) and dopamine transporter expression compared to defeated or control mice following 10 consecutive days of resident-intruder agonistic encounters (Christophersen et al. 2006; Filipenko et al. 2001; Margolis et al. 2006). In addition, dopamine transporter knock-out
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mice display augmented aggression in social hierarchy building, and an increased likelihood to initiate aggressive encounters with their home-cage mates (Rodriguez et al. 2004). Importantly, alterations in dopamine transmission have been shown to occur in response to AAS exposure. In fact, androgen plasma concentrations positively correlate with TH levels in the hypothalamus at the level of the preoptic and suprachiasmatic nuclei (Wilczynski et al. 2003). In male rats, castration resulted in a significant decrease in hypothalamic dopamine levels which were restored to baseline after injection with testosterone propionate (Putnam et al. 2003; Sanghera et al. 1991). Taken together, these results strongly suggest both an aggressive and an impulsive behavioral component to AAS-induced increased dopamine activity. These alterations likely occur through increased transmitter synthesis and/or disrupted removal from the synapse. Studies directly measuring changes in dopamine development after adolescent exposure to AAS are warranted to better understand their role in modulating aggression. Additionally, attention must be drawn to the various dopamine receptors localized to the AH and their likely role in modulating AAS-induced aggression.

$D_2$ and $D_5$ Receptors

Dopamine receptors are classified into two families, $D_1$-like receptors ($D_1, D_5$) and $D_2$-like receptors ($D_2, D_3, D_4$) (Sibley et al. 1993). While both receptor types are G-protein coupled, activation of $D_1$-like and $D_2$-like receptors produce opposing responses (Bunzow et al. 1988; Monsma et al. 1990; Sibley et al. 1993). Specifically, $D_2$-like receptors are negatively linked to adenylyl cyclase such that activation of these receptors results in neuronal inhibition (Bunzow et al. 1988). Conversely, $D_1$-like receptor activation increases adenylyl cyclase producing increased neuronal activity and excitation (Monsma et al. 1990). Both $D_2$-like and $D_1$-like receptors,
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particularly D\(_2\) and D\(_5\), are localized to the hypothalamus and implicated in aggression control in various species and animal models (Bondar and Kudryavtseva 2005; Nikulina and Kapralova 1992; Rodriguez-Arias et al. 1998; Tidey and Miczek 1992). For example, blockade of D\(_2\) receptors using haloperidol and risperidone in preclinical models has demonstrated efficacy for reducing aggression in mice (Miczek et al. 2002) and hamsters (Ricci et al. 2007a; Schwartzer et al. 2008). Moreover in clinical settings, antagonism of D\(_2\) receptors using antipsychotic medication has indication for the control of aggression (Findling et al. 2000; Findling et al. 2005; Schur et al. 2003). However, the effectiveness of these drugs for aggression reduction is accompanied by decreases in arousal and motor control (Jerrell et al. 2008). Similarly, systemic administration of selective D\(_1\)-like receptor antagonists reduce aggression with concomitant alterations in motor behavior (Arregui et al. 1993; Bondar and Kudryavtseva 2005; Gendreau et al. 1997; Miczek et al. 1994; Nikulina and Kapralova 1992; Rodriguez-Arias et al. 1998). For example, in the instance of morphine withdrawal, administration of the selective D\(_1/\)D\(_5\) antagonist SCH-23390 significantly reduced the number of aggressive acts while decreasing mobility in mice (Rodriguez-Arias et al. 1999). Additionally, in isolation induced-aggression, similar reductions in aggression and locomotor activity were reported after antagonism of D\(_1\)-like receptors (Arregui et al. 1993). While these reports demonstrate a role for D\(_2\) and D\(_5\) receptors in aggression, there is little research to date investigating the link between these receptors and AAS-induced aggression. Although several reports have shown that D\(_2\) receptor mRNA is androgen sensitive and altered in various brain regions after exposure to AAS (Birgner et al. 2008; Kindlundh et al. 2003) less is known about the sensitivity of D\(_5\) receptors to androgen levels. Considering (1) that pharmacology targeting D\(_2\) and D\(_5\) receptors alters aggression in various species and paradigms, (2) the dense distribution of D\(_5\) and D\(_2\) receptors in the
1. Background

hypothesis that increased dopamine innervation to the AH after adolescent AAS exposure activates D2 and D5 receptors facilitating the development of the elevated aggressive phenotype.

GABA

γ-aminobutric acid (GABA) is the major inhibitory neurotransmitter in the central nervous system which plays an important role in the regulation of escalated aggression (de Almeida et al. 2005). However, there are conflicting reports regarding the role of GABA in the control of aggression. For example, pharmacological manipulation of the GABAergic system revealed that activation of GABA_A receptors through intraventricular injection of a GABA_A agonist resulted in increased attack behavior while antagonism decreased the number of attacks in rats confronted in a neutral arena (Depaulis and Vergnes 1985). Conversely, Liu et al. (Liu et al. 2007) reported an increase in the number of bite attacks in mice after administration of bicuculline, a GABA_A receptor antagonist. Additionally, increases in extracellular GABA through blockade of GABA transporter 1 (GAT1) was reported to decrease the number of bites (Liu et al. 2007). Given these reported differences in GABA-mediated increases and decreases in the aggressive response, it is possible that there are multiple GABAergic pathways modulating both inhibitory (e.g. serotonin) and excitatory (e.g. AVP) neurotransmitter systems implicated in aggression control (Ferris et al. 1997; Grimes and Melloni 2002; Melloni et al. 1997b).

GABA is produced in the brain by two isoforms of the enzyme glutamic acid decarboxylase (i.e. GAD_{65} and GAD_{67}). While both GAD_{65} and GAD_{67} are enzymes involved in the synthesis of GABA in the central nervous system, the two GAD isoforms serve different
1. Background

functions and have different subcellular distributions (Erlander and Tobin 1991; Kaufman et al. 1991). Specifically, while GAD$_{65}$ appears to be localized to nerve terminals, GAD$_{67}$ is more uniformly distributed throughout the cell body. Previously, GAD$_{65}$ was shown to increase in the AH of AAS-treated animals (Grimes et al. 2003). Furthermore, AH GABA innervates magnocellular AVP neurons suppressing the excitability of these cells in the supraoptic nucleus and paraventricular nucleus (Nissen and Renaud 1994; Theodosius et al. 1986). Interestingly, GABAergic interneurons commonly express dopamine and serotonin receptors across various brain regions (de Almeida and Mengod 2007; Gerfen et al. 1990; Griffiths and Lovick 2002; Santana et al. 2008). Given the sensitivity of the GABA system to AAS exposure and the previous reports of increased GABA terminals (i.e. GAD$_{65}$) in the AH (Grimes et al. 2003; Henderson et al. 2006), it is hypothesized that GABA interneurons within the AH play an important role in integrating various neuronal inputs to the AH that directly or indirectly modulate AVP activity and that adolescent AAS-exposure disrupt this integration to produce highly aggressive responses in the Syrian hamster.

$GABA_A$ Receptors

In the central nervous system, GABA imparts its inhibitory action primarily through activation of postsynaptic GABA$_A$ receptor activity. GABA$_A$ receptors are pentameric ionotropic chloride channels and vary by 16 different receptor subunit genes (Mehta and Ticku 1999; Sieghart 1995). The most common GABA$_A$ receptor isoform expressed in the mammalian brain consists of $\alpha_1$, $\beta_2$ or $\beta_3$, and $\gamma_2$ subunits (Clark and Henderson 2003; Henderson et al. 2006; Jorge et al. 2002). Interestingly, AAS act as allosteric modulators on the GABA$_A$ receptor directly altering receptor function and demonstrating a direct link to AAS-induced aggression (Clark et
1. Background

Moreover, aggressive behaviors are mediated by alterations in GABA<sub>A</sub> receptor activity (de Almeida et al. 2005; Jorge et al. 2002; Miczek et al. 2003). Taking together the AAS-induced increase in GABA terminals (i.e. GAD<sub>65</sub>) and the link between GABA<sub>A</sub> activity and aggression control (Miczek et al. 2003), it is likely that adolescent AAS-exposure results in alterations to the expression of GABA<sub>A</sub> receptors in aggression loci of the brain, including the AH.

1.5 Hypothalamic Mechanisms Integrating AH Dopamine and Serotonin

Given the paucity of research underscoring the importance of dopaminergic, serotonergic, and GABAergic neural systems in the regulation of aggression, it is important to consider how these systems may be integrated in the AH and how adolescent AAS-exposure may disrupt the development of these systems to produce the elevated aggressive response. The hypothesis to be investigated in this dissertation is that dopamine and/or serotonin neural systems in the AH modulate local GABAergic interneurons and that adolescent AAS-exposure increases aggression through differential activation of local GABAergic circuits. Given conflicting reports as to whether GABA plays an inhibitory or facilitative role in aggression control (Depaulis and Vergnes 1985; Liu et al. 2007; Miczek et al. 2002), there likely exists multiple GABAergic pathways in the AH that are steroid sensitive and are altered in the presence of adolescent AAS-exposure to produce elevated aggressive behaviors.

The first putative mechanism to be examined is the interaction between dopamine and GABAergic neural systems. Indeed, both GABA and dopamine neural systems are present in the AH and undergo alterations after repeated exposure to AAS (Grimes et al. 2003; Ricci et al. 2009). Furthermore, inhibitory D<sub>2</sub> receptors and excitatory D<sub>5</sub> receptors are present in the AH.
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Interestingly, dopamine neurons often synapse on medium-spiny GABAergic neurons (Guzman et al. 2003; Pickel et al. 1996); these GABAergic interneurons commonly express D₂ and D₁-like receptors across various brain regions (Gerfen et al. 1990; Santana et al. 2008). It is hypothesized that there exists two populations of GABAergic interneurons that differentially express D₂ and D₅ receptors in the AH such that hypothalamic dopamine both activates and inhibits differential populations of GABA neurons. Therefore it is postulated that adolescent exposure to AAS results in increased aggression by differentially altering the number of D₂-positive and D₅-positive GABAergic neurons resulting in differential activation and inhibition of various neural systems governing aggression control.

Alternatively, serotonin afferents to the AH are hypothesized to modulate GABAergic interneurons through excitatory 5-HT₂ₐ receptors. Through excitation of GABA, serotonin is hypothesized to indirectly autoregulate its release within the AH. Indeed, activation of 5-HT₂ₐ receptors in various brain regions has been shown to increase GABA release (Aghajanian and Marek 1997; Ciranna 2006; Li et al. 2000; Munsch et al. 2003) resulting in serotonergic activation of an inhibitory system. Support for this notion stems from behavioral pharmacology studies demonstrating that 5-HT₂ₐ activation increases aggression (Sakaue et al. 2002). From a mechanistic standpoint, this would suggest that GABA mediates an inhibitory system serving to disinhibit the hypothalamus resulting in an increase in aggressive response. Indeed, research demonstrates that 5-HT₂ₐ can differentially increase or decrease GABA mediated inhibition suggesting that 5-HT₂ₐ may directly or indirectly alter GABA release (Shen and Andrade 1998). Additionally, activation of GABAₐ receptors increases aggression suggesting that both GABA and serotonin may reduce the excitability of an inhibitory system (Fish et al. 2005; Fish et al. 2001; Miczek et al. 2004; Miczek et al. 2003). Interactions between the GABAergic and
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serotonergic systems must be further investigated to better understand the effects of adolescent AAS exposure on the generation of offensive aggression.

1.6 Objectives and Experimental Design

The main objective of the research described in this dissertation is to investigate the interactions of dopamine and serotonin neural systems with GABAergic interneurons of the AH and explore how these systems are altered in the presence of adolescent AAS-exposure to elicit heightened aggressive behaviors. It is hypothesized that the exaggerated aggressive response observed in adolescent AAS-treated hamsters may be explained in part by altered dopamine development and activity in brain regions associated with the aggressive phenotype, specifically the AH. To explore this hypothesis, aggressive AAS-treated Syrian hamsters were measured for changes in aggressive behaviors and their brains were processed for immunohistochemistry to examine possible alterations in dopamine synthesis and/or the density of D2 and D5 receptor expressions. Additionally, to demonstrate a link between dopamine synthesis and aggression intensity, the aggressive behaviors of AAS-treated animals was correlated with the density of TH production.

Previous research examining the role various dopamine receptors play in the control of aggression have utilized systemic injection techniques. Given the high incidence of extrapyramidal effects associated with pharmacology used to target dopamine receptors, the findings from these studies complicate the question of whether drugs targeting D2 and D5 receptors are specific to aggression or whether they exert their anti-aggressive effects through nonspecific mechanisms, such as changes in mobility. Therefore, to examine whether D2 and D5 receptors directly modulate AAS-induced aggression in the AH, aggressive AAS-treated animals
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were locally infused with the D$_2$ receptor antagonist eticlopride or the D$_5$ receptor antagonist SCH-23390, and measured for changes in aggressive responding. Additionally, to determine whether these drugs exert their effects directly on aggression circuits or whether reductions in aggression are a result of non-specific drug effects, animals were measured for changes in non-aggression related behaviors (e.g. grooming and sniffing behaviors) as well as locomotor activity.

To investigate the putative interactions between AH dopamine and GABA neural systems, the brains of aggressive AAS-treated animals were examined for colocalization of dopamine receptors and GABAergic neurons. It is hypothesized that there exists two distinct GABAergic populations that differentially regulate excitatory (e.g. AVP) and inhibitory (e.g. serotonin) tone in the AH and that these two GABAergic populations are regulated through inhibition (i.e. D$_2$) and excitation (i.e. D$_5$) from local dopamine afferents. To explore this potential mechanism and determine whether these GABAergic neurons are altered by the presence of AAS throughout adolescent development, double immunofluorescence was used to colocalize D$_2$ and D$_5$ receptors with GAD$_{67}$ containing neurons in the AH.

An alternative hypothesis examined in this dissertation investigated the interaction of serotonin and GABA neural systems through excitatory 5-HT$_{2A}$ receptors. First, to determine whether adolescent AAS exposure altered 5-HT$_{2A}$ receptor expression in the AH, animals were treated for AAS throughout adolescence and measured for increases in aggression compared to sesame oil-treated controls. Then, their brains were processed for immunohistochemistry to visualize and quantify 5-HT$_{2A}$ receptor distribution/localization patterns in the AH and correlate these changes with the exaggerated aggressive response. Next, to determine whether AH-5-HT$_{2A}$ receptors are expressed on local GABAergic interneurons, double-label immunofluorescence
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was performed to colocalize 5-HT$_{2A}$ to GAD$_{67}$. Finally, to investigate whether the GABA neural system is compromised by adolescent AAS-exposure and explore the link between AAS-induced aggression and GABA$_A$ receptors, hamster brains were processed for immunohistochemistry and quantified for changes in GABA$_A$ in the AH.
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1.7 Compendium of Manuscripts

Chapters 3, 4, 5, and 6 were taken, in part, from the following manuscripts:


Chapter 2

Materials and Methods

2.1 Animals

Male Syrian hamsters (*Mesocricetus auratus*) were obtained from Charles River Laboratories (Wilmington, MA) individually housed in polycarbonate cages, and maintained at ambient room temperature (22-24°C, with 55% relative humidity) on a reverse light-dark cycle (14L:10D; lights off at 08:00) (Grimes and Melloni 2002). Food and water were provided *ad libitum*. For aggression testing, stimulus (intruder) males of equal size and weight to the experimental animals were obtained from Charles River Laboratories one week prior to the behavioral test, group-housed (five animals per cage) in large polycarbonate cages, and maintained as above to acclimate to the animal facility. All studies employing live animals were pre-approved by the Northeastern University Institutional Animal Care and Use Committee and all methods used were consistent with guidelines provided by the National Institutes of Health for the scientific treatment of animals.
2. Materials and Methods

2.2 Drugs

Testosterone cypionate, nandrolone decanoate, and boldenone undecylenate were purchased from Steraloids Inc. (Newport, RI) and prepared in sesame oil to produce the AAS treatment. S(-)-Eticlopride hydrochloride and SCH-23390 were purchased from Sigma Aldrich (St. Louis, MO) and dissolved in 0.9% (wt/vol) normal saline.

2.3 Experimental Procedures

Adolescent AAS-Treatment

Postnatal 27 hamsters were weighed and randomly distributed into two groups ($n = 20$ animals/group). One group of animals received daily subcutaneous (SC) injections (0.1-0.2ml) of an AAS mixture consisting of 2 mg/kg testosterone cypionate, 2 mg/kg nandrolone decanoate (19-nortestosterone), and 1 mg/kg boldenone undecylenate (1-dehydrotestosterone; Steraloids Inc., Newport, RI) dissolved in sesame oil, for 30 consecutive days during adolescent development (P27-P56). This treatment regimen, designed to mimic a chronic use regimen (Pope and Katz 1994; Pope 1988), has been shown repeatedly to produce highly aggressive animals in greater than 85% of the treatment pool (DeLeon et al. 2002; Grimes and Melloni 2005; Harrison et al. 2000). As a baseline control, a separate group of hamsters received SC injections of sesame oil alone throughout adolescent development.

Surgery

One week prior to aggression testing (P50), animals were anesthetized with isoflurane (1-4%, inhalation) and placed into a stereotaxic device for unilateral implantation of a 26-gauge guide cannula aimed at the LAH. An incision was made to expose the dorsal surface of the skull.
2. Materials and Methods

The skull surface was then wiped clean to reveal the position of lambda and bregma landmarks. A small hole was drilled into the skull at the coordinate position necessary to gain access to the LAH, (i.e., 0.7mm anterior to bregma, 0.6mm lateral to the midsagittal suture, 6.8mm ventral from dura). The cannula was placed in the brain angled at 8 degrees and anchored to the skull using dental screws and acrylic. The head-wound was then sutured closed and topical antibiotic ointment applied to the wound area.

Microinjections & Behavioral Pharmacology

For pharmacology studies, Syrian hamsters (P27) received daily SC injections of an AAS cocktail for 30 days of adolescence (P27-P56). One day following the last injection (P57), AAS-treated hamsters were randomly assigned to treatment groups (n = 12/group) and tested for offensive aggression following an injection of saline or one of four doses (0.01, 0.1, 1.0, or 10.0µg in 0.5µl) of eticlopride (i.e. a D₂ receptor antagonist) or one of three doses (0.01, 0.1, or 1.0µg in 0.5µl) of SCH-23390 (i.e. a D₁-like receptor antagonist) into the LAH. Eticlopride and SCH-23390 were selected based on there affinities for the D₂ receptor [Ki = 0.09nM (Martelle and Nader 2008; Seeman and Ulpian 1988)] and the D₅ receptor [Ki = 0.28 nM (Millan et al. 2001), respectively. The dose ranges were selected based on previous effective doses in rats (Bari and Pierce 2005; Magnusson and Fisher 2000; Sun and Rebec 2005).

A 2-µl Hamilton syringe was connected to a 33-gauge stainless injection needle via polyethylene tubing. Injection of drugs into the LAH was performed by lowering the injection needle through the guide cannula and manually delivering a final volume of 0.5µl of drug over 3 minutes using a Hamilton microsyringe (Couppis and Kennedy 2008). The injection needle was left in position for an additional minute to allow for drug diffusion away from the injection site.
2. Materials and Methods

The internal-injector cannula protruded 1mm beyond the guide cannula. After injection, animals were returned to their home cage for ten minutes before undergoing behavioral testing.

Resident Intruder Paradigm

Hamsters were tested for offensive aggression using the resident-intruder paradigm, a well-characterized and ethologically valid model of offensive aggression in Syrian hamsters (Blanchard and Blanchard 1984; Floody and Pfaff 1974; Lerwill and Makings 1971). An intruder of similar size and weight was introduced into the home cage of the experimental animal (resident) and the resident was scored for specific and targeted aggressive responses including upright offensive postures, lateral attacks, and flank/rump bites, as previously described (Grimes et al. 2003; Ricci et al. 2006). An attack was scored each time the resident animal would pursue and then either [1] lunge toward and/or [2] confine the intruder by upright and sideways threat; each generally followed by a direct attempt to bite the intruder’s dorsal rump and/or flank target area(s). A composite aggression score, used as a general measure of offensive aggression, was defined as the total number of attacks (i.e. upright offensives and lateral attacks) and bites (i.e. flank and rump bites) during the behavioral test period. Each aggression test lasted for 10 minutes and was videotaped and scored manually by two observers unaware of the hamsters’ experimental treatment. Inter-rater reliability was set at 95%. No intruder was used for more than one behavioral test, and all subjects were tested during the first 4 hours of the dark cycle under dim red illumination to control for circadian influences on behavioral responding.

For pharmacology studies, residents were measured for social interest toward intruders (i.e. contact time between resident and intruder and the frequency of social investigation), self-grooming, and cage climbing to control for nonspecific effects of receptor agents on behavior.
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Contact time was defined as the period of time during which the resident deliberately initiated contact with the intruder, through either olfactory investigation (i.e. sniffing) or aggression. To measure possible changes in motor activity due to receptor antagonism, animals were measured for changes in locomotor activity (i.e. number of line crosses) during the 10-minute agonistic encounter.

2.4 Histology

Tissue Preparation

For immunoassays and verification of cannula placement in microinfusion studies, animals were anesthetized with isoflurane one day following behavioral testing and transcardially perfused with 4% paraformaldehyde. Brains were removed, postfixed for 90 minutes in perfusion fixative, and cryoprotected overnight in 30% sucrose at 4°C. Brains were cut at 35µm on a freezing microtome in serial, coronal sections and processed for immunohistochemistry or cresyl violet staining. At the completion of immunoassays, fluorescent slides were coverslipped using fluoromount G (Southern Biotechnology, Birmingham, Alabama) and peroxidase-stained sections were mounted on gel-coated slides, air-dried, dehydrated through a series of alcohols, cleared with xylene and coverslipped with Cytoseal (Stephens Scientific, Kalamazoo, MI). To control for variability inherent in the immunohistochemical process, all brain sections were processed simultaneously including representative sections omitting either the primary or secondary antibodies to serve as negative controls for the assays. For verification of cannula placement, sections were stained with cresyl violet, dehydrated through a series of alcohols, cleared with xylene, and coverslipped with Cytoseal (Stephens
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Scientific, Kalamazoo, MI). Only animals with correctly placed cannula tips into the LAH were included in the statistical analysis.

Immunohistochemistry

*Tyrosine Hydroxylase*

Sections were washed 3 times for 10 minutes in 0.1M phosphate buffered saline (PBS) with 0.5% Triton-X (Tx), pretreated with 0.03% H$_2$O$_2$ in distilled water for 30 minutes, then rinsed thoroughly with 0.5% PBSTx. Sections were incubated in antibody buffer containing 10% normal goat serum in 0.5% PBSTx for 60 minutes. Primary antibody (TH, polyclonal, Chemicon; California) was prepared in antibody buffer diluted to a final concentration of 1:7500 and incubation with free-floating sections was carried out overnight at 4°C on a rotation wheel. Sections were then rinsed 3 times for 10 minutes with 0.5% PBSTx, incubated for 60 minutes in biotinylated secondary goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in 0.5% PBSTx and incubated for 90 minutes in avidin-biotin-complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) in 0.5% PBSTx. The peroxidase reaction was revealed using 0.5% 3,3’-diaminobenzidine in distilled water as per manufacture’s recommendations (DAB Kit, Vectastain; Vector Laboratories, Burlingame CA). The sections were mounted on gel-coated slides, air-dried, dehydrated through a series of alcohols, cleared with xylene and coverslipped with Cytoseal (Stephens Scientific, Kalamazoo, MI).

*Dopamine D$_2$ Receptors*

Free-floating sections were washed 3 times for 5 minutes each in 0.1M PBS, pretreated with 3% H$_2$O$_2$ in distilled water for 8 minutes, and rinsed thoroughly with 0.1M PBS. Sections
2. Materials and Methods

were incubated in antibody buffer composed of 10% normal goat serum in 0.1M PBS for 60 minutes. The D₂ receptor primary antibody, chosen based on its low affinity for the D₃ and D₄ receptor (Boundy et al. 1993) (D₂, polyclonal Chemicon; California) was prepared in antibody buffer diluted to a final concentration of 1:1000 and incubation with free-floating brain sections was carried out overnight at 21°C on a rotation wheel. Sections were then rinsed 3 times for 5 minutes each with 0.1M PBS, incubated for 60 minutes in biotinylated secondary goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in 0.1M PBS then rinsed again 3 times for 5 minutes in 0.1M PBS and incubated for 60 minutes in avidin-biotin-complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) in 0.1M PBS. The peroxidase reaction was revealed using 0.5% 3,3'-diaminobenzidine in distilled water as per manufacture’s recommendations (DAB Kit, Vectastain; Vector Laboratories, Burlingame, CA).

Dopamine D₃ Receptors

Sections were washed 3 times for 5 minutes each in 0.1 M PBS, pretreated with 2% H₂O₂ in distilled water for 10 minutes, and rinsed thoroughly with 0.1M PBS. Then, sections were pre-incubated in a solution containing 3% bovine serum albumin and 1% normal goat serum in 0.1M PBS for 60 minutes. Sections were then incubated in primary rabbit antiserum (1:500) generated against D₃ ([D₁b], polyclonal, Genway Biotech; San Diego, CA) overnight at 4°C. Subsequently, sections were incubated in secondary goat anti-rabbit antiserum (1:200) for 60 minutes followed by incubation for 1 hour with avidin-biotin complex (Vectastain ABC elite Kit-Rabbit, Burlingame, CA). The peroxidase reaction was revealed using 0.5% 3,3’-diaminobenzidine in distilled water, per manufacturer’s recommendations (DAB Kit, Vectastain; Vector Laboratories, Burlingame, CA).
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5-HT$_{2A}$ Receptors

Brain sections were washed in PBS with 0.6% Triton-X (Tx) 3 times for 10 minutes each, pretreated with 5% H$_2$O$_2$ in distilled water for 8 minutes, rinsed thoroughly with PBS and treated with 1% sodium borohydride in distilled water for 5 minutes. Sections were then pretreated in 10% normal goat serum, 8% bovine serum albumin in PBSTx for 90 minutes at 21°C, then incubated in primary antiserum for 5-HT$_{2A}$ (Calbiochem, Gibbstown, NJ) at a final dilution of 1:1000 at 4°C. Sections were then rinsed with PBSTx 3 times for 10 minutes each, incubated in biotinylated secondary anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in PBS and 1% bovine serum albumin for 60 minutes at room temperature, rinsed again in PBS 3 times for 10 minutes each, and then incubated in avidin-biotin-complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) with 1% bovine serum albumin in PBS for 60 minutes at room temperature. The peroxidase reaction was revealed using 0.5% 3,3′-diaminobenzidine in distilled water as per manufacture’s recommendations (DAB Kit, Vectastain; Vector Laboratories, Burlingame, CA).

GABA$_A$ Receptors

Sections were washed 3 times for 5 minutes each in 0.1M PBS, pretreated with 2% H$_2$O$_2$ in distilled water for 10 minutes, then rinsed thoroughly in 0.1M PBS 2 times for 10 minutes each. Sections were incubated in antibody buffer containing 2% bovine serum albumin in 0.4% PBSTx for 30 minutes. Primary antibody (GABA$_{AA1}$, polyclonal, Sigma-Aldrich; St. Louis, MO) was prepared in antibody buffer diluted to a final concentration of 1:1000 and incubation with free-floating sections was carried out overnight at 4°C on a rotation wheel. Sections were then rinsed 3 times for 15 minutes each in 0.4% PBSTx, incubated for 60 minutes in biotinylated
2. Materials and Methods

Secondary goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA) in 0.4% PBSTx with 2% bovine serum albumin and rinsed again 3 times for 15 minutes each in 0.4% PBSTx. Sections were incubated for 45 minutes in avidin-biotin-complex (Vectastain ABC kit; Vector Laboratories, Burlingame, CA) in 0.4% PBSTx containing 2% bovine serum albumin. The peroxidase reaction was revealed using 0.5% 3,3’-diaminobenzidine in distilled water as per manufacturer’s recommendations (DAB kit; Vectastain; Vector Laboratories, Burlingame, CA).

Double-Label Immunofluorescence

Sections were rinsed 3 times for 10 minutes each in 0.1M PBS followed by pre-incubation in antibody buffer containing 2% normal donkey serum and 5% bovine serum albumin in 0.1M PBS for 60 minutes. Combinations of primary antibodies (D₂, polyclonal, Chemicon; Billerica, MA; D₅ [D₁b], polyclonal, Genway Biotech; San Diego, CA; GAD₆₇, polyclonal, Santa Cruz; Santa Cruz, CA; 5-HT₂A, polyclonal, Calbiochem; San Diego, CA) were diluted to 1:500 (D₂, D₅, GAD₆₇) or 1:100 (5-HT₂A) and left overnight on a rotating wheel at 4°C. Sections were then washed 3 times for 10 minutes each, incubated in secondary Alexa Fluor 488 conjugated donkey anti-goat and Alexa Fluor 568 conjugated donkey anti-rabbit (Invitrogen, Carlsbad, CA) for 60 minutes at a concentration of 1:200.

2.5 Image Analysis

The density of TH-immunopositive fiber staining was determined within the aggression associated brain areas (Figure 1) using the BIOQUANT NOVA 5.8 computer-assisted microscopic image analysis software package as previously described (Grimes et al. 2006). BIOQUANT NOVA 5.8 image analysis software running on a Pentium III CSI Open PC
2. Materials and Methods

A computer (R&M Biometrics, Nashville, TN, USA) was utilized to identify the lateral septum (LS), bed nucleus of the stria terminalis (BNST), medial amygdala (MeA), central amygdala (CeA), AH, and ventrolateral hypothalamus (VLH) at low power (4X) using a Nikon E600 microscope. In addition, the somatosensory (S1) cortex was included in the analysis as a non-aggression positive control region. At 4X magnification, a standard computer-generated box was drawn to fit within the particular nuclei or region of interest (ROI). Then, each nucleus was assigned a separate and distinct ROI parcel under 10X magnification; TH-immunoreactive (ir) labeling was then calibrated within the ROI as a particular wavelength using a standard RGB-scale. Quantification of TH-ir fiber density was performed automatically using the BIOQUANT software. Two to three independent measurements were taken from several consecutive sections of each animal per treatment group. The density of TH-ir fibers was determined for each ROI and standardized per 100µm X 100µm parcel for comparison purposes. The density of TH-ir fibers was then averaged for each nucleus per hamster and used for statistical analysis. Subnuclei of the AH, at the level of the suprachiasmatic nucleus (Figure 2) were subject to further quantitative analysis consisting of TH-ir fiber density and number of cell bodies, based on previous data implicating this region as an integral part of the circuit important for aggressive behavior (Ferris et al. 1997). The nuclei within the AH analyzed consisted of the NC and the mSON as these nuclei innervate the AH and exert local effects. The number of TH-ir cell bodies examined in these nuclei was calculated manually at 10X magnification.
2. Materials and Methods

Figure 1. Diagram showing the location of the areas selected to quantify TH-ir fibers and cell bodies (shaded areas). Plates were modified from a hamster atlas (Morin and Wood 2001) and reflect specific positions in the rostral–caudal plane (i.e. distance in millimeters from bregma to the plane of section at the skull surface). Abbreviations: AH, anterior hypothalamus; BNST, medial division of the bed nucleus of the stria terminals; CeA, central amygdala; LS, intermediate part of the lateral septal nucleus; MeA, medial amygdala; mSON, medial division of the supraoptic nucleus; S1, somatosensory neocortex; SCN, suprachiasmatic nucleus; VLH, ventrolateral hypothalamus.
2. Materials and Methods

Figure 2. Diagram showing the location of the lateral anterior hypothalamus (LAH), at the level of the medial supraoptic nucleus (mSON) and nucleus circularis (NC), selected for quantification of TH, D$_2$, D$_5$, 5-HT$_2A$, GABA$_A$, and double label immunofluorescence.
2. Materials and Methods

Quantification of the number of D<sub>2</sub>, D<sub>5</sub>, and 5-HT<sub>2A</sub>, immunoreactive cell bodies were performed manually within the LAH using BIOQUANT software by highlighting them with the mouse-driven cursor within the indicated ROI. Measurements at 20X continued until all immunoreactive cell bodies throughout the entire ROI were quantified. Two to three independent measurements were taken from several consecutive sections of each animal per treatment group. The number of positively labeled cell bodies was then averaged for each brain region per hamster and used for statistical analysis. Similarly, the density of GABA<sub>A</sub> puncta was quantified automatically across all aggression nuclei (Figure 1) using BIOQUANT software as described above.

For double-label immunofluorescence, BIOQUANT NOVA 5.8 analysis software was utilized to identify the LAH (Figure 2). A standard computer generated parcel was drawn to outline the entire ROI at lower power (4×) using the darkfield settings on an Olympus BX51 microscope fitted with X-cite series 120 mercury lamp unit and Texas Red/FITC interference filters. For immunofluorescence, two images of each tissue section from the LAH were captured, one for each filter set (i.e. Texas Red and FITC), and images were overlaid for double-label analysis. For each of the three images, all positively stained cells were identified and counted using a mouse-driven cursor for quantification. Manual measurements continued at 20× until all cells were counted throughout the LAH. Four to six independent measures were taken from several consecutive sections (2–4) for each animal. All cell counts in the LAH were averaged between hemispheres and used for statistical analysis.
2. Materials and Methods

2.6 Statistical Analysis

Results from the aggression tests were compared between AAS- and sesame oil-treated groups. Nonparametric data, (total number of attacks and bites), were compared using Kruskal-Wallis followed by Mann Whitney U-test (two-tailed). Parametric data (latency to first attack and bite, and total contact time) were compared using Student’s t-test (two-tailed). Immunohistochemical and immunofluorescence data were compared between AAS and sesame oil-treated controls for all nuclei using a priori planned comparison Student’s t-tests. The α level for all comparisons was set at 0.05. LAH TH-ir, excluding cell bodies in the NC and mSON, and LAH 5-HT2A immunoreactive cell bodies were correlated with composite measures of aggressive behavior (i.e., combined total attacks and bites) and reported using the Pearson’s r correlation coefficient. For all pharmacology studies, behavioral data were compared between AAS treatment groups using one-way ANOVA followed by Fisher’s protected least significant difference post hoc test (two-tailed) when applicable. Animals with cannulae placed outside the LAH were collapsed across dose group and composite aggression scores were compared to AAS-treated hamsters injected with saline using student’s t-test (two-tailed). The alpha level was set at 0.05 for all analyses.
Chapter 3

Dopamine in the Anterior Hypothalamus

3.1 Introduction

The first set of studies was conducted to investigate the role of the dopaminergic system in the modulation of AAS-induced aggressive behavior. Hamsters were administered AAS during adolescence, scored for offensive aggression using the resident-intruder paradigm, and then examined for alterations in dopamine immunoreactivity in brain regions implicated in the aggressive phenotype, including the AH, BNST, MeA, CeA, LS and the VLH. When compared with non-aggressive sesame-oil-treated controls, aggressive AAS-treated animals showed increased TH-ir in two anterior hypothalamic subnuclei, namely the NC and mSON. In addition, AAS-treated animals showed altered D₂ receptor expression in the AH, as measured by D₂-immunoreactivity. However, no measurable differences were observed in level of D₅ receptor expression. These results suggest that alterations in dopamine synthesis and function, together with modifications in D₂ receptor expression in the AH, may underlie neuroplastic events which facilitate AAS-induced aggression.
3. Dopamine in the Anterior Hypothalamus

3.2 Results

AAS-induced Aggressive Behavior

Animals treated with AAS during their adolescent development showed significantly elevated levels of offensive aggression when presented with a weight- and sex-matched intruder (Figure 3). Specifically, AAS-treated hamsters showed a greater number of overall attacks and bites [attacks: $\chi^2(3) = 12.18, p < 0.01$; bites: $\chi^2(3) = 8.02, p < 0.05$] when compared with sesame oil-treated controls. Post-hoc analyses revealed that AAS animals demonstrated a significant increase in the number of upright offensives [$Z = 2.79, p < 0.01$] and a greater than two-fold increase in the number of lateral attacks [$Z = 2.26, p < 0.05$] compared to their oil-treated counterparts. In addition, the number of flank/rump bites was significantly increased [$Z = 2.07, p < 0.05$] in AAS-treated animals. However, there were no differences in latency to bite [$t(17) = 1.01, p > 0.05$] or attack [$t(17) = 1.47, p > 0.05$]. Overall social contact time did not differ between groups [$t(17) = 1.21, p > 0.05$].

Tyrosine Hydroxylase

AAS treatment produced a noticeable increase in TH-ir in both hypothalamic nuclei surveyed. Specifically, AAS–treatment increased the expression of TH-ir somata in the NC and the mSON (Figure 4A,B). In fact while there are relatively few TH positive cell bodies in this anterior hypothalamic brain region AAS exposure more than doubled those expressing TH in the NC and mSON nuclei. These increases were statistically significant for the NC [$t(11) = 3.36, p < 0.01$], and produced a trend toward significance in the mSON [$t(6) = 2.04, p = 0.07$]. In addition to adolescent AAS-induced increases in TH-positive cell body expression, AAS-treated animals showed significant increases in the density of TH-ir fibers in the NC [$t(9) = 2.67, p < 0.05$].
3. Dopamine in the Anterior Hypothalamus

(Figure 4A,B). There was also an AAS-induced three-fold increase in the TH-ir fibers when compared to oil-treated animals in the mSON that supported a trend toward a statistically significant increase in that region [t(8) = 2.34, p = 0.08]. In addition, increases in TH-ir within these specific subregions of the AH directly correlated with increased overall aggressive displays induced by adolescent AAS-exposure [r = 0.696, p < 0.05]. In contrast, adolescent AAS exposure failed to alter TH-ir in any of the other aggression associated brain nuclei examined [LS, t(10) = -0.29; BNST, t(11) = -0.37; MeA, t(8) = 0.57; CeA, t(11) = -0.05; VLH, t(4) = 1.61; p > 0.05 for all regions] (Figure 4C). Lastly no changes were observed in the S1 region of the sensory cortex, a non-aggression control region, as a result of AAS exposure [S1, t(9) = 0.20, p > 0.05].

Dopamine D$_2$ and D$_5$ Receptors

In the central aggression area, the AH, D$_2$-ir cell body staining increased in AAS-treated animals when compared with oil-treated controls [t(10) = 2.74, p < 0.05] (Figure 5A). Interestingly, the increase in cell body labeling was most obvious in a subregion of the AH, the lateral anterior hypothalamus, which is situated to receive local dopamine innervation from the NC and mSON (Figure 5C). At 4X, obvious gross AAS-induced increases were observed compared with oil-treated littermates. Conversely the number of cell bodies in the AH positively labeled with D$_5$ receptors was unaltered by AAS-treatment [t(8) = 1.37, p > 0.2] when compared to oil-treated controls (Figure 5B).
3. Dopamine in the Anterior Hypothalamus

Figure 3. (A) Adolescent AAS treatment increases offensive aggression. Increases were observed in the number of upright offensives, lateral attacks, and flank/rump bites. (B) No AAS-induced changes were observed for either latency to first attack, bite or for total contact time between AAS- and vehicle-treated residents (n=8-10/group). Median and Range, Mann-Whitney, two tailed (number of offensives, attacks and bites); Bars denote S.E.M. *p<0.05; **p<0.01, Student’s t-test, two-tailed (latency measures and contact time)
3. Dopamine in the Anterior Hypothalamus

**Figure 4.** (A) Adolescent AAS treatment increased TH immunoreactivity (ir) for both cell body localization and fiber density in the NC and mSON (n = 3-7/group). Bars denote S.E.M. *p<0.05; **p<0.01; Student’s t-test, two-tailed. (B) Brightfield photomicrographs showing increased TH-ir soma and fiber density in the nucleus circularis (NC) of AAS-treated and vehicle-treated hamsters. (C) Treatment with AAS produced no other changes in TH-ir in any of the other brain regions implicated in the control of aggression. Abbreviations: AH, anterior hypothalamus; BNST, bed nucleus of the stria terminalis; CeA, central amygdala; LS, lateral septum; MeA, medial amygdala; mSON, medial supraoptic nucleus; NC, nucleus circularis; OC, optic chiasm; S1, somatosensory cortex; VLH, ventrolateral hypothalamus.
3. Dopamine in the Anterior Hypothalamus

Figure 5. (A) Adolescent AAS treatment increased D$_2$ immunoreactive (ir) cell body localization in the LAH while (B) no changes were observed in the number of D$_5$-ir cell bodies. Bars denote S.E.M. *$p<0.05$, **$p<0.01$. (C) Brightfield photomicrographs showing increased D$_2$-ir somata in the anterior hypothalamus (AH), specifically in the LAH.
3. Dopamine in the Anterior Hypothalamus

3.3 Discussion

Chronic adolescent AAS exposure potentiates heightened offensive aggression in male Syrian hamsters. These enhanced displays of aggression are associated with complex neuroplastic events and alterations in the neural network and neurotransmitter systems as a result of AAS treatment (Grimes and Melloni 2002; 2006; Grimes et al. 2003; Melloni et al. 1997a; Ricci et al. 2007b; Ricci et al. 2006). The dopamine system has been widely implicated in the control of aggressive responding across several species and behavioral paradigms, and has been shown to be sensitive to sex steroids (Asmus and Newman 1993; Baggio and Ferrari 1980; Ferrari and Giuliani 1995; Filipenko et al. 2001; Lerwill and Makings 1971; Marsh et al. 2006; Sweidan et al. 1991). Activation of mesocorticolimbic dopamine has been shown to increase in response to the anticipation and execution of aggressive acts (Ferrari et al. 2003; van Erp and Miczek 2000). Accordingly, changes in TH enzymatic activity and dopamine neurotransmission have been positively correlated with heightened aggressive response (Filipenko et al. 2001; Puglisi-Allegra and Cabib 1988; Wommack and Delville 2002). For example, increased TH expression has been observed in highly aggressive and dominant animals following social isolation (Angulo et al. 1991; Matsuda et al. 2001), chronic stress (Lamprecht et al. 1972), selective aggression (Gobrogge et al. 2007), and social instigation (Filipenko et al. 2001), while decreased dopaminergic activity following TH inhibition or selective dopaminergic ablation results in reduced aggressive response and submission in animals (Ellison 1976; Serova and Naumenko 1996). Further, results from dopamine transporter knock-out mice indicate that increased aggression and impulsivity observed in these animals depends on high extracellular dopamine availability (Rodriguiz et al. 2004). Together, these results suggest a facilitative role of dopamine function and activity in aggression. Dopamine expression, as measured by TH-ir and
3. Dopamine in the Anterior Hypothalamus

mRNA, has been localized to the brain regions involved in aggression control, including different hypothalamic, septal and amygdaloid nuclei (Adams and Moghaddam 2000; Asmus et al. 1992; Gobrogge et al. 2007; Leibowitz et al. 1982; Pirnik and Kiss 2005; Wommack and Delville 2002). In the present study, it was hypothesized that alterations in the dopamine system in nuclei involved in aggressive control facilitate aggressive responding in adolescent AAS-treated hamsters. To test this hypothesis, TH-ir elements were quantified in aggressive AAS-treated animals and compared to non-aggressive sesame oil-treated controls.

The current results support the notion that AAS exposure throughout the developmentally critical period of adolescence alters TH expression in the AH, a brain nucleus critically involved in aggressive control in male Syrian hamster. Specifically, adolescent AAS exposure resulted in an increase in TH-ir fibers and somata in different AH-nuclei, with statistically significant increases observed in the NC and a trend towards significance in the mSON, possibly indicating increased dopamine synthesis and terminal density in this brain region. The AH has been shown to be the center of control of aggressive behavior and it functions by integrating inputs from other brain regions implicated in the modulation of aggressive behavior (i.e., LS, MeA, BNST) (Delville et al. 2000). Previous reports on the effects of adolescent AAS exposure have shown alterations in the function and activity of specific neurotransmitter systems such as serotonin, glutamate, and GABA in aggression nuclei connecting to the AH (Fischer et al. 2007; Grimes and Melloni 2002; Grimes et al. 2003). Interestingly, the significant increases in TH-ir elements in the NC and considerable changes measured in the mSON occurred in the absence of changes to TH-ir expression in all other aggression regions connecting the AH. These findings suggest that AAS treatment throughout adolescence alters local AH connections from the NC and mSON to the LAH resulting in the exaggerated aggressive phenotype. Indeed, increased TH-ir elements
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within subregions of the AH directly correlate with increases in aggressive responding despite the absence of AAS-induced alterations in other aggression nuclei measured. Thus, one possible mechanism by which increased AH dopamine expression might stimulate aggressive responding is by locally altering AH function through interactions with other neurotransmitter systems involved in the regulation of offensive aggression. Of particular interest is the coincidence in hypothalamic expression patterns between TH and AVP (Gobrogge et al. 2007; Grimes et al. 2006; Harrison et al. 2000). In various species and behavioral paradigms, including adolescent AAS-treated hamsters, increased AH-AVP activity has been shown to facilitate aggressive responding (Coccaro et al. 1998; Gobrogge et al. 2007; Grimes et al. 2006; Harrison et al. 2000). In fact, highly aggressive AAS-treated hamsters have an increase in the number of AH-AVP fibers compared to non-aggressive controls, which correlates with the temporal onset of aggressive displays (Grimes et al. 2007). In agreement with this notion, blockade of AH-AVP activity using a selective $V_{1A}$ antagonist has been shown to dose-dependently attenuate aggressive responding while microinjections of AVP into the AH stimulates aggressive behavior (e.g., increased number of bites and attacks) in male Syrian hamsters (Ferris et al. 1997; Ferris and Potegal 1988). Further evidence indicates that augmented AH-dopamine activity results in heightened AH-AVP release (Chernigovskaia et al. 2001; Gobrogge et al. 2007; Lindvall et al. 1984; Moos and Richard 1982). From a functional standpoint, AAS-induced increases in dopamine activity, as measured by TH-ir, in specific AH nuclei of highly aggressive animals suggests that one possible mechanism by which increased AH-dopamine activity facilitates aggression is by potentiating AH-AVP function.

The finding that adolescent AAS exposure results in increased AH-TH expression supports a facilitory role for dopamine in aggression. Interestingly, several reports indicate that
3. Dopamine in the Anterior Hypothalamus
the dopamine system facilitates aggression by its action on the G-protein coupled D2-like and D1-like receptor families (Arregui et al. 1993; Bondar and Kudryavtseva 2005; Ferrari and Giuliani 1995; Puglisi-Allegra and Cabib 1988; Ricci et al. 2007a; Sibley et al. 1993). For instance, several D2 receptor antagonists including, spiperone, (-)-sulpiride, eticlopride, risperidone, paliperidone, haloperidol and tiapride have been shown to suppress aggression in a variety of animal models and have been clinically used to diminish aggressive behavior associated with psychological and medical disorders (Arregui et al. 1993; Extein 1980; Ferrari and Giuliani 1995; Navarro and Manzaneque 1997; Puglisi-Allegra and Cabib 1988; Ricci et al. 2007a). Further evidence indicates that D2 and D1-like agonism enhances social fearfulness, defensiveness, subjugation and anxiety behaviors while diminishing dominant and offensive acts (Arregui et al. 1993; Gendreau et al. 1998; Navarro and Manzaneque 1997; Puglisi-Allegra and Cabib 1988; Sweidan et al. 1990; Tidey and Miczek 1992). In conjunction with D2 sensitivity to sex steroids and the localization of D2 and D5 receptors in brain areas involved in aggression control, the aforementioned results suggest that a likely mechanism of dopamine modulation of aggressive behavior would be through its actions at D2 and D5 receptors (Kindlundh et al. 2001; Kindlundh et al. 2003; Sweidan et al. 1991). To address this question, we examined D2 and D5 receptor expression in the AH of aggressive AAS-treated animals and compared it to non-aggressive controls.

The results revealed a significant increase in D2-ir in the AH of AAS-treated animals as compared to controls, while no changes were observed in the expression of D5-ir cell bodies. Specifically, we observed increases in D2-ir somata, suggesting augmented D2 expression in postsynaptic heteroreceptors in the AH. Functionally, increases in AH-D2 expression following chronic adolescent AAS-exposure indicate a facilitory role of dopamine acting through D2
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receptors in aggression, possibly through inhibition of an inhibitory system (e.g., GABA). Interestingly, evidence from electrophysiological recordings have shown that dopamine acting through the D$_2$ receptor family reduces inhibitory postsynaptic currents in AH-GABAergic neurons which synapse onto magnocellular cells (Azdad et al. 2003; Baimoukhametova et al. 2004). Accordingly, 40% of the total synapses onto magnocellular neurons have been shown to be GABAergic and both dopamine and GABA-mediated modulation of magnocellular neuronal activity have been found to regulate neuropeptide release, including AVP (Azdad et al. 2003; Forsling and Williams 1984; Gies and Theodosis 1994; Kimura et al. 1981; Knepel et al. 1980). From an anatomical standpoint, a very interesting observation in this report indicates that changes in D$_2$ expression occurred in a specific subregion of the AH, the lateral anterior hypothalamus (LAH). Additionally, the staining pattern of D$_5$-ir cell bodies appeared densely labeled through the LAH with more sparse staining observed in other regions of the AH. The LAH is located between the NC and mSON and thus it receives local afferent innervations from both of these hypothalamic nuclei, suggesting that the LAH is locally regulated. Given that the LAH is located between the NC and the mSON and that increases in D$_2$ expression occurred only in this subregion, it is likely that the LAH is an important hypothalamic subregion critical in AAS-facilitated aggressive responding.

In summary, these studies examined the role of the dopamine system in AAS-induced offensive aggression. Results revealed an increase in dopamine activity and alterations in D$_2$ receptor, but not D$_5$ receptor, localization in the AH of aggressive AAS-treated hamsters compared to controls. These results support an overall facilitory role of dopamine in aggression and indicate possible neuroplastic events by which chronic adolescent AAS exposure might facilitate aggressive responses. In addition, from a systems standpoint, the current results provide
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Further evidence supporting the role of the AH, and more specifically the LAH, as a key brain region involved in the regulation of adolescent AAS-induced offensive aggressive behavior.
Chapter 4

Behavioral Pharmacology

4.1 Introduction

In the AH, the dopaminergic neural system undergoes alterations after repeated exposure to AAS producing elevated aggression. Previously, systemic administration of selective dopamine receptor antagonists has reduced aggression in various species and animal models. However, these studies utilized systemic administration of drugs and reported concomitant changes in mobility. These data complicate the question of whether pharmacology targeting D_2 receptors is specific to aggression or whether they exert their anti-aggressive effects through nonspecific mechanisms. Therefore, in order to control for these systemic effects, the current studies utilized microinjection techniques to determine the effects of local antagonism of D_2 and D_3 receptors in the LAH on adolescent AAS-induced aggression. Male Syrian hamsters were treated with AAS throughout adolescence and tested for aggression after local infusion of the D_2 antagonist eticlopride, or the D_3 antagonist SCH-23390, into the LAH. Treatment with eticlopride showed dose-dependent suppression of aggressive behavior in the absence of changes in mobility. Conversely, while injection of SCH-23390 suppressed aggressive behavior, these reductions were met with alterations in social interest and locomotor behavior. These findings
4. Behavioral Pharmacology

identify a neuroanatomical locus where dopamine receptor antagonism suppresses adolescent AAS-induced aggression in the absence of alterations to general mobility.

4.2 Results

SCH-23390

There were twenty-five animals with correctly placed AH cannula included in the final analysis (0.0µg: n = 9; 0.01µg: n = 5; 0.1µg: n = 6; 1.0µg: n = 6) (Figure 6). Those animals treated with SCH-23390 outside the AH showed no statistically significant difference in AAS-induced aggression, F(3, 14) = 2.3, p > 0.1, and were excluded from behavioral analysis.

Local infusion of the D₅ receptor antagonist SCH-23390 into the LAH produced an overall decrease in offensive aggression as measured by composite aggression score, F(3, 22) = 5.48, p < 0.01. Post hoc analysis revealed that both the low (0.01µg) and moderate (0.1µg) doses of SCH-23390 failed to alter aggression compared to AAS-treated animals administered saline on test day [0.01µg, t(12) = -0.89, p > 0.3; 0.1µg, t(13) = -0.23, p > 0.5]. Only the highest dose (1.0µg) of the D₅ receptor antagonist reduced aggressive behavior compared to controls, t(13) = 3.19, p < 0.01. At this high dose, animals displayed a 4-fold reduction in aggression compared to AAS-treated animals administered saline into the LAH (Figure 7). However, these reductions in aggression occurred with concomitant alterations to locomotion and social interest. Indeed, local administration of the D₁-like receptor antagonist produced a significant main effect on the number of line crosses, F(3, 20) = 3.10, p < 0.05. While the low and moderate doses of SCH-23390 had no effect on locomotor activity, [0.01µg, t(12) = -1.37, p > 0.1; 0.1µg, t(13) = 0.21, p > 0.5], the aggression suppressing high dose resulted in a significant decrease in line crosses, t(13) = 2.11, p < 0.05. In fact, hamsters treated with 1.0µg of SCH-23390 made fewer than half
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as many line crosses when compared to saline-treated AAS controls (Table 1). Similarly, LAH injection of the D₁-like receptor antagonist altered non-aggression related interactions with a significant main effect observed in the total contact time spent between resident and intruder, F(3, 20) = 4.37, p < 0.05. Animals administered the highest dose of SCH-23390 spent significantly less time interacting with the intruder during the 10-minute period, t(13) = 3.06, p < 0.01, while the moderate and low doses had no effect on total contact time [0.01µg, t(12) = -0.6, p > 0.5; 0.1µg, t(13) = 0.1, p > 0.5].
Figure 6. Schematic (adapted from Morin and Wood 2001) and representative photomicrograph of a coronal section of the Syrian hamster brain showing the site of central administration of eticlolpride and SCH-23390 into the lateral anterior hypothalamus. Note in the photomicrograph (from the dorsal to ventral axis), the track of the guide-cannula and the track of the injection needle (arrows). 3v: third ventricle; AH: anterior hypothalamus; LAH: lateral anterior hypothalamus; oc: optic chiasm.
Figure 7. Composite aggression score of AAS-treated animals after injection of the D₅ receptor antagonist SCH-23390 (0.1µg-1.0µg/0.5µl) in the lateral anterior hypothalamus. Only the highest dose of SCH-23390 suppressed AAS-induced aggression when compared to steroid treated animals administered saline.
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Table 1. Effects of microinfusion of SCH-23390 into the AH of adolescent AAS-treated hamsters on locomotor and social interest. Data represents mean values with standard deviation.

<table>
<thead>
<tr>
<th>Behavioral Category</th>
<th>Dose (µg/0.5µl)</th>
<th>Probability associated with One-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.00</td>
<td>0.01</td>
</tr>
<tr>
<td>Contact Time</td>
<td>469 (73)</td>
<td>497 (58)</td>
</tr>
<tr>
<td>Line Crosses</td>
<td>47 (26)</td>
<td>54 (13)</td>
</tr>
</tbody>
</table>

* Differs from AAS-treated controls (0.0µg dose), p < 0.05
** Differs from AAS-treated controls (0.0µg dose), p < 0.01
4. Behavioral Pharmacology

Eticlopride

Histological Verification

In Experiment 1, twenty-three animals with correct cannula placements in the LAH were included in the behavioral analysis (0.0µg: n = 6; 0.1µg: n = 7; 1.0µg: n = 6; 10.0µg: n = 6). Some animals were injected outside the LAH (0.0µg: n = 4; 0.1µg: n = 3; 1.0µg: n = 4; 10.0µg: n = 4) with cannulas placed rostral or caudal to the AH. In Experiment 2, twenty-four animals were included in the behavioral analysis (0.0µg: n = 6; 0.01µg: n = 8; 1.0µg: n = 10) with 6 animals excluded due to placements outside the LAH (0.0µg: n = 4; 0.01µg: n = 2). Treatment with eticlopride outside the LAH had no statistically significant effect on AAS-induced aggression in either experiment [Experiment 1: t(21) = 1.9, p > 0.05; Experiment 2: t(10) = -0.9, p > 0.05].

Experiment 1

Injection of eticlopride into the LAH produced an overall effect on offensive aggression, F(3, 19) = 5.44, p < 0.01, with an effective dose beginning at 0.1µg. At this dose, eticlopride treatment significantly decreased composite aggression, t(11) = 2.62, p < 0.05, when compared to aggressive AAS-treated hamsters injected with saline into the AH. Specifically, infusion of eticlopride into the LAH produced a three-fold reduction in the frequency of aggressive behaviors during the 10-minute period (Figure 8). Similarly, eticlopride significantly reduced composite aggression scores for all other doses of eticlopride: 1.0µg, t(9) = 3.07, p < 0.01; 10.0µg, t(9) = 3.69, p < 0.01. These reductions in aggression were similar to that of non-aggressive animals administered vehicle alone throughout adolescence (data not shown) (Melloni and Ricci 2010). Further examination of aggressive behaviors showed significant reductions in the frequency of lateral attacks, F(3, 19) = 6.56, p < 0.01, upright offensive behaviors F(3, 19) =
4. Behavioral Pharmacology

3.424, p < 0.05, and the number of bites, F(3, 19) = 3.52, p < 0.05, in the 10-minute period. In fact, all doses of eticlopride reduced the number of lateral attacks [0.1µg, t(11) = 3.22, p < 0.01; 1.0µg, t(10) = 3.25, p < 0.01; 10.0µg, t(10) = 4.10, p < 0.01] and bites [0.1µg, t(11) = 2.20, p < 0.05; 1.0µg, t(10) = 2.91, p < 0.01; 10.0µg, t(10) = 2.55, p < 0.05] compared to AAS-treated animals injected with saline into the LAH. Interestingly, injection of eticlopride only altered upright offensive behaviors at the highest two doses tested, [1.0µg, t(10) = 2.33, p < 0.05; 10.0µg, t(10) = 3.01, p < 0.01] (Figure 9).
4. Behavioral Pharmacology

**Figure 8.** Composite aggression score of AAS-treated animals after injection of eticlopride (0.1µg-10.0µg/0.5µl) in the lateral anterior hypothalamus. All three doses of eticlopride significantly reduced AAS-induced aggressive behaviors producing a basement effect. * $p < 0.05$, ** $p < 0.01$ compared to AAS-treated hamsters injected with saline into the AH on test day.
Figure 9. Effects of injection of eticlopride (0.1µg-10.0µg/0.5µl) into the lateral anterior hypothalamus on the frequency of individual aggressive behaviors (i.e. upright offensive, lateral attack, chases, and bites) of adolescent AAS-treated hamsters. * $p < 0.05$, ** $p < 0.01$ compared to AAS-treated hamsters injected with saline into the LAH on test day.
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To assess any non-specific effects of LAH injections of eticlopride, animals were scored for non-aggressive behaviors during the 10-minute resident-intruder test. Interestingly, D₂ antagonism in the LAH produced a significant main effect on grooming behavior, F(3, 15) = 6.63, p < 0.01. Post hoc analysis revealed that while the 0.1µg dose had no effect on grooming behavior, t(11) = -0.91, p > 0.05, both the 1.0µg and 10.0µg dose resulted in a significant increase in the frequency of grooming when compared to AAS-treated animals injected with saline on test day: 1.0µg, t(9) = 2.79, p < 0.05; 10.0µg, t(9) = 4.02, p < 0.01. In fact, animals treated with 1.0µg and 10.0µg of eticlopride had a four- to five-fold increase in grooming frequency when compared to non-eticlopride treated AAS controls (Table 2). Finally, eticlopride failed to produce a significant effect on the frequency of sniffing, F(3, 15) = 0.28, p > 0.84, and wall climbing, F(3, 15) = 1.64, p > 0.22 (Table 2). Given the known effects of dopamine activity on mobility, animals were measured for locomotion during the 10-minute agonistic encounter. At all three doses (i.e. 0.1µg, 1.0µg, 10.0µg), eticlopride failed to produce significant changes in the number of line crosses, F(3, 15) = 1.64, p > 0.22 (Table 2).

Experiment 2

In a second study, a narrower dose range, including a replication of the effective dose from the first study (i.e. 0.01µg to 0.1µg), was conducted where animals were scored for offensive aggression to identify dose-dependent reductions in specific and targeted behaviors associated with the aggressive response. In agreement with the previous study, local infusion of eticlopride into the LAH produced an overall decrease in offensive aggression as measured by composite aggression score, F(3, 15) = 5.68, p < 0.05. Specifically, while AAS-treated animals injected with 0.1µg showed a significant reduction in composite aggression, t(14) = 3.07, p <
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0.01, hamsters administered the lower dose (i.e. 0.01µg) failed to show reductions in composite aggression $t(11) = -0.61, p > 0.05$, demonstrating a dose-dependent effect of eticlopride on aggression. To identify changes in specific aggressive behaviors post eticlopride administration, individual behaviors were broken down and examined across this dose range. Interestingly, injection of eticlopride into the LAH failed to reduce the number of upright offensive attacks at either dose, $F(2, 22) = 1.93, p > 0.17$. Conversely, blockade of D$_2$ receptors in the LAH produced an overall effect on all other aggression behaviors measured including the frequency of lateral attacks, $F(2, 22) = 4.27, p < 0.05$; chases, $F(2, 22) = 5.57, p < 0.05$; and bites, $F(2, 22) = 4.63, p < 0.05$. Post hoc analysis revealed that while the 0.01µg dose had no effect on aggression specific behaviors in a 10-minute period, the 0.1µg dose significantly reduced the number of lateral attacks, $t(14) = 2.41, p < 0.05$, chases $t(14) = 3.26, p < 0.01$, and bites $t(14) = 2.91, p < 0.01$ toward intruders (Figure 10). Lastly, neither dose tested altered the time a resident spent with an intruder (i.e. total contact time), $F(2, 22) = 0.52, p > 0.5$, nor any other non-aggression related behaviors measured: sniffing, $F(2, 22) = 0.63, p > 0.55$; grooming $F(2, 22) = 1.05, p > 0.37$; wall climbing $F(2, 22) = 0.52, p > 0.6$. 


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Table 2. Effects of microinfusion of eticlopride into the AH of adolescent AAS-treated hamsters on non-aggression related behaviors. Data represents mean values with standard error of the mean.

<table>
<thead>
<tr>
<th>Behavioral Category</th>
<th>Dose (µg/0.5µl)</th>
<th>Probability associated with One-way ANOVA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>Contact Time</td>
<td>538 (21.0)</td>
<td>480 (29.1)</td>
</tr>
<tr>
<td>Sniffing</td>
<td>9.2 (3.7)</td>
<td>9.7 (2.0)</td>
</tr>
<tr>
<td>Grooming</td>
<td>1.5 (0.6)</td>
<td>2.6 (0.6)</td>
</tr>
<tr>
<td>Wall Climes</td>
<td>8.7 (2.9)</td>
<td>11.6 (4.3)</td>
</tr>
<tr>
<td>Line Crosses</td>
<td>68 (14.4)</td>
<td>35.9 (9.3)</td>
</tr>
</tbody>
</table>

*Differs from AAS-treated controls (0.0µg dose), p < 0.05
**Differs from AAS-treated controls (0.0µg dose), p < 0.01
+Differs from 0.1µg dose, p < 0.05
++Differs from 0.1µg dose, p < 0.01
Figure 10. Broken down aggressive behavior from a second study using a narrower dose range and including a replication of the effective dose. The lowest dose of eticlopride (0.01µg) failed to alter any measure of aggression. However, consistent with the findings from the first microinjection study, injection of 0.1µg of eticlopride into the LAH reduced the frequency of lateral attacks, chases and bites while having no effect on upright offensive behaviors of AAS-treated hamsters. Dashed line indicates non-aggressive sesame-oil treated control. * $p < 0.05$, ** $p < 0.01$ compared to AAS-treated hamsters injected with saline into the LAH on test day.
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4.3 Discussion

Previous studies investigating the role of dopamine and various dopaminergic receptors in the control of aggression have resulted in varied and contradictory findings given that many of the pharmacological agents used have limited specificity to individual dopamine receptors (Bourne 2001; Hardman et al. 2001). Moreover, studies using systemic drug treatments report large variation in aggression-specificity as these agents disrupt normal striatal functions producing a myriad of extrapyramidal and locomotor side effects. To control for these variables, the present studies examined the role of hypothalamic dopamine in adolescent AAS-induced aggression utilizing local infusion techniques to limit drug/receptor interactions. We report that eticlopride, but not SCH-23390, selectively modulated AAS-induced aggression while leaving motor and other non-aggressive social behaviors unaltered.

Dopaminergic activity in the central nervous system is critical for the control of aggression across vertebrate species (Miczek et al. 2002) as systemic increases in dopamine and non-selective activation of dopaminergic receptors facilitate aggressive behaviors (Maeda et al. 1985; Sato and Wada 1974; Sweidan et al. 1990; 1991). In the AH, activation of dopamine receptors using the non-selective agonist apomorphine increases aggressive reactions supporting a local effect of dopamine on aggression control (Sweidan et al. 1991). The hypothalamus is densely innervated by dopaminergic projections and expresses high amount of excitatory D₅ and inhibitory D₂ receptors (Meador-Woodruff 1994; Meador-Woodruff et al. 1989; Zhou et al. 1999). In the Syrian hamster, the organization and expression of the hypothalamic neural system appears to be androgen sensitive and is reported to undergo alterations after adolescent exposure to AAS (for review see Melloni and Ricci 2010). Specifically, treatment with moderate doses of AAS throughout the developmentally sensitive period of adolescence increases both the
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dopaminergic innervation and the expression of D\textsubscript{2} receptors within the LAH (Ricci et al. 2009; Schwartzer et al. 2009b). This increase in the density of dopamine afferents correlates with the elevated aggressive response (Ricci et al. 2009). It is hypothesized that this increase in LAH dopamine facilitates aggression through stimulation of inhibitory D\textsubscript{2} receptors. Evidence supporting this notion stems from our finding that local blockade of D\textsubscript{2} receptors using the selective antagonist eticlopride dose-dependently decreased AAS-induced aggression. This decrease occurred in the absence of changes in motor activity and overall arousal in social interactions. Reductions in the frequency and intensity of aggressive responding in the absence of changes to non-aggression related behaviors indicate the specificity of D\textsubscript{2} receptor activity in the LAH to control aggression. Moreover, the aggression suppressing effects of eticlopride were ineffective when injected into nuclei rostral and caudal to the LAH, further supporting the notion that this brain region, and specifically dopamine within this region, modulates AAS-induced aggression.

Adolescence marks a critical developmental period where pharmacological treatment produces differential behavioral responses compared to adults. For example, adolescent rats show decreased sensitivity to catecholaminergic agonists but increased responsiveness to antagonists when compared to younger or older rats (Spear and Brake 1983). In the Syrian hamster, adolescent animals transition from juvenile displays of play fighting to the development of mature adult aggression behaviors (Wommack et al. 2003). In our animal model of adolescent AAS-induced aggression, animals display adult forms of aggression in the absence of social learning demonstrating that this treatment regimen circumvents the learning of mature fighting behavior. Here, the analysis of the effects of eticlopride on individual types of aggressive acts revealed a dose-dependent suppression of specific aggressive behaviors characterized as mature
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forms of aggression (Pellis and Pellis 1988). Conversely, D₂ antagonism only reduced the number of upright offensive behaviors at the highest doses tested (i.e. 1.0 and 10.0µg). Unlike the other aggressive behaviors measured, upright offensive postures precede both juvenile and mature bouts of aggression. Studies employing systemic activation and antagonism of D₂ receptors argue against a specific role for dopamine in the modulation of playfulness (Siviy et al. 1996), suggesting that the dopamine system may not be fully developed in the aggression circuit of juvenile hamsters. Thus it is not surprising that lower doses of eticlopride failed to reduce upright offensive postures given that this behavioral act may represent a transition from juvenile play to more serious adult displays of aggression. A more in-depth understanding of the development of the dopamine system and how adolescent AAS-exposure alters normal development in the LAH is thus warranted.

Previously, research examining the role of dopamine in aggression used systemic administration of D₂ receptor drugs to measure changes in aggressive responding. However, given the dense distribution of D₂ receptors in the dorsal striatum, systemic drug administration is often reported with concomitant decreases in mobility (Arregui et al. 1993; Navarro and Manzaneque 1997; Navarro et al. 1993). Our findings serve as the first behavioral measure of direct D₂ receptor blockade in an aggression specific locus of the AAS-treated brain. Therefore, reduced aggression was observed in the absence of altered locomotor activity. Importantly, measures of locomotor activity were observed during the 10-minute agonistic encounter. While it is possible that the resident’s level of activity may be driven, in part, by the activity of the intruder, any pharmacologically-induced alterations in extrapyramidal function and motor control would still be observable during the agonistic encounter as previously reported (Arregui et al. 1993). Interestingly, high doses of eticlopride dose-dependently increased grooming
behavior. This behavioral effect may be due to the interaction between AAS exposure and eticlopride or a direct effect of D<sub>2</sub> antagonism into the LAH. Indeed, dopamine regulates various neuroendocrine functions within the hypothalamic-pituitary axis. For example, in addition to modulating aggression, hypothalamic dopamine inhibits prolactin release through activation of inhibitory D<sub>2</sub> receptors (Fitzgerald and Dinan 2008) and these changes in prolactin result in increased grooming behavior in rodents (Drago and Lissandrello 2000). In the current study, high doses of eticlopride infused into the LAH increased grooming behavior suggesting that at these doses eticlopride likely altered other hypothalamic-mediated behaviors. Speculation aside, further research on the effects of D<sub>2</sub> antagonists of non-AAS treated animals is warranted to better understand the effects of these compounds on hypothalamic related behaviors. It is important to note that increases in grooming were only observed in the highest doses tested; that is, the lowest aggression-suppressing dose (0.1µg) failed to alter grooming behavior. Therefore administration of the effective dose of eticlopride when administered into the LAH will reduce AAS-induced aggression in the absence of changes to other hypothalamic-mediated behaviors. These findings notwithstanding, there are multiple dopaminergic receptor subtypes expressed within the LAH, so the finding that D<sub>2</sub> antagonists suppress AAS-induced aggression may only be one part of a larger dopamine mechanism within the LAH.

In addition to D<sub>2</sub> receptors, the hypothalamus is densely populated with excitatory D<sub>5</sub> receptors (Meador-Woodruff 1994; Zhou et al. 1999). It was hypothesized that increased dopamine innervation to the LAH after adolescent AAS-exposure may also facilitate aggression by increasing hypothalamic activity through activation of D<sub>5</sub> receptors. In previous studies, systemic antagonism of excitatory D<sub>1</sub>/D<sub>5</sub> receptors reduced aggression across species and animal models (Arregui et al. 1993; Bondar and Kudryavtseva 2005; Gendreau et al. 1997; Miczek et al. 2003).
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1994; Nikulina and Kapralova 1992; Rodriguez-Arias et al. 1998). However these studies were limited in clear interpretation as they produced results confounded by concomitant alterations in mobility. Moreover, the inability of current pharmacology to selectively bind either only D₁ or D₅ receptors further reduces our ability to determine which dopamine receptors are specific to aggression control. Here we suggest that these possible confounds were eliminated with the use of local drug administration techniques. Given that the hypothalamus only expresses high levels of D₅ and very low levels of D₁, local infusion of the D₁/D₅ antagonist SCH-23390 site specifically targeted D₅ receptors while leaving striatal D₁ receptors unaltered (Palermo-Neto 1997). Interestingly, only the highest dose of SCH-23390 tested suppressed AAS-induced aggression. At this high dose, animals displayed significantly decreased locomotor activity, as measured by the number of line crosses in a 10-minute period, and reductions in the total time spent interacting with a novel intruder. Therefore, the observed reductions in AAS-induced aggressive behavior are more likely explained by non-specific drug effects and suggest that LAH-D₅ receptors do not directly modulate AAS-induced aggression. The AH is involved in mediating various hormonal and behavioral responses including thermoregulation, sexual behavior, and neuroendocrine function (Cox and Lee 1977; Hull et al. 1986; MacKenzie et al. 1984). Thus, a reduction in aggression after local D₅ receptor blockade may reflect a decrease in overall arousal brought about by disruption to other hypothalamic-mediated processes.

Taking together the findings from pharmacological manipulations of LAH D₂ and D₅ receptors, it is hypothesized that D₂, but not D₅ receptors, modulate AAS-induced aggression. That is, adolescent exposure to AAS increases dopaminergic tone within the LAH resulting in increased activation of D₂ receptors. This notion is consistent with previous reports investigating hypothalamic control of aggression in felines. Increased aggressive reaction elicited by infusion
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of apomorphine into the AH can be suppressed by local injection of sulpiride, a D₂ specific antagonist, but not SCH-23390 (Sweidan et al. 1991). Similarly, cats showed increased aggression when D₂, but not D₅, receptors were activated by AH administration of selective agonists (Sweidan et al. 1991). While these reports and the findings from our pharmacology studies would suggest that LAH D₅ receptors are not implicated in the regulation of AAS-induced aggression, it is possible that their function may still be important, albeit difficult to discern. For this reason, it is important to consider the neuroanatomical localization of D₂ and D₅ receptors within the LAH and how they may be altered in adolescent AAS-treated animals to more completely implicate or eliminate these receptors in the control of AAS-induced aggression.
Chapter 5

Immunofluorescence Colocalization

5.1 Introduction

Both the GABAergic and dopaminergic neural systems are implicated in aggression control and are altered in the presence of AAS. Therefore, to elucidate a plausible mechanism whereby dopamine modulates local GABA interneurons, the following studies provide a detailed report of the interaction between D\(_2\) and D\(_5\) receptors and GABAergic neurons in the LAH, a brain region at the center of aggression control. Given the similarity in staining pattern of D\(_2\) and D\(_5\) receptors compared to GAD\(_{67}\) in the LAH (Figure 11), it was hypothesized that local GABAergic interneurons express D\(_2\) and/or D\(_5\) receptors in the LAH and that changes in the expression pattern of these receptors after adolescent AAS-exposure produces the elevated aggressive response. To test this notion, male Syrian hamsters were administered AAS throughout adolescence and their brains were processed for double-label immunofluorescence of GAD\(_{67}\) and D\(_2\) or D\(_5\) receptors. The results suggest an increase in the number of D\(_2\)-ir and GAD\(_{67}\)-ir cells in the LAH of AAS-treated animals. Although several cells in the LAH colocalized with both GAD\(_{67}\) and D\(_2\) receptors, there were no significant increases in the number
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of double-labeled GAD<sub>67</sub>/D<sub>2</sub>-ir neurons. Interestingly, there was sparse labeling of GAD<sub>67</sub> neurons colocalized with D<sub>5</sub> receptors in the LAH. While these data suggest the possibility of multiple GABAergic systems in the LAH allowing for differential inhibition of various neural systems, only one of these systems is modulated by dopamine through inhibitory D<sub>2</sub> receptors. These findings, taken together with pharmacology results from the previous chapter, indicate that the population of neurons expressing D<sub>5</sub> receptors in the LAH modulates non-GABAergic pathways that indirectly influence aggression control.
5. Immunofluorescence Colocalization

**Figure 11.** Photomicrographs depicting the staining pattern of dopamine D$_2$ and D$_5$ receptors (top) compared to GAD$_{67}$ (bottom) in the LAH. Both D$_2$ and D$_5$ receptors are localized to the LAH and conserved in a compact region of the LAH. These receptors appear in a staining pattern similar to GAD$_{67}$ suggesting that GABAergic neurons in the LAH are modulated by dopamine through expression of D$_2$ and D$_5$ receptors.
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5.2 Results

**D2 and GAD$_{67}$**

Consistent with previous findings (Ricci et al. 2009), AAS-treated animals exhibited increased D$_2$ containing cell bodies in the LAH compared to sesame oil-treated controls (Figure 12A). This increase was statistically significant [$t(9) = 2.13$, $p < 0.05$] (Figure 12A). In fact, adolescent AAS exposure produced a 2-fold increase in the number of cells positively stained for D$_2$-ir. Interestingly, AAS treatment produced a significant increase in the number of GAD$_{67}$ labeled cell bodies in the LAH [$t(9) = 2.83$, $p < 0.01$] compared to vehicle-treated littermates (Figure 12B). Animals exposed to AAS were observed to have greater than twice the number of GAD$_{67}$ containing cells compared to sesame oil-treated animals. In contrast, while a large number of cells in the LAH were colocalized with GAD$_{67}$-ir and D$_2$-ir elements (Figure 13), AAS exposure failed to produce significant changes in the number of double-labeled cell bodies in the LAH [$t(9) = 1.60$, $p > 0.05$] (Figure 12C).

**D$_5$ and GAD$_{67}$**

In agreement with previously published findings (Schwartz et al, 2009b), AAS-treated animals exhibited an increased number of cell bodies expressing GAD$_{67}$ in the LAH compared to sesame oil-treated controls (Figure 14). This increase was statistically significant, $t(10) = 2.76$, $p < 0.05$. In contrast, AAS treatment had no effect on the number of cells positively labeled for D$_5$ receptors in the LAH [$t(10) = 0.95$, $p > 0.5$] compared to vehicle-treated littermates (Figure 14). Interestingly, few cells were positively labeled for both GAD$_{67}$ and D$_5$ receptors indicating limited colocalization of these two proteins (Figure 15). This colocalized population showed no
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changes in the number of cells co-expressing GAD$_{67}$ and D$_5$ receptors between AAS and oil treated controls, $t(10) = -0.51, p > 0.05$] (Figure 14).
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Figure 12. Treatment with AAS throughout adolescence altered D<sub>2</sub> and GAD<sub>67</sub> immunofluorescent staining in the LAH. (A) Animals treated with AAS showed a 2-fold increase in the number of cells containing D<sub>2</sub> receptors. (B) Adolescent AAS exposure significantly increased the number of GABAergic neurons (i.e. GAD<sub>67</sub>). (C) AAS treatment failed to produce significant changes in the number of double-labeled D<sub>2</sub>/GAD<sub>67</sub> cells in the LAH. Bars denote S.E.M. *p<0.05; **p<0.01: Student’s t-test, two-tailed (n = 5-6/group).
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Figure 13. Darkfield photomicrographs of a coronal section through the LAH at 20x magnification. Shown are cells positively labeled with D₂ receptors (red), GAD₆₇ (green) and double-labeled D₂/GAD₆₇ cells (yellow) of sesame oil and AAS treated hamsters.
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![Graph showing immunofluorescence staining in the LAH.](image)

**Figure 14.** Treatment with AAS throughout adolescence altered GAD\textsubscript{67} but not D\textsubscript{5} immunofluorescent staining in the LAH. Animals treated with AAS showed a significant increase in the number of cells positively labeled for GAD\textsubscript{67}. However no changes were observed in the number of cells expressing D\textsubscript{5} receptors between AAS and sesame oil controls. Interestingly, few neurons were positively labeled with both GAD\textsubscript{67} and D\textsubscript{5} receptors and were unaltered by AAS-treatment. \(*p<0.05;\) Student’s \(t\)-test, two-tailed (n = 6/group).
5. Immunofluorescence Colocalization

Figure 15. Darkfield photomicrographs of a coronal section through the LAH. Shown are cells positively labeled with GAD$_{67}$ (green), D$_5$ receptors (red), and double-labeled GAD$_{67}$/D$_5$ cells (overlay) of sesame oil and AAS-treated hamsters.
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5.3 Discussion

Chronic exposure to AAS throughout adolescence produces activational and functional alterations in the brain resulting in a heightened aggressive response in Syrian hamsters (Carrillo et al. 2009; Grimes et al. 2003; Ricci et al. 2009). For example, adolescent AAS treatment produces alterations in GABA synthesis (i.e. GAD$_{65}$) in various brain regions implicated in the control of aggression, including the AH (Grimes et al. 2003). In agreement with these reported increases in GABA terminals (i.e. GAD$_{65}$), the studies reported herein suggest significant increases in the number of GABAergic cells in the LAH (i.e. GAD$_{67}$), a brain region at the center of aggression control (Delville et al. 2000; Ferris et al. 1997). This observed increase in GABA enzyme production suggests an increase in inhibition in the LAH producing an altered behavioral response. However, there are conflicting reports regarding the role of GABA in the control of aggression. For example, pharmacological manipulation of the GABAergic system revealed that activation of GABA$_A$ receptors through intraventricular injection of a GABA$_A$ agonist resulted in increased attack behavior while antagonism decreased the number of attacks in rats confronted in a neutral arena (Depaulis and Vergnes 1985). Conversely, Liu et al. reported an increase in the number of bite attacks in mice after administration of bicuculline, a GABA$_A$ antagonist (Liu et al. 2007). Additionally, increases in extracellular GABA through blockade of GABA transporter 1 (GAT1) was reported to decrease the number of bites (Liu et al. 2007). Given these reported differences in GABA-mediated increases and decreases in the aggressive response, it is possible that there are multiple GABAergic pathways modulating both inhibitory (e.g. serotonin) and excitatory (e.g. AVP) neurotransmitter systems implicated in aggression control (Ferris et al. 1997; Grimes and Melloni 2002; Melloni et al. 1997b). Studies linking GABA neural systems to both serotonin and AVP activity support this hypothesis as local infusion of various GABA$_A$
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antagonists altered AVP and serotonin release in the hypothalamus (Ghuman et al. 2007; Zagrodzka et al. 2000). However, changes in hypothalamic AVP, not serotonin, correlate with the temporal onset of AAS-induced aggression (Grimes et al. 2007). To date, it is unclear what regulates these reported differences in GABA mediated inhibition and how these GABAergic neurons functionally reorganize in the presence of AAS to facilitate the expression of the elevated aggressive phenotype. One putative mechanism for the altered GABAergic system is through altered dopamine signaling. Dopamine neurons often synapse on medium-spiny GABAergic neurons (Guzman et al. 2003; Pickel et al. 1996) and are regulated by both D1-like and D2-like receptors (Guzman et al. 2003).

Dopamine activity is correlated with increased aggression and altered in the LAH after adolescent exposure to AAS (Ricci et al. 2009). In agreement with previous findings from our laboratory (Ricci et al. 2009), these studies report that AAS exposure produced a significant increase in D2-ir elements in the LAH. Interestingly, blockade of D2 with various receptor antagonists suppresses aggression in several animal models (Arregui et al. 1993; Rodriguez-Arias et al. 1998). Given the inhibitory nature of D2 receptors (Bunzow et al. 1988), and that blockade of these receptors results in decreased aggression (Arregui et al. 1993; Rodriguez-Arias et al. 1998; Rodriguez-Arias et al. 1999), it is likely that D2 receptors in our animal model work through an indirect inhibitory mechanism in the control of aggression (e.g. GABA). Evidence from electrophysiological recordings demonstrates that dopamine, acting through the D2 receptor family, reduces inhibitory postsynaptic currents in AH-GABAergic neurons that synapse onto magnocellular cells (Azdad et al. 2003; Baimoukhametova et al. 2004). D2 receptors were colocalized to GAD67-ir neurons in the LAH suggesting that anterior hypothalamic dopamine inhibits LAH-GABA release through activation of inhibitory D2 receptors. Considering our
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results reporting AAS-induced increases in both GAD<sub>67</sub> and D<sub>2</sub> positive cell bodies, and the large number of cells in the LAH co-expressing GAD<sub>67</sub>/D<sub>2</sub>-ir, it was expected that AAS treatment would produce a significant increase in the number of double-labeled cells in the LAH. However, the data failed to demonstrate AAS-induced changes in the number of double-labeled neurons. One possible explanation is the existence of different populations of GABAergic neurons in the LAH. Specifically, while AAS increases the number of GABAergic neurons in the LAH, this increase occurs in a population of GABA neurons that do not express D<sub>2</sub> receptors. Based on conflicting reports of GABA both facilitating and inhibiting aggression (de Almeida et al. 2005; Depaulis and Vergnes 1985; Liu et al. 2007; Miczek et al. 2003), it is possible a secondary GABAergic pathway exists in the LAH similar to that proposed in other brain regions (Alexander and Crutcher 1990). For example, in the striatum, two GABAergic pathways are differentially modulated by dopamine through D<sub>1</sub> and D<sub>2</sub> receptors whereby dopamine release results in decreased activation of D<sub>2</sub>-positive GABA cells (indirect pathway) and increased activation of D<sub>1</sub>-positive GABA cells (direct pathway) (Alexander and Crutcher 1990; Gerfen et al. 1990). Similarly, changes in the number of GAD<sub>67</sub> cells in the absence of increased colocalization with D<sub>2</sub> receptors in the LAH suggests that adolescent AAS exposure may functionally reorganize the GABAergic system. These findings notwithstanding, it is clear that adolescent exposure to AAS produces complex changes to the GABAergic system.

In the Syrian hamster, aggression is modulated, in part, by the interaction between excitatory AVP and inhibitory serotonin (Ferris et al. 1997). When exposed to AAS throughout adolescence, AVP tone is increased and serotonin afferents are diminished producing the elevated aggressive response (Melloni and Ricci 2010). While dopaminergic innervation to the LAH is postulated to modulate both AVP and serotonin release (Melloni and Ricci 2010;
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Schwartzer et al. 2009b), the exact mechanisms remain unknown. Given that a subpopulation of these GABA neurons express D₂ receptors but remain unaltered after adolescent AAS treatment, it is likely that a second GABAergic system, (i.e. a non-D₂ containing population) increases in the LAH to produce the elevated aggressive response. It was hypothesized that this second population of GABA neurons express excitatory D₅ receptors and also modulate AAS-induced aggression. In this model, AH dopamine release modulates both excitation (i.e. D₅) and inhibition (i.e. D₂) of discrete GABA populations allowing for differential regulation of inhibitory (e.g. serotonin) and excitatory (e.g. AVP) inputs, respectively. Indeed, activation of GABA_A receptors and increased levels of extracellular GABA produce both inhibitory and excitatory effects on aggression (Depaulis and Vergnes 1985; Liu et al. 2007; Miczek et al. 2002), demonstrating a dual role for GABA. More specifically, dopamine activation of D₂ receptors would disinhibit LAH activity by suppressing GABA release onto aggression stimulating AVP while activation of D₅ receptors would increase GABA inhibition of inhibitory serotonin afferents. If this model were correct, then pharmacological manipulation of AH D₅ receptors would likely not effect aggressive behavior given that the development of the serotonin system is attenuated in AAS treated animals, rendering the system non-functional in suppressing the release of AVP (Melloni and Ricci 2010). Thus, the finding from the previous chapter that local administration of the D₅ receptor antagonist SCH-23390 failed to suppress AAS-induced aggression may be indicative of a weakened neural pathway that cannot be restored. To determine whether these LAH GABAergic neurons express D₅ receptors, brains from AAS and sesame oil treated controls were processed for double-label immunofluorescence.

Consistent with previously published results, adolescent exposure to AAS resulted in a significant increase in the number of GABAergic neurons in the LAH (Schwartzer et al. 2009b).
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However, no changes were reported in the number of cells expressing D₅ receptors. Moreover, analysis of the number of colocalized GABA neurons expressing D₅ receptors produced results inconsistent with our hypothesis. In fact, few neurons were colocalized with both GAD₆₇ and D₅ receptors indicating that AH GABA neurons rarely express D₅ receptors. While this evidence in conjunction with our pharmacology studies would suggest that D₅ receptors in the LAH are modulating non-GABAergic neurons that regulate hypothalamic processes indirect to aggression control, we cannot conclude that D₅ receptors are altogether unaffected by adolescent AAS exposure. Evidence from studies examining gene expression of dopamine receptors after AAS-exposure would suggest that the number of receptors is unaltered given that only D₁, D₂ and D₄, but not D₃ or D₅ receptor mRNA are sensitive to circulating androgens (Birgner et al. 2008; Kindlundh et al. 2003). Nevertheless, more sensitive protein assays are necessary to better explore whether D₅ receptors in the LAH are steroid sensitive and undergo alterations in expression after AAS exposure throughout adolescent development.

In summary, immunohistochemical analysis of dopamine receptor populations in the current study in conjunction with pharmacological results from the previous chapter, indicates that dopamine D₂ receptors in the LAH directly modulate adolescent AAS-induced aggression while the role of D₅ receptors may be unrelated to AAS-induced aggression control. Additionally, we report that these receptor pools exist on two discrete neuronal populations such that AH dopamine release regulates both GABAergic neurons through inhibitory D₂ receptors and non-GABAergic cell populations expressing excitatory D₅ receptors. Interestingly, the D₅ staining pattern observed in the present studies appears similar to the localization patterns of glutamate neurons within the AH (Fischer et al. 2007). In fact, dopamine modulates glutamatergic neurons across the neuraxis (Seamans et al. 2001; Seamans and Yang 2004) and
5. Immunofluoresence Colocalization

D₅ receptors have been colocalized with glutamatergic neurons in the AH (Schwartz & Melloni, unpublished data). This possibility notwithstanding, AAS-induced increases in hypothalamic dopamine likely impart their aggression stimulating effects through disinhibiting LAH activity. A central hypothesis currently under investigation in our laboratory is that this disinhibition occurs via increasing activation of aggression stimulating magnocellular AVP neurons. Given that 40% of synapses to magnocellular neurons are GABAergic (Gies and Theodosis 1994), we speculate that AAS-induced increases in D₂ activation in the LAH inhibits GABA mediated inhibition of magnocellular AVP resulting in elevated aggression responding. This hypothesis is supported by reports that D₂, but not D₅, receptor activation depolarize supraoptic magnocellular neurons in the hypothalamus (Yang et al. 1991). These findings warrant a narrowed approach to investigating dopamine-mediated control of AAS-induced aggression by directing a more focused understanding of hypothalamic D₂ activity with less emphasis on D₅ receptor function. Finally, given that dopamine only modulates a subpopulation of GABAergic neurons in the LAH, it is important to consider what other hypothalamic neural systems may be affected by adolescent AAS-exposure that would alter the function of this secondary (i.e. non D₂-containing) GABA population to produce exaggerated aggressive responses. A likely candidate would be through excitatory inputs from the serotonergic system.
Chapter 6

Serotonin and GABA Interactions

6.1 Introduction

Serotonin modulates aggressive behavior and is altered after chronic treatment with AAS. Furthermore, serotonin type-II receptors have been implicated in the control of aggression. For example, treatment with 5-HT$_{2A}$ receptor antagonists suppresses the generation of the offensive aggressive phenotype. However, it is unclear whether these receptors are sensitive to adolescent AAS exposure. To assess whether treatment with AAS throughout adolescence influenced the immunohistochemical localization of 5-HT$_{2A}$ in the AH, hamsters were administered AAS throughout adolescence, scored for offensive aggression, and then examined for differences in 5-HT$_{2A}$-immunoreactivity (5-HT$_{2A}$-ir). When compared with non-aggressive oil-treated controls, AAS-treated hamsters showed significant increases in 5-HT$_{2A}$-ir fibers in the lateral portion of the anterior hypothalamus (i.e. the LAH). Further analysis revealed that AAS treatment also produced a significant increase in the number of cells expressing 5-HT$_{2A}$-ir in the LAH. To test whether this population of cells expressing 5-HT$_{2A}$ receptors was GABAergic, double-label
6. Serotonin and GABA Interactions

Immunofluorescence was performed to colocalize 5-HT$_{2A}$-containing cell bodies with GAD$_{67}$. Finally, to investigate whether adolescent AAS exposure also alters the expression of GABA$_A$ receptors in the LAH, hamster brains were processed for immunohistochemistry and quantified for changes in GABA$_A$-ir. Interestingly, adolescent exposure to AAS produced a significant decrease in the number of GABA$_A$-ir elements in the LAH of aggressive hamsters. Taken together, these findings indicate the presence of multiple GABAergic populations that are differentially regulated by dopamine and serotonin through D$_2$ and 5-HT$_{2A}$ receptors.

6.2 Results

5-HT$_{2A}$ Receptors

AAS-treated hamsters exhibited a dense staining pattern of 5-HT$_{2A}$-ir fibers in the LAH (Figure 16) when compared to oil-treated controls. Quantitative analysis showed that AAS-treated animals had over an eight-fold increase in the density of 5-HT$_{2A}$-ir fibers in the LAH when compared to oil-treated littermates (Figure 17A). This difference was statistically significant [$t(10) = 3.19$, $p < 0.01$]. Similar results were observed in the number of 5-HT$_{2A}$-ir containing cell bodies in the LAH. Specifically, AAS-treated hamsters contained 2-3 times more 5-HT$_{2A}$-ir cell bodies in the LAH compared to oil-treated controls [$t(12) = 2.97$, $p < 0.05$] (Figure 17B). Conversely, no significant differences between AAS and oil-treated hamsters were observed in the dorsal-medial regions of the AH, namely AH proper [$t(10) = 1.42$, $p > 0.1$] (Figure 17A). Finally, increases in 5-HT$_{2A}$-ir within the LAH directly correlated with increased overall aggressive displays induced by adolescent AAS-exposure [$r = 0.634$, $p < 0.05$] (Figure 18).
6. Serotonin and GABA Interactions

5-HT$_{2A}$ and GAD$_{67}$

To investigate whether a population of LAH GABA interneurons expresses excitatory 5-HT$_{2A}$ receptors, 5-HT$_{2A}$ was colocalized with GAD$_{67}$. Immunofluorescence staining revealed that 5-HT$_{2A}$ receptors colocalize with a subpopulation of GABA neurons in this region (Figure 19). However, not all GAD$_{67}$ positive neurons appear to express 5-HT$_{2A}$ receptors in the LAH. Functionally, 5-HT$_{2A}$ receptor activity on LAH GABA neurons would support an excitatory role for serotonin activity on these neurons in the LAH, but an inhibitory influence overall. For example, serotonin acting through 5-HT$_{2A}$ receptors on LAH GABA interneurons would activate these neurons, enhancing the inhibitory influence of GABA within the LAH brain region.
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Figure 16. Brightfield photomicrographs of coronal sections through the Syrian hamster hypothalamus. Shown are 5-HT$_{2A}$ immunoreactive fibers and cell bodies within the anterior hypothalamus (AH; top), specifically in the lateral sub-division of the anterior hypothalamus (LAH; bottom) of AAS and oil-treated hamsters. Abbreviations: AH, anterior hypothalamus; LAH, lateral anterior hypothalamus; oc, optic chiasm; SCN suprachiasmatic nucleus.
Figure 17. 5-HT\textsubscript{2A} in brains of AAS and oil-treated hamsters. (A) Area covered by 5-HT\textsubscript{2A}-immunoreactive (5-HT\textsubscript{2A}-ir) fibers in the LAH and AH proper. (B) AAS treatment significantly increased the number of 5-HT\textsubscript{2A}-ir cell bodies in the LAH. Bars denote SEM. *p < 0.05, **p < 0.01, Student’s t-test, two-tailed.
6. Serotonin and GABA Interactions

Pearson’s r Correlation Analysis of Offensive Aggression and Lateral Anterior Hypothalamic 5-HT$_{2A}$ fibers (LAH-5-HT$_{2A}$)

<table>
<thead>
<tr>
<th>Aggression Measure</th>
<th>AH-5-HT$_{2A}$</th>
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</thead>
<tbody>
<tr>
<td>Composite Aggression</td>
<td>$r^2 = 0.402^*$</td>
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</table>

**Figure 18.** Area of 5-HT$_{2A}$-ir fibers in the LAH positively correlate with the total number of aggressive acts (composite aggression) in the Syrian hamster. *$p < 0.05$, Pearson’s r correlation coefficient*
6. Serotonin and GABA Interactions

Figure 19. Darkfield photomicrographs of a coronal section through the LAH. Shown are cells positively labeled with GAD$_{67}$ (green), 5-HT$_{2A}$ receptors (red), and double-labeled GAD$_{67}$/5-HT$_{2A}$ cells (yellow). Only a subset of GABA neurons appears to express 5-HT$_{2A}$ receptors indicating that a subpopulation of GABA neurons in the LAH is modulated by serotonin.
6. Serotonin and GABA Interactions

GABA<sub>A</sub> Receptors

In the central brain region for aggression control, the LAH, GABA<sub>A</sub>-ir elements decreased in AAS-treated animals when compared with oil-treated controls \([t(7) = 2.48, p < 0.05]\) (Figure 20A). Treatment with AAS throughout adolescence produced a greater than ten-fold decrease in the density of GABA<sub>A</sub> staining when compared to oil-treated littermates (Figure 20B). In contrast, in the MeA, adolescent AAS treatment produced a significant increase in the density of GABA<sub>A</sub>-ir elements \([t(6) = 5.06, p < 0.01]\) (Figure 20A). Finally, GABA<sub>A</sub>-ir staining remained unaltered in the various other brain regions investigated \([LS, t(6) = 0.48; BNST, t(6) = 0.09; CeA, t(5) = 1.19; VLH, t(4) = -0.55, p > 0.05 for all measures]\) (Figure 20A).
Figure 20. (A) AAS exposure throughout adolescence significantly decreases the density (in thousands) of GABA_A immunoreactivity in the LAH and increases GABA_A receptor density in the MeA. Bars denote S.E.M. *p<0.05; Student’s t-test, two-tailed (n=8-9/group). (B) Brightfield photomicrographs showing decreased GABA_A immunoreactive elements in the LAH of AAS-treated hamsters compared to vehicle-treated controls.
6. Serotonin and GABA Interactions

6.3 Discussion

Serotonin has been shown to play an inhibitory role in aggression in humans (Brown et al. 1982; Coccaro et al. 1997b; Kruesi et al. 1990; Linnoila et al. 1983) and animals (Higley et al. 1996; Kyes et al. 1995; Sakaue et al. 2002) including hamsters (Delville et al. 1996; Ferris et al. 1997; Ferris et al. 1999; Grimes and Melloni 2002). The inhibitory nature of serotonin on aggressive response has been attributed to the serotonin Type-I (i.e., 5-HT$_{1A}$ and 5-HT$_{1B}$) (Grimes and Melloni 2005; Miczek et al. 1998; Ricci et al. 2006; Sanchez et al. 1996) and Type-II (de Almeida et al. 2005; Peremans et al. 2005; Rilke et al. 2001; Sakaue et al. 2002) receptors, specifically 5-HT$_{2A}$. 5-HT$_{2A}$ is a G-protein coupled excitatory receptor implicated in schizophrenia, suicidal behavior, impulsivity, and aggression (Abdolmaleky et al. 2004; Bjork et al. 2002; Khait et al. 2005). In our laboratory, we have used the resident-intruder paradigm to study the effects of adolescent AAS exposure on the development of the offensive aggressive phenotype in hamsters. Our research supports the inhibitory nature of serotonin in the control of aggression. Specifically, reductions in 5-HT$_{1A}$ and 5-HT$_{1B}$ receptor expression mediate AAS-induced increases in aggression (Grimes and Melloni 2005; Ricci et al. 2006). Additionally, pharmacological research from our laboratory shows that antagonism of 5-HT$_{2A}$ receptors suppresses cocaine-induced offensive aggressive behavior (Ricci et al. 2007a; Schwartzer et al. 2008; Schwartzer et al. 2009a). However, it is unknown whether AAS exposure directly alters the expression of 5-HT$_{2A}$ receptors in specific aggression nuclei or if the aggression attenuating effects of 5-HT$_{2A}$ blockade are the result of alterations in non-aggression nuclei (e.g. cortical signaling). For example, in male rats, 5-HT$_{2A}$ mRNA expression in the raphe and several cortical nuclei decreased after castration and could be restored by treatment with testosterone (Sumner and Fink 1998). However, no observable differences were reported in other brain nuclei. Perhaps
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exposure to AAS throughout adolescent development stimulates aggression by altering serotonin
signaling through 5-HT$_{2A}$ expression in the anterior hypothalamus to control offensive
aggression in the hamster. To address this question, we used immunohistochemical analysis to
measure changes in 5-HT$_{2A}$ receptor immunoreactivity in the LAH of adolescent AAS-treated
aggressive animals.

Aggressive AAS-treated animals showed significant increases in 5-HT$_{2A}$-ir in the LAH, a
subregion of the AH associated with the exaggerated aggressive phenotype and a brain nucleus
sensitive to the developmental effects of AAS exposure. The changes in 5-HT$_{2A}$-ir were highly
correlated with increased aggression. The LAH has previously been shown to be important in
attack behavior in rats (Halasz et al. 2002; Haller et al. 1998) and more recently been elucidated
as an integral aggression brain region in the Syrian hamster (Fischer et al. 2007; Ricci et al.
2007b). The LAH receives local afferents from magnocellular AVP containing neurons in the
NC and mSON. AVP plays an important role in the regulation of aggressive behavior and has
been shown to be sensitive to the effects of AAS exposure (DeLeon et al. 2002; Grimes et al.
2007; Harrison et al. 2000). While serotonin activity in the AH is known for its inhibitory role
through 5-HT$_{1A}$ and 5-HT$_{1B}$ receptors, evidence suggests that some serotonin receptors in the
hypothalamus may actually stimulate the release of AVP (Jorgensen et al. 2003), including
activation of 5-HT$_{2A}$ receptors (Jorgensen et al. 2002). Increases in LAH 5-HT$_{2A}$-ir fibers and
cell bodies supports this notion and provides a possible link between increased receptor
expression and aggression, specifically through AVP release. Additionally, studies
demonstrating a reduction in aggressive response after antagonism of 5-HT$_{2A}$ receptors further
suggest a link between 5-HT$_{2A}$ activation and aggression (Ricci et al. 2007a; Schwartzer et al.
2008; Schwartzer et al. 2009a). However, it is unclear whether these receptors work directly to
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stimulate vasopressin release or activate aggression through modulation of other neurotransmitter systems.

One putative mechanism that might explain the increase in number of 5-HT$_{2A}$-ir fibers and cell bodies in the LAH stems from findings regarding GABA and serotonin reciprocal interactions. Activation of 5-HT$_{2A}$ receptors in various brain regions has been shown to increase GABA release (Aghajanian and Marek 1997; Ciranna 2006; Li et al. 2000; Munsch et al. 2003) resulting in serotonergic activation of an inhibitory system. Alternatively, activation of 5-HT$_{1A}$ receptors reduces GABA levels suggesting that serotonin can differentially modulate GABAergic neurons based on receptor expression (Shen and Andrade 1998). In our laboratory, GAD$_{67}$, the rate limiting enzymes in the synthesis of GABA, has been localized to the LAH in a staining pattern similar to 5-HT$_{2A}$. Thus it is possible that the LAH may consist of 5-HT$_{2A}$ containing GABAergic neurons. Results from the colocalization of 5-HT$_{2A}$ and GAD$_{67}$ indicate a distinct population of GABA neurons that express the excitatory 5-HT$_{2A}$ receptors. However, an increase in 5-HT$_{2A}$ receptor expression on GABAergic neurons would suggest that activation of 5-HT$_{2A}$ receptors would increase inhibition and reduce the aggressive response. Yet behavioral pharmacology studies suggest that 5-HT$_{2A}$ activation increases aggression (Sakaue et al. 2002). From a mechanistic standpoint, this would suggest that GABA mediates an inhibitory system serving to disinhibit the hypothalamus resulting in an increase in aggressive response. Research results suggest that 5-HT$_{2A}$ can differentially increase or decrease GABA mediated inhibition suggesting that 5-HT$_{2A}$ may directly or indirectly alter GABA release (Shen and Andrade 1998). Additionally, activation of GABA$_{A}$ receptors increases aggression suggesting that both GABA and serotonin may reduce the excitability of an inhibitory system (Fish et al. 2005; Fish et al. 2001; Miczek et al. 2004; Miczek et al. 2003). Interactions between the GABAergic and
6. Serotonin and GABA Interactions

Serotonergic systems must be further investigated to better understand the effects of adolescent AAS exposure on the generation of offensive aggression. Moreover, examination of GABA receptor pools is necessary to uncover the neural systems modulated by these GABA populations in the LAH.

The GABA\textsubscript{A} receptor is sensitive to the effects of sex-steroids and critical for the expression of aggressive behaviors. For example, in mice, blockade of GABA\textsubscript{A} receptors using the GABA\textsubscript{A} antagonist bicuculline significantly increases the number of bite behaviors suggesting an inhibitory role of GABA\textsubscript{A} in aggression control (Liu et al. 2007). Previous research has shown sex- and age-dependent alterations on the expression of GABA\textsubscript{A} receptors in the forebrain of mice in the presence of AAS (Penatti et al. 2005). Additionally, work by McIntyre et al. reports significant decreases in GABA subunit specific mRNA after AAS exposure (McIntyre et al. 2002). Taken together, it is likely that AAS induces aggression, in part, through alterations in the expression of GABA\textsubscript{A} receptors in the LAH. Our findings in the current report show significant decreases in the expression of the \( \alpha_1 \) subunit of GABA\textsubscript{A} receptors in the LAH of aggressive AAS-treated hamsters suggesting a decrease in receptor mediated GABAergic inhibition. Perhaps this down regulation in receptor localization is a result of AAS-induced changes in the production of GABA\textsubscript{A}\textsubscript{\( \alpha_1 \)} subunits. While McIntyre et al. previously reported no changes in \( \alpha_1 \) subunit mRNA in the presences of AAS (McIntyre et al. 2002), this hypothesis cannot be entirely ruled out as AAS also alter cell function post-transcriptionally (Ing 2005). Alternatively, these decreases in GABA\textsubscript{A} receptor localization could be a result of pharmacological down regulation due to increased GABA release as evidenced by increased GAD\textsubscript{67} and GAD\textsubscript{65} enzymes in AAS treated animals (Grimes et al. 2003). Considering this reported decrease in LAH-GABA\textsubscript{A} receptor expression, the increased aggressive response is
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likely a result of altered GABA\(_A\)-mediated inhibition, perhaps through directly altering GABA\(_A\) function. Interestingly, AAS allosterically modulate the GABA\(_A\) receptor (Clark et al. 2004; Jorge et al. 2002), and these AAS-induced changes in GABA\(_A\) function are subunit specific (Henderson et al. 2006). For example, GABA\(_A\) receptors containing the \(\alpha_2\) subunit show enhanced peak current, slowed deactivation, and diminished desensitization in the presence of the AAS 17\(\alpha\)-Methyltesosterone (17\(\alpha\)-MeT) (Yang et al. 2005), while \(\alpha_1\) containing GABA\(_A\) receptors showed no changes in function in the presence of 17\(\alpha\)-MeT (Yang et al. 2005). Thus, changes in the composition of GABA\(_A\) receptor subunits after chronic exposure to AAS may result in altered sensitivity of CNS action to AAS and endogenous sex-steroids leading to changes in various behaviors including an enhanced aggressive response.

In summary, these studies provide an examination of the interaction between the serotonergic and GABAergic neural systems in the LAH after adolescent exposure to AAS. These findings indicate that AAS-exposure during adolescent development produces increases in 5-HT\(_{2A}\)-ir in the LAH, a region of the hypothalamic attack area repeatedly shown to be integral in aggressive behavior. Moreover 5-HT\(_{2A}\) receptors colocalize with GAD\(_{67}\) suggesting that GABAergic interneurons in the LAH are modulated by serotonin. Importantly, not all cells positively labeled for GAD\(_{67}\) expressed 5-HT\(_{2A}\) receptors indicating that only a subpopulation of GABA is mediated by serotonin. While these observations support the notion that dopamine and serotonin modulate separate GABA populations in the LAH, more complex imaging modalities such as triple-label immunofluorescence are necessary to demonstrate that D\(_2\) and 5-HT\(_{2A}\) receptor pools are expressed independently of one another on separate GABA populations. Finally, examination of GABA\(_A\) receptors in the LAH revealed a significant decrease in the density of these receptors after adolescent exposure to AAS. However, it is unclear what LAH
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neural systems GABA neurons are modulating. One plausible explanation for the decreases in GABA_A receptor density may be due to their expression on serotonin afferent fibers. In this model, AH serotonin autoregulates its release through activation of local GABA interneurons. Given that AAS exposure reduces serotonin afferents in the AH, the reduction in GABA_A receptor densities may reflect this reduction in serotonin fibers within the LAH. Alternatively, reductions in GABA_A receptor density may be a result of receptor down regulation as a result of increased GABA release from excitatory 5-HT_2A receptors. Speculation aside, future investigations into the neural circuits being modulated by local GABA interneurons are necessary to provide a more complete understanding of the neural network within the LAH responsible for eliciting aggressive behaviors and how these networks are developmentally altered during adolescent AAS abuse.
Chapter 7

General Discussion

7.1 Overview

AAS abuse remains high in the adolescent population, with more than a half million 8th–10th graders in the U.S. reporting use of AAS each year (NIDACapsules 2008), and more than 2% of 12th graders reporting lifetime use 2008 (NIDACapsules 2008). These numbers are particularly concerning given the high incidence of psychological and physiological ramifications associated with taking AAS, including increased violence, rage, and aggression (Dukarm et al. 1996; Loeber and Hay 1997). Given that adolescence is a developmental period in which neurobehavioral mechanisms regulating aggressive behaviors are particularly sensitive to circulating androgens (Dabbs et al. 1991; Mattsson et al. 1980; Scerbo and Kolko 1994), the early onset of AAS use during this period may have significant effects on the developmental progression and expression of aggressive behavior in youth. For over a decade, the Syrian hamster has been used as a valid animal model to investigate the neurobiological consequences of adolescent AAS exposure (Melloni et al. 1997a). These studies have revealed that AAS-treatment produces activational and organizational alterations in distinct neurochemical systems.
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across key aggression loci of the brain (Melloni and Ricci 2010). Of particular interest is the neurochemical systems altered in the AH. The AH is reciprocally connected to all other aggression loci and its activity is essential for the control of aggressive behavior (Delville et al. 2000; Nelson and Trainor 2007). Specifically, aggression is regulated in part by activation from local magnocellular AVP release in the AH and inhibited by serotonin afferents from the dorsal raphe nucleus (Ferris et al. 1997). Animals treated with AAS undergo alterations to various neurochemical systems within the AH resulting in the display of elevated aggressive behaviors (Melloni and Ricci 2010). For example, adolescent AAS treatment increases AH-AVP release and decreases serotonin innervation to the AH resulting in elevated aggressive behaviors (Melloni and Ricci 2010). While numerous studies have demonstrated the importance of these mechanisms in the control of AAS-induced aggression (DeLeon et al. 2002; Grimes and Melloni 2002), it is unknown how these excitatory AVP and inhibitory serotonin neural systems are modulated within the AH to elicit aggressive responses.

In addition to AVP and serotonin, several other neurochemical systems are implicated in aggression control. For example, dopamine activity has been linked to aggression control with high levels of dopamine synthesis and metabolism correlated with increased incidence of aggression (de Almeida et al. 2005; Ferrari et al. 2003; Louilot et al. 1986). Moreover, dopamine receptors are localized to aggression nuclei and pharmacology targeting these receptors elicits changes in aggressive responding (Sweidan et al. 1991). Dopamine receptors are classified into two families, D1-like receptors (D1, D5) and D2-like receptors (D2, D3, D4) (Sibley et al. 1993). While both receptor types are G-protein coupled, activation of D1-like and D2-like receptors produce opposing responses (Bunzow et al. 1988; Monsma et al. 1990; Sibley et al. 1993). Specifically, D2-like receptors are negatively linked to adenylyl cyclase such that activation of
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these receptors results in neuronal inhibition (Bunzow et al. 1988). Conversely, D1-like receptor activation increases adenylyl cyclase producing increased neuronal activity and excitation (Monsma et al. 1990). Both D2-like and D1-like receptors, particularly D2 and D5, are localized to the hypothalamus and implicated in aggression control in various species and animal models (Bondar and Kudryavtseva 2005; Nikulina and Kapralova 1992; Rodriguez-Arias et al. 1998; Tidey and Miczek 1992). While both D2 and D5 receptors are localized to the LAH, it is unknown whether they work to directly modulate aggression control. Given that several dopamine receptors have previously been reported to be androgen sensitive (Birgner et al. 2008; Kindlundh et al. 2003), it is likely that the receptor pools in the LAH undergo alterations after adolescent AAS-exposure and that these changes to receptor expression modulate aggressive behaviors. If this notion were correct than AH dopamine would impart both inhibitory and excitatory effects on LAH function through D2 and D5 receptors, respectively, and would suggest that hypothalamic dopamine regulates multiple neural systems to modulate aggression control. A likely mechanism for which dopamine modulates LAH activity is through regulation of local GABAergic interneurons. The LAH is densely innervated with GABA neurons and dopamine modulation of GABAergic neurons is ubiquitous across the neuraxis. Interestingly, investigations linking GABA to aggression control has reported both an inhibitory and excitatory influence on aggressive responses (de Almeida et al. 2005; Depaulis and Vergnes 1985; Liu et al. 2007; Miczek et al. 2003). These conflicting reports of GABA’s role in modulating aggression would suggest that multiple GABAergic systems regulate both inhibitory and excitatory neural systems governing aggression. In the LAH, GABA production has been shown to increase after adolescent exposure to AAS further implicating its role in AAS-induced aggression (Grimes et al. 2003). Taking together that (1) both excitatory and inhibitory dopamine receptors are
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implicated in aggression control, (2) the conflicting reports of GABA neural systems playing both an inhibitory and excitatory role in aggression control, (3) the ubiquitous interaction between dopamine and GABA neural systems (4) the localization of these systems in the LAH, and (5) the reported sensitivity of both GABA and dopamine to AAS-exposure, it was hypothesized that dopamine modulates AAS-induced aggression in the LAH through differential regulation of multiple GABAergic systems.

A likely mechanism for this interaction would be through dopamine’s indirect regulation of both excitatory (e.g. AVP) and inhibitory (e.g. serotonin) signaling through modulation of LAH GABA. In this putative model, dopamine suppresses GABAergic inhibition of local AVP circuits through inhibitory D₂ mechanisms while increasing inhibitory tone onto an inhibitory system (e.g. serotonin) through activation of a secondary GABAergic system via excitatory D₅ receptors. Together the combined effects of dopamine in the LAH would cause a disinhibition of LAH activity resulting in increased aggressive responding. To investigate the existence of this hypothesized interaction and to identify how these systems are altered after adolescent AAS-exposure to produce the elevated aggressive response, the following studies were conducted: (1) to identify how AAS-exposure alters dopamine synthesis in aggression regions of the brain AAS-treated hamsters were processed for immunohistochemistry of TH and compared to sesame oil-treated littermates. (2) The AH of aggressive AAS-treated hamsters was processed for immunohistochemistry of D₂ and D₅ receptors to examine the distribution and localization patterns and to determine whether adolescent AAS-exposure alters the expression of these receptors. (3) To investigate whether blockade of D₂ and D₅ receptors in the LAH can suppress AAS-induced aggression, animals were locally infused with a D₂ or D₅ receptor antagonist into the LAH and measured for changes in aggressive behavior. (4) Given the similarities in
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distribution patterns of D$_2$ and D$_5$ receptors compared to GABAergic interneurons in the LAH, it
was hypothesized that these receptors are expressed on GABA neurons. Therefore, double-label
immunofluorescence techniques were utilized to colocalize dopamine D$_2$ and D$_5$ receptors with
GAD$_{67}$ in the LAH.

In addition to the plausible interactions between dopamine and GABA there are
additional neurochemical systems that are likely integrated in the LAH which must be
investigated to more completely understand the neurocircuitry of aggression and how these
systems are altered in the presence of AAS. While serotonin is well known for its inhibitory
influence on aggression through serotonin Type-I receptors (Nelson and Trainor 2007), there is a
paucity of evidence implicating the excitatory 5-HT$_{2A}$ receptor in aggression control (Sakaue et
al. 2002). Interestingly, 5-HT$_{2A}$ receptors are localized to the LAH in a staining pattern similar to
GABA and are sensitive to circulating androgens (Sumner and Fink 1998). The distribution and
staining pattern of Ah 5-HT$_{2A}$ receptors compared to that of GAD$_{67}$ might suggest an interaction
between serotonin and GABA in the LAH. Specifically, serotonin is hypothesized to increase
GABA activity through activation of stimulatory 5-HT$_{2A}$ receptors. These receptors may work in
conjunction with or in opposition to dopamine in the LAH to elicit aggressive responding. To
investigate this interaction between serotonin 5-HT$_{2A}$ receptors and GABA in the LAH and
examine how these systems are altered in the presence of adolescent AAS-exposure to produce
the elevated aggressive phenotype, several additional studies were conducted: (1) To investigate
whether 5-HT$_{2A}$ receptors in the LAH are responsive to adolescent AAS-exposure, aggressive
steroid-treated and non-aggressive oil-treated hamster brains were processed for
immunohistochemistry and measured for changes in 5-HT$_{2A}$ receptor expression. (2) In order to
discern whether changes in 5-HT$_{2A}$ receptors in the LAH are linked to levels of aggression,
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behavioral responses of AAS-treated hamsters and oil-treated controls were correlated with changes in 5-HT$_{2A}$ receptor expression patterns in the AH. (3) Given the similar staining patterns of 5-HT$_{2A}$ receptors to GABA interneurons in the LAH, double-label immunofluorescence was performed to colocalize 5-HT$_{2A}$ receptors to GAD$_{67}$ containing neurons in the LAH. (4) Immunohistochemical characterization of GABA$_A$ receptors was conducted in the LAH to uncover receptor distribution patterns that would implicate AVP and serotonin afferents as likely modulators of local GABA tone. Additionally, the density of GABA$_A$ receptor expression in the LAH was examined in adolescent AAS-treated hamsters and compared to sesame oil-treated controls to identify steroid-dependent changes in receptor expression patterns.

7.2 Summary of Findings

Hamsters exposed to a mixture of AAS throughout adolescence displayed elevated aggressive behaviors. This cocktail, designed to mimic combinations of AAS commonly abused in adolescent populations (a practice known as “stacking”), is equivalent to moderate doses of AAS mixtures abused to date. At these doses, animals exhibited increased attacking and biting behavior towards a novel intruder. These animals were observed to have a significant increase in the density of TH in the anterior hypothalamus while no differences were observed in all other aggression loci compared to vehicle-treated controls. Additionally, increases in the number of dopaminergic cell bodies were observed in the NC and mSON. This increase in the rate limiting enzyme for dopamine synthesis would suggest that this treatment regimen increases local dopamine production in the AH and that this increase in AH dopamine contributes to the highly aggressive phenotype. Indeed, the increases in TH correlated with aggression intensity in AAS-treated animals. To determine whether these dopaminergic neurons locally innervate the AH,
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Brains were processed for immunohistochemistry of dopamine D₂ and D₅ receptors. Interestingly, both D₂ and D₅ receptors were localized to cell bodies within the lateral portion of the AH (LAH). The LAH is innervated by magnocellular neurons from the NC and mSON suggesting that dopamine from these nuclei within the AH locally modulate LAH activity. Given the presence of D₂ and D₅ receptors within this subregion of the AH, it was hypothesized that increases in dopamine results in increased activation to these local receptor pools. To investigate this hypothesis, aggressive AAS-treated hamsters were microinjected with a selective D₂ or D₅ antagonist into the LAH and measured for changes in aggressive responding. The selective D₂ receptor antagonist eticlopride dose-dependently decreased aggression while leaving all other non-aggressive related behaviors intact. Conversely, only high doses of the D₅ receptor antagonist SCH-23390 suppressed AAS-induced aggression. At this high dose hamsters displayed reductions in general arousal and motor activity. While these findings suggested that dopamine’s action at D₅ receptors in the LAH might regulate other hypothalamic-mediated behaviors non-specific to aggression, it is possible that their function in the control of aggression may still be important, albeit difficult to discern. Therefore it was necessary to investigate what neural systems express these D₂ and D₅ receptors in the LAH and how they may be altered by AAS-exposure throughout adolescent development.

Dopamine is ubiquitous across the neuraxis and is often reported to regulate various neural systems through modulating local GABAergic interneurons (Gerfen et al. 1990; Santana et al. 2008). Interestingly the dopamine D₂ and D₅ receptors characterized in the AH were localized to cell bodies in a staining pattern similar to that of GAD₆₇ in the LAH. GABA is well known to influence aggressive behaviors. However, studies have reported conflicting findings as to whether GABA potentiates or attenuates aggression (de Almeida et al. 2005; Depaulis and
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Vergnes 1985; Liu et al. 2007; Miczek et al. 2003). These studies suggest that GABA may play both an inhibitory and excitatory role in aggression control. Taking together these conflicting reports and the observed similarity in stating pattern of both D\textsubscript{2} and D\textsubscript{5} receptors, it was hypothesized that dopamine modulates multiple populations of GABAergic interneurons in the LAH and that changes in the expression of these receptors after adolescent AAS-exposure would increase aggression through both inhibition and excitation of discrete GABAergic populations differentially expressing D\textsubscript{2} and D\textsubscript{5} receptors. Support for this notion stems from investigations into the dopaminergic regulation of GABA in the striatum. Specifically, two GABAergic pathways are differentially modulated by dopamine through D\textsubscript{1} and D\textsubscript{2} receptors whereby dopamine release results in decreased activation of D\textsubscript{2}-positive GABA cells (indirect pathway) and increased activation of D\textsubscript{1}-positive GABA cells (direct pathway) (Alexander and Crutcher 1990; Gerfen et al. 1990). To investigate whether a similar mechanism exists in the LAH and to examine how these systems may be altered in the presence of AAS, brains of adolescent AAS-treated hamsters were processed for double-label immunofluorescence and compared to sesame oil-treated controls.

Treatment with AAS throughout adolescence produced a significant increase in the number of cells positively labeled for D\textsubscript{2} receptors in the LAH. This finding was consistent with the reported increase observed from immunohistochemical analysis of D\textsubscript{2} receptors using the peroxidase staining method in an earlier study. Additionally, the number of cells expressing GAD\textsubscript{67} was also increased in the LAH of AAS treated hamsters indicating that this treatment regimen increases the number of GABA-producing neurons. Moreover, many of these GABA neurons were co-localized with D\textsubscript{2} receptors indicating that dopamine modulates local GABAergic neurons. Given that AAS treatment increased both GAD\textsubscript{67} and D\textsubscript{2}-positive cell
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bodies, and the large number of cells in the LAH co-expressing GAD$_{67}$ and D$_2$, it was expected that AAS treatment would produce a significant increase in the number of double-labeled cells in the LAH. However, the current data failed to demonstrate AAS-induced changes in the number of double-labeled neurons indicating the presence of a secondary non-D$_2$ containing population of GABA neurons. This secondary population was hypothesized to express excitatory D$_5$ receptors as immunohistochemical observations revealed that D$_5$ receptors in the LAH are distributed in a similar staining pattern to that of GAD$_{67}$. However, double-label immunofluorescence analysis revealed that very few neurons in the LAH are colocalized with both GAD$_{67}$ and D$_5$ receptors. In fact, while the number of GAD$_{67}$-positive neurons increased in AAS-treated animals (i.e. a replication of the previous immunofluorescence finding), the number of D$_5$-positive neurons was unaltered in AAS-treated animals compared to sesame oil-treated controls. These findings indicate that D$_5$ receptors are not expressed on GABAergic neurons in the LAH and are unaltered by AAS-exposure. Together, the immunofluorescence studies reveal two distinct neural populations of GABAergic neurons in the LAH. While one of these populations is modulated by dopamine through D$_2$ receptors, the other GABA population appears to be regulated by some other LAH neural system. Investigating which neural system modulates this GABAergic population is necessary to elucidate a more comprehensive understanding of the hypothalamic neural circuit regulating AAS-induced aggression.

A likely mechanism for modulation of this secondary non-D$_2$ expressing GABA population in the LAH is serotonin. While serotonin is well known for its inhibitory effects on aggression through its actions at inhibitory serotonin Type-I receptors (de Almeida et al. 2005; Miczek et al. 2002), there is compelling evidence implicating excitatory 5-HT$_{2A}$ receptors in the control of aggression (Giegling et al. 2006; Sakaue et al. 2002). Given the link between 5-HT$_{2A}$
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pharmacology and aggression control, and research demonstrating this receptor to be androgen sensitive, it was hypothesized that 5-HT$_{2A}$ receptors in the LAH would be altered in the presence of AAS and that these receptors are expressed on local GABA interneurons. Therefore, the brains of aggressive AAS-treated animals and sesame oil-treated controls were processed for immunohistochemistry of 5-HT$_{2A}$ receptors and changes in their receptor expression were correlated with animals’ aggression intensity. These studies indicated that AAS-exposure significantly increase the number of cells expressing 5-HT$_{2A}$ in the LAH. This increases in 5-HT$_{2A}$ receptor expression correlated with increases in aggression demonstrating a link between AAS-induced aggression and LAH 5-HT$_{2A}$ receptors. To determine whether these neurons that express 5-HT$_{2A}$ receptors are local GABA interneurons, hamster brains were processed for double-label immunofluorescence. Interestingly, many of the neurons expressing 5-HT$_{2A}$ receptors were colocalized with GAD$_{67}$. However, not all cells producing GAD$_{67}$ were observed to co-express 5-HT$_{2A}$ receptors suggesting that these colocalized proteins may represent a subpopulation of GABA interneurons in the LAH similar to that observed with colocalized D$_2$/GABA neurons. Taking together studies examining both dopamine and serotonin interactions with AH-GABA neurons, it is likely that these two neural systems differentially modulate two distinct GABA populations in the LAH and that adolescent AAS-exposure alters these systems to produce increased aggression. These findings notwithstanding, it is important to consider what neural systems GABA modulates and whether postsynaptic GABA receptors are altered in AAS-induced aggression. Immunohistochemical analysis of postsynaptic GABA$_A$ in the LAH revealed a significant decrease in the density of GABA$_A$ puncta in the brains of AAS-treated hamster indicative of a decrease in the number of terminals expressing GABA$_A$. The implications for these observed decreases in GABA$_A$ receptors along with a putative model integrating the
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dopaminergic and serotonergic modulations of local GABA interneurons in the LAH and how these systems modulate AAS-induced aggression are discussed below.

7.3 A Hypothetical Model

Investigations into the neurobiological constructs of adolescent AAS-induced aggression over the past decade and the recent findings from this dissertation have led to a plausible neural mechanism within the LAH that regulates aggressive behavior. Within the AH lies magnocellular AVP neurons from the NC and mSON that locally innervate the LAH (Figure 21, green neuron). Release of AVP into the LAH stimulates aggression through activation of excitatory AVP-responsive neurons (Figure 21, blue neuron). These aggression stimulating inputs from AVP are inhibited by serotonergic afferents from the raphe nucleus that project to the LAH (Figure 21, red neuron). Under non-aggressive conditions, LAH activity is suppressed by inhibitory inputs from serotonin and activation of inhibitory postsynaptic serotonin Type-I receptors expressed on AVP fibers and AVP-responsive neurons. Exposure to AAS throughout adolescence increases AVP synthesis/release and reduces serotonergic innervation into the LAH. This increase in excitatory inputs coupled with a decrease in inhibitory tone results in the elevated aggressive phenotype observed with AAS use.

Findings from this dissertation have added several additional neural systems to this model. In addition to AVP-release from the NC and mSON, dopaminergic neurons have been localized to these nuclei and their activity has been shown to correlate with AAS-induced aggression. These dopamine neurons (Figure 21, yellow neuron) activate postsynaptic D₂ receptors expressed on GABAergic interneurons in the LAH. Considering that increased dopamine results in elevated aggression and that inhibitory D₂ receptors are expressed on
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GABAergic neurons, it is likely that dopamine works to disinhibit LAH activity by inhibiting GABA release onto excitatory inputs to the AH (Figure 21). Research from this dissertation has also uncovered a secondary GABAergic system that is not modulated by dopamine. This secondary GABAergic system is observed to express excitatory 5-HT$_{2A}$ receptors both of which are increased in the presence of AAS. This increase in the number of GABAergic neurons expressing 5-HT$_{2A}$ would suggest that AAS treatment recruits more inhibitory neurons to elicit aggressive responding. From a mechanistic standpoint, this would suggest that GABA mediates an inhibitory system serving to disinhibit the hypothalamus resulting in an increase in aggressive response. In fact, several studies have found that serotonin receptors in the hypothalamus may actually stimulate the release of AVP (Jorgensen et al. 2003), including activation of 5-HT$_{2A}$ receptors (Jorgensen et al. 2002). Therefore, it is hypothesized that this secondary 5-HT$_{2A}$-containing GABA system reciprocally inhibits LAH serotonin to regulate the inhibitory influence on LAH activity (Figure 21). Immunohistochemical localization of inhibitory GABA$_A$ receptors in the LAH further supports this notion as the decrease in the density of receptor puncta observed after adolescent AAS-exposure is indicative of a decrease in the number of inhibitory serotonin afferents produced as a result of AAS treatment. Speculation aside, anatomical, pharmacological, and behavioral results from this dissertation reveal two distinct GABAergic pathways that differentially modulate neural inputs to the LAH (Figure 21, grey neurons). These opposing GABAergic systems provide the first direct evidence to resolve the discrepancy between conflicting reports of GABA having both an inhibitory and excitatory role in aggression control. Moreover, these GABAergic systems appear to be differentially modulated by dopamine and serotonin through inhibitory D$_2$ and excitatory 5-HT$_{2A}$ receptors, respectively. Taken together, D$_2$ and 5-HT$_{2A}$ receptors appear to be important receptor systems modulating AAS-
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induced aggression and indicate likely sites of action where targeted pharmacology could be used to manage aggression in clinical settings.
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Figure 21. A model depicting the anterior hypothalamic regulation of adolescent AAS-induced offensive aggression. Magnocellular neurons from the NC and mSON (green neuron) release AVP into the LAH eliciting the offensive aggressive response. LAH activity is normally inhibited by serotonin afferents from the raphe nucleus (red fibers) suppressing AVP release and activation of AVP-responsive neurons (blue neuron). Findings from this dissertation have identified two distinct GABAergic populations in the LAH that are postulated to differentially regulate excitatory (e.g. AVP) and inhibitory (e.g. serotonin) neurons. These two GABA populations are modulated by dopamine and serotonin through inhibitory D\textsubscript{2} and excitatory 5-HT\textsubscript{2A} receptors (grey neurons). When exposed to AAS throughout adolescence, increased dopamine from the NC and mSON (yellow neuron) activate inhibitory D\textsubscript{2} receptors suppressing
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GABAergic inhibition of AVP and/or AVP responsive neurons. Moreover, AAS-induced increases in GABAergic neurons expressing excitatory 5-HT$_{2A}$ receptors results in increased inhibition to local inhibitory tone (e.g. serotonin). AAS-induced alterations in these GABAergic systems and the neural systems modulating GABA result in increased LAH activation producing high incidence of offensive aggressive behaviors.
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7.4 Clinical Significance

Maladaptive aggression in clinically referred youngsters is a core, serious and highly prevalent comorbid symptom associated with various psychiatric disorders (Connor et al. 2002; Connor and McLaughlin 2006; Connor et al. 2004; Jensen et al. 2007). In fact, in clinical settings aggression intensity is used as a marker for psychiatric illness severity and as a prognostic indicator of treatment duration and longitudinal outcome (Connor and McLaughlin 2006; Pappadopulos et al. 2006; Werry 1997). Interestingly, despite its high incidence, clinical value, and severe social and emotional impairment among the younger population, there is no specific anti-aggressive medication currently available (Schur et al. 2003; Stewart et al. 1990; Vitiello and Stoff 1997).

In recent years, atypical antipsychotics have been used as the pharmacological treatment of choice for highly aggressive institutionalized youngsters (Jensen et al. 2007; Schur et al. 2003; Yildiz et al. 2003). Clinical studies have provided strong evidence supporting the anti-aggressive effects of different atypical antipsychotics, including risperidone, ziprasidone, olanzapine, aripiprazole, and quetiapine, in a broad spectrum of psychiatric disorders such as conduct disorder, attention deficit hyperactivity disorder (ADHD), autism, bipolar disorder, and schizophrenia (Aman et al. 2004; DelBello et al. 2006; Findling et al. 2005; Soderstrom et al. 2002). The anti-aggressive effects of these compounds depend on alterations in dopaminergic and serotonergic systems function through inhibition of D₂ and 5-HT₂A receptor activity, respectively. The involvement of D₂ and 5-HT₂A receptors in the modulation of aggression is further supported by behavioral results from preclinical studies showing increased aggressive behavior in response to pharmacological activation of D₂ and/or 5-HT₂A receptors and reduced aggression intensity following the blockade of these receptors (Arregui et al. 1993; Navarro and
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Manzaneque 1997; Sakaue et al. 2002; Sanchez et al. 1993; Shih et al. 1999; Skrebuhhova-Malmros et al. 2000). Moreover, findings from this dissertation demonstrate a direct mechanism in the aggression center of the brain (i.e. the hypothalamus) where these receptor antagonists may work directly to modulate elevated aggression.

Among the antipsychotic medications, risperidone has been the most studied atypical antipsychotic for the treatment of maladaptive aggression in emotionally disturbed children (Findling et al. 2005; Jensen et al. 2007). Indeed, risperidone has been shown to significantly reduce self-injurious and violent behaviors, irritability, agitation and emotional outbursts, as well as decrease relapse rates in children and adolescents with severe behavioral disturbances (Buitelaar 2000; McCracken et al. 2002; Scott and Dhillon 2007; Troost et al. 2005). Results from preclinical studies have provided further evidence supporting the anti–aggressive properties of risperidone. For instance, risperidone has been shown to attenuate aggression induced by social-isolation in mice and apomorphine treatment in rats (Moechars et al. 1998; Rodriguez-Arias et al. 1998; Skrebuhhova-Malmros et al. 2000). Moreover, our laboratory has previously demonstrated that acute and chronic risperidone treatment in a well-documented ethologically valid animal model of heightened offensive aggression reduced aggressive behaviors at doses comparable to those most effective in human adolescents (Ricci et al. 2007a; Schwartzer et al. 2008). While it is not yet known whether antipsychotic medications such as risperidone will effectively suppress the development of aggressive behaviors resulting from adolescent AAS-abuse, the clinical indication of these D2/5-HT2A receptor compounds is in agreement with the findings from the current dissertation and provides direct evidence supporting the hypothalamic neural model developed from these studies.
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7.4 Concluding Remarks

The studies presented in this dissertation provide a significant contribution to the field of aggression with particular emphasis on the effects of adolescent AAS-abuse. These experiments have uncovered the neurobiological consequences of adolescent AAS-abuse on the neural systems governing aggression control in the Syrian hamster. When exposed to AAS, brains of aggressive hamsters synthesize higher levels of dopamine in the LAH, a hypothalamic subregion critical for aggression control. Within the LAH, AAS-exposure increases dopamine $D_2$ but not $D_5$ receptor expression and the aggression eliciting effects of LAH dopamine can be blocked through local antagonism of $D_2$ but not $D_5$ receptors. These findings suggest that an AAS-induced increase in dopamine imparts its aggression eliciting effects through activation of inhibitory $D_2$ receptors. Double-label immunofluorescence studies described in this dissertation revealed that $D_2$, but not $D_5$, receptors are expressed on a subpopulation of GABAergic interneurons in the LAH, suggesting that AAS-induced alterations in dopamine synthesis increases aggression through inhibition of GABAergic neurons resulting in disinhibition of hypothalamic activity. The finding that only a subpopulation of GABA neurons in the LAH is modulated by dopamine led to the investigation of the serotonin neural system and how it modulates GABA through excitatory $5-HT_{2A}$ receptors. Aggressive AAS-treated hamsters showed increased expression of $5-HT_{2A}$ receptors in the LAH. These increases in receptor levels correlated with the elevated aggressive response produced by AAS-treatment. $5-HT_{2A}$ receptors were found to colocalize with a subpopulation of GABAergic neurons indicative of multiple GABAergic pathways in the LAH. Together, serotonin and dopamine are postulated to differentially modulate multiple GABAergic populations in the LAH resulting in differential
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regulation of inhibitory and excitatory neural inputs. Perturbation of these neural systems as a result of AAS-use during adolescence results in increased hypothalamic activity and exaggerated aggressive responding. Given that pharmacology used to treat aggression in clinical youth target D_2 and 5-HT_2A receptors, findings from this dissertation elucidates a plausible brain locus where these drugs may work to directly modulate aggressive behaviors and provide a putative neural mechanism whereby adolescent AAS-abuse alters brain neurochemistry resulting in increased offensive aggression.
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