THE SHR Y CHROMOSOME: INVOLVEMENT IN MECHANISMS INFLUENCING LEARNING, MEMORY, AND AGGRESSION IN THE RODENT MODEL

A dissertation submitted to Kent State University in cooperation with The University of Akron and North Eastern Ohio Universities College of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy

By

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December, 2007
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Chapter I

Introduction

Integration

Genetic mechanisms influencing behavior have increased in complexity and scope as functional genomics and newly emerging methods of genetic analysis become available. When employed in behavioral research, these tools can often shed insight as to how potential environmental factors, as well as a single gene or numerous genes, can indirectly or directly regulate hormones, neurotransmitters, and key enzymes which in turn can influence animal behavior.

The overall purpose of this study is to identify and establish novel neural, physiological and genetic mechanisms involving the Y chromosome, and more specifically, the transcription factor Sry in learning, memory, and aggression. Even though each one of these components is seemingly unrelated, they are in fact different aspects of the same study. Our lab has linked the SHR Y chromosome to various physiological phenotypes, including increased sympathetic nervous system activity, elevated pre-pubertal testosterone, hyper reactive stress responses, and decreased amygdala serotonin content [Ely et al., 1994; Ely et al., 1997; Toot et al., 2004].
Regarding aggressive behavior, animal models have shown testosterone and serotonin to be highly correlated with levels of aggression. Interestingly, gender studies also indicate that testosterone can impair memory consolidation in juvenile and aged male rodents [Fedotova, 1999; Hebbard et al., 2003]. Stress reactive models also tend to exhibit increased indices of aggression following agonist encounters, along with decreased maze performance due to a hyper-reactive stress response [Veenema and Neumann, 2007]. The hippocampus and amygdala show modulation of learning acquisition and memory consolidation in aversive and visual spatial tasks following beta adrenergic receptor activation by norepinephrine [Gliebus and Lippa, 2007; Schimanski et al., 2007]. In addition, beta adrenergic activation has also been linked to aggressive behavior [Korzan et al., 2000; Aleksidze et al., 2001]. Both of these limbic system nuclei are key components in aggression and memory/learning related behaviors, with lesions to either area resulting in decreased memory performance and increased aggression.

An additional component which makes the SHR Y chromosome animal model unique is the breeding scheme which isolates the Y chromosome in different autosomal backgrounds. The SHR Y chromosome gene, Sry, is of particular interest as a transcription factor that is able to increase sympathetic nervous system activity in vitro and following in vivo delivery of the gene [Milsted et al., 2004; Ely et al., 2007]. Sry has the potential through DNA binding sites to interact within a variety of pathways either by modulating tyrosine hydroxylase promoter activity or transactivating dopamine beta-hydroxylase, neuronal tryptophan hydroxylase, and the androgen receptor. Regulation of
any of these factors could influence specific mechanisms for aggression and/or learning and memory.

This introduction will examine how the key components pertain first to aggression and then to learning performance and memory consolidation. The topics covered include: the animal model, behavioral paradigms, neural structures, and the candidate gene, \textit{Sry}.

\textbf{Specific Aim I}: To analyze the role of the SHR Y chromosome in learning acquisition and memory retention tests.

\textbf{Hypothesis}: The hypothesis to be studied is that the SHR Y chromosome (SHR/y males) has a locus that is responsible for decreases in learning and memory performance compared to the WKY Y chromosome (WKY males).

\textbf{Specific Aim II}: To study SHR Y chromosome in relationship to different behavioral paradigms and manipulate the key modulators of aggression, testosterone and serotonin.

\textbf{Hypothesis}: The hypothesis to be tested is that the SHR Y chromosome (SHR/y males) has a locus that is responsible for increased indices of aggression compared to the WKY Y chromosome (WKY males).

\textbf{Specific Aim III}: To analyze the role of the transcription factor, Sry, in transfection studies focusing on aggression, learning acquisition, and memory retention tests.

\textbf{Hypothesis}: The hypothesis to be tested is that the transcription factor, Sry, on the SHR Y chromosome is responsible for impaired water maze performance and increased aggression.
Figure 1.1. Proposed mechanism illustrating how the transcription factor Sry can increase aggression and decrease memory performance. The schematic diagrams several interactions between physiological, genetic, and experimental components used in this study.
Aim I: Detailed Pathway for the SHR Y Chromosome, Learning Acquisition, and Memory Retention

Figure 1.2 Proposed mechanism specific to Aim I. The following pathway presents various aspects of how the SHR Y chromosome, corticosterone, stress, and the hippocampus can be responsible for impaired memory performance (increased platform latency).
Aim II: Detailed Pathway for Aggressive Behavior, Serotonin, Testosterone, and the SHR Y chromosome

Figure 1.3 Proposed mechanism specific to Aim II. The following pathway presents various aspects of how the SHR Y chromosome, testosterone, serotonin, and the amygdala can be responsible for increased aggression.
Figure 1.4 Proposed mechanism specific to Aim III. The following pathway presents various aspects of how Sry1 can influence behavior post neural transfection. Sry 1 delivery to the amygdala will increase aggression indices; whereas, Sry 1 delivered to the hippocampus will impair memory performance (increase platform latency).
### Table 1.1 Strain Derivation Overview

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The derivation of each strain indicating the parental origin of the Y chromosome, autosomes, and the X chromosome is shown in the above table.
Strain Derivation Background

In order to understand the proposed relationship between the Y chromosome and behavior, a description of the strain derivation is necessary. The animal model presented in the following research studies involves the use of normotensive WKY (Wistar Kyoto) and SHR/y (borderline hypertensive) University of Akron (UA) male rats. To develop the consomic strain SHR/y, an SHR male was crossed with a WKY female. Then a single male of this cross was crossed to a WKY female and then a male of this cross was crossed to a WKY female. These crosses have been repeated for over 20 generations creating the strain designated SHR/y. This repeated crossing replaces the autosomal and X linked loci with the alleles of the maternal strain (see Table 1.1).

The creation and phenotyping of the Y chromosome consomic strains can be used to identify genes unique to the Y chromosome or on the region of the Y chromosome that does not cross over with other chromosomes. The repeated crossing of males to females of the WKY strain produces Y chromosome consomic strains. Therefore, with a focus on the Y chromosome, any phenotypic difference between the WKY and SHR/y is consistent with a genetic basis of the Y chromosome. Since the SHR/y strain contains the SHR Y chromosome with the autosomal and X chromosome from the WKY strain, we are able to study the effects of the Y chromosome on different phenotypes.

Although this consomic strain technically only allows for the study of the phenotype in relationship to an entire chromosome and not a specific gene, this method of backcrossing and isolating the Y chromosome in a specific genetic background has been utilized in other studies. For example, Kren et al. [2001] crossed a female SHR and
a male Brown Norway (BN) rat, resulting in a consomic strain with a Y chromosome from the BN male, and a subsequent drop in blood pressure [Kren et al., 2001]. Similar work in our lab by crossing a female SHR with a male WKY resulted in the SHR/a/UA (WKY Y chromosome with SHR autosomes and X chromosome) male with a similar drop in blood pressure [Ely et al., 2000]. Human studies by Ueharra et al. [1998] have found that male offspring with normotensive mothers and hypertensive also have elevated blood pressure, while daughters remain normotensive.

**Y chromosome and Sry**

The Y chromosome is divided primarily into two regions, the pseudoautosomal region (PAR) and non-recombining or male specific region of the Y chromosome (NPAR). The NPAR region is able to recombine intrachromosomally, or within itself [Skaletsky et al., 2003]. A significant amount of research has involved the Y chromosome in mammals as being responsible for the determination of the male phenotype, specifically through the expression of the testis determining factor, called Sry [Koopman et al., 1990; Skaletsky et al., 2003]. On the Y chromosome, Sry is located in this NPAR region, specifically within the X degenerate region [Graves, 1995; Graves, 1998] and therefore does not cross over with other chromosomes.

Sry is an intronless gene [Nagamine et al., 1999] that encodes a protein that has an 80 amino acid High Mobility Group (HMG) box [Koopman, 2001] that is associated with DNA binding [Werner et al., 1995]. The HMG box of Sry shows homology to the HMG1 and HMG2 protein families, proposing a functional role as transcription factor
[McElreavey and Fellous, 1999]. This HMG box is conserved within many mammalian species, with mutations in this sequence resulting in sex reversal, typically female phenotypes [Hawkins et al., 1992; Li et al., 2001].

The Sry locus on the mammalian Y chromosome is responsible for testis determination and Sry expression occurs at approximately 12 days post coitum in the genital ridge of the embryonic tissue. At this point, the Sry protein likely interacts with the developmental genes: SOX 1, 3, 9, DAX 1, and Mullerian Inhbiting Hormone (MIS) [Goodfellow and Lovell-Badge, 1993; Werner, 1995; Graves, 1998]. Sry functions as a transcription factor [McElreavey, 1993] to initiate and facilitate the switch from a bipotential gonad into testis [Koopman, 2001]. Although this developmental role was the first known function, it is probably not the only function of Sry as a transcription factor.

Research using the previously described strains (WKY and SHR/y) has found that the SHR Y chromosome in the presence of normotensive WKY autosomes plays an important role in the hypertensive phenotype [Ely et al., 1993]. By expanding on this study of the Y chromosome and genes located on the Y chromosome, our lab has focused on Sry as a candidate gene for hypertension. Turner [et al., 2007] has identified and isolated six unique forms of Sry found at six loci on the NPAR region of the Y chromosome from the parental SHR UA strain.

Interestingly, most mammalian species only have a single Sry locus, with SHR males possessing six distinct copies. Rodents have multiple copies of Sry [Nagamine, 1994; Bullejos, 1997; Lundrigan and Tucker, 1997; Turner et al., 2007]. SHR Sry loci were isolated and characterized initially from an Sry genomic library derived from male
SHR liver tissue [Turner et al., 2007]. This screening and characterization in our lab identified three copies of Sry, which were labeled Sry 1,2,3; with further analysis using PCR and PCR related techniques identifying Sry 3B, 3B1, and 3C [Martin, 2002; Turner et al., 2007]. The multiple copies of Sry are all transcribed and translated into a potentially functional protein [Underwood, 2003; Turner et al., 2007].

The concept of Sry as a transcription factor has been studied in great deal during development through Sry’s HMG box DNA binding domain. Likely, Sry acts as a cofactor and interacts with other transcription factors, as well as being able to form dimer like structures able to further influence gene expression [Werner et al., 1995; Ukiyama et al., 2001]. Forwood [et al., 2001] indicates that Sry proteins contain two nuclear localization signals flanking the DNA binding domain, which interact with cytosolic receptors facilitating movement into the cell’s nucleus. Sry can bind to DNA response elements at the minor groove of the target sequence and thereby modulate transcription [Werner et al., 1995; Margarit et al., 1998]. Specifically, Sry can act as an antagonist with SOX3 [Graves, 1998], or to initiate transcription of MIS, Fra-1 and 2 [Goodfellow and Lovell-Badge, 1993; Werner et al., 1995], as well as its potential interactions of AP1 [Cohen et al., 1993].

Milsted [et al., 2004b] identified Sry transcripts in heart, brain, kidney, and adrenal tissue of adult male SHR/y. Studies measuring Sry mRNA indicate that the various Sry’s are in fact transcribed in unique profiles within specific brain nuclei [unpublished data], testis, adrenal and kidney [Turner et al., 2007]. If a single Sry locus was performing functions in addition to testis determination, one would expect to find
Sry expressed in tissue beyond sexual differentiation. This is consistent with the literature where Mayer [et al. 1998 and 2000] identified Sry transcripts in brain tissues of humans and mice, both of which only have one Sry copy. Other research pertaining to mouse Sry in the brain found Sry and cDNA within the substantia nigra, medial mammilary body and throughout the cortex in the same regions as tyrosine hydroxylase mRNA [Dewing et al., 2006]. Furthermore, the adult expression of Sry in various tissues supports the possibility that Sry may serve a role in the adult animal.

Therefore, besides being involved with the development of gonads, Sry could be activating transcription of genes in other pathways [Mayer, 1998; Mayer, 2000; Milsted et al., 2004b]. For example, Sry can increase tyrosine hydroxylase activity in vitro in CHO cells [Milsted et al., 2004a]. In addition, previous research in our lab has shown that Sry transfected into the adrenal gland results in increased adrenal tyrosine hydroxylase activity, blood pressure following stress and basal plasma catecholamines [Ely et al., 2007]. Additional results in our lab with in vivo renal Sry delivery show an increase in blood pressure.

**Genetics**

The choice of animal model is important in determining which genetic factors are of relevance, because of genetic differences between the models being used and the specific type of aggression being studied. Many of the previous examples involving the SHR UA strains have involved the hypertensive phenotype, thereby showing a strong link between the SHR Y chromosome and hypertension. By using this well defined
animal model and expanding it to behavioral research, similar comparisons between behavior and the SHR Y chromosome can be made and justified.

Genes products are believed to affect aggression by acting on the motivating mechanisms, cues for attack, and sensitivity to specific stimuli [Maxson, 1998]. There is also evidence in inbred and selected strains of mice that offense is heritable [Simon et al., 1998], supporting the genetic linkage of aggression. Current research has proposed roles for the effects of eleven genes, including: steroid sulfatase (Sts, steroid synthesis enzyme), monoamine oxidase (Mao, catecholamine synthesis enzyme), and the testis determining factor (Sry, developmental transcription factor) on different types of aggression in mice [Maxson, 1998]. The sequence of these genes is conserved in the mouse, rat, and human models. The effects of these genes on different types of aggression are considered as a prologue for exploring their use as accurate models for specific forms of human aggression [Maxson, 1998].

The candidate gene of particular interest in our lab, is the transcription factor Sry. Since Sry is located on the Y chromosome, as previously described, a Y chromosome link to aggression could be due to an Sry related pathway. Previous evidence for a Y chromosome effect on aggression in the SHR UA strains indicated that SHR/y males showed the increased aggression compared to WKY males in the number of attacks and scarring, to both the novel and reintroduced colony intruders [Toot et al., 2004]. Research in mice suggests that the Y chromosome influences the etiology of intermale aggression in juveniles [Maxson, 1981] and adults [Elias, 1975; Carlier, 1991; Oortmerssen et al., 1987] and that Sry may be involved [Maxson, 1998]. Other studies
involving the Y chromosome and mice further suggests that one or more genes can be attributed to differences in intermale aggression [Carlier, 1990; Sluyter, 1994; Carlier 1996] and the development of aggression in males [Oortmerssen et al., 1987; Maxson, 1998]. In addition, the Y chromosome has also been identified as being involved with other behavioral characteristics including urinary odor type distinguishing factors [Yamazaki, 1990], which influence social recognition. There is also evidence for adult sensitivity to testosterone due to a Y chromosome effect on the differential timing of the prenatal developmental occurring testosterone peak [Oortmerssen et al., 1987]. These results are similar to rodent studies where the SHR Y chromosome had an earlier pre-pubertal rise in testosterone and where SHR Y chromosome males show differences in testosterone sensitivity compared to WKY males [Ely et al., 1994].

**Housing**

The environmental concerns and overall testing situation are essential in obtaining accurate aggressive or learning/memory associated behaviors. Environmental studies dealing with social isolation [Schicknick et al., 1993], colony [Ely and Henry, 1974; McKittrick et al., 1995; Ely et al., 1997; Toot et al., 2004] and visual burrow systems [Mckittrick et al., 1995; Hardy et al., 2002] have all demonstrated the importance of the housing environment on resulting behavioral expression. Some behavioral researchers have criticized the use of rats that are specifically bred to measure one trait as being unrepresentative of the natural occurring behaviors. However, work involving established colonies of lab rats has described attack and defensive behavior for these
animals as being equivalent in both intensity and topography to the agonistic behavior seen in wild rats under similar conditions [Blanchard and Blanchard, 1977].

The colony housing model used in several experiments in this study was based on previous work involving rodent behavior in semi-natural populations. By using this colony design, male and female rats are able to interact through grooming, fighting, playing, mating, eating and sleeping. Our lab has used this particular model as a tool to cause long term social stress which increases stress responsiveness, blood pressure, and resident intruder aggression.
Figure 1.5. The colony housing model consists of a 1.25m x 1.25m center open field cage with four attached side cages 0.6m x 0.6m.
Testing Paradigms for Rodent Behavior

Aggression Paradigms

There are several types of available testing paradigms and research involving aggressive behavior, including: infanticide, resident intruder, intermale, fear induced, provoked, and drug induced. Aggressive behavior generally meets the criteria of being a harmful stimulus directed towards a subject with an evidence of intent causing arousal in the attacker with adverse reactions in the recipient [Bell and Hepper, 1987]. However, a simpler and more widely accepted definition of aggressive behavior is that it is an action by which an animal inflicts or tries to inflict damage on another animal [Hamburg, 1971]. The later definition is easier to quantify and requires less supposition by the researcher.

In an effort to measure aggression, there are a number of behavioral tests used. One of the most common and widely accepted is a form of territorial aggression referred to as the resident intruder test. The resident is the animal that has been in the particular cage for a set period of time, with the intruder being placed into this home cage setting. This is an attempt to measure behavior where one animal is invading another animal’s territory [Bell and Hepper, 1987].

Since a majority of the behavioral paradigms used to study aggression involve dyadic encounters (1 versus 1), focus on the opponents has led to some interesting data. When the neutral cage opponents of a testosterone treated male are castrated males, they are attacked less than intact and testosterone treated males [Christine and Barfield, 1979]. This is likely due to the discrimination of a conspecific as threatening or non-threatening [Breur et al., 2001]. As expected, when a homecage setting is used, there is an increase
in aggression of intact and testosterone treated males against castrated males regardless of the intruder used.

The resident intruder test is important in measuring aggression in a semi-natural environment, where the homecage environment tends to increase rodent aggression compared to neutral cages [Ferrari et al., 1997; Breuer et al., 2001; Tort et al., 2004]. In comparison, a commonly used housing manipulation to induce aggression is termed chronic social isolation which induces extreme aggression in normally non-aggressive lab mice, when the animals are forced into a social situation [Schicknick et al., 1993]. Another common type of aggression stimulus is shock induced fighting. By shocking the paws of the subject, an aggressive response results [Ulrich and Azrin, 1962]. Therefore, measuring aggression through resident intruder tests in a colony environment is more natural.

Physical provocation is a form of irritable aggression in the rat that involves the application of pressure to the distal potion of the subject’s tail. Physical provocation increases aggression in male rats with testosterone treatment, with or without dietary alcohol administration [McGinnis, 2001]. When this physical provocation paradigm is used, repeated irritation can increase aggression in testosterone treated rats, but not in castrated males [Breur et al., 2001]. Other testing examples that increase aggression through irritation or excitation of the stress response include air stress and mild footshock. Air stress simply consists of short intermittent air bursts (i.e. 30 seconds) into the face of the experimental rodent. This test, although not as robust, can increase aggression under certain conditions, i.e. testosterone treatment [Ferrari et al., 1998;
Jasnow, 2000; McGinnis et al., 2002a; McGinnis et al., 2002b]. Whereas the air stress is clearly irritable, the footshock paradigm is somewhat controversial. This paradigm is considered to be pain induced or irritable, depending of the degree and duration of foot pad stimulation. Results from increased stimulation are more consistent often showing increase aggression in rodent models [Baggio and Ferrari, 1980].

Other less commonly used paradigms involve the open field test [Bowman, 2002], which is similar to a neutral cage encounter, except the “cage” is two to three times larger with or without bedding. This test is often employed to measure exploratory behavior, as well as aggression. Animals that show decreased exploratory behavior will be less likely to encounter another conspecific, and therefore exhibit decreased aggression. Interestingly, regarding Sry and exploratory behavior, treatment with an Sry antisense oligonucleotide to rodent central nervous system show decreased exploratory behavior [Dewing et al., 2006; Gatewood et al., 2006], suggesting that this is yet another pathway involving Sry and aggression.

**Learning and Memory Testing Paradigms**

Although memory is difficult to measure directly, behavioral tests and neurotransmitter activity are important determinants. There are a variety of behavioral paradigms used to assess different components and associated brain structures of learning and memory. Several examples include the morris water maze, morris water test, modified holeboard, elevated platform, and the modified land maze [Morris et al., 1982; Diamond, 1999; Fuchs, 1999; Kaut et al., 2001].
The morris water maze (MWM), tests the ability of the hippocampus to process visual spatial information. The MWM test paradigm is a standard test, consisting of a platform hidden underneath the surface of the water filling a large tank, was used in this study to examine the associated visual spatial learning and memory related processes. In addition, various external maze cues (geometric shapes, blocks, colors, etc…) are placed on the walls of the room, with some versions also including intra-maze cues. The animal has a set number of days and trials to learn the location of the platform from several starting locations, referred to acquisition trials. Typically, there is a retention trial run several days later to measure memory performance. In addition, the MWM can also use several maze versions to examine the effects of drug, stress, lesion, and even maze version on the animal. This is of particular interest because the hippocampus is a critical brain region associated with learning and memory storage. Studies have specifically identified norepinephrine and glucocorticoids as being critically involved for hippocampal function and related task performance [Roozendaal, 2002; Roozendaal et al., 2004].

Physiological Components of Behavior

Testosterone

Androgens have been described in the regulation of sexuality, cognition, emotion, and personality [Pucilowski et al., 1985]. Specifically, aggression induced by androgens is modulated by the experimental context in which the interaction occurs [Alboetti and Farabellini, 1994]. Testosterone is commonly identified as being a primary factor
directly related to aggression, where increases in testosterone increase aggression. The ability of testosterone to facilitate the display of offensive aggression was established through investigation with a diverse group of non-primate mammals [Albert et al., 1988]. The relative importance of testosterone levels regulating aggression is evident in behavioral studies showing that levels of aggression depend on the amount of circulating testosterone, as well as the sensitivity of the androgen receptor [Ogawa et al., 1996]. Although this exact pathway is not completely understood, there is a clear positive correlation between increased testosterone and increased aggression [Edwards, 1968; Cologer-Clifford et al., 1999].

Clearly, testosterone and a variety of testosterone metabolites are able to increase aggression [Lumia, 1994; Feinberg, 1997]. The use of nandrolone, a form of testosterone involved with muscle growth, increases aggression in rats [Long et al., 1996]. Stanozolol is similar to nandrolone, but does become metabolized into estrogen [Martinez-Sanchis, 1998]. Many studies using androgen treatment often utilize a testosterone variant that is non-aromatizable, meaning the final product is testosterone or a derivative of testosterone, like dihydrotestosterone. This raises the possibility of aggressive behavior not only being regulated by testosterone, but also by both the androgenic and estrogenic metabolites of testosterone [Shrenker and Maxson, 1986].

There are multiple neuroendocrine regulatory pathways that can be activated by stimulation of distinct testosterone sensitive systems [Simon et al., 1998]. Research suggests that testosterone modulates serotonin expression as the major neurotransmitter involving aggressive behavior [Bell and Hepper, 1987], potentially through inhibiting the
rate limiting enzyme for serotonin synthesis. Testosterone has also been indirectly related to serotonin levels in the central nervous system as a whole, which lower serotonin concentrations resulting in increased aggression, specifically within the limbic system [Keleta et al., 2007].

Serotonin

The monoamine, serotonin (5HT), is grouped into the indolamine class of neurotransmitters, which are commonly associated with maintaining normal brain function in response to external and internal stimuli. These studies often include correlating 5HT with specific states of behavior such as, depression, circadian rhythms, anxiety, aggression, cognition, epilepsy, and reproduction [Clement, 1999].

Because of serotonin’s role in behavior, it has become a prominent neurotransmitter in relationship to studying neurological disorders. As well as being an important neurotransmitter in the nervous system, serotonin has also been identified in the blood. From this point of view, serum 5HT is involved in the regulation of the digestive system and certain types of muscle function [Clement, 1999]. This is probably via some intermediate or transport/chaperone molecule preventing 5HT from being quickly degraded.

As a neurotransmitter, 5HT synthesis and subsequent metabolism has been studied in great detail. The starting block of 5HT synthesis is the essential amino acid, tryptophan. This amino acid is taken in from the diet and in the case of neurotransmitters, transported to the serotonergic neurons. These neurons are found all
throughout the brain, with especially large concentrations in the raphe nucleus, and pineal gland. These neurons further support the important regulatory role of 5HT in maintaining homeostasis. From this point, tryptophan is converted into 5-hydroxytryptophan by the rate limiting enzyme tryptophan hydroxylase. Interestingly, two different isoforms of tryptophan hydroxylase have been identified as non-neuronal (TPH1) and neuronal (TPH2) [Abumaria et al., 2007; Zill et al., 2007]. Western blot and immunohistochemistry with antibodies specific for TPH1 and TPH2 show TPH2 to be present in only the hippocampus and raphe nucleus, with TPH1 being found in the pineal gland. Therefore, only a limited number of brain nuclei are capable of utilizing TPH2, which is believed to be involved with the serotonin and aggression mechanism. In the completion of the serotonergic pathway, the aromatic amino acid decarboxylase then converts 5-hydroxytryptophan into the product, serotonin. After serotonin is produced it is then release into the synaptic cleft. This pathway suggests that there is only one form of serotonin present in the body, with several subtypes of various enzymes being utilized.

Problems with this overall serotonergic mechanism can have severe consequences as seen in a number of neurological disorders, such as; schizophrenia, social phobias, obsessive compulsive disorder, post-traumatic stress, and Parkinson’s disease. This has led the pharmaceutical industry to develop new methods for controlling the end product serotonin, by directly regulating TPH activity, or through another metabolic pathway. Several examples of commonly used drugs include the use of TPH inhibitors, selective serotonin reuptake inhibitors (SSRI), and monoamine oxidase inhibitors.
Animal studies with the p-chlorophenylalanine (PCPA) show a general decrease in serotonin synthesis and elevated levels of aggression [Korpela and Sandnabba, 1998; Lesch et al., 2003]. This is because the PCPA acts to selectively inhibit function of the rate limiting enzyme TPH. Regarding neural tissue, the effect of PCPA apparently targets the raphe nucleus and to a much lesser degree the hippocampus [Korpela and Sandnabba, 1998; Mayorga, 2001]. However, many of the other pharmacological manipulations involve increasing serotonin concentrations within the neuronal synapse rather than decreasing it.

For example, animals treated with the SSRI, fluoxetine, show a larger concentration of serotonin present in the synapse, which ultimately decreases aggression [Molina et al., 1987]. Examples of other drugs that increase serotonin through similar pathways include elfoprazine, quipazine, and even a synthetic precursor of tryptophan, which all decrease aggressive behavior to varying degrees. This is supported in several animal models, where basal concentrations of serotonin present in the central nervous are increased in nonaggressive animals such as Norway rats and silver foxes [Popova et al., 1991]. Molina [et al., 1987] was able to inhibit muricidal aggression in rats treated with fluoxetine. Fluoxetine was also able to inhibit footshock induced aggression in paired rats [Datla, 1991]. Additional studies with monoamine oxidase inhibitors (pargyline or selegiline) act to slow the breakdown of serotonin into its metabolites, such as melatonin and 5 hydroxyindoacetic acid. The end result for both of these major drug types in behavioral animal models is increased synaptic serotonin and therefore decreased aggression.
The synthesis of serotonin and therefore aggression can be controlled by the rate limiting enzyme tryptophan hydroxylase. Drug treatments that act to increase serotonin levels, function to decrease aggression [Valzelli, 1982; Olivier, 1987; Eichelman, 1990]. Several studies have also shown that tryptophan dehydroxylase mRNA levels can be increased with estrogens or decreased with testosterone [Hirio et al., 2006]. The activity of TPH2 can be increased by repeated social defeat in a social context [Amstisklavskaya and Kudryavtseva, 1997; Gardner et al., 2005]. With increasing the TPH2 activity, the amount of serotonin will increase, which typically decreases aggressive tendencies [Diez et al., 1976; Kulikv et al., 1989; Amstisklavskaya and Kudryavtseva, 1997]. Therefore, TPH activity is an important indicator of potential aggressive behavior that can be regulated by both physiological and genetic components.

Drugs such as PCPA, that decrease 5HT concentration are inhibitors of serotonin synthesis, particularly by decreasing or acting as antagonists to TPH [Datla et al., 1991]. PCPA treatment increases aggression [Korpela and Sandnabba, 1998] and can also increase sexual behavior [Tsutsui et al., 1994]. When PCPA is used in conjunction with testosterone, experimental male mice show an even further increased level of aggression [Korpela and Sandnabba, 1998].

Outside of drug treatment through pharmacological agents, direct chemically induced lesions to raphe nucleus can also increase aggression [Molina et al., 1987]. Since the raphe nucleus has dense serotonergic inputs to the limbic system, specifically the amygdala, the end result is decreased serotonin and increased aggression. Lesion studies to the hippocampus have shown mixed results, either increasing or decreasing aggression
[Becker et al., 1999; Machado et al., 2006]. This could be due to the particular strain used, as well as the extensive role the raphe nucleus plays in neuronal serotonin synthesis and homeostasis.

**Serotonin Receptors**

Although serotonin receptors are not specifically manipulated in the following studies, their role in aggression and behavior warrant a brief discussion. Molecular studies have lead to the identification of seven receptor families, identified as 5HT 1-7. These receptors are present on either side of the synapse, and can therefore regulate 5HT reuptake, synaptic release, and transport.

The 5HT1 family has been the most studied because of its pathological effects and direct effect in human populations. This receptor family is currently divided into subtypes consisting of 5HT1 (A, B, Da, Db, E, and F). All members of the 5HT1 family have seven membrane spanning domains, with the amino acid sequence of these domains showing the least variability in comparison to other cloned biogenic amine receptors. In addition, the third cytoplasmic loop shows the greatest variability in its amino acid sequence, and is believed to be the key to interacting with the G-protein complex.

Estrogen is believed to decrease aggression through acting on the 5HT1A and 5HT1B receptor subtypes, in addition to increasing 5HT transporter mRNA [McQueen, 1999]. Knockout mice have been developed for the 5HT1B receptor, which show increased intermale aggression [Bruner and Hen, 1997]. These two receptor subtypes have increased in research focus, in part due to the anatomical location within the central
nervous system, particularly within the limbic system. Serotonin can bind to the 5HT$_{1A}$ receptor (somatodendritic) and the 5HT$_{1B}$ (terminal autoreceptors), allowing these receptors to modulate 5HT release from the presynaptic neuron [Veenstra-VanderWeele et al., 2000]. Serotonin antagonists and agonists that bind to 5HT$_{1B}$ receptors are also able to increase or decrease aggression [Everts, 1997]. The role of the 5HT$_{1B}$ in regulating an aspect of aggression within the limbic is further supported by *in vitro* analysis of 5HT in the raphe nucleus and hypothalamus where 5HT$_{1B}$ receptors are predominately found [Moret, 1997].

The Neuroendocrine System and Behavior Regulation

**Limbic system**

The previously discussed behaviors dealing with aggression, as well as associated specific neurotransmitters acting in discrete brain regions like the hypothalamus or amygdala are linked together through the limbic system. The limbic system is commonly referred to as the emotional or motivational “center” of the brain. This system actually composes a complex network of brain nuclei, some of which includes the thalamus, olfactory bulb, frontal cortex, amygdala, hippocampus, and hypothalamus. The hypothalamus is the first point at which an external stimulus can be observed and integrated to affect the body. How or why an animal responds to a specific situation depends if it considered to be threatening or non-threatening. Following stimulation of the hypothalamus, the hypothalamic pituitary adrenal (HPA) axis can increase in activity. This complex and well integrated system can influence specific hormone secretions such
as serotonin, corticosterone, adrenocorticotrophin hormone, and norepinephrine. In addition, this system can have whole system effects influencing reproductive, cardiovascular, immune and neuroendocrine systems. Additional systems which are of particular interesting include the sympathetic adrenal medullary (SAM) axis, which ultimately results in norepinephrine release into the systemic circulation. The two primary limbic system areas involved with aggression, learning, and memory are the amygdaloid complex and the hippocampus.

**Amygdala and Aggressive Behavior**

In terms of aggression, the amygdala is the primary neurological component of the limbic system associated with aggression. In part, due to this basic functional role, the amygdala is often referred to as the fear or rage center of the brain. There are several neural networks which connect the amygdala to other portions of the brain via neural networks, for example to the hypothalamus, cortex, and hippocampus. The concept of social intelligence and learning, involves this network of connections specifically involving the amygdala [Baron-Cohen et al., 2000]. The amygdala contributes to the initial increases in fear and blood pressure, as well as, freezing behavior [Antoniadis and McDonald, 2000]. Reduced serotonin content in the amygdala has been linked to increased aggression and the SHR Y chromosome [Toot et al., 2004]. The amygdaloid complex through decreased levels of serotonin, decreased activity of neuronal tryptophan hydroxylase, elevated stress activation, can increase aggression in a majority of previously mentioned testing paradigms.
In aggressive mice, decreased content of 5HT was found in the hypothalamus, supra-optic hypothalamic nucleus, and amygdala compared to non-aggressive animals [Devoino et al., 2004; Giammanco et al., 2005], indicating these areas of the limbic system may be interacting through neurological pathways modulating aggression. Further support for reduced serotonin in the amygdala and increased aggression was shown by stimulation of amygdala 5HT receptors that caused an inhibition of offensive and muricidal behavior in isolated rats [Pucilowski et al., 1985]. There are several neural networks (vomeronasal, corticoamygdalar, and thalamoamygdalar) that connect the amygdala to sensory systems [Adolphs and Spezio, 2006] and other portions of the limbic system [Bruchey et al., 2007; Nelson and Trainor, 2007], which are also involved in aggression. For instance, in response to threatening stimuli, the amygdala can contribute to initial increase in heart rate and blood pressure, as well as, freezing behavior in rodents [Mitsushima et al., 2006].

The amygdala, specifically the medial nuclei has connections with the olfactory system via vomeronasal input [Kevetter and Winans, 1981], while the lateral nuclei is connected to the accessory olfactory bulbs [Scalia and Winans, 1975]. Mice lacking this particular connection, displayed significantly reduced levels of copulatory behavior and intermale aggression [Scalia and Winans, 1975; Kevetter and Winans, 1981]. Connections linking the olfactory and non-chemosensory areas of the amygdala identify communication pathways of chemosensory information to the hypothalamus [Merchenthaler, 1984]. Since chemosensory input is a key mechanism for identification
in rodents, this suggests a strong role of inter and intra species identification in potential behavioral responses.

The relative importance of the amygdala as a moderator in the catecholaminergic system involved with learning and memory has been documented for over fifty years. However, only more current studies utilizing specific pharmacological agents indicate that circulating norepinephrine can enhance memory [Hatfield and McGaugh, 1999; Hatfield et al., 1999]. This is potentially through the increase in central nervous system norepinephrine content and release present under basal and stress related situations [Hatfield et al., 1999]. In addition to the commonly thought of role as the emotional response center of the brain [LeDoux, 1993], the amygdala is also involved in the attention and arousal reactivity related to memory retention and learning acquisition [Easton and Gaffan, 2000; Lang et al., 2000; Delgado et al., 2006].

Learning/Memory Performance

Just as the serotonergic pathways were important for aggression in the amygdala, there are adrenergic projections throughout the brain involving the vomeronasal organ, hippocampus, hypothalamus, and stria terminalis [Berlau et al., 2006]. Specifically, efferent pathways from the amygdala are critical for controlling other nuclei apparently involved in other types and forms of learning that are hippocampal independent [McDonald and White, 1993; McDonald and White, 1994]. This research and additional studies suggest that memory consolidation required for long term memory storage is regulated by an amygdaloid network between several limbic system brain regions, i.e.
Hippocampus, involved in memory consolidation [McDonald et al., 2006; McDonald et al., 2007].

The role of the noradrenergic system in the limbic pathway has been linked to both enhancing and impairing memory consolidation [McGaugh et al., 2002; McIntyre et al., 2002], specifically through norepinephrine release within the lateral amygdala and associated structures [Quirarte et al., 1997]. While systemically delivered norepinephrine improves memory following acute delivery [Lee and May, 1995; Miyashita and Williams, 2004], post-training infusion of norepinephrine into the amygdala or hippocampus produces dose dependent enhancements of memory retention [Hatfield and McGaugh, 1999]. Whole animal acute restraint stress also increases widespread norepinephrine release in the limbic system in a dependent manner, with chronic stress impairing memory performance [Quirarte et al., 1997] through raised peripheral catecholamines and/or increased CNS adrenergic activity. Therefore, it is clear that norepinephrine release under acute and chronic conditions resulting from stressful events and/or emotional arousal, mediates amygdala memory consolidation through the hippocampus [Roozendaal et al., 2004; Berlau and McGaugh, 2006]. However, excessive norepinephrine stimulation can ultimately lead to a decrease learning and memory performance.

**Hippocampus and Behavior**

Clearly, integration or cross talk within the various neural structures of the limbic system is responsible for a wide range of behaviors and processes. Neural connections
between the hippocampus and amygdala share various roles, with avoidance of aversive behavior or negative events activating them both. Within the central nervous system, the hippocampus is a primary site of memory formation and retrieval, along with certain areas of the cortex [Clark et al., 2002; Isaacson, 2002; Murray et al., 2007]. The medial temporal lobe is connected through fiber pathways with the hippocampal formation, entorhinal cortex and areas of the frontal, temporal, and parietal cortices [Murray et al., 2007].

Regarding aggression, hippocampal lesioned animals are more aggressive in some behavioral paradigms [Machado et al., 2006], which could be due to a decrease in available TPH2 or even deficits in memory and social recognition. Lesion studies of the hippocampus also show an increase in exploratory behavior [Gasbarri, 1996; Gerlai, 1998], thereby increasing the likelihood of a social and potentially aggressive encounter during an open field or resident intruder test. Social recognition cues, such as urinary odor types, are linked to the Y chromosome and aggression, and also connect to a variety of other brain structures via the hippocampus [Novikov, 1993; Guillet al., 1996]. Reactions to aversive situations and stress responsiveness stimulating fight or flight behaviors are attributed to interactions between the hippocampus and amygdala with potential modulation by the 5HT$_{1A}$ receptor subtype [Pare and Tegani-buit, 1996; Flugge et al., 1998].

As well as being important for visual spatial navigation and exploratory behavior, the hippocampus plays a regulatory role in possibly forming episodic memory and other spatial relationships [Rolls, 2000]. In addition, the hippocampus is also involved with
memory retrieval in spatial tasks [Morris et al., 2003], with damage to the hippocampus impairing visual spatial performance on the morris water maze [Morris et al., 1982] and raised Y-maze [Conrad et al., 2003]. Depending on the time following learning acquisition, hippocampal lesion studies do not always show impaired performance [Oliverra et al., 1997; Mair et al., 19998; Broadbent et al., 2004], because the retrieval of learned spatial tasks becomes hippocampal independent [McClelland, 1995; Manns and Squire, 2001; Clark et al., 2002]. This process may occur through a pathway believed to involve the cortex, amygdala, and long term memory storage. Deficits from drug treatment or stress can occur in the acquisition [Gouirand and Matuszewich, 2005; Labadze et al., 2006; Davis and Riley, 2007] and memory formation phases [Wise et al., 2007; Simon and Setlow, 2006] thereby inhibiting the hippocampal independent storage. When this occurs, particularly from chronic stress, it is often a result of the hippocampus having a mechanism for memory consolidation disrupted and/or CA neurons being damaged [Lathe, 2001; Bowman et al., 2006; McLaughlin et al., 2007].

The hippocampus has a dense input of adrenergic terminals suggesting a strong functional role of norepinephrine in learning and memory retention [Schroeter et al., 2000]. These adrenergic inputs travel to a majority of the hippocampus, including the dentate gyrus, CA1, CA3, and subnucleus, suggesting that the adrenergic system is crucial for learning and memory consolidation [Izquierdo and McGaugh, 2000; McGaugh and Izquierdo, 2000; Roozendaal et al., 2004], under basal conditions, and also related to emotionally demanding or stressful events. Norepinephrine concentrations increase during and following acute stressful events, which likely enhances the learning and
memory mechanisms activated in the hippocampus [Men et al., 1999]. Further real time analysis with *in vivo* microdialysis during tasks involving memory formation or retrieval; show an increased release of norepinephrine within the hippocampus [Men et al., 1999; Tzavoru et al., 2006]. Drugs stimulating norepinephrine release, by directly activating beta adrenergic receptors, or blocking norepinephrine reuptake [Lee and Ma, 1995; Izquierdo et al., 1998; Tzavora et al., 2006] all increase memory performance.

Within the hippocampus, dopamine receptor subtypes are widespread and likely play a role in learning and memory. Dopamine is considered to be a key regulator in memory inhibition and formation. For example, mice deficient in dopamine through lacking the expression of tyrosine hydroxylase show decreased active avoidance learning [Thomas and Palmiter, 1997; Glickstein et al., 2002]. Since tyrosine hydroxylase knock out mice will also show lowered norepinephrine, epinephrine, and dopamine, the exact role for dopamine in memory is not clear in the animal model. However, the use of dopamine beta-hydroxylase (DBH; enzyme that converts dopamine to norepinephrine) knock out mice show no apparent differences in spatial navigation during and immediately following acquisition, whereas testing long term memory consolidation shows impairment [Hagan et al., 1983; Thomas and Palmiter, 1997; Marino et al., 2005; Ouyang and Thomas, 2005]. Further use of DBH knock out mice support this theory that there are different mechanisms and pathways for memory retrieval compared to acquisition [Thomas and Palmiter, 1997; Marino et al., 2005]. However, additional drug studies using beta adrenergic agonists suggest that memory retrieval and acquisition requires some common signaling through beta adrenergic pathways [Garelick and Storm,
The relatively important regulatory role of norepinephrine and beta adrenergic receptor activation for proper function of the hippocampus, indicates a large degree of similarity involving learning/memory with other limbic system structures, i.e. the amygdala [Berlau and McGaugh, 2006].

Social conflicts, such as chronic and acute stress can modulate memory in animal models through not only norepinephrine release, but also through the glucocorticoid, corticosterone. In response to stress, the release of adrenocorticotrophin hormone (ACTH) from the anterior pituitary stimulates the adrenal gland to release corticosterone, which under acute conditions can enhance memory directly or indirectly via stimulating medullary norepinephrine release. However, chronically (3 months) increased corticosterone levels have been shown to decrease hippocampus volume and function, resulting in impaired retention and memory formation [McEwen, 2001; McEwen, 2005].

In regards to corticosterone and stress responsiveness, adrenalectomized male rats given supplemental corticosterone fail to show impaired retrograde spatial memory performance when subjected to stress [Wright et al., 2006]. This suggests that it is the elevated corticosterone levels following stress that are involved with inhibiting acquisition or memory consolidation. Further experimental manipulations involving the corticosterone use direct corticosterone injections or metyrapone (11 beta hydroxysteroid antagonist, inhibits corticosterone synthesis), which increase and decrease circulating corticosterone levels, respectively [Rotllant and Armerio, 2005; Wright et al., 2006].
These studies indicate that there is a physiological range at which corticosterone acts to enhance hippocampal function, where increases or decreases outside of that range result in impairment. This translates into stress induced studies which have further analyzed this corticosterone and memory pathway, where stress could either impair or enhance spatial memory performance, or even have no effect [Watanabe, 1992, Ishikawa et al., 1995; Rottlant and Armerio, 2005]. For example, studies using direct hippocampal infusions with small, acute concentrations of corticosterone show enhanced memory and acquisition performance [Sandi, 1998]. Interestingly, metyrapone showed that blocking the chronic elevation of corticosterone does indeed prevent memory impairment [Liu et al., 1999; Barrett and Gonzalez, 2004; Wright et al., 2006], but if metyrapone is given in excess the corticosterone levels drop to a point where memory retention is impaired [deQuervain et al., 1998]. Genetic studies using knockout mice for 11 beta hydroxysteroid exhibit attenuated memory performance following acute stress [Thomas and Palmiter, 1997; Marino et al., 2005], and therefore are lacking memory enhancing properties of corticosterone either present prior to or following the stress.

Regarding stress, an acute stress would tend to be a brief two to three fold increase from basal plasma corticosterone levels; whereas, chronic stress will initially show the same increase for an extended time period, followed by a subsequent decrease below basal levels. This elevated and initially persistent response following an agonistic encounter raises plasma catecholamines, ACTH, and corticosterone [Haller et al., 1998; Haller et al., 2004; Mikics et al., 2005]. This phenomenon regulated, in part, by the dysregulation of negative feedback control for corticosterone is supported by decreased
CRH mRNA, leading to a decreased CRH response, and therefore decreased corticosterone [Romeo et al., 2007]. This raises several questions regarding acute versus chronic corticosterone levels, with the first being: are there changes in the glucocorticoid receptor number in specific brain regions, or do elevated levels of corticosterone even in acute doses disrupt the negative feedback pathway and result in decreased basal levels thereby impairing acquisition and memory retention.

Externally induced stressors, such as restraint, fear induced, and social isolation all have the ability to alter hippocampal morphology [Kielstrap et al., 2002; Donohue et al., 2006; Zhao et al., 2007] in a number of animal models. Additional behavioral and stress related paradigms using chronic stress have yielded consistent results with chronic activation of the HPA axis producing hippocampal atrophy and spatial memory deficits [McKittrick et al., 2000; Blanchard et al., 2001]. Chronic stress can increase initial and basal corticosterone levels for an extended period of time, as previously mentioned, which can damage the hippocampus [Roozendaal, 2002; Roozendaal et al., 2004]. Similar results are noted in corticosterone treated rats that have decreased cognitive performance and acquisition [Haller et al., 1998; Haller et al., 2004]. Bodnoff [et al., 1995] and others describe long term stress consisting of small groups of males, which can cause learning deficits in rodents regarding spatial memory [Meaney et al., 1991; Bodnoff et al., 1995; McKittrick et al., 2000].

As suggested in several of the previous studies, low concentrations of corticosterone activating their perspective receptors are more protective in nature; whereas, excess corticosterone concentrations cause hippocampal damage. If
corticosterone is chronically elevated, the hippocampus appears to be specifically targeted for neuronal death by exhibiting an increase in atrophy of apical dendrites and pyramidal cells, thereby impeding incoming information into CA3 region of the hippocampus [McKittrick et al., 2000; Herbert et al., 2006]. This damage is consistent in a wide range of animal models where glucocorticoids lead to loss of hippocampal pyramidal neurons if persistent and at high enough levels for chronic conditions [Herbert et al., 2006], with impaired memory almost always resulting. This apparent targeting of the CA region of the hippocampus by glucocorticoids is probably due to the high density of glucocorticoid receptors and mineralocorticoids receptors present in the hippocampus [Sapolsky, 1986; Tombaugh and Sapolsky, 1992]. Although the exact mechanism for how and why elevated corticosterone leads to impaired hippocampal function is not known, research suggests that corticosterone can increase neuronal glutamate concentrations leading to an increased influx of calcium and ultimately neuronal damage [Herbert et al., 2006]. The corticosterone damage hypothesis is supported in studies showing that hippocampal cell proliferation increases following adrenalectomy, although performance on memory related tasks remains compromised [Gould et al., 1992; Cameron and Gould, 1994].

Aging and Learning/Memory

Although aging was not a component of the initial mechanism proposed in this research project, preliminary results in this area have yielded promising data. Therefore, an overview of aged related changes to physiology including stress response,
neurological decline, and changes in behavior for aggression and learning/memory are pertinent to the SHR Y chromosome and behavioral paradigms.

Several studies suggest that the limbic system is particularly vulnerable to aging, showing easily noticeable behavioral impairments [Miguez et al., 1999; Kaasinen et al., 2000]. Age related alterations in emotional responses can be potentially linked to the limbic system, specifically the hippocampus and amygdala [Pisarska et al., 2000]. Based on the previously discussed research, norepinephrine, tyrosine hydroxylase, and dopamine transmission within the limbic system contribute to the acquisition and expression of emotions [Guarraci et al., 1999], which are altered in aged animals. Tyrosine hydroxylase neurons and dopaminergic neurotransmission within the amygdala, limbic system, and central nervous system are likely involved with these changes in behavior and sympathetic nervous system activity [Barili et al., 1998; Emborg et al., 1998; Armbrechet et al., 1999].

Aged rodents, non-human primates, and humans also show other behavioral changes from younger controls. Aged populations show impairments in learning acquisition and memory performance on spatially orientated tasks [Ward et al., 1999]. This decline in performance is believed to be due to the increased neuronal atrophy during aging, which is specific to the hippocampus [Du et al., 2006]. In addition to impairing performance of memory related tasks, this neuronal decline also alters hippocampal synaptic plasticity [Foster, 1999; Rosenzweig and Barnes, 2003]. This neuronal death and altered plasticity of the hippocampus occurs in normal healthy aged populations, however only in relatively small amounts [Morrison and Hof, 1997].
Rodents, non-human primates, and humans undergoing non-pathological aging have decreased atrophy with preserved neuron number in the CA1 region of the hippocampus compared to pathologically aged individuals [Rapp, 1996; Calhoun et al., 1998]. Treatment in rodent models to attenuate this neuronal atrophy by enhancing cell proliferation and stimulating dendritic spinal formation show better performance compared to untreated control rodent and non-human primates [Williams and Herrup, 1988; Manji, 2003; Sampedro and Diaz, 2005]. This decline in overall function, and impaired performance compared to younger conspecifics is defined as the pathological state of “unsuccessful aging” [Hibbard et al., 2000].

Age related dysfunction of the autonomic nervous system is a serious problem affecting an increasing number of elderly populations [Luine et al., 1990a]. The sympathetic nervous system of an aged individual has shown a seemingly wide array of response from hyperactivated to blunted responses [Luine et al., 1990b] depending on the various stressors used. Impaired acquisition and retention in spatial memory tasks in aged rats was primarily associated with decreased norepinephrine activity and an increase in norepinephrine tissue content [Collier et al., 2004]. Changes in norepinephrine content and receptor ligand binding are associated together in a variety of age related mechanisms in performance decline [McEntee and Crook, 1990; Mabry et al., 1995]. As mice age (21months) there was a decrease in tyrosine hydroxylase neurons in the amygdala and hippocampus [Samurajski, 1973; Collier et al., 2004]. This decrease is likely via degeneration of the cell bodies in the substantia nigra and ventral tegmental area [Emborg et al., 1998; Naoi and Maruyama, 1999]. Further changes in nervous
system relating identified in these studies are to dysfunction between central and peripheral system function, showing an overall loss of postganglionic sympathetic neurons in aged individuals.

Further evidence for dysregulation of internal homeostasis is suggested by the glucocorticoid feedback hypothesis of hippocampal aging. This theory states that even minor increases in glucocorticoids, can accelerate impairment and aging of the hippocampus [Sapolsky et al., 1985; Herbert et al., 2006]. Aging can produce a variety of results related to corticosterone either increased in aged human and rodent models or no effect [Aston-Jones and Bloom, 1981]. Aged rodents have a more pronounced response of ACTH and subsequent corticosterone release to acute stressors compared to younger controls [Mclay et al., 1998]. Another factor further potentiating the basal and stressed plasma corticosterone levels is the decrease in the systemic transport for corticosterone, corticosterone binding globulin, in aged populations [Goodyear et al., 2000; Purnell et al., 2004].

Corticosterone levels, however, do clearly change in response to age, with the degree and type of change being strain related [Akirav et al., 2004]. In aged rodents, the increased glucocorticoids shows strong positive correlation with decreased or impaired spatial memory performance and a negative correlation with hippocampal volume [Landfield et al., 1978; Shanks et al., 2000].

Aged rats, besides having decreased hippocampal plasticity, also show a progressive loss of coordination in gene activity of immediate and late acting intermediate genes, following insult or trauma [Schmoll et al., 2005]. Therefore,
problems associated with aging are known to involve a dysregulation of gene transcription, activation, and an overall inability to respond properly to a normal and/or insult physiological variables [Schmoll et al., 2005]. In order for the autonomic nervous system to function properly, there is a need for precise regulation and a certain degree of neural plasticity to effectively adapt to the changing external and internal environments. In response to physiological and/or psychological stressors in aged rats, neural plasticity is partially regulated by a complicated mechanism of gene expression involving: GAP43, NF68, as well as, CREB, BDNF, and ARC [Parhad et al., 1995; Schauwecker et al., 1995; Rapp et al., 2002; Steinert et al., 2002; DelaRosa et al., 2002]. The resulting proteins are necessary for proper regulation of neural morphogenesis and organization of the hippocampus, which are in an altered phosphorylated state during the learning/acquisition phase. This phosphorylated state impairs acquisition, maze performance, and memory consolidation. [Goss and Morgan, 1995; Hirokawa et al., 1996].
Chapter II

Study 1: Long Term Social Stress Impairs Learning and Memory Retention

Introduction

The importance of how the housing environment and resulting social stress can influence behavior is evident in environmental studies dealing with social housing manipulations, such as isolation [Schicknick et al, 1993], semi-natural social colonies [Ely and Henry, 1978; McKittrick et al., 1995; Ely et al., 1997; Toot et al., 2001] and the group housed visual burrow system [McKittrick et al., 1995; Hardy et al., 2002]. Specifically, behavioral research has identified that a socially interactive environment is a useful model for studying chronic stress in relationship to impaired memory performance, aggressive behavior, and also social hierarchies [Bodnoff et al., 1995]. These physiological and psychological stress paradigms typically show that chronic stress acts to alter visual spatial processes in the hippocampus by impairing learning acquisition and memory consolidation through dysregulation or over activation of corticosterone and/or increased norepinephrine in the plasma or central nervous system (CNS) [Rich and Romero, 2005; Veenema and Neumann, 2007; Schimanski et al., 2007].

The stress hormone corticosterone can act through a variety of mechanisms in the periphery or within the CNS to inhibit hippocampal function under periods of chronic
stress [LaSarge et al., 2007; Martin and Clark 2007; von Linstow et al., 2007]. Chronic activation of the stress related pathways increase plasma norepinephrine and corticosterone resulting in impaired learning and memory performance, which was blocked following adrenalectomy [Graham et al., 2006; Spanswick et al., 2007]. In addition, direct infusion of corticosterone or norepinephrine into the hippocampus also has the ability to impair memory performance [Kobayashi and Kobayashi, 2001; Gilsbach et al., 2006]. The rise in corticosterone in response to agonistic encounters can even further potentiate the release of peripheral catecholamines and CNS adrenergic activation. The hippocampus is one of the key target areas for both peripheral corticosterone and neuronal norepinephrine release because of the large concentration of glucocorticoid receptors and the relatively dense adrenergic input.

Many studies of memory and learning performance involve chronic stress and drug delivery and have utilized the morris water maze (MWM) paradigm. Visual spatial processing which is primarily associated with the hippocampus is necessary for successful mapping and maze performance [Morris et al., 1983]. Therefore, the hippocampus is an important brain area to study in memory and learning. Several murine models (CBLA, DBH, and SAL) have further suggested the relative importance of not only basal physiological hormone levels, but also the stress response hormone levels in learning and memory performance related tasks [Kantak et al., 2001; Marino et al., 2005; Dornelles et al., 2007].

Research in our lab with a similar stress hyper-responsive rat model identifies that the Y chromosome from the spontaneously hypertensive rat (SHR) increases sympathetic
nervous system activity, adrenal tyrosine hydroxylase activity, and indices of stress responsiveness [Ely et al., 1994; Ely et al., 1997, Ely et al., 2000]. In addition, these SHR Y chromosome (SHR/y) males when housed in a social colony environment have elevations in aggression, stress arousal, cardiovascular pathology, and renal damage [Andrews et al., 1993; Ely et al., 1994; Toot et al., 2004] compared to WKY Y chromosome (WKY) males. Therefore, the hypothesis to be tested is that males under chronic social colony stress will exhibit impaired MWM learning acquisition, memory retention, and elevated stress responsiveness compared to control males. In addition, we will also test the hypothesis that the stress prone SHR/y males housed in this colony environment will show greater impaired maze performance and stress responsiveness when compared to WKY colony males.

Materials and Methods

Study Design

The objective of this study was to examine how long term social stress would differentially affect SHR/y and WKY males regarding physiology, brain neurochemistry, and maze performance. This study used a two strain (SHR/y and WKY) by two treatment (colony and noncolony) design, initially at 8 weeks of age, with n=8-10 males in each strain and treatment group. The study lasted for a total of 6 months, with behavioral testing taking place over the last two months.
**Animal Model**

The Y chromosome animal model used in this study consisted of the stress sensitive consomic borderline hypertensive (SHR/y) and normotensive Wistar-Kyoto (WKY) rats. The detailed breeding scheme has been previously described, which involves crossing an SHR male with a WKY female for over 20 generations [Ely et al., 2000]. This repeated back crossing replaces the autosomal and X linked loci with the alleles of the maternal strain, while maintaining the SHR Y chromosome. Since the SHR/y strain contains the SHR Y chromosome with the autosomal and X chromosome from the WKY strain, we are able to characterize the SHR Y chromosome [Ely et al., 1993].

**Colony environment**

The colony housing environment was strain specific for SHR/y or WKY rats, consisting of 8-10 males and 8-10 females, all initially 8 weeks of age. The colony model for social stress used in this study was based on previous work involving rodent behavior in a natural population. With this particular design, male and female rats are able to interact socially in a center open field cage (1.25m x 1.25m) center open field cage with four attached side cages (0.6m x 0.6m). The females are included only to allow for social hierarchies to be formed within the colonies. We have shown that both mice and rats in a colony design have more natural behaviors, such as defense of territory, formation of social hierarchies, and competition for females [Ely and Henry, 1973; Ely et al., 1993; Toot et al., 2004] than control males.
Morris Water Maze Protocol

The morris water maze (MWM) test paradigm was used in this study to study spatial learning and memory retention performance, with all acquisition and retention trials videotaped for later additional analysis. The MWM is a standard test consisting of a platform hidden underneath the surface of the water, with external maze cues placed around the room. Specifically, the maze consisted of a 5’ diameter tank filled with room temperature water 1” above the platform (6”diameter), and covered with packing foam to obscure view of the platform, see Figure 2.1 for a more detailed description.

The maze involved three days of acquisition with five trials per day and one retention trial for five days after acquisition day three. Based on which version of the maze was run, the starting location and sequential testing locations will change. The maze for each acquisition day includes five trials. With north (N), south (S), west (W), and east (E) being the various trial start location, therefore one trial was run twice, for example: 1 (N), 2 (S), 3 (W), 4 (E), 5 (W). With W being the first trial of the next acquisition day. Each trial was run in a consecutive order starting in one of four quadrants (N, S, E, and W) for a maximum of 90 seconds. All trials involving the water maze were timed with a timer to the nearest second. After finding the platform (6” diameter), the animal was given a 20 second mapping period before removal from the maze. This time was not necessarily consecutive, when the animal left the platform it was returned to the platform and the 20 second mapping period was continued. Following removal from the maze, each animal was dried off and placed into a cage until the next trial.
The probe trial was video recorded for later analysis of quadrant location, swim speed, and annulus crossings. The probe trial was run for a total of 2 minutes and 30 seconds, with the animal being placed back into the empty cage. For the retention trials, the platform was placed back into the tank at the correct distance. The four remaining trials were then run in the standard order following the standard 90 second swim and 20 second platform protocol.
Figure 2.1. Diagram of the morris water maze tank location and visual spatial cues located through the room at the time of each maze trial (room dimensions 11’ x 12’).
Plasma Blood samples

One week following completion of the MWM test, all animals were stressed and then anesthetized with Sodium Pentothal (50mg/kg, IP; E. Lilly, Indianapolis, IN) with a 2-3 ml retro-orbital blood sample collected between 1100 and 1700 hours and centrifuged for 5 minutes (3,000rpm) to obtain plasma from stressed animals. Animals were again anesthetized a week later prior to termination with a baseline sample being obtained, centrifuged and stored at -70C until analysis of plasma norepinephrine, corticosterone, and adrenocorticotrophin hormone.

Palkovit’s Brain Punch

Following termination, the brains were frozen on dry ice, stored at -70C, and later cut into 200 micron coronal sections at -20C (Minotome, International Equipment Company, Damon Division, Meedham Hts., Mass.). The sections were then punched [Palkovits, 1973] from the hippocampus with a modified 16 gauge needle, as previously performed in our lab [Toot et al., 2004]. Proper coronal slice and punch orientations were verified with comparison to the rostral-caudal bregma zero reference points [Pellegrino, 1981]. The punches were then placed in either mobile phase or sucrose, homogenized, centrifuged for 10 minutes at 3200rpm (Sorvall RT7, Kendro Labs, Newton Conn.) and stored at -70C for later analysis.

The norepinephrine content for the hippocampus was analyzed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters 460 Detector, Milford, MA). The mobile phase for the hippocampal punches consisted of
Citric Acid (35mM), Sodium Acetate (90mM), Octyl Sodium Sulfate, (460uM), EDTA (130uM) and 14% Methanol at a pH of 4.7. The HPLC pump, Water 510 (Waters Corp., Mildford, MA) was set at 1.3ml/min. The column was a Supelcosil LC-18, 15cm x 4.6mm, 3um which was preceded with a guard column (Discovery RP-Amide C16 2cm x 4.0mm, 5um, Supelco, Bellefonte, PA). A constant amount 2,3-DHBA was spiked into each sample and the peak height ratio was calculated electronically.

The sucrose (0.25M) solution for the hippocampal punches was used for the tyrosine hydroxylase assay. Tyrosine hydroxylase activity was calculated by measuring L-DOPA formed per milligram of tissue per minute. The homogenate was added to both a blank tube and a reaction tube. The chemical reaction was based on procedures developed by Nagatsu et al. [1979] and modified by Hooper et al. [2000] and Kumai et al. [2000] followed by extraction using the same method as for norepinephrine.

Plasma Samples

Plasma norepinephrine was analyzed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters 460 Detector, Milford, MA) in a similar manner as described above for the hippocampal brain punch homogenates.

Adrenocorticotropic hormone (ACTH) was analyzed in plasma by RIA (#24130, DiaSorin, Stillwater, Mn). The intra-assay variation was 8.1 %, the inter-assay variation was 6.7 %, with a sensitivity at 15pg/ml at the 95% confidence limit, and with
the highest cross-reactivity being Porcine ACTH 1-39 and Human ACTH 1-24 at 100%, with other peptides at <0.01%.

Corticosterone was analyzed in plasma by RIA (DSL-80100, Diagnostic Systems Laboratories, Inc., Webster, Tx.). The intra-assay variation was 3.43%, the inter-assay variation was 7.3%, with a sensitivity at 2.7ng.ml at the 95% confidence limit, and with no reported cross-reactivity.

Statistics

Statistical analysis was performed by using: Two-way RM ANOVA and a post-hoc Bonferroni test, One and Two-way ANOVAs, and Student’s t-test where applicable. Analyses were run on SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA) with significance assumed at p<0.05.

Results

Analysis of the SHR/y and WKY controls showed that maze acquisition trial performance was not significantly different (Figure 2.2). However, in both strains there was a significant improvement in acquisition performance over the three trial days (F=19.3, p<0.001). This was also noted for the SHR/y and WKY colony males, which were not significantly different from each other (Figure 2.3), but did show improvement in acquisition performance over the three trial days (F=38.0, p<0.001).

There was significant impairment in the SHR/y colony acquisition trial performance, as shown by the higher platform latency times at days one and two.
compared to the SHR/y controls (Figure 2.4, p<0.05). Although the trial performance tended to improve over the three days of testing (F=32.4, p<0.001), there was still an effect of the colony environment impairing overall acquisition trial performance (F=9.4, p<0.01).

WKY colony and control males also showed a significant impairment in acquisition trial performance, but only at day one (Figure 2.5, p<0.05). Although the trial performance improved over the three days of testing (F=21.5, p<0.001), there was a significant effect of the colony environment impairing the overall acquisition trial performance (F=4.9, p<0.05),

SHR/y and WKY control retention trial performance taken five days after acquisition day three was not significantly different between strains (Figure 2.6). This was also the noted in the retention trial performance for SHR/y and WKY colony males, which were not significantly different from each other (Figure 2.7). When comparing the SHR/y colony to control males, the SHR/y colony was significantly impaired (Figure 2.8, p<0.05). However, WKY colony to control (Figure 2.9) comparison showed no significant difference in retention trial performance.

SHR/y colony norepinephrine content in the hippocampus was elevated compared to SHR/y control (p<0.05) and WKY colony males (Figure 2.10, p<0.05). There was also an interaction effect between the Y chromosome and colony housing environment (F=5.5, p<0.05). The SHR/y control tyrosine hydroxylase activity (Figure 2.11) in the hippocampus was greater than the SHR/y colony (p<0.05) and WKY control (p<0.01). The SHR/y colony tyrosine hydroxylase activity was also greater than the WKY colony.
The SHR Y chromosome effect increased tyrosine hydroxylase activity (F=17.0, p<0.001), while the colony housing environment decreased tyrosine hydroxylase activity (F=4.3, p<0.05).

Stress increased SHR/y and WKY control male plasma corticosterone (p<0.05) from basal levels (Figure 2.12). The colony WKY males showed no change from baseline corticosterone following stress, whereas the SHR/y colony males had an increase in corticosterone following stress (p<0.05). The SHR/y colony males also showed a decrease in baseline corticosterone when compared to SHR/y baseline controls (p<0.05).

SHR/y and WKY control male stressed plasma adrenocorticotrophin hormone was increased from basal levels (Figure 2.13). The SHR/y and WKY colony males did not show this same stress effect increasing plasma adrenocorticotrophin. However, the colony males from both strains did show an increase in basal adrenocorticotrophin compared to their respective controls (p<0.05).

SHR/y and WKY male colony and control male plasma norepinephrine increased from basal levels following restraint stress (p<0.05). SHR/y colony baseline norepinephrine increased from SHR/y control and WKY colony baseline levels (Figure 2.14, p<0.05), whereas WKY colony and control baselines remained the same.

SHR/y and WKY colony males did not show significant differences in the number of hind limb scars, plasma norepinephrine, and plasma epinephrine between colony members for each strain.
Maze Acquisition Performance Improves Over Time in SHR/y and WKY Control Males

Figure 2.2. Acquisition platform latency (seconds) for SHR/y and WKY control males (means, ± SEM). There were no significant differences between SHR/y and WKY control males for each acquisition day. Maze performance did show significant improvement over the three acquisition days for both strains (Two-way RM ANOVA, F=19.3, ***p<0.001).
Maze Acquisition Performance Improves Over Time in SHR/y and WKY Colony Males

![Graph showing maze acquisition performance](image)

Figure 2.3. Acquisition platform latency (seconds) for SHR/y and WKY colony males (means, ± SEM). There were no significant differences between SHR/y and WKY colony males for each acquisition day. Maze performance did show significant improvement over the three acquisition days for both strains (Two-way RM ANOVA, F=38.0, ***p<0.001).
SHR/y Colony Male Maze Acquisition Performance is Impaired Compared to SHR/y Control Males

Figure 2.4. Morris water maze platform latency acquisition (seconds) for SHR/y control and colony males (means, ± SEM). Colony acquisition performance on days 1 and 2 were significantly impaired compared to control male acquisition (*p<0.05). There was a significant effect (Two-way RM ANOVA) of colony housing (F=9.4, **p<0.01) and trial day (F=32.9, ###p<0.001).
WKY Colony Male Maze Acquisition Performance is Impaired Compared to WKY Control Males

Figure 2.5. Morris water maze platform latency acquisition (seconds) for WKY control and colony males (means, ± SEM). Colony acquisition performance only on day 1 was significantly impaired compared to control male acquisition (*p<0.05). There was a significant effect (Two-way RM ANOVA) of the colony housing (F=4.9, *p<0.05) and trial day (F=21.5, ###p<0.001) on maze performance.
Retention Trial Performance is Similar For SHR/y and WKY Control Males

Figure 2.6. Average retention trial performance (seconds) for SHR/y and WKY control males (means, ± SEM). There was no significant difference in retention trial performance between SHR/y and WKY control males.
Retention Trial Performance is Similar For SHR/y and WKY Colony Males

Figure 2.7. Average retention trial performance (seconds) for SHR/y and WKY colony males (means, ± SEM). There was no significant difference in retention trial performance between SHR/y and WKY colony males.
SHR/y Colony Males Show Impaired Retention Trial Performance Compared to SHR/y Control Males

Figure 2.8. Average retention trial performance (seconds) for SHR/y control and colony males (means, ± SEM). SHR/y colony male retention trial performance was significantly impaired compared to control males (*p<0.05).
Retention Trial Performance is Similar Between WKY Colony and Control Males

Figure 2.9. Average retention trial performance (seconds) for WKY colony and control males (means, ± SEM). There was no significant effect of colony treatment on retention trial performance.
Hippocampal Norepinephrine Content is Increased in SHR/y Compared to WKY Colony Males

<table>
<thead>
<tr>
<th>Strain and Treatment</th>
<th>Norepinephrine Content (pg/mg tissue)</th>
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<tbody>
<tr>
<td>SHR/y Control</td>
<td>1500</td>
</tr>
<tr>
<td>SHR/y Colony</td>
<td>2250</td>
</tr>
<tr>
<td>WKY Control</td>
<td>1750</td>
</tr>
<tr>
<td>WKY Colony</td>
<td>1500</td>
</tr>
</tbody>
</table>

Figure 2.10. Norepinephrine (NE) content (pg/mg tissue) in the hippocampus of SHR/y and WKY colony and control males (means, ± SEM). SHR/y colony hippocampal NE content was significantly greater than SHR/y control (*p<0.05) and WKY colony (‡p<0.05) hippocampal NE content. Hippocampal NE content showed an interaction effect for the SHR Y chromosome and colony housing (Two-way ANOVA, F=5.48, p<0.05).
Hippocampal Tyrosine Hydroxylase Activity is Decreased in SHR/y Colony Compared to Control Males

**Figure 2.11.** Tyrosine hydroxylase (TH) activity (fmol/mg/minute) in the hippocampus of SHR/y and WKY colony and control males (means, ± SEM). SHR/y control hippocampal TH activity was greater than SHR/y colony (*p<0.05) and WKY control (**p<0.01). SHR/y colony hippocampal TH activity was greater than WKY colony (*)p<0.05). Tyrosine hydroxylase activity was effected by both the strain (Two-way ANOVA, F=17.0, p<0.001) and colony housing (F=4.3, p<0.05).
Figure 2.12. Plasma corticosterone (CORT) for both strain and treatment groups taken at baseline and following restraint stress (means, ± SEM). There was a significant effect of stress on control SHR/y and WKY males (Two-way ANOVA, F=5.6, p<0.05). The housing environment also effected the stress response in SHR/y males (F=4.1, p<0.05). SHR/y colony baseline CORT was significantly less than SHR/y control baseline and SHR/y colony stress (*p<0.05). SHR/y control baseline was significantly less than SHR/y control stress (xp<0.05). WKY control baseline was significantly less than WKY control stress (†p<0.05).
Adrenocorticotrophin Hormone Increases Following Stress in SHR/y and WKY Control Males

Figure 2.13. Plasma adrenocorticotrophin hormone (ACTH) for both strain and treatment groups taken at baseline and following restraint stress (means, ± SEM). There was a significant effect of stress elevating ACTH in control SHR/y and WKY males (Two-way ANOVA, F=5.1, p<0.05). SHR/y control baseline was significantly less than SHR/y control stress (**p<0.01) and less than colony baseline (*p<0.05). SHR/y colony baseline was higher than SHR/y colony stress (N.S., p=0.09). WKY control baseline was significantly less than WKY control stress and WKY colony baseline (+p<0.05).
Figure 2.14. Plasma norepinephrine (NE) for both strain and treatment groups taken at baseline and following restraint stress (means, ± SEM). There was a significant effect of stress on control (Two-way ANOVA, F=19.9, p<0.001) and colony males (F=9.0, p<0.05). The colony housing environment effect was also significant for stress (F=5.9, p<0.05). SHR/y control baseline was significantly less than control stress (***p<0.001) and colony baseline (*p<0.05). SHR/y colony stress was significantly greater than colony baseline (+p<0.05). WKY control and colony stress were greater than their respective controls (^p<0.05). SHR/y colony baseline was greater than WKY colony baseline (#p<0.05).
Discussion

The results of this study support the hypothesis that chronic stress from the social environment impairs learning acquisition in both SHR/y and WKY colony males. In addition, the stress prone SHR/y males when housed in this environment also show an SHR Y chromosome and housing effect with increased corticosterone sensitivity, increased hippocampal catecholamine content, as well as impaired retention trial performance compared to their respective controls. Therefore, the mechanism resulting in this impaired maze performance and altered stress responsiveness likely involves the colony housing environment and SHR Y chromosome, since the only genetic difference between SHR/y and WKY males is the Y chromosome. Of particular interesting regarding these behavioral and physiological differences are peripheral and central nervous system (CNS) mechanisms altering hippocampal function, potentially through the hypothalamic pituitary adrenal (HPA) and sympathetic adrenal medullary (SAM) axes.

Both SHR/y and WKY colony males when compared to their respective controls, showed impaired learning, as noted with the higher platform latency times. This is consistent with current literature showing impaired maze performance following chronic stress [Bowman et al., 2002; Conrad et al., 2003]. As expected, performance from acquisition day 1 to day 3 showed improvement for both strains and treatments, indicating that the location of the platform was learned and therefore spatially mapped. In addition, the largest decrease in latency time was from day 1 to day 2, which identifies this as the time period where most of the learning took place. Although not specifically
tested for, it is possible that the colony animals initially had difficulty learning the maze or the maze protocol. Deficits in this type of procedural learning have also been linked to impaired acquisition performance [LeSarge et al., 2007; von Linstow et al., 2007]. This was indicated by colony males having higher platform latencies compared to the control males, with WKY at day 1 only and SHR/y at days 1 and 2. This could possibly be due to altered stress responsiveness to the novel maze, difficulties in processing procedural memory, or a difficulty in maze navigation due to the physical obstructions by SHR/y colony males. In all likelihood, it is a combination of all three factors, since the MWM initially stimulates a stress response, is physically demanding, and requires spatial processing [Aztiria et al., 2007; Moosavi et al., 2007]. Since learning is clearly impaired in the colony males, this should translate into impaired performance.

Indeed, the effect of social colony housing also showed impairment in the retention trial, but only in the SHR/y males. This impaired memory performance could be due to an inability to retrieve stored memories, or problems with memory consolidation [Martin and Clark, 2007]. Impaired performance by SHR/y males is consistent with the literature showing that chronic stress can impair memory/retention trial performance [Bowman et al., 2002; Conrad et al., 2003]. Stress through activation of the HPA or SAM axis, especially under chronic settings, can clearly impair learning acquisition and retrieval [Bowman et al., 2002; Conrad et al., 2003]. In addition, chronic stress can also decrease overall cognitive performance and impair spatial processing [McEwen, 2001; McEwen, 2005].
Since there was memory retention impairment in SHR/y colony compared to control males, which was not present in WKY males, the time spent in the colony previous to and following acquisition trials could be a contributing factor. These results suggest that the SHR/y colony males experienced greater stress and that the increased stress responsiveness impaired memory retention. In previous behavioral studies, the colony was more stressful to SHR/y than to WKY males, as shown by an increase in aggression towards conspecifics [Toot et al., 2004]. Continually elevated levels of aggression within the colony suggests that the colony does not have a stable hierarchy. Since neither colony showed a dominant male identifiable through behavioral, scarring, and physiological data, the lack of a dominant male within the colony would increase stress and attacks between males trying to assume that position. Dominant males will normally show an increase in norepinephrine and epinephrine compared to non-dominant males, as well as a limited amount of scarring [Andrews et al., 1993; Toot et al., 2004]. In addition, the colony environment has also been shown to increase stress responsiveness and raise blood pressure more in SHR Y chromosome males, as well as, decreasing serotonin content in the amygdaloid complex [Toot et al., 2004] thereby increasing aggression.

Other limbic system structures, such as the hippocampus also showed apparent SHR Y chromosome and social housing effects for SHR/y and WKY colony males regarding norepinephrine content when compared to control males. Specifically, SHR/y colony males had increased norepinephrine content compared to control, with decreased tyrosine hydroxylase activity; whereas, WKY colony males had decreased hippocampal
norepinephrine content, with relatively similar tyrosine hydroxylase activity. The hippocampal norepinephrine is only a measure of the content present in the tissue and does not necessarily reflect the receptor number, transport, synaptic release, breakdown or activation of the post-synaptic neurons. There is also the possibility that the increased content could be compensating for increased release, or even a decreased rate of synthesis by tyrosine hydroxylase, since impaired acquisition and retention in spatial memory tasks in rats is primarily associated with decreased norepinephrine activity and an increase in norepinephrine tissue content [Collier et al., 2004]. This would then be comparable to studies showing that the hippocampal infusions of norepinephrine impair learning and memory performance [Azam and McIntosh, 2006; Gilsbech et al., 2006].

In a similar manner, the tyrosine hydroxylase activity assay does not indicate the amount, specific location, release, or degradation of the enzyme in the hippocampus. Studies with tyrosine hydroxylase knock out mice show impaired retention and memory performance [Kobayashi et al., 2001; Salaphour et al., 2007], as was noticed with SHR/y colony males that had decreased tyrosine hydroxylase activity compared to controls. Norepinephrine and tyrosine hydroxylase play an important functional role in learning and memory retention in the hippocampus [Schroeter et al., 2000]. This is evident by the dense adrenergic input throughout the hippocampus including the dentate gyrus, CA1, CA3, and subnucleus [Lee and May, 1995; McGaugh et al., 2002]. Current hypotheses state that adrenergic activation is necessary for memory consolidation [Izquerdo, 1997; McGaugh, 2000], specifically in emotionally demanding or stressful events. Changes in norepinephrine content and receptor ligand binding are associated with a variety of
performance decline mechanisms [Collier et al., 2004], including the previously discussed decrease in tyrosine hydroxylase in hippocampal neurons. This decrease in tyrosine hydroxylase is likely via degeneration of the cell bodies in the substantia nigra and ventral tegmental area [Emborg et al., 1998; Naoi and Maruyaa, 1999] potentially from chronic stress.

Stimulation of the SAM axis, results in sympathetic stimulation of the adrenal medulla and subsequent release of norepinephrine into the systemic circulation. Indeed, there was an increased peripheral plasma norepinephrine stress responsiveness associated with the SHR Y chromosome, which we have observed in other studies in our lab [Andrews et al., 1993; Ely et al., 1997]. In addition to the SAM axis activity, HPA activity also played a regulatory role involved with the stress responsiveness.

In a normal HPA axis pathway, the hypothalamus secretes corticotrophin releasing hormone, which stimulates the pituitary gland to release adrenocorticotropic hormone stimulating the adrenal cortex to release corticosterone into the systemic circulation. Since corticosterone is able to pass the blood brain barrier, it can act directly on neural tissue, such as the hippocampus or hypothalamus or stimulate peripheral norepinephrine release from the adrenal medulla. As sometimes occurs in chronic stress environments [Rich and Romero, 2005; Kosti et al., 2006], basal adrenocorticotropic hormone samples were also elevated in SHR/y and WKY colony males compared to controls. Although the baseline samples were elevated, the adrenocorticotropic hormone levels actually remained the same or decreased following stress. When these plasma adrenocorticotropic hormone levels are taken into account with the colony
corticosterone baseline and stressed samples, there was an attenuated responsiveness of
adrenocorticotrophin hormone stimulating an increased corticosterone release from the
adrenal cortex. This suggests an increased sensitivity of the HPA axis of the colony
males compared to the control males. Specifically, the SHR/y males showed decreased
adrenocorticotrophin hormone following stress, with a corresponding two fold increase in
corticosterone.

In conclusion, chronic social stress was clearly able to impair learning acquisition
performance in colony males, with the SHR Y chromosome (SHR/y) males showing
severe long term memory impairment. This SHR Y chromosome effect is potentially
through a mechanism involving alterations in hippocampal function via adrenergic
activity (increased norepinephrine content) or a more global increase in sympathetic
nervous system activity. In addition, the increased stress responsiveness of both the
SAM and HPA axes, along with a potentially increased sensitivity to corticosterone
increases the susceptibility of SHR/y males to memory impairments associated with
visual spatial processing of the hippocampus.
Chapter III

Study 2: Social Housing Alters Pup Stress Responsiveness and Impairs Water Maze Performance in the Adult SHR Y Chromosome Male

Introduction

The social colony environment was shown in the previous chapter to impair memory retention and learning acquisition, and also alter stress responsiveness in males with the SHR Y chromosome. The purpose of the colony environment was to allow for natural behavioral interactions between male and female conspecifics, including reproductive behavior. As a result, several litters were born (F₁ colony generation) and raised in this social setting. The use of social housing, although similar to environmental enrichment housing models, is a socially stressful environment that elevates blood pressure and stress responsiveness [Andrews et al., 1993; Ely et al., 1994; Ely et al., 2000]. This raises several questions regarding the impact of the potentially stressful, but yet socially enriching environment on morris water maze (MWM) performance and stress responsiveness of the F₁ colony pups later in life.
The development of mammalian neonates is dependent upon a number of maternal “interactions” for healthy normal development. Stress to the mother during either the entire gestational period or acute post-parturition pup removal can alter these maternal interactions resulting in serious negative physiological side effects on the pup [Rojo, 1985; Pawer and Moor 1988; Maccari, 1995]. These mechanisms of how maternal stress associated events impact pup development through intrauterine and neonatal interactions are complex and not clearly defined. However, a variety of physiological end point measurements have been useful in assessing this maternal influence. Developmental studies have shown that the maternal environment can effect neonatal and pre-pubertal pup gross morphology, cardiovascular function, renal function, and stress sensitivity [Chapman and Stern, 1978; Sobrian et al., 1992; Leonhardt et al., 2007]. For example, several studies using social isolation paradigms which disrupt maternal-pup interaction have demonstrated that neonatal stress can increase hippocampal atrophy [Liu et al., 2000] and impair long term potentiation [Kohoe and Bronizo 1999] of the pups later in life as adults. This paradigm has also shown that maternal pup separation can alter learning acquisition, memory consolidation; as well as, alter stress responsiveness of the adult neonate [Lehman et al., 1999; Boccia and Pederson 2001].

The environmental enrichment setting is similar to the social colony housing model, with group housing of multiple males and also the presence of objects of various sizes, shapes, and textures changed weekly, along with alternative diet supplements [Moncek, 2000]. When examining developmental effects on the pups later in life, the enriched housing environment can reverse the negative effects of reduced maternal pup
care [Bredy, 2000], by enhancing learning and memory performance [Falkenberg, 1992; Kempermann, 1997; Nilsson, 1999; Rampon, 2000]. With respect to hippocampal morphology, this enriched environment also leads to increased neurogenesis [Bruel- ungerman, 2000] and increased neuron density [Williams, 2001]. Similar studies examining the interaction of stress and the hippocampus identify a decreased time of habituation to novel learning paradigms and stressors [Schrijver, 2000], as well as a potentially lower stress arousal [Delarco, 2000] after habituation.

The altered stress response is of particular interest regarding the hypothalamic pituitary adrenal axis secretions including corticosterone and adrenocorticotrophin hormone, and also indirectly norepinephrine. Interestingly, enriched environment studies have also shown an increase in relative adrenal gland weight, as well as, elevation of corticosterone [Moncek, 2000] levels to novel stressors compared to controls. Research by our lab involving colony male members in a social colony housing model (presented in the previous chapter) identifies an SHR Y chromosome effect, resulting in impaired learning acquisition and memory retention. In addition, the SHR Y chromosome is also associated with more pronounced elevations in blood pressure, stress reactivity, plasma catecholamines, and conspecific aggression [Andrews et al., 1993; Ely et al., 2000; Toot et al., 2004].

The overall purpose of this study was to investigate the role of long term social colony living on pup behavioral development, and what impact that has on pup maze performance and stress responses later in life. Therefore, the hypothesis to be tested is that the adult F₁ colony born pups will have enhanced learning performance and memory
retention, along with decreased basal stress hormones (adrenocorticotrophin hormone, corticosterone, and norepinephrine) compared to adult non-colony born standard housed pups. In particular, the adult WKY F1 colony males will show greater learning and memory enhancement compared to controls than the SHR/y (SHR Y chromosome) F1 colony males.

Materials and Methods

Study Design

The objective of this study was to examine the role of the pre-pubertal colony housing environment for SHR/y and WKY males regarding maze performance and stress responsiveness later in life. This study utilizes a two strain by two treatment design. SHR/y and WKY colony born F1 males (n=8/strain) and control standard housed males (n=8/strain) were used for maze testing. Males were taken from the 2nd litter from colony and standard housed breeder females. All SHR/y and WKY males (control and F1 colony) were approximately 20 weeks of age upon starting behavioral testing.

Animal Model

The animal model used in this study consists of the borderline hypertensive consomic rat (SHR/y) and the normotensive rat (WKY). The detailed breeding scheme has been described previously (Chapter I). SHR/y males have the SHR Y chromosome with WKY autosomes and an X chromosome. This allows for any phenotypic
differences between SHR/y and WKY males to be attributed to the SHR Y chromosome [Ely et al., 1993; Ely et al., 2000].

**Colony Housing**

Group housing was strain specific for SHR/y or WKY rats, consisting of 8-10 intact males and females, initially 8 weeks of age. This housing environment was used in the previous chapter and has also been described as model for social stress [Ely and Henry, 1973; Toot et al., 2004]. With this design, male and female rats are able to interact socially in a center open field cage (1.2m x 1.2m), with four attached side cages (0.6m x 0.6m), with food available *ad libitum*. F1 generation colony pups were taken from their respective colonies (6 weeks old) and housed under standard conditions.

**Morris Water Maze Protocol**

The morris water maze (MWM) testing paradigm was used in this study to examine spatial learning and memory processes. The setup and validity of the test is described in the previous chapter. Briefly, the maze consisted of a 5’ diameter water filled tank to 1” above the escape platform, with external maze visual cues placed around the room. For this study, there were a total of three possible maze versions allowing for comparisons between each maze including: improvement in performance on each version (versions A, B, and C), difficulties locating the platform due to physical obstructions (version A), lack of obstructions (version B), interference effects from previous platform locations (versions B and C), stress and retrograde memory (maze version C), and also
repetition of trials (acquisition trials within each version. Specifically, version A consisted of packing foam to obscure platform location, version B consisted of murky water to obscure platform location, and version C consisted of murky water and a 30 minute restraint stress following acquisition day 1. Our previous studies have shown that the greatest improvement in water maze performance is between acquisition days 1 and 2, thereby making this time point a target for retrograde impairment with stress. Retrograde amnesia would therefore show an impairment in performance from the last trial in day 1 to the first trial in day 2, or possibly an overall impairment between day 1 to day 2 acquisition performance.

The maze consisted of a 5’ diameter tank filled with room temperature water 1” above the platform (6” diameter), and covered with packing foam to obscure the view of the platform. The maze involved three days of acquisition with 5 trials per day and 1 retention trial for 5 days after acquisition day 3. Each trial was run in a consecutive order starting in 1 of 4 quadrants (N, S, E, and W) for a maximum of 90 seconds. After finding the platform (6” diameter), the animal was given a 20 second mapping period before removal from the maze. Each version of the maze had the platform located in a different quadrant within the maze.

Plasma Blood Samples

One week following completion of the final MWM, animals were subjected to a 30 minute restraint stress using a decapicone sealed on one end, with a 5 second air burst after 15 minutes of restraint. Approximately 20 minutes post stress, the animal was then
anesthetized with Sodium Pentothal (50mg/kg, IP; E. Lilly Indianapolis, IN). A 2-3 ml retro-orbital blood sample collected between 1100 and 1700 hours, centrifuged for 20 minutes (2,000 rpm), and stored at -70°C. Animals were again anesthetized a week later and a baseline blood sample was taken prior to termination. Blood samples collected were later analyzed for stress plasma catecholamines, adrenocorticotropic hormone, and corticosterone using the techniques described below.

**Plasma Assays**

Adrenocorticotropic hormone was analyzed in plasma by RIA (#24130, DiaSorin, Stillwater, Mn). The intra-assay variation was 8.1%, the inter-assay variation was 6.7%, with a sensitivity at 15 pg/ml at the 95% confidence limit, and with the highest cross-reactivity being Porcine ACTH 1-39 and Human ACTH 1-24 at 100%, with other peptides at <0.01%.

Corticosterone was analyzed in plasma by RIA (DSL-80100, Diagnostic Systems Laboratories, Inc., Webster, Tx.). The intra-assay variation was 3.43%, the inter-assay variation was 7.3%, with a sensitivity at 2.7 ng/ml at the 95% confidence limit, and with no reported cross-reactivity.

The plasma catecholamine norepinephrine (NE) was analyzed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters 460 Detector, Milford, MA). The mobile phase consisted of Citric Acid (35 mM), Sodium Acetate (90 mM), Octyl Sodium Sulfate, (460 uM), EDTA (130 uM) and 14% Methanol at a pH of 4.7. The HPLC pump, Water 510 (Waters Corp., Milford, MA) was set at
1.3ml/min. The column was a Supelcosil LC-18, 15cm x 4.6mm, 3um which was preceded with a guard column (Discovery RP-Amide C16 2cm x 4.0mm, 5um, Supelco, Bellefonte, PA). A constant amount 2,3-DHBA was spiked into each sample and the peak height ratio was calculated electronically. The NE concentration was determined using a standard curve based on the internal standard. The minimum sensitivity of the assay was 30 pg/ml for NE.

Statistics

One-way ANOVA, two-way ANOVA, and two-way RM ANOVA analysis were used, when appropriate. Student’s t-tests were used to compare individual groups. Significance was assumed at p<0.05. Statistical analyses were run on SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA) with significance assumed at p<0.05.

Results

WKY and SHR/y F1 colony males show impaired mean acquisition performance on maze version A (Figure 3.1, p<0.05). Subsequent maze versions (B and C) showed a trend for improved WKY and SHR/y F1 colony maze performance (F=5.5, p<0.05) compared to control males. There was also a significant interaction between F1 colony treatment and maze version (F=26.1, p<0.001). Overall maze performance from the version A to version C improved for both strains and treatments (F=162.1, p<0.001). Analysis of day to day acquisition trial performance yielded similar results. In addition,
there was no significant effect of stress impairing acquisition performance in maze version C comparing day 1 trial 5 to day 2 trial 1.

SHR/y F1 colony male retention trial performance showed a significant trend of impairment compared to controls for all three maze versions (F=6.7, p<0.05). However, WKY F1 colony males showed a trend of improved retention performance compared to controls (F=4.6, p<0.05). Both strains and treatments retention performance improved over each maze version (Figure 3.2, F=9.8, p<0.001).

Plasma adrenocorticotrophin hormone levels increased from baseline following stress (p<0.001) in SHR/y and WKY males from the pre pubertal colony housing and control environment groups. Both strains treatments showed similar values for baseline and stress adrenocorticotrophin samples (Figure 3.3).

SHR/y and WKY plasma corticosterone (Figure 3.4) increased following stress in control (p<0.05) and F1 colony (p<0.001) males. Plasma corticosterone decreased compared to controls (p<0.001) in both F1 SHR/y and WKY males. The interaction effect of pre pubertal housing, stress, and the SHR Y chromosome also effected plasma corticosterone (F=12.3, p<0.001).

Plasma norepinephrine (Figure 3.5) increased from baseline following stress (p<0.001) both strains and treatment. The SHR/y control and F1 colony plasma norepinephrine were greater than their respective WKY comparisons (p<0.05). WKY stress norepinephrine increased from baseline (p<0.05). Plasma norepinephrine increased following stress in both F1 SHR/y and WKY colony males (p<0.05). There was also a
significant effect of pre pubertal housing, stress, and the SHR Y chromosome on
increasing plasma norepinephrine (F=5.3, p<0.05).
SHR/y and WKY F1 Colony Males Show Improved Mean Acquisition Performance on Later Maze Versions

Figure 3.1. Mean platform latency (seconds) over acquisition trials for each maze version from SHR/y and WKY control and F1 males (means, ± SEM). Both SHR/y and WKY F1 colony males were impaired on maze version A compared to controls (p<0.05), with an F1 strain effect impairing performance for both strains (Two-way RM ANOVA, F=10.6, ***p<0.001). WKY and SHR/y F1 maze performance was also enhanced on the subsequent maze versions, B and C (F=5.5, p<0.05). In addition, there was a significant interaction between F1 treatment and maze version (F=26.1, p<0.001). Maze performance improved (decreased platform latency) over each version for both strains and treatments (F=162.1, p<0.001).
Figure 3.2. Platform latency (seconds) on each retention trial for each maze version from SHR/y and WKY control and F1 males (means, ± SEM). SHR/y F1 colony male retention performance was significantly impaired compared to control males over three maze versions (Two-way RM ANOVA, F=6.7, *p<0.05). However, WKY F1 colony males showed slightly improved retention performance compared to control males (F=4.6, ^p<0.05). Retention performance improved over each maze version for both strains and treatments (F=9.8, p<0.001).
Plasma Adrenocorticotrophin Hormone is Increased Following Restraint Stress
For SHR/y and WKY Control and F1 Colony Males

Figure 3.3. Plasma adrenocorticotrophin hormone (ng/ml) levels for SHR/y and WKY control and F1 colony males (means, ± SEM). There was a significant stress effect (Two-way ANOVA, F=11.5, ***p<0.001) for each strain and treatment. There were no significant differences between corresponding strains and treatments.
Plasma Corticosterone Increases Following Stress in Control and F1 Males

Figure 3.4. Plasma corticosterone (pg/ml) levels for SHR/y and WKY control and F1 males (means, ± SEM). Stress increased plasma corticosterone in SHR/y and WKY control males (*p<0.05). Baseline F1 colony plasma corticosterone decreased compared to controls for both SHR/y and WKY males (***p<0.001). Stress increased plasma corticosterone in SHR/y and WKY F1 males (+++p<0.001). There was also a treatment, strain, and stress interaction (Two-way ANOVA, F=12.3, p<0.001) between both strains and treatments.
Restraint Stress Increases Plasma Norepinephrine Control and F1 Males

Figure 3.5. Plasma norepinephrine (pg/ml) for SHR/y and WKY control and F1 males (means, ± SEM). Plasma norepinephrine increased from baseline following stress (F=16.1, ***p<0.001) in both strains and treatments. There was a significant strain, F1 and stress effect on plasma norepinephrine (Two-way ANOVA, F=5.3, p<0.05). SHR/y control stress norepinephrine was greater than WKY control stress (**p<0.01). SHR/y F1 stress norepinephrine was greater than SHR/y F1 baseline (^^^p<0.001) and WKY F1 stress (+p<0.05). WKY F1 stress norepinephrine was greater than baseline (#p<0.05).
Discussion

Data from both strains (SHR/y and WKY) supported the hypothesis that the adult F\(_1\) colony born pups would have enhanced learning performance, along with elevated stress hormones compared to the adult control non-colony born pups. Specifically, the F\(_1\) males showed enhanced maze acquisition performance (versions B and C) compared to control males. In addition, the retention trial data showed an SHR Y chromosome effect, with WKY F\(_1\) colony males showing enhanced memory retrieval, but with SHR/y F\(_1\) colony males showing impaired memory retrieval. The mechanism behind the F\(_1\) acquisition trial enhancement and elevated novel stress responsiveness (i.e. norepinephrine and corticosterone) involves not only the SHR Y chromosome, but also the hypothalamic pituitary adrenal (HPA) and sympathetic medullary adrenal (SAM) axes, to be discussed in the following paragraphs.

This acquisition platform latency was enhanced in maze versions B and C for both SHR/y and WKY F\(_1\) males, but impaired in maze version A. Maze version A consisted of packing foam and probably was an obstruction thereby making swimming and platform location more difficult. In addition, this was the first version of the maze used, which suggests that impairments in procedural learning of the maze task itself may be involved [Beunieux et al., 2006; Pettitenger et al., 2006; von Linstow, 2007]. However, the increased difficulty of maze version A was not analyzed by alternating maze versions with the different treatment groups, so statistical verification of this possibility can not be confirmed. Interestingly, subsequent maze versions with SHR/y F\(_1\) and WKY F\(_1\) males showed improvement in maze acquisition performance. These
changes in later maze versions could involve improved procedural memory or habituation to the stress from the morris water maze test itself [Bert et al., 2002; Wright et al., 2004]. The acquisition performance, however, does not explain the consistent SHR Y chromosome impairment effect in the retention trial performance between adult SHR/y and WKY F1 colony born pups.

SHR/y F1 males did worse on average over all three retention trials compared to controls, while WKY F1 males showed enhancement compared to controls. Since SHR/y F1 males performed consistently worse regarding the retention trials than WKY F1 males, with the only genetic difference between the two strains lying in the origin of the Y chromosome, it can be concluded that the SHR Y chromosome can be attributed to these differences. This suggests that although acquisition performance was enhanced for both F1 strains, the impairment was specifically directed towards memory consolidation or in retrieval mechanism in the SHR Y chromosome (SHR/y F1) males. This same acquisition and retention pattern has been noted in the dopamine beta hydroxylase knock out mouse model and other stress prone animal models, which show no apparent differences in acquisition related spatial navigation [Thomas, 1995], but severe impairment in long term memory consolidation [Morris et al., 1982].

In general, F1 males showed an increased novel stress response compared to the control males with SHR/y males showing a potentiated elevation in norepinephrine. This suggests increased SAM axis activity or a more general increased sympathetic nervous system (SNS) activation in the F1 pups resulting from the maternal or peripubertal environment. Since this stress response is likely exaggerated in the SHR/y F1 males, the
impaired retention performance could therefore be a result of a locus on the SHR Y chromosome indirectly impairing memory consolidation or retrieval pathways through dopamine beta hydroxylase or altered activity in the central nervous system. Specifically, SHR Y chromosome males characteristically have increased SNS activity, increased stress arousal and increased adrenal gland tyrosine hydroxylase activity, and increased plasma norepinephrine following stress [Ely et al., 1994; Ely et al., 1997] compared to the WKY Y chromosome males. All of these components which lead to an increase in plasma norepinephrine could be an indication of increased global SNS activity and possibly even elevated adrenergic activity within the central nervous system. This correlation between increased peripheral and increased central catecholaminergic levels has been noted in similar studies [Soulage et al., 2004; Dronjak and Gavrilovic, 2006]. Since both F1 strains showed similar responses of norepinephrine following stress and similar baselines, it is possible that there is another factor related to the SNS, such as a difference in receptor number or receptor activation. This could therefore alter the sensitivity to norepinephrine thereby requiring more or less norepinephrine for similar responses between strains.

Further analysis of the stress response, specifically within the HPA axis identifies similar adrenocorticotrophin hormone levels between both strains of control and F1 males. Since adrenocorticotrophin hormone stimulates the release of corticosterone from the adrenal cortex, the levels of measured corticosterone should be similar. However, since these adrenocorticotrophin hormone levels actually corresponded to elevated corticosterone levels, this suggests a potential increased sensitivity of the HPA axis due
to differences between the F₁ pups and control males. This would also therefore point to corticosterone as being involved with the differences in water maze performance.

Indeed, the HPA axis stress hormone corticosterone was elevated in SHR/y F₁ compared to WKY F₁ stressed males. In addition, male F₁ basal corticosterone was decreased versus control, with stress in F₁ increasing 3 to 6 fold, while the control increased 0.6 to 1 fold, suggesting an increased novel stress responsiveness in F₁ males. Chronic or acute stress can modulate memory in animal models through not only peripheral norepinephrine release by the adrenal medulla, but also through glucocorticoids. Under acute conditions corticosterone can enhance memory; whereas chronically increased corticosterone levels have been shown to decrease hippocampus volume and function, resulting in impaired retention and memory formation [McEwen, 2001; McEwen, 2005; Wright et al., 2006]. Several studies have further analyzed this corticosterone and memory relationship indicating that there is a wide range of responses dependent upon the strain, gender, and age of the animal model being used [Watanabe, 1992; Galea, 1997; Sandi et al., 1997]. This could explain why the SHR/y F₁ males which have higher stressed corticosterone consistently show impaired retention performance, possibly due to a weakened habituation response in retention trials.

Since this trend of elevated stress responsiveness and enhanced acquisition performance is found in both strains of F₁ males, the final factor focused on in this study other than the SHR Y chromosome is the pre-pubertal housing environment. The social colony animal model used in this study is similar in design to environmental enrichment models used to examine peripubertal influences on pup development and behavior.
When examining developmental effects on pups later in life, this housing environment can reverse negative effects of reduced maternal pup care [Bredy, 2000], enhance learning and memory performance [Falkenberg, 1992; Kempermann, 1997; Nilsson, 1999; Rampon, 2000] through increased hippocampal neurogenesis and hippocampal neuronal cell density [Bruel-ungerman, 2000; Williams, 2000]. In addition to this increased maze performance, the pups post-puberty also have an increased stress response to novel stressors, with a decreased time of habituation to novel paradigms [Schrijver, 2000], as well as a lower basal levels of stress hormones [Delarco, 2000]. Environmental enrichment can improve visual spatial navigation and performance later in life [Rampo, 2000; Nilsson, 1999]. This pathway involving the hippocampus, stress response, and prepubertal housing environment has been supported by numerous studies showing that even short periods of environmental enrichment can offset early developmental deficits from maternal or environmental stress, as measured through spatial navigation testing of the adult pups [Vanrang, 2000].

In conclusion, F₁ males show increased stress responsiveness to novel stressors compared to controls for plasma corticosterone and norepinephrine. Since the corticosterone levels increased, while adrenocorticotrophin hormone remained unchanged between housing groups, there appears to be an increased HPA axis sensitivity. This finding in conjunction with the performance measures supports current enriched housing model research indicating that even short term enrichment early in life can have long lasting effects into adulthood. Interestingly, this enriching environment was not able to prevent the decreased retention performance in SHR Y chromosome (SHR/y males).
Therefore, the SHR Y chromosome along with alterations in the HPA and SAM axes is able to influence stress responsiveness and maze performance of the F$_1$ colony pups later in life as adults.
CHAPTER IV

Study 3: Corticosterone Manipulation Impairs Learning and Memory, While Increasing Hippocampal Norepinephrine Content

Introduction

The previous chapters have demonstrated the relative importance of the SHR Y chromosome along with the hypothalamic pituitary adrenal and sympathetic adrenal medullary axes in influencing water maze performance. Animals with the SHR Y chromosome have impaired maze performance and potentially dysregulated stress responsiveness compared to animals with the WKY Y chromosome. One of the key stress steroids, corticosterone is classified as a glucocorticoid and is associated with a variety of physiological functions. Specifically, corticosterone is released from the adrenal cortex in response to a “stressor” which releases glucose from many different sources. In addition, corticosterone plays a key role in the ability of the hippocampus to process visual spatial relationships and consolidating memory in normal and stressful situations [Manji, 2003; Hibberd et al., 2007].

Animal models indicate that either changing plasma corticosterone, can severely impair MWM learning and memory performance. There are a variety of behavioral
paradigms used to assess different components and associated processes of learning and memory pertaining to the hippocampus. In particular, the Morris water maze (MWM) tests visual spatial mapping and is often divided into two different components consisting of acquisition and retention phases [Morris et al., 1982]. Learning and memory are influenced by complex mechanisms involving a corticosterone driven hippocampal pathway regulated through glucocorticoid and mineralocorticoid receptors [Takahashi and Goh, 1998; Jameison and Dinan, 2001; Rozendaal, 2002]. Under times of either acute or chronic stress, however, corticosterone is not always adaptive or beneficial to the organism [Lupien and Lepage, 2001]. Acute stress causes a brief rise in corticosterone increasing awareness and aiding in the potential flight behavior, as mentioned previously, whereas under chronic stress conditions, this long term elevation in corticosterone will eventually impair memory performance and alter stress responsiveness. One of the hallmarks of chronic stress is not only elevated corticosterone and adrenergic activity, but also increased norepinephrine release within brain regions such as the hippocampus [McGaugh, 2002; McIntyre, 2002]. This, if persistent, commonly results in neurological atrophy of the hippocampus, cardiovascular damage, weight loss, and a decreased immune system function [Sapolsky, 1985].

In terms of the hippocampus and chronically elevated corticosterone, research indicates that hippocampal atrophy can result specific to the CA1 cells [McKittrick, 2000]. Corticosterone is able to pass through the blood brain barrier and act directly at the level of the central nervous system (CNS). This often results in impaired memory and acquisition in hippocampal dependent tasks, such as spatial navigation through a
mechanism involving glutamate and influxes of neuronal calcium resulting in cell death [Herbert et al., 2006]. This impairment is also partially explained by the glucocorticoid hypothesis suggesting that there is a decrease in glucocorticoid receptor number. Since the hippocampus has the greatest concentration of glucocorticoid and mineralocorticoids receptors in the CNS, it is particularly vulnerable to stress. However, there are other brain nuclei that are influenced by corticosterone, particularly within the limbic system, including the hypothalamus, frontal cortex, and amygdala [Sapolsky, 1985; Gould et al., 1992; Cameron and Gould, 1994]. There appears to be a preset range, dependent up the strain/animal/gender being used, as to what level of corticosterone may enhance or impair learning acquisition and memory consolidation.

Our lab has been studying the SHR Y chromosome through the SHR/y consomic strain, in relationship to stress reactivity under both acute and chronic settings. Other than the previously covered impairments in maze performance, the SHR Y chromosome has been linked to increases in: blood pressure, sympathetic nervous system activity, adrenal tyrosine hydroxylase activity, and overall stress responsiveness, when compared to the WKY rat strain [Andrews et al., 1993; Ely et al., 1994; Ely et al., 1997; Ely et al., 2000]. Therefore, the hypothesis of the following study is that corticosterone manipulation will impair MWM acquisition and retention performance, and increase norepinephrine content in the hippocampus, with SHR/y males being more sensitive to corticosterone manipulation than WKY males.
Materials and Methods:

Study Design

The objective of this study was to expand on our previous research pertaining to plasma corticosterone, maze performance, and the SHR Y chromosome. Specifically, the possibility of a difference in corticosterone levels, either increased with corticosterone injections or decreased with metyrapone treatment, manipulated via pharmacological treatment will be addressed. For this study, adult (10-14 week) SHR/y and WKY males were housed under standard conditions in the following treatment groups: control (n=6-8/strain), corticosterone injected (n=5-8/strain), and metyrapone injected (n=4-6/strain).

Corticosterone treatment

Corticosterone treatment (20mg/kg BW, IP, oil suspension) was given to SHR/y and WKY males 2 hours [McEwen, 2001; Wright et al., 2006] prior to beginning the water maze for acquisition days 1, 2, and 3, as well as before the retention trial. In addition, corticosterone treatment was also given each day during the retention period and before termination. This dose elevated plasma corticosterone was the equivalent of 700 to 1100 pg/ml at the time of maze testing.

Metyrapone (100mg/kg BW, IP, saline) inhibits 11 beta hydroxylase and was given approximately 2 hours [Wright et al., 2006; McEwen, 2001] prior to SHR/y and WKY males (n=4/dose) males beginning the water maze for acquisition days 1, 2, and 3, as well as before the retention trial. In addition, metyrapone treatment was also given
each day during the retention period and before termination. This dose was the equivalent of less than 65 pg/ml plasma corticosterone at the time of maze testing.

Maze Testing Paradigm

The morris water maze (MWM) test paradigm was used in this study to investigate spatial learning and memory processes. This is a standard test consisting of a platform hidden underneath the surface of the water. Specifically, the maze consisted of a 5’ diameter tank filled with room temperature water 1” above the platform (6” diameter), and covered with packing foam to obscure view of the platform. The maze involved three days of acquisition with 5 trials per day and 1 retention trial for 5 days after acquisition day 3. Each trial was run in a consecutive order starting in 1 of 4 quadrants (N, S, E, and W) for a maximum of 90 seconds. After finding the platform (6” diameter), the animal was given a 20 second mapping period before removal from the maze. The MWM consisted of a single maze version constituting packing foam, chapter II for a detailed description of the MWM and testing conditions.

Animal Model

The Y chromosome animal model used in this study consisted of the consomic borderline hypertensive (SHR/y), and normotensive Wistar-Kyoto (WKY) rats. This breeding scheme has been described in detail previously (Chapter I). Briefly, comparisons between WKY and SHR/y males allow us to investigate the role of the SHR Y chromosome in a WKY genetic background [Ely et al., 2000].
Palkovit’s Brain Punch

Two hours prior to termination all animals were treated with corticosterone 20 mg/kg BW or metyrapone 100mg/kg BW. Following termination, the brains were frozen on dry ice, stored at -70C, and later cut into 200 micron coronal sections at -20C (Minotome, International Equipment Company, Damon Division, Meedham Hts., Mass.). The sections were then punched [Palkovits, 1973] from hippocampus (HPC). Proper coronal slice and punch orientations were verified with comparison to the rostral-caudal bregma zero reference points [Pellegrino, 1981]. The punches were homogenized in either mobile phase, centrifuged for 10 minutes at 3200rpm (Sorvall RT7, Kendro Labs, Newton Conn.) and stored at -70C for later analysis.

The norepinephrine content for the hippocampus was analyzed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters 460 Detector, Milford, MA) and has been previously described in detail (Chapter II).

Statistics

Statistical analysis was performed by using two-way RM ANOVA and a post-hoc Bonferroni test, One-way ANOVA, Pearson correlation coefficients, and Student’s t-test was used where applicable, with significance assumed if p<0.05. Analyses were run on SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA) with significance assumed at p<0.05.
Results

WKY platform latency (Figure 4.1) was significantly impaired in metyrapone 100mg/kg BW treated males compared to controls (F=9.3, p<0.01). The WKY male corticosterone manipulations significantly effected performance (F=6.3, p<0.05), with maze performance improving with each trial day (F=8.1, p<0.01). SHR/y platform latency (Figure 4.2) was significantly impaired in metyrapone treated males compared to controls (F=8.7, p<0.01). The SHR/ platform latency was also affected by trial day (F=19.7, p<0.001), treatment (15.5, p<0.001), with an interaction between day and treatment (F=3.0, p<0.05).

SHR/y and WKY corticosterone treated (Figure 4.3) compared to control male latency performance was impaired (F=8.3, p<0.01), but with gradual improvement over the three trial days (F=17.2, p<0.001). There was no strain effect on acquisition.

However, SHR/y and WKY males treated with the metyrapone (Figure 4.4) showed a significant impairment when compared to their controls over all three trial days (F=92.9, p<0.001). The performance of both strains and treatments showed an overall trend for improvement over the three trial days (F=13.9, p<0.001), with an interaction effect between the trial day and treatment (F=5.7, p<0.001).

The SHR/y and WKY retention trial performance (Figure 4.5) following corticosterone (p<0.05) and metyrapone treatment (p<0.001) were significantly impaired when compared to the respective controls. The treatment effect of corticosterone manipulation on impairing performance in both strains was significant (F=13.9,
p<0.001), with a significant interaction between the SHR Y chromosome and treatment (F=14.8, p<0.001).

SHR/y males treated with metyrapone had significantly higher hippocampal norepinephrine tissue content than control (Figure 4.6, p<0.001). Corticosterone manipulation had a significant effect in the SHR/y males that increased hippocampal norepinephrine content (F=2.8, p<0.05). Hippocampal norepinephrine content compared to retention trial performance (Figure 4.7) in the corticosterone manipulation groups showed a significant positive correlation only in SHR/y males (R=0.626, p<0.01).
Corticosterone Manipulation Impairs Learning in WKY Males

![Graph showing the platform latency (seconds) for corticosterone manipulation treatment groups (means, ± SEM). There was a significant effect of corticosterone manipulation (Two-way RM ANOVA, F=3.5, *p<0.05), as well as improved maze performance over the three days of testing (F=12.6, ***p<0.001).](image-url)

Figure 4.1. WKY platform latency (seconds) for corticosterone manipulation treatment groups (means, ± SEM). There was a significant effect of corticosterone manipulation (Two-way RM ANOVA, F=3.5, *p<0.05), as well as improved maze performance over the three days of testing (F=12.6, ***p<0.001).
Corticosterone Manipulation Impairs Learning in SHR/y Males

Figure 4.2. SHR/y platform latency (seconds) for corticosterone manipulation treatment groups (means, ± SEM). Acquisition performance was significantly affected by corticosterone treatment (Two-way RM ANOVA, F=8.7, **p<0.01) and trial day (F=4.9, *p<0.05).
Corticosterone Treatment Impairs Learning in WKY and SHR/y Males

Figure 4.3. SHR/y and WKY platform latency (seconds) for corticosterone treatment (means, ± SEM). Learning in SHR/y and WKY males was significantly impaired by corticosterone treatment (Two-way RM ANOVA, F=8.3, **p<0.01). Over the three trial days both strains and treatments also showed a general improvement in maze performance (F=17.2, ***p<0.001).
Metyrapone Impairs Maze Performance in SHR/y and WKY Males

**Figure 4.4.** SHR/y and WKY platform latency (seconds) for control and metyrapone treatment groups (means, ± SEM). Metyrapone treatment for both strains impaired acquisition performance compared to control males for both strains, (Two-way RM ANOVA, F=14.8, ***p<0.001). Metyrapone at this dose also impaired acquisition performance compared to each respective control group (F=10.3, **p<0.01) for both strains.
Corticosterone Manipulation Impairs Retention Trial Performance More in SHR/y Males Compared to WKY Males

Figure 4.5. SHR/y and WKY platform latency (seconds) for corticosterone manipulation treatments (means, ± SEM). There was a significant treatment effect of corticosterone manipulation impairing retention performance in both strains (Two-way ANOVA, F=13.9, p<0.001), with a significant interaction effect of strain and treatment (F=2.9, p<0.05). Corticosterone treatment significantly impaired retention performance compared to control SHR/y (*p<0.05) and WKY (†p<0.05) males. Metyrapone treatment in both strains significantly impaired retention performance compared to control SHR/y (***p<0.001) and WKY (⁎⁎⁎p<0.001) males. Metyrapone impaired retention performance in SHR/y compared to WKY males (N.S., p=0.057).
Hippocampal Norepinephrine Content Increases in SHR/y Metyrapone Treated Males

![Graph showing hippocampal norepinephrine content from WKY and SHR/y males for control, corticosterone, and metyrapone treatments (means, ± SEM). SHR/y metyrapone hippocampal norepinephrine content was significantly greater than the control and castrate treatments (***p<0.001). There was a significant affect of corticosterone manipulation increasing hippocampal norepinephrine content in SHR/y and WKY males (Two-way ANOVA, F=2.8, p<0.05).]
Hippocampal Norepinephrine Significantly Correlates With Retention Trial Performance in SHR/y, But Not in WKY Males

Figure 4.7. Hippocampal norepinephrine content (pg/mg tissue) versus retention trial performance (platform latency) for SHR/y and WKY corticosterone manipulated males (means, ± SEM). SHR/y male hippocampal norepinephrine to retention performance showed a significant positive correlation (R=0.626, p<0.01). WKY male norepinephrine to retention performance was not significant (R=0.104, p=0.6)
Discussion

This study supported the hypothesis that altering corticosterone levels was able to impair both SHR/y and WKY acquisition and retention trial performance in the Morris water maze (MWM). Specifically, exogenous corticosterone (20mg/kg BW) produced minor impairment on acquisition performance, with metyrapone (100mg/kg BW) showing severe impairment. The memory retention trials appeared to be a more sensitive indicator of corticosterone manipulation with both corticosterone and metyrapone treatment producing significantly impaired memory performance. Since the only genetic difference between the SHR/y and WKY males is the SHR Y chromosome, the behavioral differences can be attributed to an SHR Y chromosome effect on memory retention and hippocampal norepinephrine content. Maze performance, as measured by acquisition and retention trial performance, along with increased SHR/y hippocampal norepinephrine content, suggests that there is an SHR Y chromosome effect involving corticosterone manipulation.

Multiple or even a single dose of corticosterone was sufficient to interfere with the visual spatial processing of the hippocampus showing a trend of impaired learning acquisition performance over all three days of testing. This process of learning involves the visual spatial information being processed initially by the entorhinal cortex. Connections between the CA, dentate gyrus, and entorhinal regions of the hippocampus allow the neural information to travel and be stored in the hippocampus associated structures. However, due to the large concentration of glucocorticoid receptors in the hippocampus, it is particularly vulnerable to corticosterone manipulation. Studies using
similarly elevated corticosterone doses (above 150 pg/ml), have also been able to interfere with this pathway and therefore impair maze performance on similar maze paradigms and cause hippocampal damage when administered under chronic conditions [McEwen, 2001; McEwen 2005].

Interestingly, slightly elevated corticosterone levels (5% to 20% increase) during fear induced maze testing paradigms increase performance, and can actually be of benefit to the animal by participating in survival strategies and predator awareness [Men et al., 1999; Wright et al., 2006]. Since this maze version consisted of packing foam and was not only physically demanding, but also difficult to navigate, this maze version may have stimulated a larger stress response. This is consistent with other MWM manipulations (i.e. decreased ambient temperature or decreased water temperature) that have also shown it to be a stressful testing paradigm causing elevations in plasma corticosterone synthesis and decreased performance compared to control settings [Livonen et al., 2003; VanDam et al., 2006].

Treatment with metyrapone, an 11 beta hydroxylase blocker, decreases plasma corticosterone and severely impaired maze acquisition in both strains. This impairing affect on acquisition was even more noticeable in the SHR/y males showing actually worse day 2 performance compared to day 1. Previous studies in our lab, as well as the other strains and treatments in this study showed slightly improved or relatively unchanged performance from day 1 to day 2. This indicates that the metyrapone was interfering with the spatial mapping or procedural memory associated with the typically large enhancement in maze performance from day 1 to day 2. Typically, decreased
plasma corticosterone is protective in nature and can actually enhance maze performance when the animal is subjected to stressors [Herbert et al., 2006]. However, the impaired acquisition performance indicates that corticosterone levels were perhaps too low and that memory performance was actually impaired, as suggested in drug treatment studies and in adrenalectomized rodent models [Quervain et al., 1990].

Just as depressed corticosterone levels can impair hippocampal function, elevated corticosterone can inhibit hippocampal plasticity, while increasing neuronal atrophy and cell death through a glutamate mediate processes [Herbert et al., 2006]. This damage to the hippocampus can result in deficits of hippocampal dependent processing, resulting in either impairment with the acquisition or retrieval aspects of learning and memory. Therefore, under most testing conditions, impaired acquisition performance in the MWM typically results in impaired retention. For this study, the corticosterone manipulation impaired retention trial performance in both strains. Although the corticosterone or metyrapone injections either increased or decreased the plasma corticosterone, it was only for a limited period of time prior to and during the MWM acquisition tests over three days. However, retention trial performance in corticosterone or metyrapone treated SHR/y and WKY males actually consisted of eight consecutive treatments and was therefore closer in nature to “chronic” studies. Studies using chronic stress can last from six days to several months, consisting of restraint, social isolation, or social defeat ranging in time from 30 seconds to 6 hours [McEwen, 2005; Rotllant and Armerio, 2005; Wright et al., 2006]. The corticosterone pathway of neural damage has been partially defined and linked to the increased glucocorticoid and mineralocorticoid receptor
population within the hippocampus. Corticosterone treatment and stress studies indicate that the hippocampus is particularly sensitive to elevated corticosterone and can therefore be damaged easily along with having an inhibitory affect on hippocampal cell proliferation [Gould et al., 1992; Watanabe et al., 1992; Cameron and Gould, 1994].

This mechanism of hippocampal damage from elevated corticosterone could also be occurring in the metyrapone treated animals through an overcompensated negative feedback effect. Since metyrapone only blocks synthesis for a short time (i.e. approximately 3 hours) and is able to decrease corticosterone five fold, the negative feedback system controlling corticosterone will have plenty of time to be activated. This will likely cause an increase in corticotrophin releasing hormone, 11 beta hydroxylase, glucocorticoid receptor number along with decreases in catabolic corticosterone enzymes. This will ultimately result in the adrenal cortex releasing increased plasma corticosterone to overcompensate and therefore, at least temporarily, be at higher than normal levels.

Although this is not necessarily long term (weeks to months), similar impairments in acute and chronic stress studies have noted decreased cognitive performance, spatial processing, and acquisition [Bodnoff et al., 1995]. Besides neuronal damage, elevated corticosterone can increase adrenergic activity [Herbert et al., 2006]. The norepinephrine content measured in the hippocampus showed an effect of corticosterone manipulation in both SHR/y and WKY males. SHR/y males treated with metyrapone had significantly increased norepinephrine content compared to controls. In addition, alterations in norepinephrine with/without corticosterone are known to modulate learning and memory when delivered systemically or infused into neural tissue. The hippocampal
norepinephrine was only a measure of the content present in the tissue and does not necessarily reflect the receptor number, transport, synaptic release, breakdown or activation of the post-synaptic neurons. Therefore, the trend noted in hippocampal norepinephrine would suggest that if corticosterone is impairing performance through a catecholaminergic pathway, it is likely involving norepinephrine and adrenergic activity within the CNS [Berlau and McGaugh, 2006].

As well as corticosterone manipulation increasing hippocampal norepinephrine, the impaired maze performance also indicated an SHR Y chromosome effect. Previous studies in our lab have identified that the SHR/y strain shows increased SNS indices of stress arousal [Ely et al., 1993; Andrews et al., 1994; Ely et al., 1997]. Although the SHR/y and WKY control males performed similarly, they could differ in response to corticosterone sensitivity.

In conclusion, maze testing identified impaired learning in both strains following corticosterone manipulation, with only the SHR/y males showing increased hippocampal norepinephrine and impaired retention performance. This suggests that the SHR/y (SHR Y chromosome) males may potentially have an increased corticosterone sensitivity, smaller number of hippocampal neurons prior to damage, increased glucocorticoid/mineralocorticoid receptor number, and also increased 11 hydroxysteroid enzyme activity. These potential factors may be involved in a mechanism involving increased tyrosine hydroxylase activity and decreased dopamine beta hydroxylase activity. These enzymes involved with catecholamine and glucocorticoid synthesis could potentially be regulated by corticosterone levels ultimately resulting in increased
hippocampal norepinephrine content and impaired maze performance. The underlying mechanism of action regarding impaired learning and memory in SHR/y males would therefore involve a locus on the Y chromosome further compounding the impairment in hippocampal function following corticosterone manipulation.
Chapter V

Study 4: Maze Acquisition and Retention Trial Performance is Impaired in Aged SHR/y and WKY Males

Introduction

The previous chapters have identified several SHR Y chromosome phenotypes including impaired maze performance and increased stress responsiveness, as well as, sensitivity to corticosterone manipulation, and a potential over-activation of the hypothalamic pituitary adrenal (HPA) axis. These alterations in stress responsiveness of control males, chronically stressed SHR Y chromosome (SHR/y) males, and the overall increased sensitivity to corticosterone manipulation suggests that SHR/y males show impaired learning acquisition and memory performance compared to WKY males. This raises several questions regarding how this SHR/y male phenotype could potentially affect learning and memory following lifelong exposure to minor perturbations, as present in aged animal models.

Aged populations often exhibit deficits in learning acquisition and memory performance on spatially orientated tasks as compared to younger controls [Oler and Mochers, 1998]. This is supported by a large body of research using a variety of models
involving elderly humans, non-human primates, and rodent, all of which indicate that performance on hippocampal or visual spatial related tasks is impaired [Morris et al., 1982; Miguez et al., 1999; Kaasinen et al., 2000; McDonald et al., 2007]. This decline in performance is believed to be due to the increased neuronal atrophy associated with aging, which is specific to the hippocampus [Markhan et al., 2005]. In addition to impairing performance on memory related tasks, this neuronal decline also alters hippocampal synaptic plasticity [Foster, 1999; Rosenzweig and Barnes, 2003]. Although neuronal death and altered plasticity of the hippocampus occurs to a small degree in normal healthy aged populations [Morrison, 1997], it is accelerated in the compromised aged populations. Of particular interest regarding this study is the neurological decline related to decreased hippocampal processing in aged populations.

The hippocampus plays a well documented role in spatial learning and memory formation [Jarrard, 1995; Tulving and Markowitsch, 1998]. Although the hippocampus exhibits a high degree of plasticity, it is a particularly vulnerable structure to psychological and physiological stressors [Squire, 1992]. Stress related deficits, especially when persisting under chronic conditions, typically elicit severe behavioral consequences resulting from hippocampal dysfunction, including: depression, deficiencies in memory, aggression, neuronal cell death, and hippocampal atrophy [Williams and Herrup, 1988; Manji, 2003; Sampecto and Diaz, 2005]. These deficiencies are thought to be due to an interaction involving the hypersecretion of stress hormones under basal and stressed settings, hippocampal atrophy, and general lack of information processing ability [Luine et al., 1990a; Luine et al., 1990b; Hibberd et al., 2007] present
in aged models. Not only is the stress response and corticosterone release altered in aged animal models, but basal levels can also be affected. Corticosterone levels do clearly change in response to age, with the degree and type of change being related to the strain, gender, and testing model used [Akirav et al., 2004]. In aged rodents, the increased corticosterone following stress bears a strong positive correlation with impaired spatial memory performance and a negative correlation with hippocampal volume [Landfield et al., 1978; Landfield et al., 1996]. Further evidence for dysregulation of internal homeostasis is suggested by the glucocorticoid feedback theory of hippocampal aging. This theory suggests that even minor increases in corticosterone, if persistent, can accelerate impairment and aging of the hippocampus through a variety of potential pathways [Sapolsky et al., 1985; Squire, 1992].

As well as these adrenocorticotrophin hormone / corticosterone related alterations, age related dysfunction of the nervous system is another serious problem affecting an increasing number of elderly populations [Kaasinen et al., 2000; Roozendaal et al., 2004; Pisaska et al., 2000]. The sympathetic nervous system (SNS) of an aged individual shows a seemingly wide array of responses from hyperactivated to attenuated depending on the various stressors used (i.e. restraint, urinary odor, or social defeat) [Lee and Ma, 1995; Men et al., 1999; Tzavaro et al., 2006]. This is of particular importance regarding memory due to the high degree of adrenergic connections involving the hippocampus [Schroeter et al., 2000]. As a result, impaired acquisition and retention in spatial memory tasks of aged rodents has been linked with decreased norepinephrine activity and an increase in norepinephrine tissue content [Collier et al., 2004]. Changes in
norepinephrine content and receptor ligand binding are also indirectly related to several of the other deficiencies involving impaired function and performance [Collier et al., 2004].

Specifically regarding learning, memory and the hippocampus, the adrenergic input and relatively important role of norepinephrine suggests a strong functional role in modulating learning and memory retention [Schroeter et al., 2000]. Therefore, the purpose of this study was to examine the role of the SNS activity and potential over-activation of adrenocorticotrophin stimulating corticosterone release. The hypothesis to be tested is that aged SHR/y males will show impaired water maze performance (acquisition and retention) and increased stress responsiveness compared to aged WKY and control males.

Materials and Methods:

Study Design

The objective of this study was to establish that aging impairs maze performance in SHR/y and WKY males. In addition, the potential role of the SHR Y chromosome in conjunction with the stress response was also of interest regarding aging effects. For this study, aged adult (13month) and control adult (3month) SHR/y and WKY males were housed under standard conditions (n=6-8/strain and age). The age of the SHR/y and WKY males used in this study was approximately 13 months, compared to other aging studies referring to aged or old adults as ranging from 18 to 24 months of age [Goss and Morgan, 1995; Rapp et al., 2002; Du et al., 2006].
**Breeding Paradigm**

The Y chromosome animal model used in this study consisted of the consomic borderline hypertensive (SHR/y), and normotensive Wistar-Kyoto (WKY) rats. SHR and WKY rats were originally obtained from Harlan Sprague-Dawley (Indianapolis, IN) in 1981 (SHR/Hsd and WKY/Hsd), and colonies have been maintained at the University of Akron research facility (SHR/UA). This breeding scheme has been described previously (Chapter I). Briefly, comparisons between WKY and SHR/y males allow us to investigate the role of the SHR Y chromosome in a WKY genetic background. For a detailed review of this SHR Y chromosome comparison, see Ely et al. [1993].

**Maze Testing Paradigm**

The morris water maze (MWM) test paradigm was used in this study to spatial learning and memory processes, see Chapter II for a detailed description of the MWM and testing conditions. This is a standard test consisting of a platform hidden underneath the surface of the water. Specifically, the maze consisted of a 5’ diameter tank, with external maze cues placed on the walls of the room. There were three days of acquisition with 5 trials per day and 1 retention trial for each maze version. Each trial was run for a maximum of 90 seconds. After finding the platform (6” diameter), the animal was given a 20second mapping period before removal from the maze. The MWM consisted of three maze versions with version A constituting packing foam, version B murky water, and version C murky water with a stressor after acquisition day 1. These versions allow for comparisons between each maze, including: general improvement in performance on
each version (versions A, B, and C), difficulties locating the platform due to physical obstructions (version A), lack of obstructions (version B), interference effects from previous platform locations (versions B and C), impairment in retrograde memory (maze version C), and also repetition of trials (acquisition trials within each version).

Blood Sampling

One week following completion of all three MWM versions animals were stressed and then anesthetized with Sodium Brevital (50mg/kg, IP; E. Lilly, Indianapolis, IN), see Chapter III for a detailed description of the stress paradigm. Following the delivery of anesthesia, a 2-3 ml retro-orbital blood sample collected between 1100 and 1700 hours and centrifuged for 5 minutes (5,000xG) to obtain stressed plasma. Animals were again anesthetized a week later with a baseline sample being obtained prior to termination and stored at -70°C until analysis.

Plasma Adrenocorticotrophin Hormone

Adrenocorticotrophin hormone was analyzed in plasma by RIA (#24130, DiaSorin, Stillwater, Mn). The intra-assay variation was 8.1 %, the inter-assay variation was 6.7 %, with a sensitivity at 15pg/ml at the 95% confidence limit, and with the highest cross-reactivity being Porcine ACTH 1-39 and Human ACTH 1-24 at 100%, with other peptides at <0.01%. 

Plasma Corticosterone

Corticosterone was analyzed in plasma by RIA (DSL-80100, Diagnostic Systems Laboratories, Inc., Webster, Tx.). The intra-assay variation was 3.43%, the inter-assay variation was 7.3%, with a sensitivity at 2.7 ng/ml at the 95% confidence limit, and with no reported cross-reactivity.

Plasma Norepinephrine

Plasma norepinephrine was analyzed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters 460 Detector, Milford, MA) and has been described previously (Chapter II).

Statistics

Statistical analysis was performed by using Two-way RM ANOVA and a post-hoc Bonferroni test, One-way ANOVA, and Student’s t-test was used where applicable. The statistical tests were run on SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA) with significance assumed at p<0.05.

Results

Aged adult males from both SHR/y and WKY strains water maze acquisition (Figure 5.1) over all three maze versions was significantly impaired compared to controls (F=21.7, p<0.001). In addition, SHR/y aged males showed the most impaired water maze acquisition performance compared to WKY aged (F=13.8, p<0.01) and SHR/y
adult males (F=44.3, p<0.001). Adult SHR/y males performed better than adult WKY males (F=13.2, p<0.01). Aged adult WKY males were impaired compared to adult WKY males (F=12.2, p<0.01).

Aged adult males from both SHR/y and WKY strains (Figure 5.2) retention trial latency was significantly impaired compared to SHR/y and WKY controls (F=54.5 p<0.001). SHR/y aged males showed severely impaired retention performance compared to aged adult WKY and adult SHR/y males (15.4, p<0.001). Adult SHR/y males showed impaired retention performance compared to adult WKY males (N.S., p=0.07). Aged adult WKY retention was impaired compared to WKY adult (F=14.1, p<0.001).

SHR/y and WKY males from both age groups stress plasma adrenocorticotrophin (Figure 5.3) was significantly increased compared to the respective baselines (F=11.3, p<0.001). There was also an interaction effect for SHR/y males between strain and age (F=4.5, p<0.05).

SHR/y and WKY males from both age groups stress plasma corticosterone (Figure 5.4) was significantly increased compared to the respective baselines (F=10.1, p<0.001). Baseline aged SHR/y male corticosterone was less than aged WKY corticosterone (p<0.05).

Aged SHR/y and WKY male stress plasma norepinephrine (Figure 5.5) were not different from each other, but showed a significant increase compared to stress adult male plasma norepinephrine (p<0.01). SHR/y adult control male plasma norepinephrine was significantly increased following stress (p<0.01).
Aged Adult SHR/y Males Show Worst Maze Performance

Figure 5.1. Average daily platform latency (seconds) over three maze versions for males from each age group and strain (means, ± SEM). Two-way ANOVA indicates that aged SHR/y males showed the most impaired maze performance (i.e., latency to platform) compared to aged WKY (F=13.8, **p=0.003) and young SHR/y males (F=44.3, ***p<0.001). Two-way ANOVA indicates that aged WKY males also showed impaired maze learning than young WKY males (F=13.2, xxp<0.01). Maze acquisition is impaired in WKY compared to SHR/y control males (F=12.2, ++p<0.01).
Figure 5.2. Average retention trial platform latency (seconds) for males from each age group and strain over all three maze versions (means, ± SEM). Two-way ANOVA indicates that aged SHR/y retention performance was impaired compared to aged adult WKY and control adult SHR/y males (F=14.5, ***p<0.001; F=55.4, ***p<0.001). Two-way ANOVA also indicates that aged adult WKY males showed impaired retention performance compared to adult WKY males (F=15.4, **p<0.001). Control adult SHR/y retention performance was less than WKY on maze version C (p=0.07).
Stress Increases Plasma Adrenocorticotrophin Hormone in Both Strains and Age Groups

Figure 5.3. Baseline and stressed plasma adrenocorticotropin hormone (ng/ml) for each age group and strain (means, ± SEM). Two-way ANOVA results indicate a significant effect of stress increasing plasma adrenocorticotrophin hormone for both age groups and strains (F= 11.3, ***p<0.001), along with a strain and age interaction effect for SHR/y males (F=4.5, *p<0.05).
Stress Increases Plasma Corticosterone in Both Strains and Age Groups

Figure 5.4. Baseline and stressed plasma corticosterone (pg/ml) for each age group and strain (means, ± SEM). Two-way ANOVA results indicate a significant effect of stress increasing plasma corticosterone for both age groups and strains (F=10.1, ***p<0.001). Aged baseline SHR/y male plasma corticosterone was significantly less than aged WKY plasma corticosterone (×p<0.05).
Aged Adults Show a Greater Increase in Plasma Norepinephrine Following Restraint Stress

Figure 5.5. Baseline and stressed plasma norepinephrine (means, ± SEM) for each age group and strain. Two-way ANOVA results indicate a significant effect of age and stress increasing plasma norepinephrine for both age groups and strains (F= 10.1, ***p<0.001). SHR/y stressed adult norepinephrine was greater than SHR/y baseline adult norepinephrine and WKY stressed adult norepinephrine (**p<0.01).
Discussion

The results of this study support the hypothesis that aged adult males (SHR/y and WKY) have impaired maze performance in acquisition and retention trials compared to the control adult males. In particular, the SHR/y aged adult males showed the most severe impairment for each acquisition and retention trial over all three maze versions. Aged SHR/y and WKY males also showed an overall increased stress responsiveness of plasma norepinephrine compared to controls. The basal and stress levels of corticosterone and adrenocorticotrophin hormone suggest a potential increased sensitivity of the SHR/y hypothalamic pituitary adrenal (HPA) axis. Based on this data, there are two likely physiological pathways that could explain the impaired maze performance. The first pathway, through HPA axis overactivation, could explain the strain differences in acquisition and memory retention between SHR/y and WKY aged males. The second pathway, involves the sympathetic adrenal medullary (SAM) axis and could explain the overall impairing age effect on impaired acquisition and retention performance in both strains.

The trend in impaired learning acquisition performance was similar over all three maze versions in both strains of aged adult males compared to the control adult males. The term “aged” is often used in the literature to represent animals between 20 and 24 months old, whereas the aged SHR/y and WKY males presented here were younger (approximately 13 months) [Goss and Morgan, 1995; McEwen, 1999; Rapp et al., 2002; Du et al., 2006]. This difference in animal age was likely not a factor for acquisition (or retention) performance, since the maze data was consistent with these published studies
showing a significant impairment in maze performance by aged animals. This impaired acquisition performance also corresponded to impairment in the retention trials, with aged males (SHR/y and WKY) consistently showing impaired retention performance compared to the control adult males.

The impaired retention trial performance in SHR/y and WKY aged males could be a result of a failure to learn the platform location, as noted in the acquisition trials. Several aging models, including: humans, non-human primates, rats, and mice all show similar results of impaired retention performance in aged or elderly populations [Aston-Jones and Bloom, 1981; Sapolsky, et al., 1985; Herbert et al., 2006]. Since the only genetic difference between the SHR/y and WKY males is the SHR Y chromosome, any behavioral difference can be attributed to this SHR Y chromosome effect. The SHR/y males have previously been shown in our lab to have an increased stress responsiveness [Ely et al., 1993; Ely et al., 1997], their impaired retention trial performance is consistent with other hyper-responsive and stress sensitive animal models showing impaired retention compared to controls [Guarraci et al., 1999; Pisceska et al., 2001;]. These memory deficits are often a result of degeneration of cell bodies and decreased plasticity mainly associated with the hippocampus [Foster, 1999; Rosenzweig and Barnes, 2003]. The hippocampal pathways for memory consolidation and/or retrieval could be damaged, inefficient, or non-functional. Much of this damage to the hippocampus can be attributed to glucocorticoids (i.e. corticosterone) accelerating the impairment and aging of the hippocampus [Jameison and Dinan, 2001; Hibberd et al., 2007]. It is important to point out, however, that even though the basal levels may be
lower or even similar between both age groups and strains, the plasma levels do not necessarily reflect receptor number, sensitivity of the HPA axis, and sensitivity corticosterone within the hippocampus.

The stress responsiveness of the HPA axis in SHR/y aged compared to control males showed a relative decrease in basal and stressed adrenocorticotrophin hormone, with an apparently over-responsive corticosterone release following stress. In comparison, the WKY aged and control males showed relatively little difference from each other in stress responsiveness of adrenocorticotrophin hormone and corticosterone. Initially, WKY males appear to be more stress responsive under basal and stress conditions. However, when taken into account with the rest of the HPA axis factors, such as adrenocorticotrophin hormone, this may not be the case. The SHR/y aged males have a decreased basal corticosterone compared to WKY aged males, but with similar stressed corticosterone levels. This would suggest either an increased sensitivity or potential dysregulation of corticosterone in the SHR/y aged males.

Several possibilities could explain this strain difference, such as a decreased receptor number, increased corticosterone binding globulin (CBG), increased receptor sensitivity, or just a lower homeostatic set point for corticosterone often noted in aged animal models [Goodyer et al., 2000; Purnell et al., 2004]. For example, following stress the CBG releases the bound corticosterone and is the primary source for the increased free plasma corticosterone. If CBG is increased, then there is the possibility for a smaller basal level followed by higher or prolonged elevations of corticosterone following stress. There is also the potential for altered mineralocortioid or glucocorticoid receptor
number/sensitivity between the control SHR/y and WKY strains, as well as, aged groups if basal or CBG levels change. An increased sensitivity to corticosterone would explain why there are similar plasma corticosterone levels between the aged males, but significant impairments in the SHR/y aged males. Previous chapters do in fact indicate that there is an increased sensitivity to corticosterone manipulation in the SHR/y strain. Therefore, the HPA axis itself or related component is likely partly responsible for the impaired differences between the SHR/y and WKY aged animals.

The second pathway that can explain the aging effects in both strains is the SAM axis. Both aged SHR/y and WKY males had elevated stressed plasma norepinephrine compared to stressed adult males, along with slight elevations in basal resting levels. This suggests that increased SAM activity in aging may be responsible for an overall decreased performance with the SHR/y aged males being more sensitive. Age related dysfunction of the nervous system is a serious problem affecting an increasing number of elderly populations [Abe et al., 2002; Lotti, 2002]. Since normal hippocampal function is crucial for proper visual spatial maze performance and the hippocampus has a dense input of adrenergic neurons, one potential mechanism for the age impairment may at least in part involve a sympathetic nervous system related deficit through the SAM axis [Luine, 1990; Squire, 1992; Foster, 1999]. This would indicate that there is at least an overactive SAM axis pathway involved with aging, which may be partly responsible for the impaired performance in aged males.

Other than these potential physiological responses contributing to impaired performance, there are several possibilities directly pertaining to the MWM itself that
could be responsible. For example, the acquisition data for the first maze version (A) was consistent between both aged adult and control adult strains, where the aged adults performed worse. Maze version A consisted of packing foam and as discussed previously, was an obstruction thereby making swimming and platform location more difficult. The MWM has been identified as a stressful paradigm, with decreased water or ambient temperatures able to stimulate HPA and SAM axis activity, along with impairing performance [Livonen et al., 2003; VanDam et al., 2006]. In addition to this potentially increased difficulty in swimming and platform location, aged animal models also show a decreased in motor function ability [Shanks et al., 2000]. Therefore, the impaired acquisition performance could be over exaggerated from a general decline in swimming ability. Since swim speed was not calculated from collected data, only observational comments can be made. For example, there were no noticeable decreases in time spent swimming or relative increases in time spent floating, with both of these possibilities leading to an increased platform latency time. In fact, some SHR/y aged males spent the entire time (90 seconds) engaged in thygmotaxis. This could be addressed in the future with a quadrant time analysis of the maze test and also software analysis of swim speed.

However, the possible decline in motor function as the only impairing factor does not explain the lack of consistent improvement from Day 1 to Day 2, specifically on maze versions A and B. Improved performance in this time period has been noted on several occasions in the previous chapters. Furthermore, if the SHR/y males also fail to show significant improvement in acquisition times in each maze version for each acquisition day, this would support a decrease in hippocampal processing and not
necessarily an inability to swim. This trend of impaired performance was particularly evident in the aged SHR/y males, whereas the aged WKY males approached WKY control male latency times as evident in Maze versions B and C. In addition, there also appeared to be a greater variability within the aged SHR/y males suggesting that some males may have a greater degree of impairment.

Although the blood samples from these SHR/y and WKY males were taken at the same time of day to avoid differences associated with the circadian rhythms, they still only represent one point in time. For example, the blood samples were taken at one time point following stress and comparisons can only be accurately be made between strains and ages at this time point. Therefore, the relative release of corticosterone or adrenocorticotrophin hormone does not specifically address the degree of stimulation, amounts released, or even the turnover rate. This could potentially explain the similar corticosterone release following stress between aged SHR/y and WKY males. The SHR/y aged male plasma corticosterone or adrenocorticotrophin hormone may have had a higher peak at an earlier time point following stress compared to the WKY aged male. There is also the possibility that even though the plasma levels may be the same, there could be an increased or decreased sensitivity, as previously mentioned. Aging can produce a variety of results related to corticosterone stress responsiveness [Aston-Jones and Bloom, 1981; Collier et al., 2004]. Aged animal models also show a more pronounced response of adrenocorticotrophin hormone and subsequent corticosterone release to acute and chronic stressors compared to younger controls [Morano et al., 1994; Miller and O’Callaghan, 2005], which were noted in SHR/y aged males. Therefore, the
HPA axis in conjunction with differences in the SAM axis could explain why SHR/y aged adults performed worse on learning and memory tests compared to WKY aged adults.

WKY and SHR/y aged males showed an increased SAM axis response, with the aged SHR/y males showing a potentially overactive HPA axis. Since increased central and peripheral norepinephrine, as well as corticosterone all have the potential to alter hippocampal function and damage neurons, this in part could explain the general impaired performance noted in aged males. In the case of SHR/y males, there is also the possibility that the impaired performance is the result of increased stress responsiveness over the lifetime of the animal. Therefore, the mechanism of learning and memory impairment is likely due to an interaction of the HPA axis and SAM axis along with the SHR Y chromosome that is responsible for the increased stress responsiveness and potentially damaged hippocampus in SHR/y males. However, future analysis of the probe and acquisition trial data involving swim speed, annulus crossings, and quadrant location will be necessary in order to distinguish between a central (i.e. hippocampal) or a more peripheral (i.e. motor decline) mechanism of impairment.
Chapter VI

Study 5: The SHR Y chromosome Increases Physiological and Behavioral Indices of Aggression in the Colony Environment

Introduction

The preceding chapters have identified the following SHR Y chromosome (SHR/y) male phenotypes: impaired maze acquisition and retention trial performance, elevated sympathetic adrenal medullary stress responsiveness, potential over-activation of the hypothalamic pituitary adrenal axis, increased hippocampal norepinephrine content, and decreased hippocampal tyrosine hydroxylase activity. This is consistent with previously published data from our lab showing that males with the SHR Y chromosome have increased sympathetic nervous system activity and stress responsiveness compared to males with the WKY Y chromosome males [Ely et al., 1993; Andrews et al., 1994; Ely et al., 1997]. Studies with this rodent model (SHR/y vs. WKY) in a semi-natural colony environment have also shown that SHR/y males have increased plasma norepinephrine and blood pressure following stress [Ely et al., 1997; Ely et al., 2000]. Additionally, males with the SHR Y chromosome also exhibit increased indices of intermale and resident intruder colony aggression, along with lower brain serotonin
and increased testosterone [Toot et al., 2004]. In terms of aggression, testosterone and serotonin are the two critical modulators of aggressive behavior.

Both testosterone and serotonin are widely studied regarding physiological mechanisms of aggressive behavior in rodent, nonhuman primates, and human models of aggression. Androgens, specifically testosterone have been directly related to increasing aggression in animal models involving physical provocation, resident intruder, and foot shock, testing paradigms [Ulrich and Azrin, 1962; Schicknick et al., 1993; Breur, 2001]. Testosterone treatment can also indirectly decrease the synthesis of serotonin in the central nervous system [Bell and Hepper, 1987; Simon et al., 1998], resulting in increased aggression. Brain serotonin levels and activity within the limbic system, specifically the amygdala are implicated in aggressive behavior, with decreased amygdala serotonin content being present in more aggressive mice [Keleta et al., 2007]. Further supporting this serotonin and amygdala relationship are serotonin receptor agonist studies, showing inhibition of offensive and muricidal behavior in adult rats [Saudua, 1994; Everts, 1997; Veenstra-VanderWeele, 2000]. Therefore, serotonin clearly has an inverse relationship with aggression, potentially driven by a testosterone mechanism.

The use of a social colony is important in the study of aggression, by allowing for a more natural housing environment in which to study this social behavior [Ely and Henry, 1978; McKittrick et al., 1995; Ely et al., 1997]. Animals housed in semi-natural colonies often form social hierarchies, and are able to engage in other social behaviors, such as territorial defense, mating, and grooming. Within the colony as a whole,
intermale and territorial conflict is increased compared to control males, suggesting that
the social environment has an important role in aggression studies [Toot et al., 2004].
The purpose of this study is to expand on our previous resident colony studies and
examine colony males in dyadic behavioral paradigms through resident intruder home
cage (partitioned colony sections) aggression tests for each resident colony male.
Therefore, the hypothesis to be tested was that SHR/y males from the experimental
colony and control standard housed environments will show elevated aggression, as well
as increased testosterone and decreased brain serotonin compared to that of WKY males.

Materials and Methods:

Study Design

The objective of this study was to study colony SHR/y and WKY males in a
dyadic testing paradigm. Specifically, examining individual differences in aggressive
behavior, physiology, and neurochemistry, the relative role of the SHR Y chromosome in
aggression can be better understood in a social context. This study uses a two strain
(SHR/y and WKY) by two treatment (colony and noncolony) design, with n=8-10 males
in each strain and treatment group. The study lasted for a total of 6 months.

Animal Model

The Y-chromosome animal model used in this study consisted of the stress
sensitive consomic borderline hypertensive (SHR/y) and normotensive Wistar-Kyoto
(WKY) rats. The detailed breeding scheme has been previously described, chapter II,
which ultimately allows for the study of the SHR Y chromosome by comparing WKY and SHR/y males [Ely et al., 2000].

**Colony environment**

The group housing environment is strain specific for SHR/y or WKY rats, consisting of 8-10 intact males and 8-10 females, all initially 8 weeks of age. The colony model for social stress used in this study is based on previous work involving rodent behavior in natural populations. With this particular design, male and female rats are able to interact socially in a center open field cage (1.25 m x 1.25 m) center open field cage with four attached side cages (0.6 m x 0.6 m).

**Standard Resident Intruder Test**

The territorial behavioral test used to elicit aggressive behavior was the resident intruder (RI) home cage paradigm [Blanchard and Blanchard, 1977]. The RI paradigm was 20 minutes in duration and consisted of placing a novel strain and gender matched intruder into a partitioned colony section of the resident colony males. Behavioral tests were conducted during the light cycle (900 to 1400 hrs) and filmed for later analysis of attack number and attack latency. In addition, vocalizations, characterized by squeaks, were counted during testing. Vocalizations have been reported to be an auditory measure of irritation in behavioral testing with rodent models [Nelson, 2004].
Physical Provocation Test

The physical provocation test consists of the standard RI test, along with physically provoking the home animal by a series of tail pinches [Breur, 2001]. Specifically, one minute after placing the intruder male into the partitioned cage, the resident male was provoked to attack by a tail pinch. The tail pinch consists of a moderate pinch to the tail for 1 second every minute for the duration of the test. Each test lasted for a total of 20 minutes.

Plasma Blood samples

Prior to termination all animals were anesthetized with Sodium Pentothal (50mg/kg, IP; E. Lilly, Indianapolis, IN) and a 2-3 ml retro-orbital blood sample was collected between 1100 and 1700 hours. The samples were then centrifuged for 5 minutes (3,000rpm), plasma was drawn off, and stored at -70°C until later analysis of plasma testosterone.

Plasma Testosterone

Testosterone was analyzed in plasma by RIA (Bio-Rad Laboratories, Hercules, CA). The correlation with another kit was r=0.991, sensitivity was 0.08 ng/mL at the 95% confidence limit, and the highest cross-reactivity with potential interfering steroids was with 5α-dihydrotestosterone (6.65%). The coefficient of variation for our sample intra-run was 7.4% to 11.6% and for inter-run was 12.5% to 16.96%.
Palkovit’s Brain Punch

Following termination, the brains were frozen on dry ice, stored at -70C, and later cut into 200 micron coronal sections at -20C (Minotome, International Equipment Company, Damon Division, Meedham Hts., Mass.). The sections were then punched [Palkovits, 1973] from the medial amygdala (AME), lateral amygdala (ABL), and anterior amygdala (AAA). Proper coronal slice and punch orientations were verified with comparison to the rostral-caudal bregma zero reference points [Pellegrino, 1981]. The punches were homogenized in either mobile phase or sucrose, centrifuged for 10 minutes at 3200rpm (Sorvall RT7, Kendro Labs, Newton Conn.) and stored at -70C for later analysis.

Brain Serotonin Assay

The serotonin content from each area of the amygdala (AME, ABL, and AAA) was analyzed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters 460 Detector, Milford, MA). The mobile phase consisted of Citric Acid (35mM), Sodium Acetate (90mM), Octyl Sodium Sulfate, (460uM), EDTA (130uM) and 14% Methanol at a pH of 4.7. The HPLC pump, Water 510 (Waters Corp., Mildford, MA) was set at 1.3ml/min. The column was a Supelcosil LC-18, 15cm x 4.6mm, 3um which was preceded with a guard column (Discovery RP-Amide C16 2cm x 4.0mm, 5um, Supelco, Bellefonte, PA). A constant amount 2,3-DHBA was spiked into each sample and the peak height ratio was calculated electronically.
Statistics

One-way ANOVA and two-way ANOVAs were used, when appropriate. Student’s t-tests were used to compare individual groups. Analyses were run on SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA), with significance assumed at p<0.05.

Results

Plasma testosterone (Figure 6.1) was elevated in SHR/y colony males compared to SHR/y control and WKY colony males (p<0.05). There was a significant interaction effect of strain and housing WKY colony and control males were not significantly different from each other.

The number of attacks following physical provocation (Figure 6.2) was higher in SHR/y control and colony males compared to WKY control and colony males (p<0.05). The SHR/y control males showed more attacks to intruders following physical provocation than the SHR/y colony (p<0.05) and WKY control males (p<0.05). In addition, SHR/y colony males were more aggressive than WKY colony males during the physical provocation testing (p<0.05).

Vocalizations for both strains and housing treatments (Figure 6.3) were increased in the physical provocation compared to the baseline resident intruder tests (F=12.8, p<0.001). The SHR/y control males vocalized more during physical provocation than the SHR/y colony (p<0.05) and WKY control males (p<0.05). In addition, SHR/y colony
males also vocalized more during physical provocation tests than WKY colony males (p<0.05).

Latency until the first attack (Figure 6.4) for both strain and housing treatments was higher in the baseline resident intruder tests compared to the physical provocation paradigms (F=15.2, p<0.001). SHR/y colony and control males attacked earlier than the respective WKY comparisons (p<0.05).

SHR/y colony medial amygdala serotonin content (Figure 6.5) was lower than SHR/y control and WKY colony (p<0.05). However, the WKY colony medial amygdala serotonin content was higher than WKY control (p<0.05). In addition, SHR/y control and colony male lateral amygdala serotonin (Figure 6.6) content was also lower than the respective WKY control and colony housing comparisons (p<0.05). Finally, WKY colony anterior amygdala serotonin (Figure 6.7) content was higher than WKY control and SHR/y colony (p<0.05).
Plasma Testosterone is Increased In SHR/y Males Compared to WKY Males

Figure 6.1. Plasma testosterone (ng/ml) for SHR/y and WKY control and colony males (means, ± SEM). SHR/y colony males plasma testosterone was higher than SHR/y control and WKY colony male plasma testosterone (*p<0.05). Two-way ANOVA indicated a significant interaction effect of the housing and SHR Y chromosome (F=8.4, p<0.01).
Figure 6.2. Average number of aggressive events over 20 minutes for SHR/y and WKY colony and control males for resident intruder tests with and without physical provocation (means, ± SEM). The SHR/y control males number of aggressive events following physical provocation was higher than the WKY control and SHR/y colony males (***p<0.001). The SHR/y colony male physical provocation aggression score was also higher than the in the WKY colony (*p<0.05). Two-way ANOVA results indicated that physical provocation increased aggression in both strains and housing groups, with the SHR/y strain being more responsive (F= 15.2, p<0.001).
Figure 6.3. Average number of vocalizations over 20 minutes for SHR/y and WKY control and colony males for resident intruder tests with and without physical provocation (means, ± SEM). The SHR/y control males number of vocalizations following physical provocation was higher than the WKY control and SHR/y colony males (**p<0.01). The SHR/y colony male physical provocation vocalization number was also higher than the in the WKY colony (#p<0.05). WKY control male vocalization number was higher than in the WKY colony (#p<0.05). Two-way ANOVA results indicated that physical provocation increased aggression in both strains and housing groups, with the SHR/y strain being more responsive (F= 18.2, p<0.001).
**Figure 6.4.** Average time for the first aggressive event (attack latency) in the 20 minute resident intruder test for SHR/y and WKY control and colony males with and without physical provocation (means, ± SEM). The SHR/y control attack latency was less than in the WKY control (*p<0.05). SHR/y control and colony attack latency times were less than the control and colony baseline times (x*p<0.05). Two-way ANOVA results indicate that physical provocation decreased attack latency in both strains and housing groups (F=4.8, p<0.05). No error bars indicate that the ceiling or maximum amount of time for the test was reached (20 minutes) without an aggressive event.
Anterior Amygdala Serotonin Content Increases in WKY Colony Males

Figure 6.5. Anterior amygdala serotonin content (pg/mg tissue) for SHR/y and WKY control and colony males (means, ± SEM). WKY colony anterior amygdala serotonin content was greater than WKY control and SHR/y colony serotonin (*p<0.05). There was no difference between SHR/y control and colony serotonin content in the anterior amygdala.
Medial Amygdala Serotonin Content Increases in WKY Colony Males

Figure 6.6. Medial amygdala serotonin content (pg/mg tissue) for SHR/y and WKY control and colony males (means, ± SEM). WKY colony male medial amygdala serotonin content was higher than SHR/y colony and WKY control medial amygdala serotonin content (**p<0.01). SHR/y colony medial amygdala serotonin content was lower than SHR/y control medial amygdala serotonin content (*p<0.05).
Figure 6.7. Lateral amygdala serotonin (pg/mg tissue) content for SHR/y and WKY control and colony males (means, ± SEM). SHR/y control male lateral amygdala serotonin content was less than WKY control lateral amygdala serotonin content (*p<0.05). SHR/y colony male lateral amygdala serotonin content was less than WKY colony lateral amygdala serotonin content (*p<0.05). Two-way ANOVA showed a significant effect of the SHR Y chromosome (F=6.2, p<0.01) and housing (F=13.6, p<0.001).
Discussion

The results of this study support the hypothesis that SHR Y chromosome (SHR/y) males are more aggressive than WKY Y chromosome (WKY) males. In addition, these results are also consistent with our previous research showing increased testosterone, increased aggression, and decreased serotonin in SHR/y colony compared to WKY colony males [Toot et al., 2004]. In addition, the contributing factor involving physical provocation induced aggression which was not present in the restraint or air stress testing paradigms (data not shown). This mechanism for increased aggression following physical provocation in chromosome colony males is influenced by the social colony housing environment, testosterone, and serotonin content in the amygdala.

SHR Y chromosome (SHR/y) males in both control and colony housing environments were more aggressive than the WKY males. This was particularly noted in the physical provocation paradigms, which showed an increase in the attack number and vocalizations by the resident males. Since both strains had similar latencies to attack, i.e. time for first attack, the increased aggression in SHR/y males was not due to an earlier provocation response, resulting in a greater total attack number over a longer time period. All of the attacks by SHR/y and WKY males were actually over the final five minutes of the resident intruder physical provocation test. This indicates that the aggression by SHR/y males is more concentrated over a similar time period compared to WKY males. The physical provocation paradigm is a type of irritation that involves the application of pressure to the distal portion of the subjects tail, simulating a rat bite or offensive type of behavior [Breur, 2001]. Although colony males show an increased stress responsiveness
to novel stressors, such as restraint or air stress, the tail pinch is indicative of normal colony behavioral interactions. This explains why colony males as a group, are less responsive to provocation or physical irritation than the control males not exposed to the social housing environment.

Physical provocation increases offensive aggression in male rats with testosterone treatment [Martinez-Sanchic, 1996; Martinez-Sanchic, 1998], similar to what was present in the SHR/y colony males that had the highest plasma testosterone. Testosterone is a contributing factor to aggression through a complex mechanism involving environmental housing, brain neurochemistry, and peripheral behavioral pathways [Bell and Hepper, 1987; Simon et al., 1998]. Levels of aggression depend not only on the amount of circulating testosterone, but also the sensitivity of the androgen receptor [Ogawa, 1996]. Although the housing environment increased plasma testosterone in colony compared to control SHR/ males, it failed to cause a similar increase in aggression. This is consistent with other social colony studies, and also indicates that environmental housing conditions and behavioral encounters are also important regulators of aggression in addition to the genotype, or presence of the SHR Y chromosome.

Testosterone, as well as behavioral experience (winning or losing) can also influence central nervous system, and more specifically limbic system serotonin levels [Amstisklavskaya and Kudryautseva, 1997; Gardner et al., 2005]. As mentioned previously, the SHR/y colony male serotonin content within the examined regions of the amygdala (medial, lateral, and anterior) were lower in serotonin content than WKY colony males. This could in part be due to the increased testosterone influencing
serotonin synthesis via tryptophan hydroxylase regulation. The decreased serotonin and increased aggression by SHR/y compared to WKY colony males is also consistent with literature showing a negative correlation between serotonin and aggression [Bell and Hepper, 1987; Simon et al., 1998; Clement, 1999]. Studies on the rate limiting enzyme of serotonin synthesis, tryptophan hydroxylase indicate that estrogen increases TPH mRNA, whereas testosterone treatment acts to decrease TPH mRNA. In addition, the activity of TPH can also be decreased by repeated social victories in a social context, directly and indirectly resulting in increased aggression [Gardner et al., 2005; Mirio et al., 2006].

Interestingly, the Y chromosome has been linked to both serotonin activity and testosterone sensitivity [Maxson, 1981; Sluyter, 1994; Maxson, 1998]. This suggests that any behavioral mechanism of male aggression is at least in part regulated by genes unique to the Y chromosome. Regarding the development of aggression, murine research also indicates that the Y chromosome influences the etiology of aggression in juveniles and adults, as well as differences in intermale aggression within each group [Maxson, 1981; Oortmerssen et al., 1987]. When taken into account with the SHR/y and WKY animal model, the only genetic difference between SHR/y and WKY males is the origin of the Y chromosome, allowing the increased aggression phenotype to be attributed to the SHR Y chromosome. In addition to testosterone and serotonin, the Y chromosome has also been identified as being involved with other behavioral characteristics, such as urinary odor type distinguishing factor [Yamazaki, 1990], which can include social recognition.
The results of study supported the hypothesis showing that the SHR Y chromosome increased aggression and testosterone, but decreased amygdala serotonin content compared to WKY Y chromosome males in the colony behavioral models. Clearly the use of the resident intruder physical provocation model in dyadic encounters showed an increased overall aggression for each colony male resident of the SHR/y and not WKY males. The colony males overall, however, did show an attenuated aggression response compared to control non colony males independent of the increased testosterone and decreased serotonin, as specifically noted in the SHR/y strain. This was likely due to the increased behavioral encounters (i.e. social acclimation) within the colony leading to more physical provocation necessary to elicit an aggressive response. In addition, this also indicates that each colony male (subordinates and dominant) was able to display aggression. Therefore, the increased aggression is due to an SHR Y chromosome mechanism involving: increased victories/defeats, decreased tryptophan hydroxylase mRNA, increased testosterone, and decreased serotonin content.
Chapter VII

Study 6: Testosterone Treatment Increases Aggression in Resident Intruder and Physical Provocation Paradigms.

Introduction

The previous chapter showed that SHR/y (SHR Y chromosome) males were more aggressive than WKY males in the social housing environment. In addition, colony male SHR/y compared to WKY testosterone was increased, along with decreased amygdala serotonin content. This physiological data, in conjunction with other studies supports the relationship between the physiological factors plasma testosterone and brain serotonin, with aggressive behavior [Shrenker and Maxson, 1986; Cologer-Clifford, 1999]. Although the exact mechanism for how testosterone increases aggression is not completely defined, testosterone is believed to act on the motivating cues associated with behavior in initiating offensive aggression and shows a positive correlation with increased aggressive acts [Albetti and Farabellini, 1994; Cologer-Clifford, 1999].

Androgens, specifically testosterone, are known to increase aggression in rodent models using physical provocation and also in other behavioral testing paradigms [Breur et al., 2001; Toot et al., 2004]. Testosterone propionate implants or daily injections
artificially elevate plasma testosterone increase and therefore aggression; whereas, castration consistently lowers aggression [Shrenker and Maxson, 1986; Bell and Hepper, 1987; Simon et al., 1998; Keleta et al., 2007]. Aromatase inhibitors can block the conversion of testosterone to estrogen and also show an increase in aggressive behavior [Trainor et al., 2006]. Treatment with dihydrotestosterone, as well as being non-aromatizable, is a metabolite of testosterone with an increased binding affinity for the androgen receptor that also increases aggressive behavior [Long et al., 1996]. Further study with the use of nandrolone or stanozolol, synthetic forms of testosterone involved with muscle growth, also increase aggression in rodents and humans [Long et al., 1996; Martinez-Sanchis 1998]. Clearly, testosterone and/or the metabolites of testosterone are involved with aggression [Ogawa, 1996], even though the exact mechanisms are not clearly defined [Cologer-clifford, 1999].

Much of the aggressive behavior resulting from testosterone treatment is potentially through testosterone or a metabolite of testosterone binding to the cytoplasmic androgen receptor and initiating an intracellular cascade stimulating gene transcription. The androgen receptor is necessary for testosterone homeostasis and the receptor mediated effects of testosterone potentially regulating behavior. Interestingly, the use of androgen receptor knockout mice have supported this line of inquiry, where these knock outs exhibit depressed aggression scores compared to normal androgen receptor male siblings [Scordalakes and Rissman, 2004].

Therefore, the hypothesis of this study is that SHR/y males will show an increased sensitivity to testosterone, with increased aggression and decreased amygdala serotonin
following testosterone treatment, compared to WKY males. With this large body of androgen related behavioral research, along with the Y chromosome, there are clearly behavioral mechanisms that warrant further study. In addition, work in our own lab regarding SHR Y chromosome (SHR/y) males has identified an earlier pre-pubertal rise in testosterone, increased aggression directed toward conspecifics, elevated steroid sulfatase activity, and increased testosterone sensitivity when compared to WKY males.

Materials and Methods

Study Design

The objective of this study was to determine if SHR/y males are more aggressive following testosterone treatment, after increasing, decreasing, or blocking the androgen receptor than WKY males. In addition, the role of testosterone on serotonin content will also be examined. SHR/y and WKY males were in the following treatment groups: castrate, castrate with testosterone implant, intact with flutamide, or gonadally intact. The treatment groups lasted for a total of 4 weeks, with n=4-6 / strain and treatment group.

Animal Model

Briefly, comparisons between WKY and SHR/y males allow us to investigate the role of the SHR Y chromosome in a WKY genetic background. This breeding scheme has been detailed previously (Chapter I).
Testosterone Implants

Testosterone implants for castrated rats at 8 weeks of age were prepared by cutting single lumen clear 50 Silastic tubing (0.062” ID x 0.125” OD) packed with 19mm of testosterone propionate (Sigma Chemical Co., St. Louis, MO). The ends of the tube were sealed with Silastic Medical Adhesive Silicone Type A (Dow Corning, Midland, MI) and allowed to dry 24 hours. Implants were primed at 4°C overnight in a solution composed of 5% bovine serum albumin, 10 mM sodium phosphate buffer (pH 7.0), 0.9% NaCl, and 0.0001% methiolate. Each implant was replaced every two weeks for the duration of the experiment.

Flutamide Treatment

Gonadally intact SHR/y and WKY males were given rat chow prepared with flutamide (83mg/kg body weight; Sigma Chemical Co., St. Louis, MO) to block the androgen receptor for the duration of the study (four weeks). Since flutamide animals were given food ad libitum, this is an approximate dose based on average body weights and food consumption in our lab.

Standard Resident Intruder Test

The territorial behavioral test used to elicit aggressive behavior was the resident intruder (RI) home cage paradigm [Blanchard and Blanchard, 1977]. The RI paradigm was 20 minutes in duration and consisted of placing a novel strain and gender matched intruder into a partitioned colony section of the resident colony males. Behavioral tests
were conducted during the light cycle (900 to 1400hrs) and filmed for later analysis of
attack number and attack latency.

**Physical Provocation Test**

The physical provocation test consists of the standard RI test, along with
physically provoking the home animal by a series of tail pinches [Breur, 2001].
Specifically, one minute after placing the intruder male into the partitioned cage, the
resident male was provoked to attack by a tail pinch. The tail pinch consists of a
moderate pinch to the tail for 1 second every minute for the duration of the test. Each test
lasted for a total of 20 minutes.

**Termination and Blood Collection**

Animals were overdosed at the conclusion of the experiment with Sodium
Pentothal (50mg/kg, IP; E. Lilly, Indianapolis, IN) and a 2-3 ml retro-orbital blood
sample was collected between 1100 and 1700 hours and centrifuged for 5 minutes
(5,000xG) to obtain plasma. Immediately following blood collection, the animals were
decapitated with the brains being stored at -70°C for later analysis.

**Testosterone Assay**

Testosterone levels were analyzed in plasma by RIA (Bio-Rad Laboratories,
Hercules, CA). The correlation with another kit was r=0.991, sensitivity was 0.08 ng/mL
at the 95% confidence limit, and the highest cross-reactivity with potential interfering
steroids was with 5α-dihydrotestosterone (6.65%). The coefficient of variation for our sample intra-run was 7.4% to 11.6% and for inter-run was 12.5% to 16.96%.

Palkovit’s Brain Punch

Following termination, the brains were frozen on dry ice, stored at -70C, and later cut into 200 micron coronal sections at -20C (Minotome, International Equipment Company, Damon Division, Meedham Hts., Mass.). The following amygdala sections were punched [Palkovits, 1973]: anterior amygdala (AAA), medial amygdala (AME), and lateral amygdala (ABL). Proper coronal slice and punch orientations were verified with comparison to the rostral-caudal bregma zero reference point [Pellegrino, 1981]. The punches were homogenized in mobile phase, centrifuged for 10 minutes at 3200rpm (Sorvall RT7, Kendro Labs, Newton Conn.) and stored at -70C for later analysis.

Brain Serotonin Assay

The serotonin content from the amygdala was analyzed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters 460 Detector, Milford, MA). The mobile phase consisted of Citric Acid (35mM), Sodium Acetate (90mM), Octyl Sodium Sulfate, (460uM), EDTA (130uM) and 14% Methanol at a pH of 4.7. The HPLC pump, Water 510 (Waters Corp., Mildford, MA) was set at 1.3ml/min. The column was a Supelcosil LC-18, 15cm x 4.6mm, 3um which was preceded with a guard column (Discovery RP-Amide C16 2cm x 4.0mm, 5um, Supelco, Bellefonte, PA).
A constant amount 2,3-DHBA was spiked into each sample and the peak height ratio was calculated electronically.

Statistics

One-way and two-way ANOVAs were used, as well as linear regressions, when appropriate. Student’s t-tests were used to compare individual groups. Statistical analysis was run on SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA) with significance assumed at p<0.05.

Results

Plasma testosterone (Figure 7.1) for both strains decreased following castration (p<0.05) and significantly increased following testosterone implantation (p<0.05) and flutamide treatment (p<0.05) compared to intact males. In addition, plasma testosterone levels showed a significant testosterone treatment effect (F=60.2, p<0.001).

Attack number was elevated in the SHR/y and WKY testosterone treated males (Figure 7.2) given testosterone or flutamide. SHR/y control and cast+Ti males were more aggressive than their respective WKY comparisons (p<0.05) during baseline testing. Physical provocation showed increased aggression in SHR/y control, cast+Ti, and flutamide treated males compared to the WKY control (p<0.01), cast+Ti (p<0.001), and flutamide (p<0.001) males. Physical provocation also showed an trend of increasing the amount of aggression in both strains (F=28.0, p<0.001).
The linear regression (Figure 7.3) for plasma testosterone and aggression during physical provocation was significant for both SHR/y (p<0.001) and WKY (p<0.001) males. As plasma testosterone increases, the number of aggressive events also increases.

Serotonin content in the anterior amygdala (Figure 7.4) increased following castration in WKY (p<0.05), but not SHR/y males compared to intact controls. The lateral amygdala (Figure 7.5) serotonin content was decreased in SHR/y and WKY cast+Ti males compared to their respective controls (p<0.05). Medial amygdala serotonin content (Figure 7.6) was less in the SHR/y control, castrate, and cast+Ti (p<0.05) males compared to the WKY comparisons. In addition, WKY flutamide treatment significantly decreased medial amygdala serotonin content compared to the WKY control (p<0.05). There was also an interaction effect of testosterone treatment with the SHR Y chromosome, showing decreased serotonin content in the medial amygdala (F=4.3, p<0.05).
Plasma Testosterone Increases Following Testosterone and Flutamide Treatment

Figure 7.1. Plasma testosterone (ng/ml) for SHR/y and WKY control, castrate (cast), castrate with testosterone replacement (cast+Ti), and flutamide (means, ± SEM). SHR/y and WKY cast+Ti plasma testosterone was higher than control and castrate SHR/y (**p<0.001) and WKY (**p<0.001) levels. The SHR/y and WKY flutamide plasma testosterone was higher than control and castrate SHR/y (++p<0.001) and WKY (++p<0.001) levels. Two-way ANOVA indicated a significant testosterone treatment affect on plasma testosterone (F=60.2, p<0.001).
Figure 7.2. Average number of aggressive events over 20 minutes for SHR/y and WKY control, castrate (cast), castrate with testosterone replacement (cast+Ti), and flutamide (means, ± SEM). The SHR/y control and cast+Ti aggression was higher than WKY control and cast+Ti during baseline resident intruder testing (*p<0.05 and xxp<0.01). During physical provocation testing, SHR/y control, cast+Ti, and flutamide males were more aggressive than the WKY control (##p<0.01), cast+Ti (^^^p<0.001), and flutamide (+++ p<0.001). Two-way ANOVA indicates a testosterone treatment effect increasing SHR/y aggression in physical provocation testing (F=28.0, p<0.001).
Figure 7.3. Linear regression for plasma testosterone (ng/ml) and attack number (number over 20 minutes) for SHR/y and WKY testosterone manipulated males. As plasma testosterone increases, aggression increases in both SHR/y \( (R=0.896, F=93.3, p<0.001) \) and WKY \( (R=0.748, F=26.6, p<0.001) \) males.
Anterior Amygdala Serotonin Content Increases Following Castration in WKY Males

Figure 7.4. Anterior amygdala serotonin content (pg/mg tissue) for SHR/y and WKY control, castrate, castrate with testosterone replacement (cast+Ti), and flutamide treated males (means, ± SEM). WKY castrate anterior amygdala serotonin content was greater than WKY control (*p<0.05).
Lateral Amygdala Serotonin Content is Not Effected By Castration

Figure 7.5. Lateral amygdala serotonin content (pg/mg tissue) for SHR/y and WKY control, castrate, castrate with testosterone replacement (cast+Ti), and flutamide treated males (means, ± SEM). The SHR/y and WKY cast+Ti lateral amygdala serotonin content was significantly less than in the SHR/y (*p<0.05) and WKY (^p<0.05) control males.
Medial Amygdala Serotonin Content is Decreased in SHR/y Compared to WKY Testosterone Treated Males

**Figure 7.6.** Medial amygdala serotonin content (pg/mg tissue) for SHR/y and WKY control, castrate, castrate with testosterone replacement (cast+Ti), and flutamide treated males (means, ± SEM). SHR/y control and cast+Ti medial amygdala serotonin content was less than WKY control (*p<0.05) and cast+Ti (^p<0.05). SHR/y castrate medial amygdala serotonin content was higher than SHR/y control medial amygdala serotonin content (#p<0.05). WKY flutamide medial amygdala serotonin content was less than WKY control medial amygdala serotonin content (x p<0.05). Two-way ANOVA indicated an interaction effect of testosterone treatment and the SHR Y chromosome decreasing medial amygdala serotonin content (F=4.3, p<0.05).
Discussion

The results of this study supported the hypothesis that castration decreases, while testosterone delivery increases indices of aggression in SHR/y and WKY males. In addition, this increased aggression in SHR Y chromosome (SHR/y) males appears to indicate that they are more behaviorally sensitive to testosterone manipulation. Although the neurochemical analysis of the amygdala was not robustly affected by testosterone treatment, there was indication of a relationship with increased aggression in both strains. Therefore, the underlying mechanism increasing aggression in SHR/y males is due primarily to an interaction between the SHR Y chromosome and testosterone, with amygdala serotonin content playing a comparably smaller role.

The increased plasma testosterone corresponded in both strains to an increase in aggressive behavior, and specifically an increase in the number of aggressive events during physical provocation testing. Physical provocation is a form of irritable aggression that involves the application of pressure to the distal portion of the subject’s tail and increases aggression with testosterone treatment [McGinnis, 2001], while having no affect in castrated males [Breur, 2001]. Our results are consistent with these findings correlating plasma testosterone (testosterone implant and flutamide treatment) with aggression.

This ability of testosterone to facilitate the display of offensive aggression was established through a wide range of animal models, ranging from rodents to non-human primates [Alberts, 1988] although the exact mechanism is not known [Edwards, 1968; Cologer-Clifford, 1999]. In addition, aggression induced by androgens is modulated by
the experimental and social settings in which the interaction occurs [Pucilowski, 1985; Alboetit and Farabellini, 1994]. Therefore, the levels of aggression depend on the amount of circulating testosterone, as well as the testing environment [Ogawa, 1996].

Although there is support for a role of the androgen receptor and subsequent receptor mediated responses of testosterone on aggression, the flutamide data presented here for both SHR/y and WKY males supports a non-androgen mediated effect. Specifically, flutamide treatment increased aggression in SHR/y and WKY males. This study design utilizing flutamide treatment initially began at eight weeks of age and continued for only four weeks. This treatment, unlike the androgen receptor knock out model, does not address the developmental aspects of how the androgen receptor is important for neurological and behavioral development, such as play behavior in juveniles and learned aggression. The androgen receptor knock out animals, show decreased aggression, whereas blocking the androgen receptor in SHR/y and WKY males produced increased aggression. This data, however, is not contradictory, because knock out mice lack all functional androgen receptors, whereas flutamide treatment only blocks a portion of the androgen receptors. In addition, flutamide treatment in adult males also increases androgen receptor mRNA levels [Kumar et al., 2002], and plasma testosterone [Toot et al., 2006]. Although both models address the androgen receptor in terms of aggression, they may do so through different pathways and mechanisms.

Multiple mechanisms for testosterone or testosterone metabolites could be responsible for the increased aggression following androgen receptor blockade. For example, dihydrotestosterone has an increased binding affinity, is present in small
concentrations, and is able to act through a limited number of receptors to increase aggression. Another possibility could be an indirect effect of testosterone involving serotonin, by decreasing synaptic transport, increasing metabolism, decreasing synthesis or even influencing the serotonergic receptor concentration. In further support of a more peripheral approach, the use of synthetic androgens and testosterone derivatives in rodent models does in fact support these potential pathways [Shrenker and Maxson, 1986; Lumia, 1994; Long et al., 1996; Feinberg, 1997].

Clearly, testosterone has the ability to act through a number of pathways influencing serotonin synthesis, breakdown and receptor number [Simon et al., 1998]. Regarding the SHR/y and WKY males, there was a serotonin response to testosterone manipulation, with castration increasing serotonin content in the amygdala. In support of a testosterone and serotonin interaction, research suggests that testosterone modulates serotonin expression as the major neurotransmitter involving aggressive behavior [Bell and Hepper, 1987] potentially through inhibiting the rate limiting enzyme for serotonin synthesis, neuronal tryptophan hydroxylase. Testosterone has also been indirectly related to decreased serotonin levels in the central nervous system as a whole, and also specifically within the amygdala. In terms of aggression, the amygdala is the primary neurological component of the limbic system associated with aggression. Specifically regarding SHR/y males, serotonin content in the amygdala has been linked to increased aggression in the SHR/y male following chronic social housing [Toot et al., 2004]. There are several neural networks which connect the amygdala to other brain areas via neural
networks, such as the hypothalamus and hippocampus [Antoniadis, 2000; Baron-Cohen, 2000].

The medial amygdala appeared to be more sensitive to testosterone manipulation than the lateral or anterior nuclei for both strains. The medial nuclei was also the most sensitive of the nuclei examined to testosterone manipulation and has connections with the olfactory system via vomeronasal input [Kevetter and Winans, 1981], while the lateral nuclei is connected to the accessory olfactory bulbs [Scalia, 1975]. Mice lacking this particular connection, displayed significantly reduced levels of copulatory behavior and intermale aggression [Kevetter and Winans, 1981]. Connections linking the olfactory and non-chemosensory areas of the amygdala identify communication pathways of chemosensory information to the hypothalamus [Merchenthaler, 1984]. Since chemosensory input is a key mechanism for identification in rodents, this suggests a strong role of inter and intra species identification in potential behavioral responses, i.e. aggression.

Several studies have also shown that neuronal tryptophan hydroxylase mRNA can be increased with estrogens or decreased with testosterone, resulting in increased aggression through decreased serotonin synthesis, particularly within the raphe nucleus, which is the primary site of serotonin synthesis. There are several neural networks that connect the amygdala to sensory systems [Adolphs and Spezio, 2006] and other portions of the limbic system and raphe nucleus [Bruchey et al., 2007; Nelson and Trainor, 2007]. For instance, in response to threatening stimuli, the amygdala can contribute to the initial increase in heart rate and blood pressure, as well as, freezing behavior in rodents.
[Mitsushima et al., 2006]. With decreased neuronal tryptophan hydroxylase activity following testosterone treatment, the amount of serotonin will decrease, which typically makes aggressive tendencies more likely in these behavioral encounters. Further support for reduced serotonin in the amygdala and increased aggression was shown by stimulation of amygdala serotonin receptors that caused an inhibition of offensive and muricidal behavior in isolated rats [Pucilowski et al., 1985; Everts, 1997]. Direct manipulation of neural serotonin in animals treated with fluoxetine show a larger concentration of serotonin present in the synapse, which ultimately decreases aggression [Molina et al., 1987]. Fluoxetine was also able to inhibit footshock induced aggression in paired rats [Valzelli, 1982; Ilivier 1987; Eichelman, 1990; Datla, 1991]. This is supported in several animal models, where basal concentrations of serotonin present in the central nervous system are increased in nonaggressive animals, such as the Norway rats and Silver Foxes [Popova et al., 1991].

In conclusion, the results of this study indicate that the SHR/y males are more aggressive than the WKY males. This increased aggression was significantly influenced by the increased plasma testosterone, potentially through the medial amygdala and not the anterior or lateral nuclei. However, the overall effect of testosterone decreasing serotonin content in the entire amygdala was not as robust as expected. This suggests, regarding serotonin content, that there may be regional amygdala differences influence by testosterone and that compensations may occur, such as increased neuronal tryptophan hydroxylase activity, decreased serotonin transport from the synapse, or even increased serotonin receptor number. These factors were not addressed specifically in this study.
since only serotonin content was measured and should be further examined. There is also
the potential that the length of testosterone treatment (four weeks) was not sufficient to
influence neural serotonin content significantly in the entire amygdaloid complex. This
does, however, raise several possibilities regarding how the SHR Y chromosome
potentially increases aggression through increased testosterone sensitivity, supporting
future studies of serotonergic activity through pharmacological and testosterone
manipulation studies.
Chapter VIII

Study 6: Testosterone and Serotonin Manipulation in Male SHR/y and WKY Rodents

Introduction

As discussed in the previous chapters, two common physiological factors that influence aggression are plasma testosterone and brain serotonin. Previous research in our lab has focused on the SHR Y chromosome in relationship to behavioral and physiological measurements of aggression. Specifically, SHR Y chromosome (SHR/y) males have increased social aggression, decreased amygdala serotonin, and increased aggression following testosterone delivery. The SHR Y chromosome has also been linked to increased stress responsiveness, increased cardiovascular disease, hypertension, and an earlier rise in pre-pubertal testosterone.

Testosterone is commonly identified as being a primary factor able to indirectly and directly regulate social behavior, where increased testosterone correlates with increased aggression [Breur, 2001; Toot et al., 2004]. The role of testosterone is particularly evident in social environments where the formation of a social hierarchy is dependent upon aggression, often due to plasma testosterone. Other testing paradigms
have shown similar results where testosterone increases aggression in physical
provocation, resident intruder, and foot shock in rodent animal models [Ulrich and Azrin,
1962; Martinez-Sanchic, 1996; Martinez-Sanchic, 1998; Breur, 2001; Toot et al., 2004].
Testosterone is related to serotonin levels in the limbic system, ie the amygdala,
potentially through indirect effects on the rate limiting enzyme in serotonin synthesis,
neuronal tryptophan hydroxylase (TPH2) [Keleta et al., 2007].

Just as testosterone has a strong positive correlation with aggression, serotonin
has a strong negative correlation, where elevated serotonin inhibits aggressive behavior
[Kulikov et al., 1989; Clement, 1994]. Increased aggression has also been linked with
decreased serotonin in specific brain nuclei, specifically in the amygdala and raphe
nucleus, well as the entire central nervous system [Molina et al., 1987]. In addition to
androgen modulation of serotonin, drugs such as p-chlorophenylalanine (PCPA) and
fluoxetine serve a more direct function on serotonin activity. Specifically, PCPA is a
TPH2 antagonist, which consistently increases aggression by decreasing TPH2 activity,
and decreasing serotonin concentration [Korpela and Sandnabba, 1998; Mayoga, 2001].
In comparison, fluoxetine decreases aggression through inhibiting activity of the
serotonin transporter, preventing reuptake of serotonin into the pre-synaptic terminal, and
therefore increasing serotonin present in the synapse.

Brain serotonin activity, as well as circulating testosterone levels, is clearly
implicated in aggressive behavior [Pucilowski et al., 1985; Devoino et al., 2004;
Giammanco et al., 2005]. Although this specific mechanism is not defined, testosterone
and serotonin likely act together and also independently of each other to modulate
aggression. The purpose of this study is to further elucidate the roles of testosterone and serotonin in conjunction with the SHR Y chromosome in order to identify how these factors contribute to aggressive behavior in SHR/y and WKY males. Therefore, the hypothesis to be tested is that SHR/y males will be more aggressive following testosterone and serotonin manipulation than WKY males.

Materials and methods

Study Design

The objective of this study was to determine the perspective roles that plasma testosterone and neural serotonin play in aggression between the SHR/y and WKY males. Adult SHR/y and WKY males utilized a two strain by four treatment design, with males being separated into two testosterone treatment groups: castrate and castrate with testosterone implants. Each testosterone treatment group was then separated into either fluoxetine or p-chlorophenylalanine drug treatment groups to increase or decrease serotonin levels respectively.

Animal Model

The Y chromosome animal model used in this study consisted of the consomic borderline hypertensive (SHR/y) and normotensive Wistar-Kyoto (WKY) rats. This breeding scheme has been described in detail previously (Chapter II). Briefly, comparisons between WKY and SHR/y males allow us to investigate the role of the SHR Y chromosome in a WKY genetic background [Ely et al., 2000].
Housing

Following surgery, all animals were housed individually and maintained in polycarbonate cages (48cm x 27cm x 20cm) with stainless steel tops and heat-treated bedding (R.J. Murphy hardwood Sani Chips). Rats were subjected to a 12h/12h light/dark cycle and maintained on a normal sodium diet (0.3% Na, Prolab 3000 Rat/chow 3000, PMI Feeds, St. Louis, MS). Food and water were accessible \textit{ad libitum}.

Testosterone Treatment Surgery

Male rats to be castrated were 6-8 weeks old, sedated with Sodium Pentothal (50 mg/kg, IP; E. Lilly, Indianapolis, IN), and both testes were removed. In a similar manner, male rats to be testosterone implanted were castrated and the implant was inserted beneath the skin parallel to the longitudinal axis of the rat.

Testosterone Implants

The protocol for making the testosterone implants was described previously (Chapter VII). Briefly, 21mm testosterone propionate implants (Sigma Chemical Co., St. Louis, MO) were used and corresponded to 12-16 ng/ml plasma testosterone (Chapter VII).

Tryptophan Hydroxylase Manipulation Protocol

Prior to each behavioral test (1.5hrs), males assigned to each testosterone treatment group were treated with either fluoxetine (Sigma Chemical Co., St. Louis, MO;
10mg/kg BW) [Molina et al., 1987] or p-chlorophenylalanine (Sigma Chemical Co., St. Louis, MO; 100mg/kg BW, PCPA) [Korpela and Sandnabba, 1998; Lesch et al., 2003].

**Resident Intruder Physical Provocation Paradigm**

The behavioral tests used to elicit aggressive behavior consisted of the resident intruder home cage physical provocation paradigm. The resident intruder home cage physical provocation paradigm consists of placing a novel strain matched intruder into the home cage of the resident male [Breur, 2001]. One minute after placing the intruder male into the cage, the resident male was provoked to attack by a tail pinch. The tail pinch consists of a moderate pinch to the tail for 1 second every minute for the duration of the test. Each test lasted for a total of 20 minutes. Behavioral tests were conducted over a four day period, and all tests were filmed for later analysis of: attack number and attack latency.

**Statistics**

Statistical analysis was performed by using two-way RM ANOVA and a post-hoc Bonferroni test. One and two-way ANOVAs, and Student’s t-test were used where applicable, with significance assumed if p<0.05. Sigmapstat and Sigmaplot statistical software were used analyze data (Jandel Scientific, San Rafael, CA).

**Results**

SHR/y castrate males treated with PCPA (Figure 8.1) showed significantly increased aggression compared to SHR/y castrate fluoxetine and WKY castrate with
PCPA males ($F=12.5$, $p<0.001$). Behavioral testing from day one to four also showed a trend of an increased number of aggressive events only in the SHR/y castrate with PCPA males ($F=8.3$, $p<0.001$).

Both SHR/y and WKY castrate with testosterone treatment males given PCPA (Figure 8.2) showed significantly increased aggression compared to fluoxetine treated males ($F=23.9$, $p<0.001$). The SHR/y castrate with testosterone treatment PCPA males were the most aggressive ($F=12.8$, $p<0.001$) and also showed increased aggression over the four days of behavioral testing ($F=5.2$, $p<0.01$).

Latency or time until the first attack (Figure 8.3) for both SHR/y and WKY castrate males treated with PCPA had earlier attack times than the fluoxetine treated males ($F=9.4$, $p<0.001$). The time until the first attack (Figure 8.4) for SHR/y and WKY castrate with testosterone treated PCPA males had earlier attack times than the fluoxetine treated males ($F=11.2$, $p<0.001$). In addition, the SHR/y castrate with testosterone PCPA males also attacked earlier than the WKY castrate with testosterone PCPA males ($F=4.8$, $p<0.05$). The PCPA treatment ($F=13.7$, $p<0.001$) and SHR Y chromosome ($F=7.3$, $p<0.001$) showed an interaction effect resulting in earlier attack times.
SHR/y Males Are More Aggressive Than WKY Males Following Castration and Serotonin Manipulation

![Figure 8.1](image_url)

Figure 8.1. Average number of aggressive events over 20 minutes for SHR/y and WKY castrate fluoxetine and p-chlorophenylalanine (PCPA) treated males (means, ± SEM). SHR/y castrate males treated with PCPA showed significantly increased aggression compared to SHR/y castrate with fluoxetine and WKY castrate with PCPA (F=12.5, ***p<0.001). Behavioral testing from day 1 to day 4 for SHR/y castrate with PCPA treatment showed a significant increase in the number of aggressive events (F=8.3, xxxp<0.001).
The image contains a graph titled "SHR/y Males are More Aggressive Following Testosterone Implantation and Serotonin Manipulation". The graph shows the average number of aggressive events over 20 minutes for SHR/y and WKY castrate males with testosterone (cast+Ti) fluoxetine and p-chlorophenylalanine (PCPA) treatment (means, ± SEM). The graph indicates that both SHR/y and WKY cast+Ti PCPA males were more aggressive than their respective cast+Ti fluoxetine comparisons (Two-way ANOVA, F=23.9, ***p<0.001). The SHR/y cast+Ti PCPA males were more aggressive than the WKY cast+Ti PCPA males (F=12.8, xxxp<0.001). Behavioral testing from day 1 to day 4 showed a significant trend for increased aggression in SHR/y cast+Ti PCPA males (Two-way RM ANOVA, F=5.2, ^^p<0.01).
SHR/y Males Attack Earlier Than WKY Males Following Castration and Serotonin Manipulation

Figure 8.3. Average time for the first aggressive event (attack latency) in the 20 minute resident intruder test for SHR/y and WKY castrate (cast) fluoxetine and p-chlorophenylalanine (PCPA) treated males with and without physical provocation (means, ± SEM). The SHR/y castrate with PCPA treatment attack latency was less than the SHR/y castrate with fluoxetine and WKY castrate with PCPA treatment (Two-way ANOVA, F=9.4; p<0.001). No error bars indicate that the ceiling or maximum amount of time for the test was reached (20 minutes) without an aggressive event.
SHR/y Males Attack Earlier Than WKY Males Following Testosterone Implantation and Serotonin Manipulation

Figure 8.4. Average time for the first aggressive event (attack latency) in the 20 minute resident intruder test for SHR/y and WKY castrate with testosterone replacement (cast+Ti) fluoxetine and p-chlorophenylalanine (PCPA) treated males with and without physical provocation (means, ± SEM). The SHR/y cast+Ti with fluoxetine treatment attack latency was less than the WKY cast+Ti fluoxetine (Two-way ANOVA, $F=11.2$, ***$p<0.001$). The SHR/y cast+Ti with PCPA treatment attack latency was less than the WKY cast+Ti PCPA (Two-way ANOVA, $F=4.8$, $xxx \ p<0.05$). Two-Way RM ANOVA indicated an effect of PCPA treatment ($F=13.7$, $p<0.001$) and also an SHR Y chromosome effect ($F=7.3$, $p<0.01$). No error bars indicate that the ceiling or maximum amount of time for the test was reached (20 minutes) without an aggressive event.
Discussion

The results of this study supported the hypothesis that SHR Y chromosome (SHR/y) males are more aggressive than WKY males following testosterone and serotonin manipulation. The behavioral data regarding attack number and latency are both consistent with previously published studies using p-chlorophenylalanine (PCPA) and fluoxetine to decrease and increase serotonergic activity, respectively [Eichlman, 1990; Datla et al., 1991]. In addition, there was also a trend for increased aggression noted in the SHR/y males over the course of behavioral testing not present in WKY males. Therefore, these results suggest that the increased aggression from testosterone and serotonin was at least partially due to the presence of the SHR Y chromosome.

Testosterone treated SHR/y and WKY males with decreased serotonin activity levels following PCPA treatment were consistently more aggressive than fluoxetine treated males. Testosterone delivery via injections or implants increased aggression in a number of animal models in a variety of testing paradigms [Hirio et al., 2006]. Likewise, decreasing serotonin levels through lesions to the raphe nucleus or drugs targeting serotonin metabolism (decrease synthesis or increasing degradation) also increased aggression [Molina et al., 1987]. Therefore, the SHR/y and WKY castrates with testosterone implants and PCPA treatment were the most aggressive, consistent with the previous literature.

The PCPA treated males compared to fluoxetine treated males identifies the relative contribution of serotonin to the aggressive behavior. For example, the SHR/y castrate with testosterone implant males with decreased serotonin activity were the most
aggressive. Increased synaptic serotonin concentration by fluoxetine treatment decreased the attack number by 75% in both SHR/y and WKY males. The relative action of PCPA and fluoxetine act via different mechanisms of serotonergic action. Specifically, PCPA blocks the serotonin synthesis through inhibition of TPH2, specifically in the raphe nucleus [Molina et al., 1987; Korpela and Sandnabba, 1998; Gardner et al., 2005]. This, over time, would result in increased TPH2 mRNA and a feedback response to increase serotonin concentration to homeostatic levels [Hirio et al., 2006]. A lack of adjustment, or control in this pathway could explain why the SHR/y male attack number increased over the 4 days (became more aggressive), while the WKY male number remained stable.

The use of fluoxetine, which inhibits reuptake of serotonin from the synapse through blocking the serotonin transporter [Molina et al., 1987; Datla et al., 1991], completely abolished the aggression in the WKY, but not in the SHR/y testosterone treated males. The increased aggression noted in fluoxetine treated SHR/y males indicates that they are less responsive to synaptic serotonin alterations than WKY males. Since this drug is acting in the synapse, it is likely that multiple factors could be affected, including the serotonergic receptors (5HT_{1A} or {1B}), as well as potentially increasing serotonin transporter mRNA and concentration. This could be do to an increase in the receptor number on the postsynaptic neuron or an over-responsive decrease in TPH2 activity resulting from a negative feedback response to the serotonin being released into the synapse, but remaining present long enough to partially inhibit aggressive behavior. Pre-synaptic receptors for serotonin may also recognize this elevated serotonin concentration, thereby decreasing serotonin synthesis to compensate.
The removal of testosterone following castration acted to decrease the overall attack number, with the SHR/y males being more aggressive than WKY males. Previous studies (Chapter VI) have also shown that the testosterone effect on aggression in the SHR/y males was increased. This suggests that the removal of serotonin by PCPA treatment was sufficient to elicit an aggressive response without the presence of testosterone. However, serotonin levels in the comparable WKY males was not sufficient to increase aggression. Expanding on this comparison would imply that serotonin, at least in the WKY males is not as important in the mechanism underlying aggressive behavior, as it is in the SHR/y males. Interestingly, the relative decrease in aggression following removal of serotonin was the same as the decrease in aggression following removal of testosterone. This suggests that was not a compound or additive effect of the two factors together, but that there may be a maximum level of aggression that can be reached following treatment.

The time until first attack or attack latency data was consistent with the number of attacks for both SHR/y and WKY testosterone and serotonin treatment groups. As expected, the most aggressive animals (SHR/y castrate with testosterone and PCPA treatment) also had the earliest time for the first attack. Likewise, WKY castrate males (PCPA or fluoxetine treated) reached the time limit during behavioral testing, indicating that there was no exhibited aggressive behavior. Although the latency to attack data supports the overall degree of aggression, the times did not necessarily follow the same trend as the attack number over the entire four days of testing. Specifically, SHR/y castrate and castrate with testosterone treatment males aggression increased from day 1 to
day 4, whereas the attack latency data remained similar. By comparison, SHR/y castrate with testosterone treated PCPA males did show an earlier time to attack along with a five fold increase in attack number. This increase in aggression and earlier time to attack is consistent with other studies indicating that both increased testosterone and decreased serotonin will increase aggressive behavior [Clement, 1999; Keleta et al., 2007].

As previously discussed, the Y chromosome has been linked to testosterone and serotonin activity, as well as, social recognition factors and differences in intermale aggression. The general Y chromosome effect, in addition to the noted behavioral differences between SHR/y and WKY males supports the mechanism that the SHR Y chromosome is responsible for increased aggression in this rodent model. This is likely not only due to an increased testosterone sensitivity as noted in the previous chapter, but also an increased sensitivity to TPH2 manipulation and blunted ability of fluoxetine to maintaining elevated levels of serotonin in the neuronal synapse.
Chapter IX

Study 8: Water Maze Performance and Brain Neurochemistry After In vivo Sry Gene Delivery to the WKY Hippocampus

Introduction

The previous chapters involving the SHR/y and WKY animal models have illustrated the relative importance of the SHR Y chromosome, hippocampal norepinephrine content, hippocampal tyrosine hydroxylase activity, and stress responsiveness in both learning acquisition and memory retention in the morris water maze. Research over the past 20 years has definitively shown the hippocampus to be involved with learning and memory retrieval in spatially oriented tasks [Morris et al., 1982; Morris, 2003], with damage to the hippocampus impairing performance on the morris water maze and raised Y-maze [Morris et al., 1982; Conrad et al., 2003]. As well as being important for visual spatial navigation and exploratory behavior, the hippocampus plays a regulatory role in forming episodic memory and forming other spatial relationships [Rolls, 2000; Murray et al., 2007].

In addition, the hippocampus has a dense input of adrenergic terminal suggesting a strong function role of norepinephrine in learning and memory retention [Schroerer et
al., 2000]. These adrenergic inputs travel to a majority of the hippocampus, including the
dentate gyrus, CA1, CA3, and subnucleus [Izquierdo and McGaugh, 2000; Roozendaal et
al., 2004]. Further neurochemical analysis also indicates that the noradrenergic system is
also crucial for the process of learning, memory, and general hippocampal function [Men
et al., 1999]. The role of the sympathetic nervous system, specifically through beta
adrenergic receptor activation in the hippocampus shows large stimulatory and inhibitory
effects on learning acquisition and memory consolidation [McGaugh and Izquierdo,
2000]. Current research identifies that adrenergic activation is necessary for memory
consolidation, specifically in emotionally demanding and aversive events.

Research in our lab has shown the SHR Y chromosome not only increases blood
pressure, but also increases sympathetic nervous system activity, stress responsiveness,
adrenal tyrosine hydroxylase activity, and norepinephrine content [Ely et al., 1993; Ely et
al., 1994; Ely et al., 1997]. With an apparent Y chromosome effect on a variety of central
and peripheral catecholaminergic dependent mechanisms, a gene on the Y chromosome is
likely involved with regulation. Specifically, Sry is a transcription factor able to
potentially regulate various aspects of this pathway. Molecular studies expanding on this
SHR Y chromosome effect with Sry have indeed shown Sry to increase promoter activity
of tyrosine hydroxylase, the rate limiting enzyme in catecholamine synthesis [Milsted et
al., 2004a]. In addition, in vivo studies delivering Sry to the adrenal medulla show an
increase in plasma norepinephrine, stress response, and medullary tyrosine hydroxylase
activity [Ely et al., 2007].
The relatively important regulatory role of hippocampal norepinephrine and adrenergic activity as well as studies identifying increased sympathetic nervous system activity, \textit{in vivo} Sry effects, and \textit{in vitro} effects of Sry on tyrosine hydroxylase promoter activity [Milsted et al., 2004a] suggests a strong potential for an SHR Y chromosome related mechanism through the actions of Sry. Based on these previous SHR Y chromosome studies, along with the corresponding brain neurochemistry and maze performance data, Sry may play a role in water maze performance through altering catecholaminergic synthesis in the central nervous system. Therefore, the hypothesis to be tested is that \textit{in vivo} delivery of the Sry gene to the WKY hippocampus will impair maze performance by increasing norepinephrine content and increasing tyrosine hydroxylase activity compared to the empty vector control WKY males.

\textbf{Materials and Methods:}

\textbf{Design}

The experimental design for this study consisted of a one strain by two treatment design. Adult WKY males (n=6/treatment) either had Sry1 pcDNA3.1 or an empty vector (pcDNA3.1) delivered to the hippocampus.

\textbf{Expression construct}

The Sry1 expression construct Sry1/pcDNA3.1 (-), was originally prepared in our lab by cloning coding sequences of SHR Sry1 (Accession Number AF274872) into the pcDNA3.1 (-) vector as previously described [Milsted et al., 2004a; Martin, 2002;
Underwood, 2003]. This expression construct includes the complete coding sequence (bp#11-520) of Sry1 from the SHR male.

**Plasmid delivery**

Based on our preliminary studies, neural tissue has an optimal electroporation protocol to yield efficient gene delivery. 10ug of either the Sry1 pcDNA3.1 or pcDNA3.1(-) was delivered to the hippocampus (AP:-4.5mm, ML: 2.5, Depth:3mm). All animals were given sodium pentothal (50mg/kg, IP) during surgery. Each animal was placed on a stereotaxic instrument, with Bregma located at the intersection of the sagittal and coronal sutures, with an elevation of 0mm. Two burr holes were drilled directly over the indicated coordinates. A total volume of 10ul (1ug/ul) was delivered over 15 minutes through a 28 gauge Hamilton syringe. Following injection of the plasmid, the needle was kept in place for an additional 10 minutes prior to removal at a rate of 1mm / minute. A modified bar electrode (BTX) was lowered to the desired depth and the pulses were delivered at (V=45V, Duration=2ms, Interval=1sec, Number=10pulses, Electrode Distance=1mm). The burr hole was then rinsed with sterile saline and closed with sterile bone wax.

**Morris Water Maze**

Approximately 18 days post surgery the morris water maze test paradigm was used in this study of spatial learning and memory processes. This is a standard test consisting of a platform hidden underneath the surface of the water. Specifically, the
maze consisted of a 5’ diameter tank filled with packing material, with external maze cues placed on the walls of the room. There were three days of acquisition (ACQ) with 5 trials per day and 1 retention trial for each maze version. Each trial was run for a maximum of 90 seconds. After finding the platform (6” diameter), the animal was given a 20 second mapping period before removal from the maze.

Palkovits Brain Punch

Following termination, the brains were frozen on dry ice, stored at -70C, and later cut into 200 μm coronal sections at -20C (Minotome, International Equipment Company, Damon Division, Meedham Hts., Mass.). The sections were then punched [Palkovits, 1973] from both the surgical and control non surgical contralateral side of the hippocampus. Proper electrode placement, coronal slice, and punch orientations were verified with comparison to the rostral-caudal bregma zero reference points [Pellegrino, 1981]. The punches were homogenized in either mobile phase (for norepinephrine) or sucrose (for tyrosine hydroxylase activity), centrifuged for 10 minutes at 3200rpm (Sorvall RT7, Kendro Labs, Newton Conn.) and stored at -70C for later analysis.

HPLC Analysis

The norepinephrine content for each brain region was analyzed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters 460 Detector, Milford, MA) and has been described in detail previously (Chapter II).
Tyrosine Hydroxylase Assay

Tyrosine hydroxylase activity was calculated by measuring L-DOPA formed per milligram of tissue per minute and has been described in detail previously (Chapter II).

Statistics

One-way ANOVA and two-way repeated measure ANOVA tests were used where appropriate. Student’s T-test was used for individual comparisons when necessary. Statistical analysis was run on SigmaStat and SigmaPlot software (Jandel Scientific, San Rafael, CA), with significance assumed at p<0.05.

Results

There were no significant differences in the mean platform latency (Figure 9.1) for hippocampal Sry1 and empty vector transfected WKY males. However, performance over the three acquisition days did improve (F=3.5, p<0.05). The average platform latency for retention trials (Figure 9.2) of hippocampal Sry1 and empty vector transfected WKY males was not significantly different.

Hippocampal norepinephrine content (Figure 9.3) for Sry1 transfected WKY males was lower in the surgical site compared to the empty vector surgical site (p<0.05). The control contralateral non surgical sides of the Sry1 and empty vector treatments showed no differences. The tyrosine hydroxylase activity in the hippocampus (Figure 9.4) for the Sry1 hippocampal surgery site was increased compared to the empty vector surgical site (p<0.05).
Mean Platform Latency Improves With Time In WKY Sry1 and Empty Vector Transfected Males

![Graph showing mean platform latency over three trial days for Sry1 and empty vector transfected males.](image)

Figure 9.1. Mean platform latency over the three acquisition trial days for hippocampal Sry1 and empty vector transfected males (means, ± SEM). There was no significant difference between treatments over the three trial days. Two-way ANOVA analysis indicated that over the three trial days there was an improvement in maze performance for both treatments (F=3.5, *p<0.05).
Retention Trial Performance is Similar Between WKY Sry1 and Empty Vector Transfected Males

Figure 9.2. Average platform latency for retention trials for both treatment groups (means, ± SEM). There was no significant difference between Sry1 and empty vector treatments.
Figure 9.3. Norepinephrine content for hippocampal transfected males with Sry1 or the empty vector at the surgery and control contralateral non surgical (means, ± SEM). The Sry1 surgical site norepinephrine content was significantly lower compared to the empty vector surgical site (**p<0.01). The control contralateral non surgical for Sry1 and empty vector treatments showed no significant differences. Two-way ANOVA indicated that there was an effect of the delivery site in Sry1 transfected males (F=4.8 p<0.05).
Sry1 Transfection Increases Hippocampal Tyrosine Hydroxylase Activity

Figure 9.4. Tyrosine hydroxylase activity for Sry1 and empty vector hippocampal surgery and control contralateral non surgical sites (means, ± SEM). Sry1 surgical side had significantly elevated tyrosine hydroxylase activity compared to empty vector surgical sites (*p<0.05). The control contralateral non surgical sites of Sry1 and empty vector treatments show no significant differences.
Discussion

The results of this study supported the hypothesis that hippocampal tyrosine hydroxylase activity would increase in WKY males following *in vivo* Sry neural delivery. However, this change in hippocampal neurochemistry did not correlate to changes in maze performance regarding acquisition or retention trial latency. Interestingly, the adrenergic activity within the hippocampus is clearly important for proper visual spatial related processing, as noted in previous chapters presented here, and also in other animal models maze performance was not directly effected [McGaugh et al., 2002; McIntyre et al., 2002; McDonald et al., 2006; McDonald et al., 2007]. Therefore, there are several pathways to be discussed in the following paragraphs that could explain the effect of the Sry gene delivered to the hippocampus and resulting changes in neurochemistry without changes in behavioral performance.

The most significant finding of this study was the increased tyrosine hydroxylase activity and decreased norepinephrine content in the hippocampus of Sry1 transfected WKY males. This increase in tyrosine hydroxylase activity is consistent with previous in *vivo* and *in vitro* studies in our lab [Ely et al., 2007]. Specifically, Sry delivered to the adrenal gland or kidney shows increased tyrosine hydroxylase activity with increases in stress responsiveness and blood pressure, respectively. In addition, in cultured cells, Sry 1 increases tyrosine hydroxylase promoter activity [Milsted et al., 2004a]. Therefore, Sry following *in vivo* transfection was acting as expected in the hippocampus.

This increased tyrosine hydroxylase activity, apparently was limited to the surgical site. This would indicate that the effect of Sry was not general to the whole
brain, or even the contralateral (control side) of the hippocampus. This is also consistent with the preliminary beta-galactosidase studies indicating that only the transfected tissue stained positive for beta-galactosidase. However, there is still a possibility for the transfected hippocampal tissue to influence the neurochemistry of the rest of the hippocampus.

Although tyrosine hydroxylase activity increased following Sry delivery to the hippocampus, the norepinephrine content decreased. This trend, however, was not as specific to the delivery site for Sry. Since Sry is likely acting on the catecholaminergic pathway by increasing tyrosine hydroxylase activity [Milsted et al., 2004a; Ely et al., 2007], there is a possibility for the increased tyrosine hydroxylase causing an increase norepinephrine content leading to increased release and increased degradation. Over several weeks, this could result in an overall depletion of norepinephrine stores within the neuronal vesicle and therefore cause a decreased norepinephrine content. Since this trend was only in the surgical site of the hippocampus, it is possible that impairments in maze performance may not be noticeable. In addition, the rest of the hippocampus and contralateral hippocampus could maintain the normal function regarding visual spatial processing.

The lack of noticeable impairments in maze performance following Sry delivery to the hippocampus is consistent with partial lesion studies that show no behavioral deficits, whereas complete lesions show severe impairments [Easton and Gaffan, 2000]. This is due to the large degree of plasticity and overall function within the hippocampus and associated structures. Further analysis with studies involving adrenergic agonists and
antagonists delivered to the systemic circulation or entire hippocampus show significant alterations in maze performance [Hatfiled and McGaugh, 1999; Roozendaal et al., 2004; Berlau and McGaugh 2006]. This would indicate that for Sry treatment to have an observable effect, a system wide or delivery to the entire hippocampal structure may be necessary. Central nervous system increases or decreases in norepinephrine content have been linked to improved acquisition and retention performance following drug treatment.

There are several pathways or potential mechanisms that could explain the changes in hippocampal neurochemistry. Although published data supports a time frame of two to three weeks for physiological changes following Sry transfection into kidney or adrenal medulla, this may not be true of neural tissue [Ely et al., 2007]. If changes in tyrosine hydroxylase activity, norepinephrine content, and maze performance do occur, they may have been at one week or even at five weeks post transfection. Although drug studies using beta adrenergic agonists and antagonists show impaired or impaired performance, respectively, such studies have not been down with chronic delivery (i.e. three weeks) [Quirarte et al., 1997; Hatfield and McGaugh, 1999]. If Sry is elevating hippocampal tyrosine hydroxylase activity in vivo for several days to weeks, this may not result in similar enhanced maze performance. Maze performance could even be impaired, as noted in knock out mice or chronically stressed animals that show elevated norepinephrine neural concentrations [Thomas and Palmiter, 1997; Glickstein et al., 2002; Ramos et al., 2007].

In all likelihood, Sry is not affecting only tyrosine hydroxylase activity in vivo, but also other enzymes. Sry has multiple potential DNA binding sites on a number of...
genes involved with catecholamine synthesis, like the enzyme dopamine beta-hydroxylase. Unfortunately, the regulating mechanism involving Sry are not known. The exogenously delivered Sry could be interacting with endogenous Sry in a number of as of yet, undefined pathways. In addition to these problems, there is relatively little known regarding Sry function outside of testicular developmental regulation.

In conclusion, Sry, potentially acting through the tyrosine hydroxylase promoter increased the relative amount of tyrosine hydroxylase and/or tyrosine hydroxylase activity. As a result of Sry gene delivery, the significant changes in brain neurochemistry following in vivo delivery indicate that Sry has the potential to influence learning and memory performance in the rat model. In addition, there are likely a number of other possible pathways through which Sry was acting, which are currently undefined. Future studies will need to address the various time constraints and issues presented here to better understand this proposed novel role for Sry, hippocampal neurochemistry, and maze performance.
Chapter X

Study 9: Aggression and Brain Serotonin Following In vivo Sry Delivery to the WKY Amygdala

Introduction

Behavioral research in the preceding chapters has focused on the role of the Y chromosome in stress arousal, social adaptation, and aggressive behavior in the male SHR/y and WKY rat strains. This and other behavioral research in our lab specifically shows that SHR males have increased aggression in resident intruder tests, increased pre-pubertal testosterone, and elevated sympathetic nervous system activity [Ely et al., 1993; Andrews et al., 1994; Ely et al., 1997]. In addition, SHR/y (SHR Y chromosome) males are more aggressive and have lower amygdala serotonin content than male rats with the WKY (WKY Y chromosome) [Toot et al., 2004].

Research in mouse models, suggests that the Y chromosome influences the etiology of intermale aggression in both juveniles [Maxson, 1981] and adults [Oortmerssen et al., 1987; Carlier, 1991]. Other studies involving the Y chromosome and mice further indicates that one or more genes can be attributed to differences in intermale aggression [Carlier, 1990; Sluyter, 1994; Carlier, 1996] and also the development of this
aggression in males [Maxson, 1998; Oortmerssen et al., 1987]. In addition, the Y chromosome has also been identified as being involved with other behavioral characteristics including urinary odor type distinguishing factors [Yamazaki, 1990], which influences social recognition and related behaviors.

In the study of animal behavior, several candidate genes have been identified as being able to influence the hormones and neurotransmitters regulating aggression, one of which is Sry [Maxson, 1998]. Previous research in our lab with Sry has shown it to increase catecholamines and tyrosine hydroxylase activity following in vivo delivery to the adrenal medulla [Ely et al., 2007]. Molecular modeling and analysis indicates the potential for Sry to act as a transcription factor in several pathways involved with behavior regulated by testosterone and serotonin, including: neuronal tryptophan hydroxylase, steroid sulfatase, 5 alpha reductase, and also the androgen receptor.

Within specific regions of the brain, such as the amygdala, artificially decreased serotonin levels through drug and/or testosterone treatment have been linked to increased aggression in numerous animal and primate studies resulting from inhibition of neuronal tryptophan hydroxylase [Keleta et al., 2007]. Therefore, if Sry can change the neuronal serotonin of the amygdala, directly or indirectly, the resulting behavior should be increased aggression. The hypothesis of this study is that in vivo delivery of the Sry gene to WKY males will increase physical indices of aggression and decrease serotonin content compared to empty vector controls.
Materials and Methods

Design

The experimental design for this study consisted of a one strain by two treatment design. Adult WKY males (n=4/treatment) either had Sry1 pcDNA3.1 or an empty vector (pcDNA3.1) delivered to the amygdaloid complex.

Expression construct

The Sry1 expression construct Sry1/pcDNA3.1 (-), was originally prepared in our lab by cloning SHR Sry1 (Accession Number AF274872) into the pcDNA3.1 (-) vector as previously described [Martin, 2002; Underwood, 2003; Milsted et al., 2004a]. This expression construct includes the complete coding sequence (bp#11-520) of Sry1 from the SHR male.

Plasmid delivery

Based on our preliminary studies, electroporation of neural tissue was optimized to yield efficient gene delivery due to high lipid content. 10ug of either the Sry1 pcDNA3.1 or pcDNA3.1(-) was delivered to the amygdaloid complex (AP:3.2mm, M:2mm, Depth:3mm). All animals were given sodium pentothal (50mg/kg, IP, E. Lilly, Indianapolis, IN) during surgery. Each animal was placed on a stereotaxic instrument, with Bregma located at the intersection of the sagittal and coronal sutures, with an elevation of 0mm. Two burr holes were drilled directly over the indicated coordinates. A total volume of 10ul (1.0ug/ul) was delivered over 15 minutes through a 28 gauge
Hamilton syringe. Following injection of the plasmid, the needle was kept in place for an additional 10 minutes prior to removal at a rate of 1mm / minute. A modified bar electrode (BTX) was lowered to the desired depth and the pulses were delivered at (V=45V, Duration=2ms, Interval=1sec, Number=10pulses, Electrode Distance=1mm). The burr hole was then rinsed with sterile saline and closed with sterile bone wax.

**Resident Intruder Test with Physical Provocation**

Approximately 18 days post surgery the resident intruder or the physical provocation paradigm was used to measure irritable aggression. This behavioral test, as shown in previous chapters, elicits consistent aggression. The resident intruder home cage physical provocation paradigm consists of placing a novel strain matched intruder into the home cage of the resident male. One minute after placing the intruder male into the cage, the resident male was provoked to attack by a tail pinch [Breur, 2001]. The tail pinch consists of a moderate pinch to the tail for 1 second every minute for the duration of the test. Each test lasted for a total of 20 minutes.

**Palkovits Brain Punch**

Following termination, the brains were frozen on dry ice, stored at -70C, and later cut into 200 micron coronal sections at -20C (Minotome, International Equipment Company, Damon Division, Meedham Hts., Mass.). The sections were then punched [Palkovits, 1973] from both the surgical and contralateral side of the amygdala complex (AMY). Proper electrode placement, coronal slice, and punch orientations were verified.
with comparison to the rostral-caudal bregma zero reference points [Pellegrino, 1981]. The punches were homogenized in either mobile phase or sucrose, centrifuged for 10 minutes at 3200rpm (Sorvall RT7, Kendro Labs, Newton Conn.) and stored at -70C for later analysis.

**Brain Serotonin Content**

The serotonin content for each brain region was analyzed using high performance liquid chromatography (HPLC) with electrochemical detection (Waters 460 Detector, Milford, MA) and has been described previously (Chapter II).

**Statistics**

One way ANOVA and two way RM ANOVA tests were used when appropriate. Student’s t-tests were used to compare specific groups with significance assumed at p<0.05. Statistical analysis was performed with SigmaPlot and SigmaStat (Jandel Scientific, San Rafael, CA).

**Results**

Attack number (Figure 10.1) and time until first attack (Figure 10.2) for the resident intruder physical provocation paradigm following amygdala transfection of Sry 1 or the control empty vector showed no change in either aspect of aggression.

Amygdala serotonin (Figure 10.3) content in Sry 1 and control empty vector amygdala transfected WKY males. Two-way ANOVA indicated a significant effect of
Sry treatment (F=4.1, p<0.05) decreasing serotonin content compared to empty vector.

There was no significant difference between brain regions within each treatment group.
Aggressive Behavior Does Not Increase Following Sry 1 Delivery to the Amygdala

Figure 10.1. Number of aggressive events for the resident intruder physical provocation paradigm in Sry1 and empty vector amygdala transfected males (means, ± SEM) showed no significant differences between treatments over 20 minutes of testing. There was no instance of aggression in the treatment or control groups. There were no significant differences in aggressive events for the standard resident intruder test, data not shown.
The Time Until First Attack Does Not Decrease Following Sry 1 Delivery to the Amygdala

Figure 10.2. Attack latency for the resident intruder physical provocation paradigm in Sry1 and empty vector amygdala transfected males (means, ± SEM) showed no significant differences between treatments. The maximum time length for the test was 20 minutes, indicating that ceiling was reach in each case. There were no significant differences in the standard resident intruder tests, data not shown. No error bars indicate that the ceiling or maximum amount of time for the test was reached (20 minutes) without an aggressive event.
Figure 10.3. Average serotonin content of the anterior, medial, and lateral portions of the amygdala for sry1 and empty vector transfected WKY males (means, ± SEM). Two-way ANOVA indicated a significant effect of treatment ($F=4.1$, $p<0.05$) with decreased serotonin content in Sry1 compared to empty vector males. There was no significant difference between brain regions within each treatment group.
Discussion

The results of this study supported the hypothesis that Sry delivery to the amygdala would decrease serotonin content. In addition, the decreased levels of serotonin content in the amygdala without increased aggression suggest that other factors are contributing to the mechanism of aggressive behavior. However, this lowered serotonin content by itself did not result in increased aggression resident intruder physical provocation. Therefore, the decreased amygdala serotonin content was due to the delivered Sry gene, and not the surgical procedure or plasmid. This suggests several mechanisms that could be acting together or independently to regulated serotonin synthesis, degradation, or behavioral modulation.

The first mechanism of action for Sry could be through a serotonergic related pathway. For example, regulation of serotonin levels could be through the direct control of neuronal tryptophan hydroxylase (TPH2) [Abumaria et al., 2007; Zill et al., 2007]. Since TPH2 has DNA binding sites for Sry, there is the potential for transactivation (upregulation or downregulation). In this case of increasing aggression, Sry would down regulate TPH2 synthesis and therefore decrease the amount of TPH2 to synthesize serotonin. Pharmacological studies with TPH2 antagonists, such as p-chlorophenylalanine, are known to increase aggression through blocking TPH2 activity and therefore decreasing neuronal serotonin levels [Korpela Sandnabba, 1998; Lesch et al., 2003]. Although the serotonergic pathway is complicated and poorly understood regarding receptor number, location, and method of activation, this is also another possible site of Sry action. There are seven different types of receptors with a variety of
subtypes depending on the animal species of interest [Veenstra-VanderWeele, 2000].

The two most common receptor types examined in aggression studies are 5HT$_{1A}$ and 1B [McQueen, 1999; Veenstra-VanderWeele, 2000]. Another potential site of action for Sry in the serotonin pathway may also involve the enzyme monoamine oxidase, which acts to break down serotonin or the serotonin transporter, which removes serotonin from the synapse [Molina et al., 1987]. For example, if Sry increased transcription and therefore the amount of the serotonin transporter, there would be an increased synaptic serotonin uptake, lowered serotonin levels binding to the post-synaptic terminal, and increased aggression.

Another possibility may involve Sry acting in vivo as expected on serotonin synthesis via inhibition of TPH2 synthesis. But, it could be that this decreased serotonin is not lowered enough to reach a point where a normally non-aggressive animal becomes aggressive. These WKY males are clearly not characteristically aggressive in nature. Therefore, the WKY males as adults may not be able to show typical aggressive responses to standard behavioral paradigms following relatively small changes in testosterone or serotonin. Our previous data reported here do in fact show that the WKY males are less aggressive than SHR/y males even with increased plasma testosterone and decreased neural serotonin levels. This “learned” behavior example is likely only one small component in the underlying mechanism.

An additional pathway that could influence behavior by through Sry regulation would involve testosterone. Testosterone not only has the ability to directly increase aggression, but also indirectly through decreasing TPH2 activity [Bell and Hepper, 1987;
Clement, 1999]. The major points of interest regarding testosterone, include synthesis and receptor activation. Several factors involved with testosterone synthesis include 5 alpha reductase, steroid sulfatase, and gonadatrophin hormone, luteinizing hormone, and the androgen receptor. Of these potential players, Sry 1 has the potential to transactivate 5 alpha reductase, steroid sulfatase and the androgen receptor through AP1 DNA binding sites. Therefore, if Sry is increasing the overall plasma testosterone or dihydrotestosterone concentrations, each of these players would likely be increased. However, since plasma testosterone samples were not measured in this study, the results can only be speculated on. This increase in plasma testosterone may then be enough to decrease serotonin content within the amygdala, but not necessarily result in increased aggression. Previous chapters have identified that relatively large increases in plasma testosterone were necessary in WKY males to elicit significant increases in aggression, even during physical provocation.

A final possibility could be that along with decreased serotonin, Sry is acting on the sympathetic nervous system in some way to inhibit aggressive behavior. Infusions of norepinephrine into the amygdala have been shown to stimulate fear responses in rodent models [Korzan et al., 2000; Alksidze et al., 2001]. Since Sry can increase tyrosine hydroxylase promoter activity in vitro, there is a possibility of altered norepinephrine release [Milsted et al., 2004a]. This could result in elevated adrenergic activation following a stress, such as physical provocation during a resident intruder paradigm [Veenema and Neumann, 2007]. However, this particular pathway in WKY males may not be as prevalent in the stress prone SHR/y males due to mechanisms already in place.
The pathway for Sry decreasing serotonin, without altering aggression following amygdala delivery could be due to any number of these proposed pathways, or some as of yet, undefined mechanism. Another possibility could be as simple as the method of delivery, time post surgery for testing, or even the nuclei transfected. However, the transactivating ability of Sry on other enzymes, cofactors, and even Sry itself is not known. In conclusion, this data did in part support the hypothesis by showing a decrease in serotonin content following Sry delivery to the amygdala. However the aggression portion of the hypothesis was not supported since there were no noticeable changes in aggressive events and attack latency. Therefore, future work will need to explore timing of Sry, alternative brain areas, and the level of serotonin content decrease needed to achieve aggression.
Chapter XI

Conclusion

The overall purpose of this research project was to identify and establish an animal model using SHR/y and WKY males to study the SHR Y chromosome in relationship to learning, memory, and aggression. In addition, these studies were also designed to examine novel neural, physiological and genetic mechanisms involving the SHR Y chromosome, as well as the transcription factor Sry, in learning, memory, and aggression.

The results for the first aim showed an SHR Y chromosome effect on learning and memory performance, and therefore supported the overall hypothesis for Aim I. In general, the SHR Y chromosome was involved in a mechanism that impaired maze performance in the following treatments: colony, corticosterone manipulated, socially enriched offspring, and aged SHR/y compared to WKY males. Specifically, SHR/y males were also more severely impaired following long term social stress, with memory retention significantly impaired. Further analysis of the role of social housing or a “socially enriched” environment also showed that SHR/y males had impaired memory performance when raised in the colony group housed environment. The aged SHR Y chromosome males also performed significantly worse than aged WKY males and
control adults regarding learning acquisition and memory retention trials. The stress hyper-responsive SHR/y males also appeared to be more sensitive to corticosterone manipulation involving memory performance tests and norepinephrine content in the hippocampus. However, future analysis of the archived probe, retention, and acquisition trials involving swim speed, annulus crossing, quadrant location, and float time may yield more significant results and better indicate mechanisms of impairment involving the SHR Y chromosome.

Likewise, the hypothesis for Aim II was also supported. In general, the SHR Y chromosome was involved in a mechanism that increased aggression in SHR/y males under chronic stress, as well as in treatments manipulating plasma testosterone and neural serotonin levels. Specifically, SHR Y chromosome males were also more sensitive to testosterone manipulation, showing increased aggression earlier following testosterone or flutamide treatment. Tryptophan hydroxylase studies also showed that SHR Y chromosome males showed increased aggression following decreased neural tryptophan hydroxylase activity in both castrate and castrate with testosterone replacement treatment groups. Interestingly, blocking serotonin reuptake from the synapse with fluoxetine failed to completely inhibit aggression in the SHR Y chromosome males, as it did in the WKY males.

The purpose of Aim III was to further investigate this SHR Y chromosome effect through in vivo transfection studies with the Y chromosome candidate gene, Sry. The neurochemistry data indicated that Sry delivery to the adult brain altered hippocampal norepinephrine, hippocampal tyrosine hydroxylase, and amygdala serotonin levels,
thereby supporting the overall hypothesis of Aim III. Pertaining to the learning and memory studies, Sry gene delivery to the hippocampus significantly altered neurochemistry without having a corresponding effect on maze performance. The transfection studies involving aggression and Sry gene delivery to amygdala showed significant decreases in amygdala serotonin content. However, this decreased serotonin content did not correspond to an increase in aggressive behavior.

The following summaries will provide an overview of the findings from each study, followed later by the presentation of future studies and potential directions for subsequent research.

**Study 1: Long term social stress impairs learning and memory retention.**

Both SHR/y and WKY colony males showed acquisition impairment on the first maze version compared to control. Retention trial performance, however, was only impaired in the SHR/y colony males. These SHR/y colony males also showed an over-responsive hypothalamic pituitary adrenal (HPA) and sympathetic adrenal medullary (SAM) axes, with altered adrenocorticotrophin hormone, corticosterone levels, as well as an increased plasma norepinephrine. The WKY colony male stress responsiveness when compared to controls exhibited only minor changes in the: corticosterone, adrenocorticotrophin, and norepinephrine profiles compared to the controls. This supports the findings that the retention trial performance was not significantly affected in WKY colony males, but was in SHR/y colony males. The maze performance data, specifically regarding retention trials, and the over-responsive HPA axis along with
elevated SAM axis suggests that the SHR/y males are more sensitive to stress than WKY males. By comparison, the WKY colony males exhibited a blunted HPA axis and SAM axis compared to control males. This blunted physiological response could be the reason why WKY colony males, in general, performed as well or better than WKY control males.

**Study 2: Social housing alters pup stress responsiveness and impairs water maze performance in the adult SHR Y chromosome male.**

As a follow up to the colony housing environment and chronic social stress results from study 1, this next study addressed the pre-pubertal and post-pubertal housing environment of the developing pups. Specifically, what effect would this housing environment have on the physiology and also the learning and memory performance of these pups later in life as adults. The results of this study suggest that the pre-pubertal environment in conjunction with the SHR Y chromosome was able to impair maze performance in SHR/y F1 and not WKY F1 males. These differences in maze performance are supported by the basal and elevated stress response of plasma norepinephrine and corticosterone in the SHR/y F1 males. This hyper-responsiveness stress profile along with potential dysregulation of adrenocorticotrophin hormone and corticosterone suggests several potential mechanisms influencing acquisition and retention performance on memory related tests.
Study 3: Corticosterone manipulation impairs learning and memory, while increasing hippocampal norepinephrine content.

With the apparent importance of the HPA axis (i.e. corticosterone) and the SHR Y chromosome in learning and memory performance, this study examined corticosterone manipulation and maze performance in SHR/y and WKY adult males. The results of this study supported the hypothesis that both elevations or decreases in plasma corticosterone would impaired water maze performance. The impaired performance was present during retention trials, with acquisition trials only showing minimum impairment. In addition to corticosterone manipulation impairing maze performance, this effect was also more noticeable in SHR Y chromosome (SHR/y) males. This study suggests that SHR/y males have a predisposed neural sensitivity through an undefined mechanism related to corticosterone as compared to WKY males potentially due to the SHR/y males have an increased sympathetic nervous system activity and overall stress response.

Study 4: Maze acquisition and retention trial performance is impaired in aged SHR/y and WKY males.

Studies involving learning and memory in aged animal models show that maze performance is typically impaired, especially in stress prone animals. The results from this study reinforce the effect of age on learning and memory, where both SHR/y and WKY aged males showed significant impairment when compared to the younger control adults. This study was consistent with previously documented research regarding the stress response and sympathetic nervous system activity in SHR/y males. The SHR Y chromosome effect was evident in the learning acquisition and memory retention trials
over multiple maze manipulations. However, the strain differences between SHR/y and WKY regarding the hypothalamic pituitary adrenal and sympathetic adrenal medullary axes did not identify a similar Y chromosome effect. Therefore, the significantly impaired maze performance by the aged SHR/y males compared to aged WKY males was likely due to a physiological, neurochemical, or anatomical variable not specifically measured in this study.

**Study 5: The SHR Y chromosome increases physiological and behavioral indices of aggression in the colony environment**

Regarding aggressive behavior in the SHR Y chromosome animal model, the results of this study indicated that colony SHR/y males, on average, were more aggressive during dyadic encounters than the WKY colony males. This would then imply that each colony member, regardless of hierarchy status in the SHR/y colony is potentially more aggressive than the WKY colony males. Along with elevated plasma testosterone, the amygdala serotonin content was lower in the SHR/y compared to WKY colony males further supporting this increased aggression and role of the SHR Y chromosome.

**Study 6: Testosterone treatment increases aggression in resident intruder and physical provocation paradigms.**

This study expanded on the previously indicated results of increased testosterone and decreased amygdala serotonin content in the SHR/y compared to WKY colony
males. The results from this study indicated that both SHR/y and WKY castrate with testosterone implant and flutamide treated males were more aggressive during physical provocation testing than control or castrate males. This also supported previous research and showed that increased plasma testosterone was positively correlated with increased aggression. Since flutamide acts to block the androgen receptor, causing an increase in plasma testosterone due to feedback inhibition, this effect on aggression may not therefore be an androgen receptor mediated process. However, the amygdala serotonin content did not follow the increased aggression to the extent as the plasma testosterone. The inability of testosterone to consistently decrease amygdala serotonin content suggests that content itself is not an overly sensitive measure for aggression. In addition, the SHR Y chromosome was also implicated in the regulating mechanism of aggression, since SHR/y males overall were more aggressive than the WKY males.

Study 7: Testosterone and serotonin manipulation in male SHR/y and WKY rodents.

The results of this study supported the hypothesis that the SHR Y chromosome was responsible for increased aggression in SHR/y males, with or without serotonin and testosterone, when compared to WKY males. In general, SHR/y and WKY castrated males were less aggressive than testosterone implanted males, with p-chlorophenylalanine (PCPA) treatment partly increasing aggression. Fluoxetine completely removed aggression in WKY males, but was only able to partly inhibit the testosterone mediated effect on aggression in SHR/y castrate males with testosterone implants. The results of this study also suggests a SHR Y chromosome effect showing
SHR/y males to be more aggressive following testosterone or serotonin treatment. Regarding the testosterone and serotonin treatments, the SHR/y males showed increased aggression indicating an elevated sensitivity to PCPA, but a blunted sensitivity to fluoxetine compared to WKY males. Therefore, these results suggest that there is a testosterone and serotonin mediated pathway that is influenced by the SHR Y chromosome.

Study 8: Water maze performance and brain neurochemistry after in vivo Sry delivery to the WKY hippocampus.

The results of the previous morris water maze studies supported the hypothesis of Aim I that the SHR Y chromosome males would have impaired maze performance compared to WKY males. Expanding on this SHR Y chromosome effect, the candidate gene Sry was delivered to the WKY adult hippocampus. The data supported the hypothesis that Sry would increase hippocampal tyrosine hydroxylase activity. However, the increased tyrosine hydroxylase activity did not correspond to increased hippocampal norepinephrine content or impaired maze performance. This could be due to several mechanisms including the time of maze testing following treatment, method of plasmid delivery, and other pathways maintaining a stable catecholaminergic environment in the hippocampus.
Study 9: Aggression and brain serotonin following *in vivo* Sry delivery to the WKY amygdala.

The previous aggression studies supported the hypothesis of Aim II, that the SHR Y chromosome males would be more aggressive than WKY males. Following delivery of the candidate gene, Sry, to the amygdaloid complex, the amygdala serotonin content decreased suggesting the potential for behavioral differences. However, the lowered serotonin content did not result in increased aggression. This suggests several mechanism that could be acting together or independently to regulate serotonin synthesis, metabolism, transport, or even behavioral processes.

**Future Directions**

The results from the previous chapters indicate that the SHR Y chromosome is involved with various aspects learning and memory performance, as well, as aggressive behavior. Due to the supporting data, there are several studies that should be addressed in the future pertaining to the mechanisms involving aggression and maze performance. Therefore, the following paragraphs will outline and give support for these future research projects.

**Future Direction 1**

Due to the relative success of the *in vivo* Sry delivery to neural tissue, a more detailed analysis is warranted. Our lab has previously identified that Sry can increase tyrosine hydroxylase promoter activity. Therefore, other players in the serotonergic
pathway should be examined in relationship to transactivation by Sry. Sequence analysis of neuronal tryptophan hydroxylase (TPH2) indicates that Sry has the potential to act via AP-1 DNA binding sites to alter TPH2 promoter activity. If Sry is able to decrease or partially suppress promoter activity, this could explain the decrease of serotonin content following \textit{in vivo} Sry transfection. Other studies should also analyze the effect of Sry on reporter constructs for the promoter of the serotonin transporter (SERT), monoamine oxidase (MAO), 5HT$_{1A}$ and 1$_B$ receptors. In addition, the role of 5 alpha reductase converting testosterone to dihydrotestosterone, steroid sulfatase, and the androgen receptor all regarding testosterone synthesis and activity should also be examined.

**Future Direction 2**

Neural \textit{in vivo} transfections of Sry into specific brain nuclei associated with aggression and maze performance along with behavioral and physiological analysis should be an additional focus. For example, the raphe nucleus is the primary site for TPH2 activity and therefore neuronal serotonin synthesis and should be transfected with Sry. In a similar manner, the testes are the primary site for testosterone production in the adult male and should also be examined in relationship to Sry transfection. Indeed, preliminary analysis of aggressive behavior following testis transfection did show increased aggression in Sry compared to empty vector transfected WKY males. This could be due to any number of these proposed pathways, or some as of yet, undefined mechanism.
Regarding maze performance and Sry delivery, there are two potential surgical sites that may yield more significant results. The first delivery site experiment should be into the entire hippocampus, instead of just one side. The second delivery site experiment should be into the adrenal gland. Since the stress response is clearly important to learning and memory, increased corticosterone or norepinephrine release following stress should have an impairing effect on acquisition and retention.

Future Direction 3

With the development of the fragment analysis technique by our lab to distinguish between the different relative copy numbers for each Sry, an Sry brain map can be developed. After establishing this baseline or control level in SHR/y and WKY males, there are several environmental and pharmacological manipulations that should be addressed. For example, the colony housing environment was shown to increase aggression and also impair maze performance. Following up on this data, Sry analysis of the limbic system structures may identify different profiles for Sry following chronic social stress. Another possibility would involve the aggression studies following testosterone treatment. The relative copy percent of total Sry copy number in the amygdala and associated limbic system structures could be compared to testosterone levels (castrate with testosterone implant, flutamide, and castration) and also behavior. Preliminary data in our lab does in fact show that castration does alter the relative percent of the total Sry copy number in various brain nuclei (amygdala, cortex, hippocampus, and hypothalamus) compared to control levels.
Future Direction 4

Since SHR/y males show an increased stress response, restraint stress followed by blood and tissue (adrenal and hypothalamus) samples at different times post stress would allow for an Sry stress profile to be established. A change Sry mRNA copy transcripts relative to each other would support the hypothesis that the SHR Y chromosome, and more specifically, Sry is involved in the stress response. In addition, the changes in relative amount of Sry would likely follow different profiles between the WKY and SHR/y strains post-stress. Since this data would specifically address the relative amount of Sry out of a predefined total Sry, the differences presented here regarding actual transcript number, mRNA turnover, or the resulting translated protein could not be addressed until real time PCR and protein analysis are performed. Preliminary results from our lab do indeed show that Sry copy profiles change following stress with several trends being evident between SHR/y and WKY males up to one hour post stress.


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