REGULATION OF TYROSINE HYDROXYLASE GENE EXPRESSION IN BRAINSTEM AND ADRENAL GLAND OF SHR/y AND WKY FEMALE RATS BY CLONIDINE TREATMENT

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REGULATION OF TYROSINE HYDROXYLASE GENE EXPRESSION IN
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I want to dedicate my Master’s thesis to the two most important little people in my life. To my two angels Anel and Isabella, my daughters, who with their smiles and presence illuminated my way and encouraged my spirit to achieve my goal. I want to thank my parents for their beautiful words in the most difficult moments in my life and the way that they taught me do not give up to get my dreams to become true. Also I want to thank to my family and friends for being very supportive and encouraging. Many thanks to my lab partners Jeff, Russ and Adam who gently helped me when I needed. To Dr. Daniel Ely who kindly helped me to take the blood pressure and Jonathan Tood who took the blood samples of the animals. To Gail Dunphy for her support, her help with catecholamines measurements and her advise in lab techniques. To Dr. Walter E.Horton’s people lab in NEOUCOM for being helpful when I had to use their Real-Time PCR equipment. To Dr. Francisco Moore in Biology Department, Dr. Richard Einsporn and Christopher Ickes in Statistics Department in The University of Akron who gently helped me in analyze my data. Finally I want to say special thanks to my advisor Dr. Amy Milsted for have believed in me to do this project, for giving me the tools to accomplish my goal and to be there when I needed. Thank you so much.
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CHAPTER I

INTRODUCTION

Hypertension is a condition with repeatedly elevated blood pressure of systolic pressure above 140 with a diastolic above 90 mmHg. An elevation of blood pressure increases the risk of developing heart and kidney disease, hardening of the arteries, eye damage and stroke. Most cases of hypertension are primary hypertension that account for almost 95% of the cases whereas secondary hypertension is less common (5-10%) and is caused by another disease or medication. When we look at the statistics, we see that men have a greater incidence of high blood pressure than women until age 55 when their respective incidences are similar. After 55, women are more likely to have high blood pressure than men are (1). Thus, this is a sex related disease and the Y chromosome has been demonstrated to play a role in its development (15).

Spontaneously hypertensive rats (SHR) and normotensive (WKY) rats have been widely used as a model of hypertension. To study the effects of the Y chromosome on blood pressure, a new substrain, SHR/y has been developed from WKY and SHR parents. SHR/y rats were obtained by crossing a male SHR with WKY female and then in following generations backcrossing the sons with female WKY rats. Thus, SHR/y males have autosomes and the X chromosome from WKY mother and Y chromosome from the original SHR father. Females SHR/y have autosomes and X chromosomes from a WKY
mother and the other X chromosome from their SHR/y father (14, 49). From this genetic background female WKY and SHR/y rats are equivalent because all the autosomes and both copies of X chromosomes in SHR/y substrain are inherited from WKY rats. However phenotypic differences have been observed in previous studies (46, 48). These differences could be associated with genomic imprinting where a gene’s expression depends on the parent that transmits it (24). During gametogenesis an allele (either from the father or the mother) is marked and it is destined to be inactive or repressed. Thus, when the maternal allele is expressed it is because the paternal allele is silenced or vice versa. This inactivated allele is called “imprinted” (59). One possible mechanism that has been proposed to explain this phenomenon is the methylation of cytosines in CG dinucleotides within the imprinted region that prevents gene’s transcription (24).

Miller analyzed phenotypic differences in SHR/y and WKY female rats under territorial stress and treatment with clonidine (blocker of the sympathetic nervous system). Blood pressure (BP), noerpinephrine (NE) levels, and organ (kidney, heart) weights were measured, showing that SHR/y females had higher BP, plasma NE levels and larger organs when compared to WKY females. These results indicated that stress induced an increase in BP and clonidine treatment lowered it, suggesting that the increase in BP could be due to the sympathetic nervous system (SNS) (48). In addition, Marcelo evaluated phenotypic differences between SHR/y and WKY female rats when they were subjected to ovariectomy and treatment with testosterone. Body and kidney weights, BP and also levels of renin and angiotensinogen messenger RNA (mRNA) were measured. The results showed higher body and kidney weight and also higher BP in SHR/y compared with WKY female rats. Moreover, levels of renin and angiotensinogen mRNA
were much higher in WKY compared with SHR/y at ten weeks of age. Additionally, the study showed no changes in systolic BP by ovariectomy but increased BP with addition of testosterone (46).

The SNS plays an important role in the development of primary hypertension. Neurogenic causes of hypertension with regard to the over activity of the SNS are well documented (11, 16, 17, 76), although the pathophysiology of the SNS dysfunction is not completely defined. Increased sympathetic nerve density, high sympathetic nerve firing rates and NE transporter dysfunction are the possible mechanisms that could explain the increased spillover to the circulation of NE in some patients with essential or primary hypertension. It is known that the locus coeruleus (LC) is the major noradrenergic nucleus in the brain from which NE is released by the sympathetic nerves. The adrenal medulla also releases NE causing vasoconstriction and subsequent increase in BP. NE is the principal neurotransmitter of sympathetic postganglionic endings and is stored in synaptic knobs of neurons that secrete it. Thus, high levels of NE are an excellent marker that supports the neurogenic basis of hypertension (76).

Evidence of the role of NE in hypertension has led to the study of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine production as a point of possible regulation of the synthesis pathway. TH catalyzes the enzymatic conversion of L-tyrosine to L-dopa, the first step in the biosynthesis of dopamine (DA), NE and E. Many different mechanisms are involved in TH regulation. TH is subjected to short term regulation (feedback inhibition by catecholamines, phosphorylation by different systems and allostERIC regulation of enzyme activity) and long term regulation at transcriptional and translational levels, as well as by mRNA stability (40).
After reviewing the importance of the *Th* gene and its possible role in the development of hypertension, I hypothesize that the *Th* gene is expressed at higher levels in brainstem and adrenal gland of SHR/y than in WKY female rats. To test my hypothesis, I will evaluate the following objectives 1) establish basal levels of *Th* mRNA in SHR/y and WKY female rats in brainstem and adrenal glands, 2) determine whether the expression of *Th* gene is reflected in levels of NE and BP, 3) determine whether SHR/y and WKY female rats respond differently to clonidine treatment and 4) establish whether clonidine regulates or changes *Th* gene expression in SHR/y and WKY female rats. I will review hypertension, SHR/y and WKY rat (animal model of hypertension), the SNS, catecholamines, tyrosine hydroxylase and its regulation. Thus, SHR/y and WKY rats are an excellent model to study hypertension. High BP has been shown to have a neurogenic component where the role of the SNS is crucial. When the SNS is activated catecholamines such as NE are released, increasing BP. Since TH is the rate-limiting enzyme in catecholamine production, it is necessary to look at TH and its regulation.
CHAPTER II

LITERATURE REVIEW

Hypertension

The pressure required to move blood through the circulatory system is provided by the pumping actions of the heart (cardiac output) and the tone of the arteries (peripheral resistance). Each of these determinants of BP is influenced by the interactions of a complex series of factors (31). High BP (hypertension), a common condition wherein the BP is chronically elevated with systolic pressure above 140 and diastolic above 90 mmHg, affects nearly one in three adults in the United States. Uncontrolled hypertension can cause commonly stroke, heart failure or heart attack, blindness and kidney failure (1). Generally, hypertension is classified according to its cause. Thus, high BP that has no known cause is called primary or essential and many factors have been associated with its etiology: age, high salt intake, sedentary life style, tobacco smoking, alcohol abuse, saturated fat in the diet, obesity, stress and diabetes. Additionally, genetics, hormones and endocrine and sympathetic nervous system have been related to the cause of primary hypertension (74). Secondary hypertension has an identifiable origin such as: renal disease, primary hyperaldosteronism, stress, sleep apnea, hyper or hypo thyroidism, pheochromocytoma, preeclampsia, hypercalcemia and aortic coarctation. This accounts for approximately 5 to 10% of all cases of hypertension.
The primary determinants of hypertension remain uncertain in the majority of the subjects and the interindividual variations have motivated the application of genetics in order to identify one of these primary determinants. Several forms of hypertension have been associated with mutations in a single gene (42). Glucocorticoid remediable aldosteronism (GRA), results from a mutation in aldosterone synthase enzyme which shows ectopic expression and aldosterone is produced from corticosterone in adrenal fasciculate instead of adrenal glomerulosa. Aldosterone allows salt and water retention that lead to plasma volume expansion. This suppresses plasma renin activity with the consequent turns off production of angiotensin II. However, this does not suppress secretion of mineralocorticoids because they are under ACTH control, this secretion of mineralocorticoids results in sustained volume expansion and hypertension (42). Lyddle syndrome is another form of hypertension where mutations in the β or γ subunit of the epithelial Na channel lead to elevation in renal Na reabsorption through this channel in the distal nephron (23). This syndrome is characterized by hypertension, hypokalemia, metabolic alkalosis, low plasma aldosterone levels and plasma renin activity (23).

Another form of hypertension where a mutation in a gene has been identified is Gordon’s syndrome (pseudohypoaldosteronism type II, familial hyperkalemia and hypertension). This is caused by a mutation in the chloride channel in the distal renal tubule which results in enhanced chloride reabsorption. The syndrome is characterized by hyperkalemia, hyperchloremic acidosis, volume expansion and normal glomerular filtration. The patients show short stature, muscle weakness, intellectual impairment, dental abnormalities impaired grow and sever hypertension. (34, 75). Variants in the angiotensinogen gene have been implicated in the pathogenesis of essential hypertension.
A highly polymorphic dinucleotide GT repeat has been identified as well as the substitution of threonine residue for methionine at codon 235. These variants act by increasing the synthesis and secretion of angiotensinogen with the subsequent elevation in BP (42, 27). Finally, there is a syndrome of apparent mineralocorticoid excess (AME) which is due to a mutation of the 11 beta-hydroxysteroid dehydrogenase type 2 gene. The enzyme catalyses the interconversion of hormonally active cortisol to inactive cortisone. In patients with congenital deficiency of 11 beta-hydroxysteroid dehydrogenase, cortisol and not aldosterone act as mineralocorticoid which results in hypokalemia and hypertension with the suppression of the renin-angiotensin-aldosterone axis (68).

Hypertension prevalence increases with age. People above 50 years old have greater risk of having high BP due to changes in endothelial functions that modulate vascular tone and structure. Moreover, excess weight and lack of physical activity have been associated with hypertension in this group of people and others. During ageing, sympathetic nervous activation occurs and the stimulation of sympathetic nervous outflow to organs involved in BP regulation contributes to the development of hypertension (17). In addition, it is known that high BP prevalence is greater in men than women until age of 55 when their incidences are similar. After 55, women are more likely to have high BP than men (1). This fact has suggested gender differences in vascular function. Female hormones such as estrogens have receptors in vascular endothelium and smooth muscle. Interaction of these receptors with estrogen stimulate endothelial cell growth and inhibit smooth muscle proliferation, moreover the activation of these receptors stimulate vascular relaxation mediated by nitric oxide, contributing to lower BP (33). Also estrogens can activate the renin-angiotensin-aldosterone system
(RAAS) by enhancing angiotensinogen synthesis and reducing the angiotensin-converting enzyme (ACE) activity. This leads to a decrease in the conversion of angiotensin I to angiotensin II decreasing vasoconstriction, increasing vasodilation and consequently lowering BP (6). Thus, this is an age and sex related disease where the Y chromosome has been shown to play an important role in its development (15).

The SHR Animal Model of Hypertension

The spontaneously hypertensive rat (SHR), a well-studied animal model of hypertension, was created as a result of selective breeding of male of the Wistar strain that showed spontaneous hypertension with a same strain of female rat with a BP slightly above the average to obtain F₁ (57). By the third generation, this breeding produced a strain with almost 100% hypertension. This SHR strain consistently and spontaneously shows moderate to severe hypertension between 7 and 15 weeks of age and has been used as a model of human hypertension (15). To study the role of the Y chromosome effects in the development of high BP, a new substrain, SHR/y, has been developed at The University of Akron from WKY and SHR parents. SHR/y rats were obtained by crossing a male SHR with WKY female and then in following generations backcrossing the sons with female WKY rats. Thus, SHR/y males have autosomes and X chromosome from WKY mother and Y chromosome from the original SHR father. Females SHR/y have autosomes and X chromosome from a WKY mother and the other X chromosome from their SHR/y father (14, 49). Therefore, based on their genetic backgrounds, SHR/y and WKY females are equivalent.
Sympathetic Nervous System (SNS)

The SNS is part of the autonomous nervous system that is located in a chain on both sides of the spinal cord and consists of ganglia which connect to skin, blood vessels and organs in the body cavity (70). This system activates the fight or flight response, also known as sympathico-adrenal response of the body, as the preganglionic sympathetic fibers that end in the adrenal medulla secrete acetylcholine which activates the secretion of E and NE. Therefore, this response that acts mainly on the cardiovascular system is mediated directly via impulses transmitted through the SNS and indirectly via catecholamines secreted from the adrenal medulla (74). The SNS can trigger changes in different parts of the body simultaneously, for instance can decrease the motility of the large intestine, cause pupil dilation, piloerection and perspiration, widen bronchial passage, accelerate heart rate, cause vasoconstriction and raise BP (70).

The role of the SNS in the regulation of arterial pressure is relevant, and there is evidence that supports the idea that essential hypertension is commonly neurogenic. The increase in the density of sympathetic innervation has been documented in SHR hypertension and remains as a possibility in humans (17). Moreover SHR Y chromosome has been demonstrated to increase indexes of SNS activity (13). Additionally, increased sympathetic nerve firing rates involves the stimulatory action of norepinephrine-releasing neurons. Another interesting fact that could explain the neurogenic causes of hypertension is that E within the sympathetic nerves may be released with NE as a cotransmitter, facilitating the release of NE through stimulation of presynaptic β adrenoreceptors on sympathetic nerves and also increasing its release per nerve impulse. The dysfunction of the neuronal NE transporter that may impairs the reuptake of this
catecholamine also has been implicated in the sympathetic nerve biology in essential hypertension (17).

Catecholamines

Catecholamines are chemical compounds products derived from the amino acid tyrosine which is concentrated in catecholaminergic neurons where it is hydroxylated by the enzyme TH to form dihydroxyphenylalanine (dopa). In the synthetic pathway, L-aromatic amino acid decarboxylase converts dopa to dopamine (DA), and then there is the conversion from DA to NE by the enzyme dopamine β-hydroxylase. In epinephrinergic neurons and in the adrenal medulla the pathway contains an additional enzyme known as phenylethanolamine-N-methyltransferase which converts NE to E (55).

From:http://web.indstate.edu/thcme/mwking/aminoacidderivatives.html

These catecholamines E, NE and DA are produced mainly in the postganglionic fibers of the SNS and adrenal medulla (55). E works as a neurotransmitter in the central nervous system and as a hormone in the blood circulation. NE is mainly a neurotransmitter in the central nervous system but is also present in the blood mostly
through spillover from the synapses of the SNS, which functions in response to short term stress. Hence, E and NE increase the heart rate as well as BP. Additionally, other actions of NE include increase in glycogenolysis in the liver, increase lipolysis in adipose tissue and relaxation of bronchial smooth muscle to open up the air passage to the lungs. These actions represent a mobilization of the body’s sources in order to meet the stressful challenge, the “fight or flight” response (4, 74).

NE is the main neurotransmitter in the SNS that regulates the function of peripheral organs in the body and is released from noradrenergic neurons during synaptic transmission (74). This catecholamine binds to α and β receptors sites in the post-synaptic membrane. Stimulation of α receptor causes vasoconstriction and subsequent increase in BP. In addition, β receptor stimulatory effects cause an increase in the rate and force of heart contractions. Several studies have searched for alterations in the interaction between NE and post synaptic receptors in hypertensive humans and animals. In this regards, 30% of hypertensive humans display sympathetic hyperactivity and increase NE release to stressful stimuli (76).

**Tyrosine hydroxylase enzyme**

Since its discovery in 1964 by Dr.Nagatsu (52), TH has been intensively studied in relation to its physiological functions and also its involvement in some pathologies, for instance hypertension. This enzyme catalyzes the initial and the rate-limiting step in the biosynthesis of the cathecholamines DA, NE and E. TH is a monooxygenase (TH, tyrosine 3-monooxygenase) that uses tyrosine, molecular oxygen and tetrahydrobiopterin (BH₄) to generate 3,4 dihydroxyphenylalanine (dopa), dihydrobiopterin and H₂O (52, 55). TH is a tetramer composed of 498 amino acids in each subunit, each of these subunits has
an N-terminal regulatory domain and C-terminal catalytic domain. Regulation of this enzyme falls into two categories: first, short term regulation that occurs at post-translational level and includes feedback inhibition, allosteric regulation and enzyme phosphorylation; second, long term regulation of gene (Th) expression including transcriptional and translational regulation and RNA stability as well (18, 40).

**TH and Th gene**

The human TH gene is located on chromosome 11 (53) and is composed of 14 exons interrupted by 13 introns, spanning approximately 8.5 kilobase pairs (kb)(35). Four types of human mRNA TH have been characterized (35, 52) resulting from alternative splicing of a single gene. The mRNAs are constant for the major part but are distinguishable from one another, by insertion/deletion of 12- and 81- bp sequences. Type 1 is the shortest and Type 4 is the longest and has 93-bp sequence composed of 12- and 81 bp sequence inserted into type 1 that does not change the reading frame of the protein-coding region. Type 2 and 3 have the 12- and 81-bp insertion sequences respectively (51). The rat Th gene is located on chromosome 1 (52). All the evidence suggests that the rat Th gene exists as a single transcript and contains 13 exons that span 7.3 kb (ranging from 51 bp to 421 bp) separated by sequences no greater than 2.0 kb and 12 introns (7).

**TH short-term regulation**

The short term regulation of TH occurs at post-translational level. Feedback inhibition is one the mechanisms involved in TH short term regulation. First, catecholamines act as feedback inhibitors of the enzyme binding to the free enzyme and preventing pterin (substrate) from binding. This is a reversible inhibition of the enzyme that functions as a sensor of the concentration of catecholamine product (51, 67).
Second, catecholamines can form an inhibitory catecholamine-metal complex within the active site of the enzyme decreasing the enzyme activity and also stabilizing it by increasing activity recovered at a later time following phosphorylation (58, 3).

TH is also subject to allosteric regulation by substances that modulate the enzyme activity at a site outside the active site of the protein (40). Among the products that have been shown to interact with TH as allosteric effectors are heparin, phospholipids and polyanions (32, 36, 43, 44, 60). These substances increase enzyme activity by decreasing the Km of the enzyme and it seems to be an electrostatic phenomenon (20, 40). Additionally, low concentrations of RNA could activate TH activity whereas higher concentrations could be inhibitory and it seems that the effects of nucleic acids are mediated by their polyanionic character (54). Although there is experimental evidence that supports the allosteric regulation of TH, polyanions probably do not exert dominant regulatory effects in vivo and the physiological relevance is questionable (40).

TH is known to be regulated by phosphorylation that leads to a covalent modification of the enzyme where the amino terminal segment contains several potential phosphorylation sites and serine site is phosphorylated by different protein kinase systems. TH phosphorylation by the different protein kinases systems can take place at five phosphorylation sites: Ser⁸, Ser¹⁹, Ser³¹ Ser⁴⁰ and Ser¹⁵³ (20, 40). Most of these events can be associated with increases in enzyme activity and the systems involved are: cAMP-dependent protein kinase A (PKA) (29), Ca²⁺/phospholipid-dependent protein kinase C (PKC) (2), Ca²⁺ cGMP-dependent protein kinase G (PKG) (62). The Ca²⁺/Calmodulin-Dependent Protein Kinase II (Ca²⁺/CaMkII) system phosphorylates TH but does not increase enzyme activity by itself due to the requirement of an activator.
protein that increase the catalytic activity (77). Also it has been suggested that 
Ca\textsuperscript{2+}/CaM\textsuperscript{II} phosphorylates only two of the subunits of the enzyme, and this is not 

enough for enzyme activation (21). In addition, there are several protein kinases 
associated with TH phosphorylation that are activated by growth factors (20). Among 
these kinases are: microtubule-activated protein kinases such as mitogen-activated 
protein kinases (MAP kinases) (69) and extra cellular signal-regulated protein kinases 
(ERKs) (25).

**TH long-term regulation**

The long-term regulation of TH occurs at gene level such as transcriptional and 
translational level and RNA stability.

Transcriptional regulation has been demonstrated by changing the physiological 
conditions of animals in vivo. For instance, immobilization stress can activate 
transcription of the *Th* gene in the locus coeruleus of the rat brain (65) and in adrenal 
gland (41). Acute air puff stress increases the *Th* gene expression in the same brain area 
of WKY but only modestly in SHR (47). Additionally, several hormones can regulate 
levels of *Th* mRNA. Androgens have been shown to increase levels of *Th* mRNA in 
adrenal medulla, contributing to the development and maintenance of hypertension in 
SHR rats (37). Also, estradiol increases *Th* mRNA expression in rat LC (66), with studies 
suggesting that the rise depends on the dose used and the mode of administration. In the 
LC the presence of estrogen receptors such as ER\textalpha and ER\textbeta has been reported, thus the 
increase in *Th* mRNA could be attributed to the direct activation of these receptors by 
estradiol injection that lead to elevated transcription (66). This study also suggests that 
the increase in *Th* mRNA could result from an indirect effect of estradiol on neurons that
innervate the LC through cAMP or AP-1 mediated pathways. Moreover, short-term estradiol injections can increase the $Th$ mRNA levels in the LC and other brain catecholaminergic regions such as ventral tegmental area and substantia nigra depending on the dose, mode of administration and the tissue examined (64). A recent study has demonstrated that transcriptional regulation of $Th$ mRNA by estrogen depends on the estrogen receptor subtype. Thus, 17 $\beta$-estradiol triggers a great increase of $Th$ levels in the presence of ER$\alpha$ but shows inhibition with ER $\beta$. Estrogen also can interact with cAMP pathway through ER $\alpha$ and ER $\beta$ to regulate $Th$ transcription (45). However, estrogen treatment in rats has been shown to decrease the level of transcription of $Th$ mRNA (5). These discrepancies in estrogen response could be explained from differences in dose, duration, mode of administration, expression of estrogen receptor subtype or animal species used (45). Glucocorticoids regulate levels of $Th$ mRNA, increasing its content by binding to a glucocortiod-responsive element (GRE) in the promoter region (22). Polypeptides such as angiotensin II stimulate $Th$ mRNA via the AngII AT1 receptor in neuronal cultures of normotensive and SHR rats (79). Also, testes determining factor Sry can increase TH transcription mainly through the AP1 response element (50). Other substances such as forskolin, adenylcyclase stimulator, can produce a great increase of $Th$ mRNA in the rat LC (63).

Certain drugs, such as reserpine have been demonstrated to increase levels of Th in the adrenal gland, peripheral sympathetic cells and the central noradrenergic cells of the LC by increasing $Th$ mRNA levels (28). In addition, cocaine induces an increase in Th activity and $Th$ mRNA levels (72). Repeated administration of morphine also increases $Th$ mRNA in the LC (20), whereas chronic treatment with antidepressants decreases the
expression of Th gene rat LC (56). Nicotine is another agent that has been implicated in the increased levels of the Th mRNA (18).

Th gene expression can be regulated at translational level. Researchers usually measure steady-state Th mRNA levels. This does not necessarily reflect Th protein levels, Th activity or catecholamine function. Several studies have demonstrated that large increases in Th mRNA levels are not accompanied by the same response in Th activity. One example of this dissociation was done in a transgenic mouse expressing 50 times the Th gene transcripts while Th immunoreactivity and enzyme activity was altered by only 2-3 fold (30). Th activity and the rate of enzyme synthesis increase additively with substances such as glucorticoids and cAMP but there was no similar increase in mRNA content (71). Based on this evidence it could be apparent that mRNA levels are not correlated with protein or activity, suggesting that there could be a lag between transcription and translation of the Th gene where processing mechanisms could produce nonfunctional mRNA forms (unspliced, nonpolyadenylated) (40).

Several studies have shown the regulation of Th mRNA stability, thus activators of PKC (73), nicotinic agonistics (8) and hypoxia (10) have been demonstrated to enhance Th mRNA stability. The mechanism to explain the stability phenomenon involves RNA-protein interaction that increases the half-life of the mRNA (9).
CHAPTER III

MATERIALS AND METHODS

Rats

The WKY (Wistar Kyoto) rat is a strain of *Rattus norvegicus* commonly used as a normotensive control for the SHR (Spontaneously Hypertensive Rat). SHR was obtained from an outbred a male of Wistar strain with marked elevation of blood pressure mated with a female with slightly elevated blood pressure (57). Rats were obtained from Harlan Sprague-Dawley (Indianapolis, Ind.), and since 1981 colonies have been maintained at the University of Akron. The SHR/y strain was developed in order to determine the genetic influence of the Y chromosome on blood pressure in SHR and WKY using genetic crosses (15). The two strains were maintained in the Biology Resource Center (BRC) at the University of Akron and treated according to NIH animal care and Institutional Animal Use and Care Committee (IACUC) regulations.

Breeders from WKY and SHR/y were provided by Dr. Amy Milsted and Dr. Daniel Ely, and housed in typical breeding boxes. The animals were maintained at room temperature (26-28 °C) with 12 hours light/dark cycle and provided with water and rodent chow (Lab Diet 5P00 Prolab RMH 3000) *ad libitum*. Female rats at three weeks of age were weaned and placed in separate cages, 2 or 3 rats per cage, and monitored three
times per week in order to see if they were in good condition. At 9 weeks, base line BP were measured using the tail-cuff sphygmomanometry (Narco Biosystems, Houston, Texas) and at 10 weeks the rats were separated into experimental (treated with clonidine) and control groups. Between 10 and 13 weeks of age BP was measured and the experimental group was given rodent chow (Lab Diet 5P00 Prolab RMH 3000) containing clonidine (clonidine hydrochloride tablets, Rugby Laboratories Inc., Rockville Center, NJ). Ten micrograms of clonidine was used per twenty grams of rodent chow consumed per day. Clonidine powder was mixed with powdered chow and combined with water. At 13 weeks, the rats were terminated with sodium pentothal (20-40 mg/kg) and brainstem, adrenal glands and kidneys were removed, placed in foil previously labeled, immediately frozen on dry ice and stored at -70°C until the RNA isolation. Blood samples to be analyzed for catecholamines, were collected by retro orbital method before termination.

RNA isolation

Total RNA from adrenal glands, brainstem and kidneys of each female rat was extracted using RNA Stat-60 reagent (Friendswood, TX). The procedure requires 0.1 grams of tissue which was homogenized with a Polytron homogenizer (Kinematica GmbH, Brinkann Instruments, Westbury, NY) in 1 ml of Stat-60 solution. The homogenate was incubated at room temperature for 5 minutes and then placed on ice. Two hundred µl of chloroform was added to each tube and shaken for 30 seconds. The tubes were placed at room temperature for approximately 3 min. The mixture was then transferred to 2 ml tubes centrifuged at 12,000 g at 4 °C for 15 minutes. The clear supernatant was transferred into a sterile tube and 500 µl of isopropanol was added. The
tubes were maintained at 4°C for 30 minutes in a cold room on ice and after that were centrifuged at 12,000 g for 15 minutes at 4°C. The supernatant was poured off, the pellet was washed with 1 ml of 75 % ethanol and vortexed gently. After this, the tubes were centrifuged at 9,500 g for 5 minutes at 4 °C and the supernatant was discarded. The pellet was dried at room temperature by inverting the tubes for 5 minutes and a sterile Q-tip was used to dry the walls of the tubes. Finally 40-65 µl (depending upon the pellet size) of sterile water was added to dissolve the pellet. The RNA isolated was kept at -70°C until quantification and use.

RNA quantification

Concentrations of RNA were determined by making a 1/100 dilution, 5µl of RNA with 495 µl of sterile water, and measured by spectrophotometry at 260 nm (Spectronic Genesys 5, Spectronic Instruments, Milton Roy Company). RNA concentrations were calculated using the following conversion factor: 260 nm reading x 40 x 100 x 1/1000= µg/µl. For RNA, 40 µg/ml=1A_{260}.

DNase treatment

Samples were treated with Turbo DNA-free reagent (Ambion, Austin TX) to remove contaminating genomic DNA from RNA preparations. RNA was used at a concentration of 0.2 µg/µl in a volume of 44 µl (RNA + H2O) in each tube. The following formulas were used:

Initial RNA concentration/0.2 µg/µl=dilution

44 / dilution= amount of RNA taken from each tube

In a DNase/RNase-free tube the correct amount of RNA previously calculated, H2O (Molecular Grade Water, Mediatech Inc), 5 µl of 10X DNase buffer and 1µl of enzyme
(2 U/µl) were added, mixed and centrifuged. Then the mixture was incubated at 37 °C for 30 minutes and briefly centrifuged. Five µl of DNase inactivating solution was added to the tubes, mixed and incubated for 2 minutes at 25 °C. The mixture was centrifuged for 90 seconds at 10,000 rpm. The supernatant was carefully removed without touching the pellet and placed in new DNase/RNase free tube. The final concentration of DNased RNA was calculated following this formula: 0.2 µg/µl x 44/ 55(final volume in each tube)= 0.160 µg/µl.

cDNA

After samples were free of DNA, cDNA was synthesized from RNA using ArrayScript Reverse Transcriptase™ (Ambion, Austin TX). The appropriate amount of DNased RNA required for 1µg of RNA in the reaction was calculated: 1µg/0.160µg/µl. The following procedure was carried out under a laminar flow hood (Nuaire Biological Safety Cabinets Class II type A/B3, Plymouth, MN). The correct amount of DNased RNA previously calculated was taken from each tube, the appropriate amount of H2O (Molecular Grade Water, Mediatech, Inc) was added up to of 11 µl and 1µl of random nonamers (50µM) (Sigma-Aldrich, St.Louis Missouri). The mixture was centrifuged and incubated at 70ºC for 10 minutes. A master mix was prepared during this time: 2µl of 10X RT buffer (500 mM Tris pH 8.3, 750 mM KCl, 30 mM MgCl2, 50 mM DTT), 4µl of dNTP (2.5mM), 1µl of SUPERase·In™RNase inhibitor (20U/µl) (Ambion, Austin TX) and 1µl of ArrayScript RT enzyme (200 U/µl). Eight µl of this RT mix was added to each tube, mixed and centrifuged briefly. Then incubation at 25ºC for 15 minutes was carried out under the laminar flow hood. After that, the samples were incubated at 44ºC for 1 hour. In order to heat inactivate the RT enzyme, the samples were incubated at 95ºC for 5
minutes in a thermocycler (PTC-100 Programmable Thermal Controller, MJ Research, Inc). Finally, 30 µl of H2O (Molecular Grade Water, Mediatech, Inc) was added to each tube to get the final volume to 50µl (5µl=100ng of cDNA). The negative RT tubes (-RT tubes) were prepared by taking half the amount of DNased RNA used for the RT tubes and the appropriate amount of H2O (Molecular Grade Water, Mediatech, Inc) to a total volume of 25µl (5µl=100 ng). The samples were stored at -70ºC until the real time PCR procedure.

**Real-Time PCR**

In order to determine relative expression levels of *Th* gene, real-time PCR reactions were performed using cDNA templates previously synthesized and the SYBRgreen PCR Master Mix (Applied Biosystem, Foster City, CA). Two different primers sets, S26 (normalizer) and *Th*, were used. The *Th* primer sequences were: THRTL 5’ GCA TTC ACC TGA GCC GGA 3’ and THRTR 5’ATG TGC GGT CAG CCA ACA T 3’, designed according to the Primer Express Software Program v 1.5 (ABI). These primers amplify a 67bp amplicon in the cDNA. The THRTL primer is complementary to (hybridizes to) exon 8, bp 4737 to 4755. The THRTR primer is complementary to (hybridizes to) exon 9, bp 4925 to 4944 in the rat *Th* transcript. The following procedure was performed under the laminar flow hood. To set up the reaction, master mixes for each set of primers were prepared. The amount of each reagent, to get the final volume of 20µl per sample, was as follows: 12.5 µl of SYBgreen PCR Master Mix (Applied Biosystem, Foster City, CA), 0.75µl (10µM) of Left (L) and 0.75µl (10µM) of Right (R) primers and 6µl of H2O (Molecular Grade Water, Mediatech, Inc.). The real-time reaction was carried out in a 96 well optical reaction plate (Applied Biosystem, Foster City, CA).
The cDNA samples were first centrifuged. Five µl of each was added to a well plus 20 µl of master mix previously prepared for each set of primers (S26 and Th). Each sample was run in triplicate. Five µl of No-RT sample was added to the appropriate well plus 20 µl of Th master mix for each sample in order to determine any genomic DNA contamination. Additionally, no template controls (NTC) were run for each set of primers, using H2O and Th and S26 master mix to check for DNA contamination. The plate was covered with optical adhesive cover (Starter Kit, Applied Biosystem, Foster City, CA) and centrifuged (Kendro Laboratory Products, Osterode, Germany) at 1,000 rpm for 10 sec. The plate was run in ABI PRISM 7700 Sequence Detector System (Applied Biosystem, Foster City, CA). The machine is located in Dr. Walter E. Horton’s lab at NEOUCOM. The real time PCR was done as follows: initial denaturation at 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds for denaturation and 60°C for 1 minute for annealing and extension. The last stage is a slow ramp that goes from 60 °C to 95 °C, takes about 20 minutes. This is the stage where data is collected to get the dissociation curve. The fluorescence emitted during the reaction was an indicator of amplicon production during each PCR cycle. The signal increases in direct proportion to the amount of PCR product in the reaction which is directly related to the initial amount of target mRNA. We set a threshold line in the exponential phase, above the background fluorescence where the samples had a linear curve. The samples were expressed in C_{T} (threshold cycle) values, which represents the PCR cycle at which the fluorescence generated by the amplicon crosses the threshold. Relative quantitation of gene expression was calculated using the comparative C_{T} method (\Delta\Delta C_{T} method)
Catecholamine Measures

Plasma levels of NE and E were measured by High Performance Liquid Chromatography (HPLC) with electrochemical detection (Waters 1515 Isocratic Pump, Waters 717 plus autosampler and the Waters 2465 electrochemical detector, Waters Corporation, Mildford, MA). The pump flow was 1.4 ml/min, voltage setting of + .65V. The column used was 4.6 x 150 mm Symmetry C-18 5µm reverse phase (Waters Corporation, Mildford, MA) and the pre-column was the Supelguard LC-18 2cm (Supleco Co, Bellefonte, PA). The column temperature was maintained at 32°C. The mobile phase consisted of 0.35 M citric acid monohydrate, 0.09 M sodium acetate trihydrate, 130 µM EDTA disodium salt, 460 µM octyl sodium sulfate and 10% methanol, pH 4.7. Briefly, a small amount of alumina (25mg) was placed in a 12x75 mm glass tube and 1.0 ml of Tris Buffer (1M Tris-EDTA, pH 8.6) was added. Next, catecholamine standard ranging 100 pg to 1600 pg, catecholamine controls (Lyphocheck Endocrine Controls, Biorad, Hercules, CA) or unknown were pipetted into each tube, followed by 100 µl 2400 pg of the internal standard (3,4 dyhydroxybenzylamine). The tubes were vortexed vigorously for 10 minutes after which the supernatant was removed. The tubes were washed 3 times with dH2O, vortexed 4 minutes and the supernatant removed after each wash. In the final step 600 µl of 100 mM perchloric acid was added and the tubes were vortexed for 5 minutes. The last supernatant was removed carefully without disturbing the alumina and placed into the appropriate autosampler vials. The chromatography peaks were identified by comparing their retention times to those of the known standards. The calculations were done by comparison of the unknown peak high
ratios with the external standard peak height and calculating it from the known standard/external standard peak height ratio. The values were in pg/ml.

Statistical Analysis

Data were analyzed using the MINITAB statistic program (14 Release). A two way analysis of variance (2 way ANOVA) was used to analyze NE, E and Th levels, using factors of strain and treatment. After ANOVA, Tukey Pairwise Comparison was performed. To analyze the results of BP, Analysis of Covariance (ANCOVA) was used. After ANCOVA Tukey Pairwise Comparison was performed. To compare the level of expression of Th gene in brainstem and adrenal gland three way analysis of variance (3 way ANOVA) was used, using factors of treatment, tissue and strain. After ANOVA Tukey Pairwise Comparison was performed. Pearson’s correlation was used to determine if the Th gene level of expression, BP and NE levels were related. Significance was assumed if P≤ 0.05.
CHAPTER IV

RESULTS

The data will be presented in three different sets from animals collected at two different times due to the absence of the author in the lab for a period of time. Group #1 was done in 2002 and BP, NE, E and Th gene levels in adrenal gland were measured. The SHR/y rats were at F_{18}. Group #2 was done in 2004 and NE, E and Th gene levels in brainstem were measured under clonidine treatment. The SHR/y rats were at F_{20}. Group #3 was done in 2004 and NE, E and Th gene levels in brainstem were measured without treatment (control for group #2), these samples were from another project in the lab. All animals were at 13 weeks of age.

Table 1 and Figure 1 show the effect of clonidine on blood pressure in WKY and SHR/y female rats in group #1. There was overall a significant difference between treated and control groups (P \leq 0.001). There was not a significant difference between strains, and both strains responded similarly to clonidine treatment. Clonidine does not affect BP in one strain more than another. When comparing control and treated groups in SHR/y and WKY, there was significant difference (P \leq 0.05). No significant difference was observed when comparing control groups or treated groups in both strains.

The effect of clonidine on NE levels is illustrated in table 2 and figure 2 for group #1. Even though there was not a statistically significant effect of clonidine in control and treated groups, there was an overall significant strain difference (P \leq 0.025). Clonidine
treatment does not affect NE levels in one strain more than another. When comparing control and treated groups of SHR/y and WKY, there was no statistically significant difference. Unexpected results were seen, levels of NE were higher in treated groups compared with controls groups. Also when comparing controls or treated groups in both strains, no statistically significant difference was observed.

Table 3 and figure 3 show the effect of clonidine on E levels in group #1. Overall there was not a significant difference between strains or between treated and control groups. Clonidine does not affect E levels in one strain more than another. When comparing treated and control groups in both strains there was no significant difference and also when comparing controls groups or treated groups. Again unexpected results were seen. Levels of E were higher in SHR/y treated group compared with control groups.

Correlation analysis indicated that levels of NE and E are not significantly correlated with BP ($P \leq 0.455$, $r = -0.201$). Our data showed that while BP decreased with clonidine treatment, NE and E levels increased in group #1.

The effect of clonidine on NE levels in group #2 and #3 is shown in table 4 and figure 4. Even though clonidine lowered NE levels in treated groups, there was not a significant difference between strains or between treated and control groups. Clonidine had the same effect in both strains. Control and treated groups did not show significant differences. When comparing control or treated groups, in both strains, there was no statistically significant difference.

Table 5 and Figure 5 show the effect of clonidine on E levels in group #2 and #3. Overall there was not a significant difference between strains or between treated and
control groups. Clonidine does not affect E levels in one strain more than another. When comparing treated and control groups in both strains there was no significant difference and also when comparing controls groups or treated groups. Unexpected results were seen, levels of E were higher in treated groups compared with control groups.
Table 1: Systolic Blood Pressure in WKY and SHR/y Female Rats. Group #1. Adrenal Gland.

Systolic Blood Pressure (mmHg)

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR/y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>122.1 ± 3.3 (n=3)</td>
<td>123.0 ± 3.0 (n=4)</td>
</tr>
<tr>
<td>Treatment (Clonidine)</td>
<td>108.2 ± 2.9 (n=4)</td>
<td>111.0 ± 2.5 (n=5)</td>
</tr>
</tbody>
</table>

BP values expressed as mean ± standard error
Figure 1: Effect of Clonidine on Blood Pressure in WKY and SHR/y Female Rats.

Group #1. Adrenal Gland.

* P ≤ 0.05 compared to treated group.
Table 2: Norepinephrine Levels in WKY and SHR/y Female Rats. Group #1. Adrenal Gland.

Norepinephrine Levels (pg/ml)

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR/y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>512.0 ± 138.5 (n=3)</td>
<td>755.5 ± 119.9 (n=4)</td>
</tr>
<tr>
<td>Treatment (Clonidine)</td>
<td>594.0 ± 119.9 (n=4)</td>
<td>973.6 ± 107.3 (n=5)</td>
</tr>
</tbody>
</table>

NE values are expressed as mean ± standard error
Effect of Clonidine on Norepinephrine Levels

Figure 2: Effect of Clonidine on NE in WKY and SHR/y Female Rats. Group #1.

Adrenal Gland.
Table 3: Epinephrine Levels in WKY and SHR/y Female Rats. Group #1. Adrenal Gland.

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR/y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>99.3 ± 42.3 (n=3)</td>
<td>128.0 ± 36.6 (n=4)</td>
</tr>
<tr>
<td>Treatment (Clonidine)</td>
<td>84.0 ± 36.6 (n=4)</td>
<td>193.2 ± 32.7 (n=5)</td>
</tr>
</tbody>
</table>

E values are expressed as mean ± standard error
Figure 3: Effect of Clonidine on E in WKY and SHR/y Female Rats. Group #1.

Adrenal Gland.
Table 4: Norepinephrine Levels in WKY and SHR/y Female Rats. Group #2 and #3. Brainstem.

Norepinephrine Levels (pg/ml)

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR/y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>379.5 ± 81.6 (n=2)</td>
<td>312.5 ± 47.1 (n=6)</td>
</tr>
<tr>
<td>Treatment (Clonidine)</td>
<td>276.0 ± 66.7 (n=3)</td>
<td>178.2 ± 47.1 (n=6)</td>
</tr>
</tbody>
</table>

NE values are expressed as mean ± standard error
Effect of Clonidine on Norepinephrine Levels

Figure 4: Effect of Clonidine on NE in WKY and SHR/y Female Rats. Group #2 and # 3.

Brainstem.
Table 5: Epinephrine Levels in WKY and SHR/y Female Rats. Group #2 and #3. Brainstem.

<table>
<thead>
<tr>
<th>Epinephrine Levels (pg/ml)</th>
<th>WKY</th>
<th>SHR/y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>74.0 ± 311.4(n=2)</td>
<td>346.5 ± 179.8(n=6)</td>
</tr>
<tr>
<td>Treatment (Clonidine)</td>
<td>352.0 ± 254.2 (n=3)</td>
<td>468.0 ± 179.8 (n=6)</td>
</tr>
</tbody>
</table>

E values are expressed as mean ± standard error
Figure 5: Effect of Clonidine on Epinephrine Levels in WKY and SHR/y Female Rats. Group #2 and #3.

Brainstem.
Table 6, figure 6 and figure 7 show relative quantitative expression of the *Th* gene in WKY and SHR/y female rats in the brainstem in group #2 and #3. Overall there was no significant difference between strains or between treated and control groups. Clonidine does not affect *Th* levels in one strain more than another. When comparing treated and control groups in both strains there was no significant difference and also when comparing controls groups or treated groups.

Relative quantitative expression of *Th* gene in WKY and SHR/y female rats in the adrenal gland in group #1 is illustrated in table 7, figure 8 and figure 9. Overall there was not a significant difference between strains or between treated and control groups. The effect of clonidine in both strains is the same. When comparing treated and control groups in both strains there was no significant difference and also when comparing controls or treated groups.

Correlation analysis showed that *Th* gene expression in adrenal gland is not significantly correlated with levels of NE (P ≤ 0.754, r = 0.085), E levels (P ≤ 0.725, r = -0.095) or BP (P ≤ 0.465, r = -0.197) in group #1.

Table 8 and figure 10 show the relative quantitative expression of *Th* in WKY female rats in brainstem and adrenal gland. There was significant difference between tissues (P ≤ 0.001). There was no statistically difference between control and treated groups and also between strains. Clonidine does not affect tissues and strains.

Relative quantitative expression of *Th* in SHR/y female rats in brainstem and adrenal gland is shown in table 9 and figure 11. There was significant difference between tissues (P ≤ 0.001), whereas no statistically difference between control and treated groups or between strains was observed.
Table #10 shows body weight in group #1 of rats. There was not significant difference between strains. There was a statistically significant difference between control and treated groups (P ≤ 0.05). Clonidine affects body weight. Even though there was a fluctuation in body weight through the time, all rats gained weight at the end of 13 weeks.
Table 6: Relative Quantitative Expression of *Th* in WKY and SHR/y Female Rats in Brainstem. Group #2 and #3

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR/y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4± 0.4  (n=3)</td>
<td>7.9± 0.3  (n=6)</td>
</tr>
<tr>
<td>Clonidine</td>
<td>8.4± 0.4  (n=3)</td>
<td>7.7± 0.3  (n=6)</td>
</tr>
</tbody>
</table>

Sample Cycle Threshold (ΔCT) values for *Th* normalized to the invariant S26 expressed as mean ΔCT ± SE.
Relative Quantitative Expression of $Th$ in WKY and SHR/y Female Rats in Brainstem

Figure 6: Relative Quantitative Expression of $Th$ in WKY and SHR/y Female Rats in Brainstem. Group #2 and #3
Relative Quantitative Levels of Expression of $Th$ in WKY and SHR/y Control and Clonidine Treated Female Rats in Brainstem

Figure 7: Relative Quantitative Levels of Expression of $Th$ in WKY and SHR/y Control and Clonidine Treated Female Rats in Brainstem. Group #2 and #3
Table 7: Relative Quantitative Expression of *Th* in WKY and SHR/y Female Rats in Adrenal Gland. Group #1

<table>
<thead>
<tr>
<th></th>
<th>WKY</th>
<th>SHR/y</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.7± 0.5 (n=3)</td>
<td>2.2± 0.4 (n=4)</td>
</tr>
<tr>
<td>Clonidine</td>
<td>2.3± 0.4 (n=4)</td>
<td>2.4± 0.3 (n=5)</td>
</tr>
</tbody>
</table>

Sample Cycle Threshold (ΔC_T) values for *Th* normalized to the invariant S26 expressed as mean ΔC_T± Standard Error.
Figure 8: Relative Quantitative Expression of \( Th \) in WKY and SHR/y Female Rats in Adrenal Gland. Group #1.
Figure 9: Relative Quantitative Expression of $Th$ in WKY and SHR/y control and clonidine treated Female Rats in Adrenal Gland. Group #1
Table 8: Relative Quantitative Expression of *Th* in WKY Female Rats in Brainstem and Adrenal Gland.

<table>
<thead>
<tr>
<th></th>
<th>WKY Brainstem</th>
<th>WKY Adrenal Gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.4± 0.4 (n=3)</td>
<td>2.7± 0.5 (n=3)</td>
</tr>
<tr>
<td>Clonidine</td>
<td>8.4± 0.4 (n=3)</td>
<td>2.3± 0.4 (n=4)</td>
</tr>
</tbody>
</table>

Sample Cycle Threshold (ΔC_T) values for *Th* normalized to the invariant S26 expressed as mean ΔC_T± Standard Error.
Relative Quantitative Expression of $Th$ in WKY Female Rats in Brainstem and Adrenal Gland

![Bar chart showing the relative quantitative expression of $Th$ in WKY female rats in brainstem and adrenal gland.]

Figure 10: Relative Quantitative Expression of $Th$ in WKY Female Rats in Brainstem and Adrenal Gland.

*** $P \leq 0.001$ compared with brainstem.
Table 9: Relative Quantitative Expression of *Th* in SHR/y Female Rats in Brainstem and Adrenal Glands.

<table>
<thead>
<tr>
<th></th>
<th>SHR/y Brainstem</th>
<th>SHR/y Adrenal Gland</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.9± 0.3 (n=6)</td>
<td>2.2± 0.4 (n=4)</td>
</tr>
<tr>
<td>Clonidine</td>
<td>7.7± 0.3 (n=6)</td>
<td>2.4± 0.3 (n=5)</td>
</tr>
</tbody>
</table>

Sample Cycle Threshold (ΔC<sub>T</sub>) values for *Th* normalized to the invariant S26 expressed as mean ΔC<sub>T</sub>± Standard Error.
**Figure 11:** Relative Quantitative Expression of *Th* in SHR/y Female Rats in Brainstem and Adrenal Gland.

*** P ≤ 0.001 compared with brainstem
Table 10: Body Weight in Group #1

<table>
<thead>
<tr>
<th>Strain</th>
<th>Group</th>
<th>9 (Before Clonidine)</th>
<th>10 (After Clonidine)</th>
<th>11</th>
<th>12</th>
<th>13</th>
</tr>
</thead>
<tbody>
<tr>
<td>SHR/y</td>
<td>Clonidine</td>
<td>152±4.6</td>
<td>166±5.6</td>
<td>162±2.9</td>
<td>167±3.0</td>
<td>168±2.6</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>149±2.3</td>
<td>157±1.7</td>
<td>172±1.4</td>
<td>181±1.2</td>
<td>188±1.7</td>
</tr>
<tr>
<td>WKY</td>
<td>Clonidine</td>
<td>150±2.4</td>
<td>164±3.5</td>
<td>159±3.2</td>
<td>165±1.9</td>
<td>168±2.9</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>157±9.2</td>
<td>168±5.6</td>
<td>174±6.6</td>
<td>180±7.6</td>
<td>189±5.1</td>
</tr>
</tbody>
</table>

BW values are expressed as mean ± standard error
CHAPTER V

DISCUSSION

The main objectives of this study were 1) establish basal levels of Th mRNA in SHR/y and WKY female rats in brainstem and adrenal gland, 2) determine whether the expression of Th gene is reflected in levels of NE and BP, 3) determine whether SHR/y and WKY female rats respond differently to clonidine treatment and 4) establish whether clonidine regulates or changes Th gene expression in SHR/y and WKY female rats.

When comparing the level of expression of Th gene in the brainstem of both strains of rats, in group #2 and #3, our results showed no statistically significant difference between them. However, Kumai et.al (38) and Reja et.al (61), showed higher levels of Th mRNA in the medulla oblongata of the brainstem in SHR compared with WKY male rats. In our study, the level of expression of the Th gene in the adrenal glands showed no statistically significant difference between strains, although elevated levels of Th mRNA in SHR compared to WKY have been reported by Kumai et.al (39). The expression of Th gene would be determined in part by the genetic origin of the autosome, and the Th gene has been mapped in chromosome 1. Although female SHR/y and WKY have the same genetic background, the regulation and the expression of the gene can still be different. A mechanism that could explain differences in expression of the same gene is
genetic imprinting where a gene’s expression depends on the parent that transmits it (24). During gametogenesis, an allele (either from the father or from the mother) is marked and it is destined to be inactive or repressed. Thus, when the maternal allele is expressed it is because the paternal allele is silenced or vice versa. This inactivated allele is called “imprinted” (59). On the other hand, the studies mentioned above were conducted in SHR and WKY males, whereas our study was conducted in females SHR/y and WKY. Thus, gender difference must be taken into account. In the regulation of $Th$ gene, testes determining factor Sry encoded by the Sry locus on the $Y$ chromosome, has been shown to increase $Th$ promoter activity (50). Sry acts as a transcription factor, which regulates a gene that is crucial for catecholamine biosynthesis and was found to be expressed in catecholaminergic regions in male rats but not in female rats (50).

Clonidine is an agent usually used to lower BP, but recently has been used to treat some types of neuropathic pain, opioid detoxification, and attention-deficit hyperactivity disorder (ADHD) (74). Clonidine is an antihypertensive drug considered an alpha 2 agonist. It binds and activates $\alpha_2$ adrenoceptors contributing to decreased levels of NE and lower BP due to negative feedback at the presynaptic neuron. This drug reduces sympathetic activity or tone causing vasodilatation and reduction in heart rate (74, 78). Clonidine also binds to imidazoline receptors I$_1$ resulting in a decrease in BP (26). One of the purposes of using clonidine in this study was to determine its effect on levels of $Th$ mRNA. When we tested the effect of clonidine on the expression of the $Th$ gene in the brainstems and adrenal glands of both WKY and SHR/y rats, we found that clonidine does not affect $Th$ transcript levels. There was no statistically significant difference in $Th$ gene levels between treated and control groups or between strains. However, in all
groups tested, significantly higher levels of *Th* gene expression were found in adrenal gland tissue as opposed to brainstem tissue. In group #1 rats, where the levels of *Th* in adrenal gland were measured, we saw higher levels of NE in the treated group compared with the control group, although we expected to see lower levels of *Th* gene in the treated groups because NE may act as a feedback inhibitor for TH enzyme (67). However, the level of expression of the *Th* gene was not affected. In previous studies, it has been shown that changes in levels of mRNA do not always affect levels of TH protein, TH activity or catecholamine function, suggesting multiple mechanisms affecting *Th* regulation (71). Some studies have shown that there is dissociation between *Th* mRNA levels and expression of the enzyme (30). In group #2 and #3 of rats, we saw lower levels of NE in treated groups, whereas the levels of *Th* gene in brainstem in these groups of rats showed no difference. There was a dissociation between *Th* mRNA and catecholamine levels and, as was mentioned above, it has been reported there can be a dissociation between *Th* mRNA levels and expression of the enzyme (30). Additionally, chronic administration of clonidine has been demonstrated not to decrease levels of tyrosine hydroxylase levels in the LC (56). The activation of the \( \alpha_2 \) adrenergic receptors by clonidine does not lead to substantial inhibition of LC neuronal activity nor does it decrease tyrosine hydroxylase expression (56). In our study, we also saw that clonidine does not affect one strain differently than another. SHR/y and WKY rats both respond in the same way to clonidine treatment. This result could be due to the genetic similarity between strains. Recall that SHR/y rats have the same autosomes and one X chromosome from WKY mother and only one X chromosome from SHR/y father.
As we know, the LC is a nucleus in the brainstem responsible for responses to stress and panic. It is located in the dorsal wall of the upper pons, under the cerebellum in the caudal midbrain and it is the major noradrenergic nucleus in the brain from which NE is released by the sympathetic nerves (74). In our study, we saw that the relative quantitative expression of $Th$ transcripts was as follows: WKY controls $8.4 \Delta C_T$ vs. WKY treated clonidine $8.4 \Delta C_T$. SHR/y controls $7.9 \Delta C_T$ vs. SHR/y treated clonidine $7.7 \Delta C_T$.

In our study, the expression of the $Th$ gene in adrenal glands was also tested. The adrenal glands are endocrine glands located at the top of the kidneys. They are responsible for regulating the stress response through the synthesis of cortisol and catecholamines. The adrenal medulla, located at the center of the gland, is the body's main source of the catecholamines E and NE (74). In our study, we saw that the relative quantitative expression of $Th$ gene in the adrenal glands of WKY controls was $2.7 \Delta C_T$ vs WKY treated with clonidine of $2.3 \Delta C_T$. In SHR/y controls the level was $2.2 \Delta C_T$ vs SHR/y treated with clonidine of $2.4 \Delta C_T$. Comparing both tissues, the level of expression of $Th$ mRNA in adrenal gland was significantly higher than in brainstem ($P \leq 0.001$) in both strains.

Clonidine decreases sympathetic activity or tone, activating the $\alpha_2$ adrenoceptors and imidazoline receptors thus decreasing the levels of NE that contribute to decrease BP. As expected, clonidine treatment lowered BP, but no significant differences were seen between strains. Animals under clonidine treatment showed significantly lower BP than controls in both strains. WKY treated group showed 108.2 vs. control 122.1 mmHg ($P \leq 0.05$) whereas SHR/y treated group 111.0 vs. control 123.0 mmHg ($P \leq 0.05$).
Additionally, in our study it was found that clonidine did not affect BP in one strain more
than another because no significant difference was observed when comparing control
groups or treated groups in both strains. This is in agreement with Miller (48) when
SHR/y control rats fed normal food had higher BP than SHR/y control rats fed clonidine
(144 vs. 126 mmHg). Likewise, WKY control rats fed normal food had higher BP than
WKY control rats fed clonidine (145 vs. 118 mmHg).

It has been shown by Miller (48) that clonidine decreased levels of NE in SHR/y
female rats. That study showed that SHR/y fed normal food had higher NE levels than
animals fed clonidine (2907 vs. 1465 vs. pg/ml). However, WKY fed normal food
showed lower levels of NE than animals fed clonidine (1411 vs 1645 pg/ml). In our
study, NE and E levels were not as expected in the group #1 of rats. Clonidine treated
group in SHR/y showed higher levels of NE than control animals (973 vs. 755 pg/ml) and
higher levels of E than the control group (193 vs. 128 pg/ml). WKY clonidine treated
group also had higher levels of NE than the control group (594 vs. 512 pg/ml), whereas
the levels of E in control group were higher than the clonidine treated group (99 vs. 84
pg/ml). One possible explanation for high levels of NE or E after clonidine treatment is
the presence of pheochromocytoma, but this is unlikely to happen in all rats. The most
likely possibility that could explain this result is that the rats may have been sick by the
time the blood samples were collected, thus affecting the levels of NE and E. However
when we looked at the body weight of the rats (Table #10), it seems that the rats were in
good condition. When we dissected the animals to remove the organs, we looked at the
organ’s condition in order to see if there were any abnormalities. We saw that SHR/y rat
#5 (clonidine treated) and WKY rat #11 and #12 (clonidine treated) had ovarian cysts
(fluid and blood in their ovaries). It has been demonstrated that an increase in NE release exists in rats with polycystic ovary (12). In the group #2 and #3 of rats, we saw that clonidine lowered NE levels in WKY and SHR/y. The clonidine treated group of SHR/y showed lower levels of NE than control group (178 vs. 312 pg/ml). Also the WKY treated group had lower levels of NE than control group (276 vs. 379 pg/ml). The results in SHR/y are in agreement with Miller (48), where animals fed clonidine had lower levels of NE than animals fed normal food (1465 vs. 2907 pg/ml). However, in Miller study WKY fed normal food had lower levels of NE than WKY fed clonidine (1411 vs. 1645 pg/ml). Our results also showed an unexpected result in E levels where clonidine groups in both strains had higher levels than control group. The SHR/y treated group had higher levels of E than control group (468 vs. 346 pg/ml). The WKY treated group also showed higher levels of E than control group (352 vs. 74 pg/ml). It may be possible that the low number of rats in WKY (n=2 in control group and n=3 in treated group) did not provide a large enough sample size and this may have affected the results. Thus, the reason for having high levels of NE or E after clonidine treatment in our study remains elusive.

When we tried to relate the level of expression of the \( Th \) gene with BP and levels of NE, we saw that these three parameters were not related. In group #1 of rats, we saw that while the \( Th \) gene expression in adrenal gland showed no statistically significant difference between strains or between control or treated groups (SHR/y control 2.2 \( \Delta C_T \) vs. SHR/y treated 2.4 \( \Delta C_T \) and WKY control 2.7 \( \Delta C_T \) vs. WKY treated 2.3 \( \Delta C_T \)), BP was significantly lower in treated group compared with control group (SHR/y control 123 vs. SHR/y treated 111 mmHg and WKY control 122.1 vs. WKY treated 108.2 mmHg). Levels of NE were higher in treated groups compared with control group (SHR/y control
However, Kumai et al. (39), showed that the Th mRNA expression in adrenal glands of SHR rats were much higher than WKY rats. In addition, this result was accompanied by higher Th activities, higher NE and E levels, as well as higher BP. This study suggested that the hypertension in SHR could be related to the high activity of Th due to high levels of Th mRNA, which, in turn, elevates NE and E levels in adrenal medulla. Kumai et al. (38) also showed increased Th mRNA in medulla oblongata of SHR compared with WKY. These results were accompanied with higher Th activities, NE, E levels and BP in SHR. The author again suggested that the hypertension of SHR might be related to the high activity of Th due to the high level of Th mRNA that increases NE levels in the medulla oblongata. In our groups # 2 and # 3 of rats where we measured levels of expression of the Th gene in brainstem and NE levels, we did not see that these two parameters were related. This difference could be because we did not dissect the specific area of LC, we used the whole brainstem where the LC is located and the Th mRNA could be diluted in the tissue. Levels of expression of the Th gene did not show statistically significant differences between strains or between control and treated group. Thus, SHR/y control showed 7.9 ΔC_T vs. SHR/y treated clonidine 7.7 ΔC_T and WKY control showed 8.4 ΔC_T vs. WKY treated 8.4 ΔC_T. Levels of NE were not statistically significant different between strains or between control and treated groups, although levels of NE in treated groups where lower than control groups. SHR/y control showed 312.5 vs. SHR/y treated 178.2 pg/ml and WKY control showed 379.5 vs. WKY treated 276.0 pg/ml). The studies cited above were done in SHR and WKY male rats and our study was conducted in females SHR/y and WKY rats. Thus, the gender difference could
be one possible explanation that may be taken into account to analyze the discrepancies between the results. Hypertension has been demonstrated to be a sex related disease where the Y chromosome has been shown to play an important role in its development (15). In the regulation of Th gene, testes determining factor Sry encoded by the Sry locus on the Y chromosome, has been shown to increase Th promoter activity. Sry acts as a transcription factor which regulates a gene that is crucial for catecholamine biosynthesis and was found to be expressed in catecholaminergic regions in male but not in females rats (50). On the other hand, female hormones such as estrogen have a protective function on BP. Estrogen stimulates endothelial cell growth, inhibit smooth muscle proliferation and stimulate vascular relaxation (33). In addition, estrogen can activate RAAS by enhancing angiotensinogen synthesis and reducing ACE activity. This leads to a decrease in the conversion of angiotensin I to angiotensin II decreasing vasoconstriction, increasing vasodilation and consequently lowering BP (6).

In our study, we analyzed the expression of the Th gene that encodes Th, the first and rate-limiting enzyme in catecholamines pathway that convert the amino acid tyrosine to DOPA. It could be very interesting to analyze the expression of other genes in the catecholamines cascade such as DOPA decarboxylase gene that encode the enzyme DOPA decarboxylase and convert DOPA in Dopamine or DBH gene that encode the enzyme dopamine β hydroxylase and convert dopamine to NE. These two genes may play a role in the neurogenic development of hypertension because they are involved in the synthesis of NE.
REFERENCES

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4. www.bartleby.com/65/no/norepine.html


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71. Tank A, Corella P and Ham L. Induction of mRNA for tyrosine hydroxylase by cAMP and glucocorticoids in a rat pheochromocytoma cell line: evidence for the regulation of tyrosine hydroxylase synthesis by multiple mechanisms in cells exposed to elevated levels of both inducing agents. *Molecular Pharmacology* 1986; 30: 497-503.


78. Ye R. Pharmacology of antihypertensive agents. UIC Department of Pharmacology. Pag.1-6

February 7, 2002

Dr. Amy Milsted
Department of Biology
The University of Akron
Akron, OH 44325

Dear Dr. Milsted:

On February 7, 2002 the Institutional Animal Care and Use Committee reviewed your protocol titled:

“Tyrosine Hydroxylase and Hypertension in Females”
IACUC # 02-02B
100 rats Faculty/Graduate Research 7/1/02–6/30/04

Your project has received unanimous approval, pending modification of 18D and 22B. For 18D please indicate criteria for administration of post-operative analgesics. The committee recommends that all animals receive a post-operative analgesic; please consult with Dr. Gary Riggs for the dosage and type. For 22B please indicate criteria e.g., significant pain, that will be used for euthanasia of an experimental animal.

You must provide the committee with documentation of updated risk information, or serious adverse reactions that occur during the course of this project. Please use the IACUC number when submitting this information to the committee.

Sincerely,

James Holda
Acting IACUC Chair

The University of Akron is an Equal Education and Employment Institution
The University of Akron
Institutional Animal Care and Use Committee

Continuation, Progress and/or Protocol Change Report

Adina Brett
Principal Investigator: Amy Hilsed
Department: Biology
Phone Extension: 7976
E mail address: anelmatton@acdl.com

IACUC Approval: # 02-02B
Original IACUC Approval date: February 2002
Estimated completion date: August 2003
Updated: 11-06-02

Project or Course Title: "Differences in Tyrosine Hydroxylase Gene Expression in Kidneys and Adrenal Glands of SHR and WKY Female Rats"
Project Status: X repeating course* _ completed
(check one) _ terminated (why)

Please list resulting papers or presentations.
None

Summarize any planned protocol changes:
1. The control group will receive normal diet whereas the other group will receive clomipine 10mg per 200g of rodent chow consumed per day for 3 weeks (10-13 week old age). The number of rats that will receive clomipine will be 20.
2. The anesthesia that will be used is Telazol i.m. 0.03 ml per 100g body weight.

Describe progress since last committee review.
None

Personnel addition:
Adina Brett, US student

PI Signature: Adina Brett
Date: 11-06-02

IACUC Approval Date:
IACUC Chair Signature: Date:

*PI must review original IACUC-approved proposal and related amendments on file in the BRC (originals may be obtained from the BRC x5845).

Animal Care & Use Training Workshop:
Amy Hilsed October 1999
Adina Brett Fall 2001

Revised 1/00
http://bevnet.iac.upenn.edu
1. **Project Director or instructor:** Dr. Milsted  
   **Phone:** 972-7576  
   **Department:** Biology  
   **4 digit mailing #:** #9068

2. List all personnel under your direction involved in the project. Also indicate when and where they attended an Animal Training Workshop. List participation in the University of Akron Occupational Health Program. All project participants with an average of 10 hours a week or more animal contact are required to participate in the Occupational Health Program provided by the University. Persons with fewer than 10 hours a week animal contact may choose to participate. See Biology Resource Center Manager for details.

<table>
<thead>
<tr>
<th>Animal Care &amp; Use Training Workshop</th>
<th>Occupational Health Program</th>
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<tbody>
<tr>
<td>Name</td>
<td>Attended workshop (yes/no)</td>
</tr>
<tr>
<td>Amy Milsted</td>
<td>Y</td>
</tr>
<tr>
<td>Dan Ely</td>
<td>Y</td>
</tr>
<tr>
<td>Gail Dunphy</td>
<td>Y</td>
</tr>
</tbody>
</table>

3. Date proposal submitted to IACUC: 1/30/02
4. Research: [] Teaching: [X]
5. Title of Research Project or course: Tyrosine hydroxylase and hypertension in females
6. Estimated starting date: 07/01/02  
   Estimated completion date: 09/30/04
7. Person providing daily care of animals (if other than Animal Facility staff): [X] Staff_____________________
8. **Species to be used:**
   - A. R. norvegicus
     - Estimated number, sex and strain: 50 female WKY  
     - Age: 13 weeks
   - B. R. norvegicus
     - Estimated number, sex and strain: 50 female SHR/y  
     - Age: 13 weeks
9. **Source of animals:**
   - a. Biology Resource Center: [X] Yes  
     [No]
   - b. If no, please name outside source: __________________________  
     Please note: project approval must be secured prior to purchasing vertebrate animals, and approval # must accompany the purchase order.
10. **Housing**
    - a. Will housing in the Biology Resource Center be required? [X] Yes  
      [No]
    - b. Has space been allocated by Biology Resource Center? [X] Yes  
      [No]
    - c. If so, state room #: B-206
11. Objective: Please provide a brief statement in plain English of overall objective of project.
   We will investigate how differences in methylation of the tyrosine hydroxylase gene affects expression of the gene and hypertension, in female SHR/y versus WKY rats. Three week old rats [30 WKY and 30 SHR/y] will be divided into 3 groups of 10. From each strain, 20 rats will be on a normal rat chow diet [control and immobilization stress groups] and 10 rats will receive clonidine (10 mg clonidine per 20 grams of rodent chow consumed per day), for 10 weeks to block SNS outflow. For immobilization stress each rat is restrained in a hardware cloth (0.054-cm squares) restrainer that is the size of the rat and does not allow the rat to turn around. The one hour stress will be performed on the same day that rats are terminated, at age 13 weeks. This procedure activates SNS activity. We will also administer Ang II for 3 weeks to 10 rats/strain [+10 controls pre strain], by osmotic minipump, to raise BP and tyrosine hydroxylase to hypertensive levels and ask whether this eliminates the strain differences in DNA methylation and gene expression. We are trying to determine the relationship between methylation of DNA and high BP.

12. Rationale: Please include a rationale for involving animals, and for the appropriateness of the species and numbers of animals used.
   Based on our previous studies we will require 8-10 rats per experimental group. Rats are needed since the entire organism is needed for both of the aims.

13. Alternatives to Animals: What alternatives to the use of animals did you consider (i.e. computer simulation, modeling, in vitro testing)? Computer simulation will not be informative and non-animal models have not been developed that can predict the variables of interest.

14. Duplication: Provide assurance that this activity does not unnecessarily duplicate previous experiments. The USDA suggests that this can be done by citing the most similar published work and then: 1) stating that you are duplicating to confirm the earlier data, or 2) describing how your proposed experiment differs from that cited, or 3) that the projects duplicative for instructional purposes.
   This is original research never done before, but both Dr. Elery's lab and mine have administered these drugs in similar experiments in male rats. My proposed experiments here differ from those because I will use females and because I am examining DNA, as well as mRNA and protein expression.

15. Pain Category: Please indicate the category (A, B, or C) of experiment for each species to be used.
   A. Animals upon which teaching, research, surgery or tests are conducted involving no pain or distress and requiring no use of pain-relieving drugs or anesthesia. Species:
   B. Animals upon which teaching, research, surgery or tests are conducted involving pain or distress to the animals and for which an appropriate anesthetic, analgesic or tranquilizing drug is to be used. Species: Rats
   C. Animals upon which teaching, research, surgery or tests are conducted involving pain or distress to the animals and for which the use of an anesthetic, analgesic or tranquilizing drug is not used because it would adversely affect the procedures, results, or interpretation of the teaching, research, experiments, surgery, or tests. An explanation of the procedures producing pain or distress in these animals and the reasons such drugs were not used must be provided:

16. Pain/discomfort: Describe the procedures designed to assure that discomfort and pain to animals will be limited to that which is unavoidable for the conduct of scientifically valuable research, e.g. include use and type of analgesic, anesthetic and tranquilizing drugs.
   Osmotic minipumps will be implanted subcutaneously in the back of the neck, and catheters will be inserted into the femoral artery, under sodium brevital or pentobarbital anesthetic and topical xylocaine applied to the exposed area. The incision is closed. After 3 weeks (osmotic minipumps) or for blood pressure measurements a 3 hr recovery, blood pressure is measured. Then the animal is euthanized with a pentobarbital overdose and tissues harvested.

17. Alternative procedures: If more than momentary or slight pain/distress is caused, (#15, category B, C) you must consider alternative procedures. Please indicate from the list below which sources you consulted.
   Index Medicus _______ Agricultural _______
   Biological Abstracts _______ Medline 1966-present _______
   Animal Welfare Information Center _______ Other (list) _______
   List key words used in the search of alternatives. animal testing alternatives, computer simulation, in vivo testing

18. Surgery: Is any type of surgical procedure to be done? Yes _______ No _______
   (All survival surgery must be performed using aseptic procedures).
   A. Describe the nature of the surgery:
   Osmotic minipumps implanted subcutaneously in back of the neck. Catheter insertion into femoral artery for
   direct blood pressure measurement [note: grant states that tail artery will be used. This was an error in the grant]
   B. Who will do the actual surgical work?
   C. Type of anesthesia (and dosage) to be used: Sodium brevital, 50 mg/kg, will be administered IP for implanting
   minipumps; pentobarbital, 50 mg/kg, for catheter
   Insertions
   D. Provisions for post-operative care (include post operative analgesics, if applicable):
   Animals are on their feet moving in 1 hr and a warm environment, 75-80 F is provided.

19. Will the animal's diet be designed to be nutritionally deficient? Yes _______ No _______

20. Will there be water deprivation? Yes _______ No _______

21. Will the animals be subjected to toxic or hazardous materials? Yes _______ No _______
   A. If yes, please identify materials:
22. Will the animals be euthanized? Yes XX No

A. If yes, describe the method: __Overdose of pentobarbital and then tissues removed.

B. Describe the criteria for euthanasia (e.g., endpoint of experiment, specific time period, tumor size, significant pain or sickness, inability to feed, etc):

Endpoint of experiment which is age [13 weeks].

23. Federal Regulations require that you consult with the facility D.V.M. (Dr. Gary Riggs) or his designee in planning this project. Please confirm that you have done so and name the person consulted:

Dr. Riggs ___________________________ Date: ________________ (Phone # 825-1637)

24. If the project involves other institutions, please list the institution along with the name and phone number of the IACUC Chairperson:

IACUC Chairperson: ________________ Phone Number: ________________

Rev. 1/2000


26. Project classification:

A. XX Faculty Research ______ Department Funded? ______ Department Name ________________

   XX External Funding Agency AHA Ohio Valley Affiliate Application Deadline 1/18/02

B. ___ Graduate Research ______ Faculty Research Application Deadline ____________ Grant #

C. ___ Undergraduate Research

D. ___ Honors Student Project

E. ___ Class Project. Please indicate the course level:

   Undergraduate nonmaj/Introductory major. Yes ___ No ___

   Senior level Undergraduate/Graduate. Yes ___ No ___

F. ___ Instruction Please indicate the course level:

   Undergraduate nonmaj/Introductory major. Yes ___ No ___

   Senior level Undergraduate/Graduate. Yes ___ No ___

1/31/02 ____________________________
Date Project Director Signature

FOR COMMITTEE USE:

Date Proposal Received: __________________ Project Director: __________________

Primary Reviewer: __________________ Project Number: __________________

Date Reviewed: __________________ Date Project Approved: __________________

Date Denial of Request: __________________

Date of yearly Review: __________________

Date of yearly Review: __________________

Reasons for denial, conditions for approval or other committee comments
Institutional Animal Care and Use Committee (IACUC) members:

Dr. J. Sinner (chair)  Dept. of Biology  Ext. 8654  +3908
Mrs. F. Bryson  Biology Resource Center  Ext. 5645  +3008
Dr. H. Ducharme  Dept. of Philosophy  Ext. 5241  +1903
Dr. D. Ely  Dept. of Biology  Ext. 7159  +3908
Ms. M. Evancho-Chapman  Father Center for Vascular Studies  375-3693
Summa Health Center P.O. Box 2090
Akron, OH 44309-2090

Dr. J. Holda  Dept. of Biology  Ext. 5116  +3008
Mr. D. Plekarz  Curator, Akron Zoological Park  375-2550
500 Edgewood Ave
Akron, OH 44307

Dr. G. Riggs  Veterinarian  625-1637
4873 Richland Ave., Norton

rev. 1/2000  hr:byron@akron-dot-bio
ANNUAL REVIEW
REQUEST TO USE ANIMALS

Please check the project status below, sign & date the form and return it to the Institutional Animal Care and Use Committee (IACUC) Coordinator. The form must be submitted by the "Submit by Date" given below in order to avoid interruptions in animal use related to a failure to obtain timely IACUC approval. If personnel are to be added to the "Request to Use Animals" (protocol), then complete and attach the "Addition of Personnel" form (file name: Add personnel). For all other proposed changes complete and attach the "Modification - Request to Use Animals" form (file name: Modification request). All changes must be approved by the IACUC before they are implemented.

PROTOCOL TITLE: Tyrosine Hydroxylase and Hypertension in Females #02-02C

PRINCIPAL INVESTIGATOR: Amy Milsted

ORIGINAL APPROVAL DATE: Feb. 7, 2002

SUBMIT BY DATE: 02-02 B

SUMMARY OF ANIMAL USE:

<table>
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<th>SPECIES</th>
<th>NUMBER APPROVED</th>
<th>NUMBER REMAINING</th>
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<tbody>
<tr>
<td>R. norvegicus</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

PROJECT STATUS:

☐ a) Project will continue with no changes

☐ b) Project will continue with changes (attach appropriate form[s])

☒ c) Project has been completed/terminated

PRINCIPAL INVESTIGATOR SIGNATURE

Date: 6/25/03

IACUC APPROVAL:

IACUC member (NEOUCOM)

Date ___________________

Attending Veterinarian (All institutions)

Date ___________________

IACUC Chairperson (All institutions)

Date ___________________