REAL-TIME PCR ANALYSIS OF AGE-DEPENDENT ALTERATIONS IN THE RVLM NEUROTRANSMITTER GENE EXPRESSION PROFILE OF F344 RATS

by

ROBIN ANN CRAIG

B.S., Kansas State University, 1999
M.S., Kansas State University, 2001

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Anatomy and Physiology
College of Veterinary Medicine

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Abstract

It is well established that normal aging is associated with progressive increases in efferent sympathetic nerve discharge (SND). Type II diabetes, obesity, heart failure, and hypertension are pathologies that have been attributed to both the processes of aging and sympathetic dysfunction, exemplifying the importance of understanding central regulation of SND during aging. However, the central mechanisms mediating altered SND with advancing age remain unclear. The rostral ventral lateral medulla (RVLM) is a brainstem region critically involved in setting the basal level of sympathetic outflow and cardiovascular function. Indeed, the RVLM is the only presympathetic region that when bilaterally inactivated results in profound reductions in both SND and arterial pressure. Glutamatergic influences in RVLM activity are powerfully inhibited by tonic GABAergic neural inputs originating from the caudal ventral lateral medulla (CVLM); effects that are mediated by $GABA_A$ receptors located on presympathetic neuronal cell bodies within the RVLM. In the present study we proposed that reductions in $GABA_A$ receptor subunit gene expression may reflect withdrawal of GABAergic tone in the RVLM thereby contributing to the basal sympathetic activation that occurs with advancing age. Therefore, the objective of the current study was to identify age-related changes in the constitutive expression of genes related to GABAergic and muscarinic, nicotinic and dopaminergic receptor systems due to their reported involvement in modulating $GABA_A$ receptor function, in the RVLM of adult young (3-5 mo. old), middle-aged (12 mo. old), weight stable presenescent (24-25 mo. old) and senescent (>24 mo. old) Fischer 344 (F344) rats using a commercially available real-time PCR array. Real-time analysis revealed nonuniform and age-associated changes in the RVLM GABA, muscarinic, nicotinic and dopaminergic neurotransmitter gene expression profile

between young and middle-aged F344 rats. Heterogenous expression of genes related to these neurotransmitters was also observed between presenescent and senescent F344 rats. Our results suggest that potential changes in neurotransmitter synthesis and degradation, uptake, transport, signaling and receptor subunit composition may account for the sympathoexcitatory state that is commonly observed in the aged.

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Acknowledgements

First and foremost I would like to gratefully acknowledge the Bank of Dad for financially supporting my undergraduate education even during times when the return on investment seemed highly questionable and downright risky. For the unconditional love, patience, unwavering support and encouragement that I received from my parents throughout my time at Kansas State. For Heather, whom I have always had tag along with me through life's adventures and unfortunate disasters. For Bryan, my math wizard who has worn himself out over the years trying his best to calm me down during times of panic and emotional unraveling- for this I am extremely appreciative and grateful. In loving memory of my mother.

CHAPTER 1 - Cellular Biology of Brain Aging

The cellular mechanisms mediating the aging process are highly complex and despite extensive investigative efforts, aging remains a poorly understood process. However, it is generally agreed that cellular mechanisms largely contribute to the gradual and progressive deleterious alterations in critical physiological processes that occur with advancing age[23-32]. Understanding aging of the brain is of particular interest because the brain provides the foundation for centrally-mediated regulation of essential physiological processes involved in the maintenance of homeostasis and the generation of adaptive responses to stress[33-35].

The biological and physiological consequences of brain aging have generated substantial interest within the research community due to the association of advancing age with neurodegenerative diseases including Alzheimer's, Parkinson's and amyotrophic lateral sclerosis (ALS)[24,28,36-41], with particular focus on understanding molecular factors that contribute to the physiological heterogeneity commonly observed among aged individuals. ***It is unclear, for example, what underlying mechanisms are responsible for an individual's increased susceptibility to the development and progression of certain neurodegenerative diseases or to the development of other centrally-mediated age-associated pathologies including hypertension. This is of critical importance because it is estimated that globally, the number of aged persons between 60 and 80 years or over will increase over eight-fold by 2050***[42]. It has been suggested that such variable responses to aging may be attributed to cellular

mechanisms such as errors in protein processing, genetic mutations, or defective repair mechanisms, all of which either independently or in combination may damage the cell, tissue or organ. Therefore, the mechanisms of aging are likely to have both a genetic and a molecular component. Indeed, age-associated reductions in cellular metabolism and oxygen consumption[28,30,43], DNA repair mechanisms[31,44], protein synthesis[25,45-48], and specific neurotransmitters[24,49]; and increases in glycation end-products[25,27,31,50-52], oxidative damage[53-55] to membrane lipids[24,32,36,56-58], proteins and DNA[24,32,59-61], and the accumulation of protein aggregates[27,62,63] have all been widely reported, a number of which have also been described in the brain. Such age-related alterations provide the foundation for over 300 theories of aging[32], demonstrating the significant investigative effort currently directed towards understanding the basic cellular mechanisms that mediate the complex process of brain aging.

CHAPTER 2 - The Sympathetic Nervous System

The autonomic nervous system plays a critical role in maintaining physiological homeostasis under basal conditions and in response to stressors and life-threatening situations[11,33-35]. While the autonomic nervous system includes both the sympathetic ("fight or flight") and parasympathetic ("rest and digest") nervous systems, it is the sympathetic nervous system that is involved in the central regulation of coordinated adaptive physiological responses to stress through the tonic activation of sympathetic nerves that innervate the heart, blood vessels, muscle, lungs, adrenal medulla and liver (*figure 2.1*)[64]. Therefore, sympathetic activation is generally associated with increased heart rate and force of myocardial contraction, peripheral vasoconstriction, blood flow to skeletal muscle,

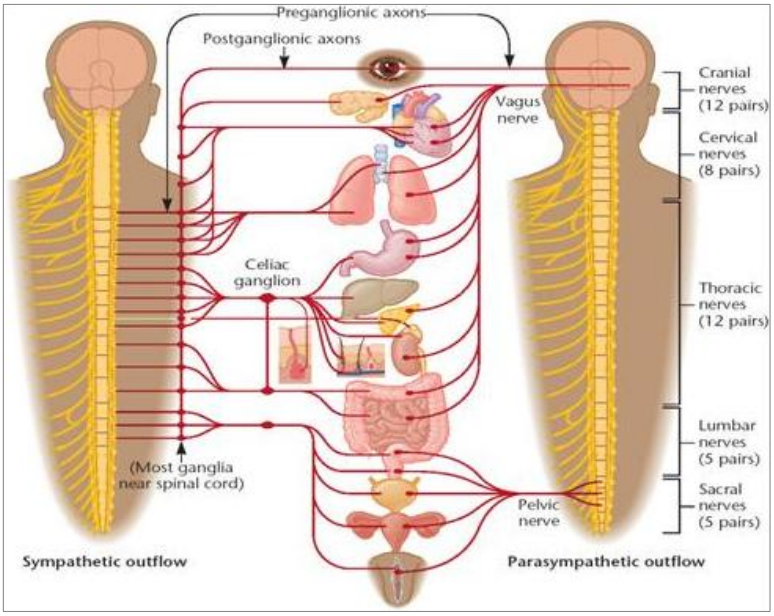

Figure 2.1 Sympathetic and Parasympathetic Arms of the Autonomic Nervous System[1]. Efferent nerves (outflow from the brain and spinal cord) and afferent nerves (incoming sensory information from the periphery) provide a link between the central nervous system and visceral effector organs, respectively, thereby providing the functional foundation of the autonomic nervous system[4]. The sympathetic nervous system is responsible for the generation of adaptive physiological responses during periods of rest, stress or life-threatening challenges whereas the parasympathetic nervous system is involved in physiological recovery in response to such situations[11].

bronchodilation, release of the “stress hormone” cortisol from the adrenal gland and energy metabolism[64].

Centrally-mediated changes in the level or pattern of efferent sympathetic outflow in response to an acute stressor are a primary means by which mammals maintain homeostasis[65-68]; however, aging is known to substantially alter sympathetic nerve responses to a variety of stressors including heat stress[65,69,70], cold stress[71], hypoxia[72,73] and footshock[74]. Sympathetic dysregulation is also associated with type II diabetes[75-82], irritable bowel syndrome[83-86], hypertension[87], obesity[88-92], chronic heart failure[93-96], insulin resistance[75,82,97], and metabolic syndrome[88,92], the incidence of which are all known to increase with advancing age. Therefore, it is has been hypothesized that ***sympathetic dysfunction may contribute in large part to the increased susceptibility of aged individuals to environmental stressors and to the development of many age-related disease states***, thereby highlighting the critical need to understand the neural mechanisms underlying alterations in centrally-mediated sympathetic activity that occur during normal aging.

Central Autonomic Pathway Organization: Descending Neural Projections

Generation of sympathetic nerve activity occurs along multiple levels of the neuraxis and involves reciprocal neural connections between the forebrain, brainstem and spinal cord[4,34] *(figure 2.2)*. Sympathetic nerve outflow is directly mediated by descending neural pathways whose cell bodies are contained in various autonomic nuclei of the forebrain and brainstem[33-35] *(figure 2.2)*. Indeed, the neural efferents that

project from these brain regions, referred to as presympathetic neurons, are considered to be the final central output directly involved in the modulation of physiological responses to internal and environmental stimuli[4,6,33,34]. Anatomical and neurochemical studies have demonstrated that glutamatergic presympathetic neurons project to the thoracolumbar intermediolateral cell column of the spinal cord (IML) and innervate preganglionic neurons[4,6,33-35] which in turn, synapse with noradrenergic-containing postganglionic neurons that directly innervate effector organs *(figures 2.1, 2.2)*.

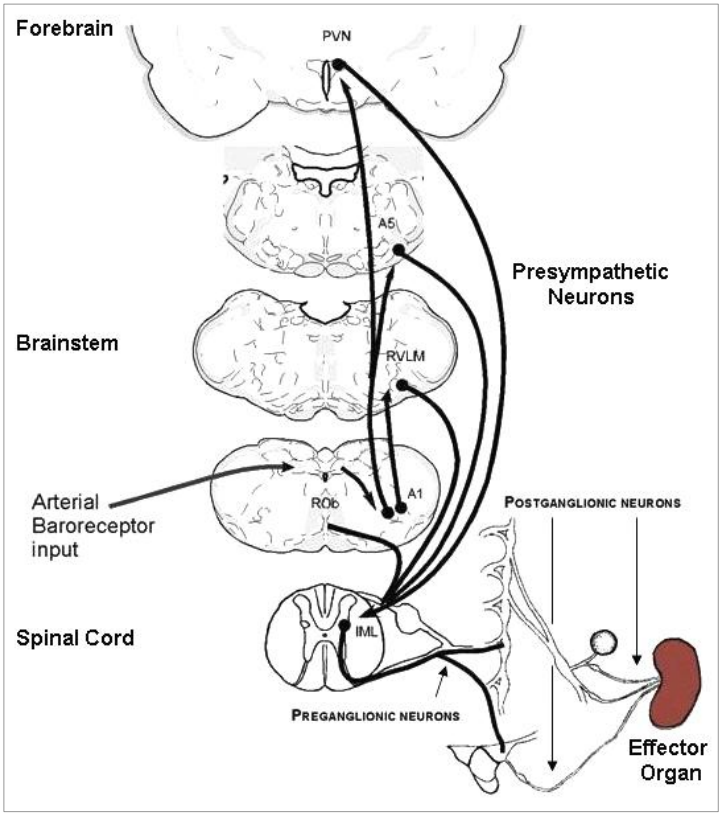

Figure 2.2. Descending Neural Projections Regulating Sympathetic Outflow[4].

In all vertebrates studied, sympathetic postganglionic neurons *in vivo* exhibit spontaneous, low levels of maintained (tonic) activity[34]. Synaptic activity is commonly

characterized by the presence of synchronized discharge bursts which contain a prominent cardiac-related component in animals with intact baroreceptors[98,99]. The entrainment of sympathetic nerve activity to the cardiac cycle (systolic and diastolic phases) provides the mechanism by which SND bursting patterns are "locked" 1:1 in relation to the arterial pulse - a hallmark of the cardiac-related discharge pattern. As *figure 2.3* demonstrates, in baroreceptor-innervated animals an inverse relationship exists between efferent sympathetic bursts (*figure 2.3, top*) and the arterial pulse (*figure 2.3, bottom*), such that decreases in blood pressure (BP) during the diastolic phase of the cardiac cycle elicit the immediate potentiation of sympathetic outflow to the heart, peripheral vasculature, adrenal gland, lumbar region of the spine, kidney *(figure 2.3A)* and spleen *(figure 2.3B)*[68].

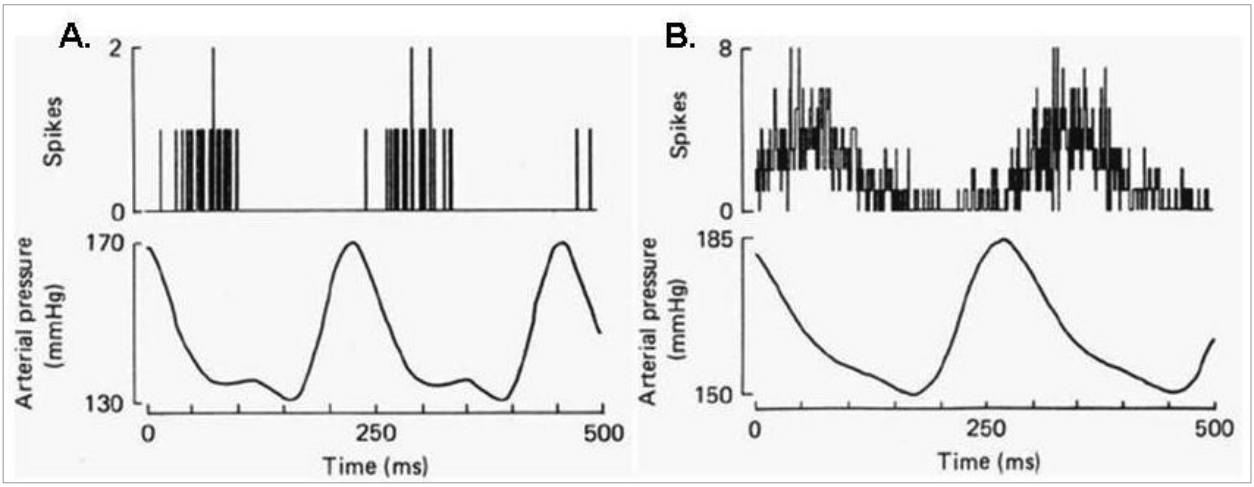

Figure 2.3. Cardiac-related Sympathetic Bursting Patterns[7]. Discharge bursts related to the arterial pulse are observed during single nerve recordings of postganglionic renal (A) and splenic (B) nerve fibers in the cat[7]. Cardiac-related rhythms have been demonstrated in many species, including humans, and are unaffected by the presence of anesthesia[4,6,7,16,18-20]. However, the correlation between the cardiac cycle and basal sympathetic nerve discharge bursts is virtually eliminated following removal of baroreceptor afferents, demonstrating an intact baroreflex is required for the generation of sympathetic activity that is coupled to the arterial pulse[21,22].

Although cardiac-related rhythmicity is considered a hallmark of most sympathetic nerves under conditions where baroreceptors remain intact[98,99], some sympathetic nerves, including those innervating brown adipose tissue (BAT) and skin do not display spontaneous, cardiac-related rhythmicity[100-104]. Therefore, sympathetic nerve discharge rhythmicity and patterns (e.g., duration and interval) may be considered non-uniform in nature[6,100], dependent in part, on the animal being studied (cat, human, rat[103,105,106]), its' physiological state at the time of direct nerve recording (advanced age, hypertension, cold or heat stress, heart failure, diabetes), and the type of nerve being recorded[11,103,105,106] (BAT, skin, sweat gland, kidney, adrenal gland, tail).

Sympathetic Nerve Discharge Patterns and Regulation of Organ Function

Sympathetic nerve discharge plays an important role in the regulation of essential physiological functions involved in the maintenance of homeostasis, although frequently the physiological relevance of oscillatory patterns displayed by a specific nerve is unclear[100]. Gebber and coworkers[107,108] have suggested that compared to random discharge bursts, rhythmic SND may provide a mechanism by which more effective neurotransmission and complex, highly differentiated physiological response patterns may be generated in response to a variety of stressors or in an effort to maintain physiological homeostasis.

Physiological response properties specific to a particular sympathetic postganglionic nerve or to its discharge bursting pattern have primarily been characterized by the direct recording of nerve activity in anesthetized animal

preparations. Insight into the functional specificity of sympathetic nerves is commonly accomplished by correlating alterations in the level or pattern of sympathetic nerve discharge with a specific end-organ function. For example, physiological experiments conducted by our laboratory routinely involve direct whole nerve recordings of sympathetic postganglionic axons that innervate the kidney and spleen because sympathetic innervation of the kidney and spleen are highly involved in the physiological processes of renal blood flow, renin release, glomerular filtration rate, salt and water retention and provide a direct, neural link between the immune system and central nervous system, respectively. Physiological processes such as these are mediated in part by tonic synchronized sympathetic neural input, the physiological relevance of which is briefly described below.

Kidney. The kidney is critically involved in the support of arterial pressure through sympathetic-mediated adjustments in blood volume and composition[33]. Adjustments are achieved by sympathetic postganglionic innervation of the juxtaglomerular apparatus, glomerulus, and epithelial cells of the nephron and renal vasculature. It has been demonstrated that cardiac-related sympathetic discharge patterns elicit increases in renin release, decreases in glomerular filtration rate, increased water and solute absorption and decreased renal blood flow, respectively[33], in an effort to maintain proper blood volume and pressure. In contrast, under conditions where cardiovascular homeostasis has been achieved, baroreceptor afferent-mediated inhibition of efferent renal SND results in the attenuation of renin release and renal tubular transport of solutes and water, and increases in renal blood flow and glomerular

filtration rate. Therefore, synchronization of renal postganglionic nerve discharge bursts is important for the generation of coordinated renal responses necessary for the maintenance of arterial pressure.

Spleen. The spleen is involved in a bi-directional communication pathway that exists between the central nervous system and the immune system[109-111]. Sympathetic innervation of the spleen modulates important immune system function including splenic T and B cell proliferation[112-114], natural killer cell activity[112,113], splenic production of immunoglobulin M[115], and splenic cytokine and chemokine gene expression[116,117]. Ganta and coworkers[116] demonstrated that acute bouts of hyperthermia transforms the cardiac-related bursting pattern of SND observed under control conditions (38°C) to a pattern that contains low frequency and non-cardiac-related bursts at 41.5°C. Of particular interest is that the increases in splenic nerve activity and pattern transformation observed at 41.5°C were associated with enhanced IL-1β, IL-6 and GRO 1 mRNA expression in the spleen. While it is unclear whether splenic transcriptional responses were the result of alterations in the level or pattern of splenic nerve discharge at 41.5°, the upregulation of these genes was abolished following splenic denervation. These results indicate that the transcriptional upregulation of splenic IL-1β, IL-6 and GRO 1 is dependent on sympathetic innervation of the spleen during acute bouts of hyperthermia.

CHAPTER 3 - Central Regulation of Sympathetic Nerve Activity and Cardiovascular Function

It is well established that the highly coordinated and integrated regulation of sympathetic nerve discharge involves numerous regions of the brain; including the paraventricular nucleus of the hypothalamus, A5 noradrenergic cell group, rostral ventral lateral medulla, ventromedial medulla, and caudal raphe nuclei[20]. Each of these brain regions contain the cell bodies of presympathetic neurons whose axons directly synapse with preganglionic neurons in the IML of the spinal cord[20] *(figure 3.1)*. Indeed, several lines of evidence support the role of supraspinal neural inputs in the generation of SND rhythms[18] and the maintenance of arterial blood pressure[4,6,15,16]. Several experimental methods exist to determine supraspinal involvement in specific physiological functions, many of which involve neuronal inactivation[18] or transection at multiple levels of the neuraxis. Such methods include chemical inactivation (kainic acid, muscimol, kynurenic acid, lidocaine) and physical inactivation of presympathetic neurons (midbrain transection, cervical

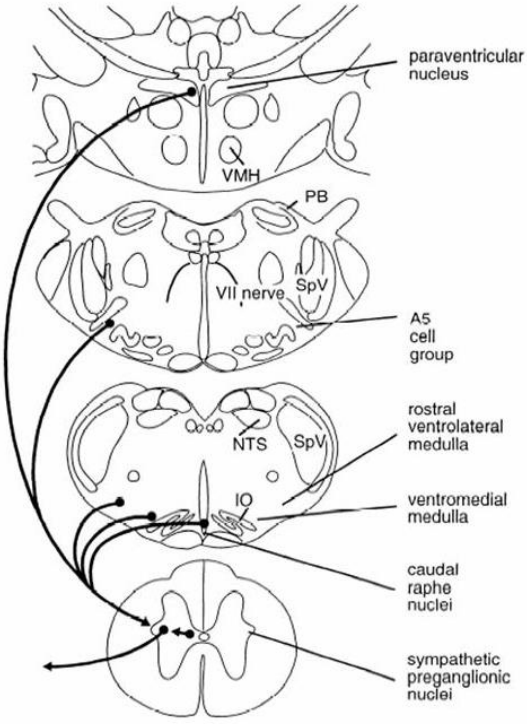

Figure 3.1. Supraspinal Neurons Synapse With Preganglionic Neurons[4,20]. Although a number of autonomic centers exist in the brain, only a few contain the cell bodies of presympathetic neurons whose axons project directly to the spinal cord and innervate preganglionic neurons[4,6,15,16]. Evidence suggests that supraspinal inputs play an important role in the regulation of cardiovascular function including the maintenance of arterial pressure[17].

transection). A variety of these methods have primarily been used to determine supraspinal neural circuit involvement in cardiovascular function and regulation of efferent SND. For example, spinal transection at the level of the first cervical vertebrae (C1) elicits profound reductions in mean arterial pressure (MAP) at levels similar to that following ganglionic blockade (prevents synaptic transmission from preganglionic neurons to postganglionic neurons) and removes the rhythmicity that is characteristic of most sympathetic postganglionic neuronal activity[118-120], ***strongly suggesting a prominent role of tonic supraspinal support in the regulation of cardiovascular function[6,16,121,122] and the modulation of efferent sympathetic nerve discharge patterns[18]***.

Regulation of Sympathetic Nerve Outflow: the RVLM

The traditional view is that that the central neurons mediating sympathetic outflow to the heart and vasculature (vasomotor neurons) are distributed throughout the brainstem[6,121-125] because removal of forebrain neural circuits by midbrain transection fails to significantly alter resting levels of MAP[66], suggesting a role of brainstem autonomic nuclei in the regulation of cardiovascular function. Such a role has been established for the rostral ventral lateral medulla (RVLM), a brainstem site that is critically involved in setting the level of basal sympathetic activity[18] of postganglionic nerves that innervate the heart[126,127] and a number of other effector organs including the peripheral

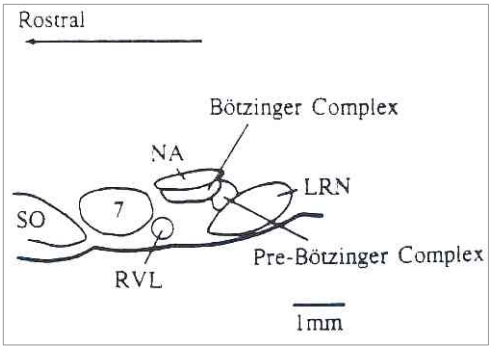

Figure 3.2. Anatomical Location of the RVLM[6]. Landmarks used to identify the location of the RVLM include the facial motor nucleus (7), nucleus ambiguus (NA) and pre-Bötzinger complex[6].

vasculature[128-133], kidney[4,128,130,134], spleen[135] and adrenal gland[128,130,136,137] through the direct regulation of spinal-projecting presympathetic nerves[138,139]. The level of basal sympathetic nerve activity is largely determined by a functional balance of both excitatory and inhibitory neural inputs to the RVLM[128,129,140-145]. Such neural modulation of RVLM activity plays an integral role in the support of arterial pressure[3,128,129,146] and the generation of adaptive physiological responses to internal and environmental stressors[134,147-151]. Located immediately caudal to the posterior end of the facial motor nucleus (7), ventral to the nucleus ambiguous (NA) and Bötzinger complex, and rostral to the pre-Bötzinger complex[6,152,153] *(figure 3.2)*, the role of the RVLM in cardiovascular function was first demonstrated by Dittmar in 1873. In this landmark study, bilateral destruction of a discrete portion of the ventral medulla was found to produce a marked reduction in arterial pressure[123]. Several lines of evidence provide additional support for the involvement of the RVLM in the modulation of vasomotor tone and the maintenance of arterial pressure. First, retrograde transport studies revealed a direct monosynaptic neural projection from the RVLM to the IML of the spinal cord[154,155] indicating direct innervation of preganglionic neurons (*figures 2.2, 3.1*). Second, direct surface application of the inhibitory amino acids γ-aminobutyric acid (GABA)[6] or glycine[156,157], or cooling[158] of the ventral areas of the medulla elicits significant reductions in arterial pressure. Third, in contrast to the medullary administration of GABA or glycine, electrical and chemical stimulation of the RVLM induces rapid and marked increases in arterial pressure and heart rate[159-162]. Fourth, chemical lesions of the RVLM permanently lowers arterial pressure in conscious and unrestrained dogs[163] while in decerebrate (midbrain-transected) anesthetized cats inhibition of RVLM neurons by

unilateral microinjections of muscimol, a $GABA_A$ receptor agonist, results in significant and lasting reductions in MAP[164]. ***Importantly, the RVLM is the only presympathetic region that when bilaterally chemically inactivated results in profound reductions in both arterial pressure and sympathetic nerve activity***[129], demonstrating its critical role in mediating the activity of postganglionic neurons that innervate the heart and other effector organs through the regulation of presympathetic nerve outflow. In contrast, experimental modulation or inactivation of supraspinal neurons in the midline medulla, A5 area, or paraventricular nucleus of the hypothalamus (PVN) does not result in significant reductions in resting arterial pressure or sympathetic activity[165,166]. Therefore, although these autonomic centers are similar to the RVLM in that they contain spinal-projecting presympathetic neurons, centrally-mediated support of arterial pressure and determination of the level of basal sympathetic nerve discharge appears to be primarily mediated by the RVLM[18]. Indeed, ***the RVLM is considered to be perhaps the single most important source of tonic excitatory drive to presympathetic neurons involved in the maintenance of arterial pressure and*** at present ***is the only autonomic region identified as being critically involved in the integration of cardiovascular reflexes***[4].

Autonomic Regulation of Sympathetic Nerve Outflow: Influence of the Baroreflex

Blood pressure is influenced by peripheral vascular resistance and cardiac output, both of which are tightly controlled by the autonomic nervous system[167]. Short term maintenance of arterial blood pressure within normal limits is achieved by the baroreflex arc that involves the afferent projections of arterial baroreceptors that

originate in the walls of the aortic arch, carotid sinus, atria and ventricles and project to supraspinal cardiovascular autonomic nuclei in the brain (*figure 3.3*). Thus, the baroreflex represents a negative feedback signaling mechanism by the heart and brain modulates blood pressure and sympathetic nerve outflow in response to the arterial pulse.

The baroreflex and therefore, tonic cardiac-related sympathetic neuronal activity are modulated primarily by a cardiovascular autonomic complex involving the nucleus of the solitary tract (NTS), caudal ventral lateral medulla (CVLM) and RVLM[128] (*figure 3.3*). However, the RVLM is considered to play a critical integrative role in facilitating the generation of physiological responses to neural inputs that arise from the periphery and from autonomic nuclei located at higher levels along the neuraxis[4]. It is also considered to be the predominant source of excitatory drive to barosensitive presympathetic neurons[7,18,19,65-68,168,169]. Therefore, the neural circuitry that comprises the baroreflex arc provides a highly regulated mechanism by which the arterial pulse may influence sympathetic nerve activity such that increases in blood pressure during the systolic phase of the cardiac cycle (contraction) elicit centrally-mediated dilation of the peripheral vasculature, reductions in cardiac contractile force and heart rate, and

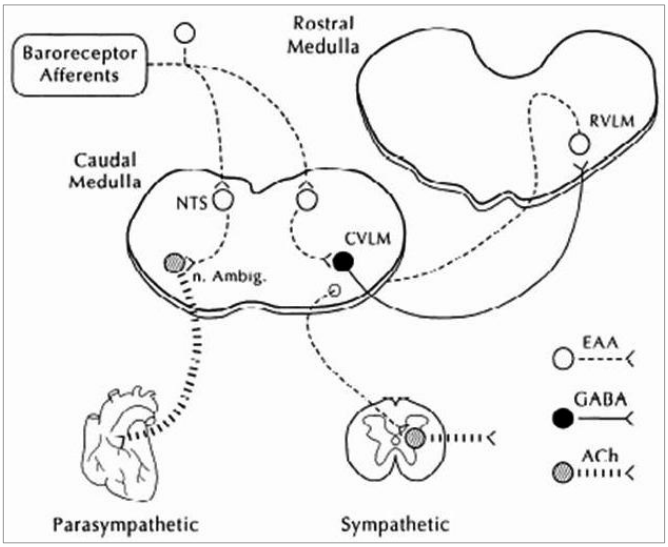

Figure 3.3. The Baroreflex Arc[3]. The baroreflex is considered to be the most important mechanism for centrally-mediated short term regulation of arterial blood pressure[12-14].

baroreceptor-mediated inhibition of RVLM presympathetic neurons[3,6,122,129,146,170-172] (*figures 2.3, 3.4* illustrate baroreflex-mediated inhibition of postganglionic nerve activity).

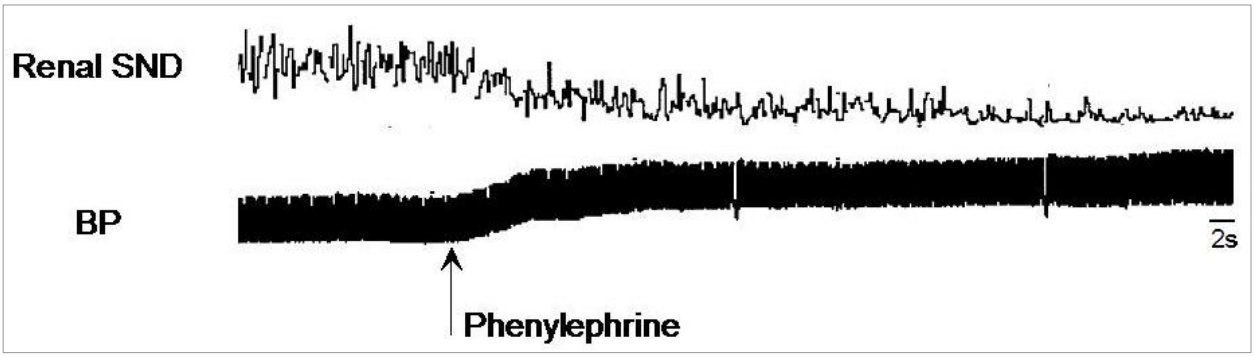

Figure 3.4. Baroreflex-mediated inhibition of Renal SND. Pharmacological activation of baroreceptor afferents is achieved by the intravenous administration of phenylephrine, a vasoconstrictive agent that is commonly used to measure arterial baroreflex function. Our laboratory has demonstrated that increases in blood pressure in response to phenylephrine administration markedly inhibits sympathetic outflow to the kidney, indicating baroreceptor-mediated inhibition of sympathetic nerve activity (R. Fels, unpublished data).

Cardiac-Related Sympathetic Nerve Discharge Bursts Are Dependent on Intact Baroreceptor Afferents

The role of the baroreflex in modulating blood pressure and cardiac-related sympathetic bursting patterns may be clearly demonstrated under experimental conditions whereby afferent baroreceptors are removed from the aortic arch and carotid region of the heart. *Figure 3.5* demonstrates the effect of sinoaortic denervation (SAD; removing baroreceptor afferents to the brainstem) on blood pressure and the cardiac-locked activity of postganglionic nerves innervating the kidney and spleen. Our laboratory and others have demonstrated that SAD increases blood pressure[17,65,173], heart rate[17,65] and the level of sympathetic outflow to a number of effector organs[17,65,174-

[176] including the kidney and spleen, compared to baroreceptor-intact animals. As *figure 3.5* demonstrates, following SAD the cardiac-related rhythm of renal and splenic postganglionic nerves is strikingly eliminated - a pattern that is not present in baroreceptor innervated animals which exhibit a 1:1 SND bursting pattern in relation to the arterial pulse (e.g., SND bursts only during the diastolic phase of the cardiac cycle when MAP is low) *(figures 2.3, 3.5)*. It is clear, therefore, that baroreceptor input to the brainstem is required for the entrainment of presympathetic neurons to the arterial pulse.

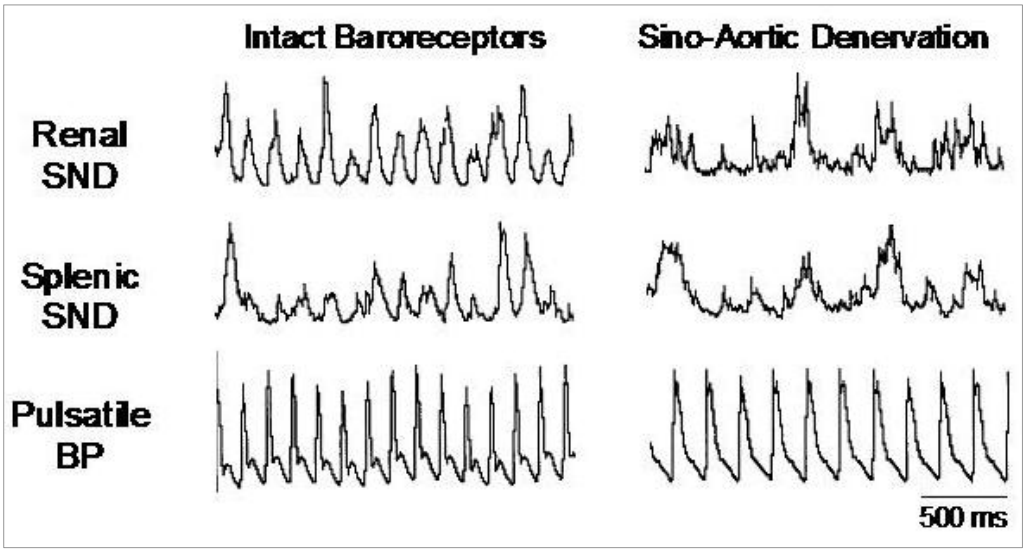

Figure 3.5. Effect of SAD on SND and MAP. In our laboratory animals in which baroreceptor afferents remain intact exhibit sympathetic nerve discharge bursts that are locked 1:1 in relation to the arterial pulse while the removal of baroreceptor afferents (SAD) eliminates this cardiac-related rhythm, demonstrating the requirement of the baroreflex in contributing to the cardiac-related rhythmicity of sympathetic nerves in baroreceptor-intact animals (R. Fels, unpublished data).

CHAPTER 4 - Modulation of RVLM Activity: Inhibitory Neurotransmitter Mechanisms

The RVLM is the only autonomic region identified as being critically involved in the integration of cardiovascular reflexes[4]. Because it is well established that under basal conditions tonic RVLM presympathetic neural activity is strongly suppressed by GABAergic influences[177-179], neurotransmitter-mediated modulation of RVLM presympathetic nerve activity is expected to play an important role in the integration of reflex responses for several reasons. First, the RVLM contains the highest concentration of presympathetic neurons[144,180-183], demonstrating its role as the final point of centrally-regulated sympathetic nerve outflow. Second, short term stabilization of blood pressure and attenuated RVLM presympathetic nerve activity in response to baroreflex activation is achieved through withdrawal or enhancement of the RVLM GABAergic neurotransmitter system. Several lines of evidence demonstrate the importance of

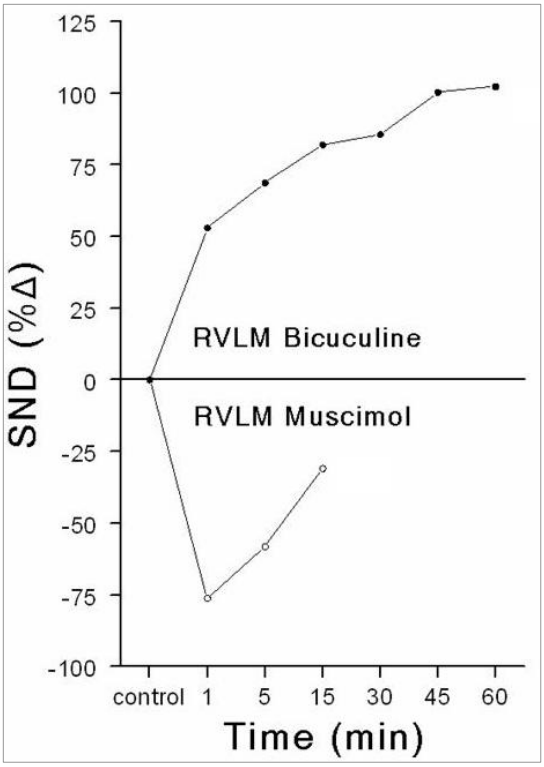

Figure 4.1. RVLM GABA Inhibits Sympathetic Outflow. Our laboratory has demonstrated that microinjection of a $GABA_A$ receptor antagonist (BIC) or agonist (MUSC) into the RVLM potentiates or attenuates SND, respectively, demonstrating that GABA exerts its inhibitory influence through $GABA_A$ receptors. Percent changes for renal and splenic SND were combined and averaged per treatment timepoint (R. Fels, unpublished data).

GABAergic neural inputs to the RVLM in influencing basal sympathetic nerve activity. Removal of inhibitory influences with the $GABA_A$ receptor antagonist bicuculline (BIC) elicits immediate increases in the firing rate and level of SND[177,179,184-186] *(figure 4.1)* and MAP[184-187] *(figure 4.2)*, indicating that GABA exerts a substantial influence on RVLM-mediated resting levels of SND and MAP. Furthermore, inhibition of local GABA synthesis within the RVLM by 3-mercaptopropionic acid (a glutamate decarboxylase inhibitor) increases MAP, demonstrating that endogenous GABA strongly inhibits RVLM vasomotor activity under resting conditions[185]. Collectively, these studies demonstrate that within the RVLM, endogenous GABA plays an essential role in the central baroreflex pathway because removal of tonic GABAergic influences attenuates baroreflex-dependent inhibition of RVLM-mediated SND (both vasomotor and presympathetic) and arterial pressure responses[172,177,179,186,187].

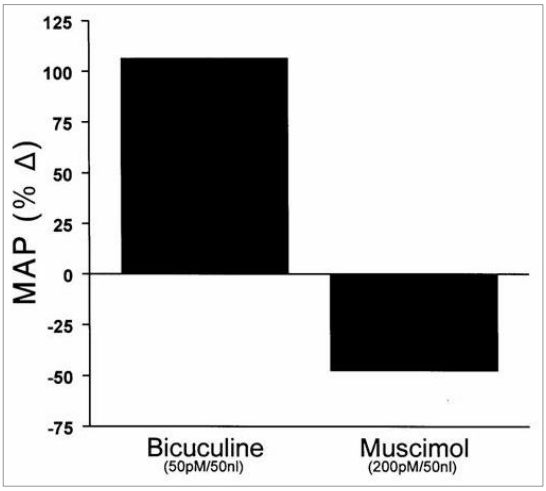

Figure 4.2. RVLM GABA Reduces Arterial Pressure. We have demonstrated that microinjections of bicuculine (BIC) and muscimol (MUSC) directly into the RVLM elicit pressor and depressor responses, respectively. These results indicate that RVLM support of arterial pressure is mediated in part by $GABA_A$ receptors located on neuronal cell bodies within the RVLM (R. Fels, unpublished data).

The CVLM Mediates GABAergic Inhibition of RVLM Vasomotor and Presympathetic Neurons

The CVLM contributes to tonic GABAergic inhibition of the RVLM such that stabilization of blood pressure is achieved through the phasic withdrawal or enhancement of CVLM GABAergic tone on RVLM barosensitive vasomotor sympathetic neurons through both baroreceptor-dependent and independent mechanisms[3,146,188-191]. Located 1.0-1.5mm caudal to the RVLM in the rat[172], the depressor neurons of the CVLM are located primarily between the nucleus ambiguous and the lateral reticular nucleus although landmarks such as the facial nucleus, obex, and calamus scriptorius are often used for reference[6,172,192,193] (see *figure 3.2* for illustration of landmarks). Such close proximity to cell bodies of presympathetic neurons in the RVLM does not allow for clear distinction between excitatory presympathetic neurons in the RVLM or inhibitory neurons in the CVLM[194,195]. However, retrograde tracing and anatomical studies have successfully established distinct, monosynaptic projections from the CVLM to the RVLM[1,4,181,182,189,190,196-199].

The role of CVLM GABAergic neurons as a source of tonic inhibition of RVLM spinal-projecting neurons has received much attention[6,191,200]. It is well established that the CVLM inhibits RVLM presympathetic and vasomotor nerve outflow[167] in order to elicit reductions in SND and MAP[172]. Such alterations in MAP are achieved through RVLM-mediated reductions in total peripheral resistance, cardiac contractility[160,196,201,202] and blood flow to the vasculature of the kidney, mesentery and hindlimb[160]. Microinjection studies have demonstrated that the activity of the CVLM may be

attenuated or potentiated by CVLM application of muscimol (MUSC; $GABA_A$ receptor agonist) or glutamate, respectively. For example, inhibition of neural cell bodies within the CVLM by MUSC microinjection increases MAP and SND[184,203] (*figure 4.3; SAD*) at levels similar to those observed following $GABA_A$ receptor blockade in the RVLM[184,203], suggesting that the CVLM inhibits efferent SND primarily through direct GABAergic neural inputs to the RVLM[177,203,204]. Antagonism of RVLM $GABA_A$ receptors with BIC prevents reductions in MAP and SND in response to glutamatergic stimulation of the CVLM[171,205-207], further demonstrating that 1) the CVLM provides a powerful source of tonic inhibition to the RVLM, 2) depressor and attenuated SND responses to CVLM stimulation are largely mediated by the RVLM and 3) tonic inhibitory neural inputs to the RVLM from the CVLM are mediated by $GABA_A$ receptors[4,177,190,205,208-212].

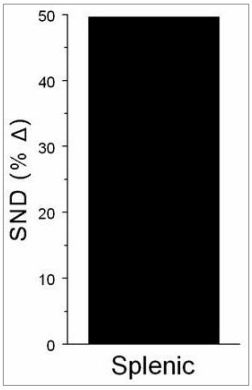

Figure 4.3. Inactivation of the CVLM Potentiates Splenic SND (R. Fels, unpublished data).

Microinjection studies conducted in the rat and rabbit in which glutamate was directed into the CVLM have demonstrated marked reductions in RVLM presympathetic SND[193,213,214] and MAP[159,193,215] (*figure 4.4*), suggesting that glutamatergic excitation of the CVLM is responsible for the SND and depressor responses observed following CVLM activation[201,205,216,217]. In contrast, chemical blockade of CVLM glutamate receptors attenuates GABAergic inhibition of RVLM neurons in response to increases in MAP or direct stimulation of the NTS[210,216,218,219], indicating that the CVLM is the principle component involved in providing direct inhibitory neural inputs to the RVLM. Collectively, these microinjection studies in which the molecular mechanisms of CVLM-

mediated GABAergic inhibition of RVLM sympathetic outflow were examined highlight the importance of RVLM $GABA_A$ receptors and demonstrate the complexity of the neural network regulating RVLM sympathetic outflow and related physiological responses.

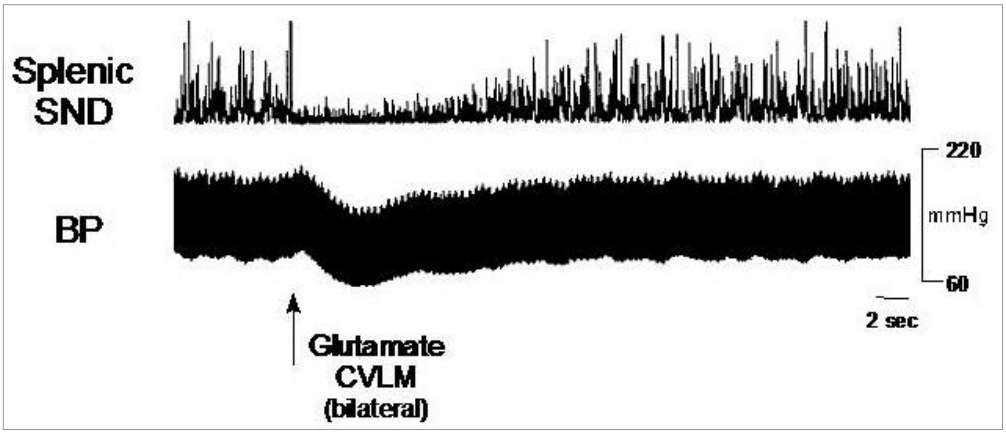

Figure 4.4. CVLM-mediated GABAergic Reductions in SND and Blood Pressure Are Dependent on Glutamate. Preliminary experiments completed in our laboratory demonstrate that CVLM glutamate microinjections markedly reduce splenic SND and blood pressure, indicating that glutamatergic neural inputs to the CVLM are the mechanism which supports CVLM-induced inhibition of efferent SND and tightly controls fluctuations in blood pressure (R. Fels, unpublished data).

CHAPTER 5 - The RVLM GABAergic Neurotransmitter System

RVLM-mediated regulation of sympathetic nerve activity is modulated by a variety of neurotransmitter systems including GABA, glutamate, serotonin, substance P, neuropeptide Y, glycine, angiotensin II, and somatostatin[6]. Spinal-projecting presympathetic neurons in the RVLM have been reported to express these neurotransmitters[6,220-223], suggesting that these neurotransmitters are involved in the regulation of efferent sympathetic nerve activity. However, ***it is well established that under basal conditions tonic RVLM presympathetic neural activity is strongly suppressed by GABAergic influences***[177-179] ***originating in large part from the CVLM***[6,167,172,191,200] ***and that such inhibition is mediated by $GABA_A$ receptors***[4,172,177,179,186,205]. This section provides a brief overview of the inhibitory neurotransmitter GABA and its ionotropic receptor subtype, $GABA_A$, in relation to GABAergic regulation of central neuronal synaptic transmission.

GABA

GABA (γ-aminobutyric acid) is the major inhibitory neurotransmitter in the brain[224-226] and $GABA_A$ receptors ($GABA_AR$) mediate the majority of fast inhibitory synaptic transmission in the central nervous system[225,227], including the RVLM. Activation of $GABA_AR$ elicits a rapid rise in intracellular levels of chloride, resulting in hyperpolarization of the postsynaptic neuronal membrane and the inhibition of postsynaptic currents[228,229]. Such powerful tonic GABAergic control of neuronal

excitability has been demonstrated in our laboratory following the microinjection of MUSC directly into the RVLM, which produces marked reductions in splenic and renal sympathetic nerve activity *(figures 4.1, 5.1)* and arterial pressure *(figure 4.2)* are observed.

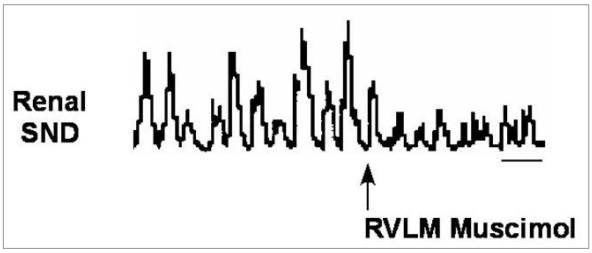

Figure 5.1. RVLM Muscimol Microinjection Reduces Renal SND. Experiments completed in our laboratory have demonstrated that microinjection of the $GABA_A$ receptor agonist muscimol (MUSC) into the RVLM mimics CVLM-mediated GABAergic inhibition of SND (R. Fels, unpublished data).

$GABA_A$ Receptors. $GABA_AR$ are members of the ligand-gated ion channel superfamily that includes nicotinic acetylcholine, glycine, glutamate and serotonin receptors[230]. X-ray crystallography studies of acetylcholine-binding protein, a protein resembling the extracellular ligand-binding domain of the nicotinic acetylcholine receptor, have provided insight into the predicted structure and identification of five separate binding sites located on the $GABA_A$ receptor including, GABA *(represented by white filled circles, figure 5.2)*, benzodiazepines *(represented by black filled circle, figure 5.2)*, barbituates, the chloride channel blocker picrotoxin, and anesthetics[9,231-235]. Similar expression studies have indicated that the $GABA_A$ receptor is composed of a fixed pentameric subunit arrangement of beta-alpha-beta-alpha-gamma[9,236-239] *(figure 5.2)*, and that a combination of alpha, beta and gamma $GABA_AR$ subunit variants are required for the

expression of fully functional and membrane localized $GABA_A$ receptors[237,240,241]. Individually, $GABA_A$ receptor subunits mediate specific aspects of $GABA_A$ receptor function. For example, $GABA_A$ receptor alpha subunits contribute to the formation of the high affinity GABA binding pocket[242,243] and determine $GABA_AR$ pharmacological properties in response to benzodiazepines[243-248]. Beta subunits are required for $GABA_A$ receptor cell surface expression[249-251] and GABA binding[244,245,248,252]. Gamma subunits influence $GABA_A$ receptor cell surface expression[253,254] and synaptic clustering[255]. Individual $GABA_A$ receptor subunit variants are differentially expressed, thereby potentially contributing to region-specific functions of certain $GABA_AR$ subtypes. $Alpha_1$ is the most widely distributed $GABA_A$ receptor subunit in the brain[256] and radioligand binding and immunohistochemical studies have indicated that at least 50% of all $GABA_A$ receptors purified from rat brain contain the $gamma_2$ subunit, 40% of which associate with $beta_2$ or $beta_3$ subunits[257]. It has also been reported that 90% of rat brain $GABA_A$ receptors contain $beta_2$ or $beta_3$[257]. Importantly, the rat RVLM reportedly expresses $alpha_{1-3}$, $beta_{2-3}$, and $gamma_2$ $GABA_A$ receptor subunits[258].

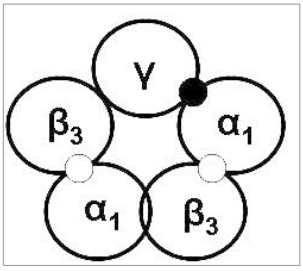

Figure 5.2. Pentameric Structure of the $GABA_A$ Receptor[9,10].

$GABA_A$ receptor compositional heterogeneity has been shown to mediate different electrophysiological and pharmacological properties of $GABA_A$ receptors[259,260]. This may explain why alterations in $GABA_AR$ function have been associated with the pathology of several neurological and psychiatric illnesses such as epilepsy, anxiety, Alzheimer's disease and schizophrenia[259,261-265]. Structural heterogeneity of mammalian $GABA_A$ receptors is attributed to the existence of nineteen known subunit variants

grouped according to seven classes of subunits (alpha$_{1-6}$, beta$_{1-3}$, gamma$_{1-3}$, delta, epsilon, phi, pi, and rho$_{1-3}$)[250,266,267], giving rise to a large number of possible subunit combinations and providing a molecular mechanism for regulating $GABA_A$ receptor function[259,268-272]. Indeed, subunit composition determines not only $GABA_A$ receptor pharmacology, channel properties[236,245,257,273,274] and modulation by endogenous ligands[275] or second messenger systems[276,277], but also cell surface localization[227,278] and intracellular targeting to different locations[271,279], which is important because functionally distinct neurons appear to express more than one $GABA_A$ receptor subtype[224,258,260,280]. Therefore, it has been suggested that differential assembly and subunit stability, subcellular compartmentalization, and temporal regulation may provide mechanisms by which $GABA_A$ receptor composition is modulated[10]. Evidence for spatial regulation comes from in situ hybridization and immunohistochemical studies which have revealed that some $GABA_A$ receptor subunits are confined to defined locations (e.g., alpha$_5$ in hippocampal pyramidal cells, alpha$_6$ in cerebellar granule cells, rho$_1$ in retinal bipolar cells, and alpha$_4$ in hippocampal dentate gyrus cells)[224,259]. Membrane insertion or internalization of $GABA_A$ receptors are a mechanism by which $GABA_A$ receptor stability at neuronal synapses may be regulated[281]. Furthermore, $GABA_A$ receptor complexity is gained due to alternative splicing of specific subunit mRNA transcripts. Each subunit has its own gene[259,282] and the existence of multiple promoters[224], which are reportedly involved in mediating developmental or tissue-specific expression[283-286], further contributes to the regulatory diversity of the $GABA_A$ receptor.

CHAPTER 6 - Aging and Central Autonomic Regulation of Blood Pressure

Advancing age is associated with abnormalities in autonomic function resulting in impaired SND regulation under basal conditions and in response to stress[33]. The consensus view is that the sympathetic dysregulation that occurs with advancing age is characterized by regionally selective, non-uniform elevations in basal sympathetic nerve activity[5,8,87,287-306]. For example, the sympathoexcitatory state in the aged is primarily attributed to increases in basal sympathetic outflow to the heart, liver-gut circulation and skeletal muscle[305,307,308] but not to the kidney, adrenal medulla[291] or skin[309].

Plasma norepinephrine (NE) levels have been routinely used as a determinant of systemic sympathetic activation in experimental subjects. Alterations in plasma NE concentrations have been reported in aged individuals at rest[310-313] *(figure 6.1)*, under acute stress (e.g., mental, exercise)[93], or in disease states (e.g., chronic heart failure)[314,315]. Esler and coworkers reported that mean plasma NE levels in healthy men between the ages of 60 and 75 were 66% higher compared to healthy, younger control subjects[291]. Cardiac NE spillover was reportedly 2- to 3-fold higher and 53% higher in the aged during mental stress and dynamic exercise, respectively, compared to young male subjects[93]. Collectively, these

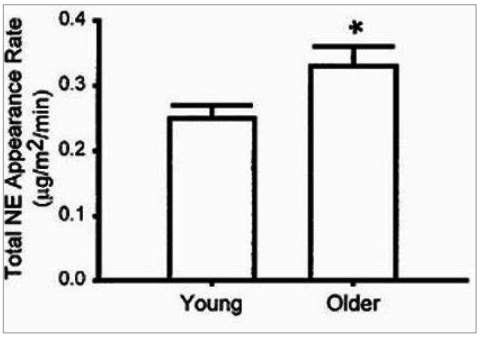

Figure 6.1. Plasma Norepinephrine Levels Increase With Advancing Age in Humans[5]**.** *p<0.05.

studies suggest higher levels of sympathetic outflow in aged experimental human subjects.

Potential Mechanisms Contributing to Age-dependent Sympathetic Activation

NE Clearance. It has been suggested that elevations in NE spillover are due to age-related increases in the release of NE from sympathetic postganglionic nerve fibers innervating the heart, liver-gut circulation and skeletal muscle[93,305]; however, reductions in NE clearance may also contribute to the total NE spillover observed in the aged[314,315]. Indeed, Esler and others[291] found that while aged men at rest had 66% higher mean plasma NE levels compared to their young counterparts, this elevation was correlated with a 22% reduction in plasma NE clearance, suggesting that NE release and uptake are augmented with advancing age.

Impaired Baroreflex Buffering. It is also possible that the progressive sympathetic activation that occurs with age may be attributed to loss of baroreflex sensitivity and may contribute to altered sympathetic support of arterial pressure. Independent of clinical disease, impaired baroreceptor reflex sensitivity is commonly associated with advancing age in healthy humans[293,305] and in experimental animal models[288,292,316,317]. Physiological consequences of depressed baroreflex responsiveness that have been reported in aged humans and rats include impaired heart rate[287,316,318,319] and SND responses to baroreceptor activation[288,292,316,317]. For example, Irigoyen and coworkers reported that renal SND responses were attenuated in aged rats when baroreflex-mediated inhibition of efferent SND was withdrawn[316]. Similarly, Hajduczok and others

demonstrated reductions in baroreflex-mediated inhibition of renal SND in senescent compared to mature, adult beagles, confirming that sensitivity to the baroreflex is altered with advanced age[301]. In order to demonstrate age-related alterations in autonomic support of arterial blood pressure in humans, Jones and coworkers administered trimethaphan to healthy young (24 ± 1 yrs) and aged (65 ± 2 yrs) men in order to produce short term blockade of ganglionic neural transmission and effectively remove baroreflex influences[8]. Although systolic (SAP), diastolic (DAP) and mean arterial pressure (MAP; *figure 6.2*) were reduced in both young and aged subjects in response to ganglionic blockade, all three parameters were reduced to a greater extent in the aged individuals examined, suggesting that at rest aged individuals have higher levels of sympathetic nerve outflow compared to young. In a similar study Jones and others reported that the depressor responses observed in the aged following ganglionic blockade was primarily due to failure to increase heart rate as would be expected following removal of baroreflex-mediated inhibition of sympathetic vasomotor outflow, demonstrating that substantial reductions in baroreflex buffering occurs with advancing age[8].

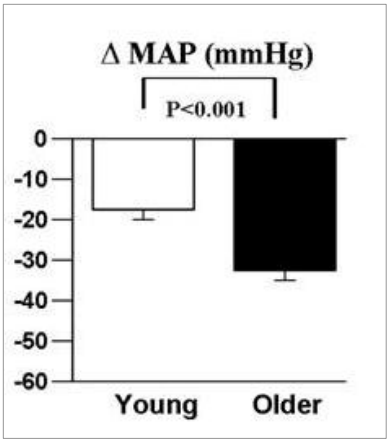

Figure 6.2. Altered Cardiovascular Support With Advanced Age[8].

Similar findings have been derived from studies using experimental animals to determine whether sympathetic support of MAP is altered with aging. Recently we have completed experiments whereby MAP and SND responses to ganglionic blockade in young and middle-aged Fischer 344 (F344) rats were determined. Prior to trimethaphan

administration the level of MAP did not differ between young (130 ± 2 mmHg) and middle-aged (133 ± 5 mmHg) F344 rats. Although ganglionic blockade with trimethaphan effectively eliminated renal and splenic SND and reduced MAP in both young and middle-aged rats, depressor responses were significantly greater in the middle-aged (86±3 mmHg) compared to young F344 rats (67 ± 4 mmHg) *(figure 6.3)*. Trimethaphan-mediated reductions in MAP were also observed in aged F344 rats (124 ± 15 mmHg control; 43 ± 3 mmHg post-blockade), demonstrating that similar to humans, outflow to sympathetic postganglionic neurons is elevated with advancing age in the rat. Therefore, these findings indicate that in both humans and animal subjects sympathetic support of MAP is altered with advancing age, a finding that is supported by evidence from experimental animal models in which a relationship between age-associated increases in SND and impairments in baroreflex-mediated inhibition of SND have been established[288].

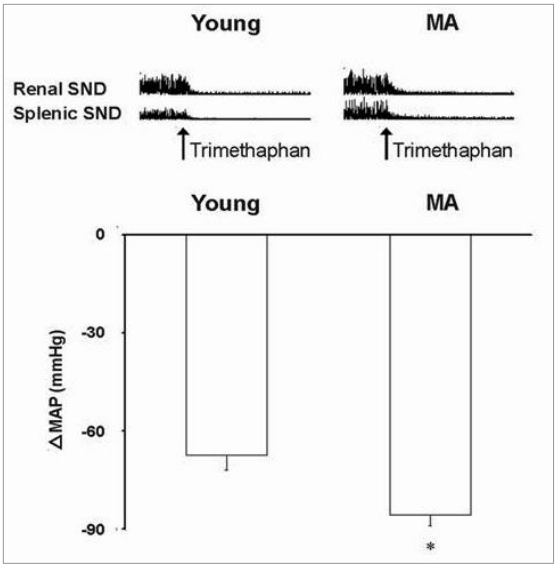

Figure 6.3. Altered Autonomic Support of Arterial Pressure With Advancing Age. Ganglionic blockade eliminated renal and splenic postganglionic nerve activity in both young and middle-aged (MA) F344 rats (*top*); however, significantly greater reductions in MAP were observed in MA compared to young F344 rats following trimethaphan administration (*bottom*). This suggests that at rest MA rats exhibit higher levels of tonic sympathetic activity compared to young rats (R. Fels, unpublished data). * $p<0.05$.

Vascular Insensitivity. Studies in which the effect of age on baroreflex buffering was examined determined that age-related impairment in baroreflex responsiveness was due in part, to reduced vascular adrenergic sensitivity[320-322]. Indeed, vasoconstriction and therefore, MAP responses to phenylephrine administration following ganglionic blockade are attenuated in 65 year old men; a response mediated by reductions in vascular epithelium alpha$_1$ adrenergic receptor sensitivity to phenylephrine[293] *(figure 6.4)*. These results indicate that markedly reduced vascular alpha1 adrenergic receptor responsiveness contributes to impairment of baroreflex buffering in the aged.

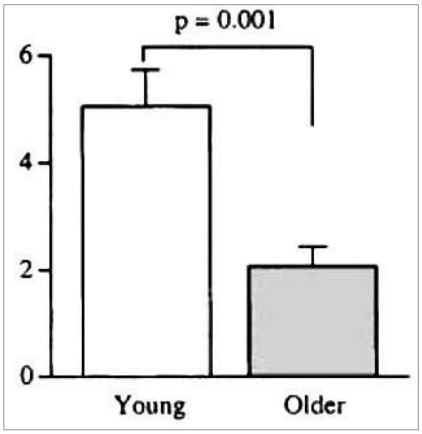

Figure 6.4. Reduced Vascular Sensitivity to an Alpha-adrenergic Receptor Agonist With Aging[8]**.** Increases in MAP responses to phenylephrine during ganglionic blockade were significantly reduced in men 65 years of age compared to those 25 years of age.

Significance

Central mechanisms underlying age-associated changes in SND regulation are not well understood. Many pathophysiological diseases including type II diabetes[75-78], hypertension[87], congestive heart failure[93-96], insulin resistance[75,82,97], and obesity[88-91] are associated with sympathetic dysfunction. Of particular interest is that the prevalence of these disease states is known to increase with advancing age. This is of critical importance because it is estimated that globally, the number of aged persons between 60 and 80 years or over will increase eight-fold by 2050[42]. Therefore, understanding the cellular and molecular mechanisms by which normal aging affects centrally-regulated SND is critical for understanding the relationships between the onset of critical disease and normal age-related changes in sympathetic neural function.

It is currently unclear how aging affects central mechanisms regulating efferent SND, although it is well established that progressive increases in sympathetic activation occur with advancing age independent of clinical disease[291]. The RVLM plays a critical role in regulating sympathetic nerve outflow and is considered the single most important source of tonic excitatory drive to sympathetic nerves involved in the maintenance of arterial pressure[4]. Both excitatory and inhibitory neural inputs serve as the predominate mechanism by which RVLM neural activity is tightly modulated[128,129,140,143]; therefore we suspect that the increase in sympathetic activation that is commonly observed in the aged is attributed to withdrawal of GABAergic inhibitory neural input to the RVLM. Because $GABA_A$ receptors are highly involved in mediating the inhibitory influence of GABAergic tone in the RVLM[4,172,177,205], the primary objective of the current study was

to examine whether aging differentially alters the RVLM GABAergic neurotransmitter gene expression profile in a rodent model of aging.

CHAPTER 7 - The Fischer 344 Rat as an Animal Model of Chronological and Biological Aging

The current understanding of physiological aging in mammals is based in large part on studies that have used young (5-6 mo. old), middle-aged (14-16 mo. old), and aged (24-25 mo. old) rats to examine the process of chronological aging[323]. Recently, studies have suggested that biological age rather than, or in addition to, chronological age may be a useful tool for evaluating the effect of age on physiological function[323-325]. Advanced stages of chronological and biological aging are associated with spontaneous reductions in body weight and food intake[325-327], collectively termed the "anorexia of aging"[326,328] or "failure to thrive"[329,330]. The development of anorexia occurs without definitive cause in healthy elderly individuals[331] or experimental rodent models of aging[326,332,333], and has been suggested to be a prelude to natural death[328,330,334,335].

Towards the end of their natural life spans, Fischer 344 (F344) rats exhibit reductions in food intake[324-326,336,337], body weight regulation[323,337,338], thermoregulation[323,337,339], and circadian rhythmicity[323,337,339]. Independent of disease, death occurs within an average of 3 weeks[326]; therefore, these deleterious events have been previously used as markers to define senescence[324-326,334,335,340,341]. Rats that display a failure to thrive and are rapidly progressing towards death are considered senescent[323-325,334,335,341]. However, similar to aging humans, the transition to senescence and subsequent progression towards death is highly variable between aged F344 rats. For example, McDonald and coworkers reported that the age at which

senescence occurs in F344 rats ranges between 24 and 31 mo. of age[323-325] and while all animals included in this particular study exhibited spontaneous rapid loss of body weight near the end of their life, the pattern of weight loss also varied[323-325]. In a separate study conducted by Coppola and others[338], F344 rats generally began spontaneous weight loss at 29 mo. of age, with an average duration of senescence before death lasting 16 ± 7 days. Therefore, biological rather than, or in addition to chronological guidelines are necessary for defining senescence. As a result, a descriptive definition of senescence was proposed by McDonald[337], describing a senescent rat as one that has lost an average of 10% total body weight over a period of 7-10 days[338].

The use of laboratory rodents as a model of human aging is well established. Importantly, it has been suggested that the underlying mechanisms mediating brain aging are similar among most mammalian species, including humans and rats[342]. A number of rodent aging models exist and have been widely used in studies involving aging; however, Fisher 344 rats have been used in the majority of studies designed to investigate the effects of aging on central regulation of SND using direct nerve recordings. Studies conducted by our laboratory revealed the presence of synchronized cardiac-related SND bursts in young (3-5 mo. old), middle-aged (12 mo. old), and weight-stable aged (24-25 mo. old) F344 rats. Direct nerve recordings of renal, adrenal, and splenic SND from a 24-mo. old (aged) F344 rat are shown in *figure 7.1*. Notably, muscle sympathetic nerve recordings from aged humans exhibit similar synchronized, cardiac-locked bursting patterns *(figure 7.1)*, thereby validating the appropriateness of

the F344 rat as an experimental model to examine age-related alterations in SND regulation.

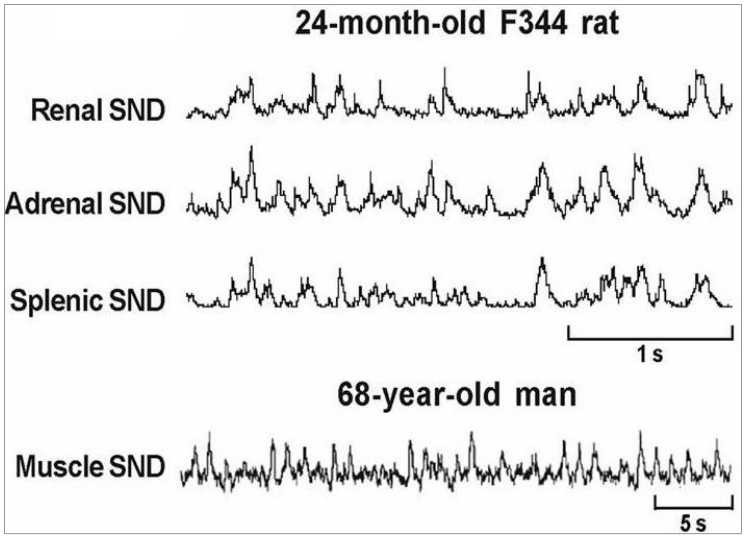

Figure 7.1. Cardiac-related Sympathetic Nerve Discharge Bursts in an Aged F344 Rat and an Aged Human. (R. Fels, unpublished data).

CHAPTER 8 - Materials and Methods

Description of Experimental Age Groups. Age-dependent alterations in RVLM constitutive gene expression profiles were examined in five healthy adult young (3-5 month old), four middle-aged (12 month old), and six aged (24-32 month old) male Fischer 344 (F344) rats obtained from the National Institutes of Health on Aging colony maintained by Harlan Sprague Dawley Laboratories. Twenty-four to thirty-two month old rats were further classified according to body weight. F344 rats demonstrating stable body weights were designated “presenescent” rats (n=3) while those exhibiting spontaneous and rapid weight loss were defined as “senescent” (n=3), following guidelines set by McDonald and Coppola[337] as previously described.

Animal Housing. Rats were housed in the animal care unit in Coles Hall in the College of Veterinary Medicine at Kansas State University. Veterinary care and animal husbandry procedures were administered by the veterinary and Animal Resource Facility staff at Kansas State University. All rats were housed in ventilated racks (Allentown) receiving 27 air exchanges per hour in 10.5 x 19 x 11-in. cages filled with Tek-fresh (Harlan) bedding and a paper towel for enrichment. All rats had free access to rack suspended standard rat chow (Laboratory Rodent Diet 5001) and water, and were maintained on a 12:12-hr. light-dark cycle. This study was approved by Kansas State University Institutional Animal Care and Use Committee and was completed in accordance with the guidelines for the care and use of laboratory animals of the National Institutes of Health and the American Physiological Society.

Health Monitoring of Aged and Senescent F344 Rats. The Fischer 344 rat is a commonly used experimental model of aging[343]. However, a high incidence of age-related pathology (e.g., testicular and pituitary adenomas, adrenal capsular fibrosis, and renal disease) has been associated with this rodent strain[326]. Of particular relevance to F344 health is the relatively high incidence of leukemia with advancing age[344-346]. Reportedly, 30 to 50% of aging F344 rats allowed to live a normal life span die from the effects of leukemia[344,346], which represents the leading natural cause of death in this strain[347-352]. Clinical indicators of leukemia include decreased redness of the eyes (anemia), spontaneous and substantial reductions in body weight, abdominal distension, weakness[345] and splenomegaly[345,346,353]. However, hematological examination with a specific focus on elevated white blood cell counts and a blood smear test[345,346] are required for confirmation. Therefore, careful health monitoring is required to distinguish healthy aged animals from unhealthy or diseased aged animals in order to reduce confounding etiological influences.

The health status of all 24-32 month old (aged) F344 rats was routinely monitored and recorded using select parameters that included body weight, food and water intake, and behavior profiles (alertness and activity, grooming and coat condition, and posture). Additionally, all aged animals were photographed upon arrival from the vendor and on the day of sacrifice in order to document any potential age-dependent changes in appearance. All aged rats were monitored for weight loss and overall health status at least once a week, because terminal weight loss is considered a marker for senescence[323-325,340]. Cages containing animals which exhibited weight loss of at least

3-5% body weight (e.g., transitioning into senescence) were immediately removed from the ventilated rack and placed on a cart within the same room to allow for more detailed observation. At this time moist standard rat chow was offered ad libitum in addition to free access of rack suspended dry rat chow. Due to the variability in the length of transition into full senescence (i.e., loss of at least 10% body weight[338]); transitional rats were monitored for weight loss and overall health status at least 2-3 times per week. During periods of observable rapid progression towards death, transitional animals were monitored on a daily basis and/or several times throughout the day when necessary. Prior to sacrifice, body condition and hydration scores were determined by the same attending veterinarian in order to prevent inter-individual variability in scoring. Spleen and brain morphology were evaluated postmortem by members of our laboratory and hematological analysis (CBC, serum chemistry) was performed by the Diagnostic Laboratory located at Kansas State University, College of Veterinary Medicine in order to identify leukemic rats or detect potential organ dysfunction. When necessary, necropsies and histopathology were performed by veterinary pathologists at Kansas State University. Collectively, this information was used to exclude aged animals that exhibited one of more of the following: dehydration, leukemia, observable brain tumors, significantly altered serum chemistry, and/or blood count profiles. Importantly, we have successfully used these criteria to detect and exclude a number of 24-32 mo. old F344 rats from our gene expression profiling studies, thereby increasing the likelihood that our findings are attributed to the aging process and not a disease state.

RVLM Tissue Micropunch. Animals were anesthetized with 5% isofluorane and decapitated by guillotine (Braintree Scientific). At this time, blood was collected and immediately submitted for hematological analysis. Brains were quickly removed, snap frozen in liquid nitrogen-cooled isopentane and stored at -80°C until further processing. RVLM tissue was isolated according to procedures established by Durgam and Mifflin[354] and Li and others[355]. Briefly, brains were gently mounted in a cooled rodent brain slicer (Braintree Scientific) using Tissue-Tek (Sakura) and cut into 500 µm coronal sections using a razor blade. Brain slices were subsequently placed onto chilled microscope slides and anatomical landmarks of the RVLM were identified as described by Paxinos and Watson[2] *(figure 8.1)* using a surgical microscope. Bilateral RVLM tissue, located at approximately -11.8 mm Bregma, was isolated using a sterile blunt-end 20 gauge needle and immediately placed in Trizol reagent (Sigma) in preparation for total RNA isolation.

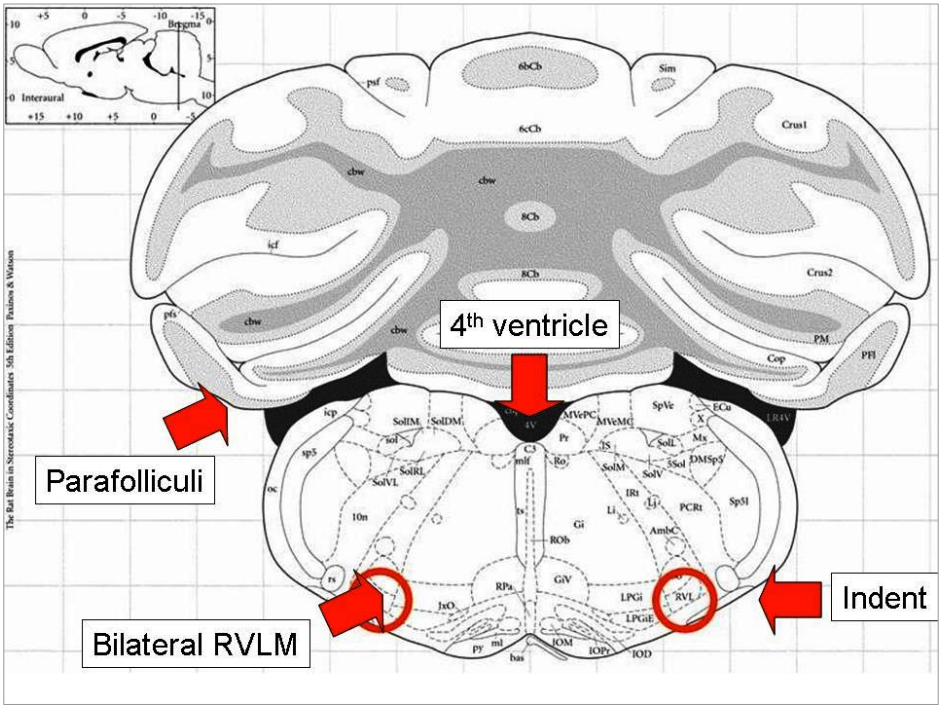

Figure 8.1. Anatomical Landmarks Used for RVLM Micropunching[2].

RNA Isolation and Reverse Transcription. Total RNA was isolated following standard protocol. RVLM tissue micropunches were homogenized in TRI Reagent (Sigma) using a battery operated hand-held pestle and total RNA purity and concentration were determined using a Nanodrop 1000 (Nanodrop). Potential genomic DNA contamination was removed and RVLM total RNA samples (1-2 µg) were reverse-transcribed using QuantiTect Reverse Transcription kit (Qiagen). Reverse transcription reactions were carried out at 42°C for 30 min. Resultant cDNA was used for individual and array-based SYBR Green® real-time PCR applications.

Array-based SYBR® Green Real Time PCR. Constitutive gene expression profiling was completed using the rat Neurotransmitter Receptors and Regulators RT^2 Profiler™ PCR array (#APRN-060A) commercially available from Superarray. Complementary DNA was used for real-time PCR based pathway-focused gene expression profiling. The expression of 84 genes involved in regulating the biological processes of neurotransmitter biosynthesis, uptake, transport, and signaling through neurotransmitter receptors, including receptors specific for GABA, acetylcholine, benzodiazepine, dopamine, glutamate, serotonin, somatostatin, and neuropeptides were included on this array in addition to five well-established housekeeping genes and three controls. Twenty-microliter reverse transcription reactions containing 1µg RVLM totRNA were mixed with an experimental cocktail containing RT^2 SYBR® green/fluorescein qPCR master mix (Superarray) and molecular grade water. Fluorescein was included as a reference dye for the purpose of normalizing the optics of the Bio-Rad iCycler. Twenty-five microliters of this cocktail were added to each well of the 96-well array so that the

final concentration of initial RVLM totRNA loaded per well was 9.0 ng. Real-time PCR reactions were performed under the following conditions on a Bio-Rad iCycler: 10 min at 95°C (cycle 1) followed by 40 cycles of 1 min at 60°C. SYBR® green fluorescence was detected and recorded from every well during the annealing step of each cycle. The efficacy of each reaction was verified by the running of a melt curve from 60°C to 95°C, increasing at 0.2°C/second. In addition, prior to commercial availability, Superarray had established that the RT^2 qPCR array had amplification efficiencies greater than 90% with amplicons ranging from 100-250 bp in size. Array-based real-time PCR results were analyzed using an Excel data analysis template available from Superarray. This format allows for the automatic calculation and interpretation of sample and control wells following the addition of an array-specific gene list and the threshold cycle data generated from the Bio-Rad iCycler. Data analysis was based on the comparative C_t method ($2^{-\Delta\Delta Ct}$) with normalization of the raw data to housekeeping genes that were included in the array. Expression levels were calculated as fold change relative to the gene expression of control samples. The threshold cycle (C_t) value for each gene was defined as the PCR cycle at which emitted fluorescence rose above a background level of fluorescence and was set at 40.4 with cycles 2-17 selected as baseline cycles using software provided with the Bio-Rad iCycler.

Data and Statistical Analysis. Results were compared between groups using Student's t-test. The overall level of statistical significance was set at $p < 0.05$.

CHAPTER 9 - Experiments and Data

Independent of clinical disease, SND is progressively activated with advancing age[5,8,87,287,288]. Virtually nothing is known about the effect of age on central neural mechanisms regulating efferent SND, although it is possible that the sympathetic activation that occurs with aging is attributed to alterations in brainstem neurotransmitters. Multiple supraspinal neural circuits are critically involved in the regulation of SND[4,6,15,16,18]; however, the RVLM serves as the final common pathway for the integration of centrally regulated pressor responses and efferent SND[4,12,18,129]. Excitatory neural inputs to the RVLM are normally balanced by powerful GABAergic inhibitory tone mediated in large part by $GABA_A$ receptors[128,129,140-143,145,356]. Therefore, the RVLM is a candidate neural substrate for investigating central molecular mechanisms regulating sympathetic outflow. In the present study ***we hypothesized that within the RVLM GABAergic inhibition of SND is withdrawn during aging, thereby shifting the balance of tonic influence towards enhanced RVLM excitation*** and potentially providing the mechanistic basis for age-related alterations in arterial pressure support. Importantly, ***nothing is known about the effect of advancing age on the role of RVLM neural circuits in SND regulation***; therefore we expect that testing of our hypothesis will provide insight into the RVLM GABAergic neurotransmitter system and further, central neural mechanisms regulating SND during advancing age.

Much of our current understanding about the diversity of central neurotransmitters has come from detailed cellular and molecular biological studies

using the rat[357]. Although F344, Brown Norway, Brown Norway x F344, and F344 x Brown Norway rodent strains have been widely used in studies involving aging, traditionally the preferred model of rodent aging and sympathetic nerve regulation is the F344 rat. Therefore, the molecular biological experiments presented hereafter were designed to examine potential age-related differences in constitutive $GABA_A$ receptor gene expression in the RVLM of F344 rats using an array-based real-time PCR approach (*figure 9.1*). Such an approach allowed for the simultaneous and comprehensive screening of 19 $GABA_A$-related genes and 65 genes related to other neurotransmitters, of which we focused on the muscarinic, nicotinic and dopaminergic receptor systems due to their reported involvement in $GABA_A$ receptor modulation. It is expected that our findings will reveal potential RVLM neurotransmitter mechanisms that may be involved in mediating age-dependent alterations in the regulation of efferent SND in the F344 rat.

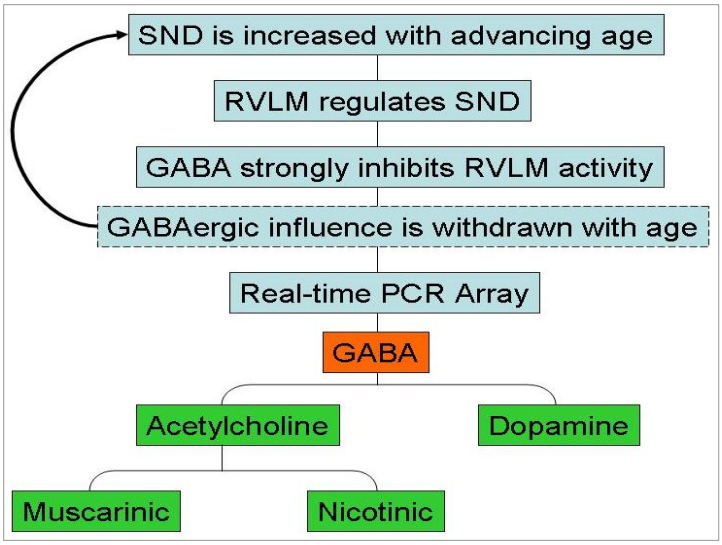

Figure 9.1. Rationale and Experimental Approach. We hypothesized that elevated SND with advanced aged may be attributed to withdrawal of GABAergic tone within the RVLM (text box with dotted lines). An array-based real-time PCR approach was used to determine the effects of aging on the expression of genes related to GABAergic, cholinergic and dopaminergic receptor systems.

Selection of F344 Rats for Real-time PCR Experiments

Five adult young (3-5 month old), four middle-aged (12 month old), and six aged (24-32 month old) F344 rats were selected to evaluate the effect of age on constitutive RVLM neurotransmitter system gene expression. In order to examine the effects of both chronological and biological age of 24-32 month old rats, aged rats were further classified as previously described, resulting in three presenescent and three senescent F344 rats available for real-time PCR analysis. Importantly, all rats were healthy and there were no indications of leukemia in any of the aged rats, thereby eliminating confounding factors that could potentially lead to misinterpretation of the data.

Real-time PCR RVLM Gene Expression Profiling: Selection of an Appropriate Housekeeping Gene

The use of real-time PCR to identify changes in gene expression has gained increasing popularity in aging research[358-360]. Relative quantification of gene expression data generated from real-time PCR experiments requires the identification of a housekeeping (HK) gene by which to normalize the expression of target genes[361,362] in order to eliminate potential experimental influences including differences in the amount and quality of starting material, differences in RNA preparation and efficiencies of reverse-transcription reactions[362]. According to Touchberry and coworkers, "housekeeping genes are essential endogenous regulatory genes that are involved in various processes in the cell, such as metabolism, cell structure, gene transcription, and homeostasis, and are therefore constitutively expressed"[363]. Ideally, the expression of HK genes should not be affected by experimental modulation[361], age[363,364], or cell and

tissue type[362]; therefore these criteria are commonly used to determine the appropriateness of candidate HK genes in real-time PCR analysis applications. However, selection of HK genes for use in real-time PCR has proved difficult as variations in HK gene expression have been reported for different tissue types[362,365-367] and several studies have reported changes in HK gene expression following a variety of experimental conditions including hypoxia[368], exercise[369,370], diet supplementation[371] and aging[363,364], demonstrating that it is highly unlikely that a "universal" HK gene exists that is appropriate for use under all experimental conditions[372,373].

The difficulty in selecting valid HK genes for aging studies was demonstrated by Chen and others, who reported large variations in the expression of five commonly used HK genes including, beta-actin (Actb), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ubiquitin C (UBC), hypoxanthine phosphoribosyl-transferase (Hprt) and cyclophilin A (CYPa). The expression of Actb, GAPDH, UBC, CYPa were found to be so variably expressed between young and aged rat livers, that only Hprt was determined to have the most stable expression suitable for accurate normalization[364]. A similar study conducted by Touchberry and coworkers examined the appropriateness of GAPDH, β_2-microglobulin (β_2M) and RNA polymerase 2a (polR2a) as HK genes in skeletal muscle from young and elderly human subjects and identified only GAPDH as the HK gene that demonstrated the most stable expression with age. Although the expression of β_2M was found to be significantly different between young and elderly age groups, it exhibited low variability within each age group, further demonstrating the difficulties of choosing valid HK genes for use in gene expression studies using aged subjects and different tissue

types[363]. To the author's knowledge, at the present time, the reports by Chen and Touchberry are the only ones that have comprehensively investigated and identified appropriate HK genes to use specifically in aging research[363,364]; however, reports that have extensively evaluated HK gene expression in the aging brain do not exist. Relatively few RVLM neurotransmitter gene expression studies using real-time PCR have been completed[374-377], none of which included aged experimental subjects. Therefore, at the present time there are no established guidelines for which HK genes are appropriate for investigating age-related changes in gene expression within the RVLM, as any differences in HK gene expression between age groups would make it difficult to make age-dependent comparisons with confidence and may inadvertently skew data such that differences are identified, when actually none exist[363]. Indeed, Chen and coworkers reported that when normalizing the expression of Cu/Zn-superoxide dismutase using a HK gene that was significantly decreased in aged rats (UBC), a significant difference in Cu/Zn-superoxide dismutase expression was detected between young and aged rat livers. However, use of Hprt, a HK gene stably expressed in the liver with age, for normalization resulted in no statistically significant differences in Cu/Zn-superoxide dismutase expression between age groups. Furthermore, when using UBC to normalize catalase levels in the livers of young and aged rats, it was determined that aged rats expressed 31.20% less catalase than young rats; however, when catalase expression was normalized to Hprt, aged rats were found to express 57.73% less catalase[363,364]. This situation clearly highlights the necessity for a stably expressed HK gene for use in studies using aged experimental subjects, as normalization of real-time PCR data with an inappropriate HK gene can result in misinterpretation of data.

Beta-actin is a well established housekeeping gene[361,366,378,379] and has been used in a number of gene expression studies using a variety of tissues and cell types including the spleen[116], brain[367,380], skeletal muscle[366], and liver[367], demonstrating the stability of this gene product across a number of tissues; an important characteristic of a suitable HK gene. In a recent study evaluating the expression of thirteen different HK genes in sixteen different tissues including the brain, spleen and heart, actin was shown to be highly expressed in all tissues examined[362]. However, none of the studies described above investigated the effect of age on brain actin gene expression, although Slagboom and others reported that age did not affect beta-actin mRNA expression in the brains of female inbred rats ranging from 6 and 24-months to 36 months of age[367]. While the work of Slagboom did not comprehensively examine the expression of a variety of HK genes in the aged rat brain, it does suggest that beta-actin may be a potential candidate for use in real-time PCR

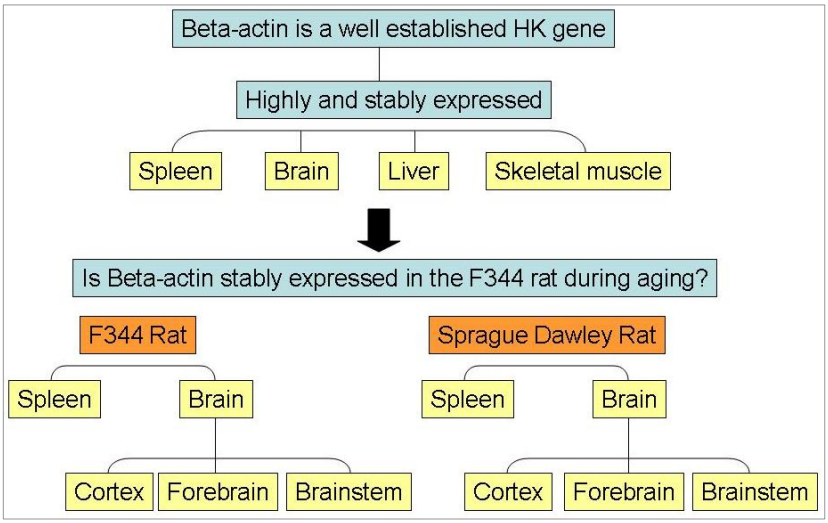

Figure 9.2. Rationale and Experimental Approach for Determining the Appropriateness of Beta-actin as a HK Gene in F344 Rats. Beta-actin has been used as a HK gene in a number of studies involving a variety of tissues and experimental treatments. Our experimental approach for confirming beta-actin as a suitable HK gene for use in aging studies involved the examination of its expression in the spleen and regionally distinct parts of the brain in F344 rats. Sprague Dawley rats were included in order to determine whether actin expression is influenced by stress.

experiments designed to identify age-related changes in gene expression in the brains of F344 rats *(figure 9.2)*.

Establishing Beta-actin as an Appropriate HK Gene for Use in Real-time PCR Experiments Using Aged Subjects

The 2-ΔΔCt method is commonly used to calculate relative changes in gene expression determined from real-time PCR experiments; however, in order for such calculations to be accurate, the amplification efficiencies of target and housekeeping

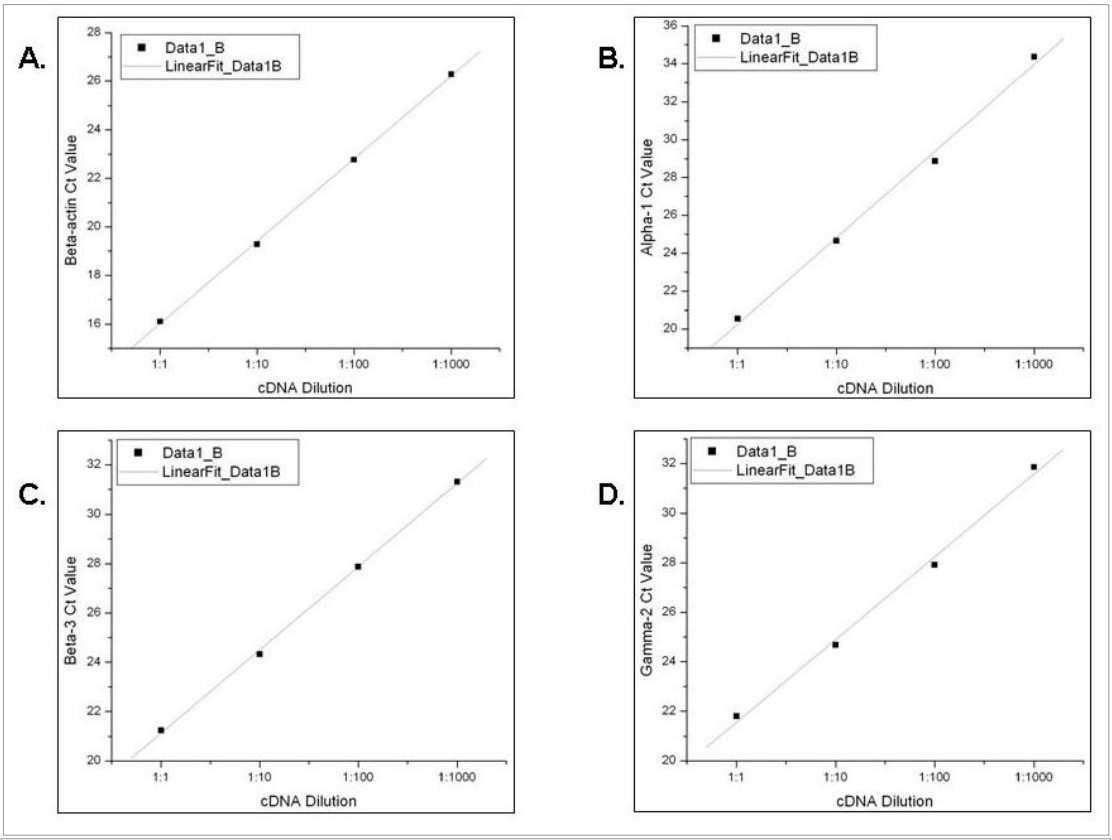

Figure 9.3. Amplification Efficiency Curves. The amplification efficiency of primers specific for beta-actin (A) and $GABA_A$ receptor subunits $alpha_1$ (B), $beta_3$ (C) and $gamma_2$ (D) was determined using a randomly selected F344 rat cortical brain sample.

genes must be equivalent[361]. Our laboratory determined the annealing efficiencies of primers specific for beta-actin as well as select target genes using commercially

available primers (Superarray) and a randomly chosen cortical brain cDNA sample from a presenescent F344 rat. Standard curves were generated using relative cDNA concentrations and threshold cycle (C_t) values generated from SYBR® Green real-time PCR. *Figure 9.3* shows representative efficiency curves for beta-actin (*A*) and $GABA_A$ receptor subunits $alpha_1$ (*B*), $beta_3$ (*C*) and $gamma_2$ (*D*). The linear correlation coefficient (R^2) of all genes examined was highly similar, with R^2 values ranging from 0.997-0.999, suggesting that potential identification of relative changes in the expression of specific genes of interest using C_t values generated from real-time PCR experiments would be accurate when normalized to beta-actin.

Because beta-actin amplification efficiency was found to be highly similar to those of select $GABA_A$ receptor subunits, we examined whether beta-actin expression was altered by aging in the spleens of young (3-5 month old) and weight stable

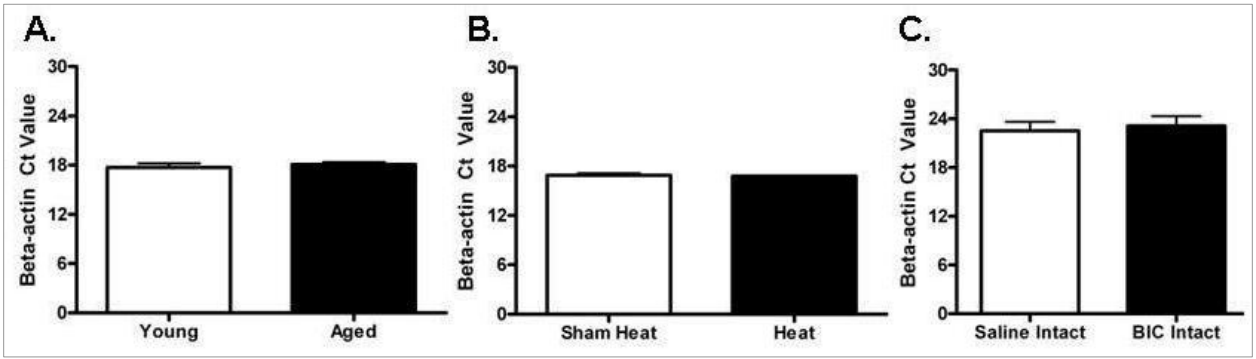

Figure 9.4. Beta-actin is Stably Expressed in the Spleen of F344 and SD Rats. The influence of age (A) and stress (B) do not markedly affect splenic actin gene expression. F344 rats were used to determine whether aging affects splenic beta-actin gene expression (A). Effects of experimental modulation on splenic beta-actin gene expression were investigated using SD rats (B, C).

presenescent (24-32 month old) F344 rats under basal conditions. We selected the spleen because beta-actin is highly expressed in this tissue and it has been used in studies involving Sprague Dawley (SD) rats previously published by our

laboratory[116,117]. As *figure 9.4(A)* demonstrates, the average C_t value of splenic beta-actin did not differ between young (17.71 ± 0.50; n=4) and presenescent (aged; 18.08 ± 0.29; n=4) F344 rats. Similarly, beta-actin expression was found to be consistent in the spleens of young SD rats exposed to short bouts of heat stress (16.75 ± 0.10; n=4) and in untreated controls (sham; 16.90 ± 0.23; n=2) (*figure 9.4B*). Splenic beta-actin expression was also unaffected following the administration of saline (22.55 ± 1.07; n=4) or the $GABA_A$ receptor antagonist bicuculline (BIC) into the RVLM in splenic nerve intact young SD rats (23.15 ± 1.20; n=4) (*figure 9.4C*). These data suggest that beta-actin is highly expressed in the spleen and is relatively unaffected by potential influences of age (young, presenescent) and experimental modulation (heat, saline or BIC RVLM), thereby meeting several criteria for what is considered an acceptable HK gene to be used for normalization purposes.

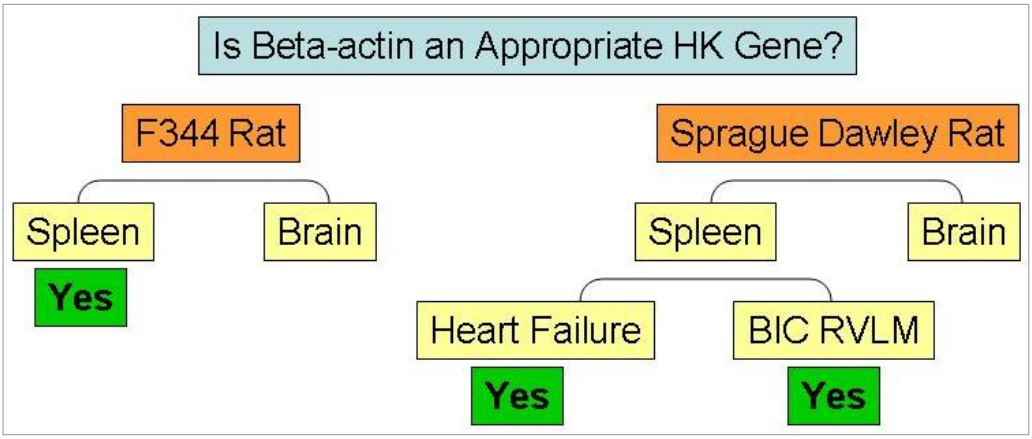

Figure 9.5. Beta-actin Gene Expression is Stable in the Spleen. Real-time PCR analysis indicated that aging does not affect beta-actin gene expression in the spleen of F344 rats. Beta-actin expression was consistently expressed in the spleen of Sprague Dawley rats with heart failure and following RVLM microinjection of bicuculine and splenic denervation.

Our real-time PCR studies using the spleens of F344 and SD rats confirmed that beta-actin is an acceptable HK gene in studies involving different ages and experimental manipulation thereby meeting the criteria of 1) abundant expression and 2) expression is unaltered with treatment (here, age may be considered a treatment, *(figure 9.5)*. Therefore, we completed experiments to determine whether beta-actin was an appropriate HK gene in tissues from distinct regions of the brain. Specifically, we examined beta-actin gene expression in the cortex, forebrain (paraventricular nucleus of the hypothalamus; PVN) and brainstem (RVLM) to establish beta-actin as an HK gene for use in real-time PCR studies designed to address the primary objective of the current study; whether aging affects neurotransmitter gene expression in the RVLM of F344 rats.

Individual real-time PCR analysis of cortical tissue micropunches revealed average actin C_t values of 19.73 ± 0.62, 19.01 ± 0.11, 20.28 ± 0.53 and 19.14 ± 0.13 from adult young (3-5 month old; n=4), middle-aged (12 month old; n=4), weight stable

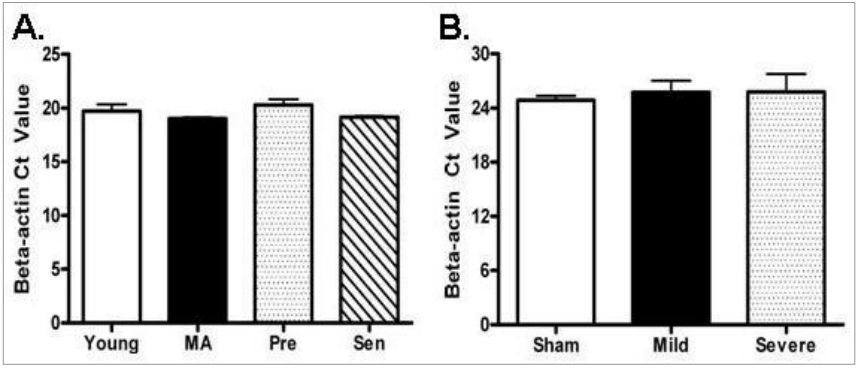

Figure 9.6. Beta-actin is Stably Expressed in the Cortex of the Rat Brain. Beta-actin expression did not differ between young, middle-aged, presenescent and senescent F344 rats (A). Sprague Dawley rats were included in the analysis to determine whether stress affected cortical beta-actin expression (B). Stress did not affect actin expression.

presenescent (24-32 month old; n=5) and senescent (24-32 month old; n=5) F344 rats, respectively (*figure 9.6A*). This suggested that similar to the spleens of F344 rats, beta-actin gene expression is not affected by advancing age in cortical brain tissue. Similarly, no difference in beta-actin expression was observed in cortical tissue micropunches from young sham operated (24.87 ± 0.47; n=3) and young SD rats suffering from mild (25.73 ± 1.29; n=3) or severe (25.80 ± 1.98; n=3) forms of heart failure (*figure 9.6B*), indicating that age or stress-related (heart failure) influences do not markedly affect cortical actin gene expression.

Importantly, our studies indicated that cortical beta-actin gene expression was not markedly influenced by stress in SD rats and that it was consistently expressed in the cortex of young, middle-aged, presenscent and senescent F344 rats, suggesting its suitability as a HK gene for normalization purposes in aging studies using F344 brain tissue. Therefore, we examined potential age-associated changes in the expression of

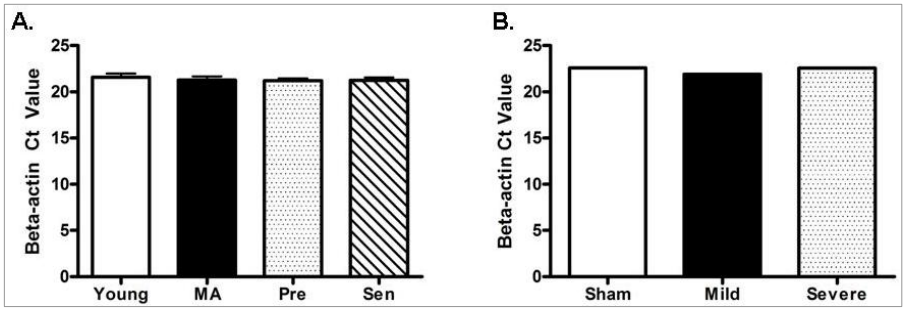

Figure 9.7. Beta-actin Gene Expression in the PVN is Not Influenced By Age or Heart Failure. Beta-actin expression did not differ between young, middle-aged, presenescent and senescent F344 rats (A) or in SD rats suffering from heart failure (B).

beta-actin in more discrete regions of the brain including the PVN and RVLM, two autonomic nuclei located in the forebrain and brainstem, respectively. Individual real-time PCR analysis of F344 paraventricular nucleus of the hypothalamus (PVN) beta-

actin gene expression revealed similarities in C_t values among young (21.58 ± 0.39; n=4), middle-aged (21.25 ± 0.40; n=4), presenescent (21.19 ± 0.27; n=5) and senescent (21.24 ± 0.31; n=5) age groups (*figure 9.7A*), confirming previous findings in the spleen and cortex of F344 rats. Importantly, no differences in PVN actin expression were observed for SD rats that were sham operated (22.6 ± 0.17; n=3) or suffering from heart failure (21.91 ± 0.36, mild n=3; 22.58 ± 0.07, severe n=3) (*figure 9.7B*), providing strong evidence that neither age or stress (heart failure) influence beta-actin expression in the rodent PVN.

Consistent beta-actin expression in the spleen, cortex and PVN of F344 and SD rats regardless of age or stressor lead us to hypothesize that aging and stress would not affect beta-actin expression in the RVLM. However, expression studies of RVLM tissue from young (n=5), middle-aged (n=4), presenescent (n=3) and senescent (n=3) F344 rats using real-time PCR provided unexpected results. In contrast to what was observed in the spleen, cortex and PVN, beta-actin was not stably expressed in the

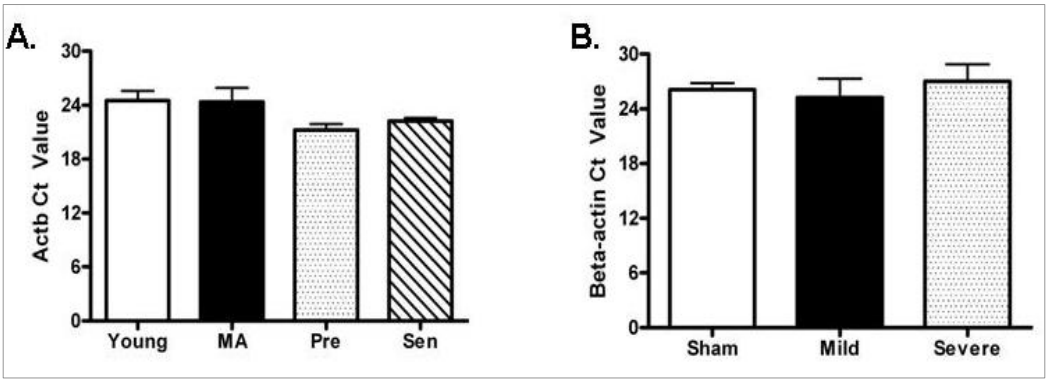

Figure 9.8. Aging Affects Beta-actin (Actb) Gene Expression in the RVLM. Presenescent and senescent F344 rats expressed higher levels of RVLM beta-actin than young and middle-aged F344 rats (A). In contrast, beta-actin expression in the RVLM was not influenced by heart failure in SD rats (B), demonstrating the changes that we observed were specific to aging.

RVLM during aging in the F344 rat although it was unaffected by heart failure in SD rats *(figure 9.8)*. Age-dependent beta-actin expression patterns were consistently observed between young and middle-aged F344 rats, and presenscent and senescent F344 rats. Young and middle-aged rats exhibited average RVLM C_t values of 24.50 ± 1.08 and 24.35 ± 1.55 while presenescent and senescent rats exhibited average C_t values of 21.23 ± 0.64 and 22.23 ± 0.35, respectively (*figure 9.8A*), demonstrating a 2.7 cycle difference in the expression of beta-actin between young/middle-aged and presenescent/senescent F344 rats. Therefore, it appears that there are fundamental differences in RVLM beta-actin gene expression with advancing age, although this was not the case when we examined RVLM beta-actin gene expression in young sham operated (n=3) or young SD rats experiencing heart failure (n=6) (*figure 9.8B*).

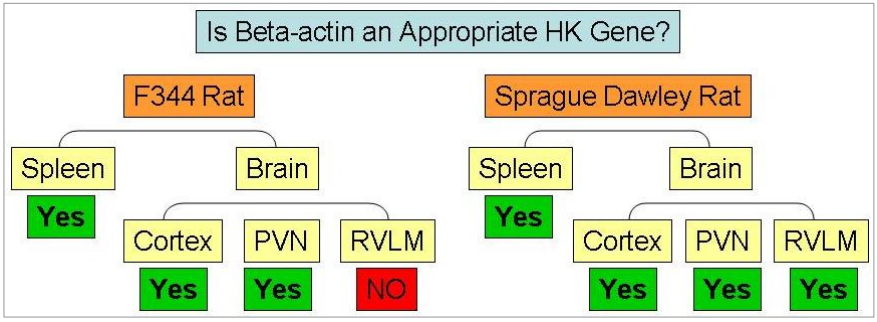

Figure 9.9. Beta-actin is an Acceptable HK Gene for the Spleen, Cortex and PVN But **Not** the RVLM in F344 Rats. We have completed studies in the F344 and SD rat to determine if beta-actin is a suitable HK gene for use in normalizing real-time PCR data. Aging did not influence beta-actin expression in the spleen, cortex or PVN of the F344 rat. Multiple stressors did not affect beta-actin gene expression in the spleen, cortex and PVN of SD rats. While beta-actin expression in the RVLM did not change during heart failure in SD rats, aging markedly increased beta-actin expression in the RVLM of F344 rats, demonstrating that beta-actin is not an acceptable HK gene for use in real-time PCR analysis of RVLM gene expression in aging studies.

The variable effect of age on actin gene expression in the RVLM was unexpected because all previous real-time PCR experiments conducted using tissue from the spleen, cortex and PVN had demonstrated stable expression despite differences in tissue type, age or stress *(figure 9.9)*. However, in the RVLM we observed similar

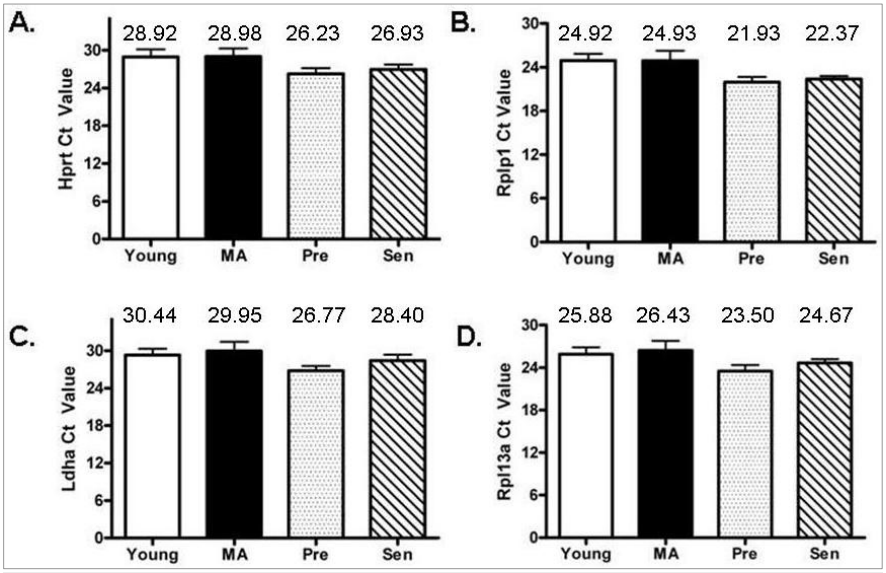

Figure 9.10. HK Gene Expression is Increased With Advancing Age in the RVLM of F344 Rats. Hprt (A), Rpl1 (B), Ldha (C) and Rpl13a (D) were higher in the RVLM of presenescent and senescent F344 rats compared to young and middle-aged F344 rats. A difference of 2.5 cycles on average exists between all presenescent/senescent and young/middle-aged HK values, indicating that between 12 and 24 months of age transcriptional regulation of genes considered to be stably expressed is substantially altered. Therefore, these housekeeping genes, in addition to beta-actin, are not appropriate for normalizing real-time PCR data generated from aging studies using the RVLM of F344 rats.

average actin C_t values between young/middle-aged and presenescent/senescent age groups but not across all age groups. In fact, we observed on average, a 2.7 cycle difference in the average actin (Actb) C_t value between young/middle-aged and presenescent/senescent age groups. Of particular interest is the fact that a 2.5 cycle difference on average, was also observed in the RVLM of four other well-established

housekeeping genes represented on the same array that generated the actin C_t value data presented here, including Hprt (hypoxanthine guanine phosphoribosyl transferase), Ldha (lactate dehydrogenase A), Rplp1 (60s acidic ribosomal protein P1) and Rpl13a (ribosomal protein L13a) *(figure 9.10)*.

Differences in housekeeping gene expression can result in the misinterpretation of real-time PCR studies. Standard criteria for an appropriate HK gene for normalization purposes are 1) abundantly expressed, 2) stably expressed in a variety of tissues, 3) unaffected by experimental conditions and 4) does not differ by more than 1 cycle between experimental groups[381]. Our initial studies indicated that beta-actin is a stably expressed gene in multiple tissues under multiple experimental conditions, including aging. However, beta-actin differed between young/middle-aged and presenescent/senescent rats by 2.7 cycles. It is standard procedure to choose an HK gene that does not vary by more than 1 cycle between experimental groups because this equates to over a 2-fold difference, the level at which many studies consider to be the benchmark for significance in differences in gene expression[363,382]. Fold change is generally calculated as $2^{\wedge}$ whereby "2" accounts for the doubling of PCR product per amplification cycle and "^" is the cycle number. Therefore, for every amplification cycle during the real-time PCR reaction, gene products double ($2^{\wedge 1}$ = 2-fold change)[382]. The 2.7 cycle difference in beta-actin expression that we observed between young/middle-aged and presenescent/senescent rats is roughly equivalent to a 6.5-fold difference ($2^{\wedge 2.7}$ = 6.5) in HK gene expression, which does not meet the requirement that a HK gene only vary between experimental groups by 1 cycle (or 2-fold). Therefore, use of a

HK gene with such a high fold difference between young/middle-aged and presenescent/senescent age groups in $2^{-\Delta\Delta Ct}$ calculations would result in the failure to detect significant changes altogether (e.g., those over 2-fold) or in the overexaggeration of the magnitude of the fold-change for true differences in target gene expression between age groups. It is estimated that in order for a target gene to overcome such a difference in fold-change HK expression and be considered significant, the target gene would need to be up- or downregulated 8.5-fold (6.5-fold difference between HK values + 2-fold benchmark for significance = 8.5-fold). This demonstrates how changes in gene expression that are significant when using a suitable HK for normalization may appear to be unchanged when using an HK gene that varies between experimental groups by more than 1 cycle because the large difference between HK genes (6.5-fold) will mask any changes that are less than 6.5-fold. The effect of using an inappropriate HK gene for normalization was demonstrated by Chen and coworkers when they normalized Cu/Zn-superoxide dismutase expression using a HK gene that was significantly decreased in aged rats (UBC). A significant difference in Cu/Zn-superoxide dismutase expression was detected between young and aged rat livers using UBC; however, use of an appropriate HK gene (Hprt) resulted in no difference in Cu/Zn-superoxide dismutase expression between age groups. A similar misinterpretation of data was demonstrated when catalase expression levels in the livers of young and aged rats were normalized to UBC. Using this approach it appeared that aged rats expressed 31.20% less catalase than young rats; however, when catalase expression was normalized to Hprt, aged rats were found to express 57.73% less catalase[363,364]. This clearly illustrates how differences in HK gene expression between age groups can

wrongly identify changes when none exist or “mask” the degree to which actual changes occur.

In conclusion, our studies examining RVLM beta-actin gene expression during aging demonstrate a limitation to our analytical approach in that we were unable to make pairwise comparisons in RVLM gene expression between young, middle-aged, presenescent and senescent F344 rats when using beta-actin as a HK gene (or Hprt, Rplp1, Ldha, Rpl13a) for real-time PCR experiments. However, we were able to quantitate RVLM gene expression profiles between young and middle-aged, and presenescent and senescent F344 rats using beta-actin as the normalization factor in $2^{-\Delta\Delta Ct}$ calculations because average actin C_t values only differed between young and middle-aged rats by 0.15 and by 1.00 between presenescent and senescent rats. This to our knowledge, is the first report that substantial changes occur in the transcriptional regulation of well established HK genes, including beta-actin, between 12 and 24 months of age in the RVLM of F344 rats.

Array-based RVLM Neurotransmitter System Gene Expression Profiling

Beta-actin was selected as an acceptable HK gene for use in identifying changes in constitutive RVLM gene expression between young and middle-aged, and presenescent and senescent F344 rats, although it was not appropriate for making pairwise comparisons between young, middle-aged, presenescent and senescent rats. Neurotransmitter system gene expression profiling was accomplished using a rat Neurotransmitter Receptors and Regulators RT^2 Profiler™ real-time PCR array

(Superarray) developed specifically for use in the Bio-Rad iCycler. This novel array system allows for the relative quantification of eighty-four genes involved in regulating the biological processes of neurotransmitter biosynthesis, uptake, transport, and signaling through neurotransmitter receptors, including receptors specific for GABA, acetylcholine, benzodiazepine, dopamine, glutamate, serotonin, somatostatin, and neuropeptides. Data analysis was based on the comparative C_t method ($2^{-\Delta\Delta Ct}$) with normalization of the raw data to beta-actin. Although Suzuki and others[379], in a review of all gene expression analyses published in 1999, found that over 90% used only one HK or reference gene, with beta-actin among the HK genes more widely used, only until recently has it been suggested that the use of more than one HK gene be included during normalization procedures to provide more accurate comparative C_t calculations[364,372,383]. Therefore, target gene expression was normalized to beta-actin alone and resultant fold-changes were validated by normalizing the data to the combined average C_t value of Hprt, Ldha, Rplp1 and Rpl13a (HK genes also represented on the array). Age-associated differences in target gene expression are presented as fold change and selection criteria for significance was set at a two-fold change, which is a widely accepted standard for the quantification of gene expression[363,364].

Young vs. MA RVLM Neurotransmitter System Gene Expression

Although the neurotransmitter array allowed us to examine the constitutive expression of 84 genes in the RVLM, we found that overall, 17 out of 84 genes (20%) were up- or down-regulated at least two-fold in magnitude between young and middle-

aged F344 rats. 16/17 genes (94%) were upregulated while 1/17 (6%) was down-regulated. Of these 17 genes, we chose a select few as genes of interest due to their known involvement in regulation of sympathetic nerve activity and grouped them into themes according to their known function in the central nervous system; therefore, results will be discussed accordingly.

$GABA_A$ Receptor Subunits: RVLM expression of $GABA_A$ receptor $alpha_5$ subunit (Gabra5) decreased and $GABA_A$ receptor subunits $alpha_2$ (LOC289606), $alpha_6$ (Gabra6) and theta (Gabrq) were increased in middle-aged compared to young F344 rats. No significant differences were detected for $GABA_A$ receptor subunits $alpha_{1,3,4}$ (Gabra1, 3, 4), $beta_{2,3}$ (Gabrb2-3), delta (Gabrd), epsilon (Gabre), $gamma_{1,2}$ (Gabrg1-2), pi (Gabrp) or $rho_{1,2}$ (Gabrr1-2).

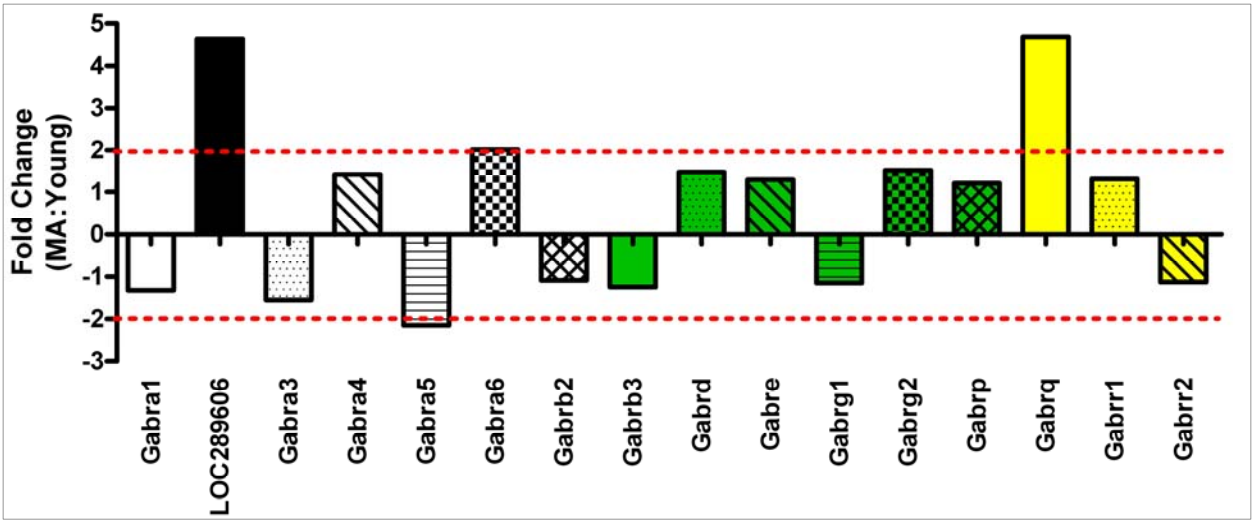

Figure 9.11. Fold-Change Differences in $GABA_A$ Receptor Subunit Expression.

Neurotransmitter Catabolism and Biosynthesis: Expression of choline acetyltransferase (Chat) and a member of the choline transporter family (Slc5a7) increased in the RVLM of middle-aged compared to young F344 rats. No significant (e.g., over 2-fold) change in 4-aminobutyrate aminotransferase (GABA-T, Abat) expression was observed.

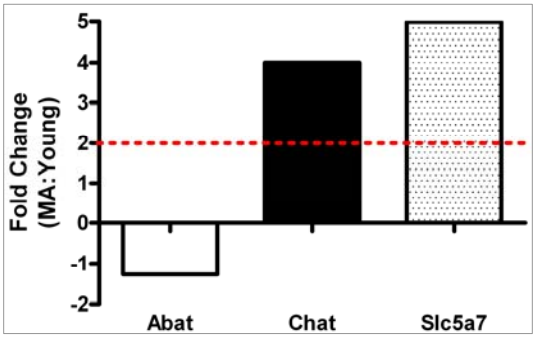

Figure 9.12. Fold-Change Differences in the Expression of Genes Involved in GABA Catabolism and Acetylcholine Synthesis.

Muscarinic Acetylcholine Receptor Subtypes: Expression of muscarinic acetylcholine receptor subtype 2 (M2, Chrm2) and 5 (M5, Chrm5) increased in the RVLM of middle-aged F344 rats compared to that of young. No significant changes were observed for subtypes M1 (Chrm1), M3 (Chrm3), and M4 (Chrm4).

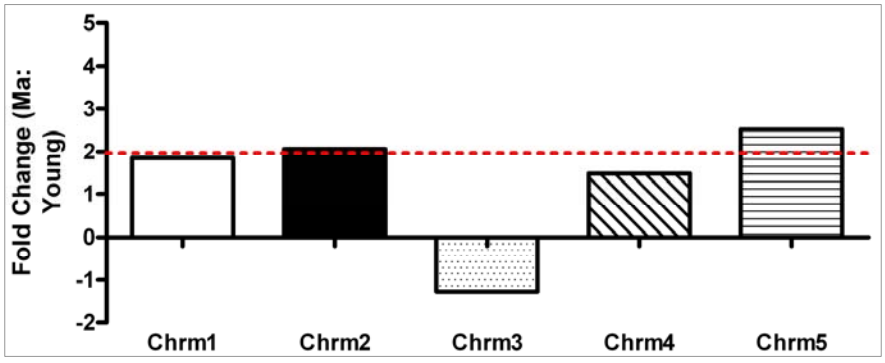

Figure 9.13. Fold-Change Differences in Muscarinic Receptor Subtype Expression.

Nicotinic Acetylcholine Receptor Subunits: Nicotinic acetylcholine receptor alpha polypeptide$_3$ (Chrna3), beta polypeptide$_1$ (Chrnb1), and beta polypeptide$_4$ (Chrnb4) gene expression increased in the RVLM of middle-aged F344 rats compared to young. No significant changes were observed in the expression of alpha polypeptide$_{1,2}$ (Chrna1-2) and 4-6 (Chrna4-6), beta polypeptide$_2$ (Chrnb2), 3 (Chrnb3), or for polypeptides delta (Chrnd), epsilon (Chrne) or gamma (Chrng) subunits.

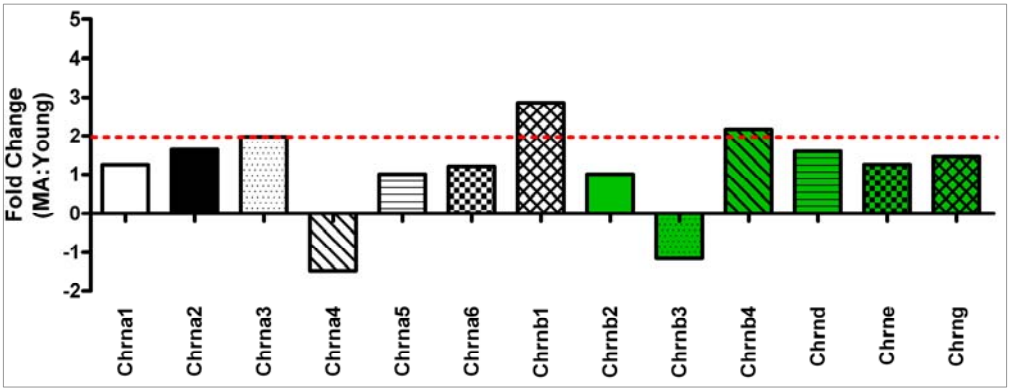

Figure 9.14. Fold-Change Differences in Nicotinic Receptor Subunit Expression.

Dopamine Receptor Subtypes: Dopamine receptor subtype 1a (Drd1a) expression increased in the RVLM of middle-aged compared to young F344 rats. No significant changes were observed for subtypes Drd2-5.

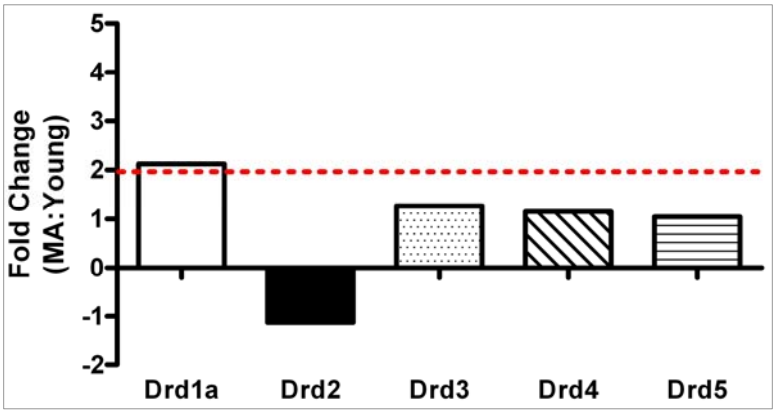

Figure 9.15. Fold-Change Differences in Dopamine Receptor Subtype Expression.

Presenescent vs. Senescent RVLM Neurotransmitter System Gene Expression

Array-based SYBR Green® real-time PCR analysis determined that overall, 36 out of 84 genes (43%) were up- or down-regulated between presenescent and senescent F344 rats by at least two-fold in magnitude compared to 17/84 genes (20%) identified in the young/middle-aged group comparison. Of these 36 genes, we chose a select few as genes of interest due to their known involvement in regulation of sympathetic nerve activity and grouped them into themes according to their known function in the central nervous system; therefore, results will be discussed accordingly.

$GABA_A$ Receptor Subunits: RVLM gene expression of $GABA_A$ receptor subunit $alpha_1$ (Gabra1), $alpha_6$ (Gabra6), $beta_3$ (Gabrb3), delta (Gabrd), $gamma_2$ (Gabrg2), pi (Gabrp), and rho_2 (Gabrr2) increased and $alpha_3$ (Gabra3) decreased in senescent compared to presenescent F344 rats. No significant differences in the expression of $alpha_2$ (LOC289606), $Gabra_{4,5}$, $Gabrb_2$, Gabre, $Gabrg_1$, Gabrq and $Gabrr_1$ were observed.

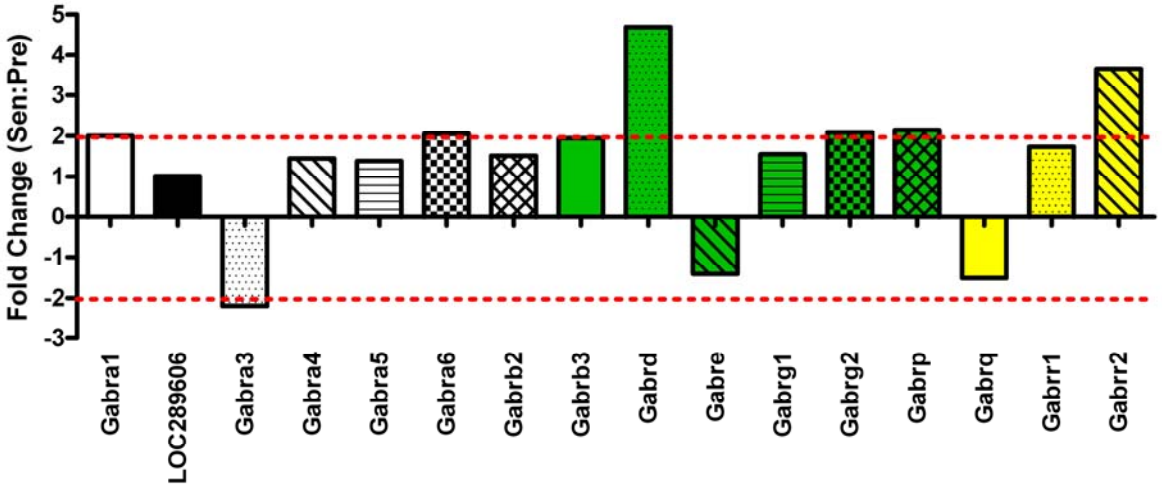

Figure 9.16. Fold-Change Differences in $GABA_A$ Receptor Subunit Expression.

Neurotransmitter Catabolism and Biosynthesis: RVLM gene expression of 4-aminobutyrate aminotransferase (GABA-T, Abat) and choline acetyltransferase (Chat) increased in senescent compared to presenescent F344 rats. No significant differences in Slc5a7 were observed.

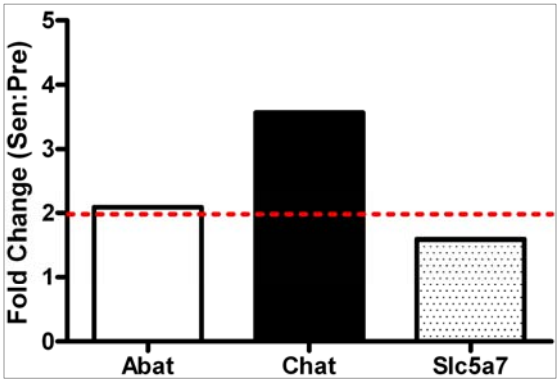

Figure 9.17. Fold-Change Differences in the Expression of Genes Involved in GABA Catabolism and Acetylcholine Synthesis.

Muscarinic Acetylcholine Receptor Subtypes: Expression of M1 (Chrm1) and M3 (Chrm3) were increased in the RVLM of senescent compared to presenescent F344 rats. No significant differences in the expression of Chrm2, 4-5 were observed.

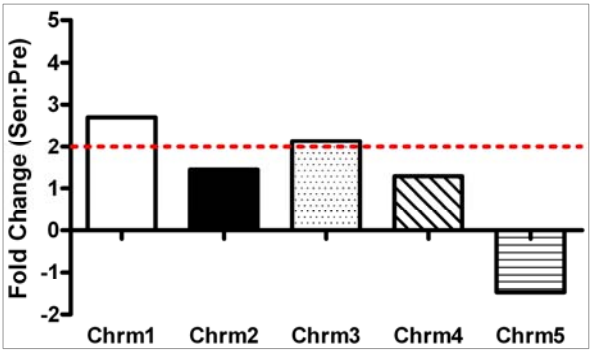

Figure 9.18. Fold-Change Differences in Muscarinic Receptor Subtype Expression.

Nicotinic Acetylcholine Receptor Subunits: Alpha polypeptide$_{1\text{-}3,5,6}$ (Chrna1-3, 5-6), beta polypeptide$_{1,3,4}$ (Chrnb1, 3-4) and polypeptide epsilon (Chrne) expression increased in the RVLM of senescent compared to presenescent F344 rats. No significant changes in Chrna$_4$, Chrnb$_2$, Chrnd and Chrng expression were observed.

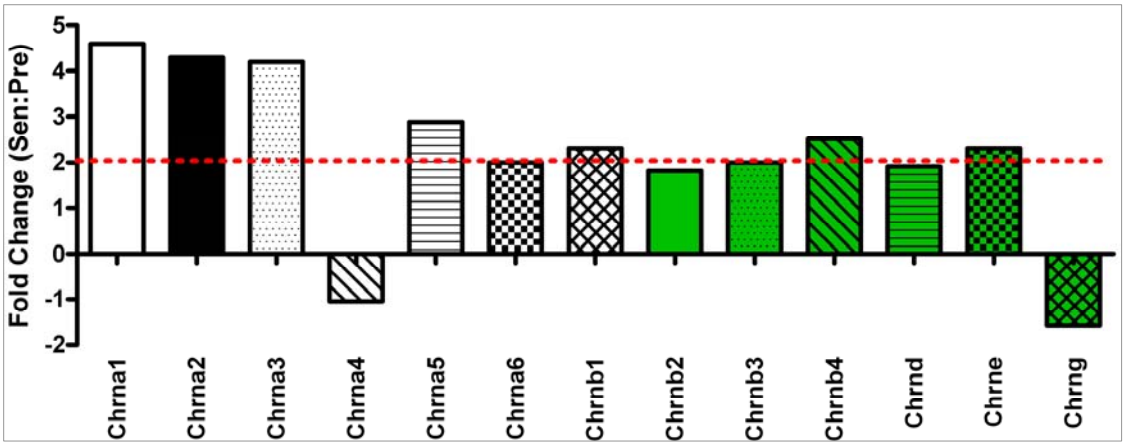

Figure 9.19. Fold-Change Differences in Nicotinic Receptor Subunit Expression.

Dopamine Receptor Subtypes: Expression of dopamine receptor subtype 1a (Drd1a) and dopamine receptor subtypes 3-5 (Drd3-5) increased in the RVLM of senescent compared to presenescent F344 rats. No significant changes in Drd2 were observed.

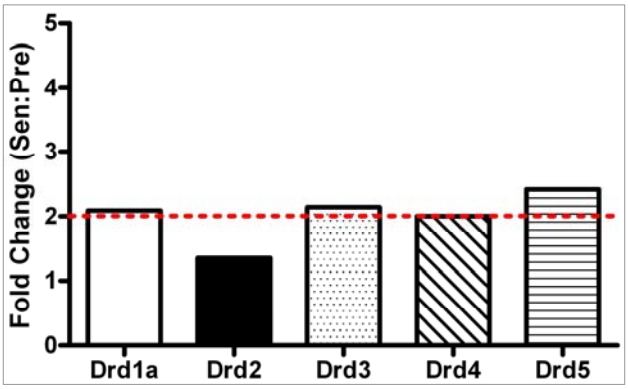

Figure 9.20. Fold-Change Differences in Dopamine Receptor Subtype Expression.

CHAPTER 10 - Discussion

Seven new findings concerning the effect of age on constitutive RVLM neurotransmitter gene expression in F344 rats are presented in the current study. First, of the five housekeeping (HK) genes that we examined under basal conditions (Actb, Hprt, Ldha, Rp1p1, Rpl13a), none demonstrated stable expression in the RVLM during the aging process, particularly between 12 and 24 months of age. Second, $alpha_1$, $alpha_6$, $beta_3$, delta, $gamma_2$, pi and rho_2 $GABA_A$ receptor subunit mRNA expression was higher while $alpha_3$ was lower in senescent compared to presenescent rats. Expression of $alpha_2$ (LOC289606), $alpha_6$ and theta transcripts was higher while $alpha_5$ was lower in middle-aged compared to young rats, demonstrating transcriptional regulation of $GABA_A$ receptor subunit expression is differentially altered during middle-age and senescence. Third, GABA-transaminase (GABA-T) mRNA expression was higher in senescent compared to presenescent rats (young and middle-aged did not differ), while glutamate decarboxylase 65 and 67 (GAD65, GAD67) remained unchanged with age. This finding suggests potential shifts in the balance between GABA synthesis and metabolism in the RVLM of senescent rats. Fourth, muscarinic receptor subtype 1 (M1) and 3 (M3) mRNA expression was higher in senescent compared to presenescent rats but remained unchanged in young and middle-aged rats, although muscarinic receptor subtype 2 (M2) and 5 (M5) were higher in middle-aged compared to young rats. Differential expression of muscarinic receptor subtypes may reflect altered muscarinic-mediated modulation of GABAergic synaptic currents in the RVLM of middle-aged and senescent rats. Fifth, nicotinic acetylcholine receptor (nAChR) subunit mRNA expression of alpha $polypeptide_{1\text{-}3,5,6}$, beta $polypeptide_{1,3,4}$ and

polypeptide epsilon was higher in senescent than presenescent rats. The expression of alpha polypeptide$_3$ and beta polypeptide$_{1,4}$ differed between middle-aged and young rats, indicating variable nAChR expression within the RVLM of middle-aged and senescent rats and possible heightened cholinergic transmission within the RVLM of senescent rats. Sixth, mRNA expression of acetylcholinesterase (Ache) was not substantially affected by advancing age. However, expression of choline acetyltransferase (Chat) and a member of the choline transporter family (Slc5a7) were higher in middle-aged compared to young rats and Chat mRNA was higher in senescent compared to presenescent rats (Slc5a7 remained unchanged). These findings demonstrate that age-related changes in the RVLM cholinergic system occur at the level of the neurotransmitter (acetylcholine) in addition to the level of the receptor (muscarinic and nicotinic); changes that collectively may enhance cholinergic transmission and influence on GABAergic synapses within the RVLM of senescent rats. Seventh, mRNA expression levels of dopamine receptor subtypes 1a (Drd1a), 3 (Drd3), 4 (Drd4) and 5 (Drd5) were higher in senescent compared to presenescent rats. Of these subtypes, all but Drd1a remained unchanged between middle-aged (Drd1a higher) compared to young rats, suggesting elevated dopaminergic transmission and potentially, attenuation of GABAergic inhibition of RVLM neurons in senescent rats. Collectively, our findings suggest that differentially expressed neurotransmitter genes in the RVLM during aging may provide the mechanistic basis for influencing GABAergic synaptic transmission within the RVLM thereby potentially contributing to the sympathetic activation that occurs with advancing age.

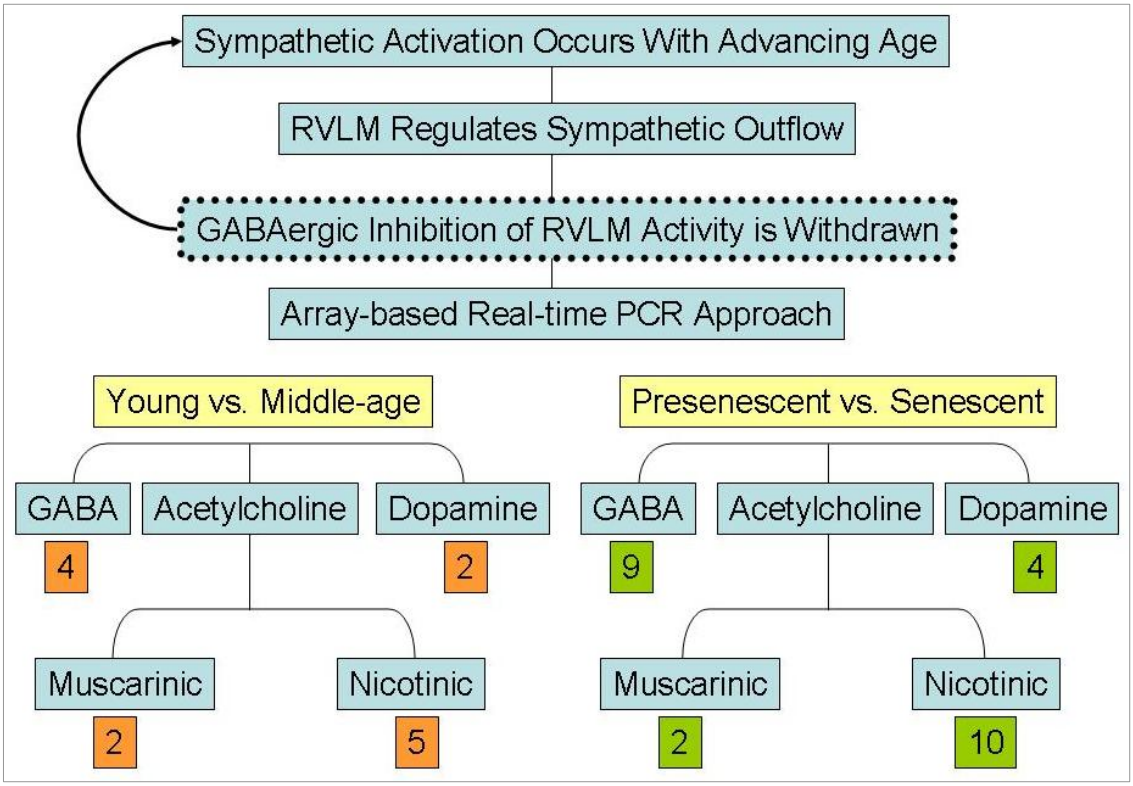

Figure 10.1. Overview of Results from the Current Study. Progressive increases in SND occur with advancing age. The RVLM is a brainstem region critically involved in the regulation of sympathetic outflow. RVLM activity is strongly inhibited by GABAergic neural inputs; therefore we hypothesized that GABAergic tone is withdrawn in the RVLM thereby contributing to the increased SND that is associated with aging (dotted line text box). An array-based real-time PCR approach was used to determine the effects of age on constitutive $GABA_A$ receptor gene expression in the RVLM of F344 rats. Because age affected beta-actin expression in the RVLM, target gene expression in young rats was compared to middle-aged rats and presenescent rats were compared to senescent rats. Cholinergic and dopaminergic receptor systems were included in our analysis due to their reported influence on $GABA_A$ receptor activity. The number of genes found to be significantly different (over 2-fold) between young/middle-aged and presenescent/senescent rats are represented in the orange and green boxes, respectively.

It is well established that normal aging is associated with progressive increases in efferent SND[87,291,296,305]. Type II diabetes[75-78], obesity[5,40,41,384], heart failure[93] and hypertension[305] are pathologies that have been attributed to both the processes of aging and sympathetic dysfunction, exemplifying the importance of understanding central

regulation of SND during aging. The RVLM is a critical brainstem region involved in setting the basal level of sympathetic outflow[18] and glutamatergic neurons provide the predominate source of excitatory input to the RVLM[125,146,385]. Therefore, it is possible that increases in sympathetic activity commonly observed in the aged are attributed to enhanced activity of excitatory amino acid (EAA) receptors in the RVLM. However, glutamatergic influences on RVLM activity are powerfully inhibited by tonic GABAergic neural inputs originating from the CVLM[3,386]; effects that are mediated by $GABA_A$ receptors located on presympathetic neuronal cell bodies within the RVLM[208]. In the present study we proposed that reductions in $GABA_A$ receptor subunit expression and/or alterations in the $GABA_A$ receptor subunit profile may reflect withdrawal of GABAergic tone in the RVLM thereby contributing to the basal sympathetic activation that occurs with advancing age. An array-based real-time PCR approach was used to investigate whether aging affects the constitutive expression of 19 $GABA_A$-related gene products. Relative quantification of gene expression data generated from the array required the use of a HK gene to normalize the expression of target genes of interest in order to eliminate differences in the amount and quality of RNA and efficiencies of reverse-transcription reactions[361,362]. A suitable HK gene for normalization purposes exhibits abundant, stable expression across a variety of tissues and experimental conditions and does not vary between experimental groups by more than one cycle[361,363,364,381]. We examined the expression of 5 well established HK genes (Actb, Hprt, Ldha, Rp1p1 and Rpl13a) in the RVLM of young, middle-aged, presenescent and senescent F344 rats under basal conditions and found that these genes, including beta-actin, are not stably expressed in the RVLM with age *(figure 10.1)*. Although in the

current study beta-actin was previously established as an acceptable HK gene for use in real-time PCR studies involving the spleen, cortex and PVN of F344 and SD rats under various experimental conditions. We report that a 2.7 cycle difference in RVLM beta-actin gene expression existed between young/middle-aged and presenescent/senescent rats, equating to a 6.5-fold difference. In contrast, the difference between young and middle-aged rats was 0.15 cycle while presenescent and senescent rats differed by 1 cycle, indicating that while gene expression could not be evaluated using pairwise comparisons (e.g., young vs. middle-aged, young vs. presenescent, young vs. senescent), it was a suitable HK gene for use in examining the differences in neurotransmitter gene expression between young and middle-aged rats, and presenescent and senescent rats. Results from array-based real-time PCR studies using beta-actin as a normalization factor will be briefly discussed.

$GABA_AR$ $Alpha_1$, $Beta_{2,3}$ and $Gamma_2$. $Alpha_1$, $beta_{2,3}$ and $gamma_2$ are the most abundantly expressed and widely distributed $GABA_A$ receptor subunits in the brain[226,230,257,258,260]. Immunohistochemistry studies have demonstrated that these subunits are frequently colocalized in the rat brain[257,258,387,388]; indicating that a large proportion of $GABA_A$ receptors in the rat brain contain $alpha_1$, $beta_{2,3}$ and $gamma_2$ and suggesting that they may be of physiological importance[226,257]. Each of these $GABA_A$ receptor subunits is critically involved in insuring proper $GABA_A$ receptor function. For example, $alpha_1$ and $beta_3$ form the GABA binding site[231], the beta subunit is required for $GABA_A$ receptor cell surface expression[249-251] and $gamma_2$ is critical for $GABA_A$ receptor clustering at the synapse[255]. Of particular relevance to the present study,

$GABA_A$ receptor subunits $alpha_1$, $beta_{2,3}$ and $gamma_2$ have been shown to exhibit substantial overlap in pattern distribution within the brainstem[258] and are expressed at appreciable levels in the RVLM[258,260].

Real-time PCR results from the current study indicate that $alpha_1$, $beta_{2,3}$ and $gamma_2$ mRNA expression did not differ between young and middle-aged rats but that each of these subunits, with the exception of $beta_2$, was higher in senescent compared to presenescent rats (+2.00, +1.95, +2.09, respectively; fold change). Senescent rats by definition are no longer thriving[326,329,330,334,335,341] and are rapidly progressing towards death[326,328,334,335,341]. Upon the transition into senescence, F344 rats exhibit reductions in food intake[323-326], body weight regulation[337,339], thermoregulation[337,339] and circadian rhythmicity[337,339]. Furthermore, senescent F344 rats die on average within three weeks of entering into senescence[326]. Therefore, it is possible that the increases in $alpha_1$, $beta_3$ and $gamma_2$ $GABA_A$ receptor subunit expression observed in senescent rats may represent a RVLM $GABA_A$ receptor profile characteristic of F344 rats which are near the end of their natural lifespan.

The increase in $alpha_1$, $beta_3$ and $gamma_2$ $GABA_A$ receptor subunits in senescent rats may also reflect enhanced GABAergic tone in the RVLM and/or altered $GABA_A$ receptor function, although mRNA expression of enzymes involved in GABA biosynthesis (glutamate decarboxylase 65 and 67; GAD65, GAD67), did not differ between presenescent and sensescent rats. This implies that the GABA biosynthetic pathway is not substantially activated during senescence; however, mRNA expression

of GABA-T, an enzyme involved in the degradation of GABA, was higher (+2.09) in senescent than presenescent rats (it did not differ between young and middle-aged rats). GABA-T has previously been identified in the medulla[389,390] and age-related increases in GABA-T have previously been demonstrated in the cerebrum and cerebellum of rats[391,392]. It is well accepted that tonic excitatory drive to the RVLM is balanced by the powerful inhibitory activity of GABA-containing axonal projections from the CVLM and that these actions are mediated by RVLM $GABA_A$ receptors. Normal aging is associated with elevated SND, suggesting potential withdrawal of CVLM GABAergic inhibition of sympathetic output. It is tempting to speculate that the transcriptional upregulation of $alpha_1$, $beta_3$ and $gamma_2$ $GABA_A$ receptor subunits in the RVLM of senescent rats occurs in response to age-associated reductions in CVLM GABAergic influence in an effort to dampen the sympathoexcitatory state during aging. On the other hand, although GABA-T does not necessarily indicate enhanced enzymatic degradation of GABA, increases in RVLM GABA-T could suggest reductions in GABA availability for inhibitory synaptic transmission. Under these circumstances, GABA-T may mediate withdrawal of CVLM inhibitory inputs to the RVLM, thereby shifting the balance towards sympathoexcitation in senescent rats *(figure 10.2)*.

Other $GABA_AR$ Subunits. $Alpha_{2-6}$, $gamma_1$, delta, epsilon, pi, rho and theta $GABA_A$ receptor subunits are not abundantly or widely expressed in the brain[230,258,393]. For example, $alpha_2$ expression is primarily confined to the forebrain and compared to $alpha_1$, is not highly expressed in the brainstem[260]. $Gamma_1$ is preferentially distributed in the amygdala, substantia nigra and inferior olive[230]. Rho and $alpha_6$ $GABA_A$ receptor

subunit expression is reportedly limited to the retina[393], and cerebellum and cochlear nucleus, respectively[230]. Immunohistochemistry studies in the adult rat brain have revealed the RVLM has little to no $alpha_{2,4}$ or $gamma_1$; low expression of $alpha_5$; moderate levels of $alpha_3$, $beta_{2,3}$ and delta, and high levels of $gamma_2$[258,260]. Results from the present study determined that of the $GABA_A$ receptor subunits that are considered to be less abundantly expressed in the brain, $alpha_2$ (LOC289606; +4.64), $alpha_6$ (+2.02) and theta (+4.69) mRNA expression was higher and $alpha_5$ (-2.15) was lower in middle-aged compared to young rats. Senescent rats displayed higher levels of $alpha_6$ (+2.05), delta (+4.70), pi (+2.14) and rho_2 (+3.65) but lower levels of $alpha_3$ (-2.19) compared to presenescent rats. These findings demonstrate differential transcriptional regulation of RVLM $GABA_A$ receptor subunits at two separate timepoints in the aging process of F344 rats (e.g., middle-age and senescence); however, the physiological implications of these age-related alterations are currently unclear.

Xenopus oocytes expressing either alpha1/beta, alpha2/beta or alpha3/beta subunit combinations have half-maximal responses at 10μM, 1μM and more than 10μM concentrations of GABA, respectively[394]; suggesting that $GABA_A$ receptors containing alpha2 subunits may exert more potent synaptic inhibition than those receptor subtypes containing $alpha_1$ or $alpha_3$. Therefore, it is possible that in our study higher $alpha_2$ mRNA levels indicate preferential expression of a more GABA-sensitive $GABA_A$ receptor subtype in the RVLM of middle-aged rats. Reportedly, $GABA_A$ receptors containing the delta subunit have intensified responses to GABA[395] and muscimol[396] and may associate with $alpha_{1,3}$, $beta_{2,3}$ and $gamma_2$ subunits. It may be considered

that the higher levels of delta, alpha$_1$, beta$_3$ and gamma$_2$ $GABA_A$ receptor subunit transcripts in senescent rats may contribute to the formation of a $GABA_A$ receptor subtype that contains a combination of delta, alpha$_1$, beta$_3$ or gamma$_2$ subunits and is capable of enhancing inhibitory responses in the RVLM. Senescent rats also expressed more $GABA_A$ receptor subunit rho transcripts. At present, rho subunits are not believed to coassemble with other $GABA_A$ receptor subtypes[393,397] but are thought to form homo- and hetero-oligomeric channels that are similar to $GABA_C$ receptors[259,398-400]. $GABA_C$ receptors are ligand-gated ion channels that are activated at lower concentrations of GABA and are less sensitive to desensitization compared to $GABA_A$ receptors[401,402]; therefore it is possible that increased rho$_2$ strengthens inhibition of synaptic activity in the RVLM of senescent rats. Our results clearly demonstrate that during senescence $GABA_A$ receptor subunits that impart increased sensitivity to GABA and resistance to receptor desensitization are transcriptionally upregulated. It is tempting to speculate that the upregulation of alpha$_1$, beta$_3$, gamma$_2$, delta and rho$_2$ subunits leads to more pronounced GABAergic synaptic transmission between the CVLM and RVLM in senescent rats. Elevations in CVLM GABAergic synaptic transmission may be mediated by increases in the expression of $GABA_A$ receptors that resist desensitization and elicit more potent responses to GABA binding, thereby enhancing GABAergic tone in the RVLM of senescent rats. The fact that we observed increases in the transcription of GABA-T may imply that this is a counter-regulatory response under conditions characterized by enhancements in both CVLM activity and $GABA_A$ receptor expression and sensitivity in the RVLM. Therefore, if inhibitory influences are not being regulated at the level of the CVLM or at the receptor level in the RVLM (e.g., downregulation of

$GABA_A$ receptors or alterations in receptor subtype in response to elevated CVLM activity), increased GABA-T may act as a "safety-switch" by which such powerful and potentially harmful inhibition may be dampened through increased enzymatic degradation of GABA at GABAergic synapses in the RVLM, an action that may effectively shift the RVLM towards a level of excitation *(figure 10.2)*.

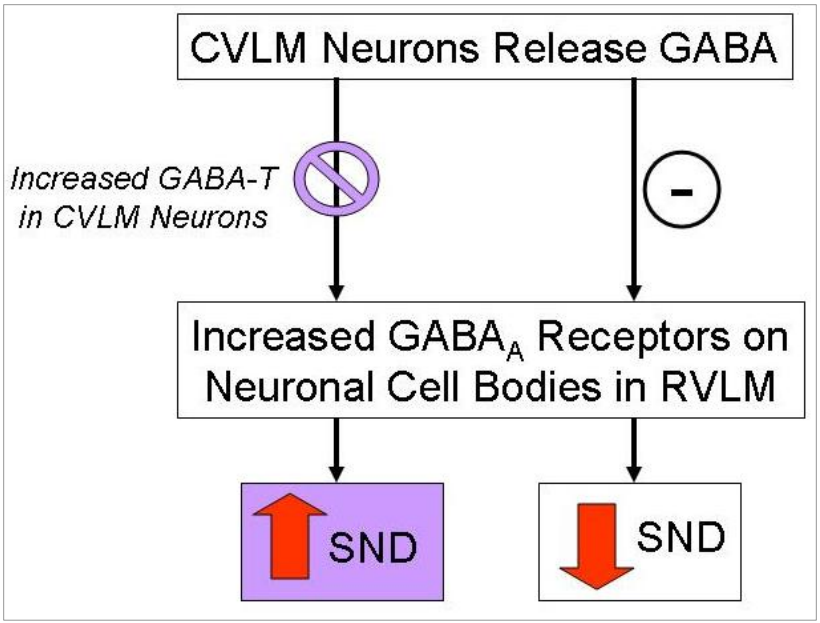

Figure 10.2. GABA-T May Contribute to Elevated SND During Aging: A Hypothetical Model. The CVLM provides tonic inhibitory input to the RVLM thereby decreasing SND. However, in the senescent rat both $GABA_A$ receptors and GABA-T were upregulated. Increased $GABA_A$ receptor expression may indicate enhanced inhibitory tone within the RVLM; however, GABA-T may effectively reduce the activity level of $GABA_A$ receptors by limiting the availability of GABA released from CVLM neurons. This may contribute to withdrawal of GABAergic tone and shift the basal level of activity towards excitation within the RVLM of senescent rats.

In the current study, the use of a commercially available real-time PCR array provided us the ability to effectively screen the RVLM transcriptome for 19 $GABA_A$-related and 65 neurotransmitter-related genes and identify those which demonstrate changes associated with age, the identities of which may provide the biological basis for central mechanisms that contribute to progressive elevations in SND associated with normal aging. Although we primarily focused on constitutive RVLM $GABA_A$ receptor expression in the present study, the array provided us the opportunity to explore several

different neurotransmitter systems reported to influence GABAergic function including, muscarinic, nicotinic and dopaminergic. Expression of genes within each of these systems was found to change with age. Those that changed by at least two-fold will be briefly discussed below.

Muscarinic Receptor Subtypes. Of the cholinergic (acetylcholine) receptors in the brain, muscarinic receptors are the most abundant[357]. Inhibitory and excitatory responses to acetylcholine are mediated by the metabotropic muscarinic receptor[357] and it is well established that central muscarinic systems undergo substantial alterations in experimental animals and humans with advancing age[403]. For example, changes in receptor binding properties and regional distribution of muscarinic receptor subtypes occur during aging[403-405], suggesting potential contributions to altered cholinergic transmission and cholinergic function[403,406]. Of particular relevance to the present study, pharmacological, immunohistochemical, real-time PCR and in situ hybridization methodologies have been used to confirm M1, M2, M3, M4 and M5 muscarinic receptor expression in the rat RVLM[374,407]. Moreover, peripheral administration of the acetylcholinesterase inhibitor physostigmine or microionotophoretic application of acetylcholine increases the firing rate of RVLM neurons, a response that is mediated in part by the M2 muscarinic receptor[408-412]. The role of acetylcholine in pressor responses is further demonstrated following microinjection of physostigmine directly into the RVLM. Increases in MAP, acetylcholine release and choline acetyltransferase (Chat, an enzyme involved in acetylcholine synthesis) activity in the RVLM are observed at greater levels in spontaneously hypertensive rats (SHR) than normotensive Wistar-

Kyoto (WKY) rats, indicating that acetylcholine in the RVLM contributes to the maintenance of elevated MAP in hypertensive animals[413,414]. Because hypertension is commonly associated with increased sympathetic outflow[305], we suggest acetylcholine may contribute in part to the sympathetic activation that occurs in the aged. Evidence supports the presence of tonically active cholinergic inputs that potentiate sympathetic outflow in the RVLM[217,407,410,411,415-420]. Our studies revealed that in the RVLM, transcripts for muscarinic receptor subtypes M2 (+2.06) and M5 (+2.52) were higher in middle-aged than in young rats while M1 (+2.70) and M3 (+2.14) were found to be higher in senescent compared to presenescent rats. In addition, we report that expression levels of Chat and a choline transporter (Slc5a7, involved in acetylcholine synthesis) were higher in middle-aged rats while Chat expression levels were higher in senescent rats. Collectively, our results indicate that the muscarinic receptor expression profile in the RVLM is variably affected with aging which may contribute to altered cholinergic transmission and elevated sympathetic outflow in middle-aged and senescent rats *(figure 10.3)*. It is also possible that the muscarinic receptor profiles represent altered modulation of sympathetic vasomotor responses to baroreflex activation with advancing age. Indeed, the baroreflex influences cholinergic responses to increases in MAP[421] because intravenous administration of phenylephrine increases acetylcholine release in the RVLM in a baroreceptor-dependent manner and glutamatergic excitation of the CVLM elicits acetylcholine release in the RVLM[421]. The presence of muscarinic receptors on GABAergic interneurons may provide yet another mechanism by which MAP is centrally regulated[422]. Burst synchronization is reportedly affected by the activation of muscarinic receptors located on GABAergic interneurons[423]

and acetylcholine has been shown to reduce GABA release and block GABAergic synaptic currents in the auditory cortex[422,424]. Of particular relevance to the current study are the findings that M1 and M2 muscarinic receptors potently attenuate GABAergic inhibition[422]. Although this phenomonen was described in corticopetal fibers[422], perhaps similar mechanisms occur in the RVLM of middle-aged and senescent rats because they expressed higher levels of M2 and M1, respectively. Therefore it is possible that these receptor subtypes in the RVLM blunt GABA signaling in the RVLM *(figure 10.3)*; however, the precise role of M1 and M2 muscarinic receptors in the RVLM is unclear. Furthermore, muscarinic receptor modulation of $GABA_A$ receptor function may be dependent on brain region or cell type due to differences in muscarinic and $GABA_A$ receptor subtype, distribution and functional properties.

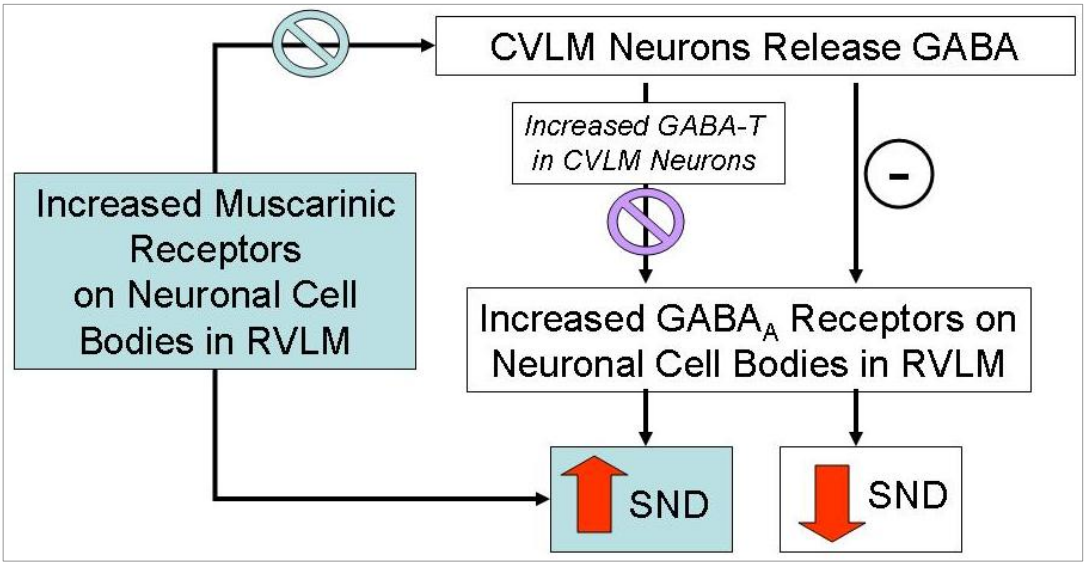

Figure 10.3. RVLM Muscarinic Receptor Expression May Mediate Increased Sympathetic Outflow in the Aged: A Hypothetical Model. Both middle-aged and senescent rats had higher levels of muscarinic receptor expression than young and presenscent rats, respectively. Muscarinic receptors mediate sympathoexcitation and pressor responses to acetylcholine in the RVLM. This may provide a mechanism by which SND is elevated with advancing age.

Nicotinic Acetylcholine Receptors. Nicotinic acetylcholine receptors are members of the super-family of ligand-gated ion channels that includes $GABA_A$ receptors[425]. Pentameric in structure, the alpha subunit contributes to the formation of a ligand binding site and determines functional properties of the receptor[357]. Diffusely distributed throughout the brain, $alpha_4$ and $beta_2$ are the most abundantly expressed nicotinic receptor subunits[426-428]. Neurons can express more than one nicotinic receptor subtype at both pre- and post-synaptic terminals[357,428], however the composition of native receptors is currently unknown[357]. Real-time PCR analysis from the current study demonstrated that alpha $polypeptide_3$ and beta $polypeptide_{1,4}$ transcript levels were higher in middle-aged than in young rats. Senescent rats expressed higher levels of alpha $polypeptide_{1\text{-}3,5,6}$, beta $polypeptide_{1,3,4}$ and polypeptide epsilon compared to presenescent rats. The impact of these changes on SND regulation in both middle-age and senescent rats is unclear because at the present time the structural composition of nicotinic receptors and their functional diversity has not been well established[357]. Although we did not detect marked changes in $alpha_4$ subunit expression, Dehkordi and others have demonstrated that $alpha_4$-containing nicotinic receptors are located on GABAergic neurons in the RVLM[429]. This may provide insight into the potential physiological relevance of elevated levels of $alpha_3$, $beta_1$ and $beta_4$ nicotinic receptor subunits in middle-aged rats and $alpha_{1\text{-}3,5,6}$, $beta_{1,3,4}$ and epsilon in senescent rats. Evidence suggests that pre- and postsynaptic nicotinic receptors can inhibit the release of GABA and inhibit $GABA_A$ receptors, respectively[428]; therefore, it is possible that nicotinic receptors located on the cell bodies of RVLM neurons may serve to regulate the degree by which GABA-

containing CVLM projections influence RVLM activity and that this mechanism may lead to elevations in efferent SND in the aged *(figure 10.4)*.

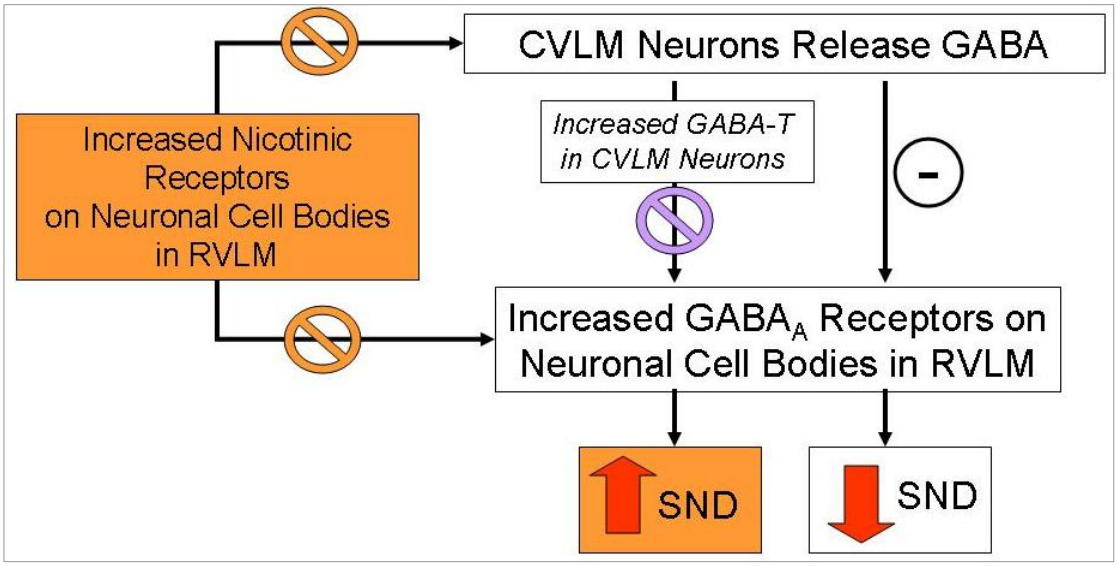

Figure 10.4. Nicotinic Receptors May Attenuate GABAergic Inhibition of Efferent SND: A Hypothetical Model. Nicotinic receptors may contribute to SND activation with advancing age by inhibiting the release of GABA from axons that project from the CVLM and synapse with presympathetic nerve cell bodies in the RVLM. The increase in $GABA_A$ receptor expression that we observed in the RVLM of senescent rats may not represent enhanced GABAergic tone in the RVLM because nicotinic receptors may also inhibit $GABA_A$ receptor activity.

Dopamine Receptor Subtypes. Metabotropic dopamine receptors mediate a number of neurochemical and physiological processes[357]. Dopamine receptor subtype expression displays regional selectivity in the adult rat brain[357] and it is believed that L-DOPA, a precursor for dopamine, is a central neurotransmitter/neuromodulator that is released from neurons to elicit pre- and post-synaptic responses[430]. Microinjection studies have demonstrated that application of L-DOPA into the RVLM increases MAP and heart rate in rats[431-433], suggesting that dopamine may modulate SND outflow and arterial pressure support. In the lower brainstem L-DOPA promotes GABA release[432] which in

turn tonically inhibits L-DOPA release and hypertensive responses to L-DOPA[430,432]. Importantly, these effects are mediated by $GABA_A$ receptors[430,432]. Results from our study indicate that dopamine receptor subtype 1A mRNA expression was higher in middle-aged rats (+2.12) compared to young rats and that dopamine receptor subtype 1A (+2.09), 3 (+2.14) and 5 (+2.41) expression was higher in senescent rats compared to presenescent rats.

While the physiological significance of these age-related alterations is currently unclear, it is possible that they may reflect dopaminergic function and/or modulation of GABA release in the RVLM of senescent rats. Several lines of evidence suggest that interactions between D_1, D_3, D_5 and $GABA_A$ receptors exist and that such interactions contribute to altered dopaminergic and GABAergic neuronal transmission. First, D_1 receptors are the most abundantly expressed dopamine receptor in the brain and have been shown to colocalize with D_3 receptors in neurons[434]. Second, D_3 and D_5 receptors have 20- and 10-fold higher affinity for dopamine than D_2 and D_1 receptors, respectively[434,435], suggesting that responses to dopamine may be markedly enhanced in senescent rats. Third, the cytoplasmic tail of D_5 receptors associates with the $GABA_A$ receptor subunit $gamma_2$[436]; an interaction that effectively inhibits the signaling capability of the other[437]. Fourth, D_3 activation attenuates the GABAergic synaptic current in the nucleus accumbuns through reductions in the cell surface expression of the $GABA_A$ receptor beta subunit, suggesting that D_3-mediated suppression of GABA responsiveness occurs through the internalization of $GABA_A$ receptors[438]. Therefore, it may be considered that increased expression of D_1, D_3 and D_5 receptors in the RVLM

reflects a point of regulation for controlling $GABA_A$ receptor responses to CVLM neural inputs in senescent rats by potentially reducing the release of GABA from CVLM axonal terminals and/or downregulating $GABA_A$ receptor cell surface expression in presympathetic neuronal cell bodies within the RVLM. It is tempting to speculate that these hypothetical consequences of dopamine-$GABA_A$ receptor interactions may serve as an additional "safety switch" (in addition to GABA-T as discussed previously) that serves to counteract potential increases in CVLM activity that may occur during senescence, thereby withdrawing inhibitory influences and shifting the balance towards the sympathoexcitatory state that is commonly observed with advanced age *(figure 10.5)*.

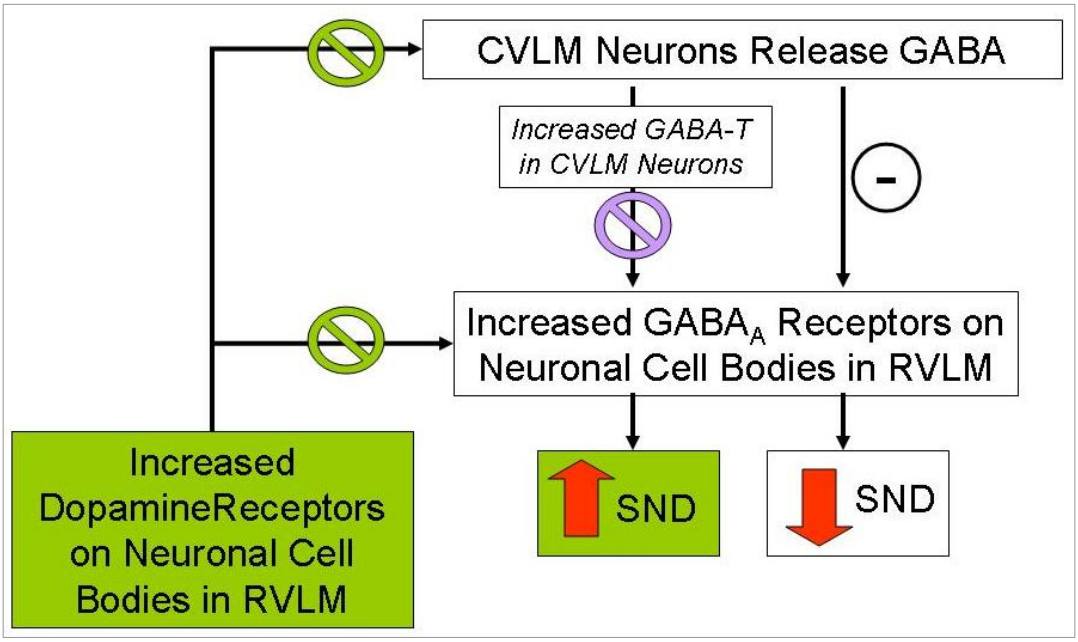

Figure 10.5. Dopamine Receptors May Increase Sympathetic Outflow by Impairing GABAergic Signaling: A Hypothetical Model. Dopamine receptors in the RVLM may inhibit GABA release from the CVLM and promote internalization of $GABA_A$ receptors which would influence the basal level of efferent SND in

The use of a "pathway-focused" (e.g., neurotransmitter-related) real-time PCR array is an attractive approach to scanning the F344 RVLM for function-specific genes

of interest. In the current study we found variable age-related alterations in the mRNA expression profiles of $GABA_A$, muscarinic, nicotinic and dopaminergic receptor systems in the RVLM of young/middle-aged and presenescent/senescent F344 rats. Several factors may explain the non-uniformity in expression patterns that we observed in the RVLM of middle-aged and senescent rats when compared to young and presenescent rats, respectively. First, specific genes may exhibit inherent differences in their sensitivity to the aging process, potentially affecting mRNA stability as well as transcriptional processes. Second, changes in the expression of individual subunits and therefore, receptor subtype expression, may reflect an age-dependent adaptation to physiological events that are occurring and/or may be specific to a particular chronological or biological age. Indeed, an important advantage to receptor compositional heterogeneity is the functional flexibility that it provides through alterations in cell surface or synaptic localization, binding kinetics, desensitization, channel gating or signal transduction properties and modulation by pharmacological agents. However, it is important to note that changes in the gene expression of a particular receptor subunit or subtype that we observed in the current study may not necessarily indicate similar changes in the protein or the incorporation of that protein into mature receptor complexes. There are at least three limitations to the present study. First, anesthesia may influence neurotransmitter system gene expression, particularly that related to $GABA_A$, although the majority of studies investigating RVLM function have used anesthetized preparations[439] and preliminary experiments completed in our laboratory have demonstrated that expression levels of $GABA_A$ receptor subunit $alpha_1$ protein do not markedly differ between young, middle-aged and

presenescent F344 rats treated with or without 5% isofluorane anesthesia. Second, the sympathetic nervous system is capable of producing regionally selective changes in sympathetic outflow and it is well established that in the brain molecular changes in neurotransmitter expression and function are often times region-specific[226,230,258,260,440-445]. Therefore, the results from the present study are only applicable to the RVLM of F344 rats and may not necessarily be indicative of similar changes in other autonomic regions. Third, because we used a focused genomic approach to examine constitutive neurotransmitter gene expression in the RVLM, our findings only represent changes that occur at the mRNA level and may not accurately reflect a similar level of protein expression[446-450]. Despite these limitations; however, our experimental approach successfully met our objective of rapidly and comprehensively screening a large number of genes related to $GABA_A$ receptor subunits, enzymes involved in GABA synthesis and metabolism, and other neurotransmitter systems in a quantitative manner. The present study, to our knowledge, is the first to undertake the task of comprehensively characterizing the constitutive expression of multiple neurotransmitter systems in the RVLM of F344 rats and to examine the effect of age on the expression of these systems. Therefore, the results presented herein provide many genes that may be of potential interest to scientists in the aging, cardiovascular physiology, endocrine and autonomic neurophysiology research communities. Future studies employing biochemical, electrophysiological, pharmacological and molecular biological methodologies will be necessary to determine the physiological relevance of these changes in the RVLM.

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Appendix A - Real-time PCR Data Supplemental Material

Gene	Young	Middle-Aged	Presenescent	Senescent
Abat	27.98 ± 0.98	28.18 ± 1.52	25.93 ± 0.78	25.87 ± 0.50
Gad1	29.30 ± 1.32	29.03 ± 1.35	26.57 ± 0.86	27.30 ± 0.93
Gad2	28.50 ± 0.69	28.20 ± 1.09	26.80 ± 0.40	26.90 ± 0.72
Gabra1	30.02 ± 1.26	30.28 ± 2.03	27.80 ± 0.97	27.80 ± 0.36
LOC289606	31.94 ± 0.94	29.58 ± 1.23	28.53 ± 1.05	29.50 ± 0.71
Gabra3	33.72 ± 0.62	34.20 ± 0.45	32.50 ± 1.07	34.63 ± 0.37
Gabra4	32.70 ± 1.02	32.05 ± 1.14	30.20 ± 1.04	30.67 ± 0.81
Gabra5	30.62 ± 0.97	31.58 ± 1.28	28.53 ± 1.06	29.07 ± 0.46
Gabra6	30.14 ± 1.03	28.98 ± 0.59	31.40	31.37 ± 2.69
Gabrb2	29.78 ± 0.96	29.75 ± 1.79	27.53 ± 1.04	27.93 ± 0.12
Gabrb3	29.24 ± 1.06	29.40 ± 1.39	27.23 ± 0.87	27.27 ± 0.64
Gabrd	33.16 ± 0.59	32.45 ± 1.12	31.73 ± 0.56	30.50 ± 1.45
Gabre	34.32 ± 0.68	33.80 ± 0.75	32.37 ± 0.89	33.87 ± 1.13
Gabrg1	30.92 ± 1.14	30.98 ± 1.46	28.63 ± 0.71	29.00 ± 0.76
Gabrg2	29.68 ± 1.16	28.93 ± 1.48	26.80 ± 0.90	26.73 ± 0.44
Gabrp	34.38 ± 0.62	33.95 ± 0.61	33.07 ± 0.61	32.97 ± 0.32
Gabrq	32.98 ± 0.86	30.60 ± 1.17	29.97 ± 0.29	31.57 ± 0.98
Gabrr1	34.12 ± 0.29	33.58 ± 0.93	34.03 ± 0.48	34.23 ± 0.41
Gabrr2	34.98 ± 0.02	35.00	35.00	34.13 ± 0.44
Ache	28.98 ± 1.10	28.23 ± 1.66	26.40 ± 1.07	27.03 ± 0.69
Chat	33.10 ± 1.43	30.95 ± 2.21	30.73 ± 2.54	29.90 ± 1.85
Slc5a7	31.72 ± 1.79	29.25 ± 2.33	28.47 ± 1.70	28.80 ± 1.96
Chrm1	34.94 ± 0.06	33.90 ± 0.67	34.57 ± 0.38	34.13 ± 0.77
Chrm2	31.34 ± 0.69	30.15 ± 1.02	30.77 ± 0.97	31.23 ± 0.74
Chrm3	32.96 ± 0.52	33.15 ± 1.21	31.47 ± 0.82	31.37 ± 0.64
Chrm4	34.92 ± 0.08	34.20 ± 0.77	33.23 ± 0.64	33.87 ± 0.98
Chrm5	33.96 ± 0.41	32.48 ± 0.55	33.23 ± 0.26	34.80 ± 0.15
Chrna1	34.42 ± 0.58	33.95 ± 1.05	33.93 ± 0.75	32.73 ± 0.97
Chrna2	34.46 ± 0.33	33.60 ± 1.04	34.03 ± 0.55	32.93 ± 0.73
Chrna3	33.88 ± 0.84	32.75 ± 1.14	32.83 ± 1.17	31.77 ± 0.88
Chrna4	31.84 ± 1.04	32.25 ± 1.52	29.43 ± 0.79	30.50 ± 0.89
Chrna5	34.06 ± 0.61	33.90 ± 0.71	33.97 ± 0.54	33.43 ± 1.27
Chrna6	35.00	34.58 ± 0.33	34.90 ± 0.10	34.90 ± 0.10
Chrnb1	33.34 ± 0.55	31.68 ± 0.67	31.93 ± 0.33	31.73 ± 0.43
Chrnb2	31.84 ± 0.93	31.68 ± 1.79	29.90 ± 1.06	30.03 ± 0.54
Chrnb3	34.84 ± 0.16	34.90 ± 0.10	35.00	35.00
Chrnb4	34.34 ± 0.66	33.08 ± 1.09	33.30 ± 1.08	32.97 ± 0.48
Chrnd	32.14 ± 0.42	31.33 ± 0.98	33.90 ± 0.59	33.97 ± 1.03

Chrne	34.68 ± 0.27	34.20 ± 0.80	33.00 ± 0.36	32.80 ± 0.49
Chrng	29.94 ± 0.56	29.25 ± 0.79	30.27 ± 0.35	31.93 ± 0.19
Comt	31.48 ± 1.25	30.65 ± 2.01	28.20 ± 0.96	28.53 ± 0.19
Maoa	30.22 ± 1.27	30.28 ± 1.70	27.23 ± 0.89	27.50 ± 0.49
Th	33.26 ± 0.75	32.13 ± 1.05	31.17 ± 0.87	32.43 ± 1.49
Drd1a	34.56 ± 0.22	33.33 ± 0.75	34.27 ± 0.53	34.20 ± 0.80
Drd2	30.68 ± 0.53	30.70 ± 0.77	29.53 ± 0.83	30.10 ± 0.66
Drd3	34.60 ± 0.26	34.10 ± 0.39	33.97 ± 0.71	33.87 ± 0.57
Drd4	35.00	34.65 ± 0.22	35.00	35.00
Drd5	34.98 ± 0.02	34.78 ± 0.23	34.77 ± 0.23	34.50 ± 0.40
Actb	24.50 ± 1.08	24.35 ± 1.55	21.23 ± 0.64	22.23 ± 0.35
Ldha	30.44 ± 1.37	29.95 ± 1.44	26.77 ± 0.77	28.40 ± 0.95
Rplp1	24.92 ± 0.91	24.93 ± 1.35	21.93 ± 0.73	22.37 ± 0.41
Rpl13a	25.88 ± 0.99	26.43 ± 1.36	23.50 ± 0.87	24.67 ± 0.55
Hprt	28.92 ± 1.20	28.98 ± 1.29	26.23 ± 0.89	26.93 ± 0.79

Table A.1. Mean threshold cycle (C_t) values for select target genes of interest and five HK genes examined in the present study. Values presented are not normalized to a HK gene. Young n=5, Middle-aged n=4, Presenescent n=3 and Senescent n=3 F344 rats.