Aging and Sympathetic Neurotransmission in Two Strains of Rats that Differ in Longevity and Immune Profiles

Sam David Perez

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Aging and Sympathetic Neurotransmission in Two Strains of Rats that Differ in Longevity and Immune Profiles

by

Sam David Perez

A Dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy in Physiology

September 2011
Each person whose signature appears below certifies that this dissertation in his/her opinion is adequate, in scope and quality as a dissertation for the degree of Doctor of Philosophy.

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ABREVIATIONS

α-AR alpha-adrenergic receptor
6-OHDA 6-hydroxydopamine
ARs adrenergic receptors
α₁-AR alpha₁-adrenergic receptor
β-ARs beta-adrenergic receptors
αMNE alpha-methylnorepinephrine
AA adjuvant-induced arthritis
ANS autonomic nervous system
BDNF brain-derived neurotrophin factor
b.i.d. twice per day
BN Brown Norway
cAMP cyclic adenosine monophosphate
Con A concanavalin A
CSF cerebrospinal fluid
CNS central nervous system
CAs catecholamines
DA dopamine
DBH dopamine β-hydroxylase
EAE experimental allergic encephalomyelitis
ELISA enzyme-linked immuno-sorbent assay
EPI epinephrine
F344 Fischer 344
HLA human leukocyte antigen
HPA hypothalamic-pituitary-adrenal
HPLC high performance liquid chromatography
IFN interferon
IL interleukin
i.p. intraperitoneal
KLH keyhole limpet hemocyanin
ABSTRACT OF THE DISSERTATION

Aging and Sympathetic Neurotransmission in Two Strains of Rats that Differ in Longevity and Immune Profiles

by

Sam David Perez

Doctor of Philosophy, Graduate Program in Physiology
Loma Linda University School of Medicine, September 2011
Dr. Denise L. Bellinger, Chairperson

Age-related changes in sympathetic neurotransmission in immune organs may be associated with immunosenescence; however no causal relationship has been established. From previous studies in Fischer rats (F344), we have found that during middle age, sympathetic nerve activity (SNA) rises in the spleen followed by a decline in noradrenergic (NA) innervation. Also, increased sympathetic signaling via beta-adrenergic receptor (β-AR) and reduced proliferation of lymphocytes are evident with increasing age. Although SNA progressively rises with age, effects of age on sympathetic factors may be different across rat strains. If causal relationships exist between sympathetic activity and immune function, they may be related to genotypic differences. Therefore, our research consisted in two major studies. First, in a longitudinal study, we characterized the age-related change in sympathetic innervation and sympathetic activity/tone in Brown Norway (BN) rats. The BN rat strain has a longer life span, a different aging pattern of NA innervation in the spleen, and a different immune profile compared to the F344 rat. Second, after establishing the time point during which sympathetic activity and NA innervation profiles in the spleens of these rats change, we investigated the role of increased SNA on neurotransmission in the spleen, as
an initial step before determining causal relationships between immune decline and altered sympathetic function with age. For the longitudinal study in BN rats, we examined NA innervation in the spleens of animals at 8, 15, 18, 24, 27, and 32 months of age (8M, 15M, 18M, 24M, 27M and 32M) by histofluorescence for catecholamines and confirmed with morphometric analysis. We measured the concentrations of splenic norepinephrine (NE) and circulating catecholamines after decapitation using high-performance liquid chromatography (HPLC). For our second study, middle-aged BN or F344 rats received a 90-day treatment with rilmenidine, a centrally acting antihypertensive drug.

In our first study, we observed a decline in NA nerve density in the splenic white pulp (45%) of BN rats at 15M. Lower splenic NE concentrations followed at 18M, and were strongly correlated with nerve density. Circulating catecholamine (CA) levels generally dropped with increasing age. In our second study, 15M male F344 and BN rats were intraperitoneally (i.p.) injected with 0, 0.5 or 1.5 µg/kg/day rilmenidine (Veh-18M, Rilmlo, Rilmhi, respectively). Untreated 3M (Crtl-3M) and 18M rats (Crtl-18M) were used to control for effects of aging and handling/injection stress. We evaluated splenic NE concentration with or without synthesis blockade, NA innervation, β-AR expression, and β-AR-stimulated c-AMP production using HPLC, fluorescence histochemistry, β-AR radioligand binding, and enzyme-linked immunoassay (EIA), respectively. We evaluated β-AR expression by β-AR radioligand binding assay. Rilmenidine reduced circulating NE without a change in epinephrine (EPI), and reduced SNA as assessed by reduced NE turnover rate after synthesis blockade in both F344 and BN rats. We observed restorative effects of rilmenidine on splenic NA innervation in both rat strains in a dose-dependent
manner, supporting the hypothesis that heightened SNA contributes to nerve destruction. Splenocyte β-AR density was increased by the optimum dose (Rilmlo), without changing affinity. β-AR-stimulated cAMP production was further augmented by either dose of rilmenidine in F344 rats; while in BN rats, Rilmlo partially reversed the age-related decline in β-AR-stimulated cAMP production. Collectively, the results from these studies demonstrate an increase in splenic SNA during middle age that affects NA neurotransmission in the aging spleen in strain-specific manner. Chronic treatment with rilmenidine protected sympathetic nerves, and further enhanced β-AR-mediated signaling in the spleen of F344 rats. No significant effects on sympathetic nerves or β-AR-mediated signaling were found in the spleen of BN rats treated with rilmenidine compared with age-matched controls. These findings suggest that rilmenidine could affect host defense against age-related diseases, by modulating sympathetic signaling. Further, they will form the foundation for further development of shorter- and longer-lived rodent models with differing immune phenotypes to investigate causal relationships between sympathetic nervous system (SNS) and immune function in aging.
CHAPTER 1
INTRODUCTION

Epidemiological projections predict a sharp rise in the elderly population in the United States over the next decade (1). The health concerns are many, particularly the incidence of chronic diseases, which will reduce the quality of life and increase the burden on the public healthcare system (2). In aging, autoimmunity, cancer and infectious disease rise, and efficacy for vaccination declines. These conditions are influenced by interactions between the immune and the central nervous systems via autonomic and endocrine signaling, primarily the hypothalamic-pituitary-adrenal (HPA) axis (3).

In this project, we investigated first, the effect of age on sympathetic innervation activity and signaling in the BN rat, an alternative rat model from the classically used F344 rat model for aging. Next, we examined whether heightened sympathetic nerve activity (SNA) in a lymphoid organ, such as the spleen, during aging is responsible for splenic NA nerve loss and altered β-AR signaling in splenocytes. NE is the main neurotransmitter/neuromodulator in lymphoid organs (3, 4). Under stimulation, NE is released from the extensive sympathetic nerve terminals and targets the adrenoreceptors expressed on the immune cells of primary and secondary lymphoid organs (4). In young adults, the SNS modulates immune function, and is critical for adequate immune function and responses (5). With aging, the SNS is dysregulated and immune function decline, but we do not know whether SNS changes in the aging spleen are consistent across strain/species. We also need to optimize methods to be able to manipulate the SNS to
study causal relationships between immune and SNS changes with advancing age.

There are no available models to investigate causal relationships, and we do not believe that there is one single strain of rat that will provide an adequate model for all scenarios of aging. For these reasons, we need to develop animal models for looking at causal relationships between the brain and the immune system in aging, because they are currently not available. To overcome this problem, we have characterized the age-related SNS changes in the spleen of two strains with different genetic background, longevity, and immune profiles. We do not believe there will be one single strain of rat that will provide an adequate model for all scenarios of aging, largely because of genetics and environment across life span interact to mediate the aging process.

*Therefore, the research objective of this doctoral dissertation was two-fold: first, to longitudinally examine the effect of age on sympathetic NA innervation and neurotransmission in the spleen of the BN rat, a rat strain with different life span and immune profile than the well studied F344 rat. Secondly, to begin investigating the role of the age-related alteration in sympathetic activity on neurotransmission in the spleen, as a first step in developing models for studying causal relationships between altered neurotransmission and immune function.* Experimental findings in both animals and humans indicate critical age-related alterations in both the SNS and the immune system. In the aging SNS, there is an increased sympathetic tone that is accompanied by elevated levels of norepinephrine in the circulation in human older adults (6, 7), and in old F344 rats (8). In rodent models for aging, changes in sympathetic innervation occur depending on the tissue/organ, species or strain, and concomitant changes occurring in the local microenvironment that regulate nerve integrity, for
example the available amount of NGF or other neurotrophic factors produced by the target cells (9). Specifically, in the F344 rat spleen, sympathetic innervation is reduced (10, 11), the density of β-ARs on lymphocytes and β-AR-stimulated cAMP production rise with age (12). But with respect to its aging immune system, the ability to mount a cellular immune response to pathogens and tumors (14, 15), as well as the capacity to respond to vaccines, declines (16). Also, a rise in proinflammatory cytokines and susceptibility to autoimmune diseases increases morbidity and mortality, as age advances (17). In old F344 rats, we have observed a decline in the density of T lymphocytes and ED3+ macrophages population in lymphoid compartments (4). Aging rats also exhibit a decline in lymphoproliferation in response to concanavalin A (Con A) that is not associated with a change in interleukin (IL)-2 production, and weakened primary antibody responses to a T-dependent antigen (18). However, other than correlative data from our laboratory, virtually no information exists either to support or to refute a causal role for sympathetic dysregulation in immunosenescence with advancing age. This doctoral project begins to investigate strain-specific changes in sympathetic innervation integrity/activity and β-ARs signaling in spleens from two well established aging rodent models, as an initial step toward establishing causality between aging SNS and immune functional changes. Without this basic information, it will be impossible to determine in a meaningful way the contribution of sympathetic dysregulation on immune function with advancing age in laboratory rat models.

Since, a relatively large body of literature indicates that heightened tonic sympathoexcitatory activity in the rostral ventral lateral medulla (RVLM) is responsible for increased SNS activity in hypertensive rodents, the studies described here target this
brain region pharmacologically, to chronically lower sympathetic outflow to the spleen. Our main hypothesis for these studies is that **heightened splenic SNA during middle age is responsible for splenic NA nerve loss and altered β-AR signaling in splenocytes, in a strain-dependent manner.**

In order to test our main hypothesis, we have conducted experiments designed to address the following specific aims:

**Specific Aim # 1:** *Evaluate the role of heightened SNA as a mechanism contributory to altered neurotransmission in the aging spleen of the BN rat.* A longitudinal study determined the age at which sympathetic activity, NA innervation and β-AR signaling changes in this strain. We considered that SNA changes in the BN rat may occur during middle, as in F344 rats. A time point for experimental interventions aimed at investigating effects of these changes on neurotransmission was determined.

**Specific Aim #2:** *Evaluate the role of heightened sympathetic activity as a mechanism responsible for altered innervation and sympathetic signaling in immune cells of secondary lymphoid organs in the short-lived F344 rat.* This aim tested the effect of age-related increased sympathetic tone on NA nerve preservation and neurotransmission in the spleen by using rilmenidine, a centrally acting antihypertensive drug, for 90 days, to reduce global sympathetic outflow during middle age. We propose that chronically lowering basal sympathetic outflow to the spleen will reduce SNA and retard or prevent the age-related changes in nerve integrity and reverse the age-related rise in β-AR-mediated signaling.

**Specific Aim # 3:** *Evaluate the role of heightened sympathetic activity as a mechanism responsible for altered innervation and sympathetic signaling in immune cells of secondary lymphoid organs in the short-lived F344 rat.*
**cells of secondary lymphoid organs in the long-lived BN rat.** Studies in this aim will provide information on the effects of a chronic reduction of SNA in the spleen of the aging BN rat, a strain that has a different immune profile and age-related β-AR signaling in the spleen than the F344 rat. We hypothesized that diminishing sympathetic outflow to the spleen will prevent the age-related rise in local SNA, and reverse the age-related alterations in NA innervation and β-AR-mediated signaling in the spleen.

**Background**

**Age-Related Changes in Immune Function**

Both arms of immunity innate and cell-mediated change during aging, but cell-mediated immunity is more affected. Proliferation, cytotoxicity, natural killer cell activity, IL-2 production, IL-2 receptor expression, and signal transduction are commonly reported to be impaired in old T cells (17). With age there is down-regulation in the expression of several genes which play important roles in the T cell activation pathway that include c-fos, c-myc, and c-jun. There is also impairment in activation of nuclear transcription factors implicated in the transcriptional regulation of IL-2 mRNA (18, 19). Also lymphocyte proliferation is generally impaired in aging, irrespective of the stimulating agent, as well as a synthesis of and response to IL-2 which hamper the proper functioning of T lymphocytes in healthy elderly people (19).

The role of IL-2 in impaired T cell function in aging is controversial, and the nature of the stimulating agent used contributes to the conflicting data. While spleen cells of both young and aged rats produce high amounts of IL-2 in response to concanavalin A (Con A) stimulation, in allogenic mixed lymphocyte tumor cell culture, spleen cells from old rats produce little IL-2 compared to spleens from young rats (20). In
mice, the frequency of responding cells decrease in aging, but the proliferative capacity and immune competency of progeny cells remain intact (21). In old human subjects fewer responsive cells are present, and fewer cells divide with each succeeding generation, but the kinetics of proliferation do not change (22). Also in old mice, the proportion of cells competent in producing IL-2 mRNA declines, but not the amount of mRNA produced by those competent cells. Similarly the proportion of cells that can express high-affinity receptors decreases, rather than the number of high affinity receptors per positive cell or the affinity of the receptor for the ligand (22). Therefore, most studies indicate an age-related decline in the proportions of T cells, which can produce IL-2, IL-2 mRNA, and IL-2 receptors after activation, accounting for the age-related loss of T cell proliferation.

Regarding cytokine production, we will not cover the subject in detail because it is beyond the scope of the studies here. However we will mention the factors that may influence the lack of coherence of data regarding cytokine production. First, the particular species tested is important. For instance, IL-4 production is up-regulated after antigenic challenge in mice but not humans (23). Second, the nature of the stimulating agent used in \textit{in vitro} culture conditions between laboratories can explain some of the controversy. With aging, IL-4 production was increased in short-term cultures, while in longer-term cultures IL-4 production by memory T cells declined under conditions that promote IL-2-drive proliferation (24). And third, most studies have been done using unfractionated T lymphocytes isolated from different tissues, such peripheral mononuclear cells (PBMC), spleen and lymph node, all of which have different accessory cells populations. Therefore, careful study using defined populations of T lymphocytes and accessory cells
should be carried out to have a better understanding of the dysregulation of cytokine production due to aging.

Aging is also associated with an increase in the CD4/CD8 ratio in human peripheral blood lymphocytes, and murine spleen (25, 26). But in murine lymph node and blood, the CD4/CD8 ratio decreases with aging (27). Also a shift towards a memory as compared to a naïve T cell population occurs in humans and experimental animals (26). Naïve CD4+ T cells are more potent in producing IL-2 compared to memory CD4+ T cells. This phenomenon is seen in both young as well as old mice, and indicates that the shift toward memory CD4+ T cells contributes to the aged-related decrease in IL-2 production. Also an age-related increase in CD8+CD28+ T cell percentage and absolute number, and an increase in anti-CD3 redirected cytotoxicity by freshly isolated T cells, suggest immune activation within the cytotoxic arm with aging (28). Meanwhile the increase in CD8+CD28- T cell with aging is consistent with an age-associated decline in the proliferative ability of aged humans, particularly prominent in the CD8 population and hyporesponsiveness to co-stimulation by CD28 in T cells from aged mice (29).

Aging and Basal Sympathetic Activity

Catecholamines, such as norepinephrine (NE) and EPI are transmitters that are released from active sympathetic nerves and the adrenal medulla respectively. While circulating NE levels reflect the net firing activity of sympathetic nerves, EPI in the blood is used as a marker of adrenomedullary activity. Indices of basal sympathetic activity, such as elevated plasma NE, have been used as markers of normal chronological aging (30). NE concentrations in the urine and cerebrospinal fluid (CSF) are also higher by 50% or more compared with young subjects (31). A rise in plasma NE levels indicates a
net elevation in sympathetic tone with subsequently increased NE spillover into the circulation (32-35). In rat models for aging (i.e. F344 rats) there are differences in the response to sympathetic activation; specifically in the time it takes for circulating NE levels to return to baseline in older rats (36, 37). Elderly humans exhibit higher plasma NE levels and a delayed return to re-stimulus levels of sympathetic activity following many stimuli that activate the SNS, including orthostasis (38). In older adult humans, mild sympathetic stressors elicit an exaggerated response, indicated by elevated plasma NE levels compared with young adults. Plasma EPI concentrations sampled under comparable conditions do not increase with age in humans (38, 39-43). This dissociation between age-related changes in NE and EPI is also evident in urine (34) and CSF (39). In aged rats, elevated plasma NE levels also persist longer in old rats after exposure to stressors that activate the SNS, such as cold water immersion for 10 minutes and immobilization stress (40). Therefore, autonomic dysfunction in these tissues may not be exposed under basal conditions, but may become apparent under stressful stimuli, where the autonomic nervous system (ANS) responds reflexively to changes from the resting state. Collectively, these findings suggest dysregulation of the SNS in aging that is similar in humans and the rodent models that we are using for this study.

**Effects of Aging on Sympathetic Neurons**

Sympathetic neurons exhibit target-specific changes with advancing age. In some target tissues, there are age-related changes in axonal, dendritic arborizations, and soma size, in response to an altered microenvironment in effector tissues (41, 42). In aged rats, the density of sympathetic nerves surrounding cerebral arteries is reduced by 50% (43). This occurs with shrinkage of adjacent smooth muscle cells (44). In the gut, there is an
age-related decline in sympathetic innervation, as well as, an extensive loss of enteric neurons (45-47). In the spleen of the aging male F344 rats, reports from our laboratory indicate that the loss of sympathetic fibers ensues concomitantly with altered splenic architecture and immunosenescence (48, 49). It is important to note that when sympathetic innervation is reduced, it usually does not result from an age-related cell death of sympathetic neurons (41-45). Apoptosis can be induced in mature neurons that are injured or lack neurotrophic support, but cell death is usually aborted (46, 47). In contrast to the target tissues mentioned above where a loss of sympathetic nerves has been reported, sympathetic neurons that supply the iris are maintained or keep growing in old age (50), and their dendrites do not atrophy during aging (51). Collectively, this literature indicates that changes in sympathetic innervation are specific to tissue type, species, and strain (9). Therefore microenvironment in the target tissue has an important influence on the integrity of sympathetic nerves that distribute to them. The latter two support an important role for genetic phenotype on aging changes in target tissues.

Age-Related Changes in Sympathetic Innervation in Lymphoid Organs

Innervation of primary and secondary lymphoid organs provides an anatomical substrate for the brain to communicate with the immune system (52). Sympathetic nerves primarily from the superior mesenteric and celiac ganglionic plexuses (53), and sparingly from the sympathetic trunk (54), enter the spleen as the splenic nerve, along with the splenic artery. NA nerves course in vascular and trabecular plexuses into the white pulp closely associated with the central arterioles and their branches (4). NA varicosities extend from these plexuses into the periarTERiolar lymphatic sheaths (PALS) (55), ending
among OX19+ T lymphocytes and interdigitating cells (52, 56). There are also NA nerves that enter the marginal, at the periphery of the white pulp, and the parafollicular zones, that distribute among IgM+, B cells and ED3+ macrophages (48). Generally, NA nerves are not present in the follicles, where resting B cells reside, and sparse in the parenchyma of the red pulp, but do course along the trabeculae, vasculature, and venous sinuses in the red pulp (49).

The mature pattern of NA innervation of the spleen in the rat is present by postnatal day 28, and adult proportions are attained by 56 days of age (57, 58). Extensive studies in our laboratory have shown that in older adults F344 rats NA innervation declines in secondary (48, 49), but not primary (59) lymphoid organs, as indicated by both neurochemical measurements of NE content and the histofluorescence for catecholamines with morphometric analysis to measure the density of NA nerve terminals. In effect, neurochemical measures have shown a greater than 50% loss in NE concentration in spleens from 27M rats compared with young adult controls (48). NA nerves are lost in lymphoid compartments of the spleen, particularly in the periarteriolar lymphatic sheath (PALS) where T cells reside. Quantitation of fluorescent varicosities has revealed an age-related decrease in the density of NA nerves greater than expected based on splenic NE concentration. This suggests that more NE is made by fewer nerves, and this measure may provide a more sensitive assessment of aging changes in sympathetic innervation than static splenic NE concentration (9). Loss of fluorescently stained nerves in the spleen may be a consequence of an inability of NA nerves to synthesize enough NE via tyrosine hydroxylase (TH), or from actual loss of nerve fibers. Studies that examined alpha-methylnorepinephrine (αMNE) (100 mg/kg body weight,
i.p.), a compound that is taken up by high affinity carriers on NA fibers and cannot be metabolized by monoamine oxidase confirmed that (a) no additional sympathetic fibers were visualized, and (b) the surviving nerve terminals were functional in aged rats (11, 60). Further, the uptake of $[^3\text{H}]$-NE by lingering fibers remains functional in old animals, and is in fact enhanced (61). Effects of aging on NA innervation patterns in lymphoid organs have been studied in various murine strains (57) and F344 rats (52, 57). In our studies we extend these findings, by examining a different rat strain, the BN rat, which lives longer and has a different immunological and behavioral profile than F344 rats, for age-related changes in sympathetic neurotransmission in the spleen.

Immune Effector Cell Responsiveness to Sympathetic Stimulation

Catecholamine stimulation of β-ARs expressed on splenocytes and peripheral blood mononuclear cells (PBMC) from both murine and humans, increase adenylate cyclase activity mostly, but not exclusively via activation of β$_2$-ARs; and suggests a potential for modulation of cellular activity at the level of gene expression (62). The primary AR subtype expressed on T cells is the β$_2$-AR (63-65), but cells of the immune system such as macrophages, thymocytes and splenocytes also express α-ARs (62, 65). In B cells, stimulation of β$_2$-ARs, either prior to, or at the time of, cell activation increased either the number of antibody-secreting cells or the amount of antibody produced per cell (65). β-AR surface expression on T cell may be influenced by cellular conditions. For instance, the level of β-AR surface expression is increased 24 h after cell activation, without alteration of affinity, by the T cell mitogen Con A (66), although epigenetic mechanisms and protein kinase C (PKC) activation may down regulate surface expression of β-AR during Con A-induced T cell activation (67). Contrasting results are
dependent on different experimental conditions (*in vitro vs. in vivo*), the time elapsed after stimulation, time of stimulation relative to mitogen stimulation, and the particular cell population examined and their activational states (67, 70). Also the subtype of CD4+ T cell subsets, such as Th1 cells, but not Th2 cells, preferentially express the β2-AR; and this feature is consistent at the mRNA level (64), as well as in Th1 cells newly generated from naïve CD4+ T cells (65). Different studies support the general hypothesis that β-AR stimulation decreases the level of mitogen- or anti-CD3 antibody-induced T cell proliferation (64, 61).

Despite increased sympathetic activity in aging, many target tissues are hyporesponsive to NA stimulation, mainly involving changes in β-AR signaling (71-72). So far in rat models, longitudinal studies in F344 rats, where splenic innervation declines, we found that compensatory mechanisms in the SNS maintained a relative effective transmission with immune cells (4), through altered NE reuptake, increased stimulated NE release, increased β-AR expression and signaling (52). In contrast, no longitudinal data in lymphoid organs were available for BN rats, but observations in aged (27M) BN rats, showed that innervation and neurochemistry were not significantly affected (73). β-AR interactions with G proteins may also regulate receptor activation by switching coupling events from Gs to Gi/Go, as reported in some cell lines and dissociated primary cells grown in tissue culture under certain conditions (74-76). Other postsynaptic effects downstream of receptor activation and the activity of adenosine cyclase, are not within the scope of these studies.
Aging and Central Autonomic Influence in the Spleen

Two studies from the same laboratory indicate that the rostral ventrolateral medulla (RVLM) provides a major input to spinal preganglionic sympathetic neurons that project to the spleen (77, 78). Reticulospinal sympathoexcitatory neurons of the C1 area of this brain stem region reduce the tonic activity of these sympathoexcitatory neurons (79). Very little is known about the role of the ventrolateral medulla in regulating sympathetic outflow to other visceral organs, including lymphoid tissues.

Sympathetic ganglia from aged humans (superior cervical and hypogastric in humans) (80-85), and animals (superior mesenteric-celiac and hypogastric, but not superior cervical ganglia in rats) (86-88) exhibit marked depletion of catecholamine histofluorescence. Similar findings are reported in sympathetic nerves along blood vessels and in other target organs (89-92). In rats (F344 rat), the effects of dysregulated sympathetic activity in a target organ, such as the spleen, may be reflected in the altered homeostatic set point for stored NE levels with advancing age (93). These findings also would be consistent with the regulation of NE content in sympathetic neurons by the local millieu of the target tissue in which their axon reside.

Significance

Strain Differences Between F344 and BN Rat Models for Aging

In our laboratory, two rat strains, the F344 and the BN rats, which are currently available through the National Institute of Aging (NIA), have been used as models to study effects of age-related changes in SNS on sympathetic neurotransmission in the spleen for several reasons that are described here. The F344 rat is the best studied model for aging. BN and F344xBN hybrid rats are becoming more commonly used as models
for aging, since the NIA has established breeding colonies for these strains. The F344 and the BN strains are genotypically different, which confers differences in life spans, and distinct behavioral stress response and immunological profiles, with consequences for age-related pathologies that are mediated via neural-immune interactions (73, 94). These differences are itemized as follows: (1) F344 rats live only 75% of the life span of BN rats on average (94, 95); the most obvious feature to consider in our studies, as it reflects the life span in the general human population. (2) Age-related pathologies that cause morbidity and mortality differ in F344 and BN rats (96). Unlike F344 rats, there is low frequency of pituitary adenomas or glomerulonephritis in BN rat with increasing age (96, 110). (3) Behavioral profiles in these rat strains suggest phenotypic difference in their limbic and stress pathways, with the F344 rat considered the hyper responsive, more vigilant strain under acute and chronic stress (97). BN rats have a low baseline level of anxiety (111), and low learning and memory performance (112). (4) Finally, differences in immune responses to mitogens, antigens and pathogens between F344 and BN rats indicate a Th1 and Th2 bias, respectively; it is not clear how age-related change in immunity affect these biases, but aging in general tends to shift an immune response toward a Th-type 2 response (98, 101). Prior to this research project, extensive studies in our laboratory were conducted to further characterize NA innervation (99), and neurotransmitter levels (NE) in the aging F344 rat spleen (9). This research also examined correlations between the SNS activation and lymphocyte proliferation patterns in the splenocytes across age (100). In chapter 2 of this dissertation, we studied the assessed net basal sympathetic activity, innervation and β-AR signaling in the spleen longitudinally across age in the BN rat. Thus, this initial investigation determined the
period when changes in SNA, β-AR signaling and sympathetic innervation in the spleen are manifested.

Specific Strain Differences in Immunity

With respect to the immune response differences, the young adult F344 rat has demonstrated a bias towards a Th1 response after immunization with ovalbumin, based on cytokine profiles (101, 102). Compared with the Lewis rat, which has identical MHC class II haplotype, the F344 rat appears to be resistant to experimentally-induced autoimmune diseases modulated by Th1 or Th2 cytokines (103, 104, and 106). Overall this low susceptibility may be largely dependent on both immunological and non-immunological factors, including the involvement of stress pathway activity (9) and local peptide concentration, such as neuropeptide (NPY) (105). These features may render young F344 rats better able to eliminate antigens using cell-mediated immunity, so that the antigens are eliminated and autoimmunity is averted. With advancing age, F344 rats may be more vulnerable to the allostatic load from heightened autonomic and neuroendocrine-mediated compensatory mechanisms, which may lead to immunosenescence as age progresses. Greater allostatic load from increased neuroendocrine outflow, particularly the HPA axis, in the face of immune senescence may be contributory to the high incidence of pituitary tumors in aged F344 rats (110). Additionally heightened SNS outflow combined with greater HPA activity may interact with immune senescence to increase the risk for the overt age-related pathology well documented in this rat strain, and contributes to the relative shorter life span of the F344 rats (96).
On the other hand, with its predominant bias toward a Th2-type of immunity when challenged with antigen (107, 109), the BN rat provides a good model for studying the mechanisms that predispose the adults to increased risk for Th2-mediated autoimmune diseases. In comparison with Lewis rats who are predisposed to Th1-type autoimmune diseases, mercuric chloride (HgCl$_2$)-induced autoimmunity can be induced in rat strains with a bias towards a Th2-mediated immunity. Induction of autoimmunity after challenge in BN rats was evident at day 7 after HgCl$_2$ exposure characterized by the anomalous low frequency of erythrocytes and CD8$^+$ with high number of B cells in the BN spleen, and the suppressed production of the Th1 cytokine, IFN-γ production (108).

Specific Strain Difference in Sympathetic Activity and Innervation

Most studies investigating the effects of sympathetic activity on NA innervation profiles have come from mice and the F344 rat. Our studies are novel in that they extend the aging-immune literature, by examining a different rat strain that has a different SNA profile and may impact sympathetic neurotransmission in aging spleen cells differently from F344 rats. Sympathetic nerve density, affected by increased SNA in the aged spleen, appears to be affected to a lesser degree by aging in BN rats (73, 96). Since, it is established that neurotrophic factors produced and released by target cells contribute to sympathetic nerve integrity (113) it seems reasonable to propose that strain differences in nerve integrity with normal aging may result from altered neurotrophic support in the splenic microenvironment. In K14-NGF-transgenic mice, high levels of NGF expression are observed in spleen and peripheral lymph nodes; and the level of innervation correlates with the level of NGF peptide (114). Therefore neurotrophic support may play an
important role in sympathetic innervation in the spleen permissive to optimum modulatory effects of CAs on lymphocyte proliferative responses, migration and homing to immune sites (114, 115). Previous reports by our laboratory have shown an age-related reduction in the density of OX19\(^+\) T cells and ED3\(^+\) macrophages in the splenic white pulp, two cellular sources of neurotrophic factors (52). Preliminary data produced in our laboratory confirm an age-related decline in nerve growth factor (NGF) in spleens from F344 rats that was not evident in BN rats (unpublished findings).

An increase in oxidative stress as a consequence of heightened SNA may provide another possible mechanism to explain age-related changes in sympathetic innervation of the spleen. The age-related autodestruction NA splenic fibers in our rat models may be induced by free radicals generated by excess oxidative catecholamine metabolites produced by high turnover rate of NE (52). Catecholaminergic neurons, both centrally and in the periphery are sensitive to oxidative stress (52, 73, 116). Therefore, heightened sympathetic activity in the aged spleen would be expected to increase oxidative metabolites, such as 6-hydroxydopamine (6-OHDA), that are toxic to sympathetic nerve fibers. Further, with age, reduced ability of target cells or neurons to absorb free radicals may lead to neuronal atrophy directly or by reduced ability to synthesize cellular components (116). \textit{In vivo}, NGF is capable to protect neurons from free radical damage in the CNS (116, 119), and \textit{in vitro} in PC12 cells (117), by elevating the activity of antioxidant enzymes. Collectively, these findings support our hypothesis for oxidative stress mediated damage, as a mechanism involved in the destruction of sympathetic nerves during aging.
Consequences for Susceptibility to Age-Related Diseases

The effects of sympathetic dysregulation in aging individuals can have detrimental effects to health, especially if these individuals have a compromised immune system concomitant with a heightened SNS activity. The dysregulated status of the SNS may profoundly affect the susceptibility of the organism to infection and/or influence disease course (4). The SNS activity in an individual can be reprogrammed by early exposure to stressful events, thereby altering the individual’s resiliency against infectious disease (118). Also later in life, both clinical and animal research reports that slower tumor growth and better patient prognosis is positively associated with good social support. Although the direct mechanisms are not clear, it is well established that optimal interactions between the nervous system, neuroendocrine, and immune system are important, as positive social interactions influence these systems to inhibit tumor growth (120). In disease progression, the incidence of tuberculosis increases and disease prognosis is worse in patients reporting stressful life event (121). Abnormalities in sympathetic activity in patients during *Helicobacter pylori* infection can be induced by psychosocial stress, and a response towards a Th2 profile may promote the onset and/or progression of the infection (122). Experimental observations in healthy rodents indicate that shifting levels of cytokine production may initially cause alterations in adequate immune activation following an increase in SNS activity. In this scenario there is a suppression of type-1 cytokines, while enhancing type-2 cytokines production. Consequently, an overall shift towards a humoral immunity over cellular immunity is observed in these animals (3). In the clinical setting this occurrence can be detrimental for the patient since cellular immunity is essential for host defense against mycobacterial infections. SNS inhibition of Th1 cell-dependent immunity may lead to the development
of a disorder, such as peptic ulcer from chronic gastritis caused by bacterial infection (123, 124, and 125).

Another example of how a deficient interface between the SNS and immune system occurs in a host is observed in HIV infection and sepsis. In HIV infection, the pathogenesis of the disease occurs in lymphoid organs and its progression is marked by increased IL-10 and reduced IL-12 indicating a bias towards Th2-type immunity (126). The proposed mechanism for the production of Th2 cytokines involves the enhanced production of intracellular cAMP via β-ARs (127, 128). During sepsis, although the activation of the SNS is important in modulating the activation of the immune system to combat infections, sustained SNS activation may have either beneficial or detrimental consequences for the affected individual by inducing changes in circulating levels of important cytokines (129, 130). In search of therapeutic interventions during septic shock, pharmacological interventions that induce anti-inflammatory effects can be achieved by targeting subpopulations of ARs with antagonists to α2-AR and β2-AR, either alone or in combination (4). Recognition of bacterial lipopolysaccharide (LPS) by the innate immune system elicits strong pro-inflammatory responses that can eventually cause a fatal sepsis syndrome in humans (131). In animal models, pharmacological manipulation of ARs in LPS-treated mice show that plasma TNF-α levels is inhibited by a β-AR agonist, and by an α2-AR blockade with CH 38083 (a highly selective α2-AR blocker), but pretreatment by blocking β-AR increases the plasma levels of TNF-α (132). Together, these data suggest a dual role for SNS activation after LPS administration that may include: a) reduction of TNFα through β-ARs, and b) inhibition of NE release through presynaptic α2-AR (133). Therefore the use of an specific catecholamine regime
to treat septic shock is impossible. For instance, the incidence of poorer survival and increased end-organ injury following the use of dobutamine, an \( \beta_1 \)-AR agonist, one of the drugs that is used to maintain adequate cardiac output under systemic septic shock. Although \( \beta_2 \)- instead of \( \beta_1 \)-AR appear to control monocyte/macrophage cytokine profiles, the use of adrenergic agents in the clinical treatment of this condition require the optimal balance between cardiovascular support and immunomodulation (134). Further an early intervention with adrenergic drugs that can suppresses IL-12 production by macrophages during cell-mediated immunity, increasing the risk uncontrolled bacterial growth (135).

In autoimmune diseases, the SNS influences the outcome of disease. If the SNS is hyperactive, a Th2 shift may potentiate and facilitate flares of systemic lupus erythematosus (SLE) (4). But if the SNS is hypoactive a Th1 shift, as in multiple sclerosis (MS) or rheumatoid arthritis (RA) may be sustained. This later observation is also evident in pregnant women during the third trimester, as proinflammatory cytokine production is suppressed (136, 137). However, the role of the SNS is more complex, as it exerts both promotional and inhibitory mechanisms under conditions where chronic inflammation is a predominant hallmark of a disease. For instance the Lewis rats, a model for rheumatoid arthritis (RA), exhibit dysregulated stress systems, and readily develop experimentally-induced Th1-mediated episodes of arthritis during experimental allergic encephalomyelitis (EAE) (132). \( \beta_2 / \beta_2 \)-AR agonists and \( \alpha_1 \)-AR blockers are reported to suppress chronic relapsing EAE in this rat model (138-140). In RA, the SNS regulates immune events locally in the inflamed joint by inducing localized inflammatory responses, such as increased blood flow, vascular permeability, release of inflammatory mediators, and mobilization of dendritic cells.
In secondary lymphoid organs, the SNS regulates Th1 vs. Th2 drive through clonal expansion of autoreactive immune cells, as well as blood flow (4). From studies in our laboratory, the timing of adrenergic drug treatment relative to the adjuvant challenge and disease course translates into different outcomes. Treatment of adjuvant-induced arthritic (AA) rats with the β2-AR agonist, terbutaline, initiated at antigen challenge, exacerbates disease severity. Otherwise, terbutaline treatment that is initiated at the onset of the disease significantly reduces the severity of the disease (141). Altered AR expression and/or signaling capacity are features of impaired SNS regulation of cellular proliferation that may lead the pathogenesis of RA. For instance, the reduction in the expression and activity of G-protein receptor-coupled kinase by PBMC, which causes deregulation of the β2-AR cell surface expression, influences the progression of disease in RA patients (142, 143). Influences of the SNS in autoimmune disorders are complex (144, 145). A hypoactive SNS may sustain the Th1, whereas a hyperactive SNS may intensify the Th2 shift and induce flares of a disease, as seen in SLE (4). F344 rats, which have a hyperactive stress system, are resistant to experimental induction of Th1-mediated autoimmune states (e.g. arthritis, uveitis, and EAE) (146). The other rat strain in our studies, the BN rat may have less reactive stress pathways, and is more sensitive to Th2-mediated autoimmune diseases such as polyarthritis, vasculitis, lupus-like syndromes (98).

Collectively, the experimental observations from humans and appropriate animal models can provide a framework from which treatment protocols can be designed in the clinical realm. Treatment regimes may become possible that tailored to each individual’s immune response to “adjust” to a more appropriate immune response to challenge with
antigens or pathogens. Our studies begin to investigate the role of heightened sympathetic activity as a mechanism responsible for altered sympathetic innervation and neurotransmission across strains. Thus we begin developing rat models for exploring mechanisms that may help explain causal relationships between sympathetic signaling and immune function across different genetically and environmentally-influenced patterns of aging. These two rat models of aging may be useful in understanding (1) racial/genetic differences that predispose individuals to certain types of age-related diseases; and (2) relative optimal vs. detrimental sympatho-immune interactions in aging. In order to understand why aging makes certain individuals vulnerable to diseases that increase frequency in aging, we need to understand how genetics, as well as epigenetic changes, interface with physiological systems like the autonomic nervous system to negatively affect host defense.
CHAPTER 2

SYMPATHETIC INNERVATION OF THE SPLEEN IN MALE BROWN NORWAY RATS: A LONGITUDINAL AGING STUDY

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Running Heading: Aging and NA Innervation in the BN Rat Spleen

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Abstract

Aging leads to reduced cellular immunity with consequent increased rates of infectious disease, cancer and autoimmunity in the elderly. The sympathetic nervous system (SNS) modulates innate and adaptive immunity via innervation of lymphoid organs. In aged Fischer 344 (F344) rats, noradrenergic (NA) nerve density in secondary lymphoid organs declines, which may contribute to immunosenescence with aging. These studies suggest there is SNS involvement in age-induced immune dysregulation.

Objectives: The purpose of this study was to longitudinally characterize age-related change in sympathetic innervation of the spleen and sympathetic activity/tone in male Brown Norway (BN) rats, which live longer and have a strikingly different immune profile than the F344 rat, the traditional animal model for aging research. Methods: Splenic sympathetic neurotransmission was evaluated between 8 and 32 months of age by (1) NA nerve fiber density; (2) splenic norepinephrine (NE) concentration; and (3) circulating catecholamines levels after decapitation. Results: We report a decline in noradrenergic nerve density in splenic white pulp (45%) at 18 months of age compared with 8 month-old (M) rats, which is followed by a much slower rate of decline between 18 and 32 months. Lower splenic NE concentrations were consistent with morphometric findings. Circulating catecholamines levels generally dropped with increasing age.

Conclusion: These findings suggest there is a sympathetic-to-immune system dysregulation beginning at middle age. Given the unique T-helper-2 bias in Brown Norway rats, altered sympathetic-immune communication may be important for understanding the age-related rise in asthma and autoimmunity.

Classification Terms: Section 6. Regulatory Systems
**Key Words:** noradrenergic nerves; secondary lymphoid organ; stress-induced plasma catecholamines; fluorescence histochemistry; cardiovascular measures; aged

**Abbreviations:** 3, 4-dihydroxybenzylamine, DHBA; adrenergic receptors, AR; Brown Norway, BN; BN X F344 (F1), BNF1; central nervous system, CNS; delayed type hypersensitivity, DTH; diastolic pressure, DP; epinephrine, EPI; ethylenediaminetetraacetic acid, EDTA; Fischer 344, F344; glyoxylic acid condensation method, SPG method; high-performance liquid chromatography with electrochemical detection, HPLC; interleukin, IL; month-old, M; natural killer, NK; nerve growth factor, NGF; neurotrophin-3, NT-3; New Zealand black, white, and black and white mice, respectively, NZB, NZW, and NZBW; noradrenergic, NA; norepinephrine, NE; periarteriolar lymphatic sheath, PALS; perchloric acid, HClO4; sympathetic-adrenal medullary system (SAM); sympathetic nervous system, SNS; T-helper-1, Th1; T-helper-2, Th2; Wistar-Kyoto, WK

**Introduction**

The central nervous system (CNS) regulates immune function, at least in part, via noradrenergic (NA) sympathetic nerves that innervate primary and secondary immune organs (reviewed in Bellinger et al., 2008b). Norepinephrine (NE) released by sympathetic nerves interacts with adrenergic receptors (AR) expressed on the surface of cells of the immune system (reviewed in Kin and Sanders, 2006; Bellinger et al., 2008b). The sympathetic nervous system (SNS) modulates many aspects of immune function. Based on *in vitro* and *in vivo* studies in young adult rodents, the roles ascribed to the SNS include: (1) limiting the magnitude of both acute and chronic inflammatory responses by
shifting the cytokine balance from a pro-inflammatory towards an anti-inflammatory cytokine profile (Vizi and Elenkov, 2002); (2) promoting T-helper-2 (Th2)-driven antibody responses via β2-AR signaling of B cells by up-regulating B cell accessory molecule expression and increasing B cell responsiveness to interleukin (IL)-4 (reviewed in Kin and Sanders, 2006); (3) enhancing cell-mediated responses, such as a delayed type hypersensitivity (DTH) reaction to a contact sensitizing agent through direct interaction with both T cells and antigen-presenting cells (Madden et al., 1989; Li et al., 2004); and (4) influencing Th1-driven antibody responses through β2-AR stimulation of Th1 cells (Madden et al., 1995; Kruszewska et al., 1995; reviewed in Kin and Sanders, 2006; Bellinger et al., 2005). In general, agents that activate the SNS tend to reduce T cell responses (Madden et al., 1989; Sanders et al., 1997; reviewed in Kin and Sanders, 2006), anti-viral immune reactivity (Dobbs et al., 1993), and natural killer (NK) cell activity (Irwin et al., 1988). Collectively, studies performed in young adult rodents demonstrate that genetic background, gender, site of immunization, type of immune cells involved in the immune response, and timing of exposure to catecholamines during the immune response all contribute to the complexity of SNS interactions with the immune system and affect the outcome of SNS modulation of the immune response (Madden et al., 1995; Kin and Sanders, 2006; Bellinger et al., 2008b). Furthermore, these complexities likely reflect an important role of the SNS in fine-tuning an immune response with the goal of effectively eliminating the threat to the host and restoring immune system homeostasis.

NA innervation of, and NE content in, secondary lymphoid organs, such as the spleen, can be affected by physiological changes (i.e., immune challenge or immunosuppression) (Yang et al., 1998; Lorton et al., 2008), immunodeficiency virus
infection (Kelley et al., 2003; Sloan et al., 2008), psychosocial stress (Sloan et al., 2007), hypertension (Purcell and Gattone, 1992), pregnancy and parturition (Bellinger et al., 2001) and with advancing age (Bellinger et al., 1987, 2001, 2002). Age-related changes in sympathetic innervation of the spleen are species- and/or strain-specific. For example, sympathetic NA innervation of spleens from aged C57Bl/6J and BALB/cJ mice is preserved (Madden et al., 1997; Bellinger et al., 2001), but declines with advancing age in male C3H, MRL-lpr/lpr (Breneman et al., 1993) and New Zealand mice (NZB, NZW, and NZBW) (Bellinger et al., 1989). In murine strains that develop autoimmune diseases (MRL lpr/lpr, NZB and NZBW) the onset of sympathetic nerve loss in the spleen occurs with, or slightly precedes the onset of the autoimmune disease (Breneman et al., 1993; Bellinger et al., 1989).

In a previous study from our laboratory (Bellinger et al., 2002) we compared sympathetic innervation of the spleen in four strains of young (3-month-old (M)) and old (21M) rats. These strains of rats were chosen for study because they are commonly used as models for human aging and/or used to study neural-immune interactions. We reported that NA innervation of spleens from male Fischer 344 (F344) and Lewis rats decline in normal aging, whereas NA innervation was preserved in age-matched Brown Norway (BN) and BN X F344 (F1; BNF1) rats. The reason for this strain difference is unclear, but maybe a result of the BN and BNF1 rats having a longer life span (median age 32M) than F344 and Lewis rats (median age 24M) (Nadon, 2004). Unlike F344 rats, the most commonly used rat model for aging, in BN rats there is low morbidity from pituitary adenomas or glomerulonephrosis with increasing age (Lipman et al., 1996, 1999; Nadon, 2004). BN and F344 rats also differ in their behavior (Rex et al., 1996;
Ramos et al., 1997), learning and memory (van der Staay et al., 1996), immune function (Sado et al., 1986; Koch 1976; Stankus and Leslie 1976; Festing, 1998; Lipman, 1996, 1999), and stress responses (Segar et al., 2008; Duclos et al., 2005; Sarrieau et al., 1998; Gómez et al., 1998), which may affect NA nerve integrity in the spleen with advancing age.

The purpose of the present study was to longitudinally examine the effect of age on sympathetic NA innervation of spleens from BN rats. Since age-related changes in SNS and sympatho-adrenal medullary system (SAM) reactivity may contribute to age-induced changes in NA nerve integrity and splenic NE content via increased local catecholamine concentrations influencing uptake into the nerve terminals from the circulation, local sympathetic nerve activity, and/or affecting leukocyte migration (Bellinger et al., 2008b; Elenkov et al., 2000; Madden et al., 1995), SNS and SAM reactivity to decapitation stress was also assessed by measuring circulating catecholamine levels (NE and epinephrine (EPI), respectively). We report here an age-related decline in NA nerve density in the splenic white pulp, evident by morphometric analysis at 18 months of age, and reduced splenic NE concentration between 18 and 32 months of age. Decapitation stress-induced plasma catecholamine levels were diminished in middle aged and aged rats. Given the well documented role of the SNS in immune modulation, altered sympathetic innervation likely affects immune competence in aging BN rats.

Results

Fluorescence Histochemistry for Catecholamines in the Spleen

In young adult rats (8M), dense plexuses of NA fibers entered the spleen with the splenic artery and its branches. The greatest density of NA nerves was found in the white
pulp associated with the central arteriole and its branches (Fig. 1A). Fluorescent NA nerve fibers extend from these vascular plexuses into the periarteriolar lymphatic sheath (PALS) among lymphocytes and macrophages, as either linear or punctate profiles. NA nerve fibers also course as less dense plexuses along the venous sinuses and trabeculae in the red pulp, but are rarely found in splenic follicles, where B cells predominantly reside.

At 15 months of age, declining NA nerve density was observed in all compartments of the spleen, mostly in the white pulp along the central arteriole (Fig. 2.1B). Between 18 and 32 months of age, the density of NA plexuses in the splenic white pulp, along the central arteriole (Fig. 2.1C-2.1F) and other regions of the spleen appeared comparable to that seen at 15 months of age. Morphometric analysis of fluorescent nerve profiles in the splenic white pulp across age (Fig. 2.2A) confirmed our qualitative assessment of NA innervation ($F(5,30) = 15.14, p < 0.0001$). A significant reduction in the mean splenic percentage of NA nerve area from 15M to 32M rats compared with 8M rats ($p < 0.001$) was found. A scatter plot showing the percent area of NA nerve fibers in splenic white pulps across age is shown in Fig. 2.2B. Linear regression analysis revealed a significant negative correlation ($p < 0.0001$) between sympathetic nerve area and increasing age.
Fig. 2.1. NA Nerves in the Splenic White Pulp across Age.
Fluorescence histochemistry for localizing catecholamines revealed a dense plexus of bluish-green fluorescent noradrenergic (NA) nerves surrounding the central arteriole (ca) and in the adjacent white pulp (wp; indicated by arrowheads) of spleens from 8M (A). In 15M (B) rats, NA innervation was diminished compared with 8M rats, a change that persisted through 32 months of age (C-F). These photographs are representative of NA innervation of spleens from all animals in each treatment group. 18M (C), 24M (D), 27M (E), and 32M (F) BN rats. Calibration bar = 100 µm.
Fig. 2.2. Effect of Age on Mean Percentage of NA Nerve Area in the Splenic White Pulp.
A. The mean percent area of noradrenergic (NA) nerves in splenic white pulps was reduced (*, $p < 0.001$) between 15 and 32 months of age. Four white pulp regions from 6 rats per age group were used to quantify nerve area and data are expressed as mean of mean ± SEM.
Fig. 2.2. Relationship between Mean Percentage of NA Nerve Area in the Splenic White Pulp across Age. (Continued).

B. A scatter plot demonstrates the distribution of splenic NA nerve expressed as a percentage of sample area in the white pulp across age. The line of best fit shows that NA nerve area is negatively associated ($r^2 = 0.344; p < 0.0001$) with age.
NE Concentration in the Spleen

There was a significant effect of age on splenic NE concentration (Fig. 2.3A) ($F(5,41) = 15.39, p < 0.0001$). In 15M and 18M rats, the mean splenic NE concentration (Fig. 2.3A) was reduced by 25 and 45% of the 8M values, respectively (18M, $p < 0.001$), followed by a more gradual decline between 18M and 32M (8 vs.24-32M, $p < 0.001$). Additionally, posthoc analysis also revealed a significant lower mean splenic NE concentration at 27M and 32M than in 15M rats (27M, $p < 0.001$; 32M, $p < 0.01$), representing a 44% drop in splenic NE concentration. In contrast, the mean spleen weight progressively increased from 15M through 32M (Fig. 2.3B) ($F(5,41) = 12.67, p < 0.0001$). Spleen weights from 24M, 27M, and 32M rats were greater than 8M rats (24M, $p < 0.01$; 27-32M, $p < 0.001$). Similarly, spleens from 24-32M rats weighed significantly more than spleens from middle aged rats (15M vs. 24-32M, $p < 0.001$; 18M vs. 32M, $p < 0.05$). Similarly, body weight progressively increased from 15M through 32M (Fig. 2.3C).
Fig. 2.3. Mean Levels of Splenic NE Concentration Across Age.
A. Mean splenic norepinephrine (NE) concentration (expressed in ng/g tissue wet weight) was slightly lower, but not significantly different at 15 months of age compared with 8M rat. Between 18 and 32 months of age, splenic NE levels significantly (*, $p < 0.01$) decreased compared with 8M levels. Splenic NE concentrations from 27M and 32M rats also significantly differed (**, $p < 0.001$) from levels in 15M rats. Error bars = SEM.
Fig. 2.3. Mean Levels of Spleen Weight Across Age (Continued).

B. Mean spleen weight (expressed in g) progressively rose between 8 and 32 months of age, with significant differences revealed by posthoc analysis in 24 through 32M rats compared with 8M and 15M rats (*, \( p < 0.01 \), 24M; and **, \( p < 0.001 \), 27-32M, respectively). Spleen weight also was significantly higher in 32M compared with 18M rats (***, \( p < 0.05 \)). Error bars = SEM.
C. Mean body weights (expressed in g) were comparable at 8 and 15 months of age, but increased between 18 and 32 months of age. *, significantly different from 8M: 18M, 24 – 32M, $p < 0.001$; **, significantly different from 15M: 24 – 32M, $p < 0.001$; ***, significantly different from 18M: 24 – 27M, $p < 0.001$. Error bars = SEM.
Fig. 2.4. Relationship between Splenic NE Concentration and NA Nerve Area or Age in the Spleen.

A. The distribution of the mean percentage of NA nerve, as demonstrated by the scatter plot area in the white pulps that were sampled per rat across splenic NE concentration (ng/g) for each age group. Linear regression analysis was used to plot the line of best fit and calculate $r^2$, and reveals a positive association ($p < 0.0001$) between NA nerve area in the white pulp and splenic NE concentration.
Fig. 2.4. Relationship between Age and NE Concentration in the Spleen (Continued).

**B.** This scatter plot represents the distribution of splenic NE concentration (ng/g) across age. Linear regression analysis reveals a negative association ($p < 0.0001$) between splenic NE concentration and increasing age ($p < 0.0001$).
Figure 4 shows the scatter plots that demonstrate the relationships between splenic NE concentration (Fig. 2.4A, 2.4B), percentage of NA nerve area in the splenic white pulps (Fig. 2.4A), and age (Fig. 2.4B). Linear regression analysis revealed a positive correlation ($r^2 = 0.444; p < 0.0001$) between splenic NE concentration and NA nerve area in the splenic white pulp. A higher percentage of sympathetic nerves in the white pulp is associated with greater splenic NE concentration (Fig. 2.4A). In contrast, increasing age is associated ($r^2 = 0.791; p < 0.0001$) with reduced splenic NE concentration (Fig. 2.4B).

### Mean Plasma Catecholamine Concentration

Plasma catecholamine levels in all age groups were significantly elevated over basal levels from previous reports (Fig. 2.5A-B). There was an effect of age on mean plasma catecholamine levels (Fig. 2.5A-B) (NE, $F(5,34) = 5.652, p < 0.0007$; EPI, $F(5,33) = 5.143, p < 0.0014$). Mean plasma NE concentration (Fig. 2.5A) was reduced by 24% in 15, 24, and 27M old rats compared with young adults (15M, $p < 0.05$; 24M, $p < 0.001$; 27M, $p < 0.01$). Similarly, plasma EPI levels (Fig. 2.5B) were highest at 8 months of age and reduced with aging (~32-58%), with a significant decline observed at 15, 24, and 32 months of age compared with young adult rats (15M, 24M, $p < 0.01$; 32M, $p < 0.05$).
Fig. 2.5. The Effect of Decapitation-Induced Stress on Plasma NE Concentration from Trunk Blood across Age.

A. Plasma norepinephrine (NE) concentrations (expressed in ng/ml as a mean ± SEM) were significantly (*, $p < 0.05$, $p < 0.001$, and $p < 0.01$, respectively) diminished at 15, 24, and 27 months of age compared with 8M rats. Dashed box represents the range of basal catecholamines levels reported in the literature based on assessments from awake, undisturbed rats from which blood was drawn via an indwelling catheter (Popper et al., 1977; Mabry et al 1995a,b,c; Paulose and Dakshinamurti, 1987; Carruba et al., 1981; Kvetnansky et al., 1978).
Fig. 2.5. The Effect of Decapitation-Induced Stress on Plasma EPI Concentration from Trunk Blood across Age (Continued).

B. Epinephrine (EPI) concentrations in BN rats are shown across age (in months). Plasma EPI levels were significantly (*, *p* < 0.01, 15 and 24M; *p* < 0.05, 32M) lower at 15, 24, and 32 months of age compared with 8M rats. Dashed box represent the range of basal catecholamines levels reported in the literature based on assessments from awake, undisturbed rats from which blood was drawn via an indwelling catheter (Popper et al., 1977; Mabry et al 1995a,b,c; Paulose and Dakshinamurti, 1987; Carruba et al., 1981; Kvetnansky et al., 1978).
Splenic NE Turnover Rates Across Age

Intraperitoneal treatment with α-MPT was used to block the activity of Tyrosine Hydroxylase, the rate limiting step enzyme in the synthetic chain of NE. The mean rate of NE loss in the spleen was measured over a 6-hour period in all age groups. Regression analysis revealed that the turnover rate and turnover time of the decline of NE was greater ($p< 0.001$) diminished ($p< 0.05$), respectively at 15 months of age, compared with young adult (8M), or older adult (18M, 27M, and 32M) rats (table 1).

Discussion

In the present study, we demonstrated a dramatic age-related decline in NA sympathetic nerve density and NE concentration in spleens from male BN rats that was evident by early middle age (15M). Splenic NE concentration positively correlated with sympathetic nerve area. In contrast, both splenic NE concentration and sympathetic nerve area in the white pulp negatively correlated with age. These findings are consistent with a loss of neurotransmitter content in the spleen as sympathetic nerves are concomitantly lost with advancing age. NA nerve density in the splenic white pulp, as well as other splenic compartments, was relatively comparable between 15 and 32 months of age despite the progressive decline in splenic NE concentration during this time period. These findings suggest a dying back of sympathetic nerves in middle age without a change in NE metabolism. Furthermore, these results suggest that there are age-related alterations in sympathetic signaling to immune cells in the aging BN rat spleen, because the amount of NE that is available to interact with cells of the immune system is reduced.
Table 2.1. Initial mean splenic NE concentrations, NE turnover rates and times in male BN rats across age as determined from regression analysis

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>Initial NE Concentrations (ng/g)</th>
<th>n</th>
<th>Rate Constant of NE Loss (k[h] ± SEM)</th>
<th>n'</th>
<th>Turnover rate (ng/g/h)</th>
<th>Turnover Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
<td>1116.54 ± 34.98</td>
<td>14</td>
<td>0.120 ± 0.003</td>
<td>16</td>
<td>83.18</td>
<td>8.31</td>
</tr>
<tr>
<td>15</td>
<td>1038.07 ± 33.03*</td>
<td>17</td>
<td>0.141 ± 0.004</td>
<td>18</td>
<td>113.88</td>
<td>7.09</td>
</tr>
<tr>
<td>18</td>
<td>710.00 ± 59.07</td>
<td>16</td>
<td>0.097 ± 0.001</td>
<td>17</td>
<td>42.78</td>
<td>10.33</td>
</tr>
<tr>
<td>27</td>
<td>487.97 ± 28.28</td>
<td>17</td>
<td>0.100 ± 0.004</td>
<td>18</td>
<td>32.43</td>
<td>9.98</td>
</tr>
<tr>
<td>32</td>
<td>463.44 ± 53.14</td>
<td>16</td>
<td>0.124 ± 0.002</td>
<td>16</td>
<td>37.10</td>
<td>8.07</td>
</tr>
</tbody>
</table>

1Expressed as a mean ± SEM
2Number of rats used to determine initial mean splenic NE concentrations
3Calculated from the slope of decline of log NE concentration. SEM determined from the variance and covariance of the rate constant and log NE concentration
4Number of rats treated with aMPT
5Time required to synthesize the steady-state tissue pool of NE

Table 2.1. Turnover rates and turnover times of NE in the spleen of BN male rats across age as determined from regression analysis of the data. Initial NE concentration was greater (p<0.01) in 15M BN rats compared with older rat groups (18M, 27M, 32M); and splenic turnover rate was significantly different (p<0.001) in 15M compared to all other age groups. Initial levels of splenic NE and the rate constants are expressed as means ± SEM. aMPT, α-methylparatyrosine NE, norepinephrine; BN, Brown Norway rats; n, number of rats per age group.
These findings are generally consistent with reports in male F344 rats (Bellinger et al., 1987, 1992a,b), the most commonly used rat model for aging. Interestingly, the age at which NA nerve loss becomes evident is comparable in both strains of rats (18 months of age) despite the fact that male BN rats live 20% longer than male F344 rats (75% mortality by 34 and 26 months of age, respectively (Nadon, 2004)). However, there are some noteworthy differences between these two strains. First, the decline in splenic nerve density in the white pulp is less extensive in BN compared with F344 rats (~56% vs. 75% at around the time of 75% mortality). This finding suggests that while the mechanisms that initiate the age-related decline in sympathetic innervation of the spleen may be similar in these two strains, the microenvironment of spleen may differ such that greater resilience is afforded to sympathetic nerve fibers in spleens from BN rats. Whether this is a reflection of the striking differences in the immune profiles of these two strains awaits further study. Similarly, how differential preservation of sympathetic innervation of secondary lymphoid tissues, and its consequences on immune regulation contributes to greater longevity, requires further investigation.

Another difference between these two strains is that the decline in nerve density in old BN rats is comparable to the loss of splenic NE concentration (approximately 56-57% decline for both parameters at 32 months of age). In contrast, the extent of NA nerve lost at 27 months of age in F344 rats is much greater (75%) than the loss in splenic NE concentration (~50%) (Bellinger et al., 1987; 1992b). These data, along with functional data in F344 rats indicating increased signaling of splenocytes via NE binding with β-AR (Bellinger et al., 2008a) suggest compensatory mechanisms in NE metabolism in response to NA nerve loss in F344 rats, which do not occur with nerve loss in BN rats.
NE turnover studies are needed to directly address this hypothesis; however preliminary findings from our laboratory (unpublished observations) showing that NE signaling of splenocytes via β-AR stimulation is significantly impaired in old BN rats indirectly supports this hypothesis.

In a previous study from our laboratory (Bellinger et al., 2002) no significant differences in splenic NE concentration between 8M and 21M male BN rats were reported. The reason for the greater effect of aging on NA nerve integrity observed in the present study is not known, but may be due to differences in the vendor source. Male BN rats in the present study were obtained through National Institute on Aging (NIA) from Harlan, whereas in the earlier study animals were purchased through NIA from Charles River Laboratories. These discrepancies are not unique as other studies have reported differences in physiological parameters depending on the vendor source (Perrotti et al., 2001; Turnbull and Rivier, 1999; Pare and Kluczynski, 1997). For example, neuroendocrine and immune responses to inflammatory stimuli differed in Sprague-Dawley rats depending on whether they were obtained through Harlan or Charles Rivers Laboratories (Turnbull and Rivier, 1999).

The loss of NA innervation of the spleen in male BN rats may be explained by (a) changes in neurotrophic support, neurotrophic receptor expression, and/or neurotrophic signal transduction in splenic sympathetic NA nerve fibers, (b) cumulative effects of oxidative stress on the NA nerve fibers, and/or (c) an increase in systemic inflammation that occurs with aging. Neurotrophic growth factors such as neurotrophin-3 (NT-3) and nerve growth factor (NGF) are important for maintaining and supporting NA nerve integrity (Levi-Montalcini, 2004). NGF, NT-3 and brain-derived neurotrophic factor are
abundantly present in the spleen (Yamamoto et al., 1996). While it is not known whether there is an age-related decrease in NGF activity in F344 and BN rats, there are reports of an age- and disease-related decline in the content of NGF in several organs. Nishizuka and coworkers reported a decline in NGF in specific brain regions with age (Nishizuka et al., 1991; Nitta et al., 1993). There is also a decline in NGF and NT3 in the spleens of Lewis rats with adjuvant-induced arthritis (Bellinger et al., 2001). NGF, an essential neurotrophin factor for sympathetic neuron survival, is extensively distributed in the parenchyma of spleen. A decline in its activity may explain the age-related disappearance of NA nerve fibers in splenic white pulp.

In support for a role for oxidative stress in NA nerve loss, administration of deprenyl, a monoamine oxidase inhibitor, reverses the 6-hydroxydopamine-induced and age-related loss of splenic NA innervation in young and old F344 rats respectively (reviewed in ThyagaRajan and Felten, 2002). The reversal in NA neuronal loss was accompanied by enhanced cell-mediated immunity especially NK cell activity and IL-2 and IFN-γ production (reviewed in ThyagaRajan and Felten, 2002). These studies suggest growth factors and cytokines are important in maintaining sympathetic nerve fibers in the splenic white pulp across life span, and altered trophic support can have functional consequences.

Alternatively, individual variability in aging of sympathetic innervation of the spleen, or survival selectivity may contribute to the differences seen; however, the individual variability we found in our study does not lend support to the former hypothesis. Other factors that may contribute include differences in animal transport to the study location, time of year, and housing conditions. Collectively, these findings
suggest that environmental factors may affect NA nerve integrity in the aging rat spleen. In support of this hypothesis, Sloan et al (2008) have shown that simian immunodeficiency virus infection decreases sympathetic innervation of primate lymph nodes, possibly due to reduced neurotrophic support.

Plasma NE and EPI levels reported here reflect a stress response to handling and decapitation, as they are ~5-10 and 15-80 fold greater, respectively, than measurements taken via indwelling catheters from awake, undisturbed rats (Popper et al., 1977; Mabry et al 1995a,b,c; Paulose and Dakshinamurti, 1987; Carruba et al., 1981; Kvetnansky et al., 1978). Basal catecholamines were not measured in the present study, however, other studies in rats commonly report no effect of age on circulating NE and EPI (McCarty, 1981, 1985; Korte et al., 1992; Mabry et al., 1995a,b,c), and some studies have found elevated levels (Michalikova et al., 1990). Plasma NE and EPI concentrations from 8M rats in our study are comparable to levels previously reported in young BN rats (Gilad and Jimerson, 1981) and other rat strains under the conditions used to obtain blood in this study (Ben-Jonathan & Porter, 1976; Roizen et al., 1975; Popper et al., 1977). One study (Gilad and Jimerman, 1981) has compared sympathetic reactivity to decapitation stress alone or with immobilization in young BN and Wistar-Kyoto (WK) rats, of which the latter strain is more reactive to stress. They found that plasma catecholamine levels immediately after decapitation and 0 or 10 min after immobilization stress were significantly higher in WK than in BN rats. No studies that we are aware of have examined the effect of decapitation stress in the long-lived BN rat across age. However, in another study from our laboratory, no age-related change in decapitation stress-induced plasma NE concentrations and reduced plasma EPI levels at 24 months were found in
male F344 rats (Bellinger et al., 2008a), a strain with a little less than 1 year shorter median life span (Nadon, 2004) and greater behavioral responses to stress generally attributed to differences in hypothalamic-adrenocortical functioning (Marissal-Arvy et al., 1999; Sarrieau et al., 1998; Gómez et al., 1996, 1998; Kusnecov et al., 1995). The age-related decline in decapitation-stress induced rise in plasma NE and EPI concentrations found in this study suggests diminished capacity of the SNS and SAM to effectively respond to an acute stressor in aging male BN rats. Relevant to the present study, Kusnecov et al. (1995) demonstrated significant differences in footshock stress during early diurnal and nocturnal periods of the day on T cell mitogen-induced lymphocyte proliferation and IL-6 response in male BN rats compared with three other strains of rats, including F344 rats.

Stress studies by other investigators (Mabry et al., 1995a,b,c; Cizza et al., 1995) have demonstrated variable age-related differences in SNS and SAM reactivity to other acute and chronic stressors, depending on the type, duration, and intensity of the stressor. For example, in contrast to our findings in F344 rats, Mabry and colleagues (1995a) reported greater plasma catecholamine responses in aged F344 rats (22M) and slower return to baseline after termination of the stressor than those of young adult rats (3M) after cold (20 and 25 ºC) swim stress, but no aging difference when the water temperature was at 30 or 35 ºC. In another aging study using Wistar rats (Michalíková et al., 1990), basal plasma catecholamines were elevated in 11 and 28M rats compared with young rats, and immobilization stress markedly increased plasma NE in 11M, but plasma NE and EPI was mildly elevated in 28M animals. Although poorly characterized in rats, stress-induced effects on sympathetic reactivity in humans are not attributable to
differences in thermoregulatory mechanisms or kinetic factors, such as neuronal uptake or plasma clearance rates of catecholamines (Linares and Halter, 1987; Morrow et al., 1987; Poehlman et al., 1990; Stromberg et al., 1991). Since visceral organs contribute very little to plasma NE levels (reviewed in Bellinger et al., 1998), it seems unlikely that reduced SNS activity in the spleen contributes to the lower plasma catecholamine levels. Collectively, these studies indicate an age-related impairment in the ability of animals, including humans, to adapt to an ever-changing environment because of defects in hypothalamic regulation of SNS and SAM activity in aged animals. Thus, whereas basal levels of circulating hormones, like NE and EPI, are often not affected by aging, defects in neuroendocrine and autonomic regulation become unmasked when aged animals are exposed to acute stressors.

Our plasma catecholamine findings are relevant to sympathetic regulation of immune function in at least two ways. First, physical and psychosocial stressors can affect an immune function by elevating circulating stress hormones [Szelenyi and Vizi, 2007; Starkie et al., 2005; Moncek et al., 2003; Giovambattista et al., 2000; Condé et al., 1999; Hasko et al., 1995; Mujika et al., 2004; Brenner et al., 1998; Pederson et al., 1997; Pyne, 1994; Hinrichsen et al., 1992]. Second, exposure to environmental antigens is and of itself a stressor, affecting the reactivity of the SNS and SAM (Sakata et al., 1994; Moncek et al., 2003; Giovambattista et al., 2000). Thus, measured plasma catecholamine levels reflect the effects of aging on the activation (i.e. decapitation) of stress pathways in normal aging laboratory BN rats. And it was important to differentiate the measured plasma NE levels from normal NE basal levels.
The functional significance of altered sympathetic reactivity to stress and sympathetic innervation of the aging rat spleen awaits further investigation. It is clear, at least in young adults, the SNS plays an important role in regulating immune function and that dysregulation of the SNS can affect immune-mediated diseases (reviewed in Kin and Sanders, 2006; Bellinger et al., 2008b; Elenkov et al., 2000). It is also well documented that as cell-mediated immunity declines with increasing age (reviewed in Chakravarti and Abraham, 1999; Shearer, 1997), there is a shift toward humoral-mediated immunity (Caruso et al., 1996; Castle et al., 1997). Given these data, it is tempting to speculate that altered NA neural signaling of the immune system may contribute to immune senescence. Whether this is true or not, SNS dysregulation in aging is likely to affect the host’s ability to optimally defend against infectious diseases, prevent autoimmunity, detect/eliminate cancerous cells, and influence circulating proinflammatory cytokine levels, which progressively rise with age. As the immune system shifts to a Th2 response with aging, tolerance mechanisms have been postulated to fail leading to the production of clinically significant autoreactive antibodies (Stacy et al., 2002; Stephan et al., 1997).

BN rats are unique, because they have a vigorous Th2 immune responses, producing cytokines IL-4, IL-6, and IL-10, along with antibody isotypes IgG1 and IgE on antigenic stimulation (Fournié et al., 2001). They also share many immunological and physical responses seen in human asthma, such as high production of IgE antibody, contraction of airway smooth muscle, airway hyperresponsiveness, involvement of leukotrienes in lung reactions, and infiltration of eosinophils and lymphocytes into the airway (Ohtsuka et al., 2005). BN rats are widely used to study several chemically-induced autoimmune syndromes such as polyarthritis, vasculitis, lupus-like syndromes,
and other types of T helper cell dependent autoimmune diseases. With their striking Th2 bias, aged BN rats may provide a good model for discovering the mechanisms that predispose the elderly to increased risk for Th2-mediated autoimmune diseases and asthma. Careful analysis of species- and strain-related differences in how the SNS and immune system changes with advancing age and their relationship with frequencies of morbidity and mortality to certain types of disease may reveal risk factors and/or aging phenotypes that strongly predict susceptibility to certain types of diseases associated with aging.

**Experimental Procedure**

**Animals**

Male, inbred, specific-pathogen-free BN/Bi (BN) rats at 8, 15, 18, 24, 27, or 32 months of age (n of 8 per age group) were purchased under a National Institute of Aging (NIA) contract from Harlan Sprague-Dawley (Indianapolis, IN). Animals were housed two per cage in the vivarium at Loma Linda University, an Accreditation of Laboratory Animal Care (ALAC)-accredited facility. The room temperature (22-25 °C) and humidity (30-40%) were controlled and maintained on a 12:12-h light-dark cycle. Rats had access to rodent chow and water *ad libitum*. Animals were closely observed for changes in physical condition and/or presence of age-related illness. All animal experiments were conducted in accordance with the principles and procedures outlined in National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee at Loma Linda University. At the time of sacrifice, all visceral organs were autopsied for evidence of gross pathologies and tissues were dissected for study. Rats with any visible lesions, tumors, and evident
pathology were removed from this study and their tissues excluded from the analysis.

Three additional animals per group at older ages in our study were purchased to compensate for loss of animals from the study due to pathology and to maintain an $n$ of 8 per treatment group.

**Study Design**

Rats were housed in the vivarium at Loma Linda University for 1 week prior to study initiation to acclimate to vivarium conditions. After acclimation to vivarium conditions, rats were sacrificed by decapitation, and spleens and trunk blood were immediately harvested. Spleens were cut cross-sectionally into 5 equally-sized pieces. The middle piece of the spleen was weighed, immediately frozen on dry ice, and stored at -80 °C until samples were prepared for neurochemical measurement of NE by high-performance liquid chromatography with electrochemical detection (HPLC). The adjacent spleen pieces were used for fluorescence histochemical staining to localize NA nerves. Trunk blood (8-10 ml per sample) was collected in 12x75 mm tubes containing 10 mmol/L disodium ethylenediaminetetraacetic acid (EDTA) kept on ice. All blood samples were collected within 1 min of decapitation. After centrifugation (1200 rpm), the plasma was collected into microfuge tubes and stored at -80 °C until the determination of catecholamine levels.

**Fluorescence Histochemistry for Catecholamines**

The glyoxylic acid method of histofluorescence for catecholamines was used to visualize NA sympathetic nerves in spleens from BN rats. Spleen blocks from each rat were sectioned at 16 µm on a cryostat at -20 °C. The sections were thaw-mounted onto
slides and stained using a modification of the glyoxylic acid condensation method (SPG method), as previously described by de la Torre (1980). Briefly, 3 sections were mounted on each slide, dipped into a solution containing 1% glyoxylic acid, 0.2 M potassium phosphate, and 0.2 M sucrose (pH 7.4), and then slides were air dried under a direct stream of cool air for 15 minutes. Spleen sections were covered with several drops of mineral oil, placed on a copper plate in an oven at 95 °C for 2.5 minutes, then coverslipped. Catecholamine-containing nerve terminals were visualized using an Olympus BH-2 fluorescence microscope equipped with epi-illumination accessories.

High-Performance Liquid Chromatography (HPLC) with Coulometric Detection

Spleen samples were transferred into labeled centrifuge tubes containing 10X volume per tissue wet weight of cold 0.1 M perchloric acid containing 0.25 µM 3, 4-dihydroxybenzylamine (DHBA) as an internal standard, sonicated using a Branson Sonifier 250, and centrifuged at 10,000 rpm for 5 min. Supernatants were transferred to microfilterfuge tubes, centrifuged at 14,000 rpm for 20 min and stored at -80 °C until assayed for NE content. Plasma samples (200 µl per sample) were pipetted into a 12x75 mm glass tube, followed by the addition of 1.0 ml of phosphate buffer (pH 7.0), 1.0 ml of 1.5 M Tris buffer (pH 8.6), 50 µl of the internal standard, DHBA, and 50 mg of acid washed alumina. Plasma samples were vortexed and placed on a shaker for 5 min at 175 rpm and then the alumina was allowed to settle. Next, the samples were aspirated, washed 3X with double distilled H2O, and centrifuged for 2 min at 14,000 rpm. The alumina was placed into a new microfilterfuge tube and vortexed in 200 µl of 0.1 M HClO4. After the samples were centrifuged again for 2 min at 9000 rpm, 50 µl of
supernatant from each sample were transferred to HPLC vials and loaded into an ESA Model 542 autosampler to quantify NE concentrations ([NE]) by HPLC using a CouleChem HPLC System (ESA, Chelmsford, MA). The mobile phase was delivered at a flow rate of 1.0 ml/min by an ESA Model 582 solvent delivery module through a reverse phase C18 5 µm, 8x100 mm Radial-Pak analytical column. The potential through the guard cell and the two detector cells in the ESA CouleChem III coulometric system were set at 400 mV, 350 mV, and -350 mV, respectively. Peak heights and area under the curves were analyzed using EZChrom Elite Software (Scientific Software Inc. Pleasanton, CA). Unknown sample catecholamine concentrations were determined by comparing peak area (peak height) with those from known standards.

Data Analysis

Morphometric analysis of splenic NA nerves in the white pulp was carried out without knowledge of the treatment groups (i.e., blinded) using the Image Pro® Plus software (version 5.0; Media Cybernetics, Bethesda, MD), as previously described (Lorton et al., 2005; Bellinger et al., 1987, 2002). The white pulp was selected for analysis, because the majority of sympathetic nerve fibers innervate this splenic compartment. One randomly selected splenic white pulp in the hilar region (the point of NA nerve entry into the spleen) of 4 spleen sections per rat from 6 animals per age group was used for analysis. The criteria for selection of white pulps for analysis were that (1) there was only one cross-section through the central arteriole in the white pulp; (2) the size of the central arteriole was comparable across all samples (80-100 µm across the largest diameter of the vessel) and (3) the arteriole was cut in true cross section. Splenic white pulps were digitally photographed at 200X and the number of pixels containing NA
nerve profiles in each image, based on size and color, were determined. At this magnification, all pixels of each image were within the white pulp. The number of positive pixels (i.e., those containing nerve fibers) was used to determine the percentage of the total area positive for sympathetic nerves in each image. The average percentage area positive for sympathetic nerves from the 4 white pulps that were sampled from each animal was calculated, and then the means from each animal per age group were averaged to determine the within group mean ± standard error of the mean (SEM).

Catecholamine concentrations, and spleen and body weights were expressed as a mean ± SEM. NE concentrations in the spleen, and plasma NE and EPI concentrations, were determined from known standards and concentrations corrected based on the recovery rate of the internal standard, DHBA. Plasma catecholamine concentrations were expressed in ng/ml. Splenic NE concentration was expressed in ng per g tissue wet weight. A one-way analysis of variance (ANOVA) was performed on all data to determine between group differences using GraphPad Prism 4.0®. Factors reaching significance levels of at least $p < 0.05$ by ANOVA were subjected to Bonferroni post-hoc analysis to determine which groups contributed to the significant ANOVA. Scatter plots and least-squares linear regression analysis were performed using GraphPad Prism 4.0® to determine correlations between splenic NE content and noradrenergic nerve density and age, and splenic NE concentration and age. Lines of best fit with a 95% confidence interval were generated. Significance levels were determined by calculating the correlation coefficients ($r^2$ values) and degrees of freedom ($n$-2); $p < 0.05$ was considered significant.
Acknowledgements

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References


Haskó, G., Elenkov, I.J., Kvetan, V., Vizi, E.S., 1995. Differential effect of selective block of alpha 2-adrenoreceptors on plasma levels of tumour necrosis factor-
alpha, interleukin-6 and corticosterone induced by bacterial lipopolysaccharide in mice. J. Endocrinol. 144, 457-462.


CHAPTER 3

CHRONICALLY LOWERING SYMPATHETIC ACTIVITY PROTECTS SYMPATHETIC NERVES FROM AN AGE-RELATED DECLINE IN SPLEENS FROM F344 RATS

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Abstract

Immune functioning declines with normal aging and with stress, increasing the risk for cancer, autoimmunity, and infectious diseases. Age-related changes in sympathetic neurotransmission in immune organs occur concomitantly with, and may be causally-related to, immunosenescence; however, no model has been developed to determine causal relationships. Splenic sympathetic activity increases throughout middle-age, followed by a decline in activity, sympathetic nerve loss, increased β-adrenergic receptor (β-AR) signaling and reduced proliferation of lymphocytes from old Fischer 344 (F344) rats. Here, we investigated the role of increased sympathetic tone during middle-age on neurotransmission in the aged rat spleen as a first step towards developing a model for determining causal relationships between immune decline and altered sympathetic functions with age. Fifteen-month-old (15M) male F344 rats received 90-day rilmenidine treatment (0, 0.5 or 1.5 mg/kg/day, i.p.; Veh-18M, Rilmlo, or Rilmhi, respectively) a centrally-acting imidazoline-1 receptor agonist that lowers sympathetic activity. Untreated 3M and 18M rats (Crtl-3M and Crtl-18M, respectively) controlled for handling/injection stress. Splenic norepinephrine (NE) concentration with or without synthesis blockade, noradrenergic (NA) innervation, β-AR expression and β-AR-stimulated c-AMP production were evaluated using high-performance liquid chromatography with coulometric detection, fluorescence histochemistry, β-AR radioligand binding, and enzyme immunoassay (EIA), respectively. Rilmenidine treatment reduced splenic NE concentration and turnover, plasma NE (but not epinephrine) concentration, and partially reversed age-related nerve loss compared with young or old control groups. Rilmlo increased β-AR density without affecting affinity compared with Veh-18M rats. Rilmlo and Rilmhi treatment further
augmented the age-related rise in β-AR-stimulated cAMP production. Collectively, these findings demonstrate that rilmenidine protected sympathetic nerves and enhanced β-AR-mediated signaling in immune organs from aging rats that display a heightened sympathetic profile. Furthermore, they suggest that rilmenidine could affect host defense against age-related diseases.

**Keywords:** rilmenidine, imidazoline-1 receptor agonist, sympathetic nervous system, β-adrenergic receptors, cAMP production, aging, splenic norepinephrine turnover
**Introduction**

The sympathetic nervous system (SNS) is a homeostatic regulator of cardiovascular, metabolic and immune functions under basal conditions, in the face of acute or chronic changes in physiological state, and in response to stress. In immune organs, the SNS regulates immune function via tonic release of its major transmitter, norepinephrine (NE) [Bellinger et al., 2008a,b]. NE binds with adrenergic receptors (AR; predominantly $\beta_2$) expressed on immunocytes to increase cAMP production [Halper et al., 1984]. The SNS modulates a wide variety of immune measures, and affects the development and progression of immune-mediated diseases [reviewed in Bellinger et al., 2008b].

With aging, basal sympathetic nerve activity (SNA) progressively rises [Docherty, 2002], and SNS responses after activation are exaggerated [McCarty et al., 1997]. The functional significance of hyperactive SNA in immune organs remains unclear and understudied. Presumably, it occurs at the expense of fine control, at the risk of over-stimulating target tissues, and places additional metabolic strain (allostatic load) on all target organs [Goldstein and McEwen, 2002]. Higher SNA can alter immune cell trafficking [Redwine et al., 2003] and host defense [Bellinger et al., 2008b], and may be a risk factor for developing immune-mediated diseases that increase in frequency with advancing age [Bellinger et al., 2008b; Cohen et al, 2001]. Furthermore, the extent to which aging changes in SNS functioning mediate, or contribute to, the aging changes in immune function, i.e. a causal relationship between these two phenomena, remains unknown. This lack of information is due, in part, to a lack of clear understanding of target-specific changes in SNS functioning across age, and the lack of model systems that
can reverse the age-induced SNS changes in immune target organs, which is necessary to test this hypothesis in vivo.

In aging male F344 rats, sympathetic innervation progressively declines in secondary lymphoid organs [Bellinger et al., 1992, 2008a,b]. Nerve loss resembles a peripheral neuropathy in that nerves recede distally to proximally from their entry sites into the spleen, the hilar region. Nerve loss precedes a significant loss in splenic NE levels, suggesting enhanced NE metabolism as an early compensatory event. This is consistent with a slight, but significant rise in splenic NE concentrations in 10M rats and increased NE turnover between 10 and 15 months. In 24M rats, splenic NE turnover is reduced compared with 8, 10, 12, and 15M rats [Bellinger et al., 2008a]. Age-related splenic denervation is associated with increased lymphocyte responsiveness to β-AR stimulation that is evident in 15M rats [Bellinger et al., 2008a] and altered immune function [Bellinger et al., 2008a,b]. Collectively, our findings indicate that splenocytes from aging F344 male rats respond to changing NE concentration in the local milieu, with compensatory regulation of β-AR expression and cAMP production to affect immune function.

As a first step in developing an aging model for investigating causal relationships between sympathetic and immune dysregulation, we investigated whether heightened splenic SNA during middle age is responsible for splenic NA nerve loss and altered β-AR signaling in splenocytes. In the brain, high catecholamine concentrations are neurotoxic due to increased reactive oxygen species (ROS) from catecholamine degradation [Chinta and Anderson, 2008; Burke et al., 2004]. We propose a similar mechanism affects NA nerve integrity, with consequences for β-AR-mediated signaling in the F344 rat spleen as
SNA rises during middle age. In this study, we investigated the effects of chronically reducing SNA centrally during middle age by treatment with rilmenidine (Rilm) on a variety of measures to assess sympathetic integrity and function. Rilm is a centrally acting antihypertensive drug that selectively inhibits sympathoexcitatory cells in the rostroventrolateral medulla (RVLM) [Reis, 1996; Monassier et al., 2004], a brainstem autonomic region that regulates SNA in the spleen [Beluli & Weaver, 1991]. This is the first study to investigate the effects of Rilm, or other drugs in its class, in normotensive rats, with endpoint in immune target organs, and nerve integrity in any organ.

Here, we report that chronic Rilm discontinuously administered during middle age reduced net and splenic SNA, protected splenic NA nerves from age-related damage, and further augmented β-AR-mediated signaling of splenic lymphocytes. Protection of splenic sympathetic nerves supports our hypothesis. Greater β-AR expression and signaling supports reduced NE available to bind with β-AR via reduced SNA and indicates functional β-AR and signaling regulation, an important criterion for developing a model for studying causality between age-related changes in SNS and immune functioning. We conclude that chronic treatment with Rilm during middle age is a strategy for reversing age-related changes in sympathetic neurotransmission in the spleen.

**Methods**

**Drug Preparation**

Rilm dihydrogenase, a centrally acting third generation antihypertensive drug, was a gift from Servier (Suresnes, France). Rilm is stable in solution at room temperature for over 24 hours [Monassier et al., 2004], therefore the drug was prepared once daily prior
to the first of two daily injections. Rilm was dissolved in sterile, endotoxin-free, physiologic saline at 0.5 mg/ml or 1.5 mg/ml. Rilm is stable in solution at room temperature for over 24 hours [Monassier et al., 2004]. α-Methyl-DL-p-tyrosine methyl ester HCl (αMPT) (Sigma-Aldrich, St. Louis, MO), an inhibitor of tyrosine hydroxylase, the rate-limiting enzyme for the synthesis of NE, was used to determine splenic NE turnover. The αMPT was dissolved in sterile, endotoxin-free, physiologic saline containing 0.1% ascorbic acid at a concentration of 0.2 mg/ml.

Animals

Seventy-six 15-month-old (15M) male, F344 rats were purchased from the National Institute on Aging (NIA) colony (Harlan Sprague-Dawley; Indianapolis, IN). All animals in the study were housed two per cage in the vivarium at Loma Linda University, given food and water *ad libitum*, and placed on a 12-h-on 12-h-off light schedule. In the animal room, the ambient temperature was maintained at 22 °C, and the humidity ranged between 30-40%. Rats were acclimated to housing conditions for one week, and then were handled daily for one week before the start of the experiment. All animal manipulations and procedures were approved by the institutional animal care and use committee at Loma Linda University prior to the start of the experiments, and followed NIH guidelines on the use and care of animals.

Study Design

The rats were randomly assigned to one of three treatment groups: low-dose Rilm (Rilmlo), high-dose Rilm (Rilmhi) or vehicle (Veh-18M) (n of 12 per treatment group).
Rats were treated with sterile, endotoxin-free saline (Veh-18M) or Rilm (500 or 1.5 mg/ml/kg/day, intraperitoneal (i.p.); Rilm\textsubscript{lo} or Rilm\textsubscript{hi}, respectively) by twice-daily injections (half the total dose per injection) at 7:30 a.m. and 3:30 p.m. for 90 days. The route of Rilm administration and Rilm\textsubscript{lo} dose used in this study were based on a previous report by Monassier et al. [2004] demonstrating that chronic treatment (30 days) with this moderate dose of Rilm (250 mg/ml/kg) administered i.p. every twelve hours (i.e., discontinuous) induces the optimum anti-hypertensive action in spontaneously hypertensive rats, when measured at the peak plasma concentration of the drug (30 ng/ml). Discontinuous treatment with 500 mg/kg/day is rapidly eliminated when delivered systemically, so it does not induce receptor desensitization, and it did not affect $\alpha_2$-AR expression in kidney membrane preparations. The Rilm\textsubscript{hi} dose was used to determine whether there were dose-response effects and/or whether a dose doubling could further potentiate drug effects on measured parameters. An additional 14 untreated 3M and 18M male F344 rats were obtained from the NIA colony 1 week before the end of the 90-day experiment, and used as controls to assess the potential effects of the stress of handling and injections. Where no differences were found between vehicle-treated and untreated 18M controls based on Student t-tests ($p<0.05$), the data were collapsed and designated as Ctrl-18M. Body weights were measured prior to starting drug treatment, and then weekly until the end of the experiment. Daily grooming, feeding and drinking behaviors were observed to monitor the general health of each rat. Based on these qualitative observations, Rilm was well tolerated.

For the turnover study, 18M rats treated with vehicle or Rilm for 90 days, and untreated 3M and 18M control rats were randomly assigned to receive either the vehicle
diluent for \( \alpha \text{MPT} \) or \( \alpha \text{MPT} \). The \( \alpha \text{MPT} \) was used to estimate basal rates of NE turnover in the spleen based on the rate of decline in splenic NE concentration after NE synthesis blockade, as previously described [Bellinger et al., 2008a]. At time zero, rats received an injection of \( \alpha \text{MPT} \) (200 mg/kg, i.p.) or an equivalent volume of sterile, endotoxin-free saline, and sacrificed 6 hr later by decapitation without prior anesthesia.

Peripheral blood and spleens were immediately harvested from each rat. Peripheral blood was collected in 12x75 mm tubes containing 10 mmol/L disodium ethylenediaminetetraacetic acid (EDTA) kept on ice. Blood samples were centrifuged at 1,200 rpm for 10 min. The plasma was collected into microfuge tubes and stored at -80 °C until used for measuring catecholamine concentrations using high-performance liquid chromatography with coulometric detection (HPLC-CD). Spleens were isolated and dissected from each animal then cut in half. Two small pieces were cut cross-sectionally from the central region of one half of the spleen (~3-4 mm thick), frozen on dry ice, and then stored at -80 °C for subsequent measurement of splenic NE concentration and fluorescence histochemistry to localize NA nerves. The other half of the spleen was used to isolate spleen cells for use in cAMP and \( \beta \)-AR binding assays.

Following the collection of plasma and spleen tissue, a gross examination of the brain and all visceral organs was completed for evidence of pathology commonly seen with age, including testicular interstitial cell hyperplasia, severe chronic nephropathy (enlarged and discolored kidneys), bile duct hyperplasia (rough surface of liver), and splenomegaly (enlarged spleen resulting from lymphoma or leukemia originating in the spleen). Rats with any visible lesions, tumors, or overt pathology were excluded from this
study. Three to four rats per chronic treatment group were removed from the study due to overt pathology.

HPLC-CD for Assessing Catecholamine Levels

Spleen samples were sonicated using a Branson Sonifier 250 in 10 times the volume/wet weight of cold perchloric acid (0.1 M) containing 3,4-dihydroxybenzylamine (DHBA) (10 ng/ml) (Sigma-Aldrich), as an internal standard. Spleen homogenates then were centrifuged at 10,000 rpm for 5 min. The resultant supernatants were transferred to microfilterfuge tubes and centrifuged at 14,000 rpm for 20 min resulting in a minimum of 50 µl of supernatant. Thawed plasma samples (200 µl) were pipetted into 12x75-mm glass test tubes containing 1.0 ml of phosphate buffer (pH 7.0), 1.0 ml of 1.5 M (pH 8.6) Tris buffer and 50 µl DHBA (10 ng/ml). After the tubes were vortexed, 50 mg of acid washed alumina was added, and the tubes were placed on a C10 platform shaker (New Brunswick Scientific, Edison, NJ) for 5 min at 175 rpm. The alumina was then allowed to settle, and the supernatant was aspirated. The alumina was washed by adding double-distilled H₂O, allowing the alumina to settle and then aspirating the supernatant for a total of 3 times. At the of the 3rd, wash, the alumina was resuspended in double-distilled H₂O and the resulting slurry was transferred in a sample tube of a microfilterfuge tube and centrifuged for 2 min at 9,000 rpm. Following centrifugation, the sample tube containing the alumina was transferred onto a new receiver tube. Two hundred microliters of 0.1 M HClO₄ were added to the alumina. The catecholamines were eluted by centrifuging the microfilterfuge tube for 2 min at 9,000 rpm and the eluate was collected. NE and DA concentrations were determined using HPLC-CD using a CouloChem III HPLC System.
(ESA, Chelmsford, MA) and an ESA Model 542 autosampler. The mobile phase was delivered at a flow rate of 1.0 ml/min through a Resolve C18 reverse phase 5-µm, 8x100-mm Radial-Pak analytical column (Canton, Massachusetts) using an ESA Model 582 solvent delivery module. The potentials of the guard cell and two detecting cells of the ESA CouleChem III coulometric detector cell system were set at 400 mV, 350 mV, and -350 mV, respectively. The signal from the detector was recorded and the peak heights and area under the curves were analyzed using EZChrom Elite Software (Scientific Software Inc. Pleasanton, CA).

**Splenic NE Turnover**

To determine splenic NE turnover, NE decay was estimated using a model in which the rate of NE reduction following synthesis blockade is defined by a single mathematical factor to which steady-state kinetics can be applied, as previously described [Bellinger et al., 2008a]. According to this model the concentration of NE is dependent on the rate of synthesis with the following relationship: \( \log[NE] = \log[NE]_0 - 0.434kt \), where \([NE]_0\) is the initial concentration of NE and \(k\) is the fraction of NE concentration formed or lost per unit time. The \(k\) of NE was calculated by least-square linear regression of the \(\log[NE]\) vs. time. To determine the rate constant, turnover time and turnover rate for NE in the spleen, the log of NE (\(\log[NE]\)) concentration was plotted versus the time following synthesis blockade. Linear regression analysis of the \(\log[NE]\) vs. time relationship was performed using individual data points obtained at 0 and 6 hr after tyrosine hydroxylase inhibition. The slope (m) and standard error of the regression coefficient (Ser) were computed by the least squares method. The rate constant for NE
disappearance (kNE defined as m/0.434), NE turnover time (1/kNE), and NE turnover rate ([NE]o x kNE) were calculated as described. The standard error of the turnover rate and the turnover time was calculated from the variance of the regression slope and the variance of [NE]o according to the delta method, using the following equation:

\[ \text{Variance } k(\text{NE}_o) = k^2 \text{var(NE}_o) + [\text{NE}_o]^2 \text{var}(k) + 2k\text{NE}_o \cdot \text{Covariance } k,\text{NE}_o. \]

Fluorescence Histochemistry for Catecholamines

A modification of the glyoxylic acid condensation method (SPG method) of histofluorescence for catecholamines was used to visualize sympathetic nerves in fresh frozen spleen tissue from F344 rats, as previously described [Bellinger et al., 1992]. From each animal, spleen blocks from the hilus (the site of blood vessel and nerve entry into the spleen) and regions distal to the hilus were mounted onto chucks with OCT compound, and 16 µm cross-sections were cut using a cryostat set at -20 °C. Sections were thaw-mounted onto glass slides, and dipped in a 0.2 M potassium phosphate solution containing 1% glyoxylic acid and 0.2 M sucrose (pH 7.4). The slides were air-dried under a direct stream of cool air for 15 min, several drops of mineral oil placed onto each section, and the slides placed on a heat-conducting copper plate in an oven at 95 °C for 2.5 min. Excess oil was drained from the slides, and the slides were coverslipped using 2 drops of fresh mineral oil. Catecholamine fluorescence was visualized using a Zeiss Axiomat fluorescence microscope equipped with epi-illumination accessories, and using a 395- to 440-nm excitation filter. Color images were captured with an Olympus image high-resolution CCD video capture system.
Spleen Cell Preparation

At sacrifice, one-half of the spleen was placed into a stomacher bag containing 10 ml Hank’s balanced salt solution (HBSS) (Mediatech Inc., Manassas, VA) containing 0.035% sodium bicarbonate (Sigma-Aldrich, St. Louis, MO) and 25 mM HEPES (Mediatech Inc., Manassas, VA) and dissociated using a stomacher Lab-Blender (Tekmar Co., Cincinnati, OH). Cell suspensions were passed through 100 µm cell strainers (BD Biosciences Discovery Labware, Bedford, MA) to remove large aggregates, then centrifuged, and resuspended in fresh HBSS. Red blood cells were removed from spleen cell suspensions by layering over Histopaque 1077 (Sigma-Aldrich), and centrifuging at 400 x g for 30 min at room temperature. Cells at the interface between Histopaque and HBSS were removed and washed three times in HBSS. Cells were counted using a Coulter Counter (Coulter Instruments, Hialeah, FL), and then resuspended to 2x10⁶ cell/ml in HBSS. Spleen cell suspensions were used for β-AR binding and cAMP assays after adjusting to the appropriate cell concentrations for each assay.

β-AR Binding Assays

The experimental design and number parameters evaluated in this study limited our ability to carry out binding studies in all treatment groups. Therefore, radioligand binding studies were performed on whole spleen cells from Veh-18M and Rilmlo-treated rats (n of 6 per group) using the ligand (-)[125I]cyanopindolol ([125I]CYP), a β-AR antagonist with equal affinity for the β₁- and β₂-AR subclasses. Rilmlo-treated rats were chosen for binding studies based on functional data from Monassier et al [2004], as described above. Spleen cells used for β-AR binding assays were resuspended to 5x10⁶ cell/ml.
cells/ml. $^{125}\text{I}\text{CYP (2200 Ci/mMole) was purchased from GE Healthcare (Piscataway, NJ), and diluted in 1% ethanol, 5 mM HCl, and 0.2% bovine serum albumin (BSA). Assays were performed in duplicate in 13x100-mm polypropylene tubes containing 1 x 10^6 spleen cells with 8 concentrations of $^{125}\text{I}\text{CYP ranging from 15.8-333 pM. Nonspecific binding was determined using parallel assays incubated in the presence of 10^{-6} M CGP-12177, a hydrophilic \( \beta \)-AR antagonist (Sigma-Aldrich). Tubes were incubated at 37 °C for 60 min in a shaking water bath (100 oscillation/min) to ensure that equilibrium was reached. The reaction was terminated by adding 3 ml of ice cold hypotonic buffer (3.8 mM KH$_2$PO$_4$, 16.2 mM K$_2$HPO$_4$, 4 mM MnSO$_4$) for 20 min to lyse the cells. The reaction mixture was filtered using a cell harvester (Brandel Corp., Gaithersburg, MD) and bound radioactivity collected on Whatman fiberglass filters (GF/B) (Brandel Corp., Gaithersburg, MD). Filters were washed with 16 ml (4x4 ml) of ice-cold Tris-EGTA buffer to remove the unbound radioligand. Filters were removed, placed in 12x75-mm tubes, and counted in a Wallac 1470 Wizard gamma counter (Long Island, Port Jefferson, NY) at 82% efficiency.

\( \beta \)-AR-Stimulated cAMP Production in Spleen Cells

Spleen cells (1x10^6) were incubated with 100 µl HBSS containing 100 µM isobutylmethylxanthine (IBMX; Sigma-Aldrich) and 0.1% BSA (EMD Chemicals, Gibbstown, NJ) (IBMX) in 12x75-mm polystyrene tubes (Thermo Fisher Scientific, Pittsburgh, PA) for 20 min at 37 °C in a shaking water bath. Spleen cells were then treated with 1 ml 10^{-5} M isoproterenol (Sigma-Aldrich) for 10 min at 37 °C. The reaction was quenched by adding 2 ml ice-cold IBMX. The tubes were centrifuged for 8 min at
1,800 rpm, and the supernatants discarded. The pellets were reconstituted with 0.5 ml of a 50 mM sodium acetate buffer. The tubes were then exposed to two cycles of boiling and freezing to lyse the cells. The cellular debris was removed by centrifugation (1,800 rpm for 8 min), the supernatants were collected into microfuge tubes, and then stored at -80 °C until assayed for cAMP. cAMP levels were determined in triplicate using a commercially available enzyme immunoassay (EIA) Kit (GE Healthcare, Piscataway, NJ) using the acetylation protocol for highest test sensitivity (lower detection limit of 14 pg/ml) according to the manufacturer’s instructions. The optical density of the samples was determined using a plate reader set at 450 nm.

Data Analysis

Body and Spleen Weights: Body and spleen weights were expressed in g ± standard error of the mean (SEM). Spleen weights were also expressed as a percentage of body weight ± SEM. These data were analyzed by one-way analysis of variance (ANOVA) with Bonferroni post-hoc testing for significant ANOVA (p<0.05).

HPLC-CD Determination of Plasma and Splenic Catecholamine Levels, and NE Turnover: Splenic and plasma catecholamine concentrations were determined based on standards of known concentrations of NE, DA, and EPI, and expressed as a mean ± SEM in ng per g tissue wet weight and ng/ml plasma, respectively. Since age and Rilm treatment affect spleen weight, total NE and DA content were also estimated based on NE concentrations/mg wet weight and whole spleen weight, and expressed as a mean ± SEM in ng/whole spleen. Similarly, turnover rate and time were expressed as means ± SEM in ng/g/h and h, respectively. All these data were analyzed using one-way ANOVA
with Bonferroni post-hoc testing for significant ANOVA \((p<0.05)\). Splenic NE and DA levels (concentration and total content) were correlated with spleen weight using linear regression analysis and the statistical software, Prism\textsuperscript{®} 4 (GraphPad, San Diego, CA).

**Morphometric Analysis of Nerve Density:** The density of sympathetic innervation of the white pulp from hilar and distal regions was estimated using the spleens of six rats from each treatment group. The white pulp was chosen for analysis, because the majority of NA nerves in the spleen reside in this compartment [Bellinger et al., 2008b]. Four randomly selected white pulps from each of the six rats per treatment group were used for analysis. Each white pulp area assessed was from a different spleen section near the hilar region or distal to the hilus. White pulps were photographed at a magnification of 200X. The criteria for selecting the white pulps for analysis were that (1) there was only one central arteriole in the white pulp; (2) the size of the central arteriole were comparable across all samples (80-100 µm across the largest diameter of the vessel) and (3) the arteriole was cut in true cross section [Bellinger et al., 2002]. Quantitation of the mean area of NA nerves in the splenic white pulp was performed using Image-Pro Plus\textsuperscript{®} imaging software (version 5.0; Media Cybernetics, Bethesda, MD). Using this software, the area of NA nerves in the total region of interest (ROI) within the white pulp was determined by the number of pixels containing NA nerve profiles in each image, based on size and color. The number of positive pixels was divided by the total number of pixels in the ROI then multiplied by 100 to calculate the % area of NA nerve profiles in each image. The mean % area of NA nerves was determined by averaging the % NA nerve area in four white pulps from each animal, and then these means were averaged within each treatment group. Data were expressed as a mean of a mean ± SEM for each
treatment group. Differences in mean % area of NA nerves among the treatment groups were analyzed using a one-way ANOVA ($p<0.05$) followed by Bonferroni post-hoc analysis.

**Radioligand Binding:** Specific binding was defined as the difference between binding of the radioligand at each concentration in the absence and in the presence of (−)CGP-12177. Nonspecific binding ranged from 10-20% of total binding. Receptor density ($B_{\text{max}}$) and affinity ($K_D$) were determined using an iterative nonlinear regression curve fitting program, Prism® 4, to a model of a single class of homogenous binding sites. Data were transformed into a linear form by Scatchard analysis. The lines of best fit were generated using the $B_{\text{max}}$ and $K_D$ to determine the X and Y axis intercepts. The maximal number of binding sites per cell was calculated based on simple stoichiometric assumptions (1 molecule of ligand bindings to 1 receptor site), expressed as sites/cell. Differences in mean $K_D$ and $B_{\text{max}}$ between groups were determined using one-way ANOVAs ($p<0.05$) followed by Bonferroni post-hoc analysis.

**β-AR-Stimulated cAMP Production:** β2-AR-stimulated cAMP production was expressed as means ± SEM in fmol/2 x 10⁶ cells/10 min. These data were analyzed using a one-way ANOVA ($p<0.05$) with Bonferroni posthoc testing.

**Results**

**Rilm Alters Body and Spleen Weights**

F344 rats appeared to tolerate Rilm or vehicle treatment well as all rats were observed to eat, drink, and groom. Prior to beginning the experiment, body weights between treatment groups were comparable (Fig. 3.1A). Mean body weights from all
Rilm treatment groups receiving twice-daily injections, were slightly reduced, but were not significantly different from initial body weights over the 90-day period or compared with body weights of vehicle-treated rats. Mean body weights from vehicle- and drug-treated groups at 12 weeks were significantly (***, p<0.001) lower than Ctrl-18M rats (Fig. 3.1A).

Spleen weights (Fig. 3.1B) were significantly greater (†††, p<0.001) in all 18M treatment groups compared with Ctrl-3M rats, and greater in the Rilmhi-treated rats compared with Rilmlo and Ctrl-18M rats (***, p<0.001). There were no differences in spleen weights between Veh-18M and untreated-18M, so the data were collapsed (collectively, Ctrl-18M). Similarly, no differences in spleen weights between Rilmlo-treated rats and Crtl-18M rats were observed. Mean spleen weight per body weight (Fig. 3.1C) was significantly greater in rats treated with Rilmhi compared with all other treatment groups (Ctrl-3M: ***, p<0.001; Ctrl-18M: *, p<0.05; Rilmlo: **, p<0.01).
Fig. 3.1. Mean Body Weights over the Time Course of the Experiment.

A. The effect of age and 90-day treatment with control (Ctrl-18M), vehicle treatment (Veh-18M), and low- or high-dose rilmenidine (Rilm_lo or Rilm_hi, respectively) on mean body as means (± SEM) in grams (g) in F344 male rats. All treatment groups maintained, but did not gain weight; Ctrl-18M rats weighed significantly more than all other age-matched treatment groups (***, *p*<0.001). There was no effect of chronic drug treatment on mean body weight over the 90-day period. Ctrl-18M, ◊; Rilm_lo, □; Rilm_hi, Δ. Low and high-dose rilmenidine, Rilm_lo and Rilm_hi, respectively; 3- or 18-month-old controls, Ctrl-3M or Ctrl-18M, respectively. Ctrl-3M, *n*=14; Ctrl-18M, *n*=22; Rilm_lo or Rilm_hi, *n*=12.
Fig. 3.1. Mean Spleen Weights over the Time Course of the Experiment (Continued).

B. The effect of age and 90-day treatment on mean spleen weight expressed as means (± SEM) in grams (g). There was a significant effect of age on spleen weight such that all 18M groups were greater than Ctrl-3M rats (†††, p<0.001), and treatment with Rilmhi, further increased (***, p<0.001) mean spleen weight compared with Ctrl-18 and Rilmlo.
Fig. 3.1. Normalized Spleen Weights over the Time Course of the Experiment (Continued).

C. Spleen weight per body weights expressed in g per g after a 90-day treatment with rilmenidine (Rilm). Treatment with Rilm_{hi} increased spleen weight per body weight compared with all other treatment groups (Ctrl-3M: *** , $p<0.001$; Ctrl-18M: *, $p<0.05$; Rilm_{lo}: **, $p<0.01$).
Rilm Reduces Plasma NE Concentrations, but not EPI Concentrations

Mean plasma NE and EPI concentrations (Figs. 3.2A and 3.2B, respectively) were similar at 3 and 18 months of age (Ctrl-3M vs. Ctrl-18M). Chronic Rilm_{lo} or Rilm_{hi} treatment significantly reduced plasma NE concentrations (Fig. 3.2A) compared with Ctrl-3M (*, p<0.05) and Ctrl-18M (**, p<0.01) control values. Plasma NE levels in Rilm_{lo}- and Rilm_{hi}-treated rats were 35% and 29% lower than the controls, respectively. Rilm had no effect on mean plasma EPI concentrations (Fig. 3.2B).
Fig. 3.2. Effect of Rilmenidine on Circulating NE.
A. The effect of rilmenidine treatment on mean plasma norepinephrine (NE) concentrations ± SEM expressed in ng/ml. Both doses of rilmenidine over the 90-day period significantly reduced plasma NE concentrations compared with Ctrl-3M and Ctrl-18M (*, p<0.05 and **, p<0.01, respectively). Low and high-dose rilmenidine, Rilmlo and Rilmhi, respectively; young, 3-month-old controls, Ctrl-3M; old, 18-month-old controls, Ctrl-18M). Ctrl-3M, n=14; Ctrl-18M, n=22; Rilmlo or Rilmhi, n=12).
Fig. 3.2. Effect of Rilmenidine on Circulating EPI.
B. The 90-day treatment with rilmenidine did not affect mean plasma epinephrine (EPI) concentrations ± SEM expressed in ng/ml. Low and high-dose rilmenidine, Rilm_lo and Rilm_hi, respectively; young, 3-month-old controls, Ctrl-3M; old, 18-month-old controls, Ctrl-18M). Ctrl-3M, n=14; Ctrl-18M, n=22; Rilm_lo or Rilm_hi, n=12).
Rilm Partially Reverses the Age-Related Decline in Splenic NA Innervation

In Ctrl-3M rats, NA nerves form a dense plexus surrounding the central arteriole in the splenic white pulp in the hilar region (Fig. 3.3A). Linear, varicose fibers extend from this vascular plexus into the surrounding periarteriolar lymphatic sheath (PALS), a predominantly T cell compartment. In contrast, while the NA nerves display a similar distribution in the white pulp, the density of fluorescent profiles was markedly reduced in this compartment in the Ctrl-18M rats (Fig. 3.3B). Rilmlo or Rilmhi treatment (Figs. 3.3C and 3.3D) increased the fluorescence intensity and density of NA nerve fibers associated with the central arteriole and in the surrounding PALS compared with the Ctrl-18M rats, an effect that appears to be augmented with Rilmlo treatment.
Fig. 3.3. Effect of Rilmenidine on Splenic NA Innervation at the Hilus.
Fluorescence histochemistry for catecholamines demonstrates abundant fluorescent linear and punctate NA nerve profiles surrounding the central arteriole (ca) in the white pulp (wp) (arrowheads) in the hilar region of the spleen in 3-month-old F344 rats (Ctrl-3M) (A.). At 18 months of age (Ctrl-18M) (B.), the density of fluorescent nerves along the central arteriole decline, an effect partially reversed by low- and high-dose rilmenidine treatment (Rilm_lo (C.) and Rilm_hi (D.), respectively. A-D. Glyoxylic acid fluorescence histochemistry. Calibration bar = 100 µm.
Morphometric analysis confirmed the age-related decline in splenic NA nerve area in the hilar region and partial restoration of nerve loss with Rilm treatment (Fig. 3.3E). Mean splenic NA nerve density in Ctrl-18M rats declined to 62.3% of Crtl-3M values (***, \( p<0.001 \)). Treatment of rats with Rilm_{lo} or Rilm_{hi} significantly increased the density of NA fibers around the central artery and in the white pulp of the spleen by 60.0% and 44.5%, respectively (**, \( p<0.001 \) and *, \( p<0.05 \), respectively). Despite the dramatic drug-induced increase in mean NA nerve area with Rilm_{lo} or Rilm_{hi} treatment, values remained significantly lower than values in Ctrl-3M rats (\( p<0.05 \) and \( p<0.01 \), respectively).
Fig. 3.3. Effect of Rilmenidine on Splenic NA Innervation at the Hilus by Morphometric Analysis (Continued).

Morphometric analysis of fluorescent profiles (E.) expressed as percent mean area is consistent with qualitative findings, showing an age-related loss of fluorescent nerves (***, p<0.001) that is attenuated by low- or high-dose rilmenidine treatment (Rilmlo: **, p<0.001; Rilmhi, *, p<0.05). Low and high-dose rilmenidine, Rilmlo and Rilmhi, respectively; young, 3-month-old controls, Ctrl-3M; old, 18-month-old controls, Ctrl-18M).
In the distal region, the effects of age and Rilm treatment on nerve density in the white pulp were similar to findings in the hilar region, although the size of the white pulps and diameter of central arterioles appear smaller than in the hilus where the splenic artery and associated sympathetic nerves enter the spleen (Figs. 3.4A-D). Similarly, as in the hilar region, the distribution of NA nerves in the white pulp in this region was not affected by Rilm treatment. Qualitatively, the abundance of NA nerves was reduced along the central arteriole and the surrounding white pulp in Ctrl-18M rats (Fig. 3.B) compared with Ctrl-3M rats (Fig. 3.4A). Both Rilmlo and Rilmhi appeared to dose-dependently increased in the presence of NA nerve fibers associated with the arteriole and in the periarteriolar lymphatic sheath compared with Ctrl-18M rats. Morphometric analysis (Fig. 3.4E) were consistent with qualitative observations, demonstrating a 66% drop in nerve density at 18 months of age compared with young controls (***, p<0.001), and Rilmlo or Rilmhi treatment reversed the aging effect by 91% or 54% (vs. Ctrl-18M: **, p<0.01 or T, p<0.1 (trend), respectively). Drug treatment did not totally restore nerve density to young adult levels; nerve densities were 35% or 47% lower with Rilmlo or Rilmhi than in young adult controls (††, p<0.01 or †††, p<0.001, respectively) (Fig. 3.4E).
Fig. 3.4. Effect of Rilmenidine on Splenic NA Innervation Distal to the Hilus.
Distal to the hilus, fluorescence histochemistry for catecholamines demonstrates fluorescent linear and punctate NA nerve profiles surrounding the central arteriole (ca) in the white pulp (wp) (arrowheads) of the spleen in 3-month-old F344 rats (Ctrl-3M) (A.). In this region of the spleen, the white pulps are smaller, central arterioles have a smaller diameter and there are fewer NA nerve surrounding the arterioles compared with the hilar region. However, the compartmentation of nerves is comparable to the hilar region. At 18 months of age (Ctrl-18M) (B.), the density of fluorescent nerves along the central arteriole decline, an effect partially reversed by low- and high-dose rilmenidine treatment (Rilm_{lo} (C.) and Rilm_{hi} (D.), respectively. Low and high-dose rilmenidine, Rilm_{lo} and Rilm_{hi}, respectively; 3- or 18-month-old controls, Ctrl-3M or Ctrl-18M, respectively). A-D. Glyoxylic acid fluorescence histochemistry. Calibration bar = 100 µm.
Fig. 3.4. Effect of Rilmenidine on Splenic NA Innervation Distal to the Hilus by Morphometric Analysis.

Morphometric analysis of fluorescent profiles (E.) expressed as percent mean area is consistent with qualitative findings, showing an age-related loss of fluorescent nerves (Ctrl-18M vs. Ctrl-3M: ***, \( p < 0.001 \)) that is attenuated by low- or high-dose rilmenidine treatment (Rilmlo or Rilmhi vs. Ctrl-18M: **, \( p < 0.01 \) or \( T, p < 0.1 \), respectively; Rilmlo or Rilmhi vs. Ctrl-3M: ††, \( p < 0.01 \) or †††, \( p < 0.001 \)).
Rilm Affects Splenic NE and DA Content and Concentration

The mean total splenic NE concentration (Fig. 3.5A) was lower in Ctrl-18M and Rilmhi than in Ctrl-3M rats (***, *p*<0.001). NE concentration in spleens from Rilmhi- treated rats also fell significantly compared with levels found in Ctrl-18M- and Rilmlo-treated rats (*, *p*<0.05 and **, *p*<0.01, respectively).

A significant age-related decline in mean total splenic NE content (Fig. 3.5B) was observed, such that Ctrl-18M rats displayed lower values than Ctrl-3M (*, *p*<0.05). The mean splenic NE content in Rilmlo-treated rats was similar to values obtained for Crtl-18M, but tended to be lower than in Ctrl-3M rats (T, *p*<0.1) (Fig. 3.5B).

No difference in mean splenic NE content was observed between the Rilmlo and Ctrl-18M rats. Mean total NE content in the spleen was reduced in Rilmhi-treated rats compared with Ctrl-3M (***, *p*<0.001), but did not differ significantly from the Ctrl-18M or Rilmlo groups. Regression analyses revealed a decrease in NE levels as spleen weight increases (Figs. 3.5C and 3.5D). Both splenic NE concentration and total content were significantly correlated with spleen weight, with the former having a greater effect (*p*<0.001 and *p*<0.01, respectively).
Fig. 3.5. Rilmenidine and Splenic NE Concentration.

A. Mean total splenic NE concentrations (expressed as ng/gm wet weight ± SEM) in 3M and 18M controls, and rats treated with low- or high-dose rilmenidine treatment (Ctrl-3M, Ctrl-18M, Rilmlo, and Rilmhi, respectively). Splenic NE concentration was reduced with advancing age (Ctrl-3M vs. Ctrl-18M: ***, $p<0.001$), an effect augmented by high-dose rilmenidine treatment (Rilmhi) (Ctrl-3M or Ctrl-18 vs. Rilmhi: *, $p<0.05$ or ***, $p<0.001$, respectively). Splenic NE concentration also was lower in Rilmhi than the Rilmlo group (**, $p<0.01$). Ctrl-3M, $n=13$; Ctrl-18M, $n=23$; Rilmlo or Rilmhi, $n=11$. 

Ctrl-3M Ctrl-18M Rilmlo Rilmhi

Splenic NE Concentration (ng/g)
Fig. 3.5. Rilmenidine and Total Splenic NE Content.

B. Mean total splenic NE content (expressed as ng/spleen ± SEM) in 3M and 18M controls, and rats treated with low- or high-dose rilmenidine treatment (Ctrl-3M, Ctrl-18M, Rilmlo, and Rilmhi, respectively). Splenic total NE content in the spleen was diminished in old (Ctrl-18M) and with a trend for lower levels in Rilmlo compared with Ctrl-3M (*, \( p<0.05 \) or **, \( p<0.1 \), respectively). Rilmhi further reduced total splenic NE content compared with young (Ctrl-3M) rats (***, \( p<0.001 \)). Ctrl-3M, \( n=13 \); Ctrl-18M, \( n=23 \); Rilmlo or Rilmhi, \( n=11 \).
Fig. 3.5. Correlation between Spleen Weight and Splenic NE Concentration (Continued).

C. Splenic NE concentration was negatively correlated with spleen weight ($R^2=0.68$).
Ctrl-3M, $n=13$; Ctrl-18M, $n=23$; Rilmlo or Rilmhi, $n=11$. 

$R^2 = 0.6806$
Fig. 3.5. Correlation between Spleen Weight and Total Splenic NE Content.
D. Splenic NE content was negatively correlated with spleen weight ($R^2 = 0.35$). Ctrl-3M, $n=13$; Ctrl-18M, $n=23$; Rilm$_{lo}$ or Rilm$_{hi}$, $n=11$. 

$$R^2 = 0.3549$$
There was no effect of either age or low dose drug treatment on splenic DA concentration (Fig. 3.6A). However, Rilmhi significantly raised DA levels in the spleen to approximately 30%, 34% or 54% of Ctrl-3M, Ctrl-18M, or Rilmlo, respectively (*, p<0.05; *, p<0.05, or **, p<0.01, respectively) (Fig. 3.6A). Similarly, Rilmhi-treated rats had a higher total splenic DA content (Fig. 3.6B) than Ctrl-3M (*, p<0.05), Ctrl-18M (*, p<0.05) and Rilmlo (**, p<0.01) treatment groups. There were no differences between the other treatment groups. In contrast to NE in the spleen, there was no correlation between either DA concentration or total content in the spleen and spleen weight (Figs. 3.6 C or 3.6D, respectively).
Fig. 3.6. Rilmenidine and Splenic DA Concentration.
A. Mean total splenic DA concentrations (expressed as ng/gm wet weight ± SEM) in 3M and 18M controls, and rats treated with low- or high-dose rilmenidine treatment (Ctrl-3M, Ctrl-18M, Rilmlo, and Rilmhi, respectively). There was no effect of age or Rilmlo on splenic DA concentration; however, Rilmhi significantly increased splenic DA concentration compared with the other treatment groups (Ctrl-3M or Ctrl-18M: *, p<0.05; Rilmlo: **, p<0.01). Ctrl-3M, n=13; Ctrl-18M, n=23; Rilmlo or Rilmhi, n=11.
Fig. 3.6. Rilmenidine and Total Splenic DA Content.
B. Mean total splenic DA content (expressed as ng/spleen ± SEM) in 3M and 18M controls, and rats treated with low- or high-dose rilmenidine treatment (Ctrl-3M, Ctrl-18M, Rilmlo, and Rilmhi, respectively). No effect of age or Rilmlo on splenic DA content was noted; however, Rilmhi significantly increased splenic DA content compared with the other treatment groups (Ctrl-3M or Ctrl-18M: *, p<0.05; Rilmlo: **, p<0.01). Ctrl-3M, n=13; Ctrl-18M, n=23; Rilmlo or Rilmhi, n=11.
Fig. 3.6. No Correlation between Splenic DA Concentration.
C. There was no correlation between splenic DA concentration and spleen weight ($R^2=0.0004$). Ctrl-3M, $n=13$; Ctrl-18M, $n=23$; Rilmlo or Rilmhi, $n=11$. 
Fig. 3.6. No Correlation between Rilmenidine and Splenic Total DA Content. 

D. Splenic DA concentration content did not correlate with spleen weight ($R^2 = 0.046$). Ctrl-3M, $n=13$; Ctrl-18M, $n=23$; Rilmlo or Rilmhi, $n=11$. 

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**Legend:**
- **Ctrl-3M**: Control group for 3 months.
- **Ctrl-18M**: Control group for 18 months.
- **Rilmlo** or **Rilmhi**: Rilmenidine low or high dose groups, respectively.
Rilm Treatment Reduced Splenic NE Turnover

Intraperitoneal injection of αMPT reduced mean concentrations of NE in spleens from Ctrl-18M and Rilmlo or Rilmhi treatment groups during the 6-h period after NE synthesis blockade (Fig. 3.7 and Table 3.1). The rate of decline in splenic NE concentration was greatest in Ctrl-18M rats and lowest in the Rilmhi treatment group (Fig. 3.7).
Fig. 3.7. Effect of Rilmenidine on NE Turnover.
The effect of rilmenidine or control on splenic NE concentrations 0 and 6 hours post-injection with αMPT to inhibit NE synthesis is shown. 90-day treatment with low- or high-dose rilmenidine (Rilmlo, □ or Rilmhi, Δ, respectively) slowed the rate of decline in splenic NE concentration over the 6-hour period compared with age-matched controls (Ctrl-18M, ◇). Each group represents an n of 12 rats.
Calculated NE turnover rate, based on the slopes of the lines (Table 3.1) and regression analysis revealed a 32.6% and 81.7% decline in splenic NE concentration 6 h after NE synthesis blockade with Rilmlo- or Rilmhi-18M treatment compared with the Ctrl-18M group, respectively. Turnover rate at 18M in this study was approximately 3.6-fold higher than in 15M rats based on a previous report from our laboratory [Bellinger et al., 2008a], and Rilmlo or Rilmhi turnover rates were 2.4-fold higher or 0.7-fold lower than reported in 15M rats, respectively (Table 3.1). Rilm dose-dependently increased turnover time (the time required to synthesize the steady-state pool of splenic NE) compared with Ctrl-18M by the 90th day of treatment (Table 3.1). There was no significant difference in NE content between age-matched and vehicle-treated controls 6 h post-treatment and rats treated with vehicle or αMPT at time 0 (data not shown).
### Table 3.1 Effect of Chronic Rilmenidine on Splenic NE Turnover Rate

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Initial Levels (ng/g±SEM)</th>
<th>n^a</th>
<th>Rate Constant of NE Loss^b k(h)</th>
<th>n^c</th>
<th>Turnover Rate^d (ng/g/h)</th>
<th>Turnover Time^e (h)</th>
<th>Fold Change in Turnover Rate from Veh-15M^f</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ctrl-18M</td>
<td>584.9 ± 50.9</td>
<td>15</td>
<td>0.42</td>
<td>7</td>
<td>246.1</td>
<td>2.4</td>
<td>3.61</td>
</tr>
<tr>
<td>Rilmlo</td>
<td>483.3 ± 53.7</td>
<td>16</td>
<td>0.34</td>
<td>8</td>
<td>165.9</td>
<td>2.9</td>
<td>2.41</td>
</tr>
<tr>
<td>Rilmhi</td>
<td>364 ± 51.6</td>
<td>13</td>
<td>0.1</td>
<td>7</td>
<td>45.1</td>
<td>10.0</td>
<td>0.71</td>
</tr>
</tbody>
</table>

^aNumber of αMPT- or vehicle-treated rats at time 0  
^bCalculated from the slope of decline of log NE content  
^cNumber of αMPT rats sacrificed at time 6  
^dNE synthesized and degraded per gram spleen tissue per hour  
^eTime required to synthesize the steady-state tissue pool of NE  
^fCalculations based on turnover rates from vehicle-treated 15M rats reported in Bellinger et al 2008c

**Table 3.1.** Turnover rates and turnover times of NE in the spleen of F344 male rats treated with low- or high-dose rilmenidine (Rilmlo or Rilmhi, respectively). Initial NE concentrations and splenic turnover rate declined in a dose-dependent manner after treatment with αMPT rats compared with age-matched controls (Ctrl-18M) rats. Rilmenidine treatment increased turnover time in a dose-dependent manner. Initial levels of splenic NE and the rate constants are expressed as means ± SEM. αMPT, α-methylparatyrosine; NE, norepinephrine; n, number of rats per age group.
Rilm Treatment Alters β-AR Binding and Affinity in Spleen Cells

**Figs. 3.8A and 3.8B** show saturation isotherms obtained from saturation binding experiments of splenocytes obtained from Veh-18M- and Rilmlo-treated rats, respectively. Binding of the radioligand was rapid, saturable, and of high affinity in both treatment groups. Specific binding was greater than 90% of the total binding at near saturating radioligand concentrations. The Scatchard plots shown in the insets of **Figs. 3.8A and 3.8B**, indicated similar $K_D$ values between the two treatment groups (similar slope of the line), but the $B_{max}$ was greater in rats treated with Rilmlo.
Fig. 3.8. Effect of Rilmenidine on β-AR Binding in Splenocytes
A. Specific binding and Scatchard plots of (-)-[125I]cyanopindolol ([125I]CYP) in whole spleen cells from age-matched vehicle-treated rats (Veh-18M). Spleen cells were incubated under equilibrium binding conditions at 37 ºC with ICYP (0.9-220 pM) for 60 min, then the reaction was stopped and the radioactivity was quantified by gamma scintillation spectrometry. Specific binding and plots represent means of duplicate determinations of specific (■) binding cpm, counts/min. This group represents an n of 6 rats.
Fig. 3.8. Effect of Rilmenidine on β-AR Binding in Splenocytes (Continued).

B. Specific binding and Scatchard plots of (-)-[\textsuperscript{125}I]cyanopindolol ([\textsuperscript{125}I]CYP) in whole spleen cells from low-dose rilmenidine (Rilm\textsubscript{lo})-treated rats. Spleen cells were incubated under equilibrium binding conditions at 37 °C with ICYP (0.9-220 pM) for 60 min, then the reaction was stopped and the radioactivity was quantified by gamma scintillation spectrometry. Specific binding and plots represent means of duplicate determinations of specific (■) binding cpm, counts/min. This group represents an \textit{n} of 6 rats.
In spleen cells from rats with Rilmlo treatment, the mean $B_{\text{max}}$ was significantly
higher (22%, *, $p<0.05$) compared with Veh-18M rats (Fig. 3.9A), but there was no
significant difference in the mean $K_D$ between these treatment groups (Fig. 3.9B). Spleen
cells from rats with Veh-18M and Rilmlo treatment expressed approximately 759 and
1080 sites/cell, respectively (95% confidence intervals were 672-846 for Veh-18M vs.
861-1298 for Rilmlo).
Fig. 3.9. Effect of Rilmlo and Vehicle treatment on Mean β-Receptor Expression (Bmax).

A. The mean density of β-AR, expressed as sites/cell on spleen cells, from vehicle- (Veh-18M) and low-dose (Rilmlo)-treated rats demonstrates that chronic treatment with Rilmlo significantly increased β-AR expression on splenocytes (*, p<0.05). Mean values were calculated from the Bmax determined from specific binding curves generated for each rat in this treatment group.
Fig. 3.9. Effect of Rilmlo and Vehicle Treatment on Mean β-Receptor Binding Affinity (K_D).

B. Affinity binding, as expressed by K_D values on spleen cells from vehicle- (Veh-18M) and low-dose rilmenidine (Rilmlo)-treated rats (n of 6 per group), demonstrates that chronic treatment with Rilmlo did not affect K_D. Mean values were calculated from the K_D determined from specific binding curves generated for each rat from each treatment group.
Rilm Treatment and β-AR-Stimulated cAMP Production in Spleen Cells

Isoproterenol-stimulated cAMP production in spleen cells rose significantly (35%; *, p<0.05) in Ctrl-18M compared with Ctrl-3M rats (Fig. 3.10). Chronic Rilmlo or Rilmhi treatment further augmented isoproterenol-induced production of cAMP (***, p<0.001 and **, p<0.01, respectively) compared with Ctrl-3M rats. In addition, there was a respective 36% (†, p<0.05) and 23% increase in cAMP production in the Rilmlo- or Rilmhi-treated compared with Ctrl-18M rats.
Fig. 3.10. Effect of Rilmenidine on Splenocyte cAMP Production
Isoproterenol-stimulated cAMP production in spleen cells *in vitro* was increased in all 18M groups compared with young controls (Ctrl-3M) (Ctrl-18M: *, $p<0.05$; Rilm$_{lo}$: ***, $p<0.001$; Rilm$_{hi}$: **, $p<0.01$). Data are expressed in fmol/10$^6$ cells/10 min. Low and high-dose rilmenidine, Rilm$_{lo}$ and Rilm$_{hi}$, respectively; 3-month-old controls, Ctrl-3M; 18-month-old controls, Ctrl-18M). Ctrl-3M, $n=11$; Ctrl-18M, $n=19$; Rilm$_{lo}$, $n=12$; Rilm$_{hi}$, $n=11$. 
Discussion

There are several salient and novel findings in this study. First, in normotensive rats [McCarty, 1985], reduced splenic NE turnover and plasma NA concentrations indicate that chronic Rilm treatment dose-dependently reduced splenic and whole body SNA, respectively. Next, chronic Rilm treatment dose-dependently protected NA nerves from age-related damage in the white pulp in both hilar and distal regions, indicating that age-related loss of sympathetic nerves can be prevented. Collectively, these two findings support our hypothesis that elevated sympathetic tone in the aging spleen causes sympathetic nerve loss. Finally, Rilm augmented β-AR signaling in splenic lymphocytes. This finding neither supports nor refutes our hypothesis, but can be explained by the drugs action on splenic SNA. Reduced SNA reduces NE availability and consequently ligand-receptor interactions, which causes a compensatory increase in β-AR expression, consistent with our findings.

The RVLM regulates SNA in a number of organs [McAllen et al., 1995], including the spleen [Beluli and Weaver, 1991]. The Rilm-induced reduction in splenic and net SNA is consistent with its stimulatory action on sympathoexcitatory neurons in the RVLM via interaction with IR₁ (and α₂-AR) ([Fig. 3.11A]) [Bruban et al., 2001]. Activation of these RVLM neurons dampens the firing rate of postganglionic NA nerves via inhibition of preganglionic neurons in the intermediolateral cell column (IML) in the spinal cord ([Fig 3.11A]). Not all of our drug effects may be mediated centrally. IR₁ are expressed in other sympathetic target tissues [Dardonville and Rozas, 2004; Dontenwill et al., 1999], but have not been reported in the spleen. Also, imidazoline binding sites are presynaptically expressed on NA nerves in other visceral organs and can block NE
Fig. 3.11. Rilmenidine Modifies the Activity of the RVLM to Affect Sympathetic Outflow to the Spleen

General schematic diagram identifying potential central neural sites involved in the regulation of sympathetic outflow to lymphoid organs. The rostral ventrolateral medulla (RVLM) is a site of a complex convergence of descending and ascending neural inputs. The excitatory drive from the RVLM is intrinsically generated, but can be modulated by excitatory and inhibitory inputs from other regions of the central nervous system (e.g., pons, hypothalamus and amygdala) that process a wide array of sensory stimuli (including baroceptive, nociceptive, and immunological) and participate in stress, emotion and behavioral responses. The neurons of the RVLM drive sympathetic outflow by synapsing onto preganglionic neurons in the intermediolateral cell column (IML) to modulate the activity of postganglionic neurons that supply lymphoid organs. Inflammatory cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) act on the circumventricular organ (CVO) and visceral afferent fibers to influence the activity of the paraventricular nucleus (PVN) and nucleus tractus solitarius (NTS), which in turn, modulates the activity of the RVLM. Rilmenidine (Rilm) acts on IR₁ and α₂-AR primarily in RVLM to inhibit the sympathoexcitatory neurons that impinge on the 2-neuron sympathetic chain that supplies the spleen. (Modified from Dampney, 1994)
release [Göthert et al., 1999]. However, α₂-ARs are expressed presynaptically in sympathetic nerves [Docherty, 2002] and splenic macrophages [Spengler et al., 1990]. Effects of Rilm via presynaptic α₂-AR would be expected to augment its central effect via presynaptic inhibition of NE release [Szabo, 2002]. It is unlikely that drug interactions with α₂-AR on immunocytes contribute to β₂-AR-mediated cAMP production in splenic lymphocytes, as baseline cAMP levels were not affected, α₂-AR or IR₁ have not been reported on splenocytes, and both receptor types signal through different intracellular pathways [Ernsberger, 1999].

The drug-induced reduction in plasma NE levels, which reflect NE spillover into the blood after its release from NA nerves, and is consistent with reduced net SNA in other studies [Monassier et al., 2004; Burke et al., 2009]. The magnitude of reduced plasma NE levels in this study is comparable to both human and animal studies using Rilm as an antihypertensive agent [Esler et al., 2004; Monassier et al., 2004]. The low doses of Rilm in this study did not change plasma EPI levels, consistent with a study in hypertensive patients [Esler et al., 2004]. These findings indicate that the SNS and the sympathoadrenal-medullary (SAM) axis are differentially regulated by autonomic centers in the brainstem, and the SNS can be functionally disconnected from the SAM axis.

The age-related loss in splenic NE concentration and content and NA nerve loss in aging is consistent with our previous findings [Bellinger et al., 2008a]. NA nerve loss contributes to reduced NE in the spleen, but other aging-related mechanisms likely play a role, like reduced NE reuptake [Docherty, 2002] (Fig. 3.11B). Tissue NE levels are normally stable even under extreme conditions, such as stress [Del Rey et al., 1982]. Tight regulation of tissue NE levels explains unaltered static splenic NE concentration
and content with Rilmlo treatment and in control groups, but at a lower set point in old rats. Precise regulation of NE reuptake, and enzymes that synthesize and catabolize NE, assures stable tissue NE concentrations (Fig. 3.11B). Altered splenic NE and DA concentrations with Rilmhi suggest dysregulation of NE homeostasis. Greater ROS from catabolized DA after release could explain the lower nerve sparing ability of Rilmhi, consistent with findings centrally in DA neurons [Chinta and Anderson, 2008]. While altered splenic NE levels provide information about homeostatic dysregulation, unaltered levels are inconclusive, and may mask altered SNS functioning. With synthesis blockade, Rilm dose-dependently reduced the rate of NE degradation and increased turnover time, consistent with reduced NE utilization and supporting reduction of SNA by Rilm.

Rilmhi, but not Rilmlo, reduced splenic NE concentration and content compared with age-matched controls. These data indicated NE synthesis did not keep pace with NE utilization in the Rilmhi treatment group, despite reduced turnover at both drug doses. In aging, higher SNA increases TH activity [Parrish et al., 2008; Morgenroth et al., 1974], an effect reduced by Rilm (Fig. 3.11B). Our finding of greater splenic DA levels with Rilmhi also supports reduced TH activity via end product inhibition (Fig. 3.11B) [Flatmark et al., 2000]. High vesicular DA concentrations drive small rates of DA into the cytosol [Reed et al., 2010; Wallace, 2007] to inhibit TH activity. Additionally, the drug-induced SNA decrease may affect TH phosphorylation, critical for its activity and the primary mechanism for maintaining tissue NE levels after secretion [Dunkley et al., 2004; Kumer and Vrana, 1996]. Still, high vesicular DA should drive DA conversion to NE by dopamine-β-hydroxylase (DBH) downstream of TH (Fig. 3.11B). Thus, reduced splenic NE levels with Rilmhi suggests reduced DBH expression or activity (Fig. 3.11B).
Preservation of NA nerves in hilar and distal regions with Rilm supports our hypothesis that SNA during middle-age contributes to nerve loss, an effect that is greater with Rilm\textsubscript{lo}. Our findings are consistent with reports in aging rats that splenic NA nerves remain plastic [ThyagaRajan et al., 2000]. This supports the feasibility for developing a model to investigate causality between aging SNS and immune functional changes. Greater protection may be afforded by earlier treatment, since altered splenic architecture and immune cell subsets occur before 15 months of age [Bellinger et al., 1992]. Also, combined Rilm treatment with trophic factors (i.e., NGF) may potentiate the Rilm effect.
Fig. 3.12. Schematic diagram of a NA junction in the spleen.
Proposed effects of age (shown in blue) and rilmenidine (shown in red) on biosynthesis of NE, and NA neurotransmission. 1. Tyrosine (Tyr) is transported into the NA varicosity by a sodium-dependent carrier (AAT). 2. Tyr is converted to DOPA via the rate-limiting enzyme, tyrosine hydroxylase (TH). 3. Decarboxylation by dopamine decarboxylase (DDC) forms DA. 4. DA is transported into the vesicle by the vesicular monoamine transporter (VMAT). This same carrier transports NE into these granules. 5. DA is converted to NE in the vesicle by dopamine-β-hydroxylase (DBH). 6. Physiological release of transmitter occurs via tonic firing of the nerve ( ). 7. Action potentials open voltage-sensitive calcium channels and increase intracellular calcium (Ca²⁺). A decline in Ca²⁺ ATPase activity in smooth endoplasmic reticulum can increase stimulation-evoke NE release in older NA nerves (blue arrow). 8. Fusion of vesicles with the surface membrane results in expulsion of NE, DA, other cotransmitters, as well as DBH. 9. After release, NE diffuses into the cleft. 10. NE binds with postsynaptic receptors, predominantly β₂-AR in lymphocytes, which increases intracellular cAMP. 11. NE binds with presynaptic autoreceptors (α₂-AR), providing feedback regulation of NE release. 12. Cotransmitters (NT) released with NE interact with presynaptic (heteroreceptors) and postsynaptic receptors; signaling via cotransmitters can affect β-AR mediated signaling. 13. Binding with heteroreceptors also regulates NE release. 14. NE degradation in the cleft occurs by monoamine oxidase (MAO) and catechol-O-methyltransferase (COMT), which increases reactive oxygen species (ROS). 15. NE is transported back into the terminal by the NE transporter (NET). 16. NE diffuses away from the cleft and into the bloodstream.
Neuroprotection occurred without reversal of splenic NE levels. However, splenic NE levels may be restored once Rilmlo is withheld, because it appears that synthetic regulatory mechanisms are intact in the aging spleen.

We propose that increased neurotoxic ROS from CA breakdown destroys splenic NA nerves (Fig. 3.11B). This mechanism of nerve loss occurs in other CA neurons with increased activity, including NA nerves in cerebral vessels [Cowen and Thrasivoulou, 1990], and locus coeruleus and nigrostriatal neurons [Jinsmaa et al., 2009; Chen et al., 2003]. Changes in the microenvironment of the aging spleen may also contribute to nerve loss via intracellular calcium dysregulation [Stirling et al., 2010; Tsai et al., 1998], altered splenocyte subset percentages/absolute numbers [Jiang et al., 1992; Wong et al., 2010; Banerjee et al., 2000; Fló and Massouh, 1997], or stromal/immunocyte mediators [Pachówka et al., 2011; Wong et al., 2010].

Greater β-AR upregulation and signaling in aging splenocytes ex vivo (Fig. 3.11B) are inconsistent with the age-related increase in SNA. However, degeneration of NA nerves increases the distance for NE diffusion, and therefore reduces ligand availability, which may explain this discrepancy (Fig. 3.11B). This interpretation is consistent with other types of neurotoxic damage to NA nerves [Tumer et al., 1990; Chatelain et al., 1983]. Also, rising free plasma corticosterone (CORT) levels with age (~70%, 9-19M) [Sabatino et al., 1991] can upregulate β-AR transcriptionally [Kumer and Vrana, 1996; Sabban et al., 2006]. Rilmlo further augmented β-AR density and signaling ex vivo, consistent with lower NE availability from reduced SNA and NE turnover (Fig. 3.11B).

Stress-induced effects on body weights in injected rats suggest hypothalamo-pituitary adrenal axis (HPA) and/or SNS activation [Kyrou and Tsigos, 2009]. There is
no supporting evidence for SNS involvement as there were no stress effects on plasma NE or EPI. However, SNA does not always increase plasma NE levels [Esler et al., 1984], and stress-induced SNA may be transient, returning to baseline before blood collection. Rilm treatment should not remarkably affect stress-induced effects, as sympathetically-mediated reflexes and psychological stressors are still elicited after Rilm treatment [Burke et al., 2010; Esler et al., 2004]. Altered hemostasis may explain the Rilm-induced increase in spleen weights. Since, the splenic red pulp is a reservoir for blood, vasodilation by Rilm would favor blood accumulation [Kimura et al., 2001; Stewart and McKenzie, 2002]. The negative correlations between spleen weight and NE concentration or content support this hypothesis.

Collectively, our data indicate that, Rilm, or other IR agonists that act in the RVLM can reduce SNA and protect NA nerves in the aging spleen. Our data support a role for heightened SNA in NA nerve loss in the aging spleen, and that Rilm can affect NE synthesis. β-AR density and signaling data support normal regulation of postsynaptic events in aging. They also suggest that drugs in this class can affect immune function indirectly by altering splenic SNA. While refinement of the timing of treatment relative to age and drug wash-out studies are needed, our data indicate that reducing central SNA may be a useful strategy for reversing age-related nerve loss. This is a necessary requirement for developing a model to study causal relationships between age-related changes in the SNS and immune system.
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References


the glucocorticoid system in aging processes and in the action of food restriction. J. Gerontol. 46, B171-179.


Wallace, L.J., 2007. A small dopamine permeability of storage vesicle membranes and end product inhibition of tyrosine hydroxylase are sufficient to explain changes occurring in dopamine synthesis and storage after inhibition of neuron firing. Synapse 61, 715-723.

CHAPTER 4

CHRONIC TREATMENT WITH RILMENIDINE, AN IMIDAZOLINE RECEPTOR AGONIST, ENHANCES SYMPATHETIC NEUROTRANSMISSION IN THE SPLEEN OF AGING BROWN NORWAY RATS

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Abstract

INTRODUCTION: Sympathetic innervation in the white pulp of the spleen, norepinephrine (NE) concentration, signaling via β-adrenergic receptor (β-AR) and mitogen-stimulated proliferation of T lymphocytes decline in the spleen of aging Brown Norway (BN) rats. Effects of these age-related changes on neurotransmission that may modulate immunity have not been explored in this rat strain. OBJECTIVE: Therefore, we begin to develop a model that explores a causal relationship between altered sympathetic activity and immunity, by investigating the role of sympathetic tone on sympathetic neurotransmission in the spleen of middle-aged BN rats. METHODS: Fifteen month-old (15M) male BN rats received intraperitoneal (i.p.) injections twice per day, for total doses of 0, 0.5 or 1.5 µg/kg/day rilmenidine for 90 days (Veh-18M, Rilmlo, and Rilmhi, respectively). Rilmenidine affects imidazoline-1 receptors, located in sympathetic centers of the brains stem, to lower sympathetic tone. Untreated 3M and 18M (Crtl-3M and Crtl-18M) male BN rats were used to control for effects of aging and stress of handling/injection. Plasma and splenic catecholamine levels were quantified using high-performance liquid chromatography (HPLC). And inhibition of NE measured with α-methyl-paratyrosine (αMPT), via i.p. injection 6 h before sacrifice, measured splenic NE turnover rate. Noradrenergic (NA) nerves, β-AR expression and β-AR-stimulated c-AMP production were determined by fluorescence histochemistry, β-AR radioligand binding, and enzyme immunoassay (EIA), respectively. RESULTS: Rilmenidine treatment reduced circulating catecholamines (NE but not EPI), and splenic NE turnover in a dose-dependent manner. Restorative effects of rilmenidine treatment were observed on NA nerves distal to the hilus dose-dependently. And the optimum dose elevated β-AR
density, without changing affinity, and partially reversed the age-related decline in \( \beta \)-AR-stimulated cAMP production. CONCLUSIONS: Overall these results revealed that rilmenidine lessened the age-related decrease in sympathetic signaling via \( \beta \)-ARs, without significantly affecting NE levels in the spleen of normotensive BN rats. Results here suggest that in age resilient rats, rilmenidine could modulate neurotransmission during exposure to pathogens or age-related diseases.

**Keywords:** imidazoline-1 receptor agonist, neurotransmission, \( \beta \)-AR- stimulated cAMP production, aging, NE turnover, Brown Norway rat.
Introduction

The sympathetic nervous system (SNS), with its wide distribution to most organ systems of the body, regulates physiological processes during basal and aroused states. In immune organs, the SNS modulates immune cells, primarily via its major transmitter, norepinephrine (NE) after release of micromolar concentrations from varicosities of adrenergic nerve fibers [Madden et al., 1995]. NE then signals lymphocytes mostly via β-adrenergic receptors (β-ARs) [Elenkov et al., 2000; Nance and Sanders, 2007; Bellinger et al., 2008a] to activate the cAMP/protein kinase A (PKA) signaling cascade to regulate lymphocyte activation, expansion, and differentiation, cytokine production, and cell trafficking [Kammer, 1988; Cole et al., 1998; Sanders and Straub, 2002]. Both the SNS and the immune system undergo an age-related change in function with adverse consequences for health [MacGilchrist et al. 1989; Petrie et al., 2000; Bellinger et al., 2008a].

In the Fischer 344 (F344) rat, we demonstrated that an age-related increase in splenic SNA was contributory to sympathetic nerve loss in the spleen [manuscript in review]. Reduced splenic NE concentration, increased β-AR expression and β-AR-stimulated cAMP production in splenocytes, suggest that adaptive mechanisms may compensate for nerve loss in order to provide continued regulation of the immune system with advancing age [Bellinger et al., 2008b]. Previously, we have reported increased SNA activity to the spleen in aging BN rats between 15 and 18M (unpublished data), consistent with reports of an age-related increase in SNS outflow [Perez et al. 2009; Docherty, 2002], and presumably contributes to dysregulation of signaling events in aging target organs, including the spleen [Bellinger et al 2008a,b]. In 18M BN rats,
sympathetic innervation declined noticeably in the hilar region, where nerves and blood vessel enter the spleen, and then maintained at this lower level of nerve density through 32 months of age. Splenic NE concentration was also reduced at 18M, but was maintained at this lower level through 32M [Perez et al., 2009]. β-AR expression spleen cells is increased in old BN rats; however, stimulation of β-AR in old spleen cells with isoproterenol reveal a significantly reduced rise in cAMP production compared with 3M controls. Taken together, these findings suggest dysregulation of sympathetic regulation of immune function in the spleen that is characterized by an uncoupling of the β-AR with its second messenger, cAMP. These findings were consistent with in vitro studies [Goonwardene and Murasco 1994; Silverman et al., 1995] demonstrating an age-related decline in concanavalin A (Con A) stimulated proliferative responses and lower IL-2 production in spleen cells.

In the present study, we focus our attention to the effects of increased sympathetic nerve activity (SNA) during middle age on nerve integrity in the aging BN rat spleen, a secondary lymphoid organ important for host defense against systemic pathogens. We hypothesized that a transient increase in SNA during middle age may be responsible for the loss of NA nerves in the spleen. Damage to sympathetic nerves due to an increase in reactive oxygen species (ROS) produced by greater NE degradation, would be consistent with mechanisms found to damage peripheral and central NA axons in aging and neurodegenerative diseases [Chen et al., 2003; Burke et al., 2004]. Our approach to test this hypothesis, was to treat middle-aged BN rats with rilmenidine, an imidazoline-1 receptor agonist (IR1) that inhibits sympathoexcitatory cells in the rostral ventrolateral medulla [RVLM] [Reis, 1996], to reduce SNA in the spleen. In addition to regulating
sympathetic outflow to the cardiovascular system, the RVLM tonically controls the
discharge of postganglionic sympathetic neurons that supply the spleen [Beluli and
Weaver 1991a, b; Perez et al., in press]. Previously, rilmenidine given discontinuously
twice daily, reduced SNA outflow in spontaneously hypertensive rats (SHR) at peak
concentration, without changing IR₁ or α₂-AR expression in the RVLM [Monassier et al.,
2004]. Therefore we used this method for pharmacologically reducing net sympathetic
outflow to test the effects of increased SNA on neurotransmission in the spleen of
middle-aged BN rats on splenic NA innervation and β-AR signaling in splenocytes.

Therefore, sympathetic neurotransmission was assessed in spleens from BN rats
treated with rilmenidine between 15 and 18 months of age. Net and splenic SNA were
assessed by circulating and splenic catecholamines, and also NE turnover studies in the
spleen. We report here, that reducing SNA in the BN rat spleen during middle age
partially increased nerve fiber density in splenic white pulps distal to the hilus but not at
the hilus, without significantly affecting splenic NE or dopamine (DA) concentration.
Collective our data demonstrate that reduced SNA during middle age had limited
neuroprotective properties on sympathetic nerves in the aging BN rat spleen, and
increased splenic NE levels and splenocyte b-AR density, but did not reverse the age-
related defect in β-AR mediated signal via cAMP. Contrasting findings reported here
compared with our previous findings in F344 rats support strain-specific differences in
mechanisms responsible for age-related changes in sympathetic regulation of the immune
system.
Methods

Drug Preparation

Rilmenidine dihydrogenase, a centrally acting, third generation, antihypertensive drug, was provided by Servier (Suresnes, France). Since rilmenidine is stable in solution at room temperature for over 24 hr [Monassier et al. 2004], the drug was prepared once daily in the morning by reconstitution in sterile, endotoxin-free, physiologic saline at 0.5 or 1.5 mg/ml. To study splenic NE turnover, $\alpha$-methyl-DL-$p$-tyrosine methyl ester HCl ($\alpha$MPT; Sigma Chemical Co., St. Louis, MO), an inhibitor of the rate-limiting enzyme for NE synthesis, tyrosine hydroxylase, was prepared in sterile, endotoxin-free, physiologic saline containing 0.1% ascorbic acid at a concentration of 0.2 mg/ml.

Animals

Eighty-three male BN rats were purchased from the National Institute on Aging (NIA) colony (Harlan Sprague-Dawley; Indianapolis, IN). Three- or 18-month-old (3M or 18M, respectively) rats (10 per age) were purchased for arrival to the vivarium 1 week before the end of the study to provide age-matched controls (18M) for stress and handling effects, and baseline young adult values (3M). The remainder of the rats were 15 months of age, and were used for a 3-month, chronic drug study. All animals in this study were housed two per cage in the vivarium at Loma Linda University, given food and water *ad libitum*, and placed on a 12-h-on 12-h-off lighting schedule. The ambient temperature of the vivarium was maintained at 22 °C and humidity ranged between 30-40%. Animals were left undisturbed to acclimate to housing conditions for one week followed by another week of daily handling to prepare them for the daily injection regimen. All
animal procedures used in this study were approved by the institutional committee for animal use and care committee at Loma Linda University; and follow NIH guidelines.

Study Design

Rats at 15 months of age were randomly assigned to one of the following treatment groups (\(n\) of 24 per treatment group): (1) vehicle treatment (Veh-18M), low-dose rilmenidine (500 \(\mu\)g/ml/kg/day; Rilmlo), or high-dose rilmenidine (1.5 mg/ml/kg/day; Rilmhi). Rats received a twice-daily injection of an equivalent volume of sterile endotoxin-free saline as the drug treatment or rilmenidine (half the total daily dose) at 7:30 a.m. and 3:30 p.m. for 90 days. The rilmenidine treatment regimen was based on a previous report demonstrating sustained anti-hypertensive action in spontaneous hypertensive rats with twice daily intraperitoneal (i.p.) injections of 250 \(\mu\)g/ml/kg rilmenidine every twelve hours [Monassier et al. 2004]. Untreated, 3M and 18M, male, control BN rats arrived one week before the end of the 90-day experiment. Body weights from vehicle or drug-treated groups were measured before starting treatment, then once every week until the conclusion of the experiment, and, for all rats, before sacrifice. Body weights were expressed in g \(\pm\) standard error of the mean (SEM). The health of the rats undergoing treatments was monitored by observing overall appearance, general activity, grooming, feeding and drinking behaviors daily, and body weight weekly.

\(\alpha\)-Methyl-p-tyrosine (\(\alpha\)MPT) Treatment. At the end of the 90-day drug treatment, rats randomly chosen from vehicle- or rilmenidine-treated (Rilmlo, or Rilmhi) groups (\(n\) of 16 rats per treatment group) were assigned to receive either vehicle or \(\alpha\)MPT. Basal rates of NE turnover in the spleen were estimated by a method previously described by
Trayhurn and Wusteman [1987], in which the rate of decline in splenic NE concentration is measured after treatment with αMPT [Trayhurn and Wusteman, 1987; Bellinger et al., 2008b]. Rats were injected with αMPT (200 mg/kg, i.p.) or an equivalent volume of sterile, endotoxin-free saline at time zero, and sacrificed 6 hr later by decapitation without prior anesthesia.

Peripheral blood from each rat was harvested in 12x75-mm tubes containing 10 mmol/L disodium ethylenediaminetetraacetic acid (EDTA) kept on ice. Blood samples were centrifuged at 1,200 rpm for 10 min, the plasma was collected into microfuge tubes, and the plasma samples stored at -80 °C until measurement of catecholamine concentrations using high-performance liquid chromatography with coulometric detection (HPLC-CD). Spleens were dissected and transversely cut into two halves. Next, two cross-sectionally pieces cut from the central region of one half of the spleen (~3-4 mm thick) were frozen on dry ice and then stored at -80 °C until measurement of splenic NE concentration, and staining with fluorescence histochemistry to localize sympathetic nerves. Spleen cells were isolated from the other half of the spleen and used to evaluated β-AR-stimulated cAMP production, and for β-AR binding assays.

Gross examination of the brain and all visceral organs was carried out after tissue collection, to assess possible age-related pathology, including splenomegaly (enlarged spleen resulting from lymphoma or leukemia originating in the spleen), bile duct hyperplasia (rough surface of liver), chronic nephropathy (enlarged and discolored kidneys), and testicular interstitial cell hyperplasia. Any rat with visible tumors, lesions or overt pathology was excluded from this study. One or two rats per chronic treatment group were removed from the study due to overt pathology.
HPLC-CD for Measuring Catecholamine Levels

Using a Branson Sonifier 250, spleen samples were sonicated after adding a 10X volume/wet weight of cold 0.1 M perchloric acid and the internal standard, 3,4-dihydroxybenzylamine (DHBA) (10 ng/ml; Sigma-Aldrich). Next, homogenates were centrifuged for 5 min at 10,000 rpm and the supernatants were harvested into microfilterfuge tubes and centrifuged for 20 min at 14,000 rpm. After centrifugation, supernatants were collected and stored at -80 °C until assayed for catecholamines.

To assess circulating catecholamine content, 200 µl of plasma from each rat was transferred into a 12x75-mm glass tube, and 50 µl of the internal standard (DHBA), 1.0 ml of a 0.1 M phosphate buffer (pH 7.0), 1.0 ml of 1.5 M Tris buffer (pH 8.6), and 50 mg of acid-washed alumina (Bioanalytical Systems Inc., West Lafayette, IN) were added. After covering the top with parafilm, the tubes were, briefly vortexed, and then placed on a C10 platform shaker (New Brunswick Scientific, Edison, NJ) for 5 min at 175 rpm. After the alumina settled, the samples were aspirated until dry, the alumina was washed with double-distilled H₂O three times, and then the resulting alumina slurry was transferred to microfilterfuge tubes. Next, the microfuge tubes were centrifuged for 2 min at 9,000 rpm, the alumina was transferred to a new microfilterfuge tube, and 200 µl of 0.1 M HClO₄ was added. Then, the samples were vortexed, the tubes were centrifuged again for 2 min at 9,000 rpm and the supernatants collected. Vials containing the supernatants were loaded into an ESA Model 542 autosampler, and NE and DA concentrations were determined by HPLC using a CouleChem HPLC System (ESA, Chelmsford, MA). An ESA Model 582 solvent delivery module delivered the mobile phase through a Resolve C18 5-µm, 8x100-mm Radial-Pak analytical column at a flow rate of 1.0 ml/min. In the
ESA CouleChem III coulometric detector cell system, the potentials of the guard cell and two detecting cells were set at 400 mV, 350 mV, and -350 mV, respectively. The signal from the detector was recorded and the peak heights and area under the curves were analyzed using EZChrom Elite Software (Scientific Software Inc. Pleasanton, CA). Standards of known concentrations of NE, Dopamine (DA) and epinephrine (EPI) were used to determine splenic and plasma catecholamine concentrations and these data were expressed as a mean ± SEM in ng per g tissue wet weight and ng/ml plasma, respectively. Total splenic NE and DA content was estimated using NE and DA concentrations/mg wet weight and whole spleen weight respectively, and expressed as a mean ± SEM in ng/whole spleen. Circulating NE and EPI were expressed as a mean ± SEM in ng/ml plasma.

Splenic NE Turnover

Splenic NE turnover was determined by calculating the rate of NE concentration decay after blocking NE synthesis using a model that applies steady-state kinetics, as previous described [Bellinger et al., 2008b]. In this model, NE concentration depends on the rate of synthesis as defined by the equation, Log[NE] = Log[NE]o – 0.434kt, where [NE]o is the initial concentration of NE and k, the rate constant, is the fraction of NE concentration formed or lost per unit time.

To determine the rate constant, turnover time and turnover rate for NE in the spleen, the log of NE (log[NE]) concentration was plotted versus the time after synthesis blockade. Linear regression analysis of the log[NE] vs. time relationship was carried out using individual data points obtained at 0 and 6 hr after tyrosine hydroxylase inhibition.
The slope (m) and standard error of the regression coefficient (Ser) were computed by the least squares method. The rate constant for NE disappearance (kNE defined as m/0.434), NE turnover time (1/kNE), and NE turnover rate ([NE]₀ x kNE) were calculated as described. The standard error of the turnover rate and the turnover time were calculated from the variance of the regression slope and the variance of [NE]₀ according to the delta method [Bishop et al. 1975], using the following equation:

\[
\text{Variance } k(\text{NE})_0 = k^2 \text{var}(\text{NE}_0) + [\text{NE}_0]^2 \text{var}(k) + 2k\text{NE}_0 \text{Covariance } k,\text{NE}_0.
\]

Splenic NE turnover rate and time were expressed as a means ± SEM in ng/g/h and h, respectively.

Fluorescence Histochemistry for Splenic Sympathetic Nerves

NA sympathetic nerves in spleens from BN rats were visualized by a modification of the glyoxylic acid condensation method (SPG method) of histofluorescence for catecholamines, as previously described [de la Torre et al, 1980; Bellinger et al, 1992]. Spleen sample blocks taken from the hilar region (zone of blood vessels and nerves entry into the spleen) and distal to the hilus, were mounted onto chucks with OCT compound, and 16 µm sections were cross-sectionally cut in a cryostat set at -20 °C. After thaw-mounting three sections onto a glass slide, the slide was dipped in a 1% glyoxylic acid solution containing 0.2 M potassium phosphate and 0.2 M sucrose (pH 7.4), and then air dried using a direct stream of cool air for 15 min. Then, spleen sections were covered with several drops of mineral oil, and the slides were placed on a heat-conducting plate in an oven at 95 °C for 2.5 min. After draining the excess mineral oil from the slides, the slides were cover slipped with fresh mineral oil. Slides were examined using a Zeiss Axiomat fluorescence microscope equipped with epi-illumination accessories and color
images were captured and digitized with an Olympus image high-resolution CCD video capture system.

Morphometric Analysis of Sympathetic Nerve Density: Estimating the mean area of NA nerves in the white pulp was carried out blinded to the treatment groups. The white pulp of the spleen was selected for analysis, because it is the compartment of the spleen with the largest proportion of sympathetic innervation [Bellinger et al., 2008b]. One splenic white pulp from each, at the hilar region (the point of NA nerve entry into the spleen), or distal to the hilar region, was randomly selected from 4 spleen sections per rat from 6 rats per treatment group for analysis. The criteria for selection of white pulps for analysis were that (1) there only one central arteriole was visible in the white pulp; (2) the size of the central arteriole was between 80-100 µm across the largest diameter of the vessel and comparable across all samples, and (3) the arteriole was cut in true cross section cut through the central arteriole, as previously described [Bellinger et al., 2002; Lorton et al., 2005]. White pulps were photographed and stored as digital images onto a computer using the 200X objective on a Zeiss fluorescent microscope containing a 395 to 440 nm excitation filter. The number of pixels containing NA nerve profiles in each image was determined, based on size and color using Image-Pro® imaging software (version 5.0; Media Cybernetic, Bethesda, MD) and the mean area of NA nerves in the white pulp was quantitated. The ratio of positive pixels and the total number of pixels in the image was multiplied by 100 to calculate the % area of NA nerve profiles in each image. The average of the % NA nerve area in the four white pulps from each animal was used to determine the mean % NA area for each animal. These mean values were
averaged for rats within each treatment group, thus, data were expressed as a mean of a mean ± SEM as a percent NA nerve area.

Spleen Cell Preparation

At the time of sacrifice, spleens were removed and weighed using sterile technique. Spleen mass was later expressed in g and as a percentage of body weight ± SEM. Spleen cells were dissociated using a stomacher Lab-Blender (Tekmar Co., Cincinnati, OH). After placing approximately half of the spleen into a stomacher bag containing 10 ml Hank’s balanced salt solution (HBSS) (Mediatech Inc., Manassas, VA) containing 0.035% sodium bicarbonate (Sigma-Aldrich, St. Louis, MO) and 25 mM HEPES (Mediatech Inc., Manassas, VA). Larger aggregates in each spleen cell suspensions were removed by passage through a fine 100 µm cell strainers (BD Biosciences Discovery Labware, Bedford, MA). Next, the cell suspensions were centrifuged, resuspended in HBSS, and then layered over Histopaque 1077 (Sigma Chemical Co.) and centrifuged at 400 x g for 30 min at room temperature to remove red blood cells. Spleen cells removed from the interface between Histopaque and HBSS were washed three times in HBSS, counted using a Coulter Counter (Coulter Instruments, Hialeah, Fl), and resuspended to 2x10^6 cell/ml in RPMI 1640 (Mediatech Inc.) media supplemented with 5% heat-inactivated fetal bovine serum (Omega Scientific, Tarzana, CA), 1mM sodium pyruvate (Hyclone Laboratories Inc., Logan UT), 2mM L-glutamate (Mediatech Inc.), 10 mM non-essential amino acids (#SM302381; Hyclone Laboratories Inc.), 4.7x10^{-5} M 2-mercaptoethanol (Sigma-Aldrich), 100 U/mol penicillin, 100 mg/ml streptomycin Mediatech Inc.), 0.2% sodium bicarbonate (Sigma-Aldrich), 0.2 mg/ml folic acid (Sigma-
Aldrich), and 10 mM HEPES (Hyclone Laboratories Inc.). The spleen cell suspensions were then used for β-AR binding and cAMP assays after adjusting to the appropriate cell concentrations for each assay.

**β-AR Binding Assays**

Resuspended whole spleen cells (5x10⁶ cells/ml) from vehicle or low-dose rilmenidine treatment groups (*n* of 6 per group) were assayed for radioligand binding studies. The radioligand ligand, (-)[¹²⁵I]cyanopindolol ([¹²⁵I]CYP) (2200 Ci/m mole) (GE Healthcare, Piscaway, NJ), was diluted in 1% ethanol, 5 mM HCl, and 0.2% bovine serum albumin (BSA). ([¹²⁵I]CYP) is a β-AR antagonist with equal affinity for the β₁ and β₂ subclasses [Sundaresan et al., 1987]. Assayed in duplicate, 1x10⁶ spleen cells were placed in 13x100-mm polypropylene tubes containing one of eight concentrations of [¹²⁵I]CYP ranging from 15.8-333 pM. Another set of tubes containing the same number of spleen cells were incubated with 10⁻⁶ M CGP-12177 (Sigma-Aldrich), a hydrophilic β-AR antagonist, were performed to control for nonspecific binding. All tubes were incubated at 37 °C for 60 min in a shaking water bath set at 100 oscillations/min, and then 3 ml of ice cold hypotonic buffer containing 3.8 mM KH₂PO₄, 16.2 mM K₂HPO₄, and 4 mM MnSO₄, for 20 min was added to lyse cells. The content of each tube was filtered through Whatman fiberglass filters (GF/B) (Brandel Corp., Gaithersburg, MD) under reduced pressure using an automatic cell harvester (Brandel Corp., Gaithersburg, MD) to collect bound radioactivity. The filters were washed with 16 ml (4x4 ml) of ice-cold Tris-EGTA buffer to remove the remaining unbound radioligand. Filters were removed from the cell harvester, placed in 12x75-mm plastic tubes, and counted in a
Wallac 1470 Wizard gamma counter (Long Island, Port Jefferson, NY) at 82% efficiency.

The difference between binding of the radioligand at each concentration in the absence and in the presence of (-) CGP-12177 was used to define specific binding. From 10-20% of total binding was due to nonspecific binding. It was assumed was made in calculating binding that $[^{125}\text{I}]\text{CYP}$ decomposed into non-β-AR active products in direct relation to radioactive decay, as previously [Doyle et al., 1984]. The maximal number of binding sites/cell was calculated based on stoichiometric assumptions that 1 molecule of ligand binds to 1 receptor An interactive nonlinear regression curve fitting program from Prism® (GraphPad, San Diego, CA) to a single class of homogeneous binding sites was used to determine receptor density ($B_{\text{max}}$) and affinity ($K_d$). Specific binding was expressed in cpm vs. the concentration of $[^{125}\text{I}]\text{CYP}$ in pM. Data also were transformed into a linear form by Scatchard analysis and shown in Scatchard plots with bound in sites/cells vs. bound/free. The lines of best fit were determined using the Bmax and K_d to determine the X and Y intercepts.

β-AR-Stimulated cAMP Production in Spleen Cells

Spleen cell suspensions ($1\times10^6$) were transferred into 12×75-mm polystyrene round bottom tubes (Thermo Fisher Scientific, Pittsburg, PA), and 100 μl HBSS containing 100 μM isobutylmethylxanthine (IBMX) (Sigma-Aldrich) and 0.1% bovine serum albumin (BSA) (EMD Chemicals, Gibbstown, NJ), was added. In a shaking water bath at 37 °C, the tubes were incubated for 20 min, and then spleen cells were treated with 1 ml $10^{-5}$ M isoproterenol (Sigma-Aldrich) for 10 min. Then, tubes were removed from the water
bath, and 2 ml ice-cold HBSS/BSA/IBMX buffer to quench the reaction. Spleen cell suspensions were centrifuged for 8 min at 1,800 rpm and the supernatant was discarded. After reconstituting the pellets with 0.5 ml of sodium acetate (50 mM) buffer, the tubes were exposed to two boiling-freezing cycles to lyse the cells. Cellular debris was removed by centrifugation at 1800 rpm for 8 min. The supernatants were collected into microfuge tubes, and then immediately stored at -80 °C. Cyclic AMP levels, were determined in triplicate with a commercially available EIA Kit (GE Healthcare, Piscataway, NJ) following manufacturer’s instructions for acetylation EIA protocol, which provides the highest sensitivity with this kit (lower detection limit of 14 pg/ml). cAMP concentration in samples was determined by measuring the optical density in each well using a plate reader set at 450 nm. β-AR-stimulated cAMP production was expressed as a mean ± SEM in fmoles/2 x 10^6 cells/10 min.

**Data Analysis**

For data where mean ± standard error of the mean (SEM) were calculated, data were analyzed using one-way analysis of variance (ANOVA) and Bonferroni post-hoc testing was used for significant ANOVA (p<0.05) to determine significant differences between treatment groups. Where no statistical differences between vehicle-treated and untreated 18M control groups were found, the data were collapsed and designated Ctrl-18M. Where no differences were found between Ctrl-18M and Veh-18M via Student t-test, the data were collapsed and designated a Ctrl-18M.
Results

Chronic Rilmenidine Treatment Did Not Alter Mean Body or Spleen Weights

BN rats appeared to tolerate the twice daily, 90-day vehicle or drug treatments, qualitatively displaying comparable activity, feeding, drinking, and grooming behaviors. There was no difference in mean body weights between the 18M treatment groups over the 90-day period (Fig. 4.1A). Similarly, within each treatment group, mean body mass did not change over the 3-month course of the experiment. Body mass from all 18M groups were significantly greater (***, $p<0.001$) than in Ctrl-3M rats (Fig. 4.1A). Spleen weights from Ctrl-18M, Veh-18M, Rilmlo (**, $p<0.001$), and Rilmhi (*, $p<0.05$) rats increased significantly compared with young control rats (Ctrl-3M) (Fig. 4.1B). There was also a trend (T, $p<0.1$) for lower spleen weights with Rilmhi treatment compared with Ctrl-18M rats. Finally, the spleen-to-body weight ratio was increased in Ctrl-18M compared with Ctrl-3M rats (††, $p<0.001$); and decreased in Veh-18M-treated rats (**, $p<0.01$), Rilmlo (*, $p<0.05$), or Rilmhi (*, $p<0.05$), compared with Ctrl-18M values (Fig. 4.1C).
Fig. 4.1. Effect of Rilmenidine Treatment on Body Weights
A. The effect of 90-day treatment with low- or high-dose rilmenidine (Rilmlo or Rilmhi, respectively) or vehicle (Veh-18M) on mean body weight of male BN rats expressed as means (± SEM) in grams. Drug treatment began at 15 months of age and continued for 12 weeks (18 months of age). Mean body weight progressively rose over the 12-week period of study, with no significant effect of vehicle or chronic drug treatment. Veh, ○; Rilmlo, □; Rilmhi, △. There was an expected age-related increase (*, p < 0.001) in body weight in all 18M rat groups compared with Ctrl-3M rats. Young, 3-month-old controls, Ctrl-3M; Old, 18-month-old controls, Ctrl-18M; Vehicle, low and high-dose rilmenidine, Veh-18M, Rilmlo and Rilmhi, respectively. Ctrl-3M, n=10; Ctrl-18M, n=10; Veh, Rilmlo or Rilmhi, n=20.
Fig. 4.1. Effect of Rilmenidine Treatment Mean Spleen Weights.
B. The effect of 90-day treatment with low- or high-dose rilmenidine (Rilm\textsubscript{lo} or Rilm\textsubscript{hi}, respectively) or vehicle (Veh-18M) on mean spleen weight of male BN rats expressed as means (± SEM) in grams. An expected increasing effect by age on spleen weight was observed (**, $p<0.001$, Ctrl-18M, Veh-18M, or Rilm\textsubscript{lo}; *, $p<0.05$ Rilm\textsubscript{hi}). Only a decreasing trend (T, $p<0.1$) in spleen weight was found in Rilm\textsubscript{hi} rats compared to Ctrl-18M rats. Veh, ◇; Rilm\textsubscript{lo}, □; Rilm\textsubscript{hi}, Δ. Young, 3-month-old controls, Ctrl-3M; Old, 18-month-old controls, Ctrl-18M; Vehicle, low and high-dose rilmenidine, Veh-18M, Rilm\textsubscript{lo} and Rilm\textsubscript{hi}, respectively. Ctrl-3M, $n=10$; Ctrl-18M, $n=10$; Veh, Rilm\textsubscript{lo} or Rilm\textsubscript{hi}, $n=20$. 
Fig 4.1. (Continued). Mean Spleen Weights/ Mean Body and vs. Time after Treatment
C. Mean spleen weight per body weight ratio was greater (††, p<0.05) in Ctrl-18M rats than in Ctrl-3M rats; and greater in Ctrl-18M rats than in Veh-18M-rats (**, p< 0.01), Rilmlo- (*, p< 0.05), or Rilmhi-treated rats (*, p< 0.05). Spleen weight per body weight in these rats is expressed in g per g.
Rilmenidine Reduced Plasma NE, Without Affecting Plasma EPI Concentrations

There was no significant effect of age on mean plasma catecholamine concentrations (NE or EPI) (Figs. 4.2A and 4.2B, respectively). Chronic treatment with Rilm_{lo} or Rilm_{hi} significantly reduced (*, p<0.05) plasma NE concentrations compared with Ctrl-3M and age-matched, Ctrl-18M rats, without an apparent dose response (Fig. 4.2A). In contrast, rilmenidine treatment had no effect on mean plasma EPI concentrations.
Fig. 4.2. Effect of Rilmenidine on Circulating NE Concentration

A. Mean plasma norepinephrine (NE) concentrations in BN rats treated with low- or high-dose rilmenidine (Rilmlo or Rilmhi), and in 3M and 18M controls. Age-matched (18M) untreated and vehicle-treated rats were not different, so data were collapsed and expressed as Ctrl-18M. Rilmenidine treatment, regardless of dose, reduced (*, p<0.05) circulating NE. Young, 3-month-old controls, Ctrl-3M; old, 18-month-old controls, Ctrl-18M; Low and high-dose rilmenidine, Rilmlo and Rilmhi, respectively. Ctrl-3M, n=10; Ctrl-18M, n=22; Rilmlo or Rilmhi, n=14.
Figure. 4.2. Effect of Rilmenidine on Circulating EPI Concentration.

B. Mean plasma epinephrine (EPI) concentrations in BN rats treated with low- or high-dose rilmenidine (Rilmlo or Rilmhi), and in 3M and 18M controls. Age-matched (18M) untreated and vehicle-treated rats were not different, so data were collapsed and expressed as Ctrl-18M. Rilmenidine treatment for 90 days, regardless of dose, did not have an effect on EPI. Young, 3-month-old controls, Ctrl-3M; old, 18-month-old controls, Ctrl-18M; Low and high-dose rilmenidine, Rilmlo and Rilmhi, respectively. Ctrl-3M, n=10; Ctrl-18M, n=22; Rilmlo or Rilmhi, n=14.
At the hilus, fluorescence histochemistry revealed a dense plexus of NA nerves surrounding the central arteriole in the white pulp of the spleen from Ctrl-3M rats (Fig. 4.3A). Noradrenergic fibers coursed into the adjacent periarteriolar lymphatic sheath (PALS) where T cells reside. A similar distribution of NA nerves was present around the central arteriole in the splenic white pulp and PALS from Ctrl-18M rats; however, the density of fluorescent NA fibers was markedly reduced compared with Ctrl-3M rats (Fig. 4.3B). There were no apparent differences in the density of NA nerves between Veh-18M (data not shown) and Ctrl-18M rats. Similarly, treatment with Rilm_{lo} or Rilm_{hi} (Figs. 4.3C and 4.3D, respectively) did not appear to affect either the distribution or the density of NA nerves in the white pulp compared with Ctrl-18M rats.

Morphometric analysis (Fig. 4.3E) confirmed an age-related decline (***, \( p < 0.001 \)) in splenic NA nerve density in the splenic white pulp of approximately 41.7% in all 18M treatment groups compared with Ctrl-3M rats, but no change in splenic NA density in the white pulp of Rilm_{lo} or Rilm_{hi} compared with Ctrl-18M rats.
Fig. 4.3. Effect of Rilmenidine on NA Innervation at the Hilus

Fluorescence histochemistry for catecholamines reveals abundant fluorescent linear and punctate (arrowheads) noradrenergic (NA) nerve profiles surrounding the central arteriole (ca) in the white pulp (wp) in the hilar region of the spleen in 3-month-old F344 rats (Ctrl-3M) (A). The density of fluorescent nerves alongside the central arteriole is diminished at 18 months of age (Ctrl-18M) (B); and maintained in spleens of rats treated with low- or high-dose rilmenidine, Rilmlo (C.) and Rilmhi (D.), respectively. The intensity and density of NA nerves in the white pulp, at the hilus, of spleens from vehicle-treated (Veh) were similar to Ctrl-18M (data not shown). A-D. Glyoxylic acid fluorescence histochemistry. Calibration bar = 100 µm.
Fig. 4.3. Effect of Rilmenidine on NA Innervation at the Hilus (Continued).

E. Morphometric analysis of fluorescent NA nerve profiles (E.) expressed as percent mean area is consistent with qualitative findings, showing an age-related loss of fluorescent nerves in the white pulp (***, $p<0.001$) in all 18M groups (Ctrl-18M, Rilmlo, or Rilmhi) compared with Ctrl-3M. Each group is representative of an $n$ of 8 rats. There was no difference in NA nerve area in Ctrl-18M and Veh rats, so the data was collapsed.
Distal from the hilus, the area of the white pulp and the diameter of the central arteriole were smaller than at the hilus (Figs. 4.4A-D). Consistent with findings in the hilar region, there was an apparent age-related loss of NA nerve fibers along the central arteriole (Figs 4A-B); fewer NA nerves were present in the white pulp from Ctrl-18M (Fig. 4.4B) compared with Ctrl-3M rats (Fig. 4.4A) effects of age, but not drug treatment on NA nerve density. The distribution and density of NA nerve in the white pulps from Rilmlo (Figs. 4.4C) were similar to that observed in Ctrl-18M rats (Fig. 4B); however, NA innervation of the central arteriole and surround PALS in the white pulp from Rilmhi rats (Figs. 4.4D) was similar to Ctrl-3M (Figs. 4.4A). Morphometric analysis (Fig. 4.4E) confirmed qualitative observations in the white pulp distal to the hilus. There was a significant (*, \( p<0.05 \)) reduction in nerve density of 25% and 21% in Ctrl-18M and Rilmho respectively, compared with Ctrl-3M rats.
Fig. 4.4. Effect of Rilmenidine on Splenic NA Innervation Distal to the Hilus

Fluorescence histochemistry for catecholamines, in the region distal to the hilus, exposes NA nerves surrounding the central arteriole (ca) in the white pulp (wp), as fluorescent punctuate and linear profiles extending to surrounding areas (arrowheads) (Ctrl-3M) (A.). In the white pulp distal to the hilus central arterioles are of smaller diameter surrounded by fewer NA nerves than in the hilar region; and compared to Ctrl-3M rats, reduced NA nerve profile density along the central arteriole and white pulp is observed at 18 months of age (Ctrl-18M) (B.) or in rats treated with a low dose of rilmenidine (Rilm_lo) (C.). Nerve density in high-dose rilmenidine-treated rats (Rilm_hi) (D.) appears similar to Ctrl-3M rats. Low and high-dose rilmenidine, Rilm_lo and Rilm_hi, respectively; young, 3-month-old controls, Ctrl-3M; old, 18-month-old controls, Ctrl-18M). A-D. Glyoxylic acid fluorescence histochemistry. Calibration bar = 100 µm.
Fig. 4.4. Effect of Rilmenidine on Splenic NA Innervation Distal to the Hilus by Morphometric Analysis (Continued).

E. Morphometric analysis confirmed the age-related decline of fluorescent NA nerves that was maintained with Rilmlo (*, \( p<0.05 \)). Low and high-dose rilmenidine, Rilmlo and Rilmhi, respectively; young, 3-month-old controls, Ctrl-3M; old, 18-month-old controls, Ctrl-18M).
Rilmenidine Affects Splenic NE and DA Concentrations and Content in a Dose-Dependent Manner

There was an age-related decline in mean splenic NE concentration and total NE content in the spleen (Ctrl-18M vs. Ctrl-3M; ***, p<0.001 and *, p<0.05) (Fig. 4.5A and Fig 4.5B, respectively). A slight dose-dependent increase in NE concentration and content resulted from Rilm treatment compared with Ctrl-18M rats. Splenic NE concentration in Rilm₉₀ rats was significantly lower than in Ctrl-3M (Fig. 4.5A), but no other treatment groups were different for splenic NE concentration or content. Regression analyses (Figs. 4.5C and 4.5D), revealed significant negative correlations between splenic NE concentration or total content and spleen weight (p<0.001 and p<0.01, respectively).
Fig. 4.5. Rilmenidine and Splenic NE Concentration.
A. Mean splenic NE concentration (mg/g wet weight ± SEM) in rats treated for 90 days with vehicle, low- or high-dose rilmenidine (Veh-18M, Rilmlo or Rilmhi, respectively). Mean splenic NE concentration was reduced in Ctrl-18M rats (***, p<0.001) and Veh-18M- or Rilmlo-treated (**, p<0.01) compared with Ctrl-3M rats. A non-significant trend toward increased splenic NE concentration with Rilmhi compared with Ctrl-18M was noted. Ctrl-3M or Ctrl-18M, respectively; and low- or high-dose rilmenidine, Rilmlo or Rilmhi, respectively. Ctrl-3M, n=10; Ctrl-18M, n=10; Rilmlo or Rilmhi, n=14.
Fig. 4.5. Rilmenidine and Splenic NE Content.
B. Mean splenic total content (ng/spleen ± SEM) in rats treated for 90 days with vehicle, low- or high-dose rilmenidine (Veh-18M, Rilm_{lo} or Rilm_{hi}, respectively). Mean splenic NE content was reduced in Ctrl-18M rats (*, \( p<0.05 \)) compared to Ctrl-3M rats. No other significant differences in splenic total NE content among treatment groups were found. Ctrl-3M or Ctrl-18M, respectively; and low- or high-dose rilmenidine, Rilm_{lo} or Rilm_{hi}, respectively. Ctrl-3M, \( n=10 \); Ctrl-18M, \( n=10 \); Rilm_{lo} or Rilm_{hi}, \( n=14 \).
Fig. 4.5. Correlation between Spleen Weight and Splenic NE Concentration.
C. A negative correlation was observed between splenic NE concentration with spleen weight ($R^2 = 0.2394$). Untreated 3M and 18M BN controls rats, Ctrl-3M or Ctrl-18M, respectively; and low- or high-dose rilmenidine, Rilm$_{lo}$ or Rilm$_{hi}$, respectively. Ctrl-3M, $n = 10$; Ctrl-18M, $n = 10$; Rilm$_{lo}$ or Rilm$_{hi}$, $n = 14$.  

$R^2 = 0.2394$
Fig. 4.5. Correlation between Rilmenidine and Splenic NE Content.

D. A negative correlation was observed between splenic NE content with spleen weight ($R^2=0.1454$). Untreated 3M and 18M BN controls rats, Ctrl-3M or Ctrl-18M, respectively; and low- or high-dose rilmenidine, Rilmlo or Rilmhi, respectively. Ctrl-3M, $n=10$; Ctrl-18M, $n=10$; Rilmlo or Rilmhi, $n=14$. 
DA concentration (Fig. 4.6A) or content (Fig. 4.6B) were not significantly affected by age, but was more variable in Ctrl-3M and tended to be lower in Ctrl-18M compared Ctrl-3M. However, treatment with Rilmlo significantly decreased DA concentration ($p<0.01$) or total content of DA ($p<0.05$) in the spleen by approximately 45% compared with Ctrl-3M rats. No correlation between either DA concentration or total content in the spleen and spleen weight was found (Figs. 4.6C and 4.6D respectively).
Fig. 4.6. Rilmenidine and Splenic DA Concentration.
A. Mean total splenic DA concentrations (expressed as ng/gm wet weight ± SEM) in 3M and 18M controls (Ctrl-3M, Ctrl-18M), and rats treated with low- or high-dose rilmenidine treatment, Rilmlo, and Rilmi, respectively). There was no effect of age on splenic DA concentration; however, Rilmlo decreased splenic DA concentration (*, \( p<0.05 \)) compared with Ctrl-3M. There was a non-significant trend for reduced DA concentration in the spleen, with Rilmlo compared with Ctrl-18M. Rilmi did not affect splenic DA concentration. Ctrl-3M, \( n=10 \); Ctrl-18M, \( n=10 \); Rilmlo or Rilmi, \( n=14 \).
**Fig. 4.6. Rilmenidine and Total Splenic DA Content.**

**B.** Mean total splenic DA content (expressed as ng/spleen ± SEM) (B.) in 3M and 18M controls (Ctrl-3M, Ctrl-18M), and rats treated with low- or high-dose rilmenidine treatment, Rilm_{lo}, and Rilm_{hi}, respectively. There was no effect of age on splenic DA content; however, Rilm_{lo} decreased splenic DA content (*, p<0.05) compared with Ctrl-3M. There was a non-significant trend for reduced total DA content in the spleen with Rilm_{lo} compared with Ctrl-18M. Rilm_{hi} did not affect splenic DA content.
Fig. 4.6. Correlation between Spleen Weight and Splenic DA Concentration.
C. No correlation was found between Spleen weight and Splenic DA concentration ($R^2 = 0.0118$). Ctrl-3M, $n=10$; Ctrl-18M, $n=10$; Rilmlo or Rilmhi, $n=14$. 

$R^2 = 0.0118$
Fig. 4.6. Correlation between Spleen Weight and Total Splenic DA Content.

D. No correlations was found between Spleen weight and Splenic DA content ($R^2=0.0068$). Ctrl-3M, $n=10$; Ctrl-18M, $n=10$; Rilmlo or Rilmhi, $n=14$. 

$D$
Rilmenidine Reduces Splenic NE Turnover

NE synthesis blockade by treatment with αMPT reduced splenic NE concentration 6 h later regardless of treatment groups (Fig. 4.7 and Table 4.1). However, a slower rate of decline was observed with rilmenidine treatment compared with Veh-18M controls, and this effect was dose-dependent (59% by Rilm$_{lo}$ and 31% Rilm$_{hi}$) (Fig. 4.7). NE turnover rates were calculated based on the slopes of the lines. Regression analysis revealed a 14.5% and 8.3% decline in splenic NE turnover rate with Rilm$_{lo}$ and Rilm$_{hi}$ treatment compared with Veh-18M rats, respectively (Table 4.1). The turnover rate in spleens from Veh-18M rats was 1.9 fold higher than previously reported in 15M rats; and Rilm$_{lo}$ or Rilm$_{hi}$ turnover rates were 0.1-fold higher and 1.7-fold lower than in 15M rats. Rilmenidine dose-dependently increased the time required to synthesize the constant pool of NE in the spleen (turnover time) compared with Ctrl-18M rats (Table 4.1).
Fig. 4.7. Effect of Rilmenidine on αMPT-Induced Decline in Splenic NE Concentration
The effect of rilmenidine on mean splenic norepinephrine (NE) concentrations at 0 and 6 hours post-injection with αMPT, an inhibitor of NE synthesis. Ninety-day treatment with low-dose (Rilmlo, □) or high-dose rilmenidine (Rilmhi, ○) slowed the rate of decline in splenic NE concentration over the 6-hour period compared with age-matched vehicle controls (Veh-18M, ◊). Data are expressed in ng/g/hr vs. time and each group represents an n of 8 rats.
Table 4.1. Turnover rates and turnover times of NE in the spleen of BN male rats treated with vehicle, low- or high-dose rilmenidine (Veh-18M, Rilm_{lo} or Rilm_{hi}, respectively). Initial NE concentrations and splenic turnover rate declined in a dose-dependent manner after treatment with αMPT rats compared with vehicle-treated rats (Veh-18M) rats. Turnover time was increased by rilmenidine treatment in a dose-dependent manner. Initial levels of splenic NE and the rate constants are expressed as means ± SEM. αMPT, α-methylparatyrosine; NE, norepinephrine; n, number of rats per age group.
Rilmenidine Treatment Increased Splenocyte \( \beta \)-AR Receptor Binding Without Affecting Affinity

Binding of the radioligand was rapid, saturable, and of high affinity in spleen cells from Veh-18M and Rilmio-treated rats (Fig. 4.8A-B, respectively). Scatchard plots (insets in both Fig. 4.8A and 4.8B, respectively) show the slopes of the line for Veh-18M-(4.8A) and Rilmio- (4.8B) treated rats, where each point represented the ratio of bound ligand per sites per cell over bound ligand. The X and Y intercepts of the line were determined by \( B_{\text{max}} \) and \( K_D \) respectively.
**Fig. 4.8. Effect of Rilmenidine on β-AR Binding in Splenocytes**

**A.** Specific binding and Scatchard plots of (-)[125I]cyanopindolol([125I]CYP) in whole spleen cells from age-matched vehicle-treated rats. Spleen cells were incubated under equilibrium binding conditions at 37 °C with ICYP (0.9-220 pM) for 60 min, then the reaction was stopped and radioactivity was quantified by gamma scintillation spectrometry. Specific binding and plots represent means of duplicate determinations of for specific binding (■), counts/min (cpm). This group represents an $n$ of 5 rats.
Fig. 4.8. Effect of Rilmenidine on β-AR Binding in Splenocytes (Continued).

B. Specific binding and Scatchard plots of (-)[125I]cyanopindolol([125I]CYP) in whole spleen cells from low-dose rilmenidine (rilm)-treated rats. Spleen cells were incubated under equilibrium binding conditions at 37 °C with ICYP (0.9-220 pM) for 60 min, then the reaction was stopped and radioactivity was quantified by gamma scintillation spectrometry. Specific binding and plots represent means of duplicate determinations of for specific binding (■), counts/min (cpm). This group represents an n of 5 rats.
The mean $B_{\text{max}}$ increased 36% (*, $p<0.001$) in spleen cells from Rilm$_{10}$-treated rats (750±65 sites/cell) over that seen with Veh-18M 560±58 sites/cell) treatment (Fig 4.9A).

The mean $K_D$ was not significantly different between Rilm$_{10}$- and with Veh-treated rats (Fig 4.9B).
Fig. 4.9. Effect of Rilm\textsubscript{lo} and Vehicle Treatment on Mean $\beta$-Receptor Expression ($B_{\text{max}}$) in BN Rats

A. Mean density of $\beta$-AR expressed as sites/cell on spleen cells from vehicle-treated (Veh-18M) and low-dose rilmenidine (Rilm\textsubscript{lo})-treated rats ($n$ of 6 per group). Treatment with Rilm\textsubscript{lo} increased $\beta$-AR expression on splenocytes (*, $p<0.05$). Mean values were calculated from the $B_{\text{max}}$ and $K_D$ determined form specific binding curves generated for each rat from each treatment group.
Fig. 4.9. Effect of Rilmlo or Veh-18M on Mean $\beta$-Receptor Binding Affinity ($K_D$).

B. Mean binding Affinity expressed as sites/cell $K_D$ on spleen cells from vehicle-treated (Veh-18M) and low-dose rilmenidine (Rilmlo)-treated rats ($n$ of 6 per group). Treatment with Rilmlo did not affect $K_D$. Mean values were calculated from the $B_{max}$ and $K_D$ determined from specific binding curves generated for each rat from each treatment group.
Rilmenidine Treatment and β-AR-Stimulated cAMP Production in Spleen Cells

The isoproterenol stimulation of β-AR increased the production of cAMP in spleen cells differentially depending on treatment group (Fig. 4.10). In spleen cells from Ctrl-18M rats cAMP production was 42% lower (**, p<0.01) in Ctrl-18M compared with Ctrl-3M rats. In rats treated with Rilm, cAMP production was comparable to Ctrl-18M levels, and Rilmlo did not differ statistically from Ctrl-3M levels. However, Rilmhi significantly reduced cAMP production levels (51%; **, p<0.001), compared with Ctrl-3M rats.
Fig. 4.10. Effect of Rilmenidine on β-AR-Stimulated cAMP Production in Splenocytes

Isoproterenol-stimulated cAMP production in spleen cells is expressed in fmol/10^6 cells/10 min ± SEM. Vehicle (Veh) and non-treated age-matched controls were not different, and therefore were collapsed (collectively Ctrl-18M). cAMP production in spleen cells was significantly lower in Ctrl-18M rats or Rilmlo-treated rats (*, p<0.05 and **, p<0.001 respectively) compared with Ctrl-3M rats. Low and high-dose rilmenidine, Rilmlo and Rilmhi, respectively; 3-month-old controls, Ctrl-3M; 18-month-old controls, Ctrl-18M. Ctrl-3M, n=10; Ctrl-18M, n=20; Rilmlo=14, Rilmhi, n=16.
Discussion

In this study, we investigated the effect of reducing SNA by treatment with rilmenidine on sympathetic neurotransmission in the spleen of aging BN rats. Reduced circulating NE concentrations and NE turnover in the spleen in Rilm-treated rats indicate lower net SNA and SNA in the spleen. Interestingly, we found that the Rilm-induced increase in β-AR binding did not affect β-AR-stimulated cAMP production in splenocytes, and minimally affected NA nerve density in the spleen at the highest dose.

Consistent with previous pharmacological studies in hypertensive [Monassier et al., 2004; El-Mas et al., 2009] and F344 rats [Perez et al., in press], rilmenidine globally reduced sympathetic outflow as indicated by the reduced circulating NE concentrations in rilmenidine-treated rats compared with age-matched controls. This effect was not dose-dependent, consistent with the findings of Raasch et al [2003]. In contrast, there we no effect of rilmenidine treatment on circulating EPI, also consistent with a previous report from our laboratory in F344 rats [Perez et al., in press]. These findings are consistent with the actions of rilmenidine on sympathoexcitatory neurons in the RVLM, via IR₁ and α₂-AR [Urban et al., 1995; Bruban et al., 2001], which causes a net inhibition of spinal preganglionic neurons that ultimately diminishes the firing rate of postganglionic NA nerves at the target site [Dampney 1994].

Importantly, rilmenidine reduced splenic NE turnover, indicating lower SNA in the spleens from BN rats treated with rilmenidine compared with age-matched controls. This finding is consistent with the spleen as a target organ of regulation by the RVLM based on anatomical and electrophysiological data [Beluli and Weaver, 1991a, b]. However, rilmenidine was not as effective in reducing circulating NE or splenic NE turnover as
seen previously in our laboratory in age-matched F344 rats with the same treatment regimen and doses [Perez et al., in press]. The reason for the this strain-related difference is not clear, but may be related to strain differences in the splenic microenvironment, immune response profile to antigens, \(\alpha_2\)-AR and/or IR\(_1\) expression or coupling efficacy with second messenger systems, and/or distribution in target tissues.

We cannot discount that some the effect of rilmenidine may be mediated peripherally, for example via \(\alpha_2\)-AR expressed on NA nerve terminals of all postganglionic nerves, including in the spleen, which act to dampen their firing when these receptors are stimulated [Docherty, 2002]. Similarly, IR\(_1\) are expressed in other tissues that are densely innervated by SNS [Dontenwill et al., 1999], and therefore may indirectly regulate the net sympathetic tone. Expression of IR\(_1\) in the spleen has not been confirmed thus far; however, rilmenidine may interact with \(\alpha_2\)-ARs expressed on cells of the immune system, such as splenic macrophages [Spengler et al., 1990]. Activation of \(\alpha_2\)-AR in splenic macrophages can increase TNF-\(\alpha\) and IL-1 [Spengler et al., 1990], cytokines which can reduced reduce splenic NE concentration [Khom and Sanders 2000] and SNA nerve activity [Madden and Felten 1995]. Future studies will investigate further the possible actions of rilmenidine on splenic SNA at sites other than the RVLM.

The small effects of rilmenidine on NA innervation in the aging spleen, which were seen only with Rilm\(_{hi}\), does not support our hypothesis that elevated SNA in the BN rat spleen contributes significantly to sympathetic nerve damage in this lymphoid organ. This finding is in contrast with previous findings in aging F344 rats from our laboratory [Bellinger et al., 2008b] demonstrating that reducing SNA activity by treatment with rilmenidine partially, but significantly, reversed NA nerve loss in the spleen [Perez et al.,
in press]. There are several key differences in age-related changing of sympathetic neurotransmission between these two strains. F344 rats exhibit a chronic increase in NE turnover rate in the spleen that begins at 10M (early middle age) and persists throughout late middle age (17M) [Bellinger et al., 2008b], whereas BN rats exhibit a transient increase in splenic NE turnover at 15M of age. Also, the decline in NA nerve density in the aging F344 rat spleen is more severe and begins early [Bellinger et al., 2008b] than in BN rats [Perez et al., 2009]. Finally, differences in immune responses to antigen challenge [Bellinger et al., 2002] and behavioral responses to stress [Gómez et al. 1998], suggest differences in stress pathways that regulate these behaviors.

The transient nature of increased SNA in spleens from aging BN rats [Perez et al., 2009] would be expected to have a lower negative impact on NA nerve integrity from oxidative stress [Tamariello et al., 2000; Lipton 1997] compared with a chronically increased sympathetic tone in F344 rats. In aging BN rats, there is an increase in NE turnover rate at 15M, followed by more than a 2-fold decline at 18M that stays reduced into old age (32M) [Perez et al., 2009]. This transitory spike in NE turnover correlates with decline in both NA innervation (at 15M) and splenic NE concentrations (at 18M), which are not further diminished in old age. The transient nature of sympathetic nerve activity in the BN rat spleen during middle age may permit better repair from insults due to increased oxidative stress on the nerves; or at 15M BN rats may have a greater inherent resilience to heightened sympathetic activity [Madden et al., 1997]. However, we cannot rule out that heighten SNA activity may occur earlier in middle age, at time points not examined in our previous studies. If this is the case, then starting treatment of rilmenidine at an earlier age may be efficacious for preventing sympathetic nerve loss in the aging
spleens of BN rats. More extensive investigation of SNA at earlier middle aged time points is needed to address this question.

Important factors that may protect sympathetic nerve integrity include better buffering capacity for eliminating free radicals, which are toxic to neurons [Madden et al., 1995] and adequate levels of neurotrophins that protect nerves from oxidative stress [Laurenzi et al., 1994]. With increasing age, there is a decrease in the antioxidant capacity in target tissues; and reduced ability of target cells or neurons to absorb free radical that may lead to neuronal atrophy [Tammariello et al., 2000]. The role of antioxidant enzymes and neurotrophic support on sympathetic nerves preservation of that supply the aging spleen, and whether there are strain differences, remains unknown and warrants investigation. A balance between positively and negatively acting neurotrophins in target tissues determine the extent of innervation [Ernfors et al., 1994]. Sympathetic nerve activity is important in regulating NGF synthesis in target tissues [Falckh et al., 1992]; and expression of neurotrophic factors may be linked to the degree of the age-related decline in NA innervation of the spleen in the BN rat strain. Unchanged concentrations in NGF or NT-3 in the spleen of old vs. young BN rats observed in our laboratory (unpublished observations) may suggest neuroprotection for sympathetic fibers by trophic factors are maintained in aging; however, whether aging affect neurotrophin receptor expression or function remains unknown.

Sympathetic nerve integrity may also be influenced by age-related changes in the composition of immune cell populations that reside in the aging spleen, which contribute to local microenvironment. For instance, in F344 rats, loss of sympathetic nerves in the spleen is accompanied by a loss of T lymphocytes and ED3+ macrophages [Bellinger et
Aging increases the numbers of anergic CD8+ T cells [Komuro et al., 1990] and memory T and B cell populations [Ernst et al., 1990], alters the CD4+/CD8+ ratio [Matour et al., 1989], and affects the functioning of lymphocytes and macrophage [Dunston and Griffith, 2010]. These aging changes may contribute to altered nerve integrity. Consistent with this hypothesis, T and B lymphocytes, eosinophils, follicular dendritic cells, reticular cells and mast cells synthesize NGF [Yoshida et al., 1992].

The minimal effect by rilmenidine on NA nerve density in BN rats is in contrast with our previous findings of a neuroprotective role in aging F344 rats. This difference may be due to greater resistance to rilmenidine effects in BN rats compared with F344 rats. This hypothesis is consistent with our finding that rilmenidine did not lower circulating NE or splenic SNA as effectively in BN rats as it did in F344 rats, and rilmenidine was better tolerated in BN rats as indicated by their weight gain over the 90 day period that was not seen in F344 rats. Studies investigating the effects of rilmenidine on sympathetic neurotransmission in the aging spleen need to be carried out with optimal doses of rilmenidine, and earlier in middle age. The difference in weight gain between BN and F344 rats may be related to strain difference in behavioral responses to the stress of injections and handling as BN rats are lower responders to acute and chronic stress compared with F344 rats [Gómez et al., 1998].

Splenic NE levels are consistent with our anatomical findings, because neurotransmitter (i.e. NE) concentration and total content in the spleen correlated with lower density of NA nerve fibers. These results suggest that neuronal NE homeostasis was maintained under the low and high rilmenidine drug condition of our study, and are consistent with no change in splenic DA concentrations and content. This finding differs
from our findings in F344 rats, where Rilmh increased splenic DA concentration and content; greater end product inhibition of DA on TH activity in F344 rats may in part explain the greater reduction in splenic NE concentration and SNA activity with high dose treatment compared with our findings in BN rats.

Despite lower splenic NE concentration in Rilmh-treated BN rats, there was no significant difference between the levels in this group compared with Ctrl-3M rats, suggesting some reversal toward young adult levels. This nonsignificant increase in NE splenic levels with Rilmh were consistent with the slight increase in NA nerve density in distal regions of the spleen toward levels in young adult rats (Ctrl-3M).

The age-related blunting of β-AR-mediated signaling via intracellular cAMP was not affected by rilmenidene treatment, suggesting that lowering SNA to the spleen does not affect aging changes in β-AR signal transduction in aging lymphocytes. However, the upregulation of β-ARs, evident by a 36% increase in β-AR expression indicates that these receptors are still responsive to changes in local concentrations of NE, with increasing receptor density as concentrations declined. Mechanisms responsible β-AR uncoupling with its second messenger, cAMP, are not understood, but may result from an uncoupling of β-AR with Gs protein, caused in part from an age-related reduction in membrane fluidity, and resulting in reduced AC activity and increased intracellular calcium [Scarpace and Abrass 1983]. In cardiac myocytes, increased availability of NE results in increased phosphorylation of MAPK and causes an uncoupling to the Gs protein [Daaka et al., 1997]. β-AR under certain conditions can switch coupling from Gs to either a Gi or Go protein, which then causes β-AR stimulation to signals through a different intracellular signaling cascade [Hausdorff et al., 1990]. It remains unknown
whether under reduced NE concentration in the spleen of the aging BN rats; this mechanism may be responsible for the decline in β-AR-stimulated cAMP production. Rilmenidine has been suggested to have direct effects on intracellular G-proteins to modulate the production of cAMP. IR₁ are coupled to cAMP pathway as its second messenger, as well as ability for cross talk between the cAMP and PC-PLC pathways, using the same intracellular machinery, and leading to complex effects [Greney et al., 2000; Separovic et al., 1996; Ho and Wang, 1998]. Collectively, our data here and from previous studies in F344 rats, suggests that age-related alterations in SNA and nerve integrity in the spleen may contribute to aging changes in NA neurotransmission in a strain-specific manner. Our data also suggest that different strategies will be needed in BN and F344 rats to restore NA signal transduction in aging lymphocytes in order to evaluate the causal relationships between aging changes in the SNS and immune function.

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References


Raasch, W., Jungbluth, B., Schäfer, U., Häuser, W., Dominiak, P. 2003. Modification of noradrenaline release in pithed spontaneously hypertensive rats by 11-
binding sites in addition to alpha2-adrenoceptors. J Pharmacol Exp Ther. 304, 1063-1071.


CHAPTER 5
CONCLUSIONS & FUTURE DIRECTIONS

Summary and Conclusions

The studies presented in this doctoral project evaluated the effects of aging on sympathetic neurotransmission in secondary lymphoid organs of F344 rats or BN rats; two rat strains that differ in SNS activity and immunity that potentially predispose them to particular types of age-related diseases. The goal of these studies was to investigate the role of the age-related altered sympathetic activity on neurotransmission in the spleen. This constituted an initial step toward developing laboratory rat models to study causal relationships between age-related changes in the SNS and immune system aging in humans. Below, we summarize and discuss the significance of our findings; and propose future specific interventions and aims to refine the feasibility of our model.

In chapter 2, a longitudinal study in BN rats was conducted to identify the effects of normal aging on basal sympathetic tone, and SNA, NA innervation as well as NE levels in the spleen. The BN rat lives an average of 25% longer than the commonly used rat model for aging, the F344 rat. We did not need to conduct a similar study in the F344 rat because we had previously characterized the age-related changes in sympathetic factors in the spleen of F344 rats, across age. Results from this study demonstrated an age-related decline in both, sympathetic nerve density and NE concentration in the spleen of BN rats, evident at 18 months of age. Unlike the F344 rat, the decline in splenic sympathetic innervation in BN rats did not progress further than middle-age (18M).
Splenic NE concentration however, continued a moderate progressive decline that leveled off by 27 months of age, well before the mean age of survival for BN rats (32M). It was clear that despite the decline in sympathetic innervation during middle age, both sympathetic postganglionic fibers and NE levels in the spleen of BN rats were relatively preserved into senescence (compared with the aging F344 rat). These observations in the BN rat suggest that as age progresses metabolism of NE was relatively maintained and may be contingent to levels of postganglionic nerves in the spleen. This assertion is supported by preliminary studies in our laboratory in which there was a decrease in NE turnover rate concomitant to reduced sympathetic signaling via β-ARs. These results suggest that in the BN rat, there is a change in sympathetic beginning at middle age (15M) that is transient in nature. Therefore these observations in the spleen suggest that in BN rats compensatory mechanisms of neurotransmission may be masked or non-existing; and reveal a clear distinction with the aging F344 rats, which exhibit a sustained increased in SNA and enhanced sympathetic signaling well into old age (147).

Additionally, these findings in the spleen of BN rats correlated with decreased basal sympathetic tone, as shown by circulating catecholamine (NE and EPI) (148) that contrasted elevated levels of these circulating CAs in aging F344 rats (147). Aged BN rats have a striking Th2 bias, and may provide a good model for discovering the mechanisms that predispose the elderly to increased risk for Th2-mediated autoimmune diseases and asthma.

Overall our longitudinal studies across age in F344 and BN rats implied that sympathetic innervation in old rats, though diminished, may still provide regulatory signals to aged target cells in the spleen that may be more sensitive to changes in NE
concentrations. In the F344 rat maintenance of adequate sympathetic signaling in old age may be achieved by enhancing and sustaining sympathetic activity with detrimental effects to sympathetic nerve integrity; whereas in the BN rat, a transient increase in SNA may have less detrimental effects on innervation with blunting effects on sympathetic signaling. The mode of age-related changes in neurotransmission in the spleen of BN rats may confer this rat strain with better sympathetic immune interaction that may contribute to its longevity.

In chapters 3-4, chronic studies were used to test the hypothesis that heightened splenic SNA during middle age is responsible for splenic NA nerve loss and altered β-AR signaling in splenocytes, in a strain-dependent manner. We tested this hypothesis by reducing sympathetic tone with rilmenidine, a centrally active antihypertensive agent that has high affinity to IR1 and α2-AR receptors in cardiovascular centers of the brain stem. We administered rilmenidine via discontinuous i.p. injections, as it previously reduced sympathetic tone in spontaneous hypertensive rats (SHR) (149).

The results from these studies demonstrated that a 90-day treatment with rilmenidine, regardless of dose, confirmed its antihypertensive properties by reducing circulating NE concentration without changing EPI levels, in both rat strains, despite acute sympathetic activation by decapitation. However, this drug treatment caused body weight gain suppression in F344 rats only, and suggests a hypothalamo-pituitary adrenal axis (HPA) and/or SNS activation (150) in this rat strain. Our observations were consistent with other reports that regarded the F344 rat as hyper-responsive to experimental periodic stress (151, 152). In some rat strains, alterations in spleen weight during the course of a chronic study are not uncommon. Increased weight can be
attributed to enhanced splenic function in compensation to, either diminished blood flow or vascular tension (153); or blood accumulation due to a drug-induced vasodilatation in the splenic red pulp (154), as it may be the case in the F344 rats in our studies.

In the spleen, rilmenidined reduced net SNA in both F344 and BN middle-aged rats, as demonstrated by the dose-dependent reduction of splenic NE turnover rate. Rilmenidine (Rilmlo and Rilmhi) treatment reversed the age-related loss of sympathetic nerves at both, the hilar (site of blood vessels and nerve entry) and distal regions of the spleen in F344 rats only. In correlation with restored sympathetic nerves, total NE content in the spleen increased in F344 rats that were treated with the optimum dose of rilmenidined (Rilmlo). NE concentration or total content in the spleen of BN rats were not significantly altered, suggesting that mechanisms promoting steady NE production and utilization were sustained in this rat strain during the drug treatment. Also in BN rats Rilmhi induced a nonsignificant increase in NE splenic levels, consistent with a small gain in more NA nerve fibers in distal regions of the spleen toward levels in young adult rats. However, there was a sharp reduction in splenic NE concentration and content in Rilmhi-F344 rats compared with age-matched control rats, despite NA nerve restoration. This data indicates that NE synthesis did not keep pace with NE utilization in the Rilmhi treatment group, despite reduced turnover with both drug doses. As our data indicated, greater splenic DA levels with Rilmhi may cause small rates of DA to be driven into the cytosol from the neurotransmitter vesicle (146) to further inhibit TH activity. At high doses, the drug-induced SNA decrease may affect TH phosphorylation, which is critical for its activity and the primary mechanism for maintaining tissue NE levels after secretion (155). And downstream of TH, reduced dopamine-β-hydroxylase (DBH)
expression or activity may have depressed the conversion of NE from high vesicular DA thereby yielding lower splenic NE levels. Incidentally, the protective effects of Rilmhi on sympathetic innervation were less significant than with Rilmlo. And alteration of NE metabolism, with elevated DA content, toxic to neurons, may be responsible for this hindrance. These results highlight the effect of age-related changes in SNA for altered NA innervation and adequate transmitter levels release from viable nerves in the spleen. And further give an insight on the strain related susceptibility for immune-mediated diseases by higher SNA that may alter activation and immune function in lymphoid organs (156).

Because the optimum dose Rilmlo was demonstrated to reduce net SNA by previous pharmacological studies (149), it was used to investigate the effects of drug treatment on β-ARs activation and signaling via β-ARs in spleen cells. In F344 rats, increased β-AR expression and β-AR-stimulated production of cAMP were consistent with lower NE availability from reduced SNA and NE turnover; and support under reduced SNA conditions there is normal regulation of postsynaptic events in aging. In BN rats, β-AR expression was increased but no significant effect on the age related decline in signaling was observed. The presence of more β-ARs without change in receptor binding affinity in both F344 and BN rats may reflect decreased phosphorylation of β-ARs, a feature in splenocytes of aged rats (74, 157). Or β-AR uncoupling with Gs protein may induce a decrease in cyclase adenylate activity, with a new set of signaling events via alternative activation of Gi (158). Of importance to our studies here, rilmenidene may directly modulate intracellular G-proteins to modulate the production of cAMP as suggested by ex vivo and in vitro studies. This mechanism consist in a cross talk between the cAMP and
PC-PLC pathways using the same intracellular machinery by which IR₁ agonists can be linked with the cAMP pathway (159, 160, and 161). These proposed mechanisms remain to be explored, as well as the effect of appropriate rilmenidine doses for BN rat, in order to investigate neurotransmission in aging.

Collectively, our data indicate that reducing central SNA may be a useful strategy for reversing age-related nerve loss. And restoration of NA nerves in the spleen of the F344 rat and supports the feasibility for developing this strain as a model to investigate causality between aging SNS and immune functional changes. Because rilmenidine did not have significant effects in NA nerve restoration or neurotransmission despite lower SNA in the spleen, we believe that fine tuning of this rat strain as a model is in need. From our results we conclude that the F344 rat may be the strain that may benefit from reduction of SNA with stronger effects in NA nerve restoration. From previous studies in the F344 rat we know that although NA nerves in the spleen diminished greatly with aging, they retain plasticity (162); and therapeutic treatments may target this property in this rat strain. Adequate NA innervation can provide the environment for more efficient immune functions such as cell proliferation, cell trafficking, clonal expansion, and cytokine production that can contribute to optimal immune responses (163). The F344 rat and murine models have demonstrated that although diminished with age, NA innervation in the spleen is not completely lost. This fact reveals that the SNS may continue to play a role in modulating immunity into well into very old age. Other studies have shown that although sympathetic innervation in the spleen of aging rodents is diminished with age, sympathetic signaling of the immune system remains viable (165, 166, and 167).
Chronic reduction of sympathetic activity in the spleen while enhancing sympathetic signaling in splenocytes is a novel finding in adult rats. Although this was true for both rat strains, this experimental approach was not effective for the aging BN rat, because the age-related decline in sympathetic innervation and $\beta$-AR activation and signaling were not reversed by rilmenidine treatment. Appropriate neurotransmission in the spleen may trigger important functional outcomes for immune cell activation in rat models. The increase in cAMP with $\beta$-AR agonists (non-selective for $\beta$-ARs or selective for $\beta_2$-AR) in adult rodents inhibits either mitogen- or anti-CD3 antibody-induced T cell proliferation (168, 169, 170, 171, and 172). And $\beta$-AR-mediated inhibition of T cell proliferation may be explained, at least in part, by induced inhibition of IL-2R expression and/or IL-2 production by activated T cells (172, 173, and 174). Agonist-stimulated increase in cAMP may also enhance $\beta$-AR-mediated cytokine production in spleen cell culture; and a similar effect can be achieved experimentally by antigen challenge (175, 176). In addition to $\beta$-AR activation and $\beta$-AR-stimulated cAMP production in immune cells, experiments in adult rodents have demonstrated that chemical sympathectomy prior to antigen challenge with a T-cell dependent antigen, can either reduce (177, 178) or enhance (179, 180) antibody production in response to the endotoxin Keyhole limpet hemocyanin (KLH), depending on Th dominance (Th1 vs Th2).

Therefore immunomodulatory effects via adequate NA innervation and NE levels are crucial for satisfactory cell signaling events in targeted immune cells (73); and this becomes more important in aging, as the SNS remains able to modulate immune functions in the spleen (178, 181). Also, the direction of sympathetically mediated changes in immune responses may vary across species and strains, dose of antigen, site of
the immune response, and type of immune response (Th1 vs. Th2) (73). Collectively, the experimental results discussed in chapter 3 and 4, as well as observations from other studies, underscores the role of the sympathetic system in the spleen across rat strains. Therapeutically reducing the age-related increase in SNA may confer specific benefits, depending on the strain of the rat model used.

**Future Directions**

From previous studies and observations presented in chapters 2 through 4, we have determined that changes in SNA, sympathetic innervation, and neurotransmitter levels in the spleen occur during middle age across rat strains. And here, we reduced the age-related increase in SNA experimentally, and assess the effects of this intervention on NA innervation and sympathetic signaling in the spleen. Future research will investigate immune functional effects from the chronic treatment with rilmenidine, in order to determine probable causal relationships of age-related changes in SNA and immunity. Future proposed studies are outlined below.

First, it will be important to determine the mechanism(s) by which chronic treatment with rilmenidine is conducive to the preservation of sympathetic nerves. Nerve destruction in the spleen of aging rats is attributed to the increased sympathetic activity in the spleen which leads to the formation of oxidative metabolites from heightened released of NE that may cause pruning of nerve terminals (60, 2008). Studies with other populations of catecholaminergic neurons support this hypothesis (182), and a similar event in the spleen will need to be confirmed. Effects of reducing SNA on the formation of oxidative metabolites by ROS concentrations, via ELISA or Western blotting, will
confirm this proposed mechanism by which NA nerves are destroyed in the spleen.

Supplementary to ROS level determination, the role of MAO can also be explored.

Increased SNA involves enhanced degradation of catecholamines by a heightened MAO activity (183, 184). Studies in both, human and rat brain (184), and adipose tissue (175), have shown inhibition of monoamine oxidase A and B (MAO-A and MAO-B) by imidazoline ligands via pathways independent of either $\alpha$-AR or $\beta$-AR pathways. *In vitro* experiments can help to determine whether incubation with rilmenidine ameliorates neuron survival. Additional experiments in the presence of cultured immune cells and platelets, which are said to express IRs (185), will measure the contribution of immune cells in the inhibition of MAOs.

Second, previously in our laboratory we were able to link sympathetic nerve plasticity to changes in the microenvironment in the splenic white pulp of aging F344 rats, by changes in the density of T cells and ED3$^+$ macrophage populations (54, 186, and 187). Macrophage recruitment and infiltration into damaged tissue influence nerve regeneration and maintenance via regulation of NGF synthesis and NGF receptor expression (188). Thus in aging reduction of splenic macrophages and T cells in the white pulp promote loss in neurotrophic factor support for sympathetic nerves in the aged F344 spleen (188, 189). Therefore correlations between sympathetic nerve preservation and density of immune cells populations following rilmenidine treatment can be addressed by immunohistochemistry labeling of tyrosine hydroxylase for NA nerves with simultaneous specific labeling for T cells and macrophages. Flow cytometric analysis can be used to confirm immunohistological experiments, and further identify possible shifts in immune cell populations in the spleen of rilmenidine-treated rats versus control rats in
both rat strains. Possible strain specific adjustments of immune cell populations in the spleen following chronic treatment with rilmenidine can be determined with this approach.

Other studies have suggested that not only reduced population of resident cells in the spleen, but possibly a gradual decline in cytokines production may be a mechanism of NA sympathetic nerve fiber loss in the aged spleen (190). Cytokines have been also referred as neurotrophic for sympathetic neurons, either by directly or indirectly stimulating the release of neurotrophic factors. In rat’s dissociated sympathetic neurons, IL-2 has been shown to stimulate neurite outgrowth via IL-2 receptors on the neurons (191). And other cytokines of macrophage origin, such as IL-1 and TNF-α, have been reported to play a critical role in regulating the synthesis of NGF and other neurotrophins in lymphoid tissues (192, 193, and 194). Measurement of cytokine levels in the spleen, by cytokine production assays, can be used as a first step for correlation to sympathetic nerve density restoration.

Third, neurotrophic factors, such as neurotrophin-3 (NT-3) and nerve growth factor (NGF), are important in supporting and maintaining sympathetic nerves (195, 196). Expression of mRNA for neurotrophic factors (NGF, NT-3, and BDNF) and their receptors (p75NGFR, trkA, trkB, and trkC) are ubiquitous in multiple cell types in the young adult mammalian spleen (197, 198, 199, and 200). Particularly in the rat spleen, p75NTR expression has been localized to dendritic cells in the PALS (201), and Trks expression on the surface of immune cells throughout various compartments of the spleen, as mentioned above has been detected (200, 201). However no studies have examined the effect of aging on the concentration and/or production of these neurotrophic
factors and their receptors in the spleen. Similarly, the effects of rilmenidine treatment on neurotrophic factors and their receptors, specially of NGF and NT3, in the spleen of aging F344 or BN rats represents an opportunity for investigation. For unknown reasons, so far at the protein level, only NT-3 has been detected in the spleen of young rats (one-month old) (202, 203). Despite its known physiological role for nerve preservation, the mature nerve growth factor has being referred as the minor species in peripheral tissues, compared to high molecular weight NGF species (205). Recent studies in old F344 rats (24M) have revealed that expression of proNGF forms (proNGF-A or proNGF-B) may serve as indicators for aged neurons that are susceptible to neuronal atrophy in the spleen (204, 205, ). Moreover, it has been suggested that some pro-neurotrophins and high molecular weight neurotrophin species can directly interact with p75 and trkA receptors in target organs (207, 208, , and 209).

Experimentally, it is crucial that the adequate extraction methods and prompt processing are used when attempting to measure total concentrations of NGF in lymphoid tissues from young adult and aged rats. Immediate assaying of harvested tissue is crucial for accurate neurotrophins concentration determination, as they readily undergo proteolytic degradation over time (206). Accurate identification as well as the distribution of loosely bound NGF and NT-3 in the spleen can be challenging but attainable by highly sensitive detection methods (i.e. ELISA or selective optical sensor chips for real-time monitoring of antibody–antigen interactions) (198, 210). In addition, complementary molecular experiments can help to determine whether the increase in proNGF-A or -B in the spleen is the result of the large-scale increase in NGF mRNA and/or due to altered post-translational processing and/or retrograde transport. The BN
rat, with significantly less splenic sympathetic nerve loss, provides an excellent alternative model to compare the effects of chronic treatment with rilmenidine on neurotrophin expression in the F344 rat spleen. Studies measuring neurotrophin levels will confirm possible reversal of age-related changes in expression of neurotrophins and/or their receptors in the spleen of rilmenidine-treated rats. As a comparative study, combined rilmenidine treatment with trophic factors (i.e., NGF, NT3 or BDNF) may confirm the potentiation effect of neurotrophins on the rilmenidine effect.

Fourth, most in vitro experiments indicate that adrenergic agonists can modulate all aspects of immune responses that include initiative, proliferative and effector phases (4). Because chronic rilmenidine treatment enhanced sympathetic signaling by altering receptor expression in responsive spleen cells in both rat strains, the functional effects of this treatment on mitogen and antigen activated clonal expansion can be investigated with cell proliferative response assays. Possible strain differences in the type of immunity (Th1 vs. Th2) prevalent in these middle-aged rats that have been treated with rilmenidine can be explored with cytokine assays and correlated with flow cytometric profiling of macrophages, T cells and B cells. Also in vivo antibody production, following treatment with rilmenidine, can be tested with an optimum dose of the endotoxin KLH; and potential connections of this antigen activation with Th predisposition under reduced basal levels of sympathetic activity can be determined. Findings from these experiments may give important insight on vaccine-induce immunity under sympathetic activity in these rats.

Fifth, we have concluded that, chronic treatment with rilmenidine may induce sympathetic nerve preservation and the enhancement of neurotransmission in the spleen.
of middle-aged F344 and BN rats, respectively. However we do not know whether reduction of SNA initiated well before changes are manifested in middle-age will be more beneficial to these animals. Rilmenidine treatment begun as early as 10M, in F344 rats, and at 12M in BN rats, may provide maintenance of young profiles rather than reversal of age-related changes in NA innervation and mechanisms involved in neurotransmission. Our studies in the BN rat revealed that this rat strain was more resistance to rilmenidine treatment at the indicated doses. We think that higher doses are appropriate for the BN rat in order to investigate accurately the effect of increase in SNA on neurotransmission in the spleen. Also an immediate confirmation of the effects observed in our experiments, can be carried out by allowing a drug wash out period at the end of the chronic treatment with rilmenidined. This experiment will determine whether the effects of this drug persist after it has clear the body of the animals completely. Thus anatomical and functional effects of rilmenidine treatment may reflect true adaptation to lower SNA in the aging spleen by these rat models.

Overall our experimental results comprise initial findings that may help further characterize therapeutic interventions tailored to individuals with different SNS profile. The immune system is the best “weapon” that the body has to fight off pathogens that can affect the body's chances of optimal survival. Although the immune system is inherent to normal physiological processed in the body, the type of immunity that it acquires are obtained differently among individuals. Perhaps changes in sympathetic measures with advancing age in rat models used in our studies may reflect an adaptation to the reshaping of immune function with age. Thus therapeutic interventions to counter the effects of aging on sympathetic signaling may enhance or interfere with adjustment in
neurotransmission mechanisms. Future studies will help to enhance the understanding of the mechanisms of age-related changes in sympathetic-immune interactions. The significance of the results reported here represent an initial step to begin understanding the link between adequate sympathetic modulation and immune function conducive to resilience towards infectious diseases, tumor immunity, and autoimmune-mediated diseases during aging.
Fig 5.1  Model for Neurotransmission in the Aging Spleen
An age related increase of sympathetic outflow driven by the rostral ventral lateral medulla (RVLM) induces an increase in sympathetic nerve activity (SNA) the spleen. This increase in local SNA is characterized by elevated levels of free radical metabolites produced by enhanced activity of metabolic enzymes (MAO-B, COMT) in the face of elevated concentration of norepinephrine (NE), via heightened enzymatic activity by tyrosine hydroxylase (TH). Excess of free radical metabolites causes deleterious damage to sympathetic nerves in the face of diminished neurothrophic support. Contributing factors to this effect may include altered immune population, reduced target cell density, reduced anti-oxidant activity, and chronic inflammation. In splenocytes expression of β-adrenergic receptors increase but subsequent coupling to alternative G protein subunits, such as G\(_i\) or G\(_o\), may induce alteration the production of cAMP by adenylate cyclase differentially in a strain-related manner. This age-related alteration in neurotransmission in the spleen may influence immune responsiveness with advancing age.
REFERENCES


156. Wallace, L.J., 2007. A small dopamine permeability of storage vesicle membranes and end product inhibition of tyrosine hydroxylase are sufficient to explain changes occurring in dopamine synthesis and storage after inhibition of neuron firing. Synapse 61, 715-723.


signals modulate the antiproliferative effects of agents that elevate cAMP in T

cAMP-dependent protein kinase (PKA) activity in T cells after stimulation of the
prostaglandin E2 or the beta-adrenergic receptors: relationship between PKA
activity and inhibition of anti-CD3 monoclonal antibody-induced T cell

169. Diamantstein, T. and Ulmer, A. 1975. The antagonistic action of cyclic GMP and
cyclic AMP on proliferation of B and T lymphocytes. Immunology 28, 113-119.

170. Ledbetter, J.A., Parsons, M., Martin, P.J., Hansen, J.A., Rabinovitch, P.S., June,
C.H. 1986. Antibody binding to CD5 (Tp67) and Tp44 T cell surface molecules:
effects on cyclic nucleotides, cytoplasmic free calcium, and cAMP-mediated

stimulation by T- or B-cell mitogens. Immunology 30, 735-739.


cAMP accumulation in T-cells inhibits anti-CD3 monoclonal antibody-induced

174. Sanders, V.M., Baker, R.A., Ramer-Quinn, D.S., Kasprowicz, D.J., Fuchs, B.A.,
Th1 and Th2 clones: implication for cytokine production and B cell help. J.
Immunol. 158, 4200-4210.

different T cell receptor-associated signaling pathways by TH1 and TH2 clones. J.
Immunol. 144, 4110-4120.

Neonatal sympathetic denervation alters the development of in vitro spleen cell
proliferation and differentiation, Brain Behav. Immun. 5, 235–261.

Involvement of peripheral and central catecholamine systems in neural-immune


