DEVELOPMENT OF THE CARDIAC BETA-ADRENERGIC SYSTEM IN BAX AND NGF KNOCKOUT MICE

A dissertation submitted to Kent State University in cooperation with the Northeastern Ohio Universities College of Medicine in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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“to God be the glory great things he has done . . .”
Approximately one-half of the neurons generated during development of the vertebrate nervous system die due to apoptosis. Neurons that survive are presumably ones that compete successfully for trophic factors secreted in limited amounts by their target tissues (Levi-Montalcini 1987). This process, described in the “Neurotrophic Factor Hypothesis,” (Purves et al. 1988) insures that an appropriate number of neurons innervate a target tissue. Therefore, hypoinnervation of an organ can result from insufficient levels of trophic factors, whereas an excess of trophic factors can result in an excess of innervation.

Studies of neurotrophin gene or receptor knockout mice have elucidated the role of neurotrophins in sympathetic neuron development. Examples of this type of targeted gene disruption are nerve growth factor (NGF) and neurotrophin-3 (NT3) knockout strains of mice. Null (-/-) NGF and NT3 mutant mice have shown significant deficits in numbers of superior cervical ganglion (SCG) neurons (Crowley et al. 1994, Farinas et al. 1994). Likewise, deletion of trkA, a NGF receptor, led to greater than 90% sympathetic neuronal cell loss at birth in the SCG (Smeyne et al. 1994). Null NGF, NT3, and trkA mutant mice die shortly after birth, thereby limiting the study of sympathetic neuronal deficits to prenatal animals. Cardiovascular sympathetic innervation matures in the first few weeks after birth, therefore sympathetic deficits induced by neurotrophin or
neurotrophin receptor insufficiency cannot be assessed completely in these mouse models.

The principal aim of this dissertation was to elucidate the role of NGF on sympathetic neuron survival and differentiation, and its importance in the postnatal animal. The underlying experimental paradigm was to block sympathetic neuron death by deletion of BAX, a proapoptotic gene, and/or deletion of a single NGF allele which presumably would reduce the amount of this trophic factor available to neurons. This paradigm thus enabled examination of the effect of these two gene deletions on the pre- and post- synaptic cells of the cardiac sympathetic nervous system. The sympathetic nervous system is believed to be the final common pathway for regulation of the chronotropic or inotropic responses of the heart to metabolic demands (Ostman and Nyback 1976). Consequently, a greater understanding of the effect of NGF on sympathetic neuron survival and differentiation is essential not only for elucidating sympathetic neuron development, but also for understanding neural regulation of cardiac function.

BACKGROUND STUDIES

NEUROTROPHINS

The homodimeric family of proteins called “neurotrophins” plays an essential role in the development of the vertebrate nervous system. Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin 4/5 (NT4/5) comprise this family of trophic factors (Barbacid 1994). Bothwell (1991) and
Barbacid (1994) showed that neurotrophins bind to two types of receptors: p75 low-affinity nerve growth factor receptor (p75<sup>LNGFR</sup>) and high-affinity tyrosine kinase receptors (trkA, trkB, trkC). All four neurotrophic factors bind to p75<sup>LNGFR</sup> (henceforth abbreviated as p75), whereas each neurotrophin preferentially binds one or two trk receptors. The survival, differentiation, and synaptic function of different classes of neurons depend on the interaction of neurotrophin and their receptors.

**Role of NGF in Development of Sympathetic Neurons**

NGF affects many cell types including neural crest derivatives such as sympathetic neurons (Levi-Montalcini 1987). Effects of NGF on cells depend upon the type of cell and level of differentiation. NGF is essential for the survival of developing sympathetic neurons (Ernfors et al. 1994, Farinas et al. 1994, Wyatt et al. 1997, Francis et al. 1999). NGF performs a trophic role in early stages of development which enhances the differentiation process and guides growing or regenerating neurites (Levi-Montalcini 1987). NGF also enhances dendritic arborization in developing sympathetic ganglion cells (Snider 1988).

The expression of NGF has been tracked in rodents through development and adulthood. By E13 in rats, NGF mRNA is weakly expressed in the thoracic region and intensifies by E15 in the developing aorta and pulmonary artery (Scarisbrick et al. 1993). At E14 in the mouse, NGF protein levels in the heart ventricles reach adult levels (Korsching and Thoenen 1988). By E16 in the mouse, most sympathetic neuroblasts are NGF-dependent (Wyatt et al. 1997). In agreement with mouse data, fetal and neonatal rat heart NGF mRNA levels are highest in the ventricles and are not affected by
sympathectomy (Clegg et al. 1999). In neonatal animals, immunosympathectomy with function blocking NGF antibodies virtually eliminate all sympathetic neurons (Zhou and Rush 1995). These effects are less damaging in young and adult animals (Levi-Montalcini 1987, Zhou and Rush 1995). In NGF-/- mice, nearly all of the sensory and sympathetic ganglia is lost in the SCG by P10 (Crowley et al. 1994). The dramatic losses of sensory and sympathetic ganglia in NGF-/- mice show that other neurotrophins are not capable of compensating in complete NGF absence (Crowley et al. 1994) and underscores the importance of NGF in the development of sympathetic neurons.

**NGF and NT3 Interactions**

Although NT3 can rescue some sympathetic neurons from death induced by NGF depletion and vice versa (Zhou et al. 1997), the dramatic losses of sensory and sympathetic ganglia in NGF-/- mice show that neither NT3 nor other neurotrophins are capable of completely compensating in NGF absence (Crowley et al. 1994). NT3 is not required for sympathetic neuroblast survival and is not needed for the onset of NGF dependence in the developing SCG (Wyatt et al. 1997). Later in development, more neurons become dependent on NT3 for survival and NT3 exerts its survival promoting effects via trkA, like NGF (Wyatt et al. 1997). It appears that the sympathetic neuroblasts do not switch dependence from NT3 to NGF, but rather become co-dependent on NT3 and NGF for survival (Wyatt et al. 1997). Thus, sympathetic neurons require NGF in the prenatal period, and both NGF and NT3 in the postnatal period for proper development. Correspondingly, the heart expresses NGF and NT3 in the postnatal period, thus suggesting a role of maintenance and maturation on cardiac sympathetic

NEUROTROPHIN RECEPTORS

Two classes of receptors are known to interact with neurotrophins. The first type of receptor is the low-affinity p75 receptor. The p75 receptor has an extracellular region for binding neurotrophin and a transmembrane region, but lacks intracellular tyrosine kinase activity (Bothwell 1991). The second type of receptor, the tyrosine kinase or trk receptor is the high affinity receptor. The trk receptor has an extracellular region for binding neurotrophins, a transmembrane region, and an intracellular region with tyrosine kinase activity (Barbacid 1994). NGF and NT3 bind to subtypes high affinity receptor as well as the low affinity p75 receptor.

The Low-Affinity Receptor: p75

The p75 receptor nonselectively binds to NGF, NT3, BDNF, and NT4/5 (Brennan et al. 1999, DeCouto et al. 2003). P75 is expressed in all cells responsive to NGF and NT3 such as sympathetic neurons (Hiltunen et al. 1996, Chao and Hempstead 1996). Knockout studies have determined that p75 promotes neuronal apoptosis, but its absence does not prevent cell death (Bamji et al. 1998, Brennan et al. 1999). The presence of p75 is necessary to promote apoptosis when insufficient levels of NGF are present (Barrett and Bartlett 1994). For example, the loss of an NGF allele in the presence of p75 leads to greater neuron deficits compared to mice lacking p75 and one NGF allele (Brennan et al. 1999). Furthermore, mice with p75 deleted have an increased number of sympathetic
neurons compared to controls, even in the absence of an NGF allele at birth; however, the increased neuron number returns to control levels by adulthood in p75-/- mice (Brennan et al. 1999). Thus, p75 is an important receptor for transducing the survival signal with sufficient NGF present or the apoptotic signal with insufficient NGF present.

**Tyrosine Kinase A Receptor**

TrkA expression correlates with NGF responsiveness in the PNS and CNS. During early neurogenesis, trkA is expressed in defined areas of the nervous system, such as in the DRG, cranial sensory ganglia, trigeminal, superior, and jugular ganglia (Smeyne et al. 1994), aortic wall, pericardium (Hiltunen et al. 1996), and fibers running along the surface of atria (Andrade-Rozental et al. 1995). In mice, trkA expression first appears at E9.5 in neural crest-derived cells including those destined to form the sympathetic nervous system (Martin-Zanca et al. 1990, Schecterson and Bothwell 1992). Similarly, neonatal and adult sympathetic neurons express high levels of trkA as well as p75 (Belliveau et al 1997, Zhou et al. 1997). Few mice lacking trkA develop to birth and none live to adulthood (Barbacid 1994, Smeyne et al. 1994). Deletion of trkA leads to greater than 90% sympathetic neuronal cell loss at P0 in SCG, similar to NGF deletion (Smeyne et al. 1994). The SCG is almost completely absent by P10 in trkA-/- mice, though the remaining sympathetic neurons appear normal in size and staining (Smeyne et al. 1994). Although p75-/- and trkA-/- mice show common phenotypic traits, the extent of neuronal cell loss is significantly greater in trkA-/- mice suggesting trkA is the primary mediator of NGF trophic actions (Barbacid 1994, Smeyne et al. 1994).
**P75 and trkA interaction**

The interaction, or lack thereof, between p75 and trkA can affect NGF function. The ratio of p75/trkA influences NGF binding to trkA (Brennan et al. 1999). Co-expression of p75 with trkA increases the high affinity site 25 times, thus enhancing NGF binding and neuron survival (LaChance et al. 1997, Chao and Hempstead 1996). Neuron populations rescued in p75/-/-NGF+/- mice succumb to apoptosis in p75/-/-NGF+/-NT3+/- supporting the notion that p75 alters trkA specificity (Brennan et al. 1999). Utilizing a novel fluorescent nerve growth factor conjugate, researchers found that both p75 and trkA are required for NGF uptake and retrograde axonal transport in adult rat sympathetic neurons (Gatzinsky et al. 2001). When NGF is blocked from binding to p75 on sympathetic neurons, the amount of NGF binding and the activation of trkA is transiently reduced (LaChance et al. 1997). A putative explanation for their interaction suggests that trkA interacts with p75 during embryogenesis to transduce the NGF survival signal and without sufficient trkA or NGF, p75 acts as a death signal (Barrett and Bartlett 1994, Brennan et al. 1999). A two-receptor system allows for tighter regulation and increased flexibility of function (Chao and Hempstead 1996).

**NEUROTROPHIN GENE DOSAGE EFFECTS ON NEURON SURVIVAL AND DIFFERENTIATION**

Knockout studies demonstrate a dosing response dependent upon the number of neurotrophin alleles present (Donovan et al. 1996, Ernfors et al. 1994, Story 2000a, Story et al. 2000b). Haplo-insufficiency, a deficit in target organ neurotrophin levels resulting
from inactivation or deletion of one copy of a gene, may affect the survival and phenotype of sympathetic neurons. Significant neuronal losses are observed in heterozygous neurotrophin gene knockout mice, approximating 50% of the deficits found in neurotrophin null mutant mice (Brennan et al. 1999, Ernfors et al. 1994, Story 2000a, Story et al. 2000b). Research studies suggest that the number of cellular copies of NT3 and NGF genes regulates the size of sympathetic neuron population. Neonatal sympathetic neurons are neurotrophic-dependent; and NT3 or NGF knockout mice both exhibit substantial neuronal population deficits (Brennan et al. 1999, Deckwerth et al. 1996, Farinas et al. 1994). For example, NT3+-/- mice show a 22% deficit in sympathetic neurons in the stellate ganglia compared to a 46% deficit observed in NT3-/- mice (Story et al. 2000b). At birth (P0), the left stellate ganglion (LSG) of NT3+-/- mice has fewer neurons than NT3+++/+ and about half that of null mutant (-/-) mice (Story et al. 2000b). Like heterozygous mutant NT3 mice, NGF+/- mice have a 50% deficit in sympathetic neurons compared to controls (Brennan et al 1999, Story 2000a). Additionally, NGF null mutant mice have a 77% deficit of sympathetic neurons (Glebova and Ginty 2004, Middleton and Davies 2001). Therefore, the number of copies of the NT3 and NGF genes affects sympathetic neuron survival.

Additionally, deletion of NT3 or NGF alleles alters phenotype. Compared to controls, NT3+-/- mice display decreased expression of TH, tyrosine hydroxylase-the rate limiting enzyme in the formation of catecholamines, at P21 and P60 (determined by binding of anti-TH), which may lead to lower ventricular NE concentration (Story et al. 2000b). Additionally, NT3-/- mice had a resting heart rate of approximately 250 beats
per minute (bpm), whereas NT3+/− had resting rates of 350 bpm and that of wild-type littermates was 350-400 bpm (Donovan et al. 1996). These measurements substantiate the abnormal autonomic tone and sympathetic neuronal deficit observed in heterozygous and null mutant NT3 mice (Donovan et al. 1996). Similar to the outcome observed in NT3+/− mice, deficits occur in cardiac sympathetic innervation and stellate ganglion neurons when one or fewer copies of NGF gene are present. NGF+/- and −/- mice show less intense anti-TH staining of the LSG at P0 and NGF+/- mice show lower intensity of staining at P60 (Crowley et al. 1994, Story 2000a, Story et al. 2000b). NGF+/- mice show similar deficits of cardiac innervation and βAR densities (Story et al. 2000b). Collectively, these studies suggest that both copies of the NT3 and NGF genes are required for full expression and function of the expressed protein.

REGULATORS OF NEURONAL APOPTOSIS

The initial excess of neurons produced during the development of the nervous system is eliminated via apoptosis. The Bcl-2 family of proteins regulate this process. The Bcl-2 family consists of three functional groups. One group has four conserved Bcl-2 homology (BH) domains, anti-apoptotic activity, and is comprised of Bcl-2, Bcl-xL, A1, Boo, Bcl-w, and Mcl-1. A second group has only three conserved BH domains, possess pro-apoptotic activity, and is comprised of Bax (BAX), Bak, and Bok. A third group contains only one conserved BH domain and is comprised of pro-apoptotic members including Bim, Bik, Bid, Bad, Bmf, Hrk, Noxa, and PUMA (Mirkes 2002). Members of the Bcl-2 family of proteins function via competing interactions to regulate death in a
variety of cell types (Deckwerth et al. 1996). In the developing sympathetic nervous system, BAX is an important apoptotic protein. Although BAX seems capable of interacting with many members of group 1, it most commonly forms heterodimers with Bcl-2 or Bcl-x<sub>L</sub>. This interaction between BAX and Bcl-2/Blc-x<sub>L</sub> is an important regulator of apoptosis in the developing sympathetic nervous system.

Numerous studies have utilized targeted gene knockout models of Bcl-2 and BAX to determine their exact roles in directing apoptosis. The ratio of apoptotic antagonist to agonist proteins is important in controlling cell susceptibility to apoptosis (Miyashita et al. 1994). For instance, when BAX and Bcl-2 are in equilibrium as occurs when BAX and Bcl-2 are both deleted (BAX-/-Bcl-2-/-), numbers of thymocytes are normal (Knudson and Korsmeyer 1997). When BAX expression is greater than Bcl-2, (such as when a single allele of BAX is deleted) apoptosis is greater than normal (Knudson and Korsmeyer 1997). When Bcl-2 expression is greater than BAX such as in mice overexpressing Bcl-2, axons do not degenerate after axotomy as occurs in wild-type mice (Dubois-Dauphin et al. 1994). Hence, the ratio of BAX to Bcl-2 can determine neuronal cell death or survival.

**BAX deletion and NGF**

If a low BAX/Bcl-2 ratio decreases apoptosis, how would this affect the developing SNS in which apoptosis is so critical? BAX-/- mice display reduced developmental sympathetic neuron death (Deckwerth et al. 1996). Neurons from BAX-/- mice retain the ability to express a distinct neuronal phenotype, to respond to trophic factors, and to display anabolic responses (Deckwerth et al. 1996). Furthermore, BAX-
deficient studies with both copies of NGF or trkA genes deleted showed an augmented number of cutaneous sensory neurons in dorsal root ganglion (DRG), although, these neurons failed to arborize in the skin (Patel et al. 2000). Thus deletion of BAX allows sympathetic neurons to survive independent of sufficient NGF levels.

CARDIAC SYMPATHETIC INNERVATION

The first functional system in the embryo, the cardiovascular system, is unique because it functions while it is developing. Sensory, sympathetic, and parasympathetic fibers innervate the heart as it matures (Hiltunen et al. 1996, Scarisbrick et al. 1993). The sympathetic and parasympathetic nervous systems, components of the autonomic nervous system, work together to regulate heart function. Most of the cardiac nerves are formed by bilateral post-ganglionic fibers of the middle cervical ganglion and stellate ganglion of the sympathetic trunks (Pardini et al. 1989, Pardini et al. 1990). Sympathetic nerve innervation of the ventricles originates mostly from the left stellate ganglion. When the heart "mature," and functionally coupling between neurons and myocytes occurs, norepinephrine (NE) concentration and βAR density reflect the density of cardiac sympathetic innervation due to functional coupling. Though some sympathetic neurons innervate their targets in prenatal development, maturation of the heart and its sympathetic innervation occur within the first months after birth in mammals (Bareis and Slotkin 1980, Cros et al. 1988, Slotkin and Slepetis 1984, Ursell et al. 1990).

A compensatory mechanism of the sympathetic system of an adult mammalian heart is fairly well established. In a normal adult heart, receptor downregulation resulting
from increased stimulation causes desensitization of the ligand-receptor system (Slotkin et al. 1995). Conversely, upregulation of receptors occurs from a decrease in sympathetic innervation or pharmacological receptor blockade and results in hypersensitivity of βAR (Slotkin et al. 1995). Although research shows that βAR are present prior to septation or establishment of sympathetic innervation, the "early" cardiovascular response to adrenergic stimulation differs from the “mature” cardiovascular response (Slotkin et al. 1995). In order for the “mature” β-adrenergic system of the heart to make proper adjustments, it must acquire a set-point or its reference point of innervation density, βAR, and NE content enabling it to make appropriate adjustments later based on this reference point. In heart tissue, receptor density and response progressively increase throughout development and neuronal input acts as a positive (upregulating) factor (Slotkin et al. 1995). During cardiac innervation, this ligand-receptor system acquires its ability to regulate by developing a “set-point” to which adjustments in the mature heart will be referenced. Immunosympathectomy in neonatal rats does not elicit compensatory upregulation of adrenoreceptors in the heart and impairs later adrenergic stimulation-induced heart hypertrophy (Slotkin et al. 1995). Therefore, the βAR compensation described for the mature adult heart is absent in the immature heart.

The early postnatal period when the sympathetic system is determining its set point may be when the system is most susceptible to outside influences that can alter the normal baseline. An absence of upregulation in the denervated fetal heart appears to be a characteristic of the developing adrenergic target cells. This lack of upregulation led Slotkin et al. (1995) to hypothesize that neural input might be required for the myocytes
to “learn” how to respond to stimulation (i.e. receptor expression). Hence, a critical period for neural input may exist for the cell to “learn” adjustments in receptor expression.

During development a reciprocal relationship may exist between cardiac myocytes and sympathetic neurons. Studies show that NGF+/- and NT3+/- mice display an attenuated cardiac sympathetic innervation in conjunction with diminished βAR densities (Story 2000a, Story et al. 2000b). Additionally Slotkin et al. (1995) using immunosympathectomized neonatal mice also saw ablation of cardiac sympathetic innervation of the heart with diminished βAR densities. Thus the coupling of the sympathetic innervation and βAR and receptor affinity in the postnatal development period is thought to establish the set point for homeostasis later in life (Cros et al. 1988). Appropriate levels of neurotrophins may be important for a proper level of sympathetic innervation, which in turn establishes an appropriate set-point for βAR expression and function.

Presumably if the sympathetic innervation to the heart is modified and the set point is altered then the functioning and response of the βAR system will be affected. One current model utilized for studying augmented cardiac sympathetic innervation is NGF overexpression. Overexpression of NGF under the control of an α-myosin heavy chain promoter leads to cardiac hypertrophy, hyperinnervation, increases in heart concentration of catecholamines, changes in potassium and calcium current, prolonged cardiac action potentials, and altered βAR density and signaling (Hassankhani et al. 1995, Heath et al. 1998). Mice overexpressing NGF demonstrate sympathetic hyperinnervation
and elevated catecholamines that develop into severe heart disease and cardiac failure (Heath et al. 1998). Though the heart mainly consists of β₁ receptors, this study found that the increase in receptor density was due to an increase in β₂AR (Heath et al. 1998); yet β₂AR were unable to functionally couple to the adenyl cyclase signaling pathway. Conversely, βAR function decreased in NGF overexpressor mice (Heath et al. 1998). Potentially, one of the intracellular signaling protein levels is a “rate-limiting” step in the β-adrenergic system of hyperinnervated hearts. If receptor numbers are upregulated, function may not be increased if the cell cannot transduce the signal. Using a BAX-/- model allows researchers to observe differences as a result of increased sympathetic innervation with the added benefit of being able to manipulate neurotrophin levels (i.e. BAX-/-, BAX-/-NGF+/-, BAX-/-NGF overexpressor). This model of attenuated neuron death might allow evaluation of the mechanisms responsible for determining the set point as well as the plasticity of the system.

**CLINICAL RELEVANCE**

The primary purpose of the study was to elucidate the role of NGF in the development and maturation of cardiac sympathetic innervation and β-adrenergic signaling. Understanding the factors that influence the development of the cardiac sympathetic nervous system may lead to better therapeutic strategies for treating dysautonomias, or imbalance of the autonomic nervous system (Robertson 2002). Both sympathetic and parasympathetic neurons innervate most organs thereby providing for tighter regulation of function. As a result of the overall function of the autonomic

Dysautonomias can be classified as having too little or an excess of sympathetic innervation (Robertson 2002). If a change in NGF or BAX results in hyper- or hypo-innervation in postnatal mice, then these two models may be appropriate for studying dysautonomia. Dysautonomia associated with too little sympathetic innervation can occur from genetic mutations as in familial dysautonomia or due to a disease such as in diabetic autonomic neuropathy (Robertson 2002, Singleton et al. 2004). The type of dysautonomia associated with decreased sympathetic input increases patient risk of serious bradyarrhythmias and sudden death (Glickstein et al. 1999). Mice with lower NGF levels in target tissues (i.e. heart) have decreased cardiac sympathetic innervation (Story 2000a) and may be a useful model in studying dysautonomias with decreased sympathetic innervation. Conversely, dysautonomia from an excess sympathetic innervation of the heart can be due to genetic mutations as Guillain-Barre syndrome or secondary to a disease such as congestive heart failure (Robertson 2002). Augmented cardiac sympathetic innervation is linked to fatal arrhythmias leading to sudden cardiac death (Cao et al. 2000a, Cao et al. 2000b, Chen et al. 2001). Deletion of BAX increases neuron survival, thus potentially increasing innervation density of target organs (Patel et al. 2000, Deckwerth et al. 1996). Thus BAX deletion model allows for manipulation of
NGF level, makes it possible to study dysautonomias with either hypo- and hyper-innervation.

**AIMS OF THE RESEARCH**

Studies of genetically altered mice have elucidated the importance of NGF in the survival and differentiation of sympathetic neurons. NGF enhances the survival of sympathetic neurons during a period of apoptosis and may also play an important role in the terminal differentiation of sympathetic neurons. Development of the sympathetic innervation of the heart occurs in the postnatal period. *The experiments outlined in this dissertation tested the central hypothesis that the sympathetic innervation and the βAR system of the heart develop in parallel in the postnatal period and that their development is NGF dependent.*

**AIM 1. Partitioning of Neuron Survival and Differentiation**

NGF is known to affect sympathetic neuron survival and differentiation into adrenergic-producing neurons (Levi-Montalcini 1987, Snider 1988, Korsching and Thoenen 1983, Ernfors et al. 1994, Farinas et al. 1994, Wyatt et al. 1997, Francis et al. 1999). One aim of the study is to partition the effects of NGF on neuron survival from its effects on differentiation. In order to accomplish this aim, cardiac sympathetic innervation will be examined in BAX knockout mice (mice in which cell death is blocked, but both NGF alleles are present) and in BAX knockout mice that lack one NGF allele (NGF concentrations are reduced but neuron death is blocked). By blocking
neuron death, the effects of NGF on the differentiation of cardiac sympathetic innervation can be assessed. For example, this aim is addressed in Chapters 2 and 3 of this dissertation. These chapters will address the following specific experimental questions.

1. *Does an increase in sympathetic neurons seen in BAX−/− mice translate into sympathetic hyperinnervation of the heart when both NGF alleles are present?*

2. *Does an increase in sympathetic neurons lead to sympathetic hyperinnervation of the heart with only one NGF allele present?*

**AIM 2. Set Point and β-adrenergic signaling**

The reference point for β-adrenergic signaling in the heart is hypothesized to be set during an early postnatal period by the amount of innervation that the heart receives during this period (Slotkin et al. 1995). The second aim of this dissertation is to test this hypothesis in BAX knockout and BAXNGF knockout mice. Deletion of BAX or BAX and NGF alleles in mice should alter the amount of sympathetic innervation that the heart receives during embryonic and postnatal development, and consequently alter the reference point for β-adrenergic signaling in the hearts of these mice. This aim, addressed in Chapter 4, is encapsulated by the following specific question.

3. *Does deleting the number of BAX alleles or a single NGF allele alter the set point and/or function of the heart’s β-adrenergic system?*

**AIM 3. Response of system to perturbation**

Adult rodent hearts upregulate the sensitivity and density of β-adrenergic receptors in response to receptor blockade or denervation. Deletion of BAX, or BAX and
NGF alleles might alter the reference point for β-adrenergic signaling independent of altering a compensatory response to receptor blockade. This hypothesis will be tested by assessing β-adrenergic signaling in BAX or BAXNGF mice that undergo chemical sympathectomy or mice that receive a non-specific β-receptor antagonist. This aim addresses the following question in Chapter 5.

4. Does the cardiac βAR system in BAX deficient mice compensate for loss of anatomical denervation or receptor blockade?

Collectively, the studies comprising this dissertation will lead to a more complete understanding of the role that NGF plays in the development of cardiac sympathetic innervation and the βAR system. Several studies have established that decreased levels of BAX increase neuron number and delay apoptosis. Numerous studies show that altering levels of neurotrophins affects neuron survival, innervation density, β adrenergic receptor density, and NE concentration. However, it is not known whether an excess of sympathetic neurons affects the maturation of sympathetic innervation and the set point of the heart βAR system when NGF concentrations are normal or reduced. By utilizing BAX deficient mice I will be able to examine the impact of NGF on the cardiac sympathetic nervous system maturation. Furthermore, these studies will further our understanding of genetic and acquired forms of cardiac sympathetic diseases (i.e. tachyarrhythmias and congestive heart failure) which arise from functional alterations of the cardiac sympathetic nervous system.
CHAPTER TWO

CARDIAC SYMPATHETIC INNERVATION IN BAX KNOCKOUT MICE

INTRODUCTION

The role of NGF in regulating the survival and differentiation of sympathetic neurons is unclear. Deletion of one or both NGF alleles significantly reduced sympathetic neuron populations in the superior cervical and stellate ganglia (Crowley et al. 1994, Brennan et al. 1999). Story (2000a) showed that numbers of sympathetic neurons in the left stellate ganglion and cardiac norepinephrine (NE) concentrations in heterozygous mutant (+/-) NGF mice were approximately 50% of those of wild type mice. It is not known if the decrement of innervation is from the decreased neuron survival or decreased neuron development. In contrast, Glebova and Ginty (2004) showed that NGF promoted the innervation of cardiac myocytes by sympathetic neurons, but was not essential for stellate ganglion formation.

The first aim of this research was to determine whether the deficits observed in the cardiac sympathetic innervation of NGF-deficient mice was the result of reduced survival of sympathetic neurons, their incomplete differentiation or both factors. The strategy employed was to block apoptotic cell death of sympathetic neurons by deletion of BAX and examine numbers of sympathetic neurons in the left stellate ganglia of these mice (as a measure of neuron survival) and the cardiac NE concentrations (as a measure
of neuron differentiation. There is no evidence that BAX affects NGF synthesis in target tissues in mice, hence BAX-deficient mice should have increased survival of neurons with normal neurotrophin levels. If differentiation of sympathetic neurons is independent of NGF concentrations in the heart, then an increase in the number of sympathetic neurons in the LSG should be paralleled by an increase in cardiac NE. Any deviation would be indicative of an effect of NGF on the differentiation of cardiac sympathetic neurons.

This study was designed to test the hypothesis that deletion of BAX allows extranumerary neurons to survive thereby eliminating neuron survival as a function; hence, determine the role of NGF on differentiation. This aim was accomplished by counting neurons and measuring norepinephrine concentrations in BAX knockout mice before, during, and after maturation of the cardiac sympathetic innervation.

**METHODS**

**BAX Mutant Mice**

All experimental procedures in this dissertation were approved by the Animal Care and Use Committee at Northeastern Ohio Universities College of Medicine, in accordance with NIH Guidelines for the Care and Use of Laboratory Animals. The BAX mutant mouse strain used was developed on a C57BL/6 background (Jackson Labs). All mice used in the experiments of this dissertation were housed in polystyrene cages, fed and watered *ad libitum*, and maintained on a 12 h: 12 h light-dark cycle. BAX+/- mice were mated to produce offspring. Mice were studied at three experimental ages: postnatal
day (0) or birth; postnatal day 21 (P21 or weaning); or as young adults at postnatal day 60 (P60).

BAX mice were genotyped by PCR amplification of tail DNA. DNA was isolated by a classic method adapted from Laird et al. (1991). A portion of mouse tail (approximately 1 cm) was excised with sterile scissors after euthanasia in P0 mice or at postnatal day 18 (P18) for weanling or adult mice. Tails were digested in 500 µl lysis buffer (100mM Tris HCl pH 8.5, 5mM EDTA, 0.2% SDS, 200 mM NaCl, 100 µg Proteinase K/ml) overnight in a shaker heated at 55°C. Tail solution was pelleted by centrifugation at 14,000 rpm for 10 min. One volume (500 µl) of isopropanol was added to the lysate for precipitation of DNA. After centrifugation at 14,000 rpm for 12 min, precipitates were resuspended in 100 µl TE buffer (10 mM Tris HCl, 0.1mM EDTA, pH 7.5) in a heated (55°C) shaker overnight. The wild-type fragment was amplified using an Applied Biosystems GeneAmp PCR machine. The wild type fragment was amplified using the oligonucleotide primers oIMR661 (5’-gttgaccagagtggcgtagg-3’) and oIMR663 (5’-gagctgatcagaaccatcatg-3’). The primers for amplification of the mutant fragment were oIMR662 (5’-ccgcttccattgctcagcgg-3’) and oIMR663. The wild-type fragment (304 bp) was differentiated from the mutant fragment (507 bp) by a difference in electrophoretic mobility on a 2% agarose gel (Figure 1). Mice were genotyped as BAX+/+ (wild type), BAX+-/- (heterozygous mutants), or BAX-/- (null mutant). Mice were genotyped after euthanasia (P0 and P21 mice) or prior to euthanasia for (P60 mice), hence most thoracic or cardiac samples were matched to mice genotyped post mortem. The genotype of P60 mice was determined prior to euthanasia and obtaining samples.
Figure 1. Genotyping of BAX mutant mice. A DNA ladder of typical PCR fragment sizes ranging from 50 to 1000 is shown in lane 1 (50, 150, 250, 500, 750, 1000 bp). Lanes 2, 3, and 4 show the mobility of mutant fragments (507 bp) or wild-type fragments (304 bp) for different mice. Lane 2 contains a 304 bp fragment, but no wild-type fragment thereby demonstrating the amplification pattern for a BAX wild-type mouse (+/+). Lane 3 contains 507 bp and 304 bp fragments indicative of the amplification pattern of a BAX heterozygous mutant mouse (+/-). Lane 4 contains a 507 bp fragment and no wild-type fragment, hence illustrating the pattern of amplification for a BAX null mutant (-/-) mouse.
**Stellate Ganglion Neuron Counts**

Numbers of cardiac sympathetic neurons in the LSG were counted in +/+ , +/- , and -/- mice at birth (P0), weaning (P21), and adulthood (P60) as an index of sympathetic neuron survival. Mice were euthanized by decapitation and the thoracic cavities were isolated. Dissected thoraxes were fixed in 4% paraformaldehyde for 24 h at 4 °C and 24 h at 25 °C. After fixation in paraformaldehyde, thoraxes were decalcified in buffered formic acid for 2-7 d. Thoraxes were then embedded in paraffin and sectioned transversely at 6 µm thickness. Sections were stained with hematoxylin and eosin and mounted on glass slides. Nuclear profiles with a prominent nucleolus were counted at 36 µm intervals throughout the ganglion. The average nucleolar diameter was estimated from 10-20 randomly sampled nucleoli. The total number of neurons in one section of the LSG was estimated by the following formula (Wright and Snider 1995):  

\[
\text{# of nuclear profiles with nucleoli} \times \text{interval length (36 µm)} \\
\quad \text{Mean nucleolar length (5 µm)}
\]

Counts of neurons in individual sections were summated in order to obtain a total count for the entire ganglion.

**Heart to body weight Ratios**

The ratios of heart weight to body weight were calculated as a crude index of relative atrophy or hypertrophy of BAX +/- and -/- mouse hearts compared to +/+ mouse hearts. Additionally, the hearts of BAX+/-NGF+/-, BAX-/-NGF+/- were compared to BAX+/-, BAX+/-, and BAX-/- as controls. Mice were weighed prior to euthanasia by
decapitation. Hearts were excised, drained of excess blood and weighed immediately following euthanasia.

**Norepinephrine Measurement**

The extent of cardiac sympathetic innervation was estimated by measuring norepinephrine (NE) concentration in the heart. At P0, P21, and P60 mice were weighed and euthanized by decapitation. Entire hearts of P0 mice or 10-20 mg of the left ventricular free wall near the apex of the heart of P21 or P60 mice were immersed in 1 mL cold 0.1M perchloric acid. Tissue samples were homogenized in a glass-walled tissue grinder and centrifuged at 3000 rpm for 5 min at 4 °C to pellet cell debris. The supernatant was aspirated through a 0.45 µm filter and the concentration of NE in the filtrate of each sample was analyzed by high performance liquid chromatography (HPLC).

**Statistical Analysis**

Numbers of sympathetic neurons, heart/body weight ratios and cardiac NE concentrations were compared among groups. Mice were grouped according to age and genotype. A SigmaStat statistical program was utilized to analyze data via multiple one-way ANOVA analyses. Significant differences denoted by ANOVA were analyzed post hoc by Student-Neuman-Keuls tests. Heart/body weight ratios were transformed by arcsin √p, where p is the HW/BW ratio, for analyses. Data are reported as untransformed values. All values are expressed as mean ± SEM.
RESULTS

Left Stellate Ganglion (LSG) Neurons in BAX mutant mice

We examined the LSG, the principal site of the soma of postganglionic neurons that innervate the ventricles to determine whether inactivation of one or both copies of the BAX gene would result in an excess number of cardiac sympathetic neurons. Number of neurons in the LSG of heterozygous (+/-) and null mutant (-/-) mice was significantly elevated compared to controls at all ages (Figure 2). Heterozygous mutant (+/-) mice had an average of 41% (40% at P0, 40% at P21, and 41% at P60) more neurons in the LSG than controls. Null mutant (-/-) mice had an average increase of 74% (81% at P0, 62% at P21, and 79% at P60) more neurons in the LSG relative to controls. BAX-/ mice had an average increase of 24% (30% at P0, 16% at P21, and 27% at P60) more neurons in the LSG than BAX+/- mice.

Heart/body weight in BAX +/-, +/-, and -/- mice

Heart weights of +/-, +/-, -/- were equivalent at birth, weaning, and adulthood, although BAX -/- mice weighed 19% less than heterozygous and wild-type mice at P21. Thus, heart to body weight ratios of BAX-/ mice were significantly (P<0.01) higher in weanling mice compared to +/- and +/- mice (Figure 3).

Elevated Cardiac NE concentration in BAX mutant mice

Norepinephrine (NE) is the principal neurotransmitter stored in the nerve terminals of cardiac sympathetic neurons; hence NE concentrations provide an index of cardiac sympathetic innervation. Mean cardiac NE concentrations were equivalent
Figure 2. Neuron counts in the LSG of BAX mice. The number of neurons decreased with age irrespective of genotype. Note that the numbers of LSG neurons in BAX +/- and -/- mice are significantly elevated at all three time points relative to BAX +/+ mice. Additionally, BAX-/- have significantly greater numbers of neurons relative to BAX+/- mice. Asterisk (*) denotes P<0.05, double asterisk (**) denotes P<0.01, triple asterisk (***) denotes P<0.001 compared to control mice; a line with one hash mark (†) denotes P<0.05, a line with two hash marks (‡) denotes P<0.01 compared to BAX+/- mice. All data points represent an n=3.
Figure 3. Heart to body weight ratio (HW/BW) in BAX knockout mice at P0, P21, and P60. The ratio is equivalent at birth and adulthood for all three genotypes. However, HW/BW of BAX null mutant mice (-/-) ratio was significantly higher at P21 than those of +/+ or +/- mice, because BAX-/- mice were lighter. Double asterisk (**) denotes P<0.01. Group sample sizes are indicated within each bar of the histogram.
Postnatal Age (days)

HW/BW (mg/g)

BAX+/+
BAX+-
BAX-/

**

25  30  22  33  39  20  37  35  17

BAX+/

BAX+-

BAX-/

Postnatal Age (days)
among +/-, +/-, and -/- mice at birth (Figure 4). Ventricular NE concentration increased with age in all three genotypes. Ventricular NE concentration of weanling mice was 12% greater in +/- mice and 25% greater in -/- mice compared to wild-type (+/+) mice. NE concentration in adult mutant (-/-) mice was significantly elevated (P<0.01) relative to +/- and +/- mice (Figure 4). In adults, [NE] was augmented by 9% in BAX +/- mice and 35% in BAX-/- mice relative to +/- mice. The average increase of [NE] from P21 to P60 was 11% in heterozygous mice and 30% for null mutant mice (Figure 5). Ventricular NE concentrations of BAX+/- mice are similar to those reported previously for wild-type rodents (Slotkin et al. 1995, Story et al. 2000).
Figure 4. Cardiac NE concentration in BAX mice. NE concentrations are equivalent among +/+, +/-, and -/- mice at P0 and P21. Note that NE concentrations are significantly augmented in adult BAX-/- mice compared to +/- and +/+ mice. Double asterisk (**) denotes a significant difference from control at P<0.01.
Figure 5. Relative cardiac [NE] (P60/P21) in BAX +/+, +/-, and -/- mice (expressed as percent increase). Note that [NE] levels off from weaning into adulthood for BAX+/+ and BAX+/- mice. However, [NE] continues to increase in BAX null mutant (-/-) mice into adulthood.
BAX+/+  BAX+/-  BAX-/-

Genotype

Percent Increase

BAX+/+  BAX+/-  BAX-/-
DISCUSSION

Due to its essential role in neuronal survival, it has not been possible to ascertain if NGF is the limiting factor of differentiation for the cardiac sympathetic nervous system. The deletion of BAX allows an analysis of the developing sympathetic nervous system to answer this question. An examination of newborn, weanling, and adult BAX mutant mice revealed three salient findings regarding neuron survival, terminal differentiation of neurons, and delay of maturation.

**Cardiac Sympathetic Neurons Survival**

Deletion of an apoptotic gene blocks the programmed cell death of supernumerary neurons formed during the development of the nervous system. Deletion of BAX allowed sympathetic neurons to escape apoptosis during development. These data agree with previous in vitro and in vivo models showing an attenuation of normal developmental death in the peripheral nervous system in BAX-/− mice (Deckwerth et al. 1996, Deshmukh and Johnson 1998, Patel et al. 2000, White et al. 1998). With normal NGF levels, BAX deletion caused increased neuron survival in a gene dosage dependent manner. For example, deletion of one BAX gene caused an average of 41% more neurons survived compared to control mice. Furthermore, when both BAX genes were deleted an average of 70% more neurons survived relative to control mice. Therefore, deletion of BAX alleles allowed sympathetic neuron survival in a manner dependent on the number of NGF alleles present.
Cardiac Sympathetic Neurons Differentiation

It is well known that NGF is important for neuron survival, but its role in regulating the terminal differentiation of sympathetic neurons that innervate the heart is not well defined. With increased neuron survival, we can determine whether normal levels of NGF limit differentiation of cardiac sympathetic neurons. Innervation increased normally from P0 into adulthood in BAX+/+ and BAX+-/ mice and is consistent with previous data on wild-type mice from the same background (Story 2000a, Story et al. 2000b). With one copy of BAX deleted, there was no significant difference in innervation relative to control mice at the ages studied. However, deletion of both copies of BAX caused a statistically significant increase in innervation density from P21 to P60 compared to controls.

The increase in neuron survival is paralleled by an increase in innervation density of BAX null mutant mice. An increased level of innervation density with normal NGF levels suggests that NGF may not set the upper limit for innervation of the heart. Other factors may be involved in determining terminal differentiation of sympathetic neurons. Alternatively, the increased innervation density may be a result of a cascade effect from the increased NE present during development. NE can increase endothelin-1 expression which is known to increase myocardial NGF (Morimoto et al. 2001, Ieda et al. 2004). Adult BAX null mutant mice had a 79% increase in neuron number, but only a 35% increase in [NE], as an index of innervation density, compared to controls. There are four possible explanations for this discrepancy. First, the excess neurons that survive produced less NE per neuron. This could occur if NGF affects the enzyme tyrosine hydroxylase
TH), the rate limiting enzyme in NE production. It has been shown that there is about a ten-fold higher requirement of NGF for maximal increases in TH mRNA, TH activity, or process outgrowth (Hefti et al. 1982, Ma et al. 1992). Second, all surviving neurons may have fewer terminals. Possibly, the number of terminals is related to the size, i.e. cross sectional area, of the neuron; it has been noted that supernumerary neurons that survive have smaller bodies and axons (Patel et al. 2000). Third, some neurons are normal, defined as producing normal amounts of NE, while supernumerary neurons produce less NE per neuron. This could be due to these neurons receiving less NGF than the normal neurons. Therefore, it may be that the amount of NGF taken up by a neuron affects TH levels (Hefti et al. 1982). Fourth, the lack of innervation density may be due to the neurons getting mixed cues from the intracellular and extracellular environment. For example, early in neuronal development BAX levels are high and decrease as the neuron matures and makes appropriate connections with target organs (Vekrellis et al. 1997). In the BAX-/- mice, a lack of the BAX protein prior to the time connections are made may affect the development and timing of proper connections. The fact that the increase in innervation density is parallel but not proportional to neuron survival suggests that NGF does not set the upper limit for tissue innervation and thus another factor might be involved.

BAX null mutant mice developed sympathetic hyperinnervation; thus, they may be a useful model for studying the development and treatment of certain cardiac anomalies. For example, atrial fibrillation, chronic MIs, dysautonomias, and CHF can all develop hyperinnervation which creates or exacerbates tachyarrhythmias (Cao et al.
2000b, Chang et al. 2001, Miyauchi et al. 2003). Further studies, however, need to be performed to determine if any of these arrhythmias are present in this hyperinnervated animal model.

**Delay in Maturation**

The BAX null mutant mice had a significantly higher heart weight to body weight ratio than wild-type controls (Figure 3). This elevated ratio was due to decreased body weight rather than increased heart weight. At weaning, the null mutants were significantly lighter than their littermates, by adulthood the differences in body size have disappeared. There are two possible explanations for these findings. First, apoptosis is a crucial process in a developing organism and deletion of a pro-apoptotic gene may lead to developmental alterations not yet explored. Therefore, BAX deletion may lead to a systemic delay in development. To support this idea, previous research found that the ventricles develop later than the atria (Stramba-Badiale et al. 1992) and this study finds that BAX null mutant mice have increased ventricular [NE] into adulthood. Second, this delay may be due to the decreased ability of the BAX null mutant pups to digest and/or absorb their mother’s milk rather than a developmental phenomenon. In addition to autonomic innervation, the digestive tract is innervated by the enteric nervous system, which is similar to the sympathetic nervous system. If BAX deletion affects the enteric nervous system in a manner similar to the sympathetic system, then this could dramatically affect peristalsis and/or glandular secretions which would decrease absorption. Further studies on the digestive tracts of BAX null mutant mice are required to test this hypothesis. The observation that innervation density continued to increase...
into adulthood in BAX-/- mice and not in wild type mice lends support to a developmental delay phenomena. Research by Ina et al. (1999) links Crohn’s disease, an inflammatory disease of the gastrointestinal tract, with an increased Bcl-2/BAX ratio. This disease is characterized by loss of appetite and weight loss. However, a difference in weight is not maintained between BAX-/- and WT mice into adulthood does not support this hypothesis. Hence, deletion of both BAX alleles leads to a developmental delay phenomenon.

**CONCLUSION**

This study finds that the increase in neuron survival and innervation is parallel but not proportional suggesting NGF is not the only factor determining terminal differentiation. Furthermore, BAX null mutant mice develop cardiac sympathetic hyperinnervation into adulthood without any known adverse affects. The results of these experiments suggest that BAX deletion is an appropriate model for sympathetic hyperinnervation.
CHAPTER THREE

CARDIAC SYMPATHETIC INNERVATION IN NGF-DEFICIENT BAX KNOCKOUT MICE

INTRODUCTION

Although NGF deletion causes dramatic losses of sensory and sympathetic neurons (Crowley et al. 1994, Story 2000a), Patel et al. (2000) found when both copies of NGF in conjunction with BAX are deleted, the dorsal root ganglion (DRG) contains increased number of cutaneous sensory neurons. Similarly, elimination of BAX concomitant with deletion of both NGF alleles preserves and increases numbers of neurons in sympathetic chain ganglia compared to wild-type mice at postnatal day 0.5 (P0.5). At the same time, BAX-/-NGF-/- mice have greatly reduced innervation at the base of the heart and an absence of innervation in the heart ventricles at P0.5 (Glebova and Ginty 2004). This study suggests that NGF is not required for stellate ganglion formation, but is essential for axon growth into the myocardium where they innervate cardiac myocytes (Glebova and Ginty 2004). Sympathetic neurons in the stellate ganglion extend their projections to the heart between embryonic days E15.5 and E18.5 (Hempstead 2004), well before the heart and its sympathetic innervation mature. The maturational processes associated with the heart occur during the first months after birth in rodents (Bareis and Slotkin 1980, Cros et al. 1988, Slotkin et al. 1984, Ursell et 1990
Story et al. 2000b). NGF null mutant mice die at birth or shortly thereafter irrespective of BAX deletion, hence the BAX-/-NGF-/- model has limited usefulness for studying the maturation of the cardiac sympathetic nervous system.

The first aim of this dissertation was to study the development of cardiac sympathetic innervation when cell death was blocked by the deletion of BAX and when both NGF alleles were present. The effects of NGF on neuron survival could be partitioned from its effects on neuron differentiation in this experimental design. A subsequent variant of this approach and a logical sequence of inquiry was to block cell death by deletion of BAX and reduce the amount of NGF available in the heart for surviving sympathetic neurons. The approach taken for this experiment was to cross-breed BAX and NGF knockout mice to produce BAX-/-NGF +/- offspring. Deletion of one of copy of the NGF gene attenuates the levels of NGF available to sympathetic neurons during development, but allows the mice to survive into adulthood (Chen et al. 2001). Measuring numbers of neurons in the LSG as an index of sympathetic neuron survival in BAX-/-NGF +/- mice and comparing these data with cardiac NE concentrations allowed testing of the hypothesis that both NGF alleles are necessary for proper development and maturation of cardiac sympathetic innervation irrespective of neuron survival.

METHODS

BAX/NGF double mutant mice

The BAX mutant strain used in this set of experiments was the same strain described in Chapter 1. The strain of NGF knockout mutant mouse was obtained as a gift
from Dr. Heidi Phillips at Genentech and has been described previously (Crowley et al. 1994). Both the BAX and NGF knockout strains were developed on a C57BL/6 background. Heterozygous mutant BAX and heterozygous mutant NGF mice were crossed to produce BAX+/-NGF+/- mice. Male and female BAX+/-NGF+/- mice were mated to produce an array of single and double mutant BAX/NGF mutant offspring for these experiments.

DNA was isolated from the tails of both strains by the methodology described previously (Laird et al. 1991). Mice were genotyped as BAX+/+, +/-, or -/- by PCR amplification and 2% agarose gel electrophoresis of tail DNA. The wild-type fragment was amplified with the oligonucleotide primers oIMR661 and oIMR663 and the primers for the mutant fragment were oIMR662 and oIMR663. The wild-type fragment (304 bp) was differentiated from the mutant fragment (507 bp) by a difference in electrophoretic mobility. The same mice were genotyped as NGF +/+, +/-, or -/- by PCR amplification and 2% agarose gel electrophoresis of tail DNA using a different set of primers. The primers used to amplify the wild-type fragment were NKO-1A (catagcgttatagtcatgt) and HSP-4A (tgaatgtccatgtt) and the mutant fragment primers were Neo490V (cggtttgatgcgtcag) and NB3 (atctggccggtcgggcatgc). A difference in electrophoretic mobility distinguished the wild-type fragment (120-135bp) from the mutant fragment (460 bp) as shown in Figure 6.
Figure 6. Genotyping of NGF mutant mice. A DNA ladder of typical PCR fragment sizes ranging from 50 to 1000 is shown in lane 1 (50, 150, 300, 500, 750, 1000 bp). Lanes 2-5 show the mobility of mutant fragments (460 bp) or wild-type fragments (120-135 bp) for two different mice. Lane 2 and 4 contain a wild type fragment and no mutant fragment respectively; hence illustrating the pattern of amplification for a NGF wild type (+/+) mouse. Lane 3 and 5 contains a 120-135 bp fragment and a 304 bp fragment respectively, thus demonstrating the amplification pattern for a NGF heterozygous (+/-) mutant mouse. Lanes 2 and 4 are from one mouse, and lanes 3 and 5 are from a different mouse.
Heart Weight/Body Weight Ratios

The ratios of heart to body weight were calculated as a crude index of relative atrophy or hypertrophy of +/- and -/- mouse hearts compared to +/- mouse hearts at P0, P21 and P60. The ratios of heart to body weight were calculated as mentioned in the methods section of the previous chapter. The hearts of BAX-/NGF+/- were compared to BAX-/ and controls (BAX+/+).

Norepinephrine Measurement

The extent of cardiac sympathetic innervation was estimated by measuring norepinephrine (NE) concentration in the heart. Cardiac NE concentrations are equivalent among null mutant, heterozygous mutant and wild type NT3 or NGF mice (Story 2000a, Story et al. 2000b, May 2005) at birth (P0), and several matings are necessary to obtain BAX-/NGF+/- mice, hence cardiac NE concentrations were measured in P21 and P60 mice, but not in P0 mice. At P21, and P60 mice were weighed and euthanized by decapitation. A piece (10-20 mg) of the free wall of the left ventricle near the apex of the heart of P21 or P60 mice were immersed in 1 mL cold 0.1M perchloric acid. The technique for NE extraction and measurement is described in Chapter 2.

Statistical Analysis

Mice were grouped according to age and genotype. A SigmaStat statistical program was utilized to analyze data via multiple one-way ANOVA analyses. Significant
differences denoted by ANOVA were analyzed *post hoc* by Student-Neuman-Keuls tests. All values are expressed as mean ± SEM.

**RESULTS**

**Neuron Counts in Superior Cervical Ganglia or Stellate Ganglia**

Neuron counts from LSG and SCG of BAX and BAXNGF mice are shown in Table 1. Mean numbers of neurons of wild type mice at birth was used as a baseline to which all other counts were compared. Deletion of an NGF allele leads to a 61% decrease in sympathetic neurons compared to NGF+/+ mice (Story 2000a). Deletion of both NGF alleles leads to an almost complete loss of sympathetic neurons in the LSG and SCG (Story 2000a, Middleton and Davies 2001). On the other hand, BAX deletion leads to an almost twofold increase of sympathetic neurons in the LSG or SCG compared to controls (May 2005, Middleton and Davies 2001, Glebova and Ginty 2004). This excess of neurons from BAX deletion persists even with both NGF alleles deleted (Glebova and Ginty 2004, Middleton and Davies 2001). Previous studies found BAX deletion allows survival of neonatal sympathetic neurons for up to 23 days in vitro (Deckwerth et al. 1996, Deshmukh and Johnson 1998, Werth et al. 2000). These data are consistent with data by Deckwerth et al. (1996) who demonstrated 100% survival of BAX−/− sympathetic neurons (from SCG) exposed to anti-NGF, compared to <5% survival of neurons from BAX+/+ mice.
Table 1. Neuron Counts expressed as means ± SEM (sample size) in BAX and BAXNGF mice. Deletion of one or both NGF alleles causes a significant decrement of neurons. Deletion of both BAX alleles allows for supernumerary neuron survival regardless of the presence or absence of NGF. “~” means approximate values.

Citation designations are as follows: ¹ May 2005 (this dissertation), ² Glebova and Ginty 2004, ³ Middleton and Davies 2001, ⁴ Story 2000a, ⁵ Brennan et al. 1999.
<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>Location</th>
<th>Neuron counts</th>
<th>% of WT</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>BAX+/+ (WT)</td>
<td>LSG</td>
<td>27,259 ± 801 (3)</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>P0.5</td>
<td>BAX+/+ (WT)</td>
<td>SCG</td>
<td>22,273 ± 777 (6)</td>
<td>100</td>
<td>2</td>
</tr>
<tr>
<td>P1</td>
<td>BAX+/+</td>
<td>SCG</td>
<td>~ 26,000 (3-5)</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td>P0</td>
<td>BAX+-</td>
<td>LSG</td>
<td>37,799 ± 2,164 (3)</td>
<td>139</td>
<td>1</td>
</tr>
<tr>
<td>P0</td>
<td>BAX-/-</td>
<td>LSG</td>
<td>49,251 ± 1,578 (3)</td>
<td>181</td>
<td>1</td>
</tr>
<tr>
<td>P0.5</td>
<td>BAX-/-</td>
<td>SCG</td>
<td>44,428 ± 2,025 (3)</td>
<td>199</td>
<td>2</td>
</tr>
<tr>
<td>P1</td>
<td>BAX-/-</td>
<td>SCG</td>
<td>~47,000 (3-5)</td>
<td>181</td>
<td>3</td>
</tr>
<tr>
<td>P21</td>
<td>BAX-/-</td>
<td>LSG</td>
<td>44,161 ± 999 (3)</td>
<td>162</td>
<td>1</td>
</tr>
<tr>
<td>P60</td>
<td>BAX-/-</td>
<td>LSG</td>
<td>42,507 ± 1,145 (3)</td>
<td>179</td>
<td>1</td>
</tr>
<tr>
<td>E16.5</td>
<td>NGF+/-</td>
<td>SCG</td>
<td>13,210 ± 2,617 (4)</td>
<td>71</td>
<td>5</td>
</tr>
<tr>
<td>P0</td>
<td>NGF+/-</td>
<td>SCG</td>
<td>8,659 ± 1,013 (5)</td>
<td>64</td>
<td>5</td>
</tr>
<tr>
<td>P60</td>
<td>NGF+/-</td>
<td>SCG</td>
<td>7,585 ± 518 (3)</td>
<td>52</td>
<td>5</td>
</tr>
<tr>
<td>P60</td>
<td>NGF+/-</td>
<td>SG</td>
<td>5,854 ± 370 (3)</td>
<td>43</td>
<td>5</td>
</tr>
<tr>
<td>P60</td>
<td>NGF+/-</td>
<td>LSG</td>
<td>12,247 ± 1314 (3)</td>
<td>61</td>
<td>4</td>
</tr>
<tr>
<td>P0.5</td>
<td>NGF-/-</td>
<td>SCG</td>
<td>5,412 ± 348 (5)</td>
<td>24</td>
<td>2</td>
</tr>
<tr>
<td>P1</td>
<td>NGF-/-</td>
<td>SCG</td>
<td>~5,500 (3-5)</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>P0.5</td>
<td>BAX-/-NGF-/-</td>
<td>SCG</td>
<td>45,905 ± 2,279 (4)</td>
<td>206</td>
<td>2</td>
</tr>
<tr>
<td>P1</td>
<td>BAX-/-NGF-/-</td>
<td>SCG</td>
<td>~47,000 (3-5)</td>
<td>181</td>
<td>3</td>
</tr>
</tbody>
</table>
Heart to body weight ratio

Mean (+SEM) heart weights of BAX-/-NGF+/- mice were significantly heavier (P<0.01) at P0, but not P21 and P60 compared to BAX+/+ mice (Table 2). BAX-/-NGF+/- mice hearts tend to remain heavier after birth: 9% larger at P21 and 5% larger at P60 than BAX+/+ and BAX-/- mice. Mean body weights of BAX-/- and BAX-/-NGF+/- mice are larger (P<0.01, P<0.001 respectively) at P0. Conversely, BAX-/- mice have significantly lighter body weights at P21 (P<0.001), while BAX-/-NGF+/- weighed slightly less (although not statistically significant) than control mice at P21. However, when heart weight was divided by body weight, there was a statistically significant elevation in BAX-/- (P<0.05) and BAX-/-NGF+/- (P<0.01) relative to wild type mice at P21, but not P0 or P60.

Elevated Cardiac NE concentration in BAX mutant mice

Measurement of NE, the principal neurotransmitter stored in sympathetic nerve terminals, provides an index of cardiac sympathetic innervation. Similar to BAX-/- mice, BAX-/-NGF+/- mice showed an increase in cardiac NE concentration with age indicative of increasing innervation density with maturation of the cardiac sympathetic nervous system (Figure 7). The simultaneous deletion of BAX with NGF resulted in innervation density to reach normal levels by adulthood (P>0.05).

At P21, ventricular NE concentration of BAX-/-NGF+/- mice is 36% less than age-matched BAX-/- mice, but only 20% less than age-matched wild-type (+/+ ) mice. At P60, BAX-/-NGF+/- mice have 38% less innervation density relative to adult BAX-/- mice, but only 15% less than wild-type (+/+ ) mice. These data demonstrate that deletion
Table 2. Heart weight (HW), Body weight (BW), and HW/BW ratios in BAX and BAXNGF mice. Heart weights are expressed in milligrams (mg). Body weights are expressed in grams (g). Values are expressed as mean ± SEM. Sample sizes are denoted in parentheses. Asterisk (*) denotes P<0.05, double asterisk (**) denotes P<0.01, triple asterisk (***) denotes P<0.001.
<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>HW (mg)</th>
<th>BW (g)</th>
<th>HW/BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0</td>
<td>BAX+/+</td>
<td>8.81 ± 0.23 (45)</td>
<td>1.37 ± 0.02 (45)</td>
<td>6.31 ± 0.21 (45)</td>
</tr>
<tr>
<td></td>
<td>BAX-/</td>
<td>9.54 ± 0.37 (39)</td>
<td>1.48 ± 0.05 (39)**</td>
<td>6.32 ± 0.25 (39)</td>
</tr>
<tr>
<td></td>
<td>BAX-/NGF+/-</td>
<td>10.32 ± 0.61 (28)**</td>
<td>1.74 ± 0.13 (28)***</td>
<td>6.20 ± 0.42 (28)</td>
</tr>
<tr>
<td>P21</td>
<td>BAX+/+</td>
<td>63.98 ± 2.22 (33)</td>
<td>8.55 ± 0.31 (33)</td>
<td>7.58 ± 0.22 (33)</td>
</tr>
<tr>
<td></td>
<td>BAX-/</td>
<td>63.82 ± 2.28 (20)</td>
<td>7.27 ± 0.25 (20)***</td>
<td>8.78 ± 0.26 (20)***</td>
</tr>
<tr>
<td></td>
<td>BAX-/NGF+/-</td>
<td>69.54 ± 4.18 (14)</td>
<td>8.14 ± 0.40 (14)</td>
<td>8.54 ± 0.42 (14) *</td>
</tr>
<tr>
<td>P60</td>
<td>BAX+/+</td>
<td>139.33 ± 3.98 (37)</td>
<td>23.12 ± 0.55 (37)</td>
<td>6.06 ± 0.11 (37)</td>
</tr>
<tr>
<td></td>
<td>BAX-/</td>
<td>138.94 ± 5.24 (17)</td>
<td>22.84 ± 0.56 (17)</td>
<td>6.36 ± 0.24 (17)</td>
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<td></td>
<td>BAX-/NGF+/-</td>
<td>146.29 ± 3.66 (17)</td>
<td>23.39 ± 0.75 (17)</td>
<td>6.39 ± 0.20 (17)</td>
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</tbody>
</table>
Figure 7. Cardiac NE concentration of BAX-/-NGF+/- mice. Values of BAX +/- and -/- mice are included in the graph for comparative purposes. A general trend of increasing cardiac innervation density with age is seen for all three genotypes. Numerals denote sample sizes. Double asterisk (**) denotes $P<0.01$. 
Postnatal Age (days)

[NE] (pg/mg)

BAX+/+
BAX-/-
BAX-/-NGF+/-
of an NGF allele limits terminal differentiation, but concomitant deletion of BAX allows for innervation density to reach near normal levels. From P21 to P60, [NE] increases by 12% in null mutant mice and 8% in BAX-/-NGF+/- mice compared to only 1% in wild type mice (Figure 8). These data suggest a delay of maturation in cardiac sympathetic innervation density when BAX is deleted regardless of the presence or absence of NGF alleles.
Figure 8. Relative cardiac NE concentration of P60/P21 (expressed as % increase) in BAX +/+,-/-, and BAX/-NGF+/- mice. Note that NE concentration levels off from weaning to adulthood in BAX+/+ mice, but continues to increase in BAX null mutant (-/-) and BAX/-NGF+/- mice. These data suggest that deletion of BAX either delays the terminal arborization of sympathetic neurons in the myocardium or less NE is produced per neuron.
BAX +/+  BAX -/-  BAX -/-NGF+/

Genotype

% Increase

BAX +/-  BAX +/-NGF+/

Genotype

% Increase
DISCUSSION

Innervation

Innervation density of BAX-/-NGF+/- mice was 37% less than BAX-/- mice. NGF+/- mice had approximately half the cardiac innervation density of age matched control mice (Story 2000a). Assuming that the deletion of one NGF allele approximately leads to one-half NGF protein expression (Bianchi et al. 1996, Chen et al. 2001) and that NGF levels determine innervation density, there should have been a 50% difference of innervation density between BAX-/- and BAX-/-NGF+/- mice. There are several possible sources of this discrepancy. First of all, the difference between a 50% decrease that should have occurred and the measured 37% decrease might be experimental error. Alternatively, another factor may contribute to determining the final innervation density of the heart. This second explanation may be supported by the discrepancy between neuron counts and innervation density of BAX-/- mice. Lastly, I cannot rule out a pleiotropic effect from the deletion of BAX itself. For instance, when NT3 was deleted to determine its role on muscle spindle development, NT3 was found to affect migration of neural crest cells and neuron survival (Donovan et al. 1996, El Shamy et al. 1996, Ernfors et al. 1994, Farinas et al. 1994). NT3 deletion caused malformation of the outflow tracts and ventricular septum (Donovan et al. 1996). Additionally, the deletion of NT3 decreased neuron populations in sensory, sympathetic, and motor ganglia/nuclei (Farinas et al. 1994).

BAX-/-NGF+/- mice had decreased innervation density compared to BAX-/- and WT mice. If the decreased innervation is coupled with decreased ßARs, then this could
have led to cardiac hypertrophy from an increase in cardiac myocyte number and size. A similar set of observations occurs in heart failure patients: decreased myocardial NGF, decreased innervation and hypertrophy (Kaye et al. 2000, Lindpaintner et al. 1987). Therefore, the BAX-/-NGF+/- mice may be a model to study CHF.

This study showed when a pro-apoptotic gene was deleted and normal NGF levels were present cardiac sympathetic hyperinnervation occurred. Furthermore, when a pro-apoptotic protein was deleted and only one NGF allele was present, cardiac sympathetic innervation returned to wild type densities. These data have clinical ramifications for conditions of hypoinnervation, as in dysautonomias. For example, insulin dependent diabetes mellitus can be complicated by a decrease in cardiac sympathetic innervation and myocardial NGF protein (Schmid et al. 1999, Faradji and Satelo 1990). Sudden Infant Death Syndrome, the leading cause of death of infants under one year of age, is related to a developmental excess of the sympathetic cardiac nervous system (Church et al. 1967, Schwartz et al. 1998). Thus, targeting the apoptosis pathway, linked to an aMHC promoter to target the heart, may allow a return of cardiac sympathetic nervous system to normal. Furthermore, a rat model of sinoaortic denervation is linked with high levels of apoptosis (Tao et al. 2004) and BAX deletion may improve this condition. One caveat is the current research involves the absence of BAX throughout embryonic and postnatal development, whereas the treatment would occur after the cardiac sympathetic nervous system is mature and established. Targeting the apoptosis pathway could be a viable strategy for treating conditions of hypoinnervation, but most likely would need to
occur prior to establishment of heart innervation and thus may only be applicable in SIDS cases.

**Delay of Growth**

The trend of decreased body weight seen in BAX null mutant mice was also seen in BAX-/-NGF+/− mice. There are two possible explanations for this finding. The first reason, as mentioned in the chapter two discussion (Maturational Delay), may be related to a decreased ability to digest and absorb nutrients due to inflammation in the digestive tract similar to Crohn’s disease (Ina et al. 1999). Additionally, previous research demonstrates a decrease of sympathetic innervation in the stomach and intestines of BAX-/-NGF-/- mice compared to BAX-/- mice (Glebova and Ginty 2004). This idea is supported by the fact that BAX-/- and BAX-/-NGF+/- mice did well in the prenatal period, evidenced by higher body weights, when they receive nutrients from the mother and did not have to digest and absorb food. Secondly, the decreased body weight may be indicative of a postnatal delay in maturation. The continual increase in innervation density from P21 into adulthood seen when BAX is deleted further supports this second idea. Lastly, these two possibilities are not mutually exclusive and both may have occurred.

**CONCLUSION**

These data find decreased levels of innervation in BAX-/-NGF+/- mice relative to the hyperinnervated BAX-/- hearts, suggesting that NGF is the primary factor determining cardiac sympathetic innervation density. Furthermore, deletion of BAX allowed the cardiac sympathetic innervation to reach control levels with only one NGF
allele present. Thus, BAX deletion enabled a normal level of cardiac sympathetic innervation with limited NGF. Hence, targeting the apoptosis pathway may be of clinical relevance for SIDS patients.
CHAPTER FOUR

CARDIAC β-ADRENERGIC RECEPTOR DEVELOPMENT
IN BAX KNOCKOUT MICE

INTRODUCTION

In a normal adult rat heart, increased stimulation of βARs caused desensitization of the ligand-receptor system by initially desensitizing the receptor followed by downregulation of receptor density (Figure 9, adapted from Slotkin et al. 1995). Conversely, denervation or pharmacological receptor blockade resulted in hypersensitivity of βAR followed by an increase in receptor density (Slotkin et al. 1995). These phenomena suggest that in order for the “mature” β-adrenergic system of the heart to make proper adjustments, it must acquire a “set-point” or reference point of innervation density, NE content, and βAR density. Appropriate adjustments in receptor sensitivity or receptor density observed in adults are based on this reference point. In heart tissue, receptor density and response to activation progressively increase throughout development, with neuronal input acting as a positive (upregulating) factor in this process. However, compensatory response of βAR’s to denervation described for the mature adult rat heart is absent in the immature heart (Slotkin et al. 1995). These observations led Slotkin et al. (1995) to hypothesize that neural input during a critical period of development might be required for the rat myocytes to “learn” the appropriate response to stimulation.
Figure 9. Schematic model of βAR system response (adapted from Slotkin 1995).

Schematic model showing the difference in response of the rat heart to denervation or pharmacological βAR blockade in the early postnatal period (P0-P35) or a later postnatal period (after P35). Note that decreasing the amount of stimulation to the βAR after P35 results in a compensatory response of upregulation of receptor sensitivity (physiological response) followed by an increase in receptor number (genomic response). However, decreased stimulation of βAR prior to P35 does not result in βAR supersensitivity or increased density of βAR. In addition, neonatal denervation induces long-lasting deficits in coupling of βAR to ornithine decarboxylase. Overstimulation of βAR in adults produces the opposite effects and overstimulation in neonates induces neither desensitization nor downregulation of βARs.
Prenatal

Early Postnatal

Late Postnatal

Age

P0

P35

Abnormal receptor/ornithine decarboxylase linkage

Denervate or Block Cardiac β-Adrenergic Receptors

No Upregulation of Receptor Numbers

No Response Supersensitivity

Response Supersensitivity

Upregulation of Receptor Numbers

Denervate or Block Cardiac β-Adrenergic Receptors

Abnormal receptor/ornithine decarboxylase linkage

No Response Supersensitivity

Response Supersensitivity

Upregulation of Receptor Numbers

Denervate or Block Cardiac β-Adrenergic Receptors
The previous chapter described how the deletion of BAX altered the innervation density, therefore a logical next step is to determine if the post-synaptic cell is altered in this model. The purpose of this research is to better understand the development of the sympathetic beta-adrenergic nervous system in a BAX knockout model with both or one NGF allele present. This study addressed how the set point of the heart’s β-adrenergic system is affected in BAX+/+, BAX-/-, and BAX-/-NGF+/- mice. It is hypothesized that the βAR system will parallel innervation density. It is hypothesized that cAMP levels will also be elevated along with the innervation and beta-adrenergic receptor density.

**METHODS**

**BAX and BAXNGF cross bred mutant mice**

The BAX and BAXNGF mutant strains used were housed, mated, and genotyped according to the protocols described in previous chapters.

**Beta-adrenergic receptor binding assays**

Mice were sacrificed by decapitation. Hearts were quickly dissected, drained of excess blood, frozen in liquid nitrogen, and stored at -70 °C until use. One adult (P60) heart was used per assay, but hearts were pooled after genotyping for P21 mice (2 hearts/sample) and P0 mice (8 hearts/sample) in order to obtain enough membrane for binding assays. Myocardial cell membranes were prepared by homogenizing hearts in 20 ml Tris buffer (50mM Tris buffer pH 7.4 at 36 °C, 10 mM MgCl₂) using a polytron tissue homogenizer. Homogenates were centrifuged at 20,000 rpm for 10 min. The supernatant
was discarded and the pellet was resuspended, homogenized, and centrifuged. This process was repeated two times. After a final resuspension and centrifugation, the resulting pellet was homogenized in 10 ml Tris buffer for P21 and P60 hearts or 5.5 ml for P0 hearts. These volumes yielded an optimal protein concentration for use in binding assays.

A βAR antagonist, $[^{125}\text{I}]$ Iodocyanopindolol (New England Nuclear) was used to radiolabel receptor sites and determine total β-AR density. Tissue homogenates were added to polystyrene plastic tubes containing Tris buffer and either $[^{125}\text{I}]$ ICYP alone to measure total binding or $[^{125}\text{I}]$ ICYP and 2 µM propranolol, to measure non-specific binding. Specific binding was calculated by subtracting the activity of tubes containing 2 µM propranolol (blank) from the activity of tubes without 2 µM propranolol (total). Total tubes contained 350 µl Tris buffer, 100 µl tissue and 50 µl $[^{125}\text{I}]$ ICYP. Blank tubes contained 300 µl Tris buffer, 100 µl tissue and 50 µl $[^{125}\text{I}]$ ICYP and 50 µl propranolol for total assay volume of 500 µl. Each assay was performed in triplicate using eight concentrations of $[^{125}\text{I}]$ ICYP (5-200 pM) for a total of 48 tubes per individual assay. Assay tubes were shaken and incubated at 36 °C for 60 min. Assays were terminated by filtration through Whatman GF/C glass fiber filters using a 48 channel Brandel filtration apparatus.

Filters containing myocardial membranes and bound $[^{125}\text{I}]$ ICYP were placed in individual gamma tubes and counted on a gamma counter. Saturation data were analyzed using Prism GraphPad curve fitting computer program in order to calculate the maximum binding ($B_{max}$) and the $[^{125}\text{I}]$ ICYP dissociation constant ($K_d$). Protein content of
individual heart samples was determined by the method of Bradford using bovine serum albumin as the standard (Bio-Rad kit). $B_{\text{max}}$ values were expressed relative to protein content of the individual assays.

**Cyclic AMP Assays**

Hearts were excised and frozen in liquid nitrogen. For P21 and P60 samples, one frozen heart was crushed with mortar and pestle and the powdered tissue was divided into three tubes. For P0 samples, 1 frozen heart was crushed per tube, therefore, requiring three hearts for the three levels of hormone. Warmed media M199 was added to each tube 1000µl for P60, 500µul for P21 and P0 hearts. Tubes were warmed in a water bath (37°C) for 5min. IBMX was added to a final concentration of 10mM and then the appropriate amount of isoproterenol was added. Three concentrations of isoproterenol were used in these assays to determine a dose response: 1) Basal level (B) with no isoproterenol, 2) 1µM isoproterenol termed “low stimulation”, and 3) 10µM isoproterenol termed “high stimulation” (Alexander et al. 1982, Xiang et al. 2002). Tubes were stimulated with isoproterenol for 15 minutes then each tube of heart tissue was lysed using a polytron. Tubes were centrifuged for 15 minutes at 14,000 rpm. Supernatant was frozen. Samples were run in duplicate on a direct cAMP EIA kit from Assay Designs, Inc.

**Statistical Analysis**

Mice were grouped according to age and genotype. A SigmaStat statistical program was utilized to analyze data. A SigmaStat statistical program was utilized to
analyze data via multiple one-way ANOVA analyses. Significant differences denoted by ANOVA were analyzed post hoc by Student-Neuman-Keuls tests. Values are expressed as mean ± SEM.

RESULTS

Binding of $[^{125}\text{I}]$ ICYP

Specific binding to $[^{125}\text{I}]$ ICYP represented approximately 65% of the total binding in mice of BAX and BAXNGF strains. Protein content of cardiac membranes were equivalent (P>0.05) among BAX+/+, BAX-/-, and BAX-/-NGF+/ mice and ranged between 0.01-0.12 mg/ml. The dissociation constant ($K_d$), which indicates the affinity to $[^{125}\text{I}]$ ICYP, was not statistically different among BAX +/+, BAX -/-, and BAX-/-NGF+/ or among ages measured (Table 3).

βAR Density

Mean values of specific binding were calculated for $[^{125}\text{I}]$ ICYP for BAX and BAXNGF mice (Figure 10). Mean specific binding, $B_{\text{max}}$, increased from P0 to P21 then decreased from P21 to P60 in BAX+/+ mice. Previous studies measured similar values of βAR in wild type adult rodents (Rohrer 1998, Rohrer et al. 1996, Tsao et al. 2000). This pattern did not occur in BAX-/- mice. The densities of cardiac βAR ($B_{\text{max}}$) remained significantly lower in BAX null mutant mice (-/-) at all three time points compared to control mice (P<0.05). However, βAR density increased from P21 to P60 by 5% in BAX null mutant mice.
Table 3. Binding affinities ($K_d$) (nM) of wild type, BAX-/-, and BAX-/-NGF+/- mice.

No significant differences ($P>0.05$) in β-AR binding affinity among mice grouped by age or genotype were observed.
<table>
<thead>
<tr>
<th>Genotype</th>
<th>P0</th>
<th>P21</th>
<th>P60</th>
</tr>
</thead>
<tbody>
<tr>
<td>BAX+/+ (WT)</td>
<td>0.12 ± 0.01 (3)</td>
<td>0.14 ± 0.02 (6)</td>
<td>0.12 ± 0.01 (6)</td>
</tr>
<tr>
<td>BAX/-</td>
<td>0.17 ± 0.02 (3)</td>
<td>0.20 ± 0.06 (4)</td>
<td>0.12 ± 0.003 (5)</td>
</tr>
<tr>
<td>BAX/-NGF+/-</td>
<td>0.13 ± 0.02 (3)</td>
<td>0.31 ± 0.19 (4)</td>
<td>0.22 ± 0.09 (3)</td>
</tr>
</tbody>
</table>
Figure 10: Cardiac β-AR Density in BAX and BAXNGF mice. BAX-/ mice have lower β-AR densities than control mice at all three ages. BAX-/NGF+/- mice have significantly lower β-AR densities than BAX+/+ and BAX-/ mice at P0 and P21; further, BAX-/NGF+/- mice have lower β-AR densities than WT, but not BAX-/ mice at P60. The BAX-/NGF+/- mice have a steady increase β-AR density into adulthood. BAX-/NGF+/- have β-AR densities at P60 equivalent to BAX-/ mice. Asterisk (*) denotes P<0.05, triple asterisk (****) denotes P<0.001; when P21 is significantly different from P0 “a” denotes P<0.05, “b” denotes P<0.01, “c” denotes P<0.001; when P60 is significantly different from P21 “A” denotes P<0.05, “C” denotes P<0.001. Numbers denote sample sizes.
Postnatal Age (days)

<table>
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<th>Bmax (fmol/mg tissue)</th>
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<tr>
<td>BAX +/+</td>
</tr>
<tr>
<td>BAX +/-</td>
</tr>
<tr>
<td>BAX +/-NGF +/-</td>
</tr>
</tbody>
</table>

- P0
- P21
- P60

Symbols:
- 3***
- 3*
- 3cC
- 4***b
- 6a
- 6A
- 4*
- 5*
Cardiac βAR ($B_{\text{max}}$) densities of BAX-/-NGF+/- mice were significantly lower at P0, P21 (P<0.001), and P60 (P<0.05) relative to wild-type mice. Furthermore, BAX-/-NGF+/- mice showed a similar pattern as BAX-/- mice by rising continuously through all three time points. There was a significant difference within BAX-/-NGF+/- among age groups, P60>P21>P0 (P<0.05).

Cyclic AMP Levels

Basal cyclic AMP levels were higher in BAX-/- and BAX-/-NGF+/- mice than wild-type mice for all three ages (Figure 11A). Further, the BAX-/-NGF+/- mice have lower basal cyclic AMP levels than BAX-/- mice at all ages. With one NGF allele (BAX-/-NGF+/-) present, the basal cyclic AMP levels are attenuated compared to when both NGF alleles present (BAX-/-NGF+/+). Basal cAMP levels in newborns and adults were similar to a previous study (Auman et al. 2002a).

Stimulation of 1µM concentration of isoproterenol, a β1 and β2 adrenergic receptor agonist, caused slightly more than a two-fold increase of cAMP above basal levels in control (+/+) mice. The ability of the heart muscle to respond to low levels of isoproterenol improved with age with almost a two and one-half fold increase above basal (an 8% increase from P0 to P21) and approximates a three-fold increase above basal level into adulthood (an increase of 12% from P21 to P60) (Figure 11B). However, BAX null mutant mice had a significantly larger response to low levels of isoproterenol at every age measured. BAX-/-NGF+/- mice had an attenuated response
Figure 11. A. Basal Cyclic AMP Levels in WT, BAX-/-, and BAX-/-NGF+/- mice. BAX-/- and BAX-/-NGF+/- mice had significantly greater basal cAMP levels than control mice. Double asterisk (**) denotes $p<0.01$, triple asterisk (***) denotes $p<0.001$ compared to WT, $a$ denotes $p<0.01$ compared to BAX-/- mice.

B. Low Stimulated Cyclic AMP Levels in WT, BAX-/-, and BAX-/-NGF+/- mice. Level of cAMP produced in cardiac tissue after being stimulated with [1uM] of Isoproterenol. Stimulated level of cAMP is expressed as fold change relative to basal cAMP values. The same trends appeared with a low level of isoproterenol stimulation, but significant increases of BAX-/- and BAX-/-NGF+/- from WT only occur at P60. Triple asterisk (***$)$ denotes $p<0.001$ compared to WT, $b$ denotes $p<0.001$ compared to BAX-/- mice.

C. High Stimulated Cyclic AMP Levels in WT, BAX-/-, and BAX-/-NGF+/- mice. Heart tissue was stimulated with a [10uM] of Isoproterenol. Level of cAMP after this higher level of stimulation was expressed as fold change from basal cAMP level. The same trends occurred with a higher level of isoproterenol. BAX-/- mice had an increased response to isoproterenol compared to WT, whereas BAX-/-NGF+/- mice had a blunted response to stimulation. Asterisk (*) denotes $p<0.05$, double asterisk (**) denotes $p<0.01$, triple asterisk (***) denotes $p<0.001$ compared to WT, $b$ denotes $p<0.001$ compared to BAX-/- mice.
A. Basal

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</tr>
<tr>
<td>BAX-/-</td>
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<td>3</td>
<td>3</td>
</tr>
<tr>
<td>BAX-/-NGF+/-</td>
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<td>3</td>
<td>5</td>
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</table>

B. Low stimulation

<table>
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<th>P21</th>
<th>P60</th>
</tr>
</thead>
<tbody>
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<td>4</td>
<td>4</td>
</tr>
<tr>
<td>BAX-/-</td>
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<tr>
<td>BAX-/-NGF+/-</td>
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<td>3</td>
<td>5</td>
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</table>

C. High stimulation

<table>
<thead>
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<th>Genotype</th>
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<th>P21</th>
<th>P60</th>
</tr>
</thead>
<tbody>
<tr>
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<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>BAX-/-</td>
<td>5</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>BAX-/-NGF+/-</td>
<td>3</td>
<td>3</td>
<td>5</td>
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</tbody>
</table>
to low levels of isoproterenol compared to BAX+/+ and -/- at P0. BAX-/-NGF+/- mice developed a more robust response to low levels of isoproterenol by P21 similar to BAX+/+ mice. The myocytes response of BAX-/-NGF+/- mice to a beta agonist increased into adulthood.

Cyclic AMP levels in all three genotypes were significantly higher than basal levels when stimulated with a higher dose of isoproterenol (10µM concentration). This concentration was used to elicit a maximal adenylate cyclase response based on a previous study (Rohrer et al. 1996). Control mice had an increasing ability to respond to high levels of isoproterenol with age (Figure 11C). BAX heterozygous and null mutant mice displayed similar levels of adenylate cyclase activity across their three age groups. BAX-/-NGF+/- had a significantly attenuated ability to respond to higher concentrations of isoproterenol compared to BAX -/- mice at P0. BAX+/-NGF+/- continued to increase adenylate cyclase activity in response to isoproterenol from P0 to P21 and into adulthood, though, still significantly decreased compared to BAX+/+, +/-, and -/- mice. BAX-/-NGF+/- show an improved ability to respond to higher concentrations of isoproterenol at P21 compared to P0 levels, but this levels off into adulthood.

There was a trend in all three genotypes to increase adenylate cyclase responsiveness with age. These data are consistent with previous studies (Auman et al. 2002a, Giannuzzi et al. 1995, Slotkin et al. 1996). Auman et al. (2002a) demonstrated increased adenylate cyclase response with age that levels off into adulthood. Additionally, the deletion of BAX led to increased cAMP production and the deletion of
NGF suppresses this increase. These relationships among age and genotype persisted irrespective of the degree of stimulation.

**DISCUSSION**

**Altered βAR expression and signaling**

BAX deletion led to a low level of βAR density relative to WT mice. Unfortunately, little is known concerning what factors affect βAR expression in young animals (an immature cardiac sympathetic system). The low βAR expression may be a result of the increased NE stimulation through development. Cardiac NE concentration in BAX-/- mice is elevated above control values at P0, P21, and P60. Prolonged treatment of cardiac myocytes with beta-adrenergic stimulatory factor leads to down-regulation of βAR density (Reithmann et al. 1990). Additionally, thyroid hormone is known to increase βAR concentration during development (Whitesett et al. 1982), while thyroidectomy prevents this increase in βAR density with no affect on the adenylate cyclase system (Maier et al. 1989). Thyrotoxicosis, a condition causing increased thyroid hormone, is associated with increased BAX levels and decreased Bcl-2 levels (Lavat-Moleur et al. 1999). If the converse is true, then an absence of BAX may lead to a hypothyroid condition. This hypothyroid condition could leads to a decreased level of βAR density and might also explain the delay in maturation observed in BAX-/- mice.

Although BAX null mutant mice maintained a significantly lower level of βAR density at all three ages, adenylate cyclase activity was increased compared to controls. Previous research has shown, βAR coupling to G-proteins is not co-ordinately regulated
early in development (Alexander et al. 1982, Chaudry and Granneman 1991, Maier et al. 1989). Since this lack of coordination between βAR and adenylate cyclase activity also occurred in adult BAX-/ mice, it was consistent with the hypothesis that the deletion of BAX leads to a developmental delay phenomena. Additionally, a study by Giannuzzi et al. (1995) demonstrated that repeated stimulation with an agonist elicited sensitization in the neonate, instead of desensitization. The sensitization occurs downstream from the receptor and can be achieved in four ways: 1) increase the ratio of β2AR: β1AR, 2) increase β1AR coupling efficiency to the G-proteins, 3) increase the number of Gas proteins, or 4) decrease in Gai. Since β2AR couple more efficiently to stimulate adenylate cyclase (AC) (Rohrer 1996), an increase in β2AR: β1AR ratio would lead to increased AC signaling. In this study, the ratio of βAR subtypes was not measured. Alternatively, if the number of Gas proteins increases or β1AR couples more efficiently to the same number of G-proteins then cAMP production would increase at the same stimulation level. Morris and Bilezikian (1986) showed that cardiac innervation increases Gsa response as well as induction of adenylate cyclase (Morris and Bilezikian 1986, Auman et al. 2002a, Auman et al. 2002b). Lastly, sympathetic stimulation leads to a decrease in Gai level which results in sensitization of adenylyl cyclase stimulation (Reithmann et al. 1990, Auman et al. 2002a, Auman et al. 2002b). Additionally, increased sympathetic stimulation, as evidenced by increased [NE] in BAX-/ mice, may decrease the concentration of muscarinic receptors (m2AChRs) which can then modulate Gi levels (Garofolo et al. 2002, Denniss et al. 1989).
The current research showed that BAX-/- mice had increased neuron survival and cardiac innervation, but lowered βAR density. BAX-/- had steady low levels of βAR density at all ages measured. Furthermore, NGF deletion concomitant with BAX deletion caused increased neuron survival, low or normal cardiac innervation, and low or normal βAR density. Interestingly, the BAX-/-NGF+/- mice had a continual increase in βAR expression which paralleled the developing cardiac sympathetic innervation. In both genotypes the absence of BAX allowed for increased neuron survival. The number of NGF alleles present, therefore accounts for the differences in cardiac innervation which in turn can alter βAR density. It is thought the NE released from pre-synaptic cells affects the βAR expression of the post-synaptic cell. However, it is unknown whether BAX or NGF directly affects βAR expression in the post-synaptic cell. To eliminate this possibility, in vitro testing could be performed on myocardial cells.

The BAX-/-NGF+/- model is consistent with Slotkin’s (1995) hypothesis that innervation is important for establishing set-point. Recent studies have demonstrated that NGF influences this system. For example NGF+/- mice had decreased βAR density (Story 2000a), whereas NGF overexpressor mice have increased βAR density (Heath et al. 1998). Excluding BAX-/- mice other mouse models (NGF knockout, NGF overexpressor, and BAXNGF double knockout mice) suggest anatomical innervation helps determine βAR density. Therefore, anatomical connectivity or some related factor determinates βAR expression.

BAX-/-NGF+/- mice had increased responsiveness to a beta-adrenergic agonist compared to controls, though, the response was attenuated compared to BAX-/- mice.
This finding suggests that 1) the deletion of BAX sensitizes the βAR signaling and 2) the level of NGF affects βAR signaling. The effect BAX deletion had on βAR signaling was counteracted by NGF deletion. A previous study demonstrated that NGF affects synaptic transmission at sympathetic neurons (Lockhart et al. 1997). The mechanism is unclear, but it is thought that NGF increased the expression, activity, or both of specific molecules, such as tyrosine hydroxylase, the rate-limiting enzyme for NE synthesis (Lockhart et al. 1997).

**CONCLUSION**

Deletion of BAX lowered the set-point of beta adrenergic receptor most likely due to augmented NE stimulation. BAX deletion enabled NGF deficient mice to achieve near normal βAR density by P60 most likely due to the increased survival of sympathetic neurons. This study demonstrated the counterbalancing effect of BAX and NGF deletion on βAR signaling. Deletion of BAX increased βAR signaling, whereas NGF deletion decreased signaling. Therefore, targeting the apoptotic pathway can be a useful tool in treating conditions with decreased beta adrenergic sensitivity, as in CHF or dysautonomias.
CHAPTER FIVE

THE EFFECTS OF PHARMACOLOGICAL SYMPATHECTOMY AND 
β-ADRENERGIC RECEPTOR BLOCKADE ON BAX AND BAXNGF 
MUTANT MICE

INTRODUCTION

Slotkin and co-workers (1995, 1996, Auman et al. 2002a, Auman et al. 2002b) showed that the hearts of neonatal and adult rats respond differently to pharmacological denervation or β-adrenergic receptor blockade (see Figure 9 on page 70). Denervation of adult rat hearts initially induces the physiological response of receptor supersensitivity followed by a genomic response of β-adrenergic receptor upregulation, whereas overstimulation of β-adrenergic receptors induces the opposite response in adults. In contrast, denervation/hyperstimulation of neonatal rat hearts induces no compensatory response in β-adrenergic receptors. Hence the difference in response was hypothesized to be related to the age of the rat (and the maturity of the β-adrenergic signaling system) at the onset of the perturbation.

One of the principal findings discussed in the second and third chapters of this dissertation was that deletion of both BAX gene alleles resulted in hyperinnervation of the heart in postnatal mice irrespective of whether one NGF allele was deleted. The fourth chapter of this dissertation tested the hypothesis that altering the neural input to the heart during development altered the setpoint of the β-adrenergic system in the heart.
This chapter examines the capacity of the cardiac sympathetic nervous system, and specifically, the capability of the cardiac myocyte, to adapt to perturbation in the form of either pre-synaptic denervation or post-synaptic β-adrenergic receptor blockade. The experimental paradigm was to either pharmacologically denervate the hearts of newborns and adult BAX-/ or BAX-/NGF+/- mice with 6-hydroxydopamine (6-OHDA) or block the β-adrenergic receptors mice with metoprolol, a β1-adrenergic receptor blocking agent, during the period of myocardial innervation by sympathetic neurons. Compensatory responses were assessed in newborn, neonatal and adult wild type and mutant mice by measuring βAR density, and binding affinity of βAR, and cAMP concentrations in myocardial membrane preparations. Collectively, these data will lead to a better understanding of whether altering the development of sympathetic neuron/β-adrenergic signaling in BAX-/ and BAX-/NGF+/- mice alters their capability of responding to perturbation.

METHODS

BAX and BAXNGF mutant mice

The BAX and NGF mutant mouse strains were housed, mated, and genotyped according to the protocols described previously. HW/BW ratios, cardiac [NE], βAR binding assay, and cAMP assays were also performed according to the procedures described previously.
**Administration of 6-OHDA**

The mice were administered a single injection of 6-hydroxydopamine (6-OHDA) (150 mg/kg ip) at P0, P18, and P57 to ablate sympathetic innervation. This drug is cytotoxic to sympathetic neurons in the periphery and ablates most of them within a few days of administration at this dosage (Story and Walro, unpublished data). Mice were euthanized by decapitation on P3, P21, and P60 for analyses of ßAR binding and adenylate cyclase activity.

**Metoprolol Pellets**

P0 mice were not utilized in this portion of the study due to the small size of the mice and the implantation procedure. Eight-day old (P8) mice were anesthetized by hypothermia. Mice were on a sterile gauze pad overlying a block of ice. Sterile techniques were used to surgically implant pellets of metoprolol, a ß<sub>1</sub>-adrenergic receptor antagonist, subcutaneously in the nape of the neck. Pellets were a 0.25 mg dose, 60-day release and 3 mm diameter. After implantation of the pellet the incision was sealed with NuSkin liquid. Pups recovered on a heating pad, after which they were returned to the mother and monitored for acceptance. Mice were euthanized by decapitation on P21 and P60 for analysis of HW, [NE], ß-adrenergic receptor density, and cAMP concentrations. Metoprolol administration caused drastic decreases in innervation density, ß-AR density, and ß-AR signaling of WT and BAX/- mice; since untreated Bax/-NGF+/- mice have shown lower innervation density, ß-AR density, and ß-AR signaling than WT and null mutant mice they were not included in this study.
Statistical Analysis

Mice were grouped according to age and genotype. A SigmaStat statistical program was utilized to analyze data. A probability level of P<0.05 was used to determine statistical significance. A series of one-way ANOVAs (SigmaStat) were used to elucidate differences among groups. Significant differences denoted by ANOVA were analyzed post hoc by Student-Neuman-Keuls tests. All values are expressed as mean ± SEM.

RESULTS

Treatment with 6-OHDA

Heart/body weight in BAX +/-, BAX-/-, and BAX-/-NGF/-/- mice with 6-OHDA

Treatment of 6-OHDA to P0 mice had different effects on heart and body weights than weanling and adult mice. In general, denervation caused increased neonatal body weight compared to intact mice of the same genotype (Table 4). At P21 and P60, however, denervation caused an average deficit of 11% in body weight compared to intact genotype-matched mice. Slotkin’s research in rats found a 10% deficit of body weight after 6-OHDA administration (Slotkin et al. 1995). WT mice had a statistically significant increase in HW at P0 with 6-OHDA compared to age and genotype-matched mice without 6-OHDA. Slotkin’s research measured initial deficits in heart weight of neonates after 6-OHDA which was not seen here. The differences in measurements may be due to the different animals used, the current study used mice while Slotkin used rats, or the BAX knockout model. In general, heart weights after weaning were unchanged in
Table 4. Heart weight (HW), Body weight (BW), and HW/BW ratios in BAX and BAXNGF mice with and without denervation. Heart weights are expressed in milligrams (mg). Body weights are expressed in grams (g). Values are expressed as mean ± SEM. Sample sizes are denoted in parentheses. All three genotypes had decreased HW/BW ratio with denervation at P0, but increased ratios at P21 and P60 compared to age/genotype-matched intact mice. HW/BW ratio was decreased at P0 due to a greater increase in HW relative to the increase in BW. At P21, there was a trend of increased HW, but decreased BW which led to increased HW/BW ratios. At P60, the trend of decreased HW and BW which caused decreased HW/BW ratios. Asterisk (*) denotes P<0.05, double asterisk (**) denotes P<0.01, and triple asterisk (***)) denotes P<0.001 compared to age-matched intact WT mice. Upper case letters represent significance of treated mice compared to age/genotype matched intact mouse: “A” denotes P<0.05, “B” denotes P<0.01, “C” denotes P<0.001. Lower case letters represent significant difference compared to P0 within genotype: “a” denotes P<0.05, “b” denotes P<0.01, “c” denotes P<0.001. Double hatched bar (‡) denotes P<0.001 compared to P21 within genotype.
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<th>HW/BW (mg/g)</th>
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<td>1.48 ± 0.05 (39)**</td>
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<td>6.86 ± 0.02 (11) b‡</td>
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</table>
response to denervation consistent with Slotkin’s work. HW/BW ratio with and without 6-OHDA administration showed had a peak at P21 regardless of genotype. Denervated neonatal WT mice had significant deficits in HW/BW ratio, while BAX/- and BAX/- NGF+/- also had decreases but these were not statistically significant compared to age/genotype matched mice without 6-OHDA. Weanling mice of all three genotypes had a significant increase in HW/BW ratio compared to intact weanling mice of the same genotype. This increase in HW/BW ratio persisted into adulthood, but was not statistically significant.

**Binding of [¹²⁵I] ICYP in mice administered 6-OHDA**

Specific binding to [¹²⁵I] ICYP represented approximately 65% of the total binding in mice of BAX and BAXNGF strains. Protein content of cardiac membranes was equivalent among all groups (data not shown). Mean (+ SEM) receptor densities ($B_{max}$) of denervated BAX+/+ mice were equivalent to untreated BAX+/+ mice at P0 and P21, but significantly (P<0.05) upregulated at P60 (Figure 12). The lack of compensatory response seen until adulthood is consistent with Slotkin et al. 1995. However, βARs were significantly (P<0.05) increased at all ages in denervated BAX/- and BAX/-NGF+/- mice compared to age- and genotype- matched intact animals (Figure 12). BAX/- and BAX/-NGF+/- mice showed an early and more robust compensatory response to chemical sympathectomy compared to WT (Figure 13). Dissociation constants ($K_d$) were equivalent (P>0.05) for BAX+/, BAX/-, and BAX/-NGF+/- mice at all three ages (Table 5).
Figure 12. Cardiac β-AR density in untreated WT, BAX-/-, and BAX-/-NGF+/- mice and mice treated with 6-OHDA (DN). Asterisk (*) denotes $P<0.05$, double asterisk (**) denotes $P<0.01$. Sample sizes are noted for mice treated with 6-OHDA. Sample sizes of untreated mice are the same as those expressed in Figure 10 on page 79 and are omitted from the figure for clarity. There was a significant upregulation of β-AR density in BAX-/- and BAX-/-NGF+/- mice at all three ages compared to age-matched untreated mice of the same genotype.
Figure 13. Percent change in β-AR density of BAX and BAXNGF mice after denervation. WT mice upregulated B\textsubscript{max} at P60 after denervation. However, BAX-/- and BAX-/-NGF+/- mice showed a greater compensatory response to denervation at P60 than wild type mice, as well as compensatory responses at P3 and P21.
WT BAX-/- BAX--NGF+-

**Change in Bmax (%)**
- P3
- P21
- P60

**Genotype**
- WT
- BAX-/-
- BAX--NGF+-

**Change in Bmax (%)**
- WT: Low
- BAX-/-: Moderate
- BAX--NGF+-: High
Table 5. Binding affinities ($K_d$) (nM) of BAX and BAXNGF mice. No statistically significant difference ($P > 0.05$) in binding affinities was observed among mice of different ages grouped by genotype, among mice of different genotype grouped by age, or between untreated mice and mice treated with 6-OHDA (DN) grouped by genotype and age.
<table>
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<th>Genotype</th>
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<th>P21</th>
<th>P60</th>
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<td>0.14 ± 0.02 (6)</td>
<td>0.12 ± 0.01 (6)</td>
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<td>BAX+/+ DN</td>
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<td>0.12 ± 0.05 (3)</td>
<td>0.09 ± 0.02 (9)</td>
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<tr>
<td>BAX/-</td>
<td>0.17 ± 0.02 (3)</td>
<td>0.20 ± 0.06 (4)</td>
<td>0.12 ± 0.003 (4)</td>
</tr>
<tr>
<td>BAX/- DN</td>
<td>0.15 ± 0.01 (3)</td>
<td>0.19 ± 0.04 (4)</td>
<td>0.16 ± 0.04 (4)</td>
</tr>
<tr>
<td>BAX/-NGF+/-</td>
<td>0.13 ± 0.02 (3)</td>
<td>0.31 ± 0.19 (3)</td>
<td>0.22 ± 0.09 (3)</td>
</tr>
<tr>
<td>BAX/-NGF+/- DN</td>
<td>0.19 ± 0.06 (3)</td>
<td>0.14 ± 0.03 (3)</td>
<td>0.23 ± 0.11 (3)</td>
</tr>
</tbody>
</table>
cAMP concentrations in preparations from mice treated with 6-OHDA

Denervated BAX-/- mice had significantly increased basal cAMP at P21 (P<0.01) and P60 (P<0.001), whereas denervated BAX+/+ mice had significantly increased basal cAMP only at P60 (P<0.001) compared to intact age- and genotype- matched animals. (Figure 14A).

No significant increases in cAMP production in response to low levels of isoproterenol (1µM concentration) after denervation were measured in the three genotypes at all ages (Figure 14B). The greatest response to isoproterenol of denervated mice occurred at P60. Increases in cAMP in response to low levels of isoproterenol were 25%, 33%, and 6% for denervated BAX+/+ , BAX-/- mice, and BAX-/-NGF+/- mice respectively. However, these data were not statistically significant (P>0.05).

With high levels of isoproterenol (10 µM concentration) stimulation after denervation, all genotypes had a significantly (P<0.01) larger response at P60 compared to intact animals of the same age (Figure 14C). At P21 denervation and high isoproterenol increased cAMP by 19% (P>0.05) in BAX-/- mice and 29% (P<0.01) in BAX-/-NGF+/- mice. Compared to similar intact animals, denervation coupled with higher levels of isoproterenol caused augmented cAMP production in BAX+/+ by 23% (P<0.01), in BAX-/- mice by 31% (P<0.001), and in BAX-/-NGF+/- mice by 28% (P<0.01). cAMP was not increased in response to high level beta agonist in denervated neonatal mice, regardless of genotype.

As with the previous cAMP measurements after stimulation (CH.4), all three genotypes displayed a trend of increased responsiveness to isoproterenol stimulation with
Figure 14. A. Percent change of basal cyclic AMP levels after denervation in BAX and BAXNGF mice. All genotypes had a measurable increase in basal cAMP after denervation into adulthood. BAX+/+, -/-, and BAX-/-NGF+/- mice had a significant (P<0.05) increase in basal cAMP levels after denervation at P60. All bars in histogram represent sample sizes of 3-5 mice/group. Double asterisk (**) denotes P<0.01, triple asterisk (***) denotes P<0.001.

B. Percent change of low stimulated [1uM Iso] cyclic AMP following denervation of BAX and BAXNGF mice. All three genotypes showed an attenuated production of cAMP following denervation at P3. Denervation and low levels of isoproterenol caused significant increases in cyclic AMP production in adult BAX+/+ (25%), BAX+/− (15%), and BAX-/− (33%) mice. Denervated BAX-/− mice had a tendency of increased cAMP production to low isoproterenol levels at P21. Denervated BAX-/− mice had an increase of 14% above intact BAX-/− mice stimulated with the same concentration of isoproterenol. All bars in histogram represent sample sizes of 3-5 mice/group.

C. Percent change in high stimulated [10uM Iso] cyclic AMP from denervation in BAX and BAXNGF mice. BAX-/− mice had an elevated ability to produce cyclic AMP at P21 (19%) compared to intact null mutant mice. BAX-/−NGF+/- also had an increased level of cAMP (29%) at P21 compared to intact animals of the same genotype (P<0.05). Most groups except BAX+/-NGF+/- had an increase in cAMP with denervation and a stimulation of 10uM isoproterenol: BAX+/+ 23% (P<0.01), BAX+/− 34% (P<0.05), BAX-/− 31% (P<0.001), and BAX-/−NGF+/- 28% (P<0.05). All data points represent group sizes of 3-5/group. Double asterisk (**) denotes P<0.01, triple asterisk denotes P<0.001.
age. Furthermore, the trend of increased responsiveness to isoproterenol stimulation in BAX null mutant mice was present after denervation at all ages; whereas, this response was blunted in BAX-/- mice that also had a deletion of a single NGF allele.

Treatment with $\beta_1$-adrenergic receptor blockade

Heart/body weight in BAX +/+, +/−, and −/− mice with metoprolol pellets

At P21 but not P60, hearts were significantly (P<0.01) heavier in wild type and null mutant mice, 20% and 26% relatively, treated with metoprolol than age/genotype-matched untreated mice (Table 6). Metoprolol treated null mutant mice, but not wild-type mice, had significantly heavier (P<0.001) body weights at P21 compared to untreated mice of the same genotype. There were no statistically significant (P>0.05) differences in adult BAX+/+ and BAX-/- mice in HW, BW, and HW/BW ratio with or without metoprolol treatments. However, there was a trend toward larger HW, BW, and HW/BW ratios in both wild type and null mutant mice treated with metoprolol compared to untreated mice.

Cardiac NE concentration with metoprolol pellets

Ventricular NE concentration increased with age in MP treated BAX mice, as occurs in untreated mice. NE concentration in weanling and adult BAX +/+, +/−, and −/− mice was significantly attenuated (Figure 15) after metoprolol treatment compared to age/genotype matched controls (P<0.001).

$\beta$AR Density with metoprolol pellets
Table 6. Heart weight (HW), body weight (BW), and HW/BW ratio in BAX untreated and treated with metoprolol. Ratios were similar in both groups at P21 and P60. Sample sizes are representative of those treated with metoprolol only for clarity. Double asterisk (**) denotes $P<0.01$, triple asterisk (***)) denotes $P<0.001$ compared to age and genotype matched mice without treatment; c denotes $P<0.001$ compared to age matched control mice, MP= treated by implantation of metoprolol pellets.
<table>
<thead>
<tr>
<th>Age</th>
<th>Genotype</th>
<th>HW (mg)</th>
<th>BW (g)</th>
<th>HW/BW (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAX+/+</td>
<td>63.98 ± 2.22 (33)</td>
<td>8.55 ± 0.31 (33)</td>
<td>7.58 ± 0.21 (33)</td>
</tr>
<tr>
<td></td>
<td>BAX+/+MP</td>
<td>76.44 ± 3.12 (16)**</td>
<td>9.50 ± 0.53 (16)</td>
<td>8.21 ± 0.80 (16)</td>
</tr>
<tr>
<td></td>
<td>BAX-/-</td>
<td>63.82 ± 2.28 (20)</td>
<td>7.27 ± 0.25 (20)</td>
<td>8.78 ± 0.26 (20) c</td>
</tr>
<tr>
<td></td>
<td>BAX-/-MP</td>
<td>80.68 ± 3.40 (13)**</td>
<td>9.30 ± 0.42 (13)***</td>
<td>8.69 ± 0.35 (13)</td>
</tr>
<tr>
<td>P21</td>
<td>BAX+/+</td>
<td>139.33 ± 3.98 (37)</td>
<td>23.12 ± 0.55 (37)</td>
<td>6.06 ± 0.11 (37)</td>
</tr>
<tr>
<td></td>
<td>BAX+/+MP</td>
<td>154.87 ± 11.65 (10)</td>
<td>24.97 ± 1.46 (10)</td>
<td>6.26 ± 0.50 (10)</td>
</tr>
<tr>
<td></td>
<td>BAX-/-</td>
<td>138.94 ± 5.24 (17)</td>
<td>22.84 ± 0.56 (17)</td>
<td>6.36 ± 0.24 (17)</td>
</tr>
<tr>
<td></td>
<td>BAX-/-MP</td>
<td>141.10 ± 9.54 (9)</td>
<td>21.73 ± 0.54 (9)</td>
<td>6.48 ± 0.32 (9)</td>
</tr>
</tbody>
</table>
Figure 15. Cardiac NE concentration in metoprolol treated BAX mice. NE concentrations were significantly attenuated in +/+ and -/- mice at P21 and P60. Triple asterisks (*** denotes P<0.001.
Figure 16. Density of β-AR in BAX mice in untreated and metoprolol treated mice.

β-AR density was significantly attenuated in BAX+/+ and BAX-/− mice implanted with metoprolol pellets (P<0.001). Numerals denote sample sizes. Asterisk (*) denotes P<0.05 compared to age-matched WT mice; triple asterisk (***) denotes P<0.001 compared to age/genotype-matched untreated mice.
Postnatal Age (days)

Bmax (fmol/mg)

P21  P60

*BAX+/+

*BAX+/+MP

*BAX-/−

*BAX-/−MP
In parallel with the norepinephrine concentration data, beta-adrenergic receptor density was decreased after metoprolol treatment (Figure 16). Beta-adrenergic receptor density was significantly attenuated at P21 and P60 in BAX+/+ and BAX-/- (P<0.001) compared to untreated age/genotype-matched controls.

Cyclic AMP Levels with metoprolol pellets

Basal cyclic AMP levels were significantly (P<0.05) different in BAX+/+ mice, but not null mutant mice, at P21 and P60 after metoprolol treatment but not in null mutant mice (Figure 17A).

A low level of stimulation with 1µM isoproterenol elicited a less robust response in animals treated with metoprolol throughout development relative to untreated age-genotype-matched mice (Figure 17B). There was a trend toward a decreased capacity to respond to low levels of isoproterenol after metoprolol administration across age (P21 to P60) and genotype, (P>0.05).

A higher level of isoproterenol stimulation (10µM) after metoprolol treatment was not able to produce cAMP levels similar to age- and genotype- matched untreated animals (Figure 17C). BAX+/+ and -/- mice had a less robust response to a high level of isoproterenol after metoprolol treatment at P21 (P<0.05) and P60 (P<0.05). Although wild-type mice had a decreased responsiveness to beta agonist stimulation, basal activity was higher. Therefore, absolute values of cAMP in wild-type mice were similar in untreated and treated mice; however, BAX-/- mice had a diminished ability to respond to a beta agonist after metoprolol treatment. Similar basal and stimulated (10µM) values have been reported previously in wild type mice (Xiang et al. 2002).
Figure 17. **A.** Basal Cyclic AMP levels in BAX mice with and without metoprolol. Slopes from P21 to P60 were similar with each genotype shifted up with metoprolol treatment. Asterisk (*) denotes P<0.05, double asterisk within genotype untreated vs. treated, “a” denotes P<0.05 comparing BAX+/+ vs. BAX-/- after treatment. All data points represent 3-5 hearts/group.

**B.** Low stimulation cAMP levels in BAX mice with and without metoprolol. After treatment with metoprolol, the heart tissue was stimulated with [1µM] isoproterenol. Both BAX+/+ and BAX-/- mice had a less robust cAMP production in response to isoproterenol after metoprolol treatment. All data points represent 3-5 hearts/group.

**C.** High stimulation cAMP levels in BAX mice with and without metoprolol. This represents heart tissue taken from metoprolol treated mice that was then stimulated with [10 µM] isoproterenol. Both genotypes had decreased βAR response to a β-agonist after treatment with metoprolol. Asterisk (*) denotes P<0.05. All data points represent 3-5 hearts/group.
DISCUSSION

Chemical Sympathectomy

In a mature organism, an increase of presynaptic activity elicits two responses: 1) desensitization, uncoupling of receptor from response elements and 2) downregulation, a process whereby receptors are internalized from the cell membrane into the cell (Hausdorff et al. 1990, Palczewski and Benovic 1991, Mayor et al. 1998, Tsao and von Zastrow 2000). In contrast, a decrease in presynaptic activity causes 1) supersensitivity and 2) upregulation of receptors (Hausdorff et al. 1990, Palczewski and Benovic 1991, Mayor et al. 1998, Tsao and von Zastrow 2000). In the developing animal, these relationships can be absent or reversed (Giannuzzi et al. 1995, Hou et al. 1989, Slotkin et al. 1996). BAX deletion induces hyperinnervation throughout development and presumably lowers the set-point. The purpose of this study was to determine if the compensatory response seen in normal mice is altered with the deletion of BAX.

In the mature cardiac sympathetic nervous system, sympathectomy upregulates beta-adrenergic receptors in the heart. Wild type mice had an upregulation of ßAR density at P60 only. This finding is consistent with previous research (Garofolo et al. 2002, Giannuzzi et al. 1995, Slotkin et al. 1995, Slotkin et al. 1996). BAX null mutant mice with both or one NGF allele present have an upregulation of receptors at all three ages. This suggests an early maturation in BAX null mutant mice. Based on the BAX knockout model, the sympathetic neurons may mature earlier than normal due to the deletion of BAX (Vekrellis et al. 1997). This state of maturation may be communicated
to the postsynaptic cell by NE: the amount release, timing of release, or a combination of the two. Therefore, when the sympathetic innervation is eliminated the system responds as a mature system even in the early postnatal period. Thus, deletion of BAX allows the sympathetic βAR system to respond as a mature system to sympathectomy (anatomical denervation).

Similar to previous research, the response of adenylate cyclase after denervation in control mice is augmented only in adult mice (Slotkin et al. 1995). The sensitization after denervation of βAR signaling in BAX null mutant mice occurred at the P21 and P60 timepoints. In Chapter 4, I proposed that the deletion of BAX may increase the sensitization of βAR signaling due to 1) increased ratio of β₂AR:β₁AR ratio, 2) increased coupling efficiency between β₁AR and G proteins, 3) increased number of Gas proteins, or 4) decreased number of Gai proteins. Possibilities 3) the increased number of Gas proteins and 4) decreased number of Gai proteins occur as a result of increased sympathetic stimulation (Morris & Bilezikian 1986, Reithmann et al. 1990, Auman et al. 2002a, Auman et al. 2002b). Since the sensitization is present after denervation, these two possibilities are most likely not the main cause of this response. Thus, the augmented sensitivity of βAR signaling in BAX-/- mice might either be from increased ratio of β₂AR:β₁AR ratio or increased coupling efficiency between β₁AR and G proteins.

Similar to BAX-/- mice, BAX-/-NGF+//- mice responded to sympathectomy with upregulation of βAR at all three ages studied. However, this sensitization to denervation in BAX-/-NGF+//- mice was less than the response observed in the BAX-/- mice. The alterations that occurred in BAX-/- mice were present, however, the loss of NGF
negatively affects these changes. The decreased βAR signaling in BAX-/−NGF+/-mice might be due to the effect of NGF on Gas and Gai protein levels (Morris & Bilezikian 1986, Reithmann et al. 1990, Auman et al. 2002a, Auman et al. 2002b). Decreased Gas and increased Gai proteins could decrease overall cAMP production. Another possibility is that NGF affects synaptic transmission. When high doses of NGF were added to neuron-myocytes cocultures there were increases in myocyte beat rate (Lockhart et al. 1997). The increased beat rate was decreased by withdrawal of NGF from the media (Lockhart et al. 1997). Additionally, the average response to stimulation (i.e. myocytes beat rate) increased more in cultures with high NGF levels compared to cultures with low NGF levels (Lockhart et al. 1997). Furthermore NGF potentiation of synaptic transmission between sympathetic neurons and cardiac myocytes acts through the trkA receptor (Lockhart et al. 1997). Therefore, levels of myocardial NGF affect the signaling between sympathetic neurons and cardiac myocytes.

βAR signaling in BAX-/−NGF+/- mice was greater than WT mice, but less than BAX-/− mice. Regardless of the presence or absence of NGF, BAX deletion caused sensitization of the βAR signaling. Furthermore, these data suggest deletion of one NGF allele decreased the response of signal transduction pathway of the βAR. These data demonstrate that post-synaptic disruption of the cardiac sympathetic nervous system leaves an intact beta adrenergic signaling pathway. Since NGF is actually produced in the heart and helps determine the level of sympathetic innervation, it would make sense that the level of NGF could be a determining factor in βAR signaling. Although, the
expression of NGF does not seem to determine βAR density, it does affect signaling through the βARs.

**Beta-adrenergic receptor blockade**

BAX null mutant mice are a model of cardiac hyperinnervation capable of an compensatory response to anatomical denervation in the early postnatal period. Administration of 6-OHDA, however, occurred only three days prior to euthanasia and thus represents an acute response to a one-time stimulus. The next logical step was to determine the response of BAX knockout mice to a long-term sympathetic perturbation, particularly during the period of cardiac sympathetic maturation. Mice were implanted with a selective β₁-adrenergic receptor blocker in the early postnatal period. Hearts were then collected at P21 (a point in the middle of cardiac sympathetic maturation) and at P60 (after cardiac sympathetic nervous system matures). Therefore, these two points represent early sympathetic perturbation 1) in the pre-maturation process and 2) in the post-maturation process.

There was a tendency of WT and BAX null mutant mice to have heavier hearts with metoprolol treatment compared to mice without treatment; these data coincide with previous research (Bostrom and Fryklund 1979). Additionally, WT and BAX-/- mice tended to be heavier with metoprolol treatment relative to genotype- and age-matched mice without beta blocker; these data coincide with data from infants receiving beta-blocker therapy in the pre-maturation period for severe congestive heart failure (Buchhorn et al. 1998). Beta blocker treatment during sympathetic development dramatically decreased cardiac innervation density. Unlike administration of 6-OHDA
which specifically damages sympathetic neurons, metoprolol should only bind to the beta receptors on the myocyte sarcolemma and affect the system post-synaptically. One possibility for the deleterious effect of metoprolol on heart innervation might be the interaction between metoprolol with endothelin-1 (ET-1) promoter activity. ET-1 is a growth-promoting peptide produced by multiple cell types including cardiac myocytes (Morimoto et al. 2001). Previous research has demonstrated that metoprolol severely blocks NE from stimulating ET-1 promoter activity (Morimoto et al. 2001). Disruption of ET-1 has been found to decrease NGF expression, sympathetic nerve density, and cardiac NE concentration (Ieda et al. 2004). The observation that metoprolol administration in mice caused significant decreases in cardiac [NE] supports this hypothesis. Therefore, metoprolol treatment during development might have blocked the establishment of the cardiac sympathetic system.

Treatment with metoprolol caused significant decreases in ßAR density of control and wild-type mice. The density of ßAR measured approximates those found in ß1/-/- mice (Rohrer et al. 1996). This suggests metoprolol treatment during development caused down-regulation of the ß1 receptors; thus, the low level of receptors measured is most likely ß2AR on myocardial cells. The level of ß2ARs is similar to a previous study (Rohrer et al. 1996), thus an increased basal production of cAMP, in BAX/-/- mice mentioned in Ch.4 is unlikely due to increased ß2AR: ß1AR ratio.

Although, metoprolol treatment decreased the density of ßAR, the basal levels of cAMP are elevated in weanling and adult control mice, and adult null mutant mice. The two remaining explanation for altered ßAR signaling associated with BAX deletion are 1)
increased Ga₃ proteins or 2) increased efficiency of coupling of β₁AR with G proteins. Beta blocker therapy enhances Ga₃ function in adult hearts (Wang et al. 1999). Since these two explanations are not mutually exclusive, increased efficiency of coupling between β₁AR and G proteins may also occur. After metoprolol treatment, which downregulated β₁AR density, the basal cAMP is not changed, but the response to an agonist is diminished.

After metoprolol treatment during development, WT and null mutant mice have a decreased responsiveness to isoproterenol. The remaining receptors are hypothesized to be β₂AR which are capable of activating Ga₁ or Ga₃ proteins (Lohse et al. 2003). At rest, the β₂AR couples more efficiently to Ga₃ leading to higher basal levels which occurs in β₁AR knockout mice and transgenic mice overexpressing cardiac β₂AR (Xiang et al. 2002, Lohse et al. 2003). When stimulated for about 15 min, as occurred in this experiment, β₂AR signaling is through Ga₁ (Xiang et al. 2002). Similar basal and stimulated cyclic AMP responses are seen in β₁AR knockout mice (Xiang et al. 2002) thereby supporting the hypothesis that this response is mediated through the remaining β₂ARs. Absolute levels of cAMP were attenuated in BAX-/- mice after isoproterenol stimulation. This data coupled with the hypothesis that the remaining receptors are β₂ARs, suggests the main cause for altered βAR signaling in BAX mice is from increased coupling efficiency of β₁ARs and G proteins.

Currently infants with CHF or congenital heart defects (CHD) that do not respond well to traditional therapies receive beta blockers, such as metoprolol (Buchhorn, et al 1998, Buchhorn et al. 2001, Shaddy et al. 1995, Shaddy et al. 1999, Ishikawa et al. 1999,
Brungs et al. 2001, Liler et al. 2002, Rusconi et al. 2002). These infants are receiving metoprolol prior to the maturation of the cardiac sympathetic nervous system, similar to the design of the metoprolol study. The decreased cardiac NE concentration, βAR density and signaling after metoprolol treatment during cardiac sympathetic development may describe significant repercussions on infants receiving beta blocker therapy. Infants and young children receiving beta blocker therapy gain weight and have decreased heart failure scores (Shaddy et al. 1999, Ishikawa et al. 1999, Bruns et al. 2001, Rusconi et al. 2002). This study found increased HW and BW with metoprolol administration in control and BAX-/- mice, or mice that develop normal and hyperinnervated hearts respectively. The present data suggests that the long term effects of metoprolol administration during cardiac sympathetic development may prevent innervation of the heart, as well as decreased βAR density and signaling. Neither survival rates nor cardiovascular function test were done for this study, therefore future studies are needed to determine if this effect of metoprolol is detrimental to the cardiac sympathetic system. As a caveat, the effects seen in the current experiments may be specific to metoprolol therapy, and therefore may not apply to other beta blocker therapies (i.e. propranolol, bisoprolol, carvedilol). Future research should focus on the long term effects on cardiac sympathetic innervation and function of infants on beta blocker therapy.

CONCLUSION

Chemical sympathectomy with 6-OHDA created a model of denervation similar to heart transplantation and demonstrated that BAX deletion sensitizes the cardiac
sympathetic system to denervation. When one NGF allele was present, βAR signaling was less sensitive. These data support previous findings that NGF is important in βAR signaling. Administration of a beta blocker during cardiac sympathetic development causes significant decreases in cardiac innervation, βAR density and signaling. It is not known whether these decreases of the cardiac beta-adrenergic system are detrimental to the function of the cardiovascular system. If these decreases are deleterious, then these data are significant for infants receiving metoprolol prior to cardiac sympathetic maturation. Currently research has not been done to determine the long-term effects of metoprolol administration on heart function of individuals whom received treatment prior to maturation of the cardiac beta-adrenergic system.
CHAPTER SIX

SUMMARY

A reduction of cardiac sympathetic innervation occurs with the deletion of one NGF allele (Table 7), and deletion of both NGF alleles leads to an almost complete loss of sympathetic innervation (Story 2000a). Conversely, overexpression of myocardial NGF leads to sympathetic hyperinnervation of the heart (Hassankhani et al. 1995). These two studies demonstrate the importance of NGF on neuron survival and differentiation. However, the role of NGF on survival and differentiation has not been partitioned. The results from the first study of this dissertation elucidated the role of NGF, as well as deletion of BAX, on cardiac sympathetic nervous system development. A parallel increase, though not a proportional increase in neuron survival and heart innervation, demonstrated that NGF may not set the upper limit of terminal differentiation. Deletion of BAX when NGF levels were normal (Table 7) leads to increased sympathetic innervation. Therefore a factor other than NGF sets upper limit. Therefore, these data suggest a factor other than NGF is involved in determining terminal differentiation. One potential factor is NT3. NT3 is produced in the heart and retrogradely transported in sympathetic neurons (Zhou & Rush 1995, Zhou et al. 1997). NT3 is known to affect survival and differentiation of sympathetic neurons, as evidenced by a loss of sympathetic neurons in NT3-/- mice (Belliveau et al. 1997, El Shamy et al. 1996, Ernfors
Table 7. Summary of NGF Overexpressor, NGF knockout, BAX knockout, and BAXNGF double knockout mice data. NGF+/-, BAX-/-, and BAX-/-NGF+/- mice compared to WT mice; denervated BAX-/- and BAX-/-NGF+/- mice relative to age- and genotype-matched intact animals; BAX-/- MP compared to untreated BAX-/- mice. NGF OE=NGF overexpressor mouse model, DN=denervated with 6-OHDA; MP=treated with metoprolol pellets; NM=not measured; ↓=decreased, ↓↓=almost complete loss, ↑=increased, “=” = equivalent to controls. All data from May 2005 unless otherwise indicated: ¹Hassankhani et al. 1995, ²Heath et al. 1998, ³Story 2000a, ⁴Glebova and Ginty 2004, *taken from BAX-/-NGF-/- mice data.
<table>
<thead>
<tr>
<th>Measurement</th>
<th>NGF OE</th>
<th>NGF +/-</th>
<th>BAX +/-</th>
<th>BAX/- DN</th>
<th>BAX/- MP</th>
<th>BAX/- NGF+/-</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Neuron Survival</td>
<td>↑¹</td>
<td>↓³</td>
<td>↑</td>
<td>NM</td>
<td>NM</td>
<td>↑³*</td>
<td>NM</td>
</tr>
<tr>
<td>Cardiac [NE]</td>
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<td>↓³</td>
<td>↑</td>
<td>↓↓</td>
<td>↓↓</td>
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<td>↓↓</td>
</tr>
<tr>
<td>β-AR Density</td>
<td>↑²</td>
<td>↓³</td>
<td>↓</td>
<td>↑</td>
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<td>↑</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
</tr>
</tbody>
</table>
et al. 1994). However, deletion of one NGF allele reduced innervation density by approximately 40% which is near WT levels. Therefore NGF is essential (which has already been established), but blocking apoptosis can compensate for NGF deficiency. Thus NGF is not the only determining factor of sympathetic neuron differentiation and BAX deletion compensates for loss of NGF.

The presence of increased sympathetic innervation makes BAX null mutant mice a potential model to study disease states associated with cardiac hyperinnervation. Deletion of BAX increases sympathetic innervation both in number of neurons and NE. Conditions such as myocardial infarction (MI) and/or congestive heart failure are characterized by increased sympathetic activity, ventricular arrhythmias, and regional hyperinnervation (Cao et al. 2000a, Cao et al. 2000b, American Heart Association website). Increased sympathetic tone is hypothesized to be a trigger for ventricular tachyarrhythmias (Cao et al. 2000a, Cao et al. 2000b, Schwartz et al. 1998, Schwartz et al. 1992, Schwartz et al. 1990, Lown et al. 1973). Whether this model spontaneously develops ventricular arrhythmias, analogous to that observed in CHF, should be examined.

Besides a role in sympathetic differentiation, this dissertation provides further evidence of the importance of NGF on beta-adrenergic signaling. With one NGF allele deleted β-AR signaling was decreased. Alterations in NGF levels might explain heart failure associated with decreased myocardial NGF and decreased β-AR signaling (Kaye et al. 2000). This hypothesis may also explain why cardiac transplant recipients, who have normal myocardial NGF levels, have no upregulation or supersensitivity of β-AR
(Denniss et al. 1989, Shimizu and McGrath 1993). This suggests that targeted NGF administration may be of therapeutic values for individuals with CHF and cardiac transplant recipient.

Future studies should focus on whether NGF administration can be targeted to the heart, and if so does this improve heart innervation and β-AR signaling. In dogs, NGF can be infused into the left stellate ganglion via an osmotic pump. In normal dogs and dogs with induced MI, NGF infusion caused cardiac nerve sprouting and hyperinnervation, but not sudden cardiac death (Chen et al. 2001, Cao et al. 2000a). However, NGF infused into dogs with induced AV block and MI, caused arrhythmias that led to sudden cardiac death (Chen et al. 2001). However, if nerve growth is stimulated in a heart with a damaged conduction system (as in AV block) the result can be sudden cardiac death. Thus NGF infusion may not be efficacious for individuals with a compromised cardiac conduction system, but may benefit other cardiac conditions.

Furthermore, these studies demonstrate the counterbalancing effect of BAX deletion and NGF deletion, as demonstrated in Figure 18. BAX deletion has a positive effect on neuron survival, innervation density and β-AR signaling, whereas, NGF deletion has a negative effect at these same features. Deletion of BAX or NGF both resulted in low levels of β-AR expression. The question arises why does increased innervation in BAX-/- mice not lead to increased β-AR density, or a higher set point. The decreased β-AR density in BAX-/- mice is a compensatory response from the increased levels of NE during development. According to Slotkin and co-workers (1995,
Figure 18. Effects of BAX or NGF deletion on the cardiac beta-adrenergic system. Effects of chemical denervation are shown on the left side. Effects of metoprolol treatment are shown on the right side. SG=Stellate Ganglion, NGF OE=NGF overexpressor mice, NE= norepinephrine, NGF= nerve growth factor, ET-1= endothelin-1. Changes from BAX deletion are shown in red, while changes from NGF deletion are shown in royal blue, and changes from NGF OE are in dark blue.
Renick et al. 1997), a compensatory mechanism, as just described, does not occur before or during the maturation period. Thus an additional question arises why a mature compensatory response occur in young BAX null mutant mice. One possibility might be that deletion of BAX causes the neurons to be in a state of early maturation. Normally, BAX levels are high and decrease as the neuron matures and makes appropriate connections with target organs (Vekrellis et al. 1997). This contention is further supported by the denervation data. The BAX/- and BAX/-NGF+/ mice have a compensatory response to denervation prior to the normal time of maturation. Together, these findings suggest that the switching of the system from immature to mature is initiated by the neurons. Therefore, blocking apoptosis might cause the neuron to mature earlier than normal. Further research need to be done to determine what factors and signals are involved in switching from an immature system without compensatory responses to a mature system able to compensate.

With BAX deleted, an early compensatory response was seen after chemical sympathectomy, but not with metoprolol administration. The early compensatory response is most likely initiated from the neurons, and the feedback loop may be specific to the $\beta_1$-ARs (Figure 18). Alternatively, the actual blocking of the beta-adrenergic receptors may have interrupted the signal from being received by the post-synaptic cell. The signal from the pre-synaptic cell that communicates the maturation response is most likely NE. NE decreases in response to decreased ET-1, and NGF was decreased in the present study. However, there was still a compensatory response in denervated mice which had extremely low levels of NE measured (data not shown). The compensatory
response is still intact because chemical sympathectomy was an acute treatment. Administration of metoprolol occurred over a longer period of time, thus, allowing time to disrupt the communication between the pre- and post-synaptic cell. These data suggest the long-term perturbation disables the early compensatory response caused by BAX deletion.

The final study demonstrated metoprolol administration during the developmental period caused drastic alterations in the cardiac β-adrenergic system. Regardless of the presence or absence of BAX, there were decrements in cardiac innervation and β-AR density (Figure 18). Administration of metoprolol is thought to cause an internalization of β₁-ARs. The decreased indices of the cardiac beta-adrenergic system in response to metoprolol administration during the developmental period have not previously been shown. These findings suggest that metoprolol administration during cardiac sympathetic development may have unwanted side effects. The human cardiac sympathetic nervous system is not mature until about six months of age. However, the use of a beta blocker during the high risk period of SIDS (second to third month of life) has been proposed as a preventative intervention (Schwartz 1976). This high risk period is the time when cardiac sympathetic innervation is being established, therefore, treatment with metoprolol during this period might be detrimental. Whether alterations in cardiac sympathetic function have deleterious physiological consequences is unknown. A future study should look at the long-term cardiovascular function of these mice treated with metoprolol. Since these changes are most likely related to metoprolol, a follow-up study can be done to determine if similar affects occur with other β-blockade therapy (i.e.
propranolol, bisoprolol, or carvedilol) during development. These decrements suggest metoprolol administration during development blocks the expression of ET-1, thus preventing NGF expression to allow innervation (Morimoto et al. 2001). These data explain the efficacy of metoprolol treatment in CHF and ischemic heart disease where regional hyperinnervation occur. The mechanisms responsible for internalization of β_{1}-ARs cannot be determined based on the current data, further study would be beneficial in this regard.

Although, NGF is a key protein involved in sympathetic differentiation, all of the factors responsible for terminal differentiation of the cardiac sympathetic system are not known. Based on this and previous research, NGF plays a role in neuron survival, innervation density, and β-AR signaling of the cardiac beta-adrenergic system. With these broad roles, targeting NGF may prove to be beneficial for cardiovascular disorders. Additionally, targeting the apoptosis pathway may have a counterbalancing effect on conditions of hypoinnervation. It has been proposed that BAX deletion also causes an early maturation of the cardiac beta-adrenergic system. Little is known about how this system switches from immature to mature with the ability to compensate to perturbation. Thus, the BAX, NGF, and BAXNGF knockout models lend insight into potential mechanism by which the cardiac beta-adrenergic system matures. These models help elucidate the roles of BAX and NGF on the development of the cardiac sympathetic nervous system. Understanding the role of BAX and NGF on the developing cardiac sympathetic system as well as the mechanisms of maturation may assist in the development of therapeutic treatment for sympathetic cardiovascular disorders.

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Appendix A. Saturation curve of beta-adrenergic receptors at P0. Curves represent neonatal BAX+/, BAX-/-, and BAX-/-NGF+/- mice.
Appendix B. Saturation curve of beta-adrenergic receptors at P21. Curves represent neonatal BAX+/+, BAX-/-, and BAX-/-NGF+/- mice.
P21

Specific Binding (fmol/mg tissue) vs [ICYP]

- +/+  
- +/-  
- -/-
Appendix C. Saturation curve of beta-adrenergic receptors at P60. Curves represent neonatal BAX+/+, BAX-/-, and BAX-/-NGF+/− mice. All saturation curves are similar to the three presented in the appendix.