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Egr3 is a Novel Transcriptional Regulator of Sympathetic Nervous System Development and Function

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ABSTRACT

Egr3 is a Novel Transcriptional Regulator of Sympathetic Nervous System Development and Function

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Early Growth Response genes (Egr1-4) are transcriptional mediators of signals governing growth and differentiation. In sympathetic neuron-derived cell lines, Egr1 is induced by NGF signaling, a pathway required for sympathetic neuron survival, differentiation and target organ innervation. In the absence of identifiable sympathetic defects in Egr1-deficient mice, we investigated the role of a closely related Egr family member Egr3 in NGF-mediated aspects of sympathetic neuron development. Here, we show that Egr3 expression is coupled to neurotrophin signaling *in vitro* and *in vivo*. Moreover, in Egr3-deficient mice, approximately 1/3 of sympathetic neurons die after birth due to increased apoptosis. These neuronal deficits manifest as decreased sympathetic innervation in a variety of target tissues as well as disrupted end-organ physiology. Indeed, loss of Egr3 results in blepharoptosis as well as abnormal cardiac and circadian physiologies. Detailed examination of Egr3-deficient mice revealed reduced axon arborization within a variety of target tissues. These results suggest that sympathetic neuron death in Egr3-deficient mice may be due to axon outgrowth defects and insufficient access to target tissue-derived trophic support since Egr3-deficient neurons do not

have NGF-dependent survival defects in vitro. In support of this hypothesis, target innervation defects identified in Egr3-deficient mice persist in Egr3/Bax double homozygous mutant mice, a model system in which sympathetic neuron death is rescued. Therefore, Egr3 plays a critical and novel role in differentiating sympathetic neurons, presumably inducing expression of genes involved in neurite branching or extension. Furthermore, Egr3-deficient mice have a sympathetic dysautonomia that resembles human disease, raising the possibility that Egr3 has an important role in human sympathetic nervous system development and/or sympathetic neuron degeneration.

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LIST OF ABBREVIATIONS

AANAT: arylalkylamine N-acetyl transferase

AraC: cytosine arabinoside

Casp3: cleaved (activated) caspase-3

DβH: dopamine beta-hydroxylase

DMSO: dimethyl sulfoxide

DKO: double knockout mouse

Egr: early growth response

FBS: fetal bovine serum

GAPDH: glyceraldehyde-3-phosphate dehydrogenase

H&E: hematoxylin and eosin

HR: heart rate

IHC: immunohistochemistry

LacZ: beta-galactosidase

MEK: mitogen-activated protein kinase kinase

MEM: minimal essential media

NGF: nerve growth factor

NT-3: neurotrophin-3

PBS: phosphate buffered saline

PN: postnatal

P/S: penicillin/streptomycin

KO: knockout (gene-deficient)

qPCR: quantitative real time RT-PCR

RT-PCR: real-time polymerase chain reaction

SCG: superior cervical ganglion

SNS: sympathetic nervous system

STG: stellate ganglion

TH: tyrosine hydroxylase

YOH: yohimbine

ZT: Zeitgeber time

DEDICATION

To my husband Brandon, my parents Jack and Mary, and my brother Jack who supported me through this incredible journey. I would not have made it to this point without you. Your confidence in me provided comfort and courage, especially when it was just the mice me at 3am.

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I. The sympathetic nervous system

Overview

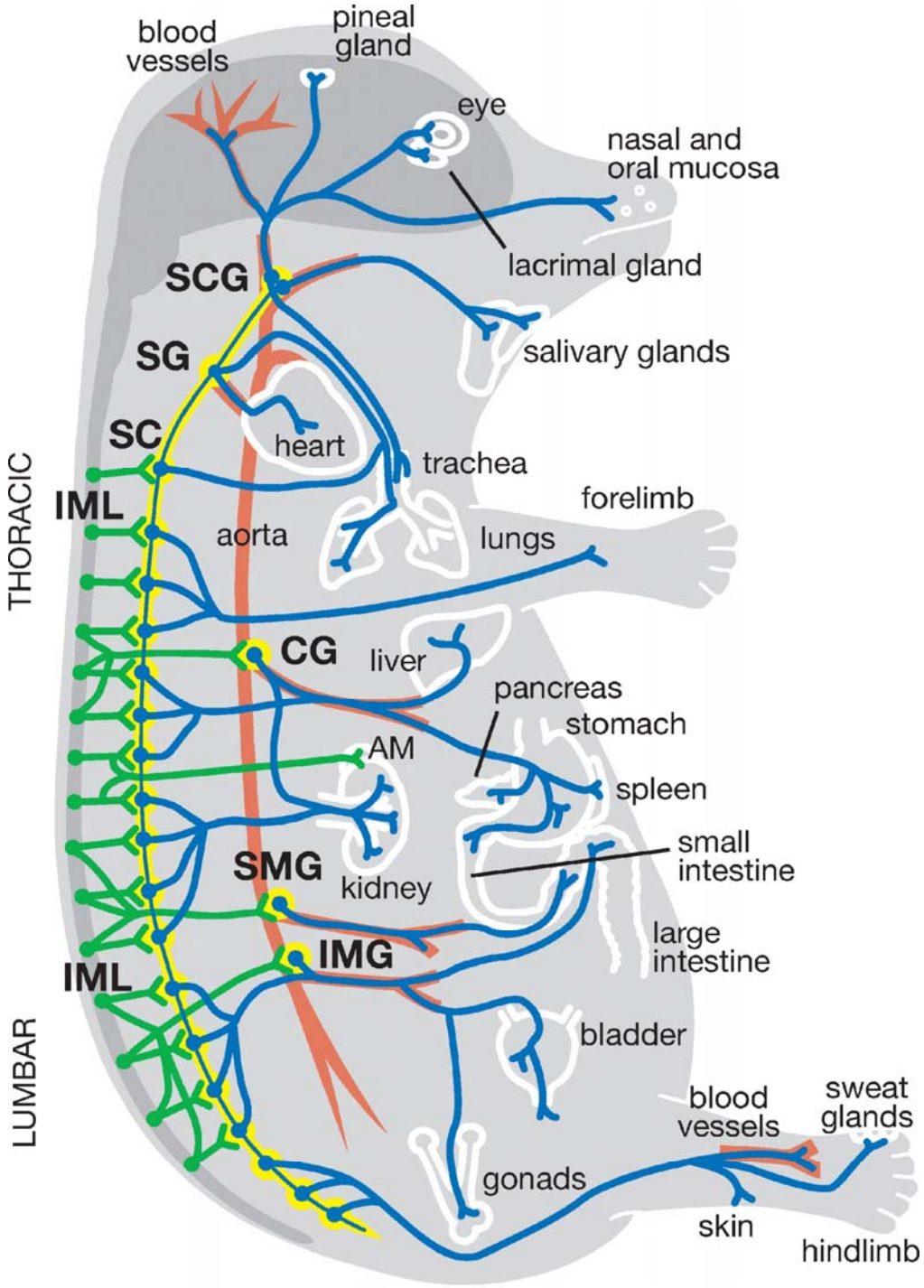
The autonomic nervous system maintains homeostasis in the body in the face of environmental changes, and is divided into the sympathetic and parasympathetic nervous systems. Balance is achieved through dual innervation of target organs by sympathetic and parasympathetic neurons that have opposing functions. Walter Cannon originally described the sympathetic nervous system contributing the "fight or flight response" while the parasympathetic nervous system directed an animal to "rest and digest" (Kandel 2000). Indeed, the sympathetic nervous system respond to stress by increasing heart rate and contractility, shunting blood from the gastrointestinal system and skin to musculature, dilating pupils to increase visual acuity, and expanding airways to improve oxygenation (Costanzo 1998). In the absence of a sympathetic nervous system, an animal is only able to survive if it is kept warm and protected from physical or emotional stress (Kandel 2000).

Anatomy

Sympathetic neurons project to innervate a variety of target tissues including, but not limited to, the eye, pineal gland, heart, salivary glands, trachea/bronchial tree, skin, kidney, bladder and intestines (Figure 1, (Glebova and Ginty 2005)). Also called the "thoracolumbar" nervous system, preganglionic sympathetic neurons extend from the T1 to L2 or L3 vertebral levels of

Figure 1. The murine sympathetic nervous system.

Preganglionic sympathetic neurons (green) extend from the intermediolateral horn of the spinal cord to synapse in the sympathetic chain of paravertebral ganglia or one of three prevertebral ganglia (CG=celiac ganglion, SMG=superior mesenteric ganglion, IMG=inferior mesenteric ganglion). The superior cervical ganglion (SCG) is the largest paravertebral ganglion and serves as an experimental source of neurons for the majority of *in vitro* studies concerning sympathetic neuron survival and differentiation. Postganglionic neurons (blue) extend axons to innervate a variety of target tissues. The chromaffin cells of the adrenal medulla (AM) receive preganglionic innervation from the spinal cord. (Figure from Glebova and Ginty, Annual Review of Neuroscience, 2005)



Glebova, NO and Ginty, DD. 2005
Annu. Rev. Neurosci. 28: 191–222

the lateral horn of the spinal cord. These preganglionic neurons synapse in paravertebral ganglia (sympathetic chain), prevertebral ganglia (celiac, superior mesenteric, inferior mesenteric ganglia) or the chromaffin cells of the adrenal medulla. Two sympathetic chains of paravertebral ganglia line the vertebrae while the three prevertebral ganglia lie between the sympathetic chain and the abdominal viscera they innervate. From these two types of ganglia, postganglionic neurons extend axons, often over great distances, to innervate a variety of target tissues.

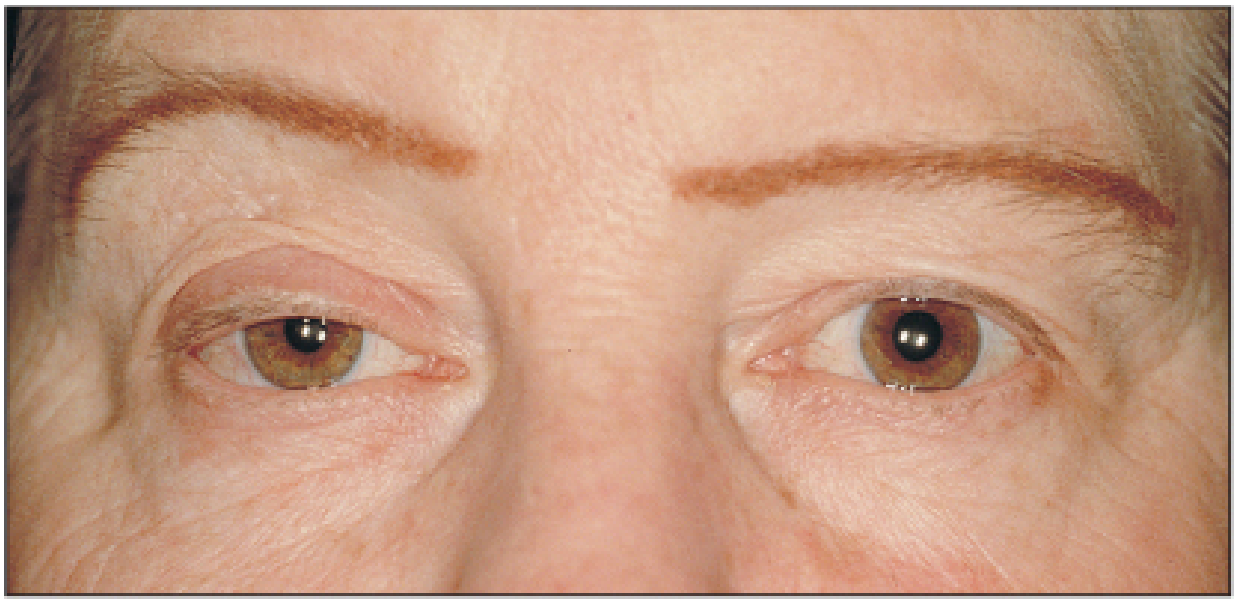
Clinical significance

Sympathetic nervous system (SNS) dysfunction results in humans and animals with perturbed cardiac, ocular, respiratory, and neurological physiologies. A relatively common example of localized sympathetic dysfunction is Horner's syndrome (Figure 2), in which injury to oculosympathetic system results in the classic triad of blepharoptosis, miosis, and anhidrosis (Gurwood 1999). A congenital form of Horner's syndrome also includes heterochromia since iris pigmentation requires migration of melanocytes along sympathetic axons (Laties 1974).

A more extensive state of sympathetic nervous system dysfunction is Familial Dysautonomia (FD) (also known as Riley-Day Syndrome and Hereditary Sensory and Autonomic Neuropathy (HSAN) type III). FD is an autosomal recessive neurodevelopmental disorder that affects the autonomic and sensory nervous systems (Axelrod 2004). 99% of FD cases share a common mutation that affects the splicing of *IKBKAP* in a tissue-specific manner (Slaugenhaupt et al. 2001; Cuajungco et al. 2003). Clinical features of FD include a variety of sympathetic, parasympathetic, and sensory dysfunctions including postural hypotension, intermittent high blood pressure, sweating while eating, corneal damage due to decreased

Figure 2. Horner's syndrome.

This patient has an interruption of the oculosympathetic system, resulting in the classic clinical triad of blepharoptosis (drooping upper eyelid), miosis (constricted pupil), and anhidrosis (lack of facial sweating) on the affected side of the face. These clinical signs are due to a lack of sympathetic innervation to the eyelid/superior tarsus muscle, iris, and facial sweat glands on the affected side of the patient. (Photograph from Yanoff: Ophthalmology, Horner's syndrome, Figure 86-4, Copyright ©2004, Mosby, Inc.)



tearing and an absent blink reflex, attenuated deep tendon reflexes, scoliosis, ataxia, seizures, hypotonia, repeated aspiration pneumonias, cyclic vomiting, decreased pain sensitivity, and the characteristic absence of fungiform papillae (Axelrod 2004).

In addition to developmental syndromes, dysfunction of the autonomic nervous system is a clinically significant problem in patients with a variety of comorbidities. Loss of sympathetic innervation to the heart occurs after cardiac transplantation. This cardiac denervation results in abnormal exercise tolerance after surgery, which is thought to be due to impaired ventricular function and chronotropic responses (Bengel et al. 2001). In addition, reinnervation of transplanted hearts is associated with improved cardiac physiology and exercise tolerance (Bengel et al. 2001). Sympathetic denervation also occurs in patients with more extensive disorders of the nervous system. Autonomic dysfunction, including orthostatic hypotension, urinary, and sexual dysfunction, is an almost universal feature of and a cause of significant morbidity in Parkinson's Disease (Poewe 2007). Moreover, autonomic dysfunction in individuals with Diabetes Mellitus is disabling and can be life threatening. Autonomic neuropathy is characteristic in diabetic neuropathy and can include orthostatic hypotension, resting tachycardia, and aberrant papillary responses as well as gastrointestinal, urinary, and sexual dysfunction (Said 2007).

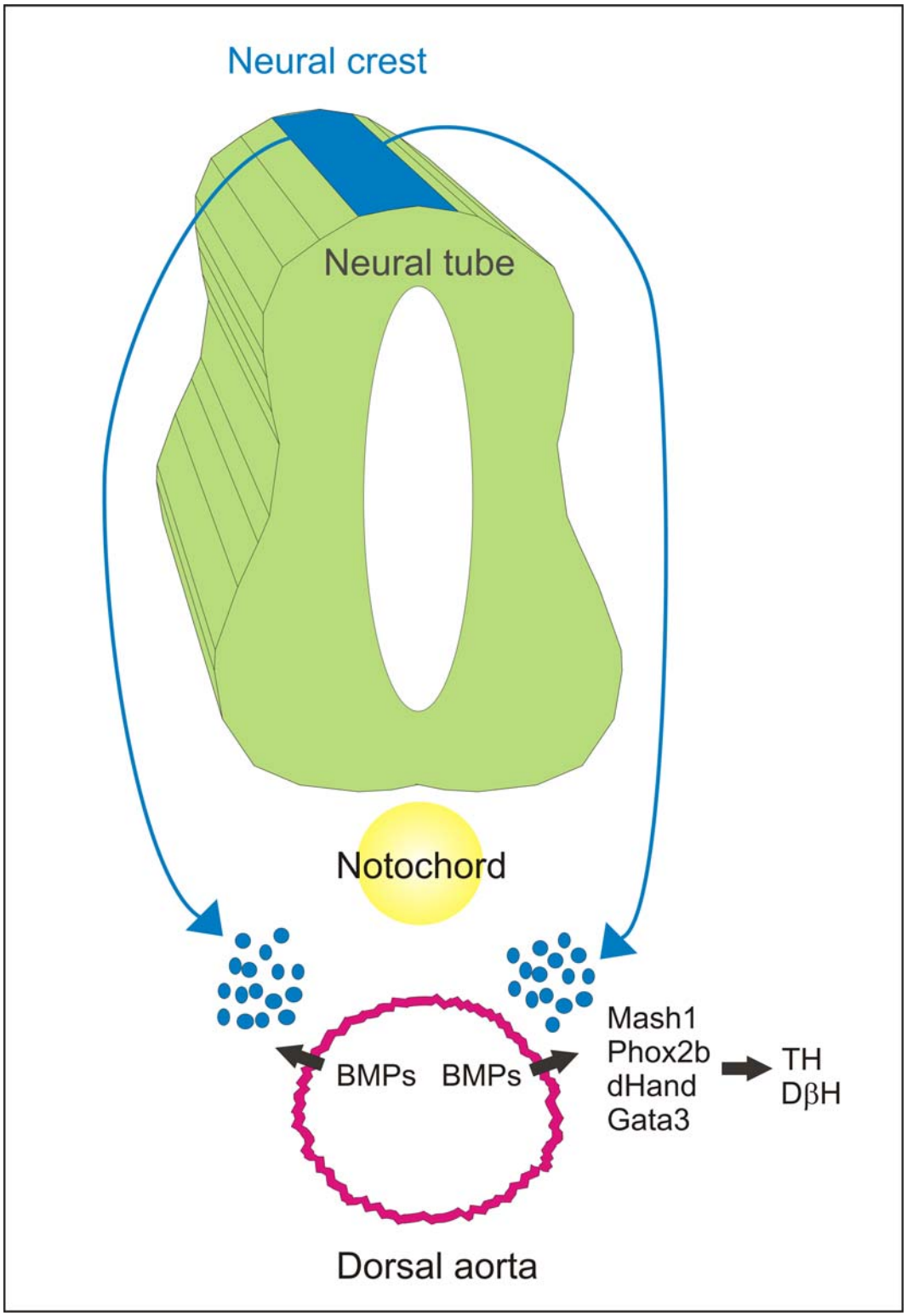
SNS dysfunction in the form of malignancy is particularly devastating. Neuroblastoma is derived from precursor sympathetic neurons, neuroblasts, which arise primarily from the adrenal medulla or the sympathetic chain of ganglia in the mediastinum. Treatment of this pediatric tumor is largely unsuccessful, and most children die within 1 year of diagnosis. However, patients with an unusual metastatic IV-S stage of disease have a relatively good prognosis because of spontaneous tumor regression and differentiation of the highly malignant

neuroblasts (Pritchard and Hickman 1994; Hedborg et al. 1995). Thus, impairment in differentiation of malignant neuroblasts appears to be a critical factor for disease progression and prognosis.

Given the morbidity and mortality associated with human SNS disorders, it is of great interest to elucidate what molecules control normal SNS development in order to understand and treat aberrant developmental and degenerative states. Moreover, greater understanding of processes governing growth and differentiation of sympathetic neurons may provide insight into the mechanisms mediating oncogenesis.

Figure 3. Migrating neural crest cells differentiate to form sympathoadrenal precursors.

Neural crest cells migrate from the top of the neural tube to the dorsal aorta where they differentiate into sympathetic neuron precursors and form primordial ganglia. This differentiation is mediated by secretion of BMPs from the dorsal aorta and subsequent induction of several transcription factors from tissue surrounding the developing sympathetic ganglia. This signaling cascade results in the neuronal precursors' acquisition of noradrenergic phenotypes including the expression of catecholaminergic enzymes tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DβH).


Neural crest
Neural tube
Notochord
BMPs
BMPs
Mash1
Phox2b
dHand
Gata3
TH
DβH
Dorsal aorta

Development

Sympathetic neurogenesis

CNS neurons and presynaptic autonomic neurons of the peripheral nervous system develop from the neural tube while cells of the sympathoadrenal (SA) lineage, sensory neurons, melanocytes, and glia develop from neural crest (Kandel 2000). SA cells migrate from the apex of the neural tube to the dorsal aorta, where they form a column of sympathetic ganglion primordia and acquire noradrenergic phenotypes (Figure 3), or to adrenal gland primordia where they differentiate to form chromaffin cells of the adrenal medulla (reviewed in (Anderson 1993; Glebova and Ginty 2005)). Bone morphogenetic proteins (BMPs) are necessary and sufficient to induce noradrenergic differentiation of neural crest cells (Schneider et al. 1999; Ernsberger 2000). In the vicinity of the developing sympathetic ganglia, BMPs induce several transcription factors including Gata2/3, Hand2, Mash1 and Phox2a/b (reviewed in (Goridis and Rohrer 2002)) that are necessary for the expression of noradrenergic enzymes tyrosine hydroxylase (TH) and dopamine beta-hydroxylase (DβH) (Figure 3).

Formation of sympathetic ganglia

Lineage-committed sympathetic neuroblasts migrate rostrally and coalesce to form the largest sympathetic ganglion: the superior cervical ganglion (SCG). Ventrally migrating sympathetic neuroblasts form the three prevertebral ganglia: the celiac, superior mesenteric, and inferior mesenteric ganglia (CG, SMG, IMG). The remaining columnar neuroblasts become the thoracolumbar sympathetic chain of ganglia.

Axon extension

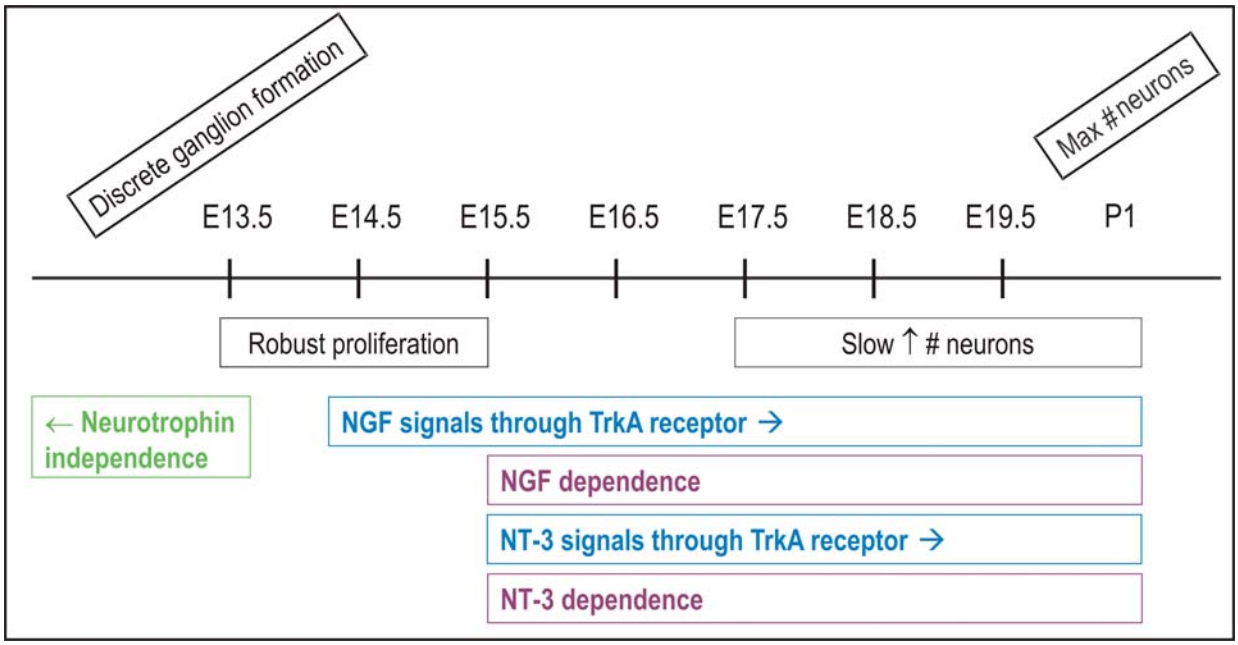
Peripheral sympathetic axons join the axons of sensory and motoneurons, and together they project along blood vessels to reach their target tissues (Young et al. 2004). Trophic support and guidance cues from blood vessels, other intermediate targets, and final target tissues are critical to these neurons finding the correct path (reviewed in (Young et al. 2004)). For example, blood vessel-derived NT-3 appears to support the survival of sympathetic neurons as they navigate toward their final targets (Kuruvilla et al. 2004). Artemin, a member of the GDNF family of neurotrophic factors, has also been shown to be a vascular-derived trophic factor for sympathetic neurons en route to their final destinations; artemin-deficient mice have defects in migration of sympathetic neuroblasts as well as misguided axonal projections (Honma et al. 2002). Additional specificity in sympathetic axonal guidance has been recently demonstrated by the discovery that endothelins (Edn3 binding to its receptor EdnrA) direct extension of axons of a subset of SCG neurons to the external carotid artery which serves as an intermediate target. The external carotid then acts as a "highway" along which axons extend to reach select sympathetic end-organs such as the salivary glands (Makita et al. 2008).

Developmental timeline

Using the SCG as the prototypical sympathetic ganglion, a developmental timeline for late sympathetic neuron development has been established (Figure 4). In the mouse, SCG neurons coalesce to resemble a discrete ganglion around embryonic day 13.5 (E13.5) (Fernholm 1971; Rubin 1985; Glebova and Ginty 2005). Robust proliferation of SCG neurons occurs between E13.5 and E15.5; another slower increase in neuron number occurs between E17.5 and birth, with maximal numbers of SCG neurons reached by postnatal day 1 (PN1) (Fagan et al. 1996). Neurotrophin signaling (see below) in sympathetic neurons begins after E13.5 when TrkA

Figure 4. Important events in murine sympathetic neuron development.

Using the SCG as the prototypical sympathetic ganglion, a developmental timeline for late sympathetic neuron development has been established. Sympathetic neurons coalesce into discrete sympathetic ganglia around E13.5. Rapid neuronal proliferation ensues, followed by a slower increase in the number of neurons; mice acquire maximal numbers of sympathetic neurons by the time of birth. Prior to E13.5, sympathetic neurons do not require neurotrophins for survival. Sympathetic neurons acquire neurotrophin dependence at approximately E15.5 as they are starting to innervate neurotrophin-secreting target tissues, a process that continues postnatally.


Discrete ganglion formation
Max #neurons
E13.5
E14.5
E15.5
E16.5
E17.5
E18.5
E19.5
P1
Robust proliferation
Slow ↑ # neurons
← Neurotrophin independence
NGF signals through TrkA receptor →
NGF dependence
NT-3 signals through TrkA receptor →
NT-3 dependence

expression is detectable in the SCG (Fagan et al. 1996). Sympathetic neurons become dependent on neurotrophins for survival around E15.5 (Francis et al. 1999).

Axonal outgrowth from the SCG begins around E13, with detectable innervation of the lacrimal glands as early as E16 (Rubin 1985). By contrast, two relatively distal targets, the pineal gland and sweat glands, are innervated postnatally (ElShamy et al. 1996). Final innervation of target tissues is still ongoing in the postnatal period, as neighboring axons arborize and compete for limited quantities of target-derived neurotrophic support (see below).

Sympathetic Neurotransmission

The neurotransmitter released by preganglionic sympathetic neurons is acetylcholine (ACh) while the majority of postganglionic sympathetic neurons secrete norepinephrine (NE, also called noradrenaline or NA) (Costanzo 1998). A minority of sympathetic neurons, such as those innervating the sweat glands of the rodent footpad, undergo a cholinergic switch to secrete ACh; this change requires signaling through the gp130 receptor (Stanke et al. 2006).

The key biosynthetic enzymes in NE synthesis are tyrosine hydroxylase (TH), which converts tyrosine to L-DOPA, and dopamine beta-hydroxylase (DβH), which converts dopamine into NE (Figure 5). Therefore, these enzymes are often used as markers of sympathetic neuron identity (ElShamy et al. 1996; Honma et al. 2002).

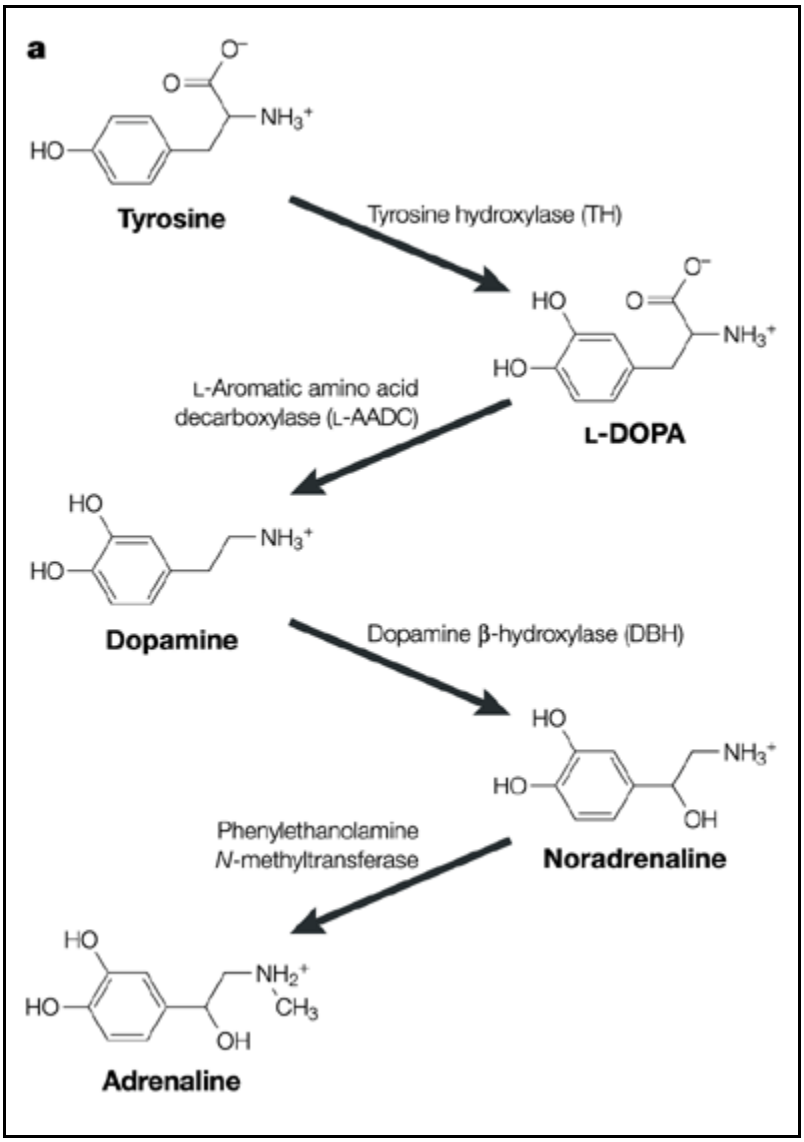
II. Sympathetic neurons require neurotrophins for survival and differentiation

Neurotrophins and their receptors

Nerve Growth Factor (NGF) was first identified as a molecule found in snake venom and subsequently in murine salivary glands (Cohen 1960; Levi-Montalcini and Booker 1960) that

Figure 5. Biosynthesis of sympathetic catecholamines.

Noradrenaline (NA, also called norepinephrine or NE), the primary neurotransmitter of postganglionic sympathetic neurons, is synthesized from the amino acid tyrosine. Two key enzymes in the NA synthesis pathway, tyrosine hydroxylase and dopamine beta-hydroxylase, are often used to identify sympathetic cell bodies and axons in innervated tissues. (Adapted from Goridis and Rohrer, Nature Reviews Neuroscience, 2002.)


a
Tyrosine
Tyrosine hydroxylase (TH)
L-DOPA
L-Aromatic amino acid decarboxylase (L-AADC)
Dopamine
Dopamine β-hydroxylase (DBH)
Noradrenaline
Phenylethanolamine N-methyltransferase
Adrenaline

increased the growth of sensory and sympathetic neurites *in vitro* and *in vivo*. Antibodies to NGF proved cytotoxic to sympathetic neurons and adrenal chromaffin cells (Cohen 1960; Aloe and Levi-Montalcini 1979). Since then, NGF has been shown to promote sympathetic axon growth *in vitro* and *in vivo*.

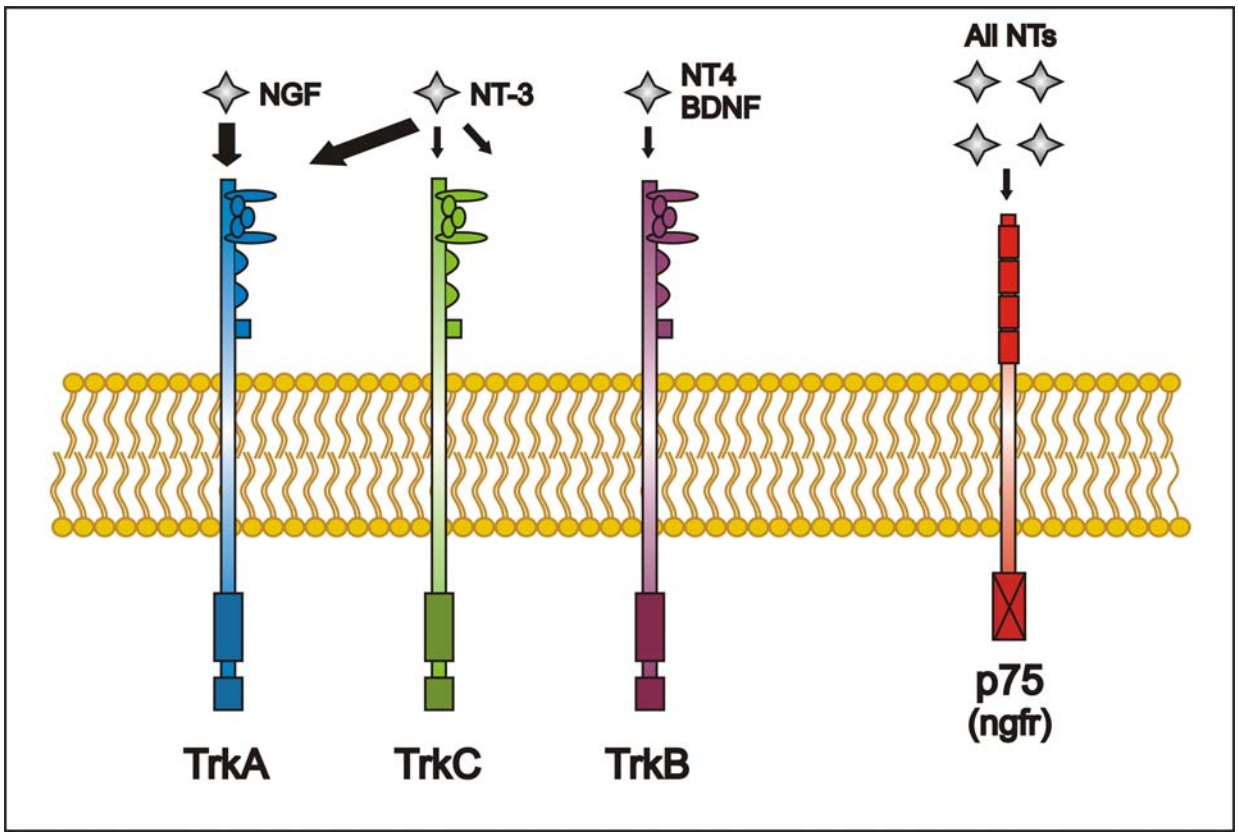
NGF was later determined to be a member of a family of neurotrophins: NGF, neurotrophin-3 (NT-3), brain-derived neurotrophic factor (BDNF), and neurotrophin-4 (NT-4). The neurotrophins are initially synthesized in as pro-neurotrophins which are then cleaved by furin or pro-convertases to their mature dimeric forms (reviewed in (Chao 2003; Reichardt 2006)). Mature neurotrophins are expressed at very low levels during development. Developing sympathetic neurons therefore must compete for trophic support, a central tenet of the neurotrophic hypothesis (see below).

The cognate receptors for the neurotrophins are the tropomyosin-related kinase (Trk) family of tyrosine kinase receptors, TrkA, TrkB, and TrkC (Figure 6, reviewed in (Kaplan and Miller 1997) for example). NGF binds with high affinity to the TrkA receptor (also known as NTRK1). NT-3 binds the TrkC neurotrophin receptor with the highest affinity but also binds and signals through TrkA (Belliveau et al. 1997). Surprisingly, this less-efficient NT-3/TrkA signaling appears to be uniquely important in sympathetic neuron survival *in vivo* since TrkC-deficient mice have no SCG neuron defects (Fagan et al. 1996). In an additional level of regulation, an alternatively spliced form of TrkA confers an enhanced response to NT-3 (Clary and Reichardt 1994). Finally, the cognate receptor for BDNF and NT-4/5 is TrkB.

$p75^{NTR}$ is the low affinity nerve growth factor receptor which binds all neurotrophins with equally low affinity; it is a member of the tumor necrosis factor (TNF) receptor superfamily.

Figure 6. Neurotrophins and their receptors.

The tropomyosin-related kinase family (Trk) of receptors are membrane spanning receptors with an intracellular tyrosine kinase domain. $p75^{NTR}$ is a member of the tumor necrosis factor (TNF) receptor superfamily and has an intracellular death domain. Pictured are neurotrophins bound to their cognate receptors: NGF binds to TrkA, NT-3 binds to TrkC, and both NT4 and BDNF bind to TrkB. In sympathetic neurons, promiscuous binding of NT-3 to TrkA (wide arrow) is critical for the survival of one-half of sympathetic neurons. Proneurotrophins (squares) bind to $p75^{NTR}$ (ngfr) with higher affinity than mature neurotrophins (diamonds), all of whom bind $p75^{NTR}$ with equally low affinity. (Adapted from M. Chao, Nature Reviews Neuroscience, 2003)


All NTs
NGF
NT-3
NT4
BDNF
p75
(ngfr)
TrkA
TrkC
TrkB

Coexpression of p75 and TrkA results in higher affinity NGF-TrkA binding (Brennan et al. 1999; Esposito et al. 2001; Chao 2003) and therefore preferential signaling of NGF through TrkA over NT-3. Conversely, NT-3 signaling through the TrkA receptor, and the ability of NT-3 to stimulate axon growth, is increased when $p75^{NTR}$ expression is decreased (Kuruvilla et al. 2004).

Neurotrophin signaling

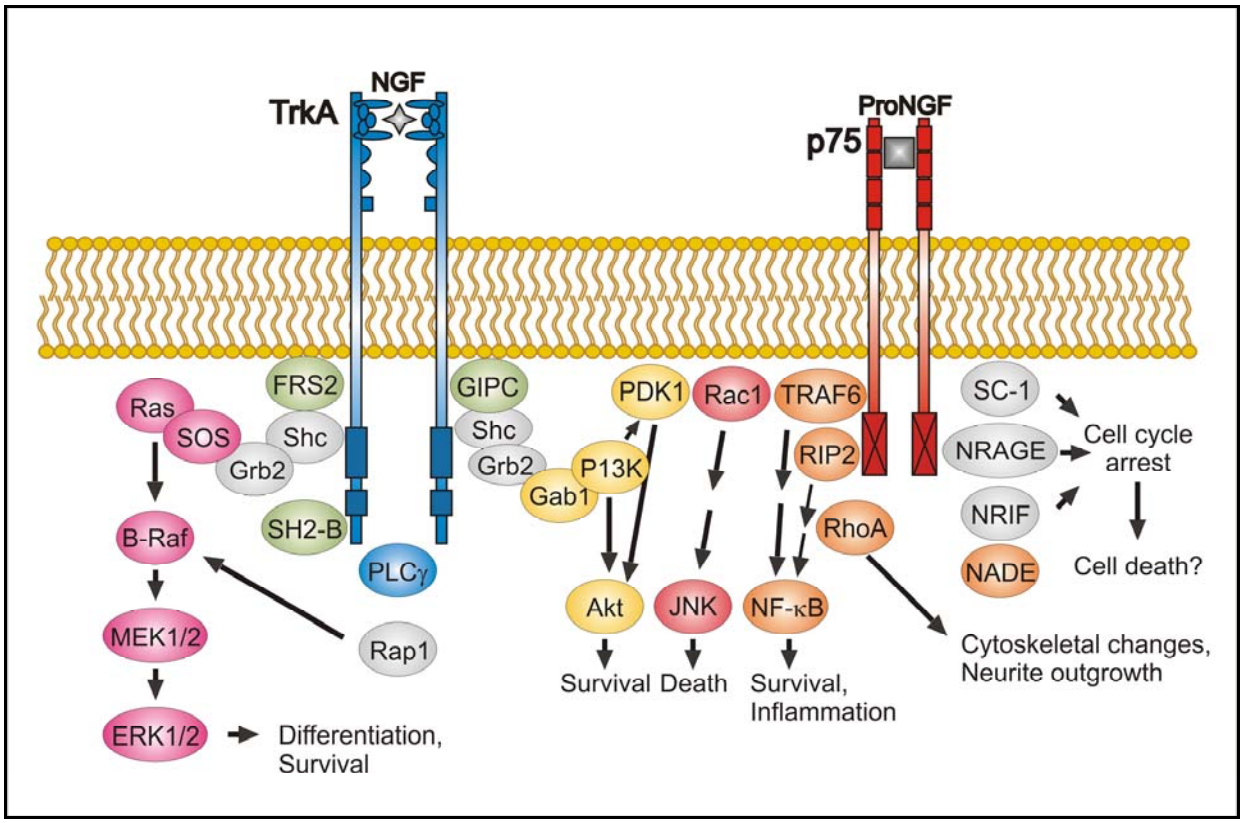
Trk receptors are receptor tyrosine kinases. Their extracellular domains bind neurotrophins, while their intracellular tyrosine kinase domains activate signal transduction pathways (Figure 7). *In vivo*, TrkA-NGF complexes are endocytosed and retrogradely transported a considerable distance from the target tissue to the sympathetic neuron cell body (Ginty and Segal 2002; Kuruvilla et al. 2004). A variety of signaling pathways are then engaged, including those affecting survival and differentiation (Figure 7). Many of the signaling pathways engaged by TrkA activation were originally identified in rat pheochromocytoma cells (PC12), an immortalized cell line that resembles sympathetic neurons (reviewed in (Sofroniew et al. 2001)). Here, the importance of Ras/MEK/ERK and the P13K/Akt pathways in sympathetic neuron development will be highlighted (reviewed in (Kaplan and Miller 1997; Huang and Reichardt 2001; Sofroniew et al. 2001; Chao 2003)).

Activation of Trk and the Ras pathway leads to sympathetic neuron differentiation

NGF binding to TrkA results in the phosphorylation of tyrosine residues within its intracellular domain. Dimerization of TrkA leads to further auto-phosphorylation; these

Figure 7. Neurotrophin signaling pathways.

Binding of NGF to TrkA results in TrkA dimerization, autophophorylation, recruitment of adaptor molecules such as Shc, and the initiation of a variety of signaling cascades resulting in neuronal survival and differentiation. Activation of the Ras/MEK/ERK pathway is critical for mediating aspects of NGF-mediated differentiation such as axonal outgrowth of sympathetic neurons and PC12 cells. In contrast, the P13K/Akt pathway mediates survival through the phosphorylation and resultant regulation of proteins involved in neuronal cell death such as the Bcl-2 molecule Bad. Signaling through the low affinity nerve growth factor receptor $p75^{NTR}$ also initiates a variety of signal transduction cascades with diverse outcomes of survival, death, inflammation, cell cycle arrest, and neurite outgrowth. (Adapted from M. Chao, Nature Reviews Neuroscience, 2003)


NGF
TrkA
ProNGF
p75
FRS2
GIPC
Ras
SOS
Shc
Shc
Grb2
Grb2
Gab1
P13K
PDK1
Rac1
TRAF6
RIP2
SC-1
NRAGE
Cell cycle arrest
NRIF
NADE
Cell death?
RhoA
SH2-B
B-Raf
PLCγ
MEK1/2
Rap1
ERK1/2
Differentiation, Survival
Akt
JNK
NF-κB
Survival
Death
Survival, Inflammation
Cytoskeletal changes, Neurite outgrowth

phosphorylated tyrosines serve as docking sites for adaptor proteins. One such adaptor is the Src homologous and collagen-like (Shc) protein which "docks" on phospho-TrkA through its src-homology-2 (SH2) and phosphotyrosine-binding (PTB) domains. Shc is then phosphorylated by Trk and binds the SH2 domain of another adaptor protein Grb2. Grb2 binds the Ras guanine-nucleotide exchange factor SOS through its SH3 domains, which leads to a cascade of activation of Ras, Raf, MEK1/2 and ERK1/2. Extracellular signal-related kinases 1 and 2 (ERK1/2) are

serine-threonine kinases and phosphorylate several substrates. One such substrate is Elk-1, which, once phosphorylated, associates with serum response factor (SRF) to induce transcription of genes with serum response elements in their promoters, such as the immediate early gene *c-fos* (Sofroniew et al. 2001). Additional transcription factors such as cAMP regulatory element binding protein (CREB) bind to their consensus response elements within the promoter of *c-fos* and thereby contribute to its regulation (Ginty et al. 1994). Regulation of *c-fos* is one of many transcriptional events involved in sympathetic neuron differentiation through the induction of late response genes (Greenberg et al. 1985; Sheng and Greenberg 1990); this dissertation will explore the role of another immediate early gene Egr3 (see below) in similar pathways leading to sympathetic neuron differentiation.

The Ras/MEK/ERK pathway is traditionally thought to be primarily important for regulating the differentiation-promoting effects of NGF rather than survival (Klesse et al. 1999; Klesse and Parada 1999). In support of this theory, the MEK inhibitors PD98059 and UO126 do not affect the survival of postnatal sympathetic neurons but severely reduce NGF-stimulated neurite extension (Thompson et al. 2004).

Activation of the PI-3 kinase pathway leads to neuronal survival

The Shc adaptor protein plays a second role in transmitting signals from the TrkA receptor to promote neuronal survival. Interestingly, mice lacking two Shc family members ShcB and ShcC lose one-third of their SCG neurons sometime after the onset of neurotrophin signaling in sympathetic neurons (Sakai et al. 2000). Binding of Shc to TrkA results in activation of phosphatidylinositol 3-kinase (PI3K) and Akt (also known as protein kinase B). Akt, in turn, phosphorylates several proteins that influence neuronal survival including BAD, IκB, the forkhead transcription factor FKHRL1, and glycogen synthase kinase 3-β (GSK3β) (Reviewed in (Huang and Reichardt 2001)).

Neurotrophin-dependent development and patterning of the sympathetic nervous system

Highly regulated patterning of sympathetic innervation within target tissues is governed by the complement of trophic molecules secreted by intermediate and final target tissues. Indeed, a target tissue's acquisition and relative density of sympathetic innervation is correlated with its timing and extent of NGF production (Korsching and Thoenen 1983; Shelton and Reichardt 1984; Korsching and Thoenen 1988). Neurotrophins are not only important for the development of the SNS but also for repair, as NGF expression is upregulated in denervated tissues (Shelton and Reichardt 1984).

Neurotrophin-deficient mice

Genetic deletion of individual neurotrophins provided crucial insight into how each molecule affects neurodevelopment. All sympathetic neurons require NGF to survive, as NGF-deficient mice lose 100% of SCG neurons by postnatal day 9 (PN9) (Crowley et al. 1994). In

contrast, Neurotrophin-3 (NT-3) is required for the survival of one-half of sympathetic neurons (Ernfors et al. 1994; Farinas et al. 1994) and for the innervation of specific tissues such as the pineal gland (ElShamy et al. 1996). Moreover, although deletion of NT-3 does not exacerbate neuron losses in NGF-deficient mice, NGF-/-NT3+/- mice have thinner TH-immunoreactive axon fascicles versus those of NGF-/-NT3+/+ mice (Francis et al. 1999). Therefore, while NGF is the major target-derived neurotrophin for sympathetic neurons, NT-3 appears to plays an independent role in the trophic support of developing neurons which may enable innervation of distal target tissues. A model was thus proposed in which NT-3 functions to assist axons en route to target tissues containing NGF (Francis et al. 1999; Kuruvilla et al. 2004).

Compromised sympathetic neuronal survival in NGF- and NT3-deficient mice initially limited investigation into other aspects of neurotrophin-regulated SNS development. However, when neuron death in neurotrophin-deficient mice was rescued by deletion of the proapoptotic molecule Bax, NGF and NT-3 were observed to have secondary roles in the sympathetic axon outgrowth. Indeed, NGF-/-;Bax-/- and NT-3-/-;Bax-/- mice have distinct but overlapping target innervation defects (Patel et al. 2000; Kuruvilla et al. 2004). Defining individual roles for NGF and NT-3 in developing sympathetic neurons is an ongoing area of investigation.

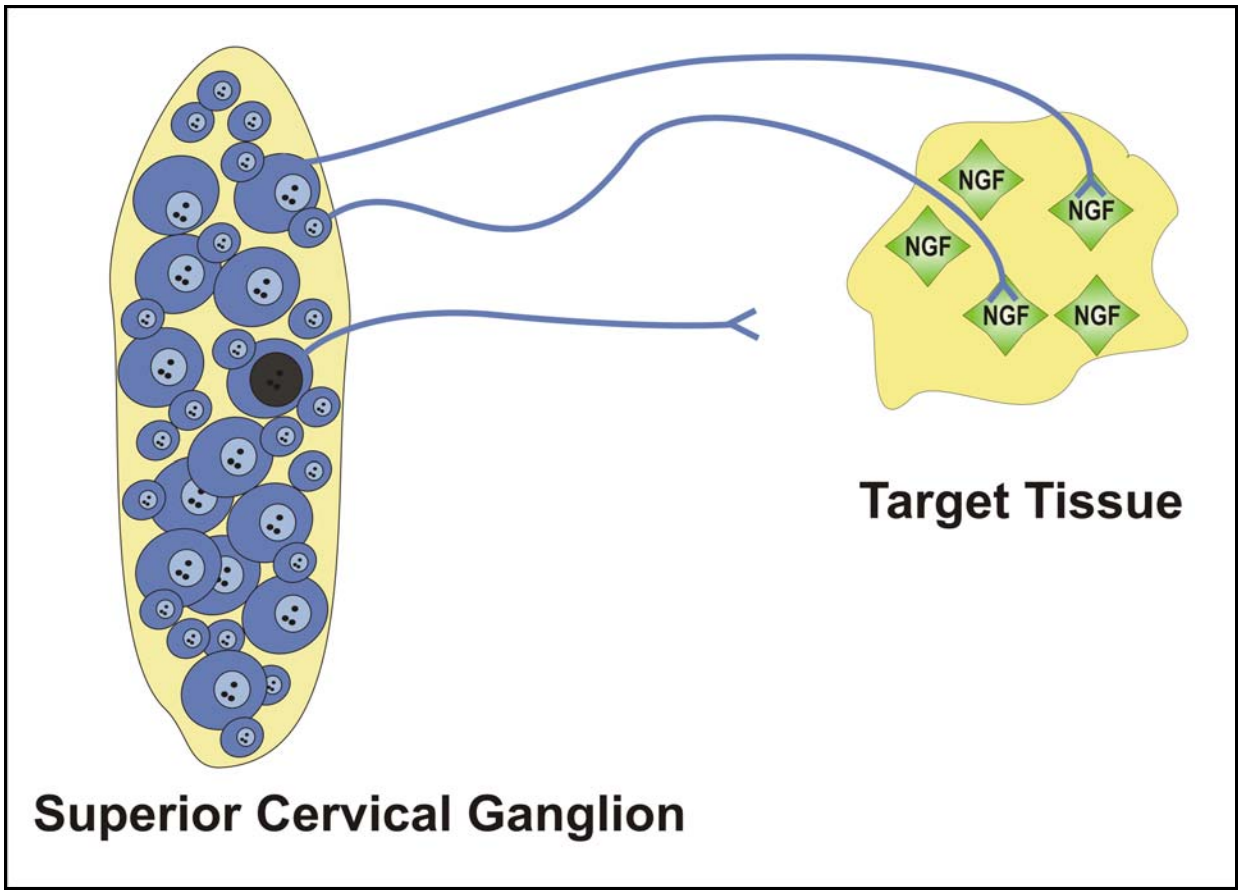
III. Developmental cell death in the sympathetic nervous system

The Neurotrophic Hypothesis

Precise development of the sympathetic nervous system results from balanced proliferation and cell death. Neurotrophins are produced and secreted by target tissues in limiting amounts, which in turn, places one neuron in competition with neighboring neurons (Oppenheim 1991).

Figure 8. The neurotrophic hypothesis.

Target tissues produce and secrete limiting quantities of neurotrophins during development. In this competitive environment, postganglionic sympathetic neurons must extend axons and acquire target tissue-derived neurotrophins to survive. Neurons that do obtain neurotrophic support undergo apoptosis (black nucleus with condensed chromatin). This naturally occurring developmental cell death occurs throughout the nervous system, deleting one-half of a neuronal population on average (Deckwerth and Johnson 1993).


NGF
NGF
NGF
NGF
NGF
Target Tissue
Superior Cervical Ganglion

Sympathetic neurons are initially overproduced, reaching a maximal number in the SCG around birth, and then undergo significant pruning (Figure 12). In order to survive this pruning, a neuron must acquire target tissue-derived neurotrophic support. Sympathetic neurons that do not obtain adequate NGF and/or NT-3 die by apoptosis (Figure 8). On average, one-half of sympathetic neurons die during this period of developmental cell death (Deckwerth and Johnson 1993).

Neurotrophin withdrawal leads to sympathetic neuron apoptosis

Withdrawal of neurotrophin support *in vitro* also leads to the death of sympathetic neurons through a well-characterized process. Loss of cultured rat SCG neurons begins 18 hours after NGF deprivation and is nearly complete (>90%) by 48 hours (Deckwerth and Johnson 1993). For the first 12 hours of neurotrophin deprivation, rat SCG neurons can be rescued by the readdition of NGF to the culture medium. After 12 hours, neurotrophin-deprived sympathetic neurons undergo degenerative changes leading to apoptosis including atrophy (decreased cell body size) and DNA fragmentation (Deckwerth and Johnson 1993). A great body of work has been focused on understanding the molecules involved in sympathetic neuron apoptosis and discovering a mechanism that is presumably common to limiting NGF levels *in vitro* and *in vivo*.

The function and utility of pro- and anti-apoptotic molecules in the sympathetic nervous system.

Bax was originally identified as a protein that heterodimerized with the anti-apoptotic protein Bcl-2 (Oltvai et al. 1993). Through this interaction, Bax counters the "death

repression" function of Bcl-2 (Oltvai et al. 1993). When in excess, Bax protein forms homodimers and accelerates the rate of cell death, albeit only after the onset of a death signal (Oltvai et al. 1993). The BCL2-related family of proteins was later shown to consist of multiple pro-apoptotic molecules such as Bax, Bad, Bak, $Bclx_S$ and anti-apoptotic molecules Bcl_2, $Bclx_L$, Mcl1, Ced9 (reviewed in (Merry and Korsmeyer 1997)).

The anti-apoptotic protein Bcl-2 is expressed at high levels in the postnatal and adult SCG postnatally, suggesting that it functions to maintain neuronal survival throughout the life of the animal (Merry et al. 1994). Additionally, forced expression of Bcl-2 by sympathetic neurons rescues them from NGF withdrawal-mediated apoptosis (Garcia et al. 1992). Conversely, Bcl-2-deficient mice exhibit increased SCG neuron loss postnatally, having 87% of the wild type complement of SCG neurons at PN3 and 60% at PN10 (Michaelidis et al. 1996). SCG neurons from Bcl-2-deficient mice die more rapidly in NGF-deprived conditions than wild type neurons, also suggesting that Bcl-2 protects neurons from apoptosis during development when NGF is limited *in vivo* (Greenlund et al. 1995).

The proapoptotic protein Bax is similarly expressed in the SCG during the time of developmental cell death in the sympathetic nervous system (Deckwerth et al. 1996). Bax-deficient mice have a variety of interesting phenotypes including aspermatogenesis and lymphoid hyperplasia (Knudson et al. 1995), proving that deletion of Bax does not necessarily confer hyperplasia in all systems. The proapoptotic role of Bax was examined in a well-tested paradigm: NGF deprivation of cultured SCG neurons. Bax-deficient SCG neurons were able to survive NGF deprivation for at least 23 days while virtually all NGF-deprived wild type neurons died within 2 days (Deckwerth et al. 1996). Similarly, Bax deficiency *in vivo* decreased normal developmental cell death in the sympathetic nervous system, as 2.5-fold more

SCG neurons were cultured from Bax-deficient SCG than from wild type SCG (Deckwerth et al. 1996). Once the effect of NGF withdrawal on neuronal survival was prevented, other functions of NGF were revealed. NGF-deprived Bax-deficient SCG neurons are atrophic and exhibit slower neurite extension, decreased glucose uptake and protein synthesis, and physiological alterations. Additionally, these parameters normalize after readdition of NGF to the culture media (Deckwerth et al. 1996; Deckwerth et al. 1998; Werth et al. 2000). Bax is nonessential for early events in neuronal cell death after NGF deprivation such as increased *c-jun* transcription, but is required for terminal events such as *c-fos* activation and caspase activation (Deckwerth et al. 1998). Importantly, since Bax-deficient neurons retain a neuronal phenotype as well as NGF-responsiveness, they are a model system for examining extra-survival aspects of sympathetic neuron development.

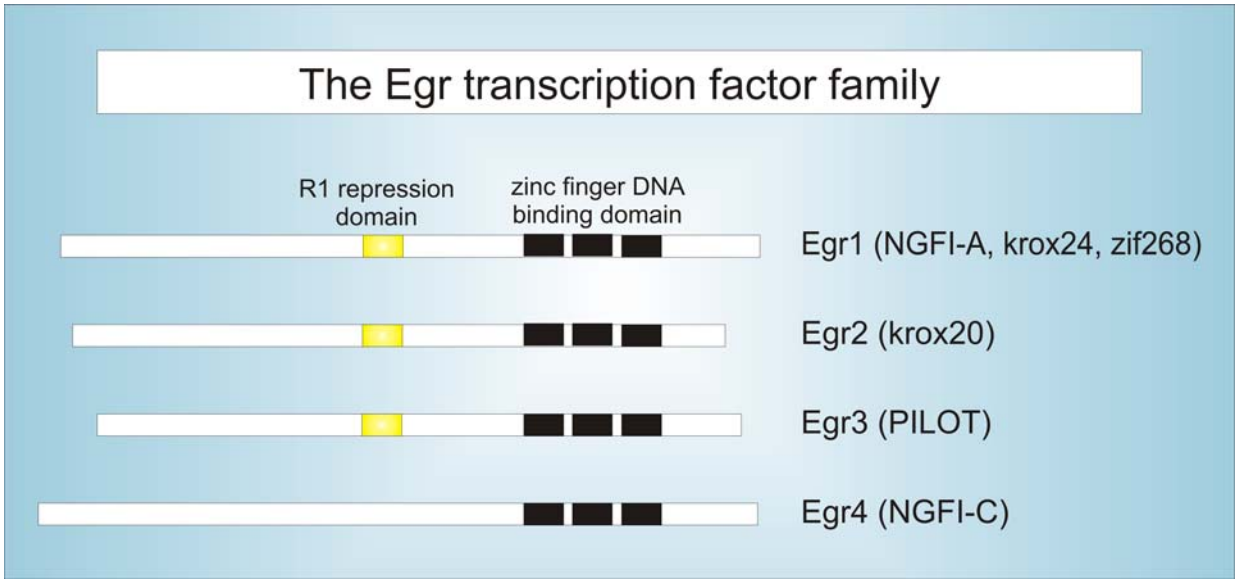
IV. Early Growth Response (Egr) transcriptional regulators

Overview

Early growth response genes are immediate early genes, and as such are rapidly induced in response to growth factors or mitogens in the absence of *de novo* protein synthesis (Sheng and Greenberg 1990). The Egr genes are therefore transcriptional regulators that are poised to rapidly influence cellular responses to environmental stimuli.

There are four members in the Egr family of transcription factors (Figure 9). Egr1, Egr2, and Egr3, and Egr4 have highly homologous DNA binding domains, each containing three zinc fingers, which may afford considerable functional redundancy through coregulation of target genes. Additionally, endogenous repressors of Egr genes, NGFI-A binding (NAB) proteins, bind to the R1 domain of Egr1, Egr2, and Egr3 (reviewed in (O'Donovan et al. 1999)).

Figure 9. The Early Growth Response (Egr) family of transcriptional regulators. Egr1, Egr2, Egr3, and Egr4 transcription factors share highly homologous DNA binding domains containing three zinc fingers. Egr1, Egr2, and Egr3 proteins have a common R1 domains to which the endogenous repressor molecules NGFI-A binding (NAB) proteins bind. Alternative gene names are included for reference. (Adapted from O'Donovan et.al, Trends Neurosci., 1999)


The Egr transcription factor family
R1 repression domain
zinc finger DNA binding domain
Egr1 (NGFI-A, krox24, zif268)
Egr2 (krox20)
Egr3 (PILOT)
Egr4 (NGFI-C)

Egr1 is the prototype of the Egr transcription factor family. Egr1 was first identified as an NGF-inducible gene in PC12 cells that was also shown to be expressed in the adult rat SCG (Milbrandt 1987). Egr genes are involved in a variety of growth and differentiation processes (O'Donovan et al. 1999; Thiel and Cibelli 2002), and in the nervous system, they may be involved in structural neuritic and synaptic changes involved in synaptic plasticity (Cole et al. 1989; Wisden et al. 1990; Mello et al. 1995; Petersohn et al. 1995; Jarvis and Nottebohm 1997; Jones et al. 2001).

Egr gene deficient mice

Multiple Egr genes may act in concert since they share conserved DNA binding domains. However, individual Egr genes have divergent transactivation domains which may be responsible for the recruitment of various coactivators and therefore individualized cellular responses. Indeed, the generation of Egr gene-deficient mice has revealed both unique and redundant functions for Egr family members (Table 1).

Egr1-deficient mice are relatively normal except for female infertility (Lee et al. 1996a; Lee et al. 1996b) due to a reduction in the level of pituitary Luteinizing Hormone (LH) and defects in learning and memory (Jones et al. 2001). Egr4-deficient mice have an autonomous germ cell defect which leads to severe oligozoospermia and male infertility (Tourtellotte et al. 1999). Egr1/Egr4 male mice are also infertile, with atrophy of androgen-dependent organs. Interestingly, Egr1/Egr4 double homozygous mutant male mice exhibit low levels of serum LH not present in either Egr1 or Egr4 single mutant mice, suggesting that functional compensation by Egr4 preserves male fertility in Egr1-deficient male mice (Tourtellotte et al. 2000).

Table 1. Egr gene deficient mice

Germline Deletion	Phenotype
Egr1	female infertility (reduction in pituitary LHβ) learning and memory defects
Egr2	defective peripheral nerve myelination (autonomous Schwann cell defect) abnormal hindbrain development
Egr3	proprioception defects (absence of muscle spindles) learning and memory defects Scoliosis resting tremor **Blepharoptosis**
Egr4	male infertility (autonomous germ cell defect)
Egr1/4	male infertility (low LH, atrophy of androgen-dependent organs)
Egr1/3	thymic atrophy (proliferation defects, impaired DN3-DN4 progression) **Blepharoptosis** failure to thrive thin peripheral myelin

Germline deletion of Egr2 results in abnormal hindbrain development with fusion of rhombomeres 3 and 5, as well as defective peripheral myelination (Schneider-Maunoury et al. 1993; Topilko et al. 1994). In addition, human mutations of Egr2 have been identified in patients with congenital hypomyelinating neuropathy (CHN) and a family with Charcot-Marie-Tooth type 1 (CMT1). This seminal discovery was the first identification of human disease resulting from a mutation in an Egr gene.

Egr3-deficient mice exhibit sensory ataxia due to the absence of muscle spindle mechanoreceptors, scoliosis, and resting tremors (Tourtellotte and Milbrandt 1998). In addition, these mice have blepharoptosis, a phenotype of humans and mice with sympathetic nervous system

defects as described above. Recently, our laboratory identified learning and memory defects in Egr3-deficient mice (Li et al. 2007). Therefore, Egr1 and Egr3 may play redundant roles in hippocampus-based learning; analysis of Egr1/Egr3 double homozygous mutant mice would be an interesting future direction for this work.

Interestingly, we have observed that Egr1/Egr3 double homozygous mutant mice appear more severely affected with respect to neurological defects than Egr1 or Egr3 singly mutant mice, providing additional evidence for functional redundancy within the Egr gene family. Egr1/Egr3 DKO mice have severe failure to thrive, in addition to the blepharoptosis, ataxia, and tremors seen in Egr3-deficient mice (Eldredge and Tourtellotte, unpublished observations). Egr1/Egr3 DKO mice also have thymic atrophy not seen in single knockout mice due to decreased progression from the double negative 3 (DN3) to double negative 4 (DN4) stage of thymocyte development (Carter et al. 2007). Finally, Egr1/Egr3 DKO mice have been shown to have decreased peripheral myelin thickness, not seen in either Egr1 nor Egr3 single mutant

mice, which may be due to deregulation of the low affinity neurotrophin receptor p75 (ngfr) since $p75^{NTR}$ expression is attenuated in Egr1/Egr3 sciatic nerves and similar myelinopathy in seen in p75-deficient mice (Song et al. 2006).

Taken together, defects in Egr gene-deficient mice provide evidence that Egr genes function in a variety of cellular contexts that are unified as systems where growth and differentiation take place. Moreover, Egr genes appear to act in a redundant fashion in some systems while they clearly have unique functions in others. Further analyses of Egr gene-deficient mice continue to reveal novel and interesting roles for these transcriptional regulators.

Egr genes and the sympathetic nervous system

Abundant *in vitro* data suggest that Egr genes are involved in the development of the sympathetic nervous system. For example, NGF treatment leads to rapid and robust expression of Egr1 and Egr4 in PC12 cells and in some neuroblastoma cell lines (Milbrandt 1987; Bogenmann et al. 1998). In these experimental paradigms, Egr1 expression parallels differentiation characterized by neurite outgrowth and sympathetic gene expression. Moreover, Egr gene induction is coupled to the Ras/MEK/Erk pathway, and MEK inhibitors abrogate NGF-mediated induction of Egr genes as well as neurite outgrowth and differentiation (Harada et al. 2001). Similarly, inhibition of Egr gene function, using the Egr corepressor NAB2 (Qu et al. 1998) or an Egr dominant negative molecule (Levkovitz et al. 2001b), abrogates neurite outgrowth and differentiation in PC12 cells. Thus, *in vitro* models of sympathetic neuron differentiation suggest that Egr genes are coupled to NGF signaling pathways and regulate important aspects of neurite outgrowth and differentiation.

An *in vivo* function for Egr genes in sympathetic nervous system development had not previously been demonstrated. Egr1-deficient mice do not have identifiable sympathetic nervous system defects. However, Egr3-deficient mice have profound blepharoptosis, and that observation in conjunction with the above *in vitro* data led us to consider whether Egr3 may play a role in sympathetic nervous system development *in vivo*.

V. Objective of the dissertation

This thesis defines a novel role for Egr3 in sympathetic nervous system development. We have determined how and when Egr3 is expressed in sympathetic neurons, how Egr3 gene deficiency affects neuron survival and target tissue innervation, and how innervation defects perturb autonomic physiology. Finally, we propose a mechanism for Egr3's critical role in sympathetic nervous system development.

Chapter 2

Materials and Methods

Animals and preparation of tissues. Egr3-deficient mice were generated from 129/SvJ ES cells and maintained on a C57BL/6J:129/SvJ hybrid genetic background (backcrossed to C57BL/6 for approximately 6 generations), and genotyped as previously described (Tourtellotte and Milbrandt 1998; Tourtellotte et al. 2001). Transgenic DβH-τlacZ reporter mice (Gao and Tourtellotte, in preparation) were generated by Xiaoguang M. Gao in the Tourtellotte lab. Since inclusion of heterologous introns can enhance expression of transgenes *in vivo* (Palmiter et al. 1991), the human DβH promoter (hDβH; 2949A12; (Hoyle et al. 1993)) was cloned upstream of the first intron of the rat insulin II gene (rInsII), followed by the τlacZ cDNA (Callahan and Thomas 1994) and mouse protamine (polyA) sequence. F_1 progeny from five transgenic founder mice were screened to establish a single reporter line with τlacz expression in >99% of sympathetic neurons, their axons, and adrenal chromaffin cells. Egr3 heterozygous mutant mice were mated to DβHτlacZ+ transgenic reporter mice and Egr3+/+;DβHτlacZ+ and Egr3-/-;DβHτlacZ+ mice were analyzed using lacZ enzyme histochemistry to evaluate target tissue innervation. For mice generated through timed matings, E0.5 was considered the day the vaginal plug was observed. Following deep anesthesia and cervical dislocation of pregnant females, embryos were isolated by cesarean section and rapidly decapitated. Adult and postnatal mice were anesthetized with intraperitoneal (IP) injection of ketamine/ xylazine, and perfused through the heart with 10mL of 0.1M phosphate buffer (PB)

followed by 100 mM phosphate-buffered 4% paraformaldehyde (PFA). Tissues harvested from adult or embryonic mice were fixed overnight at 4°C in 4% PFA. Tissues were either processed for paraffin embedding or were cryoprotected in 15%- and then 30%-phosphate-buffered sucrose for 24 hr each. Following embedding in paraffin or OCT, serial paraffin sections (16 μm) or frozen sections (12 μm) were generated. All experimental procedures complied with protocols approved by The Northwestern University Institutional Animal Care and Use Committee.

Ganglion neuron counts and volume estimates. Stereological quantification of the total number of SCG neurons was performed using the optical dissector method (StereoInvestigator, Microbrightfield). Every fifth section through SCG from a variety of developmental ages was stained with Hematoxylin and Eosin (H&E) and analyzed. Contours containing a single SCG were optically sectioned using a 100 objective (NA 1.4) and an oil substage condenser. For SCG isolated from postnatal animals, 100μm x 75μm sampling sites within contours were analyzed, and the counting frame boundaries were 40μm x 30μm. For SCG from adult animals, counting frames of 60μm x 45μm were used to reflect larger cytoplasmic areas of mature neurons. Only neurons with intact nuclear profiles (containing a nuclear membrane and at least one nucleolus) within the optical dissector counting frame boundaries were tallied. The total number of neurons per SCG was determined with the optical fractionator probe within the StereoInvestigator software and multiplied by 5 to reflect total neuron numbers.

Apoptosis and proliferation studies. PN0 mice from Egr3+/- x Egr3+/- matings were injected IP with 50mg/kg BrdU and euthanized after 2 hours. Tissues were processed as described above. 12μm sections through paraffin-embedded wild type and Egr3-/- mice were stained with H&E to identify SCG. Following antigen retrieval in citrate buffer,

immunohistochemistry for Cleaved caspase-3 **(**Rabbit anti-cleaved/activated caspase 3 (Cell Signaling, Danvers, MA) 1:500) or BrdU (Mouse anti-BrdU (Sigma, St. Louis, MO) 1:2000 was performed as described previously (Li et al. 2005). The number of cleaved-caspase 3-positive or BrdU-positive neurons and the cross-sectional area of SCG in every fifth section through the SCG of E15.5, E16.5, E17.5, E18.5, or PN0 mice were quantified using a Stereological morphometry system (StereoInvestigator, Microbrightfield, Williston, VT).

Sympathetic target tissue immunohistochemistry. Frozen sections through select target tissues were cut at 12μm, and immunohistochemistry was performed for tyrosine hydroxylase (TH) or β-galactosidase (lacZ) to identify sympathetic nerve terminals in wild type/Egr3-/- mice or Egr3+/+;lacZ+/Egr3-/-;lacZ+ tissues, respectively. Rabbit anti-tyrosine hydroxylase (TH, Chemicon, Temecula, CA) was used at 1:5000 dilution, and rabbit anti-β-galactosidase (lacZ, ICN Pharmaceuticals, Costa Mesa, CA) was used at 1:1000.

LacZ enzyme histochemistry. Animals were perfused through the heart with Phosphate Buffered Saline + 2mM $MgCl_2$ (PBS+Mg). Tissues were dissected and postfixed for 1 hour in fixative (2% PFA, 0.2% glutaraldehyde, 5mM EGTA, 0.01% NP-40 in PBS+Mg) at 4°C, followed by 5 washes in Wash Buffer (0.01% sodium deoxycholate, 0.02% NP-40 in PBS+Mg). Tissues were stained at 37°C with agitation in lacZ reaction buffer (1mg/mL X-gal (Gold Biotechnology, St. Louis, MO) in 5mM potassium ferrocyanide/5mM potassium ferricyanide-containing Wash Buffer. For some tissues, a 1 hour fixation was performed in 0.2% glutaraldehyde alone and staining was performed in lacZ reaction buffer without detergents (Table 2). Tissues were then washed, postfixed/dehydrated in Methanol, and cleared in 2:1 Benzyl Benzoate: Benzyl Alcohol. Stained tissues were photographed under an

Table 2. LacZ staining conditions

Tissue	Fixative	Perfusion	Post-fix	Reaction
eyelid	2%PFA/ 0.2%glut/ 5mMEGTA/ .01%NP40	Fixative	1.5 hr	8hr
salivary gland	2%PFA/ 0.2%glut/ 5mMEGTA/ .01%NP40	Fixative	1.5 hr	8hr
iris (eye)	2%PFA/ 0.2%glut/ 5mMEGTA/ .01%NP40	Fixative	1.5 hr	12 hr
postnatal pup	2%PFA/ 0.2%glut/ 5mMEGTA/ .01%NP40	Fixative	1.5 hr	12 hr
heart	0.2%glut	PBS+Mg	1 hr	8 hr
trachea	0.2%glut	PBS+Mg	1 hr	8 hr
pineal gland	0.2%glut	PBS+Mg	1 hr	8 hr
spleen	0.2%glut	PBS+Mg	1 hr	4 hr

Olympus SZ-PT dissection microscope connected to a digital camera (Spot Insight QE, Diagnostic Instruments, Sterling Heights, MI).

In situ hybridization. *In situ* hybridization was performed in frozen sections as previously described (Albert et al. 2005) using a digoxygenin-labeled antisense riboprobe for Egr3 (GenBank NM018781, nt 345-746). Adjacent control sections were incubated with the sense probe to control for nonspecific labeling.

Primary SCG neuron cultures. E19.5 or PN0 Swiss Webster (CFW, Charles River Laboratories, Wilmington, MA) mice were rinsed in ethanol and sterile PBS before decapitation into Leibovitz L-15 Medium (Hyclone, Logan, UT). SCG were dissected into L-15, incubated at 37°C in 1mg/mL Type IV Collagenase (Worthington Biochemical Corporation, Lakewood, NJ) for 30 minutes, 0.25%Trypsin-EDTA for 15 minutes, and washed three times in L15. Neurons were then dissociated via trituration with a fire-polished/siliconized pasteur pipet in Minimal Essential Media (MEM) with 10% Fetal Bovine Serum (FBS), 1% penicillin/streptomycin, and varying concentrations of NGF (BD Biosciences, San Jose, CA). Dissociated neurons were preplated in 35mm tissue culture dishes for 1 hour at 37°C to remove non-neuronal cells, plated in 70µl on collagen-coated 35mm dishes for 2 hours, and then covered with an additional 1.5mL of media. For signaling experiments, neurons were plated on collagen-coated 35 mm plates at a density of 2 SCG/plate in 100ng/mL of NGF for 7 days; on day 3, media was supplemented with 2µM cytosine arabinoside. The MAP Kinase Kinase (MEK) inhibitor U0126 (20µM, Promega, Madison, Wisconsin) and the Trk inhibitor K-252a (50nM, Calbiochem, San Diego, CA) were used to abrogate NGF signaling in some experiments; control cultures were treated with the inhibitor vehicle Dimethylsulfoxide (DMSO). For neuronal survival assays, neurons were plated at 1

SCG per twice collagen coated plate in 0.2, 1, 5, or 10ng/mL NGF for 24 hours. Neurons were then fixed in 4% PFA/0.12M sucrose, stained with 1:800 mouse anti-βIII tubulin (Chemicon, Temecula, CA), 1:800 cleaved caspase 3 (Cell Signaling, Danvers, MA), and Hoechst 33258. For neurite extension assays, neurons were plated at a density of 1 SCG per plate on poly-L-lysine-coated glass coverslips, and stained with mouse anti-β_{III} tubulin (1:800) and Alexa Fluor 488 phalloidin (1:40, Molecular Probes, Invitrogen, Carlsbad, CA).

Quantitative real time RT-PCR (qPCR). Total RNA was isolated from cultured sympathetic neurons or whole SCG using Trizol extraction (Invitrogen, Carlsbad, CA) and reverse transcribed with random octomer priming and Powerscript reverse transcriptase (Clontech, Mountain View, CA). All RNA samples were treated with DNAse (Invitrogen, Carlsbad, CA), and control samples were processed without reverse transcriptase to confirm the absence of DNA prior to reverse transcription. Quantitative Real time reverse-transcription PCR (qRT-PCR) was performed with SYBR green chemistry (Molecular Probes, Eugene, OR) on an SDS7300 Real time PCR System (Applied Biosystems, Foster City, CA). Non-intron spanning primers (Table 3) were designed to amplify coding sequences, and expression levels of all genes analyzed were normalized to GAPDH levels.

Circadian biology. For AANAT physiology studies, adult wild type and Egr3-deficient mice were housed in a controlled lighting environment (14 hours of light, 10 hours of dark [LD14:10]; lights on at 1400=ZT23) for at least three weeks prior to analysis. Animals that were sacrificed during the dark phase of the light-dark cycle were euthanized in darkness, and then pineal glands were rapidly dissected from mice under ambient lighting conditions for qPCR. Wheel running experiments were performed as described in collaboration with Dr. Dubocovich, Northwestern University (Benloucif et al. 1997). Briefly, mice were entrained to

Table 3. qPCR Primer Sequences

Gene	Orientation	Primer Sequence	Melting Temperature
AANAT	Forward	AGGGCTCTGTCCTCCTGTGGAGAT	84°C
AANAT	Reverse	TGACATGGGAAGTGGACCGGAG	84°C
Egr1	Forward	GCCTTCTCCAGCTGCTTCAT	83°C
Egr1	Reverse	GAGCCTTTAAGTCCTGGGAGC	83°C
Egr3	Forward	ATGGACATCGGTCTGACCAAC	83°C
Egr3	Reverse	AAAAGATTGCTGTCCAAGGCC	83°C
GAPDH	Forward	ACGGCAAATTCAACGGCACAGTCA	83°C
GAPDH	Reverse	GCTTTCCAGAGGGGCCATCCACAG	83°C
NGF	Forward	TCTAGACTTCCAGGCCCATGGTACA	84°C
NGF	Reverse	GGTGCAGTATGAGTTCCAGTGTTTGG	84°C
NT-3	Forward	CCAGGTCAGAGTTCCAGCCA	84°C
NT-3	Reverse	GTCATCAATCCCCCTGCAAC	84°C
TH	Forward	CTCTGTGAAGTTTGACCCGTACACCC	83°C
TH	Reverse	CGCATGCAGTAGTAAGATGTGGTTGA	83°C

a LD12:12 cycle for three weeks and then monitored for onsets of circadian activity (wheel running). In additional experiments, animals were placed in DD12:12 for three weeks to stabilize their free-running activity rhythms, and then light-pulsed (15 min, 300 lux) to phase-delay the onset of circadian activity. Magnitudes of phase shifts were determined for wild type and Egr3-deficient mice. Pre-pulse and post-pulse periods (tau) were assessed by a regression procedure eye-fitting the TAU guide through running onsets for 7-10 days before and 14 days after the light pulse. Phase shifts were then calculated as the difference between the steady state pre-pulse and the steady state post-pulse activity onset.

Cardiac physiology. Adult wild type and Egr3-deficient littermates of both sexes, weighting 18-23g, were used for *in vivo* functional studies. Prior to experimentation, pressure measurements from a 1.4 French micromanometer-tipped Millar pressure transducer (SPR839, Millar Instruments, Houston, TX) were calibrated against a mercury column after placing the catheter in warm 0.9% NaCl solution for 30 minutes. Mice were anesthetized via intraperitoneal injection of 0.1 mg/g ketamine and 2 mg/g acepromazine maleate, and then placed on a water-circulating heating pad adjusted to maintain a rectal temperature of 37.5± 0.5°C. The right jugular vein was cannulated for fluid administration. The catheter was surgically implanted into the right carotid under anesthesia, and then advanced into the left ventricle. Correct placement of the catheter in the ventricle was judged by loss of the arterial waveform and transition to a ventricular waveform with similar peak systolic pressure but diastolic pressure minima in the 0–5 mm Hg range. The surgical incision was then closed with 4-0 silk suture, and the mouse was allowed to stabilize for 10–15 min. Baseline heart functional measurements were compared with measurements after the injection of the α_2-

adrenoreceptor blocker, yohimbine (2 mg/kg, i.v.). Data were stored and analyzed by using Millar conductance data acquisition and analysis software.

Statistical measures. Student's unpaired, one-tailed t test was used to assign statistical significance at *=$p<0.05$ or **=$p<0.01$.

Chapter 3

Egr3 is a novel transcriptional regulator of sympathetic nervous system development.

Overview

The zinc finger transcription factor called Early Growth Response 1 (Egr1), also known as Nerve Growth Factor Inducible Factor IA (NGFI-A), krox24, and zif268, was identified as an NGF-induced gene in the rat pheochromocytoma (PC12) cell line (Milbrandt 1987). Since that time, several studies have elucidated an important role for Egr1 in NGF-mediated neurite outgrowth and differentiation of sympathetic-like cell lines including PC12 and neuroblastoma cells (Qu et al. 1998; Pignatelli et al. 1999; Levkovitz et al. 2001a; Levkovitz and Baraban 2002). However, deletion of Egr1 *in vivo* did not lead to identifiable neuronal defects in the sympathetic nervous system; Egr1-deficient mice are relatively normal except for female infertility (Lee et al. 1996a; Lee et al. 1996b) and defects in learning and memory (Jones et al. 2001). However, Egr1 is the prototype of a transcription factor family which also includes Egr2 (krox20), Egr3 (PILOT), and Egr4 (NGFI-C) (O'Donovan et al. 1999). The Egr genes have highly homologous DNA binding domains which seem to afford considerable functional redundancy in some systems (Tourtellotte et al. 2000; Li et al. 2005; Carter and Tourtellotte 2007). Therefore we hypothesized that another Egr family member may play a role in neurotrophin signaling in sympathetic neurons.

Egr3-deficient mice have blepharoptosis (Tourtellotte and Milbrandt 1998), a characteristic feature of mice and humans that have defective sympathetic nervous systems (Crowley et al.

1994; Ernfors et al. 1994; Farinas et al. 1994; Smeyne et al. 1994; Gurwood 1999; Honma et al. 2002). Moreover, we detected coexpression of Egr1 and Egr3 mRNA in lysates of sympathetic ganglia (data not shown), and given the 90% homology of the DNA binding domains of these two transcription factors (Patwardhan et al. 1991), we hypothesized that, in sympathetic neurons, Egr3 may play a role analogous to that of Egr1 in the differentiation of PC12 cells.

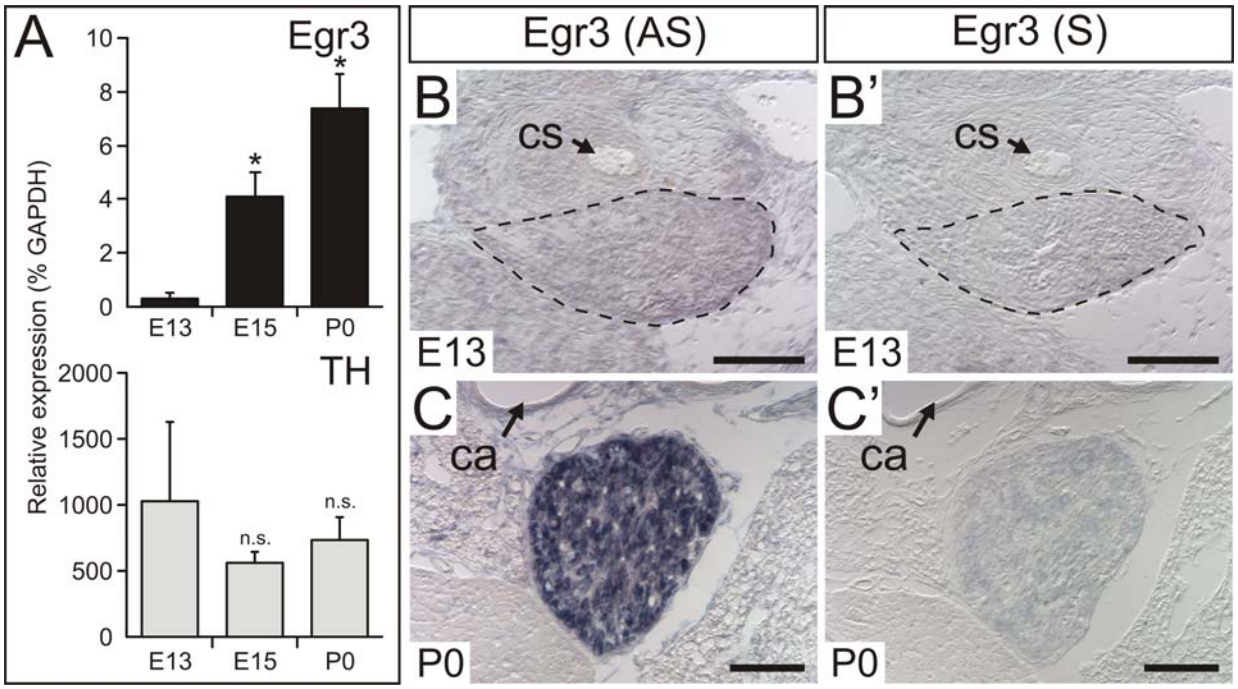
Results

Egr3 is expressed by sympathetic neurons in vivo and in vitro.

Egr1 is expressed in the developing mouse and adult rat SCG (Eldredge and Tourtellotte, unpublished observations, (Milbrandt 1987)). Likewise, we first determined where and when Egr3 is expressed in the sympathetic nervous system. SCG were isolated from wild type mice at a variety of developmental timepoints, and quantitative real time RT-PCR was performed for Egr3, tyrosine hydroxylase (TH), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). At embryonic day 13.5 (E13.5), a developmental timepoint before the onset of neurotrophin signaling *in vivo* (Wyatt and Davies 1995; Wyatt et al. 1997; Francis et al. 1999), Egr3 expression was barely detectable at less than 0.5% of the level of GAPDH expression (Figure 10A, upper panel). In contrast, Egr3 is induced by over 7-fold at E15.5 and over 11-fold at PN0, two timepoints after the onset of neurotrophin-responsiveness in sympathetic neurons *in vivo* (Figure 10A, upper panel). Tyrosine Hydroxylase (TH) was highly expressed at all timepoints analyzed, confirming the catecholaminergic identity of dissected tissues (Figure 10A, lower panel). In order to determine the localization of Egr3 expression *in vivo*, *in situ* hybridization was performed on sections through SCG from E13.5 and PN0 wild type mice.

Figure 10. Egr3 is expressed after neurotrophin stimulation of sympathetic neurons *in vivo*.

(A) Total RNA was extracted and reverse transcribed from wild type SCG isolated from E13.5, E15.5, or PN0 Swiss Webster mice. Ganglia from 5-8 mice were pooled for each timepoint. Real–time RT-PCR was performed in triplicate for Egr3 and Tyrosine Hydroxylase (TH), and data were normalized to GAPDH levels. Egr3 is induced after the onset of neurotrophin signaling *in vivo* at E15.5 and PN0, while it is present at low levels at E13.5. TH serves as a marker for dissected SCG, and TH levels are not statistically different between timepoints (n.s.). (N=5-8 SCG per timepoint; statistical significance (*) was defined as $p < 0.05$; error bars reflect +/- standard deviations) (B,C) *In Situ* hybridization for Egr3 (Antisense, AS) was performed on 12μm frozen sections through SCG from E13.5 or PN0 mice. Egr3 mRNA is much more abundant in PN0 SCG (C) than in E13.5 SCG (B), consistent with Real-time RT-PCR data from SCG lysates. Parallel sections were incubated with a nonspecific sense (S) probe (B', C') as a control for background staining. (Magnification bar=100μm, cs=carotid sinus, ca=carotid artery)


A
Egr3
Relative expression (% GAPDH)
10
8
6
4
2
0
*
*
E13
E15
P0
TH
2000
1500
1000
500
0
n.s.
n.s.
E13
E15
P0
Egr3 (AS)
Egr3 (S)
B
cs
E13
B'
cs
E13
C
ca
P0
C'
ca
P0

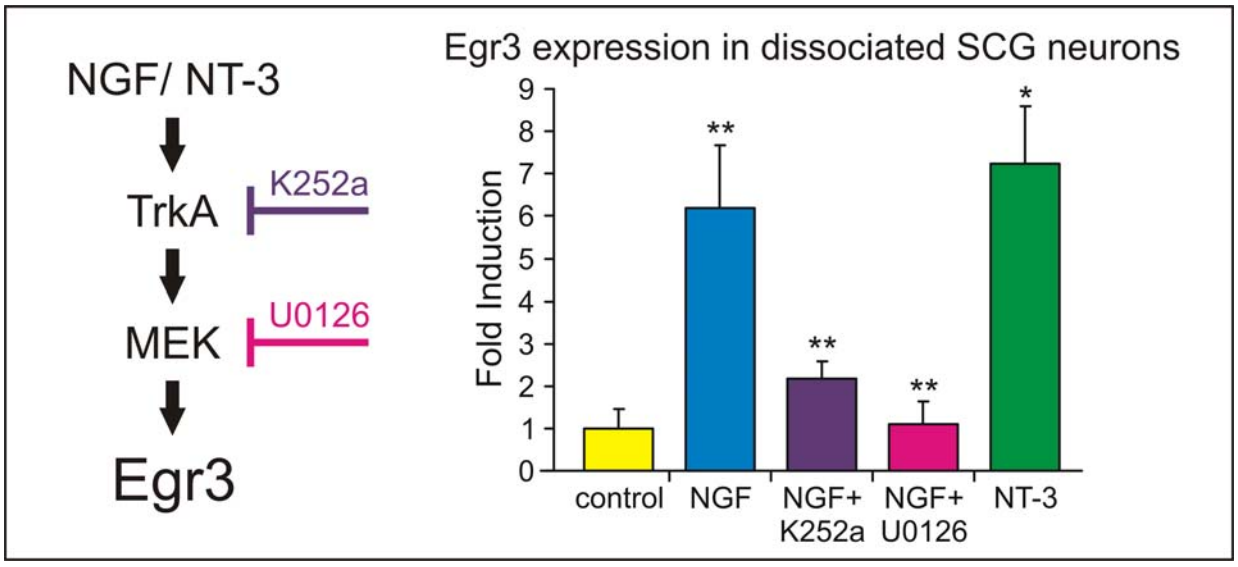
Again, minimal Egr3 expression was detected at E13.5 but robust Egr3 expression in SCG neurons was evident at PN0 (Figure 10B-C). Therefore, Egr3 expression correlates with neurotrophin signaling in sympathetic neurons *in vivo*.

To examine the signaling events leading to Egr3 expression, primary SCG neurons were cultured from E19.5 wild type mice. On day 7 of culture, neurons were starved of NGF, stimulated with 100ng/mL NGF for 45 minutes, and total RNA was isolated. Quantitative real time RT-PCR (qPCR) specific for Egr3 revealed a 7-fold induction in Egr3 mRNA in response to NGF stimulation (Figure 11). Treatment of parallel cultures with the MEK inhibitor U0126 or the TrkA tyrosine kinase inhibitor K252a reduced basal levels of Egr3 (data not shown) and prevented NGF-mediated Egr3 induction. Furthermore, neurons stimulated with 100ng/mL of NT-3 also resulted in a ~7-fold increase in Egr3 mRNA over control levels. Taken together, these results suggest that Egr3 expression in sympathetic neurons may be neurotrophin-dependent and that Egr3 is poised to be an important effector of neurotrophin signaling. Additionally, Egr3 may play a complex role in sympathetic neuron development since it is expressed in response to NGF and NT-3, two neurotrophins with overlapping but distinct roles in sympathetic neuron development.

Egr3-deficient mice lose one third of sympathetic neurons postnatally.

Egr3-deficient mice have blepharoptosis (Figure 18; (Tourtellotte and Milbrandt 1998)), a phenotype exhibited by neurotrophin-deficient mice with sympathetic neuron deficits (Crowley et al. 1994; Ernfors et al. 1994; Farinas et al. 1994; Smeyne et al. 1994). To determine if Egr3 gene deficiency resulted in sympathetic neuron loss, the number of neurons in SCG from wild type and Egr3-deficient mice was determined using an unbiased stereological approach

Figure 11. Egr3 is expressed in response to neurotrophin signaling *in vitro*. (Right) Primary SCG neurons were cultured from E19.5 wild type Swiss Webster mice for 7 days, starved of neurotrophins for 3 hours, and then stimulated with 100ng/mL NGF for 45 minutes. Total RNA was extracted from culture lysates and quantitative real time RT-PCR was performed. After NGF stimulation, SCG neurons expressed over 6-fold more Egr3 than control (dmso) treated neurons. Parallel cultures were treated with 20μm UO126 or 50nM K252a, inhibitors of MEK and TrkA respectively, for 2 hours before NGF stimulation. Both inhibitors abrogated NGF-mediated Egr3 induction in these neurons. Results for each treatment condition are representative of 3-5 experiments and 3 replicate cultures within each experiment. Real time RT-PCR reactions were performed in triplicate, with error bars reflecting standard deviations. Statistical significance was defined as *$p< 0.05$ or **$p<0.001$. (Left) Proposed signaling pathway leading to Egr3 expression in sympathetic neurons. NGF signals through the TrkA receptor, engaging the MEK/ERK pathway, and induces Egr3 expression. Inhibition of the pathway at the level of TrkA or MEK abrogates NGF-mediated induction of Egr3.


NGF/ NT-3
TrkA
K252a
MEK
U0126
Egr3
Egr3 expression in dissociated SCG neurons
Fold Induction
0
1
2
3
4
5
6
7
8
9
**
**
**
*
control
NGF
NGF+ K252a
NGF+ U0126
NT-3

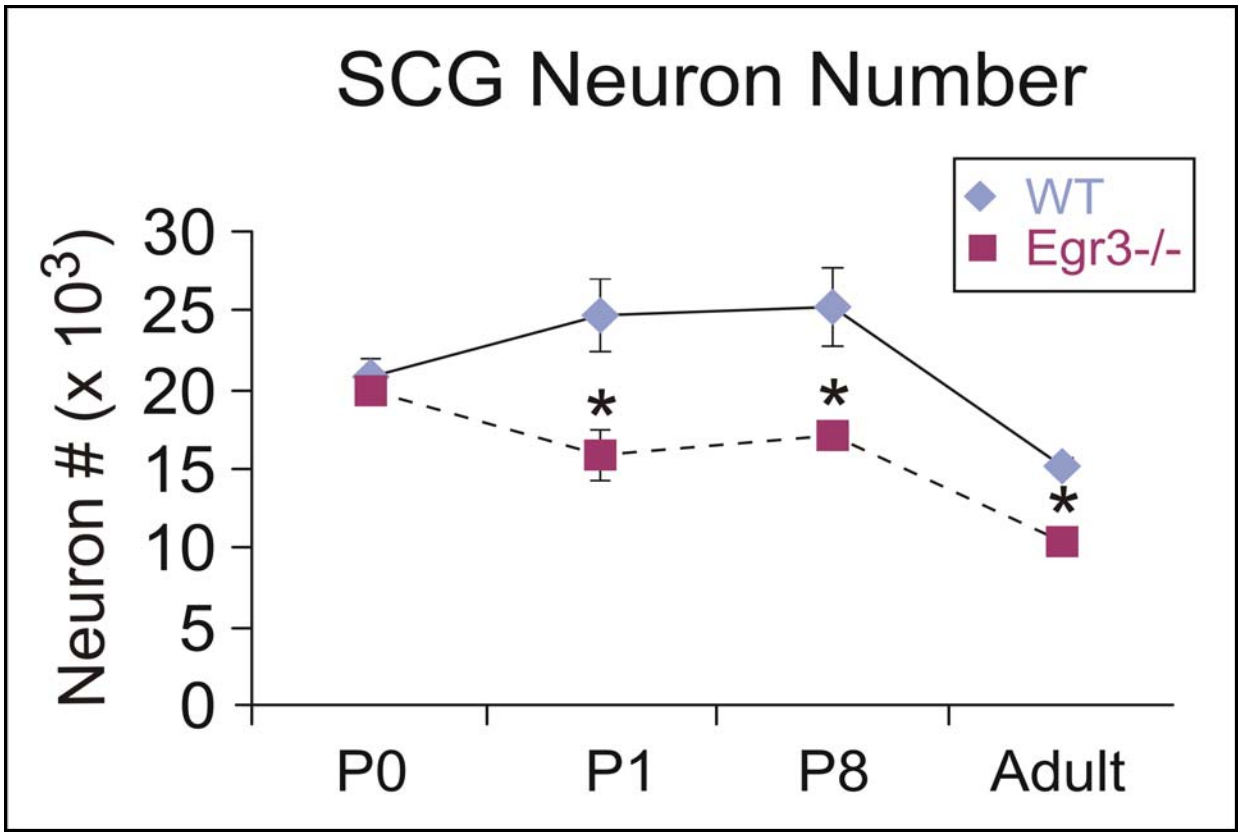
(Optical Fractionator, StereoInvestigator, Microbrightfield, Williston, VT; Figure 12). At PN0, SCG from wild type and Egr3-deficient mice have similar numbers of sympathetic neurons. Yet, at PN1, 30% of neurons were missing from Egr3-/- SCG. This reduction in neuron number persisted until PN8 and even into adulthood, after significant developmental neuron loss or "pruning."

To characterize sympathetic nervous system defects in Egr3-deficient mice, we utilized the fusion protein tau-beta-galactosidase (τlacZ, (Callahan and Thomas 1994) as a sympathetic neuron reporter molecule. Transgenic mice expressing τlacZ under the control of the dopamine Beta-hydroxylase (DβH) promoter were generated by Xiaoguang Gao in the Tourtellotte lab (Figure 13A) and bred to Egr3 heterozygous mutant mice in order to obtain Egr3+/+ and Egr3-/- mice that expressed lacZ in the cell bodies and axons of all sympathetic neurons. For example, lacZ enzyme histochemistry performed on postnatal Egr3+/+;DβHτlacZ+ mice revealed staining of SCG and STG (Figure 13B, arrowheads), thoracic sympathetic chain ganglia and intercostal nerves (Figure 13C), and sympathetic axons innervating the esophagus and kidney (Figure 13B, D). TH and β-galactosidase dual-labeling immunofluorescence was performed to confirm colocalization of the endogenous catecholaminergic enzyme and the reporter molecule within sympathetic neurons (Figure 13E-G).

Examination of the sympathetic chains from Egr3+/+;DβHτlacZ+ and Egr3-/-;DβHτlacZ+ mice revealed that Egr3-deficiency results in globally smaller sympathetic ganglia. Egr3-deficient SCG were smaller than their wild type counterparts at PN1 (Figure 14A and A'), correlating with the stereological estimates of neuron loss. Interestingly, Egr3-deficient SCG neurons were smaller than wild type neurons, most likely secondary to neuronal atrophy since a neuron diameter-frequency analysis demonstrated a skewing toward small diameter neurons in

Figure 12. One third of sympathetic neurons are lost postnatally in Egr3-deficient mice.

(A) The number of SCG neurons in every fifth section through PN0 or PN1 pups including SCG or through plucked SCG from PN8 or adult mice was counted using an unbiased stereological method (Optical Fractionator, StereoInvestigator software, Microbrightfield, Inc.) At PN0 (n=2 SCG per genotype), there was no statistically significant difference between the number of SCG neurons in wild type and Egr3-deficient mice. By PN1 and PN8 (n=3 SCG per genotype), 32-35% of SCG neurons are lost from Egr3-deficient SCG. This neuron loss persists in the adult (11 week old, n=2 SCG per genotype) SCG, despite significant attrition of neurons in wild type and Egr3-/- neurons after the postnatal period. Statistical significance was defined as $*p < 0.05$.


SCG Neuron Number
WT
Egr3-/-
Neuron # (x 10^3)
30
25
20
15
10
5
0
*
*
*
P0
P1
P8
Adult

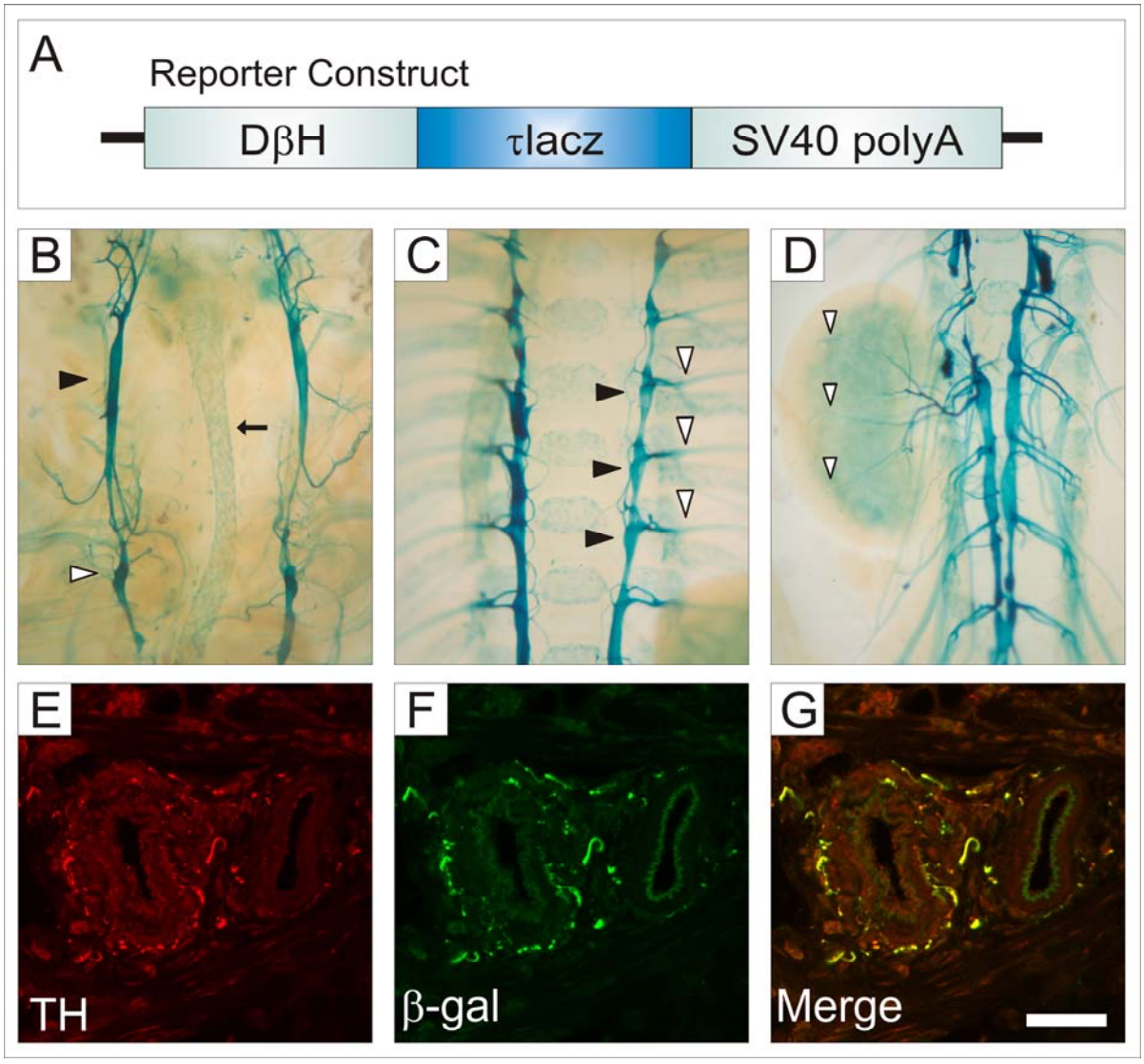
Egr3+/+;DβHτlacZ+ mice (Figure 14B-D). Interestingly, specific axonal projections from all Egr3-deficient ganglia examined were missing or reduced in caliber (white arrowheads, Figure 14A-D). Therefore, Egr3-deficient mice have sympathetic neuron defects that extend beyond the SCG. These results suggested that Egr3-deficient mice would also exhibit widespread defects in sympathetic innervation of tissues.

Egr3-deficient sympathetic axons exhibit reduced arborization in vivo.

The submaxillary salivary gland produces the most NGF of the three major salivary glands, a characteristic which results in its dense sympathetic innervation (Cohen 1960). Indeed, sympathetic neurons that project to the submaxillary gland are themselves large (in soma diameter) and contain larger dendritic arborizations than neurons projecting to smaller targets (Voyvodic 1989). In our analyses of target tissues in wild type DβH-τlacZ mice, we also observed that axons innervating the salivary gland displayed the most branching and complex pattern (Figure 16). Thus, we first examined axon outgrowth within submaxillary glands from Egr3-/-;DβHτlacZ+ mice. After staining with lacZ enzyme histochemistry, submaxillary salivary glands from PN4 Egr3-/-;DβHτlacZ+ mice (Figure 16A') exhibited decreased sympathetic innervation in comparison to glands from littermate Egr3+/+;DβHτlacZ+ mice. The submaxillary ganglion (in actuality a collection of small ganglia) within the gland itself is largely thought to be a parasympathetic ganglion, but sympathetic neurons from the superior salivatory nucleus also synapse in this "ganglion" (Huber 1896). Examination of postganglionic axons emanating from these ganglia (Figure 16, round, blue structures) revealed more numerous and thicker axon branchings (white arrowheads, Figure 16B and 16B') in the wild type gland versus

Figure 13. Expression of τlacZ in dopamine beta-hydroxylase taulacZ (DβH-τlacZ) reporter mice recapitulates expression of endogenous catecholaminergic enzymes in sympathetic neurons.

(A) Transgenic construct in which τlacZ fusion protein is driven by the human DβH promoter in all sympathetic neuronal cell bodies and axons. (B) Sympathetic ganglia and axonal projections in PN0 DβH-τlacZ+ reporter mice were labeled by whole mount lacZ enzyme histochemistry. Numerous axonal projections extend from SCG (white arrowhead), the largest sympathetic ganglion, and STG (black arrowhead). A fine meshwork of sympathetic innervation on the DβH-τlacZ+ esophagus (arrow) is also visible. (C) In the thorax, intercostal nerves (white arrowheads) extend from sympathetic chain ganglia (black arrowheads). (D) Lumbar sympathetic chain ganglia project axons to innervate the kidney (left). Note the extension of sympathetic axons within the kidney medulla (white arrowheads). Tyrosine Hydroxylase (TH, E) and β-galactosidase (β-gal, F) immunohistochemistry demonstrate colocalization (G) of TH and the τlacz reporter within sympathetic axons innervating a blood vessel from a DβH-τlacZ+ mouse. (E-G by Dr. Xiaoguang M. Gao; magnification bar=50μm)


A
Reporter Construct
DβH
τlacz
SV40 polyA
B
C
D
E
TH
F
β-gal
G
Merge

Egr3-deficient SCG (Figure 15). In addition, STG (Figure 14B'), thoracic ganglia (Figure 14C'), and caudal ganglia (Figure 14D') are smaller in postnatal Egr3-/-;DβHτlacZ+ mice than in the Egr3-/- gland. In addition, while wild type axons extended to the end of the gland, Egr3-/- axons did not (black arrowheads, Figure 16B and 16B').

Details of sympathetic innervation to additional target organs were also examined. Axons from the SCG project to form a plexus on the dorsal tracheal surface. Tracheal sympathetic innervation is concentrated in the smooth muscle between cartilaginous rings; activation of the sympathetic nervous system maximizes gas exchange through bronchodilation and the reduction of airway secretions (Mason et al. 2005). After lacZ staining and clearing, a dense meshwork of finely branched innervation was visualized on the dorsal surface of tracheae from adult Egr3+/+;DβHτlacZ mice (Figure 16C). This innervation extends to the lateral and rostral margins of the trachea. In contrast, sympathetic innervation on the Egr3-deficient trachea was vastly reduced at the lateral and rostral edges of the organ (Figure 16C'). Interestingly, innervation to the medial and caudal trachea, areas closest to the SCG projection, was largely intact. Finally, axons innervating the wild type spleen entered the parenchyma and then branched considerably (Figure 16D). In contrast, axons in Egr3-/- mice reached the spleen but failed to branch and invade the organ properly (Figure 16D'). These results suggest that Egr3-deficient sympathetic neurons may have a deficiency in their ability to undergo axonal outgrowth and/or arborization within target tissues.

Postnatal neuron loss results from increased apoptosis of Egr3-deficient SCG neurons.

Figure 14. Global reduction in the size of sympathetic ganglia and caliber of axonal projections in Egr3-deficient mice.

At PN0, SCG (black arrowheads) from Egr3-/-DβHτlacZ mice (A) appear smaller than Egr3+/+DβHτlacZ SCG (A'). In addition, several fine branches extending rostrally from the SCG (dashed circle) are absent in Egr3-deficient mice, and a branch extending caudally (likely the rostral cardiac plexus, white arrowheads) from the SCG is nearly absent in Egr3-/- mice. STG (B') and thoracic ganglia (C') from PN1 Egr3-/- mice also appear smaller than wild type ganglia (B,C). Projections from Egr3-/- STG and thoracic ganglia are thinner than wild type projections (white arrowheads). The caudal sympathetic ganglia in PN0 Egr3-deficient mice (D', black arrowheads) are most affected; they are much smaller than ganglia of their wild type littermates (D). Finally, several projections from Egr3-/- caudal sympathetic ganglia are missing and/or thinner than wild type projections (white arrowheads). (Magnification bar = 250μm.)

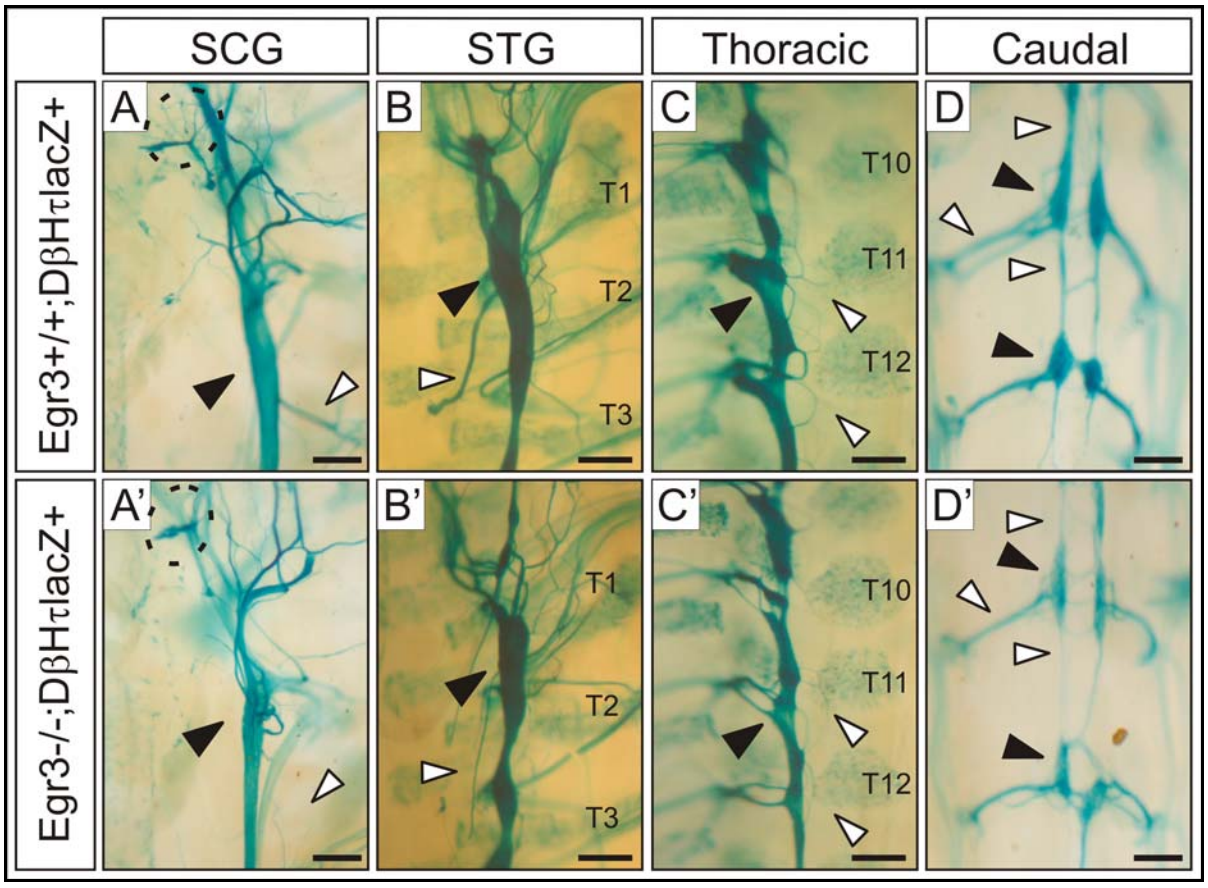
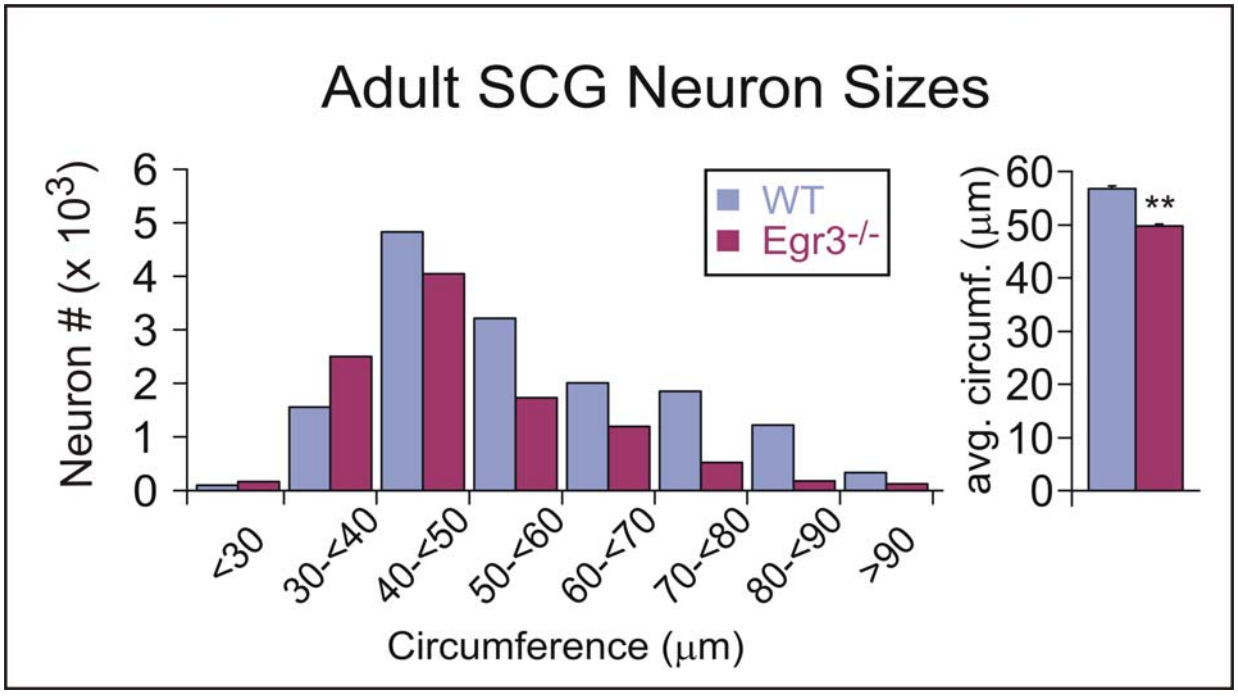

SCG
STG
Thoracic
Caudal
Egr3+/+;DβHτlacZ+
Egr3-/-;DβHτlacZ+
A
B
C
D
A'
B'
C'
D'
T1
T2
T3
T10
T11
T12

Figure 15. Egr3-deficient neurons are atrophic.

(A) The size of adult SCG neuronal cell bodies was measured by tracing the circumference of neurons using StereoInvestigator software (Microbrightfield, Inc.) Each neuron was assigned a "bin" consisting of a 10μm circumference range, and the percentage of sampled neurons in each bin was extrapolated to total neuron numbers quantified in Figure 12. Wild type neurons (n=767 neurons combined from 2 animals) fell into bins in a fairly normal distribution. In contrast, Egr3-deficient SCG (n=761 neurons combined from 3 animals) had fewer large (>40μm) neurons, but an increased number of small (less than 40μm in circumference) neurons. (B) The average neuronal circumference of Egr3-deficient neurons sampled was smaller than the average circumference of wild type neurons. Statistical significance was defined as **= $p<0.01$, error bars are +/- standard error of the mean (SEM).


Adult SCG Neuron Sizes
Neuron # (x 10^3)
0
1
2
3
4
5
6
WT
Egr3-/-
<30
30-<40
40-<50
50-<60
60-<70
70-<80
80-<90
>90
Circumference (μm)
avg. circumf. (μm)
0
10
20
30
40
50
60
**

Defective axonal arborization within target tissues would likely decrease the ability of sympathetic axons to access and retrogradely transport neurotrophins. The sympathetic neurons would then exhibit a natural form of neurotrophin withdrawal-induced cell death (Deckwerth and Johnson 1993). We therefore hypothesized that Egr3-deficient neurons would undergo excessive cell death due to their defects in axonal outgrowth (Figure 16).

Sections through SCG from mice of multiple developmental ages after the onset of neurotrophin dependence at E15.5 were obtained and analyzed for cleaved-caspase 3 immunoreactivity (Figure 17A). Cleaved (activated) caspase-3 is an early and specific marker of apoptosis and is essential to the process of sympathetic neuron apoptosis (Wright et al. 2007). There is roughly a 2-fold increase in the number of caspase 3+ apoptotic neurons in SCG from PN0 Egr3-deficient mice versus in the wild type SCG (Figure 17B). In contrast, SCG sections from PN0 animals injected with BrdU show equivalent numbers of BrdU+ proliferating neurons in wild type and Egr3-deficient ganglia (Figure 17C). Therefore, excessive apoptosis in Egr3-dependent SCG neurons results in the loss of one third of sympathetic neurons in Egr3-/- mice.

Discussion

We demonstrate that Egr3 is expressed in sympathethic neurons in response to neurotrophin stimulation *in vitro* and *in vivo*. Interestingly, Egr3 is expressed in response to stimulation of sympathetic neurons with NGF or NT-3, distinct neurotrophins signaling through a tyrosine kinase receptor TrkA. Egr3 is expressed at a very low basal level before the onset of neurotrophin signaling in sympathetic neurons *in vivo*. Similarly, pretreatment of primary

sympathetic neurons with TrkA or MAPK inhibitors blunts the induction of Egr3 in response to neurotrophins. These data place Egr3 in the correct time and place to play a role downstream of neurotrophins during sympathetic nervous system development.

Neurotrophins are required for sympathetic neuron survival and extension of axons. If Egr3 was required for sympathetic neurons to respond to neurotrophins, one would expect to see defects in Egr3-deficient sympathetic neuron survival, differentiation, or both. To this end, we have identified that about one-third of SCG neurons are lost in Egr3-deficient mice postnatally, during the time when final target innervation is taking place. In addition, we identified defects in axonal extension and/or branching within target tissues of Egr3-deficient mice. These results suggest that neuron loss may be due to insufficient axonal access to neurotrophins in target tissues and subsequent neurotrophin withdrawal-mediated apoptosis. In support of this hypothesis, we observed that Egr3-deficient neurons are atrophic. Also, Egr3-deficient neurons undergo excessive apoptosis in the Egr3-deficient SCG at PN0, a time prior to identifiable neuron loss. Therefore, we suggest that Egr3 deficiency results in secondary, rather than primary, sympathetic neuron death.

Figure 16. Decreased terminal branching of Egr3-deficient sympathetic axons *in vivo*.

Submaxillary and sublingual salivary glands, tracheae, and spleens from Egr3+/+DβHτlacZ (wild type) and Egr3-/-DβHτlacZ mice were stained with lacZ enzyme histochemistry and cleared to visualize sympathetic axons coursing through and terminating in the tissue parenchyma. PN4 wild type salivary glands (A) exhibited robust sympathetic innervation, while salivary glands from PN4 Egr3-deficient mice (A') exhibited attenuated lacZ+ innervation. Egr3-/- sublingual salivary glands (A', dashed circle) were severely affected, with very little fine innervation compared to wild type sublingual glands (A, dashed circle). Wild type sympathetic axons projected nearly to the end of the submaxillary gland (B, black arrowheads), while Egr3-deficient axons were thin and failed to reach the end of the gland (B', black arrowheads). Terminal branches of Egr3-/- axons (B', white arrowheads) were thinner than wild type axons (B, white arrowheads) or absent. Deficient arborization of Egr3-deficient axons was also apparent in the trachea. After lacZ staining, a delicate meshwork of sympathetic innervation was visible on the dorsal surface of tracheae from adult wild type mice (C). Tracheal sympathetic innervation was concentrated in the smooth muscle between cartilage rings (white arrowheads), and axons extend to the lateral edges of the organ. In contrast, the density of tracheal sympathetic innervation in adult Egr3-deficient mice was severely attenuated at the lateral and rostral portions of the trachea, farthest from the SCG (C'). Moreover, Egr3-/- sympathetic axons (D') reached the spleen but did not appear

to invade or ramify in comparison to wild type axons that invaded and branched at the spleen hilum (D). (Magnification bar= 250μm)

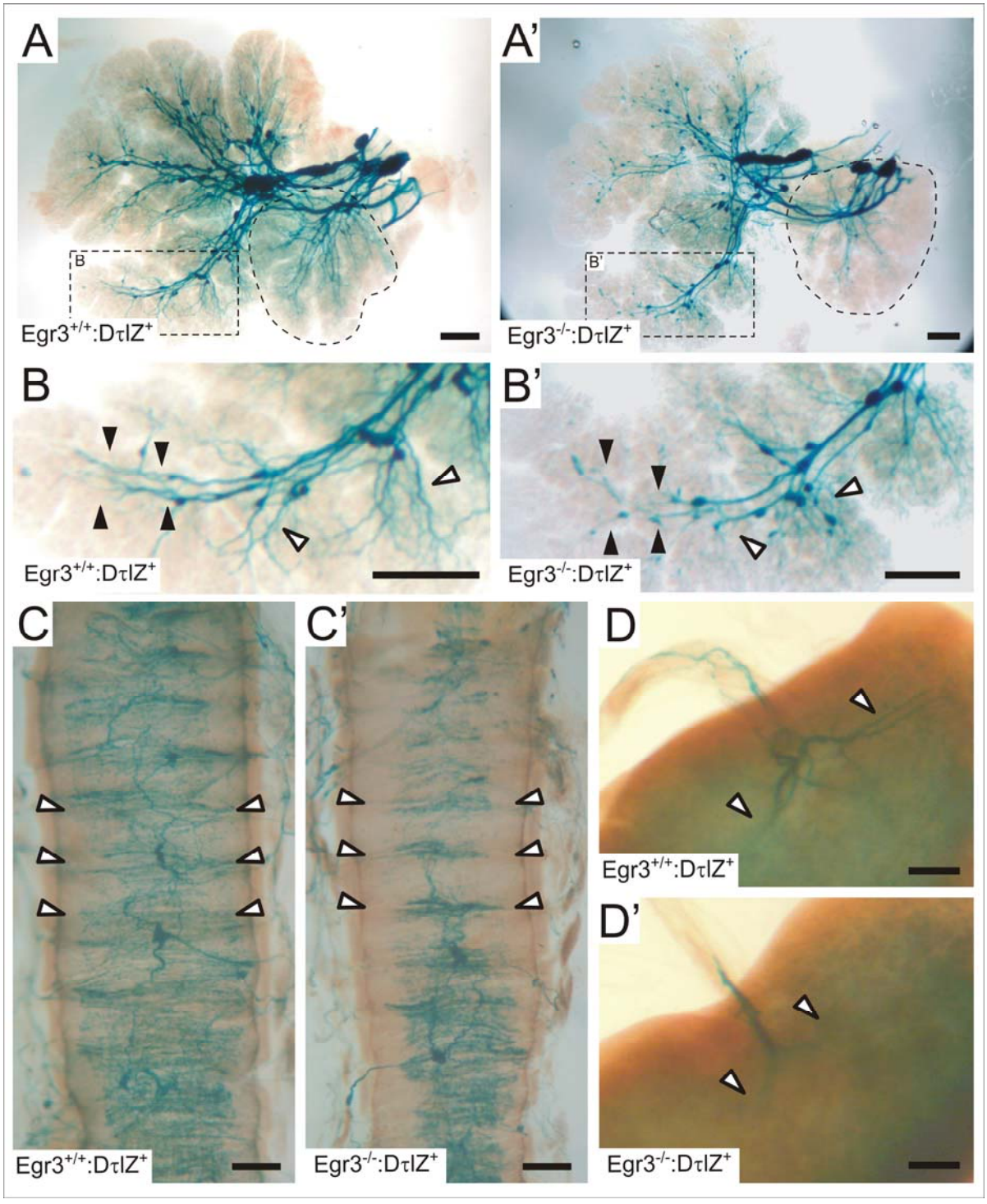
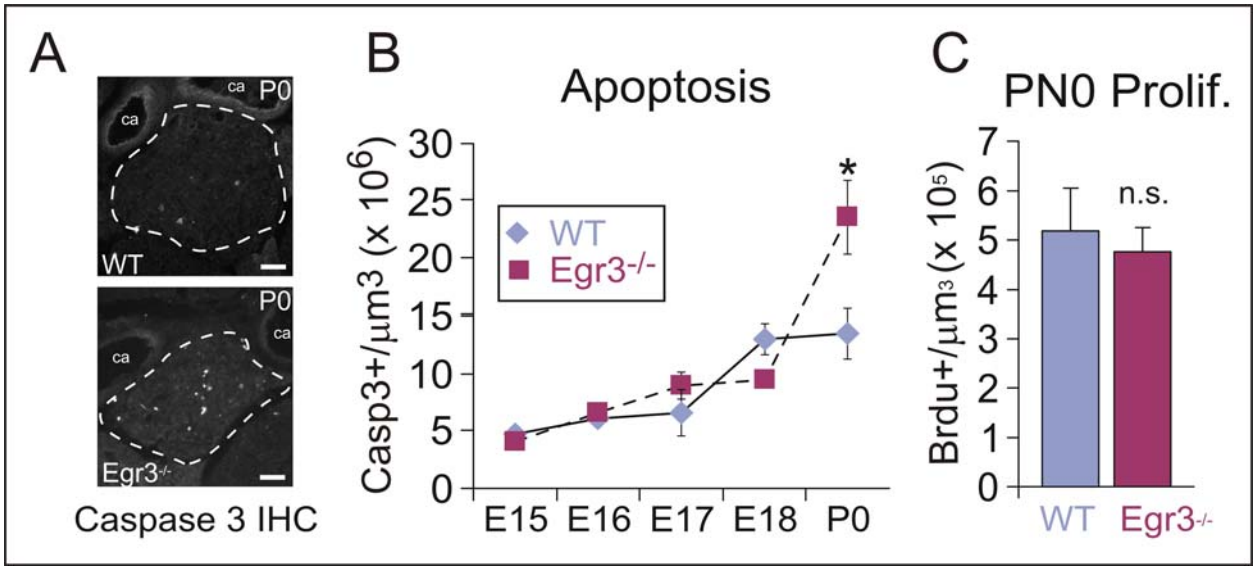

A
A'
B
B'
Egr3+/+:DτlZ+
Egr3-/-:DτlZ+
B
B'
Egr3+/+:DτlZ+
Egr3-/-:DτlZ+
C
C'
D
Egr3+/+:DτlZ+
Egr3-/-:DτlZ+
Egr3+/+:DτlZ+
D'
Egr3-/-:DτlZ+

Figure 17. Egr3-deficient SCG neurons undergo excessive apoptosis postnatally. Immunohistochemistry for the cleaved/activated form of caspase 3 was performed to identify apoptotic neurons within developing SCG. (A) Representative caspase 3 immunohistochemistry (IHC) demonstrating increased immunoreactivity in SCG from PN0 Egr3-/- mice SCG (ca= carotid artery, magnification =40μm). (B) The number of caspase 3-positive (Casp3+) neurons were counted in SCG from wild type and Egr3-deficient mice of various ages progressing from the start of neurotrophin dependence at E15.5. At PN0, before the onset of neuron death, there is a 1.8 fold increase in the number of apoptotic neurons in Egr3-deficient SCG (n=5) over the number of caspase-3-positive neurons in wild type SCG (n=6). (C) PN0 pups were injected with BrdU 2 hours prior to sacrifice to label proliferating cells, and BrdU immunohistochemistry was performed on SCG sections. There is no significant difference between the number of BrdU-positive neurons in wild type and Egr3-deficient SCG (n=4 SCG per genotype). Statistical significance was defined as *= $p<0.05$. Error bars are +/- Standard Error of the Mean (SEM).


A
ca
P0
ca
WT
P0
ca
ca
Egr3-/-
Caspase 3 IHC
B
Apoptosis
Casp3+/µm3 (x 10^6)
30
25
20
15
10
5
0
WT
Egr3-/-
*
E15
E16
E17
E18
P0
C
PN0 Prolif.
Brdu+/µm3 (x 10^5)
7
6
5
4
3
2
1
0
n.s.
WT
Egr3-/-

Chapter 4

The effect of Egr3 gene deficiency on oculosympathetic structure and function.

Overview

Sympathetic innervation to the eye functions to dilate the pupils, to retract the upper and lower eyelids, and to promote secretions from lacrimal and meibomian glands. Interruption of the oculosympathetic system results in blepharoptosis and miosis in Horner's syndrome patients (Figure 2). In addition, development and maintenance of iris pigmentation depends upon intact sympathetic innervation (Laties 1974). Indeed, individuals with congenital Horner's syndrome have heterochromia, in which iris pigmentation of the affected side does not fully develop. The goal of these studies was to determine whether the sympathetic neuron loss identified in Chapter 3 affected ocular sympathetic innervation, pigmentation, and/or physiology.

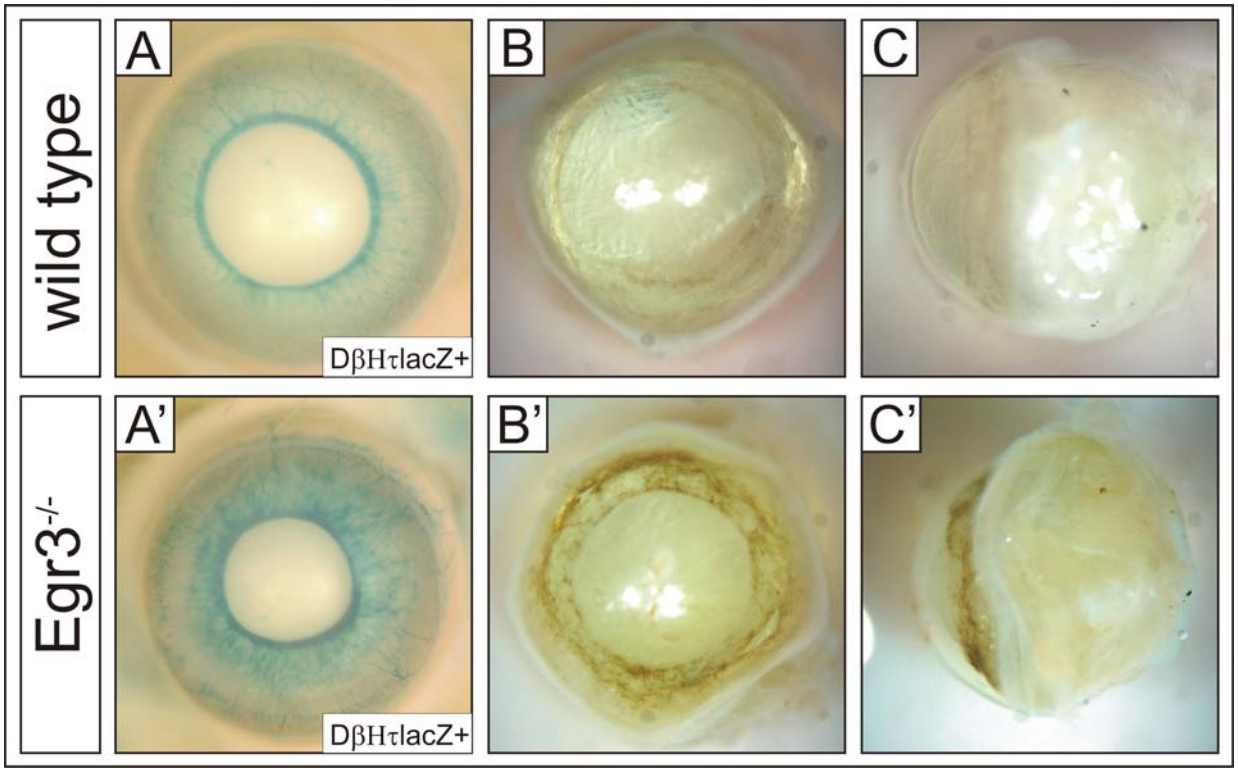
Results

Irides from Egr3-deficient mice display aberrant sympathetic innervation and pigmentation.

Whole mount lacZ enzyme histochemistry was used to assess the sympathetic innervation of the iris, a target tissue which receives innervation from SCG neurons. Surprisingly, irides from Egr3-deficient mice have disorganized rather than attenuated sympathetic innervation. In comparison to the regularly patterned innervation of wild type irides (Figure 18A), sympathetic innervation of the Egr3-deficient irides is uneven (Figure 18A'), concentrated in some areas while deficient in others.

Figure 18. Iris innervation and pigmentation in Egr3-deficient mice.

Whole mount lacZ enzyme histochemistry of Egr3+/+;DβHτlacZ and Egr3-/-; DβHτlacZ eyes revealed disorganized hyperinnervation of the Egr3-deficient iris (A') in comparison to the wild type iris (A). In addition, Egr3-deficient irides exhibit hyperpigmentation (B', front and C', lateral) in comparison to irides from wild type mice (B and C). Pictures are representative of at least 3 animals per genotype.


wild type
A
DβHτlacZ+
B
C
Egr3-/-
A'
DβHτlacZ+
B'
C'

In addition, irides from adult Egr3-deficient mice (Figure 18B' and 18C') display abnormal, patchy pigmentation in comparison to irides from their wild type littermates (Figure 18B and 18C). These results suggest that disorganized sympathetic innervation in irides of Egr3-deficient mice may result in abnormal ocular pigmentation.

Egr3-deficient mice have physiological blepharoptosis.

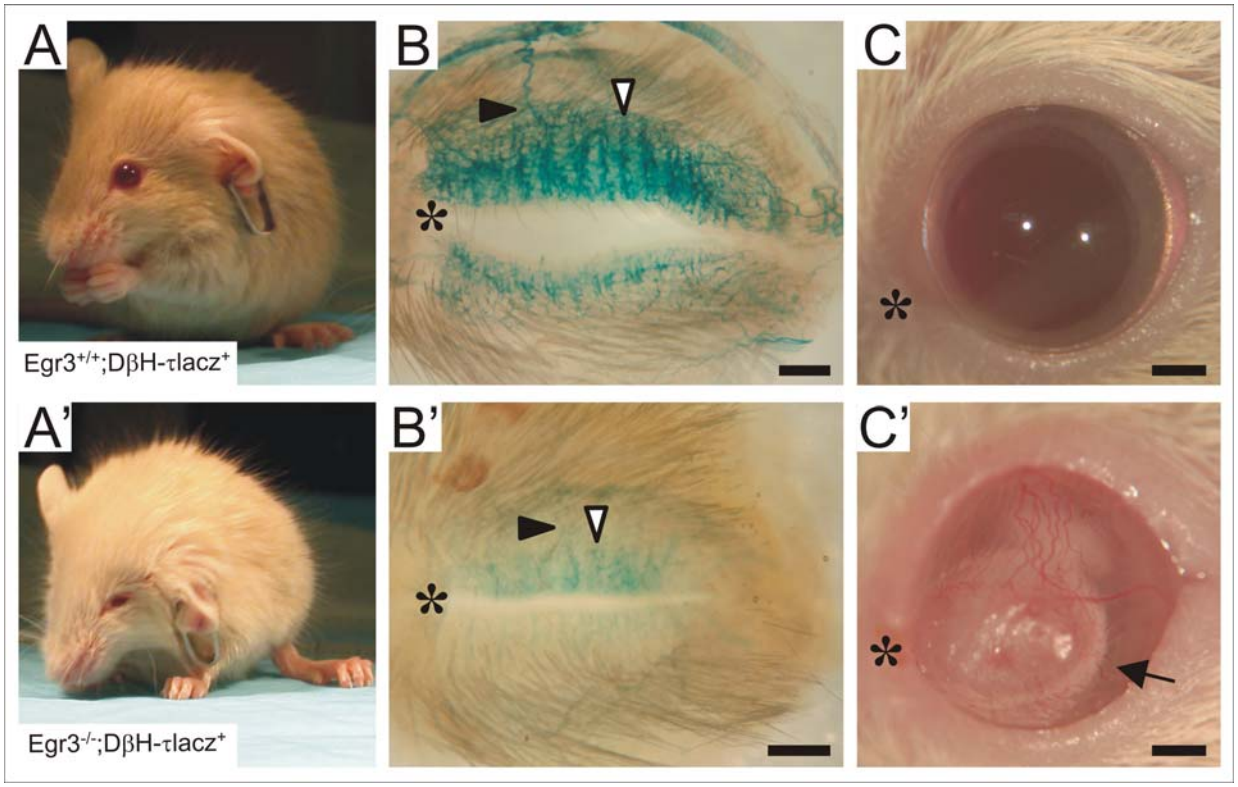
Blepharoptosis, or drooping upper eyelid, can be a manifestation of sympathetic dysfunction, as autonomic innervation of the superior tarsus muscle is critical for physiological eyelid elevation. Indeed, humans (Horner's syndrome), and mice (NGF- and TrkA-deficient mice for example) with sympathetic nervous system defects have profound blepharoptosis (Crowley et al. 1994; Gurwood 1999; Honma et al. 2002). Egr3-deficient mice display significant blepharoptosis when relaxed (Figure 19A', (Tourtellotte and Milbrandt 1998)) which we have characterized as autonomic blepharoptosis since they are able open their eyes completely when startled (Eldredge and Tourtellotte, unpublished observations).

Deficient tarsal sympathetic innervation leads to dry eye, corneal ulceration, and blepharoptosis in Egr3-deficient mice.

Aged Egr3-deficient mice frequently develop corneal neovascularization (Figure 19C'), a condition that arises in response to corneal irritation and/or damage. This observation, combined with the fact that Egr3-/- mice develop periorbital alopecia due to excessive rubbing of their eyes (Eldredge and Tourtellotte, unpublished observations), led us to question whether decreased sympathetic innervation in Egr3-deficient eyes led to "dry eyes." SCG neurons,

Figure 19. Egr3-deficient mice have physiological blepharoptosis and dry eye due to sympathetic denervation.

5 week old wild type (A) and Egr3-/- (A') littermate animals were photographed, and Egr3-deficient mice displayed profound blepharoptosis. Eyelids from adult wild type and Egr3-/- mice were stained with whole mount lacZ enzyme histochemistry, cleared, and analyzed under the dissection microscope. Robust sympathetic innervation to tarsal musculature (black arrowheads) and meibomian glands (white arrowheads) was evident in wild type eyelids (B), whereas sympathetic innervation to both targets was nearly absent in Egr3-/- eyelids (B'). Often Egr3-deficient mice have "dry eye," which leads to corneal abrasions and eventually neovascularization in aged Egr3-deficient mice (C'), while aged Egr3 wild type mice continue to have healthy eyes with no indication of corneal damage (C). (Magnification bar= 0.5mm, *= nasal canthus)


A
B
C
Egr3+/+;DβH-τlacz+
A'
B'
C'
Egr3-/-;DβH-τlacz+

whose axons innervate the superior tarsus muscle, also project to the meibomian glands of the eyelid (Simons and Smith 1994). Secretions from these glands compose the lipid component of the tear film and therefore are required for healthy ocular physiology. We performed a whole mount preparation of the eyelids from wild type and Egr3-deficient adult mice to examine the status of sympathetic innervation to tarsal musculature and glands. Wild type eyelid preparations stained with lacZ enzyme histochemistry displayed the detailed sympathetic innervation of the tarsal muscles and meibomian glands (Figure 19B). In contrast, in Egr3-deficient mice, significantly less sympathetic axons innervate the interior tarsal surface (Figure 19B'). These results suggest that the superior and inferior tarsus muscles as well as the meibomian glands are at least partially denervated in eyelids from Egr3-/- mice, leading to physiological blepharoptosis and chronic dry eye with corneal damage and neovascularization.

Discussion

Sympathetic nervous system defects in Egr3-deficient mice have profound effects on ocular physiology. Indeed, Egr3-deficient mice have abnormal sympathetic innervation in several ocular structures including the iris, tarsal muscles, and meibomian glands. These structural defects impact ocular development and physiology, resulting in abnormal iris pigmentation, blepharoptosis, dry eye, and corneal damage. Therefore, the oculosympathetic nervous system is also critical for maintaining the health of the orbit and thereby the overall health of the animal.

Chapter 5

Autonomic Cardiovascular Physiology in Egr3-deficient mice

Overview

If the prototypical sympathetic nervous system function is to mediate the "fight or flight" response, then the heart is one of its most important effector organs. In response to adrenergic stimulation, the heart increases the rate and force with which it beats to perfuse muscles with an oxygen supply adequate for exercise. Removal or attenuation of these physiological adaptations renders a human or animal less able to cope with stressful situations. Since we identified significant sympathetic denervation of other tissues in the absence of Egr3, we sought to determine whether the hearts of Egr3-deficient mice exhibited denervation and/or perturbed autonomic physiology. Experiments measuring cardiac physiological parameters were performed in collaboration with Drs. Jon Lomasney and Qiaoqiang Han.

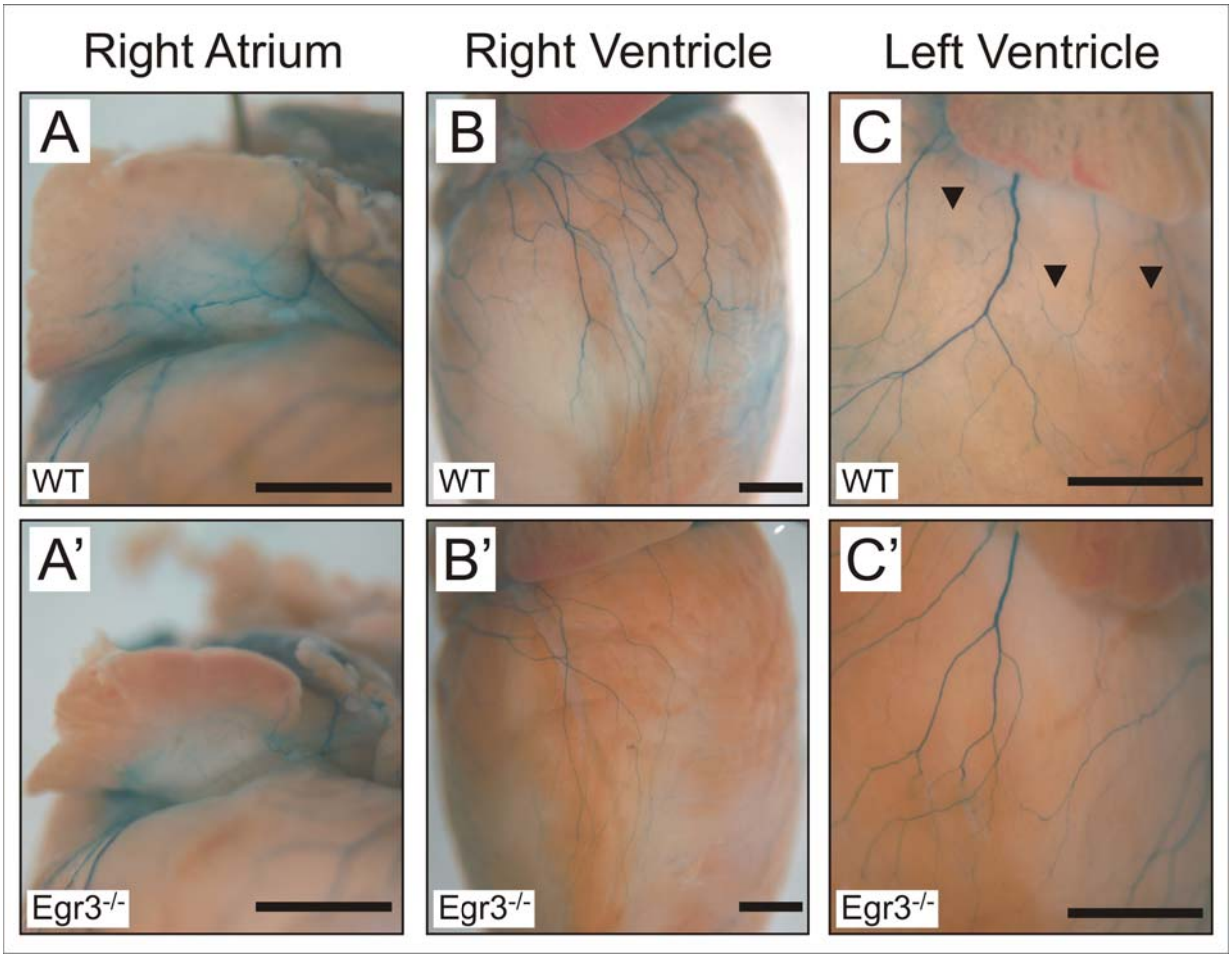
Results

Fine sympathetic innervation is attenuated in hearts from Egr3-deficient mice.

Neurons from SCG, STG, and thoracic sympathetic chain ganglia project to innervate the heart. Since we identified SCG neuron losses and smaller STG and thoracic ganglia (Figure 14), we investigated whether the hearts of Egr3-deficient mice had decreased sympathetic innervation. Hearts from wild type mice displayed complex lacZ+ innervation of the atria (Figure 20A) and ventricles (Figure 20B-C). In particular, the innervation to the left ventricular myocardium was the most finely branched (arrowheads, Figure 20C). However, lacZ+ axons

Figure 20. Hearts of Egr3-deficient mice exhibit sympathetic denervation.

Whole mount lacZ enzyme histochemistry was performed on hearts from adult wild type and Egr3-deficient mice to stain pericardial sympathetic axons. Sympathetic axons are visible on the inferior surface of the right atrium from a wild type mouse (A), while these lacZ+ fibers are absent from the Egr3-/- atrium (A'). Both right (B) and left (C) ventricles from wild type mice exhibit robust sympathetic innervation, with fine sympathetic terminals particularly evident on the left ventricle (arrowheads, C). Right ventricles (B') from Egr3-deficient mice have globally decreased lacZ+ innervation, while fine fibers are specifically absent from left ventricles (C'). (Magnification bar=1mm)

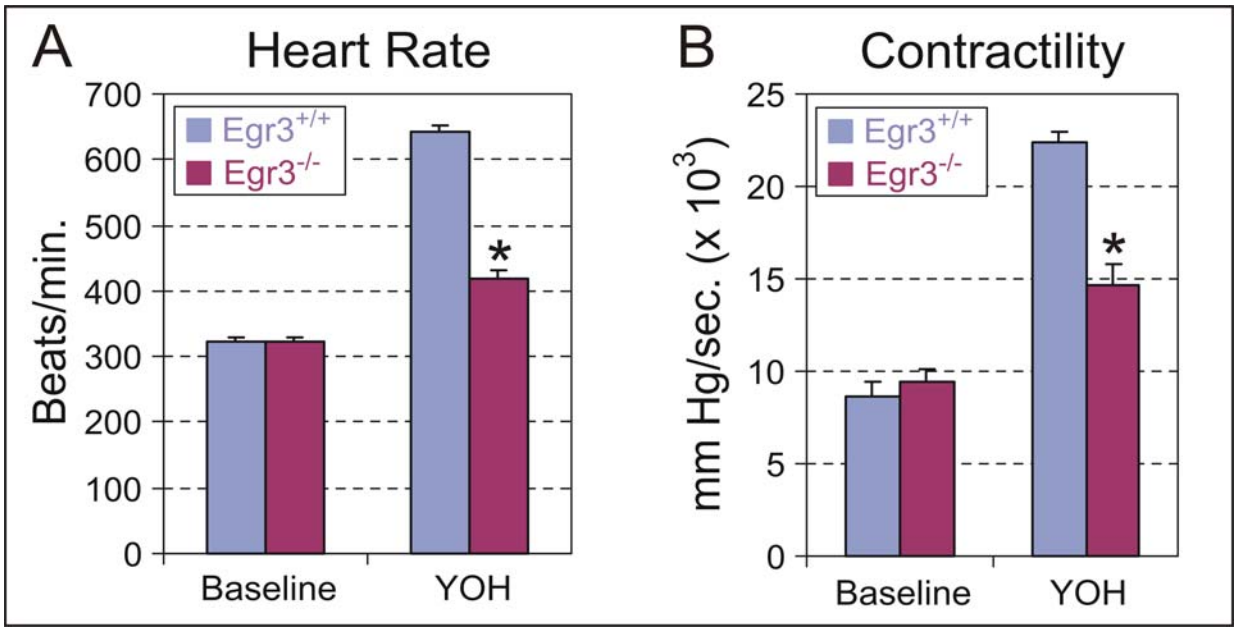

Right Atrium
Right Ventricle
Left Ventricle
A
B
C
WT
WT
WT
A'
B'
C'
Egr3-/-
Egr3-/-
Egr3-/-

were fewer in number in the Egr3-deficient right atrium and both ventricles (Figure 20A'-C'). In addition, fine processes were conspicuously absent on the ventricles of Egr3-/- hearts (Figure 20C').

Cardiac responses to adrenergic stimulation are attenuated in hearts from Egr3-deficient mice.

To determine if the attenuated innervation in Egr3-deficient hearts affected cardiac functions, we compared the physiological responses of wild type and Egr3-deficient hearts to sympathetic nervous system stimulation. Through activation of the β_1 adrenergic receptor, sympathetic neuronal activity increases heart rate and contractility of the normal heart (Costanzo 1998). Wild type and Egr3-deficient adult mice were given the α_2-adrenergic receptor blocker yohimbine as a sympathomimetic agent. Since this drug works via disinhibition in the preganglionic neurons of the central nervous system, the physiological effects are related to the number of sympathetic axons that reach and innervate the heart. After administration of yohimbine, wild type mice more than doubled their heart rates (beats per minute, Figure 21A) and contractility (mmHg/second, Figure 21B). In contrast, Egr3-deficient mice exhibited blunted cardiac responses to yohimbine. Surprisingly, despite severe innervation defects, Egr3-deficient hearts had baseline heart rate and contractility measurements that were similar to wild type hearts. We hypothesize that this basal normality is due to functional compensation by Egr3-deficient mice, perhaps due to increased levels of β_1-adrenergic receptors in the myocardium, a topic for future studies.

Figure 21. Dysfunctional cardiac autonomic physiology in Egr3-deficient mice. Heart Rate and Contractility (dP/dt) were determined in catheterized wild type (n=5) and Egr3-deficient (n=7) mice before and after administration of the sympathomimetic agent yohimbine. Baseline heart rate and contractility measurements were similar in wild type and Egr3-/- mice. After the yohimbine-mediated increase in sympathetic drive, heart rate and contractility in wild type mice more than doubled. However, Egr3-deficient mice displayed severely blunted physiological responses to increased sympathetic activity. Statistical significance was defined as *=$p<0.01$; error bars are +/- standard error of the mean.


A
Heart Rate
Beats/min.
700
600
500
400
300
200
100
0
Egr3+/+
Egr3-/-
*
Baseline
YOH
B
Contractility
mm Hg/sec. (x 10^3)
25
20
15
10
5
0
Egr3+/+
Egr3-/-
*
Baseline
YOH

Vasculature from Egr3-deficient mice exhibits intact sympathetic innervation and normal response to adrenergic stimulation.

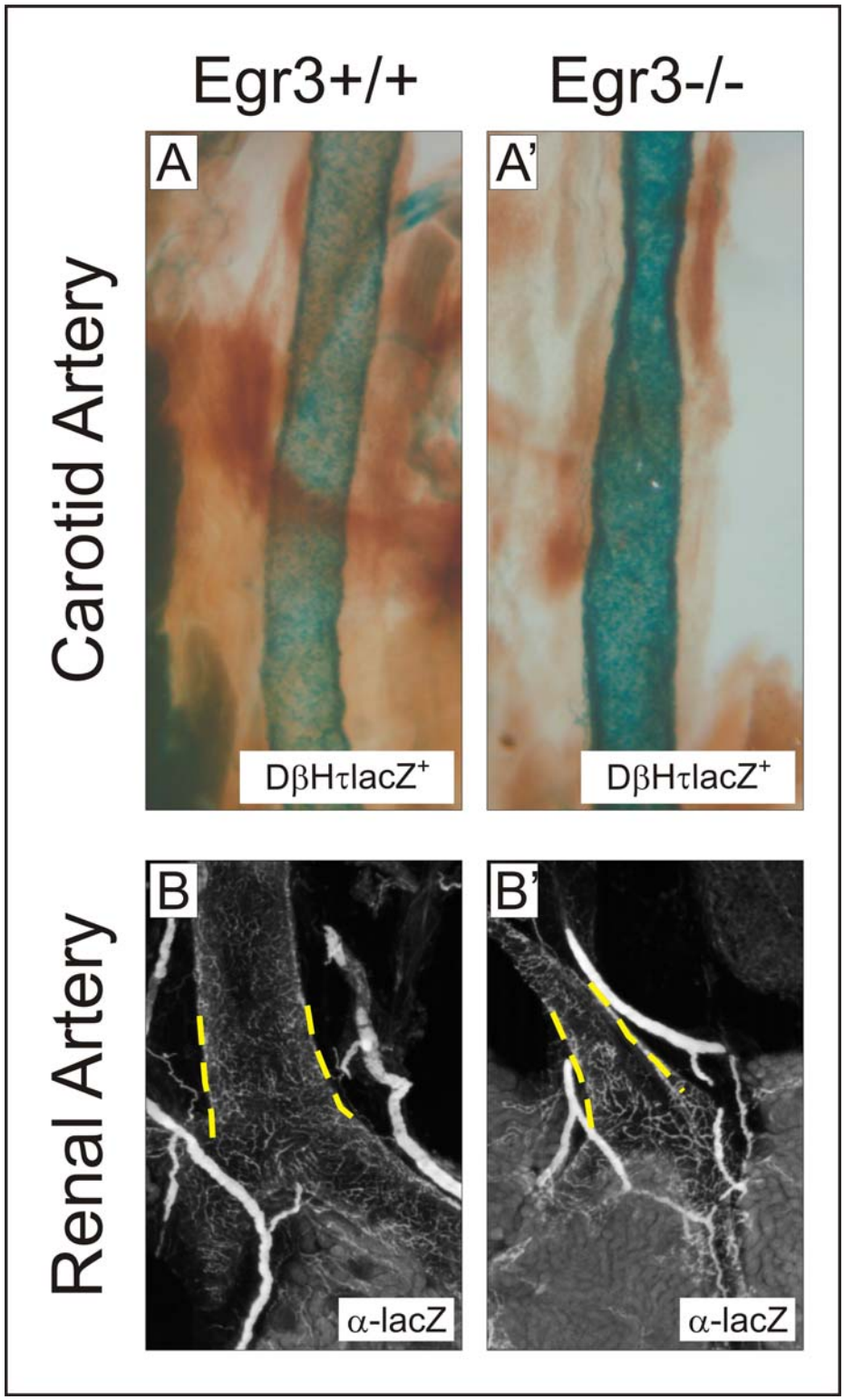
The role of the vasculature in sympathetic nervous system development is two-fold: to guide sympathetic axons to their targets during development and to regulate blood pressure through its own sympathetic innervation in the mature animal.

We next examined the sympathetic innervation of blood vessels within Egr3-deficient mice. Carotid arteries from adult Egr3+/+;DβHτlacZ+ and Egr3-/-;DβHτlacZ+ mice stained with lacZ enzyme histochemistry demonstrated no deficiencies in lacZ+ innervation in the absence of Egr3. Surprisingly, Egr3-deficient carotid arteries appear to be hyperinnervated (Figure 22A and A'). Furthermore, we examined the innervation status of the renal arteries from Egr3-deficient mice using a different approach. 100μm-thick frozen sections through the entire kidney were cut and immunostained using a lacZ antibody to visualize the plexus of fine sympathetic innervation on the renal arteries entering the kidney hila. No apparent differences in arterial innervation were appreciated between kidneys from Egr3+/+;DβHτlacZ+ and Egr3-/-;DβHτlacZ+ mice (Figure 22B and B').

Finally, along the cardiac physiology studies described above, the blood pressure of wild type and Egr3-deficient mice was determined before and after administration of the sympathomimetic yohimbine. The blood pressure measurements for wild type and Egr3-deficient mice before and after stimulation were similar (Figure 23). We hypothesize that the ability of Egr3-deficient mice to adequately regulate blood pressure may be due to the presence of intact arterial sympathetic innervation.

Figure 22. Arterial sympathetic innervation is intact in Egr3-deficient mice.

Carotid arteries from adult Egr3+/+;DβHτlacZ (A) and Egr3-/-;DβHτlacZ (A') mice were stained with whole mount lacZ enzyme histochemistry. Both wild type and Egr3-deficient carotid arteries displayed abundant sympathetic innervation, and the Egr3-deficient vessel appeared to be hyperinnervated. 50μm thick sections through kidneys from adult Egr3+/+;DβHτlacZ (B) and Egr3-/-;DβHτlacZ (B') mice were immunostained with an antibody specific for lacZ. The extent of delicate sympathetic innervation on the renal arteries entering the kidney hila (yellow dashed lines) was similar between the two genotypes.


Egr3+/+
Egr3-/-
Carotid Artery
A
A'
DβHτlacZ+
DβHτlacZ+
Renal Artery
B
B'
α-lacZ
α-lacZ

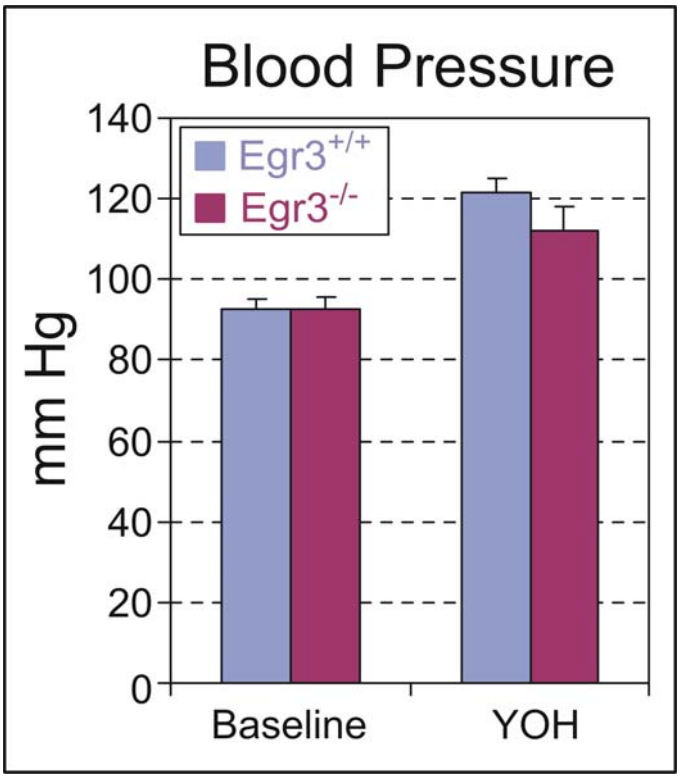
Discussion

Sympathetic innervation of the heart is extensive, with innervating axons originating in SCG, STG, and sympathetic chain ganglia. In the absence of Egr3, cardiac sympathetic innervation is attenuated. Interestingly, fine innervation on the left ventricles of Egr3-deficient hearts is conspicuously absent. It is likely that this finely branched innervation is more critical for parenchymal function rather than larger nerves which may function as axon "highways." Indeed, Egr3-deficient hearts exhibit abnormal autonomic physiology under the conditions of increased sympathetic activity.

Surprisingly, vasculature in Egr3-deficient mice is normally innervated or hyperinnervated, and these animals are able to effectively regulate their blood pressure. The cause of the disparity in cardiac and vasculature phenotypes is unclear. It is possible that heart is a more distal target and therefore hypothesized axon outgrowth defects in Egr3-deficient mice would be more detrimental to its innervation status. Alternatively, Egr3-deficient sympathetic axons may differ in their ability to respond to additional growth and guidance signals distinctly expressed by the vasculature or heart as final targets.

Figure 23. Blood pressure is unaffected by Egr3 gene deficiency.

Blood pressure measurements were obtained in the left ventricles of catheterized wild type (N=5) and Egr3-deficient mice (N=7) before and after administration of the sympathomimetic agent yohimbine. There was no statistically significant difference in the basal or stimulated blood pressures between the two genotypes.


Blood Pressure
140
120
100
80
60
40
20
0
mm Hg
Egr3+/+
Egr3-/-
Baseline
YOH

Chapter 6

A role for Egr3 in circadian physiology of the pineal gland.

Overview

Sympathetic innervation of the pineal gland serves an important role in circadian biology. Through an increase of sympathetic input to the pineal during the dark phase of the light-dark cycle, mammals synchronize their biological rhythms with the environment. In response to increased adrenergic activity, arylalkylamine N-acetyl transferase (AANAT), the rate-limiting enzyme in melatonin synthesis, is transcriptionally upregulated by innervated pinealocytes (Figure 25A, (Borjigin et al. 1995) (Foulkes et al. 1997)).

NGF and NT-3 are produced by pineal parenchyma (Randolph et al. 2007), and mice lacking NT-3 or the low affinity neurotrophin receptor p75 share a phenotype of pineal denervation (Lee et al. 1994; ElShamy et al. 1996). Since Egr3 is regulated *in vitro* by NT-3, and Egr3-deficient mice lose one-third of their SCG neurons postnatally, we investigated whether Egr3 deficiency would also result in pineal denervation and concomitant deregulation of AANAT.

Egr genes may play additional roles in the suprachiasmatic nucleus (SCN), which is the major circadian pacemaker organ (Herzog and Tosini 2001). Egr1 expression is specifically upregulated in the SCN and later in the periventricular nucleus (PVN) in response to and for the duration of photic stimulation (Tanaka et al. 1999). However, Egr1-deficient mice entrain normally to changes in the light-dark cycle, and the SCN pacemaker from these mice exhibits normal responses (i.e. induction of the immediate-early gene *c-fos*) to photic stimulation (Kilduff et al. 1998). These results suggest that another Egr family member may functionally

compensate for Egr1 in the SCN. A candidate for this redundant role is Egr3. After exposure to light, Egr3 expression is specifically induced in the ventral SCN, a structure implicated in entrainment (Morris et al. 1998). Therefore, in collaboration with Dr. Margarita Dubocovich, we investigated whether Egr3-deficient mice have abnormal circadian rhythms and light entrainment.

Results

Denervation of the pineal gland in Egr3-deficient mice.

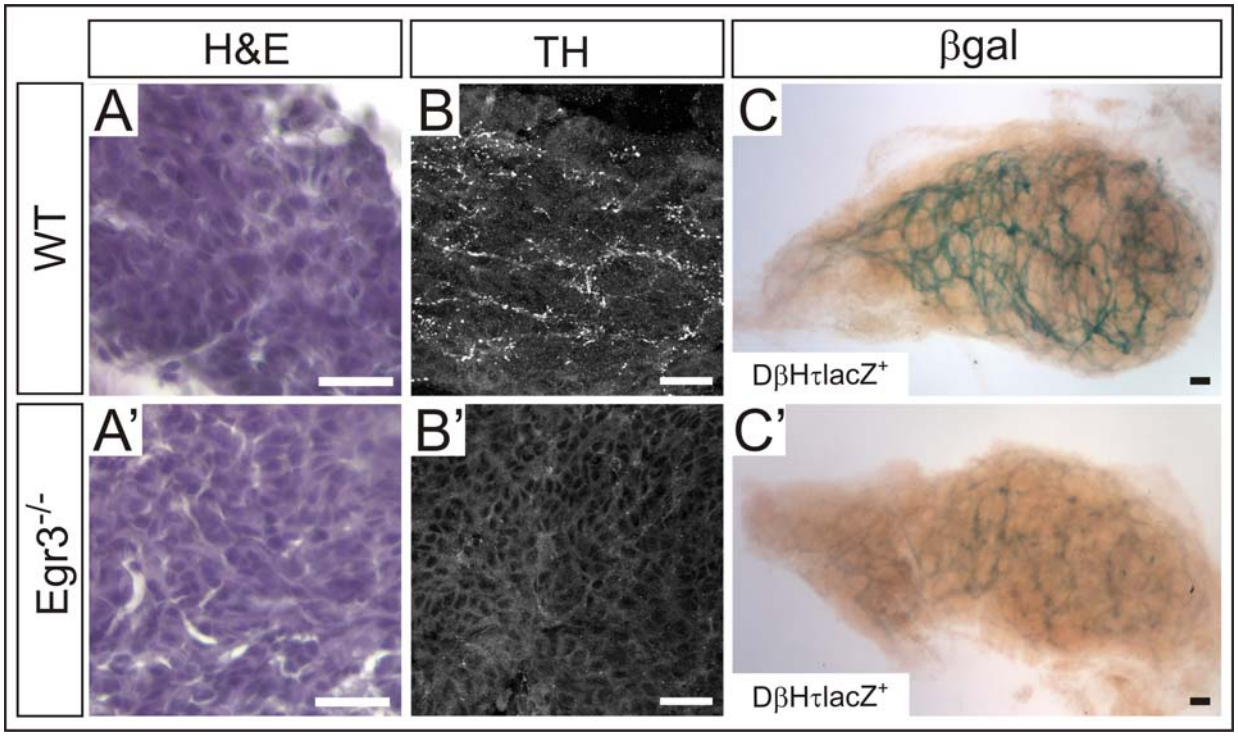
We first used TH immunohistochemistry to assess the status of pineal innervation in adult Egr3-deficient mice. When stained with hematoxylin and eosin (H&E), wild type and Egr3-deficient pineal sections appeared similar, with characteristic pineocytomatous rosettes (Figure 24A and 24A'). In contrast, there was little TH+ innervation to Egr3-/- pineal parenchyma (Figure 24B') versus the abundant innervation of the wild type gland (Figure 24B). In addition, we examined the status of pineal sympathetic innervation in Egr3+/+;DβHτlacZ and Egr3-/-;DβHτlacZ mice using a whole mount preparation of the entire pineal gland. After lacZ enzyme histochemistry and tissue clearing, wild type pineal glands displayed abundant lacZ+ terminals (Figure 24C) while Egr3-/-;DβHτlacZ pineal glands had scant sympathetic innervation (Figure 24C').

Deregulation of AANAT in the denervated Egr3-/- pineal gland.

We next asked the question whether the deficiency of sympathetic innervation in Egr3-deficient pineal glands would result in abnormal regulation of AANAT, the diurnally regulated

Figure 24. Sympathetic denervation in the Egr3-deficient pineal gland.

Pineal glands from adult wild type and Egr3-deficient mice were sectioned and stained with H&E or TH immunohistochemistry. Histologically, wild type (A) and Egr3-/- (A') pineal glands are similar, with pineocytomatous rosettes (black arrowheads). TH immunohistochemistry reveals abundant fine innervation in the wild type pineal gland (B) while innervation to Egr3-/- pineal parenchyma is nearly absent (B'). Whole mount lacZ enzyme histochemistry was also performed on Egr3+/+DβHτlacZ+ and Egr3-/-DβHτlacZ pineal glands to assess innervation status. A complicated network of lacZ+ innervation is evident in the wild type pineal gland (C) while lacZ reactivity was greatly attenuated in the Egr3-deficient pineal gland (C') reacted in parallel. (Magnification bar =25μm)


H&E
TH
βgal
WT
Egr3-/-
A
B
C
A'
B'
C'
DβHτlacZ+
DβHτlacZ+

enzyme critical for melatonin synthesis. Mice were entrained to a 14L:10D light cycle, with lights on at 1400 (also defined as Zeitgeber time ZT0). The kinetics of AANAT induction were first defined in pineal glands from wild type animals. Pineal glands were rapidly dissected at five points during the light:dark cycle: 0300 (ZT13), 0700 (ZT17), 1100 (ZT21), 1900 (ZT5) or 2300 (ZT9). qPCR was performed on total RNA extracted from the pineal lysates, and peak levels of AANAT were attained at 1100 (ZT21, Figure 25B).

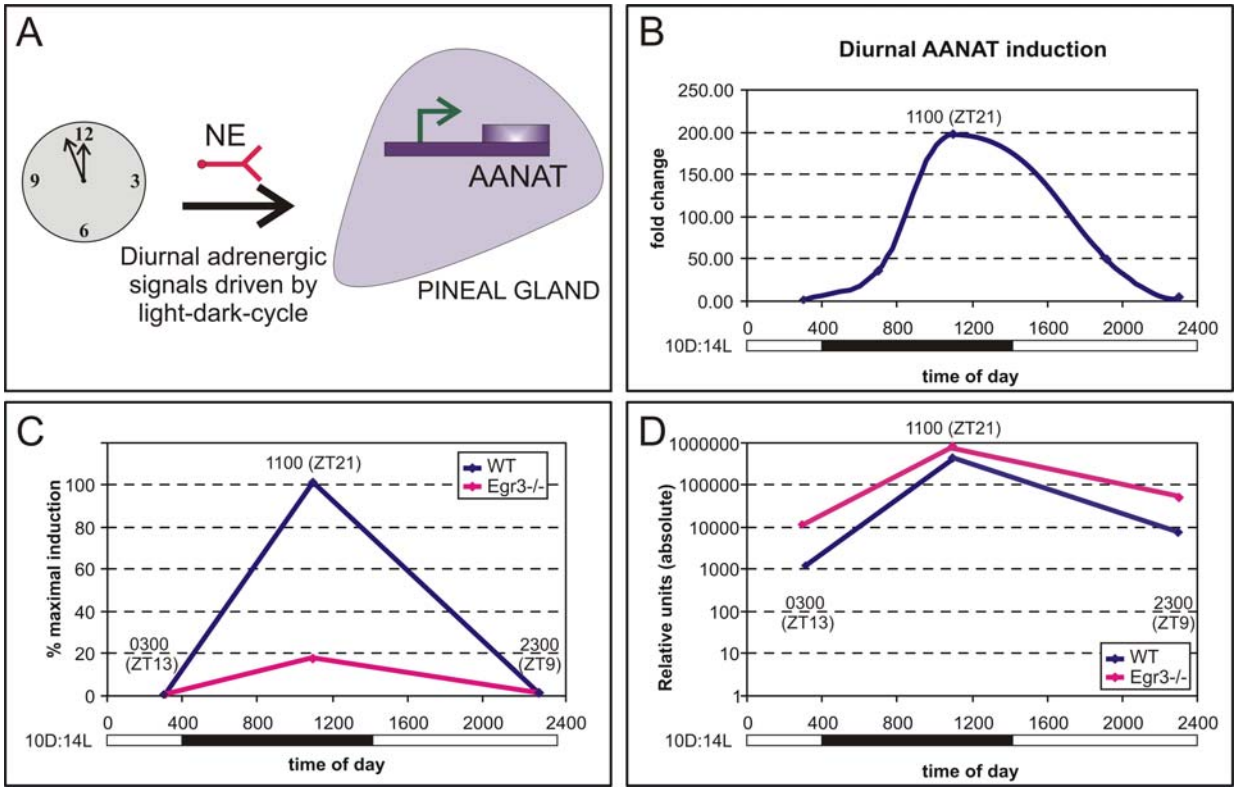
We next sought to compare the relative inductions of AANAT in wild type and Egr3-deficient (denervated) pineal glands. AANAT induction in Egr3-deficient mice was roughly 20% of the maximal induction observed in wild type mice (Figure 25C). Interestingly, however, the absolute levels of AANAT in the Egr3-deficient pineal gland were actually higher than those in the wild type pineal at all timepoints examined (Figure 25D), perhaps due to functional compensation by the mice lacking the normal adrenergic regulation mechanism. In the future, it would be interesting to determine the melatonin production by the Egr3-deficient pineal gland. If melatonin levels were higher at all timepoints in the light-dark cycle as would be expected based on the observed absolute levels of AANAT, Egr3-deficient mice may be hypersomnolent and/or exhibit entrainment defects (see below). Indeed, Egr3-deficient mice appear slower to wake from sleep than their wild type littermates (Eldredge, unpublished observations).

Diurnal regulation of Egr3 expression in the SCG

Since Egr3 is expressed in the SCG (Figure 10) and the sympathetic pathway driving AANAT synthesis that involves Egr3 is diurnally regulated, we asked the question of whether

Figure 25. Lack of sympathetic innervation to Egr3-deficient pineal glands disrupts diurnal regulation of AANAT.

Adult wild type and Egr3-deficient mice were entrained to a 10D:14L cycle in which the lights are turned off at 0400 (ZT14) and on at 1400 (defined as ZT0). (A) Diagram of the diurnal transcriptional regulation of AANAT, the rate-limiting enzyme of melatonin synthesis. (B) The kinetics of AANAT induction were determined in wild type mice. Pineal glands were rapidly dissected under ambient lighting at 0300 (ZT13), 0700 (ZT17), 1100 (ZT21), 1900 (ZT5) or 2300 (ZT9), and quantitative real time RT-PCR (qPCR) for AANAT was performed on cellular lysates. Peak levels of AANAT mRNA are normally produced in the pineal gland at 1100 (ZT21) in response to adrenergic signaling. (C) Pineal glands from cycled wild type and Egr3-deficient mice were rapidly dissected from mice at 0300 (ZT13), 0700 (ZT21), or 2300 (ZT9), and AANAT qPCR was performed. There was a ~500-fold induction of AANAT in the wild type pineal at ZT21, during the dark phase of the 14L:10D light cycle. The induction of AANAT in the Egr3-deficient pineal gland at ZT21 only reached ~20% of the maximal induction seen in wild type animals. (D) The absolute levels of AANAT mRNA in Egr3-/- pineal gland as determined by qPCR are higher the levels of the wild type animals at all timepoints, suggesting functional compensation. Results are representative of two (B) or three (C, D) independent experiments. qPCR was performed in triplicate and results were normalized to GAPDH.


A
12
9
3
6
NE
Diurnal adrenergic signals driven by light-dark-cycle
AANAT
PINEAL GLAND
B
Diurnal AANAT induction
fold change
250.00
200.00
150.00
100.00
50.00
0.00
1100 (ZT21)
0
400
800
1200
1600
2000
2400
10D:14L
time of day
C
% maximal induction
100
80
60
40
20
0
1100 (ZT21)
WT
Egr3-/-
0300
(ZT13)
2300
(ZT9)
0
400
800
1200
1600
2000
2400
10D:14L
time of day
D
Relative units (absolute)
1000000
100000
10000
1000
100
10
1
1100 (ZT21)
0300
(ZT13)
2300
(ZT9)
WT
Egr3-/-
0
400
800
1200
1600
2000
2400
10D:14L
time of day

Egr3 expression also cycles in the adult SCG. SCG were dissected from wild type adult mice that were entrained to a 10L:14D light cycle for at least 3 weeks at three timepoints: ZT21 (dark), ZT13 (light), and ZT9 (light). *In situ* hybridization for Egr3 was performed on sections through cycled SCG, and we observed that Egr3 is upregulated at ZT21 over its baseline level at ZT13 (Figure 26). This diurnal regulation parallels that of AANAT. These results suggest that Egr3 is not only critical for the development of the sympathetic nervous system but may also be coupled to synaptic activity.

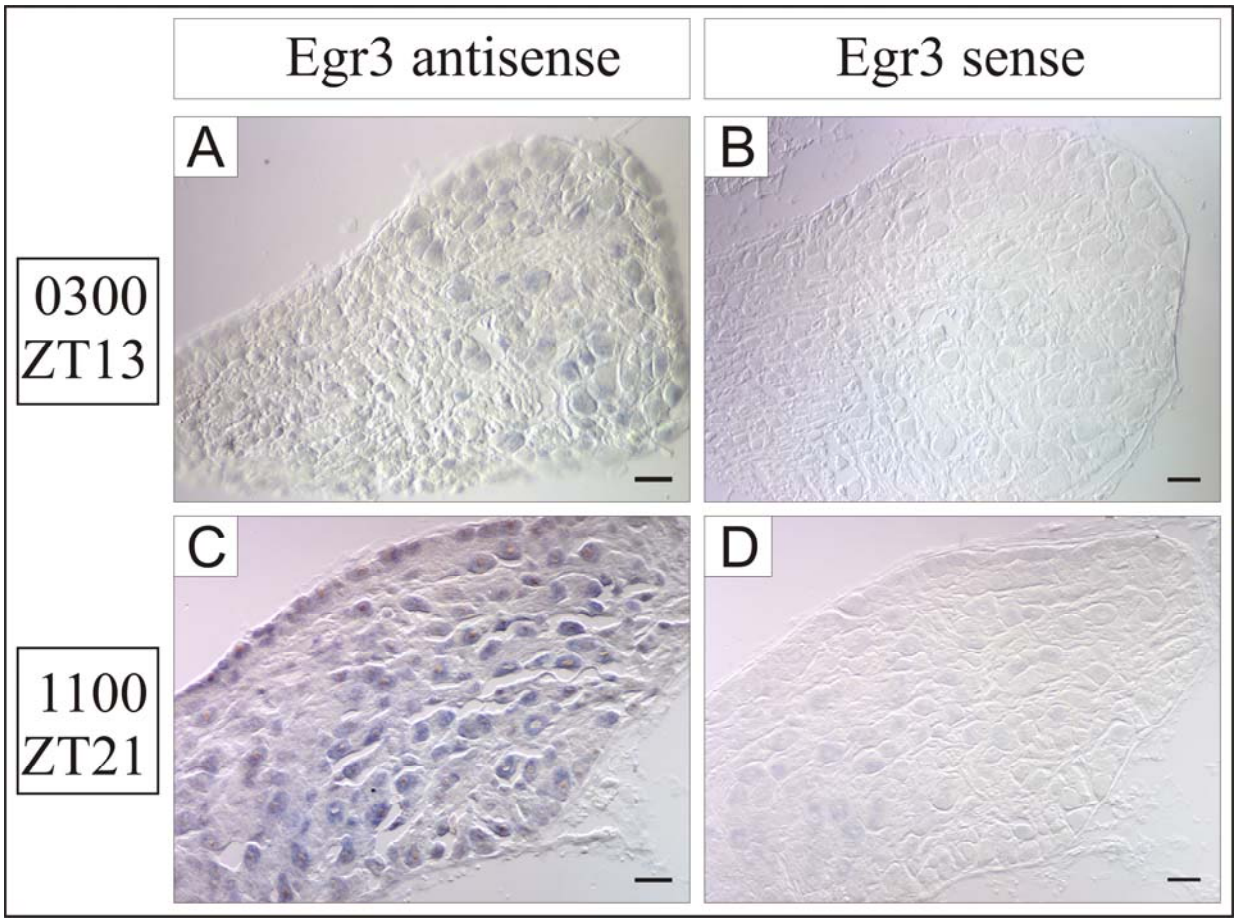
Egr3-deficient mice exhibit light entrainment defects

Since Egr3 is diurnally regulated in the SCG and Egr3-deficient mice display deregulation of AANAT mRNA in the pineal gland, we asked whether Egr3-deficient mice have abnormal circadian rhythms manifested by wheel-running behavior. Animals were entrained to a LD12:12 cycle and their circadian rhythms were plotted as a function of wheel running activity. Wild type mice are nocturnal and preferentially run during the dark phase of the light cycle (Figure 27A). Some, but not all, of the Egr3-/- mice exhibited inverted patterns of wheel running—activity in light phase of the light-dark cycle (Figure 27B). We believe that the incomplete penetrance of this phenotype may be due to genetic background effects (our Egr3-/- mice are in a hybrid C57Bl6/129 background).

In additional experiments, animals were placed in constant darkness (DD), a condition in which endogenous circadian rhythms are said to be "free running." When wild type mice were stimulated with a pulse of light under conditions of constant darkness, they experienced a phase shift to delay onset of wheel-running activity (Figure 27C). In contrast, none of the Egr3-/- mice tested shifted their wheel-running activity in response to the light pulse (Figure 27). This

Figure 26. Diurnal regulation of Egr3 mRNA in the adult SCG.

(A) *In situ* hybridization was performed on frozen sections through the adult SCG to detect Egr3 expression. SCG isolated from an entrained (10D:14L) wild type mouse shows minimal Egr3 expression at 0300 (ZT13). (B) An adjacent section from the same animal was incubated with a sense probe to control for nonspecific reactivity. (C) Upregulation of Egr3 expression in a subpopulation of SCG neurons temporally coincides with AANAT induction in the pineal gland at 1100 (ZT21, Figure 25). (D) An adjacent section incubated with control sense probe displays minimal background staining. In situ hybridization reactions were performed using a digoxygenin/alkaline phosphatase detection system, and results are representative of two independent experiments. (Magnification bar = 40μm)


Egr3 antisense
Egr3 sense
A
B
0300
ZT13
C
D
1100
ZT21

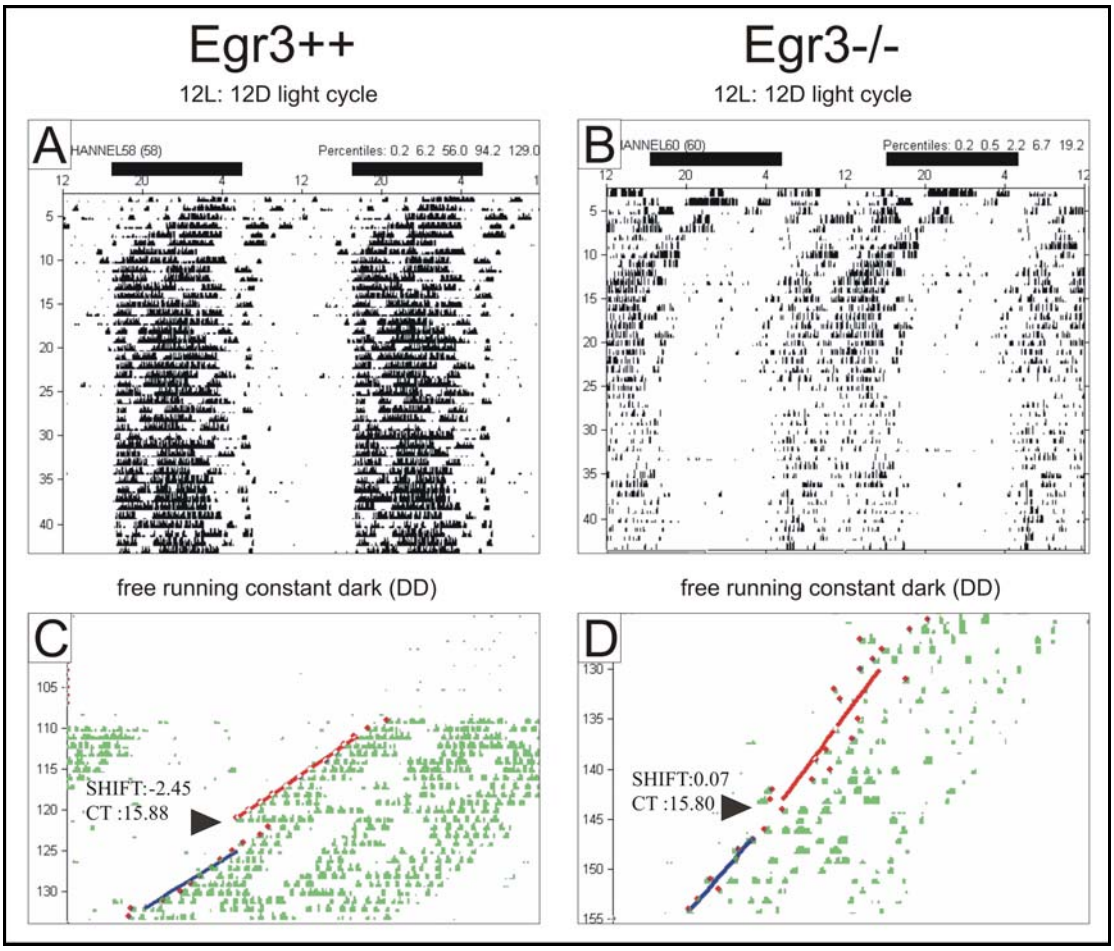
result suggests that the Egr3-/- mice are unable to respond to changing light conditions in the environment.

Discussion

Egr3 may play important roles in multiple aspects of circadian biology. First, Egr3 is diurnally expressed in the SCG, correlating with the peak of adrenergic activity that drives upregulation of AANAT in the pineal gland. Thus, Egr3 expression is coupled to sympathetic neuron activity and may be important for the function of mature sympathetic neurons. Secondly, Egr3-deficient mice exhibit severely attenuated sympathetic innervation of the pineal gland which results in the deregulation of AANAT, and presumably melatonin, synthesis. Third, Egr3-deficient mice display partially penetrant entrainment defects. At present, we cannot determine whether defective entrainment of Egr3-deficient mice may be due to the loss of the SCG-to-pineal projection or to a SCN-based defect. While the SCN is thought to be the major circadian clock, melatonin levels are highly relevant to entrainment and circadian rhythm disorders (Arendt 2005). Indeed, a sympathetic neuron-specific deletion of Egr3 will be required to clarify this issue in the future.

Figure 27. Adult Egr3-deficient mice exhibit circadian entrainment defects.

(A) Wheel-running behavior of a wild type mouse on a 12:12 light-dark cycle shows predominantly nocturnal activity (dark hours marked with black bar). (B) 2 of the 7 Egr3-/- mice studied showed a preference for wheel-running during the subjective day (light). (C) The mice were transferred to constant dark (DD) conditions for 2 weeks and then subjected to a light pulse for 15 minutes (arrowhead). Wild type mice exhibited a shift in its running pattern "away" from the time of the light pulse, while Egr3-/- mice exhibited no shift in the onset of running activity.


Egr3++
12L: 12D light cycle
Egr3-/-
12L: 12D light cycle
A
HANNEL58 (58)
Percentiles: 0.2 6.2 56.0 94.2 129.0
B
ANNEL60 (60)
Percentiles: 0.2 0.5 2.2 6.7 19.2
free running constant dark (DD)
free running constant dark (DD)
C
SHIFT:-2.45
CT :15.88
D
SHIFT:0.07
CT :15.80

Chapter 7

Early Growth Response 3 (Egr3) has a role in sympathetic neuron development in the absence of cell death

Overview

From our *in vivo* observations of sympathetic innervation defects (Figure 16) and increased neuronal apoptosis coinciding with final target innervation (Figure 17) in Egr3-deficient mice, we hypothesized that Egr3 is critical for aspects of neurite outgrowth. Therefore, we predicted that axon outgrowth deficiencies in Egr3-deficient mice would result in secondary death of sympathetic neurons via classic neurotrophin withdrawal-induced apoptosis (Deckwerth and Johnson 1993). However, from the studies described in Chapter 3, it is not clear whether the observed axon outgrowth defects are a cause or an effect of sympathetic neuron loss.

The role of neurotrophins in axonal outgrowth during sympathetic nervous system development was masked by the dependence of sympathetic neurons on NGF and NT-3 for survival (Crowley et al. 1994; Ernfors et al. 1994; ElShamy et al. 1996). Analysis of mice lacking neurotrophins and the proapoptotic molecule Bax (Knudson et al. 1995) clarified a secondary role of NGF and NT-3 (Patel et al. 2000; Kuruvilla et al. 2004) in sympathetic neuron differentiation. Mice lacking NGF or NT-3 have severely decreased sympathetic innervation of the heart, and this decreased cardiac innervation persists in mice that are doubly deficient for NGF and Bax or NT-3 and Bax (Kuruvilla et al. 2004).

Here, analysis of Egr3/Bax double homozygous mutant mice will similarly be used to separate roles of Egr3 in survival and differentiation. We hypothesized that sympathetic

innervation defects were not due to a neuron-intrinsic survival defect. Therefore, we expected that Egr3/Bax DKO animals would also exhibit sympathetic defects, even with the protection of neurons from death. This work is currently ongoing in collaboration with other additional members of the Tourtellotte Lab.

Results

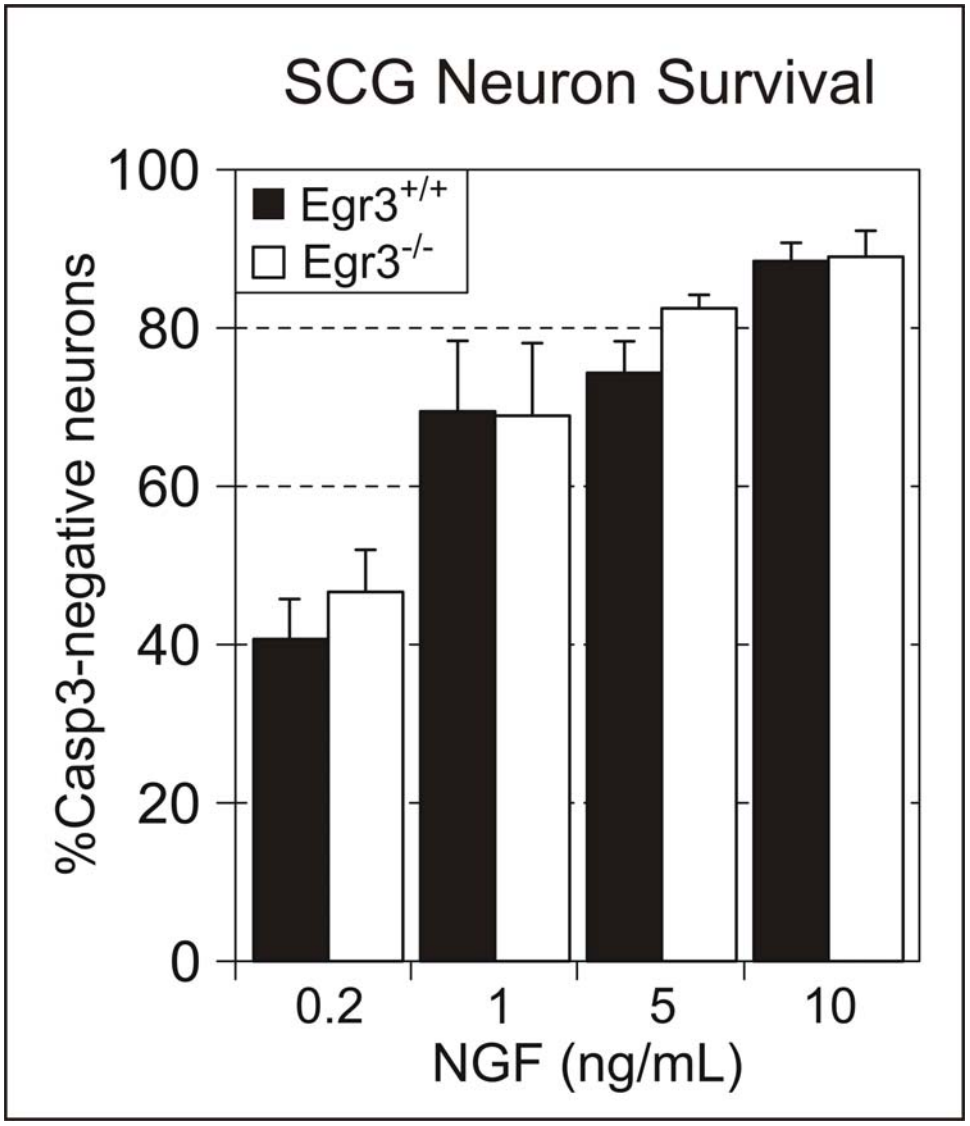
Egr3-deficient SCG neurons exhibit normal survival in vitro.

We observed postnatal SCG neuron loss in Egr3-deficient mice that was due to increased blepharoptosis (Figures 12 and 17). This neuron death could be primary (i.e., an intrinsic survival defect) or secondary (i.e. due to lack of access of the neurons to neurotrophins). In order to distinguish between these two possibilities, we cultured Egr3-deficient SCG neurons and analyzed their ability to survive limiting quantities of exogenous neurotrophins.

SCG neurons were cultured from E18.5 progeny of Egr3 heterozygous mutant females that had been mated to Egr3 heterozygous mutant male mice. This timepoint was selected because it is before the onset of increased apoptosis of Egr3-deficient SCG neurons *in vivo* (Figures 12 and 17). Total numbers of β_{III}-tubulin-positive neurons and the cleaved caspase-3-positive neurons was determined. Viable neurons were counted as caspase 3-negative. When cultured in the presence of 10ng/mL NGF (a concentration of NGF that has been reported to support the survival of 100% of SCG neurons (Belliveau et al. 1997), wild type and Egr3-deficient neurons exhibited similar rates of survival (~90%, Figure 28). Similarly, at limiting concentrations of NGF (0.2ng/mL, 1ng/mL, and 5ng/mL), there was no difference in the ability of wild type and Egr3-deficient neurons to survive. Therefore, these results support our

Figure 28. Egr3-deficient SCG neurons do not exhibit an intrinsic survival defect when administered exogenous neurotrophins *in vitro*.

E18.5 wild type and Egr3-deficient SCG neurons were cultured for 24 hours in the presence of 0.2, 1, 5, or 10ng/mL NGF, then caspase-3 and β_{III}-tubulin dual-labeling immunofluorescence was performed to identify apoptotic neurons. At all NGF concentrations used, similar numbers of wild type (n=3-7 animals and 450-2100 neurons per condition) and Egr3-deficient (n=3-4 animals and 750-2200 neurons per condition) were viable, identified as caspase3-negative neurons. Error bars are +/- standard deviations.


SCG Neuron Survival
100
80
60
40
20
0
%Casp3-negative neurons
Egr3+/+
Egr3-/-
0.2
1
5
10
NGF (ng/mL)

hypothesis that Egr3-deficient neurons do not have an intrinsic survival defect but instead may die secondarily due to insufficient access to trophic support.

Egr3/Bax double homozygous mutant (Egr3/Bax DKO) mice have blepharoptosis.

The eyelids of Egr3-/-;DβHτlacZ+ mice have dramatically attenuated sympathetic innervation. This lack of sympathetic innervation to the tarsal musculature results in physiological blepharoptosis and dry eye in Egr3-deficient mice (Figure 19). In this study, we investigated whether sympathetic innervation defects would persist in Egr3/Bax DKO in the absence of neuronal cell death.

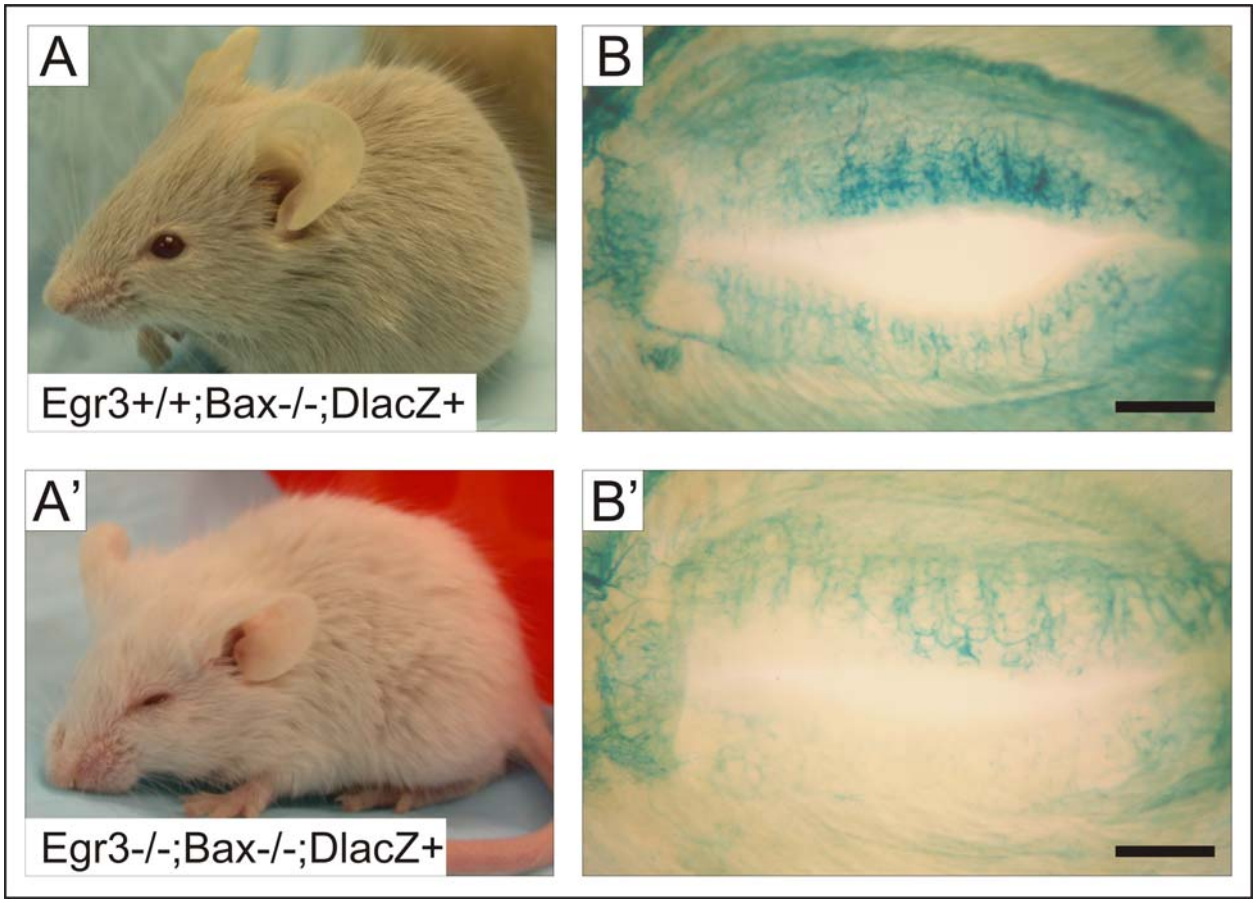
Indeed, Egr3/Bax DKO mice exhibited blepharoptosis, similar to Egr3-deficient mice (Figure 29A, A'). Furthermore, whole mount preparations stained with lacZ enzyme histochemistry revealed attenuated sympathetic innervation in Egr3-/-; Bax-/- tarsal musculature and glands (Figure 29B') compared with Egr3+/+; Bax-/-mice (Figure 29B). This finding suggests that sympathetic nervous system defects persist in the absence of Egr3 when neurons are protected from cell death.

Deficient sympathetic innervation of Egr3/Bax DKO pineal gland.

We next performed immunohistochemistry on sections through the pineal glands of Egr3+/+;Bax-/- and Egr3/Bax DKO mice. In the wild type pineal gland, Bax deficiency appears to result in a slight hyperinnervation in comparison to the TH immunoreactivity of the Egr3+/+;Bax+/+ pineal (Figure 30B,A). Pineal glands from Egr3-deficient mice are nearly devoid of TH+ sympathetic terminals (Figure 30C), and Egr3/Bax DKO pineal glands exhibit

Figure 29. Egr3/Bax double homozygous mutant mice have blepharoptosis and tarsal denervation.

Egr3/Bax DKO mice were generated to determine if sympathetic innervation defects identified in Egr3-deficient mice would persist in the absence of neuron death. Pictured are 4 week old Egr3+/+;Bax-/- (A) and Egr3-/-;Bax-/- (A') mice. Note the presence of blepharoptosis in the Egr3/Bax DKO mice. Whole mount lacZ enzyme histochemistry was performed on eyelids from mice of both genotypes. Sympathetic innervation to tarsal musculature and glands is annenuated in Egr3-/-;Bax-/- double homozygous mutant mice (B') compared to the extent of eyelid innervation in Egr+/+;Bax-/- mice (B).


A
Egr3+/+;Bax-/-;DlacZ+
B
A'
Egr3-/-;Bax-/-;DlacZ+
B'

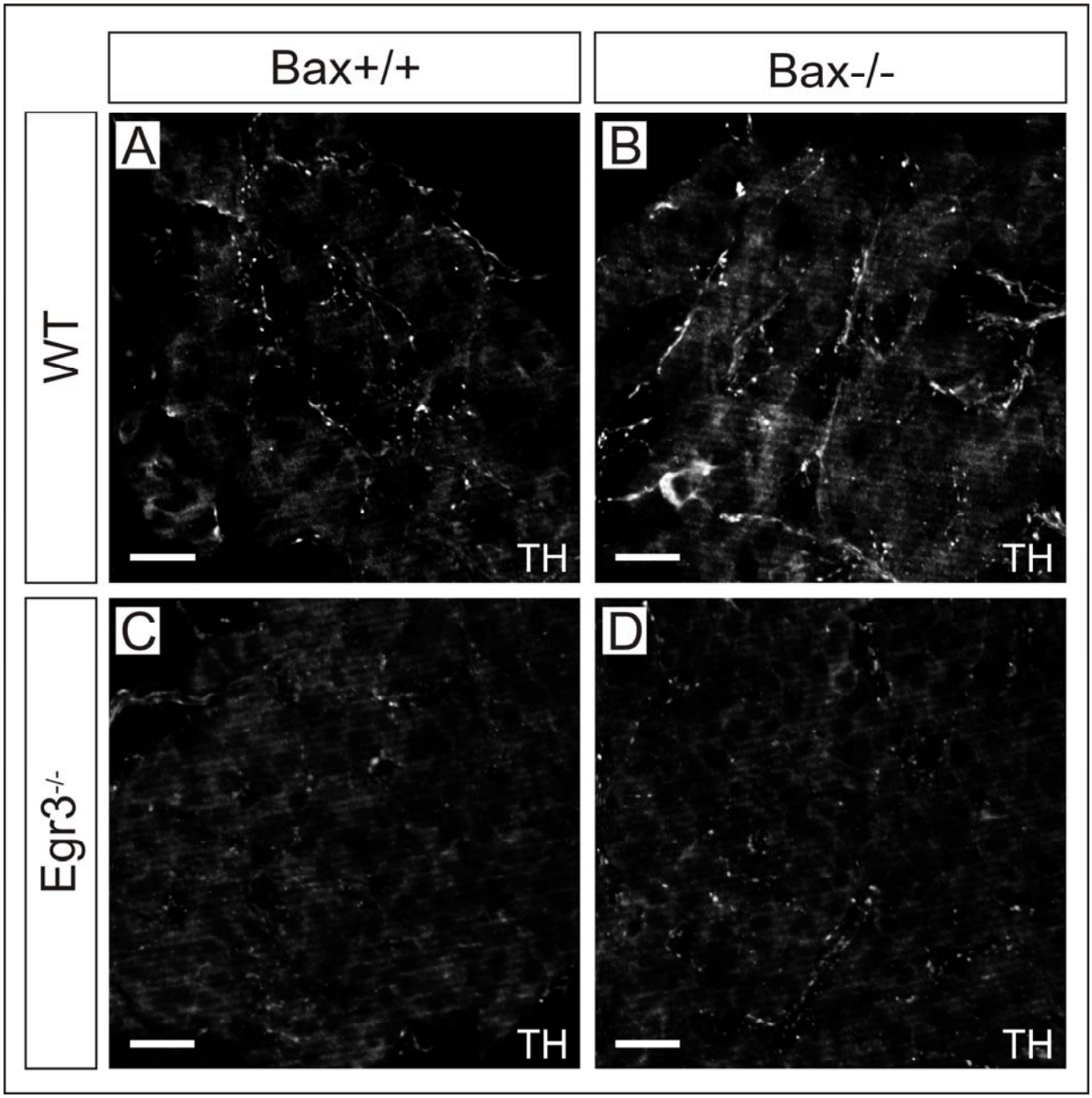
significantly attenuated innervation (Figure 30D) in comparison to the pineal glands from Egr3+/+;Bax-/- mice.

Discussion

Deletion of both Egr3 and Bax will is a strategy to separate potential roles for Egr3 in sympathetic neuron survival and differentiation. From our preliminary results, it appears that sympathetic nervous system defects such as blepharoptosis and pineal denervation persist in Egr3-deficient mice in the absence of neuronal death. Therefore, we hypothesize that Egr3 plays a role in regulating important aspects of sympathetic axon outgrowth or arborization, presumably through induction of axon guidance molecules. Identification of these molecules is an area of current research within the Tourtellotte lab.

Figure 30. Pineal denervation in Egr3/Bax DKO mice.

Tyrosine hydroxylase (TH) immunohistochemistry was performed on 12μm frozen sections cut through pineal glands of 4 week Egr3+/+;Bax+/+, Egr3+/+; Bax-/-, Egr3-/-;Bax+/+, and Egr3-/-;Bax-/- mice to assess the complement of sympathetic innervation to this SCG target tissue. Pineal glands from Egr3+/+;Bax+/+ mice (A) displayed abundant TH+ terminals, while Egr3+/+;Bax-/- pineal glands (B) were expectedly hyperinnervated. Egr3-/- ;Bax+/+ (C) pineal glands were nearly devoid of sympathetic innervation as previously described (Figure 23), while Egr3/Bax DKO (D) pineal parenchyma had slightly more but still deficient TH+ innervation. (Magnification bar =20μm)


Bax+/+
Bax-/-
WT
Egr3-/-
A
B
C
D
TH
TH
TH
TH

Chapter 8

Conclusions

Identification of Egr3 as a major transcriptional regulator of sympathetic nervous system development

An underdeveloped area of neurotrophin-related research is the elucidation of molecular mediators that function downstream of neurotrophins. In this study, we have identified Egr3 as a neurotrophin-inducible, novel transcriptional mediator of sympathetic nervous system development. A similar role was originally proposed for the closely related family member Egr1 based upon abundant *in vitro* studies of sympathetic neuron-like cell lines, but Egr1-deficient mice do not have identifiable sympathetic nervous system defects. Egr3-deficient mice have been noted to have blepharoptosis, a feature characteristic of mice with sympathetic nervous system defects, since their original generation (Tourtellotte and Milbrandt 1998), but until now *in vivo* known roles for Egr3 have been limited to muscle spindle morphogenesis, learning and memory, aggressive behavior, epilepsy, and muscle spindle morphogenesis (Tourtellotte and Milbrandt 1998).

Egr3 expression is induced by neurotrophin treatment of sympathetic neurons in a paradigm similar to that by which Egr1, the prototypical Egr transcription factor, was cloned (Milbrandt 1987). Both NGF and NT-3 stimulation of cultured SCG neurons induce Egr3 expression in a MEK-dependent manner. While the exposure of sympathetic neurons to neurotrophins is less synchronous *in vivo*, it is informative that the abundance of Egr3 message in the SCG is dramatically increased after the well-characterized onset of neurotrophin signaling. Therefore,

Egr3 is spatially and temporally poised to play a role in neurotrophin-dependent aspects of sympathetic neuron development. In support of this hypothesis, profound anatomical and physiological sympathetic nervous system defects are identifiable in postnatal Egr3-deficient mice, when neurotrophins govern the terminal axonal arborization and patterning within target tissues.

Timing, etiology, and significance of sympathetic neuron loss in Egr3-/- mice

Density and complexity of axons dramatically increase within target tissues in the early postnatal period (LE and WT, unpublished observations). It is during these later stages of "fine tuning" of innervation that Egr3-deficient neurons die, in contrast to neuron loss during late embryonic development in neurotrophin-deficient (NGF, NT-3) and neurotrophin signaling-deficient (TrkA) mice (Crowley et al. 1994; Ernfors et al. 1994; Smeyne et al. 1994; Lonze et al. 2002). Indeed, despite having a full complement of sympathetic neurons at birth, Egr3-deficient mice exhibit increased neuronal apoptosis around the time of birth. This apoptosis then results in the loss of one-third of SCG neurons in the early postnatal period. In relation to current hierarchical and sequential models of neurotrophin-dependent aspects of sympathetic nervous system development, neuronal losses Egr3-deficient mice appear during the time when NGF is the major regulator of final target innervation (Glebova and Ginty 2004; Kuruvilla et al. 2004).

Egr3-deficient neurons exhibit increased apoptosis perinatally while there they do not demonstrate decreased rates of proliferation at any of the developmental timepoints analyzed. Classic work by Deckwerth and Johnson demonstrated the induction of sympathetic neuron apoptosis in response to neurotrophin withdrawal (Deckwerth and Johnson 1993; Deckwerth and Johnson 1994). We propose that Egr3-deficient mice harbor defects in sympathetic neurite

extension/ramification (see below) that prevent access of developing sympathetic neurons to target-derived neurotrophins —effectively a "withdrawal" of trophic neurotrophin support. In further support of this hypothesis, the MEK pathway required for Egr3 expression in cultured sympathetic neurons is thought to be important for NGF-stimulated neurite extension rather than for neuronal survival (Thompson et al. 2004).

Deficient Egr3-/- sympathetic axon extension within target tissues mimics NGF deficiency

A target tissue's acquisition and relative density of sympathetic innervation is correlated with its timing and extent of NGF production (Korsching and Thoenen 1983; Shelton and Reichardt 1984; Korsching and Thoenen 1988). Similarly, NGF deficiency results in decreased invasion and arborization of sympathetic axons within targets (Glebova and Ginty 2004; Kuruvilla et al. 2004). In this study, we have found that in addition to diminished numbers of sympathetic neurons, postnatal Egr3$^{-/-}$ mice have abnormalities in sympathetic axon invasion, extension, and branching within multiple target tissues. Interestingly, in both Egr3-/- and NGF-/-;Bax-/- mice (Glebova and Ginty 2004), sympathetic axons reach organs such as the spleen but fail to invade the parenchyma. We propose that the defects we have identified in Egr3-deficient mice mimic those described in the context of NGF deficiency, and that Egr3 may be a key regulator for NGF-dependent processes of sympathetic axon outgrowth within organs.

One incidental finding in this study was that surviving Egr3-deficient sympathetic neurons are smaller in than wild type neurons in the adult mouse (Figure S4). These data suggest that there is atrophy of Egr3-deficient sympathetic neurons, which may result from dysfunctional neurotrophin signaling since surviving NGF-/- and NGF-deprived wild type sympathetic neurons are atrophic (Crowley et al. 1994; Easton et al. 1997). Therefore, Egr3 may play a

role in multiple neurotrophin-dependent processes including survival, axon outgrowth, and overall neuronal health/maintenance.

A sympathetic neuron-autonomous role for Egr3

The widespread defects in target tissue innervation observed in Egr3-deficient mice appear to be due to a sympathetic neuron-autonomous defect. To this end, Egr3 expression in target tissues (salivary gland, heart, and pineal) is near or below the level of detection by qPCR, and Egr3 deficiency does not affect the expression of NGF or NT-3 in the salivary gland or heart (Figure S3 and data not shown). However, we cannot completely rule out additional contributions of Egr3 in non-neuronal tissues. Perhaps similarly, a recent study investigating a sympathetic neuron-specific role for cAMP response element binding protein (CREB) (Parlato et al. 2007) suggests that there is a role for CREB in the periphery despite elegant studies completed earlier which described severe sympathetic neuron defects in CREB-deficient mice (Lonze et al. 2002). Indeed, a sympathetic neuron-specific deletion of Egr3 will formally address this question of autonomy. Likewise, the precise mechanism of action of Egr3 in sympathetic neurons is under investigation and a subject for future studies. Overexpression of Egr3 in myotubes revealed a number of potential Egr3 target genes . Of note, several genes in axon outgrowth and cytoskeletal remodeling are poised to be relevant targets.

Sympathetic dysautonomia in Egr3-/- mice

After identification of the structural deficits within the sympathetic nervous system of Egr3-deficient mice, we investigated their physiological consequences. Three biological systems were identified in which defective innervation resulted in dysfunctional autonomic target tissues. We demonstrate that the severe blepharoblepharoptosis previously described in Egr3-deficient mice (Tourtellotte and Milbrandt 1998) results from severely attenuated sympathetic

innervation of the tarsal musculature. In addition, the lack of sympathetic innervation to Meibomian glands on the inner tarsal surface leads to dry eye followed by corneal damage and neovascularization. Neurotrophic corneal ulcers secondary to dry eye and diminished corneal reflexes are also a salient feature in humans with Familial Dysautonomia (Axelrod 2004).

Denervation of the pineal gland in Egr3-deficient mice desynchronized production of the rate-limiting enzyme in melatonin synthesis AANAT with the light-dark cycle. We predict that this deregulation may have significant effects on the circadian rhythms of Egr3-deficient mice. Indeed, our preliminary data suggest that a subpopulation of Egr3-deficient mice exhibit abnormal entrainment to changes in the light-dark cycle (L. Eldredge, W.G. Tourtellotte, and M. Dubocovich, unpublished observations). However, the role of Egr3 in circadian biology is complicated by the fact that it was identified as a gene upregulated in the suprachiasmatic nucleus in response to light stimulation . Therefore it is possible that Egr3 deficiency may have several roles in circadian biology, including guiding sympathetic innervation of the pineal gland during development and regulating the response to changes in the light-dark cycle in mature animals.

Finally, Egr3 is required for the development of fine sympathetic innervation of the pericardium. We sought to determine the physiological relevance of this fine innervation thorough challenging wild type and Egr3-deficient mice with a centrally-acting sympathomimetic Yohimbine. Egr3-deficient mice were only able to mount a blunted response to this challenge, with modest increases in heart rate and contractility compared to wild type mice. These data suggest that Egr3-deficient mice have a decreased ability to physiologically adapt to stress. Interestingly, 40% of Egr3-deficient mice die perinatally without an obvious

cause (Tourtellotte and Milbrandt 1998). We propose that this phenomenon may be due to the lack of adaptive capabilities to rearing stresses in the cage.

Taken together, our results describe the first evidence of an *in vivo* role for an Egr transcription factor in developing sympathetic neurons. Egr3 is engaged by neurotrophin signaling, and is critical for innervation and physiological function in a variety of target tissues. In addition, while both Egr3 and Egr1 are expressed in sympathetic neurons, Egr3 is critical for normal SNS development while Egr1 appears to be dispensable. While Egr gene family members have highly homologous binding domains and exhibit functional redundancy in selected *in vivo* systems , Egr3 is a uniquely important regulator in the sympathetic nervous system.

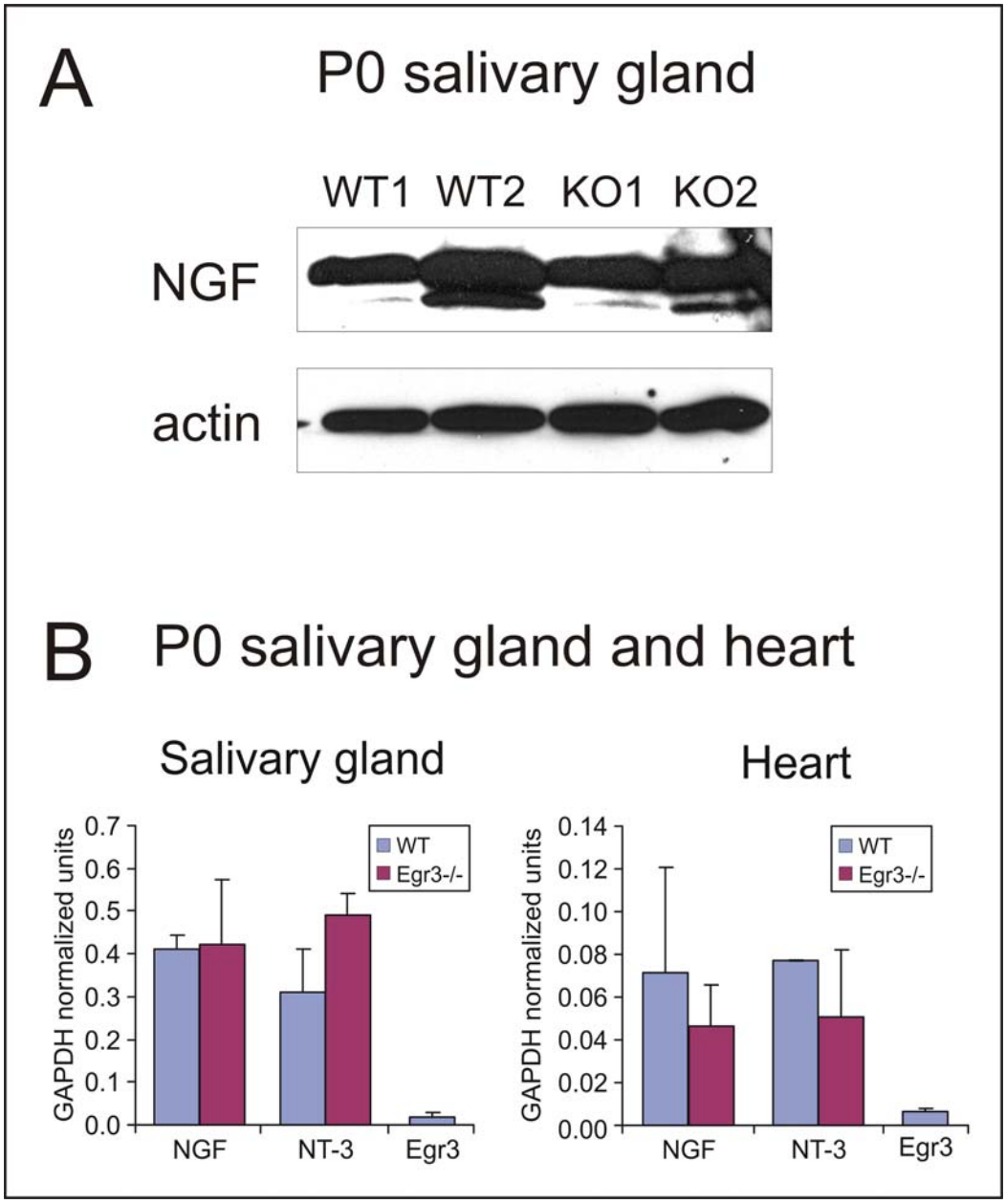
Chapter 9

Future Directions

Ongoing work utilizes Egr3/Bax DKO mice to examine whether Egr3 has a direct role in the regulation of axon extension and/or branching. Primary sympathetic neurons from Egr3/Bax DKO mice will be cultured in limiting concentrations of NGF to establish whether Egr3-deficient axons grow normally in response to growth signals. Egr3/Bax DKO neurons are protected from cell death, and therefore subsaturating levels of NGF can be used to more closely resemble *in vivo* growth conditions. In addition, experiments in NGF as well as NGF and NT-3 will be performed, since these two neurotrophins differentially regulate the patterns of axon arborization (Lentz et al. 1999).

We hypothesized that the target innervation defects seen in Egr3-deficient mice are due to a sympathetic neuron-autonomous defect in axon growth. To this end, Egr3 expression in target tissues (salivary gland, heart, and pineal) is near or below the level of detection by qPCR, and Egr3 deficiency does not affect the expression of NGF or NT-3 in the salivary gland or heart (Figure 31 and data not shown). However, we cannot completely rule out additional contributions of Egr3 in non-neuronal tissues. Perhaps similarly, a recent study investigating a sympathetic neuron-specific role for cAMP response element binding protein (CREB) (Parlato et al. 2007) suggests that there is a role for CREB in the periphery despite elegant studies completed earlier which described severe sympathetic neuron defects in CREB-deficient mice (Lonze et al. 2002). Indeed, a sympathetic neuron-specific deletion of Egr3 will formally address this question of autonomy and is an exciting area of future investigation.

Figure 31. Levels of neurotrophins are unaltered in Egr3-deficient target tissues. (A) Total protein was prepared from PN0 wild type and Egr3-deficient salivary glands, and western blotting for NGF and actin was performed. NGF levels were unaltered in Egr3-/- salivary glands. (B) Total RNA was isolated from PN0 salivary glands and hearts, and qPCR was performed for NGF, NT-3 and GAPDH. Levels of NGF and NT-3 were indistinguishable in wild type and Egr3-deficient salivary glands and hearts. In addition, Egr3 expression was low (near the cutoff for detection) in both tissues. Error bars are +/- standard deviations.


A
P0 salivary gland
WT1 WT2 KO1 KO2
NGF
actin
B
P0 salivary gland and heart
Salivary gland
Heart
GAPDH normalized units
WT
Egr3-/-
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
NGF
NT-3
Egr3

Future work will also be focused on identifying target genes regulated by Egr3 in sympathetic neurons. An Affymetrix array in which Egr3 was overexpressed in differentiating myotubes (Albert et al. 2005) identified several potential target genes involved in neurite extension and cytoskeletal reorganization. An interesting candidate target gene is the low affinity neurotrophin receptor, $p75^{NTR}$ (ngfr). Other potential Egr3 target genes are listed in Table 4. While the cellular context of the array experiment is non-neuronal, we have previously identified the activity-regulated cytoskeletal-related (Arc) gene as a direct target of Egr1 and Egr3 in the brain through this paradigm (Li et al. 2005). It will be interesting to determine whether Egr3 regulates these genes, either directly or indirectly, *in vivo* to influence sympathetic axon growth.

Table 4. Candidate Egr3 Target Genes for SCG neurons

Peripherin	prph1	28 fold	increase
rhophilin, Rho GTPase binding protein 2	rhpn2	244 fold	increase
activity regulated cytoskeletal-associated protein	arc	7.6 fold	increase
nerve growth factor receptor (p75)	ngfr	5.6 fold	increase
ephrin A3	Efna3	3 fold	increase
sema domain, immunoglobulin domain (Ig), short basic domain	sema3b	2.6 fold	increase
sema domain, immunoglobulin domain	sema4a	10 fold	increase
nectin-like 1	Necl1-pending	5.4 fold	increase
phosphatidylinositol 3-kinase, regulatory	Pik3r1	2.5 fold	increase
Rho GTPase activating protein 6	ARHGAP6	7 fold	increase
frizzled-related protein	frzb	4 fold	decrease

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