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The Role of Early Growth Response Transciptional Regulators in Neurotrophin Signaling and Sympathetic Nervous System Development

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ABSTRACT

The Role of Early Growth Response Transcriptional Regulators in Neurotrophin Signaling and Sympathetic Nervous System Development

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Early growth response genes (Egr1-4) are induced as immediate early genes by a variety of extracellular stimuli that influence cellular growth and differentiation. Egr transcriptional regulators modulate gene expression by binding to Egr response elements (ERE) in target genes.

In this study, the low affinity p75 neurotrophin receptor ($p75^{NTR}$) was identified as a target gene regulated by Egr transcriptional regulators. Both Egr1 and Egr3 were found to bind and transactivate the $p75^{NTR}$ promoter *in vitro* and *in vivo*, by using distinct EREs on $p75^{NTR}$ promoter. In the absence of Egr1 and Egr3 (Egr1/3 DKO) *in vivo*, $p75^{NTR}$ expression was greatly diminished in muscle spindle mechanoreceptors and in peripheral nerve Schwann cells. Interestingly, abnormally thin peripheral myelin was seen in Egr 1/3 DKO sciatic nerves, a phenotypic characteristic of mice lacking $p75^{NTR}$. Therefore, these results suggest a novel mechanism that Egr proteins can directly modulate $p75^{NTR}$ function *in vivo*.

Previous studies suggest that Egr1 and Egr3 may play redundant roles in sympathetic neuron development. To further investigate the cooperative roles of Egr genes in sympathetic neuron development *in vivo*, two transgenic mice and one knock-in mouse were generated. To visualize axon outgrowth and target tissue innervation of sympathetic neurons, DβH-τlacZ reporter mice were generated, in which τlacZ fusion protein was expressed in sympathetic

neurons using a human dopamine β-hydroxylase (hDβH) promoter. β-galactosidase histochemistry revealed that the fusion protein labeled almost all the sympathetic neurons and their axons innervating target organs. The reporter mice were used to characterize the sympathetic nervous system defects in $Egr3^{-/-}$ mice.

To avoid the fertility-associated phenotypes of Egr-deficient mice, I utilized a dominant negative Egr (dnEgr) molecule to block Egr-mediated transcription in sympathetic neurons. DβH-dnEgr-IRES-τlacZ transgenic mice are embryonic lethal, perhaps due to sympathetic axon outgrowth defects. To conditionally express the dnEgr molecule in sympathetic neurons, Rosa26-dnEgr (RdnE) knock-in mice were generated and bred with DβH-nlsCre-IRES-τlacZ transgenic mice, in which nuclear-localized Cre recombinase (nlsCre) and τlacZ fusion protein were expressed in sympathetic neurons under the control of the hDβH promoter. The effect of the dnEgr molecule on sympathetic neuron development will be evaluated.

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LIST OF ABBREVIATIONS

BDNF	brain-derived neurotrophic factor
β-gal	β-galactosidase
cAMP	cyclic adenosine monophosphate
ChIP	chromatin immunoprecipitation
CREB	cAMP regulatory element binding protein
DβH	dopamine β hydroxylase
DKO	double knockout
DMEM	Dulbecco's modified Eagle's media
dnEgr	dominant negative Egr
EGFP	enhanced green fluorescent protein
EMSA	electrophoretic mobility shift analysis
Egr	early growth response genes
ERK	extracellular regulated kinase
ERE	Egr response element
FBS	fetal bovine serum
IEG	immediate early gene
IRES	internal ribosome entry site
iSC	immortalized rat Schwann cells
JNK	c-Jun N-terminal kinase
KI	knockin
KO	knockout
LHβ	lutenizing hormone beta

LTD	long term depression
LTP	long term potentiation
MAPK	mitogen-activated protein kinase
MEF	mouse embryonic fibroblasts
Nab	NGFI-A binding protein
NGF	nerve growth factor
NT-3	neurotrophin-3
NT-4	neurotrophin-4
$p75^{NTR}$	low-affinity p75 neurotrophin receptor
PBS	phosphate buffered saline
PKC	protein kinase C
qPCR	quantitative polymerase chain reaction
SCG	superial cervical ganglia
Shc	Src homologous and collagen-like
SRF	serum response factor
STG	stellate ganglia
τlacZ	tau and β-galactosidase fusion protein
Tg	transgene
TH	tyrosine hydroxylase
Trk	tropomyosin-related kinase
WT	wild type

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CHAPTER 1

Introduction

1.1 The early growth response (Egr) family of transcriptional regulators

Immediate early genes (IEGs) are rapidly induced in the absence of *de novo* protein synthesis by extracellular stimuli. Due to their rapid induction kinetics, IEGs often serve as bridges connecting the stimulation at cellular membranes to the appropriate cellular responses, such as proliferation, differentiation, or apoptosis.

Early growth response (Egr) genes are rapidly induced as IEGs by a variety of extracellular signals, such as growth factors, cytokines, neurotransmitters, and membrane depolarization (O'Donovan et al., 1999). There are four members in the Egr gene family (Fig. 1), Egr1 (also known as zif-268, NGFI-A, Krox24) (Christy et al., 1988; Lemaire et al., 1988; Milbrandt, 1987; Patwardhan et al., 1991), Egr2 (Krox-20) (Christy et al., 1988), Egr3 (Pilot) (Mages et al., 1993; Patwardhan et al., 1991), and Egr4 (NGFI-C) (Crosby et al., 1991). Egr family members share highly homologous C-terminal DNA-binding domains consisting of three C_2H_2–type zinc fingers, through which they bind characteristic GC-rich sequences GCG(G/T)GGGCG (Egr response element, ERE) in target gene promoters to modulate their transcription (Fig. 1) (Chavrier et al., 1988; Christy and Nathans, 1989a; Christy et al., 1988; Crosby et al., 1991; Patwardhan et al., 1991).

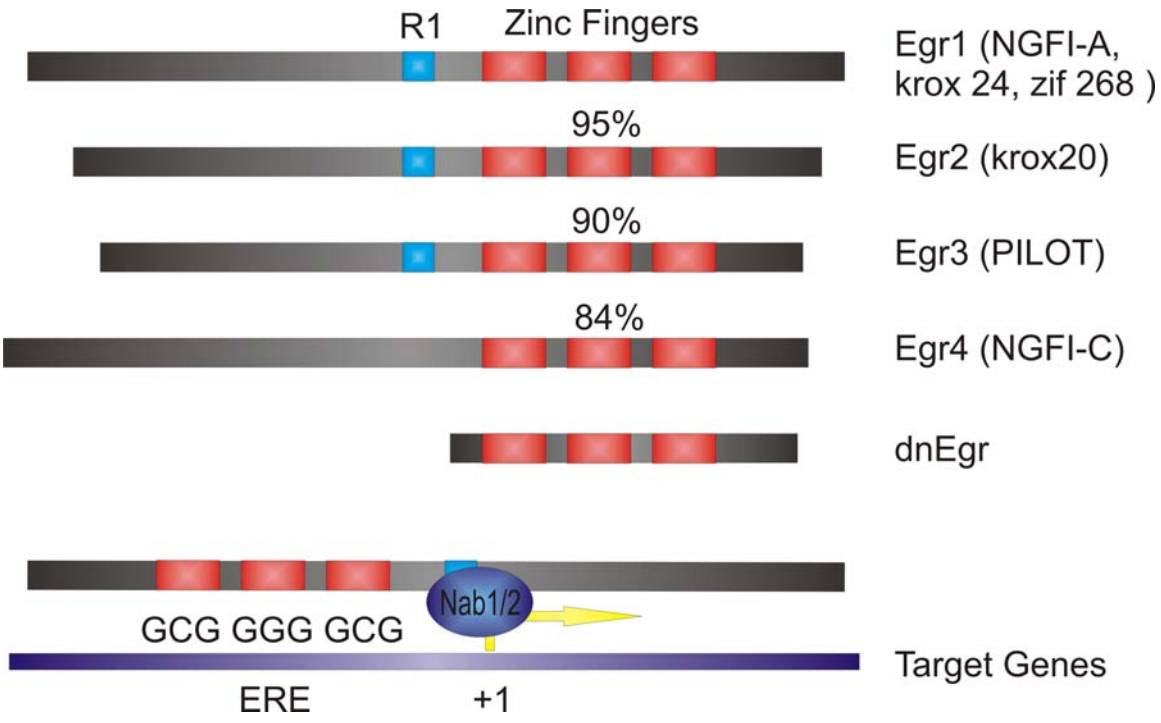

Figure 1. The structure of Egr proteins.

Egr proteins were aligned to demonstrate highly homologous DNA-binding zinc fingers of Egr1-4 and the R1 repression domain of Egr1, Egr2, and Egr3. Egr transcription factors activate target gene expression through binding to the Egr response elements (ERE) with moderately conserved sequences: GCGGGGGCG. Also shown are the Egr coregulatory proteins Nab1/Nab2, which repress Egr-mediated transcription by interacting with the R1 domain, and the artificial dominant negative Egr (dnEgr) molecule which competes with native Egr proteins for DNA-binding.

The biological functions of Egr transcription factors were studied by genetic ablation of individual or multiple Egr genes. Both Egr1- and Egr4- deficient mice are phenotypically normal except for fertility problems. Female Egr1-deficient mice are infertile due to reduced level of lutenizing hormone β subunit (LHβ) in pituitary glands (Lee et al., 1996; Tourtellotte et al., 2000). Egr4-deficient mice have male infertility due to cell-autonomous defects of male germ cell maturation (Tourtellotte et al., 1999). Egr2-deficient mice die during embryogenesis due to abnormal hindbrain segmentation and peripheral nerve hypomyelination (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993; Topilko et al., 1994). Only 60% of Egr3-deficient mice survive to adulthood, and surviving mice have ptosis, scoliosis, resting tremor, as well as sensory ataxia due to lack of muscle spindle mechanoreceptors (Tourtellotte and Milbrandt, 1998). Recent studies demonstrated that both Egr1 and Egr3-deficient mice have defective hippocampal long-term potentiation (LTP) and impaired learning and memory (Farinas et al., 1994; Li et al., 2007).

Studies from Egr double mutant mice suggest a high degree of functional redundancy among Egr family members, possibly due to homologous DNA-binding domains and co-expression patterns of Egr genes. For example, Egr4 can partially compensate for Egr1 function in regulating LHβ expression in male mice (Tourtellotte et al., 2000). Egr1 and Egr3 directly regulate activity-regualted cytoskeletal-associated protein (Arc) and are both required for normal Arc expression associated with physiological synaptic activity in brain (Li et al., 2005). Finally, Egr1 and Egr3 play functionally redundant roles in regulating normal thymocyte differentiation (Carter, 2007).

Therefore, Egr proteins have important roles in the normal development and function of many biological systems, including germ cell maturation and fertility, thymocyte differentiation,

both central and peripheral nervous system development, synaptic plasticity, and learning and memory.

1.2 Neurotrophin signaling

1.2.1 Neurotrophins and their receptors

Neurotrophins are a family of growth factors that regulate survival, differentiation, and myelination of neurons. According to the neurotrophin hypothesis, neurons are over-produced at early developmental stages, and neuron numbers are refined during competition for limiting amounts of neurotrophins secreted by target tissues. The first neurotrophin, nerve growth factor (NGF) was identified over 50 years ago as a diffusible factor that promotes sympathetic neuron growth in chicken embryos (Levi-Montalcini and Hamburger, 1951). Since then, several neurotrophins have been identified, inculding brain-derived neurotrophic factor (BDNF) (Barde et al., 1982), neurotrophin-3 (NT-3) (Ernfors et al., 1990) and neurotrophin-4 (NT-4) (Hallbook et al., 1991).

All neurotrophins are synthesized as pro-neurotrophins, which are cleaved at highly conserved sites by furin and proconvertase to release C-terminal mature proteins (Mowla et al., 2001). Mature neurotrophins are around 12KD in size, they form stable dimers and interact with two types of neurotrophin receptors: the high-affinity tropomyosin-related kinase (Trk) recptors, including TrkA, TrkB and TrkC, and the low-affinity p75 neurotrophin receptor--$p75^{NTR}$. Neurotrophins show binding selectivity for particular Trk receptors: NGF preferentially binds and signals through TrkA, BDNF and NT-4 bind and signal through TrkB, and NT-3 preferentially signals via TrkC. However, this specificity is not absolute since NT-3 can also

bind and signal through TrkA and TrkB (Chao, 2003). The other class of neurotrophin receptor--$p75^{NTR}$--nonselectively binds all four neurotrophins with equally low affinity. When coexpressed with Trk receptors on cell surface, $p75^{NTR}$ modulates the binding affinity and selectivity of Trk receptor for their preferred neurotrophins (Benedetti et al., 1993; Chao and Hempstead, 1995). Therefore, neurotrophins execute their functions by binding and signaling through Trk, $p75^{NTR}$, or both receptors.

1.2.2 Intracellular neurotrophin signaling

The intracellular signal transduction pathways mediated by Trk or $p75^{NTR}$ receptors are very different. Upon binding to neurotrophin dimers, two Trk receptors dimerize and phosphorylate each other on intracellular tyrosine residues, which in turn recruit a set of adaptor proteins, and activate three major signaling pathways: mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol 3-kinase (PI3K)/Akt pathway, and phospholipase Cγ (PLCγ)-mediated Ca^{2+} release (Chao, 2003; Segal, 2003). While the PI3K/Akt pathway is primarily involved in neuronal survival, the MAPK pathway leads to transcriptional activation of genes involved in neuron differentiation, including cell cycle withdrawal, neurite outgrowth and synaptic plasticity (Sofroniew et al., 2001).

The current model of NGF/TrkA/MAPK signaling pathway is mainly derived from *in vitro* studies in rat pheochromocytoma PC12 cells and human neuroblastoma cell lines. Upon TrkA activation, the Src homologous and collagen-like (Shc) adaptor protein is recruited to phosphorylated tyrosine residues. Phosphorylated Shc protein then recruits and activates Ras and Raf, which in turn phosphorylate MEK, Erk1/2, and Rsk sequentially. Phosphorylated Rsk and Erk translocate into the nucleus and activate cAMP regulatory element binding protein (CREB)

phosphorylation and promote the assembly of the ternary complex (SRF/TCF), both of which synergistically activate the transcription of immediate early genes (Sofroniew et al., 2001). Immediate early transcription factors activate late-response target genes, leading to global gene expression changes required for cell growth and differentiation.

When compared to Trk receptors alone, $p75^{NTR}$–Trk receptor complexes creat higher affinity binding sites for mature neurotrophins, and therefore enhance neurotrophin signaling (Benedetti et al., 1993). In addition to modulating Trk-mediated neurotrophin signaling, $p75^{NTR}$ also conveys Trk-independent signals through its intracellular death domain. Upon ligand binding, $p75^{NTR}$ recuits a different sets of adaptor proteins, and activates three main signaling pathways: ceramide, Jun N-termianl kinase (JNK), and NF-κB pathways. While NF-κB signaling is involved in neuron survival and inflammation, JNK and ceramide activation mainly lead to apoptosis (Sofroniew et al., 2001). For example, the $p75^{NTR}$ receptor forms a ternary complex with pro-neurotrophins and a coreceptor Sortilin, and triggers proapoptotic signaling in neurons and oligodendrocytes (Beattie et al., 2002; Harrington et al., 2004; Nykjaer et al., 2004). Therefore, $p75^{NTR}$ has complex roles in the life-death decision of neurons: it collaborates with Trk receptors to promote mature neurotrophin-mediated survival signaling, but also complexes with Sortilin to execute proneurotrophin-induced cell death in the absence of Trk receptors.

1.2.3 Physiological roles of neurotrophins

Genetic studies revealed that neurotrophins have a variety of essential physiological roles in nervous system development. NGF is required for survival and differentiation of sensory, sympathetic, and basal forebrain cholinergic neurons (Crowley et al., 1994). Both NGF-deficient and TrkA-deficient mice had severe sensory and sympathetic neuron loss and die perinatally

(Crowley et al., 1994; Smeyne et al., 1994). NGF heterozygous mice display defective memory acquisition and retention due to decreased cholinergic innervation of hippocampus (Chen et al., 1997).

BDNF-deficient mice have severe deficits in coordination and balance due to extensive neuron degeneration in several sensory ganglia including vestibular ganglia (Ernfors et al., 1994a). Conditional deletion of BDNF in brain after birth rescued the perinatal death of BDNF-deficient mice, but led to obesity, hyperactivity, and incrased anxiety in adult mice (Rios et al., 2001). Lack of BDNF also causes impairment in long-term potentiation and spatial memories (Korte et al., 1995; Linnarsson et al., 1997).

Loss of either NT-3 or its preferred TrkC receptor leads to severe movement defects because of substantial loss of proprioceptive sensory neurons and their sense organs in muscle---the muscle spindle mechanoreceptors (Ernfors et al., 1994b; Klein et al., 1994). Moreover, NT-3-deficient mice also have cardiovasular abnormalities and die shortly after birth (Donovan et al., 1996).

Unlike mice lacking the other three neutrophins, NT-4-deficient mice are viable despite sensory neuron loss in nodose-petrosal and geniculate ganglia (Conover et al., 1995; Liu et al., 1995). Genetic ablation of NT-4 leads to impared amygdala-mediated long-term memory and hippocampi-dependent long-lasting LTP (Xie et al., 2000).

Interestingly, TrkB-deficient mice show neonatal death and severe sensory and motor neuron deficiencies in both central and peripheral nervous systems, a more severe phenotype than $BDNF^{-/-}$, $NT\text{-}4^{-/-}$ or $BDNF^{-/-}/NT\text{-}4^{-/-}$ mice, suggesting other ligands, perhaps NT-3, can activate TrkB *in vivo* (Conover et al., 1995; Klein et al., 1993; Liu et al., 1995).

Moreover, neurotrophins are key mediators of peripheral nerve myelination. The myelin sheath is a specialized membrane structure surrounding axons to facilitate rapid saltatory conduction of action potentials along the axons (Lemke, 2001). Myelination is a complicated process that requires bidirectional interactions between the axons to be myelinated and the glia cells forming the myelin sheath. Axons are myelinated by Schwann cells in the peripheral nervous system and by oligodendrocytes in the central nervous system.

Multiple neurotrophins and their receptors are involved in the process of peripheral nerve myelination. Before myelination occurs, NT-3 signals through TrkC to inhibit Schwann cell elongation and ensheathment of axons, later the NT-3 level is decreased to remove its inhibitory function (Chan et al., 2001; Cosgaya et al., 2002). At the same time, BDNF signals through $p75^{NTR}$ to promote Schwann cell elongation and axon myelination in peripheral nerves (Chan et al., 2001; Cosgaya et al., 2002). After myelination has occurred, excessive BDNF is removed through binding to increased level of the truncated isoform of TrkB (TrkB-T1), which lacks the intracellular tyrosine kinase domain (Cosgaya et al., 2002). On the other hand, NGF/TrkA signaling in axons is required for the axonal receptivity of myelination by Schwann cells (Chan et al., 2004). Therefore, myelination is a highly regulated program, and multiple neurotrophins and their receptors function in the precise control of its progress.

1.2.4 Modulation of neurotrophin signaling by $p75^{NTR}$ receptor

As an important modulator of neurotrophin signaling, $p75^{NTR}$ plays a variety of roles in the central and peripheral nervous system. For example, $p75^{NTR}$-deficient sympathetic and sensory neurons show decreased responsiveness to NGF (Lee et al., 1994a). Consistantly, $p75^{NTR}$-deficient mice show heat insensitivity due to decreased sensory innervation to skin, and

lack of sympathetic innervation to pineal gland and some sweat glands (Lee et al., 1994a; Lee et al., 1992).

$p75^{NTR}$ also binds pro-neurotrophin ligands such as pro-NGF and pro-BDNF when in complex with Sortilin (Nykjaer et al., 2004; Nykjaer et al., 2005). In fact, mature BDNF signals through TrkB to facilitate long-term potentiation (LTP) in brain, while pro-BDNF mediates hippocampal long-term depression (LTD) via $p75^{NTR}$ receptor (Woo et al., 2005). Moreover, BDNF signals through $p75^{NTR}$ to promote Schwann cell elongation and enhance the speed and extent of axon myelination in peripheral nerves (Chan et al., 2001; Cosgaya et al., 2002). Consistently, $p75^{NTR}$-deficient mice have abnormally thin peripheral nerve myelin sheath during physiological nerve development (Cosgaya et al., 2002), and during nerve regeneration after injury (Song et al., 2006).

The $p75^{NTR}$ receptor also regulates NT-3 signaling during the development of proprioceptive sensory neurons and muscle spindles since loss of $p75^{NTR}$ on NT-3 heterozygous background significantly decreases muscle spindle numbers in many skeletal muscles when compared to NT-3 heterozygotes alone (Fan et al., 1999).

1.3 Neurotrophin signaling and sympathetic neuron development

1.3.1 Sympathetic nervous system

The sympathetic nervous system is part of the autonomic nervous system, and plays important roles in maintaining tissue and organ homeostasis. Sympathetic axons innervate endocrine and exocrine glands as well as smooth and cardiac muscles in the body, and mediate "fight-or-flight" responses to stressful environments (Glebova and Ginty, 2005).

The sympathetic nervous system is composed of pre- and post-ganglionic sympathetic neurons as well as adrenal medulla chromaffin cells. The preganglionic sympathetic neurons lie in the central nervous system and activate peripheral (postganlionic) sympathetic neurons using acetylcholine as neurotransmitter (Rubin, 1985a; Rubin, 1985b; Rubin, 1985c). Postganglionic sympathetic neurons form sympathetic ganglia along the spinal vertebrae (paravertebral ganglia) or near target tissues (prevertebral ganglia). Postganglionic sympathetic neurons are catecholaminergic neruons and secrete norepinephrine as neurotransmitters at synapses to activate adrenergic receptors on target tissues (Elenkov et al., 2000). The adrenal medulla is a specialized ganglion in the sympathetic nervous system, in which the adrenal medulla chromaffin cells secrete catecholamine (mainly epinephrine, with a small amount of norepinephrine) directly to the blood stream when activated by preganglionic sympathetic neurons (Francis and Landis, 1999). Norepinephrine is synthesized from tyrosine through a series of reactions using tyrosine hydroxylase (TH), dopa decarboxylase (DCC), and dopamine β-hydroxylase (DβH) (Anderson, 1993). The last step in catecholamine synthesis is that norepinephrine is converted to epinephrine by phenylethanolamine N-methyltransferase (PNMT) (Anderson, 1993).

1.3.2 Specification and differentiation of sympathetic neuroblasts

Sympathoadrenal precursors arise from undifferentiated neural crest cells that migrate ventrally, aggregate near the dorsal aorta, and eventually differentiate into catecholaminergic neurons and adrenal chromaffin cells (Anderson, 1993). A number of growth factors have been shown to play important roles in the migration and fate specification of sympathoadrenal progenitors. For example, hepatocyte growth factor (HGF)/Met signaling facilitates proliferation

and differentiation of sympathetic neuroblasts (Maina et al., 1998). Vascular-derived artemin signals through Ret/GFRα3 receptor complex and regulates the migration of sympathetic neuroblasts (Enomoto et al., 2001; Honma et al., 2002). Neuregulin-1/ErbB and semaphorin 3A/ neuropilin signaling pathways are also involved in the neural crest cell migration and sympathetic nervous system patterning (Britsch et al., 1998; Kawasaki et al., 2002). Moreover, bone morphogenetic proteins (BMPs) from ventral neural tube serve as instructive signals to promote the neural precursors committed to catecholaminergic lineage (Mehler et al., 1997).

Several transcription factors also play important roles in fate specification of sympathetic neurons, including basic helix-loop-helix (bHLH) transcription factors MASH1; eHAND and dHAND; homeodomain transcription factors Phox2a and Phox2b; and the zinc-finger transcription factors GATA-2 and GATA-3 (Francis and Landis, 1999). MASH1 is expressed in neural progenitor cells at very early stages and is required for their commitment to noradrenergic neurons (Sommer et al., 1995). Phox2a and Phox2b share an identical DNA-binding homeodomain and act in concert to regulate catecholaminergic traits of sympathetic neuroblasts (Pattyn et al., 1997).

1.3.3 NGF and NT-3 are required for post-mitotic sympathetic neuron survival and differentiation

Neurotrophins are not required for the migration and fate commitment of sympathetic neuroblasts, but rather are required for survival and axon outgrowth of post-mitotic sympathetic neurons. A number of *in vitro* and *in vivo* studies showed that NGF-TrkA signaling is required for sympathetic neuron survival and differentiation. First, NGF can rescue primary sympathetic neuron death in culture and is synthesized by sympathetic target tissues at levels corresponding

to the sympathetic innervation density *in vivo* (Chun and Patterson, 1977; Levi-Montalcini and Hamburger, 1951; Shelton and Reichardt, 1984). In fact, dramatic sympathetic neuron loss was seen in mice treated with an NGF blocking antibody (Levi-Montalcini, 1987). More evidence of a survival role for NGF was provided by genetically modified mouse models. Both NGF- and TrkA-deficient mice show extensive sympathetic neuron loss and die perinatally (Crowley et al., 1994; Smeyne et al., 1994). In NGF-transgenic mice, increased NGF levels lead to enlarged sympathetic ganglia and excessive axon outgrowth from these ganglia (Hoyle et al., 1993). Interestingly, in NGF/Bax double knockout mice, neuronal death is rescued by deletion of the proapoptotic protein Bax, but sympathetic innervation to many target organs is still absent or decreased, demonstrating that NGF is also required for sympathetic axon extension *in vivo* (Glebova and Ginty, 2004; Patel et al., 2000).

NT-3 signals through TrkA instead of TrkC in a subpopulation of post-mitotic sympathetic neurons, and is required for their survival since 50% of the sympathetic neurons in the superior cervical ganglia (SCG) are missing in NT-3-deficient mice (Ernfors et al., 1994b; Farinas et al., 1994; Wyatt et al., 1997). Moreover, NT-3 is also expressed by blood vessels, and may serve as guidance cues for sympathetic axon extension along the blood vessels (Carmeliet, 2003; Donovan et al., 1996).

In summary, studies from NGF-, NT-3-, or TrkA-deficient mice suggest that NGF and NT-3 both signal through TrkA and their signaling are required for post-mitotic sympathetic neuron survival and differentiation.

1.4 Egr genes are transcriptional mediators of neurotrophin signaling and sympathetic neuron development

1.4.1 Regulation of Egr transcriptional activity

Egr proteins convey signals from cell membrane to changes in gene expression profile via regulation of downstream target genes. Egr proteins are expressed at low levels in unstimulated cells, and are mainly regulated at the transcriptional level by multiple intracellular signaling pathways. Egr1, the prototypical member of the Egr proteins, was initially identified in serum-stimulated NIH3T3 fibroblasts, and showed similar induction kinetics as another immediate early gene c-fos during cell growth and differentiation (Patwardhan et al., 1991). At the same time, the rat homologue of Egr1, NGFI-A, was identified in differentiating rat pheochromocytoma PC12 cells after NGF treatment (Milbrandt, 1987). Egr2 and Egr4 were identified by similar studies in searching for novel immediate early genes in serum-stimulated NIH3T3 and NGF-treated PC12 cells (Chavrier et al., 1988; Crosby et al., 1991). Another Egr family member, Egr3 was also identified in stimulated fibroblasts by low stringency hybridization using the Egr1 zinc finger domain as the probe (Patwardhan et al., 1991).

Examination of the 5' regulatory sequences of Egr genes revealed that Egr1, Egr2, and Egr3 all have functional serum response elements (SRE) and cAMP response elements (CRE), suggesting that serum response factor (SRF) and cAMP regulatory element binding protein (CREB) may have important roles in regulating Egr gene expression (Chavrier et al., 1988; Christy and Nathans, 1989b; Crosby et al., 1991; Mages et al., 1998; Tsai-Morris et al., 1988). Indeed, SRF, ternary complex factor (TCF), CREB and CREB binding protein (CBP) cooperate to activate Egr gene expression (Ramirez et al., 1997). Similar regulatory elements may explain the coexpression pattern of Egr proteins in many systems, such as serum-stimulated NIH3T3 fibroblasts and NGF-treated PC12 cells.

Negative regulation of Egr transcripitonal activity is mediated via Egr corepressors-- the NGFI-A binding (Nab) proteins, Nab1 and Nab2. Egr1, 2, 3 share a similar R1 repression domain, through which they interact with Nab proteins (Russo et al., 1995; Svaren et al., 1996). Egr4 lacks the R1 domain, so it is not repressible by Nab proteins (Russo et al., 1995). Nab1 and Nab2 have two Nab conserved domains (NCD)-- NCD1 and NCD2. While NCD1 is responsible for Egr-binding, NCD2 actively represses the transcripitional activities of Egr proteins by recruiting repressor molecules to target promoters (Srinivasan et al., 2006; Swirnoff et al., 1998). Nab1 is expressed constitutively, whereas Nab2 is inducible by many extracellular stimuli that also induce Egr genes (Russo et al., 1995; Svaren et al., 1996). Interestingly, Nab2 is an Egr target gene, representing a potential negative feedback loop that may be involved in controlling Egr-mediated transcription (Kumbrink et al., 2005; Qu et al., 1998).

1.4.2 Egr genes are transcriptional mediators of neurotrophin signaling

A number of *in vitro* experiments in rat pheochromocytoma cells and neuroblstoma cells have shown that Egr genes play important roles in neurotrophin signaling. NGF rapidly induces robust Egr1 and Egr4 expression in rat pheochromocytoma PC12 cells, which differentiate into sympathetic-like neurons characterized by neurite outgrowth and expressing genes similar to sympathetic neurons (Crosby et al., 1991; Milbrandt, 1987). NGF-mediated Egr induction is coupled to MAPK pathway since Erk inhibitors abrogate Egr induction, neurite outgrowth and differentiation of PC12 cells (Harada et al., 2001). Moreover, blocking Egr transcriptional activity by expressing the corepressor Nab2 or a dominant negative Egr (dnEgr) molecule inhibits NGF-induced neurite outgrowth and target gene expression in PC12 cells (Levkovitz et al., 2001; Qu et al., 1998). Finally, a number of putative Egr1 target genes have been identified

in PC12 cells that may be involved in NGF-signaling and sympathetic neuron differentiation, such as the neuron-specific activator of Cdk5 (p35), nicotinic acetylcholine receptor α7 subunit, phenylethanolamine N-methyltransferase (PNMT), and transforming growth factor β1 (TGFβ1) (Harada et al., 2001; Kim et al., 1994; Nagavarapu et al., 2001; Tai et al., 2001).

Human SH-SY5Y neuroblstoma cells express very low levels of wild-type TrkA, but introducing exogenous wild-type TrkA into these cells restore their ability to differentiate into sympathetic-like neurons in response to NGF (Lavenius et al., 1995). During this process, Egr1 is induced and required for activation of the differentiation marker gene neuropeptide tyrosine (NPY) transcription (Edsjo et al., 2001; Eggert et al., 2000; Wernersson et al., 1998). Moreover, transfection of N2A neuroblastoma cells with Egr1 antisense oligonucleotides inhibits the differentiation of these cells whereas overexpression of Egr1 facilitates cell cycle withdraw and differentiation of N2A cells (Pignatelli et al., 1999).

Recently, Egr3 was identified as an important mediator of BDNF signaling in regulating type A GABA receptor α4 subunits (GABRA4) in hippocmpal neurons of epileptic brain (Roberts et al., 2006; Roberts et al., 2005). These results add Egr3, a closely related family member of Egr1, to the downstream signaling effectors of neurotrophin signaling.

1.4.3 Egr transcriptional regulators play important roles in sympathetic neuron development

Egr1 appears to play an important role in NGF signaling and sympathetic neuron differentiation based on *in vitro* studies. However, Egr1-deficient mice are phenotypically normal and have no apparent sympathetic defects (Lee et al., 1996). One possible explanation to this contradiction is that other Egr genes may compensate for Egr1 function or have more

important function *in vivo* during sympathetic neuron development. Egr3 is a good candidate since Egr3-deficient mice have ptosis, the characteristic phenotype of sympathetic nervous system defects shown in the NGF- and TrkA-deficient mice (Crowley et al., 1994; Smeyne et al., 1994). Approximately 30% of SCG neurons are lost due to excessive apoptosis in Egr3-knockout mice (Eldredge et al., 2007). Egr1/Egr3 DKO mice show more severe phenotypes: they are much smaller than their wild type littermates, have delayed eye opening with severe ptosis, and die before weaning age. These phenotypical observations suggest that Egr genes may have redundant roles in NGF signaling and sympathetic neuron development.

1.5 Egr3 is a critical transcriptional regulator of muscle spindle mechanoreceptor development

Muscle spindles are sensory mechanoreceptors that mediate stretch reflexes and proprioception. Muscle spindles consist of specialized intrafusal muscle fibers encapsulated by a fusiform capsule. Intrafusal muscle fibers are innervated by both sensory (Ia, II) and motor (γ) axons, and surgical manipulation has shown that sensory but not motor axons are required for muscle spindle development (Kucera and Walro, 1992; Kucera et al., 1993; Zelena and Soukup, 1973). The spindle capsule is derived from a ring of perineural fibroblasts surrounding the Ia-afferent innervated myotubes (Maier, 1997).

Growth factors secreted by innervating Ia-affarents transform myotubes into specialized intrafusal muscle fibers, and the surrounding fibroblasts differentiate into the spindle capsule. NT-3/TrkC signaling is required for the survival and development of proprioceptive sensory (group Ia) neurons and their peripheral sense organ—muscle spindles (Ernfors et al., 1994b; Klein et al., 1994). Ia-afferent and muscle spindles are absent in mice lacking NT-3 or TrkC

(Ernfors et al., 1994b; Klein et al., 1994). $p75^{NTR}$ also has an important role in muscle spindle development by modulating NT-3 signaling. Loss of $p75^{NTR}$ on NT-$3^{+/-}$ background dramatically decreased the number of muscle spindles in many skeletal muscles when compared to NT-$3^{+/-}$ mice (Fan et al., 1999).

The expression of Egr3 transcription factor was induced by Ia-afferent innervation in both intrafusal muscle fibers and spindle capsule cells (Tourtellotte et al., 2001; Tourtellotte and Milbrandt, 1998). Egr3 expression is required for muscle spindle morphogenesis and maintenance. The muscle spindles found in neonatal $Egr3^{-/-}$ mice lack spindle capsules and the nuclear clustering within intrafusal muscle fibers, and degenerate completely during development (Tourtellotte et al., 2001). No muscle spindle defects were observed in mice lacking Egr1, Egr2, or Egr4, suggesting that Egr3 is the only family member required for muscle spindle development. Therefore, muscle spindles offered an excellent model system to study target genes regulated by Egr3 *in vivo*. The downstream target genes regulated by Egr3 during muscle development were studied using Affymetrix microarray analysis (Albert et al., 2005). Interestingly, the low affinity p75 neurotrophin receptor-- $p75^{NTR}$--was identified as one of them.

In the first part of my dissertation, I provided both *in vitro* and *in vivo* evidence to prove that the expression of $p75^{NTR}$ is modulated by Egr transcriptional regulators. Both Egr1 and Egr3 were found to bind and transactivate the $p75^{NTR}$ promoter. In the absence of Egr1 and Egr3 (Egr1/3 DKO), $p75^{NTR}$ expression was greatly diminished in muscle spindles and in peripheral nerve Schwann cells. Interestingly, abnormally thin peripheral myelin was seen in Egr1/3 DKO sciatic nerves, a phenotypic characteristic of mice lacking $p75^{NTR}$. Therefore, these results suggest a novel mechanism that Egr proteins can modulate $p75^{NTR}$ function *in vivo*.

In the second part of my thesis, two transgenic mice and one knock-in mouse were generated to investigate the cooperative roles of Egr genes during sympathetic neuron development. DβH-τlacZ reporter mice were generated to visualize axon outgrowth and target tissue innervation of sympathetic neurons. To turn on/off a specific gene in sympathetic neurons and study its influence on sympathetic neuron development, DβH-nlsCre-IRES-τlacZ mice were generated, in which nuclear-localized Cre recombinase (nlsCre) and τlacZ fusion protein were expressed in sympathetic neurons under the control of the hDβH promoter.

To avoid the fertility-associated phenotypes of Egr-deficient mice, I utilized a dominant negative Egr (dnEgr) molecule to block Egr-mediated transcription in sympathetic neurons. DβH-dnEgr-IRES-τlacZ transgenic embryos are lethal, perhaps due to sympathetic axon outgrowth defects. To conditionally express dnEgr molecule in sympathetic neurons, Rosa26-dnEgr (RdnE) knock-in mice were generated and bred with DβH-nlsCre-IRES-τlacZ mice.

CHAPTER 2

Materials and Methods

2.1 Animals

Egr1-deficient (Lee et al., 1996) and Egr3-deficient mice (Tourtellotte and Milbrandt, 1998) were generated and genotyped as previously described. Egr1-deficient mice were backcrossed 10 generations to C57BL/6J mice and Egr3-deficient mice were backcrossed six generations to C57BL/6J mice. Male $Egr1^{-/-}$ $Egr3^{+/-}$ and female $Egr1^{+/-}$ $Egr3^{+/-}$ mice were crossed to generate $Egr1^{-/-}Egr3^{-/-}$ mice. $p75^{NTR}$ exonIII-deficient mice (Lee et al., 1992) were obtained from Jackson laboratories on C57BL/6Ctrl background and genotyped using primers listed in Table 1.

DβH-τlacZ reporter mice, DβH-nlsCre-IRES-τlacZ, DβH-HA-dnEgr-IRES-τlacZ transgenic mice, and Rosa26-dnEgr (RdnE) knock-in mice were generated with the assistance of The Transgenic and Targeted Mutagenesis Laboratory at Northwestern University. The genotyping primers are listed in Table 1. The Rosa26-rtTA-IRES-EGFP mice were purchased from Jackson Laboratories and genotyped with primers listed in Table 1.

Littermate wild type and mutant mice were used as appropriate for comparison. All experimental procedures complied with protocols approved by Northwestern University Institutional Animal Care and Use Committee.

Table 1. Genotyping primer sequences

Gene	Orientation	Primer Sequence
Egr1	forward	5'-AACCGGCCCAGCAAGACACC-3'
	WT reverse	5'-GGGCACAGGGGATGGGAATG-3'
	KO reverse	5'-CTCGTGCTTTACGGTATCGC-3'
Egr3	forward	5'-GTCAACCCACCCCCTATTACCCCA-3'
	WT reverse	5'-ATGTGAGTGGTGAGGTGGTCGCT-3'
	KO reverse	5'-ATATTGGCTGCAGGGTCGCTCGG-3'
$p75^{NTR}$ exonIII	forward	5'-TGTTACGTTCTCTGACGTGGTGAG-3'
	WT reverse	5'-TCAGCCCAGGGTGTGCACTC-3'
	KO reverse	5'-GGCCTACCCGCTTCCATTGCTCAG-3'
DβH-τlacZ Tg	forward	5'-GATTCCCCGCTAGACAAATGTGA-3'
	reverse	5'-CATGTCACCCTCCTGGTCTT-3'
DβH-nlsCre-IRES-τlacZ Tg	forward	5'-GATTCCCCGCTAGACAAATGTGA-3'
	reverse	5'-CCTCTTCTTCTTGGGTGCCA-3'
DβH-HA-dnEgr-IRES-τlacZ Tg	forward	5'-GTACGACGTCCCGGACTACGCTTC-3'
	reverse	5'-GTGGATCTTGGCGTGGCG-3'
Rosa26-rtTA-IRES-EGFP	forward	5'-GGGAGTGTTGCAATACCTTTCTG-3'
	WT reverse	5'-CGAAAATCTGTGGGAA GTCTTG-3'
	floxed reverse	5'-TGTGGAATGTGTGCGAGG-3'
Rosa26-HA-dnEgr	forward	5'-GGGAGTGTTGCAATACCTTTCTG-3'
	WT reverse	5'-CGAAAATCTGTGGGAA GTCTTG-3'
	floxed reverse	5'-TGTGGAATGTGTGCGAGG-3'
	KI reverse	5'-TGCAGATCCGACACTGGAA-3'

2.2 Cell Culture

Primary myoblasts were isolated and differentiated into myotubes for 10 days in vitro (DIV) as previously described (Albert et al., 2005). Primary mouse embryonic fibroblasts (MEF) were isolated from E16.5 C57BL/6J mice embryos. Briefly, E16.5 embryos were removed from the uterus of deeply anesthetized female mice, decapitated, eviscerated, minced, and digested in 0.25% Trypsin/EDTA (Mediatech, Herndon, VA). After digestions in 0.25% Trypsin/EDTA at 37°C for 15 minutes (twice), cells were plated in Dulbecco's modified Eagle's

media (DMEM, Mediatech, Herndon, VA) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), 1x MEM nonessential amino acids (Mediatech, Herndon, VA) and P/S (100U/ml of penicillin, 100ug/ml of streptomycin, Mediatech, Herndon, VA). The MEFs were split when they reached 60~70% confluence. All experiments were carried out with early passage (≤6) MEFs.

NIH3T3 mouse fibroblasts were purchased from American Type Culture Collection (ATCC, Manassas, VA), and maintained in DMEM supplemented with 10% calf serum (Invitrogen, Carlsbad, CA), P/S at 37°C and 5% CO_2.

Spontaneously immortalized rat Schwann cells (iSCs; a generous gift from S. Cohn, University of Chicago) were maintained in DMEM containing 10% horse serum (Invitrogen, Carlsbad, CA) and P/S at 37°C and 7.5% CO_2.

The SH-SY5Y/TrkA human neuroblastoma cells (a gift from Dr. S. Pahlman, Umea University, Sweden) were maintained in DMEM containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and P/S at 37°C and 5% CO_2.

2.3 Adenoviral infection

The generation, characterization, amplification and titration of the enhanced green fluorescent protein (EGFP), Egr1, Egr3, and truncated Egr3 (Egr3Tr) adenoviruses were described previously (Albert et al., 2005; Li et al., 2005). Primary myotubes, MEFs, or iSCs were infected with EGFP, Egr1, Egr3, or $Egr3_{Tr}$ expressing adenovirus with a multiplicity of infection of approximately 100 to obtain 100% infection efficiency with minimal identifiable toxicity. Since all adenoviruses also expressed EGFP, infection efficiency were monitored by EGFP fluorescence.

2.4 RNA preparation, RT-PCR and quantitative real–time PCR

Total RNA was isolated from tissues or cells using Trizol (Invitrogen, Carlsbad, CA). Total RNA (1ug) was reverse transcribed using random octomer priming and Powerscript reverse transcriptase according to the manufacturer's specifications (BD Biosciences Clontech, Palo Alto, CA). Quantitative real-time PCR (qPCR) was performed on an SDS5700 sequence detector (Applied Biosystems, Foster City, CA) and SYBR green (Molecular Probes, Eugene, OR) fluorescence chemistry was performed as previously described (Albert et al., 2005).

2.5 Chromatin immunoprecipitation

Cellular protein and DNA interactions were cross-linked by treating cells with 1% formaldehyde for 10 minutes at room temperature. Chromatin was isolated and sheared into ~600bp fragments using sonication as previously described (Li et al., 2005). Chromatin immunoprecipitation was performed using 5 ug of rabbit anti-Egr1 (588) or anti-Egr3 (C-14) antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or normal rabbit IgG as a non-specific binding control (Jackson Immunoresearch, West Grove, PA). To evaluate the relative enrichment of $p75^{NTR}$ promoter fragments in the ChIP libraries, qPCR was performed using primers that amplified regions of the $p75^{NTR}$ promoter near the transcription start site (proximal) and 1.5kb upstream of the transcription start site (distal). The samples were normalized to GAPDH to control for non-specific DNA bound by the antibodies and/or IgG. The fold enrichment of $p75^{NTR}$ promoter DNA represented in the Egr1 or Egr3 antibody precipitated genomic DNA was compared relative to that found in IgG precipitated (non-specific) DNA. In addition, end-point PCR was performed by using the ChIP DNA as a template, 35 cycles of amplification and agarose gel eletrophoresis.

2.6 Primer sequences used for qPCR and ChIP assays

Table 2. PCR primer sequences for qPCR and ChIP assays

Gene	Orientation	Primer Sequence
mouse/rat Egr1 mRNA	forward	5'-GCCTTCTCCAGCTGCTTCAT-3'
	reverse	5'GAGCCTTTAAGTCCTGGGAGC-3'
mouse/rat Egr3 mRNA	forward	5'-ATGGACATCGGTCTGACCAAC-3'
	reverse	5'-AAAAGATTGCTGTCCAAGGCC-3'
mouse/rat GAPDH mRNA	forward	5'-ACGGCAAATTCAACGGCACAGTCA-3'
	reverse	5'-GCTTTCCAGAGGGGCCATCCACAG-3'
mouse/rat $p75^{NTR}$ mRNA	forward	5'-CCCTCAAGGGTGATGGCAACCTCT-3'
	reverse	5'-TGTCAGCTCTCTGGATGCGTCGC-3'
mouse $p75^{NTR}$ proximal promoter	forward	5'-TGCTGCTG CTTCTAGGGGT-3'
	reverse	5'-ACCCGTCGTAGTGCTCAA-3'
mouse $p75^{NTR}$ distal promoter	forward	5'-TATTTAAATTAATGCCACACTGTCG-3'
	reverse	5'-AAACCTATCCTATGTAGCCAATAAA-3'
rat $p75^{NTR}$ proximal promoter	forward	5'-CGCTGGAGCGCATCGCAGTTC-3'
	reverse	5'-CGCCACCCGTTCCCAACA-3'
rat $p75^{NTR}$ distal promoter	forward	5'-CTAGGAAATCACCTACTTGTGAGCA-3'
	reverse	5'-CATCAAAACCTGGCACCCTTA-3'

2.7 $p75^{NTR}$ promoter reporter constructs and luciferase assays

The parental plasmid $p75^{NTR}$ minimal promoter plasmid (–250 to +170) was generated by cloning of a BamHI/ NcoI fragment of mouse $p75^{NTR}$ genomic DNA into pGL3 basic luciferase reporter vector (Promega, Madison, WI). The $p75^{NTR}$ (–124 to +170) and $p75^{NTR}$ (–9 to +170) constructs were generated by deletion of SacI/ AvrII or NheI/ NheI fragment from the parental plasmid respectively. The $p75^{NTR}$ (–9 to +77) plasmid was generated by deletion of an XhoI/ BssHII fragment from $p75^{NTR}$ (–9 to +170) and the $p75^{NTR}$ (–46 to +170) plasmid was

generated by PCR using the parental $p75^{NTR}$ (–250 to +170) plasmid as a template and cloning the amplification product into pGL3. All plasmids were sequence verified.

Site-directed mutagenesis of $p75^{NTR}$ (–250 to +170) plasmid was performed with QuikChange Site-Directed Mutagenesis Kit following the manufacturer's specifications (Stratagene, La Jolla, CA). The Egr binding site reported previously (Nikam et al., 1995) was denoted as E1 (nt –56 to –48, GAGGAGGCG) and the mutaion which abrogates Egr protein binding (GAGGAAAAG) was generated and designated as E1m. A second site, predicted by AliBaba 2.1 software (AgeLab, Germany) was designated as E2 (nt +127 to +136, GCCGGAGGCG) and the mutation which also disrupts Egr protein binding (GCCGGAAAAG) was generated and designated as E2m. In addition, a luciferase reporter construct containing both mutation E1m and E2m was generated using site-directed mutagenesis and sequence verified.

Luciferase reporter assay were performed by cotransfecting 50ng of $p75^{NTR}$ promoter reporter plasmid, 5ng of pCMV-RL and 1ug of CMV-Egr1, or CMV-Egr3 or pcDNA3 into NIH3T3 fibroblasts using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, lysates were processed for luciferase activity using the Dual Luciferase Assay Kit according to the manufacturer's specifications (Promega, Madison, WI). Relative luciferase unit (RLU) values were generated by normalizing firefly luciferase counts to Renilla luciferase counts generated from pCMV-RL. The reporter assays were repeated a minimum of three times.

2.8 Nuclear Extract and electrophoretic mobility shift analysis (EMSA)

DNA binding proteins were extracted from MEFs or iSC cells. Briefly, cells were washed once in cold PBS and resuspended in 400 μl ice-cold lysis buffer (10 mM HEPES, pH 7.9, 1.5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM dithiothreitol, 1 mM Na3VO4, 10 mM NaF, 20 mM 2-phosphoglycerate, Complete protease inhibitors [Roche, Alameda, CA]). Dissociated cells were allowed to swell on ice for 15 min, after which 25 μl of 10% NP-40 was added, and the tubes were vortexed for 10 second. After centrifugation and supernatant removal, the nuclear pellet was resuspended in 100 μl ice-cold extraction buffer (20 mM HEPES, pH 7.9, 0.4 mM NaCl, 1 mM EDTA, 1 mMDTT, 1 mMNa3VO4, 10 mM NaF, 20 mM 2-phosphoglycerate, Complete protease inhibitors) for 20 min. Binding reactions using the nuclear protein extracts were performed at room temperature for 20 min with 10 μg of nuclear extract, 0.5 ng ^{32}P-labeled oligonucleotide probe, 1 mg poly(dI-dC) in a binding buffer consisting of 10 mM Tris (pH 7.5), 26 mM KCl, 1 mM $MgCl_2$, 0.5 mM $ZnCl_2$, 1 mM EDTA, 5 mM DTT, and 5% (vol/vol) glycerol. For supershift experiments, reaction were incubated with 2 μg of anti-SP1 (sc-59x), anti-Egr1 (sc-189x), or anti-Egr3 (sc-191x) polyclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) for an additional 20 min. Electrophoresis was performed to separate the probe, protein-probe, and antibody-protein-probe complexes. Autoradiograph of the dried gel was scanned by a phosphorimager (Biorad, Hercules, CA). The sense sequence of the probes are as follows: E1: 5'-CGGGGAGGAGGCGGGGCTG-3', E1m: 5'-CGGGGAGGA**AAA**GGGGCTG-3', E2: 5'-GCTGTCGCCGGAGGCGAGCA -3', E2m: 5'- GCTGTCGCCGGA**AAA**GAGCA-3'. All probes were 5'end labeled with T4-polynucleotide kinase and [γ-^{32}P]ATP (Perkin Elmer, Boston, MA).

2.9 In situ hybridization

In situ hybridization was performed using digoxygenin-labeled riboprobes and previously published standard protocols (Darby, 2000). In situ hybridization probes were generated as previously described (Albert et al., 2005). In all cases, the sense (S) probe was used on parallel tissue sections as a control for nonspecific hybridization, and antisense (AS) probes were used to examine gene-specific expression. The probes used in this study spanned the following gene coding sequences: Egr3 (GenBank NM018781, nt 890 to 1389), p75NTR (GenBank BC038365, nt 253 to 767).

2.10 Immunohistochemistry

Deeply anesthetized mice were perfused with 4% paraformaldehyde/0.1 M phosphate buffer (pH=7.4). Tissues were dissected and frozen in OCT compound (Sakura Finetek, Torrance, CA) after 6-hour postfixation in perfusion buffer and 24-hour equilibration in 30% sucrose/0.1 M phosphate buffer (pH=7.4). Serial 16μm transverse sections were cut in a cryostat, and incubated with antibodies that recognized Egr1 (C-19), Egr3 (H-180) (both diluted 1:1000, Santa Cruz Biotechnology, Santa Cruz, CA), $p75^{NTR}$ ECD (diluted 1:2,500, R&D Systems, Minneapolis, MN), parvalbumin (R301, diluted 1:1000; a generous gift from K. G. Baimbridge), Cre (diluted 1:100, Covance, Madison, WI), TH (diluted 1:5000, Chemicon, Temecula, CA), and/or β-galactosidase (diluted 1:1000, AbD Serotec, Raleigh, NC). The primary antibodies were detected using Cy3 (Jackson Immunoresearch, West Grove, PA) or Alexa 488 (Invitrogen, Carlsbad, CA) fluorophore-conjugated secondary antibodies. The images were acquired with a digital camera (Spot RT-Slider, Diagnostic Instruments) attached to a model E600 Nikon microscope. The images were constructed into montages using Adobe Photoshop software without any further manipulation. For quantification, fluorescent images were captured with a

Zeiss LS510 confocal microscope with identical aperture and photomultiplier tube voltage settings to ensure accurate comparison between images from wild type and mutant mice. The images were assembled into montages with Adobe Photoshop software and were not further manipulated so that fluorescent intensity measurements could be compared between samples using ImageJ software (NIH).

2.11 Western blotting

Sciatic nerves were homogenized in RIPA buffer (20 mM Tris, pH 7.7, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 10 nM NaF and Complete protease inhibitors (Roche, Alameda, CA). Eighty micrograms of total cellular proteins were resolved by SDS-PAGE and transferred to a nitrocellulose membrane. Blots were probed with antibodies as follows: anti-Egr1 (sc-110) (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Egr3 (a gift of Dr Jay M. Baraban, Johns Hopkins University), anti-Egr2 (Covance Research Products, Cumberland, VA), anti-ERK (sc-1616) (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-$p75^{NTR}$ ICD (Promega, Madison, WI), then incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA) and visualized using SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL). For quantification, protein density was analyzed using ImageJ software (NIH) on scanned images and using standard curves to ensure that the film band intensities were in the linear range of detection for accurate quantification as previously described (Li et al., 2007).

2.12 Histology and peripheral myelination analysis:

Deeply anesthetized mice were perfused through the heart with 0.1M cacodylate buffer (pH=7.4), follwed by perfusion with 2.5% glutaraldehyde/2% paraformaldehyde/0.1M cacodylate buffer. Sciatic nerves were dissected and postfixed in 1% osmic acid and embedded in Eponate 12 resin (EMS, Hatfield, PA). One-micron transverse sections of nerves were collected, stained with toluidine blue and examined by light microscopy at 100x magnification using oil immersion optics. For axon diameter and g-ratio calculations, four random non-overlapping regions were imaged from each nerve and every myelinated axon in the field was analyzed. Approximately 1500-1800 axons per genotype (500-600 axons per animal, and three animals per genotype) were analyzed. Calibrated images were analyzed using DrawingSlateII digitizer (Calcomp, Columbia, MD) and SigmaScan image analysis software (Systat, San Jose, CA) to measure axon perimeter, axon ferret diameter, fiber perimeter, and fiber ferret diameter. The g-ratio, a measure of the myelin thickness of an individual axon, was calculated by dividing the axon ferret diameter by the fiber ferret diameter. Myelin thickness was calculated as ½ the fiber ferret diameter minus the axon ferret diameter.

2.13 Statistical analysis

Statistical analysis of biological replicates was performed using Student's t test. The values represent mean ± standard deviation of multiple independent experiments ($N \geq 3$). Significance was defined as $p<0.05$.

2.14 Generation of DβH-τlacZ transgenic reporter mice

The transgenic construct was generated by cloning the fusion protein τlacZ cDNA with the polyadenylation signal from Simian Virus 40 (a gift from Dr. Thomas, Salk Institute for

Biological Studies) into the BamHI site in pWT25 (a gift from Dr. Palmiter, University of Washington) (Hoyle et al., 1993), in which the 5.8kb human DβH promoter (hDβH) is located upstream of the first intron of rat insulin II (rInsII), followed by a mouse protamine polyA sequence. The whole hDβH-rInsII-τlacZ-SV40pA transgene was released from pWT25 using XbaI digestion, and purified for microinjection. The microinjection was performed at The Transgenic and Targeted Mutagenesis Laboratory at Northwestern University. Five different founders were screened by β-galactosidase enzyme histochemistry, and one reporter line with τlacZ expression in nearly all the sympathetic neurons and adrenal chromaffin cells was identified and propagated.

2.15 Generation of DβH-dnEgr-IRES-τlacZ transgenic mice

To generate the transgenic construct, τlacZ-SV40pA was cloned into the SmaI sites of pIRES (BD Biosciences). The HA-tagged dnEgr cDNA was then cloned into the EcoRI site of pIRES-τlacZ-SV40pA. HA-dnEgr-IRES-τlacZ-SV40pA was then excised and cloned into the unique BamHI site in pWT25, downstream of the hDβH promoter and rInsII first intron. The hDβH-HA-dnEgr-IRES-τlacZ-SV40pA transgene was released from pWT25 by XbaI digestion, and purified for microinjection. The microinjection was performed at The Transgenic and Targeted Mutagenesis Laboratory at Northwestern University. Due to embryonic lethality, founder analysis was performed at embryonic day 15.5, 17.5, and 19.5.

2.16 Generation of DβH-nlsCre-IRES-τlacZ transgenic mice

To generate the transgenic construct, nuclear-localized Cre recombinase (nlsCre) cDNA was cut from pTurboCre plasmid and cloned into EcoRI site of pIRES-τlacZ-SV40pA. The fragment nlsCre-IRES-τlacZ-SV40pA was excised and cloned into the unique BamHI site in pWT25, downstream of the hDβH promoter and rInsII first intron. The hDβH-nlsCre-IRES-τlacZ-SV40pA transgene was released from pWT25 by XbaI digestion, and purified for microinjection. The microinjection was performed at The Transgenic and Targeted Mutagenesis Laboratory at Northwestern University. Eleven different founders were screened by β-galactosidase enzyme histochemistry, and three transgenic lines with Cre and τlacZ expression in nearly all sympathetic neurons and adrenal chromaffin cells were identified and propagated.

2.17 Generation of Rosa26-HA-dnEgr (RdnE) knock-in mice

The targeting construct for RdnE mice was generated by targeting the Rosa26 locus according to the method of Srinivas *et al* (Srinivas et al., 2001). Briefly, HA-tagged dnEgr molecule was cloned downstream of a floxed PGK-Neo-3xpolyA cassette in pBigT plasmid (Srinivas et al., 2001). The dnEgr-containing insert was released by AscI/ PacI double digestion and cloned into pROSA26-PA vector. The targeting construct pROSA26-HA-dnEgr was linearized using XhoI and electroporated into mouse embryonic stem (ES) cells. The conditional dnEgr expression cassette was integrated into mouse genome when homologous recombination occured. Positive clones were selected for neomycin-resistance. G418-resistant ES clones were screened by PCR for correct targeting, and the results were confirmed by 5' and 3' Southern blotting. Two different ES clones were selected to generate chimeric mice, and they both showed germline transmission. RdnE mice were generated at The Transgenic and Targeted Mutagenesis

Laboratory at Northwestern University. The progeny from clone 3D5 were propagated into the RdnE line.

2.18 Southern Blotting

Genomic DNA was isolated from ES cells or mouse tails and subjected to restriction enzyme digestion with 40mM Spermidine for 4 hours. Digested DNA fragments were subjected to electrophoresis, depuration (0.25M HCl), denaturation (0.5M NaOH/1.5M NaCl), and were transferred to a positively charged nylon membrane (Zeta blot, Bio-rad, Hercules, CA) with denaturation buffer (0.5M NaOH/1.5M NaCl). After cross-link with UV light, the membrane was subjected to hydridization with ^{32}P-labeled probes. Probes were labeled using Rediprime II random prime labeling system according to manufacturer's specifications (Amersham, Pittsburgh, PA). Hybridization was performed in Ultrahyb solution (Ambion, Austin, TX) at 42 °C overnight. After multiple washes at 42 °C (twice in 2xSSC, 0.1% SDS for 5min each; twice in 0.1x SSC, 0.1% SDS for 15min each), the blot was visualized by phosphorimager (Bio-rad, Hercules, CA).

2.19 Whole-mount β-galactosidase enzyme histochemistry

Deeply anethetized mice were perfused with PBS-Mg^{2+} (pH=7.4, 2mM $MgCl_2$), followed by 2% paraformaldehyde/0.2% glutaraldehyde/5mM EGTA/PBS-Mg^{2+} (pH=7.4). Tissues were isolated and postfixed in the perfusion buffer for one hour at 4°C. After three washes in Wash Buffer (0.01% sodium deoxycholate, 0.02% NP-40, in PBS-Mg^{2+}), tissues were labeled with Staining Solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium

ferrocyanide in Wash Buffer) for 8-12 hours at 37°C. For clearing, tissues were dehydrated by three wahses of methanol, and cleared in Benzyl benzoate/Benzyl alcohol (1:2) clearing solution (Fisher, Pittsburgh, PA) .

2.20 β-galactosidase enzyme histochemistry

Deeply anethetized mice were perfused with PBS-Mg^{2+} (pH=7.4, 2mM $MgCl_2$), followed by 4% paraformaldehyde/PBS-Mg^{2+}. Tissues were frozen in OCT compound (Sakura Finetek, Torrance, CA) after postfixation in the perfusion buffer for six hours at 4°C, and cryoprotection in 30% sucrose/PBS-Mg^{2+} for 24 hours. Serial 20μm transverse sections were cut in a cryostat, stained with Staining Solution (1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide in PBS-Mg^{2+}) for 3 hours at 37°C, and counterstained with 1% neutral red.

CHAPTER 3

Modulation of Low Affinity Neurotrophin Receptor $p75^{NTR}$ Expression by Egr Transcriptional Regulators

The $p75^{NTR}$ neurotrophin receptor plays important roles in regulating neuron survival, differentiation and myelination (Barker, 2004; Nykjaer et al., 2005). $p75^{NTR}$ binds neurotrophins with relatively low affinity, but when it is coexpressed with Trk receptors, it substantially increases the binding affinity and specificity of Trk for their preferred neurotrophins (Benedetti et al., 1993; Chao and Hempstead, 1995). Similarly, $p75^{NTR}$ complex with Sortilin and bind proneurotrophin ligands such as pro-NGF to facilitate oligodendrocyte and neuron apoptosis (Beattie et al., 2002; Harrington et al., 2004; Nykjaer et al., 2004). $p75^{NTR}$ can also complex with NogoR and Lingo-1 receptors to bind myelin based growth inhibitors such as Nogo, myelin associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp) and modulate RhoA activity and cytoskeleton dynamics during axon development and regeneration (Mi et al., 2004; Wang et al., 2002; Wong et al., 2002; Yamashita et al., 1999).

Accordingly, the $p75^{NTR}$-deficient mice have a variety of neurological defects, including decreased Schwann cell number and impaired peripheral nerve myelination (Cosgaya et al., 2002; von Schack et al., 2001), lack of sympathetic nerve innervation in pineal gland and some sweat glands (Lee et al., 1994a), as well as loss of heat sensitivity due to decreased sensory innervation to skin (Lee et al., 1992).

The mechanisms by which $p75^{NTR}$ mediates such diverse cellular response are not well understood, but may be partially explained by the cellular and developmental context in which it is expressed. $p75^{NTR}$ expression is transcriptionally regulated by a variety of stimuli including growth factor signaling (such as NGF) during development (Bolin et al., 1992; Kuruvilla et al.,

2004), by central and peripheral nervous system injury (Beattie et al., 2002; Kokaia et al., 1998; Rende et al., 1993) and by disease processes such as ischemia, epilepsy, and Alzheimer's disease (Kokaia et al., 1998; Mufson and Kordower, 1992; Roux et al., 1999). The $p75^{NTR}$ promoter lacks consensus TATA and CAAT sequences, but contains multiple GC-rich motifs, suggesting functional roles for transcription factors which bind GC-rich sequences, such as Sp1, WT1, and Egr transcription factors (Poukka et al., 1996; Sehgal et al., 1988).

Egr proteins are inducible transcriptional regulators that have important roles in regulating gene expression in response to a variety of extracellular stimuli. One study suugests that Egr1 is required for $p75^{NTR}$ upregulation in Schwann cells through direct interaction with its promoter (Nikam et al., 1995). Most recently, we identified $p75^{NTR}$ as an Egr3 target gene in differentiating myotubes by Affymetrix microarray analysis (Albert et al., 2005). Therefore, we hypothesize that Egr1 and Egr3 may directly regulate $p75^{NTR}$ expression.

In this study, we first tested if Egr1 and Egr3 directly bind and transactivate $p75^{NTR}$ promoter. Next, we examined if Egr1 and Egr3 regulate $p75^{NTR}$ expression in two *in vivo* model systems: muscle spindle mechanoreceptors that mediate stretch reflexes and proprioception and Schwann cells. We chose muscle spindles as a model system because Egr3 but not other Egr proteins, is strongly expressed in muscle spindles, which is required for muscle spindle morphogenesis and maintenance (Tourtellotte et al., 2001; Tourtellotte and Milbrandt, 1998). Therefore, muscle spindles offer a good system to study Egr3 target genes. On the other hand, $p75^{NTR}$ also has a role in muscle spindle development by regulating NT-3 signaling (Fan et al., 1999). We also tested our hypothesis in Schwann cells because $p75^{NTR}$ is critical for Schwann cell biology and normal peripheral nerve myelination, and Egr1 modulates $p75^{NTR}$ expression in remyelinating Schwann cells (Cosgaya et al., 2002; Nikam et al., 1995; von Schack et al., 2001).

3.1 Enforced expression of Egr1 or Egr3 induces $p75^{NTR}$ expression in multiple cell types

The $p75^{NTR}$ gene was identified as a potential target of Egr3 regulation from a microarray analysis intended to characterize the target genes regulated by Egr3 during muscle stretch receptor development (Albert et al., 2005). The microarray result was confirmed by comparing $p75^{NTR}$ expression in primary murine myotubes infected with adenoviruses expressing either wild type (Egr3) or a transcriptionally inactive c-terminal truncation of Egr3 ($Egr3_{Tr}$). $p75^{NTR}$ expression was upregulated 14.2-fold by wild type Egr3 relative to Egr_{Tr} in primary myotubes (Fig. 2A). Egr3 protein is robustly expressed in the nuclei of both intrafusal muscle fibers and spindle capsules that are derived from perineurial fibroblasts during muscle spindle development (Maier, 1997; Tourtellotte and Milbrandt, 1998). Therefore, we also investigated whether $p75^{NTR}$ expression was upregulated in mouse embryonic fibroblasts (MEFs) by enforced expression of Egr3. In MEFs, $p75^{NTR}$ expression was induced 240-fold by wild type Egr3 when compared to $Egr3_{Tr}$ (Fig. 2B). Since $p75^{NTR}$ also has an important role in Schwann cell biology (Cosgaya et al., 2002) and it has been reported that Egr1 modulates $p75^{NTR}$ expression in Schwann cells (Nikam et al., 1995), we tested whether Egr1 and/or Egr3 can regulate $p75^{NTR}$ in spontaneously immortalized rat Schwann cells (iSCs). Enforced expression of either Egr1 or Egr3 upregulated $p75^{NTR}$ expression by 5.1-fold and 17-fold, respectively (Fig. 2C). Thus, enforced Egr1 or Egr3 expression can upregulate $p75^{NTR}$ expression in a variety of cellular contexts.

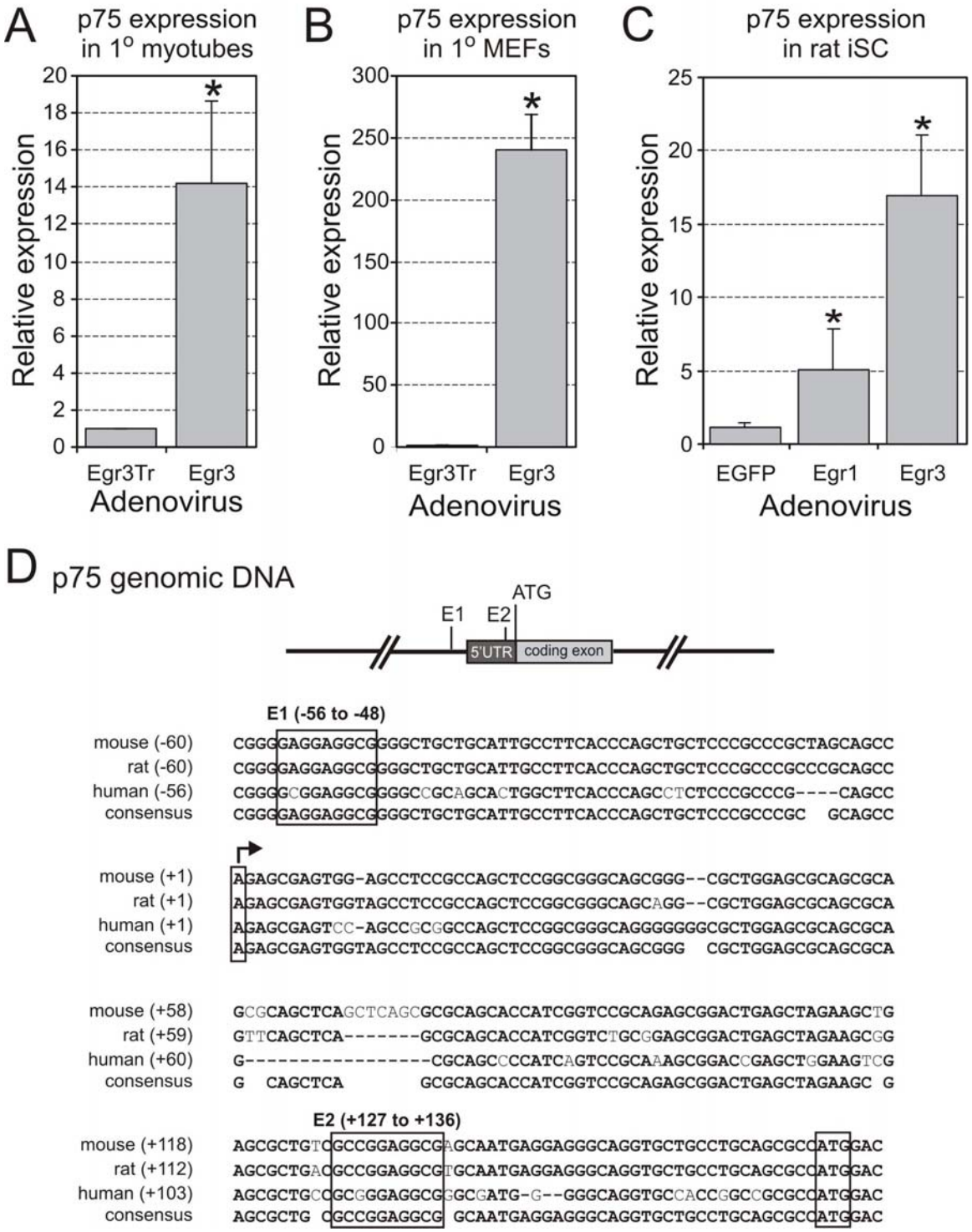

Figure 2: Egr1 and Egr3 induce p75^{NTR} expression through conserved Egr response elements (EREs) in its promoter.

(A-C) $p75^{NTR}$ expression is significantly upregulated by Egr3 (A) 14.2-fold in primary mouse myotubes, (B) 240-fold in mouse embryonic fibroblasts (MEFs) and (C) 5.1-fold by Egr1 and 17-fold by Egr3 in immortalized rat Schwann cells (iSCs). $p75^{NTR}$ expression in cells infected with either Egr1 or Egr3 expressing adenovirus is normalized to expression in cells infected with either $Egr3_{Tr}$ or EGFP expressing adenovirus as indicated. (results represent mean ± standard deviation of three independent experiments, * = $p < 0.05$, Student's t test).

(D) Alignment of a portion of the $p75^{NTR}$ 5' regulatory sequence in mouse, rat and human relative to the transcription start site (arrow) and translation start codon (ATG) shows two highly conserved potential EREs designated E1 upstream of the transcription start site (-56 to -48) and E2 within the 5' untranslated region (5'UTR) of the first exon (+127-136).

3.2 Egr1 and Egr3 bind the proximal p75^{NTR} promoter

The alignment of mouse, rat and human p75^{NTR} promoters revealed multiple conserved GC-rich motifs (Fig. 2D), suggesting potential regulatory roles for proteins that bind GC-rich sequences, such as Sp1, WT1 and Egr transcriptional regulators. Egr1 has been shown to bind to a region of the p75^{NTR} promoter which we designated as E1 (Fig. 2D) and to transactivate p75^{NTR} expression in Schwann cells (Nikam et al., 1995). Sequence analysis also revealed a second potential Egr protein binding site in the 5' UTR of p75^{NTR} which we designated E2 (Fig. 2D). Whether Egr1 and/or Egr3 directly regulate p75^{NTR} expression and through which binding sites they may confer transcriptional activation has not been examined in detail. If Egr1 and/or Egr3 directly regulate p75^{NTR} expression they should bind to its regulatory sequences. Chromatin immunoprecipitation (ChIP) combined with end-point PCR and qPCR was used to determine whether Egr1 and/or Egr3 directly bind to DNA near the p75^{NTR} locus *in vivo*. Genomic DNA bound by Egr3 was recovered by ChIP and analyzed by PCR from MEFs infected with Egr3-expressing adenovirus. Egr3 protein was bound *in vivo* near the p75^{NTR} transcription start site (Fig. 3A, Pr1/Pr2) but not to regions of the promoter 1.5-1.8kb upstream of the transcription start site (Fig. 3A, Pr3/Pr4). The end-point PCR results were confirmed by qPCR which showed a significant (10.2-fold) enrichment of Egr3 bound to proximal, but not distal, regions of the p75^{NTR} promoter (Fig. 3A). To examine whether Egr1 and/or Egr3 bound p75^{NTR} regulatory sequences in the context of endogenous (rather than supra-physiologic) levels of Egr protein, we examined immortalized rat Schwann cells (iSCs) that express p75^{NTR} (Bolin et al., 1992), Egr1 and Egr3 (Fig. 6A) in serum-containing media. ChIP-PCR showed that Egr1 and Egr3 bound proximal (Fig. 3B, Pr1'/Pr2'), but not distal (Fig. 3B, Pr3'/Pr4') , to the transcription start site of p75^{NTR}. The end-point PCR results were again confirmed by qPCR which showed a significant

4-fold enrichment of Egr1 protein and a 2.8-fold enrichment of Egr3 protein bound to regions proximal, but not distal, to the $p75^{NTR}$ transcription start site. Thus, both Egr1 and Egr3 bind to regions of the $p75^{NTR}$ promoter near the transcription start site *in vivo*.

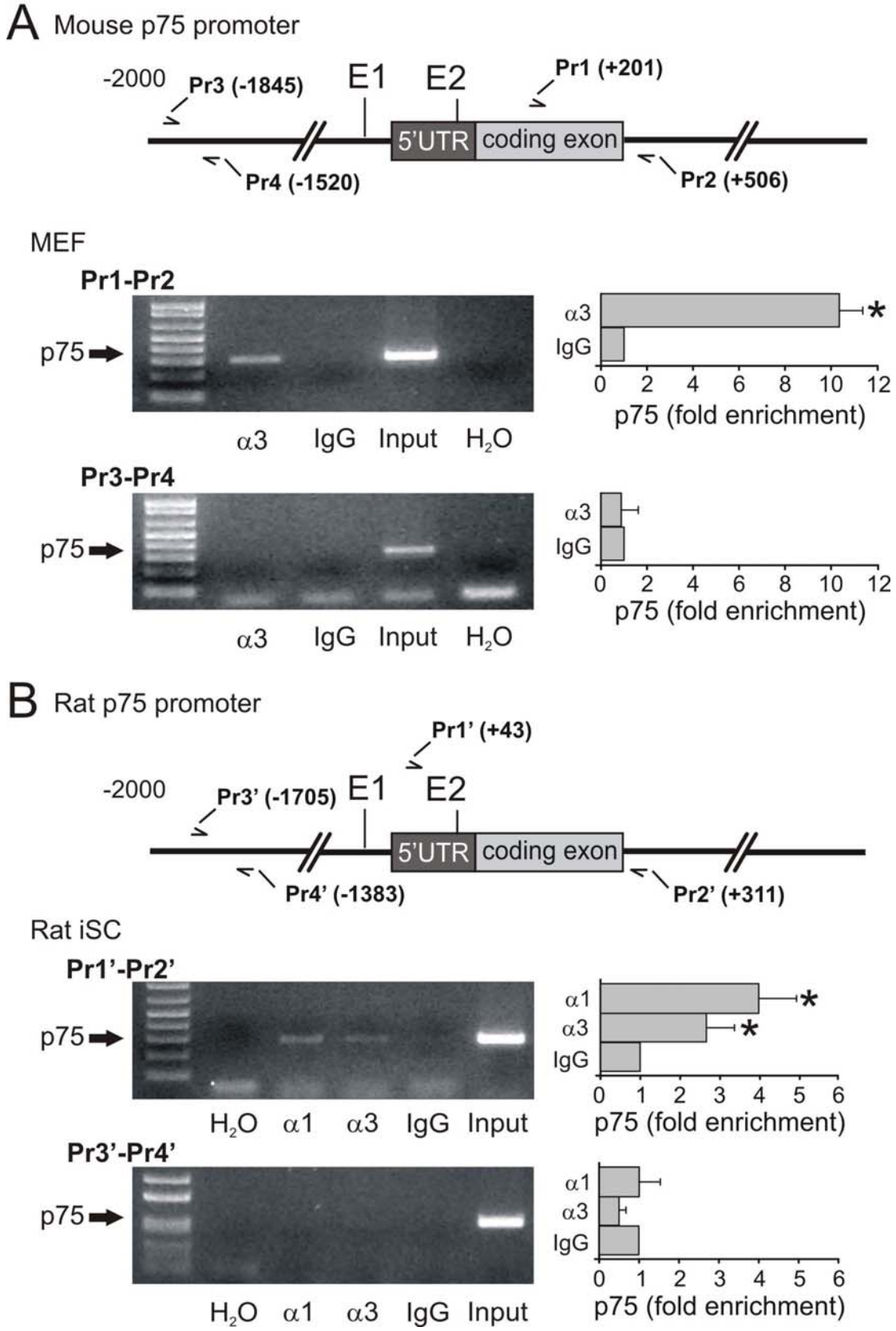
A Mouse p75 promoter
-2000
Pr3 (-1845)
E1
E2
Pr1 (+201)
5'UTR
coding exon
Pr4 (-1520)
Pr2 (+506)
MEF
Pr1-Pr2
p75
α3
IgG
Input
H2O
α3
IgG
0 2 4 6 8 10 12
p75 (fold enrichment)
Pr3-Pr4
p75
α3
IgG
Input
H2O
α3
IgG
0 2 4 6 8 10 12
p75 (fold enrichment)
B Rat p75 promoter
Pr1' (+43)
-2000
Pr3' (-1705)
E1
E2
5'UTR
coding exon
Pr4' (-1383)
Pr2' (+311)
Rat iSC
Pr1'-Pr2'
p75
H2O
α1
α3
IgG
Input
α1
α3
IgG
0 1 2 3 4 5 6
p75 (fold enrichment)
Pr3'-Pr4'
p75
H2O
α1
α3
IgG
Input
α1
α3
IgG
0 1 2 3 4 5 6
p75 (fold enrichment)

Figure 3: Both Egr1 and Egr3 bind the $p75^{NTR}$ promoter *in vivo*.

(A) ChIP performed on nuclear lysates from mouse embryonic fibroblasts (MEFs) infected with Egr3 expressing adenovirus demonstrates that Egr3 directly binds to $p75^{NTR}$ *in vivo*. Immunoprecipitation using Egr3 specific anti-serum (α3), but not non-specific IgG, shows that Egr3 directly binds to a region proximal (Pr1-Pr2) but not distal (Pr3-Pr4) to the putative E1 and E2 response elements in the $p75^{NTR}$ promoter.

(B) Similarly, in immortalized rat Schwann cells (iSCs), which express endogenous Egr1, Egr3 and $p75^{NTR}$ in serum containing growth medium, the $p75^{NTR}$ promoter is bound by endogenous Egr1 and Egr3. Immunoprecipitation using either Egr1 (α1) or Egr3 (α3) specific anti-serum, but not non-specific IgG, shows that both Egr1 and Egr3 bind proximal (Pr1'-Pr2') but not distal (Pr3'-Pr4') to the putative E1 and E2 response elements. For both MEFs and iSCs, the enrichment of chromatin precipitated by α3 or α1 relative to IgG is assessed by endpoint PCR (left) and qPCR (right). (primer locations are indicated relative to mouse and rat $p75^{NTR}$ transcriptional start sites, respectively and qPCR results represent mean ± standard deviation of three independent experiments, * = $p < 0.05$, Student's t test)

3.3 Egr1 and Egr3 preferentially transactivate the $p75^{NTR}$ promoter through different response elements

Egr transcription factors modulate target gene expression by binding to Egr response elements (EREs) in target gene promoters to facilitate gene transcription. A well conserved Egr1 binding site has been previously identified in the $p75^{NTR}$ promoter (Nikam et al., 1995) which is designated E1 at -56 to -48 relative to the transcription start site in the mouse (Fig. 2D). An analysis of the proximal 5' regulatory sequence of the $p75^{NTR}$ gene (AliBaba 2.1, AgeLab, Germany) identified a second highly conserved potential ERE which we designated E2 located at +127 to +136 in the 5'UTR of the mouse $p75^{NTR}$ gene (Fig. 2D).

Since Egr1 and Egr3 bind to the proximal $p75^{NTR}$ promoter *in vivo*, we generated luciferase reporter constructs to test whether they can transactivate it *in vitro*. A 420-nucleotide fragment of mouse genomic DNA immediately upstream of the translation initiation methionine (corresponding to -250 to +170) was fused to a luciferase reporter construct (Fig. 4, construct 1). Both Egr1 and Egr3 were capable of significantly activating this construct, but Egr3 conferred stronger transcriptional activation (14.4-fold) relative to Egr1 (3.2-fold). To map the regions of the $p75^{NTR}$ promoter that conferred Egr-mediated transcriptional activation, serial deletion constructs were generated and tested. There was no significant difference in Egr1- and Egr3-mediated transactivation between the minimal promoter construct (Fig. 4, construct 1) and a 5' deletion that retained E1 and E2 (Fig. 4, construct 2). However, most of the Egr1-mediated, but not Egr3-mediated, transactivation activity was lost on the $p75^{NTR}$ promoter when a region containing E1 was removed (Fig. 4, construct 3), and there was no significant change in transactivation when additional 5' sequence was deleted (Fig. 4, construct 4). Finally, when a

region containing E2 was also deleted, Egr3-mediated transactivation of the $p75^{NTR}$ promoter was significantly diminished (Fig. 4, construct 5).

The results from the luciferase deletion constructs suggested that E1 and E2 may represent functional EREs through which Egr1 and Egr3 preferentially transactivate $p75^{NTR}$, respectively. To test whether E1 and E2 represent functional EREs, site-directed mutagenesis was performed to generate mutations that abrogate Egr1 and Egr3 binding in E1 (E1m), E2 (E2m), or both E1 and E2 (E1m + E2m) (Fig. 6B-C). Relative to the minimal $p75^{NTR}$ luciferase promoter construct (Fig. 5, construct 1), which was significantly activated by both Egr1 and Egr3, E1m resulted in a significant decrease in Egr1-, but not Egr3-mediated transactivation (Fig. 5, construct 2). By contrast, E2m resulted in a significant decrease in Egr3-transactivation and a moderate super-activation by Egr1 (Fig. 5, construct 3). As expected, E1m+E2m resulted in abrogation of both Egr1- and Egr3-mediated transactivation (Fig. 5, construct 4). These results indicate that Egr1 preferentially transactivates $p75^{NTR}$ through E1 and Egr3 preferentially transactivates it through E2.

We next examined the binding interaction between Egr1/Egr3 and E1/E2 in vitro using iSC nuclear lysates which contain low levels of endogenous Egr1 and Egr3 (Fig. 7, lane 1). Egr1, Egr3 and Sp1 were bound to E1 in vitro and binding to E1 was increased as expected when Egr1 and Egr3 protein levels were augmented by enforced expression (Fig. 7, lane 1-6). Interestingly, Egr1 and Egr3 appeared to have a lower affinity for E2 when a comparable amount of iSC nuclear lysate and probe activity were used in the binding assays with E1 and E2. Unlike E1 (Fig. 7, lane 1), the low endogenous levels of Egr1 and Egr3 contained in the iSC nuclear lysates showed very little binding to E2 (Fig. 7, lane 7). However, when Egr1 and Egr3 protein levels were increased with adenoviral infection (Fig. 6), Egr1 (Fig. 7, lane 8) and Egr3 (Fig. 7,

lane 10) were bound to E2. The identity of the E2 binding proteins were confirmed by supershifting the complexes with specific Egr1 (Fig. 7, lane 9) and Egr3 (Fig. 7, lane 11) antibodies. These results suggest that E2 represents a relatively low affinity Egr protein binding site that is occupied when Egr1 and Egr3 proteins are induced above basal levels.

Therefore, E1 appears to represent a high-affinity binding site for Egr1, Egr3 and SP1, and may be important for regulating basal $p75^{NTR}$ expression and Egr1-mediated expression. By contrast, E2 is a low-affinity binding site for Egr1 and Egr3, and mainly converys Egr3-mediated transactivation, so E2 may represent a site through which induced Egr3 protein can preferentially modulate $p75^{NTR}$ expression. Egr proteins have highly conserved DNA-binding domain but less conserved transactivation domain. Therefore, Egr proteins may recruit different coregulators to the transcription complex and the DNA context of ERE may contribute to their interations with coregulators, which may explain how Egr1 and Egr3 can bind both E1 and E2, but differentially transactivate through them.

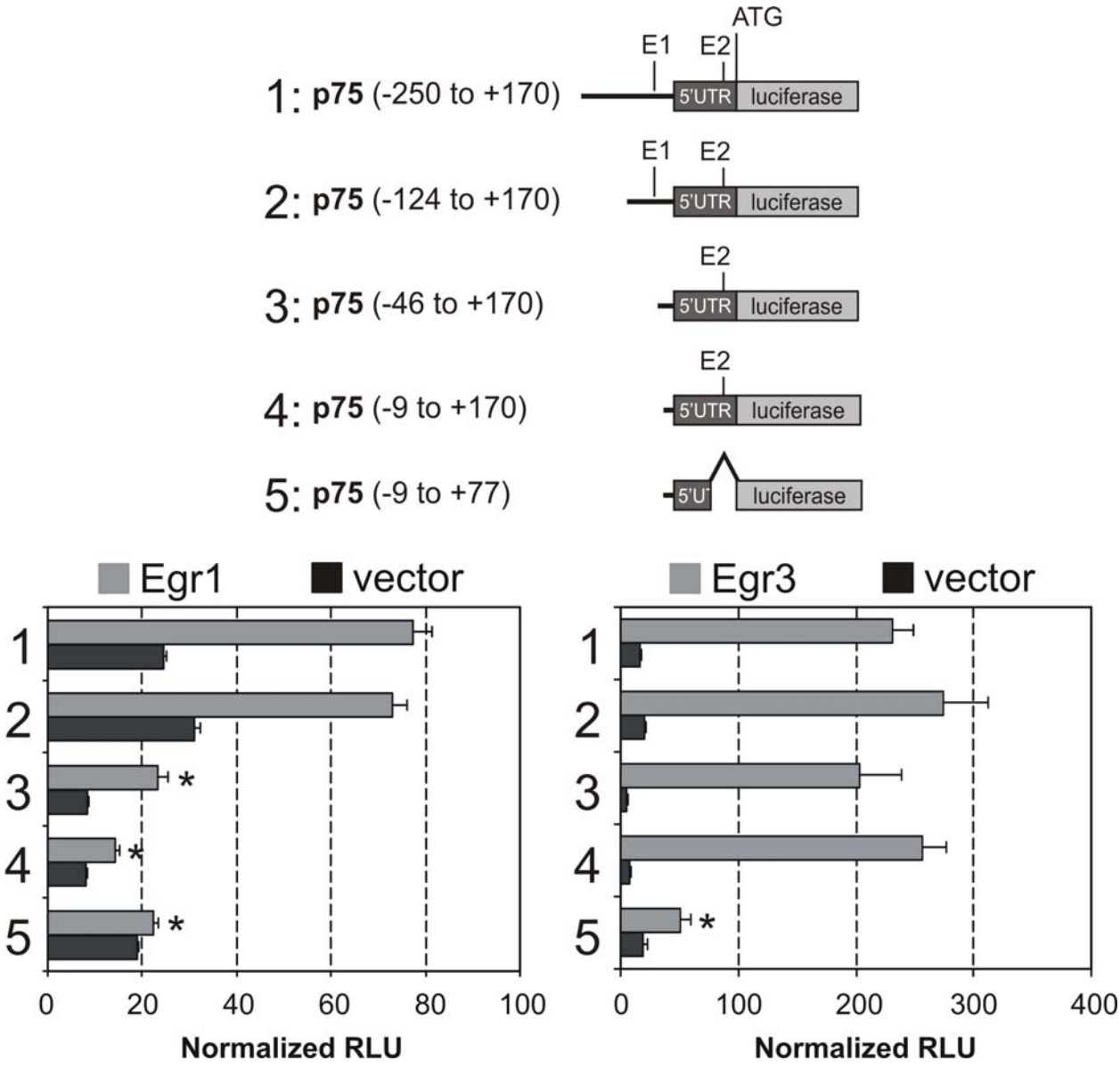

Figure 4. Egr1 and Egr3 transactivate p75^{NTR} expression through distinct DNA regions that contain E1 and E2, respectively.

NIH 3T3 fibroblasts were cotransfected with either Egr1 or Egr3-expressing plasmids and the indicated p75-luciferase reporter constructs (constructs 1-5). Both Egr1 and Egr3 are capable of significantly transactivating constructs containing E1 and E2 (constructs 1 and 2), although Egr3

transactivates the reporter constructs more efficiently than Egr1. When E1 is deleted from the 5' region of the promoter constructs (constructs 3 and 4), Egr1-induced transactivation is significantly diminished (relative to construct 1). By contrast, Egr3-induced transactivation is not affected, suggesting that the region containing E1 preferentially confers Egr1-, but not Egr3-mediated transactivation. However, when a region containing the putative E2 domain in the 5' UTR is also deleted (construct 5), transactivation by Egr3 is also significantly diminished, suggesting that the region containing E2 preferentially confers Egr3-mediated transactivation. (RLU = relative light units representing values normalized for transfection efficiency; results represent mean ± standard deviation of three independent transfection experiments; * = $p < 0.05$; Student's t test compared to construct 1.)

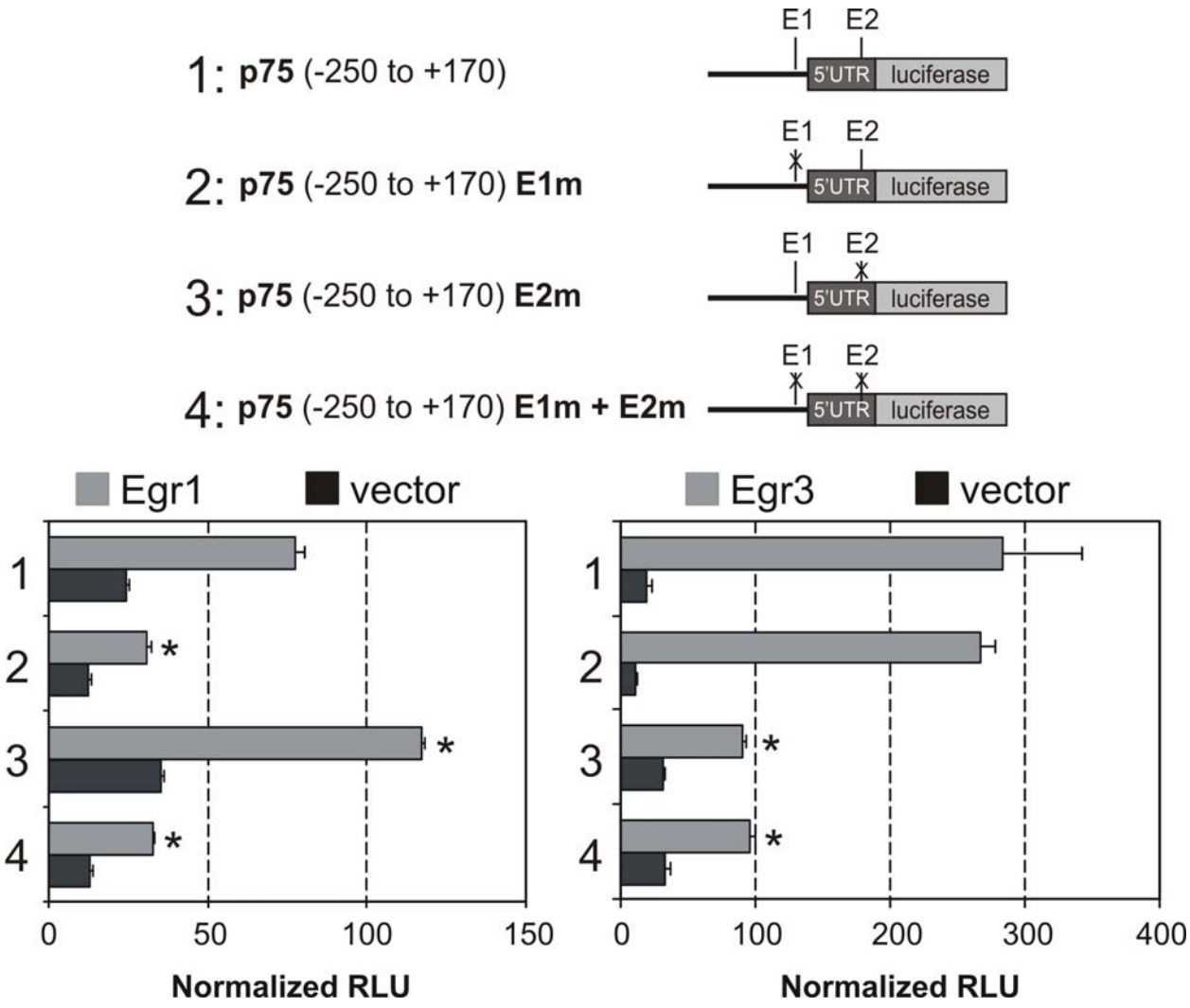

Figure 5. Egr1 and Egr3 transactivate p75^{NTR} through distinct E1 and E2 response elements, respectively.

Mutations that specifically disrupt Egr1 and Egr3 binding (Fig. 6) to E1 and E2 were systematically introduced into construct 1. While Egr1 and Egr3 markedly transactivate the minimal p75^{NTR} promoter (construct 1), transactivation by Egr1, but not Egr3, is significantly reduced when E1 is mutated (construct 2). Interestingly, while transactivation by Egr1 is slightly superactivated when E2 is mutated, transactivation by Egr3 is significantly reduced (construct 3).

When both E1 and E2 are mutated (construct 4), transactivation by either Egr1 or Egr3 is significantly impaired. These results suggest that Egr1 preferentially transactivates $p75^{NTR}$ through E1, whereas Egr3 preferentially transactivates through E2. (RLU = relative light units representing values normalized for transfection efficiency; results represent mean ± standard deviation of three independent transfection experiments; * = $p < 0.05$; Student's t test compared to construct 1.)

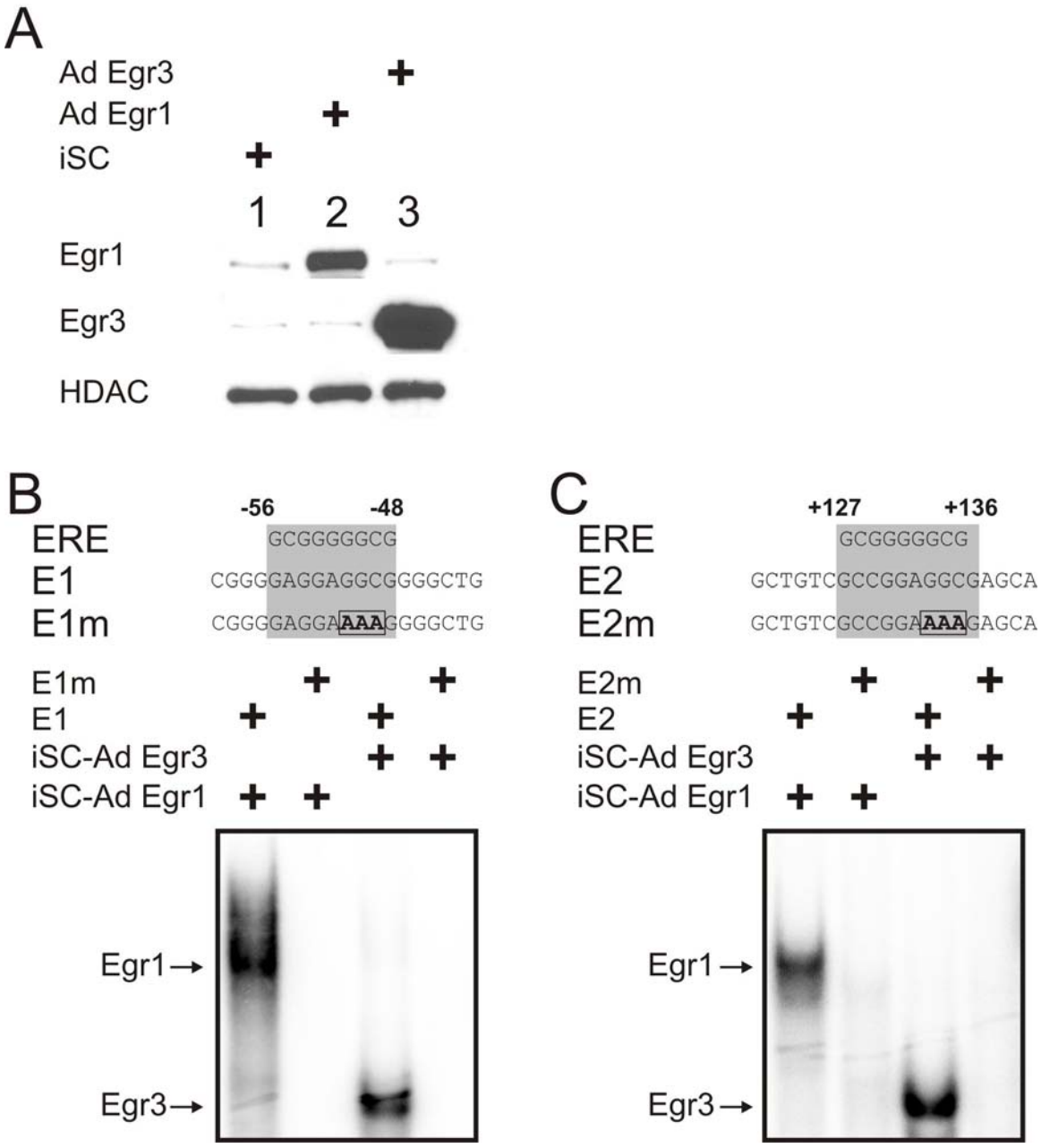

Figure 6. Egr1 and Egr3 bind to the E1 and E2 response elements in the p75^{NTR} promoter *in vitro*.

(A) Immortalized rat Schwann cells (iSCs) express low levels of both Egr1 and Egr3 in serum-containing media. Egr1 and Egr3 protein are markedly increased after infection with Egr1 or Egr3-expressing adenoviruses. Histone deacetylase (HDAC) serves as the loading control for nuclear lysates.

(B) Egr1 protein present in nuclear lysates from Egr1-expressing adenovirus infected immortalized rat Schwann cells (iSC-Ad Egr1) or Egr3 protein present in Egr3-expressing adenovirus infected immortalized rat Schwann cells (iSC-Ad Egr3) bind to a double stranded E1 oligo but not an E1m oligo which has a mutation within the core Egr binding domain (gray).

(C) Similarly, Egr1 and Egr3 bind to E2 but not E2m that contains a mutation disrupting Egr protein binding.

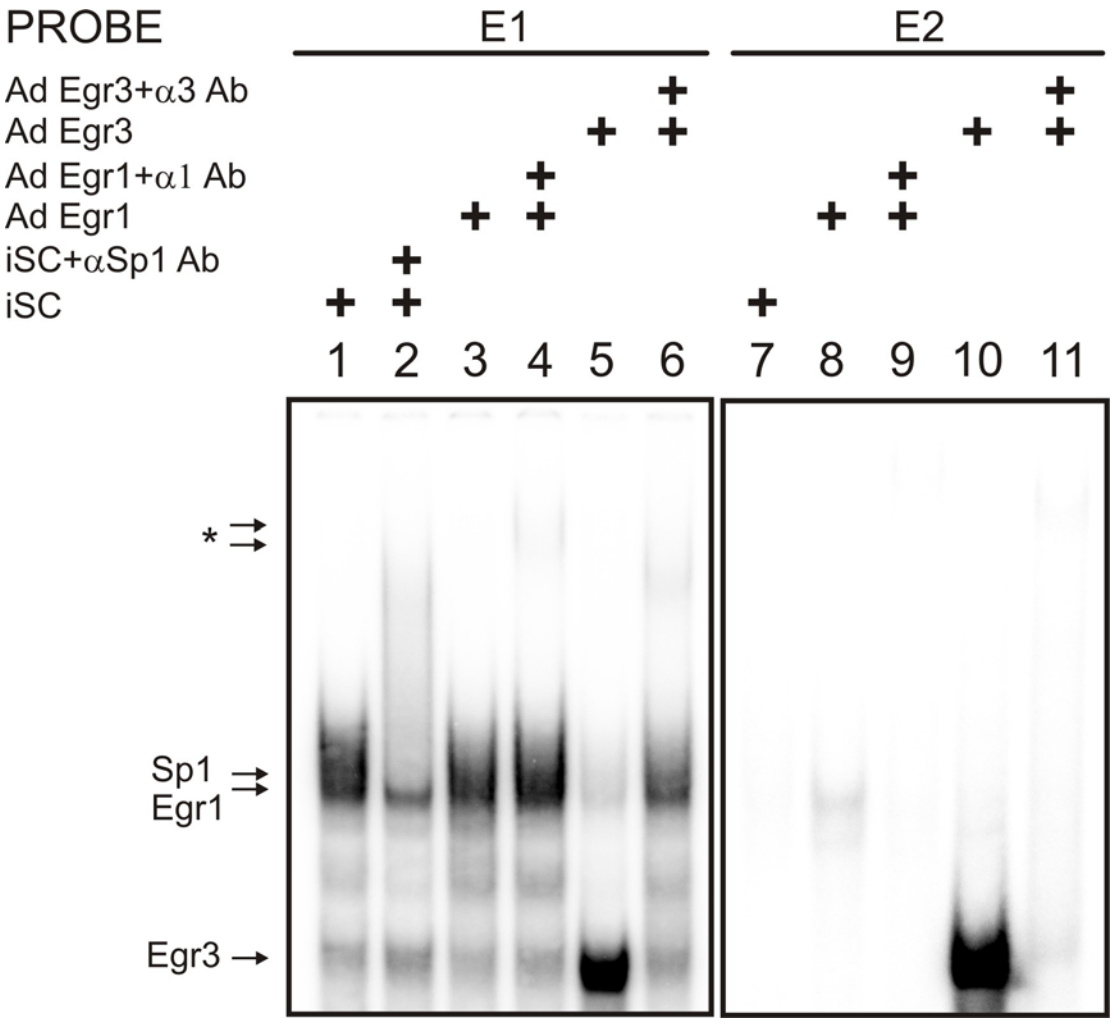

Figure 7. E1 and E2 represent high- and low-affinity Egr binding sites in the p75^{NTR} promoter, respectively.

The transcriptional regulators Egr1, Egr3 and Sp1 are present within nuclear lysates from iSCs grown in serum-containing media and all bind the E1 site (lane 1). Sp1 (lane2), Egr1 (lane 4) and Egr3 (lane 6) are identified within the shifted complexes bound to E1 by the formation of supershift complexes generated by antibodies that bind specifically to the respective proteins. The Egr1 containing complex is obscured by the intense Sp1 complex that runs with similar mobility (lanes 3 and 4). When E2 is used as a probe, the endogenous levels of Egr1 and Egr3 in

iSCs grown in serum do not form complexes with the probe (lane 7). However, when the levels of Egr1 protein are elevated by infection with Egr1 expressing adenovirus, a faint complex is formed (lane 8) which is shifted away by an Egr1 specific antibody (lane 9). Similarly, when iSCs are infected with Egr3 expressing adenovirus, a prominent complex with Egr3 is formed (lane 10) that is nearly completely shifted away by the addition of Egr3 specific antibody (lane 11). *---DNA-protein-antibody supershift complexes.

3.4 Egr3 modulates p75^{NTR} expression in muscle spindles *in vivo*

Egr1 and Egr3 directly transactivate p75^{NTR} through distinct EREs *in vitro*, but whether Egr1 and/or Egr3 modulate p75^{NTR} expression *in vivo* is not known. Egr3 is strongly expressed in developing muscle spindle stretch receptors where it is required for their normal development (Tourtellotte et al., 2001; Tourtellotte and Milbrandt, 1998). Similarly, p75^{NTR} has an important role in modulating neurotrophin-3 (NT-3) signaling which is also required for normal spindle development (Fan et al., 1999). Thus, we asked whether Egr3 may have a role in modulating p75^{NTR} expression in spindles. Interestingly, we found that Egr3 (Fig. 8A, arrowheads) and p75^{NTR} (Fig. 8A', adjacent section, arrowheads) were robustly and specifically co-expressed in spindle stretch receptors in skeletal muscle. These results were confirmed by immunofluorescence which showed that both Egr3 and p75^{NTR} protein were highly co-localized in spindle capsule cells and intrafusal muscle fibers (Fig. 8B, arrowhead) but not in skeletal muscle endomyseum where only p75^{NTR} protein is present (Fig. 8B, arrow and Fig. 8C). p75^{NTR} expression in spindles is not regulated by Egr1 since Egr1 was not expressed in spindle stretch receptors or skeletal muscle (Fig. 8D). Thus, spindle stretch receptors appear to be an excellent model system to examine whether Egr3 modulates p75^{NTR} expression *in vivo*. To examine whether Egr3 may modulate p75^{NTR} expression, the immunofluorescence intensity of p75^{NTR} protein was measured in WT and Egr3-deficient spindles which were identified in skeletal muscle by their parvalbumin (Pv)-positive sensory axon innervation (Fig. 9A, B, D, E, arrowheads). In E16.5 Egr3-deficient spindles, p75^{NTR} protein was significantly reduced by 58% (Fig. 9C) and by 69% in P0 spindles (Fig. 9F) relative to WT spindles. These results clearly demonstrated that Egr3 directly regulates p75^{NTR} expression during muscle spindle development,

and that $p75^{NTR}$ may represent one of many target genes regulated by Egr3 to facilitate muscle spindle morphogenesis.

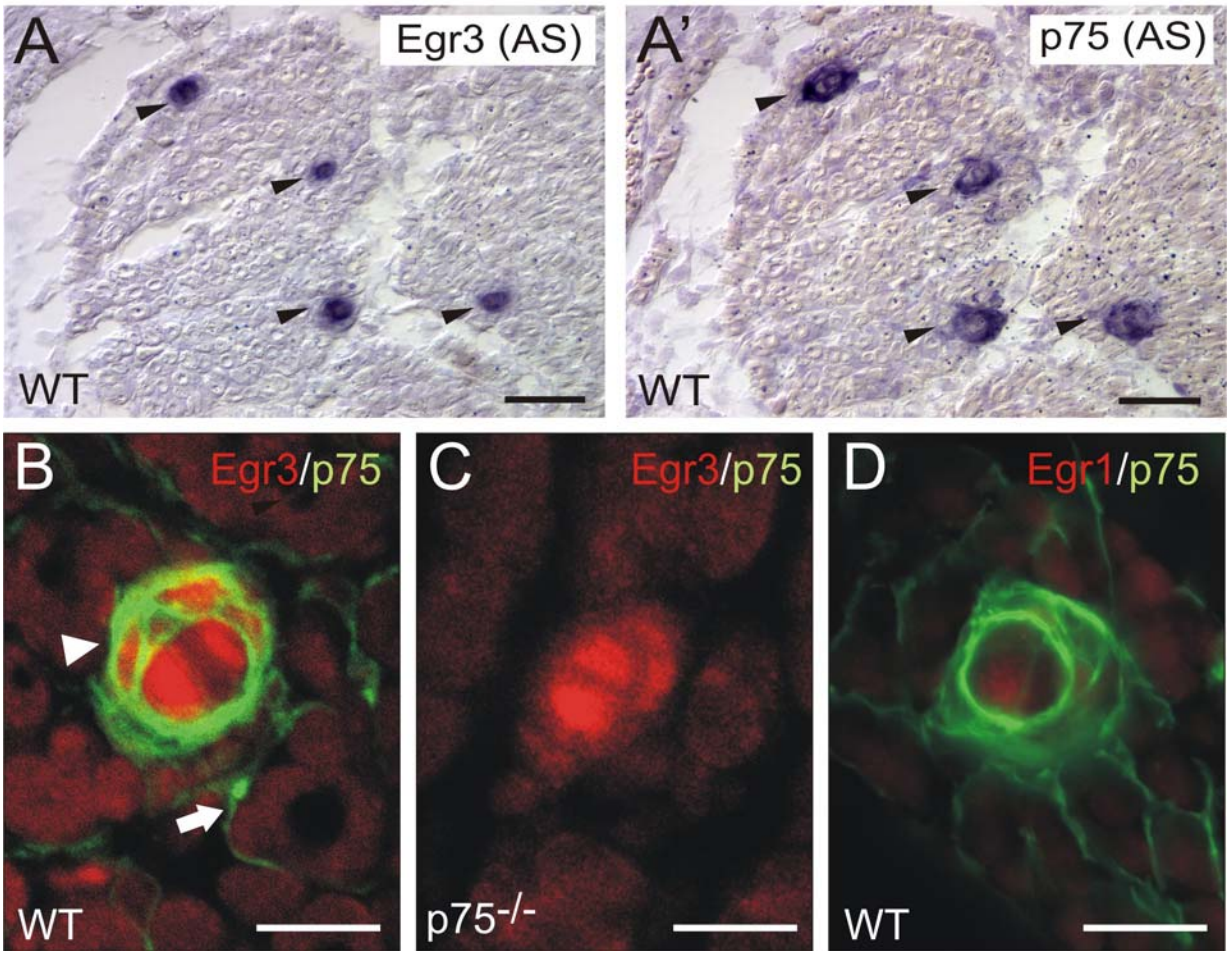

Figure 8. Egr3 expression is colocalized with $p75^{NTR}$ expression in muscle spindles.

(A-A') *In situ* hybridization on adjacent wild type (WT) newborn skeletal muscle sections demonstrates strong (A) Egr3 and (A') $p75^{NTR}$ expression specifically in muscle spindles (arrowheads).

(B) Egr3 (red) and p75^{NTR} (green) proteins are colocalized in intrafusal muscle fibers and spindle capsule cells of spindle stretch receptors (arrowhead), but only p75^{NTR} protein is present in the muscle endomyseum (arrow).

(C) The p75^{NTR} antibody is highly specific as there is complete loss of protein staining in spindles and the muscle endomyseum in p75^{NTR}-deficient skeletal muscle.

(D) Egr1 is not expressed by muscle spindles, consistent with the fact that Egr3, but not Egr1, is essential for regulating gene expression necessary for normal spindle stretch receptor morphogenesis. (Scale bar = 20μm)

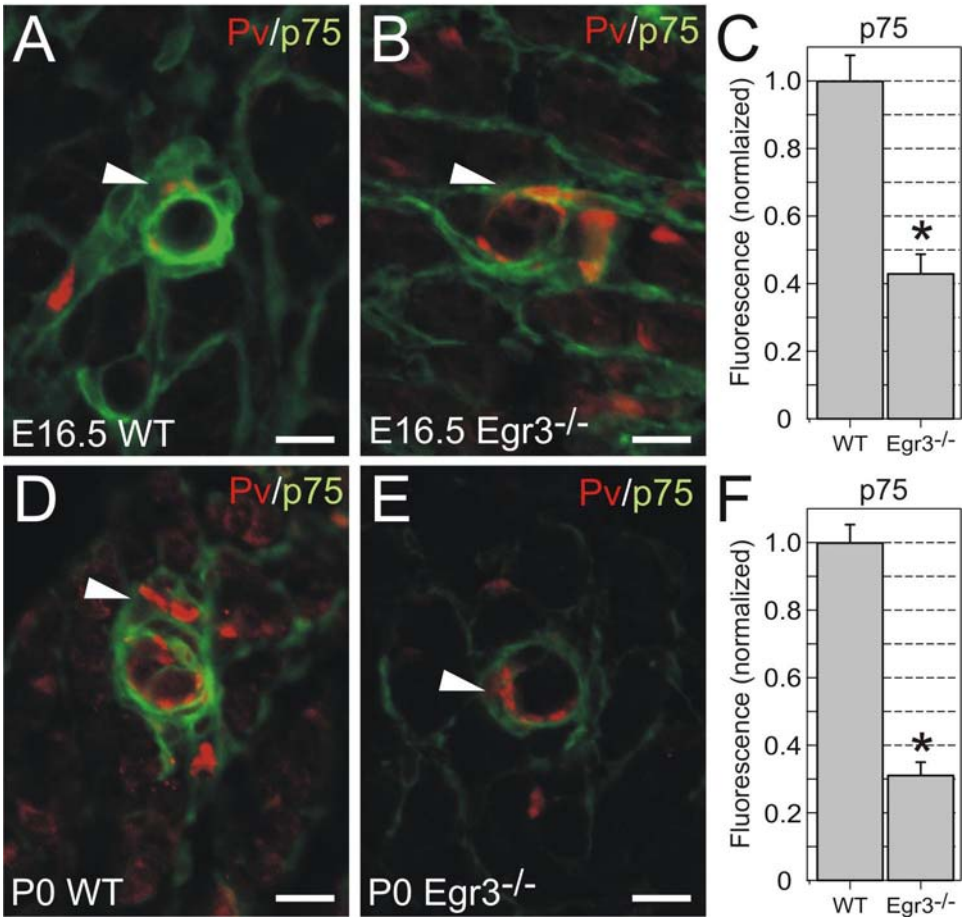

Figure 9. $p75^{NTR}$ expression is deregulated in Egr3-deficient muscle spindle stretch receptors.

(A-B, D-E) Immunohistochemistry on skeletal muscles. In (A) E16.5 wild type (WT) and (D) newborn skeletal muscle, $p75^{NTR}$ protein (green) is present in spindle stretch receptors (arrowhead) and muscle endomyseum. In Egr3-deficient (B) E16.5 and (E) newborn skeletal muscle, $p75^{NTR}$ protein is significantly decreased in spindle stretch receptors but not in muscle endomyseum.

(C, F) Comparative fluorescence analysis shows that $p75^{NTR}$ protein is reduced by 58% at E16.5 and by 69% at P0 in Egr3-deficient spindles relative to wild type. Parvalbumin (Pv) immunohistochemistry was used to localize the Ia-sensory axons that innervate the spindle stretch receptors (red). (Results represent mean ± standard deviation of fluorescence intensity measurements from 8-10 spindles per genotype per age; * = $p < 0.05$; Student's t test compared to wild type; scale bar = 20 μm).

3.5 Egr1 and Egr3 exhibit functional redundancy in modulating p75^{NTR} expression in sciatic nerves

Egr1, Egr3 and p75^{NTR} are all expressed in rat iSCs grown in serum-containing media and enforced Egr1 or Egr3 expression can upregulate p75^{NTR} expression *in vitro*. Since p75^{NTR} has an essential role in regulating peripheral axon myelin thickness (Cosgaya et al., 2002), and since Egr1 has been previously shown to regulate p75^{NTR} expression in Schwann cells *in vitro* (Nikam et al., 1995), we examined whether Egr1 and/or Egr3 could regulate p75^{NTR} expression in Schwann cells *in vivo*. Egr1 (Fig. 10A, arrowheads) and Egr3 protein (Fig. 10B, arrowheads) were present in a subpopulation of Schwann cells in WT mouse sciatic nerves that also contained p75^{NTR} protein. The extent to which Egr1 and Egr3 were co-localized in particular Schwann cells that expressed p75^{NTR} could not be determined since antibodies for Egr1 and Egr3 generated in different species were not available. Nevertheless, both Egr1 and Egr3 were required for normal expression of p75^{NTR} since p75^{NTR} expression in Egr1$^{-/-}$ and Egr3$^{-/-}$ sciatic nerves was similar to wild type, but in Egr1/3 DKO nerves, it was significantly decreased by 82.5% (Fig. 10C). Although Egr1 and Egr3 appeared to complement each other to maintain WT levels of p75^{NTR} expression in sciatic nerves, this was not due to a compensatory upregulation by either Egr1 or Egr3 expression (Fig. 10D). Accordingly, p75^{NTR} protein was also decreased by 42% in Egr1/3 DKO sciatic nerves relative to WT (Fig. 10E, F) and importantly, the loss of Egr1 and Egr3 had no effect on Egr2 protein, a closely related Egr protein that is critical for regulating myelin associated genes required for normal peripheral nerve myelination (Fig. 10E) (Topilko et al., 1994).

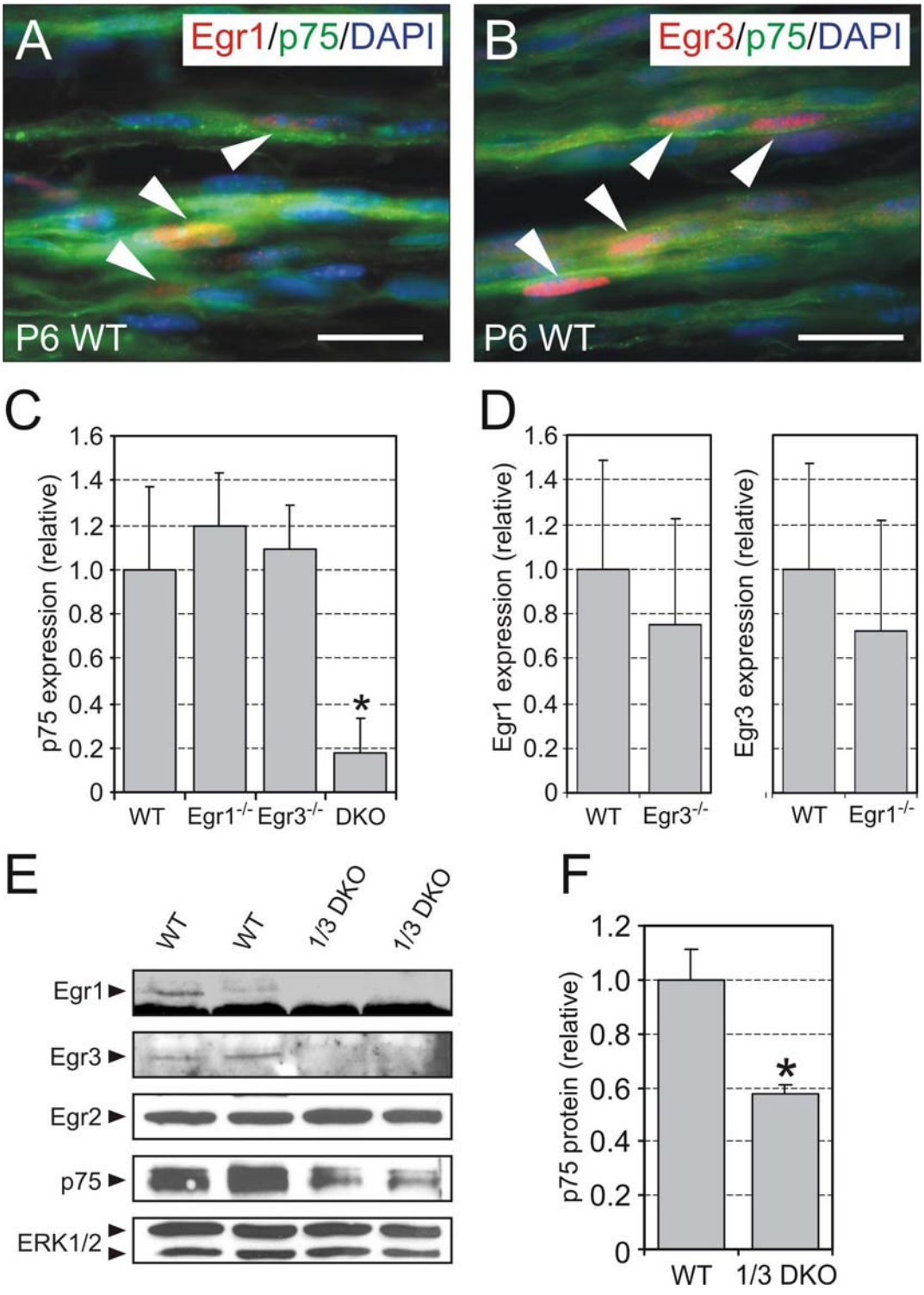
A
Egr1/p75/DAPI
P6 WT
B
Egr3/p75/DAPI
P6 WT
C
p75 expression (relative)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
*
WT
Egr1-/-
Egr3-/-
DKO
D
Egr1 expression (relative)
WT
Egr3-/-
Egr3 expression (relative)
WT
Egr1-/-
E
WT
WT
1/3 DKO
1/3 DKO
Egr1
Egr3
Egr2
p75
ERK1/2
F
p75 protein (relative)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
WT
1/3 DKO

Figure 10. Functional compensation of Egr1 and Egr3 in regulating $p75^{NTR}$ expression in sciatic nerves *in vivo.*

(A, B) Egr1 and Egr3 are co-expressed in many $p75^{NTR}$–expressing Schwann cells in P6 mouse sciatic nerves (Egr1/Egr3 (red), $p75^{NTR}$ (green), DAPI nuclear stain (blue)).

(C) qPCR analysis of $p75^{NTR}$ expression in P21 mouse sciatic nerve demonstrates that its expression is reduced by 82.5% in Egr1/3 DKO nerves compared to WT, $Egr1^{-/-}$ or $Egr3^{-/-}$ nerves. $p75^{NTR}$ expression is not altered in Egr1 or Egr3 single knockout sciatic nerves compared to WT.

(D) Although Egr1 and Egr3 complement each other to regulate $p75^{NTR}$ expression, there is no evidence of either Egr1 upregulation in $Egr3^{-/-}$ nerves or Egr3 upregulation in $Egr1^{-/-}$ nerves. (qPCR results from C and D represent mean ± standard deviation of GAPDH normalized $p75^{NTR}$ expression compared to WT levels from N=12 WT, N=5 $Egr1^{-/-}$, N=7 $Egr3^{-/-}$, and N=5 Egr 1/3 DKO sciatic nerves; * = $p < 0.05$; Student's t test compared to the level of $p75^{NTR}$ expression in WT nerves)

(E) $p75^{NTR}$ protein deregulation is confirmed in 1/3DKO mouse sciatic nerves compared to wild type. Moreover, in the absence of both Egr1 and Egr3, there is no change in the level of Egr2 protein, a closely related transcriptional regulator known to be involved in peripheral nerve myelination.

(F) Densitometry analysis confirms a 42% decrease in $p75^{NTR}$ protein levels in 1/3 DKO sciatic nerves compared to WT. (Results represent mean ± standard deviation of $p75^{NTR}$ protein compared between WT (N=4) and 1/3 DKO (N=4) sciatic nerves; * = $p < 0.05$; Student's t test; scale bar = 20 μm).

3.6 Degregulation of $p75^{NTR}$ expression correlates with peripheral myelination defects in 1/3 DKO mice

Peripheral myelin sheath is a specialized membrane produced by Schwann cells that surrounds myelinated axons and is required for normal axon development and rapid saltatory conduction of action potentials. $p75^{NTR}$ is essential to establish normal myelin sheath thickness around axons during development since germline $p75^{NTR}$-deficient mice have markedly thin (but not absent) peripheral axon myelin (Cosgaya et al., 2002). Similarly, $p75^{NTR}$ is also necessary to achieve normal myelin thickness after nerve injury and axon regeneration (Song et al., 2006). These results suggest that if decreased $p75^{NTR}$ expression in Egr1/3 DKO sciatic nerves is physiologically relevant, peripheral axon myelin thickness should be decreased. Indeed, compared to axons in WT sciatic nerves (Fig. 11A), axons in Egr1/3 DKO sciatic nerves appeared to have generally thinner myelin sheaths (Fig. 11B). Since myelin thickness positively correlates with increasing axon diameter (Gillespie and Stein, 1983), we first examined whether there was any significant difference in myelinated axon diameter between WT and Egr1/3 DKO sciatic nerves. Morphometric analysis of axon diameter showed no significant differences in the axon diameter-frequency distribution between WT and Egr1/3 DKO sciatic nerves (Fig. 11C). By contrast, there was a significant left shift in the myelin thickness-frequency distribution of Egr1/3 DKO myelinated axons, indicating that there was a significant decrease in myelin thickness when axons of similar diameter were compared (Fig. 11D). These results were confirmed by plotting the axon g-ratio (the ratio of axon diameter to fiber diameter, Fig. 11E, inset) as a function of axon diameter. The results showed a significant increase in g-ratio (thinner axon associated myelin) across all axon diameters as indicated by the upward shift of the regression line in Egr1/3 DKO sciatic nerves (Fig. 11E, solid line) relative to WT sciatic nerves

(Fig. 11E, dashed line). These results are consistent with decreased $p75^{NTR}$ expression in Egr1/3 DKO nerves that would be expected to lead to decreased myelin thickness in the absence of significant axon atrophy (Cosgaya et al., 2002).

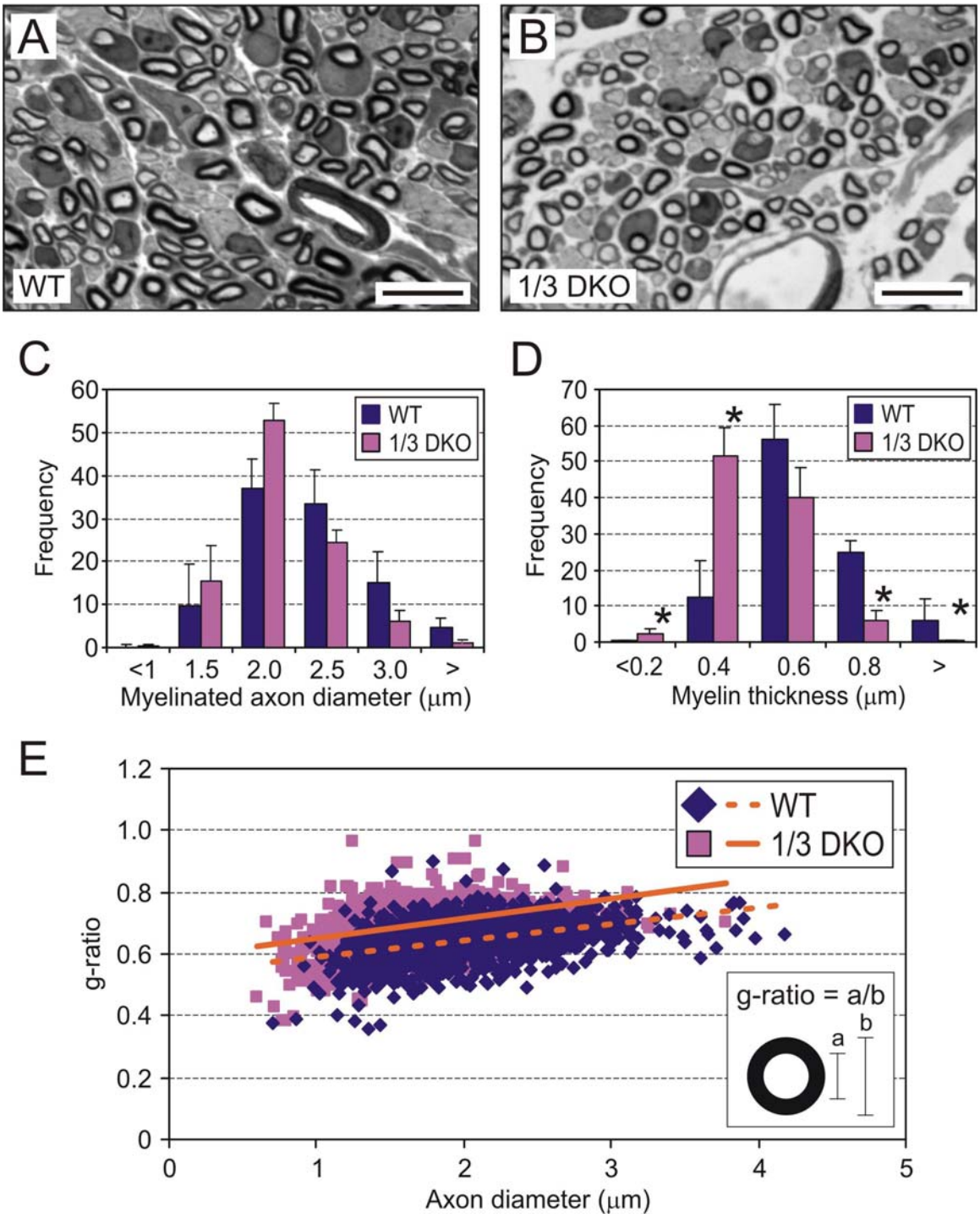
A
WT
B
1/3 DKO
C
Frequency
WT
1/3 DKO
<1
1.5
2.0
2.5
3.0
>
Myelinated axon diameter (μm)
D
Frequency
WT
1/3 DKO
<0.2
0.4
0.6
0.8
>
Myelin thickness (μm)
E
g-ratio
WT
1/3 DKO
g-ratio = a/b
a
b
Axon diameter (μm)

Figure 11. Diminished peripheral nerve myelination in Egr1/3 DKO mice.

Compared to (A) WT axons, (B) 1/3 DKO peripheral axons have thinner myelin sheaths (P6 sciatic nerve shown). (C) While axon diameter is not significantly different between WT and 1/3 DKO sciatic nerves (D) there is a significant decease in myelin thickness. (E) Morphometric analysis shows a quantitative decrease in myelin thickness (increased g-ratio) across all axon diameters. The regression lines of the data (dashed and solid lines) show a significant increase in axon g-ratio in 1/3DKO sciatic nerves compared to WT. (morphometry results of 1500-1800 axons analyzed from 3 animals of each genotype; * = $p < 0.05$; Student's t test; scale bar = 10 μm)

3.7 Conclusions

Egr transcripitonal regulators play important roles in regulating gene expression required for proper cellular response to extracellular cues. Here, we present both *in vitro* and *in vivo* evidence to support that the low affinity neurotrophin receptor ($p75^{NTR}$) expression is modulated by Egr transcriptional regulators. Both Egr1 and Egr3 were found to bind and transactivate the $p75^{NTR}$ promoter *in vitro* and *in vivo*, by using distinct response elements. Consistent with these results, $p75^{NTR}$ expression was greatly diminished in muscle spindles and in peripheral nerve Schwann cells in mice lacking both Egr1 and Egr3 (Egr 1/3 DKO). That $p75^{NTR}$ function is compromised in Egr 1/3 DKO mice was corroborated by abnormally thin peripheral myelin, a phenotypic characteristic of mice lacking $p75^{NTR}$. Taken together, these results demonstrate a physiologically relevant role for Egr proteins in regulating $p75^{NTR}$ gene expression and define a highly dynamic regulatory mechanism for modulating $p75^{NTR}$ expression during development and regeneration.

CHAPTER 4

Investigation of the Role of Egr Genes during Sympathetic Neuron Development *in vivo*

NGF treatment rapidly induces robust expression of Egr1 in a number of sympathetic-related cell lines, such as rat pheochromocytoma PC12 cells, and several neuroblastoma cell lines (GOTO, N2A, SH-SY-5Y/TrkA cells) (Bogenmann et al., 1998; Milbrandt, 1987; Pignatelli et al., 1999; Wernersson et al., 1998). Blocking Egr transcriptional activity by expressing the co-repressor Nab2 or a dominant negative Egr (dnEgr) molecule inhibits NGF-induced neurite outgrowth and delayed target gene expression in PC12 cells (Levkovitz et al., 2001; Qu et al., 1998). A number of putative Egr1 target genes have been identified in PC12 cells that may be involved in sympathetic neuron differentiation, such as the neuron-specific activator of Cdk5 (p35), nicotinic acetylcholine receptor α7 subunit, phenylethanolamine N-methyltransferase (PNMT), and transforming growth factor β1 (TGFβ1) (Harada et al., 2001; Kim et al., 1994; Nagavarapu et al., 2001; Tai et al., 2001).

Egr1 seems to play an important role in NGF signaling and sympathetic neuron differentiation based on *in vitro* studies. However, Egr1–deficient mice have no apparent sympathetic defects (Lee et al., 1996). Interestingly, our previous work showed that Egr3-deficient mice have ptosis, the characteristic phenotype of sympathetic nervous system defects shown in the $NGF^{-/-}$, $NT\text{-}3^{-/-}$ and $TrkA^{-/-}$ mice (Crowley et al., 1994; Ernfors et al., 1994b; Smeyne et al., 1994). Egr1/3 DKO mice have a more severe phenotype: they are much smaller than their wild type littermates, have delayed eye opening with severe ptosis, and die before weaning age. These phenotypic observations suggest that Egr1 and Egr3 may have redundant roles in NGF signaling and sympathetic neuron development. Therefore, I proposed to investigate the role of Egr genes in sympathetic nervous system development by generating new

genetically modified mice models. Two transgenic mice and one knock-in mouse were generated for this purpose.

4.1 Characterization of NGF-mediated Egr gene activation in differentiating SH-SY5Y-TrkA human neuroblastoma cells

Egr1 has been the prototype of Egr transcription factors to be examined in many sympathetic-related cell lines after NGF treatment. Less is known about the status of other Egr proteins in NGF signaling and sympathetic neuron differentiation. Therefore, I first examined the expression profile of all Egr proteins in human SH-SY5Y/TrkA neuroblastoma cells in response to NGF. Neuroblastoma cells resemble immature sympathetic neuroblasts or adrenal chromaffin cells arrested at different differentiation stages. TrkA, the preferred NGF receptor, is often mutated in neuroblastoma cells, and one study showed that introducing a wild type TrkA into SH-SY5Y human neuroblastoma cells restores their responsiveness to NGF (Lavenius et al., 1995).

Expression of Egr proteins was examined by Western blot in NGF-treated SH-SY5Y/TrkA cells. The expression of Egr1 proteins was undetectable in unstimulated SH-SY5Y/TrkA cells, but induced after 30-minute NGF treatment (Fig. 12A). Egr1 protein level peaked at two hours, and started to decrease four hours after NGF stimulation (Fig. 12A). Egr3 proteins showed basal expression in unstimulated SH-SY5Y/TrkA cells (Fig. 12A). The expression level of Egr3 started to increase around one hour, peaked at two hours, then gradually decreased to basal level eight hours after NGF-stimulation (Fig. 12A). No Egr2 protein was detected in SH-SY5Y/TrkA cells before or after NGF treatment (data not shown). The

expression of Egr4 protein in these cells is unknown due to the unavailability of human Egr4 antibody.

The signal transduction pathways leading to Egr activation have been partially characterized in PC12 cells. Since Egr expression is mainly activated by the MAPK pathway after NGF stimulation in PC12 cells (Harada et al., 2001), we examined whether NGF-mediated Egr activation is also coupled to the MAPK pathway in SH-SY5Y/TrkA cells. Upon NGF stimulation, extracellular signal-regulated kinase (ERK) was rapidly phosphorylated within five minutes, and stayed phosphorylated for at least 1 day in SH-SY5Y/TrkA cells (Fig. 12A). Two kinds of MEK inhibitors, PD98059 and U0126, were used to block ERK phosphorylation. Both inhibitors severely decreased, but U0126 at 20μM completely abrogated NGF-induced ERK phosphorylation and Egr1 and Egr3 activation, demonstrating that NGF-induced Egr activation is coupled to MAPK pathway in SH-SY5Y/TrkA cells (Fig. 12B). These results together with the phenotypical observations in Egr3-deficient mice suggest that Egr1 and Egr3 may collaborate in regulating target genes in sympathetic neurons.

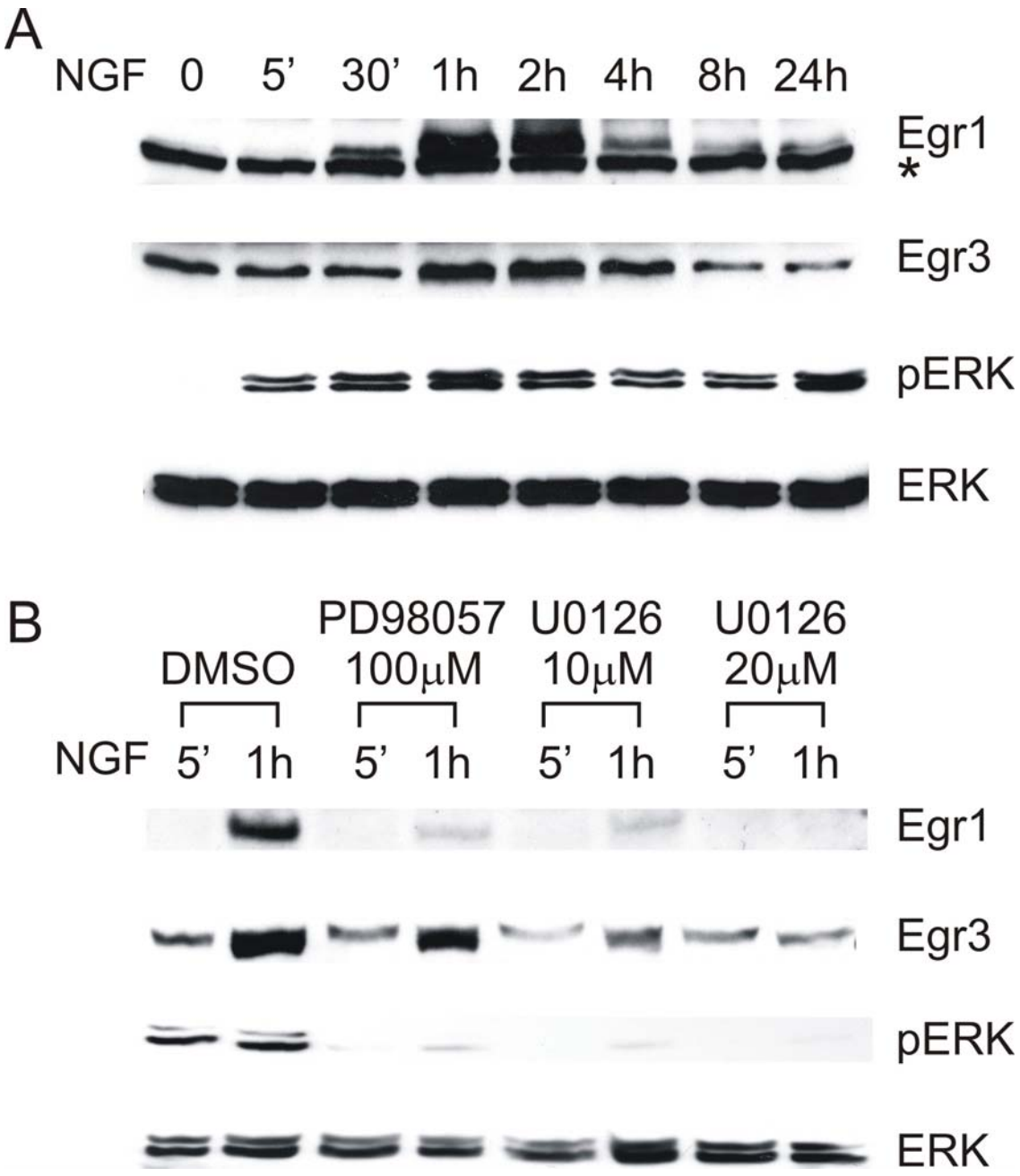

Figure 12. **NGF-mediated Egr activation is coupled to MAPK signaling pathway in human SH-SY5Y/TrkA neuroblastoma cells.**

(A) Time courses of ERK phosphorylation and Egr induction in human SH-SY5Y/TrkA neuroblastoma cells after NGF stimulation. Protein lysates were collected from SH-SY5Y/TrkA

cells stimulated with NGF for indicated time, and subjected to Western blot analysis for Egr1, Egr3 and phosphorylated ERK. ERK was phosphorylated after 5-minute NGF-stimulation, and Egr proteins were induced as immediate early genes later than ERK phosphorylation. Egr1 proteins were induced around 30 minutes, peak at two hours and greatly decreased four hours after NGF stimulation. Egr3 proteins have basal expression in these cells, the expression level increased around one-hour, peak at two hours, then gradually decreased to basal level eight hours after NGF-stimulation (* —non-specific band).

(B) MEK inhibitors abrogate NGF-induced ERK phosphorylation and Egr-induction. SH-SY5Y/TrkA cells were treated with MEK inhibitors PD98057 or U0126 or DMSO at indicated concentration before NGF-stimulation. The results of Western blots showed that both inhibitors severely decreased, but U0126 at 20μM completely abrogate NGF-induced ERK phosphorylation and Egr activation, proving that NGF-induced Egr activation is coupled to MAPK pathway.

4.2 DβH-τlacZ reporter mice

Peripheral sympathetic neurons are catecholaminergic neurons and secrete norepinephrine to activate adrenergic receptors on target tissues (Elenkov et al., 2000). Norepinephrine is synthesized from tyrosine through a series of reactions using tyrosine hydroxylase (TH), dopa decarboxylase (DCC), and dopamine β-hydroxylase (DβH) (Anderson, 1993). Therefore, DβH is often used as a characteristic marker enzyme for sympathetic neurons.

τlacZ is a fusion protein of the bovine microtubule-binding protein *tau* and *E. coli.* β-galactosidase. The expression of this fusion protein in neurons helps the transportation of β-galactosidase to axons. To visualize axon outgrowth and target tissue innervation of sympathetic neurons, τlacZ fusion protein was directed into sympathetic neurons using a human DβH promoter (Fig. 13, construct), which has been used to successfully overexpress NGF in sympathetic neurons (Hoyle et al., 1993). The first intron of rat insulin II (rInsII) was placed upstream of the transgene to enhance its expression *in vivo* (Palmiter et al., 1991). The hDβH-rInsII-τlacZ-SV40pA transgene was made as described in Chapter 2, and purified for microinjection. The microinjection was performed at The Transgenic and Targeted Mutagenesis Laboratory (TTML) at Northwestern University. Five different founders were screened by whole-mount β-galactosidase enzyme histochemistry, and one reporter line with τlacZ expression in almost all the sympathetic neurons and adrenal chromaffin cells was isolated and propagated (Table 3).

Table 3. Identification of a DβH-τlacZ transgenic line with 100% of transgene expression.

Transgenic construct	Founder ID	Gender	% Tg+ of progeny	% Exp of Tg+ mice
hDβH-rInsII-τlacZ-SV40 pA	116	F	67	50
	135	F	60	90
	138	M	55	100
	142	M	60	67
	147	F	0	0

Whole-mount β-galactosidase enzyme histochemistry revealed that the fusion protein labels the sympathetic neurons in the superior cervical ganglia (SCG, Fig. 13E), entire paravertebral sympathetic chain ganglia (Fig. 13F), sympathetic axons emanating from the ganglia (Fig. 13G), and innervating target organs, such as the heart (Fig. 13H) in transgene-positive (Tg+) but not transgene-negative (Tg-) mice (Fig. 13A-D). β-galactosidase enzyme histochemistry on 20μM frozen sections revealed that the fusion protein is expressed in almost all sympathetic neurons in SCG and stellate ganglia (STG), and adrenal chromaffin cells in DβH-τlacZ+ mice (Fig. 14A-C). The fusion protein was also detected in the sympathetic axons innervating target tissues, such as eye and lacrimal gland, nasal and oral mucosa, and gastrointestinal tract (Fig. 14D-F). Using double-labeling immunofluorescence, we proved that τlacZ expression is colocalized with TH expression in sympathtic axons *in vivo* (Fig. 15A-F). Therefore, the transgene faithfully marked almost all the sympathtic neurons and their axons *in vivo*.

To characterize the sympathetic nervous system defects in Egr3-deficient mice, DβH-τlacZ reporter mice were bred with $Egr3^{-/-}$ mice to generate double mutant mice. Whole-mount β-galactosidase enzyme histochemistry on double mutant mice revealed severe sympathetic

dysautonomia in Egr3-deficient mice, including smaller sympathetic ganglia size, less sympathetic axon branches and markedly decreased sympathetic innervation to target organs, such as eye, heart, pineal gland, salivary gland and spleen (Eldredge et al., 2007). These results confirmed that Egr3 is an important transcriptional regulator of sympathetic nervous system development.

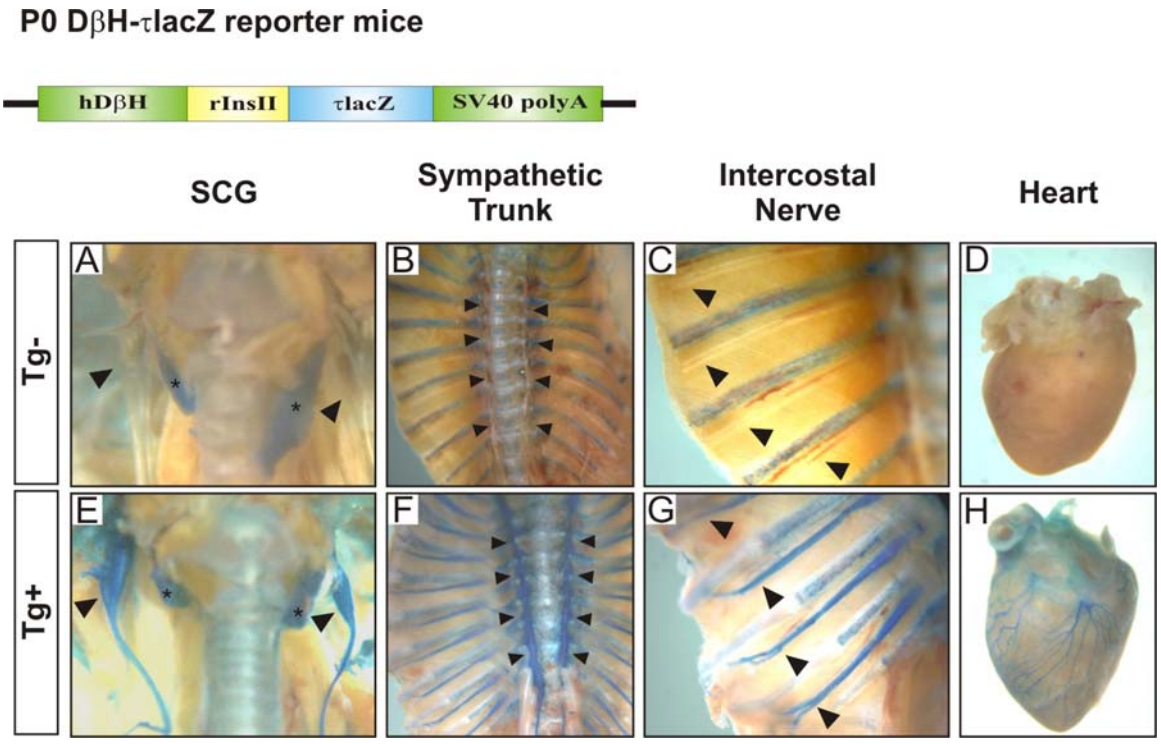

Figure 13. Generation and characterization of DβH-τlacZ transgenic reporter mice.

Expression of τlacZ fusion protein was directed to sympathetic neurons using a human dopamine β-hydroxylase (hDβH) promoter (construct, top panel). The first intron of rat insulin II was used to facilitate transgene expression *in vivo*. After screening five different founders, we isolated a

transgenic line that expresses the τlacZ fusion protein in nearly all sympathetic neurons and axons.

(A-H) Whole-mount β-galactosidase enzyme histochemistry was performed on transgene-negative (Tg-) and transgene-positive (Tg+) mice. Specific labeling of the superior cervical ganglia (SCG, E), entire paravertebral sympathetic chain (F), sympathetic axons emanating from the ganglia within the intercostal nerves (G), and terminal sympathetic axons innervating target organs, such as heart (H), was seen in Tg+ but not Tg- mice (A-D). Background staining in bones and thyroid gland (*) was observed in both Tg- and Tg+ mice.

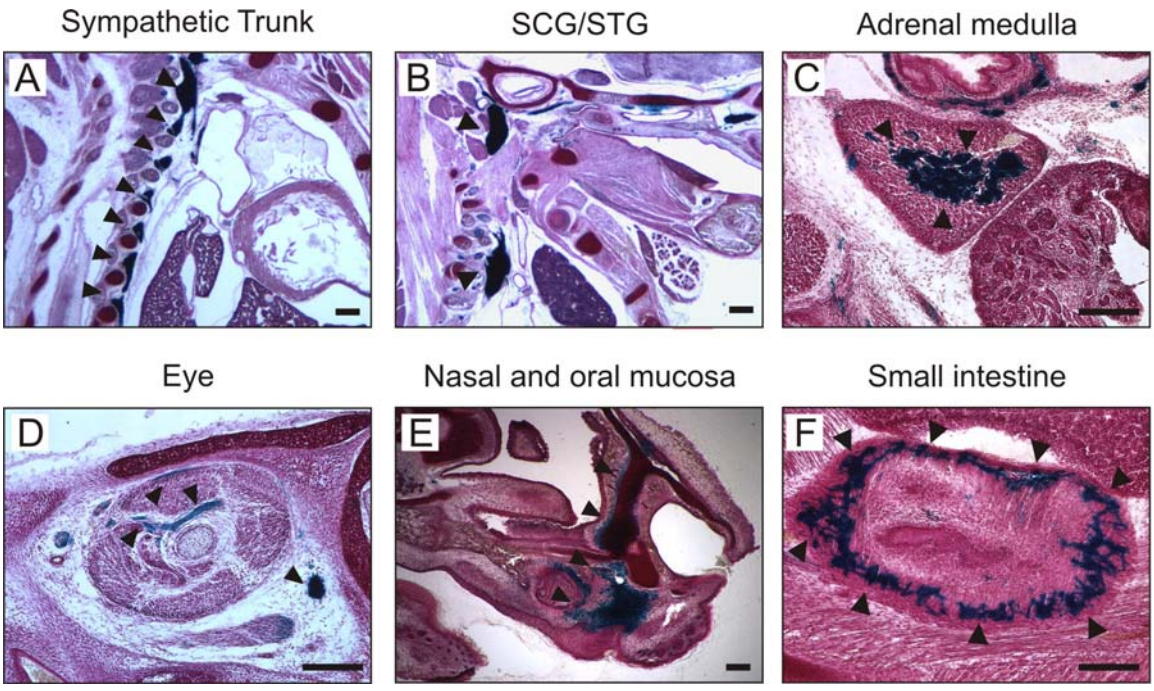

Figure 14. τlacZ fusion protein is expressed in sympathetic neurons and their axons and adrenal chromaffin cells in DβH-τlacZ transgenic reporter mice.

(A-F) β-galactosidase enzyme histochemistry on 20μM frozen sections of DβH-τlacZ+ mice revealed that the fusion protein labeled nearly all sympathetic neurons within SCG, stellate ganglia (STG) (B, arrowheads), and the entire paravertebral sympathetic chain (A, arrowheads), and nearly all the adrenal chromaffin cells (C). The τlacZ fusion protein was also identified in sympathetic axons innervating target tissues, such as eye and lacrimal gland (D), nasal and oral mucosa (E), and small intestine (F).

(Scale bar = 200μM)

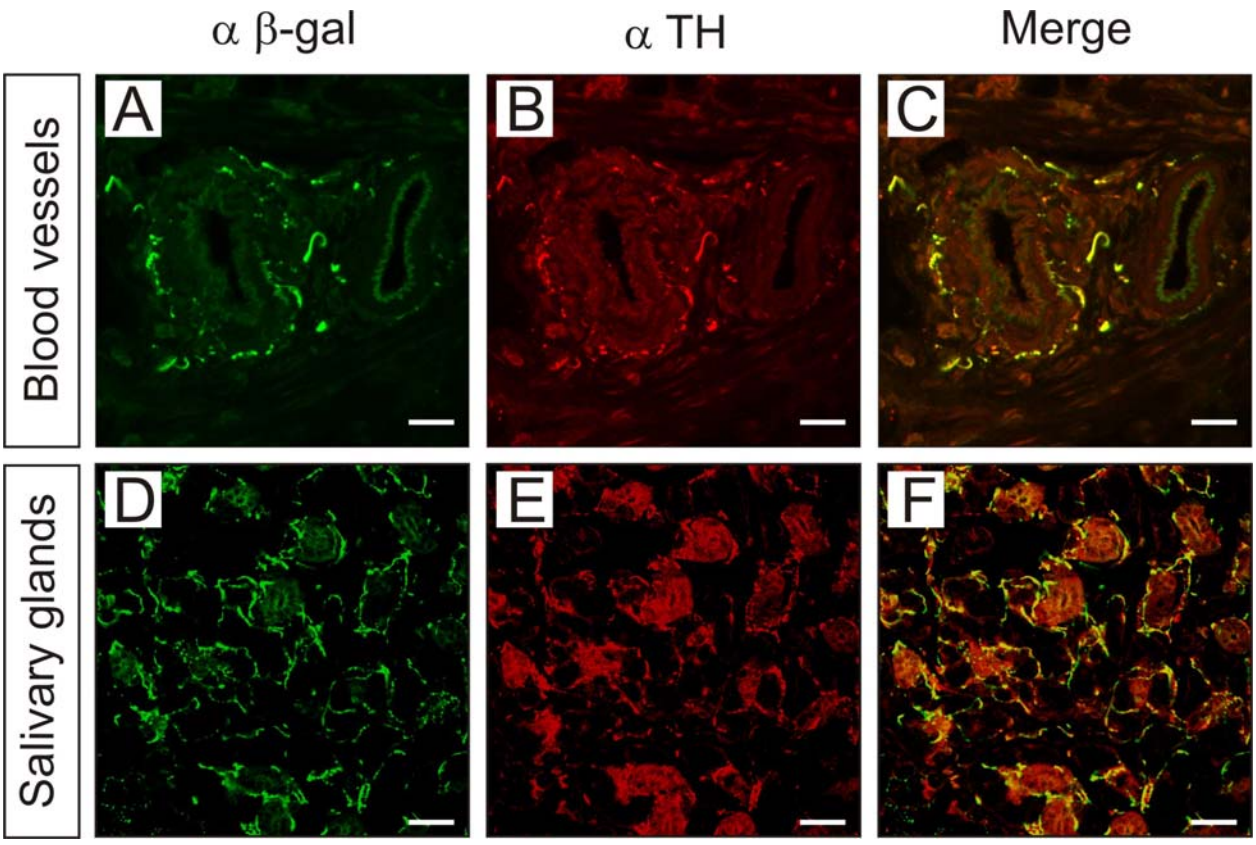

Figure 15. The τlacZ fusion protein colocalizes with tyrosine hydroxylase (TH) in sympathetic axons of DβH-τlacZ transgenic reporter mice.

Double-labeling immunofluorescence for β-galactosidase (A, D, green) and tyrosine hydroxylase (B, E, red) demonstrates colocalization of the two proteins (C, F) in sympathetic axons innervating blood vessels (A-C), and salivary glands (D-F).

(Scale bar = 50μm)

4.3 DβH-nlsCre-IRES-τlacZ transgenic mice

To specifically activate or inactivate a gene of interest in sympathetic neurons, we utilized the Cre-loxP site-directed recombination system. Nuclear-localized Cre recombinase (nlsCre) and τlacZ fusion protein were expressed in sympathetic neurons under the control of the human DβH promoter (Fig. 16, construct). Therefore, the new transgenic mice DβH-nlsCre-IRES-τlacZ express Cre recombinase in the nuclei and τlacZ fusion protein in axons of sympathetic neurons. Using this tool, we will be able to turn on/off a specific gene in sympathetic neurons and study the effect on sympathetic neuron development, axon projections and target tissue innervation.

DβH-nlsCre-IRES-τlacZ construct was generated as described in Chapter 2. The transgenic mice were made at the TTML at Northwestern University. Eleven different founders were screened by whole-mount β-galactosidase enzyme histochemistry and Cre immunohistochemistry. Three transgenic lines with Cre and τlacZ expression in most sympathetic neurons were isolated for further analysis (Table 4). The genomic integration site of DβH-nlsCre-IRES-τlacZ transgene was examined by Southern blotting on genomic DNA isolated from tails of Tg+ mice (Fig. 17A). The transgenes were integrated to a single genomic site in both line1 and line2 (Fig. 17B).

Transgenic-positive mice from line1 were further analyzed. Whole-mount β-galactosidase enzyme histochemistry confirmed the widespread expression pattern of the τlacZ fusion protein in SCG, STG, the entire paravertebral sympathetic trunk, and sympathetic axons emanating from the ganglia (Fig. 16A). Immunofluorescence showed that Cre recombinase is expressed in the nuclei of TH-positive adrenal medulla chromaffin cells (Fig. 16B). To test the

excision efficiency of Cre recombinase, we bred DβH-nlsCre-IRES-τlacZ transgenic mice with Rosa26-rtTA-IRES-EGFP knock-in mice, which conditionally express the reverse tetracycline transactivator (rtTA) and the enhanced green fluorescence protein (EGFP) from Rosa26 locus (Belteki et al., 2005). EGFP expression was evaluated in adrenal medulla of DβH-nlsCre-IRES-τlacZ+/Rosa26-rtTA-IRES-EGFP floxed/+ double transgenic mice. EGFP expression is only activated in the adrenal medulla chromaffin cells in the double transgenic mice, but not in Rosa26-rtTA-IRES-EGFP heterozygous mice alone (Fig. 16C), indicating that Cre recombinase successfully excises the floxed stop cassette placed upstream of EGFP, and activates its expression.

Table 4. Identification of three DβH-nlsCre-IRES-τlacZ transgenic lines that express Cre and τlacZ in the sympathetic nervous system.

Transgenic construct	Founder ID	Gender	% Tg+ of progeny	% Exp of Tg+ mice	Line
hDβH-rInsII-nlsCre-IRES-τlacZ	1068	F	57%	50%	
	1069	F	29%	100%	line2
	1073	M	20%	0%	
	1074	M	83%	80%	line1
	1075	M	100%	0%	
	232	F	17%	0%	
	233	F	0%	0%	
	241	M	44%	50%	
	240	M	64%	100%	line3
	1253	F	30%	0%	
	1267	F	40%	0%	

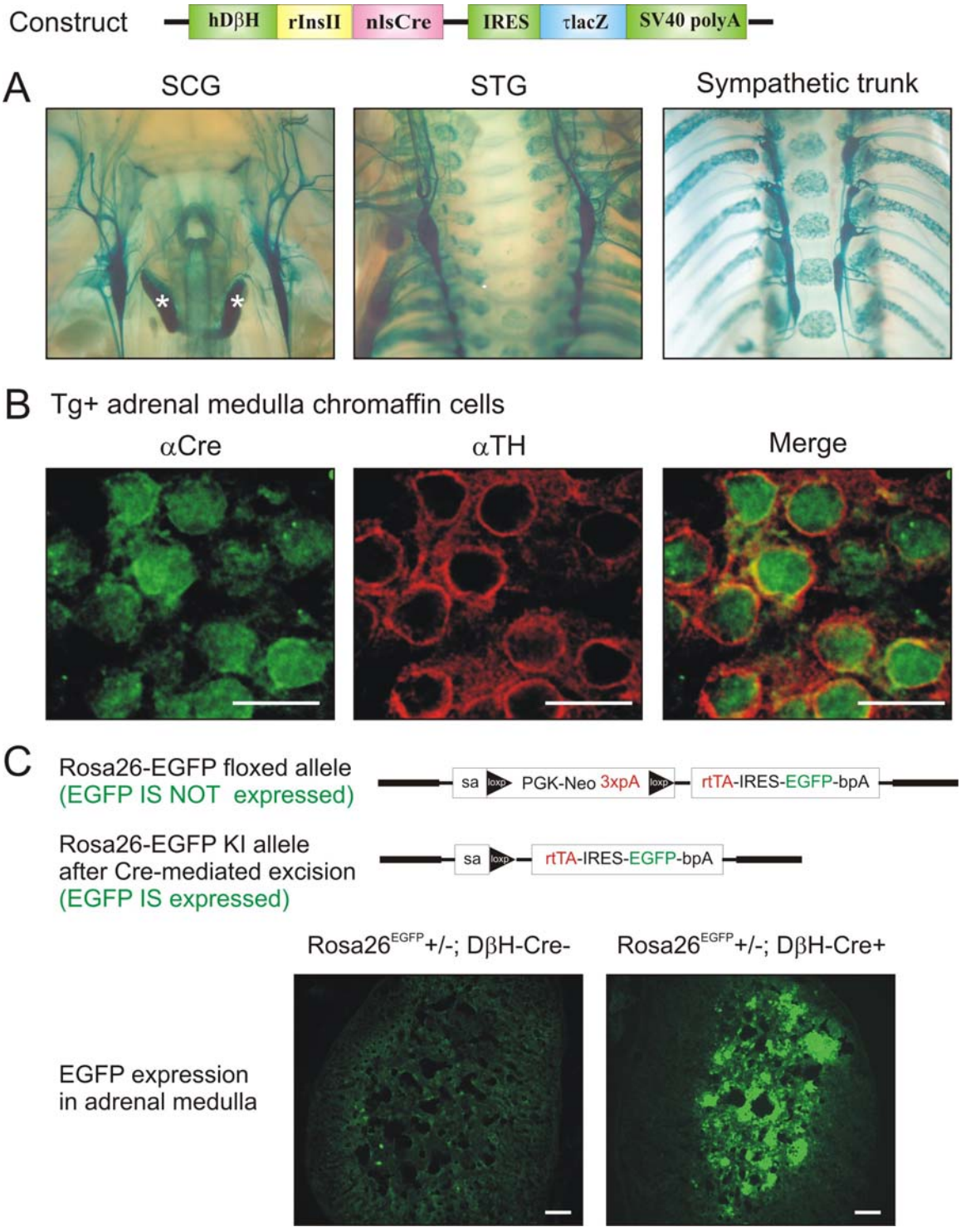

Figure 16. Generation and characterization of DβH-nlsCre-IRES-τlacZ transgenic mice.

In DβH-nlsCre-IRES-τlacZ transgenic mice, expression of nuclear localized Cre recombinase (nlsCre) and the τlacZ fusion protein were directed to sympathetic neurons using the human DβH promoter.

(A) Whole-mount β-galactosidase enzyme histochemistry confirmed the widespread expression pattern of τlacZ fusion protein in SCG, STG, sympathetic chain ganglia and axonal projections from them. Staining in bones and thyroid gland (*) was background which was also observed in Tg- mice.

(B) Cre recombinase (green) is highly expressed in the nucleus of TH-positive (red) adrenal chromaffin cells as detected by immunohistochemistry in transgenic positive mice. (Scale bar = 10μm)

(C) DβH-nlsCre-IRES-τlacZ transgenic mice were crossed with Rosa-rtTA-IRES-EGFP mice to test the function of Cre recombinase. Cre-mediated recombination activates EGFP expression in adrenal medulla chromaffin cells in $Rosa^{EGFP+/-}$; DβH-Cre+ but not $Rosa^{EGFP+/-}$; DβH-Cre- mice. (Scale bar = 50μm)

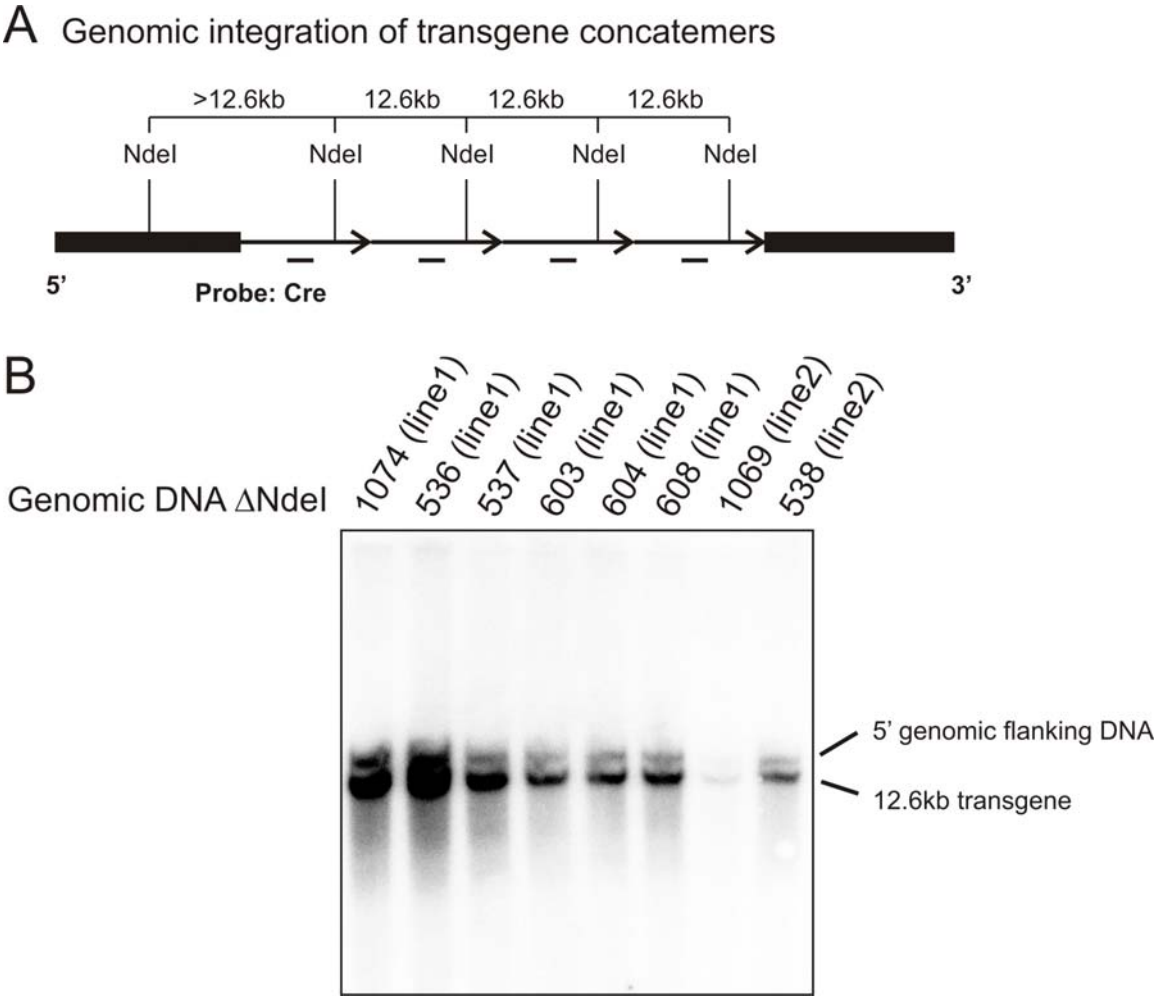

Figure 17. Characterization of the genomic integration site of DβH-nlsCre-IRES-τlacZ transgenic lines.

(A) Diagram illustrating that multiple copies of 12.6kb transgene integrate into the mouse genome as concatemers. Each copy of transgene has a single NdeI site. NdeI digestion will excise transgenes and the 5' and 3' genomic sequences flanking the integration site. A probe specific for Cre sequence will hybridize with all inserted transgenes and the 5' genomic flanking DNA.

(B) Southern blot on NdeI-digested tail genomic DNA isolated from DβH-nlsCre-IRES-τlacZ positive mice revealed a single 5' genomic flanking DNA fragment, indicating single genomic integration site of transgenes in line1 and line2.

4.4 Conditional expression of the dnEgr molecule in sympathetic neurons

Egr transcription factors appear to have redundant functions during sympathetic nervous system development. Egr1/3 DKO mice appear to have more severe sympathetic defects than $Egr1^{-/-}$ or $Egr3^{-/-}$ mice alone. It has been difficult to study the role of Egr transcription factors in sympathetic neuron development due to fertility-associated phenotypes of Egr-deficient mice. Therefore, we took another approach to study the role of Egr genes in sympathetic neuron development by directing the expression of a dominant negative Egr (dnEgr) molecule to sympathetic neurons. The dnEgr molecule is made by N-terminal truncation of a wild type Egr, so it represents the DNA-binding domain of the native Egr protein. The dnEgr molecule has been shown to compete with all endogenous Egr proteins for DNA-binding and block Egr-mediated transcription and NGF-stimulated neurite outgrowth and differentiation of PC12 cells (Levkovitz et al., 2001). A similar dnEgr molecule was made by N-terminal truncation of Egr3 and tested for its inhibitory function by luciferase reporter assay. An ERE-luciferase reporter plasmid was cotransfected into PC12 cells with CMV-Egr1, 2, 3, 4, and/or CMV-dnEgr plasmids. As expected, all four Egr proteins activate luciferase transcription through binding to ERE, and Egr-mediated transcription was inhibited by dnEgr in a dose-dependent manner (Fig. 18).

Next, we decided to express the dnEgr molecule and τlacZ fusion protein in sympathetic neurons under the control of the human DβH promoter. However, no viable DβH-dnEgr-IRES-τlacZ-positive founder mice could be identified at P0. Therefore, transient transgenic analysis was performed on embryos at different gestational stages (Table 5, Fig. 19). Whole-mount β-gal enzyme histochemistry on an E15.5 DβH-τlacZ+ reporter embryo revealed that sympathetic

axons already emanated from sympathetic chain ganglia, extending along the intercostal nerve at this gestation stage (Fig. 20B). Expression of the dnEgr molecule blocked the axon outgrowth of sympathetic neurons in an age-matched DβH-dnEgr-IRES-τlacZ+ embryo (Fig. 20C). These results implied that Egr genes may be involved in sympathetic neuron axon extension and branches, as well as target tissue innervation, consistent with our findings in Egr3-deficient mice (Eldredge et al., 2007).

Table 5. Transient transgenic analysis of DβH-HA-dnEgr-IRES-τlacZ mice.

Transgenic construct	Gestation stage	Total embryos	Tg+ embryos	Phenotype
hDβH-rInsII-HA-dnEgr-IRES-τlacZ-SV40 pA	E15.5	14	1	Decreased sympathetic axon outgrowth (Fig. 20 C)
	E17.5	5	0	N/A
	E19.5	11	1	Died in uterus, craniofacial abnormality (Fig.19 D)

To obtain enough mice with a similar genetic background to continue this study, we generated knock-in mice to conditionally express dnEgr molecule *in vivo*. We utilized the Rosa26 locus, which ubiquitously expresses non-coding transcripts with unknown function, to conditionally drive dnEgr *in vivo*. The Rosa 26 transcripts are dispensable since Rosa26-deficient mice are behaviorally normal and fertile (Zambrowicz et al., 1997). We used the Rosa26 targeting system according to Srinivas et al to target the conditional dnEgr-expressing cassette to the Rosa26 locus as described in Chapter 2 (Srinivas et al., 2001). Briefly, an HA-tagged dnEgr molecule was cloned downstream of a floxed PGK-Neo-3xpolyA cassette in pBigT plasmid.

The dnEgr-containing insert was released by AscI/ PacI double digestion and cloned into pROSA26-PA vector. The targeting construct pROSA26-HA-dnEgr was linearized using XhoI and electroporated into mouse embryonic stem (ES) cells. The conditional dnEgr expression cassette was integrated into the mouse genome when homologous recombination occurred (Fig. 21). HA-dnEgr is not transcribed from the RdnE floxed allele due to the strong polyA signal placed upstream. However, after breeding with Cre recombinase-expressing mice, the PGK-Neo-3xpolyA cassette is excised via Cre-mediated recombination between the two flanking loxP sites, allowing dnEgr expression from the RdnE knock-in (KI) allele (Fig. 21).

The RdnE targeting construct was electroporated into ES cells, and the positive clones were selected using neomycin-resistance. 31 out of 227 G418-resistant clones (13.6%) were correctly targeted as determined by PCR analysis (Fig. 22A). Five PCR-positive ES clones were randomly selected and examined for correct targeting by Southern blotting. All five PCR-positive clones were confirmed to have one floxed RdnE allele and one wild-type allele (Fig. 22B). Two heterozygous ES clones—3D5 and 3B5 were selected to generate chimeric mice.

RdnE mice were made at the TTML, and they are viable and fertile. To test the floxed RdnE allele, Mlc^{Cre} knock-in mice were used to specifically turn on HA-dnEgr in skeletal muscle fibers. The RdnE KI allele was examined by PCR in a variety of organs in $RdnE^{flx/+}/MlcCre^{KI/+}$ double mutant mice, and was detected only in skeletal muscle but not in other organs, indicating that Cre recombinase successfully excised the floxed Neo-3xpolyA cassette from the targeted Rosa26 locus in skeletal muscles (Fig. 23A). The HA-dnEgr expression was examined by RT-PCR in skeletal muscles from the littermates, and HA-dnEgr was only detected in $RdnE^{flx/+}/MlcCre^{KI/+}$ but not $RdnE^{+/+}/MlcCre^{KI/+}$ muscles (Fig. 23B), demonstrating that Cre-mediated recombination was able to activate HA-dnEgr transcription in skeletal muscles.

To restrict the dnEgr expression in sympathetic neurons, we will use DβH-nlsCre-IRES-τlacZ transgenic mice described earlier in this Chapter to activate dnEgr expression in the nuclei of sympathetic neurons. The progeny will be analyzed using whole-mount β-galactosidase enzyme histochemistry to examine the sympathetic ganglia size, axon projections from the ganglia, and target organ innervation. More detailed experimental designs and expected results are discussed in Chapter 5.

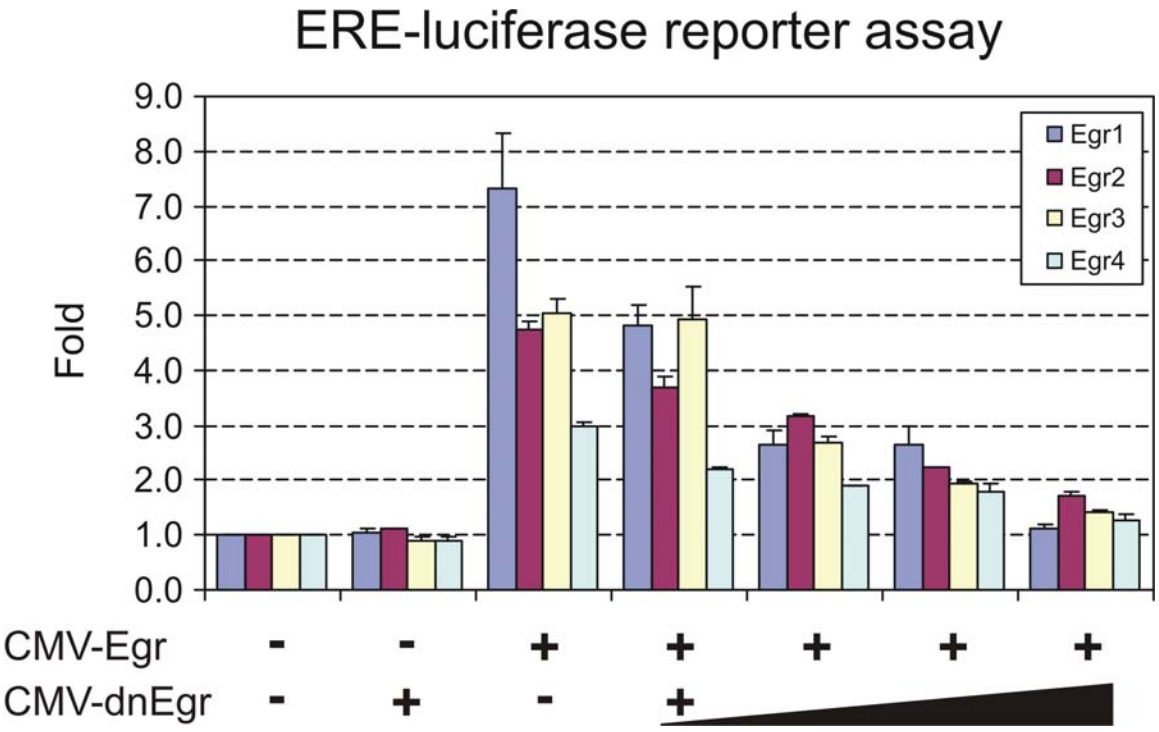

Figure 18. The dnEgr molecule inhibits Egr-mediated transcription in a dose-dependent manner.

The 4xERE-luciferase reporter plasmid was cotransfected into PC12 cells with CMV-Egr1, CMV-Egr2, CMV-Egr3, CMV-Egr4, and/or CMV-dnEgr plasmids as indicated below the graph.

As expected, all four Egr proteins activate luciferase transcription through binding to ERE. The dnEgr molecule blocks Egr-mediated transcription in a dose-dependent manner. When the mass ratio of dnEgr to Egr was increased to 8:1, nearly all Egr-mediated transcription was eliminated.

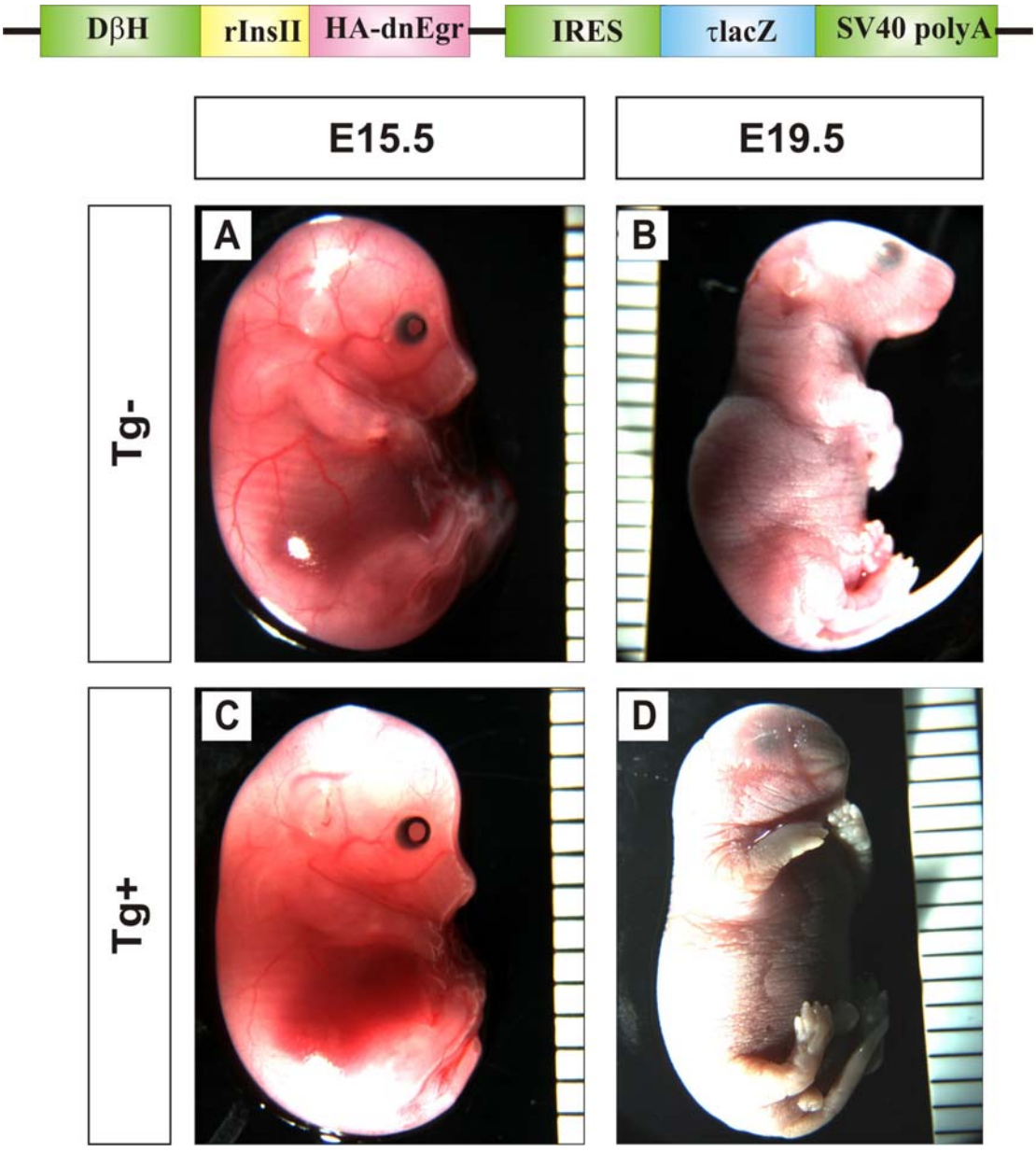

Figure 19. Transient transgenic analysis of DβH-dnEgr-IRES-τlacZ mice.

The dnEgr molecule and τlacZ fusion protein coexpressed in sympathetic neurons under the control of human DβH promoter. No viable DβH-dnEgr-IRES-τlacZ+ founder mice could be identified at P0. Therefore, transient transgenic analysis was performed at embryonic day 15.5, 17.5, and 19.5. The pictures showed the appearance of the Tg- (A, B) and Tg+ (C, D) embryos at E15.5 (A, C) and E19.5 (B, D). The Tg+ E19.5 embryo died in uterus with craniofacial abnormality (D).

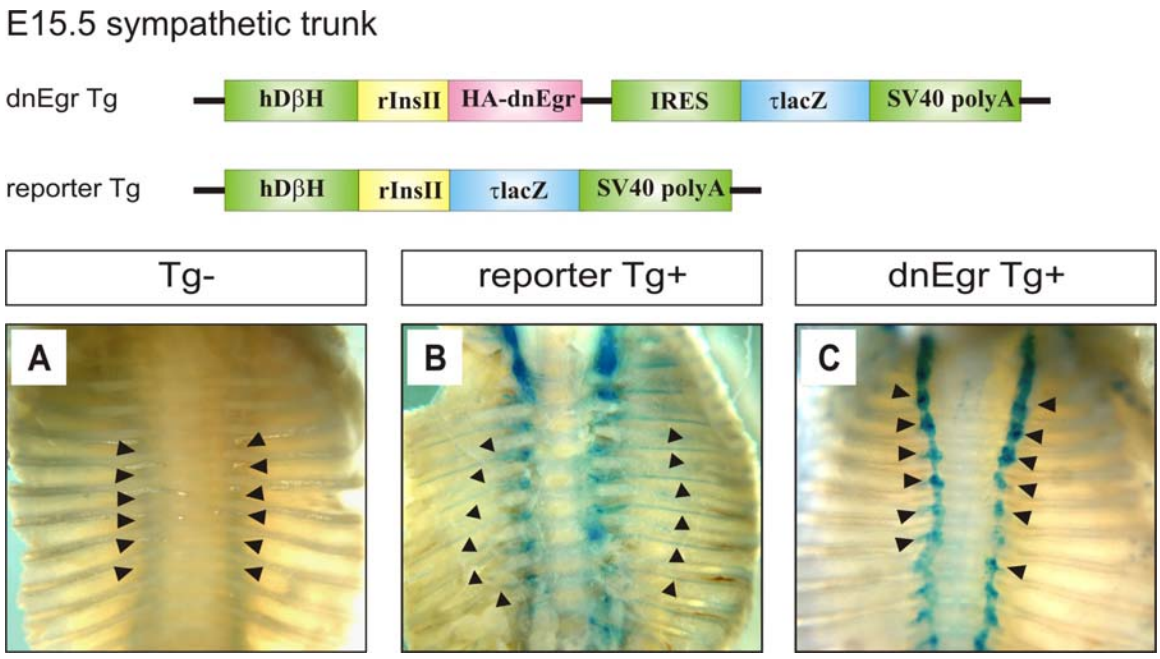

Figure 20. Expression of dnEgr molecule *in vivo* hinders axon outgrowth of sympathetic neurons.

(A-C) Whole-mount β-galactosidase enzyme histochemistry on an E15.5 reporter embryo revealed that sympathetic axons emanated from sympathetic chain ganglia, extending along the intercostal nerve in DβH-τlacZ reporter mice (B). Expression of the dnEgr molecule *in vivo*

blocked the axon outgrowth of sympathetic neurons in an age-matched dnEgr Tg+ embryo (C), suggesting that Egr genes might play a role in sympathetic neuron axon outgrowth and target tissue innervation.

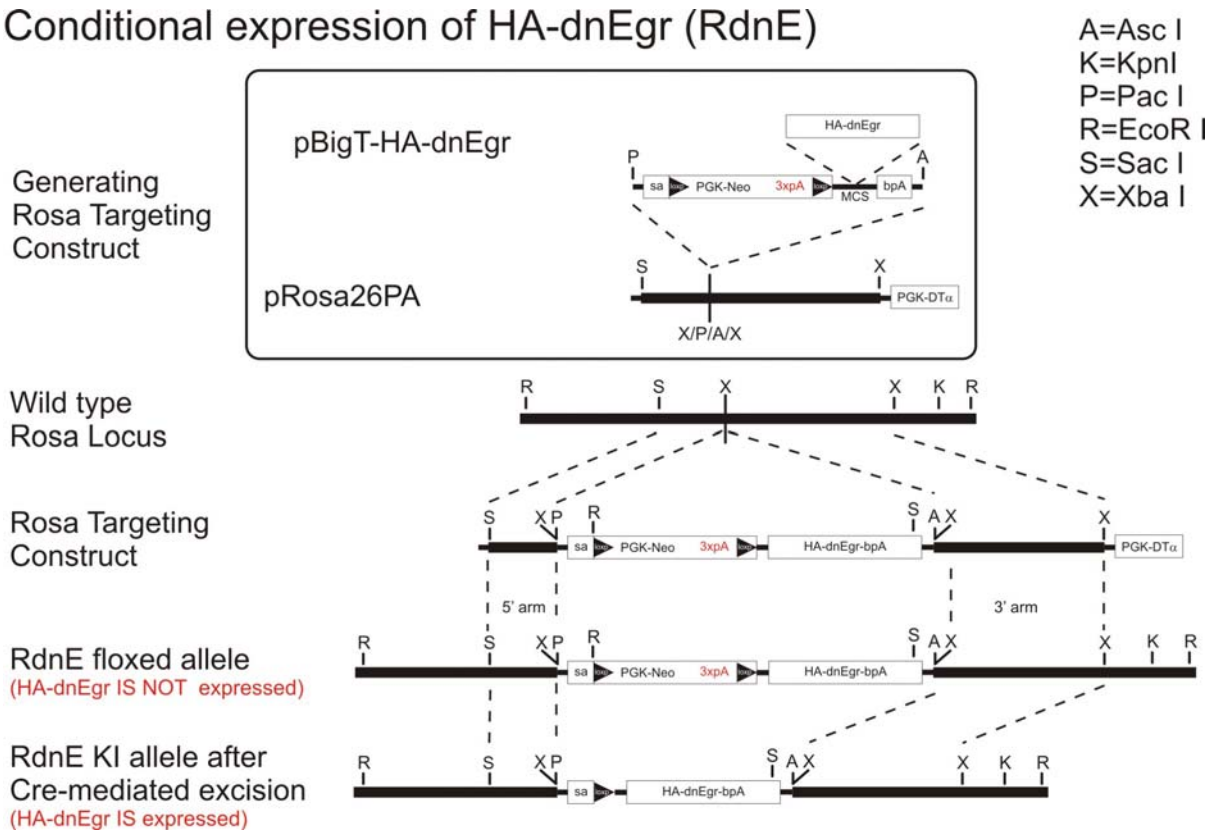

Figure 21. Conditional expression of the dnEgr molecule *in vivo*.

Briefly, the HA-tagged dnEgr molecule was cloned downstream of a floxed PGK-Neo-3xpolyA cassette in pBigT plasmid. The dnEgr-containing insert was released by AscI/ PacI double digestion and cloned into pROSA26-PA vector. The targeting construct pROSA26-HA-dnEgr was linearized using XhoI and electroporated into mouse embryonic stem (ES) cells. The conditional dnEgr expression cassette was integrated into the mouse genome when homologous

recombination occurred. HA-dnEgr is not transcribed from the RdnE floxed allele due to the strong polyA signal placed upstream. However, after breeding with Cre recombinase-expressing mice, the PGK-Neo-3xpolyA cassette is excised via Cre-mediated recombination between the two flanking loxP sites, allowing dnEgr expression from the RdnE knock-in (KI) allele.

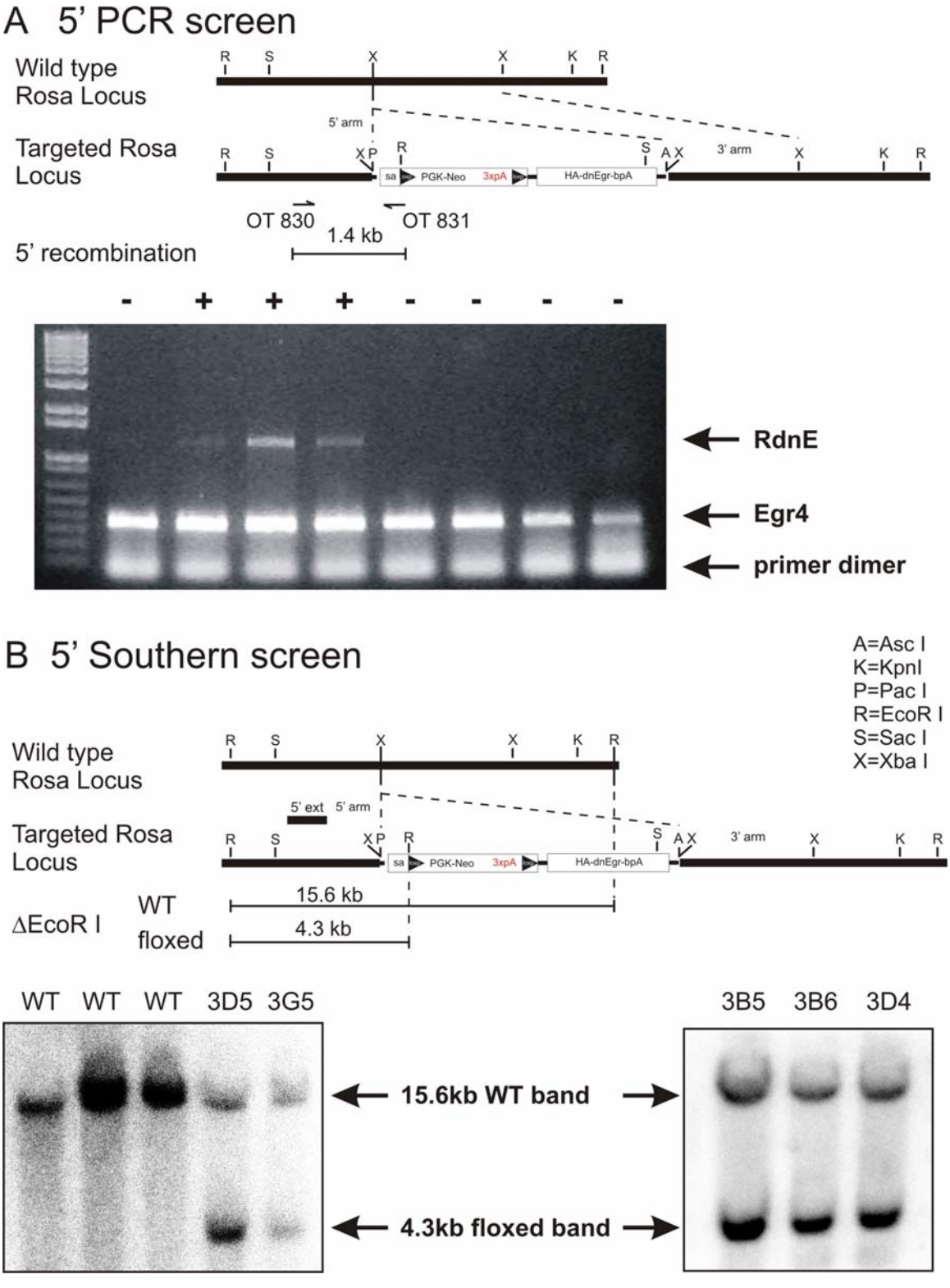
A 5' PCR screen
Wild type Rosa Locus
Targeted Rosa Locus
5' arm
3' arm
PGK-Neo
3xpA
HA-dnEgr-bpA
OT 830
OT 831
1.4 kb
5' recombination
- + + + - - - -
RdnE
Egr4
primer dimer
B 5' Southern screen
A=Asc I
K=KpnI
P=Pac I
R=EcoR I
S=Sac I
X=Xba I
5' ext
ΔEcoR I
WT
floxed
15.6 kb
4.3 kb
WT WT WT 3D5 3G5
3B5 3B6 3D4
15.6kb WT band
4.3kb floxed band

Figure 22. Screening of RdnE heterozygous ES clones.

RdnE targeting construct was electroporated into ES cells, and the positive clones were selected using neomycin-resistance. 31 out of 227 G418-resistant clones (13.6%) were correctly targeted as determined by PCR analysis (A). Five PCR-positive ES clones were randomly selected and examined for correct targeting by Southern blotting. All five PCR-positive clones were confirmed to have one floxed RdnE allele and one wild-type allele (B). Two heterozygous ES clones—3D5 and 3B5 were selected to generate chimeric mice.

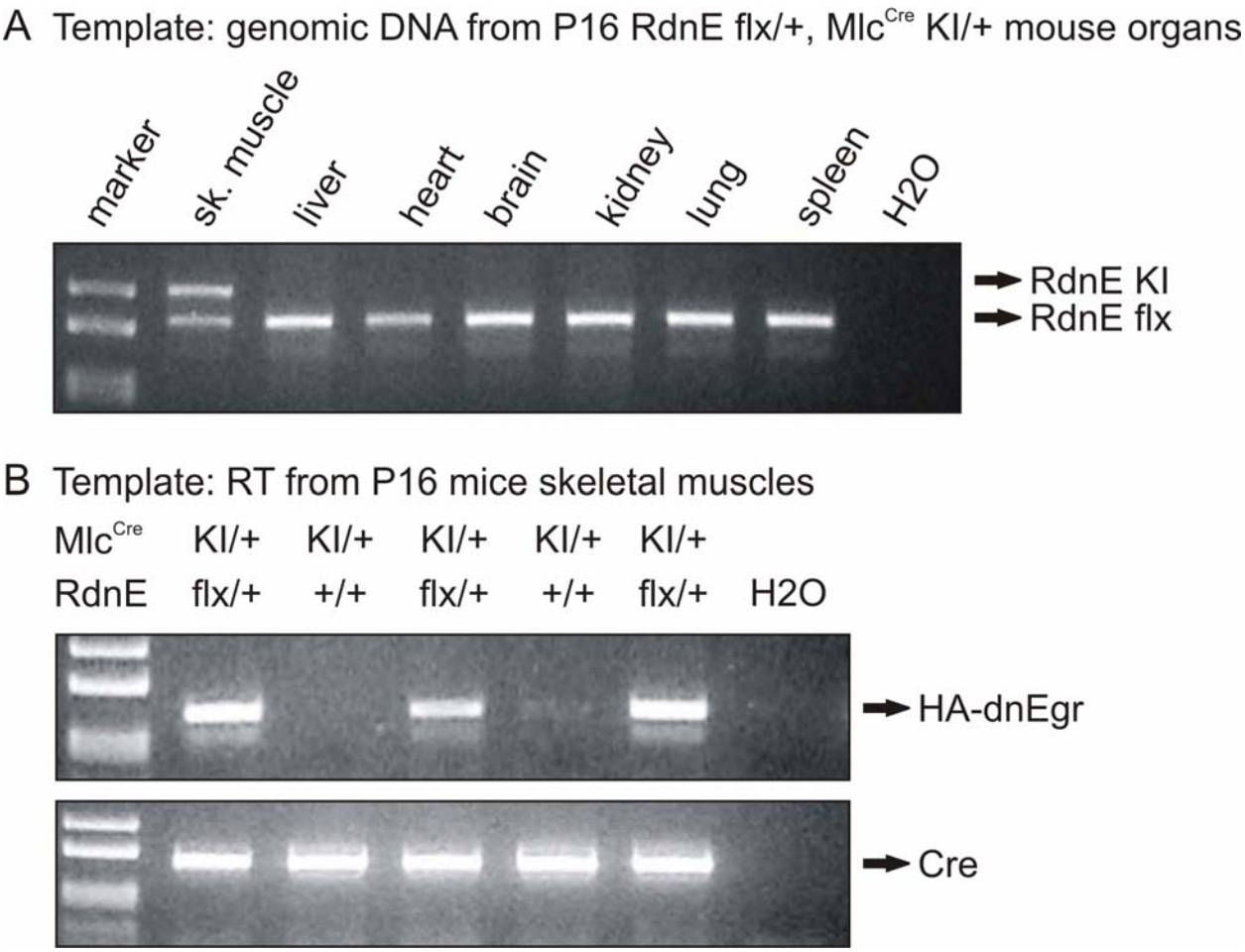

Figure 23. Characterization of RdnE knock-in mice using Mlc^{Cre} knock-in mice.

To test the floxed RdnE allele, Mlc^{Cre} knock-in mice were used to specifically turn on HA-dnEgr in skeletal muscle fibers.

(A) The RdnE knock-in (KI) allele was detected only in skeletal muscle but not in other organs examined in $RdnE^{flx/+}/MlcCre^{KI/+}$ mice.

(B) HA-dnEgr expression was detected only in $RdnE^{flx/+}/MlcCre^{KI/+}$ muscle but not in $RdnE^{+/+}/MlcCre^{KI/+}$ muscle by RT-PCR analysis.

4.5 Conclusions

Egr proteins are important transcriptional regulators of neurotrophin signaling and sympathetic neuron development. Both Egr1 and Egr3 were rapidly induced in differentiating SH-SY5Y/TrkA neuroblastoma cells after NGF stimulation, which differentiate into sympathetic-like neurons characterized by neurite outgrowth and target gene synthesis. NGF-mediated Egr activation in coupled to MAPK pathway since MEK inhibitors abrogate ERK phosphorylation and Egr induction.

To characterize the sympathetic defects of Egr3-deficient mice, DβH-τlacZ reporter mice were generated and bred with $Egr3^{-/-}$ mice, in which the τlacZ fusion protein faithfully marked almost all the sympathtic neurons and their axons *in vivo*. Whole-mount β-galactosidase enzyme histochemistry on double mutant mice revealed profound sympathetic dysautonomia in Egr3-deficient mice, including smaller sympathetic ganglia size, defective sympathetic axon branches and markedly decreased sympathetic innervation to target organs, such as eye, heart, pineal gland, salivary gland and spleen (Eldredge et al., 2007).

To turn on/off a specific gene in sympathetic neurons and study its influence on sympathetic neuron development, DβH-nlsCre-IRES-τlacZ mice were made, in which nuclear-localized Cre recombinase (nlsCre) and τlacZ fusion protein were expressed in sympathetic neurons under the control of the hDβH promoter.

To avoid the fertility-associated phenotypes of Egr-deficient mice, I utilized a dominant negative Egr (dnEgr) molecule to block Egr-mediated transcription in sympathetic neurons. DβH-dnEgr-IRES-τlacZ transgenic mice are embryonic lethal, perhaps due to sympathetic axon outgrowth defects. To conditionally express dnEgr molecule in sympathetic neurons, Rosa26-

dnEgr (RdnE) knock-in mice were generated and bred with DβH-nlsCre-IRES-τlacZ mice. The effect of the dnEgr molecule on sympathetic neuron development will be evaluated.

CHAPTER 5

Future Directions

5.1 To evaluate the effect of the dnEgr molecule on sympathetic neuron development, axon outgrowth, and target innervation.

As descibed in Chapter 4, both RdnE and DβH-nlsCre-IRES-τlacZ mice were generated, and they are viable and fertile. Breeding of these two mice will result in expressing HA-dnEgr molecule specifically in sympathetic neurons. RT-PCR of HA-dnEgr on adrenal and SCG lysates should detect HA-dnEgr expression in sympathoadrenal system in double mutant mice. Dual-labeling immunofluorescence of Cre and HA on SCG sections will examine the efficiency of Cre-mediated excision (HA+/Cre+), and HA-dnEgr protein synthesis *in vivo*. SCGs will be dissected and examined for neuron loss in double mutant mice using the StereoInvestigator morphometry system. Whole-mount β-galactosidase enzyme histochemistry will be performed on sympathetic trunk and target organs, such as eye, salivary gland, heart and pineal gland, from RdnE +/flx, DβH-nlsCre-IRES-τlacZ+ and RdnE +/+, DβH-nlsCre-IRES-τlacZ+ mice. The sympathetic ganglia size, axon projections from the ganglia, and target organ innervation will be examined and compared from these mice.

HA-dnEgr protein is expected to express in the nuclei of sympathetic neurons and block Egr-mediated transcription in sympathoadrenal systems. Assuming that the dnEgr molecule is functional *in vivo*, and that the sympathetic defects observed in Egr3-deficient mice is cell-autonomous, RdnE +/flx, DβH-nlsCre-IRES-τlacZ+ mice should show similar if not more severe sympathetic nervous system defects observed in $Egr3^{-/-}$ mice, including ptosis, sympathetic

neuron loss, smaller sympathetic ganglia, and decreased sympathetic innervation to target organs, such as eye, heart, salivary gland, and pineal gland.

5.2 To test the hypothesis that Egr transcriptional regulators modulate $p75^{NTR}$ expression in sympathetic nervous system.

$p75^{NTR}$ receptor enhances the binding affinity and selectivity of TrkA for its preferred ligand NGF over NT-3 in PC12 cells, and $p75^{NTR}$-deficient sympathetic neurons show decreased sensitivity to NGF, but increased sensitivity to NT-3 (Benedetti et al., 1993; Lee et al., 1994b). The study in Chapter 3 showed that Egr1 and Egr3 exhibit functional redundancy in regulating $p75^{NTR}$ expression in sciatic nerves where both Egr1 and Egr3 are coexpressed with $p75^{NTR}$ in Schwann cells. Moreover, both $Egr3^{-/-}$ mice and $p75^{NTR-/-}$ mice lack sympathetic innervation to the pineal gland (Eldredge et al., 2007; Lee et al., 1994a). The preliminary studies from our lab suggest that Egr1 and Egr3 have functional redundancy in sympathetic neuron development. Therefore, I hypothesize that Egr1 and Egr3 collaborate in regulating $p75^{NTR}$ expression in sympathetic neurons.

Quantative real-time PCR for $p75^{NTR}$ will be performed on P0 (physiologic apoptosis period) and P21 (after physiologic apoptosis) SCG lysates from wild type, $Egr1^{-/-}$, $Egr3^{-/-}$ and Egr1/3 DKO mice. Double labeling of Egr1/$p75^{NTR}$ and Egr3/$p75^{NTR}$ on adjacent SCG sections from wild type mice will address the colocalization of Egr1 and Egr3 with $p75^{NTR}$ in sympathetic neurons. Quantification of $p75^{NTR}$ staining intensity in wild type and Egr1/3 DKO sympathetic neurons will decide if $p75^{NTR}$ protein level is decreased in mutant mice. Moreover, dissociated SCG neurons from Egr1/3 DKO mice will be tested for survival, proliferation, axon outgrowth in

response to NGF and NT-3, and the results will be compared with wild type and $p75^{NTR}$-deficient SCG neurons.

It is expected that Egr1 and Egr3 colocalize with $p75^{NTR}$ in many if not all SCG neurons. In addition, the expression level of $p75^{NTR}$ is expected to decrease (but not be absent) in SCG lysates from Egr1/3 DKO mice since $p75^{NTR}$ expression is well-known to be modulated by multiple layers of regulatory mechanisms. Similarly, $p75^{NTR}$ protein level is expected to decrease (but not be absent) in Egr1/3 DKO SCG neurons. Finally, some phenotypical similarities between the Egr1/3 DKO and $p75^{NTR}$-deficient SCG neurons should be observed if $p75^{NTR}$ is a bona fide Egr target gene in sympathetic neurons. For example, Egr1/3 DKO SCG neurons may also show a decreased sensitivity to NGF, but increased sensitivity to NT-3, as observed in $p75^{NTR}$-deficient PC12 cells (Benedetti et al., 1993).

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CURRICULUM VITAE

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EDUCATION

- Ph.D., Integrated Graduate Program in the Life Sciences, Feinberg School of Medicine, Northwestern University, expected June 2007
- B.S., Department of Biology, Beijing Normal University, July 1998

RESEARCH EXPERIENCES

- **Graduate research assistant, Warren Tourtellotte's Lab, Northwestern University, April 2002 – June 2007**
  - Modulation of low affinity p75 neurotrophin receptor ($p75^{NTR}$) expression by Egr transcription regulators.
  - Investigate the role of Egr genes in sympathetic nervous system development by generating genetically modified mice models.
- **Graduate research assistant, Charles Brummer's Lab, Iowa State University, August 2000 - July 2001**

  Establish partially subtracted cDNA libraries from alfalfa germplasms and develop custom cDNA microarrays to identify winter-hardiness genes.
- **Graduate research assistant, Genfa Zhang's Lab, Beijing Normal University, September 1998 - July 2000**

  Investigate the genetic and biochemical factors related to cold-resistance in Jojoba, including phenotypic selection, the activities of amylase and peroxidase, the percentage of sugar and soluble proteins in the plant.

TEACHING EXPERIENCES

- **Teaching assistant, Molecular Biology, Northwestern University, Fall 2002**

  Six tutorial sessions on basic biotechniques of molecular biology, including DNA and RNA techniques, cloning, cDNA library establishment and screening, PCR and sequencing, gene expression, and protein interactions.
- **Lab instructor, Genetics, Beijing Normal University, Spring 1999**

  Twelve lab sessions of Genetics for junior college students.
- **Instructor, Biology, Beijing No.123 High School, Fall 1997**

  Biology instructor for high-school students.

PUBLICATIONS

[1] **Gao X**, Daugherty R and Tourtellotte WG. *Regulation of low affinity neurotrophin receptor ($p75^{NTR}$) expression by Egr transcriptional regulators.* Submitted.

[2] **Gao X** and Tourtellotte WG. *Selective expression of Cre recombinase with taulacZ axonal marker in catecholaminergic neurons.* Manuscript in preparation.

[3] Eldredge L, **Gao X**, Li L, Han X, Lomasney J, and Tourtellotte WG. *Egr3 is a novel transcriptional regulator of sympathetic nervous system development.* Manuscript in preparation.

[4] Li L, Carter J, **Gao X**, Whitehead J, and Tourtellotte WG. *The neuroplasticity- associated Arc gene is a direct transcriptional target of Early Growth Response (Egr) transcription factors.* Mol Cell Biol. Dec 2005; 25 (23): 10286-10300.

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PRESENTATIONS

[1] **Gao, X.** and Tourtellotte, W.G. *Low affinity neurotrophin receptor ($p75^{NTR}$) expression is regulated by early growth response (Egr) transcriptional regulators.* Oral presentation, Neuropathology session, Experimental Biology Annual Meeting. April 29th-May 2nd, 2007. Washington DC.

[2] **Gao, X.** *Low affinity neurotrophin receptor ($p75^{NTR}$) expression is regulated by early growth response (Egr) transcriptional regulators.* Invited talk at department retreat, Department of Pathology, Northwestern University. Sep 30th-Oct 1st, 2006. Chicago, IL.

[3] **Gao, X.** *Transcriptional regulation of low affinity neurotrophin receptor ($p75^{NTR}$) by early growth response gene 3 (Egr3) in muscle spindle differentiation.* Calandra Seminar Series, Department of Pathology, Northwestern University. May 16th, 2005. Chicago, IL.

[4] **Gao, X.** *The role of Egr transcription factors and target genes in sympathetic neuron differentiation and NGF signaling.* Tumor Cell Biology Seminar Series, Lurie Cancer Center, Northwestern University. May 5th, 2005. Chicago, IL.

[5] **Gao, X.** and Tourtellotte, W.G. *NGF-mediated Egr-dependent target genes in human neuroblastoma cells.* Poster session, the 34th annual meeting of Society for Neuroscience, Oct 22nd-28th, 2004, San Diego, CA.

[6] Brummer C, Luth D, Riday H, Council CL, Helland S, Alarcon B, **Gao X**, and Ryerson M. *Integrating genomics and breeding to improve yield and winter hardiness in alfalfa.* Plant & Animal Genome IX Conference, Jan 13th-17th, 2001, San Diego, CA.

HONORS AND AWARDS

- Weil Award for Best Paper on Experimental Neuropathology Presented at the Annual Meeting of American Association of Neuropathologists, May 2007
- Northwestern Conference Travel Grant for Experimental Biology Annual Meeting, Northwestern University, February 2007
- Northwestern Conference Travel Grant for the 34th annual meeting of Society for Neuroscience, Northwestern University, August 2004
- Integrated Graduate Program in the Life Sciences Fellowships, Feinberg School of Medicine, Northwestern University, September 2001 – December 2002
- Honored Graduate School Admission without Entrance Examination, Beijing Normal University, January 1998
- University Scholarship for undergraduates, Beijing Normal University, September 1995, September 1996, September 1997