Abnormal sympathetic nervous system development and physiological dysautonomia in Egr3-deficient mice

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Sympathetic nervous system development depends upon many factors that mediate neuron migration, differentiation and survival. Target tissue-derived nerve growth factor (NGF) signaling-induced gene expression is required for survival, differentiation and target tissue innervation of post-migratory sympathetic neurons. However, the transcriptional regulatory mechanisms mediated by NGF signaling are very poorly defined. Here, we identify Egr3, a member of the early growth response (Egr) family of transcriptional regulators, as having an important role in sympathetic nervous system development. Egr3 is regulated by NGF signaling and it is expressed in sympathetic neurons during development when they depend upon NGF for survival and target tissue innervation. Egr3-deficient mice have severe sympathetic target tissue innervation abnormalities and profound physiological dysautonomia. Unlike NGF, which is essential for sympathetic neuron survival and for axon branching within target tissues, Egr3 is required for normal terminal axon extension and branching, but not for neuron survival. The results indicate that Egr3 is a novel NGF signaling effector that regulates sympathetic neuron gene expression required for normal target tissue innervation and function. Egr3-deficient mice have a phenotype that is remarkably similar to humans with sympathetic nervous system disease, raising the possibility that it may have a role in some forms of human dysautonomia, most of which have no known cause.

KEY WORDS: Egr3, Sympathetic, Ptosis, Neurotrophin, Physiology, Mouse

INTRODUCTION

The sympathetic nervous system (SNS) is a division of the autonomic nervous system with an important role in maintaining organ and tissue homeostasis. A detailed understanding of the factors involved in establishing and maintaining the stability of sympathetic target tissue innervation is of considerable importance because the SNS is the target of a wide variety of debilitating developmental and degenerative diseases in many vertebrate species, including humans. Many molecules are known to have important roles in sympathoadrenal precursor migration, phenotype specification and target tissue innervation during development. For example, migration of sympathoadrenal precursors from the neural crest is dependent upon signaling through ErbB2, ErbB3 (Britsch et al., 1998) and Ret (Enomoto et al., 2001) tyrosine kinase receptors, as well as through guidance molecules such as semaphorin 3A (Kawasaki et al., 2002). In addition, specification of the noradrenergic phenotype of post-migratory sympathoadrenal precursors depends upon diffusible factors such as bone morphogenetic proteins (BMPs) and transcriptional regulators such as Mash1, Phox2a, Phox2b, Hand2 (dHand) and Gata3 (for a review, see Goridis and Rohrer, 2002; Howard, 2005). To establish their connections in the periphery, sympathetic neuroblasts also require several diffusible factors such as hepatocyte growth factor (HGF) (Maina et al., 1998), artemin (Honna et al., 2002), neurotrophin 3 (NT3) (Francis et al., 1999) and nerve growth factor (NGF) (Glebova and Ginty, 2004) that promote their survival, axon extension along blood vessels and target tissue innervation during development.

Of the diffusible factors known to be involved in establishing sympathetic neuron connections with peripheral target tissues, NGF has emerged as the most important. Its role in sympathetic neuron survival and differentiation has been known for many decades (Levi-Montalcini and Cohen, 1960), but more recently, a particularly important role in terminal axon extension and branching during target tissue innervation has been identified (Glebova and Ginty, 2004). NGF may act locally to facilitate axon extension and target tissue innervation by directly regulating neurofilament protein stabilization (Veeranna Amin et al., 1998), but it also alters gene expression in sympathetic neurons, which facilitates their survival and axon outgrowth (Milbrandt, 1987; Riccio et al., 1997). However, specific transcriptional regulators that control NGF-dependent gene expression during SNS development have not been well defined.

Early growth response 1 (Egr1) protein is among a relatively small number of transcriptional regulators induced by NGF signaling in sympathetic neurons (Milbrandt, 1987). Disruption of Egr1-mediated gene transcription using either a dominant-negative molecule (Levkovitz et al., 2001) or the Egr co-repressor molecule Nab2 (Qu et al., 1998) inhibits NGF-mediated neurite outgrowth and differentiation in sympathetic neuron-like PC12 cells. Similarly, antisense oligonucleotide knockdown of Egr1 protein translation inhibits neurite outgrowth, whereas overexpression of Egr1 enhances neurite outgrowth in N2A neuroblastoma cells (Pignatelli et al., 1999). Thus, it is surprising that Egr1-deficient mice develop normally and have no apparent SNS abnormalities (Lee et al., 1995) (L.C.E. and W.G.T., unpublished). However, considering that Egr3 and Egr4, two closely related transcriptional regulators, have been shown to functionally cooperate with Egr1 to regulate some target genes such as luteinizing hormone β-peptide (LHβ), the neuroplasticity associated protein Arc and the low-affinity neurotrophin receptor p75NTR (Gao et al., 2007; Li et al., 2005; Tourtellotte et al., 2000), it seemed plausible that other Egr proteins may have important roles in sympathetic neuron differentiation in vivo.
Here, we identify Egr3 as having an unexpected but important role in SNS development. Egr3-deficient mice exhibit sympathetic neuron loss, target tissue innervation defects and profound dysautonomia. Egr3 expression is induced by NGF signaling and it is upregulated in sympathetic neurons during a developmental period when NGF signaling is required for normal sympathetic neuron survival and target tissue innervation in vivo. Thus, Egr3 appears to be a physiologically important effector of NGF signaling with an essential role in sympathetic neuron target tissue innervation and terminal axon branching.

MATERIALS AND METHODS

Animals

Egr3-deficient mice were genotyped as previously described (Tourtellotte et al., 2001). Perinatal and adult (age 12-35 weeks) mice were used as indicated. Transgenic DBH-tdTomato (DitZ) reporter mice were generated by cloning the tdTomato cDNA (Callahan and Thomas, 1994) into the BamHI site of the previously described 2949A12 DBH promoter plasmid (Hoyle et al., 1993). F1 progeny from transgenic founder mice were screened to establish a single reporter line with tdTomato expression in over 99% of sympathetic neurons and their axons (see Fig. S3 in the Supplemental material and data not shown). DitZ transgenic reporter mice were genotyped by PCR using primers: 5'-GATTCCTCGCTAGACAAATGTGA-3' and 5'-CATGTCTCACCCTCTGGTCT-3'. All experimental procedures complied with protocols approved by The Northwestern University Institutional Animal Care and Use Committee.

Tissue preparation

Embryos from timed pregnant females were isolated by Cesarean section. Postnatal mice were perfused through the heart with 0.1 M phosphate-buffered 4% paraformaldehyde (PFA, pH 7.2) and embryos were immersion fixed. Tissues were either processed for paraffin embedding or cryoprotected overnight in graded (15-30%) phosphate-buffered sucrose and immersion fixed. Tissues were either processed for paraffin embedding or cryoprotected overnight in graded (15-30%) phosphate-buffered sucrose and embedded in OCT. Serial paraffin tissue sections (16 μm) or frozen sections (12-16 μm) were analyzed.

Ganglion neuron counts and volume estimation

SCG neuron numbers were determined using unbiased stereology and optical dissector methods (Stereoinvestigator, Microbrightfield) on every fifth serial section from various developmental ages as previously described (Albert et al., 2005). The total number of neurons per SCG was estimated using an optical fractionator probe and ganglionic volume measurements were estimated using the planimetry function of the Stereoinvestigator software.

NGF neutralization

Newborn Swiss Webster mice were injected with PBS (n=5) or antibody (n=5; 50 mg/kg, i.p., mouse anti-NGF, clone AS-18, Exalphia Biologicals) which has been previously well characterized for its specificity and NGF neutralizing effects (Wild et al., 2007). SCG were isolated 14 hours after injection and subjected to qPCR.

Primary SCG neuron cultures

For SCG neuron cultures, E19 or P0 Swiss Webster (Charles River Laboratories) mice or E18.5 Egr3+/− and littermate Egr3−/− mice were used. SCG neurons were dissociated in 1 mg/ml Type IV Collagenase (Worthington Biochemical Corporation), followed by 0.25% Trypsin-EDTA and plated on collagen-coated 35 mm dishes in Minimal Essential Media (MEM) containing 10% fetal bovine serum (FBS), 1% penicillin/ streptomycin and 100 ng/ml of NGF. For signaling studies, neurons were differentiated in the presence of 100 ng/ml of NGF for 7 days, deprived of NGF for 3 hours and then re-stimulated with either vehicle (control), NGF (100 ng/ml), NT4 (30 ng/ml) or BDNF (30 ng/ml) for 45 minutes. In some wells, DMSO (control) or the MAP kinase kinase (MEK) inhibitor U0126 (20 μM, Promega) were added to the cultures.

Immunochemistry and western blotting

Immunochemistry for tyrosine hydroxylase (TH; Chemicon) or β-galactosidase (βgal; ICN Pharmaceuticals) was performed on frozen tissue sections to identify sympathetic axons. For proliferation and apoptosis assays, tissue sections were incubated with cleaved caspase 3 antibody (Cell Signaling) to identify apoptotic neurons or anti-5-bromo-2-deoxyuridine (BrDU) antibody (Sigma) to identify proliferating cells. Species and antibody specificity is described in detail (Li et al., 2005) using the following antibodies: anti-Egr3 (sc-191), anti-ERK1/2 (sc-94), anti-phosphorylated ERK1/2 (sc-16982) and anti-actin (sc-1616) (all from Santa Cruz Biotechnology). The antibodies were detected using species appropriate horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch) and SuperSignal West Pico chemiluminescent substrate (Pierce).

lacZ enzyme histochemistry

Tissues were dissected and postfixed in 2% PFA, 0.2% glutaraldehyde, 5 mM EGTA, 0.01% NP-40 in PBS-Mg at 4°C and reacted for 6-12 hours at 37°C in reaction buffer (1 mg/ml X-gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide). After reaction, the tissues were postfixed/dehydrated in methanol, and cleared in 2:1 benzyl benzoate:benzyl alcohol.

In situ hybridization

In situ hybridization was performed on frozen sections using digoxigenin-labeled antisense and sense riboprobes for Egr3 (GenBank NM018781, nt 345-746) as described previously in detail (Albert et al., 2005).

Apopotosis and proliferation analysis

Newborn (P0) pups received injections with 50 mg/kg, i.p. BrdU (Sigma) and euthanized after 2 hours. BrdU and caspase 3 were detected by immunohistochemistry and the number of immunopositive cells in every fifth section from E15, E16, E17, E18 or P0 mice were quantified using unbiased stereology and optical fractionator methods (Stereoinvestigator, Microbrightfield, Williston, VT). The data were reported as caspase 3+ or BrdU+ neurons per unit volume of sampled ganglion.

qPCR

Total RNA was isolated from cultured sympathetic neurons or whole SCG using Trizol extraction (Invitrogen). Reverse transcription and qPCR was performed as previously described in detail (Albert et al., 2005). The primer sequences used for expression analysis are available upon request.

AANAT expression analysis and light cycling

Adult wild-type and Egr3−/− mice were housed in a controlled lighting environment [10 hours of dark, 14 hours of light (10D:14L)] for at least 3 weeks prior to analysis. ZT0 was defined as the time of dark-light transition. Some animals were sacrificed in the dark during the dark phase of the light-dark cycle and pineal glands were rapidly dissected. Gene expression was determined by qPCR and compared at three time points (ZT9, ZT13 and ZT21) between wild-type and Egr3−/− mice.

Cardiac physiology

Male and female adult wild type and Egr3−/− mice, weighing 18-23 g, were used. Pressure measurements from a 1.4 French micromanometer-tipped Millar pressure transducer (SPR839, Millar Instruments) were calibrated against a mercury manometer. The right jugular vein of anesthetized mice was cannulated for fluid administration and the pressure catheter was inserted into the right carotid artery and advanced into the left ventricle of the heart. Heart rate and myocardial contractility (change in pressure over time; dP/dt) measurements were compared before and after injection of the α2-adrenoreceptor antagonist, Yohimbine (YOH; 2 mg/kg, i.v.). Heart rate and pressure data were analyzed using Millar data acquisition and analysis software.

Statistical analysis

For SCG neuron survival assays, the data were analyzed by two-way ANOVA using Genotype (wild type and Egr3−/−) and NGF concentration as grouping factors. All values were expressed as mean±s.e.m. with P<0.05 considered to be statistically significant.
RESULTS

Egr3 is regulated during development and by NGF signaling in sympathetic neurons

Previous reports indicate that Egr1 may have a role in NGF-mediated sympathetic neuron differentiation, but the results were not confirmed in Egr1-deficient mice. We became intrigued by the possibility that Egr3 may have a role in SNS development after identifying blepharoptosis (drooping of the upper eyelid) in Egr3-deficient mice (Tourtellotte and Milbrandt, 1998), as it is often associated with abnormal sympathetic innervation to the eyelid musculature in both humans and rodents. Because nothing is known about Egr3 expression in sympathetic neurons, we first examined whether it is expressed in the superior cervical ganglion (SCG), which contains sympathetic neurons that innervate cephalic tissues including the superior and inferior tarsal muscles of the eyelids. At embryonic day 13 (E13), when sympathetic neurons are not dependent upon NGF signaling and have not yet innervated target tissues (Francis and Landis, 1999; Wyatt and Davies, 1995; Wyatt et al., 1997), Egr3 expression was low in the SCG. Egr3 expression was induced greater than 12-fold at E15 and greater than 22-fold by birth (P0), two developmental time points after which sympathetic neurons are dependent upon NGF signaling for survival and target tissue innervation (Fig. 1A). Consistent with the qPCR results, Egr3 expression was undetectable by in situ hybridization at E13 (Fig. 1B; see Fig. S1B in the supplementary material) and was markedly upregulated in most SCG neurons by birth (P0; Fig. 1C; see Fig. S1C in the supplementary material). Thus, Egr3 expression is developmentally regulated in the SCG and the timing of expression coincides with the onset of NGF dependence and target tissue innervation. Moreover, Egr3 is regulated by NGF signaling in vivo, as neutralization of NGF function significantly decreased Egr3 expression in SCG neurons (Fig. 1D).

We next examined whether NGF, the principal neurotrophin required for sympathetic neuron survival and target tissue innervation, could induce Egr3 expression in vitro. Egr3 expression was induced six- to sevenfold by NGF, but not by related neurotrophins NT4 or BDNF relative to untreated (control) neurons (Fig. 1E). Moreover, Egr3 induction was dependent upon MAPK signaling, as pretreatment with the MEK inhibitor U0126 abrogated NGF-dependent Egr3 induction (Fig. 1E). Similarly, we found that SH-SY5Y/TrkA human neuroblastoma cells, which have been previously shown to induce Egr1 expression and to differentiate in response to NGF treatment (Edsjo et al., 2001), also induced Egr3 expression after NGF treatment (see Fig. S2A in the supplementary material). Also, similar to primary sympathetic neurons, NGF-mediated Egr3 expression was completely abrogated by the MEK inhibitor U0126 (see Fig. S2B in the supplementary material). Taken together, these results indicate that Egr3 expression is modulated by NGF signaling in sympathetic neurons, consistent with a potential role in gene regulation related to their survival, differentiation and/or target tissue innervation.

Sympathetic neuron loss in postnatal Egr3-deficient mice

Egr3-deficient mice have blepharoptosis similar to mice with widespread sympathetic neuron loss in the absence of neurotrophins NGF or NT3, or their cognate tyrosine kinase receptor TrkA (Crowley et al., 1994; Ernfors et al., 1994; Farinas et al., 1994; Smeyne et al., 1994). Unlike neurotrophin signaling-deficient mice in which most sympathetic neuron loss occurs during late embryonic development, no significant neuron loss was observed in Egr3−/− mice until 1 day after birth (P1; Fig. 2A). At P1, Egr3−/− mice had ~30% fewer SCG neurons relative to wild type (Fig. 2A). Moreover, consistent with previously published observations, the total number of SCG neurons decreased with age in both wild-type and Egr3−/− mice (Gatzinsky et al., 2004; Jansen et al., 2007). Viability of senescent SCG neurons is dependent upon pro-NGF and sortilin signaling (Jansen et al., 2007), which does not appear to be disrupted in Egr3−/− mice because roughly similar amounts of neuron attrition were observed in both adult wild-type and Egr3−/− mice (Fig. 2A).

To characterize the impact of sympathetic neuron loss on target tissue innervation in Egr3−/− mice, transgenic reporter mice were generated to visualize sympathetic neurons and their axons using the human dopamine β-hydroxylase (DBH) promoter (Hoyle et al., 1993) to regulate expression of a β-galactosidase fusion protein (lacZ) (Callahan and Thomas, 1994) in all sympathetic neurons...
its rostrocaudal extent in Egr3–/–: DβτlacZ+ mice; scale bars: 250 μm.

(see Fig. S3A in the supplementary material). Whole-mount lacZ histochemistry (see Fig. S3B-D in the supplementary material) and double-labeling immunofluorescence for TH and β-gal (see Fig. S3E in the supplementary material) confirmed that the neuron/axon localized lacZ protein was a reliable and sensitive marker for all sympathetic neurons and their axons in DβH-τlacZ+ (DrtZ+) transgenic mice.

Whole-mount lacZ histochemistry performed on P1 mice showed atrophy of the paravertebral sympathetic chain ganglia throughout its rostrocaudal extent in Egr3+/+;DrtZ+ mice relative to Egr3+/-;DrtZ+ (wild type) littermates. The SCG were markedly smaller in Egr3+/- mice compared with wild-type littermates (Fig. 2B, black arrowhead), consistent with a 30% neuron loss (Fig. 2A) and a 36% decrease in ganglion volume (n=3 SCG of each genotype, P<0.01). Many axons emanating from Egr3+/- SCG were either atrophic or absent (Fig. 2B, white arrowhead and dashed contour, respectively). In addition, neurons that remained in the Egr3+/- SCG were generally smaller compared with wild type (see Fig. S4A in the supplementary material), most probably owing to atrophy, as diameter-frequency analysis of SCG neurons showed a loss of large diameter neurons that was accompanied by an increase in small diameter neurons (Fig. S4B).

Widespread abnormalities of the sympathetic nervous system were apparent as the stellate (STG; Fig. 2C, black arrowhead), thoracic (Fig. 2D, black arrowhead) and caudal paravertebral ganglia (Fig. 2E, black arrowheads) were all smaller in Egr3+/-;DrtZ+ mice, and many axon bundles emanating from them were also markedly thin or completely absent compared with wild-type mice (Fig. 2C-E, white arrowheads).

**Perinatal sympathetic neuron apoptosis in Egr3+/- mice**

In NGF-, NT3- and TrkA-deficient mice, sympathetic neurons are generated in normal numbers but undergo increased apoptosis during prenatal and postnatal development (Crowley et al., 1994; Ernfors et al., 1994; Farinas et al., 1994; Smayne et al., 1994). Sympathetic neurons were also generated in normal numbers in Egr3+/- mice as no differences in SCG neuron number were observed between wild-type and Egr3+/- newborn mice (Fig. 2A). However, significantly increased apoptosis was found in newborn Egr3+/- SCG (Fig. 3A), which correlated with differences in SCG neuron number that were detected slightly later at P1. In addition, BrdU incorporation studies showed no difference in cell proliferation between newborn wild-type and Egr3+/- SCG that could have otherwise contributed to differences in the number of sympathetic neurons in postnatal mice (Fig. 3B). Thus, similar to neurotrophin- and neurotrophin signaling-deficient mice, sympathetic neuron death in Egr3+/- mice occurs by apoptosis at a developmental time point that coincides with active target tissue innervation and acquisition of NGF dependence.

Embryonic and early postnatal sympathetic neurons depend upon NGF for their survival in vitro and in vivo, and over 90% of them can be rescued from apoptosis in vitro when 10 ng/ml of NGF is present in the culture medium (Belliveau et al., 1997). To examine whether Egr3+/- sympathetic neurons have an autonomous defect in survival, wild-type and Egr3+/- SCG were isolated at E18.5, prior to the onset of increased apoptosis that occurs in Egr3+/- mice in vivo, and sympathetic neurons were dissociated into culture media containing differing amounts of NGF. As expected, there was a highly significant effect of NGF concentration on sympathetic neuron survival for both wild-type and Egr3+/- neurons 24 hours after plating (F(1, 23)=123.9, P<0.0001). However, there was no significant effect of genotype on neuron survival (F(1, 23)=1.27, P=0.28), indicating that Egr3+/- sympathetic neurons do not have a cell autonomous defect in survival (Fig. 3C).

**Abnormal sympathetic target tissue innervation and terminal axon branching in Egr3+/- mice**

Egr3-deficient neurons do not have an autonomous survival defect and yet some of them die in vivo during a period of active target tissue innervation. These observations raise the possibility that neuron death in vivo results from a failure to normally innervate target tissues and acquire adequate trophic factor support. To address this hypothesis, sympathetic target tissue innervation was analyzed in adult Egr3+/-;DrtZ+ and Egr3+/-;DrtZ+ mice. In all Egr3+/-;DrtZ+ target tissues examined, there was a decrease in the overall sympathetic innervation compared with Egr3+/-;DrtZ+ target tissues,
consistent with sympathetic neuron loss. However, the remaining sympathetic axons also showed abnormal axon extension and branching patterns within tissues. For example, in Egr3+/+:D\textsubscript{rtlZ}\textsuperscript{+} submandibular and sublingual salivary glands (Fig. 4A), which express high levels of NGF required for normal sympathetic innervation and terminal axon branching (Glebova and Ginty, 2004), whole-mount lacZ histochemistry highlighted robust sympathetic axon innervation and axon branching deep into the glandular parenchyma (Fig. 4B, arrowheads). By contrast, in Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{+} glands, there was decreased innervation (Fig. 4A') that was accompanied by attenuated terminal axon extension and branching into the glandular parenchyma (Fig. 4B', arrowheads). Similarly, in the trachea where there is robust sympathetic innervation to smooth muscle and submucosal glands, the axons entered the dorsal midline and branched to form a dense circumferential plexus in Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{+} mice (Fig. 4C, white arrowheads). By contrast, innervation to the trachea of Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{-} mice was generally decreased but remaining axons also failed to branch efficiently to form a comparatively elaborate sympathetic plexus. In some regions of the trachea the axons appeared to barely branch at all, leaving the corresponding tracheal segments nearly devoid of sympathetic innervation (Fig. 4C', arrowheads). Sympathetic innervation to several major organs, including kidneys, bowel and spleen was also abnormal. For example, sympathetic axons in wild-type spleen entered the organ along the splenic artery (Fig. 4D, black arrowhead) and then branched considerably upon entering the splenic parenchyma (Fig. 4D, white arrowheads). By contrast, although some axons also reached the spleen along the splenic artery in Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{-} mice (Fig. 4D', black arrowhead), they failed to branch normally and invade the splenic parenchyma (Fig. 4D', white arrowheads). This correlated with an overall decrease in sympathetic innervation to target organs is accompanied by abnormalities in axon extension and terminal axon branching in Egr3\textsuperscript{+/-} mice. (A) In the submandibular gland and sublingual gland (broken outline) from Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{+} mice, lacZ histochemistry revealed robust sympathetic innervation. (B) Framed area in A: sympathetic axons branched into the distal lobules of the glands (arrowheads). (A') In Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{-} mice, there was a relative decrease in sympathetic innervation, consistent with sympathetic neuron loss. (B') Framed area in A': there was less complex axon branching and numerous axons that failed to extend to the distal lobules of the glands (arrowheads). (C) In trachea from Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{+} mice, sympathetic innervation entered along the dorsal midline and branched circumferentially to innervate smooth muscle and submucosal glands (arrowheads). (C') In trachea from Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{-} mice, however, sympathetic axon branching was consistently decreased and the branching of remaining axons was markedly diminished. (D,D') Sympathetic axons entered the splenic parenchyma along the splenic arteries (black arrowhead) and Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{-} spleens (D) the axons branched extensively after entering the organ parenchyma (white arrowheads). By contrast, in Egr3\textsuperscript{+/-}:D\textsubscript{rtlZ}\textsuperscript{-} mice (D'), sympathetic axons branched poorly as they entered the parenchyma (white arrowheads).
innervation to the spleen, as indicated by an overall decrease in the lacZ reaction product in Egr3<sup>−/−</sup>:DrtI<sup>+</sup> spleens (Fig. 4D, arrow) compared with wild type (Fig. 4D, arrow). Thus, in the absence of Egr3, there is decreased innervation to many target tissues owing to sympathetic neuron loss and innervation defects from residual axons that fail to normally branch and invade target tissues.

**Sympathetic target tissue innervation defects lead to physiologic dysautonomia in Egr3<sup>−/−</sup> mice**

We next examined the extent to which moderate sympathetic neuron loss accompanied by prominent target tissue innervation abnormalities lead to dysautonomia in several well-characterized sympathetic target tissues in Egr3<sup>−/−</sup> mice. For example, in humans with Horner’s syndrome and NGF-, NT3- and TrkA-deficient mice with SNS defects, blepharophostis results from loss of sympathetic innervation to the eye (Crowley et al., 1994; Ernfors et al., 1994; Gurwood, 1999; Smythe et al., 1994). Compared with wild type (Fig. 5A), Egr3<sup>−/−</sup> mice have prominent blepharophostis (Fig. 5A’), suggesting that sympathetic innervation to tarsal musculature may be impaired. Indeed, compared with Egr3<sup>+/+</sup>:DrtI<sup>+</sup> mice, which showed dense sympathetic innervation to the superior and inferior tarsal muscles in the eyelids (Fig. 5B, black arrowheads) and the Meibomian glands (white arrowheads) in Egr3<sup>−/−</sup>:DrtI<sup>+</sup> mice. Compared with wild type (C), adult Egr3<sup>−/−</sup> mice (C’) developed corneal ulceration (arrow), most probably owing to impaired secretomotor function of the Meibomian glands. Scale bars: 0.5 mm. *nasal canthus.

![Image of Egr3<sup>−/−</sup> mice with blepharophostis](image_url)

**Fig. 5. Physiological blepharophostis in Egr3<sup>−/−</sup> mice.** Compared with wild-type mice (A), Egr3<sup>−/−</sup> mice (A’) have prominent blepharophostis (drooping eyelids). (B) Whole-mount lacZ histochemistry of the inner eyelids from adult Egr3<sup>−/−</sup>:DrtI<sup>+</sup> mice showed dense sympathetic innervation to the superior and inferior tarsal muscles (black arrowheads) and Meibomian glands in the inner eyelids (columnar innervation, white arrowheads). (B’) Sympathetic innervation was markedly decreased to the upper and lower eyelids (black arrowheads) and the Meibomian glands (white arrowheads) in Egr3<sup>−/−</sup>:DrtI<sup>+</sup> mice. Compared with wild type (C), adult Egr3<sup>−/−</sup> mice (C’) developed corneal ulceration (arrow), most probably owing to impaired secretomotor function of the Meibomian glands. Scale bars: 0.5 mm. *nasal canthus.

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and to both ventricles (Fig. 7A). To examine whether sympathetic denervation was also associated with deregulation of AANAT expression during the light-dark cycle, wild-type and Egr3–/– mice were entrained to a 10D:14L light-dark cycle (see Materials and methods). Pineal glands were dissected for qPCR analysis at three time points during the 24-hour photo period: ZT13, ZT21 and ZT9, with ZT0 defined as the temporal onset of the dark-light transition. Wild-type pineal glands showed a characteristic pattern of AANAT expression associated with the entrained light-dark cycle. Very low basal AANAT expression was observed near the end of the light cycle (ZT13), it was highly induced during the dark phase when sympathetic activity to the pineal gland is maximal (ZT21, defined as 100% maximal induction in this paradigm) and it rapidly declined to basal levels after the onset of the entrained light phase (ZT9; Fig. 6D). By contrast, in Egr3–/– pineal glands, AANAT induction reached less than 20% of the maximal wild-type induction (ZT21) after the onset of the dark phase before returning to baseline during the light phase of the cycle (Fig. 6D). These results are consistent with decreased sympathetic innervation to the pineal gland, which leads to impaired AANAT induction during the dark phase of the light cycle in Egr3–/– mice. Moreover, the results raise the possibility that Egr3–/– mice have altered circadian rhythms as a consequence of sympathetic dysautonomia and impaired AANAT cycling and melatonin synthesis.

Neurons located in the SCG, STG and upper thoracic sympathetic chain ganglia innervate the heart. Sympathetic axon terminals release norepinephrine to activate myocardiocyte β1 adrenergic receptors and increase heart rate and contractility (Costanzo, 1998). To examine the pattern of sympathetic innervation in hearts from adult wild-type and Egr3–/– mice. In hearts from Egr3+/+:DtIZ+ mice, prominent epicardial innervation was observed in the right atrium (Fig. 7A) and ventricles (Fig. 7B,C), consistent with sympathetic neuron loss. To determine whether these abnormalities have physiological significance, heart rate and myocardial contractility were measured in cardiac catheterized adult wild-type and Egr3–/– mice. The catheterized mice were administered the central α2-adrenergic receptor antagonist yohimbine (YOH) to increase post-ganglionic sympathetic activity. Whereas the baseline heart rate and myocardial contractility of untreated wild-type and Egr3–/– mice were similar, YOH treatment of wild-type mice increased heart rate and contractility greater than twofold relative to baseline. By contrast, YOH treatment of Egr3–/– mice resulted in a significantly blunted increase in heart rate (Fig. 7D) and contractility (Fig. 7E). Thus, cardiac hemodynamic abnormalities are present in Egr3–/– mice as a consequence of impaired sympathetic innervation to the heart. Taken together, these results demonstrate that Egr3–/– mice have profound sympathetic dysautonomia associated with the structural SNS abnormalities.

**DISCUSSION**

Egr3 is a member of the early growth response family of zinc-finger transcriptional regulators that consists of four structurally similar proteins (Egr1-4). The closely related protein, Egr1 was considered to be a potential mediator of differentiation related gene transcription (Milbrandt, 1987). Egr1 was later shown to have an important role in NGF-mediated neurite outgrowth in PC12 and N2A neuroblastoma cells, but a role in SNS development was not confirmed in Egr1-deficient mice (Lee et al., 1995). Despite the fact that Egr3 is co-expressed with Egr1 in many cell types and they can cooperate to directly regulate some target genes (Carter and Tourtellotte, 2007; Gao et al., 2007; Li et al., 2005), it is surprising to find that Egr3, but not Egr1, has an essential role in SNS development in vivo.
We found that Egr3 expression was upregulated in post-migratory SCG neurons at a time point that coincides with their dependence on NGF for survival and target tissue innervation. This correlation was further substantiated by in vitro and in vivo experiments which showed that Egr3 is regulated in primary and transformed sympathetic neurons by NGF signaling. Moreover, NGF-mediated Egr3 expression was entirely dependent upon MEK (Erk1/2) signaling, which is well known to enhance NGF-dependent axon outgrowth in sympathetic neurons (Atwal et al., 2000; Lein et al., 2007; Thompson et al., 2004). Although these results do not rule out non-neurotrophin-mediated mechanisms of regulation or a non-neuron autonomous function for Egr3, they clearly demonstrate that Egr3 expression is modulated by NGF signaling and that it is expressed by sympathetic neurons at a time when it could influence gene expression involved in their differentiation and target tissue innervation.

A role for Egr3 in SNS development was confirmed in Egr3-deficient mice. Sympathetic neuron loss was first detected in SCG neurons 1 day after birth, which was preceded by a significant increase in apoptosis. Although the quantitative and in vitro studies were focused on SCG neurons, a detailed analysis of Egr3^{-/-} mice demonstrated widespread atrophy of sympathetic chain ganglia and target tissue innervation defects, indicating a global role for Egr3 in SNS development. However, unlike mice lacking NGF or its receptor TrkA, which lose most sympathetic neurons by birth (Crowley et al., 1994; Sweeney et al., 1994), we found that only about one-third of SCG neurons were lost in Egr3-deficient mice after birth, despite the fact that it is expressed in most, if not all, SCG neurons. This discrepancy between Egr3 expression and neuron survival may be explained by the fact that Egr3 does not have a neuron-autonomous role in survival as there was no difference in survival between wild-type and Egr3^{-/-} neurons when NGF was supplied in culture media in vitro. Yet, sympathetic neuron death is observed in vivo, which results in many relatively hypoinnervated target tissues that also have residual innervation with abnormal terminal axon extension and branching. Thus, partial loss of sympathetic neurons in vivo is most probably a consequence of inadequate trophic factor acquisition because of abnormal, but not completely absent, sympathetic target tissue innervation. In Egr3^{-/-} mice, blepharoptosis was a result of impaired sympathetic innervation to the tarsal musculature of the eyelid and corneal ulcerations resulted from abnormal secretomotor function of tarsal Meibomian glands, which normally provide lubrication to the surface of the eye. In addition, denervation of the pineal gland was found to disrupt light cycle-dependent and sympathetic activity-induced AANAT expression, which is required for normal melatonin synthesis and circadian rhythms. Cardiac function was also impaired by poor sympathetic terminal axon extension and branching to the myocardium, which resulted in abnormal regulation of heart rate and contractility.

Egr3 has not been previously implicated in SNS development or human dysautonomia but it is remarkable that Egr3-deficient mice have physiological abnormalities similar to humans with dysautonomia. The etiology of most human dysautonomias is unknown, but some congenital cases have been associated with abnormal NGF signaling and transcriptional regulation. For example, hereditary sensory and autonomic neuropathy (HSAN) type IV is associated with mutations in the TrkA gene (OMIM #256800), while HSAN type V is associated with mutations in the NGFB gene (OMIM #608654). Similarly, HSAN type III (Riley-Day syndrome; familial dysautonomia), the most common congenital dysautonomia, is associated with mutations in the IKBKAP gene, which encodes a protein component of the halo-transcriptional elongator complex (OMIM #223900). Taken together with this new evidence that Egr3 has an important role in SNS development and NGF signaling, it seems plausible that Egr3-dependent gene regulation could be involved in some forms of congenital and/or acquired human dysautonomia.

Egr3, a potential transcriptional effector of NGF signaling

The density of sympathetic innervation is correlated with the timing and extent of NGF production by target tissues (Korsching and Thoenen, 1983; Korsching and Thoenen, 1988; Shelton and Reichardt, 1984). Sympathetic neurons depend upon NGF for their survival (Crowley et al., 1994; Levi-Montalcini and Booker, 1960) and for terminal axon extension and branching within target tissues (Glebova and Ginty, 2004; Kuruvilla et al., 2004). Although Egr3 does not appear to have a direct role in sympathetic neuron survival like NGF, it does appear to have a similar role in sympathetic terminal axon extension and branching within target tissues. In the absence of Egr3, we observed a heterogeneous effect on sympathetic target tissue innervation with some tissues showing highly diminished innervation (e.g. eyelids and pineal gland), while in other tissues there was moderate loss of innervation associated with prominent terminal axon extension and branching defects (e.g. salivary glands, trachea, spleen and heart). These results are strikingly similar to NGF/Bax double knockout (dKO) mice, where inhibition of NGF- and Bax-dependent sympathetic neuron death (Deckwerth et al., 1996) made it possible to study target tissue innervation in the absence of NGF (Glebova and Ginty, 2004). Although NGF/Bax dKO mice died shortly after birth before the sympathetic nervous system was completely developed, sympathetic axons extended along vessels and encroached upon target tissues where they either did not innervate them or they innervated them with highly attenuated terminal axon extension and branching. Thus, although Egr3 is just one of many genes regulated by NGF signaling within sympathetic neurons, it is reasonable to assume that it has a particularly important role in NGF-mediated gene expression involved in target tissue innervation. To better understand how Egr3 mediates particular aspects of NGF signaling, it will be important for future studies to identify and characterize the target genes regulated by Egr3 in sympathetic neurons.

Sympathetic dysautonomia in Egr3-deficient mice

Unlike mice lacking NGF or TrkA, most Egr3-deficient mice survive past the perinatal period with many signs and symptoms of sympathetic dysautonomia because of abnormal, but not completely absent, sympathetic target tissue innervation. In Egr3^{-/-} mice, blepharoptosis was a result of impaired sympathetic innervation to the tarsal musculature of the eyelid and corneal ulcerations resulted from abnormal secretomotor function of tarsal Meibomian glands, which normally provide lubrication to the surface of the eye. In addition, denervation of the pineal gland was found to disrupt light cycle-dependent and sympathetic activity-induced AANAT expression, which is required for normal melatonin synthesis and circadian rhythms. Cardiac function was also impaired by poor sympathetic terminal axon extension and branching to the myocardium, which resulted in abnormal regulation of heart rate and contractility.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/17/2949/DC1
References


