Published in final edited form as:

Mol Cell Neurosci. 2011 March ; 46(3): 671–680. doi:10.1016/j.mcn.2011.01.008.

Cytokines inhibit norepinephrine transporter expression by decreasing Hand2

Michael J. Pellegrino¹, Diana C. Parrish¹, Richard E. Zigmond², and Beth A. Habecker¹

¹ Department of Physiology and Pharmacology, Oregon Health & Science University, Portland, OR, 97239, USA

² Department of Neurosciences, Case Western Reserve University, Cleveland, OH, 44106, USA

Abstract

Functional noradrenergic transmission requires the coordinate expression of enzymes involved in norepinephrine (NE) synthesis, as well as the norepinephrine transporter (NET) which removes NE from the synapse. Inflammatory cytokines acting through gp130 can suppress the noradrenergic phenotype in sympathetic neurons. This occurs in a subset of sympathetic neurons during development and also occurs in adult neurons after injury. For example, cytokines suppress noradrenergic function in sympathetic neurons after axotomy and during heart failure. The molecular basis for suppression of noradrenergic genes is not well understood, but previous studies implicated a reduction of Phox2a in cytokine suppression of dopamine beta hydroxylase. We used sympathetic neurons and neuroblastoma cells to investigate the role of Phox2a in cytokine suppression of NET transcription. Chromatin immunoprecipitation experiments revealed that Phox2a did not bind the NET promoter, and overexpression of Phox2a did not prevent cytokine suppression of NET transcription. Hand2 and Gata3 are transcription factors that induce noradrenergic genes during development and are present in mature sympathetic neurons. Both Hand2 and Gata3 were decreased by cytokines in sympathetic neurons and neuroblastoma cells. Overexpression of either Hand2 or Gata3 was sufficient to rescue NET transcription following suppression by cytokines. We examined expression of these genes following axotomy to determine if their expression was altered following nerve injury. NET and Hand2 mRNAs decreased significantly in sympathetic neurons 48 hours after axotomy, but Gata3 mRNA was unchanged. These data suggest that cytokines can inhibit NET expression through downregulation of Hand2 or Gata3 in cultured sympathetic neurons, but axotomy in adult animals selectively suppresses Hand2 expression.

Keywords

Sympathetic neurons; Cytokines; Axotomy; Phox2a; Hand2; Gata3

Address correspondence to: Beth A. Habecker, PhD, Dept. of Physiology & Pharmacology, L-334, 3181 Sam Jackson Park Rd, Portland, OR, 97239, Phone: (503) 494-0497, Fax: (503) 494-4352, habecker@ohsu.edu.

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1. Introduction

Functional noradrenergic transmission requires the synthesis, secretion, and reuptake of the neurotransmitter norepinephrine (NE). Dopamine- β -hydroxylase (DBH), the final enzyme in the NE biosynthetic pathway, and the NE transporter (NET), which terminates NE neurotransmission by reuptake of NE from the synaptic cleft, are unique to noradrenergic neurons. Their expression is coordinately regulated during development by several transcription factors including *Insm1*, *Gata3*, *Hand2*, *Phox2a*, and *Phox2b*. Regulation of noradrenergic properties in mature neurons is less clearly understood, but *Hand2* is required for maintenance of noradrenergic genes.

Although the majority of sympathetic neurons are noradrenergic, there are two well-characterized scenarios when the noradrenergic phenotype is suppressed and replaced by a cholinergic or peptidergic phenotype. First, a small sub-set of sympathetic neurons becomes cholinergic during development, using acetylcholine as their primary neurotransmitter. In addition, adult sympathetic neurons whose axons have been damaged decrease expression of noradrenergic genes and production of NE. This can occur acutely due to axotomy, or it can develop over a longer period of time after damage to the target tissue, such as the switch to a cholinergic phenotype in cardiac sympathetic neurons during the development of heart failure. Sympathetic dysfunction in heart failure includes a loss of NE uptake, which contributes to the cardiac pathology. Suppression of noradrenergic function in both of these cases is caused by inflammatory cytokines that act through the gp130 receptor.

Cytokines suppress noradrenergic genes in cultured sympathetic neurons, providing a model system to study the mechanisms underlying this phenomenon. Cytokines also suppress the homeodomain transcription factor *Phox2a* in cultured sympathetic neurons. *Phox2a* disappears from sympathetic neurons that become cholinergic during development, suggesting that it might be involved in maintenance of the noradrenergic phenotype. Two other transcription factors that have been linked to maintenance of noradrenergic identity are *Gata3* and *Hand2*. We have used neuroblastoma cell lines and sympathetic neurons to investigate which transcription factors mediate cytokine suppression of noradrenergic genes. We paid special attention to the NET gene and promoter, since very little is known about what regulates this important gene.

2. Materials & Methods

2.1. Materials

SK-N-BE(2)M17 human neuroblastoma cells and pNET4000(i)Luc luciferase promoter construct were generous gifts from Dr. Kwang-Soo Kim (McLean Hospital/Harvard Medical School, Waltham, MA). This construct includes the entire regulatory domain required to drive noradrenergic-cell specific expression of NET. The *Hand2* expression construct was a generous gift from Dr. Peter Cserjesi (Tulane University, New Orleans, LA). The 4.5TH-fLuc promoter construct was a generous gift from Dr. Dona Chikaraishi (Duke University Medical Center, Durham, NC), and includes the promoter region sufficient to drive tissue-specific expression of TH. The *Arix/Phox2a* expression construct was a generous gift from Dr. Elaine Lewis (NIH). The *Gata3* expression construct was purchased from Addgene (Cambridge, MA; deposited to Addgene by Gokhan S. Hotamisligil, Harvard University). The pcDNA 3.1 expression vector and cell culture reagents were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from ATCC (Manassas, VA). CNTF was from PeproTech (Rocky Hills, NJ) and UO126 was from Cell Signaling Technology (Beverly, MA). Nerve growth factor (NGF) was purchased from Austral Biologicals (San Ramon, CA) and from BD Biosciences (Bedford, MA). Dispase was purchased from Boehringer Mannheim (Indianapolis, IN). Collagenase type II was purchased from

Worthington Biochemicals (Freehold, NJ). Nitrocellulose membranes were purchased from Schleicher & Schuell (Dassel, Germany). Protease inhibitor cocktail tablets were purchased from Roche Applied Science (Indianapolis, IN). Phosphatase inhibitor cocktails, poly-L-lysine and phenylmethanesulfonyl fluoride (PMSF) were from Sigma Chemical Co. (St. Louis, MO), and Maxi Prep Kits were from Qiagen (Valencia, CA). SuperSignal Dura was purchased from Thermo Scientific (Rockford, IL). 4x sample buffer and 20x reducing agent were from Bio-Rad (Hercules, CA). The pRL-null *renilla* luciferase construct and the Dual-Luciferase Reporter Assay system were purchased from Promega (Madison, WI). The ChIP-IT Express Enzymatic kits were from Active Motif (Carlsbad, CA).

2.2. Antibodies

Rabbit anti-actin (A2066) was obtained from Sigma Chemical Co. (St. Louis, MO), rabbit anti-tyrosine hydroxylase (AB152) from Millipore (Temecula, CA), rabbit anti-Hand2 (H-110), rabbit anti-c-fos (H-125), goat anti-Phox2b (H-20), and rabbit anti-CREB (C-21) were from Santa Cruz Biotechnologies (Santa Cruz, CA). Rabbit anti-ERK1/2 (#9102) was from Cell Signaling Technologies (Danvers, MA). Goat anti-GATA3 (AF2605) was from R&D Systems (Minneapolis, MN). The Phox2a antibody was a generous gift from Dr Jean-Francois Brunet (Ecole Normale Supérieure, CNRS, Paris, France). Anti-rabbit secondary antibody conjugated to horseradish peroxidase (HRP) and anti-goat secondary conjugated to HRP were from Santa Cruz Biotechnologies (Santa Cruz, CA).

2.3 Animals

Pregnant Sprague-Dawley rats were purchased from Charles River (Cambridge, MA). C57Bl/6 mice were purchased from The Jackson Laboratories West (Sacramento, CA). gp130^{DBH-Cre/lox} mice were generated as previously described. All mice were kept on a 12h:12h-light dark cycle with ad libitum access to food and water.

2.4. Cell Culture

Sympathetic neurons were dissociated from the superior cervical ganglia of newborn rat pups as described. Neurons were plated onto plates coated with poly-L-lysine and mouse type IV collagen. Cultures were maintained at 37°C in 5% CO₂. Neurons were cultured in C2 media (DMEM/F12 1:1, BSA 0.5mg/ml, L-glutamate 1.4mM, selenium 30nM, transferrin 10µg/ml, insulin 10µg/ml) supplemented with 100 units/ml penicillin G, 100 µg/ml streptomycin sulfate, 50ng/ml NGF, and 3% fetal bovine serum. Neurons were cultured with the anti-mitotic agent AraC (1µM) for 2 days to deplete non-neuronal cells prior to treatment with CNTF. Half the media was changed every 2 days, including new CNTF.

The human neuroblastoma SK-N-BE(2)M17 cells and SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 units/mL penicillin G, and 100µg/mL streptomycin sulfate and maintained in 5% CO₂.

2.5. Chromatin Immunoprecipitation Assays (ChIPs)

For ChIP experiments, CNTF (100ng/ml) and UO126 (20 µM) were used for the indicated times. ChIP assays were performed with the aid of ChIP-IT Express Enzymatic kits. Briefly, for both M17 and SH5Y cells, two 10cm plates of confluent cells were used for each condition. Cells were fixed with a formaldehyde solution for 5 minutes (M17) or 8 minutes (SH5Y). Cells were washed with 1X PBS and fixation is stopped using a Glycine solution for 5 minutes. Cells were scraped, lysed, gently dounced with a homogenizer on ice for 10 strokes to aid in nuclei release. Nuclei were isolated and an enzymatic cocktail was used to shear the chromatin for 15 minutes (M17) or 10 minutes (SH5Y). Sheared chromatin was immunoprecipitated overnight at 4°C with the appropriate antibody (see antibody section) or

negative IgG. Immunoprecipitates were isolated using magnetic beads, chromatin was eluted, cross-links were reversed in a thermocycler at 95°C for 15 minutes, and Proteinase K was used to digest the protein. DNA was used in PCR reactions using the following parameters: denaturing at 94° for 3 minutes followed by 36 cycles of 94°C for 20 seconds, 59°C for 30 seconds, 72°C for 30 seconds. The following primer sets were used: DBH Forward-CCGCCTGTCTACTTCAACTCC, DBH Reverse-TGTACATGAAGGCTGCCTCC, TH Forward- CACACGGCCTGGAATCTTCTGG, TH Reverse- TCAAGAGAGCACACAGGGAGGG, NET Forward- ATCAGAGGCCTGGTGTCTTGG, NET Reverse- TGGAGCCTGTTAGCGCTAATGG. Predicted amplicon sizes are: DBH- 283 base pairs, TH- 171 base pairs, NET- 161 base pairs.

2.6. Real-time PCR

Real-time PCR was performed as described previously. Cultured neurons were harvested using the Cells-to-cDNA II kit from Ambion/ABI (Foster City, CA) and reverse transcribed. RNA was isolated from individual ganglia using the Ambion RNAqueous micro kit. For PCR amplification, 4µl of reverse transcriptase reactions were used in a total volume of 20µl. Each sample was assayed in duplicate. Real-time PCR was performed using the 7500 Real-Time PCR System from ABI, and fluorescence measurements were taken from the exponential phase of amplification for all standards and samples. TaqMan® Universal PCR Master Mix was used with pre-validated TaqMan primers to GAPDH, TH, NET, Hand2, GATA3, and Phox2a from ABI. Standard curves were generated for each primer set from known amounts of sympathetic neuron RNA (100ng-0.8ng), and each gene of interest was normalized to GAPDH mRNA from the same sample.

2.7. Transient Transfection and Reporter Assays

DNA used for transfection was purified using the Qiagen Maxiprep kit. M17 cells were plated at a density of 1.0×10^5 cells per well in 6-well plates and immediately treated with 100ng/mL CNTF. 48 hours after plating, media were removed and cells were transfected using the CaPO₄ method. Cells were transfected with 1µg of pNET4000(i)Luc or 1µg of 4.5TH-fLuc and 1µg of Hand2 expression construct, 1µg Arix/Phox2a expression construct, 1µg Gata3 expression construct, or 1µg empty pcDNA3 vector to bring the total amount of DNA to 2µg. 100ng pRL-null was used as a control for transfection efficiency. After a 4 hour incubation with DNA, cells were shocked with 15% glycerol/PBS, washed with PBS, and placed back into culture media containing CNTF. Firefly luciferase activity from promoter luciferase constructs and *Renilla* luciferase activity from the pRL-null internal control were assayed 48 hours after transfection using the Dual-Luciferase Reporter Assay system. Firefly luciferase values were normalized to *Renilla* luciferase values.

For inhibitor experiments, M17 cells were plated at a density of 1.0×10^5 cells per well in 6-well plates and allowed to adhere overnight before being treated with 100ng/mL CNTF and 10µM UO126. Transfections were performed 48 hours later as described above and cells were placed back in culture media containing CNTF and UO126. Cells were lysed 48 hours after transfection in RIPA buffer (50mM Tris, 150mM NaCl, 1mM EDTA, 5µg/mL aprotinin, 5µg/mL leupeptin, 1% Triton x-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.4) with phosphatase inhibitor cocktail I (1:1000), phosphatase inhibitor cocktail II (1:1000), phenylmethanesulfonyl fluoride (1:1000), and 25x protease inhibitor cocktail to a total volume of 200µL.

2.8. Western Blot Analysis

SK-N-BE(2)M17 cells- Transfections and cell lysis were performed as described previously. Samples were sonicated for 5 seconds on ice to reduce viscosity. Samples were diluted in

sample buffer (4x sample buffer and 20x reducing agent) to a total volume of 60 μ L before heating at 95°C for 10 minutes and separating on 4–12% Bis-Tris acrylamide gels. Membranes were blocked in 5% nonfat dry milk in TBST (100mM NaCl, 10mM Tris, and 0.1% Tween 20, pH 7.4) and incubated at 4°C overnight in antibodies against β -Actin, Hand2, or Gata3 diluted 1:1000 in TBST with 5% dry milk. After washing in TBST, membranes were incubated for 1 hour at room temperature with appropriate secondary antibody diluted 1:10,000 in TBST with 5% dry milk. Membranes were washed again in TBST and immunoreactive bands were visualized by chemiluminescence. Band intensity was recorded using a –40°C charge-coupled device camera and quantified using Labworks software (UVP, Upland, CA). Band intensities obtained for Hand2 and Gata3 were normalized to β -Actin to control for differences in cell number between the wells.

Sympathetic neurons- Neurons were placed on ice and lysed in RIPA buffer (1% Triton-X 100, 1% sodium deoxycholate, 0.2% sodium dodecyl sulfate, 125mM NaCl, 50mM Tris pH 8.0, 10% glycerol, 1mM EDTA, 25mM β -glycerolphosphate, 25mM NaF, 1mM sodium orthovanadate, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1mM phenylmethylsulfonyl fluoride, 1x complete protease inhibitor cocktail-EDTA free). Samples were sonicated for 30 seconds in a bath sonicator. Proteins were size fractionated on denaturing gels and transferred to nitrocellulose membranes for blotting. Membranes were blocked in 5% nonfat dry milk in TBST and incubated at 4°C overnight in antibodies against TH, ERK1/2, β -Actin, Phox2a, and Phox2b diluted 1:1000 in TBST with 5% dry milk. After washing in TBST, membranes were incubated for 1 hour at room temperature with appropriate secondary antibody diluted 1:10,000 in TBST with 5% dry milk. Membranes were washed again in TBST and immunoreactive bands were visualized by chemiluminescence. Bands were captured by a UVP camera and analyzed using Labworks Software.

2.9. Axotomy

Six week old mice were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), and neurons in one SCG were axotomized by unilateral transection of the major postganglionic nerves of the ganglion, the external and internal carotid nerves. Surgical procedures were approved by the Institutional Animal Care and Use Committee of Case Western Reserve University. Forty eight hours after surgery both ganglia were removed into RNAlater and mRNA levels were measured as described above.

2.10. Statistics

Statistical analyses were carried out using GraphPad Prism 5.0. Student's t-test was used for a single comparison between 2 groups. One way analysis of variance with the Dunnett post hoc test used to compare multiple treatments to the control. One way analysis of variance with a Newman-Keuls post hoc test was used to compare multiple groups to one another.

3. Results

3.1. CNTF suppresses noradrenergic genes and Phox2a

Gp130 cytokines, such as ciliary neurotrophic factor (CNTF), suppress noradrenergic genes in sympathetic neurons. In order to investigate the mechanisms of this suppression we first confirmed that CNTF decreased TH and NET mRNA in sympathetic neurons (Fig 1AB). Western blot analysis revealed significantly reduced TH protein levels 3 days after CNTF treatment, consistent with previous studies (Fig 1C).

Phox2a has been implicated in regulation of noradrenergic genes during development. Phox2a expression is suppressed in sympathetic neurons that acquire a cholinergic phenotype, and the timing is consistent with a role for Phox2a in regulating the loss of

noradrenergic gene expression. Cytokines suppress Phox2a in cultured sympathetic neurons and we confirmed that 3 and 7 days of CNTF treatment decreased Phox2a levels compared to untreated controls (Fig 2A–C). Phox2a mRNA was also significantly reduced following CNTF treatment (Fig 2D).

3.2. Phox2a does not bind the NET promoter

Using chromatin immunoprecipitation (ChIP), we tested whether Phox2a was bound to promoters of noradrenergic genes. We tested two neuroblastoma cell lines that express noradrenergic genes, and Phox2a bound the DBH and TH promoters in both M17 and SH5Y cells. Short-term (90 min and 24 hr) CNTF stimulation, which activates ERK1/2, did not alter this interaction (Fig 3A). Phox2a exhibited no binding to the NET promoter in either cell line under any conditions. The immunoprecipitation pulled down more Phox2a bound to the DBH promoter in the M17 cell line than in the SH5Y line, but no differences between cell type were observed with the TH promoter. Inputs for all primer sets were positive for both cell lines (Fig 3A). Phox2a binding to the DBH promoter is stimulated by inhibition of the ERK1/2 pathway. We tested whether pharmacological inhibition of ERK1/2 might promote Phox2a binding to the NET promoter in a fashion comparable to DBH. As expected, we observed an increase in binding of Phox2a to the DBH promoter with the MEK inhibitor UO126 compared to vehicle treated cells (Fig 3B). However, inhibition of ERK1/2 activation did not promote binding of Phox2a to the NET promoter. Both CREB and c-fos bound all 3 promoters (Fig 3C). Although there is a Phox2a/b binding site in the NET promoter, we could not detect any Phox2a bound to the promoter, in contrast to robust Phox2a binding to the TH and DBH promoters.

Although we did not observe Phox2a binding to the NET promoter using ChIP, Phox2a may still regulate NET transcription indirectly via protein-protein interactions with other transcription factors. We used a luciferase reporter assay in M17 cells to test the role of Phox2a in cytokine regulation of NET transcription. The NET-Luciferase reporter construct used included the 4Kb of upstream sequence required to drive noradrenergic-cell specific transcription of NET during development. CNTF treatment decreased NET-Luciferase transcription significantly. Addition of Phox2a did not alter NET transcription significantly (Fig 3D), although there was a trend toward decreased (not increased) transcription. The same Phox2a construct stimulated a significant increase in TH transcription, confirming expression and activity of Phox2a (Fig 3E). Blockade of ERK1/2 activation increased Phox2a-dependent basal transcription of the DBH promoter, but had no effect on basal transcription of NET-Luciferase (Fig 3F). In addition, inhibiting ERK1/2 activation did not prevent CNTF-dependent decrease in NET transcription (Fig 3G). Thus, it appears that Phox2a does not regulate the NET promoter.

3.3. CNTF does not alter expression of Phox2b

Since our results suggest Phox2a does not mediate cytokine suppression of NET, we analyzed other candidate transcription factors. Phox2b has an identical DNA binding domain and can compensate for the absence of Phox2a during sympathetic neuron development to stimulate expression of noradrenergic genes. We analyzed Phox2b protein levels after CNTF treatment and saw no significant change in Phox2b in cultured sympathetic neurons (Fig 4).

3.4. Hand2 and Gata3 are regulated by CNTF in cultured neurons and rescue NET transcription

Hand2 and Gata3 are involved in the development of the noradrenergic phenotype. To determine if these transcription factors were regulated by cytokines, we treated dissociated sympathetic neurons with CNTF for 6 days and analyzed mRNA levels using RT-PCR. Both

Gata3 (Fig. 5) and Hand2 (Fig. 6) mRNA were significantly reduced by CNTF treatment. Due to a lack of good antibodies for rodent Gata3 and Hand2, we analyzed protein levels of Hand2 and Gata3 in the M17 human neuroblastoma cells. After 4 days of CNTF, there was significantly less Gata3 (Fig. 5) and Hand2 (Fig. 6) protein when compared to untreated control cells. Using a luciferase reporter assay, we tested whether the CNTF-stimulated reduction of NET transcription could be rescued by overexpression of either Hand2 or Gata3. Both Gata3 (Fig 5D) and Hand2 (Fig 6D) rescued NET luciferase expression in cytokine-treated M17 cells. These data support a role for both Hand2 and Gata3, and not Phox2a/2b, in cytokine suppression of NET expression.

3.5 Axotomy downregulates expression of Hand2 but not Gata3

Culture studies suggested that cytokines suppress NET transcription through suppression of Gata3 and/or Hand2. To examine these transcription factors *in vivo* we carried out axotomy experiments with the SCG. Axon transection leads to the loss of TH expression and NE synthesis in sympathetic neurons due in large part to the actions of inflammatory cytokines. We asked if NET expression, which had not previously been examined in this injury paradigm, was decreased after axotomy. We found that 48 hours after injury NET mRNA levels were less than a third of sham control levels (Fig. 7). If cytokine suppression of Hand2 or Gata3 played a role in the loss of NET transcription, then their expression should also be decreased significantly. Hand2 mRNA levels were approximately 30% of sham control 48 hours after axotomy, but Gata3 levels were unchanged (Fig. 7). This suggests that Hand2, but not Gata3, plays a role in decreasing noradrenergic genes following axotomy.

4. Discussion

4.1. Phox2a/2b

Phox2a was once proposed to be a master regulator of the noradrenergic phenotype because it was expressed in all neurons that express DBH. Further evidence strengthening this hypothesis showed DBH expression was dependent on Phox2a. However, Phox2b has an identical DNA-binding domain and can also transactivate noradrenergic genes *in vitro*. Several studies carried out with the proximal DBH promoter implicated Phox2a and/or Phox2b in regulating DBH transcription. However, generation of mice lacking Phox2a or Phox2b, and more recent reciprocal replacement experiments, revealed that Phox2b plays the more important role in sympathetic neuron differentiation and expression of noradrenergic genes. The differentiation of neural crest cells into noradrenergic sympathetic neurons is complex and requires a network of transcription factors including Sox 4&11, *Insm1*, *MASH1/Ascl1*, *Phox2a/b*, *Hand2*, and *Gata3*.

We looked at Phox2a initially because expression of Phox2a is suppressed *in vivo* when noradrenergic sympathetic neurons are converted to a cholinergic phenotype. Decreased transcription of the DBH gene is tied to the loss of a transcriptional activator rather than the induction of a suppressor, and adding back Phox2a restores DBH expression in cytokine treated cells, and Phox2a binds to the DBH promoter. However, we did not detect Phox2a binding to the NET promoter, and Phox2a did not rescue cytokine suppression of NET transcription (Fig 3D). Furthermore, Phox2b levels were unchanged in cytokine-treated sympathetic neurons, suggesting the lack of Phox2 DNA binding cannot explain the loss of NET transcription. There is only one homeodomain binding site in the NET promoter, in contrast to multiple sites in the TH and DBH promoters, so we expected a weak signal at best with Phox2a binding. However, we did not detect Phox2a bound to the NET promoter even after inhibition of ERK1/2 activity, which increases Phox2a binding to the DBH promoter. We did detect CREB and c-fos binding to the NET promoter, confirming that the ChIP assay and the primers for the NET promoter were working.

4.2. Hand2

Hand2 is critical for development of the heart, the enteric nervous system, and sympathetic ganglia, in addition to its role as a key transcription factor for noradrenergic specific genes like DBH and TH. More recent studies have implicated Hand2 in the acquisition and maintenance of the noradrenergic phenotype. Our data are consistent with this model and suggest that cytokines suppress noradrenergic function, and NET in particular, through downregulation of Hand2. ChIP analysis shows that Hand2 binds the NET promoter, and our luciferase data indicate that Hand2 stimulates NET transcription. Cytokines decrease Hand2 mRNA in sympathetic neurons and Hand2 protein in M17 cells, and Hand2 overexpression was able to rescue cytokine-suppressed NET transcription. Importantly, *in vivo* studies revealed the coordinate loss of Hand2 and NET mRNAs in sympathetic ganglia after axotomy, while Gata3 was unchanged. This is the first evidence in an adult nerve injury model that Hand2 is suppressed and might play a role in the downregulation of noradrenergic genes.

Hand2 may interact directly with the NET promoter since the hNET sequence contains E-boxes, which are Hand2 binding sites. However, indirect interactions may also be involved since Hand2 can increase DBH transcription without directly binding DNA. For example, cAMP stimulates NET transcription. Our data show CREB binds the NET promoter, but Hand2 can also drive CRE-dependent transcription through interactions with CREB binding protein (CBP). The mechanisms underlying Hand2-dependent NET transcription will need further study and might involve interactions with multiple transcription factors.

4.3. Gata3

Gata3 plays a role in development of the ear, heart, T cells, and sympathoadrenal cell types. Gata3 is also required for the survival of developing sympathetic neurons and adult sympathetic neurons, but it is difficult to separate its role in survival from a role in differentiation. Our data indicate that Gata3 is still present in fully differentiated neonatal neurons, and is suppressed by treatment with cytokines. Restoring Gata3 by transfection was sufficient to rescue transcription of a NET promoter construct. This suggests Gata3 affects differentiation without affecting cell survival. It is unclear at this point if Gata3 binds directly to the NET promoter, or if it interacts with other DNA binding proteins. At least six putative Gata3 binding sites [WGATAR W=A/T, R=A/G] are present in 5Kb of the human NET promoter sequence (Genbank AF061198.1), but Gata3 can also interact with other transcription factors to regulate noradrenergic genes.

During development Hand2 and Gata3 are part of a network of transcription factors that can regulate each other in addition to having direct effects on noradrenergic genes. Since overexpression of Gata3 and Hand2 both rescued NET transcription, but only Hand2 was downregulated *in vivo* following axotomy, we wondered if Gata3 was upstream of Hand2 in this context. We were unable to detect an increase in Hand2 protein after overexpression of Gata3 in neuroblastoma cells. However, we cannot conclude that Gata3 did not stimulate Hand2 expression because we were unable to control for transfection efficiency with the western blot procedure.

4.4. Concluding remarks

The differentiation of sympathetic neurons during development requires the coordinate expression of specific transcription factors to regulate noradrenergic genes. We asked if any of these transcription factors are involved in the suppression of these noradrenergic genes by gp130 cytokines. We focused on NET since little is known about regulation of NET and it is one of the genes specific to the noradrenergic phenotype.

Our data identified several differences in transcription factor binding and stimulation of the NET promoter compared to the synthetic enzymes TH and DBH. Although we have examined NET transcription indirectly through NET-luciferase, the 4Kb promoter used in our experiments is sufficient to drive noradrenergic-specific expression of NET *in vivo*, and encompasses a polymorphism site linked to attention-deficit hyperactivity disorder. Therefore, we believe that regulation of this construct accurately reflects regulation of the NET gene *in vivo*. Similarly, the TH-luciferase construct used here contains the full promoter region necessary for tissue-specific expression. Inflammatory cytokines acting through gp130 decreased Hand2, Gata3, and Phox2a in sympathetic neurons, but NET transcription was only regulated by Hand2 and Gata3. Our new data in an adult injury model, together with recent studies by Schmidt and co-workers, implicate Hand2 as a key injury-regulated transcription factor that may control the suppression of noradrenergic function. An interesting new study has identified Satb2 as a cytokine-induced transcription factor that stimulates cholinergic function and suppresses NET expression in sympathetic neurons. It's not known if Satb2 is increased in sympathetic neurons after axotomy. If Satb2 is elevated after injury *in vivo*, it might act together with decreased expression of Hand2 to suppress NET transcription. However, our data indicate that restoring Hand2 is sufficient to bring NET transcription back to control levels. Understanding the factors that regulate NET expression after injury is important. The loss of NE uptake in the face of increased NE release is a key factor of heart failure, and sympathetic dysfunction occurs in other pathologies that include inflammation, such as metabolic syndrome. Thus, these culture and axotomy studies lay the groundwork for further *in vivo* studies investigating the regulation of NET and noradrenergic transmission in other pathological conditions.

Acknowledgments

This work was supported by RO1 HL068231 (BAH) and RO1 NS17512 (REZ). We would like to thank Dr. Kwang-Soo Kim, Dr. Peter Cserjesi, Dr. Dona Chikaraishi, Dr. Elaine Lewis, and Dr. Gokhan Hotamisligil for reagents.

Abbreviations

NE	Norepinephrine
NET	Norepinephrine Transporter
DBH	Dopamine- β -hydroxylase
TH	Tyrosine Hydroxylase
CNTF	Ciliary Neurotrophic Factor
ChIP	Chromatin Immunoprecipitation

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Fig 1. Cytokines suppress noradrenergic genes. (A) Sympathetic neurons were treated for 6 days with CNTF. Real-time PCR analysis of TH (A) and NET (B) mRNA normalized to GAPDH. (C) A representative western blot showing TH (top panel) and ERK1/2 (bottom panel). Data are mean values $\pm$ SEM of triplicate values. Each experiment was repeated at least 3 times. * $p < 0.05$. ** $p < 0.01$.

Fig 2. Cytokines suppress expression of Phox2a. (A,B) Sympathetic neurons were treated with vehicle (control) or CNTF for 3 (A) or 7 (B) days. Phox2a was quantified by western blot. (C) A representative western blot of sympathetic neurons treated with vehicle (Un) or CNTF (CF) for 7 days. Two samples from each condition are shown, each blotted for Phox2a (top panel) and β-Actin (bottom panel). (D) Phox2a mRNA normalized to GAPDH mRNA in sympathetic neurons treated with vehicle or CNTF for 6 days. Data are mean values ± SEM (n=3). *p<0.05.

Fig 3.

Phox2a does not regulate transcription of the NET gene. (A) M17 and SH5Y cells were untreated or treated with CNTF and ChIP analysis was performed with a Phox2a antibody or a negative IgG. PCR reactions were done with primers specific for TH, NET, and DBH. Positive signals from input DNA are shown from both M17 cells (M) and SH5Y cells (S). (B) ChIP analysis was performed with M17 cells that were treated with DMSO (Con) or UO126 (UO) for 90 minutes and PCR reactions were done with primers specific for NET and DBH. (C) M17 cells were subjected to ChIP analysis using antibodies against CREB or c-fos as a positive control. Positive signals from input DNA are shown. PCR reactions were performed with primers specific for TH, NET and DBH, and all three promoters were pulled down with CREB and c-fos antibodies. (D) Cells were treated with control medium (Control) or CNTF (CNTF) for 2 days prior to transfection with luciferase reporter constructs (NET-Luc+pRLNull) and a control vector or Phox2a (Phox2a + CNTF). Cells were maintained for an additional two days in vehicle or CNTF prior to analysis of luciferase. Data are a ratio of firefly luciferase/renilla luciferase. There was no significant difference between CNTF treated cells and CNTF+Phox2a. (E) Cells were treated with vehicle for 2 days prior to transfection with luciferase reporter constructs (TH-fLuc +pRLNull) and a control vector or Phox2a (Phox2a). Cells were maintained for an additional two days prior to analysis of luciferase. Data are a ratio of firefly luciferase/renilla luciferase. (F) Cells were treated with DMSO (Vehicle) or UO126 for 2 days prior to transfection with NET-Luc and pRL-Null. Cells were maintained in vehicle or UO126 for two more days prior to analysis of luciferase. Data is shown as a ratio of firefly luciferase/renilla luciferase. (G) Cells were treated with DMSO (all conditions), CNTF, or CNTF + UO126 for 2 days prior to transfection and then maintained in drug treatments for two days after transfection. Data are a ratio of firefly luciferase/renilla luciferase. Data are mean values $\pm$ SEM of triplicate values. Each experiment was repeated at least 3 times. * $p < 0.05$, ** $p < 0.01$ vs control.

Fig 4. Cytokines do not suppress Phox2b. (A, B) Phox2b protein was quantified in control and CNTF treated sympathetic neurons. (C) A representative western blot showing Phox2b (top panel) and β -Actin (bottom panel) in untreated (Un) and treated with CNTF (CF) for 3 days. Data are mean values $\pm$ SEM (n=3).

Fig 5.

Gata3 is suppressed by CNTF and rescues NET transcription. CNTF suppresses Gata3 mRNA in sympathetic neurons (A) and decreases Gata3 protein (B) in M17 cells. Gata3 levels were normalized to β -Actin levels and shown as a percent of control (mean values $\pm$ SEM, n=3). (C) A representative western blot showing Gata3 (top panel) and β -Actin (bottom) protein levels. (D) M17 cells were treated for 2 days with vehicle (Control) or CNTF. Cells were then transfected with NET-Luc+pRL-Null to monitor transcription, together with a control vector or Gata3 expression plasmid (Gata3 + CNTF) and maintained for two more days in CNTF or control medium. Data are a ratio of firefly luciferase/renilla luciferase. Data are mean values $\pm$ SEM of triplicate values. Each experiment was repeated at least 3 times. *p<0.05, **p<0.01, ***p<0.001.

Fig 6.

Hand2 is suppressed by CNTF and rescues NET transcription. CNTF suppresses Hand2 mRNA in sympathetic neurons (A) and decreases Hand2 protein (B) in M17 cells. Hand2 levels were quantified in the same cells analyzed for Gata3, and Hand2 protein was normalized to the β -Actin shown in Fig 5C. Data shown are percent of control (mean values $\pm$ SEM, n=3). (C) A representative western blot showing Hand2 (top panel). (D) M17 cells were treated for 2 days with vehicle (Control) or CNTF. Cells were then transfected with NET-Luc+pRL-Null to monitor transcription, together with a control vector or Hand2 expression plasmid (Hand2 + CNTF) and maintained for two more days in CNTF or control medium. Data are a ratio of firefly luciferase/renilla luciferase. Data are mean values $\pm$ SEM of triplicate values. Each experiment was repeated at least 3 times. * $p < 0.05$, ** $p < 0.01$.

Fig. 7.

Axotomy regulates NET and Hand2, but not Gata3. NET (A), Hand2 (B), and Gata3 (C) mRNAs were quantified in SCG 48 hrs after axotomy. The contralateral SCG from the same animal served as a control. NET and Hand2 mRNA were decreased significantly after axotomy, but Gata3 mRNA was unchanged. Data are mean values $\pm$ SEM (n=4), * $p < 0.05$, *** $p < 0.001$.