Dataset Paper

Neurogenomics of the Sympathetic Neurotransmitter Switch Indicates That Different Mechanisms Steer Cholinergic Differentiation in Rat and Chicken Models

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Vertebrate sympathetic neurons have the remarkable potential to switch their neurotransmitter phenotype from noradrenergic to cholinergic—a phenomenon that has been intensively studied in rat and chicken models. In both species, loss of noradrenergic markers and concomitant upregulation of cholinergic markers occurs in response to neurotrophic cytokines such as ciliary neurotrophic factor (CNTF). However, other aspects of the neurotransmitter switch including developmental timing, target tissues of cholinergic neurons, and dependence on neurotrophic factors differ between the two species. Here we compare CNTF-triggered transcriptome changes in both species by using DNA microarrays. CNTF induced changes in 1130 out of 16084 analyzed genomic loci in rat sympathetic neurons. When this set of genes was compared to CNTF-induced changes in the chicken transcriptome, a surprisingly small overlap was found—only 94 genes were regulated in the same direction in chicken and rat. The differential responses of the transcriptome to neurotrophic cytokines provide additional evidence that the cholinergic switch, although conserved during vertebrate evolution, is a heterogeneous phenomenon and may result from differential cellular mechanisms.

1. Introduction

Transmitter phenotypes are specified at defined stages during neuronal development through coordinated expression of complex sets of gene products, involved in the synthesis and transport of transmitters [1]. Once specified, the neurotransmitter phenotype remains permanently unaltered for most cell types. Rodent sympathetic neurons are a notable exception since they can transdifferentiate from a fully functional noradrenergic to a cholinergic phenotype *in vitro* and *in vivo* [2, 3]. This neurotransmitter switch serves as a textbook example of plasticity in postmitotic neurons and depends on growth factor signalling [4, 5]. The first identified cholinergic differentiation factors are ligands of the gp130/LIFR β receptor complex and belong to the family of neurotrophic cytokines [6]. *In vivo* gp130/LIFR β ligands are secreted from the target structure of cholinergic sympathetic neurons such as eccrine sweat glands [7]. *In vitro*, the sympathetic superior cervical ganglion (SCG) neurons dissected from postnatal rats represent the best studied model for cholinergic sympathetic development. These noradrenergic

cells, upon exposure to neurotrophic cytokines such as ciliary neurotrophic factor (CNTF) or leukaemia inhibitory factor (LIF), downregulate markers for the noradrenalin synthesis including the *tyrosine hydroxylase* (*Th*) or the *norepinephrine transporter* (*Net*). At the same time, cholinergic markers, for example, *choline acetyltransferase* (*Chat*) and the *vesicular acetylcholine transporter* (*Vacht*) are massively upregulated. We have recently identified a signalling module that is essential for this process [8, 9]. It comprises activation of p38 MAPK and upregulation of the nuclear matrix protein *Satb2* downstream of gp130/LIFR β .

While progress has been made in the mechanistic understanding of how neurotrophic cytokines trigger cholinergic transdifferentiation *in vitro* and *in vivo*, a number of questions concerning other aspects of cholinergic differentiation still remain open. It has become evident that apparently distinct types of rodent sympathetic cholinergic neurons can be discriminated by their mode of development (reviewed in [10]). In particular, a group of cholinergic cells develops early, before neurons establish contact with their target tissue. The development of these cells depends on the Ret receptor

tyrosine kinase which is a component of the receptor for neurotrophic factors of the GDNF family. Therefore, different classes of growth factors appear to regulate cholinergic differentiation of rodent sympathetic neurons before and after target tissues innervation. This concept is consistent with findings in another well-investigated model of sympathetic development—the chicken embryo. Cholinergic differentiation in avian sympathetic neurons *in vitro* is induced not only by neurotrophic cytokines but also by GDNF family ligands (GFLs) and the neurotrophin NT3 [11, 12]. *In vivo*, the GDNF receptor Ret and the NT3 receptor TrkC are coexpressed with cholinergic markers in the maturing avian sympathetic nervous system indicating a function of their ligands [12]. Thus, commonalities as well as differences exist as to how the cholinergic differentiation is brought about in rat and chicken models. It remains to be established whether the differences are caused by divergent evolution (300 million years) or whether they mainly reflect the ontogenetic differences of cells analyzed at different stages of sympathetic development.

In the current study, we employed DNA microarrays to analyze the transcriptome changes that occur in rat sympathetic neurons cultured for 7 days in the presence of CNTF, a treatment which triggers cholinergic differentiation through the p38 MAPK/Satb2 signalling module. A comparison of the resulting dataset with the effect of procholinergic treatments on the chicken transcriptome revealed surprisingly large differences. A small set of genes was identified as coregulated with neurotransmitter markers in both species under all experimental conditions and thus represents the evolutionary conserved neurotransmitter synexpression group of sympathetic neurons. The newly identified marker genes provide a resource for future functional analysis and stratification of neurotransmitter phenotypes of the sympathetic nervous system.

2. Methodology

Primary cell culture was prepared as follows. Sympathetic neurons from superior cervical ganglia (SCG) of newborn rats (P0-P2) were dissociated and plated at a density of 100 000 cells per well onto 6-well plates previously coated with poly-L-ornithine (Sigma) and laminin (Sigma). Cells were grown for 7 days in Ham's F-14 medium (Invitrogen) supplemented with N2 (Invitrogen) in the presence of either recombinant human NGF (20 ng/mL) alone or CNTF (25 ng/mL, Peprotech) and NGF (5 ng/mL). Cultures were maintained at 37°C in a 3% CO₂ atmosphere and were fed every second day. The proliferation of nonneuronal cells was suppressed by treatment with 15 mM aphidicolin (Sigma) starting from day 2 of the culture.

Total RNA was extracted by using TRIzol Reagent (Invitrogen) and cleaned up with RNeasy MinElute Kit (QIAGEN). The quality and size distribution of extracted RNA were evaluated by RNA Nano Kit on the Agilent's Bioanalyzer.

Microarray experiments and analysis of gene expression data were performed as follows. Affymetrix Rat Genome 230 2.0 and Chicken Genome Arrays were used for genome-wide expression profiling experiments. RNA samples extracted from three independent cell cultures were used for each

experimental group. Sample labelling, hybridization, and scanning were carried out at the Expression Profiling Unit (Innsbruck Medical University) according to the Affymetrix standard protocols. Normalization and computation of expression values were performed by using GC-RMA method included in *afflmGUI* module of the Bioconductor open source software [13, 14]. For the assessment of differential expression, significance analysis of microarray (SAM) algorithm was applied [15]. A false discovery rate (FDR) of <1% and 1.5-fold change were used as a cut-off for statistical significance. GO-term enrichment analysis was done by using the DAVID Functional Annotation Clustering Tool [16, 17]. As a statistical measure for overrepresentation, the EASE score, a modified Fisher exact *P* value, was used. For the identification of overrepresented clusters of functional categories, the chicken dataset consisted only of genes that had the corresponding rat orthologue.

Quantitative RT-PCR (qRT-PCR) was used as follows. One microgram of total RNA from each sample was reverse transcribed in the presence of oligo-dT primer in a total volume of 20 µL. After dilution with 30 µL of water, 1 µL of the diluted cDNA was used as a template for amplification with iQ SYBR Green Supermix (BIO-RAD). qRT-PCR quantification was performed on an iCycler iQ Optical System (BIO-RAD) with the following thermal cycling conditions: initial denaturation at 95°C for 10 min followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and fluorescence detection at 72°C for 60 s. All amplification reactions were conducted in triplicates. The gene-specific primers used in this study are listed in Table 1. For each primer pair, the amplicon size was confirmed by agarose gel electrophoresis. Relative expression ratios were calculated by using $\Delta\Delta C_t$ method and GAPDH as a reference gene.

Tissue preparation and immunohistochemistry were as follows. Stellate ganglia from E18 rat embryos and P40 rats were dissected and fixed in 4% paraformaldehyde in PBS for 15 min. The ganglia were cryoprotected by immersion in 25% sucrose solution in PBS at 4°C and mounted in Tissue-Tek medium (Sakura Finetek). Fourteen-micrometer cryostat sections were mounted on gelatine-coated slides and air dried for 1 h at 37°C. The sections were fixed for 5 min in precooled acetone, washed 2 × 5 min each in TBS, and permeabilized for 10 min in 0.25% Triton-X-100 in TBS (TBST). After blocking in 10% normal serum, 1% BSA in TBST, sections were incubated with primary antibodies, rabbit anti-Vacht (1 : 2000, Sigma); mouse anti-ALK (1 : 500; a generous gift from Marc Vigny); and rabbit anti-CGRP (1 : 300; AbD Serotec) diluted in blocking solution at 4°C overnight. Subsequently, sections were rinsed in TBST and incubated in Alexa-Fluor-conjugated secondary antibodies (Invitrogen) for 1 h at room temperature. Finally, the sections were incubated for 2 min with 300 nM DAPI in PBS, rinsed again in TBS and distilled water, and mounted with Mowiol. Images were taken with an ApoTome Imaging System based on Axiovert 200M (Zeiss) using AxioVision software.

Differences in gene expression between the noradrenergic and cholinergic condition were studied in cultures of neonatal rat SCG primary neurons. In the noradrenergic condition, neuronal cultures were exposed to NGF alone whereas

TABLE 1

Gene Symbol	Forward Sequence	Reverse Sequence
<i>Arhgap22</i>	GATTGAGTACCAGAGGAGCC	ATGGCATACTTCTTCTTCTCTCGG
<i>Bai3</i>	ACAGATTTTCGGGACATACCAAC	ATTGAGACACTTCTCCCACTCC
<i>Bdnf</i>	AAACCATAAGGACGCGGACTT	AGGCTCCAAAGGCACTTGACT
<i>Cbln2</i>	AAGGTGGCCTTCTCCGCTAC	GCTACAAATATACTCGAGGCAAG
<i>Gapdh</i>	CTGGAGAAACCTGCCAAGTATG	ACAACCTGGTCCTCAGTGTAG
<i>Hmx1</i>	TCTCAGCAAAGTCTGGGTACC	TCCATGACCCTGCTCTCTCTC
<i>Hpcal1</i>	CGAACAGACAAGATCTTCAGGC	GTGTTTCTCACAACGTGCCAAAAG
<i>Lmo3</i>	TACCTGAGGCTGTTTGGTGTG	CGAAGCAGTCCAGGTGGTAC
<i>Pcdh7</i>	GCTTCTAAACCGAATGGACAGAG	GGGAACTGGATTTGTAATGCCTTG
<i>Pnoc</i>	AGATGCAGCACCTGAAGAGAATG	TTCTGCAGCTGCTTCTGCTC
<i>Rgs9</i>	CAGGCTGAAACCCAAGAAAGTAG	TGACTTCCTTTTCCGTGGTCTG
<i>Zfyve28</i>	ACGAACTATGCTAGCGACCTAAG	CTGGCACAAATGCACAGTCTTC

TABLE 2: Fold changes measured by Affymetrix Gene Chip and qRT-PCR analysis for 11 randomly picked genes.

Gene Symbol	Affymetrix Fold Change	Real-Time RT-PCR Fold Change	S.E.M.
<i>Hmx1</i>	-1.9	-2.9	0.6
<i>Bai3</i>	-1.9	-3.0	0.3
<i>Rgs9</i>	-1.8	-2.3	0.3
<i>Arhgap22</i>	-1.8	-2.1	0.4
<i>Zfyve28</i>	1.8	4.5	1.6
<i>Hpcal1</i>	2.3	3.8	1.1
<i>Pnoc</i>	2.6	3.4	0.9
<i>Pcdh7</i>	10.3	25.4	5.5
<i>Lmo3</i>	-1.5	-2.9	0.5
<i>Cbln2</i>	3.0	15.3	2.7
<i>Bdnf</i>	4.2	4.6	0.5

in the cholinergic condition primary neurons were treated with a combination of NGF and CNTF. The presence of NGF is required for neuronal survival. RNA was extracted after 7 days *in vitro* when maximal expression of the cholinergic locus is reached after CNTF treatment. Transcripts obtained from three independent culture experiments per treatment were analyzed on Affymetrix GeneChip Rat Genome 230 2.0 Arrays. A total of 1120 genes were found to be differentially regulated (FDR < 1%, 1.5-fold change cut-off)—transcripts of 656 genes were more abundantly expressed in the cholinergic condition whereas transcripts of 464 genes were enriched in the noradrenergic condition. A list of those genes, including their fold changes following CNTF treatment, is given in Dataset Item 1 (Table). Since 16084 annotated genes are represented on the array, we conclude that 7.03% of all tested genes were differentially regulated by CNTF compared to NGF in rat SCG primary neurons. Essentially, all classical neurotransmitter phenotypic markers were amongst the identified set of differentially expressed genes and showed expression changes as expected—the levels of the noradrenergic markers *Th*, *Net*, *dopamine beta hydroxylase (Dbh)*, *GTP cyclohydrolase 1 (Gch1)*, and somatostatin were reduced

FIGURE 1: Correlation between fold changes measured by Affymetrix Gene Chip and qRT-PCR analysis for selected genes.

after CNTF treatment whereas the transcripts for *Chat*, *Vacht*, *vasointestinal polypeptide (Vip)*, and substance P were enriched.

To validate the microarray results, we analyzed the expression of 11 randomly picked genes by qRT-PCR using RNA samples isolated from independent SCG cultures. Table 2 shows the fold changes measured by Affymetrix Gene Chip and qRT-PCR analysis for selected genes. A strong positive correlation between the two measurements was detected (Figure 1).

Thus, we conclude that our results appropriately reflect the response of sympathetic neurons to CNTF since they can be validated by an independent method and the identified set of differentially expressed genes contains all known noradrenergic and cholinergic marker genes. However, the large number of identified differentially expressed genes indicates that neuropoietic cytokines exert a pleiotropic effect on the neurons—lots of cellular processes are likely to be activated downstream of CNTF, the cholinergic switch being only one of them. Thus, additional filtering mechanisms are

required for identifying the genes that are coregulated with the neurotransmitter phenotype.

To define more precisely the transcriptional changes relevant to the sympathetic neurotransmitter switch, we used an interspecies comparison as a filter. We compared the set of CNTF-regulated genes in rat SGC neurons, identified in this study, with previously obtained expression profiles of CNTF-regulated genes in chick E12 sympathetic neurons [18]. A pool of homologous neurotransmitter marker genes is regulated in both chick and rodent neurons exposed to CNTF, indicating that essential regulatory pathways of the neurotransmitter switch have remained conserved during vertebrate evolution. For the interspecies comparison, the genomic annotation of the chicken dataset was updated and identical stringency conditions were applied to both sets of raw data (1.5-fold change threshold; FDR < 1%). Under these conditions, 982 annotated genes out of 18043 testable loci (5.4%) were found to be regulated by CNTF in the chicken model (listed in Dataset Item 2 (Table))—425 genes were downregulated and 557 were upregulated.

First, we characterized the response to CNTF in both species by GO categorization of the differentially regulated genes (GO term Biological Process). The results of the GO analysis, including a statistical rating for the overrepresented Functional Annotation Clusters in both rat and chicken sets of genes, are given in Table 3. We found that identical GO categories are overrepresented in the two groups of differentially expressed genes including such as synaptic transmission, neuron differentiation, neuron development, cell adhesion, and cell migration (followed by asterisks). These findings indicate that the global effect and overall set of functions exerted by CNTF is similar in both species.

Next, we defined how many orthologous genes are present in both datasets (Table 4 and Figure 2). Of the 1120 regulated genes after CNTF treatment in the rat model, 802 genes (71.6%) had a probe set for the orthologous gene on the chicken chip, and 318 (28.4%) genes had no chicken orthologue or feature. For the chicken dataset, we found that 618 orthologues of the 982 loci regulated in chick (62.9%) can be compared with the rat. 364 avian CNTF-regulated genes could not be compared because either no rat orthologue is known or the Affymetrix chip does not contain a corresponding probe set.

Of the 802 comparable loci, 643 (80.2%) were not differentially regulated by CNTF in the chicken model and 65 genes (8.1%) were regulated in the opposite direction in the two species (Table 4 and Figure 2). Thus, although CNTF responses in both species are very similar in terms of the GO categorization of the genes, the actual sets of regulated genes are surprisingly different. Only 94 genes (11.7%) were regulated consistently in both species by CNTF, that is, in the same direction. Expression of 36 genes was downregulated and that of 58 was upregulated by the cytokine. A list of those genes, including their fold changes following CNTF treatment in both species, is given in Dataset Item 3 (Table).

In the avian *in vitro* model cholinergic marker gene expression is not only regulated by neurotrophic cytokines but also by NT3 and GFLs GDNF and NRTN [11, 12, 18].

In order to arrive at the group of genes which are strictly coregulated with the noradrenergic and cholinergic markers, we compared the list of CNTF-regulated genes in rat to the set of avian genes, commonly regulated by CNTF, NT3, and GDNF, described in a previous study [18]. Identical stringency criteria were applied for both groups of genes. Only 33 genes were found to be consistently coregulated with the neurotransmitter phenotype in both species under all experimental conditions applied (Dataset Item 4 (Table)). This evolutionary conserved synexpression group contains the noradrenergic markers *Th*, *Gch1*, and *Net* as well as the cholinergic markers *Chat*, *Vacht*, *Vip*, and substance P.

The unexpected, very limited overlap, observed in the interspecies comparison, raised the question whether it is explained by differences in the experimental conditions of the two model systems. To address this question, we randomly chose several differentially expressed genes as diagnostic markers and determined their expression after varying individual parameters of the culture conditions, for example, dissociated versus ganglionic explants cultures, presence versus absence of NGF in the CNTF condition, and anatomical localization—using SCG versus paravertebral ganglia. None of these parameters explained the differences between the species for the tested marker set.

An alternative explanation for the differential response to cholinergic differentiation factors is that the two *in vitro* culture systems, in essence, model two different developmental events: (1) the neurotrophic cytokine-dependent, target-induced neurotransmitter switch that occurs in rodent sudomotor neurons postnatally, after establishing contact with the sweat gland (rat model); and (2) the cholinergic differentiation process in chick that occurs during embryonic development, does not depend on gp130 cytokines and at least *in vitro* can also be induced by NT3 and GDNF (chick model). Furthermore, a number of similarities exist between the cholinergic differentiation process in chick and the acquisition of early embryonic cholinergic properties in rodents, for example, RET signalling seems to play a role in both processes [19, 20] as opposing to target-derived neurotrophic cytokines [21, 22]. In support of this hypothesis, we tested the *in vivo* expression of representative genes from the two datasets relative to cholinergic markers, and their dependence on GFLs. *ALK* (anaplastic lymphoma kinase receptor) and *Satb2* were chosen as examples from the chicken and rat dataset correspondingly. To label the cholinergic sympathetic neurons *Vacht* immunostaining was used since in the peripheral nervous system *Vacht* is expressed at much higher levels relative to *Chat*; hence *Vacht* expression is readily detectable. At late developmental stages, the noradrenergic and cholinergic populations are clearly segregated within chick paravertebral ganglia [12, 21]. *ALK* has already been shown to be selectively expressed in the RET-positive, cholinergic population [18]. Interestingly, *ALK* is also expressed by *Vacht*-positive, E18 embryonic rat cholinergic neurons (Figure 3(a)) but is absent from the CGRP-positive, sudomotor neurons, undergoing the classical target-dependent, gp130 cytokine-induced neurotransmitter switch (Figure 3(b)). On the other hand, *Satb2*, as differentially expressed gene identified in the rat model system, is not expressed by embryonic cholinergic

TABLE 3: Results of the GO analysis including a statistical rating for the overrepresented Functional Annotation Clusters in both rat and chicken sets of genes.

Category	Chick			Rat		
	Term	Enrichment	Benjamini	Category	Term	Benjamini
Annotation Cluster 1						
Enrichment Score: 6.29						
GOTERM_BP_FAT	GO:0007267~cell-cell signaling*	2.5	0.00014	GOTERM_BP_FAT	GO:0007267~cell-cell signaling*	2.0
GOTERM_BP_FAT	GO:0019226~transmission of nerve impulse	2.6	0.00016	GOTERM_BP_FAT	GO:0019226~transmission of nerve impulse	1.8
GOTERM_BP_FAT	GO:0050877~neurological system process	2.0	0.00013	GOTERM_BP_FAT	GO:0050877~neurological system process	1.4
GOTERM_BP_FAT	GO:0007268~synaptic transmission	2.5	0.00171	GOTERM_BP_FAT	GO:0007268~synaptic transmission	1.7
Annotation Cluster 2						
Enrichment Score: 4.82						
GOTERM_BP_FAT	GO:0048545~response to steroid hormone stimulus*	2.5	0.00013	GOTERM_BP_FAT	GO:0009719~response to endogenous stimulus*	1.7
GOTERM_BP_FAT	GO:0032355~response to estradiol stimulus	4.1	0.00052	GOTERM_BP_FAT	GO:0010033~response to organic substance	1.5
GOTERM_BP_FAT	GO:0009725~response to hormone stimulus	2.0	0.00093	GOTERM_BP_FAT	GO:0009725~response to hormone stimulus	1.7
GOTERM_BP_FAT	GO:0010033~response to organic substance	1.7	0.00097	GOTERM_BP_FAT	GO:0048545~response to steroid hormone stimulus	1.8
GOTERM_BP_FAT	GO:0043627~response to estrogen stimulus	3.0	0.00163			
GOTERM_BP_FAT	GO:0009719~response to endogenous stimulus*	1.8	0.00283			
GOTERM_BP_FAT	GO:0031960~response to corticosteroid stimulus	2.7	0.01533			
GOTERM_BP_FAT	GO:0051384~response to glucocorticoid stimulus	2.7	0.02100			
Annotation Cluster 3						
Enrichment Score: 5.63						
GOTERM_BP_FAT	GO:0022610~biological adhesion*	2.2	0.00052	GOTERM_BP_FAT	GO:0007155~cell adhesion*	2.0
GOTERM_BP_FAT	GO:0007155~cell adhesion*	2.2	0.00052	GOTERM_BP_FAT	GO:0022610~biological adhesion*	2.0
GOTERM_BP_FAT	GO:0016337~cell-cell adhesion	1.9	0.19556	GOTERM_BP_FAT	GO:0016337~cell-cell adhesion	2.0

TABLE 3: Continued.

Chick				Rat			
Category	Term	Fold Enrichment	Benjamini	Category	Term	Fold Enrichment	Benjamini
Annotation Cluster 7				Annotation Cluster 2			
GOTERM_BP_FAT	GO:0030182~neuron differentiation*	2.1	0.00049	GOTERM_BP_FAT	GO:0030182~neuron differentiation*	2.1	0.00001
GOTERM_BP_FAT	GO:0048812~neuron projection morphogenesis	2.7	0.00119	GOTERM_BP_FAT	GO:0048666~neuron development	2.3	0.00002
GOTERM_BP_FAT	GO:0032989~cellular component morphogenesis	2.1	0.00162	GOTERM_BP_FAT	GO:0031175~neuron projection development	2.3	0.00008
GOTERM_BP_FAT	GO:0048858~cell projection morphogenesis	2.5	0.00173	GOTERM_BP_FAT	GO:0030030~cell projection organization	2.1	0.00006
GOTERM_BP_FAT	GO:0000904~cell morphogenesis involved in differentiation	2.5	0.00191	GOTERM_BP_FAT	GO:0048812~neuron projection morphogenesis	2.5	0.00014
GOTERM_BP_FAT	GO:0000902~cell morphogenesis	2.2	0.00193	GOTERM_BP_FAT	GO:0048858~cell projection morphogenesis	2.4	0.00018
GOTERM_BP_FAT	GO:0048666~neuron development	2.1	0.00303	GOTERM_BP_FAT	GO:0032990~cell part morphogenesis	2.3	0.00047
GOTERM_BP_FAT	GO:0032990~cell part morphogenesis	2.4	0.00297	GOTERM_BP_FAT	GO:0000902~cell morphogenesis	2.0	0.00060
GOTERM_BP_FAT	GO:0048667~cell morphogenesis involved in neuron differentiation	2.4	0.01158	GOTERM_BP_FAT	GO:0000904~cell morphogenesis involved in differentiation	2.2	0.00099
GOTERM_BP_FAT	GO:0031175~neuron projection development	2.1	0.01179	GOTERM_BP_FAT	GO:0007409~axonogenesis	2.4	0.00095
GOTERM_BP_FAT	GO:0006928~cell motion	1.9	0.01572	GOTERM_BP_FAT	GO:0032989~cellular component morphogenesis	1.9	0.00168
GOTERM_BP_FAT	GO:0030030~cell projection organization	1.9	0.01716	GOTERM_BP_FAT	GO:0048667~cell morphogenesis involved in neuron differentiation	2.2	0.00545
GOTERM_BP_FAT	GO:0007409~axonogenesis	2.3	0.03259	GOTERM_BP_FAT	GO:0007411~axon guidance	1.9	0.39703
GOTERM_BP_FAT	GO:0007411~axon guidance	2.3	0.25197				
Annotation Cluster 8				Annotation Cluster 8			
GOTERM_BP_FAT	GO:0006928~cell motion*	1.9	0.01572	GOTERM_BP_FAT	GO:0006928~cell motion*	1.75553	0.014535426
GOTERM_BP_FAT	GO:0016477~cell migration	2.1	0.02150	GOTERM_BP_FAT	GO:0016477~cell migration	1.8	0.04952
GOTERM_BP_FAT	GO:0051674~localization of cell	2.0	0.03139	GOTERM_BP_FAT	GO:0051674~localization of cell	1.7	0.09427
GOTERM_BP_FAT	GO:0048870~cell motility	2.0	0.03139	GOTERM_BP_FAT	GO:0048870~cell motility	1.7	0.09427
Annotation Cluster 11				Annotation Cluster 1			
GOTERM_BP_FAT	GO:0001944~vasculature development*	2.2	0.01832	GOTERM_BP_FAT	GO:0001944~vasculature development*	2.5	0.00005
GOTERM_BP_FAT	GO:0048514~blood vessel morphogenesis	2.3	0.024790429	GOTERM_BP_FAT	GO:0001568~blood vessel development	2.5	0.00006
GOTERM_BP_FAT	GO:0001568~blood vessel development	2.1	0.026514792	GOTERM_BP_FAT	GO:0048514~blood vessel morphogenesis	2.6	0.00008
GOTERM_BP_FAT	GO:0001525~angiogenesis	2.3	0.118658169	GOTERM_BP_FAT	GO:0001525~angiogenesis	2.4	0.02003

* Identical GO categories overrepresented in the two groups of differentially expressed genes including synaptic transmission, neuron differentiation, neuron development, cell adhesion, and cell migration.

TABLE 4

	Chick		Rat	
	Number	Percentage (%)	Number	Percentage (%)
Probe sets	37693		31042	
Genes	18043		16084	
Annotated CNTF-regulated genes (FDR < 1%, 1.5-fold change threshold)	982	5.4	1120	7.0
Without orthologue or probe set on the other chip	364	37.1	318	28.4
With orthologue/probe set	618	62.9	802	71.6
Regulated in one species and not in the other	459	74.3	643	80.2
Regulated in both species—opposite direction	65	10.5	65	8.1
Regulated in both species—same direction	94	15.2	94	11.7

FIGURE 2

neurons (E15/E18); *Satb2* expression in the stellate ganglion is induced only after contact with the target tissue and strictly correlates with the sudomotor phenotype (colocalizes with CGRP, a marker of sudomotor cholinergic neurons); *in vitro* *Satb2* is strongly induced by neurotrophic cytokines but not by GFLs [8].

3. Dataset Description

The dataset associated with this Dataset Paper consists of 4 items which are described as follows.

Dataset Item 1 (Table). A list of 1120 genes identified as differentially expressed following CNTF treatment in rat SCG neurons (FDR < 1%; 1.5-fold change cut-off): transcripts of 656 genes were more abundantly expressed in the cholinergic condition (fold change from 1.50 to 174.85) whereas

transcripts of 464 genes were enriched in the noradrenergic condition (fold change from 0.01 to 0.73).

- Column 1: Gene Title
- Column 2: Gene Symbol
- Column 3: Fold Change
- Column 4: Ensembl ID
- Column 5: Entrez Gene ID
- Column 6: Affy ID

Dataset Item 2 (Table). A list of 982 annotated genes out of 18043 testable loci (5.4%) that were found to be regulated by CNTF in the chicken model: 425 genes were downregulated

FIGURE 3: Representative images of sections of E18 (a) and P40 (b) rat stellate ganglia immunostained for *ALK* and *Vacht* (a) and *ALK* and *CGRP* (b); scale bar, 20 μ m; nuclei are stained with DAPI.

(fold change from 0.07 to 0.61) and 557 were upregulated (fold change from 1.63 to 31.21).

Column 1: Gene Title
Column 2: Gene Symbol

Column 3: Fold Change
Column 4: Ensembl ID
Column 5: Entrez Gene ID
Column 6: Affy ID

Dataset Item 3 (Table). A list of 94 genes including their fold changes that were regulated in the same direction in both chick and rat species by CNTF. Expression of 36 genes was downregulated (chick fold change: from 0.09 to 0.61; rat fold change: from 0.50 to 0.61) and that of 58 was upregulated (chick fold change: from 1.65 to 31.21; rat fold change: from 1.62 to 174.85) by the cytokine.

Column 1: Gene Title
Column 2: Gene Symbol
Column 3: Chick Fold Change
Column 4: Rat Fold Change
Column 5: Rat Affy ID
Column 6: Chick Affy ID

Dataset Item 4 (Table). A list of 33 genes that were found to be consistently coregulated with the neurotransmitter phenotype in both chick and rat species under all experimental conditions applied. This evolutionary conserved synexpression group contains the noradrenergic markers *Th*, *Gch1*, and *Net* as well as the cholinergic markers *Chat*, *Vacht*, *Vip*, and substance P (followed by asterisks).

Column 1: Gene Title
Column 2: Chick Gene Symbol
Column 3: Rat Gene Symbol
Column 4: Chick Fold Change

Column 5: Rat Fold Change

Column 6: Chick Ensembl

Column 7: Rat Ensembl

Column 8: Chick Entrez Gene

Column 9: Rat Entrez Gene

Column 10: Rat Affy ID

Column 11: Chick Affy ID

4. Concluding Remarks

In this work, we analyzed the global transcriptome changes that occur in rat SCG sympathetic neurons after stimulation with CNTF—the classical cell-culture model of the neurotrophic cytokine-dependent neurotransmitter switch. The comparison of the genes identified in this study with the expression changes induced by cholinergic differentiation factors in avian sympathetic neurons revealed major interspecies differences and a surprisingly limited overlap. Since the differences in the CNTF-induced gene programs between the two species are not attributable to differences in the experimental conditions, it can be speculated that they in fact reflect the heterogeneity in the mechanisms of cholinergic differentiation in the sympathetic nervous system. At least two mechanisms seem to be at work at different developmental stages with regard to the acquisition of the cholinergic properties [10, 20]: (1) early, target-independent induction/selection process whereby the cholinergic phenotype is selected from initially bimodal neurons upon the influence of external cues (neurotrophins and GFLs being the primary candidates)—likely to be modelled by the avian *in vitro* system in which the cholinergic phenotype can be induced by several growth factors—GDNF, NT3, and CNTF; and (2) late, target-dependent transdifferentiation of noradrenergic neurons into cholinergic neurons—strictly dependent on p38MAPK/Satb2 signalling module and modelled *in vitro* by CNTF-triggered cholinergic differentiation in rat SCG-derived sympathetic neurons.

Dataset Availability

The dataset associated with this Dataset Paper is dedicated to the public domain using the CC0 waiver and is available at <http://dx.doi.org/10.7167/2013/520930/dataset>.

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