Expression profiling and Ingenuity biological function analyses of interleukin-6- versus nerve growth factor-stimulated PC12 cells

Dieter Kunz*†1, Gaby Walker†2, Marc Bedoucha3, Ulrich Certa4, Pia März-Weiss2, Beatrice Dimitriades-Schmutz1 and Uwe Otten1

Address: 1Department of Biomedicine, Institute of Physiology, University of Basel, Pestalozzistrasse 25, CH-4056 Basel, Switzerland, 2Molecular Medicine Laboratories (MML), Hoffmann-La Roche Ltd., Grenzacherstrasse 2, CH-4002 Basel, Switzerland, 3Discovery Research (PRBD), Hoffmann-La Roche Ltd., Grenzacherstrasse 2, CH-4002 Basel, Switzerland and 4Non-Clinical Drug Safety (NCS), Hoffmann-La Roche Ltd., Grenzacherstrasse 2, CH-4002 Basel, Switzerland

Email: Dieter Kunz* - dieter.kunz@unibas.ch; Gaby Walker - gaby.walker@roche.com; Marc Bedoucha - marc.bedoucha@roche.com; Ulrich Certa - ulrich.certa@roche.com; Pia März-Weiss - pia.maerz-weiss@roche.com; Beatrice Dimitriades-Schmutz - beatrice.dimitriades@unibas.ch; Uwe Otten - uwe.otten@unibas.ch

* Corresponding author    †Equal contributors

Abstract

Background: The major goal of the study was to compare the genetic programs utilized by the neuropoietic cytokine Interleukin-6 (IL-6) and the neurotrophin (NT) Nerve Growth Factor (NGF) for neuronal differentiation.

Results: The designer cytokine Hyper-IL-6 in which IL-6 is covalently linked to its soluble receptor s-IL-6R as well as NGF were used to stimulate PC12 cells for 24 hours. Changes in gene expression levels were monitored using Affymetrix GeneChip technology. We found different expression for 130 genes in IL-6- and 102 genes in NGF-treated PC12 cells as compared to unstimulated controls. The gene set shared by both stimuli comprises only 16 genes.

A key step is upregulation of growth factors and functionally related external molecules known to play important roles in neuronal differentiation. In particular, IL-6 enhances gene expression of regenerating islet-derived 3 alpha (REG3A; 1084-fold), regenerating islet-derived 3 beta (REG3B/PAPI; 672-fold), growth differentiation factor 15 (GDF15; 80-fold), platelet-derived growth factor alpha (PDGFA; 69-fold), growth hormone releasing hormone (GHRH; 30-fold), adenylate cyclase activating polypeptide (PACAP; 20-fold) and hepatocyte growth factor (HGF; 5-fold). NGF recruits GDF15 (131-fold), transforming growth factor beta 1 (TGFB1; 101-fold) and brain-derived neurotrophic factor (BDNF; 89-fold). Both stimuli activate growth-associated protein 43 (GAP-43) indicating that PC12 cells undergo substantial neuronal differentiation.

Moreover, IL-6 activates the transcription factors retinoic acid receptor alpha (RARα; 20-fold) and early growth response 1 (Egr1/Zif268; 3-fold) known to play key roles in neuronal differentiation.

Ingenuity biological function analysis revealed that completely different repertoires of molecules are recruited to exert the same biological functions in neuronal differentiation. Major sub-categories include cellular growth and differentiation, cell migration, chemotaxis, cell adhesion, small molecule biochemistry aiming at changing intracellular concentrations of second messengers such as Ca2+ and cAMP as well as expression of enzymes involved in posttranslational modification of proteins.

Conclusion: The current data provide novel candidate genes involved in neuronal differentiation, notably for the neuropoietic cytokine IL-6. Our findings may also have impact on the clinical treatment of peripheral nerve injury. Local application of a designer cytokine such as H-IL-6 with drastically enhanced bioactivity in combination with NTs may generate a potent reparative microenvironment.
Background
Interleukin-6 (IL-6) is the prototype member of the IL-6 cytokine family, also termed neuropoietic cytokines, including IL-6, IL-11, IL-27, ciliary neurotrophic factor (CNTF), leukemia inhibitory factor (LIF), oncostatin M, cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC; also known as novel neurotrophin 1, NNT1), neuropoietin and B cell stimulatory factor 3 (BSF3) [1,2]. A common feature of all family members is the signaling through a specific receptor that is associated to the intracellularly located transduction component gp130. Subsequently, the Janus-activated kinase-signal transducer, activator of transcription (JAK-STAT) and mitogen-activated protein kinase (MAPK) signal transduction pathways are activated. Neuropoietic cytokines display multiple functions in the peripheral (PNS) and central nervous systems (CNS), including the developing and adult brain, synaptic plasticity as well as the brain’s response to injury and disease. In particular these molecules control cell fate and differentiation of neural stem and progenitor cells during development; due to their neurotrophic and regenerative actions they crucially affect injury-induced neurogenesis, neuronal survival and regeneration; moreover, these molecules can also influence neuronal activity and are implicated in long-term potentiation (LTP; reviewed in [2]).

Cellular functions of IL-6 are mediated by two specific receptors, the membrane-bound 80 KDa IL-6 receptor (IL-6R) or the soluble form of IL-6R (s-IL-6R) which can be generated either by shedding of IL-6R or by alternative splicing of the IL-6R mRNA [3,4]. Using s-IL-6R, IL-6 responsiveness may be conferred to cells expressing the transduction component gp130, but are devoid of membrane-bound IL-6R in the process of transsignaling [5-7]. The transsignaling mechanism led to the development of a fusion protein in which IL-6 is covalently linked to s-IL-6R thereby creating a unimolecular protein with enhanced biological activities. The fusion protein, termed Hyper-IL-6 (H-IL-6), turned out to be fully active at 100–1000-fold lower concentrations as compared to the combination of the two separate molecules [8,9].

The neurotrophin (NT) family of growth factors including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and NT-4/5 is important for development, maintenance and survival of many different cell types in the PNS and the CNS [10]. NTs are also involved in regulating adult neurogenesis [11,12], learning and memory [13,14]. NTs are synthesized as proNT precursors that may be processed to mature NTs intra- and extracellularly by specific proteases [15]. NTs exert their effects via two different types of cellular receptors: pan-neurotrophin receptor p75 (p75NTR) which binds all NTs with a similar affinity, and the family of high affinity tyrosine kinase receptors (Trk). The interactions of proNTs and NTs with the NT-receptors comprise a complex signaling system thus generating a broad variety of biological effects [16,17].

In the first report of IL-6 actions on neural cells rat pheochromocytoma cells (PC12), a well-characterised cellular model for neuronal differentiation, were incubated for up to 6 days with B-cell stimulatory factor BSF-2/IL-6 thereby inducing significant neurite outgrowth [18]. PC12 cells that were differentiated either using irradiation [19] or the well-known hypoxia mimetic agent CoCl2 [20] require IL-6 expression. We have demonstrated that primary sympathetic neurons [21] and PC12 cells [22] can strongly respond to IL-6 by transsignaling, and that the potential of IL-6 to induce neuronal differentiation in PC12 cells is in close correlation to the availability of s-IL-6R [22,23]. PC12 cell differentiation is accompanied by enhanced expression of GAP-43 mRNA at 24 hours after stimulation with IL-6/s-IL-6R [22]. Moreover, we found that the fusion protein H-IL-6 is a highly active molecule in inducing survival of cultured sympathetic neurons, comparable to the effects of NGF [21,22]. Recently, IL6RIL6, a fusion protein in which IL-6 is directly linked to the extracellular domain of the IL-6 specific receptor, has been used for expression profiling studies in primary cultures of dorsal root ganglia. In these cells, IL6RIL6 strongly increases axonal network and expression of neural genes [24].

A significant problem in the clinical treatment of peripheral nerve injury is that the currently used therapeutic approaches do not allow complete neuronal recovery [25]. Mixtures comprising neuropoietic cytokines, glial cell-line derived neurotrophic factor ligands (GFLs) and NTs are being tested for the suitability to generate a microenvironment with a high reparative potential upon local administration at the site of the lesion [26].

In the present study we monitored changes in neuronal gene expression induced by incubation of PC12 cells for 24 hours with H-IL-6 as well as NGF, and compared the genetic programs utilized by these stimuli for neuronal differentiation.

Results
Overall changes in gene expression patterns in IL-6- and NGF-stimulated PC12 cells
Affymetrix Gene Chip U34A arrays were used to analyse global changes in gene transcripts using a cutoff in the change of gene expression of > 2-fold. In PC12 cells stimulated for 24 h with 10 ng/ml H-IL-6, we found 130 differently expressed genes as compared to unstimulated controls. Of them, 94 genes were upregulated with gene expression values from 2-fold to 1085-fold, whereas 36 genes were found to be downregulated in the range from
-fold to -61-fold. The genes are further classified into major functional categories including cytokines (2 up-regulated/0 down-regulated), enzymes (20/8), G-protein coupled receptors (2/3), growth factors (7/1), ion channels (2/0), kinases (4/4), nuclear receptors (2/1), peptidases (3/1), phosphatases (0/2), transcription regulators (8/4), transmembrane receptors (5/0), transporters (8/3) and molecules with other functions (31/9; Table 1).

In PC12 cells stimulated for 24 hours with 50 ng/ml NGF, we identified 102 differently expressed genes as compared to unstimulated controls. Of them, 71 genes were upregulated with gene expression values from 2-fold to 303-fold, whereas 31 genes were found to be downregulated by -2-fold to -20-fold. Major functional categories include enzymes (18 up-regulated/9 down-regulated), G-Protein coupled receptors (2/2), growth factors (3/1), ion channels (7/2), kinases (6/2), peptidases (4/1), phosphatases (2/1), transcription regulators (0/2), transmembrane receptors (1/0), transporters (9/2) and molecules with other functions (21/9; Table 2).

Only a small overlapping gene subset is shared by IL-6 and NGF comprising a total of 16 genes and including the major functional categories enzymes (3 genes), G-Protein coupled receptors (1), growth factors (1), ion channels (2), kinases (1), peptidases (2), transporters (1) and molecules with other functions (5; Table 3). All genes are regulated in a parallel fashion except for caspase 1 with an opposite expression pattern of IL-6 (40-fold) as compared to NGF (-5-fold; Table 3). Tables 1, 2, 3 summarize gene description names, Genbank accession numbers and changes in expression levels derived from the Chip analyses, gene symbols and abbreviations derived from the IPA Tool.

Exemplary validation of microarray data using LightCycler quantitative RT-PCR analyses (qRT-PCR) on GAP-43 and REG3B mRNA expression

For an exemplary validation of the microarray data, qRT-PCR using LightCycler was performed on GAP-43 and REG3B mRNA expression. In the microarray analyses, GAP-43 mRNA was found to be upregulated 3-fold by IL-6 (Table 1), whereas qRT-PCR revealed an induction of about 20-fold (Figure 1, left). In NGF-treated PC12 cells, GAP-43 mRNA was found to be upregulated by < 2-fold and therefore did not meet the exclusion criteria applied in the current work. However, qRT-PCR analyses revealed a 10-fold induction of GAP-43 mRNA levels induced by NGF in PC12 cells (Figure 2). Thus, PC12 cells treated with IL-6 or NGF undergo substantial neuronal differentiation. REG3B mRNA expression in the microarray analysis was found to be induced to 672-fold by IL-6 (Table 1), whereas qRT-PCR revealed an induction of REG3B mRNA by about 955-fold (Figure 1, right). In NGF-treated PC12 cells, neither microarray nor qRT-PCR analyses revealed changes in REG3B expression.

Ingenuity biological functional analyses of the gene sets regulated by IL-6 and NGF in PC12 cells

The criteria applied for the search of major biological function categories were maximum number of genes and the p-value of significance. As shown in Table 4, top biological functions found to be regulated by IL-6 include cancer (61 genes), cellular growth and proliferation (54 genes), cell death (47 genes), cell-to-cell signalling and interaction (46 genes), tissue development (45 genes) and others. A further gene set is involved in nervous system development and function (24 genes). The p-values in the range from $2.26 \times 10^{-7}$ to $3.77 \times 10^{-3}$ indicate statistical significance.

Similarly, in NGF-treated PC12 cells top biological functions deal with the overall topics on cellular growth and proliferation (37 genes), cell-to-cell signalling and interaction (31 genes), molecular transport (30 genes), cancer (30 genes), cellular movement (29 genes) and others. One gene set is involved in nervous system development and function (29 genes). The p-values in the range from $8.89 \times 10^{-6}$ to $7.43 \times 10^{-3}$ indicate statistical significance (Table 4).

More detailed analyses for functional sub-categories are summarized in Table 5. Both stimuli utilize different repertoires of genes to exert the same biological functions that are all crucial for neuronal differentiation and nervous system development. Among others, important functional sub-categories include cellular growth (IL-6, 33 genes; NGF, 24 genes), differentiation (IL-6, 45 genes; NGF, 16 genes), cell movement (IL-6, 39 genes; NGF, 27 genes), chemotaxis (IL-6, 13 genes; NGF, 13 genes), adhesion of cells (IL-6, 26 genes; NGF, 18 genes), cellular signalling and small molecule biochemistry aiming at changing intracellular concentrations of second messengers such as Ca$^{2+}$ (IL-6, 16 genes; NGF, 16 genes) as well as cAMP (IL-6, 12 genes; NGF, 9 genes) as well as expression of posttranslational processing enzymes (IL-6, 23 genes; NGF, 15 genes). Table 5 (bottom) summarizes genes involved in specialized sub-categories of nervous system and development as far as they are represented in the IPKB.

Discussion

In a previous study, we have used PC12 cells to examine the effects of IL-6/s-IL6R on neuronal differentiation in comparison to NGF [22]. Already after 24 hours of exposure to IL-6/s-IL6R or NGF PC12 cells are highly active in cellular growth and proliferation displaying pronounced formation of extending neurites. Combined incubation with IL-6/s-IL6 plus NGF drastically enhanced cell
Table 1: List of gene set regulated by IL-6 in PC12 cells

| Gene                          | Accession no. | Fold change | Subcellular location      |
|-------------------------------|---------------|-------------|---------------------------|
| **Cytokines**                 |               |             |                           |
| chemokine ligand 13           | CXCL13        | AF044196    | 43 Extracellular Space    |
| chemokine ligand 10           | CXCL10        | U17035      | 7 Extracellular Space     |
| **Enzymes**                   |               |             |                           |
| cytochrome P450, 4f16         | CYP4F16       | U39207      | 424 Cytoplasm             |
| ceruloplasmin                 | CP            | AF202115    | 191 Extracellular Space   |
| peptidyl arginine deiminase, type III | PADI3    | D88034      | 142 Cytoplasm             |
| acyl-CoA synthetase, member I | ACSL1         | D90109      | 102 Cytoplasm             |
| transglutaminase 1            | TGM1          | M57263      | 93 Plasma Membrane        |
| nitric oxide synthase 2A      | NO2A          | U03699      | 58 Cytoplasm              |
| ornithine carbamoyltransferase| OTC           | M11266      | 43 Cytoplasm              |
| Similar to Lysophospholipase  | LOC374569     | AB009372    | 37 Unknown                |
| trehalase                     | Treh           | AF038043    | 35 Plasma Membrane        |
| kynureninase                  | KYNU          | U68168      | 25 Cytoplasm              |
| nitric oxide synthase 3       | NOS3          | A011115     | 21 Cytoplasm              |
| glycine amidinotransferase    | GATM          | U07971      | 14 Cytoplasm              |
| guanine nucleotide binding protein, alpha z | GNAZ | U77485 | 14 Plasma Membrane         |
| ST6 galactosamidase alpha-2,6-sialyltransferase I | ST6GAL1 | M83143 | 14 Cytoplasm               |
| aldo-keto reductase, 1C1      | AKR1C1        | BAA92883    | 12 Cytoplasm              |
| myxovirus resistance 1        | MX1           | P20591      | 9 Nucleus                 |
| aldolase C                    | ALDOC         | X06984      | 3 Cytoplasm               |
| 2',5'-oligoadenylate synthetase | OAS1    | Z18877      | 3 Cytoplasm               |
| protein disulfide isomerase, A2 | PDI2     | AAC50401    | 3 Cytoplasm               |
| RNA (guanine-7-) methyltransferase | RNMT  | BAA82447    | 3 Nucleus                 |
| polymerase, alpha 2           | POLA2         | AI45648     | -2 Nucleus                |
| steroid-5-alpha-reductase, alpha 1 | SRDSA1 | J05235 | -2 Cytoplasm               |
| aminolevulinate, delta-, synthase 2 | ALAS2 | D86297 | -3 Cytoplasm               |
| glutathione S-transferase A3  | GSTA3         | X78847      | -3 Cytoplasm              |
| UDP glycosyltransferase 8     | UGT8          | BC075069    | -3 Cytoplasm              |
| cell division cycle 42       | CDC42         | U37720      | -4 Cytoplasm              |
| cysteine dioxygenase, type I  | CDO1          | M35266      | -4 Cytoplasm              |
| ST8 alpha-2,8-sialyltransferase 3 | ST8A3   | X80502      | -5 Cytoplasm              |
| **G-protein coupled receptors** |               |             |                           |
| adrenergic receptor, alpha-2B | ADRA2B        | M32061      | 26 Plasma Membrane        |
| arginine vasopressin receptor 2 | AVPR2   | AAB87678    | 5 Plasma Membrane         |
| vasoactive intestinal peptide receptor 1 | VIPR1 | M86835 | -2 Plasma Membrane        |
| cholinergic receptor, muscarinic 3 | CHRM3 | AB017656 | -3 Plasma Membrane       |
| cholinergic receptor, muscarinic 4 | CHRM4 | M16409 | -10 Plasma Membrane      |
| **Growth factors**            |               |             |                           |
| regenerating islet-derived 3 alpha | REG3A | L10229 | 1084 Extracellular Space  |
| regenerating islet-derived 3 beta | REG3B | S43715 | 672 Extracellular Space   |
| growth differentiation factor 15 | GDF15 | A011970 | 80 Extracellular Space    |
| platelet-derived growth factor alpha | PDGFA | M29464 | 69 Extracellular Space    |
| nudix-type motif 6            | NUDT6         | AF188995    | 22 Extracellular Space    |
| jagged 2                      | JAG2          | U70050      | 5 Extracellular Space     |
| hepatocyte growth factor      | HGF           | X84064      | 4 Extracellular Space     |
| macrophage stimulating 1      | MST1          | X95096      | -4 Extracellular Space    |
| **Ion channels**              |               |             |                           |
| glutamate receptor, ionotropic, delta 2 | GRID2 | U08256 | 91 Plasma Membrane        |
| purinergic receptor P2X       | P2RX2         | Y10475      | 11 Plasma Membrane        |
| **Kinases**                   |               |             |                           |
| fyn-related kinase            | FRK           | U02888      | 122 Nucleus               |
| Janus kinase 2                | JAK2          | U13396      | 120 Cytoplasm             |
| phosphatidylinositol 4-kinase beta | PI4KB | D84667 | 2 Cytoplasm               |
| Gene Set                                      | Gene Symbol | Identification  | Location  |
|----------------------------------------------|-------------|-----------------|-----------|
| **Peptidases**                               |             |                 |           |
| Peptidases                                   |             |                 |           |
| Complement component Is                      | C1S         | D88250          | 230       |
| Caspase 1                                    | CASP1       | U14647          | 40        |
| Proteasome subunit, alpha 1                  | PSMA1       | M29859          | 5         |
| Kallikrein-related peptidase 8               | KLK8        | AJ005641        | -5        |
| **Phosphatases**                             |             |                 |           |
| Phosphatases                                 |             |                 |           |
| Pyruvate dehydrogenase phosphatase 2         | PDP2        | AF062741        | -4        |
| Protein tyrosine phosphatase receptor D      | PTPRD       | U57502          | -9        |
| **Transcription regulators**                 |             |                 |           |
| Transcription regulators                     |             |                 |           |
| Signal transducer and activator of transcription 1 | STAT1     | AF205604        | 579       |
| Kruppel-like factor 6                        | KLK6        | AF072403        | 249       |
| HIV-1 Tat interacting protein                | HTATIP      | AA818236        | 159       |
| HIV enhancer binding protein 2               | HIVEP2      | D37951          | 65        |
| Upstream transcription factor 1              | USF1        | U41741          | 22        |
| Early growth response 1                      | EGR1        | M18416          | 3         |
| Interferon regulatory factor 1               | IRF1        | M34353          | 3         |
| Signal transducer and activator of transcription 2 | STAT2     | AF206162        | 3         |
| Breast cancer 1                              | BRCA1       | U36475          | -2        |
| D site of albumin promoter binding protein   | DBP         | J03179          | -2        |
| Nuclear factor 1B                            | NF1B        | Y07685          | -2        |
| Transcription elongation factor A 2          | TCEA2       | D12927          | -5        |
| **Transporters**                             |             |                 |           |
| Transporters                                 |             |                 |           |
| Cadherin 17                                  | CDH17       | X78997          | 273       |
| Solute carrier family 6, member 3            | SLC6A3      | M80570          | 90        |
| Nucleoporin 153kDa                           | NUP153      | L06821          | 83        |
| Solute carrier family 9, member 2            | SLC9A2      | L11004          | 32        |
| Cadherin 17                                  | CDH17       | L46874          | 13        |
| Lipocalin 2                                  | LCN2        | X13395          | 9         |
| Syntaxin 4                                   | STX4        | L20821          | 3         |
| Secretory carrier membrane protein 2         | SCAMP2      | AF295405        | 2         |
| Solute carrier family 12, member 5           | SLC12A5     | U55816          | -3        |
| Solute carrier family 30, member 2           | SLC30A2     | U50927          | -5        |
| Syntaxin 5                                   | STX5        | U87971          | -8        |
| **Others**                                   |             |                 |           |
| Others                                       |             |                 |           |
| Regenerating islet-derived 1 alpha           | REG1A       | J05722          | 796       |
| TIMP metallopeptidase inhibitor 1            | TIMP1       | L31883          | 210       |
| Calcitonin-related polypeptide beta          | CALC1B      | M11596          | 195       |
| Fibrinogen gamma chain                       | FGG         | J00734          | 164       |
| Trans-Golgi network protein 2                | TGOLN2      | X53565          | 113       |
| LIM and senescent cell antigen-like domains  | LIMS1       | AAA20086        | 94        |
| Alpha-2-HS-glycoprotein                      | AHSN        | M29758          | 80        |
Table 1: List of gene set regulated by IL-6 in PC12 cells (Continued)

| Gene Description | Gene Symbol | Accession no. | Fold change | Subcellular Location |
|-------------------|-------------|---------------|-------------|----------------------|
| ribosomal protein L3-like | RPL3L | AAC50777 | 60 | Unknown |
| collagen, type IV, alpha 5 | COL4A5 | AB041350 | 59 | Extracellular Space |
| parvalbumin | LOC4951 | J02705 | 58 | Unknown |
| YTH domain containing 1 | YTHDC1 | AF144731 | 39 | Cytoplasm |
| growth hormone releasing hormone | GHRH | Z34092 | 31 | Extracellular Space |
| annexin A1 | ANXA1 | M19967 | 29 | Plasma Membrane |
| collagen, type XII, alpha 1 | COL12A1 | U57362 | 26 | Extracellular Space |
| regenerating islet-derived 3 gamma | REG3G | L20869 | 24 | Extracellular Space |
| adenylate cyclase activating polypeptide 1 | ADCYAP1 | S83513 | 20 | Extracellular Space |
| heat shock protein 90 kDa, alpha B 1 | HSP90B1 | S45392 | 20 | Cytoplasm |
| luteinizing hormone beta | LHB | U25653 | 17 | Extracellular Space |
| galectin 5 | LGALS5 | L36862 | 8 | Extracellular Space |
| myocilin | MYOC | AF093567 | 8 | Cytoplasm |
| prolactin family 8a1 | PRL8A | AB000107 | 8 | Extracellular Space |
| troponin C type 2 | TNNC2 | J05598 | 8 | Unknown |
| ribosomal protein L18a | RPL18A | X14181 | 7 | Cytoplasm |
| fibrinogen beta chain | FGB | U05675 | 6 | Extracellular Space |
| tropomyosin 3 | TPM3 | X72859 | 4 | Cytoplasm |
| tubulin, beta | TUB8 | AB011679 | 4 | Cytoplasm |
| extracellular proteinase inhibitor | EXPI | X13039 | 3 | Extracellular Space |
| growth associated protein 43 | GAP43 | M16736 | 3 | Plasma Membrane |
| galectin 9 | LGALS9 | U72741 | 3 | Extracellular Space |
| tubulin, alpha 4a | TUBA4A | M13444 | 3 | Cytoplasm |
| BCL2-like 11 | BCL2L11 | AF136927 | 2 | Cytoplasm |
| integrin alpha 7 | ITGA7 | X65036 | 2 | Plasma Membrane |
| syndecan 2 | SDCC2 | M81687 | 2 | Plasma Membrane |
| zinc finger protein 260 | ZNF260 | U56862 | 2 | Nucleus |
| filamin C | FLNC | AF119148 | -3 | Cytoplasm |
| metallothionein 3 | MT3 | S65838 | -3 | Cytoplasm |
| arginine vasopressin | AVP | M25646 | -4 | Extracellular Space |
| fasciculation and elongation protein zeta 1 | FEZ1 | U63740 | -4 | Cytoplasm |
| crystallin, alpha B | CRYAB | U04320 | -6 | Nucleus |
| neurofascin | NFASC | U81036 | -7 | Plasma Membrane |

Gene description names, gene symbols are from IPA Tool; accession numbers are from GenBank.

Table 2: List of gene set regulated by NGF in PC12 cells

| Gene Description | Gene Symbol | Accession no. | Fold change | Subcellular Location |
|-------------------|-------------|---------------|-------------|----------------------|
| rat senescence marker protein 2A gene | SMP2A | X63410 | 303 | Cytoplasm |
| myosin, heavy chain 3 | MYH3 | K03468 | 133 | Cytoplasm |
| lecithin-cholesterol acyltransferase | LCAT | X54096 | 101 | Extracellular Space |
| UDP glucuronosyltransferase 2, polypeptide A1 | UGT2A1 | X57565 | 63 | Cytoplasm |
| contactin 4 | CNTN4 | U35371 | 44 | Plasma Membrane |
| phosphodiesterase 4B, | PDE4B | J04563 | 37 | Cytoplasm |
| guanone oxidase | GULO | J03536 | 34 | Cytoplasm |
| superoxide dismutase 3 | SOD3 | Z24721 | 28 | Extracellular Space |
| fibronectin 1 | FN1 | X15906 | 28 | Plasma Membrane |
| acetylcholinesterase | ACHE | S50879 | 28 | Plasma Membrane |
| tryptophan hydroxylase 1 | TPH1 | X53501 | 24 | Cytoplasm |
| aldo-keto reductase family 1, member C1 | AKR1C1 | BAA92883 | 10 | Cytoplasm |
| guanine nucleotide binding protein, alpha z | GNAA | U77485 | 9 | Plasma Membrane |
| aminoadipate aminotransferase | AADAT | Z30144 | 5 | Cytoplasm |
| phosphophate D2 | PLD2 | D38672 | 4 | Cytoplasm |
| N-deacetylase/N-sulfotransferase 1 | NDST1 | M92042 | 3 | Cytoplasm |
| phosphate cytidylyltransferase 2 | PCYT2 | A080564 | 2 | Cytoplasm |
| peptidylprolyl isomerase A | PPIA | M19523 | -2 | Cytoplasm |
| Rab geranylgeranyltransferase alpha | RABGGTA | L10415 | -3 | Cytoplasm |
| glutathione S-transferase A3 | GSTA3 | X78847 | -3 | Cytoplasm |
| cytochrome P450, 4F4 | CYP4F4 | U39206 | -3 | Cytoplasm |

Gene description names, gene symbols are from IPA Tool; accession numbers are from GenBank.
| Gene Name                                      | Accession | Log2 Fold Change | Location     |
|-----------------------------------------------|-----------|-----------------|--------------|
| 3-hydroxyanthranilate 3,4-dioxygenase (HAAO)  | D28327    | -3              | Cytoplasm    |
| Stearoyl-Coenzyme A desaturase 2 (SCD2)       | A8032243  | -4              | Cytoplasm    |
| Aldo-keto reductase family 1, member C3 (AKR1C3) | L32601    | -6              | Cytoplasm    |
| Myxovirus resistance 2 (MX2)                 | X52711    | -10             | Nucleus      |
| Serine dehydratase (SDS)                     | M38617    | -11             | Cytoplasm    |

**G-protein coupled receptors**

- Calcitonin/calcitonin-related polypeptide alpha (CALCA) - V01228 - 136 Plasma Membrane
- Angiotensin II receptor 1 (AGTR1) - NM_009585 - 50 Plasma Membrane
- Cholinergic receptor, muscarinic 3 (CHRM3) - A8017656 - 2 Plasma Membrane
- Parathyroid hormone receptor 1 (PTHR1) - M77184 - 3 Plasma Membrane

**Growth factors**

- Growth differentiation factor 15 (GDF15) - A011970 - 131 Extracellular Space
- Transforming growth factor beta 1 (TGFBI) - X52498 - 101 Extracellular Space
- Brain-derived neurotrophic factor (BDNF) - X67108 - 89 Extracellular Space
- Neuregulin 1 (NRG1) - U02324 - 3 Extracellular Space

**Ion channels**

- Calcium channel, voltage-dependent, beta 2 (CACNB2) - M80545 - 90 Plasma Membrane
- Glutamate receptor, ionotropic, delta 2 (GRID2) - U08256 - 78 Plasma Membrane
- Sodium channel, voltage-gated, type II, beta (SCN2B) - U37147 - 73 Plasma Membrane
- Potassium inwardly-rectifying channel J4 (KCNJ4) - X97635 - 51 Plasma Membrane
- Solute carrier family 9 member 3 (SLC9A3) - MB5300 - 40 Plasma Membrane
- Purinergic receptor P2X, ligand-gated ion channel 2 (P2RX2) - Y10475 - 13 Plasma Membrane
- Sodium channel, voltage-gated, type I, alpha (SCN1A) - M22553 - 12 Plasma Membrane
- Purinergic receptor P2X-like 1 (P2XXL) - X92070 - 2 Plasma Membrane
- Gamma-aminobutyric acid A receptor gamma 2 (GABRG2) - X56313 - 19 Plasma Membrane

**Kinases**

- G protein-coupled receptor kinase 5 (GRK5) - NM_005308 - 131 Plasma Membrane
- Protein kinase, cGMP-dependent, type II (PRKG2) - Z36276 - 68 Cytoplasm
- Mitogen-activated protein kinase kinase kinase kinase 1 (MAP4K1) - Y09010 - 25 Cytoplasm
- Calcium/calmodulin-dependent serine protein kinase (CASK) - U47110 - 3 Plasma Membrane
- Discs, large homolog 1 (DLG1) - U14950 - 3 Plasma Membrane
- Phosphatidylinositol 4-kinase beta (PI4KB) - D84667 - 3 Cytoplasm
- Discoidin domain receptor family member 1 (DDR1) - L26525 - 8 Plasma Membrane
- Non-metastatic cells 6 (NME6) - AF051943 - 14 Extracellular Space

**Peptidases**

- Carboxypeptidase A3 (CPA3) - U67914 - 5 Extracellular Space
- Adam metallopeptidase domain 17 (ADAM17) - A012603 - 4 Plasma Membrane
- Proteasome subunit alpha 1 (PSMA1) - M29859 - 3 Cytoplasm
- Protein disulfide isomerase family A member 3 (PDIA3) - D63378 - 2 Cytoplasm
- Caspase 1 (CASP1) - U14647 - 5 Cytoplasm

**Phosphatases**

- Dual specificity phosphatase 6 (DUSP6) - U42627 - 53 Cytoplasm
- Protein phosphatase 1 subunit 1A (PPP1R1A) - A027693 - 18 Cytoplasm
- Protein tyrosine phosphatase type 11 (PTPN11) - U09307 - 2 Cytoplasm

**Transcription regulators**

- Jun dimerization protein 2 (JDP2) - U53449 - 2 Nucleus
- CAMP responsive element modulator (CREM) - Z15158 - 4 Nucleus

**Transmembrane receptors**

- Cholinergic receptor, nicotinic, beta 1 (CHRNA1) - X74833 - 39 Plasma Membrane

**Transporters**

- Solute carrier family 1 member 1 (SLC1A1) - U21104 - 238 Plasma Membrane
- Solute carrier family 22, member 3 (SLC22A3) - A003286 - 95 Plasma Membrane
- Gap junction protein, beta 2 (GJB2) - X51615 - 55 Plasma Membrane
- Solute carrier family 22, member 3 (SLC22A3) - U35245 - 4 Cytoplasm
- Solute carrier family 30, member 1 (SLC30A1) - U17133 - 3 Plasma Membrane
- Mating type protein (MUP) - J05352 - 2 Extracellular Space
- ATPase, Cu++ transporting, beta polypeptide (ATPB) - AF120492 - 6 Cytoplasm
number and neurite outgrowth arguing for an additive effect of both stimuli on neuronal differentiation. In the current study we have chosen this time point to perform microarray analyses in order to monitor changes in gene expression and to compare the genetic programs utilized for neuronal differentiation by IL-6 versus NGF.

An important aspect in gene expression profiling using microarrays is the accuracy of the measurements in the relative changes in mRNA expression. Thus, alternative technologies such as qRT-PCR are used for the validation of microarray data [27]. Several systematic studies comparing the changes in gene expression obtained from oligonucleotide- or cDNA arrays to data from qRT-PCR revealed that a good correlation exists for genes exhibiting fold-change differences in expression of > 2-fold [28,29]. Therefore, in our datasets all genes displaying changes in expression levels of < 2-fold were excluded. Moreover, our exemplary validation data on GAP-43- and REG3B-expression are in line with other previous reports confirming that it is rather the magnitude of fold change varying between qRT-PCR and Affymetrix-analysis, but not the direction.

Detailed Ingenuity biological function analyses reveal that IL-6 and NGF activate gene sets that regulate the same process in neuronal differentiation and nervous system development, however, utilizing completely distinguished sets of individual molecules. This may explain our previous observation that combined application of IL-6/s-IL-6R plus NGF generates an additive effect on PC12 cell differentiation. Important processes in neuronal differentiation and nervous tissue development include cellular growth and proliferation in order to enhance cell number. Neurite outgrowth and network generation requires migration of neurons or nerve growth cones. Neuronal navigation is guided by the interaction of the neuron with its local environment, in particular by chemotaxis as the key mechanism. This process involves three major steps including directional sensing along a gradient of chemotactic factors, cellular motility i.e. the cell’s movement by changes in cytoskeletal organisation and

| Gene | Fold change IL-6 | Fold change NGF |
|------|------------------|-----------------|
| G-protein coupled receptors | | |
| cholinergic receptor, muscarinic 3 | CHRM3 | - 3 | - 2 |
| Growth factors | | |
| growth differentiation factor 15 | GDF15 | 80 | 131 |
| Ion channels | | |
| glutamate receptor, ionotropic, delta 2 | GRID2 | 91 | 78 |
| purinergic receptor P2X, ligand-gated ion channel | P2RX2 | 11 | 13 |
| Kinases | | |
| phosphatidylinositol 4-kinase beta | PI4KB | 2 | 3 |
| Peptidases | | |
| caspase 1 | CASP1 | 40 | - 5 |
| proteasome subunit alpha 1 | PSMA1 | 5 | 3 |
| Transporters | | |
| syntaxin 4 | STX4 | 3 | 2 |

Gene description names, gene symbols are from IPA Tool.
cellular adhesion and cellular polarisation [30-32]. Certainly, a key step in the regulation of these processes is the increased gene expression of growth factors and functionally related external molecules, indicating convergence of several different signaling pathways (Table 5). In IL-6 stimulated PC12 cells these tasks may be taken by growth differentiation factor 15 (GDF15), platelet-derived growth factor alpha (PDGFA), hepatocyte growth factor (HGF), regenerating islet-derived 3 alpha (REG3A), regenerating islet-derived 3 beta/pancreatitis-associated protein 1 (REG3B/PAPI), growth hormone releasing hormone (GHRH) and adenylate cyclase activating polypeptide (PACAP). NGF recruits GDF15 (131-fold), transforming growth factor beta 1 (TGFβ1; 101-fold) and brain-derived neurotrophic factor (BDNF; 89-fold). TGFβ1 is the prototype member of the TGFβ-superfamily comprising multifunctional growth factors with numerous cell and tissue functions such as cell cycle control, regulation of early development, differentiation, extracellular matrix (ECM) formation and chemotaxis. In the nervous system, TGFβ1 has been shown to regulate neuroprotection against glutamate cytotoxicity, ECM production, and cell migration in the cerebral cortex, control of neuronal death as well as survival of neurons (reviewed in [33]). GDF15 is a member of the TGFβ-superfamily and has been shown to be a potent trophic factor in the brain (reviewed in [34]). Hepatocyte growth factor (HGF) is a chemoattractant and a survival factor for embryonic motor neurons. In addition, sensory and sympathetic neurons and their precursors respond to HGF with increased differentiation, survival and axonal outgrowth [35]. Moreover, HGF may synergize with other neurotrophic factors to potentiate the response of developing neurons to specific signals [36]. Platelet derived growth factor (PDGF) has been suggested to support neuronal differentiation [37], and has previously been reported to act as a mitogen for immature neurons [38] and neural progenitor cells [39]. REG3A and REG3B/PAPI are members of the regenerating protein (REG)/pancreatitis-associated protein (PAP) family representing a complex group of small secretory proteins which display many different functions, among them growth factor activity for neural cells [40]. So far, only limited knowledge is available about the role and function of PAP/REG-proteins in the nervous system. REG3B/PAPI expression is induced in spinal motor neurons as well as subsets of the dorsal root ganglion neurons [41]. Moreover, in vitro REG3B/PAPI has a mitogenic effect on Schwann cells [42]. In a hypoglossal nerve injury model in rats, expression of REG3B/PAPI mRNA was found to be enhanced in injured motor neurons after axotomy and a marked induction of REG3G/PAPIII mRNA was observed in the distal part of the injured nerve [43]. More recently, REG3G/PAPIII has been identified as a macrophage chemoattractant that is induced in and released from injured nerves [44]. With REG1A/PSP and REG3G/PAPIII, two further members of the REG/PAP family are induced by IL-6 in PC12 cells. It is noteworthy that these genes are up-regulated at the highest levels obtained in the entire dataset for IL-6. In NGF-treated PC12 cells, no up-regulation of the PAP/REG protein genes was observed. The results in our study are in line with an earlier report demonstrating up-regulation of PAP/REG gene family members in PC12 cells upon stimulation with IL-6/s-IL-6R [45].

So far various studies have investigated gene expression profiles in NGF-treated PC12 cells applying different experimental protocols in respect to time points and periods of NGF administration [46-51]. From most studies, it is obvious that PC12 cells require at least 3 to 5 days of NGF-treatment to obtain the fully differentiated neuronal phenotype. The most significant morphological changes occur within the first 2 days, reaching a plateau phase at day 3 [51]. Redundant data sets as well as unique genes have been identified and followed. Our study provides novel candidate genes activated in the early phase of the differentiation process and thus may enlarge the repertoire of known NGF-regulated genes.

The current study reveals novel aspects of IL-6 action, notably that it applies several major routes to direct PC12 cell differentiation. Besides up-regulation of growth factors known to act in autocrine and paracrine fashion to take over further tasks in the differentiation process, these include induction of PACAP, a pleiotropic molecule with a broad spectrum of biological functions. Among them are actions as a neurotrophic factor similar to NGF as well as induction of transcription factors known to be of key importance in neuronal differentiation [52].
Upregulation of PACAP could have an important impact on IL-6-induced PC12 cell differentiation. A recent report provided data from microarray analyses of PACAP-regulated gene transcripts in primary cultures of sympathetic neurons at 6 hours and 92 hours of stimulation [53]. A comparison with our data reveals that many gene families that are activated by PACAP in primary sympathetic neurons are also induced by IL-6 in PC12 cells (Table 6). Thus, many of the effects of IL-6 on PC12 cells are likely to be mediated by the intermediate autocrine and/or paracrine action of PACAP. PACAP is a member of a family of neuropeptides known to activate class II G-protein coupled receptors (GPCRs; reviewed in [54]). Other family members include growth hormone releasing hormone (GHRH) and calcitonin-related peptide beta (CALCB) which are activated by IL-6 in PC12 cells by 31-and 195-fold, respectively. All members of the class II GPCR superfamily regulate intracellular cAMP-levels by receptor coupling to the Gs-adenylate cyclase-cAMP signaling pathway [54]. A further mechanism of PACAP action in PC12 cells could be a transactivation of TrkA receptors [55]. However, in light that the overlap in the datasets of IL-6 versus NGF is rather small, TrkA activation may not be a primary event at all or at the time point of our study.

A further key step in IL-6 actions on PC12 cell differentiation is the induction of RARA and EGR-1/Zif268, two transcription factors known to be of crucial importance in neuronal differentiation. Among the genes regulated by retinoic acid is GAP-43, a neuron specific protein frequently used as a marker of neuronal differentiation as it is expressed in most neurons during neuronal development, nerve regeneration and LTP [56-60]. The data herein are confirmative to our previous study in which we have found induction of GAP-43 mRNA upon stimulation of PC12 cells with IL-6/s-IL-6R [22]. EGR-1/Zif268 is induced in nearly every model of long-lasting synaptic plasticity in the CNS [61-64] and suppression of Zif268 prevents neurite outgrowth in PC12 cells [65]. Recently candidate target genes of Zif268 in PC12 cells were identified suggesting that a key component of the long-lasting effects of Zif268 on CNS plasticity is the regulation of proteasome activity [66,67].

Signal transducer and activator of transcription 1/2 (STAT1/2), two members of the STAT family of transcription factors involved in signaling by Interferons (IFN) [68] are activated by stimulation of the PC12 cells with IL-6. As we could not detect changes in IFN gene expression, an autocrine action of PDGF is the most likely candidate for upregulation of STAT1/2 as described for neural progenitor cells [39]. STAT1/2 may upregulate interferon regulatory factor 1 (IRF1)-expression, a further transcription factor of IFN-signaling. Breast cancer 1 (BRCA1) encodes a tumour suppressor gene whose germ line mutations in women are associated with a genetic predisposition to breast and ovarian cancer. STAT1 transcriptional activity is decreased by a physical interaction with BRCA1 as a key step in the regulation of IFN-induced cellular growth arrest [69]. By the action of IL-6, BRCA1 gene expression is down-regulated thus supporting STAT1 mediated PC12 cell growth. We failed to detect STAT3 expression, the key transcription factor of IL-6 signaling. This is most likely due to the fact that STAT3 gene transcription occurs very early in IL-6-stimulation and is already terminated at the time point of the analysis, or the expression levels are below 2-fold and thus did not meet the exclusion criteria.

The morphological changes during nervous system development are controlled by interactions of individual neurons with the ECM. Signals from the ECM into a particular neuron are mediated by integrins via associated adapter molecules. In this way growth factor induced receptor tyrosine kinase (RTK)- and integrin-mediated signalling determine the fate of a particular cell, notably differentiation, cell shape, adhesion, polarity, migration, as well as proliferation versus apoptotic cell death (reviewed in [70]). LIM and senescent cell antigen-like domains1/PINCH (LIMS1/PINCH) is an intracellular adaptor molecule providing the molecular link of an integrin-RTK network. LIMS1 physically connects integrin-linked kinase (ILK) to non-catalytic (region of) tyrosine kinase adaptor protein 2 (Nck2), an adapter molecule of the growth factor receptor (RTK) [70]. LIMS1 is activated by IL-6 as well as NGF and thus is one of few genes regulated in the common subset. In contrast to IL-6, NGF simultaneously upregulates major components of the ECM including collagen, type XI, alpha1 (COL11A1), COL12A1, fibronectin1 (FN1) as well as fibrillin2 (FN2) (Table 2).
In contrast to NGF, only one publication provided expression profiling data analysing gene sets regulated by IL-6 upon neuronal differentiation. Primary cultures of rat dorsal root ganglia (DRG) were treated with IL6RIL6 for 2 and 4 days, respectively. A detailed comparison reveals that only a small number of commonly regulated genes may be identified in the datasets that are regulated in parallel or opposite direction. These include Egr-1 (upregulated in PC12 cells; downregulated in DRG cells), TGFA (upregulated in PC12 cells and DRG cells), TGFB (upregulated in PC12 cells; downregulated in DRG cells), PDGFA (upregulated in PC12 cells; downregulated in DRG cells) and IRF-1 (upregulated in PC12 cells and in DRG cells) [24].

The results obtained from our study may also have impact into clinical treatments of injured peripheral nerves which, in contrast to central nerves, have the ability to recover from damage. Currently the therapy of choice is the use of autologous grafts where the defect is bridged with a section of autologous nerve tissue, mostly a sensory nerve [71]. Alternatively, nerve conduits or decellularized nerve grafts can be used; however, no therapy could yield a satisfactory functional recovery [72]. Various combinations of NTs, neuropoietic cytokines and GFLs have been shown to generate a microenvironment suitable to improve nerve repair [26]. The results of our study may provide novel aspects for the treatment of peripheral nerve injury as the local application of a designer cytokine such as H-IL-6 with a strongly enhanced bioactivity on neuronal development and neurite outgrowth in combination with NTs and/or GFLs may create a microenvironment with a strong reparative potency.

**Conclusion**

IL-6 and NGF utilize different genetic programs to exert the same biological functions in neuronal differentiation. An important step is the recruitment of many growth factors that may act in autocrine and/or paracrine fashion and may control the long-term effects on growth, neuronal differentiation or survival.

**Methods**

**Reagents, buffers and cells**

DMEM medium, horse serum, fetal bovine serum and other cell culture supplements were obtained from Gibco-BRL. TRIZOL reagent and Superscript reverse transcriptase were purchased Life Technologies. PC12 cells were obtained from ATCC, Manassas (VA), USA. Hyper-IL-6 was generated as described [8]. The LightCycler PCR kit was from Roche Diagnostics, Mannheim, Germany.

**Cell culture**

PC12 cells were cultured in DMEM medium containing 10% fetal bovine serum and 100 U/ml penicillin and streptomycin at 37°C in humidified 5% CO₂/95% air. For stimulation confluent cells were washed once with PBS and cultured in cell culture medium containing 10 ng/ml H-IL-6 or 50 ng/ml recombinant human NGF for 24 hours. Control cells were incubated in cell culture medium alone for 24 hours.

**Table 4: Top high-level functions identified by Ingenuity global function analysis of regulated genes in IL-6-versus NGF- stimulated PC 12 cells**

| Biological function classification | Number of genes | Significance (p-value)         |
|-----------------------------------|-----------------|-------------------------------|
| **IL-6-regulated genes**          |                 |                               |
| Cancer                            | 61              | 2.98 × 10⁻⁶ to 5.16 × 10⁻³     |
| Cellular Growth and Proliferation  | 54              | 1.14 × 10⁻⁶ to 5.16 × 10⁻³     |
| Cell Death                        | 47              | 4.54 × 10⁻⁶ to 5.16 × 10⁻³     |
| Cell-to-Cell Signalling and Interaction | 46          | 2.26 × 10⁻⁷ to 5.16 × 10⁻³     |
| Tissue Development                | 45              | 2.26 × 10⁻⁷ to 5.16 × 10⁻³     |
| Cellular Movement                 | 39              | 9.19 × 10⁻⁶ to 5.16 × 10⁻³     |
| Cellular Development              | 38              | 8.56 × 10⁻⁶ to 4.85 × 10⁻³     |
| Small Molecule Biochemistry       | 37              | 1.32 × 10⁻⁵ to 4.47 × 10⁻³     |
| Nervous system development and function | 24           | 2.83 × 10⁻⁵ to 3.77 × 10⁻³     |

| **NGF-regulated genes**           |                 |                               |
| Cellular growth and proliferation | 37              | 7.86 × 10⁻⁵ to 8.88 × 10⁻³     |
| Cell-to-cell signalling and interaction | 31          | 1.03 × 10⁻⁴ to 7.43 × 10⁻³     |
| Molecular transport               | 30              | 8.89 × 10⁻⁶ to 8.70 × 10⁻³     |
| Cancer                            | 30              | 1.03 × 10⁻⁴ to 7.43 × 10⁻³     |
| Cellular movement                 | 29              | 2.41 × 10⁻⁵ to 8.70 × 10⁻³     |
| Cell death                        | 29              | 2.73 × 10⁻⁵ to 8.77 × 10⁻³     |
| Neurological diseases             | 29              | 1.07 × 10⁻⁴ to 8.70 × 10⁻³     |
| Nervous system development and function | 29           | 1.60 × 10⁻⁴ to 8.70 × 10⁻³     |

p-values are from IPA Tool
Table 5: Ingenuity biological function analyses of IL-6-versus NGF-regulated genes in PC12 cells (selected)

| Category                          | IL-6 regulated genes in PC12 cells | NGF-regulated genes in PC12 cells |
|-----------------------------------|------------------------------------|-----------------------------------|
|                                   | p-value   | Molecules | p-value   | Molecules |
| **Cellular Growth and Proliferation** |          |           |          |           |
| Growth of cells                   | 2.27 × 10^{-4} | ACVR2A, AHSG, ANXA1, BCL2L1, BRCA1, CASP1, CDC42, CHRM3, CXCL10, EGR1, FGFR2, CGAP43, GDF15, GHR, GRD2, HGF, IGFR2, IRF1, ITGA7, JAK2, MAP2K5, MST1, MT3, MX1, NOS3, NOS2A, PI3, RARA, SCAMP2, SDCC2, STAT1, TIMP1, VDR | 8.82 × 10^{-3} | ACHE, AGTR1, BDNF, BNP3, CASP1, CD44, CHRM3, CREM, DDR1, DUSP6, FB2N, FIN1, GDF15, GJ2, GRD2, MYL9, NRG1, PIA3, PTNP11, SLCO3A1, TGFBI, TPM1, VPS33B, ZMAT3 |
| Proliferation of cells            | 9.06 × 10^{-7} | ACVR2A, ADYCAPI, ANXA1, AVP, BCL2L1, BRCA1, CALCB, CDC42, CHRM3, CHRM4, CRYAB, CXCL10, EGR1, FGFR1, FRK, GDF15, GHR, GHRH, HGF, IGFR2, IRF1, JAK2, KLF6, K18, LCN2, MAP2K3, MT3, NFIB, NOS3, NOS2A, NXR3C2, PDGFA, RARA, REG1A, REG3A, RNMT, SDC2, SDGAL1, STAT1, TIMP1, TPM3, USFI, VDR, VIPR1 | 3.82 × 10^{-3} | AGTR1, AKR1C3, BDNF, CALCA, CD44, CHRM3, DDR1, FIN1, GDF15, GRK5, NPCC, NRG1, PIA, PTNP11, TACI, TGFBI |
| **Cellular Movement**             |          |           |          |           |
| Cell movement                     | 2.18 × 10^{-8} | ADCYAP1, ANXA1, CASP1, CDC42, CHRM3, CHRM4, CXCL10, CXCL13, EGR1, FGGR2A, FER, FGB, FGFR1, GNAZ, GRD2, HGF, HLA-G, HSP90A1, IGFR2, JAK2, LCN2, LGALS9, LMS1, MAP2K3, MST1, NOS3, NOS2A, OLRI, PDGFA, RARA, REG3A, SDC2, SDGAL1, STAT1, TIMP1, TPM3, TBB, VDR, VIPR1 | 7.96 × 10^{-9} | ADAM17, AGTR1, APCS, BDNF, CALCA, CASP1, CD44, CHRM3, DDR1, F11, GJB2, GNAZ, GRID2, LCAT, LMS1, NAP1L4, NPCC, NRG1, PDE4B, PIA, PTNP11, SCN2B, SELP, SLCO3A3, TACI, TGFBI, TPM1 |
| Chemotaxis                        | 4.05 × 10^{-4} | ANXA1, CDC42, CXCL10, CXCL13, FGGR2A, FER, FGFR1, GNAZ, HGF, IGFR2, LGALS9, NOS3, VIPR1 | 6.29 × 10^{-5} | AGTR1, BDNF, CALCA, CD44, FIN1, GNAZ, NAP1L4, PDE4B, PIA, PTNP11, SCN2B, TACI, TGFBI |
| **Cell-to-Cell Signaling and Interaction** |          |           |          |           |
| Adhesion of cells                 | 1.47 × 10^{-7} | ANXA1, CDC42, CD4H7, CXCL10, EGR1, FGGR2A, FER, FEB1, FGB, FGFR1, GNAZ, GRD2, HGF, IGFR2, ITGA7, JAK2, LGALS9, LMS1, NOS3, OLRI, REG3A, SDC2, SDGAL1, STAT1, STX4, TIMP1 | 1.34 × 10^{-4} | ACH, ADAM17, CASK, CD44, CNT4, DDR1, DLGI, FGB, F11, GRID2, LMS1, NRG1, PTPNP11, SELP, STX4, TACI, TGFBI, TPH |
| **Cell Signaling**                |          |           |          |           |
| Quantity of calcium               | 3.25 × 10^{-3} | ADCYAP1, AVP, CHRM3, CXCL10, CXCL13, FGGR2A, GRHRI, HGF, NOS3, NOS2A, VDR | 8.89 × 10^{-6} | AGTR1, BDNF, CALCA, CHRM3, FIN1, GRK5, NPCC, PLD2, PIA, PTHR1, PTNP11, SELP, TACI, TGFBI |
| Production of nitric oxide         | 1.33 × 10^{-3} | IRF1, JAK2, MST1, NOS3, NOS2A, STAT1 | - | - |
| Flux of calcium                   | 1.67 × 10^{-3} | ADCYAP1, ANXA1, AVP, CHRM3, CXCL10, CXCL13, FGGR2A, P2X2 | 2.20 × 10^{-3} | CALCA, CHRM3, FIN1, NPCC, P2X2, PPIA, TGFBI |
| Cell surface receptor linked signal transduction | 1.45 × 10^{-3} | ACVR2A, ANXA1, CDC42, CXCL10, FGGR2A, FGRF1, ITGA7, JAK2, KLF6, LMS1, PDGFA, PTPRD, STAT1 | - | - |
| **Small Molecule Biochemistry**   |          |           |          |           |
| Quantity of cyclic AMP            | 1.00 × 10^{-5} | ADCYAP1, AVP, CHRM4, CXCL10, GAP43, GRHRI, GNAZ, NOS3, VIPR1 | 6.03 × 10^{-3} | BDNF, CALCA, GNAZ, NPCC, PTHR1 |
| Production of cyclic AMP          | 2.17 × 10^{-4} | ADCYAP1, AVP, CHRM4, CXCL10, GAP43, GRHRI, GNAZ, NOS3, NOS2A, VIPR1 | 4.35 × 10^{-4} | CALCA, CHRM3, GRK5, PTHR1, TACI, TGFBI |
| Accumulation of cyclic AMP        | 1.21 × 10^{-3} | ADCYAP1, AVP, AVPR2, CHRM3, GRHRI, VIPR1 | - | - |
| Formation of cyclic AMP           | 1.28 × 10^{-4} | ADCYAP1, AVP, AVPR2, GRHRI, GANZ | 7.26 × 10^{-4} | CALCA, GNAZ, PTHR1, TACI |
| Release of Ca2+                   | 9.82 × 10^{-5} | ANXA1, AVP, CHRM3, FGGR2A, FGB, GNG | - | - |
| Quantity of cholesterol           | - | - | 2.85 × 10^{-3} | ATP7B, BDNF, CALCA, GULO, LCAT |
RNA Preparation

Total RNA from unstimulated (control), H-IL-6- and NGF-stimulated PC12 cells was isolated using TRIZOL reagent according to the manufacturer's instructions. RNA was quantified spectrophotometrically by measuring the absorbance at 260 nm and the integrity was checked by formaldehyde agarose gel electrophoresis. The extracted RNA was stored at -80°C.

GeneChip analysis

20 μg of total RNA was used for each experiment and the target cRNA for Affymetrix Gene Chip analysis was prepared according to the manufacturer's instructions. Affymetrix GeneChip Rat Genome U34A arrays containing each 8799 probes including full-length or annotated rat genes and several thousands of rat EST clusters consisting of redundant probes spanning an identical transcript were hybridized with the target cRNAs at 45°C for 16 h, washed and stained by using the Gene Chip Fluidics Station. The arrays were scanned with the Gene Array scanner (Affymetrix), and the fluorescence images obtained were processed by the Expression Analysis algorithm in Affymetrix Microarray Suite (ver. 4.0) and Microsoft Excel. Data were imported into GeneSpring® analysis software (ver. 4.1.3, Silicon Genetics, Redwood City, CA) for further analysis. Genes that showed substantial up- or down-regulation after stimulation by fold changes > 2 were selected from three independent experiments. Genes whose fold change was < 2 and expressed sequence tags (ESTs) that were not fully identified were excluded from the gene list. Thus, only genes with a change fold cutoff > 2 were considered to be significantly differentially regulated. Values are given as round off numbers. For each condition (unstimulated control- and H-IL-6-simulated PC12 cells or unstimulated control and NGF-stimulated PC12 cells) 3 independent microarray analyses (n = 3) were performed using RNA samples derived from independently prepared cell culture batches.

Quantitative Real Time PCR (qRT-PCR)

Total RNA (10 μg) from individual samples cultured separately from those used for microarray analyses was processed by the Expression Analysis algorithm in Affymetrix Microarray Suite (ver. 4.0) and Microsoft Excel. Data were imported into GeneSpring® analysis software (ver. 4.1.3, Silicon Genetics, Redwood City, CA) for further analysis. Genes that showed substantial up- or down-regulation after stimulation by fold changes > 2 were selected from three independent experiments. Genes whose fold change was < 2 and expressed sequence tags (ESTs) that were not fully identified were excluded from the gene list. Thus, only genes with a change fold cutoff > 2 were considered to be significantly differentially regulated. Values are given as round off numbers. For each condition (unstimulated control- and H-IL-6-stimulated PC12 cells or unstimulated control and NGF-stimulated PC12 cells) 3 independent microarray analyses (n = 3) were performed using RNA samples derived from independently prepared cell culture batches.
Table 6: Comparison of commonly regulated gene families in PACAP-stimulated sympathetic primary neurons versus IL-induced PC12 cells (data derived from [53])

| PACAP-stimulated sympathetic neurons (data are from [53]) | 9 hours | 96 hours | Gene family | IL-6-stimulated PC12 cells | 24 hours |
|----------------------------------------------------------|---------|----------|-------------|-----------------------------|---------|
| Pituitary adenylate cyclase activating polypeptide        |         |          | ADCYAP1     | +                           |         |
| BCL2-like protein                                         | +       | n.c.     | BCL2L11     | +                           |         |
| Chemokine Ligands                                         | +       | +        | CXCL1       | +                           |         |
| Cytochrome P450 proteins                                  | +       | +        | CYP1B1      | +                           |         |
| Early growth response                                     | +       | n.c.     | EGR1        | +                           |         |
| Glutathione S-transferase                                 | +       | n.c.     | GSTA3       | -                           |         |
| Heat shock proteins                                       | +       | n.c.     | HSP70B1     | +                           |         |
| Janus kinase                                              |         |          | JAK2        | +                           |         |
| Kruppel-like factors                                      | +       | n.c.     | KLF4        | +                           |         |
| Nuclear factors                                           | +       | n.c.     | NFIA        | +                           |         |
| Nuclear receptors                                         | +       | n.c.     | NR4A3       | +                           |         |
| Sialytransferases                                         | +       | +        | ST8SIA1     | +                           |         |
| Solute carrier proteins                                   | +       | +        | SLC1A3      | +                           |         |
| SLC2A1                                                   | +       | +        | SLC6A3      | -                           |         |
| SLC2A3                                                   | +       | +        | SLC12A5     | -                           |         |
| SLC7A1                                                   | +       | +        | SLC30A2     | -                           |         |
| SLC7A3                                                   | +       | +        | +           |                             |         |
| SLC18A2                                                  | +       | +        | +           |                             |         |
| SLC24A2                                                  | +       | +        | +           |                             |         |
| Tubulins                                                 | -       | n.c.     | TUBA1       | +                           |         |
| Tissue Inhibitor of metalloproteinase                     | +       | +        | TIMP1       | +                           |         |

*, upregulated –, downregulated; n.c., not changed from control cultures; gene symbols are from IPA Tool
reverse-transcribed using Superscript II Reverse Transcriptase (GibcoBRL) according to the manufacturer’s instructions.

PCR reactions were performed in glass capillaries with the LightCycler thermal cycler system (Software version 3.5; Roche Diagnostics, Mannheim, Germany) using the LightCycler DNA Master SYBR Green 1 kit (Roche Diagnostics, Mannheim) according to the manufacturer’s instructions. The primers used for RT-PCR analyses were rat S12 forward: 5′-GGG ATA GCT GCT GGA GGT GTA A-3′; rat S12 reverse: 5′-CCT TGG CCT GAG ATT CTT TG-3′; rat REG3B forward: 5′-GGT TTG ATG CAC TGG CCT-3′; rat REG3B reverse: 5′-TGA CAA GCT GCC ACA GAA TCC-3′; rat GAP-43 forward: 5′-CGT TCG TGG TGT GGA GAA-3′; rat GAP-43 reverse: 5′-GCA GCC ACA TCG GCT TGT TTA-3′. PCR conditions were 50 cycles with denaturation at 95 °C for 8 seconds, annealing at 57 °C for 8 seconds, and extension at 72 °C for 14 seconds. Negative controls without cDNA (non-template controls; ntc) were run concomitantly. Specificity of amplified PCR products was confirmed by melting curve analysis after completion of the PCR run. Each PCR was performed in 3 independent experiments (n = 3) using different cell-culture batches.

**Quantification of LightCycler qRT-PCR data**

Quantification of data was performed with the LightCycler software 3.3 (Roche Diagnostics) using the ΔΔC\textscript{p} method. The difference between the crossing points (CPs; ΔC\textscript{p} values) for the target mRNA samples and reference S12 RNA samples (ΔΔC\textscript{p}) was used to calculate the expression values of the target mRNAs (2^{-ΔΔC\textscript{p}}).

**Ingenuity global functional analyses**

To investigate possible biological interactions of differently regulated genes, datasets representing genes with altered expression profile derived from microarray analyses were imported into the Ingenuity Pathway Analysis Tool (IPA Tool; Ingenuity®Systems, Redwood City, CA, USA; http://www.ingenuity.com). The basis of the IPA-program consists of the Ingenuity Pathway Knowledge Base (IPKB) which is derived from known functions and interactions of genes published in the literature. Thus, the IPA Tool allows the identification of biological networks, global functions and functional pathways of a particular dataset. The complete dataset containing gene identifiers (Genbank accession numbers) and corresponding expression values was uploaded into the application. Each gene identifier is mapped to its corresponding gene object in the IPKB. Each gene product is assigned to functional (e.g. "cellular growth and proliferation") and sub-functional (e.g. "colony formation") categories. The biological functions that are most significant to the dataset are identified by the use of Fischer’s exact test to calculate a p-value that determines the probability that each biological function assigned to that data set is due to chance alone.

**Statistical analysis**

Differences were tested by Welch’s t-test based on three independent experiments, and p-values less than 0.05 were considered statistically significant. Values are expressed as means ± SEM.

**Authors’ contributions**

DK and GW performed the microarray data and drafted the manuscript. UC provided the microarray facility. MB performed the statistical analyses of the microarrays. BD and PM performed the cell-culture of PC12 cells. DK and UO provided support, direction and oversight of the experiments and revised the final manuscript. UO holds the SNF grant.

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