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Differential and Synergistic Effect of Nerve Growth Factor and cAMP on the Regulation of Early Response Genes during Neuronal Differentiation

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Key Words
Cyclic AMP · Dibutyryl cAMP · Microarray · Neurite formation · Nerve growth factor

Abstract
Neurotrophin (NT)-driven differentiation is a process involving activation of multiple signalling events. Treatment of PC12 cells with the prototypic NT nerve growth factor (NGF) induces PC12 cell differentiation characterized by neurite outgrowth and expression of differentiation genes. Cyclic AMP (cAMP), one of the second messengers of NGF stimulation, has also been observed to induce neuronal differentiation in PC12 cells. Interestingly, co-treatment of NGF and dibutyryl cAMP (DBcAMP) exhibits a synergistic effect on neurite outgrowth in PC12 cells, but the mechanisms underlying this synergism remain unknown. In the current study, we compared the gene expression profiles of PC12 cells treated with NGF, DBcAMP or both for 12 h to identify differentially regulated genes during the early stage of differentiation. We found that the genes that were differentially regulated by NGF, DBcAMP or both include genes for acquiring neuronal phenotypes, cytoskeleton-binding proteins and cell cycle proteins. Importantly, we identified a subset of genes that was specifically regulated during co-treatment of NGF and cAMP, suggesting that the synergistic effect of NGF and DBcAMP on neurite outgrowth is possibly mediated through transcription regulation. Our observations provide novel insights on the signalling mechanisms underlying the regulation of neuronal differentiation by NGF and cAMP.

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Introduction

Neurotrophins (NTs) are a family of trophic factors critical for the survival and development of neurons within the peripheral and central nervous systems [1–3]. Members of the family include the prototypic member nerve growth factor (NGF), brain-derived neurotrophic factor, NT-3 and NT-4/5 in mammals, and NT-6/7 in fish species. The actions of NTs are mediated by tropomyosin-receptor-kinase (Trks), a family of receptor tyrosine kinases and p75, the low affinity NT receptor. Binding of NTs to Trk receptors such as TrkA, TrkB, or TrkC induces rapid tyrosine phosphorylation of the receptors, resulting in their transactivation. Phosphorylated tyrosine residues on the receptors then serve as docking sites for var-

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ious adaptor molecules, thereby activating multiple intracellular signal transduction pathways including Ras/mitogen-activated protein kinase, phosphatidylinositol 3-kinase, and phospholipase Cγ [1–3]. The activated signalling cascade can directly regulate the downstream functions of NTs, as well as initiate gene transcriptions for complex biological responses. Among the transcription factors triggered by Trk signalling, induction of AP-1 activity is implicated in the pro-survival property of NGF in serum-deprived PC12 cells [4]. In addition, phosphorylation of CREB (cAMP-response element-binding protein) is involved in NT-mediated neuronal survival and differentiation [5]. Activation of Egr-1, on the other hand, has been demonstrated to play crucial roles in NGF-induced neuronal differentiation in PC12 cells [6–8]. More recently, activation of STAT3, a member of the signal transducer and activator of transcription family, has been observed after activation of Trk receptors [9]. These observations collectively reveal the importance of gene transcription regulation in mediating the downstream functions of NTs. It is therefore pivotal to examine the global changes in gene expression during NT-induced neuronal differentiation in order to understand the molecular mechanisms by which NTs regulate neuronal differentiation.

The effects of NTs on gene transcription during neuronal differentiation have been extensively studied using PC12 cells as a model. NGF treatment induces differentiation of PC12 cells characterized by the expression of differentiation-specific genes and neurite outgrowth, with PC12 cells changing their phenotype from that of proliferating chromaffin-like cells to one resembling non-proliferating, sympathetic neuron-like cells [10]. The approaches that have been taken to comprehensively analyze the global changes in gene expression in PC12 cells include the sequence tag approach [11], restriction landmark cDNA scanning [12], target display [13], serial analysis of gene expression [14] and cDNA microarray analysis [15]. However, most models focus on the genes that are associated with acquisition of neuronal phenotypes in PC12 cells. These cells were thus exposed to NGF for several days, a time frame when NGF-induced differentiation is almost complete. Our focus, in contrast, is on changes in gene expression following exposure to NGF for 12 h to examine the early changes that will ultimately influence the end stage of differentiation.

Among the signalling pathways initiated by NGF stimulation, it has been demonstrated that the intracellular cAMP level increases during the first few minutes of NGF exposure in PC12 cells [16, 17]. We and others have previously shown that activation of cAMP pathway is essential for neuronal differentiation in PC12 cells [8, 18–21]. Interestingly, it has been suggested that co-treatment of NGF and dibutyryl cAMP (DBcAMP, an analogue of cAMP) exhibits a synergistic effect on neurite outgrowth in PC12 cells [22, 23]. Nonetheless, little is known concerning the global effect of DBcAMP, or NGF and DBcAMP combined, on gene transcription during NGF-induced neuronal differentiation. We therefore also examined the changes in gene transcription in PC12 cells after treatment with DBcAMP alone and in combination with NGF for 12 h to examine their effects on gene transcription during differentiation.

Materials and Methods

Cell Cultures

PC12 cells were cultured and maintained according to the previously published protocols [24]. In brief, PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 6% heat-inactivated horse serum, 6% heat-inactivated fetal bovine serum, penicillin (50 U/ml), and streptomycin (100 μg/ml, Invitrogen). Cells were routinely grown on 100-mm tissue culture dishes (Falcon) at 37°C in a humidified atmosphere with 7.5% CO_2.

To delineate the role of the cAMP pathway in the regulation of gene expression, PC12 cells were treated with NGF (50 ng/ml, Alomone Labs Ltd, Jerusalem, Israel), DBcAMP (100 μM) or treated with a combination of the two in DMEM supplemented with 1% heat-inactivated horse serum, 1% heat-inactivated fetal bovine serum, penicillin and streptomycin for 12 h. Total RNA was then extracted for microarray analysis. For morphological analysis of PC12 cell differentiation, PC12 cells were similarly treated with NGF, DBcAMP, or co-treatment of NGF and DBcAMP for 2 days. Images of 5 random fields of each culture were acquired with an inverted microscope (Zeiss). The length of the longest neurite was traced using MetaMorph Version 5.0r1 software (Universal Imaging Corp., Downingtown, Pa., USA). For each measurement, at least 50 cells in each treatment were counted from 3 separate cultures. Each experiment was repeated 3 times.

Microarrays and Data Analysis

Total RNA was isolated from untreated and NGF and/or DBcAMP-treated PC12 cell cultures with an RNA extraction kit according to the protocol supplied by the manufacturer (Qiagen). The RNA was labeled according to the one-cycle eukaryotic target labeling protocol from Affymetrix (Santa Clara, Calif., USA). The samples were hybridized to Affymetrix Murine RAE 230A GENEChips. There are 30,248 transcripts on each array, representing 15,923 genes. Each microarray contains oligonucleotide probes with 25-mers which are complementary to each corresponding sequence and are synthesized in situ on the arrays. The array contains a set of rat maintenance genes to facilitate normalization and scaling of array experiments. Multiple (up to 5) microarray analyses were performed for each treatment.

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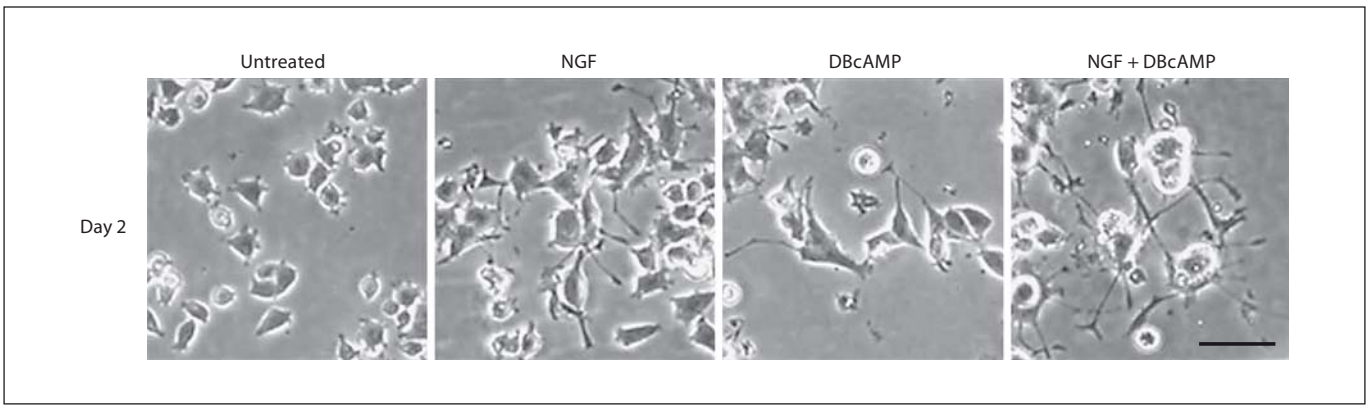

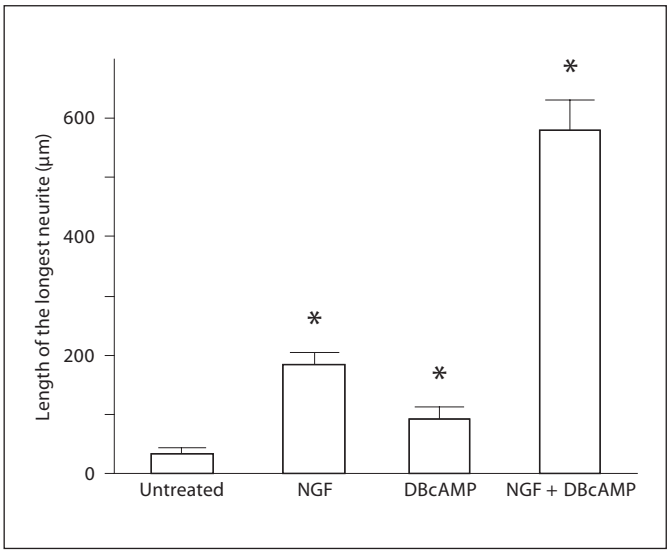

Fig. 1. Neurite outgrowth induced by DBcAMP. PC12 cells were treated with NGF (50 ng/ml), DBcAMP (100 μM) or co-treated with NGF and DBcAMP for 2 days. Quantitation of the length of the longest neurites was presented. Scale = 50 μm, mean ± SD, n = 3, * $p < 0.05$.

Data analysis was performed using GeneSpring software (Agilent Technologies). The dataset from the treated samples were normalized against untreated samples with the p value of Student's t test set at 0.05. The selected genes were then examined in the context of pre-established pathways such as Gene Ontology (GO) pathways, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and Database for Annotation, Visualization and Integrated Discovery (DAVID).

RT-PCR Analysis

Total RNA of PC12 cells was extracted using the RNA extraction kit according to the protocol supplied by the manufacturer (Qiagen). For the synthesis of cDNA, 5 μg total RNA was mixed with 0.5 μM oligo-dT primers (Invitrogen) and denatured at 70°C for 10 min. After cooling on ice, reverse transcription mixture (1 PCR buffer, 2.5 mM $MgCl_2$, 0.5 mM dNTPs, 10 mM DTT and 10 U/μl Superscript II reverse transcriptase) was added. Reaction was carried out at 42°C for 50 min and terminated at 70°C for 15 min. One tenth of the cDNA mixture was used as the template for the subsequent PCR amplification. PCR products were separated on 1% agarose gel.

Results

Identification of Differentially Regulated Genes in Response to 12 h of NGF Stimulation in PC12 Cells

We first examined the global changes in gene transcription following 12 h of NGF treatment in PC12 cells. Consistent with earlier literature, treatment with NGF induced outgrowth of neurites from PC12 cells (fig. 1). Analysis of gene expression using microarray revealed that expression of 253 genes changed by 1.5-fold or more in response to 12 h of NGF treatment. Of these, 145 genes have been matched to named genes and 108 to currently novel, unmatched expressed sequence tags. 192 genes were identified to be up-regulated by 1.5-fold or more upon the NGF treatment, and 61 genes were down-regulated to less than 0.5-fold. Results have been cataloged into known functions (see Materials and Methods) as shown in table 1. Since we aimed to identify early chang-

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Table 1. Analysis of differential gene expression in PC12 cells upon 12 h of NGF or DBcAMP treatment

Functional classification	Fold change by	
	NGF	DBcAMP
Neuronal differentiation		
Actinin, α1	1.59	–
Activated leukocyte cell adhesion molecule	2.79	2.86
Angiotensin II receptor, type 2	0.34	0.42
cAMP-responsive element-binding protein 1	–	0.35
CCAAT/enhancer-binding protein (C/EBP), β	–	2.13
CD9 antigen	2.11	–
Early growth response 1	23.81	–
Ephexin 1	3.44	3.57
Fasciculation and elongation protein ζ1 (zygin 1)	–	0.35
Growth-associated protein 43	4.12	–
Nerve growth factor receptor (p75)	2.03	–
Neurofilament 3, medium	1.95	–
Protein tyrosine phosphatase, receptor type R	1.61	0.47
Similar to vasodilator-stimulated phosphoprotein	1.87	–
Stahmin-like 2	1.58	–
Tumor necrosis factor receptor superfamily, member 12a	4.07	3.33
UDP-N-acetyl-α-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 14	–	0.39
UNC-5 homolog C *(C. elegans)*	–	0.31
VGF nerve growth factor inducible	8.54	4.55
Neurotransmission		
Activity and neurotransmitter-induced early gene protein 4	13.68	–
Calcium-dependent secretion activator	–	0.45
Chloride channel 2	–	2.22
G-protein-coupled receptor 50	2.38	2.38
Glutamate receptor, ionotropic, 2	–	0.42
Glutamine synthase 1	–	2.22
Potassium inwardly rectifying channel, subfamily j, member 11	–	0.48
Prepronociceptin	10.36	14.29
RAB3C, member RAS oncogene family	2.15	2.15
Sodium channel, voltage-gated, type 6, α polypeptide	17.83	25.00
Solute carrier family 1 (glial high-affinity glutamate transporter), member 3	8.55	6.25
Solute carrier family 6, (neurotransmitter transporter, taurine), member 6	–	2.00
Neuroactive ligand receptor function		
Adenosine A2a receptor	0.53	–
Corticotropin-releasing hormone	10.08	9.52
Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2	3.50	–
Gastric inhibitory peptide receptor	0.66	–
Immediate early response 3	2.99	–
Secretin	2.00	–
Secretin receptor	0.45	0.41
Tachykinin 1	8.17	9.52
Cytoskeleton		
Breast cancer 1	0.60	–
Coronin, actin-binding protein 1A	1.62	–
Kelch-like 5 *(Drosophila)*	–	0.39
Kinesin-like 7	0.61	–
Microtubule-associated protein 2	–	0.47
Myosin regulatory light chain	1.70	–
Nephronophthisis 1	–	0.49
Protein kinase C-binding protein ζ 1	0.63	–
Serine/threonine kinase 12	0.59	–
Sperm-associated antigen 5	0.65	–
Similar to four and half LIM domain protein 3	1.78	–
Similar to KIFC1	0.61	–
Syntenin	1.57	–
Cell growth		
Annexin A1	22.88	19.84
Adenylate cyclase-activating polypeptide 1	2.49	3.39
Cell division cycle 2 homolog A	0.65	–
Cyclin D1	–	2.86
Cyclin G2	0.58	–
DNA ligase 1	0.63	–
Epidermal growth factor receptor	–	2.17
Epithelial membrane protein 3	2.22	–
G0/G1 switch gene 2	–	0.35
Growth arrest specific 6	–	0.40
Minichromosome maintenance deficient 6	0.54	–
Mismatch repair protein	0.60	–
Placental growth factor	0.67	–
Protease, serine, 11	3.38	–
Protein kinase Chk2	0.48	–
Retinoblastoma-like 2	0.66	–
S-100 related protein, clone 42c	2.41	–
Transforming growth factor, β_1	1.89	–
Cytokine and local hormone signalling		
Chemokine (C-C motif) ligand 2	21.80	22.47
Cytokine inducible SH2-containing protein	1.70	–
Interferon-related developmental regulator 1	2.05	2.05
Interferon-stimulated protein	–	2.63
Interleukin-18	–	2.56
Interleukin-6 signal transducer	0.70	–
Interferon regulatory factor 6	–	2.09
Nuclear factor, interleukin-3 regulated	–	2.35
Prostaglandin-endoperoxide synthase 1	2.83	–
Small inducible cytokine subfamily A11	3.42	–
Calcium signalling		
ATPase, Ca^{2+} transporting, plasma membrane 2	0.62	–
Bradykinin receptor B2	2.04	2.54
Calcium/calmodulin-dependent protein kinase II β subunit	1.84	–
Protein kinase Cα	2.03	–
Protein tyrosine phosphatase, receptor type N	1.60	2.07
cAMP and MAPK signalling		
cAMP-responsive element-binding protein 1	–	2.03
cAMP-responsive element-binding protein 3-like 2	–	5.05
cAMP-responsive element modulator	–	23.09
MAP kinase-activated protein kinase 2	–	3.01
Protein dephosphorylation		
Dual specificity phosphatase 1	–	3.12
Dual specificity phosphatase 2	10.78	6.99
Protein phosphatase 1, regulatory (inhibitor) subunit 3C	0.36	0.43
Protein tyrosine phosphatase 4a3	–	0.41
Protein tyrosine phosphatase, receptor type F	–	2.49
Wnt signaling		
Adenomatosis polyposis coli	1.45	–
Conductin	0.77	–
Fos-like antigen 1	16.05	4.58
Frizzled homolog 4	2.07	–
Frizzled homolog 5	–	0.20

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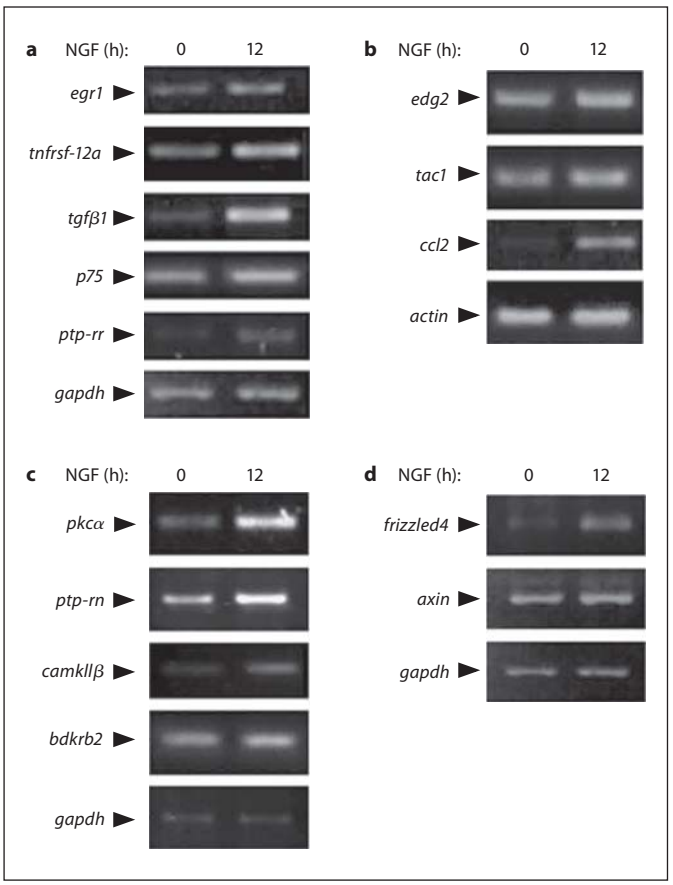

Fig. 2. Verification of genes identified by microarray analysis. RT-PCR of RNAs from untreated and 12 h NGF-treated PC12 cells. Following PCR amplification with individual primer pairs, products were resolved on agarose gels. **a** Representative genes showing the validation of the treatment. **b** Regulation of gene expression in neuroactive ligand and cytokine responses. **c** Genes regulated in calcium signalling pathway. **d** Gene regulation in Wnt signalling. *actin* and *gapdh* serve as equal loading control. Bradykinin receptor B2 *(bdkrb2)*, calcium/calmodulin-dependent protein kinase II β-subunit *(camkIIβ)*, chemokine (C-C motif) ligand 2 *(ccl2)*, endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor, 2 *(edg2)*, early growth factor 1 *(egr1)*, frizzled homolog 4 *(frizzled 4)*, glyceraldehyde 3-phosphate dehydrogenase *(gapdh)*, protein kinase C α *(pkcα)*, protein tyrosine phosphatase, receptor type R *(ptp-rn)*, protein tyrosine phosphatase, receptor type R *(ptp-rr)*, tachykinin 1 *(tac1)*, tumor necrosis factor receptor superfamily, member 12a *(tnfrsf-12a)*, transforming growth factor β_1 *(tgfβ1)*.

es in gene expression which might affect the subsequent process of NGF-induced neuronal differentiation, our choice of genes for further study was based on their relevance to the neuronal differentiation process, such as cytoskeleton rearrangement and cell cycle regulation (table 1). We also identified genes for other cellular functions, such as energy metabolism and biosynthesis, which were regulated during the NGF treatment (data not shown). To substantiate the microarray results, a set of regulated transcripts was subjected to semiquantitative RT-PCR using RNA from naive and NGF-treated cultures (fig. 2).

Consistent with previous reports, we have identified a number of differentiation-related genes in NGF-stimulated PC12 cells, including *egr1* [9], *p75* [25] and neurofilament 3 [26], all of which were found to be induced following NGF treatment. Some of the genes (e.g. tumor necrosis factor receptor superfamily member 12a; *tnfrs-12a*) were identified for the first time as NGF-regulated genes in PC12 cells. The up-regulations of *egr1*, *p75*, and *tnfrs-12a* were confirmed by RT-PCR analyses (fig. 2a). Furthermore, in agreement with previous reports suggesting that NGF treatment promotes cell cycle withdrawal during differentiation of PC12 cells, several cell cycle-related proteins such as cyclin G2 and cell division cycle 2 homolog 1 were down-regulated after 12 h of NGF treatment. In addition, genes contributing to other cellular functions such as neuroactive ligand and cytokine responses (table 1; fig. 2b) and intracellular signalling (table 1; fig. 2c) were also differentially regulated after 12 h of NGF treatment in PC12 cells. For example, tachykinin 1 *(tac1)*, which encodes neurokinin A, neuropeptide K, neuropeptide γ and substance P through differential splicing and posttranslational processing, was induced after 12 h of NGF treatment, similar to earlier observations [27, 28]. Other examples include transforming growth factor β_1 *(tgfβ1)*, endothelial differentiation lysophosphatidic acid G-protein-coupled receptor 2 *(edg2)*, CC chemokine ligand 2 *(ccl2)*, bradykinin receptor B2 *(bkrb2)*, protein kinase C α2 *(pkcα2)*, calcium/calmodulin-dependent protein kinase II β subunit *(camkIIβ)*, and protein tyrosine phosphatase receptor type N and R *(ptp-rn* and *ptp-rr)*. Again, differential changes of these genes were confirmed by semiquantitative RT-PCR (fig. 2a–c). Interestingly, some novel pathways that have not been reported in the NGF signalling cascade were also identified. In particular, several components in the Wnt pathway were induced, suggesting that Wnt signalling may also be actively involved during the differentiation process (table 1). The up-regulations of Frizzled 4 *(frizzled4)* and Axin *(axin)* was confirmed by semiquantitative RT-PCR (fig. 2d).

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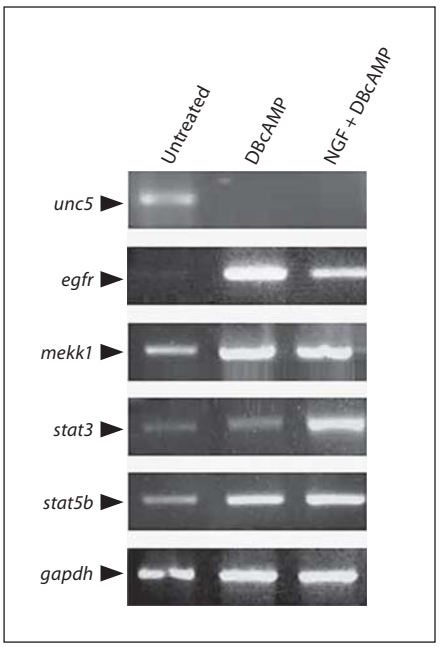

Fig. 3. Verification of genes regulated by cAMP pathway identified by microarray analysis. PC12 cells were treated with DBcAMP alone or co-treated with NGF and DBcAMP for 12 h. Total RNAs were collected for RT-PCR analysis with individual primer pairs. PCR products were resolved on agarose gels. *gapdh* serves as equal loading control. Epidermal growth factor receptor *(egfr)*, mitogen-activated protein kinase kinase 1 *(mekk1)*, signal transducer and activator of transcription 3 *(stat3)*, signal transducer and activator of transcription 5b *(stat5b)*, UNC-5 homolog C *(C. elegans) (unc5)*.

Gene Regulation in PC12 Cells after 12 h of DBcAMP Treatment

We next examined the genes that were differentially regulated by activation of cAMP pathway with the use of DBcAMP. Consistent with an earlier report [8], we found that treatment with DBcAMP, similar to the treatment of NGF, induced differentiation of PC12 cells (fig. 1). The extent of DBcAMP-induced differentiation of PC12 cells was less pronounced than that induced by NGF, with shorter neurite length. The global gene expression profile of PC12 culture treated with DBcAMP for 12 h was examined by microarray, resulting in 331 genes with changes of 1.5-fold or more. Among these 331 genes, 195 are known genes and 136 are genes with unknown functions. Expression of 231 genes was induced upon the 12-hour DBcAMP treatment while 100 genes were down-regulated after the treatment. Some identified genes have been cataloged into known functions, such as acquisition of neuronal phenotypes, neurotransmission, cytoskeleton rearrangement, regulation of cell cycle and intracellular signalling, as shown in table 1. The expression of representative genes including the epidermal growth factor receptor *(egfr)* and UNC-5 homolog C *(unc5)* were confirmed using semiquantitative RT-PCR (fig. 3); *egfr* and *unc5* were, respectively, up- and down-regulated. In agreement with an earlier study [8], protein tyrosine phosphatase, receptor type F and MAP kinase-activated protein kinase 2 were regulated by DBcAMP (table 1). Consistent with the induction of neuronal differentiation by DBcAMP in PC12 cells, a number of genes related to neuronal differentiation and functions and intracellular signalling pathways were also identified as cAMP-regulated genes (table 1).

Interestingly, a total of 113 NGF-mediated genes were also observed to be differentially regulated by DBcAMP treatment. Among these genes, 68 genes are genes with known functions and 45 are novel genes; the expression of 95 genes was increased, while 18 genes were shown being down-regulated. Most of the common genes had compatible level of expression in the two treatments (table 1), suggesting that these genes may contribute to the induction of neuronal differentiation by NGF through the downstream activation of the cAMP pathway. Notable examples of gene products related to the cAMP signalling pathway include adenylate cyclase activating polypeptide 1, secretin receptor and corticotrophin-releasing hormone.

Collaboration of NGF and cAMP in the Regulation of Gene Transcription during PC12 Cell Differentiation

Consistent with previous findings [22, 23], we also observed that co-treatment of NGF and DBcAMP exhibits a synergistic effect on neurite outgrowth in PC12 cells (fig. 1). Upon co-stimulation with NGF and DBcAMP, the neurite length was significantly longer than the arithmetic sum of those produced by either treatment alone. We next examined the genes that were differentially regulated in the presence of both NGF and DBcAMP in PC12 cells during the initiation of neuronal differentiation using cDNA microarray analysis. This result was obtained by filtering the datasets from PC12 cells treated with NGF, DBcAMP and both to identify genes differentially regulated in the presence of both NGF and DBcAMP. Surprisingly, 559 genes were differentially regulated only in the presence of both NGF and DBcAMP. Of the 559 genes, 295 genes have known functions while 264 genes are unknown genes; expression of 171 genes was induced upon the treatment, while 388 genes were down-regulated in the presence of both factors. Some

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Table 2. Genes regulated only in the presence of both NGF and DBcAMP

Functional classification/gene name	Fold change
Neuron differentiation	
Ahnak nucleoprotein (desmoyokin)	2.44
Amphoterin-induced gene and ORF 3	2.22
Cadherin 22	0.37
CBP/p300-interacting transactivator, Glu/Asp-rich C-terminal domain, 2	5.00
Ephrin A1	0.45
Hairy/enhancer-of-split related with YRPW motif 1	0.32
Mitogen-activated protein kinase kinase 1	2.27
Neurturin	0.34
PDZ and Lim domain 7	2.86
Phytanoyl-CoA hydroxylase	2.60
Signal transducer and activator of transcription 3	2.06
Signal transducer and activator of transcription 5B	2.04
Neurotransmission	
Calcium channel, voltage-dependent, T type, α1G subunit	0.46
Choline transporter	2.22
Cholinergic receptor, nicotinic, α polypeptide 3	0.29
Monoamine oxidase	0.35
Neurexophilin 4	0.43
Purinergic receptor P2X, ligand-gated ion channel	0.45
Potassium channel, subfamily K, member 2	0.20
Solute carrier family 38, member 1	2.38
Cytoskeleton	
Adducin 3γ	0.28
A disintegrin and metalloprotease domain 8	0.48
A disintegrin and metalloprotease domain 23	0.45
Coronin relative protein	0.42
Myosin viia and rab interacting protein	2.70
Minichromosome maintenance protein 7	0.46
Parvin, α	0.43
Tropomyosin isoform 6	2.77
Cell growth	
Adenylate kinase 1	0.24
Megakaryocyte-associated tyrosine kinase	0.48
MutS homolog 2 *(E. coli)*	0.43
Pericentriolar material 1	0.40
Platelet-activating factor acetylhydrolase, isoform Ib, α subunit 45 kDa	2.22
S100 calcium binding protein A11 (calizzarin)	2.59
Tumor protein p53 inducible protein 11	0.44
Cytokine signalling	
Erythropoietin receptor	2.03
Interleukin-6 receptor	2.28
Interlukin-11 receptor, α-chain 1	0.50
Calcium signalling	
Transient receptor potential cation channel, subfamily c, member 3	0.27
Protein dephosphorylation	
Dual specificity phosphatase 11	2.34
Wnt signalling	
Dickkopf homolog 3	0.24
Frizzled homolog 7	2.39

of the known genes were listed based on their relevance to neuronal differentiation and functions as shown in table 2. For example, expression of mitogen-activated protein kinase kinase 1 *(mekk1)* and STAT3 *(stat3)* was induced and enhanced upon co-treatment of NGF and DBcAMP (fig. 3). Both molecules have previously been shown to be necessary for neurite outgrowth in PC12 cells [9, 29]. We also found that NGF and DBcAMP regulated expression of tropomyosins, which are integral components of neurites and growth cones [30]. Expression of tropomyosin isoform 6 was induced after the treatment of NGF and DBcAMP. The presence of genes that were differentially regulated only by concurrent stimulation of NGF and cAMP suggests that transcription of 'new' genes serves as one of the mechanisms to enable the synergistic effect of NGF and cAMP on neuronal differentiation.

Discussion

Study of neuronal differentiation necessitates cellular models that can be compared before and after factor exposure. Since neuronal differentiation in cell line models rely on factor stimulation or receptor activation, different stages of neuronal differentiation can be timed and examined in these cell line models. One of the commonly used models for examining neuronal differentiation is NGF-treated PC12 cells. Beyond the initial signalling events, NGF stimulates both transcription-dependent and transcription-independent pathways to achieve the terminal differentiation phenotype of PC12 cells. Several studies using different approaches have characterized the differential gene expression of PC12 cells before and after several days of NGF treatment [11–15]. In this study, we have identified early response genes that are potentially relevant to the early stages of neuronal differentiation.

Among the differentially regulated genes are transcription factors like *egr1* and *Fos-like antigen 1*, which may trigger expression of genes related to later stages of neuronal differentiation. For example, *egr1* have been demonstrated to induce genes for neuronal cytoskeleton proteins such as neurofilament 3, genes involved in regulating actin reorganization such as coronin, and microtubulin assembly like myosin light chain [6]. They are also involved in the induction of genes required for neuronal functions such as voltage-gated sodium channel polypeptides, glutamate receptor subunit, neuropeptides and their receptors. Cessation of proliferation is believed to be a prerequisite of cell differentiation [9,

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31]. We also observed down-regulation of transcripts encoding cell cycle proteins, such as cyclin G2, protein kinase checkpoint kinase 2 (Chk2) and cell division cycle 2 homolog A after 12 h of NGF treatment. Moreover, during our pathway analysis of the microarray data, we have identified novel components such as those in the Wnt signalling pathway for the initiation of differentiation. The induction of Wnt signalling components, like Fos-like antigen, adenomatosis polyposis coli and frizzled homolog 4, suggests that Wnt signalling pathway could be actively involved during NGF-induced neuronal differentiation. These observations will facilitate the construction of signalling networks in the differentiation program.

Several reports have already described the induction effect of cAMP on neuronal differentiation. Pituitary adenylate cyclase-activating polypeptide (PACAP) stimulation leads to the activation of $G\alpha_s$-coupled receptors to regulate neuronal differentiation via cAMP pathways [32]. Ravni et al. [8] have compared gene expression profiles after exposure of PC12 cells to either forskolin, DBcAMP, PACAP or NGF to examine cAMP-dependent target genes, and reported a cAMP-dependent, PKA-independent pathway involving the up-regulation of two genes: *egr1* and *vil2*, to mediate PACAP-dependent neuritogenesis. Our group has also previously revealed that two herb extracts induce PC12 cell differentiation in a cAMP-dependent manner [18, 19]. Nevertheless, unlike NGF, DBcAMP-induced neurites are unstable and do not sustain neurite extension [22]. Therefore, it is of interest to further understand the role of cAMP in neuronal differentiation. In the current study, we found that the mRNA expression of several protein phosphatases and cytokine signalling proteins was regulated by DBcAMP treatment. More importantly, several genes for neuron differentiation and functions were regulated by DBcAMP, suggesting that cAMP treatment alone could induce differentiation-specific genes.

Upon comparison of the genes regulated by DBcAMP with those regulated by NGF, 113 genes appeared to be similarly regulated by the two treatments. For example, *ccl2* was significantly induced by NGF or DBcAMP (table 1). The targets of *ccl2* are G-protein-coupled chemokine receptors and the involvement of G_i proteins in NGF signalling has already been demonstrated [33]. Strikingly, some examples of genes regulated by either NGF or cAMP including ephexin 1, G-protein-coupled receptor 50, prepronociceptin, voltage-gated sodium channel type 6 α-polypeptide, corticotrophin-releasing hormone, secretin receptor, tachykinin 1, annexin A1, and bradykinin receptor B2 showed comparable levels of regulation, suggesting that these NGF-induced genes could be induced through a cAMP-dependent pathway. Our observations further support the notion that cAMP-dependent gene transcription is necessary for NGF-induced neuronal differentiation [17]. However, why cAMP alone cannot sustain neurite outgrowth despite the induction of considerable number of neuronal differentiation genes requires more investigation. In this regard, it is noteworthy that more 'neuronal differentiation genes' are differentially regulated by NGF than by DBcAMP (table 1).

Potentiation of neurite outgrowth in the presence of NGF and DBcAMP is observed in PC12 cells [22]. It has been proposed that during the co-treatment, DBcAMP induces a rapid but unstable cytoskeleton rearrangement to initiate neurite formation, which is RNA synthesis-independent. At the same time, NGF sustains the reorganization of the PC12 cytoskeleton by an RNA synthesis-dependent mechanism, resulting in an overall synergistic effect to neurite outgrowth. Here we filtered genechip results from cultures treated with NGF, DBcAMP and the combination of the two to isolate genes that were regulated only in the presence of both NGF and DBcAMP (table 3). We found that a lot of the known signalling molecules, including MEKK1, STAT3 and STAT5, were induced in the presence of both NGF and DBcAMP. Furthermore, functional genes in Wnt signalling pathway, e.g frizzled homolog 7 and dickkopf homolog 3, were regulated in the presence of both NGF and DBcAMP, suggesting that activation of Wnt signalling during NGF-induced neuronal differentiation in PC12 cells could be dependent on both NGF and cAMP pathways. Interestingly, some genes that were induced by NGF treatment alone (table 1), like kinesin-like 7, placental growth factor, cyclin G2, cytokine inducible SH2-containing protein were identified with enhanced degree of gene regulation when cells were treated with both NGF and DBcAMP (data not shown). This group of genes likely also contributes to the synergistic effect of NGF and DBcAMP on neuronal differentiation. Nonetheless, it should be noted that some ion channels, including potassium channel, glutamate receptor, nicotinic cholinergic receptor and purinergic receptor, were down-regulated by the co-treatment of NGF and DBcAMP. How this contributes to the synergistic effect of NGF and DBcAMP on neuronal differentiation remains to be explored.

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In the present work, we have verified some known NGF-mediated genes, and introduced new candidates to the gene list contributing to NGF-induced differentiation. Moreover, we have identified genes that are regulated only in the presence of both NGF and cAMP, suggesting that in addition to functioning as a downstream mediator of NGF signalling, cAMP may also cooperate with NGF to trigger gene transcription. Our study provides an example of using pathway-focused microarray systems to dissect the mechanisms of complex signalling pathways in NGF-induced neuronal differentiation. Understanding how these early response genes are being regulated provides further insights on the neuroblast-neuron transition and regulation of neuroblastoma development. It is also interesting to note that these genes could be potential therapeutic targets for regeneration processes in damaged neurons.

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