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Dibutyryl-cAMP up-regulates nur77 expression via histone modification during neurite outgrowth in PC12 cells

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An elevated level of cyclic AMP (cAMP) within cells activates gene expression through the cAMP-PKA-CREB pathway. Among the CREB target genes, some immediate early genes exist that are responsive to cAMP including the nur77 and c-fos genes. Treatment with dibutyryl-cAMP (dbcAMP) as well as nerve growth factor (NGF) induces neurite outgrowth in PC12 cells. Here, we report that acetylation of histone H3 was gradually stimulated after treatment with dbcAMP in PC12 cells and peaked 1 h after treatment. As the result of reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR (qPCR) experiments, both nur77 and c-fos gene expression were found to have peak 1 h after treatment. Knock-down with siRNA against nur77 mRNA inhibited the neurite outgrowth induced by dbcAMP, whereas knock-down with siRNA against c-fos mRNA did not inhibit the dbcAMP-induced neurite outgrowth. A chromatin immunoprecipitation (ChIP) assay revealed that the nur77 gene was associated with the acetylated Lys14 of histone H3 after treatment with dbcAMP. However, the amount of c-fos gene associated with acetylated histone H3 was not changed after treatment with dbcAMP. These results suggest that the expression of nur77, which is essential for the neuronal differentiation induced by dbcAMP, is up-regulated via dbcAMP-induced acetylation of the Lys14 of histone H3 in PC12 cells.

Keywords: cAMP/Differentiation/immediate early gene/local chromatin structure/PC12 cell.

Many hormones and growth factors activate transcription by raising the level of cyclic AMP (cAMP) within cells and thereby regulate proliferation, differentiation, survival and plasticity by triggering programmes of gene expression. cAMP activates cAMP-dependent protein kinase (PKA), which phosphorylates and regulates a variety of cellular proteins (*1–3*). In order to optimize the responsiveness to a wide variety of signals, the cAMP/PKA pathway may interact with other signalling pathways.

PKA phosphorylates the transcription factor CRE-binding protein (CREB), which then binds to the cAMP-responsive element (CRE), a consensus sequence found in promoter regions of many target genes. There are more than 100 CREB target genes including immediate early genes (IEG). IEG were some of the first identified CREB target genes, the expression of which is induced in a CRE-dependent manner in response to certain stimuli that activate CREB.

Some of the products of IEG are transcriptional regulators of a number of downstream genes and may have important roles in neurite outgrowth of neuronal cells. For example, the NGF-induced neurite outgrowth observed following the activation of TrkA may be regulated by the products of IEG such as c-fos and nur77 family genes (*4–6*). On the other hand, the mechanism by which the cAMP/PKA pathway induces the expression of immediate early genes causing neurite outgrowth is largely unknown.

The nur77 gene is an immediate early gene. Its product, Nur77, is known to be an orphan nuclear receptor and is also known as NGFI-B, TR3 and Nr4a1. Nur77 is a member of the Nur77 family, which also contains the orphan nuclear transcription factors Nurr-1 and NOR-1. A particularly instructive cell system for studying immediate early gene expression is the PC12 cell line, in which it can be stimulated by various agents to produce various cellular responses (*7*, *8*). The transcription of nur77 has been shown to be stimulated by treatment with forskolin, which elevates the level of cAMP and activates PKA, which in turn phosphorylates and activates CREB. It has also been suggested that in some cell types, both PKA and extracellular signal-regulated kinase (ERK) 1/2 are involved in the regulation of nur77 transcription. However, the detailed mechanisms of nur77 transcription have not been fully elucidated. In this study, we investigated the mechanism underlying the regulation of nur77 transcription during neurite outgrowth induced by dbcAMP in PC12 cells. We found that the cAMP-PKA-nur77 pathway is essential for the induction of differentiation by dbcAMP in PC12 cells.

Transcription of some genes is known to be activated by covalent histone modifications. Such covalent histone modifications are brought about by proteins that, upon recruitment, can phosphorylate, acetylate

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and methylate histone tails (*9*, *10*). The best-studied histone modification involved in transcriptional activation is acetylation. The level of acetylated histone H3 has been shown to be correlated with the transcription status of many genes: transcriptionally active euchromatin regions of the genome are often associated with hyperacetylated histones, whereas transcriptionally silent regions are associated with hypoacetylated histones. Histone-modifying complexes are thought to cooperate with chromatin-remodelling complexes to re-configurate chromatin, thereby establishing a local chromatin structure that allows the subsequent assembly of an active pre-initiation complex (PIC) at the promoter. The *in vivo* sequence of histone-modifying and chromatin remodelling events relative to this PIC formation and transcriptional activation has been studied previously (*9*, *10*).

Acetylation of histone tails is increased by histone acetyl transferase (HAT) and decreased by histone deacetylase (HDAC). The level of histone acetylation is regulated by the balance of these two enzymes and other factors associated with histones in nucleosomes. However, nothing is currently known regarding the involvement of histone acetylation in nur77 gene expression during dbcAMP-induced neurite outgrowth in PC12 cells. Here, we report that nur77 transcription is up-regulated via histone acetylation induced by dbcAMP in PC12 cells.

Materials and Methods

Cell culture

PC12 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% (v/v) fetal bovine serum, 5% (v/v) heat-inactivated horse serum and 0.1% (v/v) penicillin–streptmycin solution (Gibcco BRL, USA).

Measurement of neurite outgrowth

PC12 cells were plated in 24-well plates at 0.5×10^4 cells/cm^2, the medium was changed to serum-free DMEM, and the cells were incubated for 16 h. Then, the cells were treated with 300 nM dbcAMP alone or with 10 μM H89 in serum-free DMEM supplemented with TIP (insulin, transferrin, and progesterone) for 72 h. After the treatment, the cells were fixed with 4% paraformaldehyde for 30 min. For analysis of neurite outgrowth, cells (200 cells/well) were randomly photographed using a Nikon microscope (TE-2000U). Neurite lengths were measured using the Lumina Vision software (Mitani Corporation, Tokyo, Japan) running on a Fujitsu FMV computer (Fujitsu, Kanagawa, Japan). Differences between groups were examined for statistical significance using the Kolmogorov–Smirnov test. A *P*-value <0.05 denoted the presence of a statistically significant difference.

Immunoblot analysis

PC12 cells were seeded onto 6-cm-diameter dishes, and the medium was changed to serum-free DMEM, and cells were incubated for 16 h. Then, the cells were untreated or treated with 300 nM dbcAMP in the absence or presence of 10 μM H89 for 0, 0.5, 1, 2 or 4 h. The cells were lysed in a lysis buffer containing 50 mM Tris–HCl (pH7.8), 150 mM NaCl, 1% sodium dodecylsulphate (SDS), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml leupeptin, 2 μg/ml of aprotinin, 1 mM Na_3VO_4 and 10 mM sodium butyrate (*11*). Then, the lysate (15 μg of protein per lane) was loaded on SDS-polyacrylamide gel for electrophoresis (PAGE) after being added to Laemmli buffer and blotted onto a PVDF membrane using a semi-dry blotter (Atto, Japan). The first antibody (anti-histone H3 (monoclonal) (Upstate Biotechnology, USA) or anti-acetylated histone H3 (Upstate Biotechnology) was loaded onto the membrane after blocking treatment with 5% skimmed-milk (Nakarai, Japan), and then the horse radish peroxidase-conjugated second antibody (Santa Cruz, USA) was loaded. The bands were detected by enhanced chemiluminescence (SuperSignal West Femt Maximum Sensitivity Substrate; Pierce, USA) and visualized with a light-capture system (Atto, Japan).

RNA preparation and reverse transcription-polymerase chain reaction

PC12 cells were seeded onto 6-cm-diameter dishes, the medium was changed to serum-free DMEM, and the cells were incubated for 16 h. The cells were left untreated or pre-treated with 10 μM H89 for 15 min. Then, cells were untreated or treated with 300 nM dbcAMP in the absence or presence of H89 for 0, 1 or 4 h. Total RNA was collected with Isogen (Nippongene, Japan) and extracted with phenol–chloroform. cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Amersham, USA). PCR primers were synthesized as described previously for c-fos (sense: GGAGCTGAC AGATACGCTCCA anti-sense: AATGTTCTTGACGGCTCCA) (*12*), nur77 (sense: CCGGTGACGTGCAGCAATTTTATGAC anti-sense: GGCTAGAATGTTGTCTATCCAGTCACC) (*13*) and beta-actin (sense: TGTGATGGACTCCGGTGACGG anti-sense: A CAGCTTCTCTTTGATGTCACGC) (*14*). cDNA were amplified with TaKaRa *Taq* polymerase for 30 (nur77), 25 (c-fos) and 35 (beta-actin) cycles.

Quantitative real-time PCR (qPCR) analysis

Total RNA was collected with Isogen (Nippongene) and extracted with phenol–chloroform. cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Amersham). One-hundred nanograms of cDNA was used as template for StepOnePlus™ real-time PCR system (Applied Biosystems, USA) using TaqMan® MGB probe. PCR primers used were those of TaqMan® gene expression assay kits for c-fos (Assay ID: Rn02396760_g1), nur77 (Assay ID: Rn00666994_g1) and beta-actin (Assay ID: Rn00667869_m1). Differences between groups were examined for statistical significance using the two-way analysis of variance (ANOVA). A *P*-value <0.05 denoted the presence of a statistically significant difference.

Knock-down of nur77 and c-fos

A siRNA targeting the 25-nt sequence, (sense: UCCAGUGGCUCU GAUUACUAUGGAA anti-sense: UUCCAUAGUAAUCAGAG CCACUGGA) of nur77, the control scrambled siRNA (scRNA) (sense: UCCCGGUGUCUAUUAUAUCGGAGAA anti-sense: U UCUCCGAUAUAAUAGACACCGGGA), a siRNA targeting the c-fos gene (sense: UCUUUCAGUAGAUUGGCAAUCUCG G anti-sense: CCGAGAUUGCCAAUCUACUGAAAGA) and the other control scRNA (sense: UCUCUUUGACGAUUUACG GUAACGG anti-sense: CCGUUACCGUAAAUCGUCAAAGA GA) were obtained from Invitrogen, USA. PC12 cells were seeded onto six-well plates and transfected with 100 nM siRNA against nur77, c-fos, or control scRNA using Lipofectamine®RNAiMAX (Invitrogen). After 1 day, the medium was changed to serum-free DMEM and incubated for 16 h. Then, the cells were treated with 300 nM dbcAMP in serum-free DMEM supplemented with TIP for 1 h. For measurement of the neurite outgrowth of PC12 cells transfected with siRNA against nur77 or c-fos, the cells were plated onto 24-well plates at 0.5×10^4 cells/cm^2. Then, 100 nM siRNA against nur77 or c-fos, and 20 nM pCX-EGFP were co-introduced into cells with Lipofectamine®RNAiMAX (Invitrogen). After 24 h, the cells were treated with 300 nM dbcAMP in serum-free DMEM supplemented with TIP for 48 h, and were then fixed with 4% paraformaldehyde for 30 min. For analysis of neurite outgrowth, GFP-expressed cells (200 cells/well) were randomly photographed 48 h after the initial treatment with dbcAMP using a Nikon photo microscope. Neurite length was measured using the Lumina Vision software (MITANI CORPORATION) running on a Fujitsu FMV computer.

Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) was performed as described previously (*15*) with some modifications. The cells were cross-linked at a concentration of 2×10^7 cells/ml (in PBS) with 1% formaldehyde for 10 min at 37°C. After quenching of the cross-linking reaction with 125 mM glycine, the fixed cells were washed with PBS containing protease inhibitors (1 mM PMSF, 2 μg/ml aprotinin

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and 2 μg/ml leupeptin), before being suspended in 400 μl of SDS lysis buffer (1% SDS, 10 mM EDTA, and 50 mM Tris–HCl at pH 8.0) and sonicated to make a mean fragment size of 200–1000 bp. The solubilized chromatin was obtained by centrifugation for 10 min at 15,000 rpm at 4°C and diluted 10-fold in ChIP dilution buffer (1% Triton X-100, 1 mM EDTA, 150 mM NaCl and 15 mM Tris–HCl at pH 8.0). The diluted chromatin of 5×10^6 cells was incubated with anti-acetyl-histone H3 (Upstate Biotechnology), anti-acetyl-histone H3 (Lys14) (Upstate Biotechnology), or normal rabbit IgG (Santa Cruz) for 12–16 h at 4°C. Immune complexes were bound to Protein G sepharose beads preblocked with salmon sperm DNA and BSA for 90 min at 4°C. The beads were washed once each with low-salt wash buffer (0.1% SDS, 1% Triton-X100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris–HCl at pH 8.0), high-salt wash buffer (500 mM NaCl wash buffer), LiCl wash buffer (0.25 M LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA and 10 mM Tris–HCl at pH 8.0), and twice with TE buffer (10 mM Tris–HCl at pH 8.0 and 1 mM EDTA). Immune complexes bound to Protein G beads were incubated overnight at 65°C and treated with 100 μg/ml Proteinase K for 2 h at 56°C, before being extracted once with phenol/chloroform, and then the DNA was precipitated with ethanol plus glycogen as a carrier, and the precipitated DNA was resuspended in 20 μl water. The following primers were used to amplify the c-fos and nur77 promoters in 30 cycle PCR with TaKaRa *Taq* polymerase: TCCTACATGCGGAGGGTCCAGGAGAC (c-fos forward primer); GAGTAGTAGGCGCCTCAGCTGGCCG (c-fos reverse primer); TCACGCGCGCAGACATTCCA (nur77 forward primer); and CACCTCTTAAGCGCTCCGTGA (nur77 reverse primer) (*16*). The PCR products were electrophoresed on agarose gels and detected with ethidium bromide.

Results

In order to analyse the differentiation-inducing effect of cAMP on PC12 cells, we first measured the length of neurites produced 72 h after treatment with dbcAMP. We confirmed that dbcAMP induced neurite outgrowth in PC12 cells (Fig. 1A and B). Neurite length from PC12 cells treated with dbcAMP was significantly increased in comparison with that from untreated cells when statistical analysis was performed using the Kolmogorov-Smirnov test. H89, an inhibitor of protein kinase A, significantly decreased the length of the neurites induced by dbcAMP to near the control level (Fig. 1B), indicating that the cAMP–PKA pathway is involved in this differentiation.

It is well established that promoter-associated chromatin can be remodelled by protein complexes that covalently modify histone tails in the nucleosomes that surround promoter DNA (*9*, *10*). In this broadly accepted model, the acetylation of N-terminal lysines in the core histone H3 is associated with the recruitment of HAT-containing protein complexes and enhanced gene expression. CREB-binding protein (CBP)/p300 then interacts with the holoenzyme complex bringing Pol II and other general transcriptional machinery into contact with the promoter. In order to determine whether histone H3 acetylation is necessary for dbcAMP-induced early differentiation of PC12, we analysed global histone H3 modifications in PC12 cells. We detected histone H3 acetylation 30 min after dbcAMP addition, and it reached to its peak at 1 h and then declined to the basal level by 4 h (Fig. 2). In addition, H89 decreased the histone H3 acetylation induced by dbcAMP, suggesting that histone H3 acetylation is regulated by the dbcAMP–PKA pathway during the early period of differentiation 0–1 h after dbcAMP treatment.

It has been reported that many immediate early genes including nur77 and c-fos are regulated by cAMP, using DNA microarray and serial analysis of chromatin occupancy (SACO) in PC12 cells (*17*). Pap and Szeberenyi (*18*) revealed that the expression of the nur77 and c-fos genes was efficiently increased by dbcAMP. Then, we searched for the immediate early gene needed for the transition from the undifferentiated state to the differentiated state 0–4 h after the dbcAMP addition, and for this we focused on the nur77 and c-fos genes. Total RNA isolated from control cells, cells treated with 300 nM dbcAMP, and cells treated with 300 nM dbcAMP and 10 μM H89 were subjected to RT-PCR and qPCR analysis. As shown in Fig. 3A and B, the expression of the nur77 and c-fos genes peaked 1 h after the dbcAMP treatment, and H89 decreased the expression of nur77 and c-fos genes induced by dbcAMP, indicating that the expression of the nur77 and c-fos genes is regulated by the dbcAMP–PKA pathway during the early period of differentiation 0–1 h after dbcAMP treatment.

Next, in order to determine which of the nur77 and c-fos genes was necessary for the dbcAMP-induced differentiation of PC12 cells, knockdown experiments were performed. Knockdown with siRNA against nur77 mRNA inhibited the neurite outgrowth induced by dbcAMP (Fig. 4B and C). Neurite length from PC12 cells treated with siRNA against nur77 mRNA was significantly decreased in comparison with that from cells treated with scrambled RNA (scRNA) when statistical analysis was performed using the Kolmogorov–Smirnov test. On the other hand, treatment with siRNA against c-fos mRNA did not significantly reduce the dbcAMP-induced neurite outgrowth compared with treatment with scRNA (Fig. 5B and C). These results indicate that the up-regulation of the expression of nur77, but not c-fos, by dbcAMP is essential for the differentiation induced by dbcAMP.

The dbcAMP-induced H3 acetylation gradually increased during the early period of differentiation 0–1 h after dbcAMP induction (Fig. 2). The increase in H3 acetylation may serve as a link from the upstream dbcAMP–PKA pathway to the up-regulation of nur77 expression in PC12 cells (Fig. 3A and B). The dbcAMP–PKA pathway is activated during the early differentiation period and may be responsible for the increase in histone H3 acetylation. Accordingly, we considered that the increase in histone H3 acetylation may be closely related to the up-regulation of nur77 expression, which serves as a critical mediator of differentiation induced by dbcAMP.

In order to clarify the contribution of histone H3 acetylation to the dbcAMP-induced up-regulation of nur77 expression, ChIP assays were carried out. ChIP assays with anti-acetylated histone H3 antibody revealed that the nur77 gene promoter was associated with the acetylated histone H3 after treatment with dbcAMP (Fig. 6A). In addition, we found that H89 decreased the association between the nur77 gene promoter and the acetylated histone H3 induced by dbcAMP, indicating that the regulation of the nur77 promoter via histone H3 acetylation occurs through the dbcAMP–PKA pathway during the early period

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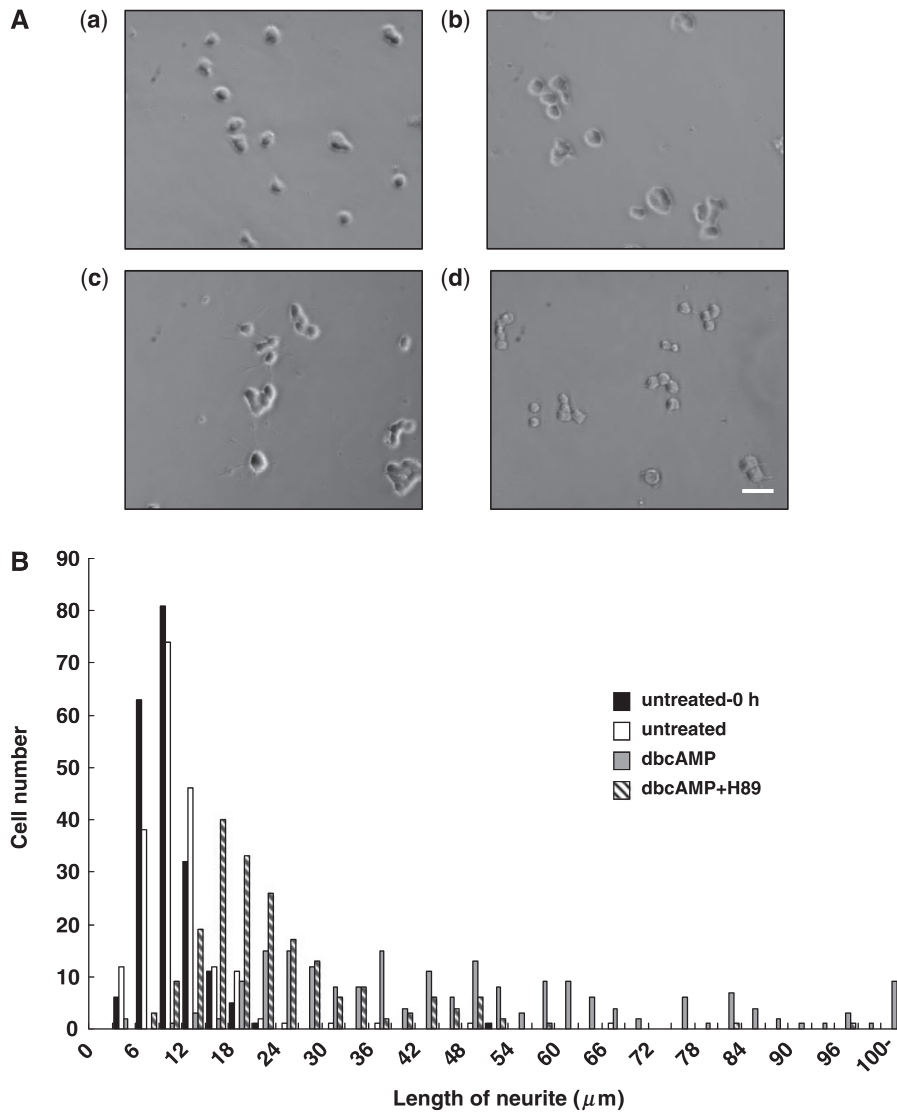

Fig. 1 PKA is required for dbcAMP-induced neurite outgrowth in PC12 cells. (A) Photomicrographs of PC12 cells treated with dbcAMP. PC12 cells were cultured for 0 h (a) and 72 h (b–d) without (a and b) or with 300 nM dbcAMP (c and d) in the absence (a–c) or presence of 10 μM H89 (d). The results are representative of three independent experiments. Scale bar: 30 μm. (B) Histogram of neurite length of dbcAMP-treated PC12 cells. PC12 cells were cultured for 0 h (closed bars) and 72 h (open, grey and striped bars) without (closed and open bars) or with 300 nM dbcAMP (grey and striped bars) in the absence (closed, open and grey bars) or presence of 10 μM H89 (striped bars). Neurite length was measured using the Lumina Vision software. Differences between groups were examined for statistical significance using the Kolmogorov–Smirnov test. $P < 0.001$ (untreated *versus* dbcAMP); $P < 0.001$ (dbcAMP *versus* dbcAMP + H89).

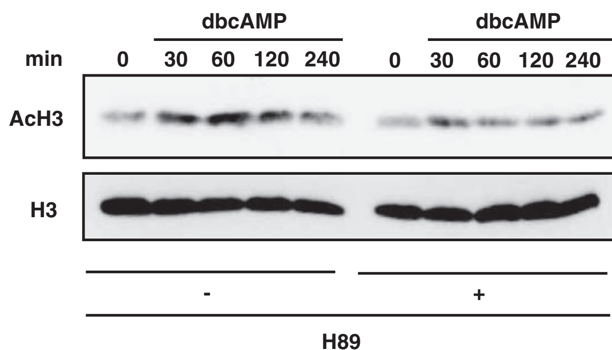

Fig. 2 Effect of dbcAMP on global acetylation of histone H3 in PC12 cells. Western blot analysis to analyze dbcAMP-induced global acetylation of histone H3 in PC12 cells. The cells were untreated or treated with 300 nM dbcAMP in the absence or presence of 10 μM H89 for the indicated times in serum-free DMEM. The results are representative of six independent experiments. H3: total histone H3 as a control; AcH3: acetylated histone H3.

of differentiation 0–1 h after dbcAMP treatment. Furthermore, in order to identify the detailed place of the histone H3 acetylation, we analysed whether the nur77 gene promoter was associated with the acetylated Lys14 of histone H3 after treatment with dbcAMP (Fig. 6A), and we discovered it was. In addition, we revealed that H89 decreased the association between the nur77 gene promoter and the acetylated Lys14 of histone H3 induced by dbcAMP, indicating that the regulation of the nur77 promoter via the acetylated Lys14 of histone H3 is mediated by the dbcAMP–PKA pathway during the early period of differentiation 0–1 h after dbcAMP treatment.

We also analysed whether the association of the c-fos gene promoter with the acetylated histone H3 was increased after treatment with dbcAMP. As shown in Fig. 6B, the amount of c-fos gene promoter

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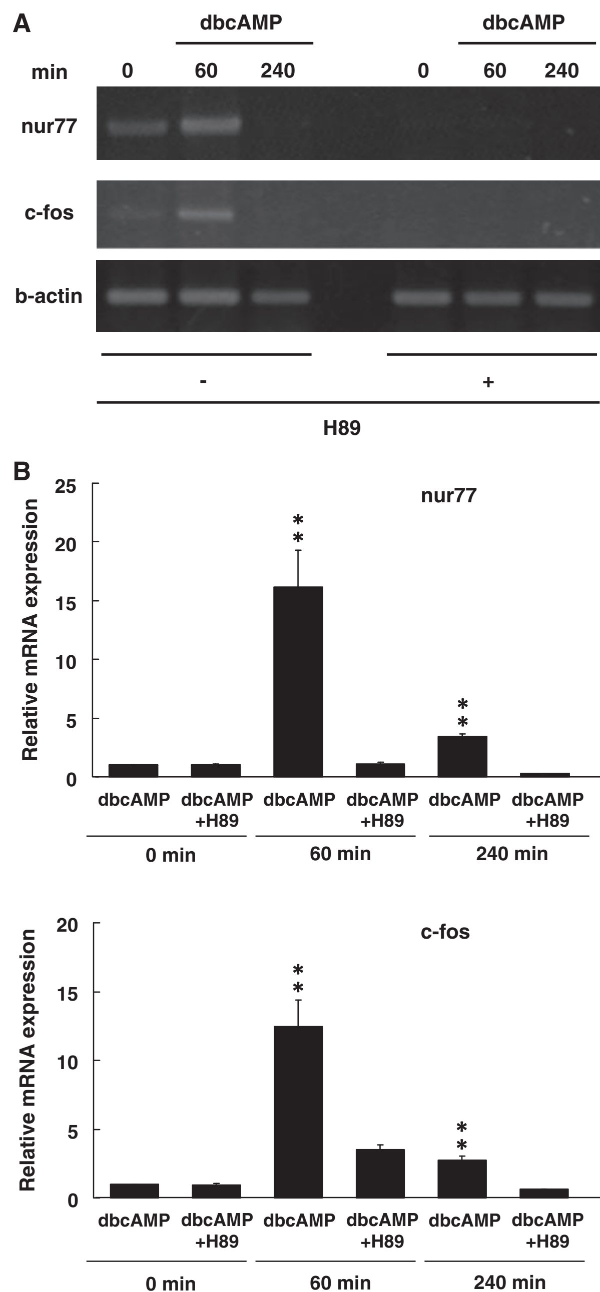

Fig. 3 PKA is involved in the expression of nur77 and c-fos genes in dbcAMP-treated PC12 cells. (A) PC12 cells were untreated or treated with 300 nM dbcAMP in the absence or presence of 10 μM H89 for the indicated times in serum-free DMEM. nur77, c-fos and beta-actin (b-actin) mRNA were detected by RT-PCR as described in Materials and Methods section. The results are representative of three independent experiments. (B) PC12 cells were untreated or treated with dbcAMP in the absence or presence of H89 as described above. nur77, c-fos and beta-actin (b-actin) mRNA were detected by quantitative real-time PCR (qPCR) as described in Materials and Methods section. nur77 and c-fos mRNA levels were normalized against beta-actin mRNA levels and against the initial time point (0 min). Means ± SEM ($n = 3$) are shown, and differences between groups were examined for statistical significance using the two-way ANOVA. $^{**}P < 0.01$ (dbcAMP-0, 60, 240 min versus dbcAMP + H89-0, 60, 240 min).

associated with acetylated histone H3 was not changed after treatment with dbcAMP, and H89 did not change the association between the c-fos gene promoter and the acetylated histone H3 induced by dbcAMP, indicating that the expression of c-fos promoter is not regulated via histone H3 acetylation downstream of the dbcAMP–PKA pathway. We also found that the association of the c-fos gene promoter and the acetylated Lys14 of histone H3 was not changed after treatment with dbcAMP, by using anti-acetylated Lys14 of histone H3 antibody. These results indicate that during the dbcAMP-induced activation of nur77 gene, histone modification including acetylation of Lys14 of histone H3 occurs at the promoter of nur77 gene. Taken together, our results show that local changes in chromatin structure associated with Lys14 of histone H3 modification play an important role in mediating the transcriptional activation of the nur77 gene, but not the c-fos gene, during dbcAMP-induced neurite outgrowth in PC12 cells.

In conclusion, our findings suggest that the expression of nur77, which is essential for the differentiation induced by dbcAMP, is up-regulated via Lys14 of histone H3 the acetylation of which is induced by dbcAMP in PC12 cells.

Discussion

One of the critical steps in neural differentiation is the outgrowth of neurites (*19*, *20*). Neurite outgrowth induced by growth factors, neurotrophins, depolarization and other extracellular agents leads to the transcriptional activation of many immediate early genes (IEG). In this report, we investigated the expression of the nur77 gene as a key step during the early phase of dbcAMP-induced neurite outgrowth in PC12 cells.

A previous study revealed that the nur77 and c-fos genes were activated within 1 h of forskolin treatment, and these activations were regulated by CREB in PC12 cells (*16*). In this study, we reported that in dbcAMP-treated PC12 cells, the expression of nur77 was regulated by PKA within 1 h of treatment with dbcAMP (Fig. 3A and B), and knockdown with siRNA against nur77 mRNA drastically inhibited the neurite outgrowth (Fig. 4B and C). Because there is a possibility of off-target effect of nur77 siRNA used, we performed knockdown experiments with siRNA against other region of nur77 mRNA. As a result, we observed similar knockdown effect on neurite outgrowth in dbcAMP-treated PC12 cells (data not shown). These results suggest that the PKA-CREB-Nur77 signalling pathway is one of the main pathways of neurite outgrowth in cAMP-treated PC12 cells. This is the first report showing that the PKA-CREB-Nur77 signalling pathway participates in cAMP-induced neurite outgrowth.

In addition of the role of the nur77 gene, we also investigated the role of the c-fos gene in cAMP-induced transcription. Transcriptional regulation of the c-fos gene has been extensively studied in PC12 cells and serves as a paradigm for the regulation of IEGs. Our study revealed that in the early phase of dbcAMP-treated PC12 cells, the expression of nur77 and c-fos genes was regulated by PKA (Fig. 3A and B) and that the association of the nur77 gene promoter and acetylated Lys14 of histone H3 was also regulated by PKA (Fig.6A), but the association of c-fos gene

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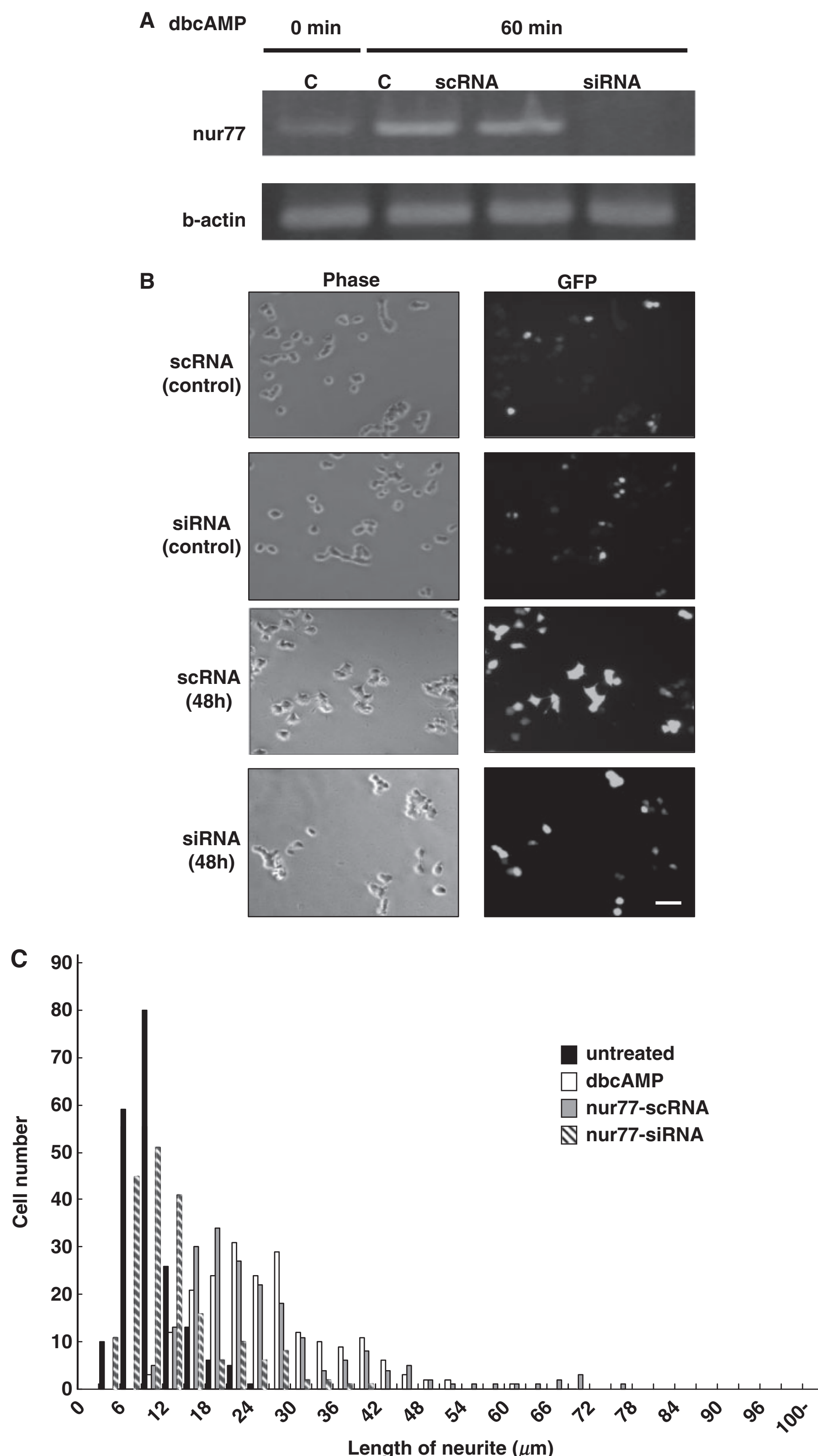

Fig. 4 Expression of the nur77 gene is required for dbcAMP-induced neurite outgrowth in PC12 cells. (A) PC12 cells transfected with siRNA against nur77 mRNA were treated or untreated with 300 nM dbcAMP for 1 h. nur77 and beta-actin (b-actin) mRNA were detected by RT-PCR as described in Materials and Methods section. The results are representative of three independent experiments. (B) Photomicrographs of PC12 cells transfected with siRNA against nur77 mRNA and the GFP-expressing plasmid for 24 h. After transfection, the cells were treated with 300 nM dbcAMP in serum-free DMEM supplemented with TIP for 48 h. PC12 cells were cultured without (scRNA control, siRNA control) or with 300 nM dbcAMP (scRNA 48 h, siRNA 48 h). Phase contrast (Phase) and GFP-expressed (GFP) images were photographed. Representative images of two independent experiments are shown. Scale bar: 30 μm. (C) Histogram of neurite length of dbcAMP-treated PC12 cells pre-treated with siRNA (striped bars) against nur77 mRNA and scRNA (gray bars). PC12 cells pretreated with siRNA and the GFP-expressing plasmid for 24 h were treated or untreated with 300 nM dbcAMP for 48 h. PC12 cells were cultured without (closed bars) or with 300 nM dbcAMP (open, grey and striped bars). Neurite length was measured using the Lumina Vision software. Differences between groups were examined for statistical significance using the Kolmogorov–Smirnov test. $P < 0.01$ (nur77 siRNA *versus* nur77 scRNA).

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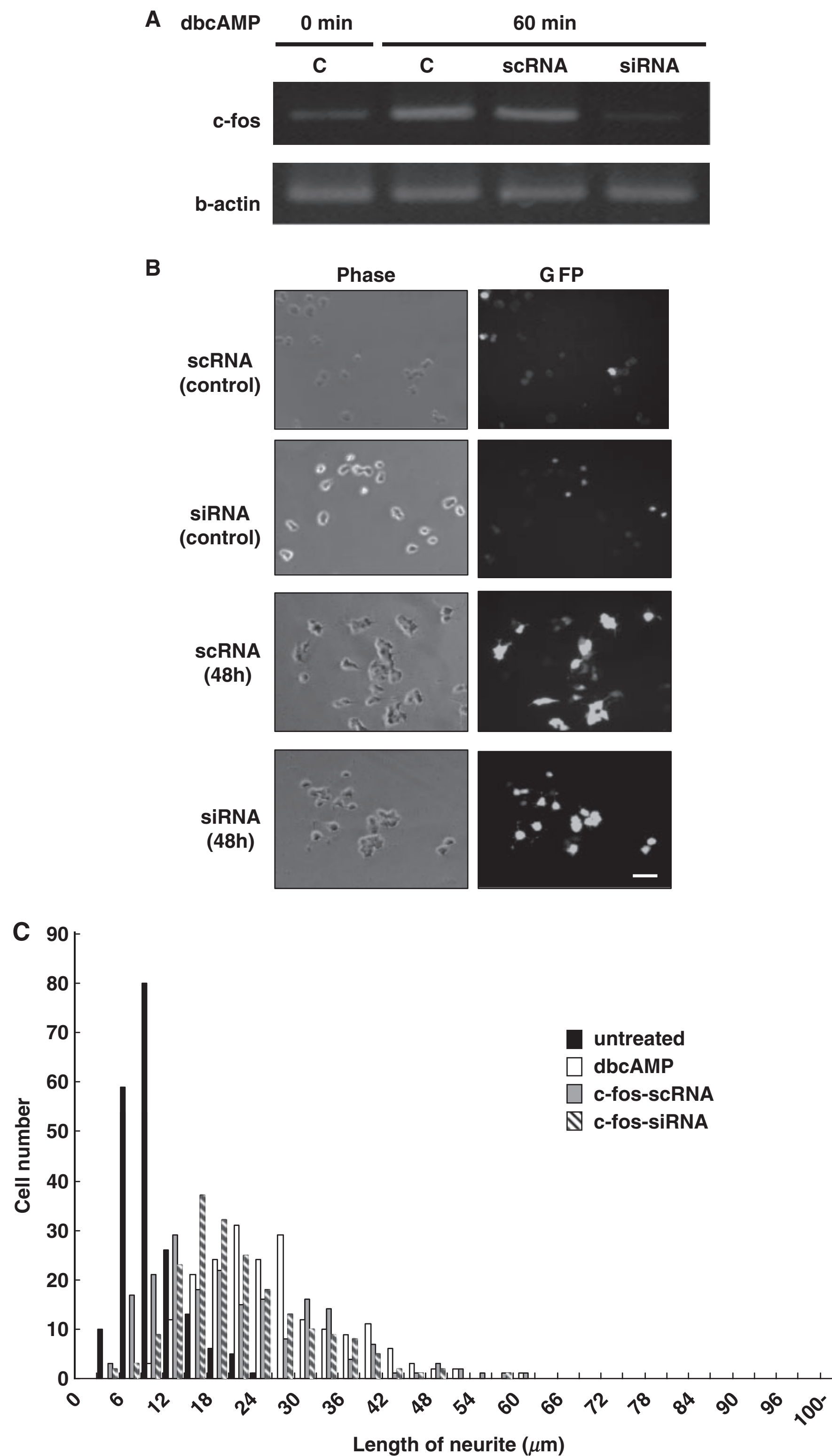

Fig. 5 Expression of the c-fos gene is not required for dbcAMP-induced neurite outgrowth in PC12 cells. (A) PC12 cells transfected with siRNA against c-fos mRNA were treated or untreated with 300 nM dbcAMP for 1 h. c-fos and beta-actin (b-actin) mRNAs were detected by RT-PCR as described in Materials and Methods section. The results are representative of three independent experiments. (B) Photomicrographs of PC12 cells transfected with siRNA against c-fos mRNA and the GFP-expressing plasmid for 24 h. After transfection, the cells were treated with 300 nM dbcAMP in serum-free DMEM supplemented with TIP for 48 h. PC12 cells were cultured without (scRNA control, siRNA control) or with 300 nM dbcAMP (scRNA 48 h, siRNA 48 h). Phase contrast (Phase) and GFP-expressed (GFP) images were photographed. Representative images of two independent experiments are shown. Scale bar: 30 μm. (C) Histogram of neurite length of dbcAMP-treated PC12 cells pre-treated with siRNA (striped bars) against c-fos mRNA and scRNA (grey bars). PC12 cells pre-treated with siRNA and the GFP-expressing plasmid for 24 h were treated or untreated with 300 nM dbcAMP for 48 h. PC12 cells were cultured without (closed bars) or with 300 nM dbcAMP (open, grey and striped bars). Neurite length was measured using the Lumina Vision software.

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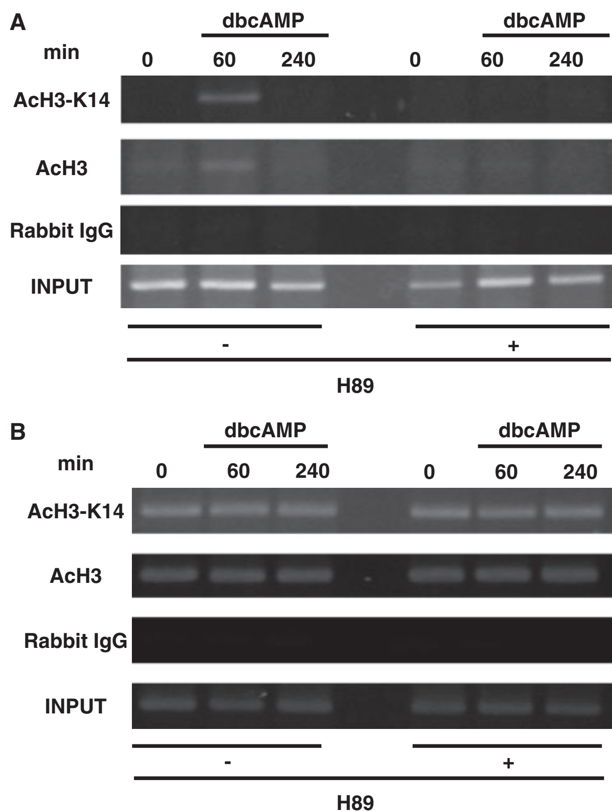

Fig. 6 dbcAMP triggers differential association of nur77 and c-fos promoters with acetylated histone H3. (A and B) PC12 cells were untreated or treated with 300 nM dbcAMP in the absence or presence of 10 µM H89 for the indicated times. The cells were subjected to ChIP assays with antibodies against acetylated histone H3 (AcH3), acetylated Lys14 of histone H3 (AcH3-K14) and normal rabbit IgG (control) at the nur77 (A) and c-fos (B) promoters. The results are representative of three independent experiments.

promoter and acetylated Lys14 of histone H3 was not regulated by PKA (Fig. 6B). These results suggest that in dbcAMP-induced neurite outgrowth, the association of the nur77 gene promoter, but not the c-fos gene promoter, and the acetylated Lys14 of histone H3 are regulated by PKA, and the regulation of c-fos gene expression is carried out by a process without PKA-mediated association with the acetylated Lys14 of histone H3.

What distinguishes the mechanism of c-fos expression from nur77 expression? We considered two possibilities that might explain the differences between the two genes. The first possibility is that both the nur77 and c-fos genes are regulated by PKA, but c-fos is greatly influenced by other signal pathways at the same time. In PC12 cells, c-fos induced by depolarization is mediated by the MAP kinase (MAPK) pathway (*21*). On the other hand, nur77 expression induced by depolarization is not mediated by the MAPK pathway but is mediated by both PKA and CAMKII (*22*). As the MAPK pathway has an important role in the proliferation and differentiation of dbcAMP-treated PC12 cells, it is thought that the c-fos gene is regulated by the MAPK pathway under basal conditions. Our study revealed that knockdown with siRNA against c-fos mRNA did not inhibit dbcAMP-induced neurite outgrowth (Fig. 5C). These results suggest that c-fos gene hardly participates in dbcAMP-induced neurite outgrowth and that the PKA-CREB-cFos signalling pathway participates in a process other than neurite outgrowth in PC12 cells.

As the second possibility, we considered the different states of HAT complex at nur77 and c-fos promoters. We observed differences in the nur77 and c-fos promoters from our ChIP assay data (Fig. 6A and B). Under basal conditions, although the nur77 gene promoter did not associate with the acetylated Lys14 of histone H3, the c-fos gene promoter was found to associate with the acetylated Lys14 of histone H3 (Fig. 6A and B). In addition, a previous study (*16*) revealed a different mechanism between nur77 and c-fos gene promoters under basal conditions in PC12 cells. They reported that at the c-fos gene promoter, RNA Polymerase II and TFIIB were assembled under basal conditions and did not change even when forskolin was added. At the nur77 gene promoter, these proteins existed under basal conditions, and their expression is increased by forskolin treatment (*16*). Fass *et al.* (*16*) also suggested that under basal conditions, the nur77 promoter exists in a state ready to bind to the PIC. With regard to the c-fos promoter, Pinaud *et al.* (*23*) revealed that short and abortive transcripts from the promoter proximal region of the c-fos gene were continuously produced in the absence of stimuli. From these results, it is thought that the difference in the state of the PIC on each promoter under basal conditions is related to differences in gene expression.

At present, we do not understand the processes that occur after the binding between nur77 gene promoter and acetylated Lys14 of histone H3. Regarding binding to the nur77 gene promoter, we consider that not only the acetylated Lys14 of histone H3 but also other modified residues, including the acetylated Lys9 and phosphorylated Ser10 of histone H3 may be required for binding.

Previous studies (*24*, *25*) revealed that phosphorylation of the Ser10 of histone H3 has been linked to the acetylation of Lys14 of histone H3 and that this modification is important for the transcriptional activation of several genes. Phosphorylation of the Ser10 of histone H3 was found to be combined with acetylated Lys14 or Lys9 of histone H3 using antibodies against multiple histone modifications (*24*, *25*). Cheung *et al.* (*25*) proposed that phosphorylation of histone H3 at IEGs promoters precedes and directs its subsequent acetylation and the 'synergistic phosphorylation and acetylation' model. This model illustrated that histone H3 modifications; *i.e.* the phosphorylation of Ser10 and then rapid acetylation of Lys14 are tightly coupled in EGF-stimulated cells (*25*). In addition, the phosphorylation of Ser10 of histone H3 has been shown to be increased during activation of cAMP-dependent PKA responsive genes (*26*). In this study, we identified the association of the nur77 gene promoter with acetylated Lys14 of histone H3 in dbcAMP-treated PC12 cells, but further analysis of histone modifications other than the acetylation of Lys14 of histone H3 is necessary to elucidate the mechanism of nur77 gene expression.

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Our findings in this study suggest that the expression of nur77, which is essential for the differentiation induced by dbcAMP, is up-regulated via dbcAMP-induced Lys14 acetylation of histone H3 in PC12 cells. Further studies are necessary to elucidate the detailed mechanisms of histone modifications and related gene expression during dbcAMP-induced neurite outgrowth.

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Conflict of interest

None declared.

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