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Comparison of Alzheimer Disease-related Gene Expression in Differentiated and Undifferentiated NG108-15 Cells

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ABSTRACT: The pathogenesis of Alzheimer's disease (AD) is complicated and multifactorial, and is under the influence of genetic regulation. The NG108-15 cell line, a neuronal cell line, once differentiated exhibits neuron-like in morphology and intercellular synaptic junction formation. We, thus, hypothesize that the differentiation process may alter the expression patterns of AD-related genes in this cell line. This study primarily aimed to compare the expression patterns of five AD-related genes, namely AChE, COX-2, α7 nAChR, γ-secretase, and APP between undifferentiated and differentiated NG108-15 cells by RT-PCR techniques. The cyclic AMP analogue dibutyryl cAMP (Bt_2cAMP) and 12-O-tetradecanoyl-phorbol 13-acetate (TPA) were used to induce neuronal differentiation. The differentiated NG108-15 cells exhibited neuron-like morphology and intercellular network formation. The level of AChE mRNA in differentiated cells was significantly greater than in undifferentiated cells at 2 and 3 day post-induction. Also, COX-2 mRNA expression in differentiated cells at day 1 post-induction was significantly greater than that in undifferentiated cells. In contrast, the expression of α7 nAChR mRNA was significantly down-regulated in differentiated cells at 1 and 3 days post-induction, and the expression level of γ-secretase mRNA was also significantly reduced in differentiated cells at 3 days post-induction. Interestingly, no significant difference of the expression of APP mRNA was detected. Our findings in this study indicate that the patterns of mRNA expression of AD related-genes in NG108-15 cells are changed after differentiation induction by Bt_2cAMP and TPA.

KEYWORDS: Alzheimer's disease, NG108-15 cells, AChE, COX-2, α7 nAChR, γ-secretase, APP.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and has staggering economic impact on families, health care systems and society in general. Hebert *et al*[1]. estimated that AD will be diagnosed in 13.2 million Americans by 2050. The annual health care costs were approximately US $9,711 for AD patients[2]. The pathology of AD is characterized by an accumulation of extracellular amyloid plaques and intracellular neurofibrillary tangles of the brain[3]. The actual causes of AD are still unknown but strong risk factors are correlated to age over 65 years, sex and family history. The mechanisms of AD at the molecular level have been investigated and revealed. Several hypotheses have been proposed for the causes of AD. These include decrease in cholinergic activity due to reduction of acetylcholine (ACh) levels, alteration of nicotinic receptor numbers, accumulation of β-amyloid peptides (Aβ), and inflammation of neurons[3,4,5].

The cholinergic hypothesis can be stated as the deficit of cholinergic neurotransmission and alteration of nicotinic receptors in the central nervous system. An autopsy analysis of AD brains showed a reduction of ACh levels and choline acetyl transferase (CAT) and acetylcholinesterase (AChE) activities compared to normal brains[4]. Immunohistochemical studies of AD hippocampus discovered that both $A\beta_{1-42}$ peptide and α7 nicotinic acetylcholine receptor (α7 nAChR) were present in neuritic plaques and co-localized in cortical neurons[6].

The amyloid cascade hypothesis is widely accepted in AD pathogenesis. Aβ peptides are derived from the processing of amyloid precursor protein (APP) by two proteases, β-secretase and γ-secretase. The Aβ, especially highly fibrillogenic $A\beta_{1-42}$, (produced via the amyloidogenic processing of APP) can trigger a neurotoxic cascade, thereby causing neurodegeneration and finally AD[3]. The relationship between AChE and amyloid formation in AD has been vigorously studied. It has been reported that AChE promotes aggregation of the β-amyloid protein in the cerebral cortex in AD patients[7].

Several mechanisms of Aβ neurotoxicity have been

proposed, and it is likely to be complicated and multifactorial. These mechanisms include microglia and astrocyte activation and induction of inflammation mediators, such as cytokines, complement proteins and cyclooxygenase-2 (COX-2). Nogawa *et al*[5]. demonstrated that COX-2 was transiently and rapidly induced upon inflammatory stimulus. The autopsy of AD brains also showed that COX-2 immunoreactivity was detected between senile plaques in pyramidal neurons in the cerebral cortex and hippocampus[5].

The NG108-15 cell line is a hybrid cell line of mouse neuroblastoma (N18TG-2) and rat glioma (C6BU-1)[8]. The NG108-15 cell line was employed in this study to compare mRNA expression of AD-related genes, since this cell line is able to release ACh and form cholinergic neuromuscular synapses[8]. A high level of the AChE mRNA expression was detected in differentiated cells[9]. Furthermore, its availability, ease of handling and culturing, and lack of the ethical associated with animal models are also advantages. Thus, this study aimed to compare patterns of mRNA expression of AD-related genes including AChE, COX-2, α7 nAChR, γ-secretase and APP in undifferentiated and differentiated NG108-15 cells.

Materials and Methods

Cell Culture

NG108-15 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 0.1% of glucose and supplemented with 0.1 mM hypoxanthine, 10.8 mM aminopterine, 0.01 mM thymidine and 6% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2 by a method modified from that of Tojima and colleagues[9]. Cells were plated in 25-cm^3 flasks at 3×10^5 cells/flask. The cells were seeded for 1 day and then the culture medium was changed. In the undifferentiated group, the cells were allowed to grow for 1, 2 and 3 days. To induce neuronal cells differentiation, 1 mM dibutyryl cAMP (Bt_2cAMP) and 100 nM 12-O-tetradecanoyl-phorbol 13-acetate (TPA) were added to the culture medium[10] and the cells were cultured for 1, 2 and 3 days before harvesting.

Isolation of RNA

Total RNA from the cells was isolated by the method of Chomezynski and Sacchi[11]. RNA of the cells was isolated from 2×10^6 cells by using 1 ml of Trizol®. The cell lysate was transferred to a microtube. Chloroform was added and centrifuged. The aqueous phase was transferred into a new tube and re-extracted with 0.3 ml of phenol:chloroform (5:1). RNA in the aqueous phase was precipitated with 0.5 ml of isopropanol. The pellets were washed with 75% ethanol and dissolved in 25 µl of DEPC water. The RNA concentration was spectrophotometrically measured at 260 nm and the quality was certified by a wavelength scan.

Reverse Transcription - Polymerase Chain Reaction

A reverse transcription-polymerase chain reaction (RT-PCR) was used to synthesize and amplify cDNA. First strand cDNA was synthesized from 2 µg of total RNA using 200 units of Superscript III (Invitrogen, California, USA) and 10 µM oligo (dT) primer in 20 µl.

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was carried out in a 10 µl reaction containing 1 µl of the first strand cDNA, 1 µM each of sense and antisense primers specific to each mRNA as shown in Table 1., 250 µM dNTPs, and 2 units of *Taq* DNA polymerase in a reaction buffer containing 2.5 mM $MgCl_2$. Thermocycling was performed using the following protocol: (1) 94°C for 10 min, (2) cycles of 94°C for 1 min, 56°C for 2 min and 72°C for 2 min, (for cycle numbers according to each primer as shown in Table 1), and (3) 72°C for 10 min before cooling down to 4°C. Primers used in the PCR were specific to specific segments of the AChE, COX-2, α7 nAChR, γ-secretase and APP genes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) PCR product was used as an internal control. The PCR products were electrophoresed in a 6% polyacrylamide gel and then stained with ethidium bromide. The DNA bands were visualized under UV

Table 1. Specific primers, number of cycles and expected product sizes applied in Polymerase Chain Reactions for specific genes*.

Gene	PubMed accession number	No. of cycles	PCR product size (bp)	Primers	
				Sense sequence	Anti-sense sequence
GAPDH	BC059110	18	247	5'-CAAGTTCAACGGCACAGTCA-3'	5'-GGTTCACACCCATCACAAAC-3'
AChE	NM_172009	32	261	5'-TCGAGAGCTGAGTGAAGACT-3'	5'-TGATCCAGCAGGCCTACATT-3'
COX-2	S67722	35	249	5'-ATGTCTTCCAGCTCACGGTA-3'	5'-GAGCTACACAGTAGTCCTGA-3'
α7 nAChR	NM_007390	30	340	5'-AAACAGGAGCATCCTGAGTG-3'	5'-GTGTTCAAGGAGGATGGAGT-3'
γ-secretase	NM_019163	24	323	5'-TTGCCATTCACTGGAAAGGC-3'	5'-CTTGTGTGCTATACTTGGGG-3'
APP	BC062082	28	259	5'-AATCCTGCAGTACTGCCAAG-3'	5'-TGGCAACAGTATGCCAGTGA-3'

* Thermocycling was performed by the following protocol: (1) 94°C for 10 min, (2) the designated number of cycles of 94°C for 1 min, 56°C for 2 min and 72°C for 2 min, and (3) 72°C for 10 min before cooling down to 4°C. Primers used in the PCR were specific to segments of GAPDH, AChE, COX-2, α7 nAChR, γ-secretase and APP genes.

light and quantified with gel documentation using Quantity One Version 4.4 (BIO-RAD, Hercules, CA, USA).

Data Analysis

All mRNA expression measurements were normalized with the internal control of GAPDH mRNA expression. The expression density of each mRNA of the undifferentiated NG108-15 cells at day 1 was calculated as 100%. The percentage of mRNA expression was compared with the mRNA of undifferentiated NG108-15 cells at day 1. The results are described as mean ± SEM. Significance levels were tested by two-way ANOVA (among groups and incubation times) and one-way ANOVA (within the same group at different incubation times) followed by LSD test. The mRNA expression levels between undifferentiated and differentiated cells in each day were compared by an unpaired student's t-test.

Results

The morphological characteristics of the undifferentiated and differentiated NG108-15 cells were examined at days 1, 2 and 3 of the incubation periods. The undifferentiated cells were flat, asterisk in shape and displayed only a few neurites, as shown in Fig. 1 (A, B and C). After exposure to Bt_2cAMP and TPA,

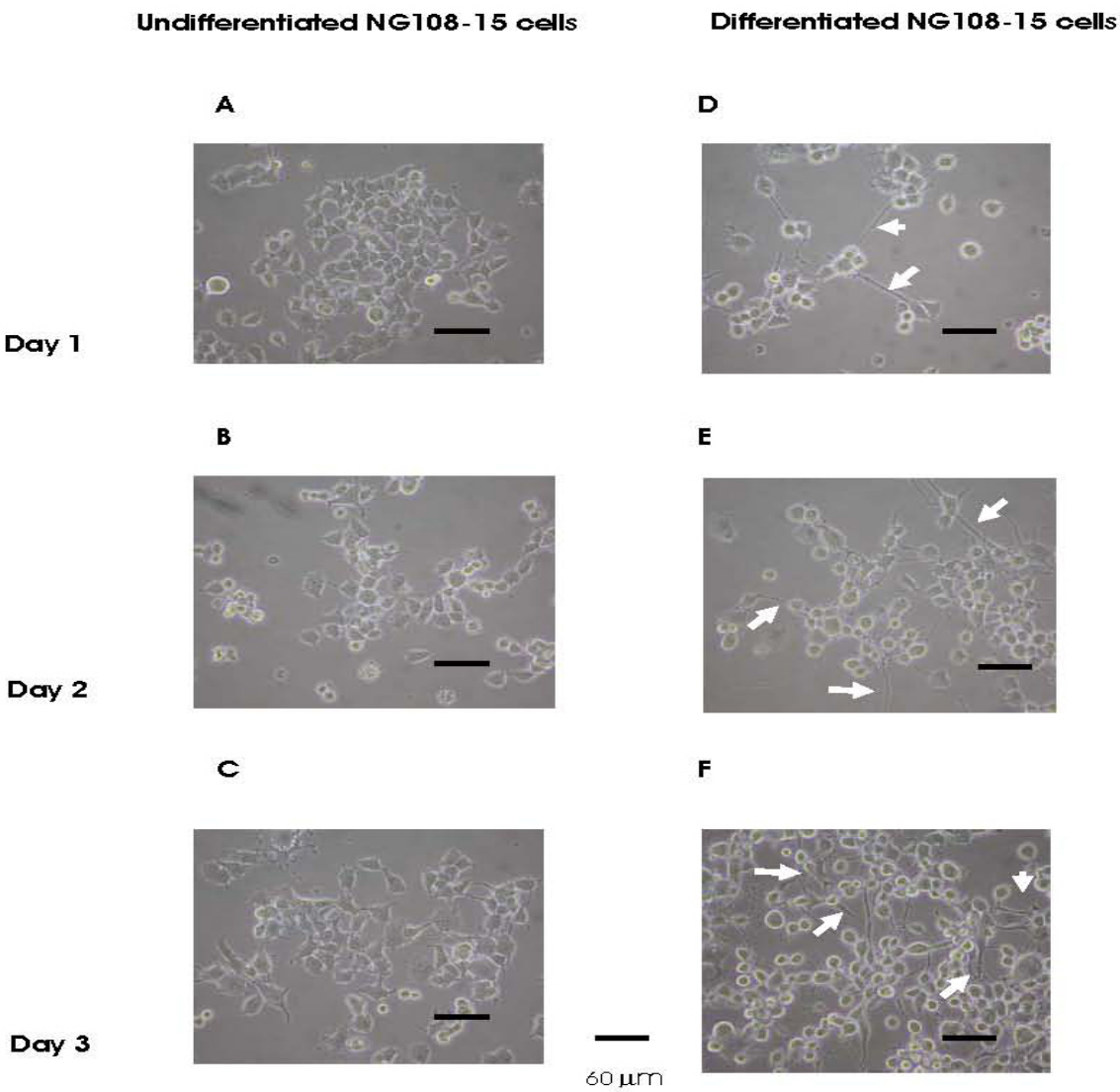

Fig 1. Pictures of undifferentiated NG108-15 cells (A, B, C) and NG108-15 cells (D, E, F) induced to differentiate with Bt_2cAMP and TPA at 1, 2 and 3 day incubation periods. The neurite outgrowth (arrowed) is more obviously seen in differentiated cells than undifferentiated cells from the first day post Bt_2cAMP and TPA exposure. Network formation is more pronounced in the induced group at 2 and 3 day incubation periods. Scale bars = 60 µm (A-F).

the differentiated cells exhibited neuron-like morphology, as shown in Fig. 1 (D, E and F). They extended neurites (defined as cellular processes which are longer than the diameter of the cell body) with abundant varicosities. The number of neurites increased rapidly and reached its maximum at day 3.

The mRNA was then isolated from both undifferentiated and differentiated NG108-15 cells at days 1, 2 and 3. The expression levels of GAPDH, AChE, COX-2, α7 nAChR, γ-secretase, and APP mRNA in the undifferentiated and differentiated NG108-15 cells were measured and statistically compared, as shown in histograms in Fig. 2.

It was clearly seen in Fig. 2A that there was no statistically significant difference between differentiated and undifferentiated cells in the expression levels of GAPDH mRNA, which served as an internal control group.

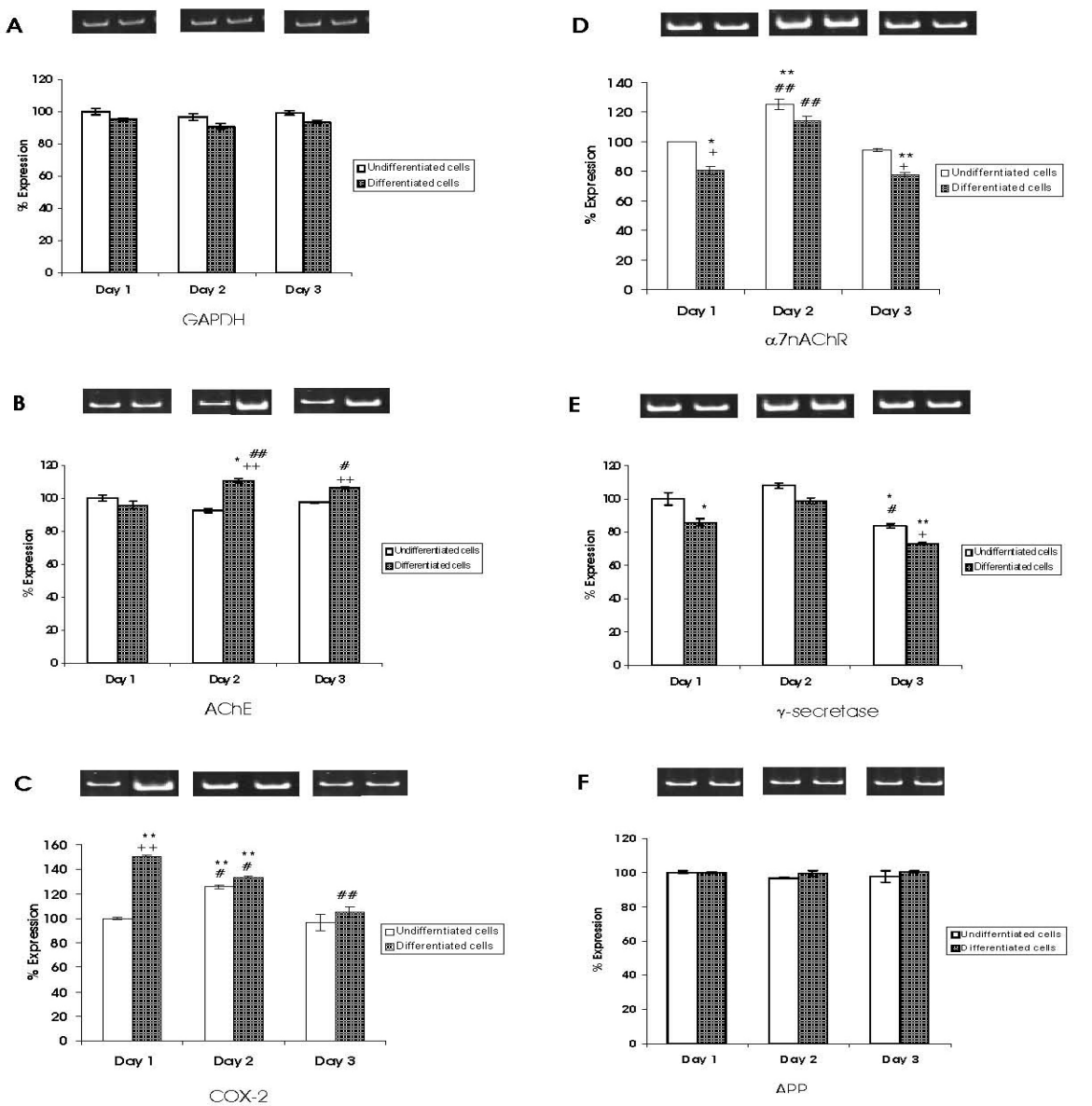

Fig 2. The mRNA expression patterns of Alzheimer's disease related genes in undifferentiated and differentiated NG108-15 cells at various incubation periods (A = GAPDH, B = AChE, C = COX-2, D = α7 nAChR, E = γ-secretase, F = APP). The expression of the undifferentiated NG108-15 cells on day 1 was set as 100%. The results are in mean ± SEM. The sample number of each group was three. Significance levels were tested by two-way ANOVA followed by LSD test: * $P<0.05$, ** $P<0.01$; one-way ANOVA followed by LSD test: # $P<0.05$, ## $P<0.01$. Difference between the expression of undifferentiated and differentiated cells on the same day was tested by unpaired t-test: + $P<0.05$, ++$P<0.01$. The PCR products (bands) are shown above the corresponded histograms.

As shown in Fig. 2B, the AChE mRNA expression in the undifferentiated cells remained unchanged at all incubation periods (P>0.05, one-way and two-way ANOVA). In contrast, the differentiated cells which were induced by Bt_2cAMP and TPA significantly expressed the highest amount of AChE mRNA at day 2 (P<0.05, two-way ANOVA) and it dropped slightly at day 3. The AChE mRNA expression of the differentiated cells at day 2 (P<0.01) and day 3 (P<0.05) were significantly higher than the expression at day 1 (one-way ANOVA). Comparison of the AChE mRNA expression between differentiated and undifferentiated cells at a specific incubation period by unpaired t-test analysis found that the mRNA expression levels of AChE in the differentiated cells at day 2 (P<0.01, t-test) and day 3 (P<0.01, t-test) were significantly higher than those of the undifferentiated cells while the expression in differentiated and undifferentiated cells at day 1 was similar.

In Fig. 2C, the COX-2 mRNA expression in the differentiated cells was significantly high at day 1 when compared to different incubation periods and to the undifferentiated group (P<0.01, two-way ANOVA). The expression then gradually and steadily declined at days 2 and 3. In the undifferentiated cells, the mRNA expression of COX-2 at day 2 was significantly greater than the mRNA expression at days 1 and 3 (P< 0.05, one-way ANOVA). However, it was found that the COX-2 mRNA levels in differentiated and undifferentiated cells at days 2 and 3 were not different (t-test).

The mRNA expression of α7 nAChR of NG108-15 cells was significantly down-regulated after differentiation induction compared to undifferentiated cells (P<0.05, t-test) at day 1 and day 3 (Fig. 2D)(P<0.01, one-way ANOVA). In both undifferentiated and differentiated cells, the a7 nAChR mRNA expression at day 2 (P<0.01) was significantly greater than the mRNA expression at days 1 and 3 (P<0.01, one-way ANOVA).

The γ-secretase mRNA expression levels of the differentiated cells were slightly lower than those of the undifferentiated cells (P<0.05, t-test) (Fig. 2E). The decrease was more pronounced in day 3 of incubation post-differentiation induction (P<0.01, two-way ANOVA). In the undifferentiated cells, the mRNA expression at day 3 (P<0.05, one-way ANOVA) was significantly lower than the expression at days 1 and 2.

Interestingly, as shown in Fig. 2F, the expression levels of APP mRNA in both the undifferentiated and differentiated cells remained statistically unchanged (two-way ANOVA, one-way ANOVA and t-test).

Discussion

The morphology of undifferentiated NG108-15 cells is flat, polygonal in shape, resembling primary-cultured non-neuron-like cells. After neuronal differentiation induction by Bt_2cAMP and TPA, the cells display the neuron-like morphology. In this study, when Bt_2cAMP and TPA were applied, there was evidence for neuronal differentiation, which was visualized by neurite outgrowth. Bt_2cAMP and TPA are known as agents that increase intracellular calcium ions and activate protein kinase C, resulting in neuronal cell differentiation, as seen as neurite outgrowth and network formation[10]. Tojima *et al*[12] reported that cAMP-induced neuritogenesis was dependent on MAPK/ERK kinase activity. The simultaneous application of Bt_2cAMP and TPA induces neurite outgrowth, as was also reported by other research groups[10]. Some cellular characteristics of differentiated NG108-15 cells have also been demonstrated in previous studies, such as the presence of a wide range of voltage-dependent membrane currents[13], the high expression of choline acetyl transferase (CAT) activity[14], the expression of numerous cell membrane receptors for various neurotransmitters[15,16,17], the formation of cholinergic synapses with cultured myotubes[18] and the high expression of AChE and serotonin mRNAs[9,17]. Nevertheless, there was no comparative information of the levels of expression of AD related genes in non-differentiated and differentiated NG108-15 cells. Therefore, we have conducted RT-PCR to demonstrate the differences in the expression patterns of AD related genes between undifferentiated and differentiated NG108-15 cells and at different culture periods.

GAPDH, which is one of the important enzymes in the glycolytic pathway, was used in this experiment as an internal control. GADPH is considered to be a constitutive, stable, housekeeping gene and is widely used as an internal control gene in RT-PCR analysis[19]. As demonstrated in Fig. 2A, the mRNA expression of GAPDH in both undifferentiated and differentiated cells was at the statistically comparable levels, thereby confirming that similar amount of RNA were employed in all experiments.

Previous autopsy studies of AD brains demonstrated severe neuronal and synaptic loss, along with the accumulation of senile plaques[20]. In addition, the AD brains also showed reduced ACh levels and decreased CAT and AChE activity[4]. With the onset of AD, the loss of neurons and axons, together with the reduction of ACh synthesis, leads to the release of less ACh at synaptic clefts; therefore, the brain finds it more difficult to maintain nerve impulses and transmission of information. On the other hand, AChE, which is an enzyme that catalyzes the breakdown of ACh into acetate and choline, may play an important role in AD. The high expression level of this enzyme may lead to less ACh in the synaptic cleft. Tojima *et al*[9]. reported that high expression of AChE mRNA was detected in

differentiated NG108-15 cells by RT-PCR analysis. Our study, as shown in Fig. 2B supports this finding, as the AChE mRNA expression in the undifferentiated cells remained unchanged during the incubation periods, but the differentiated cells expressed the greatest amount of AChE mRNA at day 2 and it dropped slightly at day 3. It was also found that the mRNA expression levels of AChE in the differentiated cells at days 2 and 3 were significantly greater than those of the undifferentiated cells. The work of Thoda *et al*[10] and Yu *et al* [21] supports our findings by demonstrating that both Bt_2cAMP and TPA can activate PKC and finally induce c-Jun/AP-1 mRNA expression. AP-1 and c-Fos are known as transcriptional factors which specifically bind to a DNA sequence in the proximal AChE gene promoter, and finally lead to the up-regulation of AChE mRNA[22]. Recently treatment for AD has been focused on enhancing cholinergic function with AChE inhibitors, such as donepezil, rivastigmine and galantamine. Several studies showed that increasing ACh by inhibiting AChE activity to some degree ameliorates the cognitive deficits in AD patients[23,24]. Another way to increase the ACh level is to give ACh substitutes, but none is currently available.

The link between COX-2 and AD pathology has been demonstrated in previous studies. It has been reported that COX-2 expression was up-regulated at the sites of inflammation in AD frontal cortex[25]. Interestingly, the COX-2 immunoreactivity was prominently detected between senile plaques at the cerebral cortex and hippocampus in the autopsy of AD brain[5]. As clearly seen in this study (Fig. 2C), the COX-2 mRNA expression in the differentiated cells was significantly elevated. The study by Mestre and his colleages[26] gives us a clue to explain our results. They demonstrated that treatment of oral epithelial cells with TPA for 5 hours is associated with the enhancement of COX-2 transcription. It has also been reported that TPA is able to induce COX-2 mRNA expression through extracellular signal-regulated kinase activity (ERK) and nuclear factor–kappa B (NF-kB) activation in a mouse skin model[27]. Therefore, the up-regulation of COX-2 mRNA expression in the differentiated cells in our study may result from the TPA-activated ERK pathway-regulated NF-kB signaling cascade, and NF-kB then binds to a specific binding site in the COX-2 promoter, resulting in the increase of COX-2 mRNA expression. Previous clinical studies have confirmed the efficacy of some non-steroid anti-inflammatory drugs (NSAIDs) that could delay or slow down the clinical development of AD[28]. Moreover, a COX inhibitor, indomethacin, has been shown to suppress the production of the secreted form of processed APP and Aβ[28]. These findings encourage the search for selective COX-2 inhibitory agents for the treatment of AD.

The α7 nAChR is pentameric in structure, consisting of homodimer of α subunits and various heteromeric combinations of β, γ and δ subunits. The α7 subunit receptors are distributed in cerebral cortex, hippocampus, hypothalamus and amygdala of the brain. The α7 nAChR subtype is highly permeable to the Ca^{2+} ion, which acts not only as a neurotransmitter stimulator, but also as a secondary messenger through stimulation of various calcium-dependent intracellular enzymes, such as protein kinases and nitric oxide synthetase. Calcium ion also plays an important role in brain function, especially memory and cognitive abilities, which are closely related to AD clinical pathology. It was suggested that stimulation of α7 nAChR enhances cognitive ability in a variety of behavior tasks[29]. Moreover, Guan *et al*[30]. reported a reduction of the α7 nAChR by 36% in hippocampus of the AD brains (in postmortem brain of patients with AD and age-matched controls). Koninck and Cooper also demonstrated that the α7 nAChR mRNA expression level in superior cervical ganglion (SCG) neurons dropped by 60-75% within the first 48 hour and remained low for at least 2 weeks[31]. Our result in Fig. 2D suggests the same trend in that the mRNA expression of α7 nAChR of NG108-15 cells is down-regulated after differentiation induction compared with the undifferentiated cells. The down–regulation of α7 nAChR mRNA may result from the inhibition of CaM IV kinase activity by Bt_2cAMP treatment leading to cAMP response element binding protein (CREB) signaling cascade suppression. High levels of PKA activity have been shown to inhibit CaM Kinase IV (Ca^{2+}/calmodulin-dependent protein kinase IV) activity[32]. Enslen *et al*[33] demonstrated that CaM kinase IV involved in α7 nAChR transcriptional regulation through phosphorylation of Ser133 in CREB. Thus, Bt_2cAMP may activate PKA and lead to inhibition CaM Kinase IV activity leading to down-regulation of α7 nAChR expression via the reduction of phosphorylation of Ser133 in CREB.

The γ-secretase is one of the intramembrane-cleaving proteases that (together with β-secretase) hydrolyzes APP and generates Aβ peptides, a crucial marker of AD[3]. It has been reported that γ-secretase inhibitors can reduce β-amyloid burden in an animal model[34]. As can be seen in Fig. 2E, our study demonstrated that the expression of γ-secretase mRNA of the differentiated NG105-18 cells was slightly lower than that of the undifferentiated cells, with the difference being more pronounced in day 3 after being differentiated. Hung and his group[35] have demonstrated that PKC activators such as TPA are able to decrease the release of Aβ peptides. So, taken together, our results and their work suggest that the differentiated cells generate less γ-secretase mRNA due to TPA treatment, leading to less Aβ peptides processing.

APP is a single transmembrane domain protein and is metabolized rapidly in the cells. The proteolytic processing of APP by the β-amyloidogenic pathway is strongly related to AD[3]. Our study interestingly showed that there was no significant difference in APP mRNA expression between the differentiated and the undifferentiated NG108-15 cells (Fig. 2F). Shekarabi *et al*[36] also demonstrated that the APP mRNA expression in both undifferentiated and differentiated NG108-15 cells induced by only Bt_2cAMP are at the same levels at early days, and then gradual increased after day 3 and continue up to day 6. However, due to the complexity of the Aβ processing cascade, the differentiation process may not directly affect the expression level of APP mRNA, but may rather affect other Aβ processing genes such as β- or γ-secretases and/or other AD-related genes. For instance, Berezovska *et al*[37]. demonsrated that Notch1 and APP are competitive substrates for γ-secretase cleavage. Activation of Notch1 or related substrates of γ-secretase cleavage may result in Aβ reduction without any changes in the amount of APP expression.

Our study has demonstrated the changes of mRNA expression patterns of the AD related genes AChE, COX-2, α7 nAChR, γ-secretase and APP in NG108-15 cells after differentiation induction by Bt_2cAMP and TPA at different incubation periods. The differentiated NG108-15 cells exhibited neuron-like morphology. The expression levels of AChE and COX-2 mRNA in the differentiated cells were significantly greater than those in the undifferentiated cells. On the other hand, the expressions of α7 nAChR and γ-secretase mRNA were significantly down-regulated in the differentiated cells, while the APP mRNA expression was unchanged. This study clearly shows that the NG108-15 cells express the AD-related genes and their expression can be modified by the differentiation process.

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