

## Upregulatory Effects of Nobiletin, a Citrus Flavonoid with Anti-dementia Activity, on the Gene Expression of mAChR, ChAT, and CBP

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### Abstract

We previously showed that nobiletin, a polymethoxylated flavone from the peels of *Citrus depressa* Hayata, improves impaired memory in dementia model animals. It was also reported that nobiletin stimulates cAMP response element-dependent transcription through activation of the cAMP response element-binding protein signaling pathway. To determine whether nobiletin alters the expression of genes involved in learning and memory, we performed real-time polymerase chain reaction after treating PC12 cells with nobiletin. Nobiletin upregulated the expression of muscarinic acetylcholine receptor subtype M1, choline acetyltransferase, and cAMP response element-binding protein genes in the cells. The muscarinic acetylcholine receptor M1 and choline acetyltransferase mRNA levels reached maximum values at 6 h after nobiletin treatment, whereas the cAMP response element-binding protein mRNA levels peaked at 12 h. These results suggest that nobiletin enhanced the expression of genes involved in learning and memory by stimulating the cAMP response element-dependent transcription.

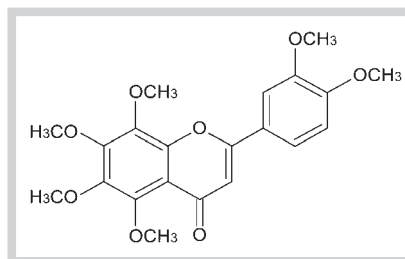
### Key words

Nobiletin · *Citrus depressa* · Rutaceae · gene expression · muscarinic acetylcholine receptor · choline acetyltransferase

### Abbreviations

CRE:	cAMP response element
PKA:	protein kinase A
ERK:	extracellular signal-regulated kinase
CREB:	CRE-binding protein
mAChR:	muscarinic acetylcholine receptor
ChAT:	choline acetyltransferase
CBP:	CREB-binding protein

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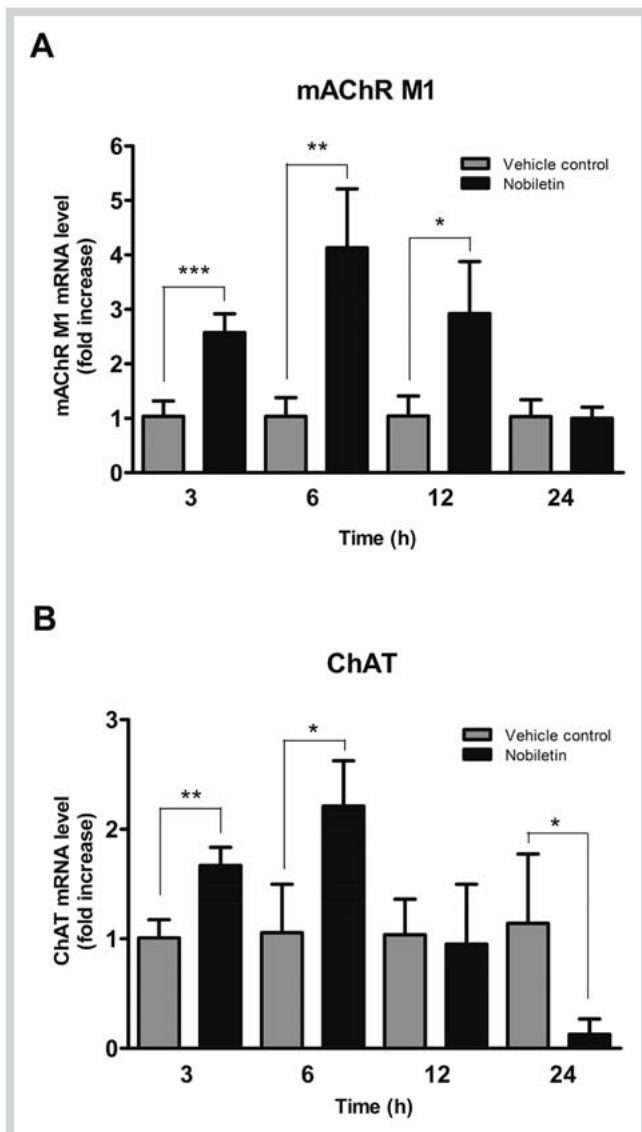


**Fig. 1** Chemical structure of nobiletin isolated from the peels of *C. depressa* Hayata.

Several studies have shown that memory formation requires the altered expression of numerous genes. Previous studies of ours indicated that nobiletin (● Fig. 1), a polymethoxylated flavone isolated from *Citrus depressa* Hayata (Rutaceae) peels, has the ability to improve learning and memory impairment in dementia model animals such as olfactory-bulbectomized (OBX) mice [1–6]. Furthermore, nobiletin also markedly stimulates CRE-dependent transcription by activating the PKA/ERK/CREB signaling pathway in PC12D cells (a subclone of the rat pheochromocytoma cell line PC12) [2,7]. However, it remains unclear whether the expression of genes associated with learning and memory is altered by nobiletin. In this study, we focused on the expression of cholinergic system- and gene transcription-associated genes in PC12 cells, which are frequently used as a suitable model for biochemical studies on neurodifferentiation.

PC12 cells were treated with 100 μM nobiletin for the desired time periods (3 h, 6 h, 12 h, and 24 h), after which the mRNA levels of mAChR subtype M1, ChAT, and CBP were evaluated by real-time polymerase chain reaction (PCR). Three hours from the initiation of nobiletin treatment, the mAChR M1 mRNA level in the cells was significantly upregulated by approximately 2.5-fold ( $p < 0.001$ ) and significantly higher than that of the vehicle control at 6 h (4.0-fold,  $p < 0.01$ ) and 12 h (2.8-fold,  $p < 0.05$ ; ● Fig. 2A). Similarly, an increase in the ChAT mRNA level was observed at 3 h (1.7-fold increase,  $p < 0.01$ ), and which peaked at 6 h (2.1-fold increase over the vehicle control,  $p < 0.05$ ; ● Fig. 2B). Thereafter, by 24 h, the level of ChAT mRNA decreased to one below the control value (0.1-fold,  $p < 0.05$ ). As a possible reason for this phenomenon, it has been suggested that the depression of CRE-dependent transcription might occur through the expression of the inducible cAMP element repressor (ICER), which is a repressor of CRE-dependent transcription [8,9]. It has also been reported that the activation of CRE-dependent transcription associated with an elevated cAMP concentration is probably attenuated by the expression of ICER in PC12 cells [9]. However, we previously showed that the intraperitoneal administration of nobiletin (50 mg/kg/day) to OBX mice consecutively for 11 days ameliorates the OBX-induced decrease in ChAT protein levels and memory impairment [3]. Hence, in view of the clinical outcome, the long-term administration of nobiletin should be effective. Furthermore, a nobiletin-induced change in the gene expression of CBP was evaluated. Six hours from the initiation of nobiletin treatment, the CBP mRNA level in the cells began to increase, and it peaked at 12 h, becoming approximately 2.1-fold ( $p < 0.01$ ) greater than that of the vehicle control (● Fig. 3). However, by 24 h, the mRNA level of this gene had decreased to less than or equal to that of the vehicle control, thus showing a transient effect of nobiletin on the expression of this gene.

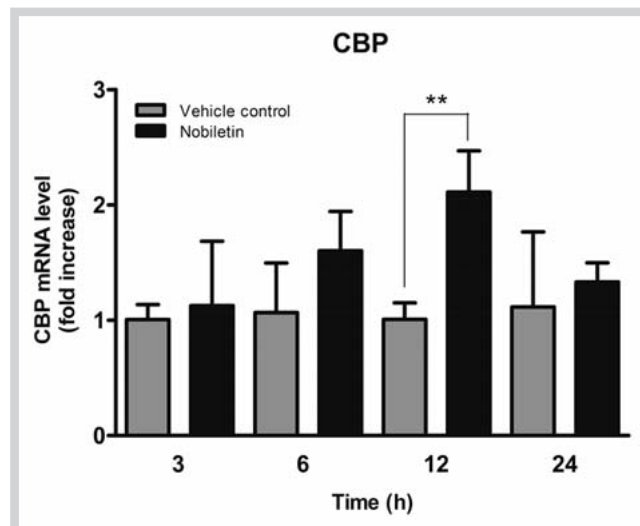
The mAChR is a well-known cholinergic receptor. In particular, its M1 subtype is important in learning and memory, and its mRNAs are abundantly distributed in the hippocampus [10]. A previous



**Fig. 2** Effects of nobiletin on the expression of mAChR M1 (A) and ChAT (B) genes in PC12 cells. The values are expressed as the mean  $\pm$  SD (n = 3–4). \*P < 0.05, \*\*p < 0.01, and \*\*\*p < 0.001 vs. vehicle control.

study of ours showed that nobiletin improves impaired memory in mice subjected to olfactory bulbectomy, whose impairment is characterized by a decreased expression of the ChAT protein, suggesting that the prevention of cholinergic neurodegeneration would be an effective strategy [3]. In the present study, we showed that the ChAT mRNA level in the PC12 cells was also up-regulated by nobiletin (● Fig. 2B). These results suggest that the enhanced gene expression of mAChR M1 and ChAT plays a key role in the mechanisms underlying the restorative action of nobiletin in memory-impaired animals with cholinergic neurodegeneration.

The expression of many genes is associated with phosphorylated CREB, which interacts with CBP, a transcriptional coactivator that has histone acetyltransferase activity [11, 12]. CBP-conditioned KO mice exhibit a robust impairment of memory formation [12]. The present results showed that the CBP mRNA expression in the



**Fig. 3** Effect of nobiletin on CBP gene expression in PC12 cells. The values are expressed as the mean  $\pm$  SD (n = 3–4). \*\*P < 0.01 vs. vehicle control.

PC12 cells was increased by nobiletin (● Fig. 3), suggesting that the effect of nobiletin on CBP gene expression reflected the activation of CRE-dependent transcription.

The findings of the present study suggest that nobiletin upregulated the expression of genes involved in learning and memory by stimulating CRE-dependent transcription via the activation of the PKA/ERK/CREB signaling pathway. Future studies may confirm this effect of nobiletin by using inhibitors of CREB signaling. In conclusion, these results support the potential utility of nobiletin for developing citrus peel-derived functional foods to prevent and ameliorate dementia.

### Materials and Methods

Nobiletin (purity > 99%, ● Fig. 1) was obtained from the peels of *C. depressa* Hayata (Rutaceae). Extraction and isolation of nobiletin were performed as previously described [2]. The purity was confirmed by HPLC using a Shimadzu LC-20AD pump, Tosoh UV-8020 detector, Capcell Pak C18 UG120 column (250  $\times$  4.6 mm; Shiseido), MeOH:H<sub>2</sub>O:H<sub>3</sub>PO<sub>4</sub> solvent (65:35:0.05, v/v/v), 1.0 mL/min flow rate, and 340 nm detection and by inspecting <sup>1</sup>H (500 MHz) and <sup>13</sup>C NMR (125 MHz) spectral features (Bruker AV-500). Samples of *C. depressa* Hayata were kindly provided by Dr. Masamichi Yano, National Institute of Fruit Tree Science, Shizuoka, Japan. The purified nobiletin was dissolved in DMSO (Sigma-Aldrich Co. LLC). PC12 cells were plated at a cell density of 1.5  $\times$  10<sup>4</sup> cells/well (BioCoat Poly-D-Lysine 96-multiwell plate; Beckton-Dickinson) and grown in DMEM in 5% CO<sub>2</sub> at 37°C for 24 h. The medium was then replaced with fresh DMEM with low serum (0.5% heat-inactivated horse serum). At 24 h, the medium was replaced with low-serum DMEM containing 100  $\mu$ M nobiletin or 0.1% DMSO as the vehicle control. The cells were then incubated for 3, 6, 12, or 24 h in low-serum DMEM containing 100  $\mu$ M nobiletin or 0.1% DMSO as the vehicle control. Next, cell lysates for real-time PCR were prepared from the treated cells by using an SYBR® Green Cells-to-CT™ Kit (Applied Biosystems), following the manufacturer's protocol.

## Supporting information

Detailed descriptions of the methods of cell culture, reverse transcription, real-time PCR, and statistical analysis are available as Supporting Information.

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## Conflict of Interest

The authors declare no conflict of interest.

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## Bibliography

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