Original Article

Cellular level of coenzyme Q increases with neuronal differentiation, playing an important role in neural elongations

Akari Nakamura,[1] Mizuho Okamoto,[1] Ayaka Maeda,[1] Huiyu Jiang,[1] Kyosuke Sugawara,[1] Kanae Kitatani,[2] Susumu Takekoshi,[2] Akio Fujisawa,[1] Yorihiro Yamamoto,[1] and Misato Kashiba[1,]*

[1]School of Bionics, Tokyo University of Technology, 1404-1 Katakura-machi, Hachioji, Tokyo 192-0982, Japan
[2]School of Medicine, Tokai University, 143 Shimokasuya, Isehara, Kanagawa 259-1193, Japan

Deficiency of coenzyme Q has been reported in various neurological diseases, and the behavior of this lipid in neurons has attracted attention. However, the behavior of this lipid in normal neurons remains unclear. In this study, we analyzed the concentration of coenzyme Q before and after neuronal differentiation. Nerve growth factor treatment of PC12 cells caused neurite outgrowth and neuronal differentiation, and the amount of intracellular coenzyme Q increased dramatically during this process. In addition, when the serum was removed from the culture medium of N1E-115 cells and the neurite outgrowth was confirmed, the intracellular coenzyme Q level also increased. To elucidate the role of the increased coenzyme Q, we administered nerve growth factor to PC12 cells with coenzyme Q synthesis inhibitors and found that coenzyme Q levels decreased, neurite outgrowth was impaired, and differentiation markers were reduced. These results indicate that coenzyme Q levels increase during neuronal differentiation and that this increase is important for neurite outgrowth.

Key Words: coenzyme Q, neural differentiation, neurite outgrowth, NGF, PC12 cells

Coenzyme Q (CoQ) is an important free radical-scavenging antioxidant. There are two redox states of CoQ: oxidized (ubiquinone) and reduced (ubiquinol). The reduced form of CoQ, ubiquinol, is an important lipid-soluble antioxidant.[1–3] CoQ is also an essential lipid in the mitochondrial respiratory chain.[4]

Levels of CoQ in neuronal cells require particular attention. Several neuronal diseases are reported to be caused by the mutation of the enzymes to synthesize CoQ. Tsuji *et al.*[5] reported a homozygous mutation in COQ2 in patients with multiple system atrophy. PDSS1-2, which is also called COQ1 mutation, has been identified in patients presenting with either multi-organ involvement or Leigh syndrome and nephropathy.[6] COQ8 gene mutations have been reported to present with a progressive neurological disorder with cerebellar atrophy, developmental delay, and hyperlactatemia.[7] In these cases, enzymological studies pointed to ubiquinone deficiency.[8,9]

Furthermore, plasma and platelet levels of CoQ10, especially its reduced form, are reported to be decreased in Parkinson's disease.[10] The administration of CoQ was reported to be effective in neural diseases. McDonald *et al.*[11] reported that concurrent administration of CoQ10 and alpha-tocopherol improves learning in aged mice. Kasparová *et al.*[12] reported the effect of CoQ10 and vitamin E on brain energy metabolism in the animal model of Huntington's disease.

Apart from other antioxidants such as vitamins C and E, CoQ is synthesized *in vivo*.[13] In mammalian cells, the biosynthesis of CoQ is from acetyl-CoA via the mevalonate pathway, as biosynthesis of cholesterol is. The brain has complete enzymatic systems for the biosynthesis of the mevalonate pathway.[14] Andersson *et al.*[15] previously reported that the rates of biosynthesis of cholesterol and ubiquinone in brain slices were 5.5 and 0.25 nmol/h/g, respectively. They also reported that the rates of biosynthesis of cholesterol and ubiquinone in liver slices were 416 and 1.8 nmol/h/g, respectively. Although the rate of CoQ biosynthesis in the brain is lower than that in the liver, it is important to maintain neuronal function as observed in the diseases caused by the mutations in CoQ biosynthesis enzymes. It should be noted that, in contrast to several other organs, the uptake of lipids from the circulation by the brain is limited;[16] thus, the endogenous synthesis of this lipid is considered to be very important in neuronal systems.

CoQ has a phytyl side chain. All cells synthesize their own CoQ, and the tail length varies from 6 to 10 isoprene units (CoQ6–CoQ10) depending on the organism. In mammalian cells, *Homo sapiens* synthesize CoQ10, whereas rats and mice synthesize CoQ9.

The concentration of CoQ in the body has been shown to increase with the growth of the organism.[17] For example, in the human heart, it has been reported that CoQ levels increase from the first day of life, peak at 20 years of age, and then decrease with age. The amount of CoQ in neurons has received much attention, but the changes in CoQ levels during differentiation have not been investigated. In this study, CoQ levels before and after neurite elongation is studied with cultured neuronal cell lines. We used PC12 cells, which are derived from rat, and N1E-115 cells, which are derived from mouse. Both rat and mouse produce CoQ9. We found that CoQ levels dramatically increased during the differentiation of neuronal cells into mature neurons. Neurite elongation is an important event to determine the fate and quality of the neuron. The effect of the administration of CoQ biosynthesis inhibitor is also studied.

Materials and Methods

Cell culture. The PC12 cells were cultured in a DMEM/F-12 medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% horse serum (Thermo Fisher Scientific), 5% fetal bovine serum (FBS; #SH30396.03, Thermo Fisher Scientific), and 1% penicillin/streptomycin at 37°C and 5% CO_2 in an incubator.

For differentiating PC12 cells, nerve growth factor (NGF) is

*To whom correspondence should be addressed.
E-mail: kashiba@stf.teu.ac.jp

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added.[18] The PC12 cells were seeded at 1×10^5 cells/ml on collagen-coated plates. After incubation for 48 h, the culture medium was replaced with a fresh DMEM/F-12 medium containing NGF (20 ng/ml) (Alomone Labs, Jerusalem, Israel). After incubation for 48 h, the medium was changed again. After another 48 h of incubation, cells were analysed for CoQ and free cholesterol (FC) levels. The expression of mRNA was also measured. The levels of CoQ, FC and mRNA of NGF treatment were also measured over time (1, 2 and 4 days of NGF treatment).

Cultures of N1E-115 cells were maintained in DMEM (SIGMA, St. Louis, MO) supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C and 5% CO_2 in an incubator.

For differentiating N1E-115 cells, FBS is removed from the medium.[19,20] The N1E-115 cells were seeded at 1×10^5 cells/ml on collagen-coated plates. After 48 h of incubation, the culture medium was replaced with a serum-free DMEM. After another 48 h of incubation, the medium was changed again. Then, 48 h later incubation, the cells were analysed for CoQ and FC levels.

Neurite length measurement. Cultured cells were observed at 100× magnification with an inverted microscope equipped with a phase contrast microscope (#CKX41, OLYMPUS, Tokyo, Japan) and photographed with a camera (#IX71, OLYMPUS). The resulting image files were printed on A4 size paper. The length of the neurites of the cells in the printed picture was measured with a ruler.

For the measurement of the change in neurite length over time, we used the photographs taken after 1 day of NGF treatment, which include 185 ± 76 neurites. For 2 days samples, 417 ± 96 neurites in photographs, and for 4 days samples, 315 ± 84 neurites were included in each picture. We measured 6 photographs each. The graphs are expressed relative to 1-day of NGF treatment.

In the 4-nitrobenzoate (4-NB) treatment experiment, 111 ± 3 neurites of control and 33 ± 10 neurites of 4-NB were included per photograph and we measured 3 photographs each.

Mitochondrial fractionation. Mitochondrial fractions were obtained using the method of Wallace.[21] In brief, cells were collected in isolation buffer [210 mM mannitol, 70 mM sucrose, 0.1 mM EDTA, 0.5% BSA (fatty acid-free), and 5 mM HEPES, pH 7.2]. The suspension was then lysed with a glass homogenizer and centrifuged at $1,000 \times g$ for 10 min at 4°C. The supernatant was collected and centrifuged at $8,500 \times g$ for 15 min at 4°C, and the pellet was used as the mitochondrial fraction.

Lipid analysis. Concentrations of CoQ and FC in cells were determined using HPLC, as reported previously, with minor modifications.[22] Briefly, cells collected with 2-propanol were centrifuged, and thus, the obtained supernatant was injected into the HPLC system. Two separation columns (Ascentis® C8, 5 μm, 250 mm × 4.6 mm i.d. and Supelcosil™ LC-18, 3 μm, 5 cm × 4.6 mm i.d.; Supelco Japan, Tokyo, Japan) and a reduction column (RC-10, 15 mm × 4 mm i.d.; IRICA, Kyoto, Japan) were employed. The samples were then detected by ECD and UV. The mobile phase for the separation columns was 50 mM sodium $NaClO_4$ in methanol/2-propanol (85/15, v/v) and was delivered at a flow rate of 0.8 ml/min. The columns were maintained at 25°C.

RNA measurement. Total RNA was prepared using TRIzol reagent and Phasemaker™ Tubes (Thermo Fisher Scientific) from cultured PC12 cells. cDNA was synthesized by reverse transcription using QuantiTect Reverse Transcription Kit (QIAGEN, Venlo, The Netherlands). qPCR (95°C for 15 min followed by 40 cycles of 95°C for 15 s and 72.0°C for 30 s, with a final extension step of 60°C for 30 s) was conducted using a QuantStudio® 5 (Thermo Fisher Scientific). The gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.[23,24] The sequences of PCR primers are shown in Table 1.

Establishment of CoQ-deficient cell. For CoQ depletion, a CoQ biosynthesis inhibitor, 4-NB, was used.[25,26] PC12 cells were cultured with 4-NB at a final concentration of 1 mM. 4-NB was dissolved in DMSO, and the same volume of DMSO was added to the control cell line.

Statistical analysis. All results are presented as means and SDs. Student's t test and the one-way analysis of variance (ANOVA) were used to assess the association between variables. P values <0.05 were considered statistically significant.

Results

Levels of CoQ in differentiated and undifferentiated neuronal cells. As shown in Fig. 1A, the addition of NGF significantly affects cell morphology in PC12 cells. As judged from its cell shapes, PC12 cells are differentiated into neural cells. PC12 cell is derived from rats, it contains CoQ9. As shown in Fig. 1B, both reduced and oxidized forms of CoQ9 increased by the administration of NGF, and thus the total CoQ9 level was significantly increased in differentiated neural cells. In this figure, cellular CoQ9 level is normalized with cellular total protein level. It was quite surprising to see such a large and

Table 1. Primer used in real time PCR analysis

Gene	Forward Sequence (5'-3')	Reverse Sequence (5'-3')
TH	GTGAACCAATTCCCCATGTG	CAGTACCGTTCCAGAAGCTG
GAP-43	CGACAGGATGAGGGTAAAGAA	GACAGGAGAGGAAACTTCAGAG
GAPDH	GTTACCAGGGCTGCCTTCTC	GATGGTGATGGGTTTCCCGT
PDSS1	GAAAGGTTTGCCCACTACCT	CATCTGGTCAGAACATGAGGTG
PDSS2	CTTCAGATCTCTCGACACCATC	CAGTGGTAAGCAGTGGGTG
coq2	GATGATGCTCTGATTGGCCT	GGTGTAAATCTGGTGAGCCA
coq3	GGATGAAGATTCTCGACGTTGG	CTCATTCAAGGTCTCCTCCAG
coq4	CGGAGAAGTTGTGGTAAAGTGG	CTCCCAACGCTGTTCATAGTAG
coq5	AGTACCAGAGTAAAGAGGACCC	TGACATTCCGGATCCCAAAG
coq6	CTGCTCAGAGGCCTTGATAATG	CCATCACCTAGGGTAATATGGACC
coq7	CCTCAGGAATCACTTTTGGCTG	GGAATGTCCTATGTAGACCAGG
coq8	GATCTGTCAGAGTGGAGACGTA	CTATGGGGGTCTGTTGCATT
coq9	AGAACTGTTCTCTAGGAGTGGG	CACTATGTGTTGCCCTTTGGACC
FDFT1	AGCCACAAGGATGGAGTTCG	GAGTTCCGGTCCATCTTGGG
PSAP	CATCCTGGACATGATTAAGGGG	GAGAGGGATGTTGGACATGAAG

TH, tyrosine hydroxylase; GAP-43, growth associated protein-43; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; FDFT1, farnesyl-diphosphate farnesyltransferase 1; PSAP, prosaposin.
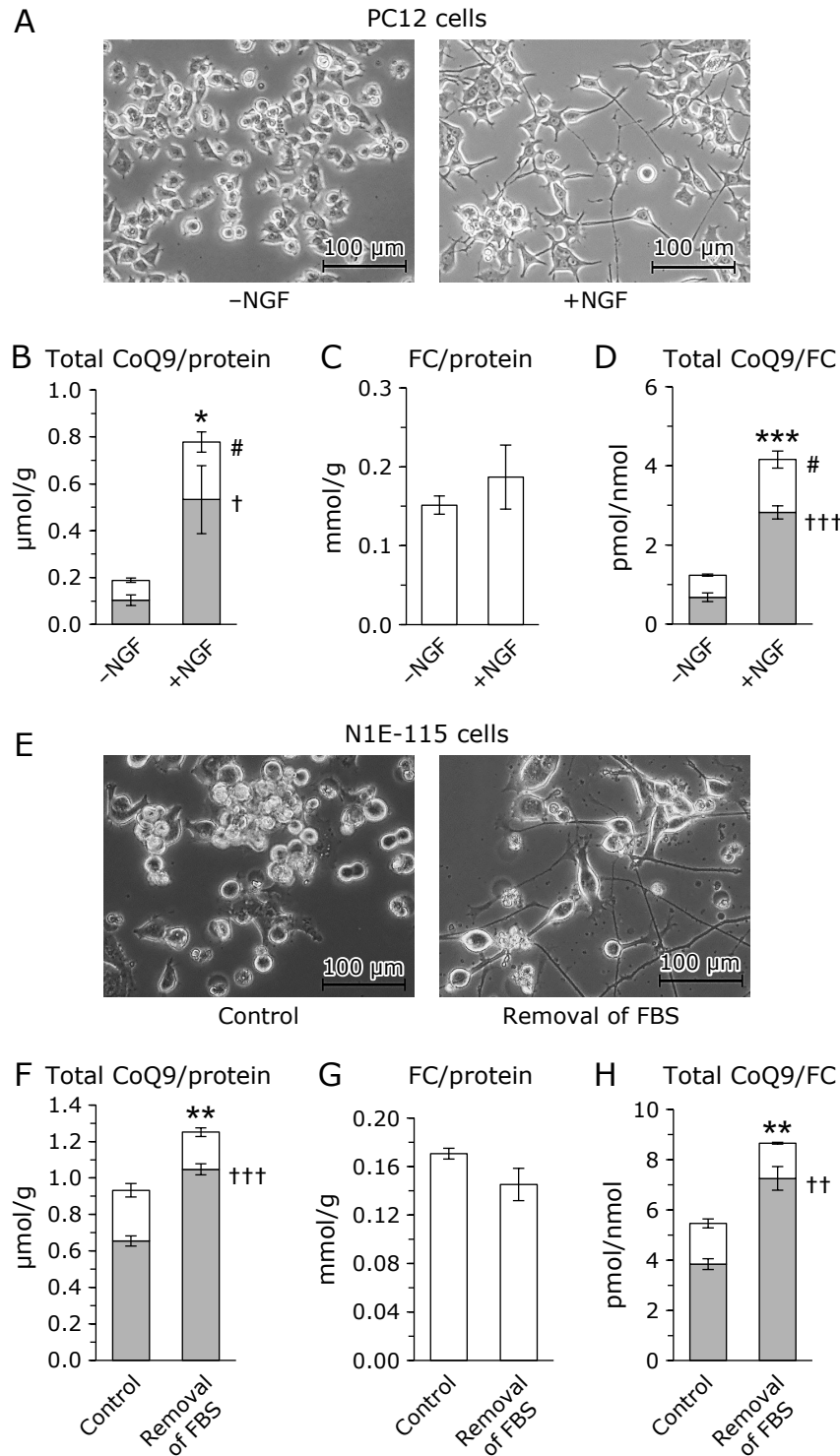
Fig. 1. The amount of CoQ in the differentiated neurons is increased. (A) Microscopic photographs of PC12 cells treated with and without NGF. (B) CoQ9 levels corrected for protein in PC12 cells. The white graph shows the amount of reduced CoQ, and the gray graph shows the amount of oxidized CoQ. (C) FC levels corrected for protein in PC12 cells. (D) CoQ9 levels corrected for FC content. The white graph shows the amount of reduced CoQ, and the gray graph shows the amount of oxidized CoQ. (E) Microscopic photographs of control and removal of FBS N1E-115 cells. (F) CoQ9 levels corrected for protein in N1E-115 cells. The white graph shows the amount of reduced CoQ, and the grey graph shows the amount of oxidized CoQ. (G) FC levels corrected for protein in N1E-115 cells. (H) CoQ9 levels corrected for FC in N1E-115 cells. The white graph shows the amount of reduced CoQ, and the grey graph shows the amount of oxidized CoQ. Levels of CoQ in differentiated cells were compared with those in controls using Student's t test. Values are represented as mean ± SD ($n = 3$) of the data obtained from three independent experiments. *, **, and *** indicate significant differences ($p<0.05$, 0.01, and 0.001, respectively) compared with control Total CoQ values. # indicates significant differences ($p<0.05$, 0.01, and 0.001, respectively) compared with control reduced CoQ values. †, ††, and ††† indicate significant differences ($p<0.05$, 0.01, and 0.001, respectively) compared with control oxidized CoQ values.
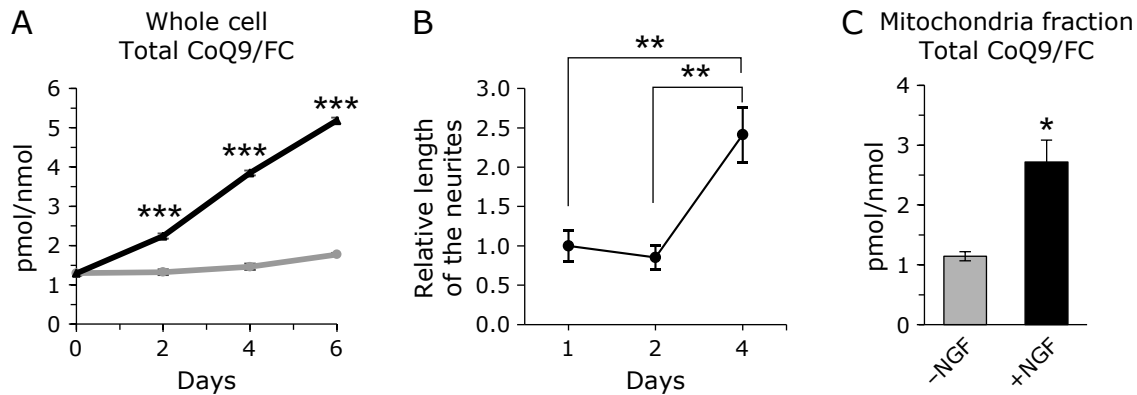
Fig. 2. (A) Time course of CoQ9 levels in PC12 cells treated with (black line) and without (grey line) NGF (***p<0.001). (B) Temporal changes in neurite length. Statistics were analysed using ANOVA. Values are represented as mean ± SD (*n* = 6) of the data obtained from three independent experiments (**p<0.01). (C) Levels of CoQ9 in mitochondria fraction also increased in NGF-treated cells. Statistics were analysed using Student's *t* test. Values are represented as mean ± SD (*n* = 3) of the data obtained from three independent experiments (*p<0.05).

pronounced increase in the value of CoQ9. To confirm this increase, we normalized the CoQ9 values not only by the amount of protein, but also by the amount of free cholesterol (FC). CoQ9 is a lipid that is synthesized from acetyl-CoA via the mevalonate pathway, this lipid's level was compared with the FC level, which is also formed from the mevalonate pathway. Cellular FC level normalized with cellular total protein level was not altered by the administration of NGF (Fig. 1C). Cellular CoQ9 levels, reduced, oxidized and total CoQ9, increased when corrected for the levels of FC (Fig. 1D). These data show that the differentiation of PC12 cells by the administration of NGF increased cellular CoQ9 levels.

Figure 1E shows cell morphological changes observed with N1E-115 cells. As shown in Fig. 1E, the removal of FBS from its medium caused a change in cell morphology: neurite outgrowth was observed. As shown in Fig. 1F and H, cell differentiation increased CoQ9 levels both normalized with cellular total protein level and FC level.

We then analyzed the time-course changes of cellular CoQ levels after the addition of NGF in PC12 cells. As shown in Fig. 2A, the level of CoQ9 increased with time.

Changes in neurite outgrowth over time are shown in Fig. 2B. The results in Fig. 2A and B show that CoQ9 levels increased with neurite length over time.

CoQ is present in all membranes, including the plasma, nuclear, and other membranes.[2] CoQ in mitochondria is important as an electron carrier. Thus, the level of CoQ in the mitochondria is being studied. As shown in Fig. 2C, the level of CoQ9 also increased with NGF treatment in the mitochondrial fraction.

These results imply that the level of CoQ increased during neural differentiation. We then tried to assess the mechanism of CoQ level increase.

Levels of mRNA expressions of CoQ biosynthesis enzymes and CoQ binding protein. Figure 3A illustrates the reported mechanisms of the biosynthesis CoQ. As shown in Fig. 3B–D, expression levels of these genes were not significantly altered by the treatment of PC12 cells with NGF. Thus, the increased CoQ9 level during the neuronal differentiation is due to the change in protein activities of these CoQ biosynthesis enzymes, or it may be due to a completely new CoQ synthesis mechanism. Prosaposin is a CoQ binding protein.[27,28] The level of prosaposin mRNA did not change with the addition of NGF (Fig. 3D). FDFT1, an enzyme that synthesizes squalene, was also unchanged (Fig. 3D). Mechanism(s) to increase cellular CoQ9 levels in neural differentiation requires further investigation.

Since the administration of NGF to PC12 cells increased cellular CoQ9 levels and resulted in the elongation of neurons, next, we assessed the role of increased CoQ9 levels on the cell differentiation processes.

Effect of CoQ synthesis inhibitors on the elongation of neural processes. To address this question, we used a CoQ biosynthesis inhibitor known as 4-NB. 4-NB competitively inhibits 4-hydroxybenzoate: polyprenyl transferase (Coq2) and dose-dependently decreases CoQ in mammalian cells.[25,26] As shown in Fig. 4A, the administration of 4-NB reduced cellular CoQ9 levels in PC12 cells with NGF administration. We assessed the mRNA expressions of neuronal differentiation markers: growth-associated protein-43 (GAP-43) and tyrosine hydroxylase (TH). As shown in Fig. 4B and C, the addition of NGF to control PC12 cells increased the mRNA expression levels of both GAP-43 and TH. Conversely, the addition of NGF to PC12 cells treated with 4-NB failed to increase these neuronal differentiation markers. That is, CoQ-reduced PC12 cells failed to differentiate normally.

Because the administration of 4-NB successfully reduces cellular CoQ9 levels and the expression of differentiation markers, we compared the neurite length with 4-NB administration to that without 4-NB administration. The length of the neuronal elongation process was measured using photographs taken of the process (Fig. 4D). As shown in Fig. 4E, the administration of 4-NB significantly reduced the neurite length.

Discussion

To understand brain function, knowledge regarding the molecular mechanisms involved in neural differentiation is necessary. In this study, we newly reported that cellular neural differentiations increased CoQ9 level in PC12 and N1E-115 cells. Neuronal differentiation has been reported to be accompanied by metabolic reprogramming to meet the increasing energy demand.[29,30] Glucose metabolism shift with increased lactate production and elevated glycolytic enzyme expression at the mRNA level was observed in mouse RGC differentiation.[31] Zheng *et al.*[29] reported that the loss of hexokinase and lactate dehydrogenase expression, together with a switch in the pyruvate kinase gene splicing from PKM2 to PKM1, marks the transition from aerobic glycolysis in neural progenitor cells to oxidative phosphorylation in neuronal cells. Agostini *et al.*[30] reported that mitochondrial biogenesis is increased during neuronal differentiation. Concomitant with mitochondrial biogenesis, they also observed an increase in glucose and glutamate–glutamine metabolisms.
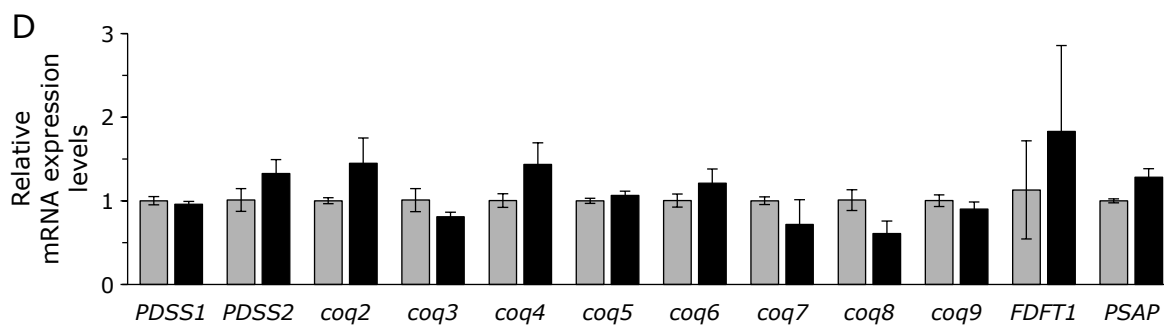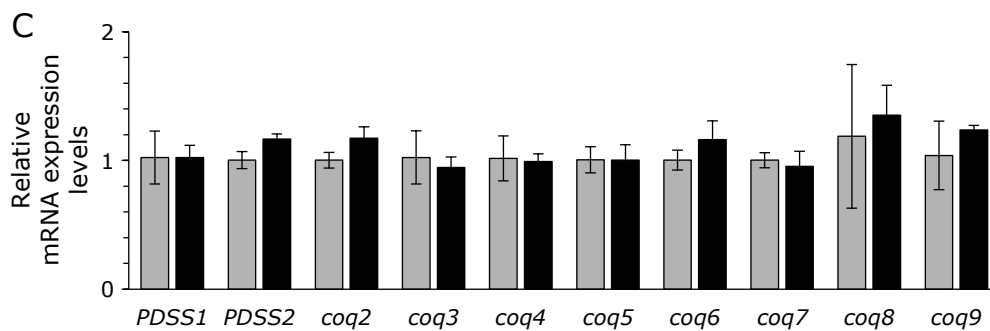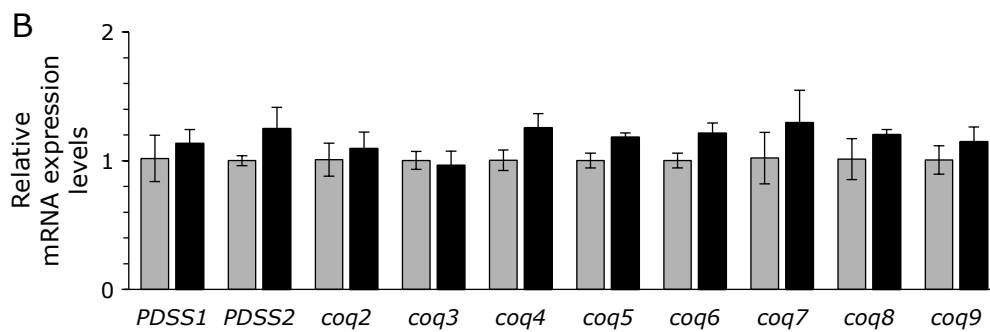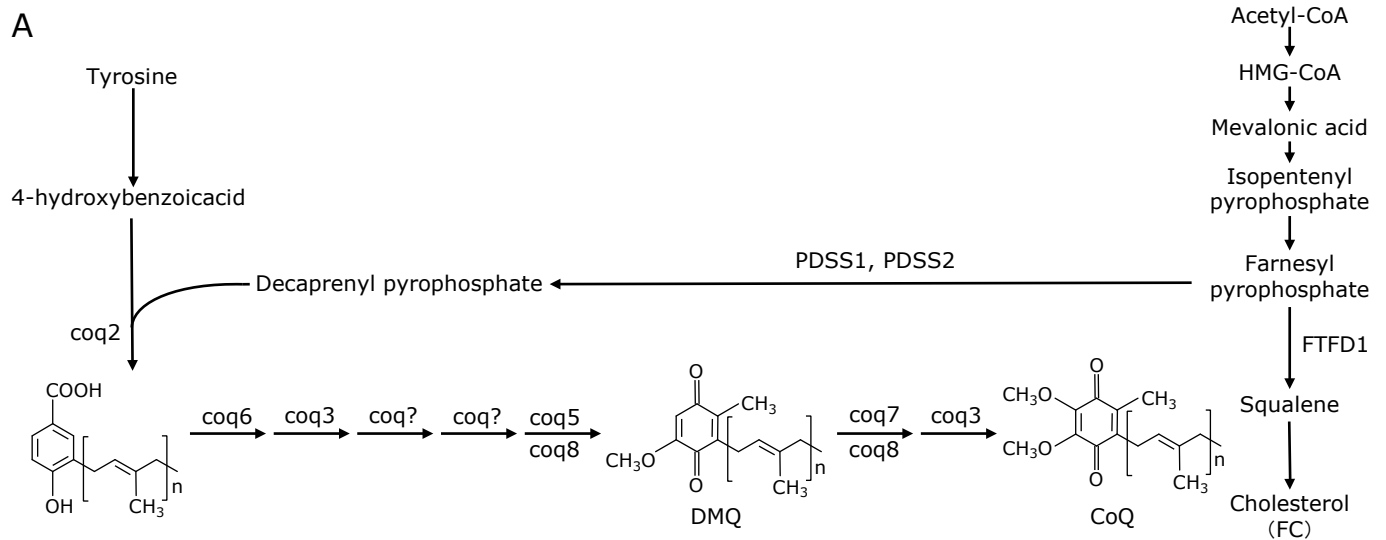
Fig. 3. Outline of the CoQ synthesis pathway and expression levels of each gene. (A) Illustrated schemes of CoQ and cholesterol biosynthetic pathways. The CoQ and cholesterol synthesis pathways share some similarities. (B–D) Gene expression levels of coq1–9, FDFT1, and PSAP with (black bar) and without (grey bar) NGF treatment [1 day (B), 2 days (C), 4 days (D)]. Values are represented as mean ± SD ($n = 3$) of the data obtained from three independent experiments.
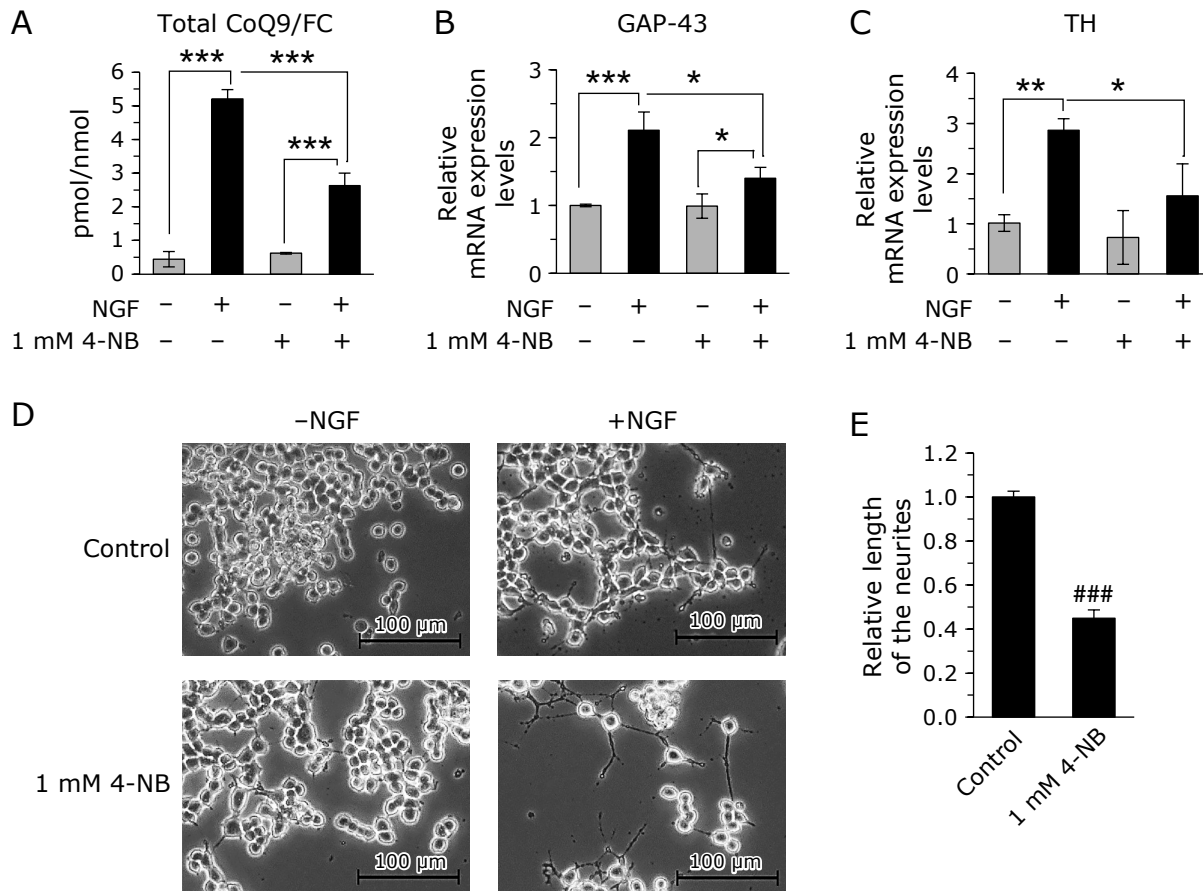
Fig. 4. Neuronal differentiation with 4-nitrobenzoate (4-NB), an inhibitor of CoQ synthesis. (A) Intracellular CoQ9 levels were reduced by the administration of 4-NB, both with (black bar) and without (grey bar) NGF treatment. (B) mRNA expression of differentiation marker GAP-43 increased with the addition of NGF. NGF treatment failed to increase the mRNA expression of GAP-43 in 4-NB treated PC12 cells. Grey bar: without NGF, Black bar: with NGF. (C) mRNA expression of differentiation marker TH increased with the addition of NGF. NGF treatment failed to increase the mRNA expression of TH in 4-NB treated PC12 cells. Statistics were analysed using ANOVA. Values are represented as mean ± SD ($n = 3$) of the data obtained from three independent experiments (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (D) Microscopic photographs of undifferentiated and differentiated added 4-NB in PC12 cells. (E) Administration of 4-NB reduced the length of the neuronal elongation. Statistics were analysed using Student's t test. Values are represented as mean ± SD ($n = 3$) of the data obtained from three independent experiments (###$p<0.001$).

Notably, CoQ in the mitochondrial electron transport system is an essential lipid for ATP production; thus, increased CoQ level in neural differentiation is crucial for maintaining cellular energy during differentiation.

To match the higher energy demand, reorganization of the mitochondrial network plays a pivotal role in neuronal differentiation. Mitochondrial mass, morphology, and function were changed during the differentiation of cortical neurons.[30] NGF-induced differentiation increases mitochondrial fission and fusion proteins and induces *Sirt3*, the transcription factors mtTFA and PPARγ, which regulate mitochondrial biogenesis and metabolism.[32] As shown in Fig. 2D, the level of CoQ9, normalized by FC in the mitochondrial fraction, was also increased by NGF administration. CoQ9 level normalized by the protein level also increased in the mitochondria. This shows that the increase in CoQ9 level during neural differentiation is more than that of the mitochondria.

NGF is a member of the Neurotrophin family of proteins that regulates the development, maintenance, and recovery of neurons from injury.[33] NGF treatment protects cells against oxidative stress induced by hydrogen peroxide treatment by stimulating GSH, the enzymes of the GSH metabolic cycle, and the antioxidant redox system of GSH/GSH peroxidase.[34] NGF-induced cell differentiation increases catalase and glutathione peroxidase

activities.[35] Our study revealed that NGF administration increased the CoQ9 level. Because the reduced form of CoQ is an important lipid-soluble antioxidant, this increase also plays an important role in protecting the cell from oxidative stress.

As mentioned above, in the process of neuronal differentiation, more efficient energy production in the mitochondria seems to be purposeful, considering the characteristics of the cell. It would also be desirable to increase the amount of reduced CoQ when increasing the antioxidant activity of the cell. The finding that CoQ9 levels are increased during neuronal differentiation suggests that CoQ9 may play an important role in regulating cellular energy production and altering antioxidant mechanisms. Further investigation of the physiological significance of increased CoQ levels during neuronal differentiation is warranted.

The mechanism(s) of increase in the CoQ level in neural differentiations remains debatable. We reported that cellular neural differentiations increased CoQ9 level both in PC12 and N1E-115 cells. Since these two neurite outgrowth models are by different stimuli, we do not believe that the mechanism of CoQ increase is exactly the same. There are some parts of the signal transduction system for neurite outgrowth that are common to all of them.[36,37] This may affect the CoQ production system, but the details are unknown at this time. This will be an issue for further study. We focus on PC12 cells and analysed CoQ synthesis

genes. We found no gene's expression was increased even if the sampling time is varied. The increase in CoQ levels after NGF administration may occur through a mechanism independent of the amount of these genes. Notably, CoQ synthesis proteins, COQ1–9, are reported to form supercomplex and synthesize CoQ.[38,39] This biosynthetic complex termed complex Q. Complex Q is located on the matrix face of the inner mitochondrial membrane. Previous studies demonstrated that mutation of COQ8 and COQ9 in mice causes selective and significant depletion of numerous COQ proteins.[40] Immunoprecipitation studies also shows the existence of supercomplex to synthesize CoQ.[41] COQ proteins form a supercomplex, and the degree of supercomplex formation is thought to affect CoQ synthesis activity. CoQ synthesis activity is not defined only simply by COQ gene expression. NGF administration could affect the formation of this complex Q and the activity to produce CoQ. This possibility should be explored in future studies.

Several neurological diseases are caused by mutations in the enzyme that synthesizes CoQ.[39] In these cases, CoQ level has been reported to be decreased. Our results revealed that CoQ level increased during neural differentiations and the inhibition of CoQ level during this process failed to produce normal neurite. Therefore, further studies regarding the relationship between this mechanism and diseases associated with CoQ synthase deficiency are warranted.

Author Contributions

MK, YY, and AF conceived the project and designed the experiments. AN, MO, AM, HJ, KS, and KK performed the experiments. MK, AN, and ST wrote the paper. MK coordinated and directed the project.

Abbreviations

CoQ	coenzyme Q
FC	free cholesterol
GAP-43	growth-associated protein-43
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
4-NB	4-nitrobenzoate
NGF	nerve growth factor
TH	tyrosine hydroxylase

Conflict of Interest

No potential conflicts of interest were disclosed.

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