Brief Report

DOI: 10.5582/ddt.2023.01003

Docosahexaenoic acid contributes to increased CaMKII protein expression and a tendency to increase nNOS protein expression in differentiated NG108-15 cells

Daisuke Miyazawa*, Kinari Suzuki, Hikari Sato, Natsumi Katsurayama, Tomoko Tahira, Hideki Mizutani, Naoki Ohara

College of Pharmacy, Kinjo Gakuin University, Nagoya, Japan.

SUMMARY Docosahexaenoic acid (DHA; 22:6*n*-3), an *n*-3 polyunsaturated fatty acid, has various important roles in brain functions. Nitric oxide (NO) produced by neuronal NO synthase (nNOS) and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is also involved in brain functions. We investigated the influence of DHA on nNOS and CaMKII protein expression in differentiated NG108-15 cells. NG108-15 cells were seeded in 12-well plates, and after 24 h, the medium was replaced with Dulbecco's modified Eagle's medium containing 1% fetal bovine serum, 0.2 mM dibutyryl cyclic adenosine monophosphate and 100 nM dexamethasone as differentiation-inducing medium. When cells were cultured in differentiation-inducing medium, neurite-like outgrowths were observed on days 5 and 6. However, no significant difference in morphology was observed in cells with or without DHA treatment. With or without DHA addition, nNOS protein expression was increased on days 5 and 6 compared with day 0. This increase tended to be enhanced by DHA. CaMKII protein expression did not change after differentiation without DHA, but was significantly increased on day 6 compared with day 0 with DHA addition. These data indicate that DHA is involved in brain functions by regulating CaMKII and nNOS protein expression.

Keywords DHA, *n*-3 polyunsaturated fatty acids, neuronal cell, nNOS, CaMKII

1. Introduction

In the brain, nitric oxide (NO) plays important roles in neural development, neuroprotection, synaptic plasticity, long-term potentiation, learning, and memory (*1-4*). NO is synthesized from L-arginine by NO synthases (NOSs). Three NOS isoforms have been characterized, namely neuronal NOS (nNOS), endothelial NOS, and inducible NOS (*5,6*). In particular, nNOS has been found in neuronal tissue (*7,8*). The activity of this enzyme requires Ca^{2+} and calmodulin (*8*). Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) is highly expressed in the brain (*9-11*). Neuronal activation induces CaMKII to translocate from the cytoplasm to the active zone at presynaptic membranes and the postsynaptic density (*12,13*). Presynaptic CaMKII regulates neurotransmitter synthesis and release (*14*). CaMKII is an important protein of synaptic plasticity (*15*).

Polyunsaturated fatty acids are essential components of membrane lipids in the mammalian brain. Docosahexaenoic acid (DHA), an *n*-3 polyunsaturated fatty acid, is a phospholipid component of the cell membrane, which is particularly abundant in neuronal tissues and essential for neuronal functions including neurite outgrowth, synaptic plasticity, behavior, mood regulation, learning, and memory (*11,16-20*).

In this study, we examined the effect of DHA addition to differentiation-inducing medium on nNOS and CaMKII protein expression in the neuroblastoma - glioma hybrid cell line NG108-15.

2. Materials and Methods

2.1. Materials

NG108-15 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Cell culture medium and dexamethasone (DEX) were purchased from Fujifilm Wako (Osaka, Japan). Fetal bovine serum (FBS) was purchased from Gibco (Grand Island, NY, USA). Penicillin and streptomycin were purchased from Nacalai Tesque (Kyoto, Japan). HAT supplement (hypoxanthine, aminopterin, and thymidine, 50×) was purchased from MP biomedicals (Santa

Ana, CA, USA). DHA was purchased from Cayman (Ann Arbor, MI, USA). Dibutyryl cyclic adenosine monophosphate (dbcAMP) was purchased from Sigma (St. Louis, MO, USA).

2.2. Cell culture and microscopy

NG108-15 cells were grown and maintained in high-glucose Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS, HAT (0.1 mM hypoxanthine, 0.4 µM aminopterin, and 16 µM thymidine), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C with 5% CO_2. Figure 1 shows the study design. Cells were seeded in 12-well plates 5000 cells/cm^2. After 24 h, the medium was replaced with DMEM supplemented with 1% FBS, 1% HAT, 100 U/mL penicillin, 100 µg/mL streptomycin, 10 µM α-tocopherol, 0.2 mM dbcAMP, and 100 nM DEX, which was added to induce differentiation (*21,22*). DHA (2 µM) bound to 0.05% fatty acid-free bovine serum albumin was added to the appropriate treatment groups. Then, the cells were cultured for 5 or 6 days. Microscopy performed under an Axiovert200 (Carl Zeiss, Oberkochen, Germany).

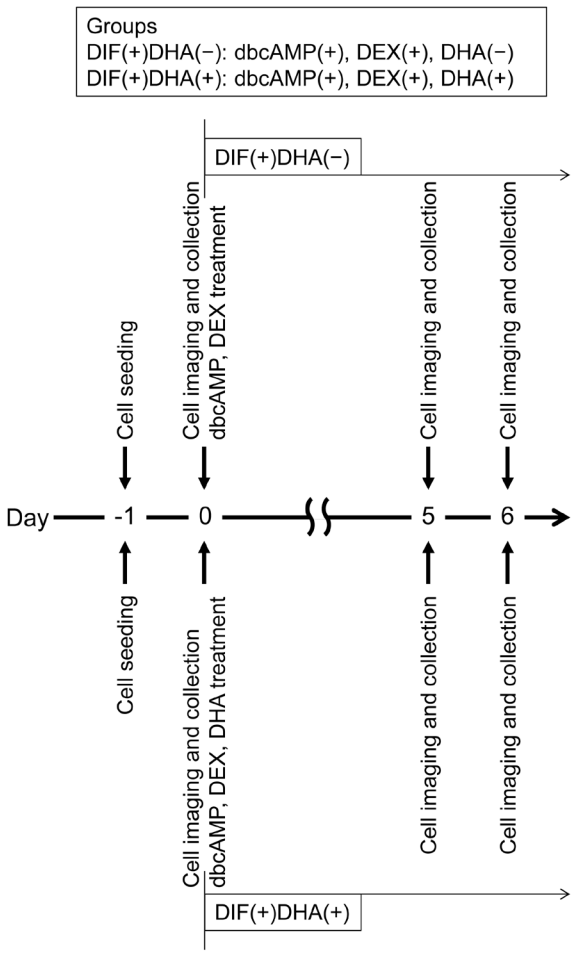

Figure 1. Cell culture and treatment. The day after seeding cells (day 0), differentiation medium and/or docosahexaenoic acid were added. At day 6, cells were observed by microscopy and collected. DIF, differentiation medium; DHA, docosahexaenoic acid; DEX, dexamethasone; dbcAMP, dibutyryl cyclic AMP.

2.3. Preparation of samples for western blotting

Cells were harvested in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/mL leupeptin, and 1 mM PMSF), and the samples were sonicated. The protein concentration was determined with a BCA protein assay kit (Pierce, Rockford, IL, USA) using bovine serum albumin as the standard (*23*).

Aliquots were mixed with concentrated sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (final concentrations: 62.5 mM Tris-HCl, pH 6.8, 2% 2-mercaptoethanol, 10% glycerol, 2% SDS, and 10% bromophenol blue).

2.4. Western blotting

For SDS-PAGE, samples containing equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with PVDF blocking reagent (Toyobo, Osaka, Japan) and then incubated overnight at 4°C with primary antibodies [nNOS (#4236, Cell Signaling Technology, Danvers, MA, USA), CaMKII (#3362, Cell Signaling Technology), and β-actin (Sigma)]. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) and developed with SuperSignal West Pico (Thermo Fisher, Waltham, MA, USA) or ImmunoStar LD reagents (Fujifilm Wako). Signal detection and quantification of the band intensity were performed using an Amersham Imager 680 (Cytiva, Marlborough, MA, USA).

2.5. Statistical analysis

Significant differences among day 0, 5, and 6 values within each group were evaluated by Fisher's test and between the DIF(+)DHA(−) and DIF(+)DHA(+) groups within each day were evaluated by Student's t test. $p < 0.05$ was considered significant. Excel-Toukei software (2012, Social Survey Research Information Co., Ltd., Tokyo, Japan) was used for the statistical analysis.

3. Results and Discussion

To determine the effects of DHA on differentiated NG108-15 cells, we investigated nNOS and CaMKII protein expression on day 0 (undifferentiated) and days 5 and 6 (differentiated). This cell line is known to differentiate on days 4-6 of induced differentiation,

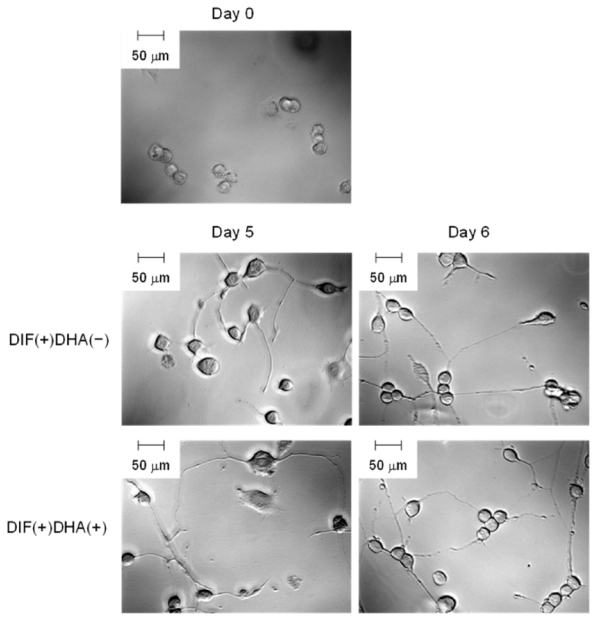

Figure 2. Microscopy observation of NG108-15 cells. The upper micrograph shows the day 0 (undifferentiated), the middle micrograph shows the DIF(+)DHA(−) group, and the lower micrograph shows the DIF(+)DHA(+) group.

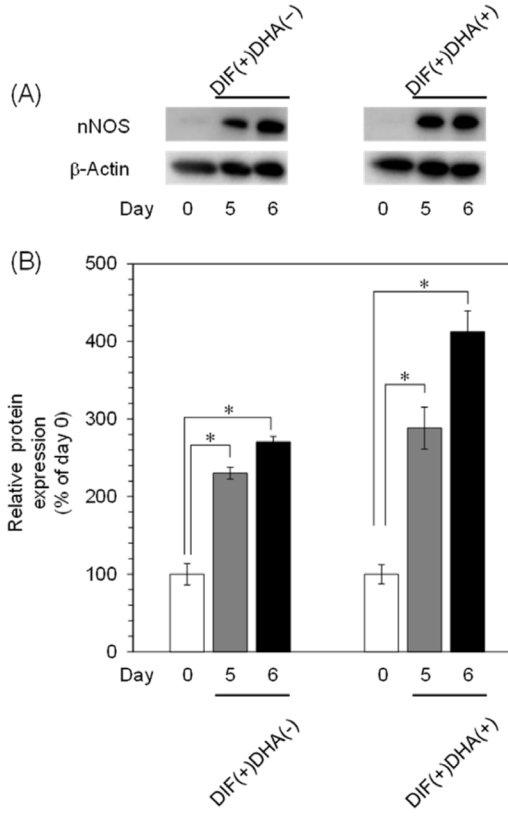

Figure 3. Western blot analysis of nNOS expression in NG108-15 cells on days 0 (undifferentiated), 5, and 6 of differentiation. (**A**) Representative western blots of nNOS and β-actin. (**B**) Semiquantitative analysis of nNOS/β-actin. Each column and bar represents the mean and SEM of four individual experiments. (* $p < 0.05$) nNOS, neuronal NO synthase; DIF, differentiation medium; DHA, docosahexaenoic acid; DEX, dexamethasone; dbcAMP, dibutyryl cyclic AMP.

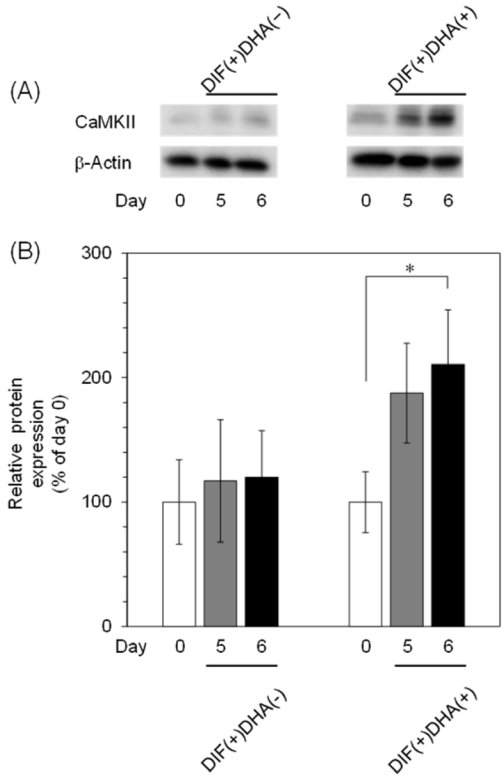

Figure 4. Western blot analysis of CaMKII expression in NG108-15 cells on days 0 (undifferentiated), 5, and 6 of differentiation. (**A**) Representative western blots of CaMKII and β-actin. (**B**) Semiquantitative analysis of CaMKII/β-actin. Each column and bar represents the mean and SEM of four individual experiments (* $p < 0.05$). CaMKII Ca^{2+}/calmodulin-dependent protein kinase II; DIF, differentiation medium; DHA, docosahexaenoic acid; DEX, dexamethasone; dbcAMP, dibutyryl cyclic AMP.

and the changes on days 0, 5, and 6 were confirmed to assess time dependency.

Figure 2 shows micrographs of the cell morphology. Neurite outgrowth occurred on days 5 and 6 of differentiation. However, no significant morphological changes due to DHA addition were observed on days 5 or 6.

Figure 3 shows nNOS protein expression in NG108-15 cells after incubation for 5 or 6 days in differentiation-inducing medium. In the DIF(+)DHA(−) group, nNOS protein expression was increased on days 5 and 6 compared with day 0. Additionally, in the DIF(+)DHA(+) group, nNOS protein expression was increased on days 5 and 6 compared with day 0. These increases tended to be enhanced by DHA addition.

Figure 4 shows CaMKII protein expression in NG108-15 cells. No significant difference was observed in the DIF(+)DHA(−) group even on days 5 and 6 after treatment. However, in the DIF(+)DHA(+) group, a significant increase was observed on day 6 compared with day 0, and a non-significant tendency to increase was observed on day 5 compared with day 0. However. the expressions of nNOS and CaMKII were not significantly different between DIF(+)DHA(-) and

DIF(+)DHA(+) groups on day 5 and 6.

nNOS protein expression was increased on days 5 and 6 compared with day 0 by differentiation without DHA. These increases tended to be enhanced by DHA (Figure 3). CaMKII protein expression was increased on day 6 compared with day 0 by differentiation with DHA, but its expression in the DHA(−) group did not change from day 0 to days 5 and 6. (Figure 4). Therefore, nNOS and CaMKII protein expression in NG108-15 cells was simultaneously affected by DHA.

It has been reported that DHA regulates the expression of several transcription factors. For example, it has been reported that DHA increases the expression of NeuroD, a transcription factor, which is involved in neural differentiation (*24*). It has also been reported that DHA increases the expression of several proteins *via* the tyrosine kinase receptor B (trkB) – extracellular signal-regulated kinase (ERK)1/2 – cAMP-response element binding protein (CREB) pathway (*25*). In this study, it may be that the protein levels of CaMKII and nNOS were changed by being involved in these pathways and/or other transcriptional regulation.

Long-term potentiation (LTP) of hippocampal synaptic transmission plays a role in the mechanism of learning memory. Glutamate receptors are involved in LTP induction. Glutamate receptors are ionotropic or G protein-coupled receptors, and the ionotropic NMDA receptor and its downstream CaMKII pathway play an important role in LTP induction (*26*), learning, and memory (*27*). Additionally, nNOS/endothelial NOS double mutations reduce hippocampal LTP in mice (*4*). It has also been reported that the NMDA receptor-nNOS pathway acts in the induction of presynaptic plasticity (*28*). Several studies have reported that CaMKII and nNOS act in concert with each other (*29,30*), and colocalization of CaMKII, nNOS, and postsynaptic density 95 is important for acting together in a coordinated manner (*31*). nNOS activity is suppressed by phosphorylation of Ser845 by CaMKII (*29*). NO overproduced during ischemia suppresses CaMKII activity *via* S-nitrosylation of Cys6 (*30*). It has been reported that nNOS and CaMKII may perform appropriate functions by coordinating their activities with each other (*32*).

Several studies have reported that DHA is involved in neuroplasticity, LTP, learning, and memory (*11,19,20*). Although our experiments were performed in cells, addition of DHA caused a significant increase in CaMKII protein expression and a tendency to increase nNOS protein expression. Our findings and those in other reports (*11,19,20*) suggest that recovery of synaptic plasticity and learning memory by *n*-3 fatty acids is affected by regulation of nNOS and/or CaMKII expression by *n*-3 fatty acids. We believe that further research, including gene expression, phosphorylation, and intracellular localization of nNOS and CaMKII *in vitro* and *in vivo*, may reveal the mechanisms by which DHA plays several roles in brain functions.

Acknowledgements

We thank Mitchell Arico from Edanz (https://jp.edanz.com/ac) for editing a draft of this manuscript.

Funding: This work was supported by JSPS KAKENHI Grant Number JP16K00876.

Conflict of Interest: The authors have no conflicts of interest to disclose.

References

1. Garthwaite J. NO as a multimodal transmitter in the brain: Discovery and current status. Br J Pharmacol. 2019; 176:197-211.
2. Garthwaite J, Boulton CL. Nitric oxide signaling in the central nervous system. Annu Rev Physiol. 1995; 57:683-706.
3. Hawkins RD. NO honey, I don't remember. Neuron. 1996; 16:465-467.
4. Son H, Hawkins RD, Martin K, Kiebler M, Huang PL, Fishman MC, Kandel ER. Long-term potentiation is reduced in mice that are doubly mutant in endothelial and neuronal nitric oxide synthase. Cell. 1996; 87:1015-1023.
5. Knowles RG, Moncada S. Nitric oxide synthases in mammals. Biochem J. 1994; 298 (Pt 2):249-258.
6. Moncada S, Bolaños JP. Nitric oxide, cell bioenergetics and neurodegeneration. J Neurochem. 2006; 97:1676-1689.
7. Bredt DS, Hwang PM, Glatt CE, Lowenstein C, Reed RR, Snyder SH. Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. Nature. 1991; 351:714-718.
8. Bredt DS, Snyder SH. Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proc Natl Acad Sci U S A. 1990; 87:682-685.
9. Erondu NE, Kennedy MB. Regional distribution of type II Ca^{2+}/calmodulin-dependent protein kinase in rat brain. J Neurosci. 1985; 5:3270-3277.
10. Peng J, Kim MJ, Cheng D, Duong DM, Gygi SP, Sheng M. Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. J Biol Chem. 2004; 279:21003-21011.
11. Fedorova I, Salem N, Jr. Omega-3 fatty acids and rodent behavior. Prostaglandins Leukot Essent Fatty Acids. 2006; 75:271-289.
12. Tao-Cheng JH, Dosemeci A, Winters CA, Reese TS. Changes in the distribution of calcium calmodulin-dependent protein kinase II at the presynaptic bouton after depolarization. Brain Cell Biol. 2006; 35:117-124.
13. Strack S, Choi S, Lovinger DM, Colbran RJ. Translocation of autophosphorylated calcium/calmodulin-dependent protein kinase II to the postsynaptic density. J Biol Chem. 1997; 272:13467-13470.
14. Liu Q, Chen B, Ge Q, Wang ZW. Presynaptic Ca^{2+}/calmodulin-dependent protein kinase II modulates neurotransmitter release by activating BK channels at Caenorhabditis elegans neuromuscular junction. J Neurosci. 2007; 27:10404-10413.
15. Lisman J. Criteria for identifying the molecular basis of

the engram (CaMKII, PKMzeta). Mol Brain. 2017; 10:55.
16. Harauma A, Sagisaka T, Horii T, Watanabe Y, Moriguchi T. The influence of *n*-3 fatty acids on maternal behavior and brain monoamines in the perinatal period. Prostaglandins Leukot Essent Fatty Acids. 2016; 107:1-7.
17. Ikemoto A, Kobayashi T, Emoto K, Umeda M, Watanabe S, Okuyama H. Effects of docosahexaenoic and arachidonic acids on the synthesis and distribution of aminophospholipids during neuronal differentiation of PC12 cells. Arch Biochem Biophys. 1999; 364:67-74.
18. Ikemoto A, Kobayashi T, Watanabe S, Okuyama H. Membrane fatty acid modifications of PC12 cells by arachidonate or docosahexaenoate affect neurite outgrowth but not norepinephrine release. Neurochem Res. 1997; 22:671-678.
19. Ikemoto A, Ohishi M, Sato Y, Hata N, Misawa Y, Fujii Y, Okuyama H. Reversibility of *n*-3 fatty acid deficiency-induced alterations of learning behavior in the rat: level of *n*-6 fatty acids as another critical factor. J Lipid Res. 2001; 42:1655-1663.
20. Fujita S, Ikegaya Y, Nishikawa M, Nishiyama N, Matsuki N. Docosahexaenoic acid improves long-term potentiation attenuated by phospholipase A(2) inhibitor in rat hippocampal slices. Br J Pharmacol. 2001; 132:1417-1422.
21. Machová E, Málková B, Lisá V, Nováková J, Dolezal V. The increase of choline acetyltransferase activity by docosahexaenoic acid in NG108-15 cells grown in serum-free medium is independent of its effect on cell growth. Neurochem Res. 2006; 31:1239-1246.
22. Machová E, Nováková J, Lisá V, Dolezal V. Docosahexaenoic acid supports cell growth and expression of choline acetyltransferase and muscarinic receptors in NG108-15 cell line. J Mol Neurosci. 2006; 30:25-26.
23. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. Anal Biochem. 1985; 150:76-85.
24. Katakura M, Hashimoto M, Shahdat HM, Gamoh S, Okui T, Matsuzaki K, Shido O. Docosahexaenoic acid promotes neuronal differentiation by regulating basic helix-loop-helix transcription factors and cell cycle in neural stem cells. Neuroscience. 2009; 160:651-660.
25. Bie N, Feng X, Li C, Meng M, Wang C. The protective effect of docosahexaenoic acid on PC12 cells in oxidative stress induced by H_2O_2 through the TrkB-Erk1/2-CREB Pathway. ACS Chem Neurosci. 2021; 12:3433-3444.
26. Silva AJ, Stevens CF, Tonegawa S, Wang Y. Deficient hippocampal long-term potentiation in alpha-calcium-calmodulin kinase II mutant mice. Science. 1992; 257:201-206.
27. Silva AJ, Paylor R, Wehner JM, Tonegawa S. Impaired spatial learning in alpha-calcium-calmodulin kinase II mutant mice. Science. 1992; 257:206-211.
28. Hardingham N, Dachtler J, Fox K. The role of nitric oxide in pre-synaptic plasticity and homeostasis. Front Cell Neurosci. 2013; 7:190.
29. Komeima K, Hayashi Y, Naito Y, Watanabe Y. Inhibition of neuronal nitric-oxide synthase by calcium/ calmodulin-dependent protein kinase IIalpha through Ser847 phosphorylation in NG108-15 neuronal cells. J Biol Chem. 2000; 275:28139-28143.
30. Song T, Hatano N, Kambe T, Miyamoto Y, Ihara H, Yamamoto H, Sugimoto K, Kume K, Yamaguchi F, Tokuda M, Watanabe Y. Nitric oxide-mediated modulation of calcium/calmodulin-dependent protein kinase II. Biochem J. 2008; 412:223-231.
31. Watanabe Y, Song T, Sugimoto K, Horii M, Araki N, Tokumitsu H, Tezuka T, Yamamoto T, Tokuda M. Post-synaptic density-95 promotes calcium/calmodulin-dependent protein kinase II-mediated Ser847 phosphorylation of neuronal nitric oxide synthase. Biochem J. 2003; 372:465-471.
32. Araki S, Osuka K, Takata T, Tsuchiya Y, Watanabe Y. Coordination between calcium/calmodulin-dependent protein kinase II and neuronal nitric oxide synthase in neurons. Int J Mol Sci. 2020; 21:7997.

Received January 16, 2023; Revised May 2, 2023; Accepted May 21, 2023.

**Address correspondence to:*
Daisuke Miyazawa, College of Pharmacy, Kinjo Gakuin University, 2-1723 Omori, Moriyama-ku, Nagoya 463-8521, Japan.
E-mail: miyazawa@kinjo-u.ac.jp

Released online in J-STAGE as advance publication May 27, 2023.