Note

Docosahexaenoic Acid Increases Vesicular Glutamate Transporter 2 Protein Levels in Differentiated NG108-15 Cells

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Docosahexaenoic acid (DHA; 22:6n-3), which is enriched in the neuronal membrane, plays a variety of roles in the brain. Vesicular glutamate transporters (VGLUTs) are responsible for incorporating glutamine into synaptic vesicles. We investigated the influence of DHA on the fatty acid profile and the levels of VGLUT1 and VGLUT2 proteins in differentiated NG108-15 cells, a neuroblastoma–glioma hybrid cell line. NG108-15 cells were plated and 24 h later the medium was replaced with Dulbecco's modified Eagle's medium supplemented with 1% fetal bovine serum, 0.2 mM dibutyryl cAMP, and 100 nM dexamethasone, which was added to induce differentiation. After 6 d, the amount of DHA in the cells was increased by addition of DHA to the medium. VGLUT2 levels were increased by the addition of DHA. These data indicate that DHA affected the levels of VGLUT2 in NG108-15 cells under differentiation-promoting conditions, suggesting that DHA affects brain functions involving VGLUT2.

Key words docosahexaenoic acid (DHA); vesicular glutamate transporter 2 (VGLUT2); NG108-15 cell

INTRODUCTION

Glutamate is a major excitatory neurotransmitter in the central nervous system and is released by many neurons. It is involved in numerous neuronal functions including perception, learning, and memory[1] and is loaded into synaptic vesicles by vesicular glutamate transporters (VGLUTs). Three isoforms of VGLUT (VGLUT1–3) have been identified and are expressed in the brain.[2–5] VGLUT1 and VGLUT2 are brain-specific Na^+-dependent inorganic phosphate transporters. VGLUT1 and VGLUT2 have complementary distributions in the central nervous system.[2,6] VGLUT1 is mainly expressed in the cerebral cortex and hippocampus, while VGLUT2 is expressed in the thalamus, hypothalamus, and brain stem.[2,6] VGLUT3 is expressed in cholinergic and serotoninergic neurons.[4] Polyunsaturated fatty acids have several functions in the brain. Docosahexaenoic acid (DHA), an n-3 polyunsaturated fatty acid, is a phospholipid component of the cell membrane, is particularly abundant in neuronal tissues, and is essential for neuronal functions *in vitro* and *in vivo* (including neurite outgrowth, synaptic plasticity, behavior, mood regulation, and learning and memory).[7–10] It has been reported that NG108-15 cells, a neuroblastoma–glioma hybrid cell line, exhibit neuronal-like morphology and properties by inducing differentiation.[11–14] To investigate the possibility that VGLUT is associated with various brain functions affected by DHA, we examined the effect of adding DHA to differentiation medium on the levels of VGLUT1 and VGLUT2 proteins using the neuroblastoma–glioma hybrid cell line, NG108-15.

MATERIALS AND METHODS

Cell Culture NG108-15 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) (high-glucose) containing 10% fetal bovine serum (FBS) (Gibco, Grand island, NY, U.S.A.), 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine (Kohjin Bio, Sakado, Japan), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in 5% CO_2. Figure 1 shows the study design. Cells were plated in 12-well plates at a density of 5000 cells/cm^2. After 24 h, the medium was replaced with DMEM supplemented with 1% FBS, 0.1 mM hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 μM α-tocopherol, 0.2 mM dibutyryl cAMP (dbcAMP) (Sigma, St. Louis, MO, U.S.A.), and 100 nM dexamethasone (DEX) (FUJIFILM Wako, Osaka, Japan), which was added to induce differentiation (DIF).[11–14] DHA (Cayman, Ann Arbor, MI, U.S.A.) (20 μM) bound to 0.05% bovine serum albumin (fatty acid-free) was added to the medium. Two groups, DIF(+)DHA(−) [dbcAMP(+), DEX(+), DHA(−)] and DIF(+)DHA(+) [dbcAMP(+), DEX(+), DHA(+)], were prepared. Then, the cells were cultured for 6 d.

Analysis of Fatty Acid Composition Total lipids were extracted from FBS or cells in chloroform/methanol solution according to the method of Bligh and Dyer.[15] The combined extracts were dried under a stream of nitrogen gas. Then, fatty acid residues were converted into methyl esters using 0.5% HCl in methanol and quantified using capillary column (DB-255; J&W Scientific, Folsom, CA, U.S.A.) gas chromatography (Shimadzu, Kyoto, Japan) as described previously.[16] Heptadecanoic acid (Sigma) was added as an internal standard.

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_2 ethylenediaminetetraacetic acid, 1 mM ethylene glycol bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1 μg/mL leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF)] and the samples were sonicated. The protein concentration was determined with a bicinchoninic acid (BCA) protein assay

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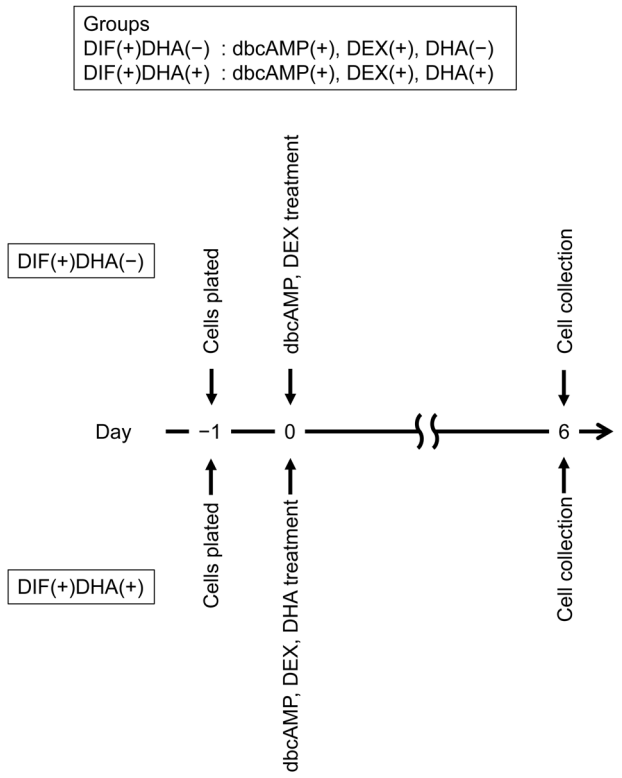

Fig. 1. Cell Culture and Treatment

The day after cell seeding (day 0), differentiation medium and docosahexaenoic acid were added. At day 6, cells were collected. DIF, differentiation medium; DHA, docosahexaenoic acid; DEX, dexamethasone; dbcAMP, dibutyryl cyclic AMP; FBS, fetal bovine serum.

kit (Thermo Fisher Scientific, Waltham, MA, U.S.A.) using bovine serum albumin as the standard.[17] Aliquots were mixed with concentrated sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer [final concentration: 62.5 mM Tris–HCl (pH 6.8), 2% 2-mercaptoethanol, 10% glycerol, 2% SDS, 10% bromophenol blue].

Western Blotting For SDS-PAGE, samples containing equal amounts of protein were loaded onto 10% SDS-polyacrylamide gels[18] and subsequently transferred to polyvinylidene fluoride (PVDF) membranes. The membranes were blocked with PVDF blocking reagent (Toyobo, Tokyo, Japan) and then incubated overnight at 4 °C with primary antibodies against VGLUT1 (Cell Signaling Technology, Danvers, MA, U.S.A.), VGLUT2 (Cell Signaling Technology), and β-actin (Sigma). The membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (Dako, Glostrup, Denmark) and developed with SuperSignal West Pico (Thermo Fisher Scientific) or ImmunoStar LD reagents (FUJIFILM Wako). Signal detection and quantification of the band intensity were performed using an Amersham Imager 680 (Cytiva, Tokyo, Japan).

Statistical Analysis The mean differences between the DIF(+)DHA(−) and DIF(+)DHA(+) groups were evaluated by Student's *t*-test. A *p*-value <0.05 was considered significant. Excel-Toukei software (2012, Social Survey Research Information, Tokyo, Japan) was used for the statistical analysis.

RESULTS

Fatty Acid Profile Table 1 shows the fatty acid con-

Table 1. Fatty Acid Profiles of FBS and 1% FBS in DMEM

	FBS			DMEM (1% FBS)
	(μg/mL)	(% w/w)	(μM)	(μM)
Saturated FA				
14 : 0	3.46	1.64	15.15	0.1515
16 : 0	66.29	31.32	258.51	2.5851
18 : 0	32.02	15.13	112.55	1.1255
22 : 0	2.80	1.32	8.22	0.0822
Monounsaturated FA				
16 : 1	8.88	4.19	34.90	0.3490
18 : 1	58.73	27.75	207.91	2.0791
20 : 1	0.98	0.47	3.17	0.0317
N-6 Polyunsaturated FA				
18 : 2 n-6	12.15	5.74	43.34	0.4334
20 : 4 n-6	15.54	7.34	51.04	0.5104
N-3 Polyunsaturated FA				
22 : 5 n-3	5.11	2.42	15.47	0.1547
22 6 n-3	5.69	2.69	17.33	0.1733
	211.65	100	767.59	7.6759

Fatty acids (FAs) are designated by the carbon chain length : number of double bonds and the position of the first double bond numbered from the methyl terminus (n-6 or n-3). The FA content in DMEM containing 1% FBS was calculated based on the FA content of FBS. FBS, fetal bovine serum; DMEM, Dulbecco's modified Eagle's medium.

Table 2. Fatty Acid Profile of NG108-15 Cells

Fatty acid (FA)	DIF(+)DHA(−)	DIF(+)DHA(+)
Saturated FA	mg/g Protein	
14 : 0	1.51 ± 0.38	1.07 ± 0.29
16 : 0 DMA	2.29 ± 0.48	2.29 ± 0.88
16 : 0	21.25 ± 1.26	22.41 ± 4.00
18 : 0 DMA	1.70 ± 0.86	3.11 ± 1.12
18 : 0	33.58 ± 5.18	36.78 ± 6.47
20 : 0	1.40 ± 0.35	1.03 ± 0.85
22 : 0	0.89 ± 1.87	0.06 ± 0.14
Total saturated FA	62.61 ± 6.07	66.74 ± 10.49
Monounsaturated FA		
16 : 1	2.46 ± 0.18	1.38 ± 0.29**
18 : 1	63.20 ± 9.57	50.60 ± 11.75
20 : 1	6.73 ± 1.59	4.59 ± 1.42*
22 : 1	0.58 ± 0.31	0.07 ± 0.17**
Total monounsaturated FA	72.97 ± 10.98	56.64 ± 13.24
N-6 Polyunsaturated FA		
18 : 2 n-6	2.12 ± 0.42	2.11 ± 0.44
20 : 3 n-6	0.38 ± 0.22	1.27 ± 1.52*
20 : 4 n-6	4.61 ± 1.17	4.16 ± 2.18
Total n-6 FA	7.12 ± 1.64	7.54 ± 1.30
N-3 Polyunsaturated FA		
18 : 3 n-3	0.99 ± 0.36	1.43 ± 0.29
22 : 5 n-3	1.39 ± 0.89	3.18 ± 0.26**
22 : 6 n-3	1.42 ± 0.91	32.07 ± 3.91**
Total n-3 FA	3.80 ± 1.53	36.67 ± 4.10**
n-6/n-3	2.45 ± 2.10	0.20 ± 0.02*
Total FA (mg/g protein)	146.51 ± 18.85	167.59 ± 26.21

Values represent the mean ± standard deviation ($n = 6$). Asterisks indicate a significant difference between the two groups (*$p < 0.05$; **$p < 0.01$). DMA, dimethyl acetal derived from plasmalogen; DHA, docosahexaenoic acid; DIF, differentiation medium.

tent of FBS. The calculated fatty acid content of 1% FBS in DMEM before the addition of DHA is also shown. Before DHA addition, the DHA concentration in DMEM containing 1% FBS was 0.17 μM. Table 2 shows the fatty acid contents in NG108-15 cells after incubation for 6 d in differentiation-inducing medium. The DHA and docosapentaenoic acid n-3 (DPA n-3; 22:5n-3) contents were significantly higher in the DIF(+)DHA(+) group than in the DIF(+)DHA(−) group. In addition, the increases in DPA n-3 were considered to be caused by the addition of DHA because the α-linolenic acid contents in the DIF(+)DHA(−) group were not different from those of the DIF(+)DHA(+) group. The palmitoleic acid (16 : 1), eicosenoic acid (20 : 1), and docosenoic acid (22 : 1) contents were significantly lower in the DIF(+)DHA(+) group than in the DIF(+)DHA(−) group.

VGLUT1 and VGLUT2 Levels Figure 2 shows the VGLUT1 and VGLUT2 protein levels in NG108-15 cells after incubation for 6 d in differentiation-inducing medium. There was no significant difference in VGLUT1 levels between the two groups (Fig. 2B). The VGLUT2 levels in the DIF(+)DHA(+) group were significantly higher than those in the DIF(+)DHA(−) group (Fig. 2C).

DISCUSSION

We investigated the fatty acid profile and VGLUT protein levels in NG108-15 cells to determine the effects of the addition of DHA to the differentiation medium at 6 d after treatment. The palmitoleic acid (16 : 1), eicosenoic acid (20 : 1) and docosenoic acid (22 : 1) contents were decreased by the addition of DHA. Changes in fatty acid profiles resulting from the addition of DHA may be involved in changes of neuronal

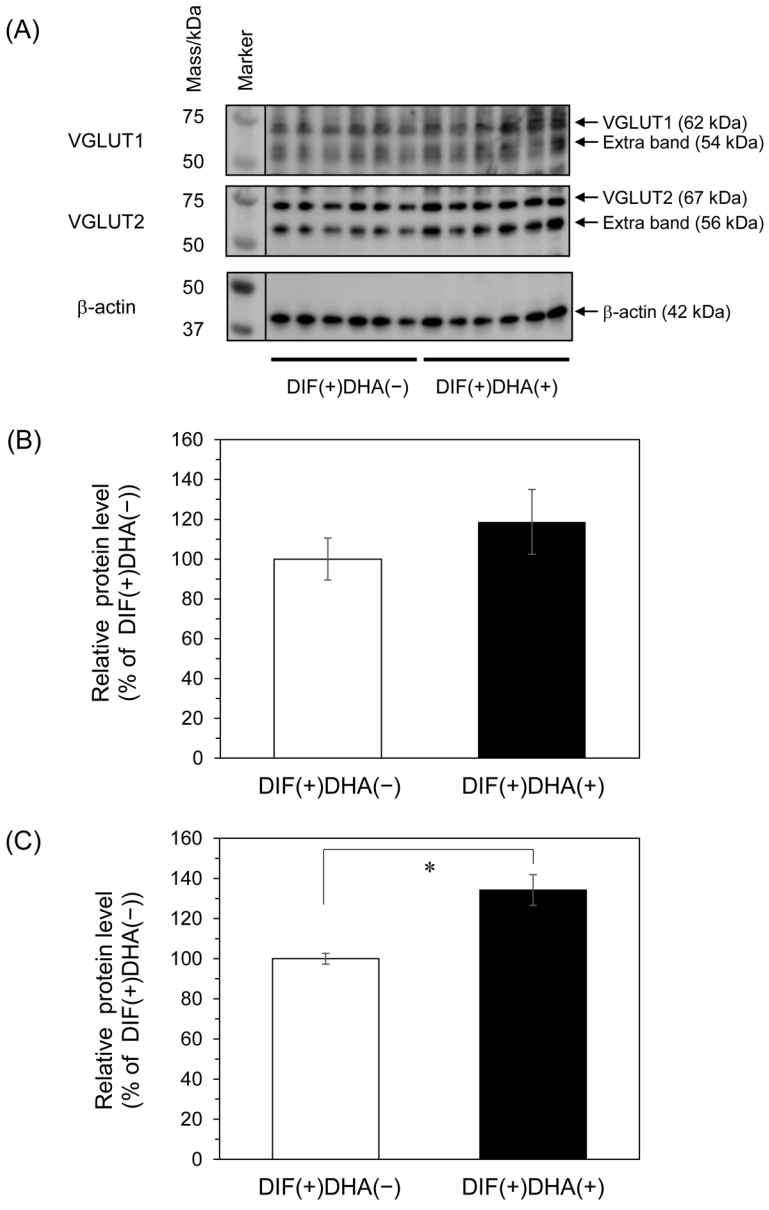

Fig. 2. Western Blot Analysis of VGLUT1 and VGLUT2 in NG108-15 Cells at 6 d after Treatment

(A) Representative Western blot bands of VGLUT1, VGLUT2 and β-actin. The molecular masses of the samples were calculated based on the calibration curve using Excel. The identity of the extra bands has not been identified. (B) Relative protein level of VGLUT1/β-actin. Each column and bar represent the mean and SEM from six separate experiments. SEM, standard error of the mean; VGLUT, vesicular glutamate transporter. (C) Relative protein level of VGLUT2/β-actin. The asterisk indicates a significant difference between the two groups ($*p < 0.05$). Each column and bar represent the mean and SEM from six separate experiments.

functions. Our study showed that VGLUT2 levels were significantly increased after adding DHA to NG108-15 cell culture medium (Fig. 2C), but that the level of VGLUT1 was not altered (Fig. 2B). VGLUT3 was not detected (data not shown).

VGLUTs are associated with various psychiatric disorders. Preadolescent mice that lack VGLUT2 in the cortex and amygdala exhibit schizophrenia-like behavioral deficits.[19] Several studies have reported that a low level of n-3 polyunsaturated essential fatty acids is associated with mental illness (*e.g.*, depression and schizophrenia).[8,20] In clinical trials, 26-week administration of eicosapentaenoic acid + DHA improved or contributed to the improvement of mental health in patients with first episode schizophrenia.[21,22]

Although our experiments were performed on cells *in vitro*, addition of DHA increased VGLUT2 levels. Our findings, when considered with other reports,[8,19,20] may indicate that schizophrenia, which is associated with a decrease in n-3 fatty acids, is affected by a decrease in VGLUT2 levels. Therefore, adequate intake of n-3 fatty acids may play a beneficial role in synaptic vesicle function. We think that further research (including intracellular localization and changes in expression levels *in vivo*) will help reveal the mechanisms by which DHA prevents schizophrenia and other psychiatric disorders.

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Conflict of Interest The authors declare no conflict of interest.

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