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Modulation of Fear Memory by Dietary Polyunsaturated Fatty Acids via Cannabinoid Receptors

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Although the underlying mechanism remains unknown, several studies have suggested benefits of n-3 long-chain polyunsaturated fatty acid (PUFA) for patients with anxiety disorders. Elevated fear is thought to contribute to the pathogenesis of particular anxiety disorders. The aim of the present study was to evaluate whether the dietary n-3 to n-6 PUFA (3:6) ratio influences fear memory. For this purpose, the effects of various dietary 3:6 ratios on fear memory were examined in mice using contextual fear conditioning, and the effects of these diets on central synaptic transmission were examined to elucidate the mechanism of action of PUFA. We found that fear memory correlated negatively with dietary, serum, and brain 3:6 ratios in mice. The low fear memory in mice fed a high 3:6 ratio diet was increased by the cannabinoid CB_1 receptor antagonist rimonabant, reaching a level seen in mice fed a low 3:6 ratio diet. The agonist sensitivity of CB_1 receptor was enhanced in the basolateral nucleus of the amygdala (BLA) of mice fed a high 3:6 ratio diet, compared with that of mice fed a low 3:6 ratio diet. Similar enhancement was induced by pharmacological expulsion of cholesterol in the neuronal membrane of brain slices from mice fed a low 3:6 ratio diet. CB_1 receptor-mediated short-term synaptic plasticity was facilitated in pyramidal neurons of the BLA in mice fed a high 3:6 ratio diet. These results suggest that the ratio of n-3 to n-6 PUFA is a factor regulating fear memory via cannabinoid CB_1 receptors.
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INTRODUCTION

Long-chain polyunsaturated fatty acids (PUFAs) are a major nutrient essential for human health (Horrocks and Farooqui, 2004). In the mammalian brain, there are two major classes of PUFA, n-3 and n-6. Mammals cannot convert PUFAs from one family into those from the other; n-3 and n-6 PUFAs are directly ingested or biosynthesized from their precursor PUFAs. The fatty acid composition of the brain can be extensively modulated by dietary lipids (Youdim *et al*, 2000). PUFAs are major components of the plasma membrane, where they influence membrane fluidity (Salem and Niebylski, 1995; Yang *et al*, 2011). Lifelong insufficiency of n-3 PUFAs induces changes in rodent behaviors (Lafourcade *et al*, 2011; Larrieu *et al*, 2012; Takeuchi *et al*, 2003) and cannabinoid signaling in the brain (Lafourcade *et al*, 2011; Larrieu *et al*, 2012). However, the effect of n-3 PUFA supplementation on brain function in rodents not deficient in n-3 is not yet known.

We conducted a literature search for studies examining PUFA involvement in anxiety disorders, excluding depression, which yielded four results. Supplementation with n-3 PUFA, docosahexaenoic acid (22:6n-3; DHA), and another n-3 PUFA, eicosapentaenoic acid (20:5n-3; EPA), after a traumatic experience prevented the development of post-traumatic stress disorder (PTSD) (Matsuoka *et al*, 2010). Similar supplementation given to female members of rescue groups in a severe disaster reportedly reduced the incidence of PTSD (Nishi *et al*, 2012). Furthermore, DHA, EPA, and the n-3 PUFA α-linolenic acid (18:3n-3) are significantly decreased (by 18%, 36%, and 32%, respectively) in red blood cell membranes of patients with social anxiety disorder compared with controls (Green *et al*, 2006); in the same study, the ratio of n-6 to n-3 PUFA differed significantly between patients and controls. An association between dietary intake of DHA and clinically determined anxiety disorders was demonstrated in a cross-sectional, community-based study in women (Jacka *et al*, 2013). The biological mechanism underlying the contribution of the ratio of n-3 to n-6 PUFA (3:6) in anxiety disorders is unknown.

It is widely accepted that fear memory and its abnormal processing are involved in the pathogenesis of anxiety

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disorders in which fear is a contributory factor (Cuthbert *et al*, 2003). For example, PTSD develops following exposure to a life-threatening event, and the persistence of traumatic memories in PTSD is often explained in terms of a trauma-induced enhancement of fear memory encoding (Debiec and LeDoux, 2006; O'Donnell *et al*, 2004). It is also known that PTSD involves an impairment of fear extinction (Milad *et al*, 2006; Rauch *et al*, 2006).

Fear conditioning is an experimental model for conditioned fear memory (LeDoux, 2000), which is essential for an individual's survival. Many studies suggest that this pivotal memory is influenced by manipulation of cannabinoid signaling that, in the brain, represses neurotransmitter release through cannabinoid CB_1 receptors. For example, extinction of auditory fear memory is impaired in CB_1 receptor knockout mice without any changes in memory acquisition or consolidation (Marsicano *et al*, 2002). Furthermore, the CB_1 receptor neutral antagonist, AM4113, exerts no effect on the expression of contextual fear memory in rats (Sink *et al*, 2010). On the other hand, systemic administration of the CB_1 agonists WIN55,212-2 (WIN) or HU-210 impairs contextual fear conditioning in rats (Mackowiak *et al*, 2009; Pamplona and Takahashi, 2006). In addition, pre-test microinjection of another CB_1 agonist, anandamide, into the dorsolateral periaqueductal gray (PAG) or ventromedial prefrontal cortex (vmPFC) impairs contextual fear conditioning in rats (Lisboa *et al*, 2010; Resstel *et al*, 2008). These results consistently suggest that activation of CB_1 receptors reduces contextual fear memory.

The aim of the present study was to evaluate whether dietary 3:6 ratio influences fear memory in mice not deficient in n-3, and, if so, to identify the underlying mechanism(s). Such information would be valuable for the clinical utilization of PUFAs. For this purpose, we examined the effects of various dietary 3:6 ratios on mouse fear memory in contextual fear conditioning experiments.

MATERIALS AND METHODS

Animals and Feeding of Diets

Male C57BL/6J mice were purchased at 4–5 weeks of age and fed a solid standard mouse diet CE-2 (Clea, Tokyo, Japan; see Supplementary Table S2) for 1 week. The mice subsequently received chow containing various amounts of n-3 and n-6 PUFAs for 6 weeks (see Results for dietary PUFA analysis). The mice were housed four or five per cage under controlled temperature (25 ± 1 °C) and lighting (12 h light–dark cycle) conditions, and water was provided *ad libitum*. Animal procedures were in strict accordance with the guidelines of the National Institute of Neuroscience, National Center of Neurology and Psychiatry (Tokyo, Japan) and were approved by the Institutional Animal Investigation Committee.

Contextual Fear Conditioning

Immediately after the 6-week feeding period, a contextual fear conditioning test and extinction session were conducted as described previously (Yamada *et al*, 2009). The freezing response is expressed as the percentage of time that the mouse spent freezing during a 180 s time window. See Supplementary Methods for detailed descriptions.

Other Behavioral Tests

The open-field, shock-sensitivity, and light–dark transition tests were performed as described previously (Sakurai *et al*, 2008). The procedure for the Y-maze test was based on a previous report (Sarter *et al*, 1988).

Analysis of Fatty Acids

The fatty acid content of the diets and the mouse serum and brain were determined using gas chromatography (Lepage and Roy, 1986). See Supplementary Methods for detailed descriptions.

Quantitative Real-Time PCR

See Supplementary Methods.

Electrophysiology

Whole-cell patch-clamp recordings from pyramidal neurons in the basolateral nucleus of the amygdala (BLA) were performed as described previously (Yamada *et al*, 2012; Zushida *et al*, 2007). See Supplementary Methods for detailed descriptions.

Drugs

Rimonabant (RIM; SR141716A) was purchased from Cayman Chemicals (Ann Arbor, MI). Naloxone (NLX), WIN, and methyl-β-cyclodextrin (MCD) were purchased from Sigma (St Louis, MO). For subcutaneous administration, RIM was dissolved in vehicle (VEH) containing 2.5% dimethyl sulfoxide (DMSO) and 1.0% Tween-80 in saline solution. NLX was dissolved in saline. For electrophysiological experiments, RIM and WIN were dissolved in DMSO at 5 and 10 mM, respectively, and diluted in artificial cerebrospinal fluid (aCSF) at final concentrations as indicated in Results and Figure legends.

Statistical Analysis

All data are shown as mean $\pm$ SEM. The data were analyzed by one- or two-way analysis of variance (ANOVA) for comparisons among three or more groups. If the ANOVA results were significant, *post hoc* Bonferroni multiple comparisons were performed. The two-tailed unpaired *t*-test was used for statistical comparisons between two groups. Spearman's rank correlation coefficient (ρ) was calculated for correlation analyses. $P<0.05$ was considered statistically significant.

RESULTS

We prepared eight varieties of chow (Supplementary Table S1). Mice (5–6 weeks of age at the start of the experiment) were fed one variety for 6 weeks. Behavioral, biochemical, and electrophysiological experiments followed. The control 1 diet was similar to the n-3 non-deficient standard AIN-93 diet (Reeves *et al*, 1993), with soybean oil as the fat

component (Supplementary Table S1). The test diets were prepared by replacing soybean oil with oils rich in n-3 PUFAs (2.5% krill oil, 5% krill oil, 5% krill oil + linoleic acid (18:2n-6, LA; n-6 PUFA), 2.5% fish oil, 5% fish oil, 2.5% fish oil + olive oil (OL)). Krill oil was prepared from Antarctic krill and fish oil was prepared from sardines. The oils were mixed with the other chow components before solidification. Total fat contents did not differ among the diets that were compared. The fatty acid compositions and the 3:6 ratios of all diets are listed in Supplementary Table S2.

Dietary, Brain, and Serum Ratios of n-3 to n-6 PUFAs Influence Contextual Fear Memory

Figure 1a shows the fraction of time that mice ($n = 12$ for all groups) spent freezing during a 3-min behavioral test session (re-exposure of mice to a conditioning box 24 h after contextual fear conditioning). The freezing rate was highest in mice fed the control 1 diet (3:6 = 0.14; n-3 PUFA content, 0.44%; Figure 1a), lower in those fed the 2.5% krill diet (3:6 = 0.33; n-3 PUFA content, 0.89%), and significantly lower (compared with the control 1 diet group) in those fed the 5% krill diet (3:6 = 0.97; n-3 PUFA content, 1.32%; $F(2, 33) = 8.58$, $P = 0.001$ by ANOVA, $P < 0.001$ with *post hoc* Bonferroni comparison). Figure 1b shows the time course of the freezing rate in Figure 1a. On the other hand, when the test was conducted 1 h after the conditioning (short-term memory test), freezing rates were almost identical in mice fed control 1 and 5% krill diets (Figures 1a and b (inset)). These results suggest that mice fed diets with a higher 3:6-ratio or n-3 PUFA content showed reduction of long-term contextual fear memory.

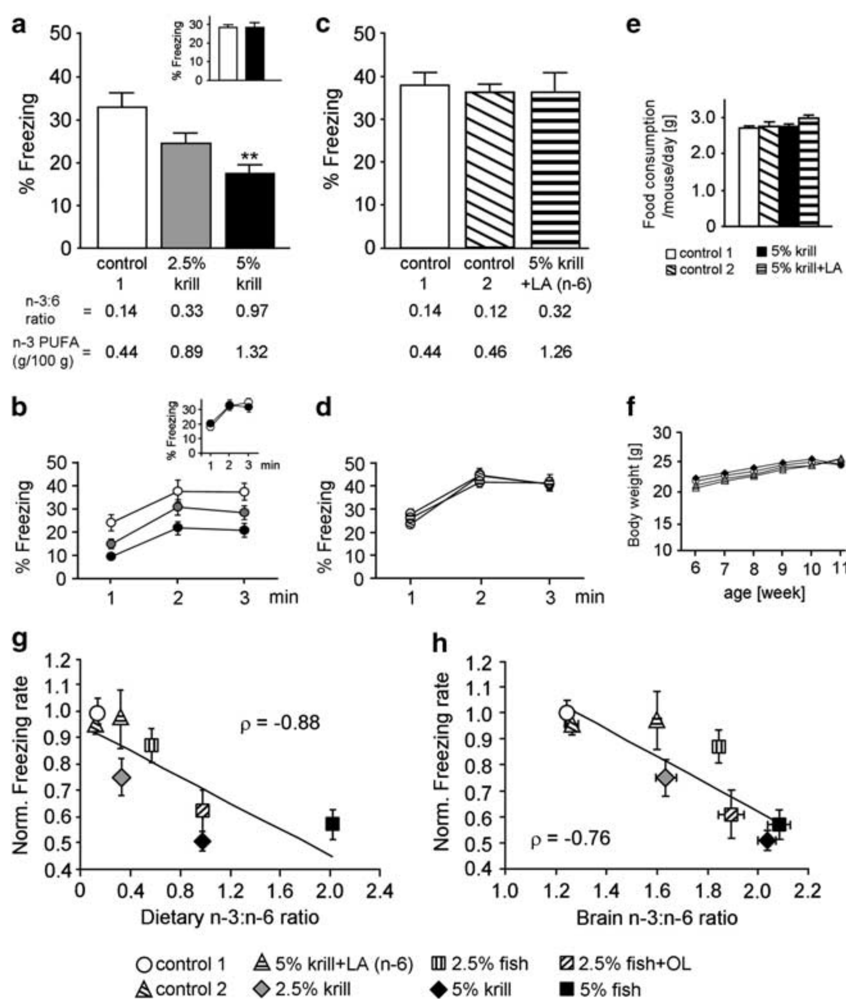

Figure 1 Dietary ratio of n-3 to n-6 polyunsaturated fatty acids (PUFAs) influences contextual fear memory. (a) Dose-dependent reduction of contextual fear memory (24 h after conditioning) induced by krill diets ($n = 12$ per group). (a, inset) Short-term memory test, conducted 1 h after conditioning ($n = 10$ per group). (b) Time course of the freezing rate during the test session in (a). (b, inset) Time course of the freezing rate during test session in (a, inset) ($n = 10$ per group). (c) Increasing the content of linoleic acid (LA) abolished the fear-attenuating effect of the 5% krill diet (control 1, $n = 7$; control 2, $n = 10$; 5% krill + LA, $n = 10$). Control 2 diet was used to compensate for the higher total fat content (9.5%) after addition of LA to the 5% krill diet (see Supplementary Table S1). (d) Time course of the freezing rate during test session in (c). (e) Food consumption measured during the 11th week in mice fed the control 1 ($n = 12$), control 2 ($n = 10$), 5% krill ($n = 12$), and 5% krill + LA ($n = 10$) diets. (f) Body weight changes in mice fed the control 1 ($n = 12$), control 2 ($n = 10$), 5% krill ($n = 12$), and 5% krill + LA ($n = 10$) diets. (g and h) Correlation between dietary (g) or brain (h) 3:6 ratio and freezing rate. ρ, Spearman's rank correlation coefficient. All data are shown as means ± SEM. **$P < 0.01$, one-way analysis of variance (ANOVA) and *post hoc* Bonferroni comparison.

To determine the factor(s) governing fear memory, mice were fed the 5% krill diet with added LA to change the dietary 3:6-ratio (3:6 = 0.32; n-3 PUFA content, 1.26%; see also Supplementary Tables S1 and S2). LA is an n-6 PUFA and a precursor of arachidonic acid (20:4n-6, AA; n-6 PUFA). Because total fat content was increased to 9.5% by adding LA, we prepared a new control diet, control 2, to compensate for the higher total fat content (3:6 = 0.12; n-3 PUFA content, 0.46%; see also Supplementary Tables S1 and S2). The freezing rate in mice fed this 5% krill + LA diet ($n = 10$) was almost identical to that in mice fed the control 2 diet ($n = 10$; Figure 1c). Time course of the freezing rate is shown in Figure 1d. We also confirmed that freezing rates did not differ between the control 1 ($n = 7$) and control 2 diet-fed mice (Figure 1c). These results indicate that the dietary 3:6 ratio is an important factor in determining fear response: fear memory in mice is poorer if the dietary 3:6 ratio is high. In addition, the behavioral results after the 5% krill + LA diet also confirmed that the effect of the 5% krill diet on fear memory was not due to trace impurities in the krill oil, because the content of krill oil was the same in the 5% krill and 5% krill + LA diets. To verify this, we measured food consumption ($n = 10$–12; Figure 1e) and body weight ($n = 10$–12; Figure 1f). No significant changes were observed in daily dietary consumption among mice fed the control 1, control 2, 5% krill, and 5% krill + LA diets (Figure 1e). Similarly, there were no major differences in body weight gain from the start of feeding (postnatal week 6) until the end of feeding (postnatal week 11) (Figure 1f).

These results are summarized in Figure 1g, together with the results obtained with fish oil-containing diets, plotting dietary 3:6 ratios *vs* freezing rates normalized by the rate obtained with the control 1 diet ($n = 6$ for each diet). The plot reveals a negative correlation ($\rho = -0.88$, $P = 0.007$), suggesting that the dietary 3:6 ratio and contextual fear memory in mice are inversely correlated. Namely, mice fed a 5% fish oil diet (3:6 = 2.02) showed low freezing rates and those fed a 2.5% fish oil diet (3:6 = 0.57) showed high freezing rates (Figure 1g). Furthermore, we prepared a 2.5% fish oil diet in which the 3:6 ratio was increased to 0.97 by replacing soybean oil with OL. n-6 PUFA is abundant in soybean oil (Supplementary Table S2) but not in OL (in which oleic acid, 18:1n-9, is abundant). Thus, this substitution reduces the content of n-6 PUFA, thereby increasing the 3:6 ratio. Mice fed the 2.5% fish oil + OL diet showed lower freezing rates than those fed the equivalent diet without OL, indicating that the effects of the 3:6 ratio do not depend on the dietary n-3 source (fish or krill).

We quantified the fatty acid contents of the brain and serum (see Table 1 for brain and Supplementary Table S3 for serum) and examined the relationship between 3:6 ratio and freezing rate ($n = 6$ for each quantification). The brain sample analyzed was composed of the remaining tissue after removal of the basal ganglia and hippocampus from the

Table 1 Composition of Fatty Acids in the Brain (Remaining Tissue after Removal of Basal Ganglia and Hippocampus from the Cerebrum)

Numerical symbol	Control 1	2.5% Krill	5% Krill	Control 2	5% Krill + LA	2.5% Fish	5% Fish	2.5% Fish + OL
Total SFA	41.74 ± 0.25	41.94 ± 0.13	41.69 ± 0.21	41.57 ± 0.16	41.98 ± 0.15	41.80 ± 0.14	41.62 ± 0.43	42.00 ± 0.15
Total MUFA	18.07 ± 0.05	17.32 ± 0.10	19.00 ± 0.18	17.82 ± 0.08	18.04 ± 0.05	19.41 ± 0.06	19.02 ± 0.26	19.29 ± 0.31
Total n-3 PUFA	**17.23 ± 0.20**	19.45 ± 0.13	**20.05 ± 0.21**	17.29 ± 0.34	18.97 ± 0.21	19.58 ± 0.16	20.09 ± 0.30	19.58 ± 0.26
18:3n-3	—	—	—	0.01 ± 0.01	—	—	—	—
20:3n-3	—	—	—	—	0.17 ± 0.11	—	—	—
20:5n-3	—	0.16 ± 0.01	0.54 ± 0.09	0.57 ± 0.26	0.20 ± 0.02	0.27 ± 0.02	0.50 ± 0.02	0.33 ± 0.01
22:5n-3	0.08 ± 0.04	0.39 ± 0.02	0.57 ± 0.01	0.16 ± 0.01	0.42 ± 0.01	0.46 ± 0.00	0.63 ± 0.02	0.50 ± 0.01
22:6n-3	17.15 ± 0.16	18.90 ± 0.10	18.94 ± 0.11	16.55 ± 0.07	18.18 ± 0.07	18.85 ± 0.14	18.96 ± 0.26	18.75 ± 0.24
Total n-6 PUFA	**12.98 ± 0.20**	11.92 ± 0.31	**9.82 ± 0.16**	13.71 ± 0.09	11.87 ± 0.16	10.62 ± 0.09	9.69 ± 0.26	10.35 ± 0.26
18:2n-6	0.75 ± 0.01	0.52 ± 0.01	0.49 ± 0.02	0.77 ± 0.0	0.77 ± 0.03	0.69 ± 0.02	0.37 ± 0.02	0.46 ± 0.02
18:3n-6	—	—	—	0.01 ± 0.01	0.01 ± 0.01	—	0.02 ± 0.01	—
20:2n-6	0.06 ± 0.03	0.14 ± 0.13	0.05 ± 0.03	0.13 ± 0.00	0.06 ± 0.03	—	0.10 ± 0.05	0.07 ± 0.03
20:3n-6	0.44 ± 0.01	0.66 ± 0.01	0.54 ± 0.01	0.43 ± 0.01	0.65 ± 0.01	0.53 ± 0.01	0.36 ± 0.01	0.49 ± 0.01
20:4n-6	9.61 ± 0.10	8.86 ± 0.10	7.40 ± 0.08	10.02 ± 0.04	8.67 ± 0.06	7.96 ± 0.04	7.55 ± 0.12	7.87 ± 0.16
22:2n-6	0.02 ± 0.02	—	—	0.07 ± 0.02	—	—	0.03 ± 0.02	—
22:4n-6	2.10 ± 0.03	1.74 ± 0.06	1.34 ± 0.02	2.28 ± 0.01	1.71 ± 0.02	1.44 ± 0.02	1.26 ± 0.03	1.46 ± 0.04
Unknown	9.98 ± 0.09	9.37 ± 0.10	9.44 ± 0.14	9.61 ± 0.07	9.14 ± 0.08	8.59 ± 0.10	9.58 ± 0.73	8.78 ± 0.52
n-3:n-6	**1.33 ± 0.02**	1.63 ± 0.04	**2.04 ± 0.03**	1.26 ± 0.03	1.60 ± 0.02	1.84 ± 0.02	2.07 ± 0.05	1.89 ± 0.05

Abbreviation: LA, linoleic acid; MUFA, monounsaturated fatty acid (FA); OL, olive oil; SFA, saturated FA; Value, % of total FA; 20:5n-3, eicosapentaenoic acid (EPA); 22:6n-3, docosahexaenoic acid (DHA); 18:2n-6, LA; 20:4n-6, arachidonic acid (AA); —, not detected.
Bold numerals represent significant difference ($P < 0.05$) between mice fed control 1 and 5% krill diets.

cerebrum (mainly consisting of the cortex and amygdala). Brain and serum 3:6 ratios both correlated negatively with the freezing rate (Figures 1h, $\rho = -0.76$, $P = 0.037$ for brain; Supplementary Figure S1, $\rho = -0.86$, $P = 0.011$ for serum).

Taken together, these results suggest that ingestion of diets high in n-3 and low in n-6 PUFAs enhances brain and serum 3:6 ratios and attenuates contextual fear memory in mice.

Extinction of Contextual Fear Memory and Other Behavioral Tests

We tested the effects of a 5% krill diet on extinction of contextual fear memory and other behaviors, and found that no significant changes were evident in extinction ($n = 8$ for each diet; Figures 2a–d), the open-field test (locomotor activity and percent center time; $n = 12$ for each diet; Figure 2e), the shock-sensitivity test ($n = 8$ for each diet; Figure 2f), the light–dark transition test (anxiety; $n = 8$ for each diet; Figure 2g), or the Y-maze test (working memory; $n = 10$ for each diet; Figure 2h). Therefore, it seems that changes in the 3:6 ratio do not alter contextual fear extinction or global behavior.

Involvement of Cannabinoid CB_1 Receptors

Next, we sought to elucidate the underlying mechanism by which n-3 PUFA supplementation influences brain function. PUFAs are directly incorporated into the plasma membrane as a constituent of phospholipids (Salem and Niebylski, 1995; Youdim *et al*, 2000). Numerous biophysiological studies investigating n-3 PUFA incorporation into membranes have shown that (i) uptake of DHA into phospholipids results in exclusion of cholesterol from raft domains in the reconstituted membrane (Stillwell and Wassall, 2003; Stillwell *et al*, 2005), and (ii) reduction of cholesterol from the membrane enhances the activities of cannabinoid CB_1 receptors (Bari *et al*, 2005a; Maccarone et al, 2009; Oddi *et al*, 2011). Thus, we hypothesized that endocannabinoid signaling would participate in 3:6 ratio-dependent changes in fear memory. To test this hypothesis, we subcutaneously administered RIM (3 mg/kg), a blocker of CB_1 receptors (Rinaldi-Carmona *et al*, 1994), to mice 60 min before the exposure test in fear conditioning. RIM abolished the fear-attenuating effect of the 5% krill diet, as revealed by comparison of the 5% krill + VEH and 5% krill + RIM groups ($F(1,36) = 3.45$, $P < 0.05$ for diet, $F(1,36) = 6.95$, $P < 0.05$ for drug, and $F(1,36) = 12.14$, $P < 0.01$ for interaction with two-way ANOVA; $P < 0.01$ with *post hoc* Bonferroni comparison; $n = 10$ for all four groups; Figure 3a). The time course of the freezing rate is shown in Figure 3b. The effect of RIM on fear memory was seen only in mice fed the 5% krill diet, whereas there was no difference between the freezing rates of VEH- and RIM-injected mice fed the control 1 diet. This suggests that an elevated 3:6 ratio upregulates the endocannabinoid system.

RIM, however, is thought to have multiple sites of action in the central nervous system (CNS). In particular, a recent report demonstrated that RIM also acts on μ-opioid receptors at a concentration comparable to that effective at the CB_1 receptor (Zádor *et al*, 2012). To determine whether the μ-opioid receptor is involved in the fear-attenuating effect of the 5% krill diet, we administered the μ-opioid antagonist NLX (3 mg/kg, subcutaneously). NLX failed to abolish the fear response attenuation in 5% krill diet-fed mice when administered 60 min before the exposure test (comparison of 5% krill + VEH and 5% krill + NLX; NLX had no effect on mice fed the control 1 diet; $F(1,36) = 18.25$, $P < 0.001$ for diet, $F(1,36) = 0.49$, $P > 0.05$ for drug, $F(1,36) = 0.11$, $P > 0.05$ for interaction with two-way ANOVA; $n = 10$ for all four groups; Figure 3c). Figure 3d shows the time course of the freezing rate in Figure 3c. These results indicate that the CB_1 receptor, and not the μ-opioid receptor, is involved in the effect of RIM on the behavior of mice fed the 5% krill diet.

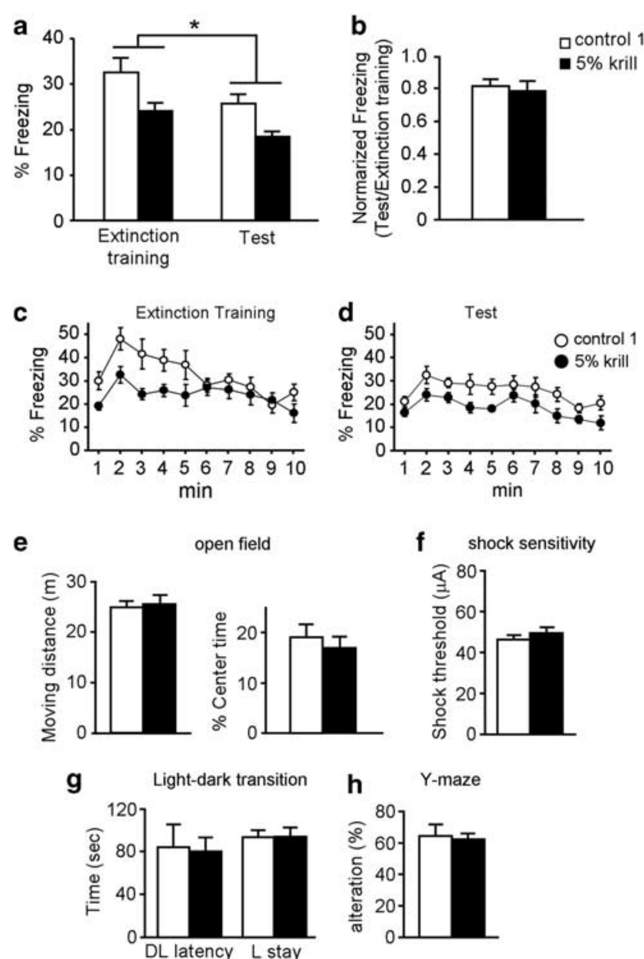

Figure 2 (a–d) Extinction of contextual fear memory. (a) Freezing rates during 10 min extinction training and test sessions (24 and 48 h after conditioning, $n = 10$ per group). (b) Freezing rate of each animal in the test session of (a) was normalized to its freezing rate in extinction training. (c and d) Time course of the freezing rate during extinction training (c) and test (d). (e–h) Mouse behavior in the open-field test (e; $n = 12$), shock-sensitivity test (f; $n = 8$), light–dark transition test (g; $n = 8$), and Y-maze test (h; $n = 10$). All data are shown as means ± SEM. $^*P < 0.05$, one-way analysis of variance (ANOVA) and *post hoc* Bonferroni comparison.

mRNA Levels

We compared CB_1 receptor mRNA levels in the brain (the remaining tissue after removal of the basal ganglia and hippocampus from the cerebrum) of mice fed the control 1

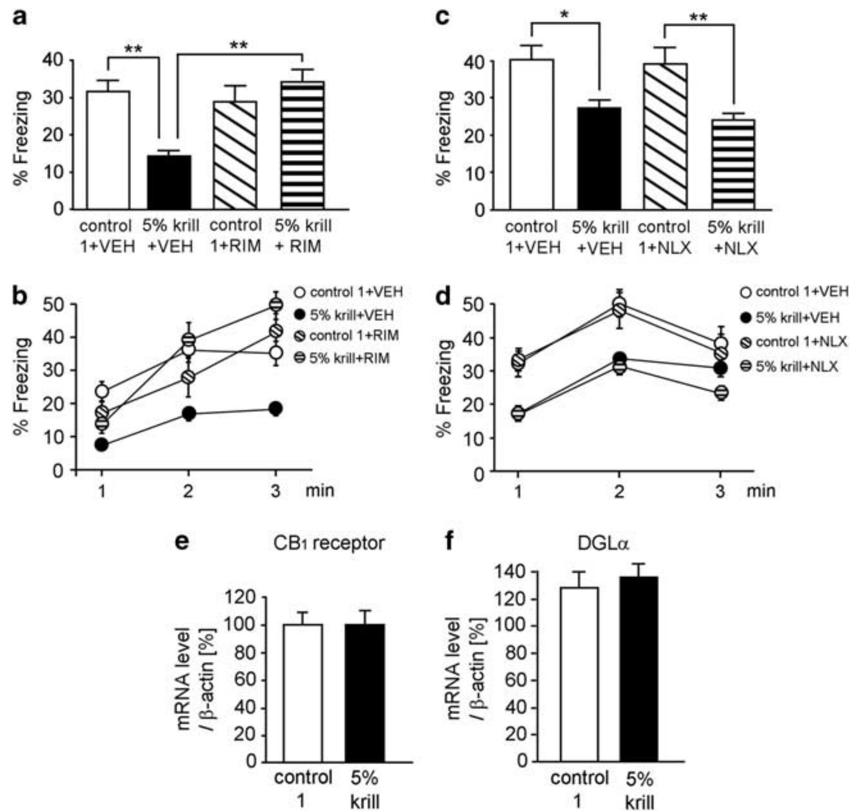

Figure 3 Involvement of type I cannabinoid (CB_1) receptors in the fear-attenuating effect of the high 3:6 ratio diet. (a) Administration of the CB_1 receptor antagonist rimonabant (RIM, 3 mg/kg) 60 min before the test abolished the effect of the 5% krill diet on fear memory ($n = 10$ per group). (b) Time course of the freezing rate during the test session in (a). (c) Administration of the μ-opioid receptor antagonist naloxone (NLX, 3 mg/kg) 60 min before the test did not change the effect of the 5% krill diet on fear memory ($n = 10$ per group). (d) Time course of the freezing rate during the test session in (c). (e and f) Quantitative polymerase chain reaction (PCR) for CB_1 receptor (e) and diacylglycerol lipase α (DGLα; f) analyses of brain extracts from mice fed the control 1 and 5% krill diets ($n = 6$ per group). VEH, vehicle. All data are shown as means ± SEM.*$P < 0.05$, **$P < 0.01$, two-way analysis of variance (ANOVA) and *post hoc* Bonferroni comparison.

and 5% krill diets ($n = 6$ per group) using quantitative PCR (Figure 3e), but no difference in expression level was detected ($P > 0.05$ with t-test). We also analyzed mRNA levels of diacylglycerol lipase α, a key enzyme in the biosynthesis of endocannabinoid 2-arachidonoylglycerol (Kano *et al*, 2009), but there was no significant difference in levels between the mice fed the control 1 and 5% krill diets ($P > 0.05$ with t-test; $n = 6$ per group; Figure 3f).

Agonist Sensitivity of CB_1 Receptors in the BLA

Next, we examined the function of CB_1 receptors in mice fed the 5% krill diet compared with that in control 1 diet-fed mice. First, to examine the enhancement of CB_1 receptor activity, we compared the effect of the CB_1 agonist WIN (0.1, 0.3, 1.0, 3.0, and 10 μM) on the amplitude of evoked excitatory postsynaptic currents (eEPSCs) in BLA pyramidal neurons between mice fed 5% krill and control 1 diets. It is known that the eEPSCs are inhibited by WIN (Yoshida *et al*, 2011). The importance of the BLA in contextual fear conditioning is well established (LeDoux, 2000). The dose–response curve for WIN was shifted to the left in mice fed the 5% krill diet compared with those fed the control 1 diet (Figures 4a and b), suggesting that CB_1 receptor activity was enhanced in mice fed the 5% krill diet.

CB_1 Receptor Activity and Cholesterol Expulsion in the BLA

To identify the mechanism underlying aforementioned enhancement of CB_1 receptor activity, we applied MCD, a drug that excludes membrane cholesterol (Bari *et al*, 2005a; Maccarone et al, 2009). Treatment of slices with MCD (5 mM) resulted in significantly greater suppression of eEPSC amplitude in control 1 diet-fed mice, but not in mice fed the 5% krill diet (Figures 4a and b). These results suggest that cholesterol is involved in the mechanism of action of the 5% krill diet.

Facilitation of Depolarization-Induced Suppression of Excitation

In the CNS, the CB_1 receptor is involved in depolarization-induced suppression of excitation (DSE) (Ohno-Shosaku *et al*, 2002). We examined DSE to confirm enhancement of CB_1 receptor activity. For this purpose, we recorded eEPSCs from BLA pyramidal neurons in brain slices (12 cells from five mice fed the control 1 diet and 11 cells from five mice fed the 5% krill diet). The eEPSCs were more strongly suppressed after 10 s of depolarization in mice fed the 5% krill diet than in those fed the control 1 diet (Figure 4c; the lower graph shows the time course of DSE). The DSE in the

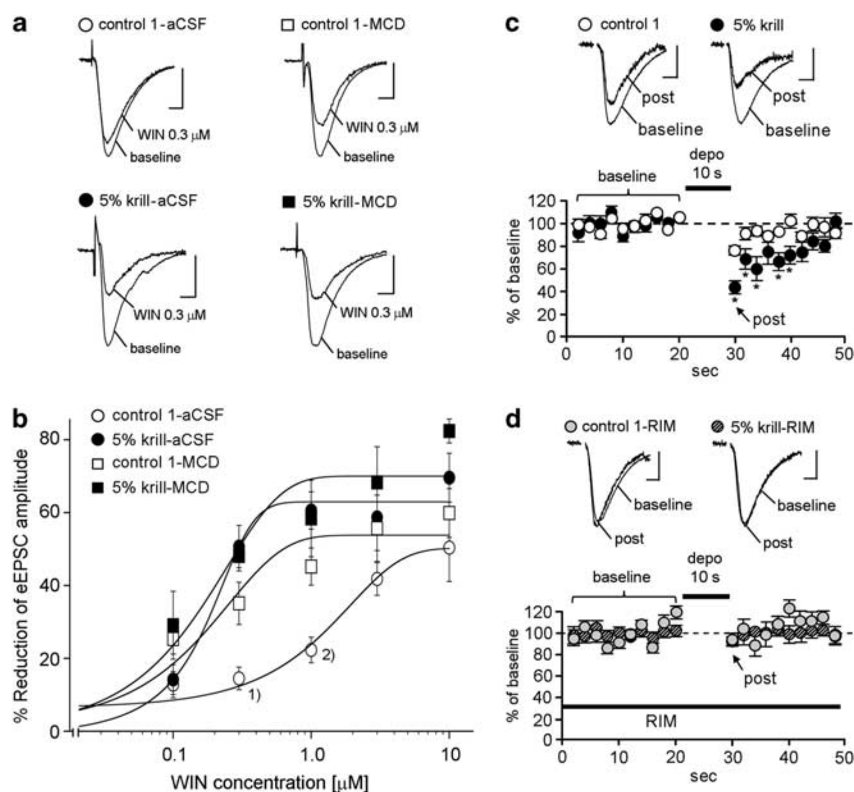

Figure 4 Electrophysiological analysis of type I cannabinoid (CB_1) receptors. (a) Representative recordings in individual neurons before (baseline) and after the application of 0.3 μM WIN55,212-2 (WIN). (b) Reduction of evoked excitatory postsynaptic current (eEPSC) amplitude by application of various concentrations of WIN (0.1, 0.3, 1.0, 3.0, and 10 μM; $n = 4$–6). Data were fitted using sigmoidal function (eight cells from seven mice for control-artificial cerebrospinal fluid (aCSF); 10 cells from seven mice for control-methyl-β-cyclodextrin (MCD); 10 cells from six mice for 5% krill-aCSF, and eight cells from six mice for 5% krill-MCD). Note that the dose–response curve for WIN was shifted to the left in mice fed 5% krill diet ('5% krill-aCSF', half-maximal effective concentration (EC_{50}) = 0.15 ± 0.08 μM) compared with mice fed control 1 diet ('control 1-aCSF', EC_{50} = 1.52 ± 0.19 μM), and that MCD treatment resulted in a leftward shift of the curve in mice fed control 1 diet ('control 1-MCD', EC_{50} = 0.23 ± 0.05 μM) but not in mice fed 5% krill diet ('5% krill-MCD', EC_{50} = 0.21 ± 0.09 μM). (c) Lower graph, time course of depolarization-induced suppression of excitation (DSE) in the basolateral nucleus of the amygdala (BLA) of mice fed the control 1 and 5% krill diets (12 cells from five mice fed the control 1 diet; 11 cells from five mice fed the 5% krill diet); above, traces show representative recordings in individual neurons. (d) Lower graph, rimonabant (RIM, 5 μM) abolishes the effect of the 5% krill diet on DSE (seven cells from four mice fed the control 1 diet; six cells from three mice fed the 5% krill diet); above, traces show representative recordings in individual neurons. Scale: 5 ms and 50 pA. *$P < 0.05$, one-way analysis of variance (ANOVA) and *post hoc* Bonferroni comparison. [1)]$P < 0.05$ between control-aCSF and control-MCD, $P < 0.001$ between control-aCSF and 5% krill-aCSF; [2)]$P < 0.05$ between control-aCSF and control-MCD, $P < 0.01$ between control-aCSF and 5% krill-aCSF with one-way ANOVA and *post hoc* Bonferroni comparison. All experiments in Figure 4 were carried out in the mice to which no behavioral experiments were conducted.

control 1 diet group was weaker than the DSE previously reported in the BLA (Yoshida *et al*, 2011). The DSE was abolished by preincubation of the slices (control 1, $n = 7$; 5% krill diet, $n = 6$) with RIM (5 μM; Figure 4d), indicating that the investigated DSE was almost completely dependent on CB_1 receptors. These results suggest that CB_1 receptor-mediated short-term synaptic plasticity is increased in the BLA of mice fed the 5% krill diet.

DISCUSSION

In the present study, we found that the ratio of n-3 to n-6 PUFA is a factor regulating contextual fear memory via cannabinoid CB_1 receptors. Previous reports suggest characteristic concentration-dependent actions of WIN (intraperitoneal injection) upon extinction of contextual fear memory (Pamplona *et al*, 2006). WIN enhances extinction of contextual fear memory in a low concentration (0.25 mg/kg), but higher concentrations, for example, 2.5 mg/kg, show no facilitation of extinction (Pamplona *et al*, 2006). In a similar experiment by the same authors on the other hand, a high concentration of WIN applied before conditioning suppresses the expression of contextual fear memory itself (Pamplona and Takahashi, 2006). Thus, the selective behavioral effects of 5% krill diet upon contextual fear memory are similar to those previously reported for high concentrations of WIN: extinction remains unchanged but the expression of fear memory itself is suppressed. In our electrophysiological experiments, we confirmed higher sensitivity to a CB_1 receptor agonist in BLA neurons of mice fed 5% krill diet compared with controls. We therefore hypothesize that the effects of 5% krill diet on fear memory are propagated by this increased sensitivity of CB_1 receptors.

Because the BLA has an essential role in contextual fear conditioning (LeDoux, 2000), this brain region can be regarded as one of the candidates mediating the effects of 5% krill diet. However, the possibility of a contribution of other regions such as the hippocampus, PAG (Resstel *et al*,

2008), and vmPFC (Lisboa *et al*, 2010) to the observed effects should be examined in a future study. In addition, because this memory is essential for individuals' survival, it seems that a warning is necessary in excessive reduction of this memory. Furthermore, our analysis of basal anxiety was conducted by the light–dark transition test alone. In mice fed 5% krill diet, it could be that basal anxiety levels may be changed in other behavioral tests, such as the elevated plus-maze test. Further experiments should be carried out to elucidate basal anxiety levels in mice fed 5% krill diet.

The mechanism linking PUFAs to CB_1 receptors may be explained by enhancement of CB_1 receptor activity in the plasma membrane, in which PUFAs are incorporated as the acyl groups in phospholipids. It is known that uptake of DHA into phospholipids results in the exclusion of cholesterol from the rafts in the membrane (Stillwell *et al*, 2005; Stillwell and Wassall, 2003). Other studies have suggested that the CB_1 receptor is distributed in lipid rafts in the plasma membrane of cultured human breast cancer cells (Sarnataro *et al*, 2005) and C6 glioma cells (Bari *et al*, 2008), and that the CB_1 receptor has a cholesterol-binding domain that suppresses receptor activity upon binding of cholesterol (Oddi *et al*, 2011). Therefore, the exclusion of cholesterol by DHA from the raft would enhance CB_1 receptor activity, as the suppressive effect exerted by cholesterol on the CB_1 receptor is reduced or even abolished (Bari *et al*, 2005a, b). Our electrophysiological findings on WIN-sensitivity and MCD action are in agreement with these biophysical and biochemical findings, although we cannot yet rule out the possibility that high 3:6 ratio diets alter levels of endocannabinoids in brain regions relevant to fear memory.

On the basis of previous findings and our results, we propose that the following molecular mechanism underlies the effects of a high 3:6 ratio diet on fear memory: (i) ingestion of a high 3:6 ratio diet increases DHA in neuronal plasma membranes in the brain; (ii) this excludes cholesterol from rafts in the plasma membrane; and (iii) this, in turn, facilitates the activity of CB_1 receptors and alters the synaptic plasticity that is important for fear memory retrieval.

The lower solubility of cholesterol in the DHA-rich membrane is considered to be due to expansion of the space between acyl groups in the membrane with high levels of unsaturation (Wassall and Stillwell, 2008). The expansion of this space would be more prominent in DHA (with six double bonds) than LA (two double bonds) or AA (four double bonds) (Shaikh *et al*, 2006). This could explain why LA suppresses the action of DHA when a high 3:6 ratio diet supplemented with LA is consumed (AA is a major n-6 PUFA in the brain and is synthesized from LA). However, this does not rule out the possibility that n-6 PUFA compensates for the loss of n-3 PUFA when n-3 is deficient.

To summarize, the primary aim of the present study was to evaluate whether dietary 3:6 ratio influences fear memory. Indeed, dietary 3:6 ratio influenced contextual fear memory in mice, and the effects were abolished by a CB_1 receptor antagonist. Consistent with this observation, the activity of CB_1 receptors, as measured by WIN-sensitivity, was enhanced in the BLA of mice fed a high 3:6 ratio diet. In these mice, DSE was also enhanced in the BLA. We suggest that cholesterol is involved in one of the possible mechanisms underlying the observed enhancement of CB_1 activity. Our findings provide biological evidence that PUFA influences fear memory in fear conditioning.

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