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N-3 PUFA improved pup separation-induced postpartum depression *via* serotonergic pathway regulated by miRNA☆

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Abstract

Stress and ovarian hormone fluctuation are risk factors for postpartum depression (PPD). Previous studies suggested antidepressant-like effects of n-3 polyunsaturated fatty acids (PUFA), but their effect on dam animal with additional stress were not clear. The purpose of the present study was to investigate the hypothesis that n-3 PUFA improved PPD through the serotonergic and glutamatergic pathways by modulating miRNA. Rats were fed n-3 PUFA or control diet from gestation, with pup separation (PS) on postpartum days 2–14 and non-PS controls. N-3 PUFA reversed PS-induced depressive behaviors, including increased immobility, latencies to contact first pup and retrieve all pups, and decreased sucrose preference. N-3 PUFA also modulated the hypothalamic–pituitary–adrenal (HPA) axis by decreasing circulating levels of adrenocorticotropic hormone and corticosterone and expression of hypothalamic corticotrophin releasing factor and hippocampal miRNA-218 but increasing the hippocampal expression of glucocorticoid receptor. N-3 PUFA inhibited neuroinflammation by decreasing circulating levels of prostaglandin E_2 and hippocampal expression of tumor necrosis factor-α, interleukin-6, and miRNA-155. In addition, n-3 PUFA up-regulated the serotonergic pathway by increasing circulating levels of serotonin and hippocampal expression of serotonin-1A receptor, cAMP response element binding protein (CREB), pCREB, brain-derived neurotrophic factor, and miRNA-182 but did not affect the glutamatergic pathway according to the hippocampal expression of N-methyl-D-aspartate receptor-2B. The present study suggested that n-3 PUFA improved PPD through the serotonergic pathway by modifying the HPA axis, neuroinflammation, and related miRNAs.

Keywords: MiRNA; N-3 PUFA; NMDA receptor; Postpartum depression; Rats; Serotonin

1. Introduction

Epidemiologic study showed the negative association between consumption of seafood and the prevalence of postpartum depression (PPD) [1]. Clinical studies also reported that supplementation with n-3 polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA; 20:5n3) and docosahexaenoic acid (DHA; 22:6n3), improved depression in postpartum women [2].

Stress and fluctuations in ovarian hormones have been shown to induce PPD through disruptions of the hypothalamic–pituitary–adrenal (HPA) axis, including corticotrophin releasing factor (CRF), corticosterone, and glucocorticoid receptor (GR) [3]. Impairment of the GR inhibits the cAMP response element binding protein (CREB)/brain-derived neurotrophic factor (BDNF) pathway and increases neuroinflammation, which affects serotonergic and glutamatergic neurotransmission [4].

Previously, circulating level of corticosterone was increased by n-3 PUFA-deficient diet in non-stressed male rats [5], but decreased by supplementation with n-3 PUFA in a rat model of PPD induced by hormone simulated pregnancy [6]. Furthermore, previous studies observed that activity of the HPA axis and serotonergic neurotransmission deteriorated more in PPD dam rats with pup separation (PS) than without PS, suggesting that the effects of n-3 PUFA could be greater in PPD dams with PS [7,8]. In addition, our previous studies showed that supplementation with n-3 PUFA modulated circulating levels of serotonin and hippocampal expression of CREB, BDNF, TNF-α, and interleukin-6 (IL-6) in depressed rats under acute stress induced by a forced swimming test (FST) [9–11]. As compared with acute stress, activity of the HPA axis, neuroinflammation, and CREB/BDNF-serotonergic neurotransmission were more aggravated in rodents with chronic stress [12,13]. However, there have been no studies that investigated the antidepressive effects and underlying mechanisms of

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n-3 PUFA in PPD dam animal with PS, which induces a combination of chronic stress and fluctuations in ovarian hormones.

Although the associations between miRNA and PPD have not been studied, miRNA expression was altered in depressed rodents with acute or chronic stress [14]. Previous studies suggested that the GR, CREB, and BDNF are regulated by miRNA-218, −132, −182, and−155 in depressed humans [15,16] and rodents [17,18]. Treatment with EPA increased miRNA-155 expression in human macrocytes [19], and treatment with EPA and DHA lowered miRNA-17 expression in rabbitfish hepatocytes [20], but no studies have shown an association between treatment with n-3 PUFA and miRNAs related with depression. Therefore, the purpose of the present study was to investigate the hypotheses that n-3 PUFA have anti-depressive effects in a rat model of PPD with PS, and if they do, that the mechanism involves effects on serotonergic and glutamatergic neurotransmission, *via* regulating the HPA axis through miRNA modulation.

2. Materials and methods

2.1. Animals and diet

The experimental protocol was approved by the Institutional Animal Care and Use Committee of Hanyang University (HY-IACUC-18-0030). Ten-week-old female and 14-week-old male Wistar rats (Orient Bio Inc., Seongnam, Korea) were housed individually in a ventilated air-conditioned room maintained at 22±1°C with a 12 h light–dark cycle and 40–50% humidity. Diet consisted of isocaloric modified American Institute of Nutrition-93G diets with either 0 energy percent (en%) of n-3 PUFA (control diet) or 1 en% of n-3 PUFA (N-3 PUFA diet) **(Supplemental Table S1)**. The control and n-3 PUFA diet were made up of grape seed oil (Sajo Haepyo, Seoul, Korea) containing 0 g or 8.1 g of fish oil (Cenovis Health Company, Turrella, Australia), respectively. In total, the control diet had 0.07% of n-3 PUFA, and the n-3 PUFA diet had 5.09% of EPA (0.8 en%) and 3.25% of DHA (0.5 en%) in total fatty acids.

2.2. Experimental design

After a week of acclimatization, the phase of the estrous cycle was identified in each female rat for 1 week by vaginal cytology, as previously described [21] **(**Fig. 1**)**. Thirty-four female rats were individually mated with male rats for 5–6 consecutive days in order to include at least two estrous days. After mating, the female rats were separated and randomly assigned to the control diet or n-3 PUFA diet for 6 weeks (n=17 per diet). The day after mating was considered the first day of gestation, and the day of parturition was assigned as postpartum day 0. One rat from each group had not given birth by the fifth week of gestation and was evaluated as not pregnant and euthanized. Rats after birth (dams) were randomly assigned to the non-PS (n=16) or PS (n=16) groups on postpartum day 2–14. There were four total groups (n=8 per group): non-PS with control diet (Con) and with n-3 PUFA diet (N-3); PS with control diet (Con+PS) and with n-3 PUFA diet (N-3 + PS).

2.3. Pup separation

As previously described [22], the procedure for PS was performed for 180 min (9:30–12:30) on the PS group, and for 15 min (9:30–9:45) on the non-PS group during postpartum day 2 through 14. The dams were first removed from the nest to a separate new cage, and the pups remained in their original cage were transferred to a room adjacent to the colony, and lined with bedding material and placed atop a heating pad (Ilwoul, Gwangju, Korea) maintained at a temperature of 32±1°C. After separation, the dams were returned to the home cage. All dams and pups were left undisturbed until weaning on postpartum day 20.

2.4. Pup retrieval test

Retrieval behavior was measured to assess maternal behavior of care for the pups in PPD animal with PS [23]. On postpartum day 6, five pups were spread throughout the cage during their regular separation, using a previously described protocol with slight modifications [7]. After separation, dams were returned to their pups and observed to measure time to contact the first pup and latency to return all pups to the nest.

2.5. Sucrose preference test

Anhedonia, a core symptom of depression, was assessed using a standard two-bottle choice procedure to measure voluntary consumption of sucrose solution, as previously described [24]. On postpartum day 20, the standard age of weaning, rats were

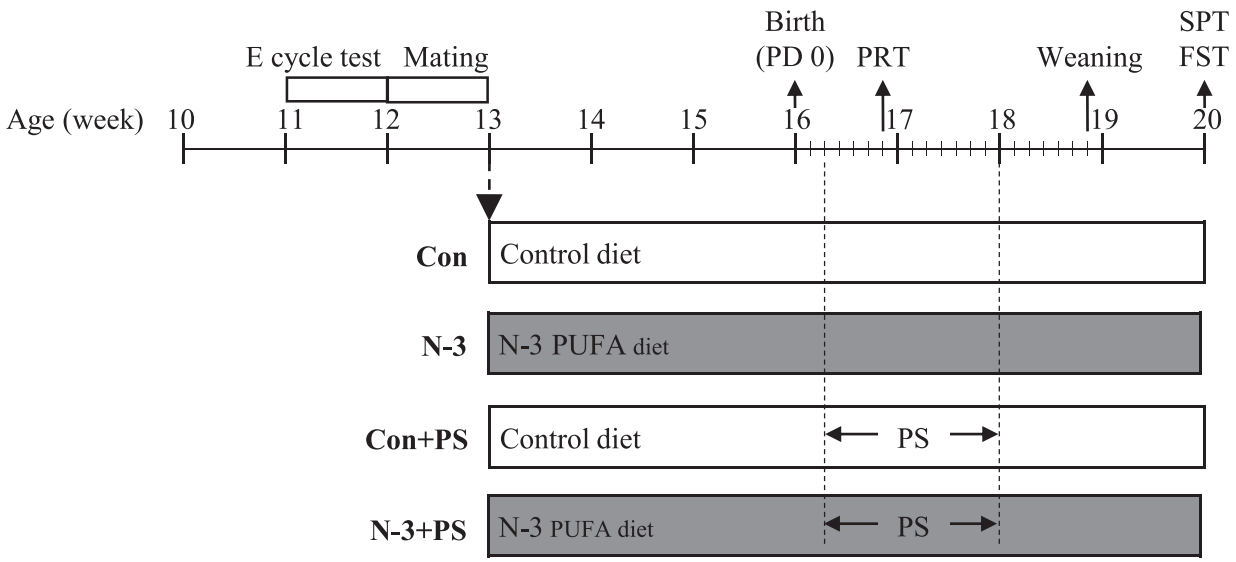

Fig. 1. Schematic representation of the experimental design and groups. Con and N-3, 0% and 1% n-3 PUFA without pup separation (PS); Con+PS and N-3 + PS, 0% and 1% n-3 PUFA with PS; E, estrous; PD, postpartum day; PRT, pup retrieval test; SPT, sucrose preference test; FST, forced swimming test.

individually housed after separation from pups, and were acclimated to two bottles containing tap water and 1% sucrose solution for 4 days. Positions of the bottles within each cage were switched daily to avoid side bias. On postpartum day 25, rats were given a choice of tap water and sucrose solution during 12 h after 10 h of food/water deprivation. The percentage of sucrose intake to total fluid intake was expressed as the sucrose preference index (%) calculated as ([grams of sucrose solution consumption]/[grams of sucrose solution consumption + tap water consumption]) × 100.

2.6. Forced swimming test

Rats underwent a FST as previously described to measure depression-related behavior [25]. Briefly, rats were individually placed inside a cylinder (height 50 cm, diameter 20 cm) containing 25±1°C water up to a height of 30 cm. During the pre-test, animals were forced to swim for 15 min, and 24 h later, each animal was re-exposed to a FST for 5 min. The test was videotaped with a camera positioned at the side of the cylinder, and the duration of immobility, swimming, and climbing were measured by three raters who were unaware of the treatment conditions.

2.7. Tissue and blood collection

Serum and plasma were obtained by using SST and EDTA blood tubes, respectively, after centrifugation at 3000 rpm for 10 min. Dissected organs were rinsed with saline, weighed, and frozen in liquid nitrogen. Blood and tissue samples were stored at−80°C for further analysis.

2.8. Gas chromatography

Brain fatty acid content was measured as previously described [26]. Brain tissue (100 mg) was homogenized with 5 mL of chloroform:methanol:distilled water (2:2:1 v/v). The homogenates were centrifuged at 4000 rpm (Hanil Science Inc., Gimpo, Korea) for 10 min, and the bottom layer was redissolved in *n*-hexane and separated by thin layer chromatography (SIL G-25; Macherey-nagel GmbH & Co, Duren, Germany) in *n*-hexane:diethyl ether:acetic acid (40:10:1, v/v). After developing the TLC plate, phospholipids were collected, and brain phospholipids were directly methylated by adding boron trifluoride methanol (B1252; Sigma-Aldrich, St. Louis, MO, USA) and heated at 100°C for either 10 min. Fatty acid methyl esters from brain phospholipids were analyzed using gas chromatography (Shimadzu 2010AF; Shimadzu Scientific Instrument, Tokyo, Japan) with a 100 m×0.25 mm inner diameter and 0.20 μm film capillary column (SP2560; Supelco, Bellefonte, PA, USA). Hydrogen was used as the carrier gas at a flow rate of 40 mL/min. The injection and detection temperatures were 230°C and 240°C, respectively. The run temperature began at 180°C, then was increased by 5°C/min up to 200°C, and by 10°C/min to 240°C. The split ratio was 10:1. Gas chromatography was calibrated using an external standard composed of defined fatty acids (GLC-OQA; Nu-Check Prep, Elysian, MN, USA). Qualified GC evaluation was carried out by measuring the coefficient of variation of the sum of EPA and DHA composition in the quality control material; the coefficient of variation was 2.8%.

2.9. Measurement of hormone levels

Serum levels of serotonin (IBL, Hamburg, Germany) and plasma levels of corticosterone (Enzo Life Science, Farmingdale, NY, USA) and adrenocorticotropic hormone (ACTH; Phoenix Pharmaceuticals, Belmont, CA, USA) were measured using an ELISA kit according to the manufacturer's instructions. All measurements were performed in duplicate and quantified using standards in a spectrophotometer (Multiscan GO, Thermo Scientific, Waltham, MA, USA).

2.10. Measurement of prostaglandin E_2

After homogenization of brain tissues in homogenization buffer at pH 7.4, the supernatant was purified using polypropylene mini-columns C-18 (Waters Corp. Milford, MA, USA). The concentration of PGE_2 in homogenized brain tissue was determined by enzyme immunoassay (Abcam, Cambridge, UK) according to the manufacturer's protocol. Measurements were quantified using the spectrophotometer standard (Multiscan GO, Thermo Scientific, Waltham, MA, USA).

2.11. Western blot analysis

Hypothalamic and hippocampal tissues were homogenized in an ice-cold radio immunoprecipitation buffer containing Complete EDTA-free protease and PhosSTOP inhibitor cocktails (Roche Diagnostics GmbH, Mannheim, Germany). The homogenates were centrifuged at 10,000 g for 15 min at 4°C, and a bicinchoninic acid assay (Pierce Biotechnology, Rockford, IL, USA) was utilized to quantify protein content in the supernatant. Protein (30 μg) was separated by 12% SDS-PAGE, transferred to polyvinylidene fluoride membranes (0.45 μm, Merck Millipore, Billerica, MA, USA), and blocked for 1 h at room temperature with 5% skim milk or 3–5% bovine serum albumin in Tris-buffered saline with 0.1% Tween 20 (TBST). The membrane was then incubated with primary antibody against the GR (1:500), CRF (1:2000), CREB (1:1000), phosphorylated CREB (pCREB; 1:1000), BDNF (1:2000), TNF-α (1:500), IL-6 (1:250), serotonin 1A receptor (5-HT_{1A}R; 1:5000), or N-methyl-D-aspartate receptor 2B subtype (NMDAR-2B; 1:5000) with 5% skim milk in TBST overnight at 4°C. Antibodies against CREB and pCREB were purchased from Cell signaling (New England Biolabs, Beverly, MA, USA), and antibodies against the GR, CRF, TNF-α, IL-6, BDNF, 5-HT_{1A}R, and NMDAR-2B were purchased from Abcam (Cambridge, UK). After washing in TBST, membranes were incubated with horseradish-peroxidase-conjugated secondary antibody, either anti-rabbit IgG (Cell signaling, New England Biolabs, Beverly, MA, USA) or anti-mouse IgG (Enzo Life Science, Farmingdale, NY, USA), with 5% skim milk in TBST for 1 h at room temperature. Immunoreactive bands were visualized with the UV setting on the Chemidoc MP Imaging System (Bio-rad, Hercules, CA, USA) to estimate total protein per lane, and β-actin was used for normalization. The hypothalamus was used to analyze CRF expression, while the hippocampus was used for all other proteins. Each Western blot was performed in triplicate.

2.12. Immunofluorescence staining

Rats were anesthetized and perfused through the aorta with saline followed by 4% paraformaldehyde. Brains were extracted and placed in the same fixative at 4°C overnight. After fixation, tissues were dehydrated in using an alcohol gradient, cleared with xylene, then embedded in paraffin. Tissues were cut into 10 μm sections and mounted on glass slides. The sections were immersed in xylene followed by alcohol and rehydrated in wash buffer. Antigen retrieval was performed by heating the sections in sodium citrate buffer (0.01 M, pH 6.0). To block endogenous peroxidase activity, DAKO peroxidase block solution (DAKO, Produktionsvej, Denmark) was applied and anti-GR antibody (1:30, Abcam, Cambridge, UK) was added and incubated at 4°C overnight. The sections were incubated for 2 h at room temperature with goat anti-rabbit IgG conjugated with DyLight 488 (Abcam, Cambridge, UK) to detect the GR. After washing, the slides were mounted with fluoroshield containing 4′, 6-diamidino-2-phenylindole (DAPI; Abcam, Cambridge, UK). A Leica AF6000

fluorescent microscope and Leica AF imaging software (Leica Microsystems, Wetzlar, Germany) were used to evaluate immunofluorescence images of the CA1, CA2, CA3, and dentate gyrus regions of the hippocampus.

2.13. Quantitative real time-polymerase chain reaction (RT-PCR) of microRNA

Total RNA was extracted from the hippocampus using the NucleoSpin miRNA kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions. RNA concentration was determined using a micro-spectrohotometer (Nano-200, Hangzhou Allsheng Instruments, Hangzhou, China). Complementary DNA was synthesized from small RNA-enriched total RNA using the iScript Select cDNA synthesis kit (Bio-rad, Hercules, CA, USA) and gene-specific primers for miRNA-218 (Assay ID: 000521), miRNA-17 (Assay ID: 002308), miRNA-155 (Assay ID: 002571), miRNA-132 (Assay ID: 000457), miRNA-182 (Assay ID: 002599), and small nuclear RNA U6 (U6 snRNA; Assay ID: 001973) (Applied Biosystems, Carlsbad, CA, USA). Quantitative RT-PCR reactions for the detection of miRNA-218, −17, −155, −132, and−182, and U6 snRNA were performed using the Thunderbird Probe qPCR mix (Toyobo, Osaka, Japan) and CFX96 Real-Time Detection system instrument (Bio-rad, Hercules, CA, USA). The PCR conditions were as follows; 95°C for 60 s, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Each sample was analyzed in triplicate. The relative expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method using the U6 snRNA as an internal control.

2.14. Statistical analyses

Values are expressed as the mean and standard deviation, and differences were considered significant at $P<.05$. Data were analyzed using a two-way analysis of variance (ANOVA) for the factors of diet and PS, followed by a Least Significant Difference *post hoc* test. Analyses were done using SPSS for Windows, version 24.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Fatty acid composition of brain phospholipids

There were no significant effects of PS and diet on body weight, or weights of the liver, kidney and hippocampus (**Supplemental Table S2**). Independent of PS, n-3 PUFA supplementation increased n-3 fatty acid content of brain phospholipids, including 20:5n3, 22:5n3, and 22:6n3, but decreased n-6 fatty acid content, including 18:2n6, 20:2n6, 20:3n6, 20:4n6, 22:4n6, and 22:5n6 (Table 1).

3.2. Animal behavior, circulating levels of hormones, and brain levels of prostaglandin E_2

Regardless of diet, PS increased duration of immobility during FST and latencies to contact the first pup and to retrieve all pups, while decreasing the duration of climbing during FST and sucrose preference (Table 2). Independent of PS, n-3 PUFA supplementation decreased duration of immobility during FST and latencies to contact the first pup and to retrieve all pups, while increasing the duration of climbing during FST and sucrose preference. Regardless of diet, PS increased plasma levels of corticosterone and ACTH and brain levels of PGE_2, but decreased the serum serotonin level (Table 2). Independent of PS, supplementation with n-3 PUFA decreased plasma levels of corticosterone and ACTH and brain levels of PGE_2, while increasing serum levels of serotonin.

3.3. The expression of regulators of the hypothalamic–pituitary–adrenal axis and serotonergic neurotransmitters, cytokines, and the N-methyl-D-aspartate receptor

Regardless of diet, PS decreased hippocampal expression of the GR, CREB, pCREB, BDNF, and 5-HT_{1A}R, but increased hypothalamic expression of CRF and hippocampal expression of TNF-α, IL-6, and NMDAR-2B (Fig. 2). Independent of PS, n-3 PUFA supplementation increased hippocampal expression of the GR, CREB, pCREB, BDNF, and 5-HT_{1A}R, but decreased hypothalamic expression of CRF and hippocampal expression of TNF-α and IL-6. However, n-3 PUFA supplementation had no significant effect on hippocampal expression of NMDAR-2B.

Table 1
The fatty acid composition of brain phospholipids[a]

Fatty acid (%)	Con	N-3	Con+PS	N-3 + PS	Diet	PS	Diet×PS
C16:0	22.72±0.81	22.95±1.10	22.24±0.60	23.09±1.39	0.145	0.641	0.387
C17:0	4.32±0.48	3.85±0.78	4.48±0.34	4.26±0.51	0.089	0.160	0.517
C18:0	21.15±0.58	21.26±0.53	20.91±0.23	21.35±0.42	0.103	0.669	0.326
SFA	48.95±0.82	48.86±0.75	48.44±0.41	49.43±1.11	0.125	0.931	0.071
C16:1n7	0.34±0.02	0.34±0.03	0.32±0.02	0.34±0.02	0.251	0.186	0.326
C18:1n9	21.28±1.07	21.67±1.45	22.08±0.64	21.14±1.82	0.563	0.768	0.166
C20:1n9	0.34±0.06	0.36±0.06	0.36±0.04	0.36±0.06	0.700	0.477	0.599
MUFA	22.16±1.17	22.56±1.55	22.98±0.64	22.01±1.93	0.570	0.795	0.179
C18:2n6	0.75±0.04	0.69±0.06*	0.75±0.04	0.65±0.08*	<0.001	0.341	0.333
C20:2n6	0.18±0.01	0.14±0.02*	0.19±0.02	0.12±0.02*	<0.001	0.548	0.083
C20:3n6	0.37±0.04	0.32±0.03*	0.37±0.04	0.33±0.03*	0.001	0.831	0.834
C20:4n6	9.29±0.48	8.38±0.57*	8.98±0.31	8.22±0.64*	<0.001	0.206	0.673
C22:4n6	3.36± 0.20	2.96±0.23*	3.33±0.24	2.96±0.31*	<0.001	0.856	0.831
C22:5n6	1.41± 0.20	0.30±0.04*	1.25±0.31	0.34±0.18*	<0.001	0.419	0.195
n-6 PUFA	15.38±0.74	12.79±0.78*	14.88±0.36	12.63±0.97*	<0.001	0.220	0.535
C20:5n3	0.31±0.08	0.52±0.15*	0.32±0.09	0.54±0.13*	<0.001	0.741	0.978
C22:5n3	0.08±0.01	0.23±0.02*	0.08±0.02	0.24±0.01*	<0.001	0.375	0.837
C22:6n3	12.91±0.65	14.70±0.95*	13.06±0.55	14.66±0.62*	<0.001	0.815	0.703
n-3 PUFA	13.31±0.59	15.48±0.86*	13.48±0.52	15.46±0.64*	<0.001	0.745	0.684

[a] Values are mean and standard deviation ($n=8$); Con and N-3, 0% and 1% n-3 PUFA without pup separation (PS); Con+PS and N-3 + PS, 0% and 1% n-3 PUFA with PS; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; Trans, trans fatty acids; H, highly unsaturated fatty acid.
* Values are significantly different between Con and N-3, with or without PS, respectively.

Table 2
Maternal and depressive behaviors, circulating levels of hormones and serotonin, and brain levels of prostaglandin E_2[a]

Parameter	Con	N-3	Con+PS	N-3 + PS	Diet	PS	Diet × PS
Immobility (s)	36.20±5.37	8.76±2.51*	63.65±30.68†	27.15±8.68*, †	<0.001	<0.001	0.436
Climbing (s)	60.28±8.30	84.78±17.58*	29.97±7.99†	55.05±11.40*, †	<0.001	<0.001	0.946
Swimming (s)	203.51±10.66	210.18±25.81	206.37±27.22	217.80±18.13	0.244	0.497	0.757
Sucrose preference index (%)	55.72±15.51	92.08±7.47*	31.59±21.39†	63.86±19.25*, †	<0.001	<0.001	0.733
Latency of the first contact (s)	13.46±1.99	7.97±2.64*	22.20±4.67†	14.40±2.94*, †	<0.001	<0.001	0.319
Latency to retrieve (s)	730.00±401.12	273.75±177.92*	1156.87±433.16†	663.75±427.55*, †	0.001	0.005	0.890
Corticosterone (nmol/L)	95.84±10.62	13.77±9.26*	131.89±64.70†	49.06±12.41*, †	0.001	0.006	0.975
ACTH (pmol/L)	36.54±4.54	26.33±5.78*	43.51±7.38†	32.29±3.52*, †	<0.001	0.002	0.798
Serotonin (pmol/L)	5.39±1.03	6.88±0.39*	4.45±1.10†	5.42±0.89*, †	0.001	0.001	0.414
PGE_2 (pmol/g tissue)	19.71±2.24	13.03±1.85*	26.15±3.99†	17.62±3.13*, †	<0.001	<0.001	0.380

[a] Values are mean and standard deviation (n=8); Con and N-3, 0% and 1% n-3 PUFA without pup separation (PS); Con+PS and N-3 + PS, 0% and 1% n-3 PUFA with PS; ACTH, adrenocorticotropic hormone; PGE_2, prostaglandin E_2.
* Values are significantly different between Con and N-3, with or without PS, respectively.
† Values are significantly different between PS and non-PS within the same dietary groups.

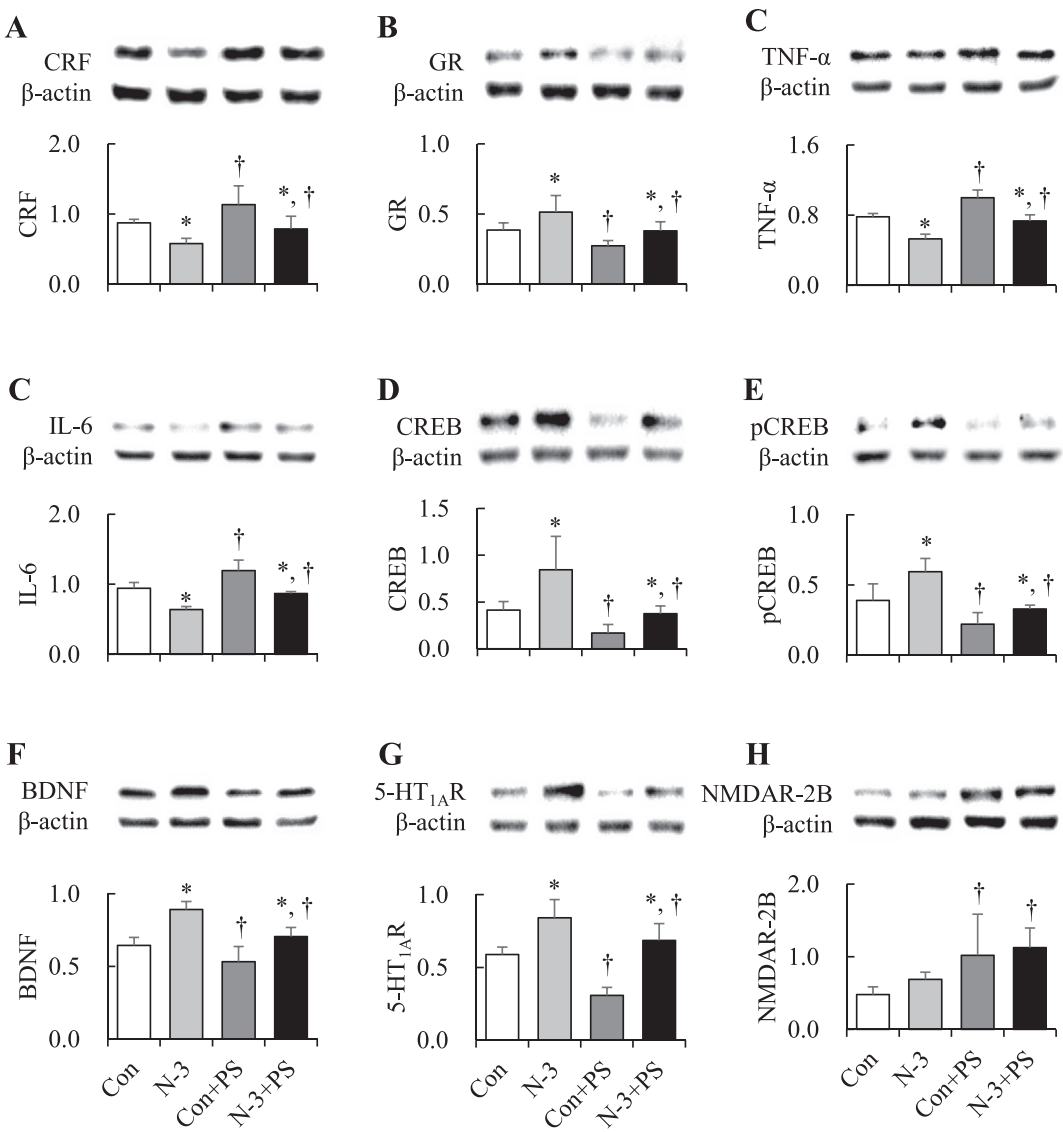

Fig. 2. Effect of n-3 PUFA on the expression of regulators of the hypothalamic–pituitary–adrenal axis, neuroinflammation, and serotonergic and glutamatergic neurotransmission. A: hypothalamic corticotropin releasing factor (CRF). B-H: hippocampal glucocorticoid receptor (GR), tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), cAMP response element binding protein (CREB), phosphorylated CREB (pCREB), brain derived neurotrophic factor (BDNF), serotonin 1A receptor (5-HT1AR), and N-methyl-D-aspartate receptor 2B subtype (NMDAR-2B). Values are mean and standard deviation (n=8); Con and N-3, 0% and 1% n-3 PUFA without pup separation (PS); Con+PS and N-3 + PS, 0% and 1% n-3 PUFA with PS; *Values are significantly different between Con and N-3, with or without PS, respectively; †Values are significantly different between PS and non-PS within the same dietary groups.

Consistent with these results, immunofluorescence staining showed that PS decreased, but n-3 PUFA increased, the expression of the GR in the CA1, CA2, CA3, and dentate gyrus regions of the hippocampus (Fig. 3).

3.4. Expression of microRNAs in the hippocampus

PS increased hippocampal expression of miRNA-218 and -132 but decreased miRNA-155 in all rats, fed with both control and n-3 PUFA diets (Fig. 4). However, PS increased hippocampal expression of miRNA-182 in rats fed the control diet only, confirming a significant PS × n-3 PUFA interaction. Supplementation with n-3 PUFA decreased hippocampal expression of miRNA-218 and -182, but increased miRNA-155. There was no significant effect of PS or diet on hippocampal expression of miRNA-17 (data not shown).

4. Discussion

The present study demonstrated first that n-3 PUFA supplementation can diminish the psychological detriment of PS on dams. Second, we found evidence to suggest that the mechanism of serotonergic neurotransmission *via* regulation of the HPA axis through modulation of miRNA-218, −182, and−155 (Fig. 5). To date, there have been no studies that investigated the effects of n-3 PUFA supplementation on depressive behavior in PPD animals with additional stress. Tang et al. [27] reported that oral administration of n-3 PUFA increased sucrose preference in rats with chronic mild stress (CMS), consistent with our study. Harauma et al. [28] also reported that a n-3 PUFA sufficient diet containing α-linolenic acid (ALA) increased brain levels of DHA and improved nursing behavior, a marker for maternal behavior in PPD dam mice, as compared with a n-3 PUFA deficient diet. However, Tang et al. [29,30] reported that supplementation with n-3 PUFA had no effect on sucrose preference in PPD dam rats, as compared to control diet containing ALA. Since rats can convert ALA into EPA and DHA [31], ALA in the control diet could have blunted the effect of n-3 PUFA on sucrose preference in the studies by Tang et al. [29,30].

Consistent with the present study, previous studies have reported that n-3 PUFA decreased the duration of immobility, called behavioral despair, in PPD dam rats [30], PPD rats with hormone simulated pregnancy [6], depressed rats with chronic restraint stress [32], CMS, [27] and acute stress [9–11]. In addition, previous studies showed that supplementation with n-3 PUFA increased the duration of climbing, but not swimming, in depressed rats with acute stress [9–11] or without stress [33]. However, Ferraz et al. [32] reported that oral administration of n-3 PUFA increased swimming, but not climbing, in depressed rats with chronic restraint stress. Climbing behavior is known to be affected by norepinephrine, the release of which is elevated by postsynaptic 5-HT_{1A}R in the hippocampus [34,35], while swimming is increased by inhibition of glutamatergic neurotransmission in depressed rats with severe CMS [36]. Previous studies also reported that n-3 PUFA increased serotonin level, but not glutamate level in PPD dam rats [29] or depressed rats with acute stress [11], suggesting that n-3 PUFA modulated climbing through up-regulating serotonin.

Furthermore, previous studies showed that n-3 PUFA had an antidepressive effect in both PPD and virgin rats [29,30], and in both depressed rats and normal rats [32]. A meta-analysis also reported that n-3 PUFA supplementation provided clinical benefit for both patients diagnosed with major depression and people with depressive symptoms [37]. Consist with this, the present study showed that the effect of n-3 PUFA was similar in PPD dam rats with and without PS, suggesting that the antidepressant-like effect of n-3 PUFA was independent of severity of depression. In the present study, 1 en% of n-3 PUFA was equivalent to 3 g/day in human [38], which was considered safe by the U.S. Food and Drug Administration [39], and effective to protect depression by the American Psychiatric Association [40] and the Canadian Network for Mood and Anxiety Treatments [41].

Depression is suggested to occur through dysregulation of the serotonergic and glutamatergic pathways [3]. Previous studies reported that n-3 PUFA increased serotonin levels in PPD dam mice [28], depressed mice with CMS [42], and depressed rats with acute stress [9–11]. It is known that serotonin levels are raised by BDNF though increased expression of tryptophan hydroxylase, a key enzyme for serotonin biosynthesis, in mice with a genetically determined depression-like state [43]. In depressed rats with acute stress [9–11] and rats without stress [44,45], n-3 PUFA consistently increased hippocampal levels of BDNF and CREB, a transcription factor contributing to BDNF up-regulation [46]. In addition, n-3 PUFA up-regulated hippocampal expression of 5-HT_{1A}R in rats without stress [44,45], which contributed to activation of CREB by increasing CREB phosphorylation [46]. On the other hand, Levant et al. [47] showed that increases in brain DHA level from ALA supplementation did not affect hippocampal levels of BDNF or serotonin, and decreased hippocampal density of 5-HT_{1A}R in PPD dam rats that underwent two sequential reproductive cycles (gestation and lactation). Levant et al. [47] also reported that hippocampal density of 5-HT_{1A}R was increased in PPD rats compared to virgin rats, but it has been shown that hippocampal expression and density of 5-HT_{1A}R were decreased in depressed rodents and patients [48]. Our previous study showed that ALA supplementation increased DHA levels in the brain (to a lesser extent than diets supplemented with EPA and DHA), but had no significant effect on the expression of CREB and BDNF, and level of serotonin [11], suggesting that conversion of ALA into EPA and DHA was not sufficient to up-regulate CREB/BDNF-serotonergic pathway in the study by Levant et al. [47]. It has also been shown that an elevated n-3/n-6 PUFA ratio in the neuronal membrane affects membrane fluidity, which in turn increased the density and binding affinity of 5-HT_{1A}R [49]. In the present study, n-3 PUFA was significantly increased, while n-6 PUFA was decreased, as a percent of brain phospholipids. Thus, the present study suggests that n-3 PUFA increased serotonin levels through the up-regulation of the 5-HT_{1A}R/CREB/BDNF pathway, which is consistent with previous studies.

Serotonin degradation has also been shown to be modulated by pro-inflammatory cytokines which activate the tryptophan-degrading enzyme indoleamine 2,3-dioxygenase [50]. Pro-inflammatory cytokines, mainly TNF-α and IL-6, were increased in depressed patients compared with healthy controls [51], and related with up-regulated synthesis of PGE_2 [52]. Previous studies showed that n-3 PUFA attenuated levels of TNF-α and IL-6 in PPD rats with hormone simulated pregnancy [6], PPD dam rats [29], and depressed rats with acute stress [9–11]. In addition, n-3 PUFA supplementation decreased brain levels of PGE_2 in rats with acute stress [10,11] and rats administered IL-1 [53], because n-3 PUFA compete with n-6 PUFA, such as 20:4n6, for eicosanoid synthesis [49]. Consistent with this, the present study reported that n-3 PUFA reduced hippocampal expression of TNF-α and IL-6 and brain levels of PGE_2. In addition, PGE_2 is known to increase the activity of the HPA axis through increasing hypothalamic release of CRF [54], and secretion of pituitary ACTH and adrenal corticosterone, which down-regulates the GR [3], a modulator of CREB phosphorylation and cytokine transcription [4]. N-3 PUFA decreased circulating levels of corticosterone in PPD rats with hormone simulated pregnancy [6], and depressed rodents with chronic restraint stress [32] and chronic social defeat stress [55], while increasing hippocampal expression of the GR in PPD dam rats [30]. In addition, DHA activated the GR through binding to the GR [56], suggesting that n-3 PUFA could regulate the GR expression not only through decreasing HPA axis activity, but also directly modulating the function of the GR. Consistent with the above, the present study showed that n-3 PUFA decreased hypothalamic expression of CRF, and circulating levels of ACTH and corticosterone, while increasing hippocampal expression of the GR.

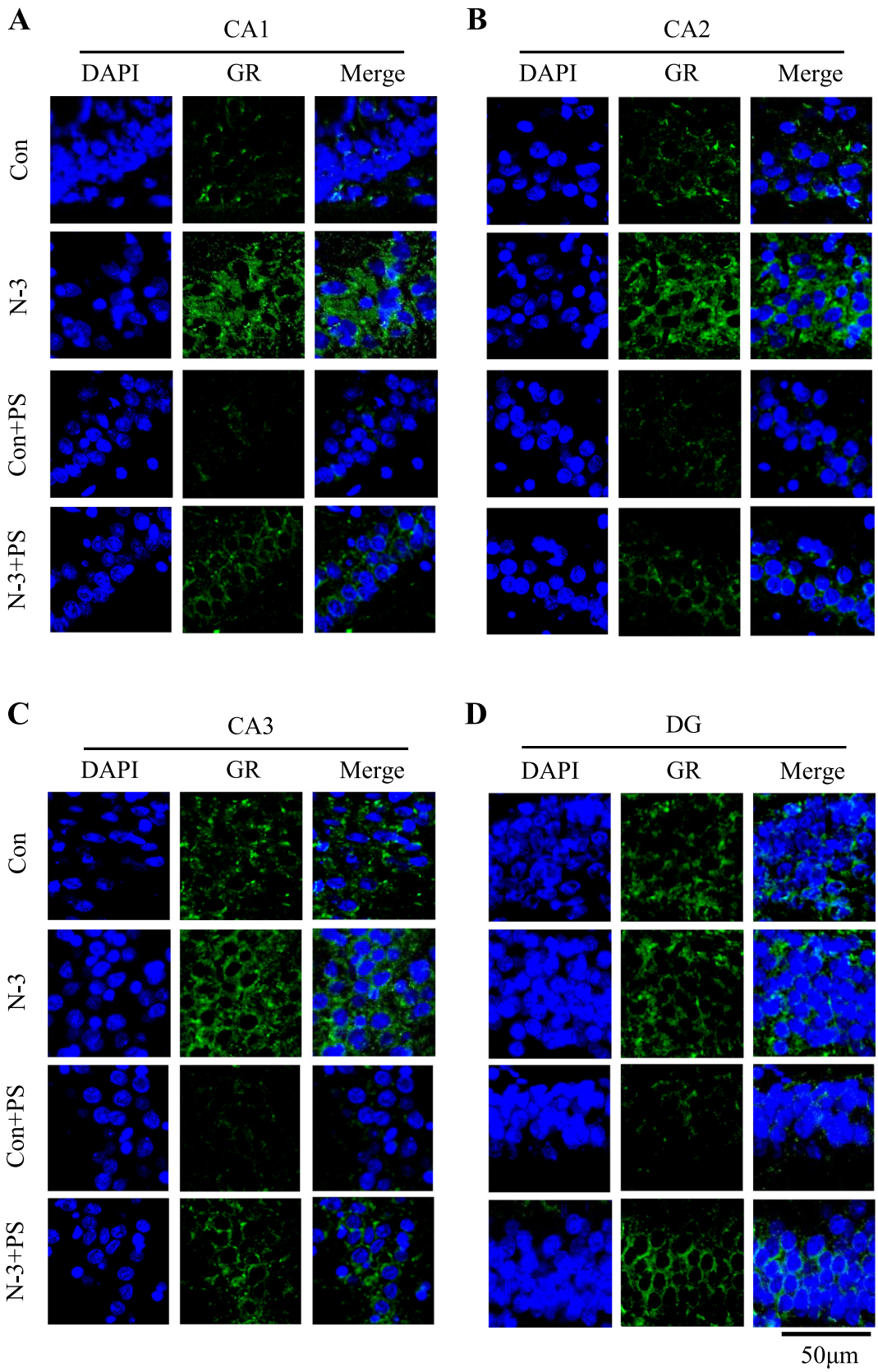

Fig. 3. Immunofluorescence staining of glucocorticoid receptor (GR) in the hippocampus of rats. A-D: CA1, CA2, CA3 and dentate gyrus (DG) regions. GR expression was visualized with DyLight 488 (green), and DNA was stained blue (DAPI). Each staining of GR and DAPI, and merged images are shown. Scale bars=50 μm. Con and N-3, 0% and 1% n-3 PUFA without pup separation (PS); Con+PS and N-3 + PS, 0% and 1% n-3 PUFA with PS.

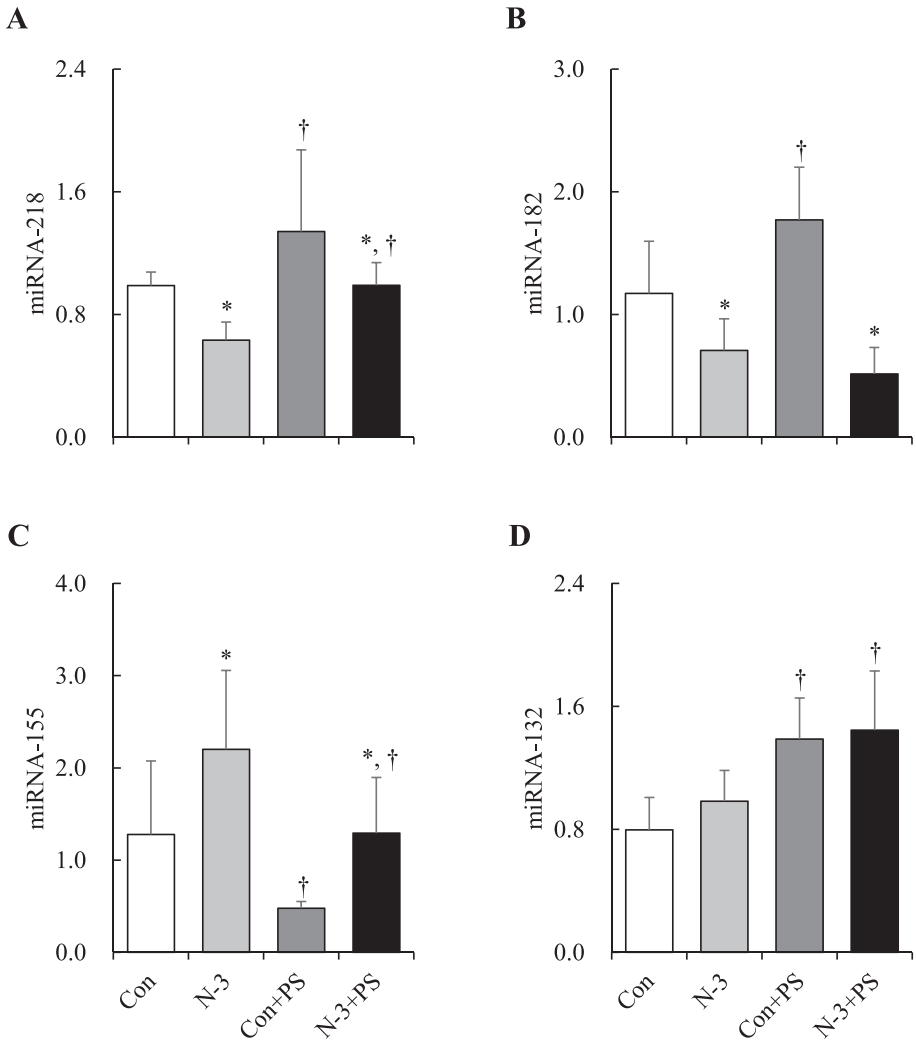

Fig. 4. Effect of n-3 PUFA on the hippocampal expression of miRNA normalized to small nuclear RNA U6. A-D: miRNA-218, −182, −155, and−132. Values are mean and standard deviation (n=6); Con and N-3, 0% and 1% n-3 PUFA without pup separation (PS); Con+PS and N-3 + PS, 0% and 1% n-3 PUFA with PS; *Values are significantly different between Con and N-3, with or without PS, respectively; †Values are significantly different between PS and non-PS within the same dietary groups.

Beside serotonergic neurotransmission, neuroinflammation and the HPA axis have also been shown to modulate glutamatergic neurotransmission through down-regulation of glutamate uptake [57]. Previous studies suggested that injection of EPA- and DHA-derived resolvins prevented activation of the extrasynaptic glutamate receptor NMDAR-2B in rodents with chemically induced inflammation [58,59]. However, the present study reported that n-3 PUFA supplementation had no effect on hippocampal expression of NMDAR-2B, suggesting that the anti-depressive effects of n-3 PUFA were not related with glutamatergic neurotransmission. Previous studies also showed that supplementation with n-3 PUFA had no effect on hippocampal levels of glutamate in PPD dam rats [29] or depressed rats with acute stress [11]. Xu et al. [59] reported that resolvins had 10,000 times greater anti-inflammatory effects than EPA and DHA in mice with chemically induced inflammation. In addition, NMDAR-2B is an ionotropic receptor [57], and is not related with membrane fluidity caused by changes in membrane fatty acid composition [60].

MiRNA is a key post-transcriptional regulator of gene expression, and certain miRNAs have been reported to be related to the glutamatergic and serotonergic pathways in depression [14]. In the present study, n-3 PUFA influenced hippocampal expression of miRNA-155, −218, and −182, but not of miRNA-17 or−132. Previous *in vitro* studies also reported that EPA elevated the expression of miRNA-155 [19] but not miRNA-17 [20]. It has been shown that synthesis of pro-inflammatory cytokines is inhibited by miRNA-155 through reductions in inflammatory transcription factor NF-κB [61], but increased by miRNA-17 *via* up-regulation of signal transducer and activator of transcription pathways [62]. There has been no study regarding the effect of n-3 PUFA on miRNA-218, −182, and−132, which were elevated in both depressed rats with corticosterone injection [17] and CMS [18], and depressed patients [63]. Consistent with the above, the present study showed that miRNA-218, −182, and−132 were increased in PPD dam rats. Since miRNA-218 and -182 have been shown to modulate the GR and BDNF [17,18], and miRNA-132 to increase NMDAR-2B [64], the results from these miRNAs suggest that n-3 PUFA dominantly regulates the serotonergic pathway.

There are a few limitations to the present study. Other monoaminergic pathways such as the norepinephrinergic and dopaminergic systems were not investigated, but previous studies reported that n-3 PUFA had no effect on cortical levels of norepinephrine and dopamine in PPD mice [28] or depressed mice with CMS [42]. In addition, the expression of genes targeted by the studied miRNAs was not measured. However, to our knowledge, this is the first study suggesting that n-3 PUFA may affect the up-regulation of serotonergic pathway through the alterations in HPA axis and neuroinflammation at the miRNA level in a

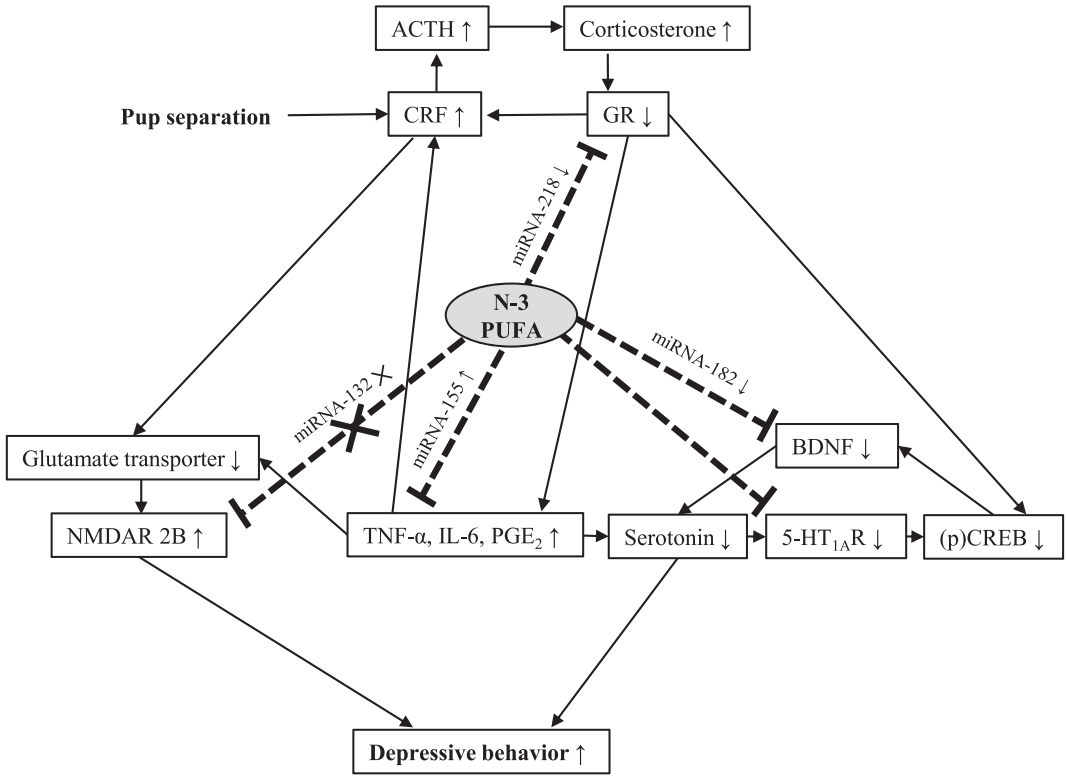

Fig. 5. Abridged general view of the potential pathway for the antidepressant effect of n-3 PUFA. N-3 PUFA increased the expression of glucocorticoid receptor (GR), and decreased the expression of miRNA-218, leading to down-regulation of corticotrophin releasing factor (CRF)- adrenocorticotropic hormone (ACTH)-corticosterone, and modulation of cAMP response element binding protein (CREB) phosphorylation and cytokine transcription. N-3 PUFA increased expression of 5-HT_{1A}R, which in turn up-regulated CREB phosphorylation followed by increase in BDNF and serotonin, while decreased expression of miRNA-182, a negative modulator of BDNF. In addition, n-3 PUFA decreased level of PGE_2, and expression of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α), while increased expression of miRNA-155, which inhibited production of pro-inflammatory cytokines. However, n-3 PUFA had no effect on the expression of NMDAR-2B and miRNA-132, a regulator of NMDAR-2B.

PPD rat model with PS. Further studies are warranted to prove the mechanisms of the antidepressive effects of n-3 PUFA by using knockout models for serotonin or glutamate receptors.

Acknowledgments

Jeong-Eun Choi performed experiments and prepared the manuscript. Eun-Young Kim conducted the statistical analyses. Yongsoon Park supervised the study design and data analysis, and revised the manuscript. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jnutbio.2020.108417.

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