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ORIGINAL RESEARCH COMMUNICATION

Maternal Docosahexaenoic Acid Feeding Protects Against Impairment of Learning and Memory and Oxidative Stress in Prenatally Stressed Rats: Possible Role of Neuronal Mitochondria Metabolism

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Abstract

Aims: Docosahexaenoic acid (22:6n-3; DHA) is known to play a critical role in postnatal brain development. However, no study has been performed to investigate its preventive effect on prenatal stress-induced behavioral and molecular alterations in offspring. In the present study, rats were exposed to restraint stress on days 14–20 of pregnancy, three times a day, 2 hours each time; DHA was given at the doses of 100 and 300 mg/kg/day for two weeks. ***Results:*** We showed that prenatal restraint stress caused (1) learning and memory impairment, (2) BDNF mRNA level decrease, (3) oxidative damage to proteins, (4) enhanced expression of nitric oxide synthase and apoptosis, and (5) abnormalities in mitochondrial metabolism that included changes in mitochondrial complexes I–V, and enhancement of expression of proteins involved in mitochondrial fusion/fission (Mfn-1, Mfn-2, Drp-1) and autophagy (Atg3, Atg7, Beclin-1, p-Akt, and p-mTOR) in the hippocampus of offspring. ***Innovation:*** Besides the well-known role in child brain development, we reported the novel finding of DHA in protecting prenatal stress-induced cognitive dysfunction involving the modulation of mitochondrial function and dynamics. ***Conclusion:*** Maternal feeding of DHA significantly prevented prenatal stress-induced impairment of learning and memory and normalized the biomarkers of oxidative damage, apoptosis, and mitochondrial metabolism in the hippocampus of both male and female offspring. These results suggest that maternal feeding of DHA exerts preventive effects on prenatal stress-induced brain dysfunction and that modulation of mitochondrial metabolism may play critical role in DHA protection. *Antioxid. Redox Signal.* 16, 275–289.

Introduction

Prenatal stress causes widespread alterations in brain morphology, behavior, and cognitive ability of offspring (53, 56). Prenatal restraint stress (PRS) in pregnant rats is a valid model of stress with neurobiological and behavioral consequences (17, 63). Increasing evidence shows that loss of spatial learning and memory is a severe consequence of PRS (56). Although the detailed mechanisms have not been elucidated, it has been proposed that oxidative stress and mitochondrial dysfunction may play important roles in PRS-induced abnormality of hippocampal function, loss of neurons, and memory impairment (56, 61). New studies indicate that mitochondria dynamically undergo fusion and fission processes when under oxidative stress (26). Mfn-1 and Mfn-2 are important factors for facilitating fusion and Drp-1 is an important factor for promoting fission. However, the relationship of mitochondrial dynamics and hippocampal function in response to prenatal stress has not been explored.

Nutritional intervention has been used for stress management, such as with specific vitamins including ascorbic acid, vitamins B1 and B6, the coenzyme forms of vitamin

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Innovation

DHA is the most prevalent polyunsaturated fatty acid in the central nervous system. The most important role for DHA has been indicated as promoting brain development, and most researchers focused on its effect after birth. Here, we reported that DHA has a significant role in preventing prenatal stress-induced offspring cognitive dysfunction in both male and female. The data suggest that DHA is important not only in postnatal brain development, but is important in the prenatal period as well. Mitochondrial dysfunction is connected to the cognitive function in this study, and for the first time we find that DHA might modulate mitochondrial metabolism and function to exert cognitive protection.

B5 (pantothenate) and B12 (methylcobalamin), the amino acid tyrosine, and other nutrients such as alpha-lipoic acid, carnitine, phosphatidylserine, the antioxidant GSH, and docosahexaenoic acid (22:6n-3; DHA) (27, 32). DHA is the most abundant polyunsaturated fatty acid in the central nervous system (59). DHA maintenance is necessary for the processes of neuronal differentiation (25), neurite growth (11), synapse formation, and photo receptor biogenesis (19). The protective effects of DHA supplementation on oxidative stress in animals have not been well studied; however, many reports have indicated that DHA protects against oxidative stress in several kinds of cells (5, 46, 49). Considering the high sensitivity of DHA to ROS, we propose that mitochondrial membranes with high levels of DHA incorporation should be more resistant to ROS attack.

During the last period of gestation, DHA is preferentially transported from maternal resources to infant circulation through the placenta; it quickly accumulates in the fetal brain and plays an important role in the first period of brain development (22). Compared with age and sex-matched normal children, infants with prenatal DHA deficiency have lower DHA levels in their cerebella and show significantly higher incidence of impulsivity, learning disabilities, language impairments, and anxiety (38). Experimental prenatal DHA deficiency in rodents causes neuronal loss in the hippocampus and impairment of hippocampus-dependent spatial learning and memory (38). However, no studies have been carried out to study the effects of maternal DHA feeding on prenatally-stressed rat offspring. In the present study, we propose that DHA may protect against brain impairment and regulate the redox system by modulating mitochondrial metabolism. Therefore, we examined the effects of maternal DHA feeding on prenatal-stress-induced cognitive impairment in male and female offspring and also investigated the possible mechanisms involving the reduction of oxidative damage and apoptosis and the improvement in mitochondrial metabolism in the hippocampus of prenatally-stressed rats.

Results

Maternal DHA feeding prevented PRS-induced cognitive impairment in both female and male offspring

The Morris water maze was used to assess spatial learning for four and a half consecutive days; two duplicate trials from each starting point (N, E, S, W) were performed each day and their results averaged together for each of two sessions per day (9 sessions total). PRS significantly increased the escape latency of both female and male offspring, compared to the relevant controls (Figs. 1 and 2).

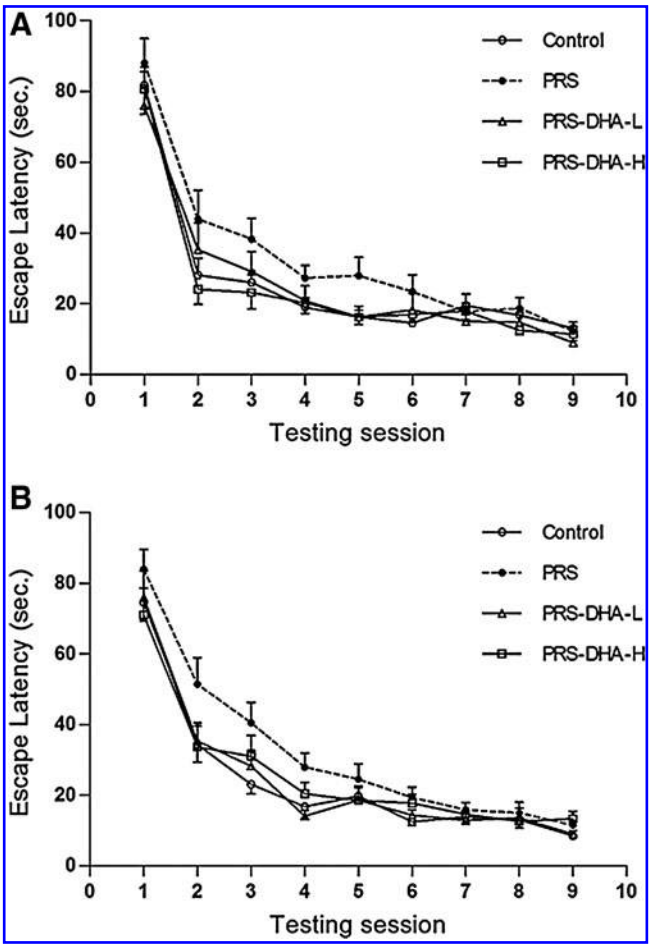

FIG. 1. Effects of prenatal restraint stress (PRS) and maternal DHA feeding on cognitive impairment in offspring rats. (A) Females and **(B)** Males. Cognitive function (spatial memory) was tested with the Morris Water Maze. All groups of animals were able to learn the task through consecutive trials. The latency per testing session represents the average of four trials of all animals in each group. Data are means ± S.E.M of animals (Females, $n \geq 9$ for each group; Males, $n \geq 10$ for each group). Statistical significance of differences was determined by two-way ANOVA (general linear model) repeated measures followed by LSD *post hoc* analysis. Control vs. PRS: $^{*}p < 0.05$; Low-dose DHA feeding vs. PRS: $^{\wedge}p < 0.05$; High-dose DHA feeding vs. PRS: $^{\wedge\wedge}p < 0.01$.

As shown in Fig. 1A, two-way ANOVA (general linear model) repeated measures revealed that DHA produced a significant effect in females ($F_{(3,41)} = 3.481$, $p = 0.024$, $n \geq 9$ for each group). LSD *post hoc* analysis showed that PRS induced poorer learning ability than the controls in female offspring ($p < 0.05$). While both low-dose and high-dose feeding significantly decreased the escape latency time compared with the PRS-only group ($p < 0.05$), there is no significant difference between the low-dose and high-dose groups. Two-way ANOVA (general linear model) multivariate measures revealed a significant effect for DHA at session 5, $F_{(3,41)} = 3.113$, $p = 0.037$. LSD *post hoc* showed significant differences at sessions 2, 3, and 5.

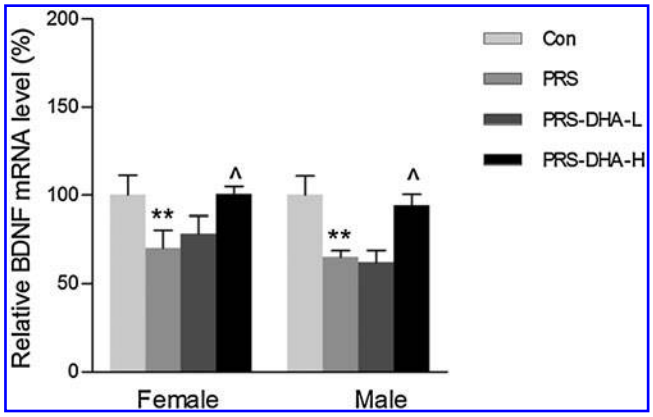

FIG. 2. Effects of PRS and maternal DHA feeding on mRNA level of brain-derived neurotrophic factor (BDNF). Hippocampal mRNA was extracted and BDNF mRNA contents were analyzed by real time PCR in both male and female offspring. Data are means ± S.E.M. Seven animals were used in each group of both male and female. Statistical significance of differences was determined by one-way ANOVA followed by LSD *post hoc* analysis. $^{**}p < 0.01$ vs. control, $^{\wedge}p < 0.05$ vs. PRS.

In males, as shown in Fig. 1B, ANOVA indicated a similar significant effect of DHA as in females ($F_{(3,51)} = 4.307$, $p < 0.01$, $n \geq 10$ for each group). LSD *post hoc* showed poorer learning ability induced by PRS compared with controls ($p < 0.05$). For low-dose DHA, a significance level of $p < 0.05$ vs. the PRS-only model was achieved; for high-dose DHA, $p < 0.01$ vs. the PRS-only model. Again, there was no significant difference between the low-dose and high-dose groups. Two-way ANOVA (general linear model) multivariate measures revealed a significant effect for DHA at session 3, $F_{(3,51)} = 2.958$, $p = 0.041$, session 4, $F_{(3,51)} = 2.967$, $p = 0.041$. LSD *post hoc* showed significant differences at sessions 2, 3, 4, and 6. Spatial learning was not significantly affected by either pup gender or DHA dosage.

Maternal DHA feeding increased total DHA content in offspring hippocampus

The total DHA contents in hippocampus of both female and male offspring were summarized in Table 1. PRS didn't show significant effect on DHA content in both female and male (Table 1). Low dose DHA maternal feeding was prone to increase DHA content in offspring but no significance was found. However, high dose DHA feeding significantly elevated the offspring DHA content nearly 20% in both female and male (Table 1).

Maternal DHA feeding inhibited PRS-induced decrease in BDNF mRNA level

PRS significantly decreased brain-derived neurotrophic factor (BDNF) mRNA content in both male and female offspring. As shown in Figure 2, low dose DHA supplement has no apparent effect while high dose DHA significantly increased BDNF mRNA level as compared with PRS group.

Maternal DHA feeding inhibited PRS-induced increases in nNOS and iNOS expression

PRS significantly increased not only nNOS levels, but also iNOS levels in both female and male offspring. As shown in Figures 3A and 3B, PRS significantly induced high levels of nNOS expression in both female ($p < 0.01$) and male ($p < 0.01$) offspring, compared with controls. In female offspring, both low- and high-dose DHA maternal feeding efficiently inhibited nNOS expression compared with the PRS-only group ($p < 0.01$). In male offspring, however, only high-dose DHA feeding led to significant inhibition of nNOS expression ($p < 0.01$). As shown in Figures 3C and 3D, PRS significantly increased iNOS expression in both female ($p < 0.01$) and male ($p < 0.01$) offspring. Both low- and high-dose DHA feedings significantly inhibited the increase in iNOS expression in both female ($p < 0.01$) and male ($p < 0.01$) offspring compared with the PRS-only group.

Maternal DHA feeding inhibited PRS-induced increases in oxidative damage to proteins

PRS significantly increased protein carbonyl levels ($p < 0.01$) in female offspring, and both low and high-dose DHA maternal feeding inhibited PRS-induced increases in protein carbonyl levels, although only the low dose produced a statistically significant difference ($p < 0.05$) (Figs. 4A and 4C). In male offspring, just as in female offspring, PRS significantly increased protein carbonyl levels ($p < 0.01$), and both low-dose and high-dose DHA maternal feeding efficiently inhibited PRS-induced increases in protein carbonyls ($p < 0.05$, $p < 0.01$, respectively) (Figs. 4B and 4D).

Maternal DHA feeding prevented PRS-induced dysfunction of mitochondrial respiratory chain complexes

Mitochondria are the source and also the target of reactive oxygen species. Mitochondrial dysfunction has been

Table 1. Total Docosahexaeonic Acid, 22:6 (n-3) Content in Female and Male Offspring Hippocampus

		PRS		
DHA (mg/Kg/day)	*0*	*0*	*100*	*300*
Female (%)	0.391 ± 0.017	0.396 ± 0.016	0.410 ± 0.019	0.462 ± 0.020*,#
Male (%)	0.386 ± 0.025	0.375 ± 0.032	0.445 ± 0.030	0.463 ± 0.015*,#

Data are means of 8 animals in each group. Statistical significance of difference was determined by one-way ANOVA followed by LSD *post hoc* analysis.

*$p < 0.05$ vs. control; #$p < 0.05$ vs. PRS.

DHA, docosahexaeonic acid; PRS, prenatal restraint stress.

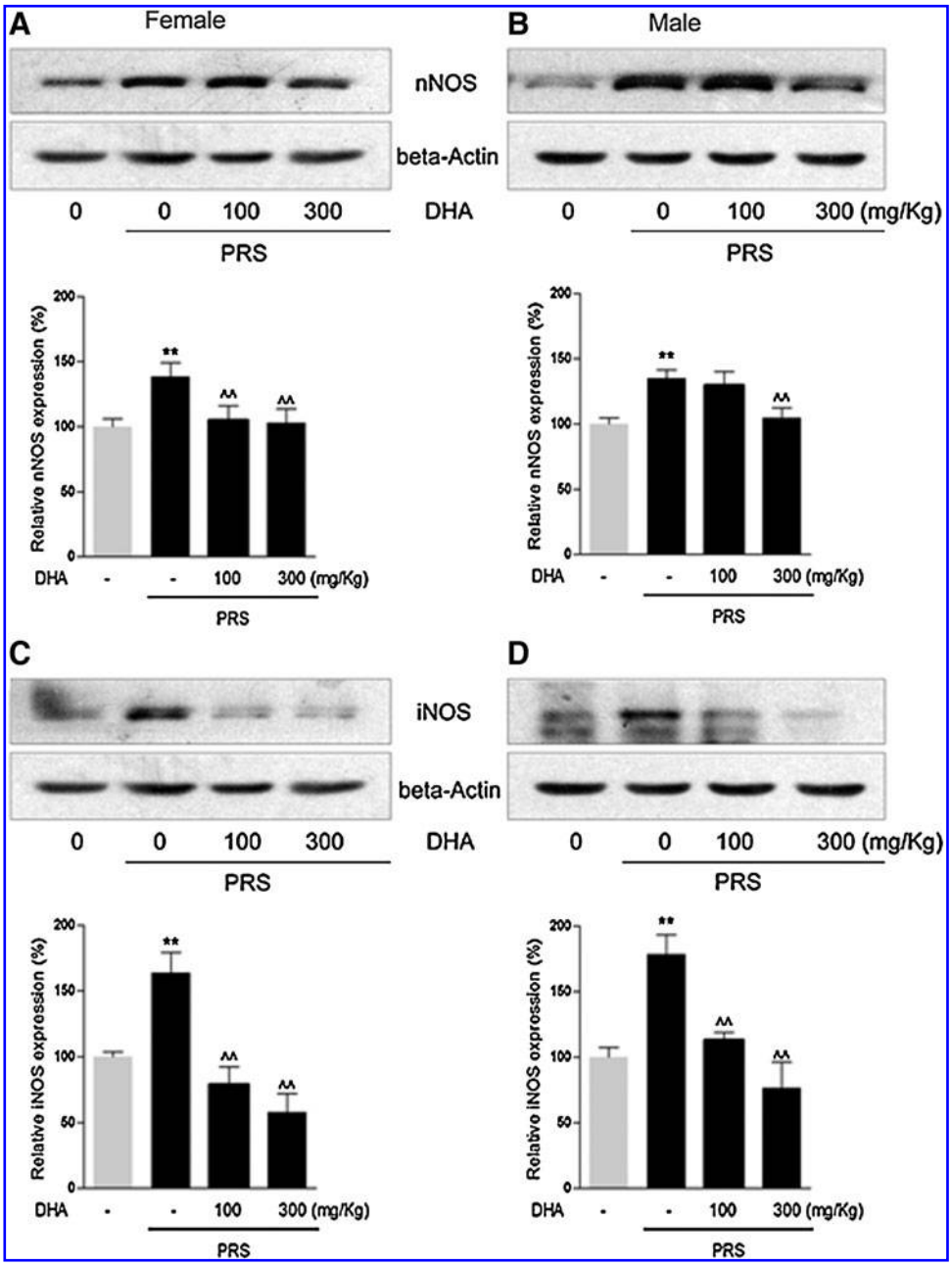

FIG. 3. Effects of PRS and maternal DHA feeding on expressions of neuronal nitric oxide synthase (nNOS) and inducible nitric oxide synthase (iNOS) in offspring hippocampus. Total protein extracts were prepared from brain hippocampi of both male and female offspring in each group. **(A)** nNOS expression in females, **(B)** nNOS in males; **(C)** iNOS in females, and **(D)** iNOS in males (*top*: representative Western blots, and *bottom*: quantitative results of Western blot image densities). Data are means ± S.E.M. In female offspring, 7 animals were used in each group, and in male offspring, 8 animals were used in each group. Statistical significance of differences was determined by one-way ANOVA, followed by LSD *post hoc* analysis. $^{**}p < 0.01$ vs. control, $^{\wedge\wedge}p < 0.01$ vs. PRS.

suggested to play an important role in stress-related disease (32). We found that PRS reduced complex I and complex II activities in both female and male offspring. In female offspring (Table 2), PRS caused a significant decrease in complex I and II activities ($p < 0.01$); both low and high-dose DHA treatments significantly prevented the decrease in complex I activity ($p < 0.01$ for both low and high doses) and complex II activity ($p < 0.05$ for low dose and $p < 0.01$ for high dose, respectively). In male offspring (Table 3) complex I and II activities were also decreased by PRS; however, only high-dose DHA feeding significantly inhibited the PRS-induced decrease in activity of complexes I and II ($p < 0.01$ for both complexes I and II). Only male offspring showed a reduction in complex V in the PRS-only group ($p < 0.05$) (Table 3), but activity was unaffected in this group of females (Table 2). In female offspring (Table 2), high-dose DHA maternal feeding caused a decrease in complex V activity compared with both control ($p < 0.01$) and PRS-only groups ($p < 0.05$). In male offspring (Table 3) both low- and high-dose DHA feeding significantly inhibited the PRS-induced decrease in activity of complex V ($p < 0.01$ for low dose and $p < 0.05$ high dose).

Unlike the decreases produced in complex I, II, and V activities, PRS induced increases in complex III and IV activities in both female and male offspring ($p < 0.01$ for complex III and $p < 0.05$ for complex IV). In female offspring (Table 2), only high-dose DHA feeding inhibited the PRS-induced increase in complex III activity ($p < 0.01$); both low ($p < 0.05$) and high ($p < 0.01$) dose DHA feedings significantly inhibited the PRS-induced increase in complex IV activity. In male offspring (Table 3), low dose DHA feeding actually significantly enhanced ($p < 0.01$ compared with control group), while the high dose DHA feeding effectively inhibited the PRS-induced increase in complex III

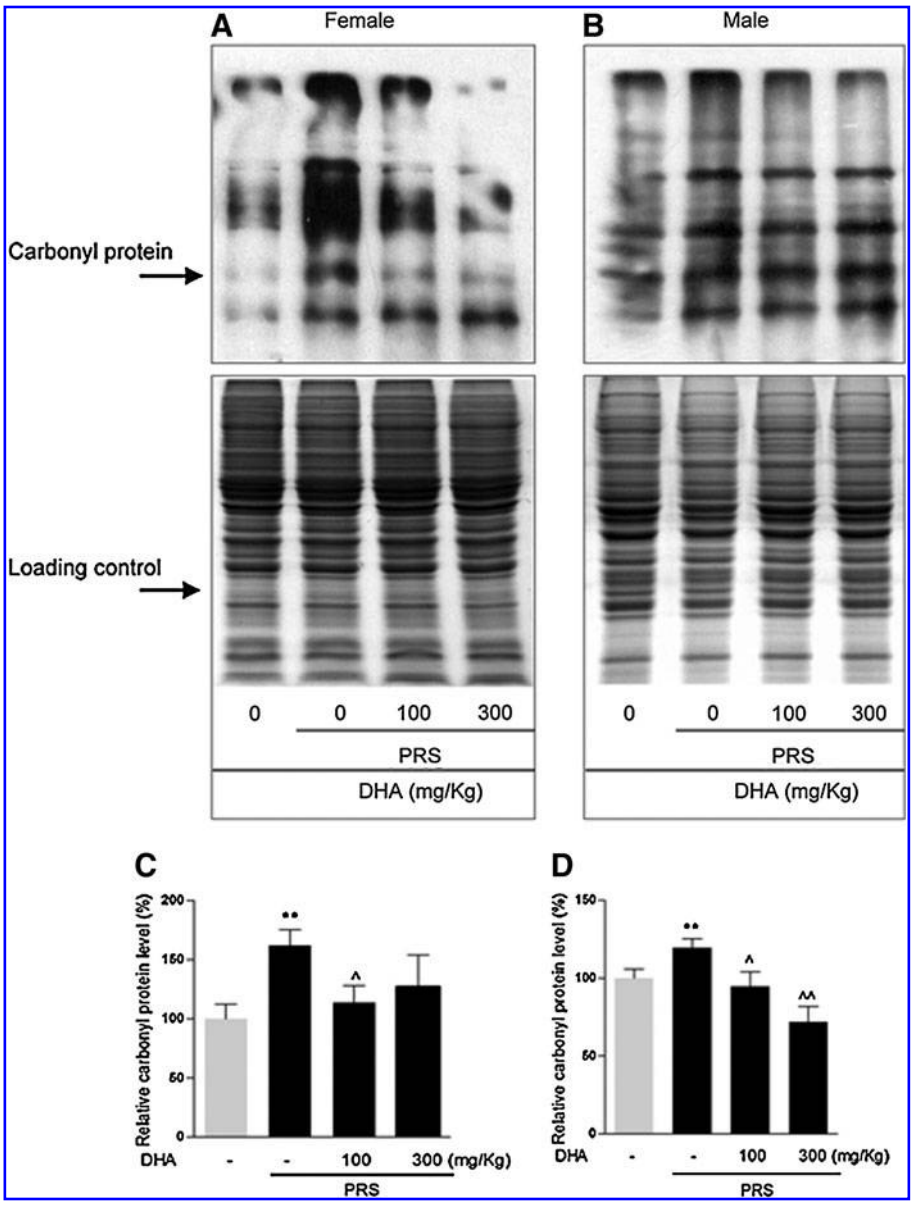

FIG. 4. Effects of PRS and maternal DHA feeding on oxidative damage to proteins in offspring hippocampus. Protein carbonyl levels were detected by Western blot as an indication of oxidative damage to proteins. **(A)** and **(B)** Representative Western blot images for females and males, respectively. *Bottom*: quantitative results of Western blot densities for females **(C)** and males **(D)**. Data are means ± S.E.M. of 7 animals in each group. Statistical significance of differences was determined by one-way ANOVA followed by LSD *post hoc* analysis. $^{**}p < 0.01$ vs. control, $^{\wedge}p < 0.05$ and $^{\wedge\wedge}p < 0.01$ vs. PRS.

Table 2. Effects of Prenatal Restraint Stress and Maternal Docosahexaeonic Acid Feeding on the Activities of Mitochondrial Respiratory Chain Complexes in Female Offspring Hippocampus

		PRS		
DHA (mg/Kg/day)	*0*	*0*	*100*	*300*
Complex I (%)	100 ± 2.68	86.46 ± 1.68**	96.63 ± 2.19##	98.54 ± 1.99##
Complex II (%)	100 ± 4.16	77.25 ± 2.27**	94.14 ± 5.24#	101.06 ± 2.31##
Complex III (%)	100 ± 2.57	119.61 ± 3.86**	112.47 ± 5.75	103.45 ± 2.56##
Complex IV (%)	100 ± 2.12	108.09 ± 2.15*	100.32 ± 2.26#	98.02 ± 2.11##
Complex V (%)	100 ± 4.14	98.54 ± 4.64	93.01 ± 5.32	86.22 ± 3.00

Data are means of 9 animals in each group. Statistical significance of differences was determined by one-way ANOVA followed by LSD *post hoc* analysis.

$^{*}p < 0.05$ and $^{**}p < 0.01$ vs. control; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. PRS.

Table 3. Effects of Prenatal Restraint Stress and Maternal Docosahexaeonic Acid Feeding on the Activities of Mitochondrial Respiratory Chain Complexes in Male Offspring Hippocampus

		PRS		
DHA (mg/Kg/day)	*0*	*0*	*100*	*300*
Complex I (%)	100 ± 3.94	89.33 ± 1.53*	95.09 ± 3.94	101.33 ± 2.46##
Complex II (%)	100 ± 5.19	80.71 ± 1.67**	83.58 ± 4.00	96.07 ± 2.82##
Complex III (%)	100 ± 3.81	118.08 ± 4.79*	129.72 ± 6.84	107.50 ± 1.95#
Complex IV (%)	100 ± 2.05	115.24 ± 5.18*	98.93 ± 2.74#	95.92 ± 2.24##
Complex V (%)	100 ± 7.04	79.52 ± 5.09*	106.57 ± 4.83	105.32 ± 7.00#

Data are means of 9 animals in each group. Statistical significance of differences was determined by one-way ANOVA followed by LSD *post hoc* analysis.
*$p < 0.05$ and **$p < 0.01$ vs. control; #$p < 0.05$ and ##$p < 0.01$ vs. PRS.

activity ($p < 0.05$ vs. PRS). In contrast, both low ($p < 0.05$) and high ($p < 0.01$) dose DHA feeding significantly inhibited the PRS-induced increase in complex IV activity.

Maternal DHA feeding prevented the PRS-induced increase in expression of mitochondrial fusion/fission proteins in offspring hippocampus

As shown in Figs. 5A and 5C, PRS caused a significant increase in the expression of Mfn-1, Mfn-2, and Drp-1 in the hippocampus of female offspring, suggesting an increase in mitochondrial fusion and fission processes. Maternal feeding of DHA showed a dose-dependent inhibition of the PRS-induced increase in protein expression of Mfn-1, Mfn-2, and Drp-1, and the high dose significantly reduced expressions of these proteins from their PRS-induced levels. In contrast to the changes in females, neither PRS, nor maternal feeding of DHA, had any obvious effect on the expressions of Mfn-1, Mfn-2, and Drp-1 in the hippocampus of male offspring (Figs. 5B and 5D).

Maternal DHA feeding prevented the PRS-induced increase in expression of cytochrome c in offspring hippocampus

Since in our tests we have shown that high complex III and complex IV activities are induced by PRS, we sought to

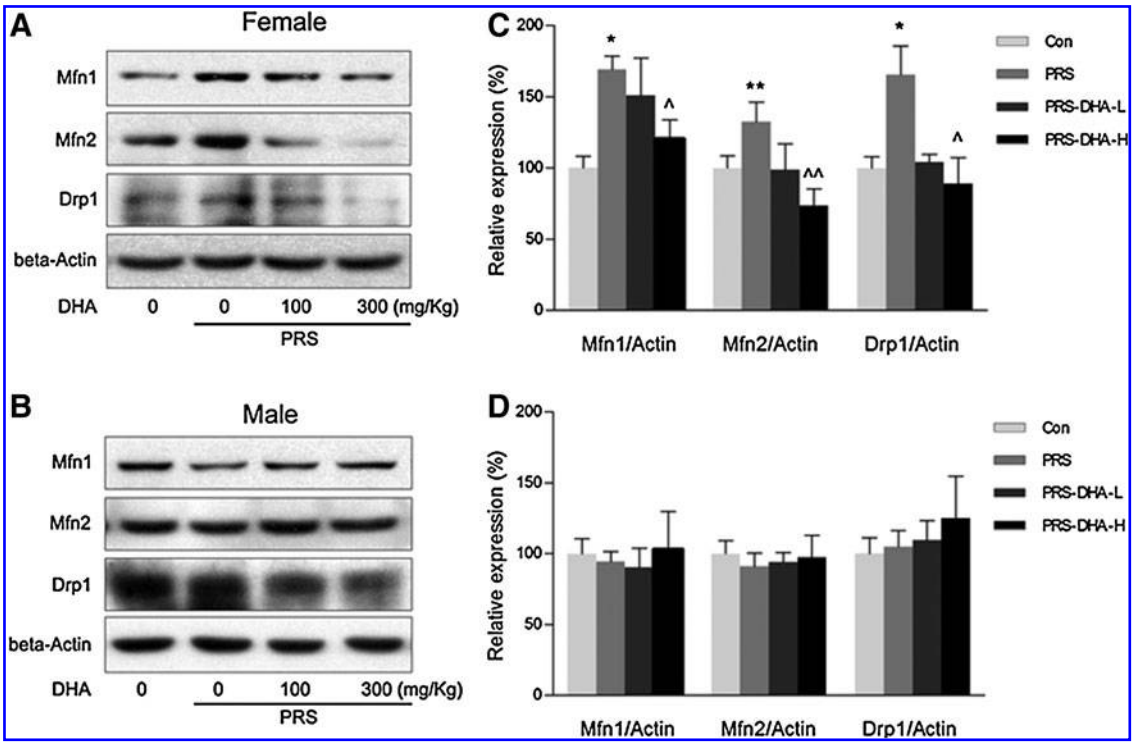

FIG. 5. Effects of PRS and maternal DHA feeding on expressions of Mfn1, Mfn2, and Drp1 in offspring hippocampus. Total protein extracts were prepared from brain hippocampi of both male and female offspring in each group. **(A)** Representative Western blot images of females; **(C)** Quantitative results of Western blot densities of Mfn1, Mfn2, and Drp1 in females. **(B)** Representative Western blot images of males; **(D)** Quantitative results of western blot densities of Mfn1, Mfn2, and Drp1 in males. Data are means ± S.E.M. of at least 6 animals in each group. Statistical significance of differences was determined by one-way ANOVA followed by LSD *post hoc* analysis. *$p < 0.05$, **$p < 0.01$ vs. control, and ^$p < 0.05$, ^^$p < 0.01$ vs. PRS.

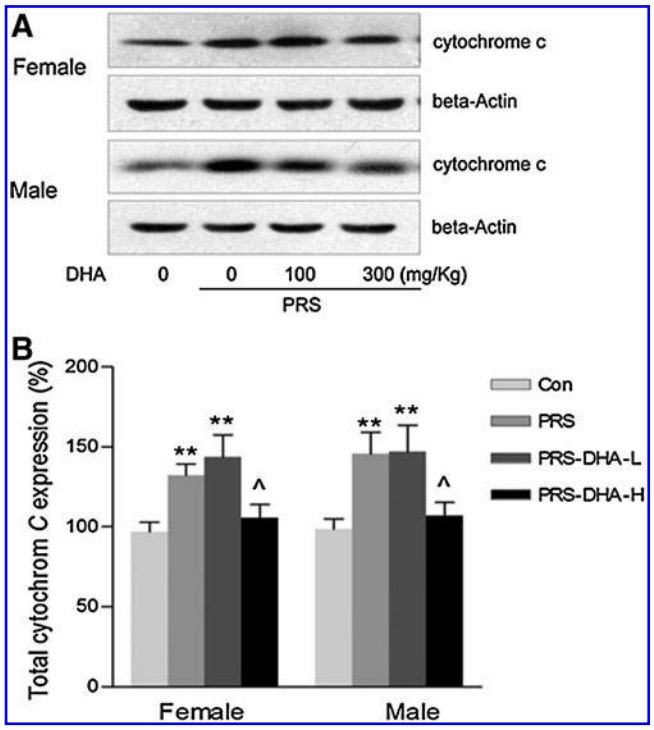

FIG. 6. Maternal DHA feeding inhibited PRS-induced increases in expression of cytochrome *c* in offspring hippocampus. Total protein extracts were prepared from brain hippocampi of female and male offspring in each group; cytochrome *c* expression was detected by Western blot. **(A)** Representative western blots. **(B)** Quantitative results of western blot image densities. Data are means ± S.E.M. of 8 animals in each group. Statistical significance of differences was determined by one-way ANOVA followed by LSD *post hoc* analysis. $^{**}p < 0.01$ vs. control, and $^{\wedge}p < 0.05$ vs. PRS.

discover whether cytochrome *c* levels were affected by the high activities of complex III and complex IV. PRS enhanced expression of cytochrome *c* in both female offspring ($p < 0.01$) and male offspring ($p < 0.01$) (Figs. 6A and 6B). Only high-dose DHA maternal feeding significantly reduced cytochrome *c* expression in female ($p < 0.05$) and male offspring ($p < 0.05$), while low-dose DHA feeding did not show any inhibiting effects in either the female or male offspring (Figs. 6A and 6B).

Maternal DHA feeding prevented the PRS-induced increase in apoptosis signaling (increased expression of Bax, pro-caspase-9, and pro-caspase-3)

In female offspring, neither PRS nor DHA feeding had any effect on Bax expression (Figs. 7A and 7C). In male offspring, PRS significantly enhanced expression of Bax ($p < 0.01$), and both low- ($p < 0.01$) and high- ($p < 0.01$) dose DHA feeding inhibited the PRS-induced increase in Bax expression (Figs. 7B and 7E).

In female offspring, PRS significantly enhanced expression of Bcl2 ($p < 0.05$), and both low- ($p < 0.05$) and high- ($p < 0.05$) dose DHA feeding inhibited the PRS-induced increase in Bcl2 expression (Figs. 7A and 7D). In male offspring, neither PRS nor DHA feeding had any effect on Bcl2 expression (Figs. 7B and 7F).

PRS caused significant increases in pro-caspase-9 expression in the hippocampi of both female ($p < 0.05$) (Figs. 7A and 7G) and male offspring ($p < 0.05$) (Figs. 7B and 7I); both low and high dose DHA feeding efficiently inhibited the PRS-induced increase in expression of pro-caspase-9 in both female and male offspring (low dose: $p < 0.05$ for females and males; high dose: $p < 0.01$ for females and $p < 0.05$ for males).

Similar to the results for pro-caspase-9, PRS caused significant increases in pro-caspase-3 expression in the hippocampi of both females ($p < 0.01$) (Figs. 7A and 7H) and males ($p < 0.01$) (Figs. 7B and 7J); both low and high-dose DHA feedings significantly inhibited the PRS-induced increase in expression of pro-caspase-3 in both female and male offspring ($p < 0.05$ for all).

Maternal DHA feeding prevented the PRS-induced increase in expression of proteins related to autophagy in offspring hippocampus

PRS induced a significant increase in Atg3 and Beclin-1 in the hippocampi of both female and male offspring, and the maternal DHS feeding showed significant inhibition of the increase in Atg3 and Beclin-1 (Fig. 8). PRS also caused a significant increase in the expression of Atg7 in the hippocampus of female offspring, and the maternal DHA feeding showed significant inhibition of the Atg7 increase. However, in male offspring neither PRS nor the maternal DHA feeding showed any effect on the expression of Atg7 (Fig 8).

PRS induced obvious decreases in the phosphorylation of Akt and mTOR in both female (Fig. 9A) and male (Fig. 9B) offspring. Maternal DHA feeding showed protection against the PRS-induced decrease in the phosphorylation of both Akt and mTOR in the hippocampi of female (Fig. 9A) and male (Fig. 9B) offspring.

Discussion

PRS leads to long-term behavioral and neurobiological abnormalities, including vulnerability to anxiety and depression-like behavior, drug administration, and memory impairment in the offspring (10, 36, 56). Mechanisms underlying the effects of PRS on offspring remain largely unknown. However, previous studies have demonstrated that reduced hippocampal plasticity, decreased neurogenesis, metabotropic and glutamate glucocorticoid receptors, and increased Fos expression might be involved in the adaptation of offspring to PRS (17). The hippocampus is known to play a critical role in the processing of spatial learning and memory. Restraint stress was reported to cause a highly significant increase in lipid peroxidation in the hippocampal region, compared with other regions (33). Moreover, prenatal stress induced an increase in the number of nNOS-positive cells in the hippocampus, a significant decrease in the number of hippocampal neurons, and significant increases in calcium content and oxidant generation in the hippocampal CA3 region (61). A recent study shows that feeding of DHA-enriched omega 3 fatty acids to rats results in their significant incorporation into phospholipids of hippocampus, striatum, and cortex, among which hippocampus and striatum show higher amounts of DHA incorporation (29). In our study we found that high dose maternal DHA feeding significantly increased offspring hippocampus DHA content. Therefore, in our study all tests were performed on the hippocampus.

BDNF was the second neurotrophic factor to be characterized after nerve growth factor. It acts on certain neurons of

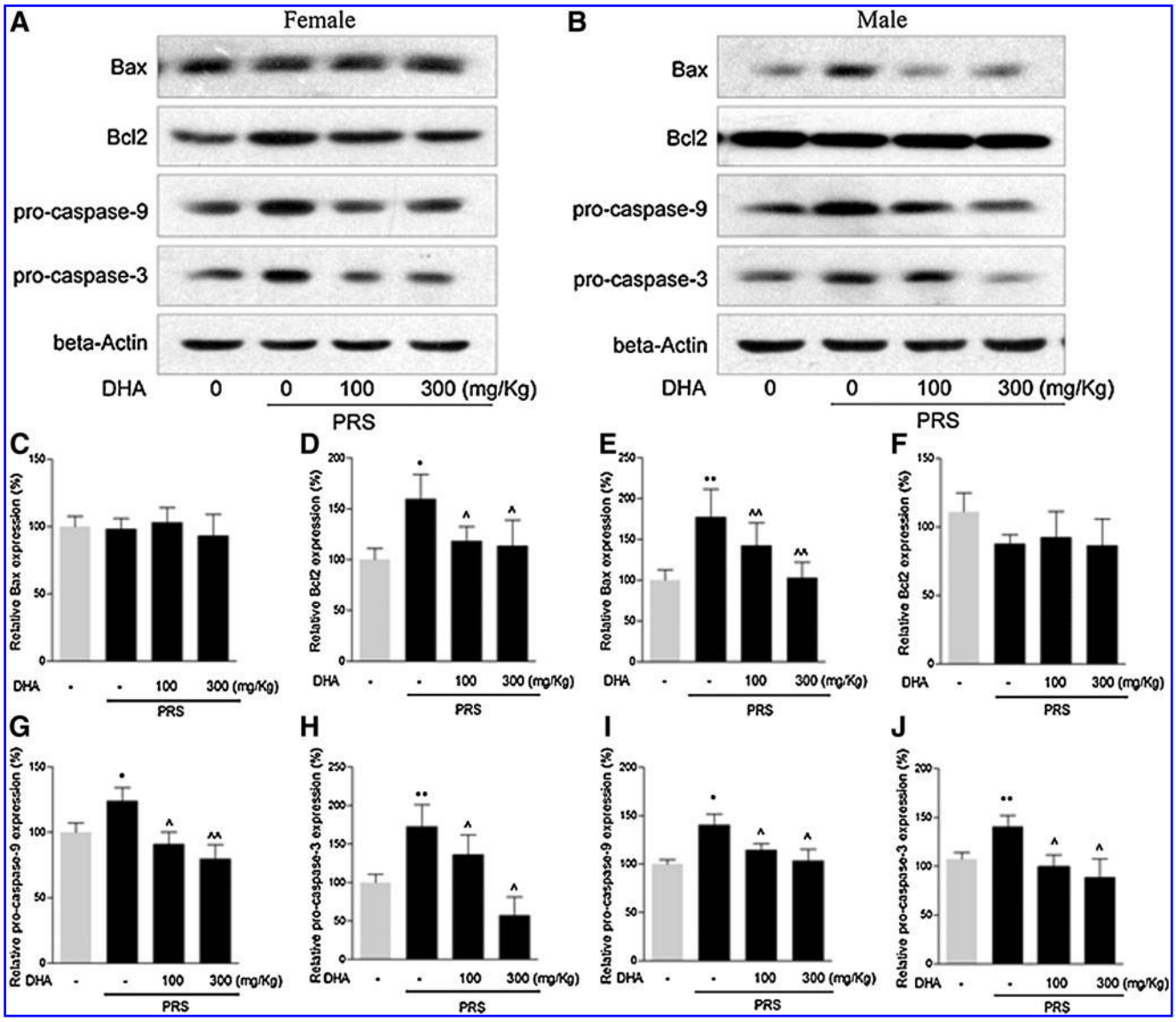

FIG. 7. Maternal DHA feeding prevented PRS-induced increases in apoptosis signaling (Bax, Bcl2, pro-caspase-9, and pro-caspase-3 expression) in offspring hippocampus. Total protein extracts were prepared from brain hippocampi and expression levels of Bax, Bcl2, pro-caspase-9, and pro-caspase-3 were detected by Western blot. **(A)** Representative Western blot images of females; **(C, D, G,** and **H)** Quantitative results of Western blot densities of Bax, Bcl2, pro-caspase-9, and pro-caspase-3 in females. **(B)** Representative Western blot images of males; **(E, F, I,** and **J)** Quantitative results of Western blot densities of Bax, Bcl2, pro-caspase-9, and pro-caspase-3 in males. Data are means ± S.E.M. of 8 animals in each group. Statistical significance of differences was determined by one-way ANOVA followed by LSD *post hoc* analysis. $^{**}p < 0.01$ vs. control, and $\wedge p < 0.05$ vs. PRS.

central nervous system and the peripheral nervous system, helping to support the survival of existing neurons and encourage the growth and differentiation of new neurons and synapases (1, 24). Also, BDNF is present in high concentration and activation in hippocampus (57). We found that PRS could significantly decrease BDNF mRNA level in both male and female offspring, while high dose DHA feeding could effectively prevent the decrease indicating that protective effect of maternal DHA feeding on offspring neural development.

NO is thought to be involved in neuronal plasticity in the hippocampus (14). Nitric oxide synthase (NOS) regulates NO levels and plays a critical role in neuronal plasticity. Various studies have shown that NOS inhibitors block the induction of LTP (8, 23, 62). Spatial learning has also been found to be impaired in rats after administration of NOS inhibitors using the radial-arm maze (7, 62). A recent study showed that NOS inhibition induced delay-dependent performance deficits in the object location task in rats (44). It seemed that lack of NOS function could noticeably impair learning ability. On the other hand, high levels of nNOS expression were detected in hippocampus of PRS offspring rats, which also suffered impairment of cognitive function (61). In our study, we found PRS induced high expression levels of nNOS in both male and female offspring. Moreover, iNOS was also increased in both male and female PRS offspring. In this model, DHA maternal feeding efficiently improved cognitive function in both male

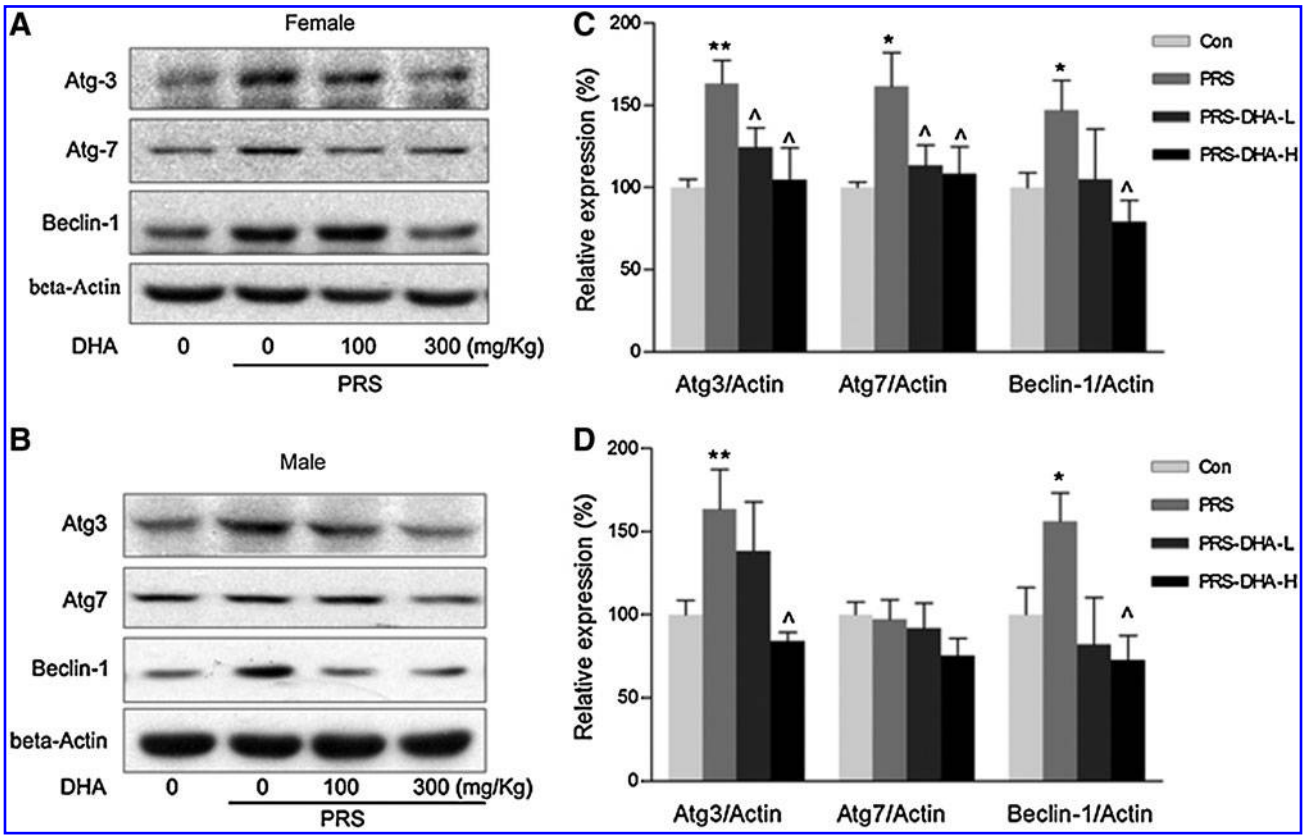

FIG. 8. Effects of PRS and maternal DHA feeding on expressions of proteins related to autophagy (Atg3, Atg7, and Beclin-1) in offspring hippocampus. Total protein extracts were prepared from brain hippocampi of both male and female offspring in each group. **(A)** Representative Western blot images of Atg3, Atg7, and Beclin-1 in females; **(C)** Quantitative results of Western blot densities in females. **(B)** Representative Western blot images of Atg3, Atg7, and Beclin-1 in males; **(D)** Quantitative results of Western blot densities in males. $*p < 0.05$, $**p < 0.01$ vs. control, and $\wedge p < 0.05$ vs. PRS.

and female PRS offspring; nNOS and iNOS expression also decreased in hippocampus of both male and female offspring. These results indicate (1) that PRS-induced elevation of NOS expression might disturb the process of neuronal plasticity and impair rats' spatial learning ability, and (2) that maternal feeding of DHA could possibly modulate NOS and protect the spatial learning ability in offspring.

Protein carbonyl levels indicate oxidative damage to proteins. Our study is the first to report that PRS can cause high protein carbonyl levels in both male and female offspring. Maternal DHA feeding can significantly reduce protein carbonyl levels compared to untreated PRS controls, suggesting that DHA feeding can prevent PRS-induced protein damage in the brain. Mitochondria are the major source of ROS and various oxidative stresses are induced by mitochondrial dysfunction. Multiple lines of evidence suggest that progressive oxidative damage caused by mitochondrial dysfunction might be a conserved, central mechanism of age- or environmentally-related cognitive decline (6, 16, 18, 20, 21, 39). We have also demonstrated that age-related cognitive decline is closely associated with structural and functional disorder in hippocampal mitochondria (3, 31, 48). However, no reports show how changes induced in mitochondria by prenatal stress cause cognitive decline in offspring. In our

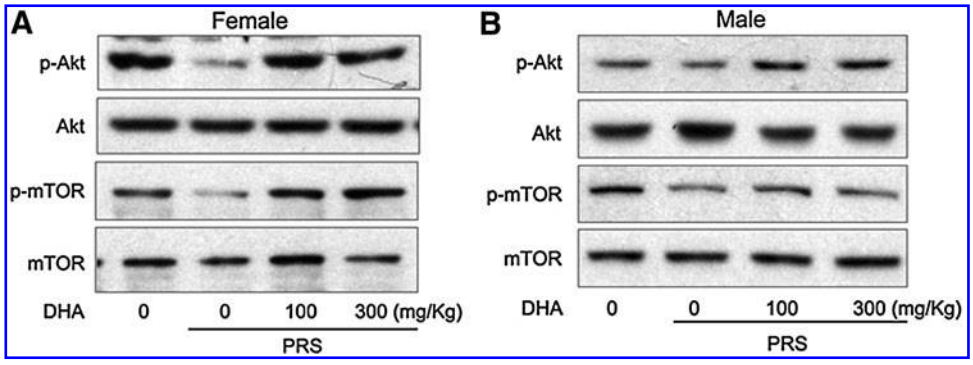

FIG. 9. Effects of PRS and maternal DHA feeding on PI3K/Akt and mTOR activation. Total protein extracts were prepared from brain hippocampi of both male and female offspring in each group; Akt and mTOR activation were analyzed by Western blot. **(A)** Representative Western blot images of p-Akt, Akt, p-mTOR, and mTOR in females. **(B)** Representative Western blot images of p-Akt, Akt, p-mTOR, and mTOR in males.

current tests, mitochondrial complex activities were found to be abnormal consequent to PRS, in both male and female offspring. DHA feeding inhibited these PRS-induced abnormalities. Meanwhile, mitochondria dynamically undergo fusion and fission processes. Since we have observed increased oxidative stress in the brain of offspring (increased NOS and protein carbonyls), we also hypothesized that maternal stress would affect mitochondrial morphology by causing alterations in fusion proteins such as MFN-1 and MFN-2 and fission proteins such as DRP-1. As shown in Figure 7, for the first time, we report significant stress-induced increases in fusion proteins Mfn-1 and Mfn-2, and fission protein Drp-1, in female offspring. DHA efficiently blocked these alterations, suggesting that alteration of mitochondrial dynamics was involved in the cognitive decline. However, mitochondrial dynamics were not affected by PRS in male offspring. Again, the cause for the differences between male and female animals is unknown and warrants further study. Cardiolipin (CL) is a diphospholipid required for mitochondrial structural integrity and for the proper function of the electron transport chain (37). CL is susceptible to ROS and is easily peroxidized by ROS, which results in a decrease in CL levels in the mitochondrial membrane and finally causes a drop in mitochondrial membrane potential and initiation of apoptosis (41–43). Study also shows that DHA is preferentially incorporated in CL (55), suggesting that DHA may directly interact with the mitochondria or lodge in mitochondrial membranes. Therefore, the mitochondrial membrane might be the primary or a specific target where DHA may modulate mitochondrial function by maintaining membrane integrity to produce less ROS and to become more resistant to the oxidative stress.

Apoptosis is one of the main types of programmed cell death and involves a series of biochemical events leading to a characteristic cell morphology and death. Numerous studies have revealed involvement of mitochondria function in oxidative stress induced apoptosis (9, 54). Recently, studies indicated that PRS apparently can induce loss of hippocampal neurons in offspring (30, 61). Also, study showed that DHA supplementation increased both DHA and EPA and decreased ARA in mitochondrial phospholipid, and significantly delayed mitochondrial permeability transition pore (MPTP) opening as assessed by increased Ca^{2+} retention capacity and decreased ROS production and Ca^{2+}-induced mitochondria swelling (28, 40). Taken together, we hypothesized that PRS-induced oxidative stress could activate an apoptotic pathway that might lead to loss of hippocampal neurons and cause impairment of cognitive function. In our study, we tested apoptosis-related protein levels. Although there were some differences between male and female changes induced by PRS, no matter in which direction expression of these proteins changed, maternal DHA feeding efficiently eliminated the PRS effect and maintained normal expression levels of these proteins.

Autophagy is a homeostatic and dynamic process for the intracellular recycling of bulk proteins, lipids, and aging organelles, such as mitochondria (4). Disruption of autophagy results in accumulation of unwanted proteins and neurodegeneration; acute brain injury has been found to enhance autophagy (4). We detected the involvement of autophagy by examining three proteins: (1) Atg3, an E2-like enzyme that catalyzes the conjugation of Atg8 and phosphatidylethanolamine (58), (2) Atg7, an E1-like enzyme binding to Atg 3 (58), and (3) beclin-1, which plays a role in initiating formation of autophagosomes (47). As shown in Figure 8, PRS caused significant increases in expression of Atg3, Atg7, and Beclin-1 in both female and male offspring, suggesting that autophagy is greatly enhanced as a defense mechanism for clearing the accumulation of damaged mitochondria and unwanted proteins. Maternal DHA administration effectively prevented the increases in Atg3, Atg7, and Beclin-1, suggesting that no significant accumulation of damaged mitochondria and unwanted proteins in the neurons of the brain had occurred. The reason for the unchanged Atg7 in male brain is unknown and needs to be studied further.

Autophagy is known to be regulated by mTOR proteins (60). Target of rapamycin (TOR) signaling plays a critical role in brain development, learning, and memory formation (45). Also, macroautophagy is the sequestrating of organelles such as mitochondria and long-lived proteins in *autophagosomes*; the formation of autophagosomes is initiated by class III PI3K and Beclin-1 (47). Carloni et al. (13) have shown that in neonatal rats subjected to hypoxia-ischemia (HI), rapamycin administration increases autophagy, decreases apoptosis and significantly reduces brain damage. We have examined phosphorylation of mTOR and PI3K/Akt in the hippocampus. As shown in Figures 9A and 9B, PRS caused significant decreases in phosphorylation of both Akt and mTOR and maternal administration of DHA effectively prevented these decreases, suggesting that mTOR and PI3K/Akt play important roles in the PRS-induced increase in autophagy. These results also suggest that mTOR and PI3K/Akt are possible therapeutic targets for protecting brain development, and learning and memory. Many neurodegenerative diseases are characterized by neuronal death via apoptosis, while mTOR has been associated with the inhibition of apoptosis (60). Carloni et al. (13) also found that the activation of autophagy and Akt/CREB signaling play equivalent roles with respect to the neuroprotective effect of rapamycin in neonatal hypoxia-ischemia, indicating that autophagy can be part of integrated prosurvival signaling which includes the PI3K–Akt– mTOR axis. From our results, the PRS-induced increase in apoptosis in the hippocampus is apparently associated with the decrease in mTOR. Accordingly, maternal DHA administration ameliorated the learning and memory impairment and inhibited apoptosis, possibly by targeting the PI3K–Akt–mTOR pathway.

In conclusion, our study demonstrates that both male and female offspring suffered cognitive functional impairment from PRS, and maternal DHA feeding efficiently improved cognitive function. All the results indicate that DHA is important not only in postnatal brain development, but is important in the prenatal period as well. These results also might suggest that DHA feeding is helpful for preventing schizophrenia because the prenatal stress model has been found to produce behavioral abnormalities that are thought to be consistent with schizophrenia. However, the detailed mechanism of DHA protection is not clear. Based on our studies and other reports, we may assume that (a) PRS-induced ROS production and calcium increase might trigger autophagy and mitochondria dysfunction induced apoptosis in offspring hippocampus; (b) The apoptosis and autophagy will cause neuron death and impair cognitive function; (c) Maternal feeding may cause DHA accumulation in hippocampus mitochondria; and (d) DHA-enriched mitochondrial membrane is able to produce less ROS or to become more resistant to ROS and Ca^{2+}-induced consequent to maternal PRS. Therefore,

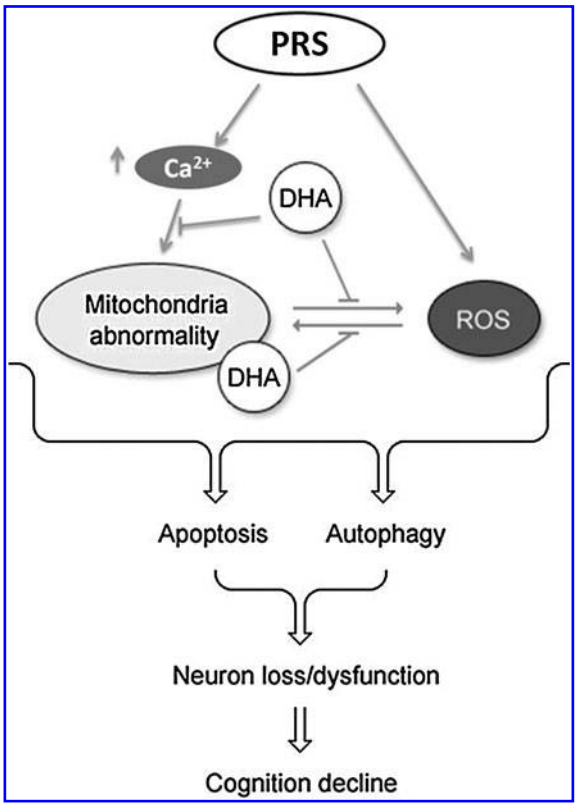

FIG. 10. Possible role of maternal DHA feeding in the protection against cognitive decline in prenatally stressed rats. PRS-induced ROS production and calcium increase might trigger autophagy and mitochondria dysfunction-induced apoptosis in offspring hippocampus. The apoptosis and autophagy will cause neuron death and impair cognitive function. Maternal feeding may cause DHA accumulation in hippocampus mitochondria. DHA-enriched mitochondrial membrane is able to produce less ROS or to become more resistant to ROS and Ca^{2+} induced consequent to maternal PRS. Therefore, offspring hippocampal neuron loss and cognitive function decline are well protected by maternal DHA feeding.

offspring hippocampal neuron loss and cognitive function decline are well protected by maternal DHA feeding (Fig. 10). The proposed mechanism warrants further study with many different tests, and lipoperoxides and DHA content in dam brain and blood are definitely two of them. Hopefully, our study and proposed mechanism could help other investigators furthering this field.

Materials and Methods

Reagents

DHA (5% in sunflower oil) was provided by Nestlé Research Center, Lausanne, Switzerland. The DHA used was tuna-derived DHA-rich fish oil. The oil is thoroughly chemically refined by being degummed, neutralized, bleached, deodorized, and stabilized with delta-enriched mixed tocopherols. Autooxidation was minimized by shipping the oil at 4°C and storage at −25°C. Cytochrome c, coenzyme Q_1, $NADP^+$, antimycin A, and dithiothreitol were purchased from Sigma Chemical Co. (St. Louis, MO); Tris base and NADH from Amersco Inc. (Palm Harbor, FL); 2,6-dichlorophenol indophenol (DCPIP) from Merck & Co. Inc.; and rotenone from Riedel De Haen Seelze (Hannover, Germany). Anti-nNOS, anti-iNOS, anti-cytochrome c, anti-Mfn-1, anti-Mfn-2, and anti-Drp-1 were obtained from Santa Cruz Biotechnology, Santa Cruz, CA. Anti-caspase 9, anti-beta-actin, anti-caspase 3, anti-Bax, anti-Bcl2, anti-Atg3, anti-Atg7, anti-Beclin-1, anti-Akt, anti-p-Akt, anti-mTOR, and anti-p-mTOR were from Cell Signaling Technology, Danvers, MA. BCA Protein Assay kits and ECL Western blotting Detection kits were from Pierce, Rockford, IL. Oxyblot protein oxidation detection kits were from Cell Biolabs, San Diego, CA. Other chemicals were all reagents of analytical purity from local vendors.

Animals

Sprague–Dawley female rats were purchased from a commercial breeder (SLAC, Shanghai). The rats were housed in a temperature- (22–28°C) and humidity-controlled (60%) animal room and maintained on a 12-h light/12-h dark cycle (lights on from 08:00 AM to 08:00 PM) with free access to food and water throughout the experiments. Female rats weighing 230–250 g and male rats weighing 280–350 g were used. At the beginning of experiments, female rats were divided into the following groups: Control, Stress, and two DHA treatment groups (100 mg/kg/day and 300 mg/kg/day denoted as Low and High groups, respectively). DHA doses were derived from recommended human doses and adjusted for the 10-fold higher metabolic rate of mice (2) (*i.e.*, 100 and 300 mg/kg/day for rats equal approximately 700 and 2100 mg daily for humans, respectively). DHA was administered by gavage (2.5 mL/day). The Control and Stress groups were given the same volume of sunflower oil. The normal rat chow contained linoleic acid (>1.14%), and linolenic acid (>0.15%). Fourteen female rats in each group were pretreated with DHA or sunflower oil for 2 weeks before mating. For every experimental group, a total of 14 female and 3 male rats were used. Virgin female rats were placed overnight with adult male rats (3:1) for mating. Vaginal smears were examined on the following morning. The day on which a smear was determined to be sperm-positive was set as embryonic day 0. Each pregnant rat was then housed separately.

Stress procedure

Each group of pregnant rats except the Control group was exposed to restraint stress on days 14–20 of pregnancy, three times a day, 2 h each time. To prevent habituation of animals to the daily procedure, restraint periods were randomly shifted to different time periods (08:00 AM to 11:00 AM, 11:00 AM to 02:00 PM, and 04:00 PM to 07:00 PM). The restraint device was a transparent plastic tube (6.8 cm in diameter) with closed ends and air holes for breathing. The length could be adjusted to accommodate the size of the animal. After birth, offspring of all groups were housed in the same animal room, and kept together with their biologic mothers. The pregnant rats of the Control group were left undisturbed. On day 21, after all offspring were weaned, male and female pups were housed separately until testing at 1 month of age.

At the end of postnatal day 30, two male and female offspring rats from the same dam were selected randomly for testing of learning and memory, using the Morris water maze (MWM). The remaining untested offspring were used for

other assays. In each group, at least one male and female offspring were selected randomly from the same litter, at least 6 litters were used for other assays. The stress procedure has been used previously and validated by several laboratories in various parts of the world (10, 12, 15, 50, 56, 63). All procedures were carried out in accordance with the United States Public Health Services Guide for Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the Shanghai Institute for Nutritional Sciences. All efforts were made to minimize the number of animals used and their suffering.

Morris water maze

The MWM apparatus consisted of a white circular pool, 180 cm in diameter and 50 cm in height, placed in an experimental room and filled with opaque water, mounted with four different visual cues at four directions, kept at room temperature (20°–23°C). A 10 cm diameter platform was submerged 2 cm under the water surface. The day before beginning the MWM test, each rat was allowed to swim freely for 120 s, in order to become familiar with the novel environment of the maze and to locate and climb onto the escape platform. The purpose of this procedure was to lower the animal's stress level during the MWM task. For four and half consecutive days, each animal performed eight swimming trials per day, with two trials beginning at each of the four start positions (N, E, S, W). The average result of the four start positions was defined to be the result for that session. The time lapse between 2 sessions was 6 hours. The order of start positions was randomized each day, and across days, for all animals. Latency and swim patterns were digitally tracked by HVS Image Water 2020™. For each trial, rats were allowed to swim until they reached the platform and climbed onto it, subject to a 120 sec cut-off. Any animal which failed was guided to find the platform and allowed to stay on it for 3 sec.

The variable recorded was the escape latency, a measure of the time required for an animal to reach the platform and remain there for more than 3 s. For cases in which rats did not reach the platform, the latency value was set at 120 s. Data were recorded and then analyzed by parametric ANOVA (repeated measures and multivariate measures).

Hippocampal mitochondrial preparation

One-month-old pups were sacrificed and hippocampi were dissected on ice and preserved at −80°C. Mitochondria were prepared using a standard method for brain mitochondria as described previously (2, 34). Protein concentrations were determined using the BCA Protein Assay kit (Pierce 23225) using bovine serum albumin (BSA) as a standard.

Total DHA content analysis in offspring hippocampus

The hippocampal tissues of the offspring (wet weight 30–50 mg) were homogenized in MTBE and directly transesterified with methanolic sodium methoxide (52). Fatty acid methyl esters were analyzed on a gas chromatograph (Agilent5980, Santa Clara, CA) equipped with a flame ionization detector. Peaks were identified by comparison of their retention times with standards. The area under the curve (AUC) of the peak was determined by using Chrom-Quest software. The quantification of DHA (C-22:6) was calculated from the ratio of AUC of the tested sample and AUC of the internal standard methyl undecanoate (C-11:0). The results were presented as a percentage of weight (g DHA/100g tissue).

Activity assays for mitochondrial enzyme complexes

Activities of NADH-ubiquinone oxidoreductase (complex I), succinate-CoQ oxidoreductase (complex II), ubiquinol cytochrome c reductase (complex III), cytochrome c oxidase (complex IV), and ATP synthase (complex V) were measured spectrometrically using conventional assays as described (35, 51).

Detection of protein carbonyls

Hippocampal samples were lysed with Western and IP lysis buffer (Beyotime, Jiangsu, China). The lysates were homogenized and protein concentrations were determined by the BCA Protein Assay kit. Protein carbonyls in soluble hippocampal proteins were assayed with the Oxyblot protein oxidation detection kit (Chemicon International, Temecula, CA). Protein carbonyls were labeled with 2,4-dinitrophenylhydrazine (DNPH) and detected by Western blot.

Real time PCR

Total RNA was extracted from 30 mg of tissue using Trizol reagent (Invitrogen) according to the manufacturer's protocol. 2 μg of RNA was reverse transcribed into cDNA. Quantitative PCR was performed using a real-time PCR system (Eppendorf, Germany). Reactions were performed with SYBR-Green Master Mix (TaKaRa, DaLian, China) with gene-specific primers. The primers are as follows:

18SRNA: 5-CGAACGTCTGCCCTATCAACTT-3 (forward) and

5-CTTGGATGTGGTAGCCGTTTCT-3 (reverse);

BDNF: 5-TCCCTTCTACACTTTACCTC-3 (forward) and

5-GTTTCACCCTTTCCACTC-3 (reverse)

mRNA contents were normalized to mRNA of 18SRNA as housekeeping gene and expressed as relative values using the $2^{-\Delta\Delta CT}$ method.

Western blot assays

Hippocampal samples were lysed with Western and IP lysis buffer (Beyotime, Jiangsu, China). The lysates were homogenized and the homogenates were centrifuged at 13,000 *g* for 15 min at 4°C. The supernatants were collected and protein concentrations were determined by the BCA Protein Assay kit. Equal aliquots (20 μg) of protein samples were applied to 10% SDS-PAGE gels, transferred to pure nitrocellulose membranes (PerkinElmer Life Sciences, Boston, MA), and blocked with 5% non-fat milk. The membranes were incubated with anti-cytochrome c (1:5000), monoclonal mouse (1:5000); anti-beta-actin (1:5000) monoclonal mouse (1:5000); anti-caspase 9 (1:1000), monoclonal mouse (1:1000); anti-caspase 3 (1:1000), polyclonal rabbit (1:1000); anti-Bax (1:1000), polyclonal rabbit (1:1000); anti-Bcl2 (1:5000), monoclonal rabbit (1:5000); anti-nNOS (1:1000), monoclonal mouse (1:1000); anti-iNOS (1:1000), polyclonal, rabbit (1:1000); anti-Mfn-1 (1:1000), polyclonal rabbit (1:1000); anti-Mfn-2 (1:1000), polyclonal rabbit (1:1000); anti-Drp1 (1:1000), polyclonal goat (1:1000); anti-Atg3 (1:1000), polyclonal rabbit (1:1000); anti-Atg7 (1:1000), polyclonal rabbit (1:1000); anti-Beclin-1 (1:1000), polyclonal rabbit (1:1000); anti-Akt (1:1000),

polyclonal rabbit (1:1000); anti-p-Akt (1:1000), monoclonal rabbit (1:1000); anti-mTOR (1:1000), polyclonal rabbit (1:1000); anti-p-mTOR (1:1000), polyclonal rabbit (1:1000) at 4°C overnight. Then the membranes were incubated with anti-rabbit or anti-mouse secondary antibodies at room temperature for 1 h. Chemiluminescent detection was performed by an ECL Western blotting detection kit. The results were analyzed by Quantity One software to obtain the optical density ratio of target protein to beta- Actin.

Statistical analysis

Data are presented as means ± S.E.M. Latencies in the Morris water maze were analyzed by two-way ANOVA for repeated measures and multivariate measures. The ANOVA analyses were always followed by LSD *post-hoc* comparisons. The data of the other assays were analyzed by one-way ANOVA which was followed by LSD *post-hoc* comparisons. In all comparisons, the level of significance was set at $p < 0.05$.

Author Disclosure Statement

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Abbreviations Used

BDNF = brain-derived neurotrophic factor
BSA = bovine serum albumin
DCPIP = 2,6-dichlorophenol indophenol
DHA = docosahexaenoic acid
DNPH = 2,4-dinitrophenylhydrazine
iNOS = inducible nitric oxide synthase
MTBE = tert-butyl methyl ether
nNOS = neuronal nitric oxide synthase
PRS = prenatal restraint stress

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