Original Article

Postnatal Maternal Separation and n3 Fatty Acid Supplementation on Cognitive Performance in d21 Rat Offspring

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Abstract

Background: Early life stress affects brain development in later life. n3 fatty acids are associated with cognitive function. **Objective:** The study in rats assesses whether maternal separation (MS) alters pup brain fatty acids (Docosahexaenoic acid (DHA)), nucleopeptide Y, and cognitive performance. It explores the influence of postnatal n3 fatty acid supplementation on these indices. **Methods:** Pregnant Wistar rats ($n = 18$) were fed chow throughout pregnancy. On delivery, dams and pups were separated into three groups Group 1: Dams fed a control diet only with no maternal separation; Group 2: Dams fed a control diet and pups were separated from dams daily for 3 hours from d2-d21 of lactation; Group 3 Dams fed a control diet supplemented with n3 fatty acids and pups were separated from dams daily for 3 hours from d2-d21 of lactation. In the present study, 6 offspring from each group were used to assess the cognitive performance and then dissected to collect brain tissue for biochemical estimations. **Results:** Weight gain in pregnancy, litter weight, and size were similar between groups. Pup weight was lower in Group 2 in comparison to Group 1 ($P = 0.014$) and Group 3 ($P = 0.028$) on d21 of lactation. MS did not affect brain fatty acids and neuropeptide Y. MS and n3 supplement did not affect the cognition of pups. **Conclusion:** The findings imply that a maternal separation did not affect the cognitive performance of the offspring at the end of lactation, n3 fatty acid supplementation increased brain DHA levels but did not influence other parameters.

Keywords: Cognition, maternal separation, n3 fatty acid, neuropeptide Y

Introduction

The importance of the mother–child relationship is reported to be critical and positively influences the mental and physical health of both mother and child.[1] Maternal separation (MS) using an animal model of early life stress (ELS) is used to study the consequences of a disturbed mother-offspring bond.[2] It is reported that early life stressful events may adversely affect the brain health.[3] Stress-related diseases and mental illness are reported to be the second-leading cause of disabilities globally, as predicted by the World Health Organization.[4]

The MS paradigm uses shorter (15 min–½ h) or longer (3 h) duration of separation between postnatal d1-d21. MS in rodents affects the behavior of the offspring, leading to cognitive dysfunction[5] in later life. However, studies examining the effect of MS report contradictory findings since alterations in hippocampal neurosteroids, serum hormones, and neurotransmitters are dependent on the exposure times of the stressor in early life.[6]

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It is reported that the use of moderate stressors may or may not influence the cognitive function. Maternal restraint daily for 3 h affects the time taken by the rodents to reach the platform in the Morris water maze test.[7] In contrast, Weinstock report that prenatal stress did not affect the time the animals remained in the trained quadrant in the test session.[8] Further, it is reported that despite of extensive research over the last three decades[9] reports are inconsistent and it is unclear whether sex (male or female offspring) alters the effects of MS and whether mice and rats are similarly affected.

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It is reported that the effects of ELS on brain and behavior may be modulated by nutrition. n3 polyunsaturated fatty acids possess anti-inflammatory effects and may be an effective nutrient in preventing disorders caused due to stress.[10]

In view of the above background, the present study was undertaken to study if a MS paradigm for 3 h has any impact on the pup brain fatty acids, neuropeptide Y (NPY) levels, and cognition. We further investigated if supplementation of n3 fatty acids to the MS dams affects these parameters.

Methods

Study design

The study was conducted after seeking ethics approval at Bharati Vidyapeeth animal house facility.

A total of 25 female Wistar rats weighing 170–180 g were used in this study. Of which, 18 rats got pregnant and were allowed to deliver normally on day 22 of gestation. On delivery, the dams and their pups were assigned to three dietary groups ($n = 6$ dams/group) and their litter size was culled to eight/dam in each group and were as follows: Group 1: Dams fed a control diet only with no MS; Group 2: Dams fed a control diet only and the pups were separated from the dams daily for 3 h; and Group 3: Dams fed a control diet and supplemented with omega-3 fatty acids and the pups were separated from the dams daily for 3 h. The dose of the oral supplement of omega-3 fatty acids was (fish oil 1.2 mg/day). The n3 fatty acid supplementation (fish oil capsule (Maxepa, Procter and Gamble Health Ltd) containing eicosapentaenoic acid (EPA) 180 mg and docosahexaenoic acid (DHA) 120 mg was given at a dosage of 1.2 mg/day.[11]

Dams from Groups 2 and 3 and their pups were subjected to a daily separation from 10.00 am to 1.00 pm. After 3 h, the dams and their pups were caged together. This separation was followed from d2 to d21 of lactation. At the end of lactation, cognitive assessments were carried out on 6 offspring from each group (3 males and 3 females). After which the offspring were dissected to collect brain tissue, put in liquid nitrogen and then stored at − 800°C for fatty acid and NPY estimation. The data from the study was blinded to the person carrying out the experimental procedures and then analysed after the completion of the study.

Brain weights

Pup brain was collected upon dissection and was weighed on an electronic balance (Shimadzu: range from 0.001 g to 220 g).

Fatty acid estimation

The protocol for fatty acid estimation of 15 fatty acids from the rat brain tissue has been used by authors earlier.[12] The Folch method was used for lipid extraction[13] followed by the transesterification process. A gas chromatogram (PerkinElmer) and a capillary column (SD 2330; PA, USA) were used for fatty acid estimation. Fifteen fatty acids, such as saturated (myristate, palmitate, and stearate), monounsaturated (myristeolate, palmiteolate, oleate, and nervonic acid), n3 fatty acids (alpha linoleic acid, EPA, docosapentaenoic acid, DHA), and omega 6 fatty acids (linolenic acid, dihomogamma linolenic acid, gamma linolenic acid, arachidonic acid, and docosapentaenoic acid), were analyzed and reported as g/100 g fatty acids.

Neuropeptide Y levels

ELISA kits procured from CUSABIO CSB-E13431r, USA, were used for these estimations. The assay uses the quantitative sandwich enzyme immunoassay technique. Microplates provided were precoated with NPY-specific antibody to which any NPY present in the standard or sample is bound. The unbound substances were washed off, and a biotin-conjugated antibody specific for NPY was added to the wells. After washing, the horseradish peroxidase conjugate was added to the wells. Following a wash to remove any unbound conjugate, a substrate solution was added to the wells, and the color developed was proportional to the amount of NPY bound in the initial step. The color development was stopped, and the intensity of the color was measured at 450 nm. Total protein from the homogenates was estimated by the method of Folch *et al*.[13] and NPY concentration was reported as pg/mg total protein.

Cognitive tests

Morris water maze

The protocol using the Morris water maze for cognitive assessment has been used by authors in another study, which examined the effect of maternal vitamin B_{12} deficiency and omega 3 fatty acid supplementation on rat offspring cognition by us earlier.[14] A circular tank heighted 62 and 115 cm in diameter was used as the maze. This test is carried out for 5 consecutive days the animals have to locate a platform, which is submerged under the water surface. In each trial, the escape latency, i.e., the time the animal takes to locate the platform, was noted. The trial lasted for 5 min, after which the animal was placed in its cage. Day 1 was training day, followed by 4 days of trials. Spatial learning memory was assessed by measuring escape latency, which was the time the animal takes to locate the platform.

Radial eight-arm maze

The radial eight-arm maze test was performed for 5 consecutive days.[14] An octagonal platform at the center with eight radiating arms was the maze. Before the test, all animals were fasted overnight. Animals were kept on the central platform and allowed to explore the maze for 300s. A food pellet (bait) was kept at the end of alternate arms. The path traveled by the animal in the maze for 5 min was tracked using Anymaze Software, Gentech. The following was recorded: Correct choice: Initial entry in a baited arm; Reference memory error: visit to an unbaited; Working memory error (WME): repeat entry to a baited arm; and Reference and WME: re-entry into an unbaited arm.

Statistical analysis

Fatty Acids, Neuropeptide Y levels and Cognitive Assessments are reported as mean ± standard deviation (SD). The mean

values for the treatment groups were compared with the control group at conventional levels of significance, $P < 0.05$ using analysis of variance. SPSS (SPSS Inc., Wacker Drive, Chicago, IL)/PC+ package Version 20, Chicago IL, USA was used for analysis.

Results

Reproductive performance

The total weight gain of dams during pregnancy was similar between groups: (1) Group 1: 140.5 ± 10.55 g; (2) Group 2: 146.17 ± 29.728 g, and (3) Group 3: 146.83 ± 33.820 g. The litter size and litter weight were also similar between groups. However, the pup weight was lower in Group 2 compared to both Group 1 ($P = 0.014$) and Group 3 ($P = 0.028$) on d21 of lactation.

Fatty acid levels

MS had no effect on brain fatty acids. n3 fatty acid supplementation improved brain DHA in Group 3 pups in comparison to Group 1 ($P < 0.05$) and Group 2 ($P < 0.01$). All other fatty acids were similar between groups [Table 1].

Pup brain neuropeptide Y levels

The NPY levels in the pup brain in different groups were as follows: Group 1: (8.30 ± 3.48 pg/mg protein); Group 2: (5.97 ± 1.21 pg/mg protein); and Group 3: (8.78 ± 2.85 pg/mg protein) and did not differ between groups [Figure 1].

Table 1: Pup brain fatty acids at the end of lactation

Brain FA (g/100g)	Group 1	Group 2	Group 3
Myristic acid	0.01±0	0.01±0	0.01±0
Myristoleate	1.51±1.7	0.77±0.89	0.47±0.30
Palmitic acid	24.9±2.36	26.13±1.51	25.7±1.29
Palmitoleate	0.84±0.8	0.54±0.22	0.67±0.5
Stearic acid	21.95±0.98	23.03±1.86	21.69±1.43
Oleic acid	13.04±1.73	12.92±1.48	13.43±1.53
Linoleic acid	3.07±1.43	2.24±0.54	2.49±0.79
Gamma linolenic acid	0.28±0.13	0.51±0.15	0.45±0.11
Alpha linolenic acid	0.16±0.12	0.16±0.17	0.24±0.06
Di homo gamma linoleic acid	2.01±1.58	1.41±1.57	1.55±1.18
Arachidonic acid	12.68±0.98	12.42±1.79	12.83±1.56
Eicosapentaenoic acid	0.85±0.76	0.49±0.38	0.26±0.12
Nervonic acid	3.15±0.38	3.09±0.56	2.99±0.28
N6 docosapentaenoic acid	2.4±0.79	2.27±0.28	1.26±0.25
n3 docosapentaenoic acid	0.4±0.15	0.35±0.1	0.39±0.10
Docosahexaenoic acid	11.33±1.39	10.32±1.06	13.78±2.05*,##
Saturated fatty acids	46.86±3.03	49.17±1.94	47.41±2.05
Monounsaturated fatty acids	18.54±1.35	17.32±1.35	17.57±1.51
N3 polyunsaturated fatty acids	12.75±1.75	11.32±1.11	14.67±2.16##
N6 polyunsaturated fatty acids	20.44±1.84	18.85±2.38	18.57±1.17

*P<0.05 as compared to control, ##P<0.01, as compared to MS. Data are expressed as mean±SD. Group 1: Dams fed a control diet with no maternal separation; Group 2: Dams fed a control diet and the pups were separated from the dams daily for 3 h; Group 3: Dams fed a control diet and an oral supplement of omega-3 fatty acids (fish oil 1.2 mg/day) and the pups were separated from the dams daily for 3 h. MS: Maternal separation, MS + O: Maternal separation supplemented with omega-3 fatty acids, SD: Standard deviation, FA: Fatty acid

Cognitive performance

Cognition of the offspring was similar between groups [Figures 2 and 3].

Discussion

This present study reports the effect of MS on brain fatty acids and NPY levels and cognitive performance of pups at the end of lactation. The findings of the study are: (1) MS lowered the pup weight at the end of lactation as compared to both Groups 1 and 3, (2) MS does not alter levels of fatty acids and NPY in the brain of the offspring, (3) Cognition was similar across all groups, and (4) n3 fatty acid supplementation to dams in Group 3 increased the levels of brain DHA as compared to both Groups 1 and 2. However, n3 fatty acid supplementation did not influence NPY levels and cognitive performance of the offspring at the end of lactation.

Maternal separation resulted in lower pup weight during the period of lactation and is similar to the findings reported by other studies examining the effect of maternal separation (early life stress) on cognitive performance in the offspring[15-17] and have attributed it to poor feed intake. In the current study, the feed intake of dams was not recorded. In contrast, a few earlier studies report no change in pup body weight as a consequence of MS.[18] In the present study, brain fatty acids were unaltered in offspring subjected to MS. There is limited literature on MS and brain fatty acids. In contrast, to our findings, an earlier study by Yam *et al*. reports changes in fatty acids in both central and peripheral tissues in an ELS mouse model subjected to chronic stress.[19] In the MS + O group, there was an increase in DHA in the pup brain as compared to both the control and MS groups. Our findings are in line with several studies reported by others[20] and by us earlier where maternal n3 fatty

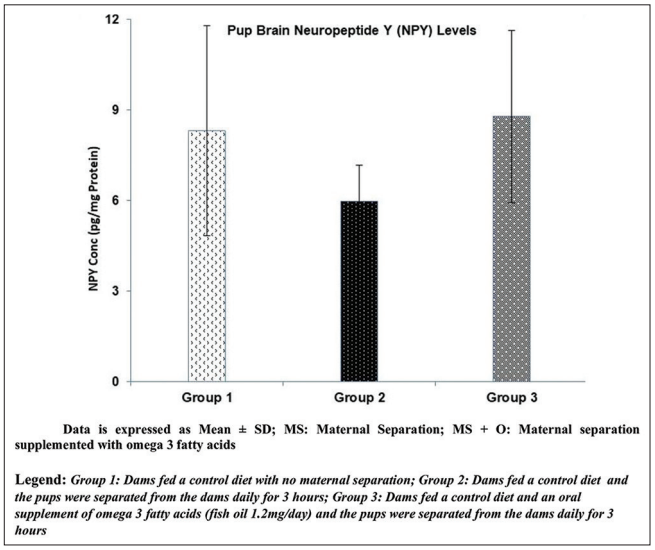

Figure 1: Group 1: Dams fed a control diet with no maternal separation; Group 2: Dams fed a control diet and the pups were separated from the dams daily for 3 hours; Group 3: Dams fed a control diet and an oral supplement of omega 3 fatty acids (fish oil 1.2 mg/day) and the pups were separated from the dams daily for 3 hours

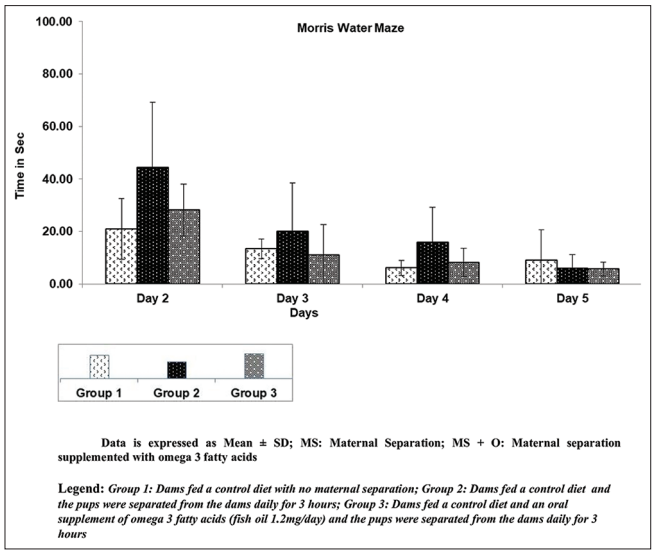

Figure 2: Group 1: Dams fed a control diet with no maternal separation; Group 2: Dams fed a control diet and the pups were separated from the dams daily for 3 hours; Group 3: Dams fed a control diet and an oral supplement of omega 3 fatty acids (fish oil 1.2mg/day) and the pups were separated from the dams daily for 3 hours

acid supplementation is reported to increase the pup brain DHA levels to dams fed a high fat diet deficient in Vitamin B12;[12] dams induced with preeclampsia and supplemented with n3 fatty acids;[21] and dams fed Vitamin B12 deficient diet and supplemented with n3 fatty acids.[14] It is reported that consumption of omega-3 improved learning, memory ability, and cognition in humans.[22] Our earlier animal studies have reported the beneficial effects of n3 fatty acids on cognitive functions in pups born to dams fed a micronutrient imbalanced diet supplemented with n3 fatty acids;[23] in pups born to dams fed a Vitamin B12 deficient diet supplemented with n3 fatty acids;[14] and pups born to dams induced preeclampsia and supplemented with n3 fatty acids.[21]

In addition to n3 fatty acids, it is reported that NPY modulates neuroplasticity and learning.[24] The present study reports no effect of MS on levels of NPY2 in the brain of pups. Similar to our findings, a review article compiles findings of Autio *et al*., who report no change in learning and memory abilities between NPY gene-deficient and wild-type mice.[25] In contrast, maternal deprivation for 24 h has shown a slow reduction of NPY in the arcuate nucleus of the hypothalamus.[26] MS lowered NPY in female pups compared to male pups.[16] NPY is abundant in the central nervous system[27] and regulates cognitive processes[28] by interacting with its presynaptic Y2 (Y2R) receptors.[24] In the present study, n3 fatty acid supplementation to MS animals did not affect NPY, and they remained comparable to control. In contrast to our findings, lower NPY 1 receptor and BDNF levels are reported in the brain regions of rats fed a high-fat diet, thereby increasing anxiety-like behavior.[29] Bhatia *et al*. report lower levels of NPY1 receptor and BDNF in the hypothalamus and hippocampus of rat male offspring when fed a n3-deficient diet.[30] Since we do not report any changes in the present study in the supplementation (n3 fatty acid) group, suggesting that the dose and duration of n3 fatty acid supplementation need to be modified.

It is reported that rodents are highly dependent on maternal care after birth and any disturbance in the mother and pup interactions leads to detrimental alterations in the offspring. Thus, maternal separation (MS) is an accepted model for investigating disruption of mother and pup relationship.[31]

Several studies have reported cognitive testing on postnatal d21 of rats using both the Morris water maze and the radial eight arm maze.[32-37] In this study, MS did not influence the cognitive performance of the pups at the end of lactation. Similar to our finding, a study by Maghami *et al*. reports no change in cognitive performance as a consequence of MS in the pups using the Barnes maze test,[15] possibly since although these pups were exposed to the Barnes maze test, they were not habituated to its environment. While a recent study reports that MS increased memory performance as tested using the Morris water maze test and also reports an increase in the BDNF gene expression.[38] On the other hand, MS reported poor cognitive outcomes in adult rodents in whom the expression level of the N-methyl-d-aspartate receptor subunit GluN1, known to affect cognitive processes, was lowered.[39] The discrepancies in findings may be due to differences in rat strain, MS procedures, and anxiety-testing methods.[40] Since in the present study we report no change in the cognitive testing, it may be possible that the duration of separation needs to be increased since prolonged separation, i.e., more than 3 h is reported to reduce learning and memory abilities in adults.[41] Second, it may be possible that the age at which the cognitive testing was performed may not be appropriate since rats have shown that MS effects may be more prominent after postnatal day 30.[42]

Similar to the MS group, MS + n3 fatty acid supplementation also did not affect the cognitive performance of the pups in the present study. Similar to our finding, Pusceddu *et al*.[43] reported that EPA and DHA supplementation improved cognition and anxiety in rats with no separation but not with MS. It is suggested that lifetime supplementation of n3PUFA had greater effect rather than supplementing during preweaning or postweaning period.[44]

The study has a limitation, i.e., it did not explore the effect of maternal separation on cognitive performance of the offspring at adult age.

Conclusion

In conclusion, the present study reports that maternal separation of pups for 3 hours during lactation does not affect the brain fatty acids, NPY levels, and cognition in offspring at d21. n3 fatty acid supplementation to the MS dams increased the pup brain DHA levels but did not affect the NPY levels and cognitive performance of the offspring.

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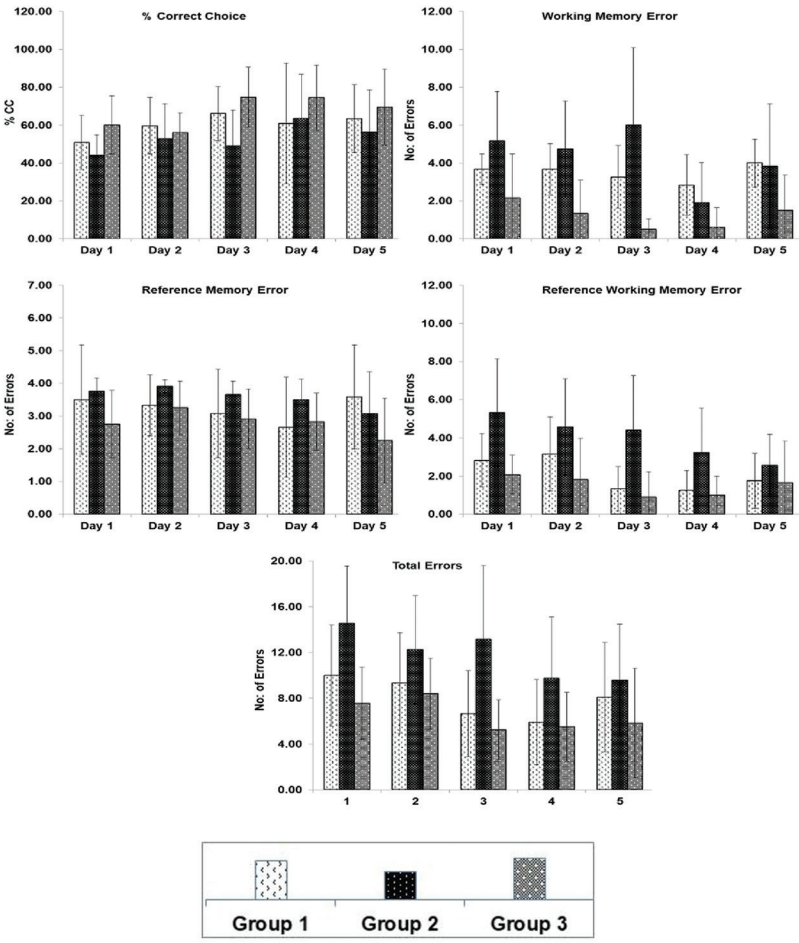

Data is expressed as Mean ± SD; MS: Maternal Separation; MS + O: Maternal separation supplemented with omega 3 fatty acids

Legend: ***Group 1: Dams fed a control diet with no maternal separation; Group 2: Dams fed a control diet and the pups were separated from the dams daily for 3 hours; Group 3: Dams fed a control diet and an oral supplement of omega 3 fatty acids (fish oil 1.2mg/day) and the pups were separated from the dams daily for 3 hours***

Figure 3: Cognitive assessment using Eight Arm Radial Maze Test

Ethical consideration

The study was initiated after seeking ethical approval on 5/2/2022. The ethical approval number is: BVDUMC/4I}2022/00 1/001.

Author's contribution statement

AK designed and conducted the study; AK collected and analyzed the data and contributed to writing the manuscript.

Data availability statement

Data will be available with corresponding author on request.

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Conflicts of interest

There are no conflicts of interest.

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