Early life diet containing prebiotics and bioactive whey protein fractions increased dendritic spine density of rat hippocampal neurons

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**Abstract**

Early life nutrition plays an important role in brain development. Emerging research in rodents, piglets and humans suggest that prebiotics, milk fat globule membrane and lactoferrin may each play unique roles in brain development and cognitive functions. However, knowledge of their combined impact is lacking. We show here that providing weanling rats with a diet containing milk fat globule membrane, lactoferrin and a polydextrose/galactooligosaccharide prebiotic blend led to a significant increase in total dendritic spine density in hippocampal dentate gyrus neurons. Region-specific alterations in dendritic spine density and morphology could provide a mechanistic basis underlying broader cognitive benefits, but further research is required to demonstrate functional consequences of these observations.

**Introduction**

Optimal nutrition early in life is exceptionally important when rapid brain development occurs. In human infants, breast milk is recognized as the optimal form of nutrition due to its many roles in supporting infant growth and development. Some human milk bioactives thought to exert beneficial effects on developing brain such as docosahexaenoic acid (DHA), milk fat globule membrane (MFGM), and lactoferrin have been individually studied (Clandinin et al., 2005; Diersen-Schade and Boettcher, 2005; Timby et al., 2014; Chen et al., 2014), but knowledge of their combined impact is lacking. Aside from milk bioactives, prebiotics have been reported to help maintain normal microbial communities in infants (Scalabrini et al., 2012) and modulate central nervous system molecular signalling pathways and behavioral responses to stress in rodent models (Savignac et al., 2016; Savignac et al., 2013). While a reductionist approach would isolate each of these to study their specific effects, we have chosen a more translational approach in this animal model to evaluate their combined effects as would be expected within the diet of a developing infant.

Although nutrition can influence many aspects of brain development, recent reports suggest differential vulnerability across neural pathways. In the absence of normal gut microbiota, germ free mice showed altered brain development and behavior compared with conventional mice (Heijtz et al., 2011). The effects were associated with dysregulation of growth factor gene expression narrowly expressed across neuroanatomical regions, but highlighted by changes in prefrontal cortex and dorsal hippocampus. Following on this, we targeted these regions in the current study where we investigated dendritic spine density and morphology, key synaptic plasticity-related indicators which respond to treatments for psychiatric and affective disorders (Li et al., 2010; Kitahara et al., 2016). The observation that nutrition can increase the density of dendritic spines, membranous protrusions extending from dendrites, was first reported by Sakamoto et al. (2007) using a combination of DHA and uridine. Importantly, here we investigated mechanisms of action for a mixture of MFGM, lactoferrin, and prebiotics beyond what is provided by DHA since DHA was included in both control and test diets.

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1. Introduction

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Although nutrition can influence many aspects of brain development, recent reports suggest differential vulnerability across neural pathways. In the absence of normal gut microbiota, germ free mice showed altered brain development and behavior compared with conventional mice (Heijtz et al., 2011). The effects were associated with dysregulation of growth factor gene expression narrowly expressed across neuroanatomical regions, but highlighted by changes in prefrontal cortex and dorsal hippocampus. Following on this, we targeted these regions in the current study where we investigated dendritic spine density and morphology, key synaptic plasticity-related indicators which respond to treatments for psychiatric and affective disorders (Li et al., 2010; Kitahara et al., 2016). The observation that nutrition can increase the density of dendritic spines, membranous protrusions extending from dendrites, was first reported by Sakamoto et al. (2007) using a combination of DHA and uridine. Importantly, here we investigated mechanisms of action for a mixture of MFGM, lactoferrin, and prebiotics beyond what is provided by DHA since DHA was included in both control and test diets.
2. Materials and methods

2.1. Animals

Weanling male Long Evans rats (Harlan Lab., Indianapolis, IN) were housed in groups of three in a climate-controlled environment and maintained on a 12-h light/dark cycles. Each rat was randomly assigned across control and experimental groups to assure equivalent body weight distribution between groups. For the entire study, 15 rats were assigned per diet with five rats per treatment group utilized for spine density analysis. Control (CONT) animals received a modified AIN-93 diet containing DHA/ARA (0.7 g/kg and 1.4 g/kg body weight, respectively). The experimental (TEST) group received a similar AIN-93 based diet containing polydextrose (PDX, 6.05 g/kg, Danisco, Terre Haute, IN, USA), galactooligosaccharide (GOS, 6.05 g/kg, FrieslandCampina, Zwolle, Netherlands), lactoferrin (1.8 g/kg, Tatua Cooperative Dairy Company, Morrinsville, New Zealand), and milk fat globule membrane (Lacpradon MFGM-10®, 15.1 g/kg, Arla Food Ingredients, Aarhus, Denmark) that was adjusted to ensure it delivered equivalent calories, protein, carbohydrates, lipids and micronutrients. This ensured that the only differences between the diets were from the bioactive components themselves. Diets and water were provided ad libitum starting at weaning (postnatal day 21) for 40 days until study completion when the rats were approximately 9 weeks old (61 days old). Body weight and food consumption were measured three times a week for the duration of feeding. All experimental animal procedures were performed according to regulations of the Animal Welfare Act, principles of the Public Health Service Policy on Humane Care and Use of Laboratory Animals (PHS Policy), and in accordance with the Institutional Animal Care and Use Committee approved protocols.

2.2. Brain collections, ballistic dye labeling, and microscopy

Rats were deeply anesthetized while perfusion fluid and 0.1 M PBS was administered at a rate of 25 ml/min. This was followed by the perfusion of 4% paraformaldehyde/PBS solution for fixation for 10 min at the same rate. Upon completion of the perfusion procedure, the brains were removed, post-fixed in 4% paraformaldehyde for 30 min and then placed in 0.1 M PBS. The brains were sectioned using a tissue vibratome (Leica VT1000) to collect sections (300 μm thick) from the anterior to posterior extremes of each brain. Ballistic dye labeling (DiI) was performed according to protocols developed by Afraxis Inc. to label target neurons (Ota et al., 2014). Sections were slide mounted and cover slipped. Laser-scanning confocal microscopy (Olympus FV1000) was performed using a 63× objective (1.42 NA) to scan individually labeled neurons at high resolution (0.103 x 0.103 x 0.33 μm voxels). Target neurons were identified in the brain region of interest by anatomical location and cell morphology. Microscopy was performed blind to experimental conditions. A minimum of 5 cells per animal were sampled (both left and right hemispheres included) from each of the two target brain regions: the medial prefrontal cortex (mPFC) and dorsal hippocampus. For multiple dendritic targets identified from the same neurons, independent micrographs (>50 μm dendritic length) (1 x/cell) were collected for each sample.

2.3. Assessment of dendritic spine morphology

Blind deconvolution (AutoQuant) was applied to raw three-dimensional digital images. Dendritic spine morphometry was analyzed from 2 sample positions on primary apical dendrites of Layer 5 pyramidal neurons (200 and 300 μm from the soma) in mPFC. A third mPFC sample position was collected from proximal tufts on the same neurons. Additionally, two positions from dentate granule neurons in dorsal hippocampus (DG) were selected: (1) primary dendrites within the inner molecular layer (IML), and (2) secondary dendrites from the middle molecular layer (MML). Analysis for spine density and morphology was performed on deconvolved Z-series micrographs by trained analysts as described (Burgdorf et al., 2015; Ota et al., 2014). Briefly, individual spines were measured manually for (a) head diameter, (b) length, and (c) neck diameter from image Z-stacks using custom-built Afraxis ESP software. Each dendrite was analyzed by 2–3 independent analysts. The mean spine-by-spine accuracy, calculated as the percent of co-detected spines versus total spines detected by a given analyst, was 80.48%. Automated image assignment software (C++, Java) distributed images to analysts in a randomized manner and ensured that each analyst performed measurements of near equal numbers of dendrites per group. Analysts were blinded to all experimental conditions (including treatment, brain region, and cell type). Spine classification was performed by custom-built software (Matlab) and followed an established evaluation workflow (Rodriguez et al., 2008): head-to-neck ratio criterion = 1.1 and HD criterion = 0.37 μm. Detached spine heads from dendritic shaft were excluded; these are nearly non-existent when labeling neurons with a membrane dye. Statistical analysis of inter-analyst variability for each dendrite was examined on-line and used to eliminate dendrites that did not meet inter-analyst reliability criteria: A dendrite was incorporated into the final analysis only if measurement distributions for all three measures failed to be significantly different between analysts.

2.4. Statistical analysis

For spine density and spine morphological classification, data across analysts were averaged to report data for each dendrite. Data population values (N’s) were reported for dendrites collected equally from all rats. Values are reported as group means ± standard errors of the mean (SEMs). For all group comparisons of parametric values, statistical significance was determined using the multivariate analysis of variance analysis of variance test (MANOVA; IBM SPSS Statistics v. 24). For both brain regions, homogeneity of variance-covariance matrices was observed (Box’s test of equality of covariance matrices: p > 0.003). Post-hoc comparisons were assessed using the one-way MANOVA.

3. Results and discussion

It is well understood that neuroanatomical development does not progress uniformly across all regions and pathways and that differential maturation contributes to differential development of cognitive capacities (Kolb et al., 2012). Therefore, we selected two brain regions for investigation: the medial prefrontal cortex (mPFC), which mediates a broad set of executive functions, and dorsal hippocampus, which is critical for memory formation and has demonstrated responses to a variety of early life interventions (Grigoryan and Degal, 2016). Furthermore the specific sample positions chosen from mPFC and DG were selected based on well-understood discriminative connectivity between these laminae (Llorens-Martin et al., 2014). Representative laser-scanning confocal micrographs from both brain areas are shown in Fig. 1A. Raw measures of spine morphological features (head diameter, spine length, and neck diameter) were used to classify each spine as thin, mushroom, or stubby (Hara et al., 2012). This classification set has been used to characterize dendritic spines across the lifespan and associated with a variety of cognitive states Bourne and Harris (2007). Densities of these subclasses, as well as total spine density, were assessed for all sample positions described above. In hippocampus, a significant interaction of diet and sample position (IML vs. MML) was detected, F(4, 93) = 3.078, p = 0.02; Wilks’ Λ = 0.883;
Fig. 1. Representative laser scanning confocal micrographs (maximum projections shown) of dendritic segments and dendritic spines in neurons from rats administered TEST or CONT diets. Micrographs from medial prefrontal cortex (mPFC) show primary apical (left, Apical; 200 μm from soma) and tuft (right) dendrites. Micrographs from dorsal hippocampus show samples from inner molecular layer (left, IML) and middle molecular layer (right, MML). High magnification insets show the presence of various spine morphological types, including thin (arrow) and mushroom spines (arrowhead).

Table 1

Dendritic spines measurement raw value medians (group mean ± SEM; see Methods for details). Raw spine length and head diameter median values are shown for CON vs. TEST conditions. For medial prefrontal cortex (mPFC), sample positions were derived from layer 5 pyramidal neurons as follows: (#1) 1° apical dendrite, 200 μm from soma, (#2) 2° apical dendrite, 300 μm from soma, and (#3) proximal tufts. For dorsal hippocampus, dentate granule neurons were sampled as follows: (#1) 1° apical dendrite within inner molecular layer, and (#2) 2° apical dendrite within middle molecular layer. A significant interaction of group vs. sample position was observed for mPFC, F(6, 284)=3.463, p=0.003; Wilks’ Λ = 0.868; partial η² = 0.068. Bolded values indicate significant difference from CON group (p=0.013, 1-way MANOVA post-hoc test). No main effect or interaction was observed for dorsal hippocampus, F(3, 94)=1.151, p=0.33; Wilks’ Λ = 0.965; partial η² = 0.035.

| Sample position #1 | Sample position #2 | Sample position #3 |
|---------------------|---------------------|---------------------|
| CON | TEST | CON | TEST | CON | TEST |
| CON | TEST | CON | TEST | CON | TEST |
| CON | TEST | CON | TEST | CON | TEST |
| CON | TEST | CON | TEST | CON | TEST |

| Sample position #1 | Sample position #2 | Sample position #3 |
|---------------------|---------------------|---------------------|
| CON | TEST | CON | TEST | CON | TEST |
| CON | TEST | CON | TEST | CON | TEST |
| CON | TEST | CON | TEST | CON | TEST |
| CON | TEST | CON | TEST | CON | TEST |

| Sample position #1 | Sample position #2 | Sample position #3 |
|---------------------|---------------------|---------------------|
| CON | TEST | CON | TEST | CON | TEST |
| CON | TEST | CON | TEST | CON | TEST |
| CON | TEST | CON | TEST | CON | TEST |
| CON | TEST | CON | TEST | CON | TEST |
modification by diet was a selective increase in total spine density within the hippocampus. The total density difference was driven largely by thin and mushroom spine types. The former class, informally referred to as “learning spines” and associated with memory acquisition (Bourne and Harris, 2007), expresses dramatic losses during aging and are implicated in senescence. Losses of large-diameter and mushroom-type spines, in contrast, are more closely related to impairments of long-term memory storage and neurodegeneration, especially in hippocampus (Hara et al., 2012), as observed in Alzheimer’s Disease rodent models (Penazzi et al., 2016). Evidence for the inverse exists as well: elevations of both long-thin and mushroom spines correlate with multiple forms of cognitive enhancement (Kitahara et al., 2016; Lauterborn et al., 2016). This effect was observed within the MML of the dentate gyrus, which along with the outer molecular layer, receives perforant pathway inputs from the entorhinal cortex. Afferent projections to the MML largely derive from the medial entorhinal cortex and are known to mediate spatial navigation and information coherence among others (Cauter et al., 2013). The IML, in contrast, predominantly receives inputs via the commissural/associational fiber system from the contralateral mossy cells. The differential impact of diet on dendritic spines between hippocampal lamina supports the notion that nutritional supplementation facilitates neuronal maturation heterogeneously across neuroanatomical pathways. This differential effect following prolonged treatment likely results either from direct modification of the target circuits, including modifications to gene expression, synaptic targeting, and more (Routtenberg et al., 2000) or alternatively, the nutritional regimen may induce changes to the regions tested here through alteration of distant brain regions through modified afferent activity (Steward et al., 2007).

In contrast to effects in the hippocampus, relatively little impact by diet was observed in layer 5 pyramidal neurons of the medial prefrontal cortex, a dendritic arborization with demonstrated rapid and long-term spine responses to neuropsychiatric therapeutics (Ampuero et al., 2010; Ota et al., 2014). Here we observed a spine subtype effect, although not to the more mature classes of spines nor to total spine density. The mPFC also expressed the only significant change to raw structural features: an increase in both head diameter and spine length. Together, these effects do not indicate complimentary modifications that would provide clear interpretation of the results for mPFC. It is unclear at this time why mPFC neurons failed to express robust responses to diet similar to DG; this may be related to intrinsic differences in spine maturation over development (Kolb et al., 2012), differences in responses to activity (Zhang et al., 2011), or other factors. In total, the findings here warrant further study with prolonged or varied feeding of the nutritional ingredients.

Formation of dendritic spines requires a significant expansion of cell membrane area (Bryan et al., 2004). DHA has been shown to support increased spine formation by promoting the synthesis of membrane phospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) (Sakamoto et al., 2007). The quantity of the synthesized membrane from these phosphatides significantly increased per brain cell and in whole brain (Wurtman et al., 2006). Since these membrane phosphatides are among the major constituents of polar lipids found in MFGM (Lopez and Ménard, 2011), it is possible that the presence of membrane components in the diet contributed to the markedly increased dendritic spine formation observed here. In addition, dietary lactoferrin was recently shown...
to significantly alter the level of molecules that critically mediate activity-related synaptic structural modifications, including brain derived neurotrophic factor (BDNF) and poly-sialic acid neural cell adhesion molecule (PSA-NCAM) in neonatal piglets (Chen et al., 2014). Although not evaluated in the current study, it is possible that lactoferrin could also contribute to changes in dendritic spines through these mechanisms.

While a specific combination of nutrient has been reported to increase spine density and promote synaptogenesis (Wurtman et al., 2009), undifferentiation showed a reversed effect on spine density and postsynaptic potentials (Zhang et al., 2013) highlighting the importance of optimal nutrition in supporting brain development. Although it is not possible to determine the individual contribution of all components of the diet to the increased spine density, other very recent studies utilizing this combination of nutrients have shown effects on structural brain development (Mudd et al., 2016), neurotransmitter expression and gut development (Berding et al., 2016) in young piglets. Collectively, these results suggest that a combination of bioactive ingredients in early life diet can influence structural brain development.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijdevneu.2016.09.001.

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