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Neonatal Growth Rate and Development of Mice Raised on Milk Transgenically Enriched With Omega-3 Fatty Acids

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ABSTRACT: The n-3 polyunsaturated fatty acid (PUFA) status of the neonatal brain has been associated with cognitive capability in mice. Previously, transgenic mice expressing the *Caenorhabditis elegans* (*C. elegans*) n-3 fatty acid (FA) desaturase gene under the control of a lactation-induced mammary gland promoter were found to produce milk containing significantly elevated levels of α-linolenic (ALA) and eicosapentaenoic (EPA) acid. In this study, the preweaning growth rate and development of mouse pups consuming elevated n-3 PUFA milk produced by transgenic dams were evaluated using the Wahlsten observational test battery and the object novelty preference test. Brains were collected at weaning and analyzed for FA composition. Pups nursed on transgenic dams had earlier eye opening, higher visual placement scores, and 1.6-fold more docosahexaenoic acid (DHA) in their brains compared with pups raised on wildtype dams. There was no significant effect of milk treatment (transgenic *versus* control) on other developmental parameters or novelty preference behavior. The pups consuming the elevated n-3 PUFA transgenic milk had slower preweaning growth rates such that pups reared on wildtype dams producing control milk were heavier than pups reared on transgenic dams producing high n-3 PUFA milk by postnatal day 18. This transgenic model enables the provision of a high n-3 PUFA lactational environment independent of maternal diet or gestational FA status. **(*Pediatr Res* 62: 412–416, 2007)**

The health benefits of n-3 polyunsaturated fatty acids (PUFAs) have been demonstrated for a variety of diseases and disorders (1–3). N-3 PUFAs have also been shown to play a role in brain development. Studies found a positive correlation between the amount of alpha linolenic acid (18:3n-3, ALA) supplied in infant formula and neurodevelopment scores at 1 y of age (4). Improved visual acuity and cognitive development were also observed in infants supplemented with dietary n-3 highly unsaturated fatty acids (FAs) [FAs with ≥20 carbon atoms and four or more double bonds (HUFAs)] (5). Other studies reported no effect of n-3 HUFA supplementation on cognition, motor development, or growth when compared with infants fed on nonsupplemented formula and breast-fed infants (6). Although there is dissent in the literature as to the exact extent to which n-3 HUFA supplementation influences brain development, the majority of studies suggested that there is a weak association between the amount of time that infants are breast-fed and their scores on performance tests (7).

In addition to research conducted on human infants, numerous studies have used model species to investigate the effects of n-3 PUFAs on behavior and cognitive capability as recently reviewed by Fedorova and Salem (8). The most widely used approach for the study of the effects of n-3 PUFA on rodent behavior has been to induce dietary n-3 PUFA deficiency. The majority of such studies have reported behavioral testing scores with statistically significant deficits attributable to dietary n-3 FA restriction (7,9,10). Evidence of a dose-response effect of FA supplementation has also been shown in some studies, and this finding is important because many rodent studies involve n-3 FA deficiencies of such severity that they are unlikely to be encountered in human populations under normal circumstances (7,11).

Because vertebrates lack the n-3 FA desaturase responsible for synthesizing n-3 PUFAs, they must derive these essential FAs from fish and other dietary sources rich in n-3 FAs. Due to concerns about the sustainability (12,13) and safety of fish and shellfish consumption (14), alternative sources of n-3 HUFAs have been the subject of much research. The manipulation of desaturase genes in plants to create PUFA "factories" is a possible solution. Researchers have attempted to alter the FA content of plants but achieved mixed results depending on the specific desaturase gene and gene source selected for expression (15). Although transgenic manipulation of plant FA production has great potential, the number of enzymes required to be transgenically expressed to produce HUFAs is a potential barrier to success. Greater control over substrates and products is needed for optimization and to increase efficiency (16).

Mammalian expression of FA desaturases to produce essential PUFAs is an intriguing prospect. Mammals possess the endogenous ability to convert ALA to eicosapentaenoic (20:5n-3) acid (EPA) and docosahexaenoic (22:6n-3) acid (DHA), so fewer exogenous enzymes would be needed to produce HUFAs than with plants. Expression of the *C.*

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Abbreviations: ALA, α-linolenic acid, 18:3n-3; ***C. elegans,*** *Caenorhabditis elegans*; **DHA,** docosahexaenoic acid, 22:6n-3; **EPA,** eicosapentaenoic acid, 20:5n-3; **FAs,** fatty acids; **HUFAs,** fatty acids with 20 or more carbon atoms and four or more double bonds; **PND,** postnatal day; **PUFAs,** polyunsaturated fatty acids; **transgenic-fed,** pups reared on transgenic dams producing high n-3 PUFA milk; **wildtype–fed,** pups reared on wildtype dams producing control milk

elegans n-3 FA desaturase gene under the control of a constitutively expressed chicken β-actin promoter in transgenic mice and pigs enabled the successful conversion of n-6 FA to n-3 FA and accumulation of n-3 PUFA in their tissues (17–19). The *C. elegans* n-3 FA desaturase recognizes a variety of 18- and 20-carbon n-6 substrates (20), and mammals can further convert the 18- and 20-carbon n-3 FA products of this enzymatic reaction into n-3 HUFAs including EPA and DHA.

Transgenic mice expressing the *C. elegans* n-3 FA desaturase gene under the control of the mammary gland–specific β-casein promoter produced milk with an increase in n-3 PUFAs, a decrease in n-6 PUFAs, and an overall decrease in the n-6:n-3 PUFA ratio compared with milk derived from control mice (21). This transgene was found to be expressed in the lactating mammary gland but not the brain of animals carrying the transgene, and the brain lipid profile of pups raised on transgenic dams showed a significant increase in DHA and EPA levels compared with controls (22). This mouse model is useful for the evaluation of the role of n-3 PUFAs in cognitive development of neonates because it allows dietary manipulation of n-3 PUFAs in the gestational environment to occur independently of the n-3 PUFA sufficiency of the lactational environment. In comparison, models using maternal dietary manipulations subsequently require artificial rearing methods or cross-fostering to disassociate the n-3 FA content of the gestational environment from the lactational environment (23). These interventions introduce additional variables (*e.g.* feeding through a gastrostomy tube) that may influence pup development and behavior.

The objective of this study was to compare the neonatal growth rate and development of pups suckling on milk produced by either wildtype dams consuming a high n-6 FA diet or transgenic dams consuming the same diet but expressing an n-3 FA desaturase in the mammary gland and thereby endogenously producing milk with elevated levels of n-3 PUFAs. We hypothesized that pups nursed on transgenic dams would obtain sufficient n-3 PUFAs during the lactational period to counteract the effects of maternal gestational n-3 deficiency on pup brain FA composition and neonatal development.

METHODS

Generation of mouse study population. Experimentation on animal subjects was conducted in accordance with the regulations of the American Association for Accreditation of Laboratory Animal Care in fully accredited facilities at the University of California, Davis. Transgenic mice (C57BL/6J × DBA) expressing the *C. elegans* n-3 FA desaturase gene (GenBank accession number L41807) under the control of the goat β-casein promoter of the pBC1 mammary expression vector (Invitrogen, Carlsbad, CA) were generated by pronuclear microinjection (21).

Eight heterozygous transgenic and 10 wildtype C57BL/6J female mice were placed on a high n-6 and low n-3 FA mouse diet (Diet D03092902, Research Diets, New Brunswick, NJ) and paired with C57BL/6J males. The FA composition of this diet and the milk produced by wildtype and transgenic dams consuming it was previously reported (22). The FA breakdown of the experimental diet was 3.2% C18:0, 17.8% C18:1, 69.2% C18:2n-6, 0.17% C18:3n-3; 0.0% C20:4n-6, and C22:6n-3, and the n-6:n-3 ratio was 407. The transgenic females were fifth-generation descendants of a transgenic founder male. Cage enrichment included a nestlet and plastic sleeping house through weaning. Litters were equalized with regard to sex and number (four to six pups per litter) on postnatal day (PND) 3. Home cages were initially changed on PND 7 and weekly thereafter. The light cycle was 0700–2100 h (14-h light cycle). A single pup from each litter was euthanized at PND 22 *via* carbon dioxide asphyxiation and the brain removed and analyzed for FA composition (22). Toe samples for genotyping were also taken from all pups at the completion of the experiment (21).

Mouse assessment. The Wahlsten observational test battery is a neurobehavioral test series that measures growth rate and development (24). Measurements were taken daily from PND 8 to 18 for all pups, and except for the animal's weight, each test was graded on a scale of 0–3. Measures included daily body weights, nest observation and definition, righting speed and ability, cliff perception and aversion, needle grasp and grip strength (hindlimb and forelimb), vibrissa sensing and reaction, ear maturation, ear twitch response, vertical screen resistance pull, vertical screen clinging strength, vertical screen climbing ability, narrow stick perception and grasp response, wide stick perception and grasp response, eye maturation, visual sensing and reaction, auditory startle response, muscle tone, motor coordination, and excitability. Litters were not disturbed until the first day of testing. Pup order was randomized, and the test series was performed in the same order for all animals. All testing was conducted between 1400 and 1600 h. Each test session was 20 min from mouse retrieval to return, and each session was 3 min per mouse. Tests were repeated if an animal had previously been observed to be able to perform at a higher level. If tests were repeated, the highest score attained was recorded.

The object novelty preference test was performed on one male and one female per litter on PND 22–23 (25). This widely used task typically consists of an association trial during which mice explore two similar objects followed by a recognition trial in which a novel object is presented together with one familiar object already presented during the association trial. It is generally found that rats and mice spend more time exploring the novel object, indicating that the familiar object was recognized.

In this experiment, each session was 2 d long with 1 d of habituation (PND 22) in which the mouse was placed in an empty apparatus (60 × 39 × 22-cm plastic box) without any objects for a 5-min period, and 1 d of testing (both association and recognition trials, PND 23). In the association trial, the animal was placed in the testing apparatus with two never before seen identical objects (known as the familiar objects) and given 5 min to investigate the objects. The animal was then removed from the apparatus and placed in a holding cage for 30 s and the apparatus was cleaned with 70% alcohol solution.

For the recognition trial, one of the objects was replaced with a new object known as the novel object (the novel object position was randomized to either left or right). The animal was then placed back in the apparatus with the familiar and novel object and given 5 min to investigate the objects. At the end of the trial, the animal was removed from the apparatus and placed back in its home cage. Time away from the home cage on each day was kept to a maximum of 20 min from mouse retrieval to mouse return, and each test session was a maximum of 10 min. The test sessions were recorded (Cleversystem TopScan software, Reston, VA) and analyzed for locomotion activity, distance traveled, and duration and frequency of object investigation.

Statistical analysis. All statistical analyses were conducted using SAS Version 8 (SAS Institute Inc., Cary, NC). Data are expressed as mean ± SEM. Factorial and two-way repeated-measures analysis of variance (with the main effects of dietary treatment group, sex, pup genotype, and PND) were used to analyze data from the Wahlsten observational test battery and the object novelty preference test. The *t* test was used to analyze brain FA data.

RESULTS

FA analysis. Gas chromatography analysis of mouse brain tissue (Table 1) showed significant differences in the levels of several FAs when comparing brains collected from pups reared on transgenic dams producing high n-3 PUFA milk (transgenic-fed) to pups reared on wildtype dams producing control milk (wildtype–fed). The PND 22 brains from transgenic-fed pups had higher levels of EPA ($p < 0.05$) and DHA ($p < 0.001$) and lower ($p < 0.001$) levels of adrenic acid (22:4n-6) and docosapentaenoic acid (22:5n-6).

Wahlsten observational test battery. All pups ($n = 55$) raised on wildtype dams and pups ($n = 33$) raised on transgenic dams were evaluated using the Wahlsten observational test battery. The wildtype–fed mice showed a trend for higher weights throughout the testing session, with the difference becoming significant by PND 18 (least significant means, $p <$

Table 1. *FA analysis (g/100 g FA) of mouse brains derived from a single pup per litter at weaning from transgenic dams providing milk high in n-3 FAs (transgenic-fed) or control dams (wildtype–fed)*

FA	Transgenic-fed ($n = 8$)	Wildtype–fed ($n = 8$)
C14:0	0.50 (0.04)	0.47 (0.02)
C16:0	23.90 (0.26)	23.84 (0.37)
C18:0	20.02 (0.09)	19.74 (0.16)
C18:1 cis9 and 10	13.90 (0.21)	13.54 (0.19)
C18:2 (n-6)	1.15 (0.02)	1.13 (0.03)
C20:0	0.51 (0.03)	0.52 (0.02)
C18:3 (n-3)	1.18 (0.09)	1.15 (0.05)
C20:4 (n-6)	11.66 (0.24)	12.12 (0.12)
C20:5 (n-3)	0.01 (0.00)*	0.00 (0.00)
C22:4 (n-6)	3.59 (0.08)†	4.34 (0.08)
C22:5 (n-6)	2.08 (0.24)†	6.69 (0.22)
C22:5 (n-3)	0.29 (0.05)	0.15 (0.04)
C22:6 (n-3)	12.24 (0.32)†	7.35 (0.42)
Total n-6	18.47 (0.43)†	24.26 (0.29)
Total n-3	13.71 (0.31)†	8.62 (0.41)
22:5n-6/22:6n-3	0.17 (0.02)†	0.95 (0.08)
22:5n-6+22:6n-3	14.32 (0.14)	14.04 (0.27)
n-6/n-3 ratio	1.36 (0.06)†	2.86 (0.16)

Data are mean (SEM). Significant differences between values in the same row are noted: * $p < 0.05$; † $p < 0.001$.

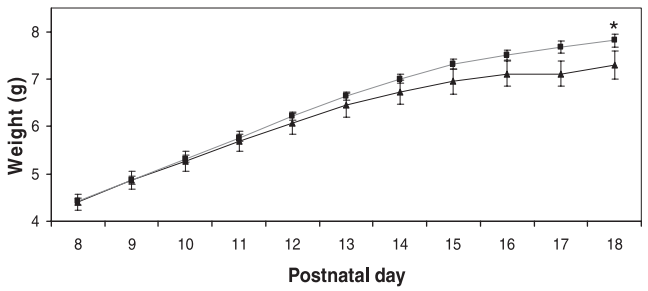

Figure 1. Weight (g) of mouse pups raised on milk from transgenic or control dams. Data are mean ± SEM (bars). The difference between the transgenic-fed treatment group (*triangles*, $n = 33$) and the wildtype–fed treatment group (*squares*, $n = 55$) was not significant, however group effect did attain significance by postnatal day 18 (*$p < 0.05$).

0.05; Fig. 1). Mice in the transgenic-fed group opened their eyes ($p < 0.01$, Fig. 2) and had higher visual placing scores ($p < 0.05$, data not shown) at an earlier age than controls. There were no other significant effects of milk source (*i.e.* transgenic or control dams) treatment for any of the remaining variables

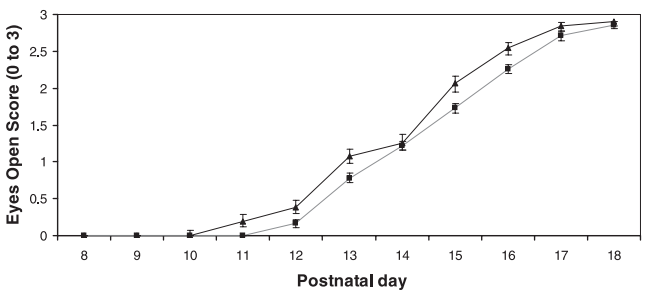

Figure 2. Wahlsten observational test battery eyes opening score. The degree of eye opening was scored from 0 to 3, and is shown according postnatal day. Data are mean ± SEM (*bars*). The transgenic-fed treatment group (*triangles*, $n = 33$) opened their eyes earlier ($p < 0.01$) than the wildtype–fed treatment group (*squares*, $n = 55$).

evaluated in the Wahlsten observational test battery, nor was pup genotype a significant effect for any of the Wahlsten observational test battery variables. As expected for a developmental test battery, there was a large effect of PND on all tests ($p < 0.0001$) as the animals matured from PND 8 to PND 18.

Object novelty preference test. The object novelty preference test was conducted on the mice immediately after weaning (PND 22–23). No treatment effects were observed for the duration, frequency, or percentage of time spent in the novel or familiar half of the area, in the immediate area of the novel or familiar object, or in sniffing the novel or familiar object (data not shown). Likewise, there were no statistically significant values for these measurements when examined by sex or pup genotype (data not shown).

DISCUSSION

The neonatal development and weight gain of pups raised on milk produced by transgenic female mice endogenously producing n-3 PUFA–enriched milk or control females were compared. From pairing onward, all dams received a 10% safflower oil diet containing high levels of n-6 FAs (69.2 g 18:2n-6/100 g FAs) and low levels of n-3 FAs (0.17 g ALA/100 g FAs). The brain FA composition at weaning differed between pups in the two treatment groups (transgenic-fed and wildtype–fed). The mice raised on transgenic dams had 1.6-fold more n-3 FAs in their brains compared with mice raised on wildtype dams. However, these pups had a brain FA profile very similar to that of wildtype pups previously raised in our laboratory (21) on milk from dams consuming a standard mouse chow (Purina 5015, Purina Co., St. Louis, MO). This suggests that pups raised on the transgenic milk in this study had relatively normal brain FA profiles despite gestational exposure to n-3 deficiency, whereas wildtype–fed pups remained n-3 PUFA deficient. This contention is supported by the finding that pups in the wildtype–fed group exhibited a brain FA profile (decreased DHA and a reciprocal increase in 22:5n-6) that has been previously associated with dietary n-3 FA deficiency (26). This transgenic model of perinatal n-3 supplementation is simpler than other approaches of supplementation such as feeding milk substitutes through a gastrostomy tube and may prove useful for studies focused on the response of the CNS to the lactational provision of n-3 PUFAs.

In the Wahlsten observational test battery, transgenic-fed pups opened their eyes and had higher visual placing scores at an earlier age than pups nursing on wildtype dams. The effect of n-3 FAs on retinal development has been extensively investigated in human infants, and many studies have shown that n-3 FAs play an important role in eye development (27–29). In a study conducted on rhesus macaque infants, dietary n-3 FA supplementation led to improved visual orientation toward objects outside their cage, which was interpreted to indicate earlier maturation of visual abilities (30). Previous studies showed that n-3 FA deficiency, as was seen in the control group in the current study, resulted in decreased visual acuity in infants (31). By the end of the Wahlsten observa-

tional test battery monitoring period (PND 18), mice from both the transgenic and wildtype treatment groups showed similar visual scores suggesting that n-3 FA supplementation provided pups with an early visual maturation benefit.

No group differences were detected for any of the other variables evaluated in the Wahlsten observational test battery or in the object novelty preference test. This agrees with other studies, which suggest that dietary restriction of only n-3 FAs does not grossly affect development or growth (8). Previous studies demonstrated that the ability to discriminate between n-3 FA–supplemented and –deficient animals in behavioral and cognitive tests was dependent on the magnitude of the FA difference present in the brain. At PND 22, there were highly significant differences in the amounts of n-3 and n-6 brain FAs between the transgenic-fed and wildtype–fed treatment groups; however, the percentage of reduction in DHA and total n-3 FA content in the control group was only 40% and 38%, respectively. Previous rodent studies with significant differences in Morris Water Maze test performance had reductions in brain n-3 FA composition (DHA and total n-3 FA content) >80% (9,32,33). Researchers using rodents with less extreme n-3 PUFA reductions (≤60%), as was the case in this study, have generally shown fewer statistically significant differences between treatment groups (10,34). Although less dramatic perturbations of brain FA composition make it difficult to see significant changes in behavior and cognition (7,9,35), they may more accurately reflect the range of variation in brain FA composition typically seen in human infants.

Interestingly, the wildtype–fed mice gained weight more rapidly and were heavier than the transgenic-fed mice by the end of the testing session. No significant difference in nesting behavior was observed between transgenic and wildtype dams in the Wahlsten observational test battery. Other researchers have reported that the ratio of n-6:n-3 PUFA in maternal milk rather than the total level of n-6 or n-3 FAs can greatly affect growth of adipose tissue and adipocyte size (36). Differences in the perinatal dietary ratio of n-6:n-3 FA have also been linked to changes in body fat and adult energy metabolism (37,38). N-6 PUFA–mediated increases in the expression of peroxisome proliferator–activated receptors (PPAR$\alpha/\delta/\gamma$) and other genes in white adipose tissue have been found to increase adipogenesis (adipocyte differentiation) and adipocyte growth, with potential ramifications for increased development of obesity (39). Future studies with this transgenic model will examine the long-term physiologic effects of ingesting lipids of differing qualitative FA content during the suckling period by investigating the relationship between neonatal diet and postweaning growth and energy metabolism.

In summary, pups raised on milk from transgenic mice endogenously producing n-3 PUFAs in their milk had an increased proportion of DHA in their brain, accelerated visual development, and a slower rate of preweaning growth when compared with pups raised on wildtype dams. No other components of the Wahlsten observational test battery or novelty preference behavior differed between the two treatment groups. The β-casein n-3 desaturase transgenic mouse model offers a unique approach to study the effects of neonatal consumption of milk high in n-3 FAs independent of the need for a similarly high n-3 gestational environment.

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