Effects of essential fatty acid deficiency during late gestation on brain N-acetylenuraminic acid metabolism and behaviour in the progeny

BY BRIAN L. G. MORGAN, JOHN OPPENHEIMER AND MYRON WINICK

Institute of Human Nutrition, Columbia University, College of Physicians and Surgeons, 701 West 168th Street, New York, New York 10032, USA

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1. Rat dams given a diet containing 100 g maize oil/kg for approximately 2 weeks before mating and during the first 14 d of gestation, were given the same diet or one containing 100 g hydrogenated coconut oil/kg (essential fatty acid (EFA)-deficient) in place of maize oil until parturition. After parturition the dams were given the same diets and all progeny were weaned on the maize oil diet at 21 d of age. Brain N-acetylenuraminic acid (NeuNAc) content as well as neuraminidase (sialidase; (EC 3.2.1.18)) and cytidine monophosphate N-acetylenuraminic acid synthetase (CMP-NeuNAc synthetase) activities were measured at days, 7, 14, 21 and 168 in the progeny. Y-maze learning was measured at 168 d.

2. Brain weight was independent of dietary fat at all ages.

3. Lack of EFA in the maternal diet during gestation and lactation depressed ganglioside and glycoprotein NeuNAc levels and the activities of sialidase and CMP-NeuNAc synthetase.


Much evidence has accrued to show that maternal malnutrition during gestation or lactation or both has permanent effects on the development of the central nervous system in the progeny (Svennerholm et al. 1972; Hseuh et al. 1974; Winick, 1976). More recently, it has been found that a nutritional insult applied during the postnatal growth spurt of the rat pup brain reduces dendritic arborization as measured by the total brain ganglioside and glycoprotein N-acetylenuraminic acid (NeuNAc) content (Merat & Dickerson, 1974). Several workers have specifically linked brain ganglioside content and behaviour (Dunn & Hogan, 1975; Rahmann et al. 1976; Bogoch, 1976). It has been demonstrated both that environmental stimulation in malnourished animals causes an improvement in their behavioural performance and also an increase in their brain NeuNAc (Morgan & Winick, 1980a). Further, intraperitoneal injection of NeuNAc during the brain growth spurt has been shown to improve behavioural performance and increase brain NeuNAc concentrations (Morgan & Winick, 1980b). This evidence suggests that there might be an association between brain NeuNAc content and behaviour.

A diet containing an inadequate amount of essential fatty acids (EFA; general formula EFA — y:yWz, fatty acid with x carbon atoms and y double bonds with the terminal double bond z C atoms from the methyl group) fed to rat dams during the latter part of gestation has been found to be associated with the development of offspring that have permanently impaired learning abilities (Caldwell & Churchill, 1966) and reduced total brain lipid contents (Lamptey & Walker, 1978a). This effect was accentuated if the diet was fed to the deprived dams during lactation as well as during gestation (Lamptey & Walker, 1978b).

In this study, we were interested in ascertaining whether the impaired learning ability characteristic of pups born to dams suffering from a lack of dietary EFA was associated with a change in glycoprotein metabolism. Thus, we measured total brain ganglioside and glycoprotein NeuNAc contents, as well as the activities, of cytidine monophosphate N-acetylneuraminic acid synthetase (EC 3.2.1.18) and neuraminidase (Svennerholm et al. 1972).
Table 1. Composition (g/kg) of experimental diets

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Control</th>
<th>EFA-deficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (vitamin-free)</td>
<td>200.00</td>
<td>200.00</td>
</tr>
<tr>
<td>Maize</td>
<td>637.50</td>
<td>637.50</td>
</tr>
<tr>
<td>Maize-oil*</td>
<td>100.00</td>
<td></td>
</tr>
<tr>
<td>Hydrogenated coconut oil†</td>
<td>-</td>
<td>100.00</td>
</tr>
<tr>
<td>D,L-methionine</td>
<td>2.50</td>
<td>2.50</td>
</tr>
<tr>
<td>Salt mix‡</td>
<td>40.00</td>
<td>40.00</td>
</tr>
<tr>
<td>Vitamin mix¶</td>
<td>20.00</td>
<td>20.00</td>
</tr>
</tbody>
</table>

* Maize oil contained the following fatty acids (g/kg): 16:0 100, 18:0 210, 18:1 230, 18:2 633, 18:3 10, other constituents 6.
† The major fatty acids in the hydrogenated coconut oil were (g/kg): 8:0 60, 10:0 75, 12:0 529, 14:0 176, 16:0 74, 18:0 92, 18:1 2, 18:2 2.
‡ Salt mix formulated after Bernhart & Tomarelli (1966).
¶ Vitamin mix previously published by Morgan & Winick (1980a).

N-acetylneuraminic acid synthetase (CMP-NeuNAc synthetase) a key enzyme in glyco-compound synthesis, and sialidase (EC 3.2.1.18), which is involved in the degradation of these compounds in progeny of dams deprived of linoleic acid during the last week of gestation.

METHODS AND MATERIALS

Forty-eight Sprague Dawley rat dams of approximately 250 g in weight were given a diet containing 100 g maize oil/kg (Table 1) for approximately 2 weeks. They were then mated. Rats were considered to be pregnant when a vaginal plug was found at the bottom of the cage on which was considered to be day 1 of pregnancy. All such positive matings culminated in successful parturitions.

After mating, they were paired off into two groups of twenty-four. One group, the control group, was given the maize-oil diet for the period of pregnancy and lactation and the offspring from this group were weaned to the same diet. The second group, the experimental group, was pair-fed to the first group. The mode of pair-feeding used in the experiment was to pair one rat from each group with a counterpart of approximately equal weight in the second group. Then the animal that consumed the most food in each pair was presented with the amount of food that had been eaten by its counterpart in the other group on the previous day. The animals consumed progressively more food as they advanced through gestation and then lactation, and hence, the amount of food presented daily gradually increased during the experiment. Food was presented twice daily at 10.00 hours and 18.00 hours. In each instance the animal being pair-fed always consumed the amount of food offered. The experimental group, received the maize-oil diet for the first 2 weeks of gestation at which time they were given a diet containing 100 g hydrogenated coconut oil (EFA-deficient) in place of the maize oil throughout the remainder of gestation. After birth, this group was transferred back to the maize-oil diet and received this until the end of the period of lactation. Their offspring were also given the same diet ad lib.

At birth all litters were culled to eight pups per litter, ensuring that there were four male and four female rats in each. This was possible in all litters and it was not found necessary to substitute pups from other litters. One pup from each litter was killed by decapitation at the following ages: 5, 6, 7, 14, 16 and 21 d. Thus twelve female and twelve male pups were killed in each group at each time-point. The brains were removed, freed from the dura and blood vessels, weighed and analysed for various biochemical indices, i.e. DNA, protein, ganglioside NeuNac, glycoprotein NeuNac as well as CMP-NeuNac synthetase and sialidase activities. At day 21 of lactation, the remaining pups from each litter were weaned.
and housed individually. At 25 d of age, a further rat from each of the original litters was killed and its brain analysed for the previously-mentioned indices. At 168 d of age the remaining rat from each litter was assessed for ability to learn a Y-maze in a blind study (Morgan & Winick, 1980a). These too were then decapitated and their brains analysed for the neurochemical indices described previously.

**Biochemical estimations**

The brains were homogenized in 9 vol. distilled water. DNA was extracted from the homogenate by the method of Klemperer (1963) and estimated by the diphenylamine reaction of Burton (1956). Protein was measured using the method of Lowry et al. (1951). Gangliosides and glycoproteins were extracted from samples of homogenized brain by the Suzuki (1964) modification of the method of Folch et al. (1957) as adapted by Roukema & Heijlman (1970). Total gangliosides were determined as NeuNAc by a modification (Miettinen & Takk-Luukalnen, 1959) of the resorcinol method of Svennerholm (1957). Glycoproteins were also determined by measurement of the NeuNAc content of the chloroform–methanol insoluble residue left after lipid extraction. Sialidase activity was estimated at pH 4.0 using gangliosides as substrate (Roukema & Heijlman, 1970). CMP-NeuNAc synthetase activity was assayed in the homogenate using the method reported by Roukema et al. (1964).

**RESULTS**

The results were statistically analysed using paired t-tests for differences between the two groups and analysis of variance for differences between time points (Bruning & Kintz, 1968).

Food intake was independent of dietary fat as was the change in weight of the rat dams during gestation or lactation or both. Rats in both groups ate steadily through the day and so despite the poor feeding both groups of rats were effectively fed ad lib. Litter sizes were not statistically different being 12.3 ± 1.05 and 12.8 ± 1.03 pups per litter for the controls and EFA-deficient groups respectively. Furthermore birth weights of the pups were also approximately the same, i.e. 4.93 ± 0.28 for controls and 4.90 ± 0.32 for the EFA-deficient group. There were no differences between sexes for any variable measured and so the values for males and females have been pooled.

Table 2 shows that there were no differences between groups with respect to brain weight, DNA (cell number) or protein (cell size) at any of the time-points measured.

The results of the analysis of brain gangliosides and glycoproteins are shown in Fig. 1. The timing of synthesis of both gangliosides and glycoproteins in the control and EFA-deficient groups is the same. There was a rapid increase in brain glycocompound content during the first 21 d of postnatal life at which time the rate of accretion slows down rapidly to reach mature levels by day 25. After this time, there was very little change in brain content. At every time point measured, the controls had a significantly higher brain content of glycocompounds than the EFA-deficient groups and they ended up with approximately 25% more gangliosides and 30% more glycoproteins than the EFA-deficient group at maturity.

CMP-NeuNAc synthetase is an enzyme that participates in ganglioside synthesis. The activity of the enzyme measured in both groups of animals appeared to rise until day 6 (Fig. 2). There followed a decline in activity until day 16, then a small rise to the stable mature level. In the EFA-deficient group, enzyme activity was significantly lower at all time-points measured.

The results of the sialidase estimations are shown in Fig. 3. Here we see that there was a rapid increase in activity from a low level at day 5 to reach a maximum value at day 9. The decline in activity was almost as rapid, levelling out at day 16. The activity profile
Table 2. Brain composition of offspring from rat dams receiving either a complete diet or one excluding essential fatty acids (EFA)

<table>
<thead>
<tr>
<th>Age (d)</th>
<th>Brain DNA (mg)</th>
<th>Brain protein (mg)</th>
<th>Brain wt (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>EFA-deficient</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SE</td>
<td>Mean</td>
</tr>
<tr>
<td>5</td>
<td>0.40</td>
<td>0.03</td>
<td>0.41</td>
</tr>
<tr>
<td>6</td>
<td>0.47</td>
<td>0.06</td>
<td>0.47</td>
</tr>
<tr>
<td>7</td>
<td>0.51</td>
<td>0.04</td>
<td>0.51</td>
</tr>
<tr>
<td>14</td>
<td>0.73</td>
<td>0.02</td>
<td>0.72</td>
</tr>
<tr>
<td>16</td>
<td>0.87</td>
<td>0.03</td>
<td>0.88</td>
</tr>
<tr>
<td>21</td>
<td>0.99</td>
<td>0.04</td>
<td>0.98</td>
</tr>
<tr>
<td>168</td>
<td>1.46</td>
<td>0.23</td>
<td>1.51</td>
</tr>
</tbody>
</table>

Fig. 1. Effects of essential fatty acids (EFA) deficiency on brain glycompound (μmol N-acetylneuraminic acid (NeuNAc)) content in rats of various ages. (●) and (○) represent brain ganglioside levels for control and EFA-deficient rats respectively. (■) and (□) represent brain glycoprotein levels for control and EFA-deficient rats respectively. Points represent mean values of twenty-four determinations; one on each pup. The differences between the two groups are statistically significant at all time-points measured: days 5, 6 and 7 (P < 0.01), the other time-points (P < 0.001) (Bruning & Kintz, 1968).

DISCUSSION

The only source of EFA that the rat foetus has access to is the maternal circulation. Only during the last week of gestation do polyunsaturated fatty acids cross the placenta (Popjak & Beekman, 1950), and thus feeding a pregnant dam a diet devoid of EFA during the last week of gestation deprives the progeny of EFA.
Essential fatty acids and brain glycompounds

Fig. 2. Effects of essential fatty acid (EFA) deficiency on brain CMP-NeuNAc synthetase activity. (●) and (○) represent values for control and EFA-deficient rats respectively. Points represent the mean values of twenty-four determinations: one on each pup. The differences between the two groups are statistically significant at all ages measured \( (P < 0.001) \) (Bruning & Kintz, 1968).

Fig. 3. Effects of essential fatty acid (EFA) deficiency on brain (sialidase EC 3.2.1.18). (●) and (○) represent values for control and EFA-deficient rats respectively. Each point represents the mean of twenty-four determinations; one on each pup. The differences between the two groups are statistically significant at all ages measured \( (P < 0.001) \) (Bruning & Kintz, 1968).
Our results clearly show that such deprivation during the last 7 d of gestation gave rise to reduced brain ganglioside and glycoprotein NeuNAc concentrations in the offspring. Glycocompounds are located primarily in the neuronal membranes (Ledeen, 1978), and it has been proposed that sialo compounds have a role in the structural and functional establishment of synaptic pathways (Rahmann et al. 1976). Schengrund and her co-workers (Schengrund & Rosenberg, 1970) have postulated that NeuNAc may have a direct role in affecting the movement of positive neurotransmitters by virtue of its electronegativity. Furthermore, Weseman et al. (1971) have suggested that the negative charge associated with NeuNAc may be involved in the process of binding neurotransmitters to neuronal membranes.

Accompanying this reduction in glycocompounds we also found a reduced capacity to learn a Y-maze in aversion to shock in adult animals that had been exposed to EFA-deficiency in utero. We have previously reported (Morgan & Winick 1980b) that learning disabilities characteristic of malnourished animals can be alleviated by an intraperitoneal injection of NeuNAc provided that it is given during the postnatal growth spurt when the neurons develop their axons and dendrites and form synaptic interconnections. Furthermore, environmental stimulation of malnourished animals during the brain growth spurt also caused the same improvement in behaviour as well as being accompanied by an increase in brain NeuNAc (Morgan & Winick, 1980a). Other workers (Savaki & Levis, 1977) have shown that brain ganglioside synthesis increases after active avoidance conditioning. Moreover, certain behavioural patterns have been shown to be inhibited by antiserum to brain gangliosides (Karpiak et al. 1976). Even after short-term behavioural stimulation, small changes in ganglioside metabolism seem to occur (Irwin & Samson, 1971). These observations suggest that brain NeuNAc has a key role to play in determining behaviour.

Although the last week of gestation in the rat is the major time period for neuronal division, we would not expect an insult at this time to have a direct effect on the development of neuronal processes in the postnatal period. However, the effects of late gestational EFA-deficiency could have been carried over to the postnatal period and failed to be reversed by refeeding the animals on a complete diet from birth onwards. Several authorities have made the same observation. Caldwell & Churchill (1966) showed that pups from dams given a fat-free diet throughout gestation did not learn a Y-maze as well as controls. Once again, the effect was not reversed by transferring the dams to a 'lab-chow' diet at birth. They cited pathological evidence for a decrease in ganglion cell size from the hippocampus of the brain as a possible mechanism for the phenomenon. However, in our experiment we were not able to show any change in total brain cell number, cell size or weight. Paoletti & Galli (1972) demonstrated that progeny from dams given a fat-free diet during the last week of gestation and throughout lactation also had inferior avoidance behaviour. Lamptey & Walker (1978b) showed impaired performance in a Y-maze learning situation in pups born to dams given an EFA-deficient diet in the last week of gestation. Once again, this could not be reversed by feeding a diet with a good content of EFA from birth onwards. They suggested that the altered pattern of brain $W_3$ and $W_6$ polyunsaturated acids in these pups could be partly responsible for altering the aforesaid behaviour.

Our results really only show that dietary deficiency of EFA during pregnancy is associated with impaired learning and in no way does this study provide evidence of causality. Deficient diets provided during pregnancy alter interaction between mother and offspring, which also impairs behaviour of the progeny (Levitsky et al. 1975). In order to discern to what extent the latter has affected the behavioural results reported here we should have to cross-foster all pups to 'normal' mothers at birth.

CMP-NeuNAc synthetase was shown to have a reduced activity in the pups deprived of EFA in utero compared to control animals. This difference in enzyme activity between
the two groups of animals suggests that the production of glycoproteins and gangliosides in the deprived group would be reduced in amount.

The sialidase measurements also show that the deprived pups had lower enzyme activities at all ages measured up to and including adult levels. Sialidase seems to have a key role in development of the central nervous system (Suzuki, 1967; Schengrund & Rosenberg, 1971) as well as possibly playing a role in the process of neurotransmission (Schengrund & Nelson, 1975). Hence, the lower sialidase activity expressed in the deprived group provides evidence of an irregular process of brain maturation and possible impaired synaptic transmission.

As progeny in each litter were killed sequentially leaving the dam with progressively fewer pups to feed, the developmental patterns of the variables measured in this work may be affected by the changing plane of nutrition of the surviving pups. However, this factor affects both groups equally and so it is true to say that the activities of these enzymes may provide a possible explanation for why EFA-deficiency during the last week of gestation should affect postnatal brain development. As polyunsaturated fatty acids constitute an integral part of all cell membranes, their absence during the time of greatest neuronal proliferation, and hence cell membrane synthesis, might lead to the production of qualitative changes in the cell membrane. This then could alter the cell’s future ability to take up substrates for biosynthesis of the glycompounds themselves or the enzyme involved in their metabolism.
REFERENCES


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