Learning Deficits in Rats with Malnourished Grandmothers

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Female rats (F_0) were maintained on a protein-restricted or a normal diet 1 month prior to mating and throughout pregnancy. Their female offspring (F_1) were maintained on a normal protein diet and mated with normal males. In previously reported studies, the 2nd generation offspring (F_2) of the malnourished rats have been found at birth to have significantly lower cerebral DNA (a measure of cell number), cerebral weight, and cerebral protein than normal controls. We now report that these F_2 animals show marked learning deficits at maturity on 2 different successive reversal tasks, even though they themselves have never directly experienced malnutrition. Thus, certain behavioral as well as biochemical effects of malnutrition appear in the next generation of animals.

The deleterious effects of malnutrition on learning ability are well documented; considerable evidence indicates that the brain is particularly susceptible to damage resulting from malnutrition during the earliest periods of life when it is growing most rapidly. For example, in rats irreversible changes in brain weight and total brain deoxyribonucleic acid (DNA) are produced by a restricted diet in the early portions of postnatal life. If the same dietary restriction occurs after weaning, the resulting deficits can be repaired by subsequent adequate feeding (Winick & Noble, 1966). These results may be applicable to humans (e.g., Cabak & Najdanvic, 1965).

Less is known, however, about the effects of prenatal dietary restriction on later brain development. Yet in many species the rate of neuroblast division in the central nervous system (as measured by the accumulation of DNA in the brain, an indication of the number of brain cells present) peaks prior to birth (Dickerson & Dobbing, 1967; Margolis, 1969) suggesting possibilities of even more serious impairments from improper dietary conditions at this stage of development. Several have shown that rats born and suckled by malnourished mothers are deficient in thier learning ability, even though subsequently raised on an adequate diet (Barnes, Cunnold, Zimmerman, Simmons, MacLeod, & Krook, 1966; Cowley & Griesel, 1966).

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We have previously reported that when female rats (F_0) are maintained on a low-protein diet 1 month prior to mating and throughout gestation, the brains of the resulting infants (F_1) have significantly lower levels of DNA at birth than do normal infant rats (Zamenhof, van Marthens, & Margolis, 1968). Because the rat brain at birth is composed primarily of immature neurons (Brizzee, Vogt, & Kharetchko, 1964) the level of DNA present indicates that prenatal protein restriction results in a decreased total number of brain neurons at birth. Because other research (e.g., Altman & Das, 1966) has indicated that relatively few brain cells divide after birth, this level of DNA further indicates that prenatal dietary restriction produces a brain deficiency that persists through the life of the animal.

We have reported also that the effects of prenatal protein deprivation upon brain DNA levels at birth can pass into the 2nd generation of animals (Zamenhof, van Marthens, & Grauel, 1971). In these experiments, female rats (F_0) were maintained on a low-protein diet prior to and throughout gestation, but upon reaching term were immediately switched to a normal laboratory chow diet and permitted to raise their own newborns (F_1). Upon reaching maturity, the F_1 females were mated with normal (i.e., standard colony) males. Both following weaning and throughout their pregnancy, F_1 females were maintained on standard laboratory chow. At term, the offspring (F_2) of these mothers were sacrificed and the levels of total brain DNA compared with those of normal offspring from the standard colony. We found that total brain DNA was significantly lower in these 2nd generation (F_2) animals, even though their mothers (F_1) had received adequate nutrition throughout.

The findings may be summarized as follows: Following severe prenatal protein deprivation, the number of brain cells is markedly reduced at birth and may be permanently altered. Furthermore, the offspring of these animals may have a permanent brain deficiency even though they were never directly exposed to a low-protein diet.

Consider the relevance of total brain DNA at birth (the measure upon which most preceding arguments are based) to the capacity of the brain to process information. For example, the F_1 infants of protein-deprived animals may be born at a less developed stage, but may reach the same capacity as normal animals at a later time. Some neurons do proliferate after birth (Altman & Das, 1966) and perhaps a large number of neurons develops postnatally in these animals. Such a possibility is very difficult to test on a biochemical level for the brain after birth becomes increasingly difficult to quantify; neuroglial cells proliferate until weaning (Winick & Noble, 1966) and neuronal and glial DNA cannot be distinguished at this point. Thus, any changes in total brain DNA could be attributed merely to differences in supportive cell (i.e., glial) number, rather than numbers of neurons. Other indices such as brain weight and size largely reflect lipid and water content in the brain and any differences between experimental and control animals would be uninterpretable. Furthermore, any real differences in number of neurons present might very possibly be masked by some nonneuronal elements which would leave the basic deficit present but unobservable.

The most direct method for studying the relationship between neonatal DNA and cognitive ability at maturity is to conduct behavioral tests of "intelligence" in these animals, that is, of behavior regulated to meet the changing demands of new environments (i.e., to adapt to new circumstances and to solve problems). One behavioral test of such flexibility is habit reversal, in which the animals are first trained

to respond to 1 of 2 stimulus choices for food reward. After reaching a criterion of learning, the positive and negative stimuli are reversed and the animals must relearn the new problem. When the criterion is again reached the stimuli are reversed once more and so on. In the present study, both "visual" and "spatial" tasks were used to control for specific olfactory, proprioceptive, and visual differences which might be related to malnutrition.

Method

Subjects

Sprague-Dawley derived albino rats (*Rattus norvegicus*) bred as a closed colony for 20 generations served as subjects as in the previous biochemical studies (Zamenhof, van Marthens, & Grauel, 1971, 1972; Zamenhof *et al.*, 1968). Sixty-day-old virgin females (F_0) were maintained on either a low (8%) protein-restricted diet or a standard control (20.5% protein) pellet diet. At 90 days of age, all females were mated with normal males and maintained on their respective diets throughout gestation. At birth, the newborns (F_1) were nursed by their own mothers, all of whom were then given the control diet. At weaning all animals were coded to eliminate any possibility of bias. After weaning all F_1 animals were maintained on the control diet, ad lib.

Upon reaching maturity, F_1 females were mated with normal males. The female offspring of these matings (F_2) were also maintained on the control diet and became the subjects for this experiment. Thus, 2 groups of F_2 animals were obtained: the experimental (E) animals whose grandmothers were fed a protein-restricted diet 1 month prior to and throughout pregnancy, and the control (C) animals who had no history of malnutrition. One set of F_2 animals (E: $n = 63$; C: $n = 74$) was sacrificed immediately after birth for biochemical studies whereas a 2nd set (E: $n = 16$; C: $n = 19$) was reared to maturity for behavioral testing. Two animals per litter were selected for behavioral tests. The selection was at random. After the completion of the behavioral tests, these animals were used for biochemical analysis, forming the Mature group.

Behavioral Procedure

Animals were tested on both spatial and visual habit reversal tasks using an automated 2-choice discrimination apparatus which consisted of 2 identical discrimination units (D. E. Bresler, unpublished data[1]). The units were connected in series via ramps to form a closed loop such that the goal box of one unit served as the start box of the other. Each unit consisted of 2 Y-mazes connected back-to-back (i.e., the arms of the 2 mazes were joined together). Motor-driven doors were placed at the ends of the Y-maze arms (choice-point doors) and stems (goal/start box doors), and testing and data acquisition were fully automated. Animals were coded and tested using a "blind" procedure (i.e., the investigators testing the animals and analyzing the data did not know to which group any individual animal belonged).

Pretraining. At 4 months of age, both groups of animals were shifted to a food-deprivation schedule and maintained at 85% of their ad lib body weights (which

were not significantly different for the 2 groups). Water was available at all times in their home cages. During the first 3 days of pretraining, each animal was placed into the goal/start box of one unit and following a 15-sec intertrial interval all doors in that unit were raised automatically allowing the rat to explore freely. When it reached the goal/start box of the other unit, all doors in the 1st unit closed and a 45-mg Noyes food pellet was automatically dispensed. Fifteen seconds later, all doors in the 2nd unit were raised and when the rat reached the original goal/start box, all doors closed and food was again delivered. Twently such trials were given on the 1st day and 40 trials were given on Days 2 and 3. On Days 4 and 5, 40 trials per day were again given. Now the choice-point doors were closed and the rats were trained to open them by touching them with their noses or paws, thus closing a contact circuit. (Both choice-point doors opened automatically when touched, allowing the animal to reach the goal/start box to receive food.)

Testing. Following pretraining, rats in the C and E groups were matched according to their position preference and to their rate of learning (during pretraining), split into 2 subgroups, and tested in visual and spatial habit reversal tasks. In one subgroup (Visuals), E ($n = 10$) and C ($n = 11$), animals were trained on a visual discrimination problem in which the lighted choice-point door was correct and the dark door was incorrect. In the other subgroup (Spatials), E ($n = 6$) and C ($n = 8$) animals were trained on a spatial discrimination problem in which the right choice-point door was correct and the left door was incorrect.

A response to the correct door permitted passage through the maze to the goal/start box where a food pellet was automatically delivered. Incorrect responses were recorded as "errors" and did not permit passage but had to be followed by a correct response. Daily testing on this original problem (R_0) was continued for each rat until a criterion of learning was reached (6 or less errors during a daily 40-trial session) after which the stimulus contingencies were reversed (i.e., dark door now correct for visuals, left door now correct for spatials). Upon reaching criterion in the 1st reversal (R_1), each animal was reversed again and so on until the visual animals were run through 5 reversals and the spatial animals through 10 reversals.

Biochemical Procedure

One F_2 set of E and C groups was decapitated immediately after birth. Another F_2 set was reared to maturity, tested behaviorally as described above, and then decapitated. After decapitation, the brains (cerebral hemispheres, without cerebellum and olfactory lobes) were immediately removed and weighed; they were then frozen and subsequently used for analysis. DNA was determined by a modification of the diphenylamine colorimetric method (Zamenhof, Grauel, van Marthens, & Stillinger, 1971); protein was determined by a modification of the Lowry colorimetric method (Lowry, Rosebrough, Farr, & Randall, 1951).

Results

Differences in errors between the E and C groups on the original spatial problem were not significant (Fig. 1). The C animals demonstrated an initial increase in errors

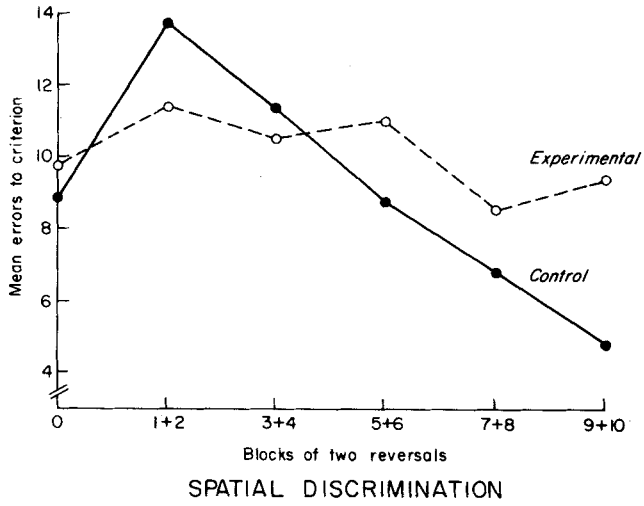

Fig. 1. Mean errors to criterion on successive reversals of the spatial discrimination task. The controls show the initial rise in errors on the 1st reversal and the subsequent progressive improvement typically reported for the rat. The 2nd generation offspring of malnourished animals shows a relatively flat curve with little progressive improvement.

on the 1st reversal and a subsequent progressive improvement. The E animals, however, failed to show either of these 2 behaviors and exhibited a significantly smaller decrease in errors across reversals than did the controls ($F = 7.0; df = 1/48; p < .05$). By the 9th and 10th reversals, C's made significantly fewer errors than did E's. The visual discrimination task was much more difficult; although progressive improvement was not seen in either group over 5 reversals (Fig. 2), clear differences again emerged. The

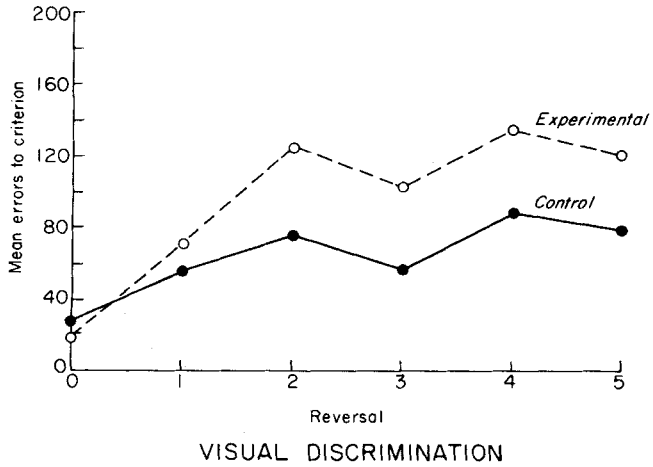

Fig. 2. Mean errors to criterion on successive reversals of the visual discrimination task. This task was much more difficult, and even the controls failed to show progressive improvement across 5 habit reversals. However, the 2nd generation offspring of malnourished rats required more trials to reach criterion on each reversal than did controls.

C animals started from an initial baseline similar to the E's but showed fewer total errors across successive reversals ($t = 2.05; p < .05$, 1-tailed). Taken together, these results suggest that E animals show both quantitative (greater number of errors to criterion) and qualitative (no progressive improvement across spatial reversals) deficits in complex discrimination learning tasks).

Elsewhere, we have obtained almost identical results in pilot studies of visual habit reversal learning in 2nd generation animals using a water maze. We conclude that complex learning deficits can be demonstrated in the 2nd generation offspring of malnourished animals in a variety of learning tasks and that these deficits are not specific for sensory modality or reinforcement condition. That is, rats whose grandmothers had experienced severe protein malnourishment while pregnant, but who had never themselves experienced direct malnutrition, shown significant and persisting learning deficits, and thus, the deleterious effects of malnutrition seem to be transmitted to the next generation of animals.

The results of biochemical measurements on newborn animals show similar effects (Table 1). The newborn F_2 animals (from prenatally malnourished F_1), exhibited a signficant decrease in neonatal body weight, cerebral weight, cerebral DNA (cell number), and cerebral protein.

Discussion

When tested at birth, the F_2 experimental animals showed clearly significant decreases in a number of brain and body measures. As reported previously, (Clark & Zamenhof, 1973; Clark, Zamenhof, van Marthens, Grauel, & Kruger, 1973; Zamenhof, *et al.,* 1972), these differences are no longer demonstratable in mature animals in that they apparently become obscured by general brain and body growth (Table 1). Yet, the behavioral manifestations of grandmaternal malnutrition persist.

Several possible explanations of these results should be considered. Malnutrition may have produced a variety of chromosomal abnormalities as has been recently

TABLE 1. The Effect of Maternal (F_0) Protein Restriction on F_2 Offspring.

	Group[a]	Number of Animals	Body Weight[b] (g)	Cerebrum[b] Weight (mg)	Cerebrum[b] DNA (μg)	Cerebrum[b] Protein (mg)
Neonatal	Control	74	6.1 ± .5	170 ± 13	598 ± 33	8.8 ± .8
	Experimental	63	5.7 ± .5[c]	158 ± 15[c]	565 ± 35[c]	8.3 ± .8[d]
Mature	Control	19	269 ± 38	1220 ± 40	1085 ± 46	100.9 ± 7.6
	Experimental	28	271 ± 32	1210 ± 60	1087 ± 47	98.9 ± 7.7

[a]All comparisons made to the control. The data for neonatal (both groups) and mature animals (experimentals) are from Zamenhof, van Marthens, & Grauel, 1972.

[b]Each value represents the mean ± SD.

[c]Significant at $p < .001$ level.

[d]Significant at $p < .01$ level. Student's *t*-test.

reported (Armendares, Salamanca & Frenk, 1971). This is highly unlikely here in that the correlated biochemical abnormalities are inherited only through the F_1 female animals (Zamenhof, van Marthens, & Grauel, 1971, 1972). These effects, therefore, cannot be due to any dominant trait, and if it were a sex-linked recessive trait no F_1 females would show deficits and only half of the F_2 male animals would be affected. None of these postulated results were obtained.

Behaviors can also be nongenetically inherited. For example, the F_2 offspring of rats subjected to stress during pregnancy show altered emotionality in the face of stress (Denenberg & Rosenberg, 1967; Wehmer, Porter, & Scales, 1970). These effects are not due to alterations in rearing of the infants by the stressed mothers because the alterations are retained with cross fostering, and because similar effects can be produced solely by injections of stress hormones into the pregnant F_0 females (Thompson, 1957; see review by Archer & Blackman, 1971). In humans, recent studies have shown high correlations of personality factors between grandmothers and their grandchildren, perhaps due to maternal nongenetic inheritability (Insel, 1972).

In any case, the precise nature of the mechanisms underlying these phenomena is essentially unknown. The effects of protein malnutrition on the quality of lactation and nursing behavior is probably unimportant as shown by cross-fostering studies (Zamenhof *et al.*, 1972). However, a variety of biochemical abnormalities in the F_1 animals may be responsible for the F_2 deficits. In addition to retardation in brain development (Zamenhof *et al.*, 1968), the offspring of protein-restricted rats show abnormalities in kidney development and kidney function (Hall & Zeman, 1968; Zeman, 1968), reduced feeding efficiency, low nitrogen balance, and excessive amino acid excretion (Lee & Chow, 1968), and endocrine dysfunction (Stephen, Chow, Frohman, & Chow, 1971) which perhaps may affect placental and subsequent brain development (Zamenhof *et al.*, 1972). Thus, cryptic malnutrition may result in inheritable learning deficits due to a variety of biochemical abnormalities which may be nongenetically transmitted to the next generation of animals.

The implications of these findings must be carefully considered in studies of genetic factors and intelligence. Although alleged differences in intelligence due to genetic factors would not be affected by environmental manipulations, the nongenetic inheritable differences due to cryptic malnutrition may perhaps be countered by remedial feeding programs. For example, animals who are continuously malnourished show progressively greater learning deficits across successive generations that disappear after several generations of adequate feeding (Cowley & Griesel, 1966). If this analogy is correct, the beneficial results of feeding programs for the offspring of malnourished members of our society may become progressively more apparent only after several generations have passed.

Notes

[1] An unpublished manuscript entitled "An automated two-choice discrimination apparatus for rats" is available from the senior author.

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References

Altman, J., and Das, G. D. (1966). Autoradiographic and histologic studies of postnatal neurogenesis. *J. Comp. Neurol., 126*: 337-390.

Archer, J., and Blackman, D. (1971). Prenatal psychological stress and offspring behavior in rats and mice. *Dev. Psychobiol., 4*: 193-248.

Armendares, S., Salamanca, F., and Frenk, F. (1971). Chromosome abnormalities in severe protein calorie malnutrition. *Nature (Lond.), 232*: 271-273.

Barnes, R. H., Cunnold, S. R., Zimmerman, R. R., Simmons, H., MacLeod, R. B., and Krook, C. (1966). Influence of nutritional deprivation in early life on learning behavior of rats as measured by performance in a water maze. *J. Nutr., 89*: 399-410.

Brizzee, K. R., Vogt, J., and Kharetchko, X. (1964). Postnatal changes in glia/neuron index with a comparison of methods of cell enumeration in the white rat. *Prog. Brain Res., 4*: 136-149.

Cabak, V., and Najdanvic, R. (1965). Effect of undernutrition in early life on physical and mental development. *Arch. Dis. Child., 40*: 532-534.

Clark, G. M., and Zamenhof, S. (1973). Correlations between cerebral and cortical parameters in the developing and mature rat brain. *Int. J. Neurosci., 5*: 223-229.

Clark, G. M., Zamenhof, S., van Marthens, E., Grauel, L., and Kruger, L. (1973). The effect of prenatal malnutrition on dimensions of cerebral cortex. *Brain Res., 54*: 397-402.

Cowley, J. J., and Griesel, R. D. (1966). The effect on growth and behavior of rehabilitating first and second generation low protein rats. *Anim. Behav., 14*: 506-517.

Denenberg, V. H., and Rosenberg, K. (1967). Nongenetic transmission of information. *Nature (Lond.), 216*: 549-550.

Dickerson, J. W. T., and Dobbing J. (1967). Prenatal and postnatal growth and development of the central nervous system of the pig. *Proc. R. Soc. Lond. [Biol.], 166*: 384-395.

Hall, S. M., and Zeman, F. J. (1968). Kidney function of the progeny of rats fed a low protein diet. *J. Nutr., 95*: 49-54.

Insel, P. (1972). The grandmother effect in personality. *New Sci., 53*: 132-134.

Lee, C. J., and Chow, B. F. (1968). Metabolism of proteins by progeny of underfed mother rats. *J. Nutr., 94*: 20-26.

Lowry, O. M., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem., 193*: 265-275.

Margolis, F. L. (1969). DNA and DNA-polymerase activity in chicken brain regions during ontogeny. *J. Neurochem., 16*: 447-456.

Stephan, J. K., Chow, B., Frohman, L. A., and Chow, B. F. (1971). Relationship of growth hormone to the growth retardation associated with maternal dietary restriction. *J. Nutr., 101*: 1453-1458.

Thompson, R. (1957). Influence of prenatal maternal anxiety on emotionality in young rats. *Science, 125*: 698-699.

Wehmer, F., Porter, R. H. and Scales, B. (1970). Premating and pregnancy stress in rats affects behavior of grandpups. *Nature (Lond.), 227*: 622.

Winick, M., and Noble, A. (1966). Cellular response in rats during malnutrition at various ages. *J. Nutr., 89*: 300-306.

Zamenhof, S., Grauel, L., van Marthens, E., and Stillinger, R. A. (1971). Quantitative determination of DNA in preserved brains and brain sections. *J. Neurochem., 19*: 61-68.

Zamenhof, S., van Marthens, E., and Grauel, L. (1971). DNA (cell number) in neonatal brain. Second generation (F_2) alteration by maternal (F_0) dietary protein restriction. *Science, 172*: 850-851.

Zamenhof, S., van Marthens, E., and Grauel, L. (1972). DNA (cell number) and protein in rat brain. Second generation (F_2) alteration by maternal (F_0) dietary protein restriction. *Nutr. Metab., 14*: 262-270.

Zamenhof, S., van Marthens, E., and Margolis, F. L. (1968). DNA (cell number) and protein in neonatal brain: Alteration by maternal dietary protein restriction. *Science, 160*: 322-323.

Zeman, F. J. (1968). Effect of maternal protein restriction on the kidney of the newborn young of rats. *J. Nutr., 94*: 111-116.