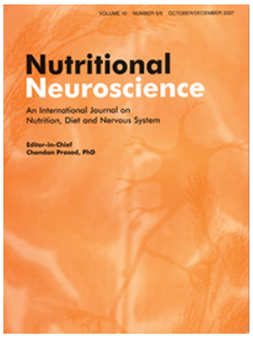

Taylor & Francis
Taylor & Francis Group

Nutritional Neuroscience

An International Journal on Nutrition, Diet and Nervous System

ISSN: 1028-415X (Print) 1476-8305 (Online) Journal homepage: http://www.tandfonline.com/loi/ynns20

Intergenerational transmission of maternal care deficiency and offspring development delay induced by perinatal protein malnutrition

Octavio Gianatiempo, Silvina V. Sonzogni, Estefanía A. Fesser, Laura M. Belluscio, Ezequiel Smucler, Mariela R. Sued & Eduardo T. Cánepa

To cite this article: Octavio Gianatiempo, Silvina V. Sonzogni, Estefanía A. Fesser, Laura M. Belluscio, Ezequiel Smucler, Mariela R. Sued & Eduardo T. Cánepa (2018): Intergenerational transmission of maternal care deficiency and offspring development delay induced by perinatal protein malnutrition, Nutritional Neuroscience, DOI: 10.1080/1028415X.2018.1509178

To link to this article: https://doi.org/10.1080/1028415X.2018.1509178

Intergenerational transmission of maternal care deficiency and offspring development delay induced by perinatal protein malnutrition

Octavio Gianatiempo [1†], Silvina V. Sonzogni[1†], Estefanía A. Fesser[1], Laura M. Belluscio[1], Ezequiel Smucler[2], Mariela R. Sued[2], Eduardo T. Cánepa [1]

[1]Laboratorio de Neuroepigenética, Departamento de Química Biológica, Universidad de Buenos Aires, and Consejo Nacional de Investigaciones Científicas y Técnicas, Ciudad de Buenos Aires, Argentina, [2]Instituto de Cálculo, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, and Consejo Nacional de Investigaciones Científicas y Técnicas, Ciudad de Buenos Aires, Argentina

Objectives: Early life represents a sensitive and critical period for an individual. Nutrition plays a crucial role in the maturation and functional development of the central nervous system. Inadequate nutrition before birth and during the postnatal life can seriously interfere with brain development and lead to behavioral and neurological disorders such as learning disabilities and psychiatric diseases. In addition, the quality of mother–infant interactions represents an important adaptive pathway that prepares offspring for the conditions of life. In this work, we asked if protein malnutrition alters maternal care and offspring development and if these phenotypes can be transmitted to next generation.
Methods: Female mice were fed with a normal or hypoproteic diet during pregnancy and lactation. Nurturing behaviors, i.e. arched, blanket and passive nursing, and liking and grooming of the pups, were evaluated from postnatal day 1 (PD1) to postnatal day 7 (PD7). The same protocol was employed to evaluate maternal behavior for filial generation 1 (F1) and filial generation 2 (F2) dams. Offspring development was evaluated for F1, F2, and F3 generations. Developmental landmarks and neurological reflexes were assessed from PD8 until complete development of the landmark or acquisition of the reflex.
Results: Our results show that malnourished dams provide a lesser and more fragmented maternal care than their normally fed counterparts. This altered maternal behavior as well as the delay in the physical and neurological development observed in the offspring from malnourished mothers was transmitted up to two generations at least.
Conclusion: These results highlight the harmful effects of protein malnutrition even for generations that are not directly exposed to this environmental adversity.

KEYWORDS: Early-life stress, Environmental adversity, Intergenerational transmission, Maternal behavior, Neurodevelopment, Nurturing

Introduction

Early life experiences impact strongly on brain architecture and can have long-lasting consequences on cognitive performance and emotional responses. Thus, the first years of life represent a period of both great opportunity and great vulnerability for child development.[1,2] An enriched environment with social stimulation, suitable nutrition and parental care, provides the opportunity for optimal brain development. Conversely, adverse experiences like poverty, nutritional deficiency or social deprivation increase the risk for the onset of psychopathologies through the entire life.[3,4]

Maternal malnutrition remains one of the most widespread non-genetic factors affecting the development of the new-born's brain.[5,6] In particular, maternal protein malnutrition has been associated with behavioral and neurological deficiencies such as impaired development of cognitive abilities and linked to both acute and long-term health damaging effects.[7,8] Experimental studies on rodents have shown that protein malnutrition in early stages of life could alter neuronal plasticity.[9,10] In addition, reported effects of prenatal and/or postnatal protein

†These authors contributed equally to this work.
Correspondence to: Eduardo T. Cánepa, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria Pabellón II piso 4, C1428EGA Ciudad de Buenos Aires, Argentina. Email: ecanepa@qb.fcen.uba.ar

DOI 10.1080/1028415X.2018.1509178

malnutrition on the offspring include delay in physical growth and neurological development, changes in exploratory activity and anxiety-related behaviors, alterations in learning and memory and differences in the response to aversive stimuli.[11–14]

Parent–offspring interactions are important developmental signal to environmental suitability and have the capacity to modulate physiology and behavior of the offspring.[15,16] In rodents, as in most mammals, these interactions are primarily through the mother. During gestation, interactions mediated by the mother's neuroendocrine system and physiology shapes the neurodevelopment of the fetus including those pathways that mediate responses to stress.[17] Similarly, the care received by an infant during early life can produce changes in the development of neural systems regulating social behavior. Several studies have demonstrated the impact of mother-infant interactions on the hypothalamic–pituitary–adrenal axis, the hypothalamic–pituitary–gonadal axis, and the mesolimbic dopamine system.[18–20] Thereby, the maternal environment surrounding the offspring plays an important role in modulating cognitive and social skills. Furthermore, the effects of maternal behavior can be transmitted to subsequent generations through alterations in the reproductive behavior of offspring.[21] Several evidences point out that the mechanisms mediating this transmission involve epigenetic alterations to steroid receptor genes that produce long-term changes in gene expression and behavior.[22,23]

Besides the naturally occurring variations in maternal care across species, environmental adversities heavily impact on maternal behavior. In rats, dams exposed to stress during pregnancy showed increased pup retrieval latencies, a finding that would seem to reflect an effect of stress on maternal responsivity.[24] Other factors like physical restraint or chronic corticosterone administration induce a decreased frequency of maternal licking/grooming and arched-back nursing.[25,26] More recently, it was observed that exposure of dams to chronic social stress, an ethological model of postpartum depression and anxiety, during lactation impairs maternal care and exerts similar effects on the filial generation 1 (F1) female offspring.[27] Taken together, these findings suggest that environmental adversity alters the mother–offspring interaction.

The critical question we have addressed in this study is whether protein malnutrition alters the mother–offspring relationship and if this were the case, if disrupted maternal behavior is transmitted to the following generations. Our results show that malnourished dams provide a lesser and more fragmented maternal care than their normally fed counterparts. This altered maternal behavior as well as the delay in the physical and neurological development observed in the offspring from malnourished mothers was transmitted up to two generations at least. These results highlight the harmful effects of protein malnutrition even for generations that are not directly exposed to this environmental adversity.

Material and methods

Animals and diets

CF-1 mice from the colony of the Bioterio Central, (removed to protect anonymity), were used for all experiments. The diets employed in this study were prepared according to the AIN-93 Final Report[28] and purchased from Research Diets Inc. (New Brunswick, NJ, USA). NP (normal protein) and LP (low protein) diets contain 20 and 8% of casein as the sole source of proteins, respectively (Supplementary Table 1).[29]

Animals were kept in a 12:12 h light:dark cycle with lights on at 6 a.m., and food and water were administered ad libitum. Experiments were performed in accordance with (removed to protect anonymity).

Experimental design

Ten to twelve weeks old female and male mice (F0) were fed with standard laboratory chow diet (SC) until seven days prior to mating when they were switched to NP diet. Three days prior to mating female mice were switched to the corresponding diet: NP or LP. Male mice continued to be fed with NP diet and were only fed LP diet during mating with LP diet fed females. After mating, dams were kept on the same diet (NP or LP) during pregnancy and lactation (Supplementary Fig. 1A). At postnatal day 21 (PD21) pups were weaned (Supplementary Fig. 1A). After weaning, offspring (F1), as well as the following offspring generations (F2 and F3), were fed with normal SC diet. Therefore, for all generations excluding F0, whenever a dietary treatment is used to designate a group (i.e. F1-LP), this treatment represents the diet received by the corresponding F0 ancestor.

To obtain the F2 generation, NP and LP F1 females between 10 and 12 weeks old were mated following the same protocol as F0. The mating was performed with non-sibling F1-NP or F1-LP males to generate four experimental F2 groups (Supplementary Fig. 1B). These groups were termed: F2-NN, F2-NL, F2-LN, and F2-LL (Each letter designates the F1 mother's experimental group and the F1 father's experimental group respectively). Next, to obtain the F3 generation, NN and LL females between 10 and 12 weeks old from the F2 were mated, following the same protocol as F0, with non-sibling males from the same experimental group to generate only two experimental F3 groups termed F3-NN and F3-LL (Supplementary Fig. 1B). In all generations, efforts were made to

balance the litter size within a range of 10 to 12 pups per dam and the sex ratio near 1 on postnatal day 1 (PD1).

Assessment of maternal behavior

The same protocol was employed to evaluate maternal behavior for F0, F1, and F2 dams. Maternal care was evaluated from PD1 to PD7. Every day, dams were observed for three periods of 1 hour at 9 a.m., 13 p.m., and 16 p.m. During each period, mother's activity was recorded sequentially by an observer every 3 minutes and classified according to the following categories: arched-back nursing, blanket nursing, passive nursing, licking and grooming of the pups, carrying pups, mother/pup contact (excluding nursing posture), nest building, eating, drinking, self-grooming and other non-relevant behaviors without the pups (e.g. sleeping, exploring, rearing, sniffing). The assessment of every generation was made by three observers trained to a high level of inter-rater reliability.[30–32]

Developmental landmark acquisition and neurological reflexes evaluation

Offspring development was evaluated using the same protocol for F1, F2, and F3 generations. Developmental landmarks and neurological reflexes were assessed from PD8 until complete development of the landmark or acquisition of the reflex. Two female and two male pups from each litter were randomly selected and marked on PD8. These pups were weighed every day until weaning (Supplementary Fig. 2). For the assessment of physical growth, the following changes were observed and recorded: ventral fur emergence, opening of the auditory canal and eyes opening. For neurological reflex evaluation, the sound startle reflex and the air righting reflex were analyzed.[13,33] The sound startle reflex test was performed every day from PD8 until acquisition. Pups were held by the scruff of the neck and presented with a sound of 80 dB in a 74 dB background. The reflex was considered present when a sudden and transient freezing was detected immediately after the sound and was considered acquired when it was present for two consecutive days. The air righting reflex test was performed every day from PD12 until acquisition. Mice were held upside-down at a height of 50 cm over a padded surface and then dropped. The reflex was considered present if the pup successfully righted itself during the fall and was considered acquired when it was present in two consecutive days.

Statistical analysis

Statistical analysis was performed using R Statistical Software version 3.4.0.[34] The Shapiro–Wilks (R-base) and the Levene (car package version 2.1-6) tests were used to test the normality and homogeneity of variances, respectively. Normal samples were compared using unpaired Student's *t*-tests with Welch's correction for heteroscedasticity if necessary (R-base) or ANOVA with Tukey's posttests (car package) when comparing more than two samples. For samples that did not pass normality criteria, Wilcoxon–Mann–Whitney tests were used or Kruskal–Wallis tests with Dunn's posttests (dunn.test package version 1.3.5) when comparing more than two samples.

For the analysis of maternal behavior through the days, we used lme4 package version 1.1-15 to perform a generalized linear mixed effects analysis of the relationship between nurturing behavior and diet throughout the postnatal days.[35] We modeled the number of observations of nurturing behavior as a binomial variable. An 'inside-out' model building approach was employed, as suggested by Pinheiro and Bates.[36] For the three generations of dams (F0, F1, and F2) as fixed effects, we entered diet and PD into the model. For the F0 we added an effect to model the final two PDs of LP mothers. For the F1 generation we added PD squared for LP mothers. As random effect we used an independent intercept for each mother to account for the correlated observations. Visual inspection of residual plots did not reveal deviations from homoscedasticity or normality. The significance of fixed-effects was obtained by likelihood ratio tests of the full model against a model without the effect in question and corroborated by parametric bootstrap of the confidence intervals.

For the analysis of food intake of dams during lactation Graphpad Prism 5.01 (La Jolla, CA, USA) was used to perform a repeated measures ANOVA followed by Bonferroni post hoc tests to compare both treatments on each postnatal day.

Principal components analysis (PCA, R-base) was used to formulate an index that characterized the delay in development of each mouse based on the postnatal day of acquisition for each developmental landmark and reflex, and the weight at weaning. PCA was undertaken for scaled variables. The six loadings for the first component, which explains 47.2% of the variance, were used to construct the developmental index as indicated by various conventions: its variance was the only one that was greater than both the mean variance and the variance predicted by the broken stick model and was the only one to the left of the break point in the scree plot (Supplementary Fig. 3A). Moreover, its loadings were easily interpreted: All the developmental landmarks had a negative loading while weight at weaning had a positive one, thus a lower score indicates developmental delays and lower weight (Supplementary Table 2). Finally, this one-

dimensional representation of the data retained the separation of the samples according to the dietary treatment in each generation (Supplementary Fig. 3B).

Results

Protein malnourished dams display disturbed maternal care

To evaluate the global difference in maternal care between NP and LP dams we analyzed lactation and pup licking and grooming events. Lactation, regardless of the mother's position, is the only food source during the first week of postnatal life and represents the main positive contribution to offspring development. Pup licking and grooming is also well established as a beneficial stimulus for the pups during this period. Therefore, lactation events and licking and grooming events were grouped in a single category termed nurturing behavior. This category encompasses all positive behaviors directed towards the pups, which were aggregated from PD1 to PD7 for each mother. Analyzing this parameter, we found that the parental generation (F0) of LP mothers spent less time providing nurturing behavior than NP mothers ($t_{25} = -2.14$, $P = 0.04$) (Fig. 1A upper panel).

Nurturing behavior events within each observational period were clustered in discrete epochs of consecutive events termed bouts (Fig. 1B, upper panel). A larger number of bouts with shorter duration results in fragmented nurturing behavior which has been associated with changes in neuroendocrine functions in offspring.[15,23] Therefore, we analyzed the total number of bouts per mother and their duration, represented by the average of the maximum bout length per period for each mother. The number of nurturing behavior bouts was unchanged between NP and LP F0 mothers ($t_{25} = 0.77$, $P = 0.45$ (Fig. 1C upper panel), but the average maximum bout length was smaller in LP mothers ($t_{25} = -3.01$, $P = 0.006$) (Fig. 1D upper panel). These results indicate that the decrease in the global amount of nurturing behavior observed in LP mothers is explained by a reduction of bout length rather than a decrease in the number of bouts.

Examining the temporal dynamics of maternal behavior, we observed that maternal care declines over the first postnatal week in both NP and LP dams ($\chi^2_1 = 114.01$, $P < 0.001$) (Fig. 2A and Supplementary Fig. 4A). However, in agreement with the results observed with aggregated nurturing

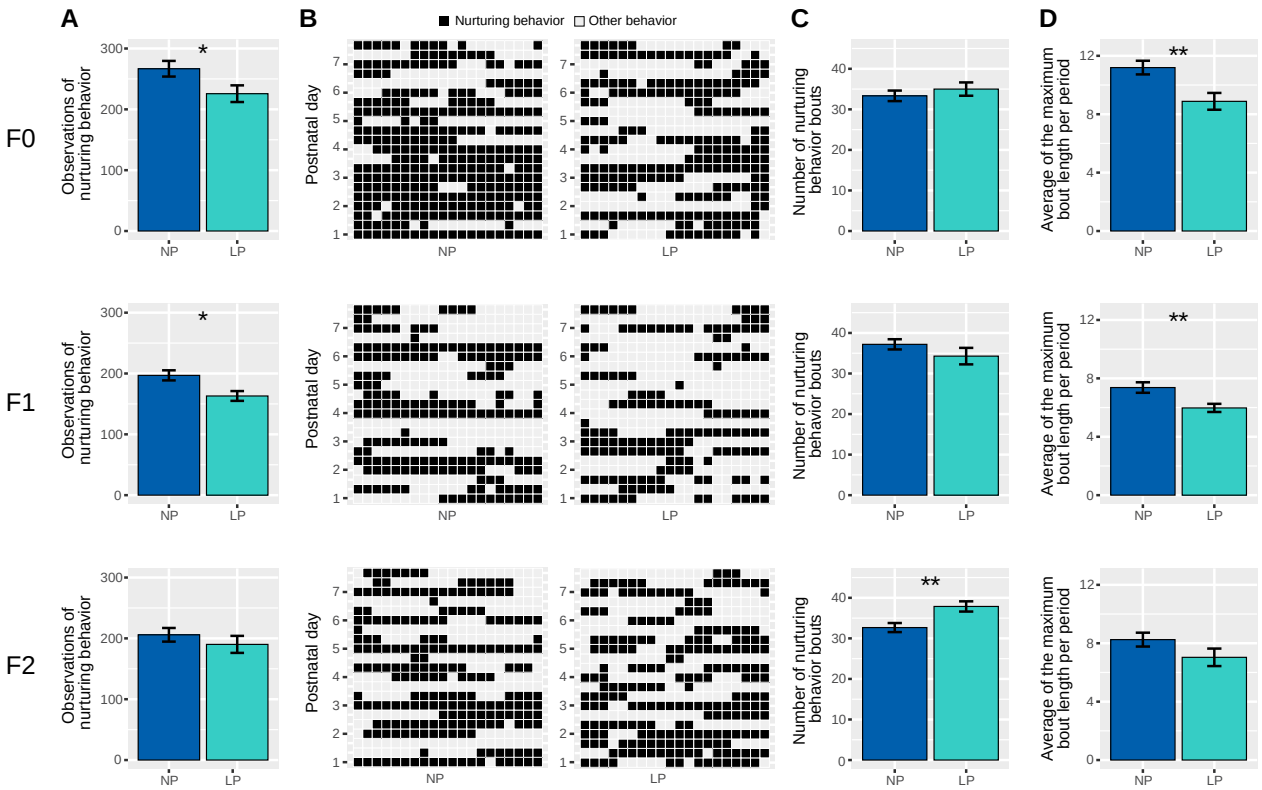

Figure 1 Maternal care global evaluation. (A) Lactation events and licking and grooming events were grouped in a single category termed nurturing behavior and aggregated from PD1 to PD7 for each mother. Bars represent mean ± S.E. of total observations of nurturing behavior performed by dams. (B) Representative observational periods. Each black square represents one observation of nurturing behavior. Nurturing behavior events within each observational period were clustered in discrete epochs of consecutive events termed bouts. (C) Bars represent the mean number ± S.E. of nurturing behavior bouts. (D) Bars represent the mean ± S.E. of the maximum bout length per observational period. (A–D) F0 (upper panel), F1 (middle panel), and F2 (lower panel) dams. (n_{F0}= 12–15, n_{F1}= 14–26, n_{F2}= 8–9). Student's *t*-test or Mann–Whitney test, **P* < 0.05, ***P* < 0.01. NP: normal protein diet, LP: low protein diet

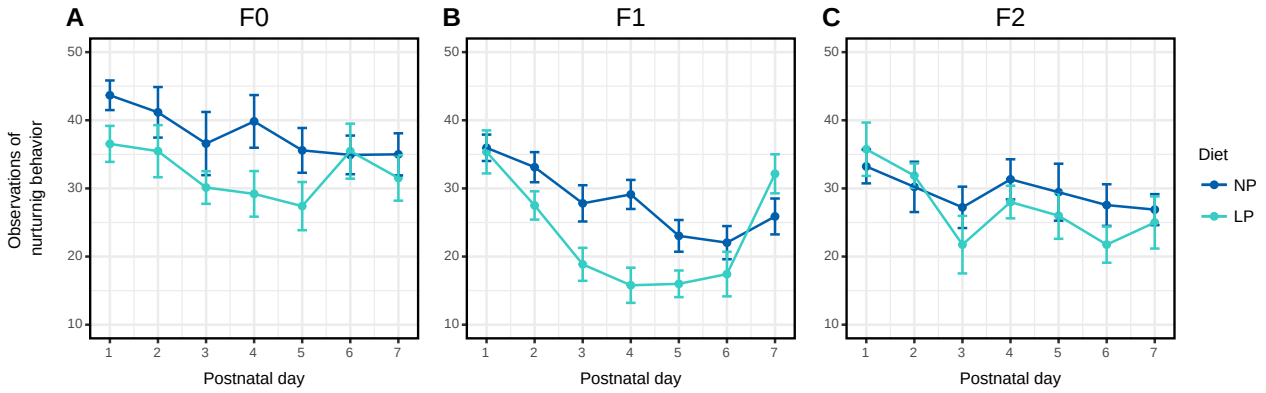

Figure 2 Temporal dynamics of maternal care. Nurturing behavior was aggregated daily from PD1 to PD7 for each mother. Points represent the mean ± S.E. of total observations of nurturing behavior per day for F0 (A), F1 (B), F2 (C) dams. (n_{F0}= 12–15, n_{F1}= 14–26, n_{F2}= 8–9). NP: normal protein diet, LP: low protein diet.

behavior, LP diet was associated with a significant reduction of maternal care compared to NP diet ($\chi^2_1 = 8.23$, $P = 0.004$). Notably, a significant recovery in the amount of nurturing behavior was observed following PD5 for the LP group ($\chi^2_1 = 65.85$, $P < 0.001$) (Fig. 2A). As a whole, these results show that maternal care is significantly impaired by protein malnutrition.

Disturbed maternal care of malnourished dams is intergenerationally transmitted

Several reports have pointed out the pervasive impact of early life adversities since its long-lasting effects.[37,38] Therefore, we wonder if the disturbed maternal behavior observed in F0 malnourished dams was transmitted to the next generations.

To do this, we analyzed the nurturing behavior, the number of bouts and the average of the maximum bout length in F1 and F2 dams. Remarkably, despite not having been exposed directly to LP diet, the female offspring of F0 LP dams also showed a decrease in the overall amount of nurturing behavior ($t_{38} = -2.65$, $P = 0.012$) (Fig. 1A, middle panel) accompanied by a reduction in the average maximum bout length ($t_{38} = -3.07$, $P = 0.004$) (Fig. 1D, middle panel), while the number of bouts remained unchanged ($t_{38} = -1.28$, $P = 0.21$) (Fig. 1C, middle panel). As well as in the F0 generation, this suggests a reduction of maternal care characterized by shorter epochs of nurturing behavior with no change in the number of bout. The temporal dynamics of maternal behavior of F1 generation resemble the F0 (Fig. 2B and Supplementary Fig. 4B). There was a decline in nurturing behavior through the days ($\chi^2_1 = 236.70$, $P < 0.001$) and maternal exposure to LP diet was associated with a reduction in maternal care ($\chi^2_1 = 34.04$, $P < 0.001$). Once more, a recovery of nurturing behavior was also observed following PD6 for the LP group ($\chi^2_1 = 343.54$, $P < 0.001$).

In the second filial generation, we observed a partial recovery of the maternal care quality since the global amount of nurturing behavior was not affected ($W = 25.5$, $P = 0.34$) (Fig. 1A, lower panel). Unexpectedly, the number of nurturing behavior bouts was significantly increased in the LP group of this generation ($t_{15} = 3.10$, $P = 0.007$) (Fig. 1C, lower panel) but the maximum bout length was not affected by the dietary treatment ($t_{15} = -1.61$, $P = 0.13$) (Fig. 1D, lower panel). This could imply that the recovery of the quality of maternal care is not complete, since LP dams seem to compensate not only by increasing bout length but also by increasing the number of bouts which may lead to a fragmented maternal care. As expected, in F2 generation there was a general reduction of maternal care across the postnatal period in both groups ($\chi^2_1 = 49.45$, $P < 0.001$) and the decrease associated with the LP diet was absent ($\chi^2_1 = 0.86$, $P = 0.35$) (Fig. 2C and Supplementary Fig. 4C).

In summary, the impact of protein malnutrition on maternal behavior could be observed up to a second generation of offspring suggesting the intergenerational nature of its transmission.

Distribution of maternal activities across generations

To further strengthen our hypothesis about the disrupting effects of protein malnutrition on maternal care we quantified and analyzed all the activities performed by NP and LP mothers during the first week postpartum. The dam activities –described in section 2.3– were classified in three categories: activities directly beneficial for pups (nursing, licking and grooming the pups, carrying the pups, mother–pup contact, nest building), activities indirectly beneficial for pups (mother eating or drinking) and activities not beneficial for the pups (self-grooming and any other activity outside the nest).

The frequency of activities in each category for every mother was analyzed employing a ternary

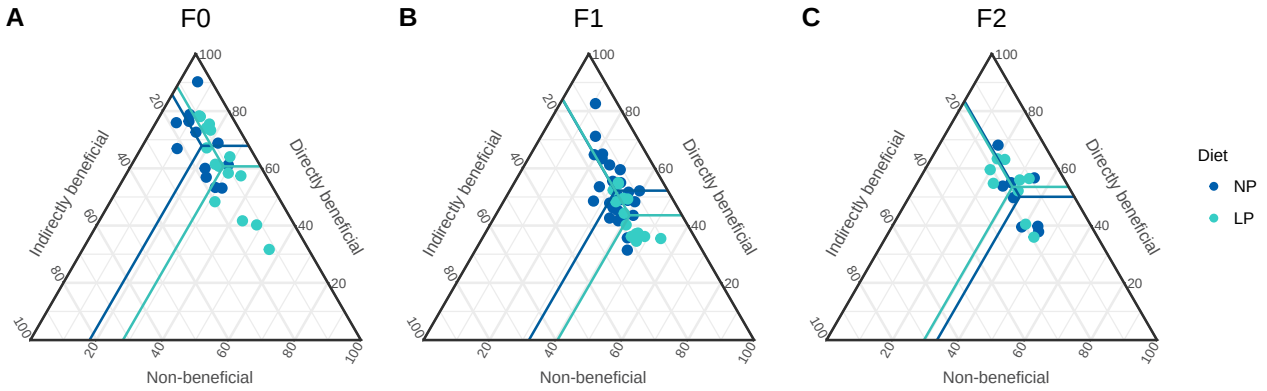

Figure 3 Distribution of maternal activities. The distribution of maternal activities was represented in a ternary plot graph. Maternal activities were classified in three categories: directly beneficial for pups (nursing, licking and grooming of the pups, carrying the pups, mother–pup contact, nest building), indirectly beneficial for pups (mother eating or drinking) and not beneficial for the pups (self-grooming and any other activity outside the nest). Each category is represented in a side of the triangle and the corners indicate the maximum value for a category. Each point represents a mother. Lines are the mean value for each group. (n_{F0}= 12–15, n_{F1}= 14–26, n_{F2}= 8–9)

graph (Fig. 3). In this type of graph each point corresponds to a mother and each corner represents the case when all observations for a particular mother fall in a single category. For the three generations studied the decrease in beneficial activities was associated with an increase in non-beneficial activities. Whilst in F0 and F1 the LP mothers showed a tendency to separate from NP mothers, in the F2 there is no clear separation between groups.

Food intake of dams during pregnancy and lactation

Offspring's nutritional condition depends almost exclusively on mother's nutrients and caloric intake. Therefore, we measured the food intake of F0 dams from both groups during pregnancy and lactation. We observed that, during pregnancy, LP dams consumed 38% more food per day than their NP counterparts ($t_{17} = 2.87$, $P = 0.01$) (Supplementary Fig. 5A). However, since the protein content of the LP diet is 60% lower than that of the NP diet, we conclude that the higher food intake of the LP dams during this period does not compensate for the protein deficiency in their diet.

A repeated measures two-way ANOVA was used to evaluate consumption during lactation and a significant effect was found the interaction between time and treatment ($F_{12,144} = 3.65$, $P < 0.001$). Bonferroni's post hoc tests showed that NP dams consumed more food per day than their LP counterparts during this period ($\alpha = 0.05$) (Supplementary Fig. 5B). In the last postnatal days there was an increase of consumption in the NP group that can be explained by the pups directly eating the chow. The relative food intake, i.e. total quantity of food per day relative to the number of lactating pups, was also analyzed with a repeated measures two-way ANOVA. Interaction between time and treatment ($F_{12,144} = 2.75$, $P = 0.002$) was significant. Bonferroni's post hoc test showed no difference between groups in the relative food intake ($\alpha = 0.05$) (Supplementary Fig. 5C), suggesting that the difference in consumption can be explained by the number of pups in each litter. Since both diets are isocaloric, these results strongly suggest that the differences between LP and NP mice should be due to the protein deficiency and not to a lesser quantity of food and/or calories consumed by the mothers.

Impaired physical and neurological offspring development caused by early protein malnutrition affects multiple generations

To characterize whether protein malnutrition affects pup's development and especially if these conditions were maintained during the next generations, principal component analysis (PCA) was used to construct a developmental index that characterized the delay in development for each mouse based on the postnatal day of acquisition for each developmental landmark and reflex (eye opening, ear canal opening, ventral fur emergence, air righting reflex and sound startle reflex), and the weight at weaning. A lower score indicates later acquisition of landmarks and lower weight at weaning (Supplementary Table 2). The developmental score was obtained from the first principal component of the PCA and an average score was calculated for each combination of generation, sex and treatment.

In the case of the first generation, we observed a significant decrease in the developmental index for both F1 LP males and females when compared with control mice (Males: $t_{48} = -7.31$, $P < 0.001$, Females: $t_{31.11} = -4.85$, $P < 0.001$) (Fig. 4A). For the F2 generation males (Fig. 4B) we observed a significant effect of the diet on developmental score

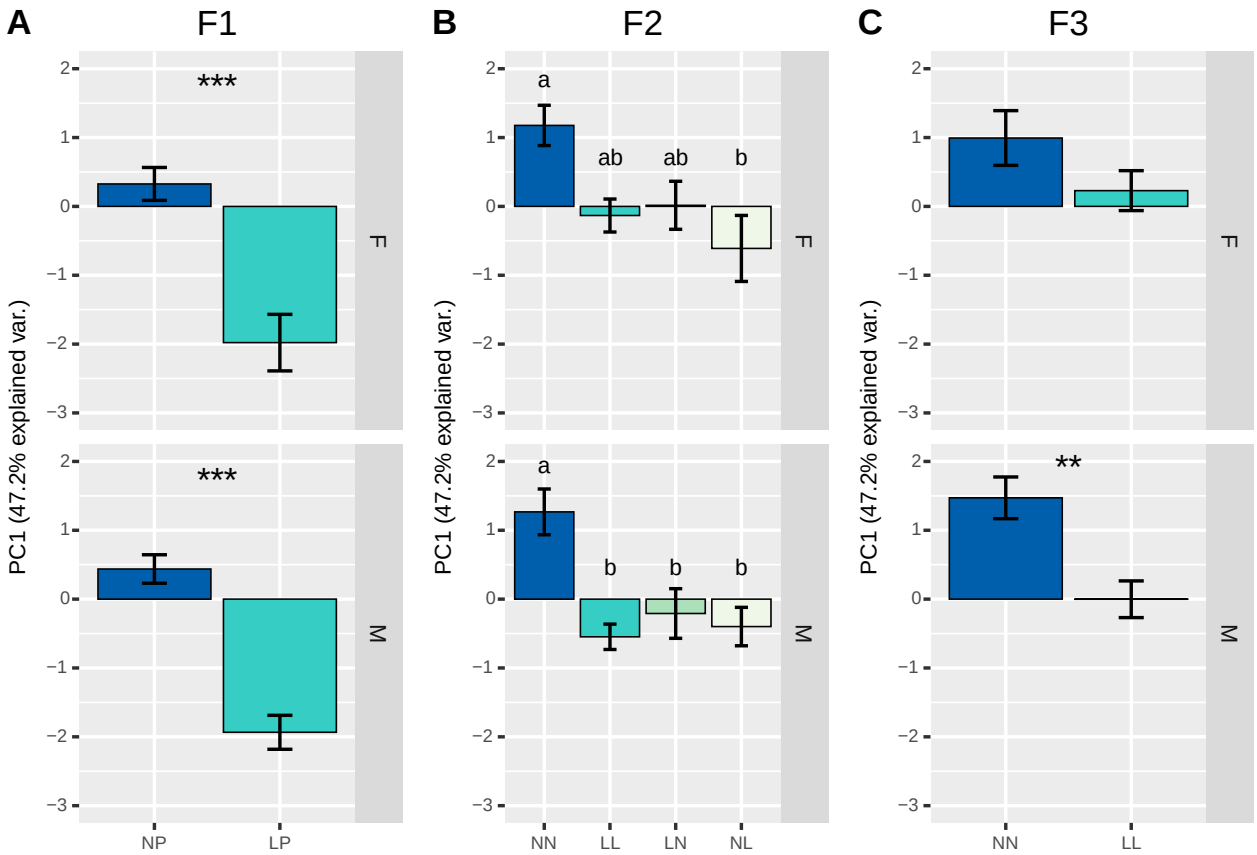

Figure 4 Developmental score analysis. Principal components analysis (PCA) was used to obtain a developmental score for each mouse based on the postnatal day of acquisition for each developmental landmark evaluated and the weight at weaning as described in section 2.4. The average score was calculated for each combination of generation, sex and treatment. Bars represent mean ± S.E. of scores for F1 (A), F2 (B), F3 (C) offspring generations. ($n_{F1} = 20–26$, $n_{F2} = 13–22$, $n_{F3} = 13–18$). Student's *t*-test or Mann–Whitney test for F1 and F3. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. ANOVA with Tukey's post hoc test or Kruskal–Wallis with Dunn's post hoc test for F2. Different letters indicate significant differences between groups. F (female, upper panels); M (male, lower panels); NP: normal protein diet; LP: Low protein diet; NN and LL: both parents NP or LP, respectively; LN and NL, mother LP and father NP or vice versa, respectively

($\chi^2_3 = 16.47$, $P < 0.001$). Dunn's post hoc test showed that the delay was attenuated but present in LL, LN and NL groups when compared with NN group, but there were no significative differences between treated groups ($\alpha = 0.05$). For F2 females (Fig. 4B) the effect of the diet on the developmental score was also significant ($F_{3,67} = 4.93$, $P = 0.004$) but Tukey's post hoc contrasts found differences only when comparing the NL group with the NN group ($\alpha = 0.05$). Regarding the F3 generation (Fig. 4C) the delay was only present in males (Males: $t_{32} = -3.61$, $P = 0.001$, Females: $t_{27} = -1.49$, $P = 0.15$).

In summary, these results show that the developmental delays associated with F0 exposure to perinatal protein malnutrition were sex dependent with higher prevalence in males and declined in each generation. The fact this effect could be observed in the third generation of LP males suggest a possible transgenerational transmission of the delays. Nevertheless, the transgenerational nature of this transmission cannot be assured due to the incomplete recovery of F2 maternal behavior.

Discussion

The results obtained in the present work indicate that dams fed with low protein diet during the perinatal period display abnormal maternal behavior. The main feature of this anomalous maternal behavior was that less time was spent performing nurturing behavior, also the nurturing bout length was shorter without change in the number of nurturing bouts.

In agreement with our results, previous reports have indicated that low-protein fed mothers may, under certain circumstances, show reduced attention to their offspring and lick their pups less often than control fed dams.[13,39–42] Conversely, several works have shown opposite results, indicating that exposure to perinatal protein malnutrition and others types of malnutrition may induce an increase in active pup nursing in comparison with control fed mothers.[43–46] Regardless of using a perinatal malnutrition treatment, similar to the one applied in our model, Robert Hall has observed that low-protein fed mothers spent more time nursing their pups than well-nourished controls.[47] On the contrary, the

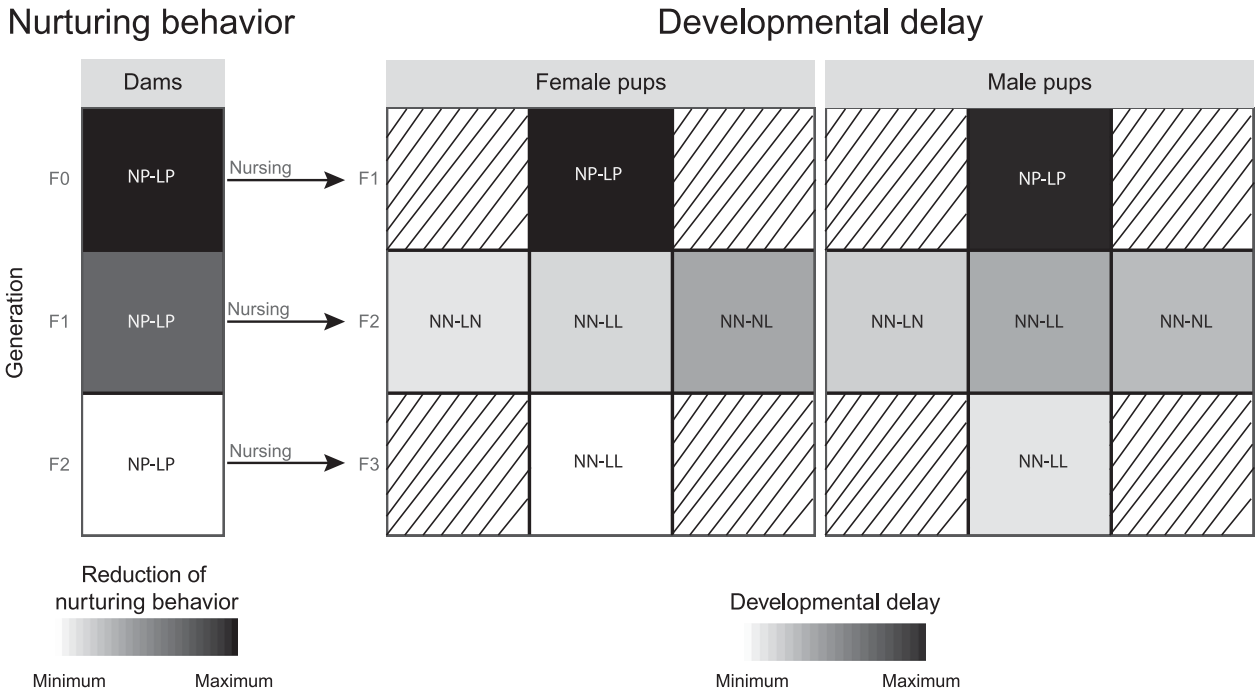

Figure 5 Summary of the obtained results. On the left side, the difference in nurturing behavior between NP and LP mothers for each generation evaluated is shown. A darker color indicates a greater difference between NP and LP maternal behavior, i.e. a greater reduction in nurturing behavior associated to the dietary treatment. For example, the reduction of nurturing behavior present in F0 LP dams when compared with F0 NP dams is greater than the reduction of nurturing behavior displayed by F1 LP dams when compared with F1 NP dams. On the right side, the difference in the developmental score between the offspring of each treated group and the corresponding control group is shown. Again, a darker color indicates a greater difference between the developmental score of the treated and control groups, i.e. a greater delay in development for the treated group with respect to the control group. For example, the developmental delay of F3 LL male pups when compared with F3 NN male pups is greater than the developmental delay of F3 LL female pups when compared with F3 NN female pups., Offspring: F1: NP: both parents NP, LP: both parents LP. F2: NN: both parents NP, LL: both parents LP, NL: NP mother and LP father, LN: LP mother and NP father. F3: NN: both parents NN, LL: both parents LL

maternal care observation protocol employed by Hall had important differences with our protocol. They performed many observations in a short lapse of time (one observation per minute of a 5-min period every 3 hours) from PD 1 to PD 20 and they did not group into a single category the nursing and licking and grooming behaviors. These differences might be the cause of the discrepancies observed between both works.

The demonstration that the abnormal maternal care caused by protein malnutrition was not limited to the first and directly exposed generation (F0) was one of the most interesting observations of the present work. We have observed that it was transmitted to the following generation (F1) in spite of being fed with normal food after weaning. In F2 generation the LP mothers showed a recovery in the global amount of maternal care mainly driven by an increase in the number of bouts rather than an enlargement of bout length. Even though this increase in nurturing may seem beneficial, several works have described this form of maternal behavior as fragmented and unpredictable and have shown that can also have long-lasting negative consequences on offspring.[48–50] All comparisons were made between groups within the same generation because nurturing behavior decreases in F1 and F2 compared to F0. One possible explanation for this decline is that the F0 generation dams were reared undisturbed in the breeding sector of the animal facility, on the contrary, the F1 and F2 females were handled by us, including noninvasive manipulations such as lifting, cleaning and moving of the cages and developmental tests that were performed from PD 7 until weaning. Routine handling is a cause of stress for experimental animals.[51] Furthermore, the quality of maternal care in rodents, particularly during sensitive periods early in life, is susceptible to any type of stress.[24] Thus, we hypothesize that the routine handling and the development tests performed could represent a source of stress for F1 and F2 pups of both groups (NP and LP) affecting the maternal care they display as adults.

While numerous studies propose that the amount and nature of maternal care can be transferred to next generation,[22,52,53] the transmission of abnormal maternal care due to protein malnutrition to the following generations has not yet been explored. Studies performed by Galler's lab[54] address a related

question with a different experimental approach. In their work, maternal behavior was studied in rat dams maintained during 15–20 generations with malnourished diet and then restored with a well-nourished diet for three generations. This work has shown, as opposed to our results, an increment in active maternal care in malnourished dams. This contradictory outcome could be explained by the dissimilarities in experimental design. In our work, the exposure to malnutrition was only during the first generation. This is not a trivial difference since the question and the experimental procedure is not the same.

Little is known about how protein malnutrition impacts on offspring's postnatal development and its contribution to later life physiology. Previous work from our laboratory has shown that protein malnutrition affects the progeny phenotype.[13,29] In the current work, we confirmed this affectation and showed the transgenerational effects of protein malnutrition. The LP offspring presented a significant developmental delay measured by the developmental index. The index was significantly different across three generations in males and across two generations for females. In our paradigm, the exposure to protein malnutrition occurs in pregnant and lactating dams. Thus, protein malnutrition affects the forming fetus (F1) and its developing germ cells during pregnancy, and the F1 pups and their gametes during lactation. In this way, only the effects present in the third generation of offspring can be deemed transgenerational. Recently, it has been reported that epigenetic mechanisms are involved in the inter- or transgenerational transmission of environmental modulated phenotypes.[22] Since alterations in maternal care and delays in offspring's development are maintained until the third generation, our results suggest the participation of epigenetic mechanisms in the transmission of protein malnutrition cues. When considering these results, it is important to remember that our model does not allow us to study independently the effect of protein malnutrition on maternal care and on offspring development. It is, therefore, possible that the effects of protein malnutrition on preweaning growth and development may have been exacerbated by the reduction in maternal behavior caused by the aforementioned treatment.[55] The inverse case is also feasible, as delays in offspring development might encompass the belated appearance of necessary cues that trigger a normal maternal behavior.[56]

Fig. 5 summarizes the results obtained in this work (Fig. 5). On the left side is represented the difference in nurturing behavior between NP and LP mothers. For example, a big difference indicates a reduction of maternal care performed by LP mothers (black color). The biggest difference is observed in F0 generation, when protein malnutrition occurs. In F1, the difference is still present despite of the offspring has been feeding with normal food after weaning. In F2 the difference is negligible; however the maternal care is not fully recovered since LP mothers showed a fragmented nature of maternal behavior. On the right-side offspring of LP mothers is represented in a sex specific manner. The color scale indicates the difference in developmental score with the control group within each generation. The developmental delay decreased across the different generations, differentially affecting male and female offspring. Perinatal protein malnutrition seems to have a transgenerational effect in male offspring and an intergenerational effect in female offspring. The figure shows how the protein malnutrition occurring only in F0 mothers and in consequence the maternal care received by pups affects the pup's development.

Supplemental data

Supplemental data for this article can be accessed 10.1080/1028415X.2018.1509178

Disclaimer statements

Funding This work was supported by Agencia Nacional de Promoción Científica y Tecnológica: [Grant Number PICT-2013-0653]; Consejo Nacional de Investigaciones Científicas y Técnicas [Grant Number PIP 2012-0476] and Universidad de Buenos Aires [Grant Number UBACYT2014-011].

Disclosure of interest The authors declare no conflict of interest.

Ethics approval None

ORCID

Octavio Gianatiempo http://orcid.org/0000-0001-5660-942X

Eduardo T. Cánepa http://orcid.org/0000-0002-4322-1650

References

1 Hoeijmakers L, Lucassen PJ, Korosi A. The interplay of early-life stress, nutrition, and immune activation programs adult hippocampal structure and function. Front Mol Neurosci 2015;7 (January):1–16.
2 Hoffmann A, Spengler D. DNA memories of early social life. Neuroscience 2014;264:64–75.
3 Fox SE, Levitt P, Nelson CA. How the timing and quality of early experiences influence the development of brain architecture. Child Dev 2010;81(1):28–40.
4 Krugers HJ, Arp JM, Xiong H, Kanatsou S, Lesuis SL, Korosi A, et al. Early life adversity: lasting consequences for emotional learning. Neurobiol Stress 2017;6:14–21.
5 Food Security Information Network. Global report on food crises. 2017.
6 Walker SP, Wachs TD, Meeks Gardner J, Lozoff B, Wasserman GA, Pollitt E, et al. Child development: risk factors for adverse outcomes in developing countries. Lancet 2016;369(9556): 145–57.

7 Morgane PJ, Mokler DJ, Galler JR. Effects of prenatal protein malnutrition on the hippocampal formation. Neurosci Biobehav Rev 2002;26(4):471–83.
8 Kar BR, Rao SL, Chandramouli BA. Cognitive development in children with chronic protein energy malnutrition. Behav Brain Funct 2008;4:31.
9 Godoy MA, Souza AS, Lobo MA, Sampaio OV, Moraes L, Baldanza MR, et al. Effects of protein restriction during gestation and lactation on cell proliferation in the hippocampus and subventricular zone: functional implications. protein restriction alters hippocampal/SVZ cell proliferation. Brain Res 2013;1496:10–27.
10 Ranade SC, Rose A, Rao M, Gallego J, Gressens P, Mani S. Different types of nutritional deficiencies affect different domains of spatial memory function checked in a radial arm maze. Neuroscience 2008;152(4):859–66.
11 Maria Moreira Camargo L, de Sousa Almeida S. Early postnatal protein malnutrition changes the development of social play in rats. Physiol Behav 2005;85(3):246–51.
12 Reyes-Castro LA, Rodriguez JS, Charco R, Bautista CJ, Larrea F, Nathanielsz PW, et al. Maternal protein restriction in the rat during pregnancy and/or lactation alters cognitive and anxiety behaviors of female offspring. Int J Dev Neurosci 2012;30(1):39–45.
13 Belluscio LM, Berardino BG, Ferroni NM, Ceruti JM, Cánepa ET. Early protein malnutrition negatively impacts physical growth and neurological reflexes and evokes anxiety and depressive-like behaviors. Physiol Behav 2014;129:237–54.
14 Belluscio LM, Alberca CD, Pregi N, Cánepa ET. Altered gene expression in hippocampus and depressive-like behavior in young adult female mice by early protein malnutrition. Genes, Brain Behav 2016;15(8):741–9.
15 Curley JP, Champagne FA. Influence of maternal care on the developing brain: mechanisms, temporal dynamics and sensitive periods. Front Neuroendocrinol 2016;40:52–66.
16 Meaney MJ, Szyf M. Maternal care as a model for experience-dependent chromatin plasticity? Trends Neurosci 2005;28(9):456–63.
17 Champagne FA, Meaney MJ. Stress during gestation alters postpartum maternal care and the development of the offspring in a rodent model. Biol Psychiatry 2006;59(12):1227–35.
18 Buschdorf JP, Meaney MJ. Epigenetics/programming in the HPA axis. Compr Physiol 2015;6(1):87–110.
19 Cameron N, Del Corpo A, Diorio J, McAllister K, Sharma S, Meaney MJ. Maternal programming of sexual behavior and hypothalamic-pituitary-gonadal function in the female rat. PLoS One 2008;3(5):e2210.
20 Peña CJ, Neugut YD, Calarco CA, Champagne FA. Effects of maternal care on the development of midbrain dopamine pathways and reward-directed behavior in female offspring. Eur J Neurosci 2014;39(6):946–56.
21 Meaney MJ. Maternal care, gene expression, and the transmission of individual differences in stress reactivity across generations. Annu Rev Neurosci 2001;24:1161–92.
22 Champagne FA. Epigenetic mechanisms and the transgenerational effects of maternal care. Front Neuroendocrinol 2008;29(3):386–97.
23 Peña CJ, Neugut YD, Champagne FA. Developmental timing of the effects of maternal care on gene expression and epigenetic regulation of hormone receptor levels in female rats. Endocrinology 2013;154(11):4340–51.
24 Champagne FA, Francis DD, Mar A, Meaney MJ. Variations in maternal care in the rat as a mediating influence for the effects of environment on development. Physiol Behav 2003;79(3):359–71.
25 Baker S, Chebli M, Rees S, LeMarec N, Godbout R, Bielajew C. Effects of gestational stress: 1. evaluation of maternal and juvenile offspring behavior. Brain Res 2008;1213:98–110.
26 Brummelte S, Galea LAM. Chronic corticosterone during pregnancy and postpartum affects maternal care, cell proliferation and depressive-like behavior in the dam. Horm Behav 2010;58(5):769–79.
27 Nephew BC, Carini LM, Sallah S, Cotino C, Alyamani RAS, Pittet F, et al. Intergenerational accumulation of impairments in maternal behavior following postnatal social stress. Psychoneuroendocrinology 2017;82(May):98–106.
28 Reeves PG, Nielsen FH, Fahey GC. AIN-93 purified diets for laboratory rodents: final report of the American institute of nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet. J Nutr 1993;123(11):1939–51.
29 Berardino BG, Fesser EA, Cánepa ET. Perinatal protein malnutrition alters expression of miRNA biogenesis genes Xpo5 and Ago2 in mice brain. Neurosci Lett 2017;647:38–44.
30 Cohen J. A coefficient of agreement for nominal scales. Educ Psychol Meas 1960;20(1):37–46.
31 Hallgren KA. Computing inter-rater reliability for observational data: an overview and tutorial. Tutor Quant Methods Psychol 2012;8(1):23–34.
32 Champagne FA, Curley JP, Keverne EB, Bateson PPG. Natural variations in postpartum maternal care in inbred and outbred mice. Physiol Behav 2007;91(2–3):325–34.
33 Ohishi T, Wang L, Akane H, Shiraki A, Sato A, Uematsu M, et al. Adolescent hyperactivity of offspring after maternal protein restriction during the second half of gestation and lactation periods in rats. J Toxicol Sci 2012;37(2):345–52.
34 R Development Core Team. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vol. 0. Vienna: Austria; 2015. ISBN 3-900051-07-0.
35 Bates D, Mächler M, Bolker B, Walker S. Fitting linear mixed-effects models using lme4. J Stat Softw 2015;67(1):51.
36 Pinheiro JC, Bates DM. Mixed-Effects models in S and S-PLUS. New York: Springer-Verlag; 2000. (Statistics and Computing).
37 Gutièrrez-Mecinas M, Trollope AF, Collins A, Morfett H, Hesketh SA, Kersanté F, et al. Long-lasting behavioral responses to stress involve a direct interaction of glucocorticoid receptors with ERK1/2-MSK1-Elk-1 signaling. Proc Natl Acad Sci USA 2011;108(33):13806–11.
38 Pérez-García G, Guzmán-Quevedo O, Da Silva Aragão R, Bolaños-Jiménez F. Early malnutrition results in long-lasting impairments in pattern-separation for overlapping novel object and novel location memories and reduced hippocampal neurogenesis. Sci Rep 2016;6(August 2015):21275.
39 Franková S. Relationship between nutrition during lactation and maternal behaviour of rats. Act Nerv Super (Praha) 1971;13(1):1–8.
40 Smart JL, Preece J. Maternal behavior of undernourished mother rats. Anim Behav 1973;21(3):613–9.
41 Chaplin TM, Visconti KJ, Molfese PJ, Susman EJ, Klein LC, Sinha R, et al. Prenatal cocaine exposure differentially affects stress responses in girls and boys: associations with future substance use. Dev Psychopathol 2015;27(01):163–80.
42 Regalado M, Torrero C, Salas M. Maternal responsiveness of neonatally undernourished and sensory stimulated rats: rehabilitation of maternal behavior. Nutr Neurosci 1999;2(1):7–18.
43 Massaro TF, Levitsky DA, Barnes RH. Protein malnutrition in the rat: its effects on maternal behavior and pup development. Dev Psychobiol 1974;7(6):551–61.
44 Crnic LS. Models of infantile malnutrition in rats: effects on maternal behavior. Dev Psychobiol 1980;13(6):615–28.
45 Rocha JBT, Soares FAA, de Mello CF. Influence of the test situation on pup retrieval behavior of normal and undernourished lactating rats. Brazilian J Med Biol Res 2002;35(1):91–7.
46 Batista TH, Veronesi VB, Ribeiro ACAF, Giusti-Paiva A, Vilela FC. Protein malnutrition during pregnancy alters maternal behavior and anxiety-like behavior in offspring. Nutr Neurosci 2016;8305(May):1–6.
47 Hall RD, Leahy JP, Robertson WM. The effects of protein malnutrition on the behavior of rats during the suckling period. Dev Psychobiol 1979;12(5):455–66.
48 Baram TZ, Davis EP, Obenaus A, Sandman CA, Small SL, Solodkin A, et al. Fragmentation and unpredictability of early-life experience in mental disorders. Am J Psychiatry 2012;169(9):907–15.
49 Ivy a S, Brunson KL, Sandman C, Baram TZ. Dysfunctional nurturing behavior in rat dams with limited access to nesting material: a clinically relevant model for early-life stress. Neuroscience 2008;154(3):1132–42.
50 Heun-Johnson H, Levitt P. Early-life stress paradigm transiently alters maternal behavior, dam-pup interactions, and offspring vocalizations in mice. Front Behav Neurosci 2016;10:142.
51 Balcombe JP, Barnard ND, Sandusky C. Laboratory routines cause animal stress. Contemp Top Lab Anim Sci 2004;43(6):42–51.
52 Champagne FA, Meaney MJ. Transgenerational effects of social environment on variations in maternal care and behavioral response to novelty. Behav Neurosci 2007;121(6):1353–63.
53 Curley JP, Davidson S, Bateson P, Champagne FA. Social enrichment during postnatal development induces transgenerational

effects on emotional and reproductive behavior in mice. Front Behav Neurosci 2009;3:25.
54 Galler JR, Propert KJ. Early maternal behaviors predictive of the survival of suckling rats with intergenerational malnutrition. J Nutr 1982;112(2):332–7.
55 Weaver ICG, Cervoni N, Champagne FA, D'Alessio AC, Sharma S, Seckl JR, et al. Epigenetic programming by maternal behavior. Nat Neurosci 2004;7(8):847–54.
56 Stern JM. Offspring-induced nurturance; animal-human parallels. Dev Psychobiol 1997;31(1):19–37.