

Support for the Committee for Aid and Education in Neurochemistry (CAEN)

Report

CATEGORY 1B: Research supplies for use in the applicant's home laboratory International Society for Neurochemistry

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“Protein malnutrition and premature aging: impact on cognitive skills and neuronal plasticity”

Background

Early-life adversity increases the vulnerability to develop psychopathologies and cognitive decline later in life. In mammals (including humans), nutrition in early life (including in utero) can have lasting effects on health during aging, often referred to as developmental programming. Heart disease, diabetes, obesity, depression, substance abuse, school success, premature mortality, disability at retirement, and accelerated aging and memory loss all have determinants in early life ¹. This association is supported by clinical and preclinical studies. Remarkably, experiences of stress during this sensitive period, like malnutrition elicit long-term effects on brain structure and function ². Aging is an inevitable natural phenomenon mainly characterized by increased oxidative stress (OS), elevated inflammatory response, accelerated cellular senescence, and progressive organ dysfunction that lead to a gradual decline in physical and mental faculties of individuals ³. Brain is arguably the most multifaceted tissue in complex organisms, controlling processes that are vital not only to life but also at the heart of cognition and personality. Aging brain is characterized by low level of chronic inflammation and increased OS, both seems to be the damaging agents in age-related neurodegeneration. An extensive literature shows that the aging process is linked to the accumulation of nuclear DNA damage, especially in the tissues with limited cell proliferation, e.g. brain and heart. In the brain, both single- and double-strand breaks in DNA accumulate during aging, particularly in neurons ⁴. Recent studies have suggested an association between an adverse intrauterine environment and premature aging. However, little is known about the molecular mechanisms underlying interaction between diet and aging trajectory. Given that, the perinatal malnutrition can induce deleterious changes in brain structure and function and they can persist into adulthood, thus the general objective of this project is to analyze the role of protein malnutrition during pregnancy and lactation, as an early

adversity agent that would cause stress and its relation to the establishment of senescence and premature aging.

In our laboratory, we have set up a perinatal malnutrition model in mice by feeding dams with a low protein diet (LP group) or normal protein diet (NP group) during pregnancy and lactation. The offspring (fed with normal diet after weaning) of the malnourished dams exhibited a delay in both physical and neurological development, traits of anxiety-like behavior ⁵ and impairment in long term memory as measured by the object recognition task (unpublished data). We have observed that malnutrition affects the neuromuscular coordination in matured mice. This deterioration was detected earlier in mice from the LP group. In the CA1, CA3 and dentate gyrus regions fewer differentiated neurons were observed in LP mice. In addition, preliminary results obtained by culturing in vitro mouse embryonic fibroblasts (MEFs) extracted from females NP and LP showed that, MEFs from malnourished mothers undergo senescence prematurely. We observed an increase expression of Senescence-associated beta-galactosidase (SA- β gal) activity, reduced percentages of BrdU-positive and increased ROS at early passage in LP MEFs.

Paradigm of protein malnutrition

CF-1 mice (crlfcm:CF1) from the colony of the Bioterio Central, Facultad de Ciencias Exactas y Naturales (University of Buenos Aires), were used for all experiments. The diets used in this study were formulated by Research Diets Inc. (New Jersey, USA). Normal protein (NP) diet contains 20% casein and low protein (LP) diet contains 8% casein, both diets are isocaloric. Female mice (F0) were fed with NP diet for four days and then switched to the assigned diet (NP or LP) for three days prior mating. Male mice from the F0 were only fed with NP diet for three days prior mating. For mating, one male was housed with two nulliparous females for five consecutive days. Ten days after the end of the mating period female mice were individually housed and kept under the same diet until weaning (P21). Weight was regularly measured during both pregnancy and lactation. On PD2 litters were adjusted to 8-9 pups per litter with a 1:1 male:female ratio when possible. Offspring (F1) was fed with regular laboratory chow after weaning.

To evaluate long- term effects of perinatal protein malnutrition, associated with premature aging, NP or LP mice of 2 month (young), 7 month (adult) and 12 month (old) were use in all experiment.

1. Brain Function

To investigate the effect of protein malnutrition on brain functions during aging three different techniques were setup: two tests to measure olfactory function and one test to measure cognition (working memory).

1.1 Buried pellet test: which relies on the animal's natural tendency to use olfactory cues for foraging, is used to confirm ability to smell volatile odors. The main parameter is the latency to uncover a small piece of chow, cookie, or other palatable food, hidden beneath a layer of cage bedding in a maximum of five minutes. Three different pellets were tested: chocolate cookie, cheese pellet and strawberry pellet. Considering the pellet, the mice always preferred the chocolate cookie. Related to the amount of cage bedding and time of fasting, different conditions were tested and have been shown in table 1.

Table 1

Protocol	Pellet	Fasting Time (hours)	Amount of cage bedding	Latency to uncover the pellet
1	chocolate cookie	20	3 cm	1:42 min.
2	chocolate cookie	24	5 cm	0:53 min.
3	chocolate cookie	19	5 cm	1:43 min.

Among three options (table 1) we decided to use 5 cm of cage bedding and 19 hours of fasting, since it was a balance between fasting time and the challenge of finding the pellet.

The test was performed in males and females from both groups (NP and LP) in two different ages (three months and seven month).

Related to results obtained, the first age is complete (figure 1), the second age remains to be analyzed and the third age mice are waiting in the animal facility until one year of age.

In figure 1 the measured parameter was the latency to uncover the chocolate cookie, using a non-parametric Mann-Whitney test, no significant difference was observed between two groups. At early ages we did not expect to observe significant differences between both groups, mainly because the olfactory system deteriorates during aging.

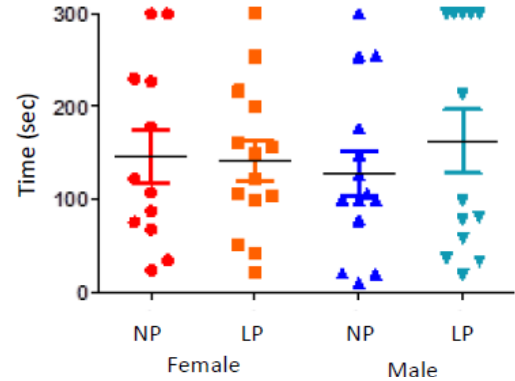


Figure 1: Buried pellet test, mice males and females from NP and LP groups. Using Mann-Whitney test no statistically significant difference was found between two groups

1.2 Olfactory sensitivity test: relies on the animal's tendency to investigate novel smells, is used to assess whether the animal can detect and differentiate different odors, including both non-social and social odors ⁶. The mouse is placed in a transparent box with a special lid which contains a swab. The odor (or water) is presented in consecutive trials. The main parameter analyzed is the cumulative time spent sniffing the swab during the 2-min trial.

First, we had tried with fragrances like chocolate, almond, pineapple, cinnamon and vanilla without obtaining favorable results. After that, social odors were used obtaining favorable results. The final protocol consists: during two consecutive days, a habituation was performed with the swab embedded in water for 20 minutes. On the third day, the swab was first presented with water for 3 minutes, and then, for another 3 minutes, the swab (embedded in a social odor) was present. The time of sniffing was measured. To obtain the social odor, cages containing mice (the same sex as the analyzed subject) which have not been changed for at least one or three days were used. The swab was swiped inside the cage bottom in a zigzag fashion several times.

The figure 2 showed that the sniffing time was significantly higher when the social odor is presented, especially when the social odor came from the cage which have not been changed for at least one day. Currently we are

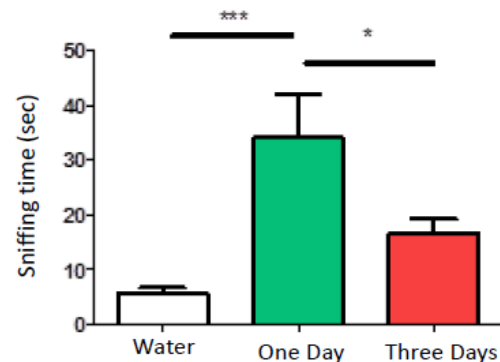


Figure 2: Representative results for a session of olfactory sensitivity test, Total time spent sniffing was assessed, repeated measures ANOVA, Bonferroni post hoc test. n = 10 for each treatment. *p < 0.05 and ***p < 0.001

analyzing the results obtained from the first and second age. Nonetheless, with this test we expect corroborate the results achieved with the buried pellet test, it will permit to obtain more robust conclusions related to the effect of protein malnutrition in the precipitation of aging phenotype, specially the disturbance of the olfactory system.

1.3 Novel place recognition test: place recognition memory will be examined through this test, it depends on the integrity of hippocampus which is severely affected with aging. The test consists in different phases: habituation, training and test phases. First day the habituation phase is performed during 20 minutes with the empty arena which has a white band like spatial cue. On the second day, the training is performed with the objects located in a specific way. This training is repeated two more days. Finally, the test session is performed on the fourth day, where one of the object location is changed (figure 3).

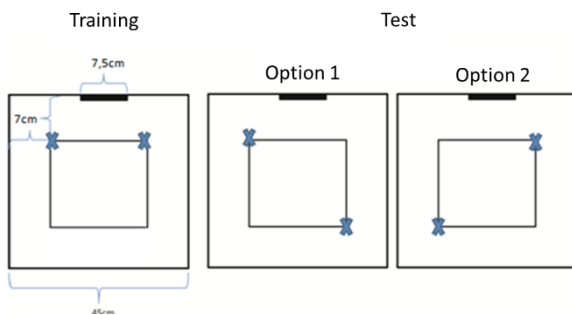


Figure 3: Arena and objects positions of novel object location recognition memory test, during the training (left) and testing (center and right) sessions.

Several parameters have been optimized in order to obtain the best results. Table 2 summarizes the different conditions used.

We recorded total time spent by the animal exploring both objects during the test and analyzed percentage of time exploring novel place location object and discrimination index (DI). DI is calculated as follows: $(\text{time exploring the novel location object} - \text{time exploring the familiar}) / (\text{time exploring novel} + \text{familiar}) * 100$. A positive score indicates more time spent with the novel object, a negative score indicates more time spent with the familiar object, and a zero score indicates a null preference.

Table 2

	Proof 1	Proof 2	Proof 3	Proof 4
Total exploration time	11.56 sec	19.48 sec	24.79 sec	32.42 sec
ID (Discrimination Index) (%)	-35	-3.87	15.2355 *	22.12 *
Percentage of time exploring the novel place object	32%	48.06%	57.61% *	61.06 %*
Objects	Metal cylinders	Metal cylinders	Glass bottle 20ml	Glass bottle 20ml
Protocol	Day 1: Hab 20min; Days 2,3,4: Training 10min; Day 5: Test 5min	Day 1: Hab 20min; Days 2,3,4: Training 10min; Day 5: Test 5min	Day 1: Hab 20min; Days 2,3,4: Training 10min; Day 5: Test 5min	Day 1: Hab 20min; Days 2,3,4: Training 10min; Day 5: Test 5min

Analyzing the results obtained with the proofs 1 and 2 we did note that mice, in general, explored very little the objects. Changing the objects for glass bottle we finally obtained a positive ID and the percent of time exploring the novel object place was above of 50% indicating a preference for the new object location. Actually, we are analyzing the result obtained with mice of two and seven month.

2. Senescence phenotype

To evaluate the effect of protein malnutrition on the establishment of senescence in mice of different ages we have been setting up two different assays, the first one measures the Senescence Associated β galactosidase activity (SA- β gal activity) and the second one measures adult neurogenesis using BrdU incorporation. Both techniques were analyzed in brain slides particularly in the hippocampus dentate gyrus because it is a region that presents adult neurogenesis (cell proliferation). Mice were deeply anesthetized with avertin (0.4 mg avertin/g i.p.) and perfused transcardially with 0.9% NaCl followed by 4% paraformaldehyde. Brains were post fixed overnight in 4% paraformaldehyde and coronal 40 μ m sections were cut. Slices were stored in cryoprotectant solution (25% glycerol, 25% ethylene glycol, 50% phosphate buffer 0.1 M, pH 7.4) at -20 °C.

2.1 Senescence Associated β galactosidase activity (SA- β gal activity)

SA- β -gal staining was performed incubating the brain slides in phosphate buffer pH 6.0 containing potassium ferrocyanure, potassium ferricyanure, NaCl, MgCl₂, X-gal for 16-18 hours at 37°C. After X-gal incubation the slides were stained with hematoxylin and eosin. To identify clearly the positive cells, different options related to brain slide size and time of incubation with hematoxylin and eosin were tested. We obtained the better results using brain slides of 40 μ m, 12 minutes of incubation with hematoxylin and 30 seconds of incubation with eosin. Representative images of the final protocol are showed in figure 4. A positive control was made incubating with the x-gal solution at pH=4. Actually, we are taking the pictures of first and second age. It remains to analyze and quantify the positive cells in each hippocampus region, particularly the dentate gyrus.

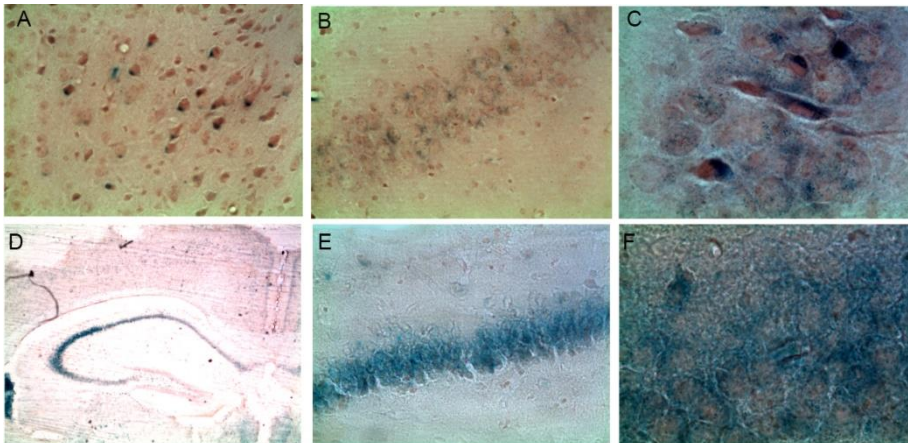


Figure 4: SA- β -gal staining, A, B y C are representative images of 40 μ m slides, corresponding to dentate gyrus of the hippocampus, using the x-gal solution at pH=6. D, E y F are representative image of the positive control (pH=4). Both treatments were incubated with hematoxylin-eosin.

2.2 Cell Proliferation and Neurogenesis in Adult Mouse Brain

To measure adult neurogenesis two protocols of BrdU injection were tested. The first protocol includes three intraperitoneal injection with 150mg/Kg of BrdU on one day every 4 hours and 24 hours after injections the mouse is perfused. The second one involves seven days of BrdU injections (50mg/Kg) and the mouse is perfused 24 hours after the last injection. With the second protocol better results were obtained, thus we use this protocol at the different ages. Immunofluorescence was made free floating sections spanning hippocampal region. In figure 5 are representative images obtained from a

control mouse group of two month of age. Actually, we are processing the samples (first and second age) and the quantification of BrdU positive cells still be done.

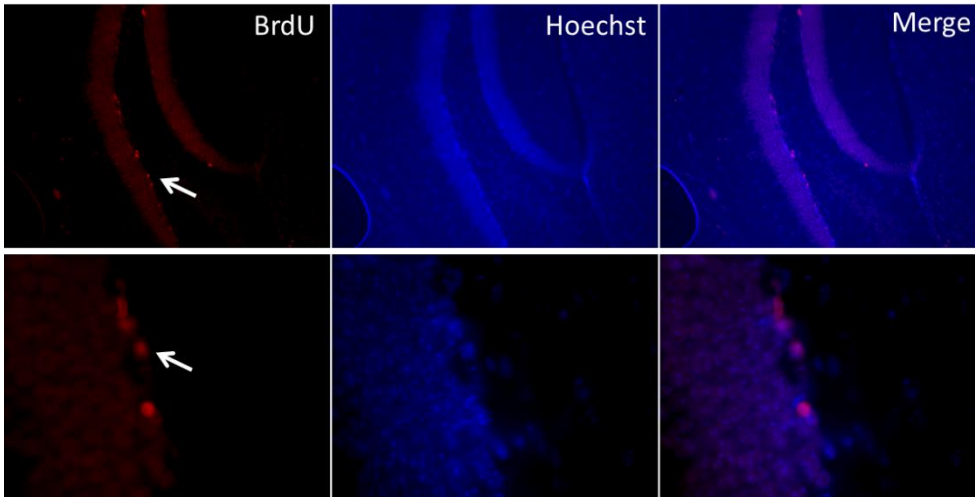


Figure 4: BrdU immunofluorescence in brain slides counterstained with Hoechst, the upper panel showed BrdU positive cells in the dentate gyrus region of the hippocampus (white arrow) 10X magnification. The lower panel showed 400X magnification of BrdU positive cells.

3. Oxidative Stress

To analyze the potential causes associated with premature aging, reactive oxygen species (ROS) levels were measured by fluorimetry (ex/em: 485/520 nm) in freshly prepared prefrontal cortex, olfactory bulb and hippocampal homogenates employing H₂DCFDA as substrate. ROS levels were measured in presence or not of 2,2'-azobis (2 methylpropionamide) dihydrochloride (ABAP). This compound is a peroxy radical generator by thermal decomposition at 35 °C. Total antioxidant capacity against peroxy radicals was estimated as the difference in ROS area with and without ABAP. A big difference between with and without ABAP means that the tissue cannot neutralized high ROS levels indicating a low antioxidant capacity.

In figure 5 raw data is shown from the first age in different tissue with or without ABAP. The fluorometer measured every 3 minutes for 50 minutes. The basal ROS levels in OB and PFC in female mice were higher in LP group compared to NP, also the area seems to be a little bit different. We observed a slight upward trend in ROS levels in young mice females subjected to protein malnutrition during perinatal period, nonetheless a more detailed analysis is necessary to obtain a more robust conclusion. In male mice, we did not observe an upward trend comparing both groups, moreover in PFC we observed the opposite trend. Actually, we are analyzing more deeply the results obtained from the first and second age making linear mixed model.

4. Conclusion

In summary, we did not observe significant differences between NP and LP group in young mice analyzed so far, but rest to analyze a lot of data obtained from age one and two related to behavioral test, BrdU incorporation, SA-b-Gal activity and ROS levels. The mice of the third age are waiting in the animal facility until one year of age. In October-November of this year we will processes all samples. This report reflects all the work and troubleshooting that we have to deal with setting up of the different techniques. This could not be possible without the economic help received from ISN, because it allows us buying research supplies necessary to start with this project.

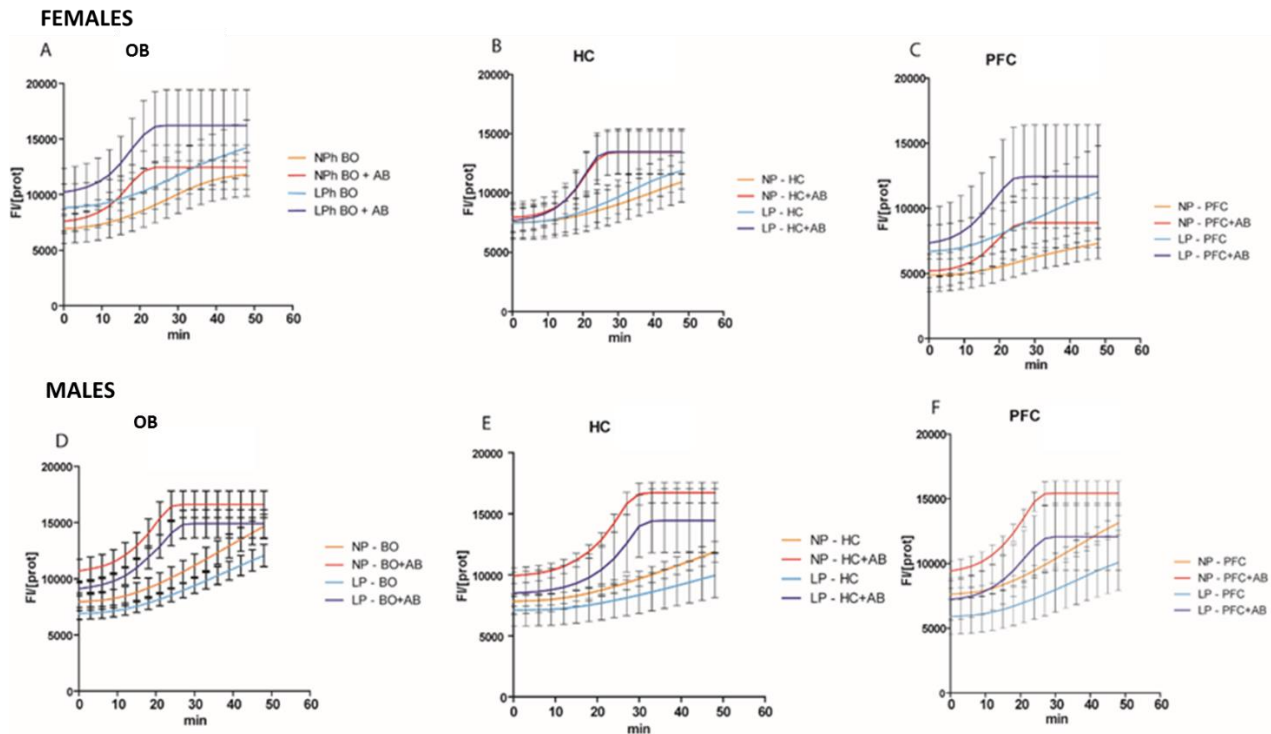


Figure 5: Indirect measure of ROS levels and antioxidant capacity (with ABAP) in brain homogenates of olfactory bulb, hippocampus and prefrontal cortex. The homogenates have been extracted from male and females mice of two month age, the graphs have shown the fluorescence in function of time, the florescence relative to protein concentration of each sample. N=6 for each group.

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Financial Report ISN CAEN Award

Dr. Silvina Sonzogni

Date	Concept	Amount
09/03/2016	Office supplies - printing and binding (La Copia)	USD 51,18
09/03/2016	Office supplies - biding (La Mutual Heliográfica)	USD 6,95
09/03/2016	Office supplies - printing (La Copia)	USD 6,46
20/04/2016	Office supplies - printing and binding (La Copia)	USD 85,05
20/04/2016	Sharping of laboratory knives (Cuchillería Villa Crespo)	USD 13,90
25/04/2016	Laboratory reagents (Científica Parque)	USD 156,85
15/06/2016	Laboratory supplies - anti-body, reagents, etc. (Sero-Immuno Diagnostic, Inc.)	USD 3.716,00
15/06/2016	Wire fee to Sero-Immuno Diagnostic, Inc.	USD 30,00
01/07/2016	Animal facilities costs (Bioterio FCEN)	USD 695,11
22/09/2016	Animal facilities costs (Bioterio FCEN)	USD 252,79
	TOTAL	USD 5.014,29