Maternal protein restriction during the lactation period disrupts the ontogenetic development of behavioral traits in male Wistar rat offspring

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SUPPLEMENTARY MATERIAL

METHODS

Behavioral assessment

All behavioral tests were conducted in a testing room adapted and exclusively used for such tests. Temperature (22±1°C) and humidity (50±5%) were controlled. Animals were allowed at least 30 min to habituate to the testing room prior to behavioral testing. Experimenters were blind as to group allocation. All animals assigned to the experiments were tested. All available data points were used in the statistical analyses.

Elevated Plus Maze (EPM)

It is shaped like a plus sign and consists of two "open" arms (OA, 50 cm long × 10 cm wide, no walls) and two "closed" arms (CA, 50 cm long × 10 cm wide, with 40 cm high walls) arranged perpendicularly and elevated 40 cm above the floor. Each test began with the animal being placed on the center of the equipment facing an open arm. Animals were allowed 5 min to explore. All tests were video-recorded and the recorded images were used to assess the

animal's behavior. The total time (in seconds) spent in and the number of entries into the open and closed arms, and the central area (CN) of the maze were noted. The percentage of time spent in the open arms [%Time OA: Time OA / (Time OA + Time CA)] and the percentage of open arms entries [%Entries OA: Entries OA / (Entries OA + Entries CA)] were used as anxiety measures[38,51,52,54]. Increases in values regarding these variables are considered as evidence of a lowered anxiety-like state[58,59]. The number of CA entries (Entries CA) was used as a measure of activity and the percentage of time spent in the center of the maze (%Time CN: Time CN / total time, used as a measure of decision-making[52,61]. Ethologically derived measures were also evaluated and included the following behaviors: Stretching, Rearing, Grooming, Head Dipping and Return CA[52,59]. The EPM was cleaned with paper towels soaked in 50% ethanol and dried before each test. Animals were immediately returned to their home cages after the test.

Open Field (OF)

The OF consists of a white wooden box (37.6 cm long × 30.4 cm wide × 17 cm high) and its floor is divided by lines in to 16 same-sized rectangles (7.6 cm × 9.4 cm) that allow for the definition of peripheral (PER: 12 outer rectangles) and central areas (CNT: 4 inner rectangles). At the beginning of the session, each rat was placed in the center of the arena and its activity was recorded for 5 min. A valid crossing was considered when the animal crossed a line with all four paws. The number of entries into and the time spent on each square type (PER or CNT) was noted; Total locomotor activity (Total Entries) reflected the number of rectangles crossed by the animals in the center as well as in the periphery[55,56]. In addition, the number of entries into the central rectangles corrected by total ambulation [%Entries CNT: CNT / (CNT + PER)] was evaluated; the activity in the center is inversely related to levels of anxiety[55,63]. The following ethologically derived measures[53,59] were also evaluated: Rearing and Grooming. At

the end of each test, the OF was cleaned with paper towels soaked in 50% ethanol and dried. Animals were immediately returned to their home cages after the test.

Radial Arm Water Maze (RAWM)

The RAWM is shaped like an asterisk and consists of eight swim paths (arms: 29 cm long × 13 cm wide × 40 cm high) extending out of an open central area (41 cm diameter). The RAWM was filled with water (26±1°C) to a depth of 34 cm so that rats could not touch its floor with their feet or tails while swimming on the surface. An escape platform (8 cm long × 10 cm wide) was placed at the end of one of the arms, 1 cm below the surface. Non-toxic white paint was used to make the water opaque, hiding the platform. Several extra-maze cues were present in the testing room and their positions remained fixed throughout the entire experiment. A test began by placing the animal at the center of the arena facing away from the arm that had the escape platform. All tests were video-recorded and the recorded images were used to assess the behavior in the RAWM. Animals were tested for 5 consecutive days and 4 trials per day (inter-trial interval: 15 min). Animals were allowed 2 min per trial to explore the maze and find the hidden platform. Whenever they failed to find the platform in a given trial during the allotted time, they were gently guided to it and allowed to stay on top of it for 20 s. The escape platform was not moved from its initial position during the first 4 days (D1-4). On the 5th day (Reversal Day - RD), the platform was placed in the arm opposite to the one in which it was located during the previous 4 days. The latency to find the hidden platform on each trial of each testing day was the main variable used to assess performance in the RAWM[51,64]. The following variables were also measured[51,52,64]: 1) Latency to first arm entry (Lat. FA); 2) Number of first entries into any arm that didn't have the platform (reference memory errors, Ref. Err.); 3) Number of re-entries into any arm that did not have the platform (working memory errors - incorrect arms, W. Mem.). The pool was cleaned (bedding residue and fecal boli were removed)

and the water thoroughly mixed between tests. The water was completely changed every day. Animals were dried with paper towels and immediately returned to their home cages after the test.

Hormones measurements

Total corticosterone was measured using a specific murine RIA kit (ImmuChemTM 125I, double antibody; ICN Biomedicals, Inc.)[67]. The assay sensitivity was 50 ng/mL and the intra-assay variation was 7.1%. Plasma free triiodothyronine (FT3) and free thyroxin (FT4) were determined by RIA, using commercial kits (MP Biomedicals Diagnostics Division, Orangeburg, NY, USA)[68]. The intra-assay variation coefficients for FT3 and FT4 were 5.6% and 5.7% respectively, and the lower limits of detection were 0.69 pg/dL and 0.3 ng/dL respectively.

Total catecholamines (epinephrine and norepinephrine) content in the adrenal gland was quantified using the trihydroxyindole method[69]. Left adrenal glands were homogenized in 10% acetic acid centrifuged (1,120×g for 5 min) and the resulting supernatants were kept frozen for later analysis. Epinephrine was used as the standard. Briefly, 50 µL of the standard/supernatant were mixed with 250 µL of 0.5 M buffer phosphate, pH 7.0, and 25 µL of 0.5% potassium ferricyanate, followed by incubation (20 min, ice bath). The reaction was stopped with 500 µL of 60 mg/mL ascorbic acid / 10 N NaOH (1:19 proportion). The parameters used in the fluorometer (Victor³, PerkinElmer) were 420 nm for excitation and 510 nm for emission. Results were obtained by plotting the values into a linear regression of the standard adrenaline curve. Data were expressed as µM of catecholamines / adrenal mass (g).

RESULTS - Additional offspring's behavioral data

Results of the ANOVAs that included Age and Diet as factors are shown in table S1. Regarding RAWM analyses, repeated-measures ANOVAs, which also included Day as the within-subjects factor, were used: rANOVA results concerning Day effects or interactions will be shown in the text below.

Supplementary Table S1: ANOVA results of additional behavioral variables.

	Age		Diet		Age × Diet	
	F (d.f.)	***p***	***F (d.f.)***	***p***	***F (d.f.)***	***p***
Elevated Plus Maze						
Entries OA	9.3 (3,136)	< 0.001	7.0 (1,136)	= 0.009	---	
Time OA	4.0 (3,136)	= 0.009	5.2 (1,136)	= 0.024	---	
%Time CN	12.0 (3,136)	< 0.001	4.6 (1,136)	= 0.033	---	
Return CA	10.3 (3,136)	< 0.001	---		---	
Rearing	106 (3,136)	< 0.001	---		---	
Grooming	---		---		---	
Stretching	---		---		---	
Head dipping	5.0 (3,136)	= 0.002	---		---	
Open Field						
Time CNT	4.6 (3,136)	= 0.004	---		---	
Entries CNT	9.8 (3,136)	< 0.001	---		---	
Entries PER	19.2 (3,136)	< 0.001	---		6.9 (3,136)	< 0.001
Rearing	15.7 (3,136)	< 0.001	9.1 (1,136)	= 0.003	9.3 (3,136)	< 0.001
Grooming	---		---		---	
Radial Arm Water Maze						
Lat. FA (D1-4)	5.4 (3,136)	= 0.002	---		---	
Lat. FA (RD)	4.5 (3,136)	= 0.005	---		---	
Ref. Err. (D1-4)	11.8 (3,136)	< 0.001	---		---	
Ref. Err. (RD)	5.3 (3,136)	= 0.002	---		---	
W. Mem. (D1-4) $	37.5	< 0.001	---			
W. Mem. (RD) $	26.2	< 0.001	---			

D1-4: Sum of the results of the initial 4 days (escape platform in the same position). RD: Reversal Day - platform placed on the opposite arm. --- no effects or interactions were identified. $ Kruskal-Wallis test was used: Data in F column are Chi-Square values.

Elevated Plus Maze

For the additional variables of the EPM that showed a significant Diet effect (table S1), the protein-restricted offspring had increases in values when compared to the normoprotein ones for the following variables: Entries OA (+31%), Time OA (+35%) and %Time CN (+16%). These increases did not affect the ontogenetic variations of values since no Age × Diet

interactions. Except for the ethological variables Grooming and Stretching, all other EPM variables showed significant Age effects (table S1). For most of these variables, the magnitude of change from the lowest observed value to the highest one, irrespective of age, ranged from 2-fold to 3-fold increases, albeit the ethological variable Rearing showed a 31-fold increase (table S2).

Open Field

The only additional variable of the OF that showed a significant Diet effect was the ethological variable Rearing (table S1), with a 22% increase in protein-restricted offspring when compared to normoprotein ones. Diet interfered with the profile of ontogenesis for both Rearing (-77% in PR offspring vs. controls at PN90) and Entries PER (3.3-fold increase in PR offspring vs. controls at PN21) (table S2). Except for Grooming, all additional variables showed variations from the lowest to the highest values, irrespective of age, that ranged from 2- to 3-fold increases (table S2).

Radial Arm Water Maze

All additional variables of RAWM showed only Age effects, indicating that significant ontogenetic variations were present (table S1). From the lowest to the highest values observed for a given variable, irrespective of age, increases ranged from +40% to 6-fold. Diet neither had an overall effect on the aforementioned parameters nor it changed the profiles of the results as the animals aged (table S1).

Supplementary Table S2: Ontogenesis of behavior - additional variables in the Elevated Plus Maze, Open Filed and Radial Arm Water Maze.

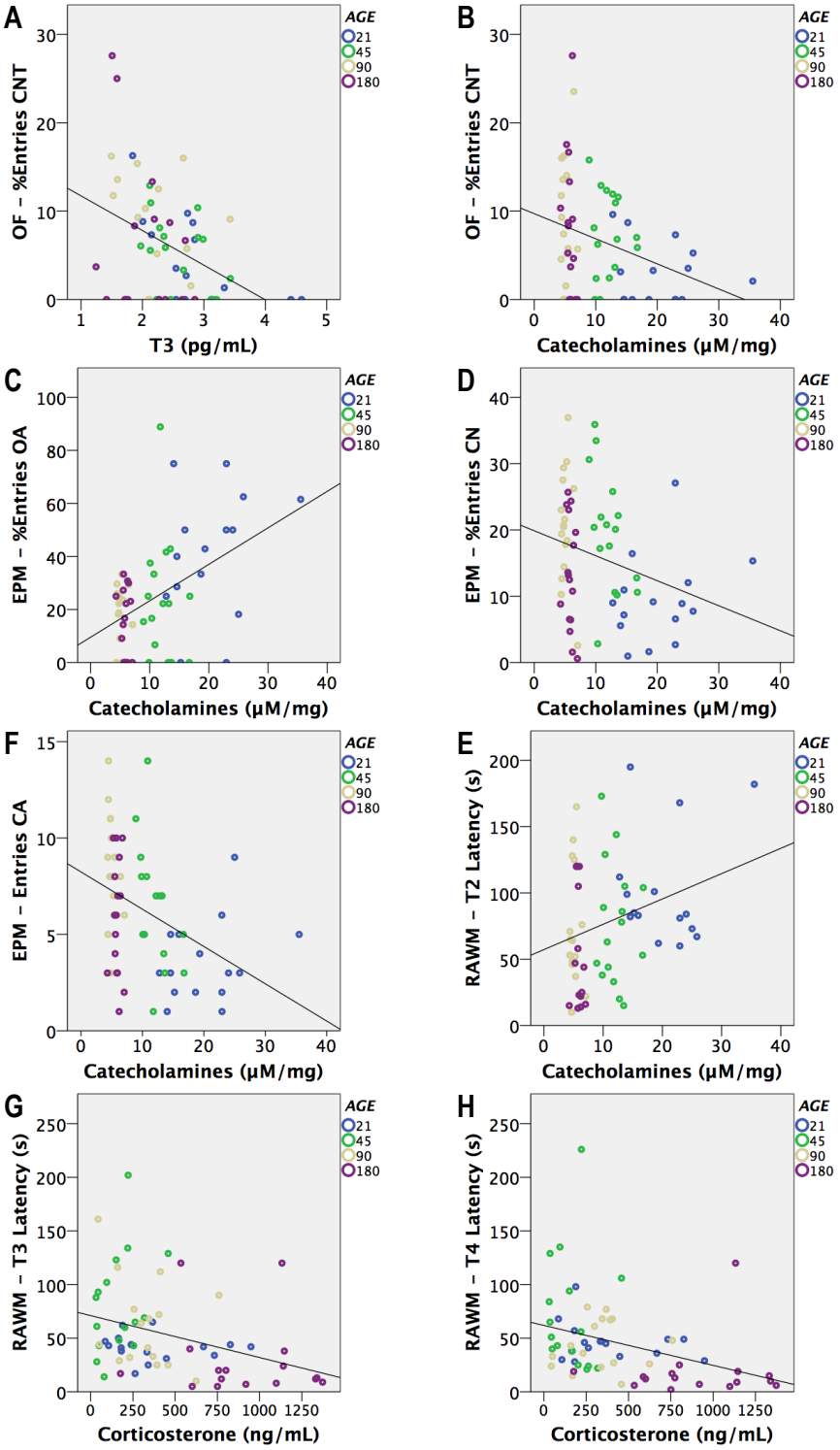
Postnatal day	**Elevated Plus Maze**							
	Entries OA	*Time OA*	*%Time CN*	*Return CA*	*Rearing*	*Grooming*	*Stretching*	*Head dipping*
	Age effect	*Age effect*	*Age effect*	*Age effect*	*Age effect*	---	---	*Age effect*
	$F_{3,136} = 9.3$; $p < .001$	$F_{3,136} = 3.0$; $p = .009$	$F_{3,136} = 12.0$; $p < .001$	$F_{3,136} = 10.2$; $p < .001$	$F_{3,136} = 106$; $p < .001$			$F_{3,136} = 5.0$; $p = .002$
PN21[(a)]	2.61 ± 0.30	34.9 ± 5.5	9.6 ± 1.0	1.14 ± 0.18	5.97 ± 0.50	3.92 ± 0.28	1.69 ± 0.25	1.61 ± 0.20
PN45[(b)]	1.86 ± 0.27	19.4 ± 3.6 [a]	14.6 ± 1.1 [a]	2.64 ± 0.23 [a]	0.22 ± 0.07 [a]	4.11 ± 0.34	1.44 ± 0.20	1.89 ± 0.27
PN90[(c)]	2.97 ± 0.40 [b]	34.2 ± 6.2 [b]	18.7 ± 1.2 [a,b]	1.97 ± 0.23 [a,b]	0.47 ± 0.11 [a]	3.67 ± 0.32	1.81 ± 0.22	2.78 ± 0.37 [a,b]
PN180	0.94 ± 0.19 [a,b,c]	16.3 ± 4.1 [a,c]	11.5 ± 1.3 [c]	1.31 ± 0.19 [b,c]	7.03 ± 0.47 [a,b,c]	4.17 ± 0.33	1.78 ± 0.24	1.42 ± 0.22 [c]
	Open Field							
	Time CNT	*Entries CNT*	*Entries PER*		*Rearing*		*Grooming*	
	Age effect	*Age effect*	C Diet	PR Diet	C Diet	PR Diet	---	
	$F_{3,136} = 4.6$; $p = .004$	$F_{3,136} = 9.8$; $p < .001$	$F_{3,68} = 6.9$; $p < .001$	$F_{3,68} = 4.3$; $p = .008$	$F_{3,68} = 17.2$; $p < .001$	$F_{3,68} = 9.3$; $p < .001$		
PN21[(a)]	3.28 ± 0.85	1.47 ± 0.29	28.6 ± 5.8	42.7 ± 4.9	3.4 ± 0.6	11.3 ± 1.1 *	5.89 ± 0.45	
PN45[(b)]	5.66 ± 1.23	3.22 ± 0.55 [a]	41.4 ± 3.9	51.5 ± 3.8	11.2 ± 1.3 [a]	11.3 ± 1.1	5.39 ± 0.46	
PN90[(c)]	5.83 ± 0.69 [a]	4.19 ± 0.47 [a]	52.7 ± 4.5 [a]	34.5 ± 2.7 [b,*]	8.1 ± 0.7 [a,b]	8.0 ± 1.0 [a,b]	4.69 ± 0.44	
PN180	2.10 ± 0.55 [b,c]	1.58 ± 0.34 [b,c]	18.1 ± 1.2 [a,b,c]	21.3 ± 2.0 [a,b,c]	5.1 ± 0.6 [b,c]	4.9 ± 0.7 [a,b,c]	6.25 ± 0.37	
	Radial Arm Water Maze							
	Lat. FA (D1-4)	*Lat. FA (RD)*	*Ref. Err. (D1-4)*	*Ref. Err. (RD)*	*W. Mem. (D1-4)*	*W. Mem. (RD)*		
	Age effect	*Age effect*	*Age effect*	*Age effect*	*Age effect*	*Age effect*		
	$F_{3,136} = 5.8$; $p = .001$	$F_{3,136} = 4.6$; $p = .004$	$F_{3,136} = 11.8$; $p < .001$	$F_{3,136} = 5.3$; $p = .002$	$\chi^2 = 37.5$; $p < .001$	$\chi^2 = 26.2$; $p < .001$		
PN21[(a)]	56 ± 4	19 ± 2	32 ± 1	11 ± 1	12 (17.5)	11 (8.0)		
PN45[(b)]	112 ± 13 [a]	41 ± 6 [a]	25 ± 1 [a]	8 ± 1 [a]	4 (7.0) [a]	3 (9.8) [a]		
PN90[(c)]	83 ± 6	30 ± 3	23 ± 1 [a]	9 ± 1 [a]	4 (5.8) [a]	5 (6.8) [a]		
PN180	107 ± 17 [a]	36 ± 5 [a]	23 ± 2 [a]	*8 ± 1* [a]	2 (5.0) [a,b]	4 (5.8) [a]		

All animals (C and PR offspring) were used to calculate the avarage for each age when only Age effects were present. When Age x Diet interactions were identified for a given variable, data at each age for each group are shown separately. D1-4: Sum of the results of the initial 4 days (escape platform in the same position). RD: Reversal Day - platform placed on the opposite arm. Data are shown either as mean ± SEM or median (interquartile range). [a], [b] or [c] represent $p < 0.05$ within-group pairwise age comparisons. * $p < 0.05$ C *vs* PR (same age). --- no effects or interactions were identified.

Correlation between variables

Correlation analyses were carried out between the hormonal parameters and the main behavioral data. When using all available data (not segmented by age or group), most analyses failed to show significant associations between parameters. However, some hormone/behavior pairs resulted in significant correlations: Open Field – T3 × %Entries CNT ($r = -0.40$, $p = .001$; figure S1A), catecholamines × %Entries CNT ($r = -0.31$, $p = .014$; figure S1B); Elevated Plus Maze – catecholamines × %Entries OA ($r = +0.46$, $p < .001$; figure S1C), catecholamines × %Entries CN ($r = -0.28$, $p = .026$; figure S1D), catecholamines × Entries CA ($r = -0.40$, $p = .001$; figure S1E); Radial Arm Water Maze – catecholamines × T2 ($r = +0.28$, $p = .025$; figure S1F), corticosterone × T3 ($r = +0.35$, $p = .005$; figure S1G), corticosterone × T4 ($r = +0.36$, $p = .004$; figure S1H). As seen in figure S1, in which each dot had a color that corresponded to a specific age, all these significant correlations are clearly age-dependent.

Figure S1: Correlation analyses between selected behaviors and hormones.



Graphs showing examples of analyses for which significant correlations between behaviors and hormones were identified. OF: Open Field; EPM: Elevated Plus Maze; RAWM: Radial Arm Water Maze. RAWM–T2, –T3 and –T4, 2nd (D2), 3rd (D3), and 4th (D4) testing day, respectively. A different color was attributed to each age. All correlations were age-dependent since clusters of same-color data can be clearly identified.