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LITTER SIZE, AGE-RELATED MEMORY IMPAIRMENTS, AND MICROGLIAL CHANGES IN RAT DENTATE GYRUS: STEREOLOGICAL ANALYSIS AND THREE DIMENSIONAL MORPHOMETRY

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Abstract—It has been demonstrated that rat litter size affects the immune cell response, but it is not known whether the long-term effects aggravate age-related memory impairments or microglial-associated changes. To that end, we raised sedentary Wistar rats that were first suckled in small or large litters (6 or 12 pups/dam, respectively), then separated into groups of 2–3 rats from the 21st post-natal day to study end. At 4 months (young adult) or 23 months (aged), all individual rats were submitted to spatial memory and object identity recognition tests, and then sacrificed. Brain sections were immunolabeled with anti-IBA-1 antibodies to selectively identify microglia/macrophages. Microglial morphological changes in the molecular layer of the dentate gyrus were estimated based on three-dimensional reconstructions. The cell number and laminar distribution in the dentate gyrus was estimated with the stereological optical fractionator method. We found that, compared to young rat groups, aged rats from large litters showed significant increases in the number of microglia in all layers of the dentate gyrus. Compared to the microglia in all other groups, microglia in aged individuals from large litters showed a significantly higher degree of tree volume expansion, branch base diameter thickening, and cell soma enlargement. These morphological changes were correlated with an increase in the number of microglia in the molecular layer. Young adult individuals from small litters exhibited preserved intact object identity recognition memory and all other groups showed reduced performance in both spatial and object identity recognition tasks. We found that, in large litters, brain development was, on average, associated with permanent changes in the innate immune system in the brain, with a significant impact on the microglial homeostasis of aged rats.

Key words: litter size, aging, microglia, dentate gyrus, stereology, morphometry.

INTRODUCTION

In rodents, the early postnatal environment has long-term physiological and behavioral consequences late in life (Pfeifer et al., 1976; Stanton et al., 1987; Kaffman and Meaney, 2007; Langer, 2008; Uriarte et al., 2008), as described in a recent review (Walker, 2010). Indeed, a series of well-described experiments designed to measure the impact of reduced maternal care have demonstrated important detrimental effects on the rat central nervous system (CNS), both in developmental and adult brain functions (Liu et al., 1997; Caldji et al., 1998; Menard et al., 2004; van Hasselt et al., 2012a,b). In addition, epigenetic changes (e.g., in DNA methylation) caused altered glucocorticoid receptor expression, which changed the stress responses of the offspring (Meaney and Szyf, 2005).

A number of studies have shown that, when litter sizes were extremely small (3–4 pups per litter; Plagemann et al., 1999; Velkoska et al., 2005; Rodrigues et al., 2009) or extremely large (16–18 pups per litter; Davidowa et al., 2002; Velkoska et al., 2008), suckling conditions may induce permanent changes in the metabolic profiles of adult rats. These permanent changes were associated with epigenetic changes of acquired alterations in the DNA methylation pattern (Plagemann et al., 2010).

Another series of experiments in a variety of small mammals demonstrated that a large litter size was potentially life threatening during early post-natal life. The degree of sibling competition within litters depends

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Abbreviations: 3D, three dimensions; AL, aged, large litter size; ANOVA, analysis of variance; AS, aged, small litter size; CE, coefficient of error; CV, coefficient of variation; DF, depth of focus; G-CSF, granulocyte colony-stimulating factor; IBA-1, ionized calcium binding adapter molecule 1; MCSF-1, macrophage colony-stimulating factor-1; NIH, national institute of health; PBS, phosphate-buffered saline; YL, young, large litter size; YS, young, small litter size.

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on the number of offspring that must compete for access to milk from each nipple (Stockley and Parker, 2002; Zhang et al., 2011). Indeed, in extreme cases, where the number of pups far exceeds the number of nipples, the consequences of this competition may be fatal (Cameron, 1973). However, non-lethal consequences of this natural competition may induce important differences in an individual's postnatal life. For example, growth rates are favored for subjects with greater access to maternal milk; these individuals may achieve higher reproductive success and longer lives; thus, a small litter size may represent an evolutionary advantage (Azzam et al., 1984; van Engelen et al., 1995; Stockley and Parker, 2002; Rodel et al., 2008; Rodel et al., 2010). Furthermore, it has been demonstrated that spontaneous litter size changes may affect emotionality in adulthood; these changes could not be explained by concomitant changes in maternal care (Dimitsantos et al., 2007).

A previous report (Celedon et al., 1979) described the long-term effects of early undernutrition and environmental stimulation on learning performance in mature rats (11-week-old). Rats had been maintained in litters of 18 pups/dam or 6 pups/dam during the weaning period. The performance of adult rats from large litters was compared to the performance of age-matched control rats from small litters. They found that the induced undernourishment did not impair Hebb–Williams performance, and environmental stimulation improved learning performance, both in control and previously undernourished groups.

However, very little is known about the possible long-term consequences of litter size on the immune system (Cortes-Barberena et al., 2008). Indeed, this is a single report describing the effects of moderate and severe malnutrition induced by litter size changes on lymphocyte proportions and activation markers of T cells in experimentally malnourished rats during lactation. Although recent evidence has indicated that litter size affected corticosterone levels at early postnatal stages (Prager et al., 2010; Walker, 2010; Hudson et al., 2011), very little is known about the long-term consequences of that condition on microglia populations in adult life (Tapia-Gonzalez et al., 2011).

In the present study, we investigated the long-term effects of two different litter sizes (6 and 12 pups/dam) on microglia morphology, laminar distribution, and number in the dentate gyrus of adult, mature (4-month-old) and aged (23-month-old) rats. Furthermore, we determined whether litter size changes were associated with object recognition and spatial memory deficits later in life.

EXPERIMENTAL PROCEDURES

All procedures applied to this investigation were submitted to and approved by the institutional animal care committee of the Federal University of Pernambuco, Brazil. Animals were handled in accordance with the "Principles of Laboratory Animal Care" (National Institute of Health NIH).

Experimental groups

All experiments were performed with the offspring of an outbred colony strain of Wistar rats obtained from the Department of Nutrition of the Federal University of Pernambuco. Wistar female rats were fed a rodent laboratory chow diet (Purina do Brazil Ltd.), with 23% protein, *ad libitum*. Rats were maintained in groups of 2 or 3 subjects. After mating and gestation, the pregnant rats delivered 7–12 pups per litter. The pups from 4 to 6 litters (both females and males), born in the same day, were first joined in a big pool. Pups from this pool were randomly assigned to form the small- and large-litters of this study but only males were selected to the present study at the end of the lactation period. In order to manipulate maternal care and the level of competition for suckling, a pup-to-dam ratio of either 6:1 (small litter size) or 12:1 (large litter size) was established 48 h after birth. The assumption was that the competition for milk access and maternal care would be significantly different between the two groups. We also assumed, as previously demonstrated, that body weight differences between these two litter sizes would not be accompanied by under nourishment (Morag et al., 1975; Yagil et al., 1976; Jans and Woodside, 1987). Body weights were measured at different time windows to follow growth in the different experimental conditions. Rats underwent cognitive testing, followed by sacrifice for brain sectioning and analyses, at two ages: young adult ($N = 10$; 4 months) or aged ($N = 10$; 23 months).

The time schedule of the experimental procedures for pre- and post-weaned Wistar rats is represented in Fig. 1.

After the suckling period (21 post-natal days), rats in both experimental groups were transferred to cages in groups of 2 or 3 subjects/cage and fed a rodent laboratory chow diet (Purina do Brazil Ltd.), with 23% protein, *ad libitum*. Weaned rats were maintained in polypropylene cages (51 × 35.5 × 18.5 cm), under a light–dark cycle (12/12 h; lights on at 6 am) at and room temperature (23 ± 1 °C). These conditions were similar to the standard housing conditions in most laboratories. All subjects were housed under these standard conditions, from 21 days (end of the weaning period) to the day of sacrifice. To compare the impact of litter size on aged and adult rats we sacrificed subjects at correspondent ages after behavioral tests. All selected individuals for the present study were males.

Behavioral tests

At 4 months (young adult) or 23 months (aged), individual rats were submitted to spatial memory and object recognition tests. Fig. 2 shows schematic diagrams of the object recognition and object placement apparatuses and test procedures. In the present work, we used single-trial tests to assess object identity and object placement recognition memory.

The apparatus for the single-trial object recognition and spatial memory tests consisted of an open circular box (1-m diameter) made of painted, varnished wood. The floor was painted with lines to distinguish four quadrants, and the luminance at the center of the circular box floor was 2.4 cd/m^2. Detailed protocols and the rationale for the test choices provided are discussed elsewhere (Tulving, 2001; Ennaceur et al., 2005; Dere et al., 2007). In brief, behavioral assays were performed over 5 days: 1 day for open field habituation, 2 days for object habituation, and 2 days for testing: one test per day.

To minimize the influence of natural preferences for particular objects or materials, we chose objects made of the same plastic material, with similar possibilities for interaction, but with different geometries that could be easily discriminated (Dere et al., 2005). Thus, objects had different shapes, heights, and colors. Before each rat entered the arena, the arena and objects were cleaned with 75% ethanol to minimize distinguishing olfactory cues.

Open field habituation: each subject was placed in the arena, free of objects, for 5 min to explore the open field.

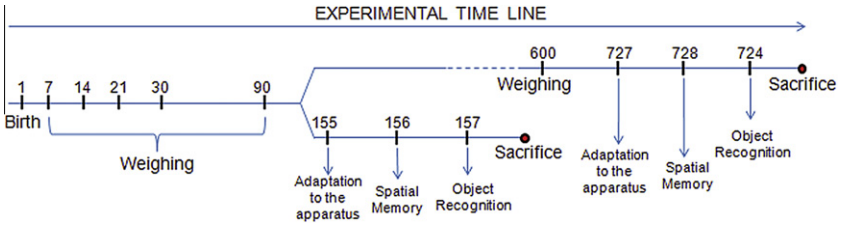

Fig. 1. Experimental timeline. Time sequence (days) of experiments conducted with Wistar rats that had been placed in small (6 pups/dam) or large litters (12 pups/dam) at 48 h postnatally. Body weight was measured during suckling (< 21 post-natal days) and post-weaning (⩾21 postnatal days). Each litter-size group underwent cognitive testing at a young adult age (lower branch; 4 months; $N = 16$; YS = 7, YL = 9) and at an older age (upper branch: 23 months; $N = 13$; AS = 9, AL = 4). After testing, rats were sacrificed for immunohistochemical analyses of brain sections.

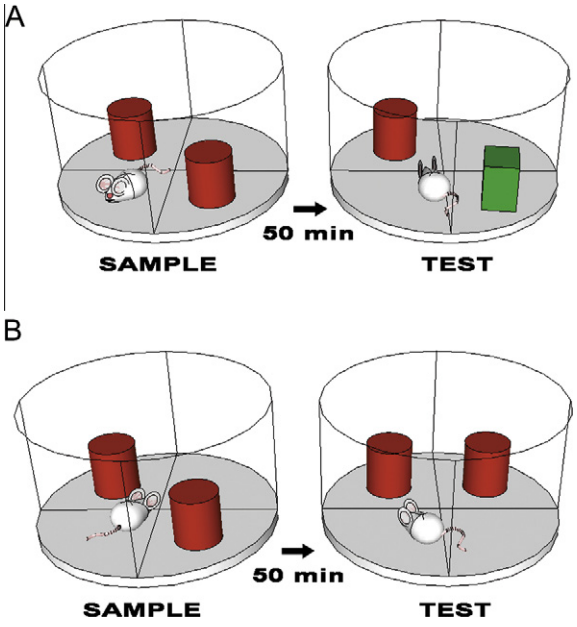

Fig. 2. Diagrams of the experimental designs for object recognition and spatial memory tests. Sample (left) and test (right) conditions are shown. (A) Object identity recognition:identical objects (left) are presented; then, after 50 min, a novel object (green) is presented. (B) Spatial memory: identical objects are presented; then, after 50 min, one object is moved to a new position. Modified from (Ennaceur et al., 2005). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Object habituation: each subject was exposed to two identical objects placed in the same quadrants of the arena for 5 min, three times, with 50 min between exposures. These objects were not used on the test days.

Testing: one-trial, recognition tests included the object identity test and the object placement test. These tests were administered on two consecutive days.

One-trial, object identity recognition test consisted of a 5-min sample trial, during which subjects explored two identical objects in a familiar arena, followed by a 50-min intermission. Then, the 5-min test trial consisted of a ''novel'' object presented together with one ''familiar'' object that had been explored during the sample trial. Objects differed in form, dimensions, color, and texture, and they had no ethological significance for rats. It was expected that rats would spend more time with the ''novel object than with the ''familiar'' one.

One-trial object placement recognition followed the same procedure as above, except that, for the test trial, one of the two identical objects was shifted to a novel location (''displaced'' object). It was expected that rats would spend more time with the ''displaced'' object than with the ''stationary'' one.

The basic measurement was the time a rat spent exploring each object during the test trial. Scores were determined for object identity recognition (novel vs. familiar) and for placement (displaced vs. stationary) memory. In these tests, the exploration of an object was assumed when a rat approached an object, the head was directed toward it, and the head was placed within 0–3 cm from the object. This definition required that each object be fixed to the apparatus floor; thus, we chose heavy objects for interaction.

Immunohistochemistry

After the behavioral tests, all rats were weighed and anesthetized with an intraperitoneal injection of 2,2,2-tribromoethanol (0.04 ml/g of body weight). Then, rats were transcardially perfused with heparinized saline, followed by 4% paraformaldehyde in 0.1 Molar phosphate buffer (pH 7.2–7.4). Tissue collection and processing for adult and aged occurred on the same day after behavioral tests. Alternate serial sections (70-µm thickness) were obtained with a Vibratome (Micron). Sections were immunolabeled with a polyclonal antibody against the ionized calcium-binding adapter molecule 1 (IBA-1) to detect microglia and/or macrophages (anti-IBA-1, #019-19741; Wako Pure Chemical Industries Ltd., Osaka, Japan). All chemicals used in this investigation were supplied by Sigma–Aldrich (Poole, UK) or Vector Labs (Burlingame, CA, USA).

For immunolabeling, free-floating sections were pre-treated with 0.2 Molar boric acid (pH 9) at 65–70 °C for 60 min to improve antigen retrieval. Then, sections were washed in 5% phosphate-buffered saline (PBS), immersed for 20 min in 10% normal goat serum and incubated with anti-IBA-1 (2 µg/ml in PBS, diluted in 0.1 Molar PBS; pH 7.2–7.4), for 3 days at 4 °C, with gentle, continuous agitation. Washed sections were then incubated overnight with a biotinylated secondary antibody (goat anti-rabbit for IBA-1; 1:250 in PBS, Vector Laboratories). We inactivated endogenous peroxidases by immersing the sections in 3% H_2O_2 in PBS, then washed the sections in PBS, and transferred them to a solution of avidin–biotin–peroxidase complex (VECTASTAIN ABC kit; Vector Laboratories) for 1 h. The sections were washed again before incubation in 0.1 Molar acetate buffer (pH 6.0) for 3 min. Finally, sections were developed in a solution of 0.6 mg/ml diaminobenzidine, 2.5 mg/ml ammonium nickel chloride, and 0.1 mg/ml glucose oxidase (Shu et al., 1988). We confirmed the specificity of the immunohistochemical pattern by omitting the primary antibody (Saper and Sawchenko, 2003). This negative control resulted in the absence of immunolabeling in all structures.

Microscopy, three-dimensional reconstruction, and cell counts

A total of 120 microglial cells were digitally reconstructed in three dimensions (3D) with the aid of a NIKON Eclipse 80i microscope (Nikon, Japan), equipped with a motorized stage (MAC200, Ludl

Electronic Products, Hawthorne, NY, USA). Images were acquired under oil immersion, with a high-resolution, 100× oil immersion, a plan fluoride objective (Nikon, NA 1.3, (Depth of Focus) DF = 0.19 μm), and a computer with Neurolucida software (MBF Bioscience Inc., Frederick, MD, USA). Only cells with trees that were unequivocally complete were included for analysis (cells were discarded when branches seemed to be artificially cut or were not fully impregnated). Terminal branches were typically thin. The cells (30 for each experimental group, six from each animal) were selected from the middle molecular layer of the dentate gyrus, from both ventral and dorsal dentate sections. In these chosen sections, the margins of the molecular layer of the dentate gyrus could be clearly distinguished from the granular layer. Twelve microglial parameters were evaluated, as follows: (1) segment length (μm); (2) tree surface area (μm^2); (3) tree volume (μm^3); (4) number of segments/mm; (5) tortuosity; (6) complexity (fractal dimension; k-dim); (7) soma area (μm^2); (8) soma perimeter; (9) soma convexity; (10) base diameter of the primary branch (μm); (11) number of trees per microglia; and (12) number of protrusions per microglia (defined as true ends, shorter than 4 μm). All parameters were determined with the Neuroexplorer software (MBF Bioscience Inc., USA).

The optical fractionator is an accurate, stereological method of quantification that combines the properties of an optical dissector and a fractionator; it has been used in a variety of studies to determine cell numbers in multiple brain regions (West, 1999, 2002; Bonthius et al., 2004). The optical fractionator is unaffected by histological changes, shrinkage, or damage-induced expansion with injury (West et al., 1991). We tested the hypothesis that the microglial changes associated with litter size would be influenced by aging. At all levels in the histological sections, we delineated the layers of the region of interest (dentate gyrus) by placing counting probes on the section and digitizing directly from the sections with a low-resolution, 4× objective on a NIKON, Eclipse 80i microscope (Nikon, Japan), equipped with a motorized stage (MAC200, Ludl Electronic Products, Hawthorne, NY, USA). This system was coupled to a computer that ran the Stereo Investigator software (MicroBrightField, Williston, VT, USA), which could store and analyze the *x*, *y*, and *z* coordinates of the digitized points. For unambiguous detection, and to facilitate counting the microglia with the dissector probe, the low-resolution objective was replaced with a high-resolution, 100× oil immersion, plan fluoride objective (Nikon, NA 1.3, DF = 0.19 μm).

The thickness of the section was carefully assessed at each counting site with the high-resolution objective, and the fine focus of the microscope was used to define the immediate layers at the top and bottom of the section. Because the section thickness and distribution of cells in the section were variable, the total number of objects of interest was weighted by the section thickness. This variability may be a consequence of different degrees of tissue fixation, dehydration and method of sectioning (Gardella et al., 2003) that may change thickness between sections of the same individual, different individuals or between experimental groups (e.g. young vs. old brains). All microglial cell bodies that came into focus inside the counting frame were counted and added to the total number, provided they were entirely within the counting frame, or they intersected the acceptance line without touching the rejection line (Gundersen and Jensen, 1987). The counting boxes were randomly, systematically placed within a grid.

Photomicrographic documentation and processing

To obtain digital photomicrographs, we used a digital camera (Microfire, Optronics, CA, USA) coupled to a NIKON Eclipse 80i microscope. Digital photomicrographs were processed with Adobe Photoshop software; scaling and adjustment of the brightness and contrast levels were applied to the whole image. To display representative sections from each experimental group, we selected micrographs with the number of microglia in the region of interest closest to the mean number of microglia for that group.

Statistical analyses

Behavioral data were analyzed with parametric statistics. The two-tailed *t*-test for dependent groups was used to detect significant differences between the percentages of time spent on different objects during the total time of exploration in the test trial. The performance was defined as the time of exploration for each object, expressed as a proportion (percentage) of the total time of exploration. Possible significant differences were detected with the two-tailed *t*-test for dependent groups (Dix and Aggleton, 1999). In all statistical tests, the threshold for significance was set at $p < 0.05$. All tested subjects from different experimental groups were included in the sample (young, small litter size (YS) = 7, young, large litter size (YL) = 9, aged, small litter size (AS) = 9, aged, large litter size (AL) = 4) and as a criteria to decide whether the objects were distinguished by the tested subjects we established that the average time spent on one of the objects should be equal or higher than 60% of the total time of exploration.

Stereological and morphological data are reported as the mean ± the standard error of the mean. See Tables 1–3 for details on the experimental parameters and the average counting results from the optical fractionator. The grid size was adapted to achieve an acceptable coefficient of error (CE). For the CE of the total microglial counts for each rat, we adopted the one-stage, systematic sampling procedure (the Scheaffer CE) described previously (Glaser and Wilson, 1998).

The acceptable CE level for stereological estimations was defined as the ratio of the intrinsic error introduced by the methodology, to the coefficient of variation (CV) (Glaser and Wilson, 1998). The CE expresses the accuracy of the cell number estimates; a $CE \leqslant 0.05$ was deemed appropriate for the present study, because the variance introduced by the estimation procedure contributed little to the observed group variance (Table 3) (Slomianka and West, 2005). Theoretically, the ratio of CE^2:CV^2 should not be higher than 0.5, although there are exceptions and strict adherence to this rule is not advised (Slomianka and West, 2005). We detected an exception in the present investigation, where CE^2/CV^2 value was higher than the rule recommends. However, it is demonstrated in Table 1 that when CE^2/CV^2 is higher than 0.5 (aged subjects from small litters in Table 1), both the CV = 6% and the CE = 7% are small. In that case it is neither meaningful nor practical to strictly adhere to the rule (Slomianka and West, 2005).

Stereological estimations of all groups were compared with the two-way analysis of variance (ANOVA), Bonferroni post-tests, the Pearson linear correlation test, and cluster and discriminant analyses with Bioestat 5.0, the free statistical software (Ayres et al., 2007). Significant levels were set to $p < 0.05$.

RESULTS

Body weights and dentate gyrus volumes

Fig. 3 shows a comparative survey of the mean values and the respective standard errors of body weight measured at 7, 14, 21, 30, 90, and 600 post-natal days. We observed progressive, significant body weight gain, independent of the litter size, in all time windows. Compared to animals from small litters, the animals from large litters showed significantly smaller mean

Table 1. Microglial granular layer estimates for aged and young adult rats raised in large and small litters. Experimental parameters, optical fractionator counting results and individual unilateral microglial numbers (*N*) and mean groups with the coefficient of error (CE)

Subjects	Section thickness (μm)	*N*	CE	tsf	No. of counting frames	ΣQ^-	Subjects	Section thickness (μm)	*N*	CE	tsf	No. of counting frames	ΣQ^-
Aged from large litters							*Young adult from large litters*						
SMG20 EX62	31.5 ± 7.55	7354.49	0.070	0.258 ± 0.037	198	126	SM G39 EXP 96	20.4 ± 1.07	6845.66	0.063	0.349 ± 0.02	204	174
VIE G21 EX66	23.5 ± 0.22	8337.7	0.059	0.298 ± 0.003	206	184	VIDE G38 EXP 86	19.2 ± 1.09	4337.82	0.073	0.372 ± 0.02	193	117
VSDE G21 EX64	32.7 ± 5.33	9083.46	0.0615	0.236 ± 0.034	199	146	VIE G39 EXP 94	21.8 ± 0.96	4777.27	0.074	0.325 ± 0.02	183	114
VSDE G29EX119	26.7 ± 0.39	7679.83	0.068	0.263 ± 0.004	201	150	VSD G38 EXP 89	19.7 ± 0.87	5219.27	0.064	0.359 ± 0.01	189	137
VSDEG29EX120	32.2 ± 3.10	5493.52	0.085	0.224 ± 0.017	200	90	VSE + VID G39 EXP 92	19.9 ± 1.75	4554.58	0.072	0.362 ± 0.03	197	126
Mean	29.3 ± 1.80	7589.8	0.069				Mean	20.2 ± 0.44	5146.92	0.069			
SD		1346.128					SD		1004.129				
CV^2 = (SD/Mean)^2		0.031					CV^2 = (SD/Mean)^2		0.038				
CE^2		0.005					CE^2		0.005				
CE^2/CV^2		0.1507					CE^2/CV^2		0.1257				
CVB^2		0.027					CVB^2		0.033				
CVB^2 (% of CV^2)		85%					CVB^2 (% of CV^2)		87%				
Aged from small litters							*Young adult from small litters*						
DOR EXP 122	27.6 ± 4.06	4501.15	0.077	0.284 ± 0.049	155	88	PAD G52 EXP 136	15.6 ± 0.35	3193.78	0.075834	0.450 ± 0.010	181	106
SM G01B	18.1 ± 0.06	4644.68	0.071	0.388 ± 0.001	171	134	PPE G52 EXP 135	13.9 ± 0.90	3322.62	0.076046	0.515 ± 0.034	200	123
VME G04B	19.2 ± 0.40	4642.5	0.071	0.366 ± 0.007	194	125	SM G32 EXP 148	15.5 ± 0.37	3090.66	0.079999	0.453 ± 0.011	186	103
VSD G04B	21.5 ± 0.69	4862.41	0.068	0.327 ± 0.011	173	118	SM G52 EXP 134	18.9 ± 0.82	4153.19	0.1	0.375 ± 0.015	206	113
VSE G01	20.2 ± 0.34	4112.38	0.074	0.348 ± 0.006	190	106	VSDE G37 EXP 71	14.8 ± 0.68	3928.22	0.067753	0.479 ± 0.021	187	137
Mean	21.3 ± 1.66	4552.62	0.072				Mean	15.7 ± 0.84	3537.694	0.079926			
S.D.		277.93					S.D.		473.21				
CV^2 = (D.P./Mean)^2		0.004					CV^2 = (D.P./Mean)^2	0.018					
CE^2		0.005					CE^2		0.006				
CE^2/CV^2		1.296					CE^2/CV^2		0.35				
CVB^2		-0.001					CVB^2		0.011				
CVB^2 (% of CV^2)		-25%					CVB^2 (% of CV^2)		66.6%				

All evaluations were performed using a 100X objective lens (Nikon, NA 1.3, DF = 0.19 μm). a(frame), area of the optical dissector counting frame = 60 × 60 μm^2; A(*x*, *y* step), *x* and *y* step sizes = 90 × 90; asf, area sampling fraction [a(frame)/A(*x*, *y* step)] = 0.44; tsf, thickness sampling fraction, calculated by the height of optical dissector = 7 μm divided by section thickness, *h*/section thickness; ssf, section sampling fraction = 1/6; number of sections = 5; $\sum Q^-$, counted microglial markers.

Table 2. Microglial molecular layer estimates for aged and young adult Wistar rats raised in large and small litters. Experimental parameters, optical fractionator counting results and individual unilateral microglial numbers (*N*) and mean groups with the coefficient of error (CE)

Subjects	Section thickness (μm)	*N*	CE	tsf	No. of counting frames	ΣQ^-	Subjects	Section thickness (μm)	*N*	CE	tsf	No. of counting frames	ΣQ^-
Aged from large litters							*Young adult from large litters*						
SMG20 EX62	31.8 ± 7.43	31315.52	0.047	0.253 ± 0.036	199	305	SM G39 EXP 96	21.2 ± 1.55	30981	0.05	0.341 ± 0.031	241	426
VIE G21 EX66	23.4 ± 0.24	35491.54	0.040	0.299 ± 0.003	213	439	VIDE G38 EXP 86	19.7 ± 1.09	21664.67	0.05	0.363 ± 0.019	208	322
VSDE G21 EX64	33.1 ± 5.21	36923.36	0.044	0.233 ± 0.034	203	338	VIE G39 EXP 94	22.5 ± 1.16	27878.07	0.04	0.316 ± 0.017	211	368
VSDE G29EX119	26.7 ± 0.41	32348.03	0.045	0.262 ± 0.004	203	351	VSD G38 EXP 89	19.6 ± 0.69	34254.02	0.03	0.360 ± 0.013	228	509
VSDEG29EX120	32.4 ± 1.80	34783.94	0.045	0.219 ± 0.011	216	311	VID G39 EXP 92	20.3 ± 1.23	26981.75	0.04	0.342 ± 0.020	205	395
Mean	29.5 ± 1.89	34172.48	0.044				Mean	20.7 ± 0.54	28351.9	0.04			
SD		2300.65108					SD		4705.53				
$CV^2 = (SD/Mean)^2$		0.005					$CV^2 = (SD/Mean)^2$		0.028				
CE^2		0.002					CE^2		0.002				
CE^2/CV^2		0.4288					CE^2/CV^2		0.0703				
CVB^2		0.003					CVB^2		0.026				
CVB^2 (% of CV^2)		57%					CVB^2 (% of CV^2)		93%				
Aged from small litters							*Young adult from small litters*						
DOR EXP 122	29.5 ± 3.89	29682.22	0.043	0.262 ± 0.047	197	304	PAD G52 EXP 136	16.0 ± 0.44	16082.51	0.045	0.441 ± 0.013	213	291
SM G01B	18.6 ± 0.31	24145.84	0.039	0.378 ± 0.006	198	380	PPE G52 EXP 135	14.1 ± 0.92	16272.66	0.051	0.506 ± 0.033	226	327
VME G04B	18.9 ± 0.29	21524.84	0.044	0.370 ± 0.006	207	330	SM G32 EXP 148	15.9 ± 0.19	15834.46	0.049	0.442 ± 0.005	201	292
VSD G04B	21.6 ± 0.64	22572.45	0.046	0.325 ± 0.010	212	304	SM G52 EXP 134	19.4 ± 0.98	24809.92	0.038	0.365 ± 0.018	268	373
VSE G01	19.8 ± 0.58	21415.26	0.045	0.356 ± 0.010	211	316	VSDE G37 EXP 71	15.8 ± 0.92	18657.07	0.048	0.452 ± 0.026	216	340
Mean	21.7 ± 2.02	23868.122	0.044				Mean	16.2 ± 0.86	18331.32	0.046			
S.D.		3430.27445					S.D.		3794.99				
$CV^2 = (D.P./Mean)^2$		0.021					$CV^2 = (D.P./Mean)^2$						
CE^2		0.002					CE^2		0.002				
CE^2/CV^2		0.0921					CE^2/CV^2		0.0499				
CVB^2		0.019					CVB^2		0.041				
CVB^2 (% of CV^2)		91%					CVB^2 (% of CV^2)		95%				

All evaluations were performed using a 100× objective lens (Nikon, NA 1.3, DF = 0.19 μm). a(frame), area of the optical dissector counting frame = 60 × 60 μm^2; A(*x*, *y* step), *x* and *y* step sizes = 90 × 90; asf, area sampling fraction [a(frame)/A(*x*, *y* step)] = 0.44; tsf, thickness sampling fraction, calculated by the height of optical dissector = 7 μm divided by section thickness, *h*/section thickness; ssf, section sampling fraction = 1/6; number of sections = 5; $\sum Q^-$, counted microglial markers.

Table 3. Microglial polymorphic layer estimates for aged and young adult Wistar rats raised in large and small litters. Experimental parameters, optical fractionator counting results and individual unilateral microglial numbers (*N*) and mean groups with the coefficient of error (CE)

Subjects	Section thickness (μm)	*N*	CE	tsf	No. of counting frames	ΣQ^-	Subjects	Section thickness (μm)	*N*	CE	tsf	No. of counting frames	ΣQ^-
Aged from large litters							*Young adult from large litters*						
SMG20 EX62	32.4 ± 7.69	27768.73	0.045	0.249 ± 0.035	208	265	SM G39 EXP 96	21.0 ± 0.95	22123.43	0.044	0.338 ± 0.017	207	305
VIE G21 EX66	24.4 ± 0.30	30496.62	0.041	0.288 ± 0.003	200	363	VIDE G38 EXP 86	19.6 ± 1.06	16861.75	0.047	0.363 ± 0.018	198	253
VSDE G21 EX64	33.9 ± 5.18	33614.91	0.042	0.227 ± 0.032	200	302	VIE G39 EXP 94	22.2 ± 0.96	20119.39	0.050	0.318 ± 0.033	207	263
VSDE G29EX119	27.1 ± 0.28	33714.75	0.048	0.258 ± 0.002	214	362	VSD G38 EXP 89	19.0 ± 0.57	21918.56	0.043	0.370 ± 0.011	209	336
VSDEG29EX120	33.4 ± 2.57	26736.54	0.052	0.214 ± 0.014	215	234	VID G39 EXP 92	20.8 ± 1.79	18787.87	0.048	0.347 ± 0.028	203	274
Mean	30.2 ± 1.89	30466.31	0.046				Mean	20.5 ± 0.56	19962.2	0.046			
SD		3227.037					SD		2208.778				
$CV^2 = (SD/Mean)^2$		0.011					$CV^2 = (SD/Mean)^2$		0.012				
CE^2		0.002					CE^2		0.002				
CE^2/CV^2		0.1861					CE^2/CV^2		0.1739				
CVB^2		0.009					CVB^2		0.010				
CVB^2 (% of CV^2)		81%					CVB^2 (% of CV^2)		83%				
Aged from small litters							*Young adult from small litters*						
DOR EXP 122	30.4 ± 3.91	18690.85	0.053	0.257 ± 0.049	161	187	PAD G52 EXP 136	16.3 ± 0.30	11971.18	0.054	0.441 ± 0.013	202	214
SM G01B	19.3 ± 0.20	15951.26	0.055	0.364 ± 0.004	165	239	PPE G52 EXP 135	14.5 ± 0.91	13099.32	0.047	0.506 ± 0.033	200	259
VME G04B	19.4 ± 0.52	16177.82	0.050	0.362 ± 0.009	191	243	SM G32 EXP 148	15.3 ± 0.29	11548.52	0.050	0.442 ± 0.005	201	220
VSD G04B	22.2 ± 0.72	17137.98	0.047	0.317 ± 0.011	212	227	SM G52 EXP 134	18.9 ± 0.92	15869.08	0.046	0.365 ± 0.018	222	243
VSE G01	21.2 ± 0.33	21281.11	0.042	0.331 ± 0.005	219	290	VSDE G37 EXP 71	15.6 ± 0.75	14771.88	0.047	0.452 ± 0.026	212	275
Mean	22.5 ± 2.04	17847.8	0.049				Mean	16.1 ± 0.75	13452	0.049			
S.D.		2201.584					S.D.		1838.804				
$CV^2 = (D.P./Mean)^2$		0.015					$CV^2 = (D.P./Mean)^2$	0.019					
CE^2		0.002					CE^2		0.002				
CE^2/CV^2		0.1593					CE^2/CV^2		0.1268				
CVB^2		0.013					CVB^2		0.016				
CVB^2 (% of CV^2)		84%					CVB^2 (% of CV^2)		87%				

All evaluations were performed using a 100× objective lens (Nikon, NA 1.3, DF = 0.19 μm). a(frame), area of the optical dissector counting frame = 60 × 60 μm^2; A(*x*,*y* step), *x* and *y* step sizes = 120 × 120; asf, area sampling fraction [a(frame)/A(*x*,*y* step)] = 0.25; tsf, thickness sampling fraction, calculated by the height of optical dissector = 7 μm divided by section thickness, *h*/section thickness; ssf, section sampling fraction = 1/6; number of sections = 5; $\sum Q^-$, counted microglial markers.

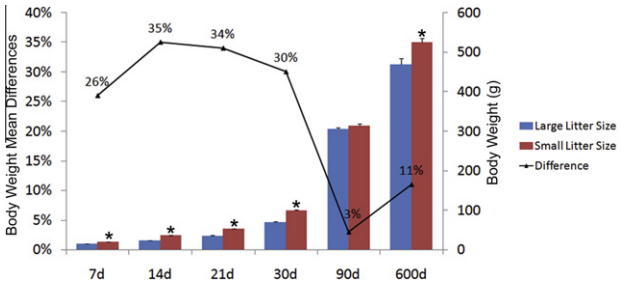

Fig. 3. Body weight evolution. The mean body weight (mean ± s.e.m., right *Y*-axis) is shown over time (age) for rats reared in small or large litters. The left *Y*-axis indicates the percent differences in body weights between the two groups (dotted line). (∗) indicates significant differences between groups, based on a two tailed *t*-test, $p < 0.05$.

body weights from the 7th to the 30th postnatal day; this was a critical time for the establishment of neural circuitry and for the establishment of the adult pattern and distribution of microglia. At 30 days, the mean body weight of subjects from large and small litters were indistinguishable; on the 600th postnatal day, subjects from small litters showed higher body weights than subjects from large litters.

Stereological estimates of microglial numbers in the dentate gyrus

Fig. 4 illustrates a series of low- and high-power photomicrographs from sections of the dentate gyrus immunolabeled for detection of IBA-1. The bar graphs (top row) show the estimated numbers of microglial cells in the molecular, granular, and polymorphic layers. Tables 1–3 give microglial granular, molecular and polymorphic layer estimates respectively for aged and young adult rats raised in large and small litters. The experimental parameters, optical fractionator counting results and individual unilateral microglial numbers (*N*) and mean groups with the CE are indicated.

Note that, independent of age, the large litter group, on average, was associated with a large number of microglia in all layers (molecular: 4 M, $t = 4.328$, $p < 0.01$; 23 M, $t = 4.451$, $p < 0.001$; granular: 4 M, $t = 2.799$, $p < 0.05$; 23 M, $t = 5.422$, $p < 0.001$; polymorphic: 4 M, $t = 4.245$, $p < 0.01$; 23 M – $t = 8.228$, $p < 0.001$). A high degree of microgliosis in all layers was observed in the aged, large litter group (molecular: $t = 2.514$, $p < 0.05$; granular: $t = 4.361$, $p < 0.001$; polymorphic: $t = 6.849$, $p < 0.001$). In contrast, the aged, small litter group showed

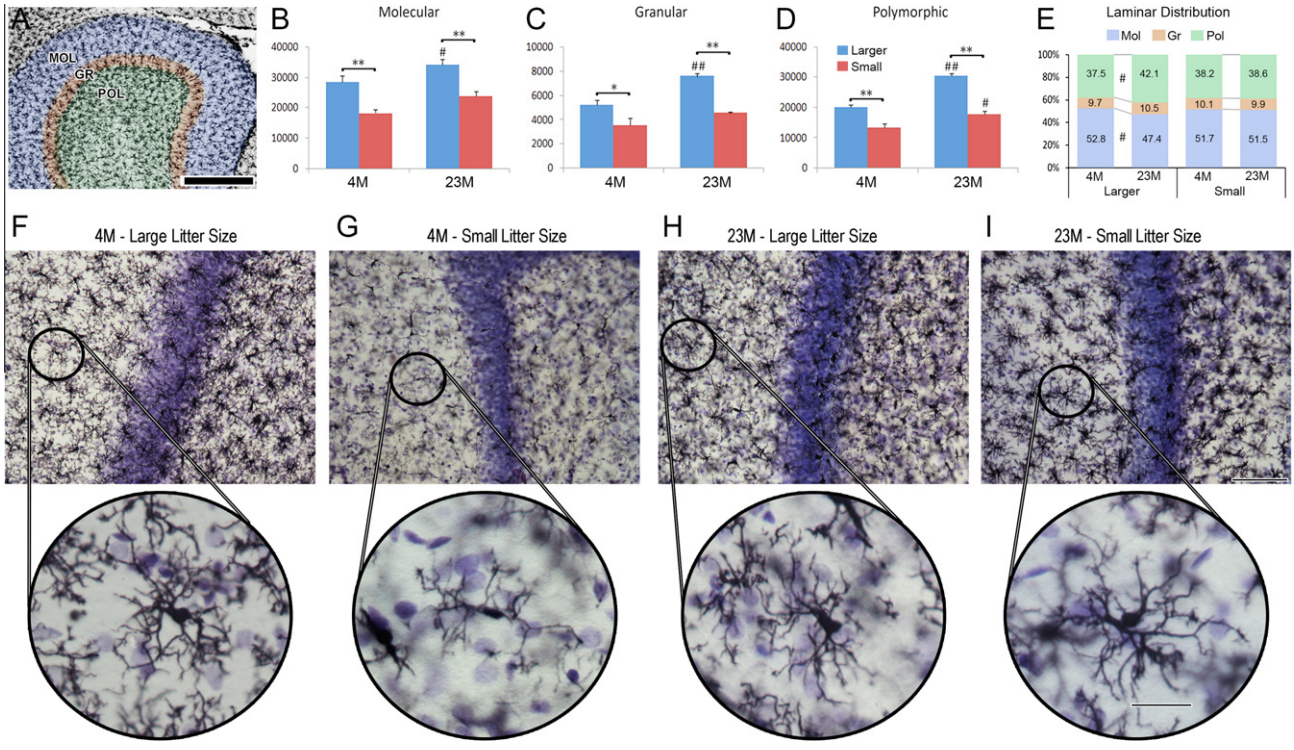

Fig. 4. Laminar distribution and morphology of microglia in the dentate gyrus of young adult (4 M = 4 months old, $N = 10$, YS = 5, YL = 5) and aged (23 M = 23 months old, $N = 10$, AS = 5, AL = 5) rats raised in small (6 pups/dam) or large litters (12 pups/dam). (A) The very low power image exhibits the delimitations of the dentate layers; MOL: molecular layer; GR: granular layer; POL: polymorphic layer; middle panels: bar graphs show stereological estimates of the mean (s.e.m.) numbers of microglia in the dentate gyrus molecular (B), granular (C), and polymorphic (D) layers; bar graph shows a comparison of the numbers of microglia in each laminar layer as a percentage of the total dentate gyrus, for each experimental group (E). (Middle row) representative photomicrographs of immunolabeled sections prepared at 4 and 23 months from subjects raised in large (F, H) and small (G, I) litters. Nissl staining indicates the limits of the granular layer; (bottom row): high-power photomicrographs show molecular layer microglia for each experimental group, to illustrate the morphological changes as a function of age and litter size. Images are representative of the mean microglia number and morphology for each experimental group. $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant differences between litter size groups, based on 2-way ANOVA, with Bonferroni post-tests; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ indicate significant differences between age groups; Scale bars = top, 500 μm; middle, 100 μm; bottom, 25 μm.

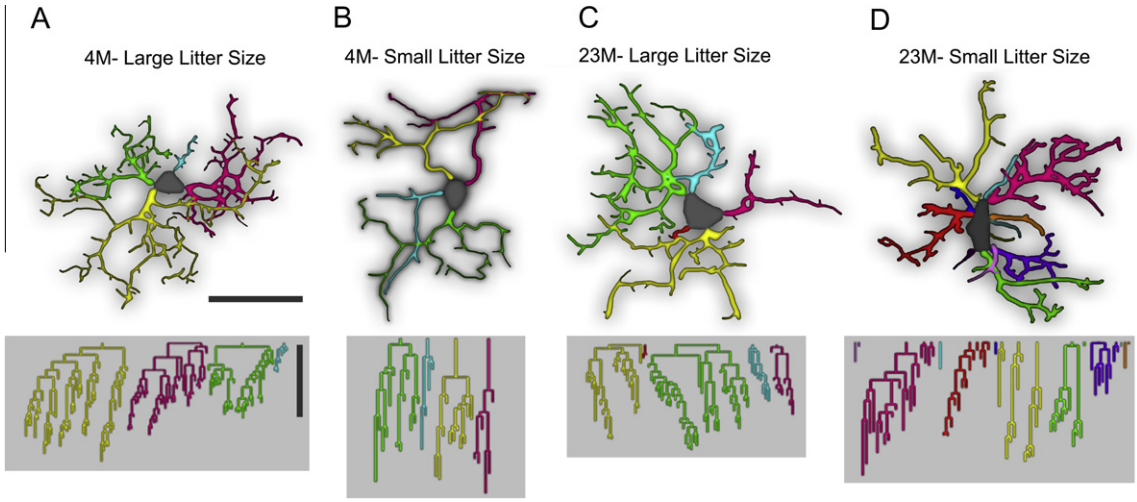

Fig. 5. Three-dimensional reconstructions of representative microglia show differences in morphology. Top, representative microglia from the young adult (4 M; A, B) and aged (23 M; C, D) Wistar rats from small and large litters. Individual branches are distinctly colored to facilitate examination. Bottom: linear dendrograms of microglia arbors; the length of each branch segment is displayed to scale; sister branches are horizontally displaced. Branch colors correspond to the 3D reconstructions above. The dendrograms were plotted and analyzed with Neuroexplorer (MicroBrightField). Scale bars = 30 μm.

microgliosis only in the polymorphic layer ($t = 2.866$, $p < 0.05$). Statistical analyses with a 2-way ANOVA showed that the number of microglial cells in all layers of the dentate gyrus was influenced by aging (molecular: $F_{1.16} = 12.03$, $p = 0.003$; granular: $F_{1.16=}18.6$, $p = 0.0005$; polymorphic: $F_{1.16} = 47.19$, $p = 0.0001$) and litter size (molecular: $F_{1.16} = 38.53$, $p = 0.0001$; granular: $F_{1.16} = 33.79$, $p = 0.0001$; polymorphic: $F_{1.16} = 77.78$, $p = 0.0001$) A significant interaction between age and litter size was only detected for the polymorphic layer ($F_{1.16} = 7.93$, $p = 0.012$). Detailed stereological data for each layer, in each individual, and for each experimental group are shown in Tables 1–3. The bar graph in Fig. 4 (top right) shows the influences of litter size and age on the laminar distribution of microglia in the dentate gyrus. Note that only the large litters showed a laminar redistribution of microglia associated with aging; the redistribution primarily affected the molecular ($F_{1.16} = 4.65$, $p = 0.046$; post test, $t = 2.91$, $p < 0.05$) and polymorphic layers ($F_{1.16} = 4.51$, $p = 0.049$; post test, $t = 2.77$, $p < 0.05$).

Morphometric analysis of microglia in the molecular layer of the dentate gyrus

Fig. 5 illustrates the 3D reconstructions of the ‘‘average microglia’’ in the molecular layers of different groups at different ages. The corresponding dendrograms show the extent of branching for each experimental group. Note that the microglia from aged rats exhibited thicker branches and larger somas than those from young adults. Also, the microglia from large litters appeared to be more branched than those from small litters.

A quantitative analysis of these changes is shown in Fig. 6. Note that, on average, independent of the litter size, aging was associated with significant increases in the soma area (large litter: $t = 7.08$, $p < 0.001$. Small litter: $t = 3.05$, $p < 0.016$), the soma perimeter (large litter: $t = 4.61$, $p < 0.002$. Small litter: $t = 2.3$, $p < 0.0503$), the base diameter of the primary branch (large litter: $t = 11.32$, $p < 0.001$. Small litter: $t = 5.89$, $p < 0.001$), the number of trees/microglia (large litter: $t = 4.00$, $p < 0.004$. Small litter: $t = 5.28$, $p < 0.007$), the tree surface area (large litter: $t = 2.89$, $p < 0.02$. Small litter: $t = 6.37$, $p < 0.002$), and the tree volume (larger: $t = 6.05$, $p < 0.003$. Small: $t = 7.68$, $p < 0.001$). However, microglia in individuals from the large litters showed enhanced effects of aging, with significantly greater expansion in tree volume ($t = 2.38$, $p < 0.044$), thickening of base diameter of primary branches ($t = 5.202$, $p < 0.001$), and enlargement of cell soma area ($t = 3.15$, $p < 0.013$), but they showed a reduced effect of age on the number of segments/mm ($t = 2.55$, $p < 0.05$). Litter size also affected the morphological parameters in young adult individuals; compared to the small litter group, the large litter group showed greater segment lengths ($t = 3.07$, $p < 0.015$), surface areas ($t = 4.28$, $p < 0.0027$), tree volumes ($t = 4.32$, $p < 0.0025$), number of segments/mm ($t = 2.55$, $p < 0.05$), protrusions ($t = 3.96$, $p < 0.01$), complexity (fractal dimension)($t = 3.12$, $p < 0.014$), and number of trees per microglia ($t = 2.58$, $p < 0.032$), but reduced tortuosity ($t = 2.76$, $p < 0.024$).

Linear regressions showed that tree volumes and tree surface areas (Fig. 7A, B) were correlated with the estimated numbers of microglial cells in the molecular layer, regardless of age or litter size. Other morphological variables were also significantly correlated with the number of molecular layer microglia (tortuosity: $r = -0.542$, $p = 0.02$; base diameter: $r = 0.668$, $p = 0.002$; protrusions: $r = 0.587$, $p = 0.01$; soma perimeter: $r = 0.59$, $p = 0.009$; soma area: $r = 0.655$, $p = 0.003$). A cluster analysis, based on metric features and stereological estimates with correlation indices above 65% (soma area, base

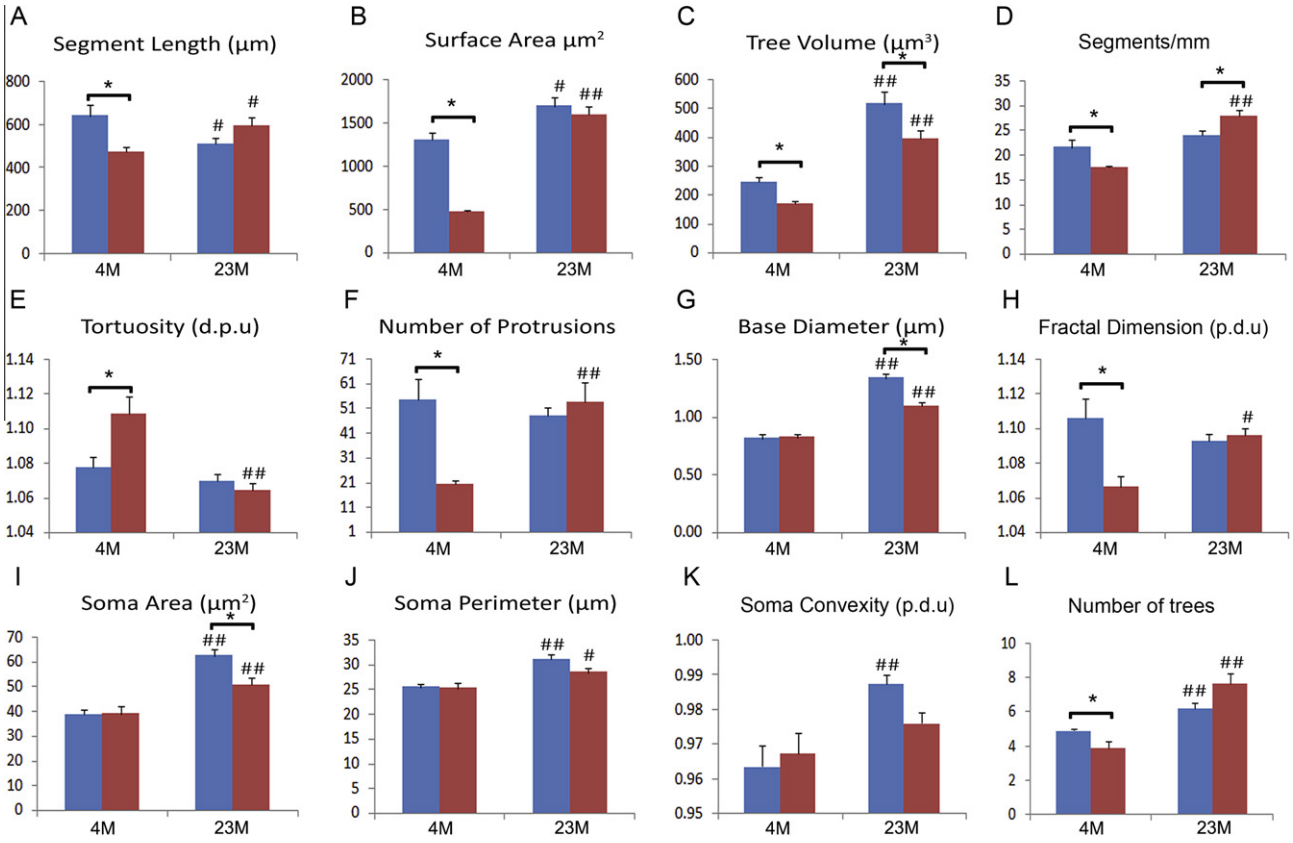

Fig. 6. Parameter measurements, based on microglial 3D morphometry. Morphologic parameters of representative microglia were measured from 3D reconstructions. Means (s.e.m.) are shown for large (blue) and small (red) litter size groups, measured at 4 months (4 M) and 23 months (23 M) of age. The parameters were segment length (A), total surface area (B), tree volume (C), number of segments/mm (D), tortuosity (E), number of protrusions (F), tree base diameter (G), branch complexity (fractal dimensions) (H), soma area (I), soma perimeter (J), soma convexity (K), and the number of trees per microglia (L). Note that aged individuals from large litters showed an enhancement of aging effects, with a significantly higher degree of tree volume expansion, thickening of branch base diameter, and enlargement of cell somas. On the other hand, the litter size affected microglial morphology in young adult individuals. Compared to the small litter group, those from large litters showed increases in segment length, surface area, tree volume, number of segments/mm, protrusions, complexity (fractal dimension), and the number of trees. $^{*}p < 0.05$, $^{**}p < 0.01$ indicate significant differences between litter size groups, based on 2-way ANOVA, with Bonferroni post-tests; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ indicate significant differences between age groups; 4 M = four-month-old, 23 M = twenty-three-month-old; p.d.u. = procedure defined unit (arbitrary units). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

diameter, tree area, and tree volume), revealed that young and aged subjects were clearly distinguishable in a dendrogram. A discriminant analysis indicated that the number of cells and tree area were the principal components for distinguishing between the experimental groups (Fig. 7D).

A graphic representation that illustrates the results obtained is shown in Fig. 8. Note that litter size effects mainly influence the branching pattern of microglia. On the other hand, aging causes enlargement of the soma and tree morphometric features. The combination of litter size and aging interacts additively to increase soma area, base diameter of primary branches, and tree surface area.

Behavioral assays

The results of the object identity and placement recognition tests are shown in Fig. 9A, B. After *t*-tests for related samples it was demonstrated that all groups showed impairments in both object placement and object identity recognition, except young adults from small litters that showed preserved object identity recognition memory (YS, $t = -2.57$, $p = 0.041$) with a rate of discrimination above chance (66%). All other groups showed discrimination rate between 51% and 58%. These results demonstrate that both young adult and aged Wistar rats, raised sedentarily in standard laboratory cages, showed impaired memory, independent of the litter size.

DISCUSSION

It has been proposed that aging is associated with inflammation in the central nervous system, but it is not known whether microglial changes induced by aging are affected by early-life litter size. We investigated the influences of litter size on memory loss and dentate gyrus microglia morphometry in young adult and aged Wistar rats. We found that a large litter size enhanced microglial morphological changes and increased the number of microglia induced by aging; moreover, these

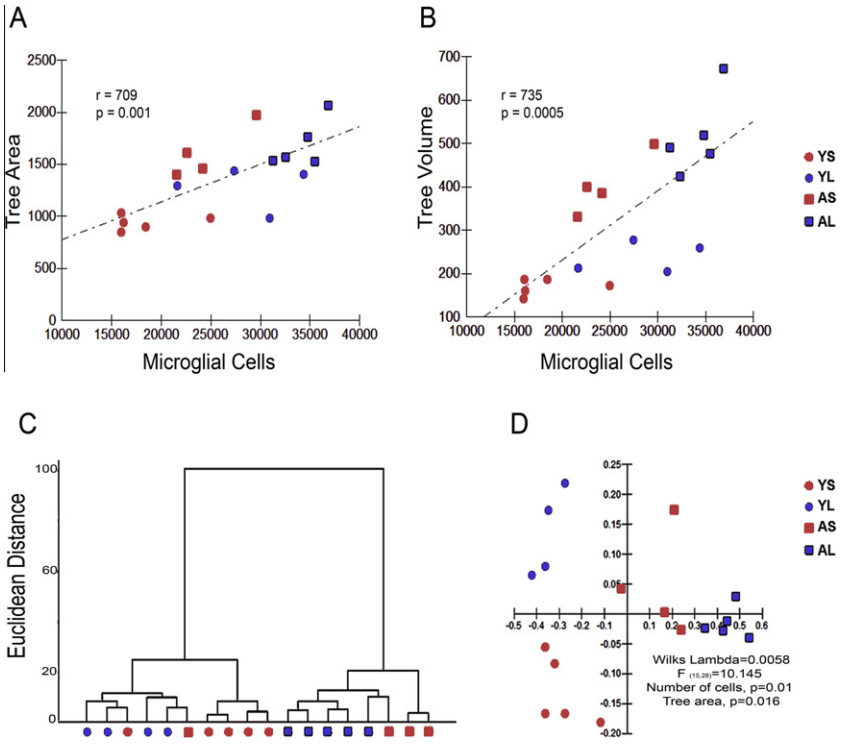

Fig. 7. Microglia cell number, morphometry, clusters, and discriminants associated with age and litter size effects in Wistar rats. (A, B) Linear correlations are shown between the number of microglia and (A) tree areas or (B) tree volumes for different ages and litter size groups. (C) Cluster analysis revealed distinct groups for aged and young adults, except for one aged subject. (D) Discriminant analysis indicates a clear litter size effect on age-related changes. YS: young, small litter size; YL: young, large litter size; AS: aged, small litter size; AL: aged, large litter size.

changes were associated with low performance in an object recognition task.

Litter size, growth, and somatic maturation

Our experimental manipulation was based on the protocol of Celedon et al. (1979), with an adaptation. We reduced the number of pups in the large litter size to 12 pups per dam to avoid undernourishment. In our model, pups from different dams were pooled, then divided among the dams to yield different ratios of pups per dam (12:1 or 6:1). We assumed that, on average, a high ratio of pups to dam would result in a low number of suckling opportunities per pup. As a consequence of these differences in litter size, we detected a difference in the body weight curve from 7 to 30 days, a critical time for the establishment of both neural circuitry and the adult pattern and distribution of microglia. After 30 days, there was no obvious difference in growth; age-matched subjects from small and large litters were indistinguishable one from another. From the 600th postnatal day on, individuals from small litters were, on average, 10% heavier than those from large litters. Despite these significant differences in the weight curves, previous reports demonstrated that, among normal litter sizes for Wistar rats (1–13 pups/dam), no undernutrition was produced during the weaning period (Chahoud and Paumgartten, 2009; Bulfin et al., 2011). Consistent with those observations, (Chahoud and Paumgartten, 2009) showed that, among three classes of natural litter sizes in Wistar rats, only minor differences were observed in pup growth and somatic maturation. Class 1: litters with six, seven, and eight pups (representing 27% of all pups), Class 2: litters with nine and 10 pups (42% of all pups), Class 3: litters with 11 and 12 pups (23% of all pups). Therefore, our experimental design was not expected to cause body weight differences due to nutritional imbalances. This suggested that the differences in maternal care induced by the different number of pups/dam may be relevant to our results. It is highly likely that pups in the large litters were exposed to less licking and grooming by the mother compared to those in the small litters and a reduction in maternal care has been shown to interfere with the innate immune response in adulthood and in aging due to imprinting that causes permanent alterations on the offspring's immune system (Barja-Fidalgo et al., 2003; Silva et al., 2010).

Litter size and microglial changes

Considerable research has explored the epigenetic effects of maternal behavior (licking and grooming) on pups. Much of that work was pioneered by Meaney's group (Meaney and Szyf, 2005), who showed that maternal behavior in the first weeks of life had profound epigenetic effects on the genes that regulate the Hypothalamus/Pituitary/Adrenal axis. It has been also demonstrated that survival and differentiation of microglia, its number and cell processes are strongly influenced by thyroid hormones during postnatal development (Lima et al., 2001). Glucocorticoids have

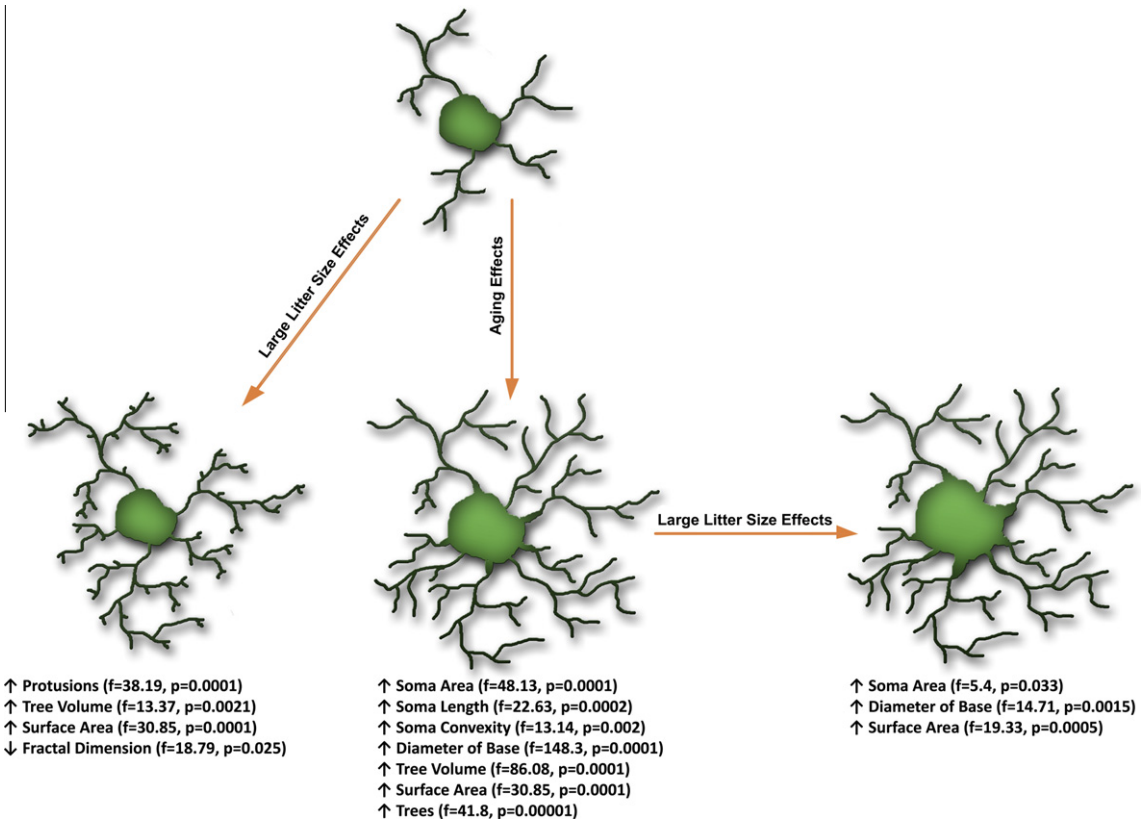

Fig. 8. Graphic representation of litter-size and aging effects on molecular layer microglial morphology. An average microglia (top) is shown before the separate and combined effects of litter size and aging. (Bottom) left: Litter-size effects influence mainly the branching pattern of microglia; middle: aging effects influence both soma and tree morphometric features; right: combined litter-size and aging effects interact addictively to influence soma area, base diameter of primary branches, and tree surface area.

profound influences on microglia throughout life (Nichols et al., 2005; Li et al., 2007), and litter size was shown to affect corticosterone levels (Rodel et al., 2010). Coherently optical density analyses of IBA-1 immunoreactivity in the dentate gyrus of adult, but not aged, gerbils with or without chronic restraint stress. Compared to controls, the stressed gerbils showed significant differences in dentate gyrus morphology; moreover, these differences were associated with higher levels of corticosterone in the adult, stressed compared to control groups (Park et al., 2011). Taken these findings together it is reasonable to postulate that microglia will change as a function of litter size. Because we did not assess hormone levels early in life in the present report, it remains to be investigated whether these changes are connected with early postnatal stress.

It has been suggested that litter size may affect the early-life development of social interactions between offspring; this may lead to a variety of permanent changes in anxiety, exploration of novelty, and adaptation to stressful situations, that cannot be directly explained by differences in maternal care (Dimitsantos et al., 2007). However, the relative contributions of reduced maternal care and infant-infant interactions remain to be quantified, and it has not been determined whether these variables interact to affect cognition and emotion later in life.

To date, no information has been available regarding the impact of litter size on microglia number and morphology later in life. Here, we demonstrated a vigorous, long-term effect of litter size on the laminar distribution and morphology of microglia in the dentate gyrus of young and aged rats. As previously reported in mice (Mouton et al., 2002; Lei et al., 2003; Kohman et al., 2012), stereological estimates of the present report revealed a significant increase in the number of microglia of all dentate layers in aged rats and this effect was higher in the individuals from larger litter sizes. However this aging effect seems to be influenced by gender with an increase in the number of dentate microglia only in female mice (Mouton et al., 2002). Similarly *t* has been also demonstrated using non-stereological procedure that there is not an increased number of microglia in the dentate gyrus of aged rats (VanGuilder et al., 2011). We have no explanation for these differences but it may possible that distinct procedures for cell counting, degree of immunolabeling and ambiguities in the definition of the objects and limits of the area of interest may explain at least part of these conflicting results. Unfortunately no stereological estimates are available for dentate microglia in aged

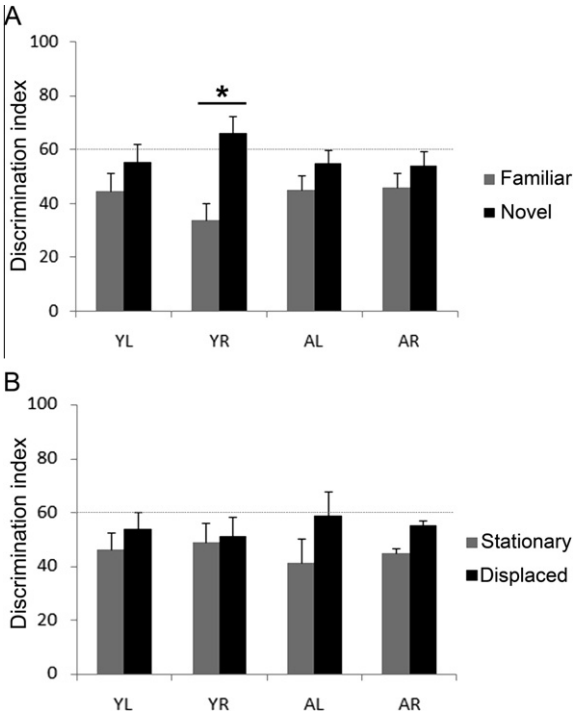

Fig. 9. Object identity and placement recognition tests. Performance scores for different age and litter size groups show the percentage of time spent on object exploration during (A) object identity recognition and (B) object placement recognition tests. When no significant difference is observed between the times spent on the two objects, it is an indication of memory impairment in either object recognition (A) or spatial memory (B). Dotted line indicates 60% of the total time of object exploration. $^{*}p < 0.05$ significant difference based on two-tailed *t*-tests for related events. YS: young, small litter size; YL: young, large litter size; AS: aged, small litter size; AL: aged, large litter size.

rats to be compared with our findings. It remains to be established whether there is a direct correlation between litter size, age related effects on dentate gyrus plasticity, and changes in microglial numbers and morphology.

Litter size, aging, and cognitive decline

Under homeostatic conditions, microglia across different regions of the central nervous system exhibit a typical branching morphology that distinguishes them from tissue macrophages (Ransohoff and Perry, 2009). However, it was previously demonstrated that, even in the absence of neurological disease, highly-reactive astrocytes and microglia phenotypes are expressed during aging as part of an elevated, persistent, pro-inflammatory profile (Ogura et al., 1994; Godbout and Johnson, 2009). Age-related physiological changes in microglia include altered cytokine production (Ye and Johnson, 2001; Sierra et al., 2007), altered activation marker expression (Perry et al., 1993; Kullberg et al., 2001), and dystrophic morphologies (Streit et al., 2004). In addition, neuron-microglia crosstalk during aging appears to be deregulated, with a concomitant loss of neuronal-derived factors that control microglial activation (Jurgens and Johnson, 2012). However, a marked, aged-related induction of pro-inflammatory microglial profiles in the hippocampus and dentate gyrus are not necessarily associated with cognitive impairment (VanGuilder et al., 2011). Moreover, these profiles in the dentate gyrus are found more frequently in sedentary subjects than in active subjects (Kohman et al., 2012).

More recently it has been demonstrated that morphological microglial changes seem to be dynamically influenced by extracellular signals such as ATP and chemokines arising from other neighboring cells (Davalos et al., 2005; Liang et al., 2009). These influences mediated via extracellular ATP, released in response to glutamatergic neurotransmission is done through probenecid-sensitive pannexin hemichannels (Fontainhas et al., 2011) and aging seem to affect this microglial dynamic behavior (Damani et al., 2011). In the present report we have quantified a series of microglial morphometric changes that seem to be affected by both litter size and aging and many of these changes we have found can be recognized in previous referred reports. For example we have found a higher number of protrusions (spine-like structures) and thicker branches on microglia from aged subjects raised in larger litters as compared to the other groups. However, because we have not measured extracellular signals such as ATP or chemokines it is difficult to directly correlate our findings with these extracellular signs. It is also difficult to directly associate microglial changes with memory impairments. However, it has also been demonstrated that exercise attenuates microglia proliferation and enhances the expression of a pro-neurogenic phenotype in the hippocampus and dentate gyrus (Kohman et al., 2012). Our finding that only the young adult rats from small litters preserved object recognition memory suggested that, as previously described in mice (Kohman et al., 2012), there may be two phenotypes of microglia (those with pro-neurogenic and those with pro-inflammatory profiles) in the dentate gyrus of Wistar rats. Thus, the young adult individuals from small litters may have exhibited the dominant effects of pro-neurogenic phenotypes; this could explain the superior performance compared to other rat groups in the identity object recognition task. Furthermore, we have previously demonstrated that standard laboratory cages provided an impoverished environment that impaired the spatial memory of both adult and aged mice raised in sedentary conditions (Diniz et al., 2010). Coherently aged rats raised in similar conditions showed losses in both spatial memory (Long et al., 2009; Bergado et al., 2011) and object recognition memory (Platano et al., 2008). Consistent with this view, it has been demonstrated that an enriched environment and physical activity reduced microglial numbers, suggesting that microglial homeostasis may be also modulated by activity-dependent, functional alterations (Ehninger et al., 2011; Kohman et al., 2012).

The mechanisms that link litter size to microglial changes and cognitive decline remain to be determined. Microglial survival is governed by two key molecules: granulocyte colony-stimulating factor (G-CSF) and macrophage-colony-stimulating factor-1 (MCSF-1).

Osteopetrotic mice (op/op), which lack MCSF-1, have been shown to harbor 24% fewer microglia in the cerebral cortex than wild-type controls; however, the microglial morphology was relatively normal (Kondo and Duncan, 2009). After an injury, microgliosis was significantly inhibited in MCSF-1 deficient mice compared to wild-type mice controls, and microglial morphology in both mutant and control mice changed to an activated profile (Kondo and Duncan, 2009). Recently, another ligand for the MCSF-receptor was discovered, called IL-34. At the mRNA level, this ligand was highly expressed in the brain and, to a lesser extent, in other tissues (Burns and Wilks, 2011). In contrast, G-CSF controls bone marrow production of circulating blood cells. G-CSF has been also implicated in the modulation of systemic immune responses by its inhibition of pro-inflammatory cytokines (Hartung et al., 1998). Interestingly, studies have shown that low plasma levels of G-CSF were associated with cognitive dysfunction in transgenic Alzheimer's mice (Sanchez-Ramos et al., 2009); moreover, low G-CSF levels in human plasma were predictive of the conversion from mild cognitive decline to an Alzheimer's type dementia (Ray et al., 2007).

Whether the morphological changes we have described in the present report are related with microglial activation and/or accelerated differentiation remains to be established. In particular, if altered morphology is related with microglial activation, it would be interesting to investigate cytokine production and activation marker expression, particularly M-CSF and IL34 (Wang et al., 2012). Coherently with microglial activation hypothesis it was observed a reduction in the expression of CD11b, a marker of microglial activation, in the fornix and parietal cortex of aged rats and restoration of microglial morphology to that in young adult rats after inhibition of cholinesterase (ChE) and monoamine oxidase (MAO) activities, and this anti-aging effect was associated with the preservation of novel object recognition (Weinstock et al., 2013). However it remains unknown whether MCSF or IL-34 are influenced by litter size or whether G-CSF might be reduced in sedentary, young adult and aged rats from large litters. These questions remain to be further investigated.

Technical limitations

It is difficult to estimate the number of objects in histological sections with stereological methods, due to ambiguities in definition and areas of interest (Mouton et al., 2002). To reduce these potential sources of error, when comparing animal groups, we processed all samples with the same protocols, and all data were collected and analyzed with the same stereological method, software, and hardware. To detect possible variations in the criteria for identifying the objects of interest, we performed checking procedures of the objects of interest by having different investigators count the same regions with the same anti-IBA 1 antibody as a microglial marker. As a result, we were able to reduce possible variations associated with non-biological sources to acceptable levels. On the other hand, microscopic, 3D reconstructions may be affected by mechanical factors associated with the vibratome sectioning and the dehydration procedure, which can induce non-uniform shrinkage in the *z*-axis of the sections (Hosseini-Sharifabad and Nyengaard, 2007). Thus, estimates of modifications in the *x*/*y* dimensions during tissue processing cannot be linearly extrapolated to the *z* dimension. These methodological constraints imposed limitations that must be taken into consideration when interpreting the results of the present study.

One important question to be considered is that if the subjects put into a particular condition were from the same or different litters. As previously described in methods the pups from 4 to 6 litters (on average 10–12 pups per litter) both females and males, born in the same day, were first joined in a big pool of 40–72 pups. Pups from this pool were randomly assigned to form the small- and large-litters of this study. Because of the random selection and distribution of the pups per mother it is reasonable to assume that all litters may have a mixture of siblings and non-siblings, minimizing the possibility of the potential confound effect associated to the performance of correlated littermates in behavioral tests artificially increase the *F* value. Finally, microglial plasticity may be affected by corticosteroids, which inhibit microglial activation (Nichols, 1999). In the present report, it could be argued that manipulation-induced stress during the behavioral tests may have altered the plasma corticosteroid levels, and thus, affected the microglial numbers. Although all individuals were submitted to the same procedures, we did not measure plasma corticosteroid levels after the behavioral tests; therefore, we cannot exclude the possibility that different levels of corticosteroids may have contributed to our observations.

CONCLUSIONS

We have shown that, in Wistar rats, litter size changes during the suckling period followed by *ad libitum* access to a conventional laboratory diet in conventional, sedentary laboratory conditions had significant effects on the morphology and laminar distribution of microglia in the dentate gyrus. These effects occurred in both young adult and aged animals, and the changes were associated with cognitive decline. The comparison of our data with those of human and animal studies, relating early nutritional condition to cognitive development (Laus et al., 2011; Lutter and Lutter, 2012), may help in pointing to the importance of controlling litter size in studies involving neural and glial cells and behavioral variables.

Future research should investigate the molecular factors that contribute to the number and morphological phenotype of microglial changes and their possible influence on how dentate gyrus development contributes to spatial and object recognition memories in adulthood and aging.

AUTHORS' CONTRIBUTIONS

C.W.P.D., R.C.A.G., and A.P. designed the study; L.C.V., C.M.L., C.R.F., M.A.O., R.P.B., T.T.C., I.N.F.A., D.G.D., J.B.T., M.B.O., A.A.C.L., R.F.M.S., R.A.G., A.A.S., and D.S.L. carried out most of the lab work and analyzed the data. L.C.V. was responsible for the stereological measurements. J.B.T. carried out the statistical analysis and prepared the figures. C.W.P.D., R.C.A.G., C.C., and P.F.C.V. participated in writing the manuscript. All authors read and approved the final manuscript.

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