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Influence of environmental enrichment on the volume of brain regions sensitive to early life stress by maternal separation in rats

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Abstract

Background: Exposure to maternal separation (MS) in rodents may have long-lasting consequences for the structure and function of several brain regions, eventually associated with alterations in cognition and emotion later in life. Post-weaning environmental enrichment (EE) has been reported to ameliorate the detrimental effects of exposure to early life stress mainly in the hippocampus. **Method:** In vivo magnetic resonance imaging (MRI) was applied to evaluate possible volumetric changes in the dorsal and ventral hippocampus, the medial prefrontal cortex and the dorsal striatum of 90-day-old male rats after daily MS for 240 min from postnatal days 2-21. **Results:** No significant volume changes were found in the selected brain regions in MS animals as compared with an age-matched control group. However, additional groups of control and MS animals with EE from days 21-60 showed significant volume increases in the medial prefrontal cortex and the ventral hippocampus as compared to the groups without EE. In addition, general hemispheric asymmetry was found in the volume of the brain regions measured. **Conclusions:** Our results demonstrate that EE could have differential effects depending on previous exposure to MS and on the development of brain lateralization.

Keywords: Brain volume, MRI, early life stress, environmental enrichment, brain asymmetries.

Resumen

Influencia del enriquecimiento ambiental en el volumen de regiones cerebrales sensibles a estrés vital temprano por separación materna en ratas. **Antecedentes:** la exposición a separación materna (MS) en roedores puede tener consecuencias a largo plazo en la estructura y función de regiones cerebrales, particularmente asociadas con alteraciones cognitivas y emocionales. El enriquecimiento ambiental (EE) tras la lactancia ha mostrado contrarrestar los efectos adversos de la exposición a estrés temprano principalmente en el hipocampo. **Método:** se obtuvieron imágenes por resonancia magnética (IRM) in vivo para evaluar los posibles cambios volumétricos en el hipocampo dorsal y ventral, la corteza prefrontal medial y el estriado dorsal en ratas macho de 90 días de edad tras MS durante 240 min diarios entre los días 2 y 21. **Resultados:** no hallamos cambios significativos de volumen en las regiones cerebrales seleccionadas de animales MS, frente a un grupo control. Sin embargo, grupos adicionales de animales control y MS con EE entre los días 21-60 mostraron incrementos volumétricos significativos en la corteza prefrontal medial y el hipocampo ventral, frente a grupos sin EE. Asimismo, se encontró asimetría hemisférica en el volumen de las regiones cerebrales medidas. **Conclusiones:** nuestros resultados demuestran que el EE tendría efectos diferenciales dependiendo de la exposición previa a la MS y en el desarrollo de la lateralización cerebral.

Palabras clave: volumen cerebral, IRM, estrés vital temprano, enriquecimiento ambiental, asimetrías cerebrales.

The novel concept of 'psychoexposome' would suggest that the psychosocial environmental exposures shape our behavior and body functions by epigenetic mechanisms (Colomina et al., 2018; González-Pardo & Pérez Álvarez, 2013). It is well known that early life stress (ELS) is a critical environmental factor for the development of mental disorders. In this regard, it has been reported that exposure to a wide range of traumatic social experiences like parental neglect and/or maltreatment increases the risk for the development of impaired cognitive and emotional functioning during adulthood (Banqueri & Arias, 2017a; Gilmer & McKinney, 2003; Leeb, Paulozzi, Melanson, Simon, & Arias, 2008; Nelson et al., 2007; Taylor, Way, & Seeman, 2011). Maternal separation is one of the most used models of ELS in rodents that could be generally considered to mimic the adverse psychosocial influences on brain development and behavior. Moreover, there is some evidence about the effects of MS on the development of regional brain lateralization and asymmetry in experimental animals (Luo et al., 2014; Sullivan, 2004).

The hypothalamus-pituitary-adrenal (HPA) axis is the main neuroendocrine system involved in the stress response mediated by the glucocorticoid hormone cortisol (corticosterone in rodents). High levels of circulating cortisol could decrease neuronal survival, neuronal processes and neurogenesis in particular brain regions expressing a high density of glucocorticoid receptors like the hippocampus (van Bodegom, Homberg, & Henckens, 2017). Accordingly, it has been consistently reported reduced hippocampal volumes in patients with major depression or posttraumatic stress

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disorder, but not in animal studies of ELS exposure (Hui et al., 2011; O'Doherty, Chitty, Saddiqui, Bennett, & Lagopoulos, 2015; Videbech & Ravnkilde, 2004).

On the other hand, exposure to ELS can also decrease the volume of non-hippocampal regions like the prefrontal cortex and the striatum as reported in human studies (Dannlowski, Stuhrmann, & Beutelmann, 2012; Gorka, Hanson, Radtke, & Hariri, 2014; Saleh et al., 2017). However, it is not clear whether these anatomical changes caused by ELS could be reversed by environmental manipulations. In this regard, environmental enrichment (EE) in rodents has been reported to increase brain plasticity, cortical thickness, neuronal processes and brain weight, and to partially reverse the behavioral and endocrine effects of stress, if applied particularly during the post-weaning period (Bennet, Rosenzweig, & Diamond, 1969; Diamond, Krech, & Rosenzweig, 1964; Hirashe & Sinohara, 2014; van Praag, Kempermann, & Gage, 2000). One of the first experiments evaluating the effects of housing conditions in rats on learning and memory was performed by the Canadian neuropsychologist Donald Hebb in 1947. EE protocols in rodents differ greatly, but commonly involve housing rodents in large cages and large groups with different toys, tunnels, running wheels and nesting materials in order to increase social interaction and exposure to complex stimuli (van Praag, Kemperman, & Gage, 2000).

The current study aimed to evaluate the effects of prolonged MS in rats on the volume of the dorsal and ventral hippocampus, the striatum and the prefrontal cortex measured by structural MRI. Further, we also tested the hypothesis of a possible reversal effect by EE of the anatomical changes caused by MS in the same brain regions.

Method

Participants

Six timed-pregnant Wistar rats (central vivarium facility, University of Seville, Spain) were maintained in a 12:12 h light–dark schedule (with lights on at 07:00 h) room temperature at 22±2 °C and with ad libitum access to food and tap water. Experimental procedures carried out with animals used for this study strictly followed European guidelines (2010/63/EU) and Spanish regulations (Royal Decree 53/2013 of the Ministry of the Presidency) regarding the manipulation and use of animals for experimentation and other scientific purposes.

Instruments

Animals were scanned using a 3 T MRI scanner (MR Solutions Ltd., Guildford, Surrey, UK) by technicians unaware of the experimental groups used (Preclinical Imaging laboratory, University of Oviedo, Spain). Scout imaging in three planes with a fast spin-echo pulse sequence was first acquired to control for rat head positioning. T1-weighted and T2-weighted sequences were used to obtain axial brain images using the following parameters: field of view (FOV) = 60 × 60 mm, flip angle: 90 degrees, slice thickness = 1 mm, slice GAP = 0.1 mm, 12 slices, total anesthesia and acquisition time per animal was 35 min per subject. For T1-weighted sequence: TR = 720 ms, echo time = 11 ms, echo train length = 5, matrix size = 256 × 252. For T2-weighted sequence: TR = 4800 ms, echo time = 68 ms, echo train length = 8, matrix size = 256 × 248.

Procedure

Maternal separation and environmental enrichment

On gestational day 19, pregnant females were individually housed and observed daily for parturition. The day of birth was considered as postnatal day (PND) 0. On PND 1, all pups were weighted, randomized and adjusted to 5 males and 5 females (10 pups per dam). On PND 2, half of the litters (with 20 male and 20 female pups in total) were assigned to maternal separation group (MS). Each MS litter was separated daily from the mother and pups in each litter maintained together in an incubator at 30ºC with continuous ventilation and controlled humidity at 65±5% (FIEM, Italy) to maintain body temperature for 4 hours during the first 3 weeks of life according to a previously published standardized protocol (Banqueri, Méndez, & Arias, 2017b; Wang et al., 2015). Control dams remained undisturbed and their litters were assigned to a standard facility rearing group (SA, 20 male and 20 female pups in total) and kept undisturbed during the entire lactation period until PND 21.

On PND 21, male pups were weaned, weighted and housed with same-rearing condition groups under either standard (20 males) or enriched (20 males) conditions with food and water available ad libitum. Female pups were not studied in order to avoid confounding effects of estrous cycle on the effects of stress and EE on brain development. Animals in the environmental enrichment condition (SA-EE, MS-EE) were housed in groups of ten males in a large enrichment cage (80 cm long × 75 cm wide × 86.5 cm high, FerPlast, Castelgomberto, Italy) located in the same rearing room where the animals were kept since birth. The cage contained different objects (tunnels, cylinders, different objects made of metal or plastic and a running wheel) and nesting material that were replaced regularly. The objects in the cage were rearranged and changed weekly. Non-enriched groups (SA, MS) were kept during the same period (PND 21-60) in clear plastic cages (41 cm × 34 cm × 18 cm) under standard rearing conditions in groups of 4 rats per cage and they were kept undisturbed except for weekly cage cleaning (10 males per group). At 60 days of age, all rats were housed together with same-treatment groups, with five animals per cage until day 90.

MRI analysis

Five 90-day old male rats of each experimental group (SA, MS, SA-EE, MS-EE) were anaesthetized using inhaled isoflurane/O2 (3% for induction and 1.5-2% for maintenance). The volume of dorsal and ventral hippocampus, striatum and prefrontal cortex was independently calculated by different researchers from the Preclinical Molecular Imaging Unit (Instituto de Investigaciones Sanitarias, IDIS, University of Santiago de Compostela, Spain). For this purpose, all T1- and T2-weighted images were spatially normalized to the W. Schiffer-T2 template using PMOD 3.7 software (PMOD Technologies LLC, Zurich, Switzerland) and then co-registered to the VOI (volume of interest) W. Schiffer rat brain atlas (Schiffer et al., 2006), which includes accurate delineations of dorsal and ventral hippocampus, striatum and prefrontal cortex. Inverse spatial transformations were applied to the selected VOIs corrected for total brain volume in each brain hemisphere and then overlapped into the original MRI using MRIcro software (McCausland Center for Brain Imaging, University of South Carolina, Columbia, USA). Finally, the volumes of dorsal and ventral hippocampus, striatum and prefrontal cortex

were calculated by summing products of area measurements and slice thickness.

Data analysis

In order to take into account the small sample size and type I errors in multiple comparisons of related brain regions, ROI volumes were analyzed using SAS Software, version 9.2 (Cary NC, USA). Linear mixed effects model (PROC MIXED) was used to evaluate the effects of the four experimental groups (as between-subject variable) and laterality (right or left hemisphere as within-subject variable) on the volume of the each selected ROI. Post-hoc analysis of significant differences for main effects or interactions was performed by Benjamini-Hochberg procedure. This procedure adjusts the significance level ($\alpha = 0.05$) for multiple comparisons using the false discovery rate (FDR) p-level (FDR-p) correction approach (Benjamini & Hochberg, 1995).

Differences in body weight between experimental groups were analyzed using a repeated-measures two-way ANOVA with age (21 or 90 days) as repeated measure and experimental treatment (SA, MS, SA-EE, MS-EE) as main factor. Post-hoc analysis in case of significant differences in ANOVA results ($p < 0.05$) was performed with Tukey's HSD tests.

Results

Mean body weight

There was a significant interaction between age and treatment ($F(3,39) = 15.39$; $p < 0.001$). Post-hoc analysis of the interaction showed that body weight of 3-week-old MS male rats (PND 21) was significantly higher than the SA group (Tukey's HSD test, $p < 0.05$) (Figure 1). Body weight increased with age in both SA and MS groups at 90 days of age ($p < 0.001$) with increased body weight of MS group versus the MA group at PND90 ($p < 0.001$). In addition, EE groups had increased body weight as compared to groups SA ($p < 0.001$) and MS ($p < 0.001$) at PND 90. However, no significant differences were found between SA-EE and MS-EE groups (Figure 1).

Volumetry

Figure 2A shows a sample T2-weighted MR image showing a horizontal or axial plane of a 90-day-old rat brain, with the boundaries of the ROI manually delimited according to the W. Schiffer anatomical template (Figure 2B).

Results from the mixed effects model applied to the estimated volume of ROI by MRI are summarized in Table 1. Statistical

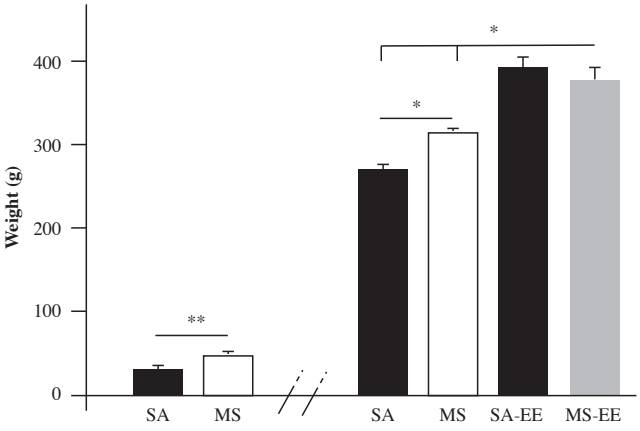

Figure 1. Mean body weight (±S.E.M.) of male rats at 21 days (PND21) and 90 days of age (PND90) in the different experimental groups. See Materials and Methods section for abbreviations used
*$p < 0.002$, **$p < 0.001$

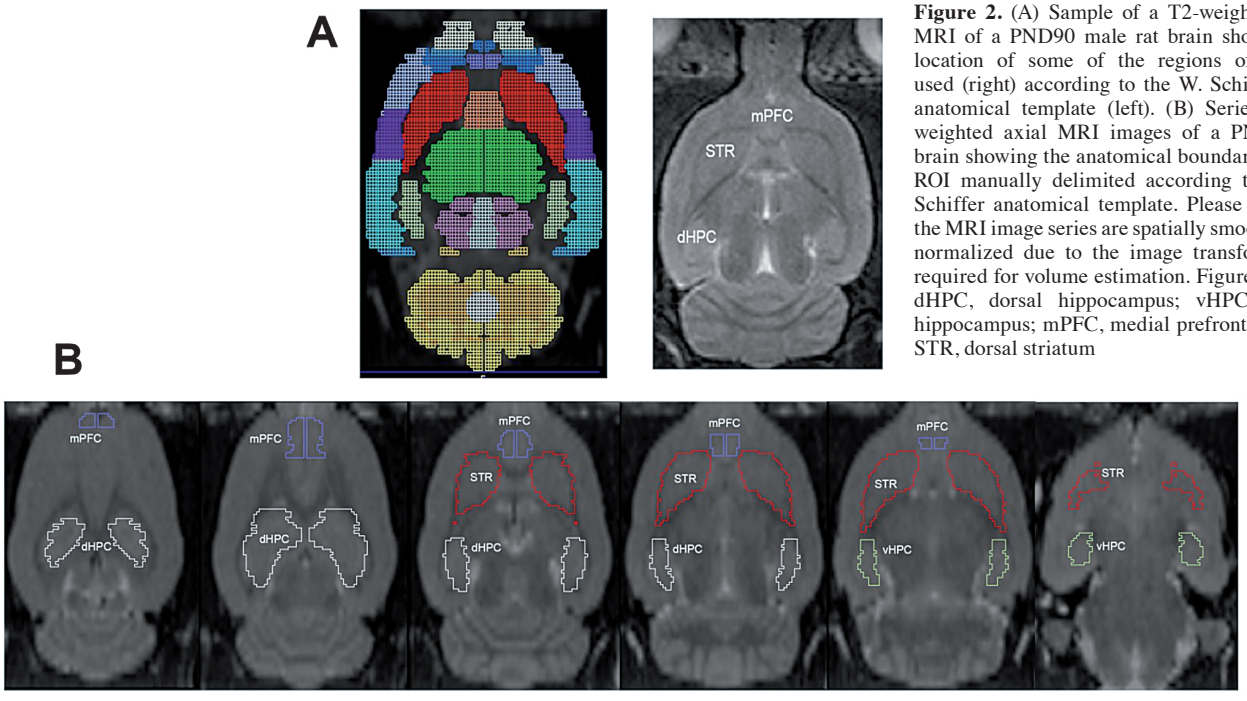

Figure 2. (A) Sample of a T2-weighted axial MRI of a PND90 male rat brain showing the location of some of the regions of interest used (right) according to the W. Schiffer MRI anatomical template (left). (B) Series of T2-weighted axial MRI images of a PND90 rat brain showing the anatomical boundaries of the ROI manually delimited according to the W. Schiffer anatomical template. Please note that the MRI image series are spatially smoothed and normalized due to the image transformations required for volume estimation. Figure legends: dHPC, dorsal hippocampus; vHPC, ventral hippocampus; mPFC, medial prefrontal cortex; STR, dorsal striatum

analysis revealed significant main effects of treatment group ($p < 0.05$) and laterality ($p < 0.05$) in the prefrontal cortex, without group × laterality interaction. Post-hoc analyses (Benjamini-Hochberg tests) showed significant prefrontal cortex volume increase in EE groups. In particular, increased volume was found in the SA-EE group as compared to the SA group (FDR-$p <$ 0.05).

No significant group differences were found in the volume of the dorsal striatum, but there were significant differences between hemispheres ($p <0.05$).

However, significant differences were found between experimental groups ($p < 0.05$) and laterality ($p <0.05$) in the dorsal hippocampus. In this case, only increased dorsal hippocampal volume was found between the SA-EE group and the SA group (FDR-$p <0.05$). No significant group × laterality interaction was found in the dorsal striatum.

On the other hand, highly significant group differences were found between groups in ventral hippocampal volume ($p <0.0001$) and laterality ($p <0.05$). Post-hoc analysis of pairwise differences between groups showed increased volume of the MS-EE group as compared to the rest of groups (FDR-$p <0.01$). In contrast, the SA-EE group showed decreased ventral hippocampal volume as compared to the SA group (FDR-$p <0.02$) and the MS group (FDR-$p <0.01$). No significant group × laterality interaction was found in the dorsal striatum.

Lastly, complementary mixed effects model analysis of the lateralization effects only in the groups submitted to EE (Table 2) showed only significant group × laterality interaction in the dorsal hippocampus when volume was estimated in the right hemisphere ($p <0.05$) but not in the left hemisphere.

Table 1
Mean estimated volume (mm^3) of the selected ROI by MRI volumetry of the experimental groups

	Prefrontal cortex	Dorsal striatum	Dorsal hippocampus	Ventral hippocampus
SA	7.67 ± 0.30	47.39 ± 1.90	28.51 ± 0.19	15.16 ± 0.54
MS	8.30 ± 0.32	47.56 ± 2.67	28.85 ± 0.65	15.21 ± 0.54
SA-EE	8.54 ± 0.15*	47.03 ± 0.98	30.72 ± 0.92*	13.64 ± 0.28^{+}
MS-EE	8.53 ± 0.54	47.83 ± 1.78	32.61 ± 1.90	17.36 ± 0.46$^{\#}$

Note: Mean ± S.E.M. *significantly higher vs. SA group (FDR-$p < 0.05$), $^{+}$significantly lower vs. MS and SA groups (FDR-$p<0.01$), $^{\#}$significantly higher vs. MS and SA groups (FDR-$p < 0.001$)

Discussion

Exposure to early MS increased body weight in 21-day-old male rats as compared to standard facility reared (SA) rats, a result that persisted in 90-day-old MS rats (see Fig. 2). Accordingly, it has been reported that early life stress induced by MS increases both food intake and body weight in rats, an effect probably mediated by dysfunctions in both the HPA axis related with impaired stress response (Ryu, Yoo, Kang, Lee, & Jahng, 2009). Conversely, body weight increases in MS rats could be caused by increased maternal care in these animals (Macrì, Chiarotti, & Würbel, 2008). In addition, environmental enrichment increased body weight in both MS and SA rats at 90 days of age. Body weight gain associated with EE could be related with stress-related behavior and HPA reactivity caused by increased social interactions and physical activity as previously reported (Konkle, Kentner, Baker, Stewart, & Bielajew, 2010; Morley-Fletcher, Rea, Maccari, & Laviola, 2003). EE would also induce increased body weight derived from higher muscle mass associated with physical exercise under EE as previously reported (Konkle et al., 2010). Alternatively, standard facility rearing conditions of MS and SA rats (groups of four animals per cage) would cause reduced weight gain as compared to EE rats, caused by increased social stress and decreased physical activity of small groups of rats under standard facility conditions. Therefore, standard facility rearing could also be considered as an 'impoverished' rearing condition (Simpson & Kelly, 2011; Würbel, 2001).

On the other hand, early MS did not affect the volume of the selected brain regions in male 90-day-old rats as measured by MRI. Accordingly, a previous MRI study reported that MS did not change hippocampal volume in 70-day-old rats (Hui et al., 2011). Another MRI study also reported reduced hippocampal volume after MS in young mice, but it was normalized in adulthood (70 days) and hippocampal neurogenesis was also unaffected, although hippocampal synaptic plasticity assessed by long-term potentiation was impaired in adult mice after MS (Herpfer et al., 2012). Conversely, several studies in humans showed that early life stress caused by childhood emotional or sexual maltreatment was associated with decreased volume of the prefrontal cortex and/or the hippocampus during adulthood (Bremner et al., 2003; Saleh et al., 2017; Teicher & Samson, 2016). However, other studies report volume reductions only in the basal ganglia or the anterior cingulate cortex (Cohen et al., 2006).

The reasons for this discrepancy between human and animal findings could lie in the different causes of early life stress in

Table 2
Mean estimated volume (mm^3) of the selected ROI in the right (RH) or left hemisphere (LH) by MRI volumetry of the experimental groups

		Prefrontal cortex	Dorsal striatum	Dorsal hippocampus	Ventral hippocampus
SA	LH	8.15 ± 0.51	48.59 ± 1.07	29.27 ± 0.51	15.10 ± 0.56
	RH	7.20 ± 0.12	46.19 ± 1.97	27.75 ± 0.75	15.01 ± 0.39
MS	LH	8.85 ± 1.17	46.50 ± 2.42	29.53 ± 0.11	15.75 ± 0.94
	RH	7.74 ± 0.54	48.63 ± 3.27	28.16 ± 0.53	14.66 ± 0.62
SA-EE	LH	8.89 ± 0.18	48.75 ± 1.13	32.58 ± 1.04	13.87 ± 0.32
	RH	8.19 ± 0.19	45.71 ± 1.46	28.85 ± 1.04	13.41 ± 0.73
MS-EE	LH	8.53 ± 0.39	47.12 ± 3.67	31.83 ± 1.96	18.40 ± 0.56
	RH	8.54 ±0.39	44.32 ± 3.32	32.58 ± 2.76^{+}	16.32 ± 0.72

Note: mean ± s.e.m. *significantly higher vs. SA-EE group in the RH (FDR $p < 0.05$)

children (e.g. sexual abuse, physical or emotional maltreatment, severe family conflict, etc.) that could have specific effects on adult brain morphology (Saleh et al., 2017). Additionally, the number and timing of early life stress events during early or late childhood would also differentially affect regional brain volumes (Baker et al., 2013).

In addition, the different methodologies used in animal models used to emulate the effects of early life stress in humans would also explain these differences. Another possibility to explain the absence of effects of maternal separation on the regional brain volume would be related with the effects of maternal care as related with the HPA development. In this regard, increased levels of maternal care could counteract the adverse effects of maternal separation, especially during long periods of maternal separation as used here (Macrì et al., 2008). Accordingly, increased maternal care of pups after MS by body grooming and licking would support the 'mismatch stress' hypothesis stating that early exposure to stress would prepare the individuals to optimally cope with future exposure to stressful stimuli later in life (van Bodegom et al., 2017).

Environmental enrichment (EE) generally increased regional brain volume as compared to SA rats in most regions studied. Accordingly, early EE could increase the volume of particular brain regions like the cortex and the hippocampus by promoting a major structural reorganization in these regions involving at least increases in dendritic spine density, synaptogenesis, glial cell numbers, neurogenesis in the hippocampus, myelination, including increased capillary density (He, Tsipis, LaManna, & Xu, 2017; Hirashe & Shinohara, 2014). These morphological changes induced by EE would support the general beneficial effects of EE on standard facility reared rats, but the benefits of EE on MS rats to counteract the negative behavioral and emotional consequences of early exposure to stress seem to be modest and region-specific as suggested by our results. Conversely, other authors did not find volume changes after EE using MRI volumetry in rat hippocampus (Hui et al., 2011) whereas a MRI study in adult mice reported volume increases in the dorsal hippocampus, the striatum and several cortical and subcortical regions after 3 weeks of EE or nonsignificant increases even after 24h of EE (Scholz, Allemang-Grand, Dazai, & Lerch, 2015). Large differences in the protocols and materials used for EE could be one of the main reasons that would be related with the inconsistencies across EE studies, as already reported (Simpson & Kelly, 2011; Toth et al., 2011). However, an unexpected result found was a significant decrease in the volume of the ventral hippocampus in the group of standard facility reared rats after EE (SA-EE group) as compared with SA rats. Conversely, the MS-EE group had increased ventral hippocampal volume as compared to the MS group. These results suggest that the effects of EE on hippocampal volume are not only region-specific, but also that they are closely related with previous early exposure to stressors in rats.

Additional reasons for these discrepancies would be related with the MRI methodology used to estimate regional brain volumes. For example, the study by Hui et al. (2011) did not apparently distinguish between dorsal and ventral hippocampus, and hippocampal volume was normalized to intracranial volume estimated with a specific method. Although differences across species should also be considered, the MRI method used in mice by Scholz et al. (2015) involved also a specific method to obtain and align brain templates. It is therefore likely that methodological differences across studies would be the main reason that would explain different findings using a similar experimental approach.

Moreover, significant volume increases in the prefrontal cortex and the dorsal hippocampus were found after EE in standard facility reared (SA) animals. However, nonsignificant volume increases after EE were found in these regions of MS rats, probably due to the small sample of animals used and the manual method used to delimitate brain regions. These results would support the hypothesis of beneficial effects of EE on emotional and cognitive aspects of behavior that would be impaired after early exposure to stress as previously described. Although our results are limited by the use of an animal model, it is likely that the benefits of EE would be seen in similar human populations. In fact, animal models of EE may be simulating standard human conditions, whereas control environments may be considered as impoverished. However, the EE procedure used here involves early sensory-motor stimulation, social interaction, play, and physical exercise, which could be used as potential interventions in children at high risk for developing neurodevelopmental disorders or mental disorders.

Finally, we found general hemispheric asymmetries in the effects of MS and/or EE regarding the volume of the prefrontal cortex, the dorsal striatum and the dorsal and ventral hippocampi. Significant volume differences between hemispheres were found in all brain regions measured. Accordingly, a previous MRI study in mice reported also volume increases after three weeks of EE in the left or right part of several brain regions (Scholz et al., 2015). We do not have a clear explanation for this laterality in the effects of MS or EE, although lateralized effects in brain volume have been previously reported in mice striatum after water maze training (Lerch et al., 2011). Another MRI study suggests that the volumes of the left and right hippocampus differentially decrease with chronic exposure to mild stress in rats (Luo et al., 2014). In this regard, we found that EE was associated with a greater volume increase in the right dorsal hippocampus, probably related with the beneficial effects of EE after stress derived from MS. Therefore, the present study would support that the effects of early stress and EE are lateralized in particular brain regions like the hippocampus.

In conclusion, although this study demonstrates the differential effects of EE on prefrontal cortex and hippocampal volumes, there are several methodological limitations to generalize our results, like the reduced number of animals used, the cross-sectional design of this study and the possible errors derived from the manual delimitation of the regions of interest in MRI images. Future neuroimaging studies should address the effects of different types of early life stressors on additional brain regions, in order to better understand the specific effects of early stress and environmental enrichment procedures on brain development and its standardization and therapeutic use in children.

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