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Research report

Long-term environmental modifications affect BDNF concentrations in rat hippocampus, but not in serum

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ABSTRACT

The role of mBDNF on the beneficial effects of cognitive stimulation on the brain remains controversial, as well as the potential of peripheral mBDNF as a biomarker of environmental effects on its central status. We investigated the effect of different environmental conditions on recognition memory, proBDNF, mBDNF and synaptophysin levels in the hippocampus, and on mBDNF levels in blood. Male Wistar rats (6 and 17 months-old) were assigned to cognitively enriched (EE), standard (SE) and impoverished (IE) environmental conditions for twelve weeks. Novel object recognition was performed at week 10. When the animals were 9 and 20-months old, hippocampus was collected for mBDNF, proBDNF and synaptophysin analysis; serum was analyzed for mBDNF levels. The cognitively EE improved recognition memory, resulted in a trend to increased hippocampal mBDNF and augmented synaptophysin levels. Accordingly, hippocampal mBDNF, proBDNF and synaptophysin were significantly higher in EE than IE animals. Hippocampal mBDNF was positively correlated to proBDNF, cellular and behavioral plasticity markers. No effect of age was seen on the studied variables. Moreover, no significant effects of EE or IE on serum mBDNF were observed. Serum mBDNF also failed to correlate with hippocampal mBDNF, proBDNF and with the cellular and behavioral plasticity markers. These findings indicate that mBDNF is involved in neuronal and behavioral plasticity mechanisms induced by cognitively enriched environments, and that peripheral mBDNF may not always be a reliable biomarker of the effects of environmental settings on central mBDNF and plasticity, which is of special interest from a translational research perspective.

1. Introduction

The brain-derived neurotrophic factor (BDNF) has attracted great interest in the last decades because of its broad effects on brain functioning. BDNF is associated with the modulation of neurogenesis, neuroplasticity and neuronal survival [1–3]. As a common trait for all neurotrophins, BDNF is produced as a pro-neurotrophin (proBDNF), being cleaved into its mature form (mBDNF) both at intra- and extracellular compartments [4]. While mBDNF facilitates neuroplasticity, neurogenesis and neuronal survival by means of its interaction with TrkB receptors, binding of proBDNF to the low-affinity p75 neurotrophin receptor (p75NTR) was shown to negatively regulate these functions [5,6]. Thus, since mBDNF and proBDNF may have opposite roles, it is likely that the balance between them plays an important role in physiological and pathological conditions [7–11].

BDNF is thought to have a key role in the beneficial effects of interventions aimed to prevent or rehabilitate age-related cognitive decline [12–14]. As current techniques fail to assess mBDNF levels in the

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living human brain, peripheral (serum and plasma) measures of mBDNF have been used as an indicator of central (brain) alterations of this neurotrophin. Although physical activity and cognitive stimulation are known to improve cognitive functioning, only physical activity was shown to be associated with increased peripheral levels of mBDNF [15,16]. Thus, there are doubts about the role of mBDNF in the beneficial effects of cognitive stimulation interventions. Besides, the use of peripheral mBDNF as an indicator of brain mBDNF levels, or of mBDNF effects on neuronal and behavioral plasticity, is still seen with skepticism. The dynamics of mBDNF exchange across the blood-brain barrier is poorly understood [17–19]. Moreover, various peripheral tissues, such as the skeletal muscles and the cardiovascular system, are also capable of producing BDNF and contribute to its circulating levels [20–22]. Thus, animal models are needed to properly investigate the effects of cognitive stimulation on brain mBDNF, as well as the relation between central and peripheral mBDNF.

Environmental enrichment protocols for rodents, aimed to simulate cognitive stimulation interventions for humans, include the traditional components used to create enriched environments (special bedding, toys, tunnels, social interaction), whit the exception of running wheels [23]. Although this cognitively enriched environment was shown to confer benefits on learning and memory in young and old adult animals [23–25], its association with central mBDNF alterations is still a matter of debate and was investigated only in young adult animals [23,26].

Environment manipulation is also an interesting paradigm to investigate the relation between central and peripheral mBDNF levels. In opposition to environmental enrichment, environmental impoverishment (social isolation, lack of sensory-motor stimuli) impairs memory performance and was shown to decrease the expression and protein levels of mBDNF in the hippocampus, a key structure for memory processing [27–29].Thus, the comparison of experimental conditions expected to have opposite effects on mBDNF levels in brain, such as enriched and impoverished environments, would be advantageous to evaluate the relations between central and peripheral mBDNF levels.

The present study investigated the effect of three months of enriched and impoverished environmental conditions in younger (6 month-old) and middle-aged (17 month-old) adult rats on: I) central proBDNF and mBDNF levels, as measured in the hippocampus; (II) neuronal plasticity, as assessed by the synaptophysin levels of hippocampal homogenates, considered an indirect biomarker for neuroplasticity [30–32]; (III) behavioral plasticity, evaluated whit the novel object recognition task, which is hippocampal-dependent and sensitive to environmental conditions and aging [29,33,34]; (IV) peripheral mBDNF levels, as measured in serum.

It is hypothesized that the cognitively enriched environment will increase central levels of mBDNF, and that this alteration will be accompanied by increased proBDNF levels, neuronal and behavioral plasticity, improving memory performance in younger and middle-aged adult animals. It is also hypothesized that, if central and peripheral mBDNF levels are correlated, then serum mBDNF will also be a reliable biomarker of the environmental effects on hippocampal neuronal and behavioral plasticity.

2. Material and methods

2.1. Animals

Male Wistar (CrlCembe:WI) rats were bred and housed in the Centro de Modelos Biológicos Experimentais (CeMBE) of the Pontifical Catholic University of Rio Grande do Sul (PUCRS) until the beginning of experimental manipulation (at 6 or 17 months of age). Animals were maintained in standard transparent individually ventilated cages (Tecniplast), the floor covered with sawdust, under controlled temperature (24 ± 1 °C), humidity (55%), circadian cycle of 12/12 h (lights on at 7 P M) and ad libitum access to standardized pellet food and water. All experiments were carried out in conformity with the Guide for the Care and Use of Laboratory Animals and performed according to the recommendations of the Brazilian Guidelines for the Care and Use of Animals in Research and Teaching (DBCA, published by CONCEA, MCTI). Experimental protocols were approved by the Ethics Committee for the Use of Animals of the Pontifical Catholic University of Rio Grande do Sul (CEUA, registration No. 7142). All efforts were made to reduce sample size and minimize animal suffering.

2.2. Experimental conditions

At the age of 6 or 17 months, animals were moved to CEMBE's research facility and randomly distributed into three groups: standard environment (SE), enriched environment (EE) and impoverished environment (IE). All groups were housed in the same room during the experimental protocol, which lasted 12 weeks.

In the SE condition young adult rats (6–9 month-old) were housed at three animals/cage, while middle-aged rats (17–20 month-old) were housed at two per cage. This difference in the number of animals per cage was required in order to maintain the cage area per animal constant between the groups. Animals of the IE group were single housed for the entire experimental protocol.

The EE condition was adapted from Bruel-Jungerman and colleagues [34] considering the findings of Simpsom and Kelly [35]. Animals were housed in groups as described for the SE condition and placed in a large, one square meter cage-like apparatus with sensory and motor stimuli (e.g.: mazes, toys, bedding material) for 90 min per day, six days per week for 12 weeks. Stimuli were changed every week to encourage exploration. As the middle-aged rats were housed in groups of two animals per cage, we combined animals from two housing cages into one large apparatus for the environmental enrichment. As the introduction of new animals can be considered a mild stressor, animal welfare was constantly monitored for signs of fighting and stress. The environmental enrichment exposure was always initiated between 4 PM and 5 PM to avoid circadian influences. After the 90 min period, animals were returned to their home cages. In the IE condition animals were single-housed.

Thus, in the SE conditions animals had social interaction, in the EE condition increased opportunity for sensory-motor experiences was added to the social interaction, whereas in the IE condition animals had no social interaction and sensory-motor experiences were not stimulated.

2.3. Behavioral assessment

In the tenth week after the beginning of the experimental protocol, the animals were submitted to the behavioral analysis. Locomotor, exploratory and anxiety behaviors were assessed with the open field test (OF). Memory, an indicator of behavioral plasticity, was evaluated with the novel object recognition (NOR) test. One day before the behavioral tests all animals were handled for 90 s for habituation to the experimenter (Fig. 1).

The OF arena consisted of a square box (40 × 40 × 60) with three wooden walls and one glass wall for animal observation. The floor was divided in 16 symmetrical squares. The four squares in the center of the apparatus were considered the inner zone, while the remaining 12 squares were called outer zone. The animal was placed with its head towards the glass wall and allowed to explore the open field for 5 min. The number of squares the animal crossed (number of crossings), the number of rearings and the proportion of time spent in the inner zone were determined as measures of locomotion, exploratory behavior and anxiety, respectively.

The NOR task uses the natural preference for novel objects displayed by rats and was used to assess the effects of environmental condition and age on long term memory. On the first day, animals were habituated to the open field box filled with sawdust. In the training session, performed 24 h after habituation, the animal was allowed to

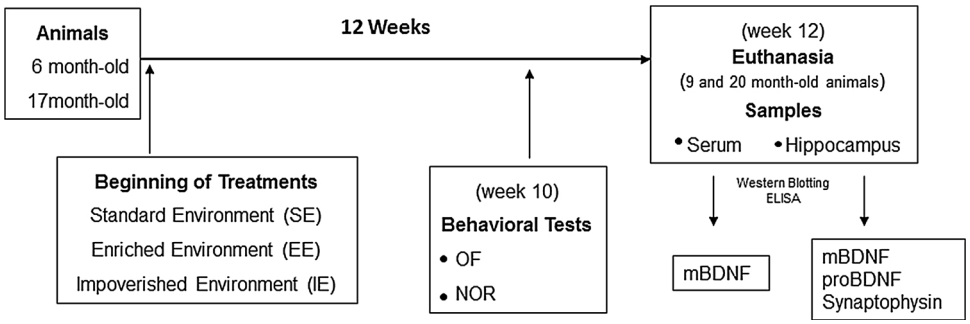

Fig. 1. Timeline of experimental procedures. Animals of 6 and 17 months of age were submitted to three different environmental conditions: SE (standard environment), EE (enriched environment), and IE (impoverished environment) for 12 weeks. Behavioral testing on the open field (OF) and novel object recognition (NOR) task was performed during the 10th week after the beginning of the treatments. At the end of the different treatments 9 and 20-month-old animals were euthanized to collect samples for mBDNF, proBDNF and synaptophysin analysis.

explore two identical objects (A and A, Duplo Lego toys) positioned in two adjacent corners, 9 cm from the walls. Animals were left to explore the objects until they had accumulated 30 s of total object exploration time or for a maximum of 10 min. Animals were tested for retention 24 h after training (long-term memory). In the retention test trial, the rats explored the open field for 5 min in the presence of one familiar (A) and one novel (B) object. Keeping the nose or nostrils on the object, and poking and sniffing of the object were considered as signs of exploration. Trials were videotaped and object exploration was measured by an experimenter blind to group assignment, using two stopwatches to record the time spent exploring the objects. All objects presented similar textures, colors, and sizes, but distinctive shapes. A recognition index calculated for each animal was expressed by the ratio TB/(TA + TB) [TA = time spent exploring the familiar object A; TB = time spent exploring the novel object B]. Between trials the objects were washed with 10% ethanol solution [36].

2.4. Blood and tissue sampling and processing

Animals were euthanized by decapitation 12 weeks after the beginning of exposure to the different environmental conditions and trunk blood and the hippocampus were collected for analysis. Blood was kept at room temperature for 1 h before centrifugation at 1000 *g* for 10 min and the supernatant was collected. Hippocampus was separated from whole brain and snap frozen in liquid nitrogen. All samples were stored at −80 °C for further analysis.

Blood and hippocampus mature BDNF (mBDNF) were measured by ELISA (CYT306 ChemiKine, Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Hippocampal and serum mBDNF concentrations were corrected for the total amount of protein, since it is known that small variations in the levels of proteins can have a significant effect on mBDNF levels, especially in serum samples, which have lower protein levels than hippocampus samples. Total protein levels were measured using the Bradford assay [37] with bovine serum albumin as standard, and performed according to a protocol previously described by our laboratory [38]. Briefly, tissue homogenates were prepared by gently grinding hippocampus samples in 0.1 M phosphate-buffered saline solution with protease inhibitor at room temperature. The samples were immediately centrifuged at 2000 *g* for 5 min and the supernatant was frozen at −80 °C until further analysis. For mBDNF analysis, 25 µL of samples of serum or supernatant of brain homogenates (in duplicate) and reference standards of mBDNF with concentrations ranging from 15.63 to 500 pg/mL were added to 96-well flat-bottom microtiter plates. After 24 h incubating at 4 °C, plates were rinsed four times with the wash buffer provided by the manufacturer. Biotinylated mouse anti-human mBNDF monoclonal antibody (diluted 1:1000 in sample diluent) was added to each well and incubated for 3 h at room temperature. Wells were once again washed and then incubated with streptavidin–horseradish peroxidase conjugate solution (diluted 1:1000) for 1 h at room temperature. After the addition of substrate and stop solution (CYT306 ChemiKine, Millipore, Darmstadt, Germany), the amount of mBDNF was determined by a plate reader. Absorbance was set at 450 nm.

Synpatophysin and proBDNF concentrations in the hippocampus were measured by Western Blot with a method previously reported [32]. Briefly, proteins were extracted in homogenization buffer containing 10 mM Tris–HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, protease inhibitor cocktail (Sigma-Aldrich: 104 mM 4-(2-Aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF), 80µM Aprotinin, 4 mM Bestatin, 1.4 mM E-64, 2 mM Leupeptin, 1.5 mM Pepstatin-A), 0.5% Triton X-100, and 0.1% SDS. After 30 min on ice, samples were centrifuged at 14,000 *g* for 10 min. The supernatant was collected and the protein content was determined using a Bradford assay. Aliquots were stored at −80 °C until further analysis. 25 µg of protein was separated on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were blocked with 5% nonfat dry milk in TBS containing 0.05% Tween 20 and were incubated overnight with either anti-synaptophysin (1:2500; Abcam, Cambridge, UK) or anti-proBDNF (1:1000; Sigma-Aldrich, São Paulo Brazil). Goat anti-mouse IgG and goat polyclonal anti-rabbit IgG (both from Abcam, Cambridge, UK) secondary antibodies were used and detected using the ECL Western blot Substrate Kit (Abcam, Cambridge, UK). After that, membranes were washed twice with a mild stripping buffer (containing 15 g glycine, 1 g SDS, 10 ml Tween20, pH 2.2, final volume of 1,0 L) for 30 min, and twice with PBS for 10 min at room temperature, before reprobing for loading controls (anti-tubulin, 1:2500; Abcam, Cambridge, UK, for synaptophysin analysis or anti-β-actin, 1:1500; Sigma-Aldrich, São Paulo Brazil, for proBDNF analysis). Pre-stained molecular weight protein markers (SuperSignal Molecular Weight Protein Ladder, Thermo Scientific, Rockford, USA) were used to determine molecular weight corresponding to the detected bands. The densitometric quantification was performed using Chemiluminescent photo finder (Kodak/Carestream, model GL2200). The target-to-control protein ratio was calculated (i.e. synaptophysin/tubulin and proBDNF/β-actin).

2.5. Statistical analysis

Data were analyzed using two-way ANOVAs., with environment (standard, enriched and impoverished) and age (9 and 20-month-old animals) as between group factors and Bonferroni as *post hoc* test whenever appropriate. Pearson's correlation analyzes were conducted to verify if the peripheral and central levels of mBDNF could be associated with each other and with proBDNF levels, cellular and behavioral plasticity markers. Results are expressed as mean ± standard error (SE). For all statistical analyses, significance was set at $p < 0.05$.

3. Results

3.1. Open Field (OF) behaviour

Statistical analysis of the OF results identified significant main effects of environment on locomotion [number of squares crossed: $F(2, 61) = 4.119$; $p = 0.021$] and anxiety [time spent in the center of the field: $F(2, 61) = 4.691$, $p = 0.013$]. However, no significant effects of

Table 1
Open field behavior.

Young Adult rats (9 m)				
Group	Number of crossings	Number of rearings	Duration in centre (s)	n
SE	55.93 ± 5.35	26.47 ± 2.60#	55.87 ± 10.61	15
EE	54.40 ± 4.40	27.50 ± 3.48#	57.00 ± 10.11	10
IE	70.83 ± 6.49*	30.00 ± 1.94 #	27.75 ± 6.19**	12
Middle-Aged Adult rats (20 m)				
SE	52.44 ± 5.39	22.89 ± 3.12	29.78 ± 8.44	9
EE	49.09 ± 3.71	14.18 ± 1.42	47.41 ± 8.25	11
IE	56.65 ± 5.06*	21.70 ± 3.48	24.85 ± 4.43**	10

Open-field behavior was analyzed during the habituation session for the object recognition task. Data are expressed as mean ± SEM. Data were analyzed by general linear models (GLMs), with environment (standard - SE, enriched – EE, and impoverished - IE) and age (9 and 20-month-old animals) as between group factors and Bonferroni as post hoc test. * indicates $p < 0.05$ when the number of crossings of IE was compared to SE and EE. ** indicates $p < 0.05$ when the time at the centre of IE was compared to EE. # $p < 0.05$ indicating a main effect of age when the number of rearings of 9 m and 20 m rats was compared.

age, or interaction between age and environment were found when number of crossings and time spent in the center of the arena were compared between groups (all $p > 0.05$, Table 1). As can be seen in Table 1, further analysis of the environment effects showed that IE animals showed an increased number of crossings compared to the other two groups ($p = 0.048$ for IE vs SE; $p = 0.035$ for IE vs EE), and spent significantly less time in the center of the arena than EE animals ($p = 0.012$). On the other hand, the analysis of the exploratory behavior showed a main effect of age, indicating that younger animals performed more rearings than older animals [$F(2, 61) = 13.46$, $p < 0.001$]. However, no significant effects of environment or interactions between environment and age (all $p > 0.05$) were found for this behavioral parameter.

3.2. Behavioral plasticity evaluated by the Novel Object Recognition Task (NOR)

The long-term memory on the Novel Object Recognition (NOR) task was used as an indicator of the effects of environmental conditions and age on behavioral plasticity. The statistical analysis revealed a significant main effect of environmental condition on recognition memory retention [$F(2, 61) = 15.548$; $p < 0.001$]. However, there was no effect of age or any interaction between age and environment on memory (all $p > 0.05$). As can be seen in Fig. 2, the *post hoc* analysis of the environmental effects on memory indicated that the EE group had the highest recognition memory retention index when compared to the other groups ($p = 0.003$ for EE vs. ES; $p < 0.001$ for EE vs. IE). On the other hand, the IE group had the lowest recognition index when compared to the other two groups ($p = 0.041$ for IE vs. SE; $p < 0.001$ for IE vs. EE). (Fig. 2). No statistically significant main effects or interactions of age and environmental conditions were found when recognition indexes of the training session were compared (all $p > 0.05$).

3.3. Hippocampal mBDNF and proBDNF levels

As can be seen in Fig. 3A, the statistical analysis indicated significant effects of environment [$F(2, 34) = 13.31$; $p < 0.001$], but not of age, or age x environment interactions (all $p > 0.05$), on hippocampal mBDNF levels. Further analysis indicated higher levels of mBDNF in the EE group in comparison to the SE and IE groups. However, only the difference between EE and EI groups reached statistical significance ($p < 0.001$). EI animals showed significantly lower mBDNF levels than SE animals ($p = 0.008$).

The general pattern of the results obtained for the hippocampal proBDNF levels resembled the results obtained for the hippocampal mBDNF levels. Thus, significant effects of environment [$F(2, 20) = 7.468$; $p = 0.004$], but not of age, or age x environment interactions (all $p > 0.05$), were identified on hippocampal proBDNF levels. Moreover, as previously described for mBDNF, the levels of hippocampal proBDNF were higher in the EE group in comparison to the SE and IE groups. However, in the case of proBDNF, statistical significance was reached both for the differences between the EE and IE groups ($p = 0.004$) and for the differences between the EE and ES groups ($p = 0.018$) (Fig. 3B).

3.4. Cellular plasticity evaluated by hippocampal synaptophysin levels

As can be seen in Fig. 4, the hippocampal synaptophysin levels showed the same pattern of results as found for the proBDNF levels. Hence, significant effects of environment [$F(2,21) = 12.066$; $p < 0.001$], but not of age, or age x environment interactions (all $p > 0.05$), were identified on hippocampal synaptophysin levels. *Post hoc* analysis indicated significantly higher levels of synaptophysin in EE animals than in the other groups ($p = 0.043$ for EE vs. SE; $p < 0.001$ for EE vs. EI).

3.5. Serum mBDNF

The results obtained for serum mBDNF levels can be seen in Fig. 5. Despite the environmental effects seen on hippocampal mBDNF, the two-way ANOVA failed to identify significant effects of environment [$F(2,29) = 1.518$; $p = 0.236$], age [$F(2,29) = 0.485$; $p = 0.492$], or interactions between age and environment [$F(2,29) = 0.311$; $p = 0.735$]

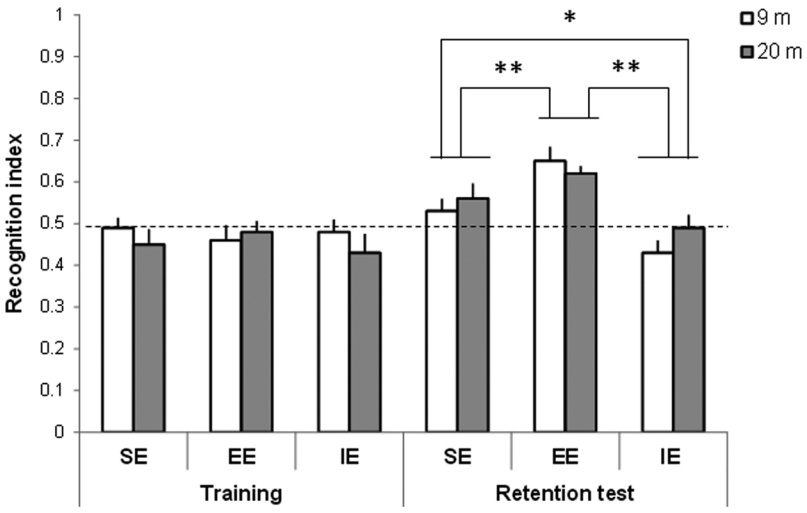

Fig. 2. Effects of environmental conditions on recognition memory tested at 9 and 20 months of age.
The long-term memory on the Novel Object Recognition task was evaluated in 9- or 20-month-old rats submitted to three different environmental conditions: SE (standard environment), EE (enriched environment), and IE (impoverished environment) for 12 weeks. Behavioral testing was performed after 10 weeks of exposure to environmental conditions. Statistical analysis was performed using two-way analysis of variance (2-way ANOVA) with age (9 and 20 months of age) and environmental conditions (SE, EE, and IE) as fixed factors, followed by Bonferroni *post hoc* test. Data are expressed as mean ± standard error of the mean (SEM). n = 9–15 per group. Statistically significant differences between EE vs. SE and IE are indicated as ** $p < 0.001$ and between IE vs. SE is indicated as * $p < 0.05$.

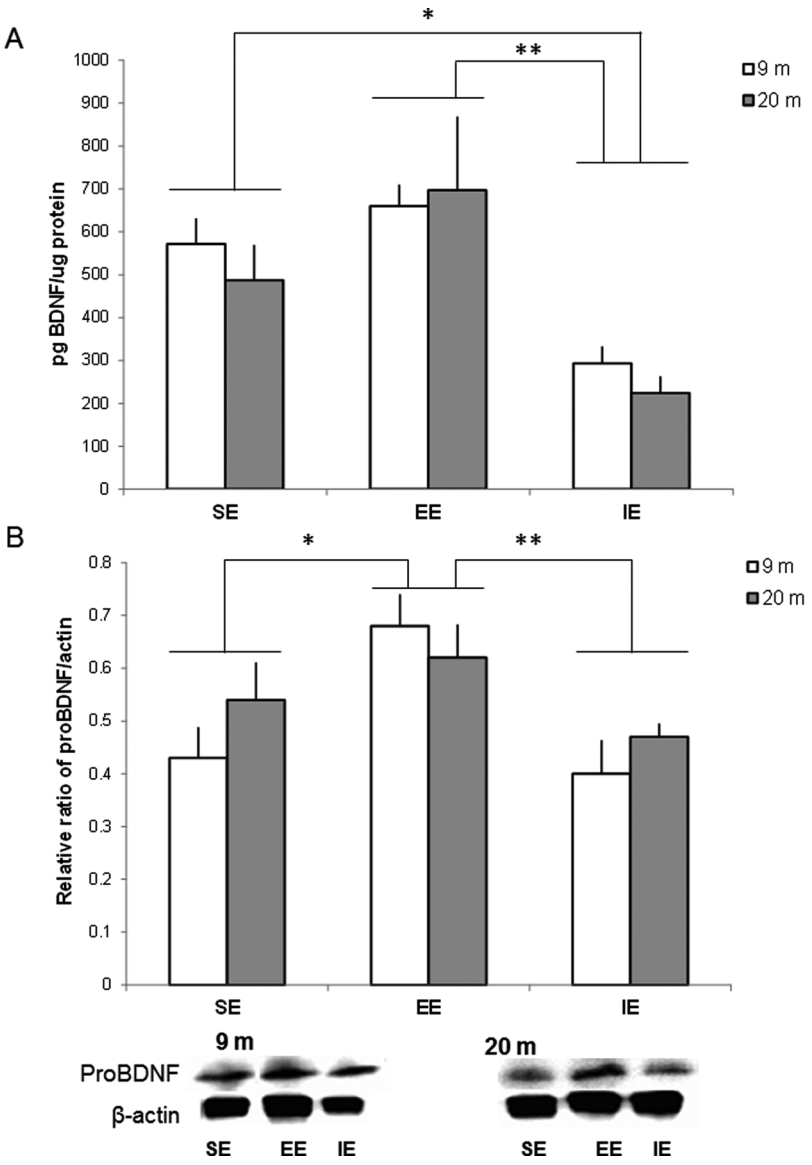

Fig. 3. Effects of environmental conditions on hippocampal mature brain-derived neurotrophic factor (mBDNF) (A) and proBDNF (B) levels in 9 or 20 month-old rats.
Rats were euthanized for mBDNF and proBDNF measurements after 12 weeks of exposure to the three different environmental conditions: SE (standard environment), EE (enriched environment), and IE (impoverished environment). mBDNF was quantified by anti-BDNF sandwich-ELISA assay (ChemiKine, Millipore, Darmstadt, Germany) according to the manufacturer's instructions. ProBDNF was measured by Western blot. Twenty five μg of protein were separated on SDS-PAGE and probed with a specific antibody and normalized to β-actin. Representative Western blots for proBDNF and β-actin are shown in the lower panel. Data are presented as mean ± SEM and expressed as pg of mBDNF/ug of protein or the relative ratio of proBDNF/β-actin, $n = 6–8$ (mBDNF) or $n = 4–5$ (proBDNF). Statistical analysis was performed using two-way analysis of variance (2-way ANOVA) with age (9 and 20 months of age) and environmental conditions (SE, EE, and IE) as fixed factors, followed by a Bonferroni *post hoc* test. Statistically significant differences in mBDNF levels between EE vs. IE is indicated as ** $p < 0.001$ and IE vs. SE is indicated as * $p < 0.01$. Significant differences in proBDNF levels between EE vs. IE is indicated as ** $p < 0.01$ and EE vs SE is indicated as * $p < 0.05$.

on serum mBDNF levels.

3.6. Correlation analysis between peripheral and central mBDNF levels and between them and proBDNF, cellular and behavioral plasticity markers

Separate Pearson's correlation analysis, including data of all experimental groups, were run to evaluate the relations between (I) hippocampal mBDNF levels and proBDNF, synaptophysin and recognition index in the NOR task; (II) serum mBDNF and hippocampal mBDNF; (III) serum mBDNF and proBDNF, synaptophysin and recognition index in the NOR task. As can be seen in Table 2, hippocampal mBDNF levels were significantly correlated to proBDNF and synaptophysin levels, as well as to the recognition index in the NOR task (all $p < 0.05$). In clear opposition to these results, no significant associations were found between serum mBDNF levels and hippocampal proBDNF and synaptophysin levels, nor between serum mBDNF levels and the memory index (all $p > 0.05$). As already expected from the pattern of the results described above, no significant correlation was found between serum mBDNF levels and hippocampal mBDNF levels ($p = 0.48$)

4. Discussion

The present study investigated the effects of enriched and impoverished environmental conditions on hippocampal proBDNF, mBDNF, synaptophysin and long-term memory, as well as the relation between hippocampal and serum mBDNF levels, analyzing the potential of peripheral mBDNF as a biomarker of central mBDNF, neuronal and behavioral plasticity. Consistent with the working hypothesis, the EE increased central levels of proBDNF and mBDNF, and these alterations were accompanied by increased neuronal and behavioral plasticity, improving memory performance in younger and middle-aged animals. As expected, IE animals had impaired memory and lower levels of proBDNF, mBDNF and synaptophysin than EE animals. However, no significant effects of environmental conditions were observed on serum mBDNF and no correlation was found between central and peripheral mBDNF levels. Accordingly, serum mBDNF also failed to be a reliable biomarker of the environmental effects on hippocampal neuronal and behavioral plasticity.

In this study we reproduced findings from other research groups, showing that long-term memory for object recognition was improved by the cognitively EE protocol and impaired by the IE condition [23,39–41]. Although the critical elements of the EE condition responsible for the improved performance on the NOR task are difficult to identify, the daily exposure of animals to a variety of toys, objects, and mazes provides an opportunity to improve multiple cognitive abilities, including object memorization [34]. On the other hand, in the IE

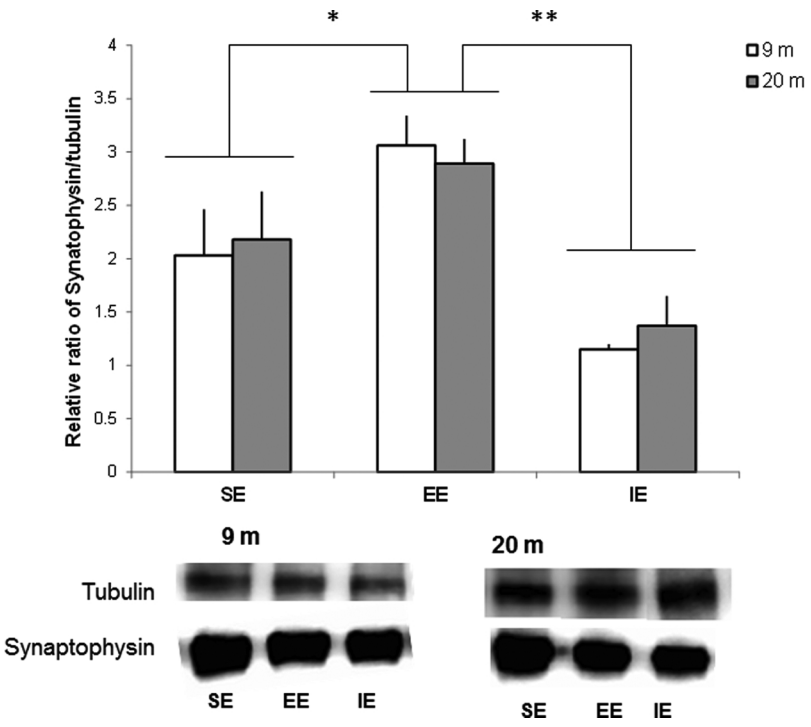

Fig. 4. Effects of environmental conditions on hippocampal Synaptophysin levels in 9 or 20 month-old rats.
Rats were euthanized for synaptophysin measurements after 12 weeks of exposure to the three different environmental conditions: SE (standard environment), EE (enriched environment), and IE (impoverished environment). Synaptophysin was measured by Western blot. Twenty five μg of protein were separated on SDS-PAGE and probed with specific antibody and normalized to tubulin. Representative Western blots for synaptophysin and tubulin are shown in the lower panel. Data are presented as mean ± SEM of the relative ratio of synaptophysin/tubulin, $n = 4–5$. Statistical analysis was performed using two-way analysis of variance (2-way ANOVA) with age (9 and 20 months of age) and environmental conditions (SE, EE, and IE) as fixed factors, followed by Bonferroni *post hoc* test. Statistically significant difference between EE vs. IE is indicated as ** $p < 0.001$ and between EE vs. SE is indicated as * $p < 0.05$.

condition animals are moved from their social environment to a situation characterized by the lack of social interaction with other individuals. It has been demonstrated that social isolation acts as a stressor, affecting multiple behavioral domains, including anxiety behaviors [39,42,43], as suggested in this study by the increased locomotor activity (a sign of hyperactivity) and decreased time spent in the inner zone of the OF. Thus, a role of anxiety on the impairment seen in the NOR task cannot be ruled out for IE animals.

Neurobiological changes associated with beneficial effects of EE and detrimental effects of IE on memory have been extensively studied. Overall, studies suggest that EE increases, while IE decreases the dendritic branching, spine and synapse numbers, as well as the weigh and thickness of the cortex and hippocampus [44–46]. EE and IE also have antagonistic effects on neurogenesis and synaptogenesis [42,47,48]. The mature form of BDNF is one of the main candidates to orchestrate all these alterations, because of its multiple functions in neuronal and behavioral plasticity, neurogenesis and neuronal survival [1–3]. In accordance with this concept, studies have demonstrated that hippocampal mBDNF increases in EE [30,31,49,50] and decreases in IE animals [27–29]. However, most of the EE studies include running wheels in the EE cages and the most prevalent view in recent literature is that the physical component of environmental enrichment is responsible for the increase in mBDNF expression and protein levels in brain [23,26,51]. In fact, physical activity can also increase the levels of other neurotrophins. Twenty-two-months old rats receiving moderate treadmill training had better spatial memory performance associated with increased BDNF and NT3 levels in the hippocampus [52]. Our study shows, for the first time, that a cognitively EE also has the potential to increase hippocampal mBDNF levels.

Although our EE animals showed only a trend towards increased hippocampal mBDNF in comparison to SE animals, it was accompanied by increased proBDNF levels. Moreover, a significant positive correlation was found between hippocampal mBDNF and proBDNF levels. Together these results suggest an increased synthesis of the precursor protein, and thus of mBDNF. Environmental enrichment protocols with running wheels were already shown to increase mRNA levels for BDNF and promote higher conversion rates of proBDNF to mBDNF [49,53,54]. Prior studies comparing enriched environments with and

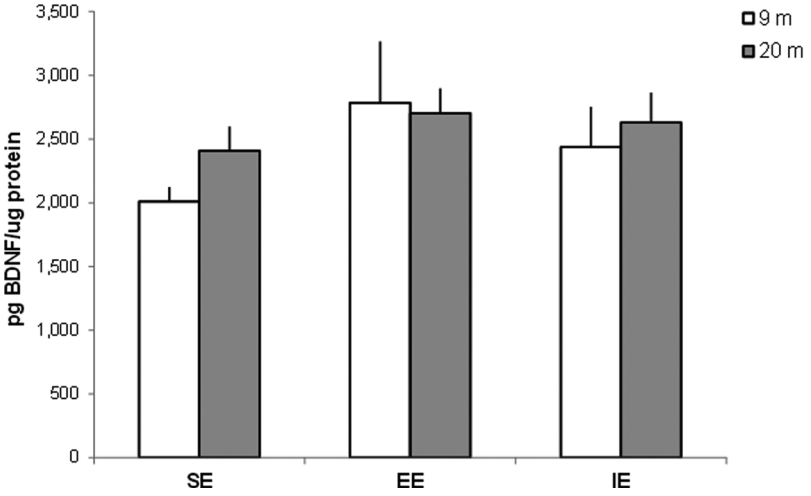

Fig. 5. Effects of environmental conditions on serum mature brain-derived neurotrophic factor (mBDNF) levels in 9 or 20 month-old rats.
Rats were euthanized for mBDNF measurements after 12 weeks of exposure to the three different environmental conditions: SE (standard environment), EE (enriched environment), and IE (impoverished environment). mBDNF was quantified by anti-BDNF sandwich-ELISA assay (ChemiKine, Millipore, Darmstadt, Germany) according to the manufacturer's instructions. Data are presented as mean ± SEM of pg mBDNF/ug of protein, $n = 5-7$. Statistical analysis was performed using two-way analysis of variance (2-way ANOVA) with age (9 and 20 months of age) and environmental conditions (SE, EE, and IE) as fixed factors. No statistically significant differences were found.

Table 2
Pearson correlation analyses.

	Hippocampal mBDNF	
	R	p
proBDNF	0.82	< 0.001
Synaptophysin	0.50	0.047
Recognition Index	0.42	0.004
	Serum mBDNF	
	R	p
Hippocampal mBDNF	0.15	0.48
	Serum mBDNF	
	R	p
proBDNF	0.57	0.56
Synaptophysin	0.46	0.086
Recognition Index	0.23	0.280

Separate Pearson's correlation analysis, including data of all experimental groups, were run to evaluate the relations between (I) hippocampal mBDNF levels and proBDNF, synapthophysin and recognition index in the NOR task; (II) serum mBDNF and hippocampal mBDNF; (III) serum mBDNF and proBDNF, synapthophysin and recognition index in the NOR task.
Abbreviations: R Pearson's correlation coefficient; p statistical significance.

without the physical activity component failed to find alterations in BDNF expression and protein levels in the cognitively enriched environments [23,26]. However, they analyzed younger animals and submitted them to a shorter time of enrichment than the present study, making direct comparisons difficult.

The IE condition showed opposed and more pronounced effects on hippocampal mBDNF levels than the EE condition, as indicated by the significant decrease of mBDNF in the IE group in comparison to the EE and SE groups. Although social isolation was already shown to reduce BDNF transcription and mRNA expression [42], its effect on proBDNF levels and conversion rates to mBDNF was never explored. However, from studies that evaluated the effects of stress on the proBDNF/mBDNF balance, we can expect that IE, as a stressful condition (for rats are social mammals), also modulates the proBDNF/mBDNF balance [55,56]. Here we show, for the first time, that the decrease in hippocampal mBDNF levels by IE is not accompanied by a significant reduction of proBDNF levels. Thus, it is possible that the conversion rate of proBDNF to mBDNF, catalyzed by intracellular matrix metalloproteinases (MMP) and extracellula plasmin (tPA) [4], is reduced in the hippocampus of IE animals, increasing the proBDNF/mBDNF ratio. However, our experimental design does not allow final conclusions to be drawn about this issue.

The alterations seen for hippocampal mBDNF in animals exposed to the EE and IE conditions were positively correlated with their long-term memory indexes and proBDNF levels. Moreover, hippocampal mBDNF alterations were accompanied by reciprocal modifications of synapthophysin levels. As a synaptic vesicle protein involved in neurotransmission [57], synaptophysin of brain tissue sections or homogenates is commonly used as an indirect marker of neuronal plasticity [30–32]. It is also worth to mention that the hippocampal mBDNF and synaptophysin levels were significantly and positively correlated, further supporting the role of mBDNF on environmental induced neuronal plasticity [30]. Neuroplasticity studies are relatively scarce on cognitively EE protocols, but there are evidences for changes on neuronal morphology and plasticity [58–60]. Our results on hippocampal synaptophysin levels help in expanding the knowledge about the mechanisms proposed to mediate the beneficial effects of this EE protocol on long-term memory retention. Even so, the results obtained for the synaptophysin levels should be interpreted with caution, since only the analysis of this protein in preparations of purified synaptic fractions can be considered a reliable proof of alterations in synaptic density.

The present findings suggest an association between the environmental condition, hippocampal mBDNF levels, behavioral and cellular plasticity. Thus, a clear difference was observed in these parameters when comparing EE and IE groups. However, these alterations were not accompanied by modifications in serum mBDNF levels. Moreover, no significant correlations were found between serum mBDNF and hippocampal mBDNF, proBDNF, synaptophysin or performance on the NOR task. These results are in clear contrast with the positive correlations found between hippocampal mBDNF and proBDNF, synaptophysin and object recognition performance. Thus, the experimental design of this study compares, for the first time, the different roles of central and peripheral mBDNF levels as biomarkers of neuronal and behavioral plasticity in the hippocampus. Peripheral mBDNF was not able to indicate the ongoing alterations that were occurring in the hippocampus as a result of the different environmental conditions. Therefore, our results suggest that changes in central mBDNF are not always reflected by changes in peripheral mBDNF levels or, in other words, that the lack of alterations on peripheral mBDNF does not signify that mBDNF is not involved in brain mechanisms that induce cellular and behavioral plasticity, having considerable implications from a translational perspective. However, the causes of the discrepancies between central and peripheral mBDNF in the present conditions are not fully understood, and future studies are warranted in order to address this question. The inclusion of a positive control for serum mBDNF levels in these studies would be advantageous to strengthening the interpretation of results and draw stronger conclusions.

In fact, only a few studies have tried to correlate peripheral and central BDNF levels in experimental conditions, and the exchanges of central and peripheral mBDNF across the blood-brain barrier are still a matter of debate. Lanz and colleagues induced robust increases of mBDNF in the brain but failed to find detectable changes in plasma [61]. In fact, mBDNF efflux from brain was measured only under extenuating physical activity [18]. However, other studies suggest that mBDNF influx in the brain is faster than its efflux [17], raising the possibility that blood may function as a mBDNF reserve [62]. Actually, mBDNF can be released by different peripheral tissues [20,21], including skeletal muscle. This tissue could contribute to build up of the blood reserve of mBDNF, as well as stimulate BDNF synthesis in brain via proteins released by the active muscle that can cross the blood-brain barrier [63]. These findings could explain why physical activity is more efficient than cognitive stimulation to induce increased central and peripheral mBDNF levels, as already shown by different studies with animal models and humans [15,16,26].

Furthermore, it must be taken into consideration that the biological functions of mBDNF in the brain and its activity-dependent synthesis and secretion by neurons suggest that, under physiological conditions, this neurotrophin is secreted only in amounts necessary to modulate surrounding neuronal populations [3]. Thus, only situations that induce excessive increases in brain mBDNF levels can be expected to contribute to alterations of this neurotrophin in peripheral blood. This seems not to be the case in our cognitively EE condition, as described above. Finally, it cannot be ruled out that the lack of an association between central and peripheral mBDNF levels is caused, at least partially, by the comparison of two different pools of mBDNF, one mostly intracellular (hippocampal BDNF) and the other mainly extracellular (serum mBDNF).

It is also important to note that neither a main effect of age, nor an interaction between age and environmental condition, was seen for the variables investigated in this study. Our results are in line with evidence that there is no age-related change in brain (hippocampus and frontal cortex) and serum levels for this neurotrophin in rats between two months and two years of age [64]. Moreover, former studies suggest that environmental enrichment and social isolation protocols have a conserved pattern of effects on cellular and behavioral plasticity from weaning throughout adulthood in animal models [42,65].

In conclusion, the present findings show for the first time that the beneficial effects of cognitively EE protocols on memory are associated to increased central mBDNF levels and neuronal plasticity. Remarkably, peripheral mBDNF failed to correlate whit the central levels of this

neurotrophin and with the neuronal and behavioral plasticity induced by the EE and IE protocols. These results suggest that blood/serum mBDNF levels may not always be reliable biomarkers of environmental effects on brain, which is of special interest from a translational research perspective.

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