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Enriched environment increases the total number of CNPase positive cells in the corpus callosum of middle-aged rats

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It had been reported that enriched environment was beneficial for the brain cognition, neurons and synapses in cortex and hippocampus. With diffusion tensor imaging (DTI), several studies recently found the trained-induced larger corpus callosum. However, the effect of enriched environment on the oligodendrocytes in corpus callosum has not been explored with the unbiased stereological methods. In current study, the effect of enriched environment on the total number of 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) positive cells in middle-aged rat corpus callosum was investigated by means of immunohistochemical techniques and the unbiased stereological methods. We found that, when compared to standard rats, the spatial learning capacity of enriched-environment rats was significantly increased. The total number of the CNPase positive cells in the corpus callosum of enriched-environment middle-aged rats was significantly increased when compared to standard rats. The present study provided, to the best of our knowledge, the first evidence of environmental enrichment-induced increases in the total number of CNPase positive cells in the corpus callosum of middle-aged rats.

Key words: 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), corpus callosum, enriched environment, middle-aged rats, oligodendrocyte, spatial learning capacity, stereology

INTRODUCTION

The corpus callosum is the major white matter tract that crosses the interhemispheric fissure in the brain of mammals including the human brain (Pandya and Seltzer 1986). Using MRI techniques, the morphology of the corpus callosum has been extensively studied. Such studies showed that abnormalities of the corpus callosum were correlated with abnormalities in cognition (Duara et al. 1991) and behavior (Yazgan and Kinsbourne 2003). The corpus callosum was of particular interest in this context because of its pivotal involvement in the age-related changes of the brain (Peters and Sethares 2002) and its importance for a continuous exchange of information which might underlie both the transfer of more digested information, such as memory engrams, as well as the bilateral synergy of the two hemispheres (Bloom and Hynd 2005, Glickstein and Berlucchi 2008). The midsagittal area of corpus callosum became thinning from young to middle-aged (Pfefferbaum et al. 1996) and accelerated thinning during old-aged (Driesen and Raz 1995, Salat et al. 1997). Diffusion-tensor imaging (DTI) studies found that the total volume of corpus callosum was expanded in adulthood and then decreased with aging (Hasan et al. 2010). The structural bases of aged-related anatomical changes in corpus callosum included the loss of myelinated nerve fibers (Aboitiz et al. 1996, Peters and Sethares 2002, Hasan et al. 2010).

It is known that gliogenesis does not cease in the mature mammalian central nervous system, and oligodendrocytes are preferentially generated with increas-

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ing age (Levison et al. 1999, Parnavelas 1999). Oligodendrogenesis and differentiation occured in rodent brain during adulthood (Cerghet et al. 2006) but the turnover decreased with increasing age (Sim et al. 2002). Previous studies found that enriched environment could decrease the incidence of cognitive decline and structural changes in aged rodents (Kempermann et al. 1998, Lores-Arnaiz et al. 2006, Harburger et al. 2007). Using DTI, Bengtsson and coworkers (2005) found that piano practicing during childhood and adolescence was correlated with the increase of axon caliber and myelination in human corpus callosum. Oligodendrocytes are the myelin-forming cells of central nervous system. Knowledge about the glia cellular basis of this effect, however, was contradictory. Some studies indicated that environmental enrichment induced increased number of oligodendrocytes in defined cortex area (Diamond et al. 1966) and a significant increase of volume fraction of oligodendrocytes in the upper 4 layers of occipital cortex in weaning rats (Sirevaag and Greenough 1987). By contrast, in the corpus callosum, Szeligo and Leblond (1977) found that the number of oligodendrocytes per field in rats after housing in enriched environment did not differ from that in controls. Bhide and Bedi (1984) reported that there was no significant difference in the volume fraction of the oligodendrocytes in the visual cortex between enriched-environment rats and isolated rats.

The 3-demensional unbiased stereological technique is optimal for quantitatively investigating the total number of cells in brain (Gundersen et al. 1988). Until now, to the best of our knowledge, there has been no study investigating the effect of enriched environment on the number of the oligodendrocytes in the corpus callosum. In the present study, the Morris water maze was firstly used to test the effect of the enriched environment on the spatial learning of middle-aged female rats. Then, the effect of the enriched environment on the total number of the oligodendrocytes in aged corpus callosum was investigated with the unbiased stereological methods.

METHODS

Housing conditions

Twenty four 14-month-old female SD rats from the Chongqing Medical University were used. They were divided randomly into enriched environment (EE) group and standard environment (SE) group. Rats in the EE group were housed in group of 12 in a large cage (120 cm × 60 cm × 50 cm high) containing wood shavings, various toys and small constructions. The toys and constructions were changed once a week at the time of cage cleaning. Rats in the SE group (4 rats/cage) were reared in an ordinary cage (40 cm × 30 cm × 30 cm high) containing only wood shavings. The rats were group-housed for 4 months in a temperature-controlled room, with a constant 12-h light/dark cycle. Food and water could be obtained ad libitum. Animal care and treatment followed the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23).

Morris water-maze test

Morris water maze test used a circular tank with a diameter of 200 cm, a height of 80–90 cm, which was filled with water (24 ± 2°C). The water was made opaque with nontoxic paint. The tank was divided into four quadrants. The four start positions were located at the intersections of the quadrants. A platform with a diameter of 10 cm was hidden 2 cm below the water level. Rats were first placed on the platform for 30 seconds and then placed into water. Each rat received four trials per day for 4 consecutive days. The start position was changed on every trials. The experiment researchers used a stopwatch to record the time to reach the platform. If a rat failed to reach the platform in 3 minutes, it was taken out and placed on the platform for 30 seconds. On day 5th, the time to reach the visible-platform was measured (Morris 1983, Brandeis et al. 1989).

Tissue preparation

The rats were anaesthetized by 4% chloral hydrate intraperitoneally injected and were perfusion-fixed with 4% paraformaldehyde (1 ml/100 g) in 0.01 M phosphate buffered saline (pH 7.4). Two brains of EE rats and one brain of SE rats were not well fixed during perfusion so that they were removed from this study. Then, 5 rats were randomly selected from SE rats and EE rats for the stereological analysis. After perfusion, the cerebrum was cut and taken out from the skull. The left or right hemisphere was sampled at random. The sampled hemisphere was coronally cut into 2-mm-thick slabs from rostral to caudal, starting randomly at

the rostral pole. Seven to eight slabs were obtained from each hemisphere. The slabs of the randomly selected hemisphere were post-fixed in 4% paraformaldehyde for at least 2 hours. Then, they were embedded in paraffin with the caudal surface being faced down. Serial coronal sections with thickness of 14 μm were cut with a sliding microtome (Leica, Germany) and mounted onto slides from the rostral end to caudal end of rat hemisphere. One section was systematically sampled every 30th section, the first section being sampled randomly from first 30 sections (Gundersen et al. 1999). Therefore, the section sampling fraction was 1/30. On average, 13~15 sections were sampled per rat.

Immunohistochemistry

One robust marker of near-mature and mature oligodendrocytes, the myelin-associated enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase), was used to label the myelin sheath forming oligodendrocytes (Gravel et al. 1996, Yin et al. 1997, Lyck et al. 2008, Rivers et al. 2008). The 14-μm paraffin sections were deparaffinized in xylol and rehydrated in graded alcohol series. Sections were washed three times for 5 min in phosphate buffered saline (PBS, 0.01M, pH 7.4) before being immersed in citrate buffer (0.01M, pH 6.0) and heated in a microwave oven for 15 min for antigen retrieval. After being cooled, sections were washed three times for 5 min in PBS. Endogenous peroxidase was inhibited by incubation with 3% H_2O_2 for 10 min and then washed in PBS three times for 5 min. Non-specific binding sites were blocked with normal goat serum for 60 min at 37°C. Sections were incubated with primary antibody (MAB326R, Millipore) diluted 1:300 in PBS at 4°C over 48 hours and then 37°C for 1 hour. After three 5 min washes in PBS, sections were incubated with biotinylated goat anti-mouse IgG for 30 min at 37°C, which was followed by three additional 5 min washes in PBS. Then, the specimens were incubated with S-A/HRP (Hstostain TM-Plus SP/9002 kit from ZYMED, GB; Beijing, China) for 30 min at 37°C, which was followed by repeated washes as described previously. Diaminobenzidine (DAB, ZLI-9032, ZSGB; Beijing, China) was used as a chromogen. The nuclei were satined by haematoxylin. Then, sections were dehydrated by sequential immersion in gradient ethanol and xylene and then coverslipped.

Stereological equipment

A stereology workstation consists of an Olympus Optiphot microscope, stereology software (New CAST; Olympus, Glostrup, Denmark) and a 17″-television screen monitor. The Olympus Opitiphot microscope was equipped with a motorized stage for precise, automatic movements in the X and Y directions, a video camera to project images onto the computer screen, and a microcator (Heidenhain, USA) attached to the stage for precise measurement of the focal depth (in 0.1 μm). Counting was performed using a ×100 (N.A.1.40) oil objective lens.

Stereological analysis

Region definition was based on the Paxinos and Watson (1982) rat brain atlas. Under the low magnification, a contour was traced around the edge of the corpus callosum. Under oil objective, the area of the unbiased counting frame was set as 2235 μm² for CNPase positive cells in the corpus callosum. The step length, which was the distance of disector moving in the x axis and y axis from one sampled place to the next one, was set as 346 μm and 258 μm in x axis and y axis for CNPase positive cells in EE rats, respectively. The step length was set as 245 μm and 183 μm in x axis and y axis for positive cells in SE rats, respectively. The new computer assistance stereological™ (New CAST) systematically randomly sampled the counting places from the delineated regions. The unbiased counting frame was placed over the sampled places. The ratio between the area of the unbiased counting frame and the rectangle area that was obtained by multiplying the step length in x axis and the step length in y axis represented the area sampling fraction (asf). Therefore, asf was 2235 / (346 × 258) = 0.025 for CNPase positive cells in EE rats and 2235/ (245 × 183) = 0.05 for cells in SE rats (West et al. 1991). Postprocessing section thickness, t, was measured at 30 random locations in each region of each rat using the oil-immersion objective (100 ×objective) to focus on the top and bottom of sections (1–3 locations per section). The spread of values showed low variation of section thickness. Thickness sampling fraction was calculated by dividing mean disector height (h) by section thickness (t) for each rat (tsf = h/t). In present study, the height of the optical disector was set at 7 μm. The mean section thickness of SE rats and EE rats was 10.0 ± 0.1 μm (Mean ± SEM)

and 10.4 ± 0.3 μm, respectively. Therefore, the section thickness sampling fraction was 7/10.2.

In each of the counting places, the number of positive cells was counted with an oil immersion objective lens (numerical aperture of 1.4), and the optical disector counting rules were used when counting (West et al. 1991). Within the optical disector height, a CNPase positive cell was counted when its nucleus first came into focus, and at this focal plane, the nucleus was completely within the 2-dimensional counting frame or partly within the counting frame but not touching any exclusion lines or its extensions (Fig. 1). Based on the above parameters and counts, the total number of the CNPase positive cells was calculated with the following formula (West et al. 1991).

$$N = \Sigma\bar{Q} \times (1/ssf) \times (1/asf) \times (1/tsf) \quad (1)$$

Where N equals the total number of CNPase positive cells, $\Sigma\bar{Q}$ denotes the total number of the CNPase positive cells counted per rat corpus callosum, ssf is the section sampling fraction, asf is the area sampling fraction, and tsf is the thickness sampling fraction.

Statistics

All statistical analyses were performed with SPSS 16.0. Morris water maze data between the two groups from day 1 to day 4 were analyzed by ANOVA with repeated measures, followed to test for Huynh-Feld. The outcome of day 5 was analyzed with an analysis of variance (one-way ANOVA).

Unpaired, two-tailed Student's *t*-test was used to analyze the stereological results. A significant difference was considered when $P<0.05$. The mean coefficient of error (CE) for the estimation of the total number of the CNPase positive cells in the corpus callosum of two group rats was calculated according to Gundersen and coworkers (1999).

RESULTS

Morris water-maze tests for spatial memory

For hidden-platform test, the mean latency of enriched environment (EE) group from day 1 to 4 was significantly shortened compared to standard environment (SE) group, and there was no significant change for visible-platform test between them (Fig 2).

Stereological Estimation

The quantitative estimations of corpus callosum and CNPase positive cells in corpus callosum from two groups were presented in Table I.

The mean total number of CNPase positive cells in the corpus callosum of SE rats and EE rats were 0.94 ± 0.08 × 10^6 (Mean ± SEM) and 4.16 ± 0.54 × 10^6, respectively. The total number of CNPase-positive cells in the corpus callosum in EE rats was signifi-

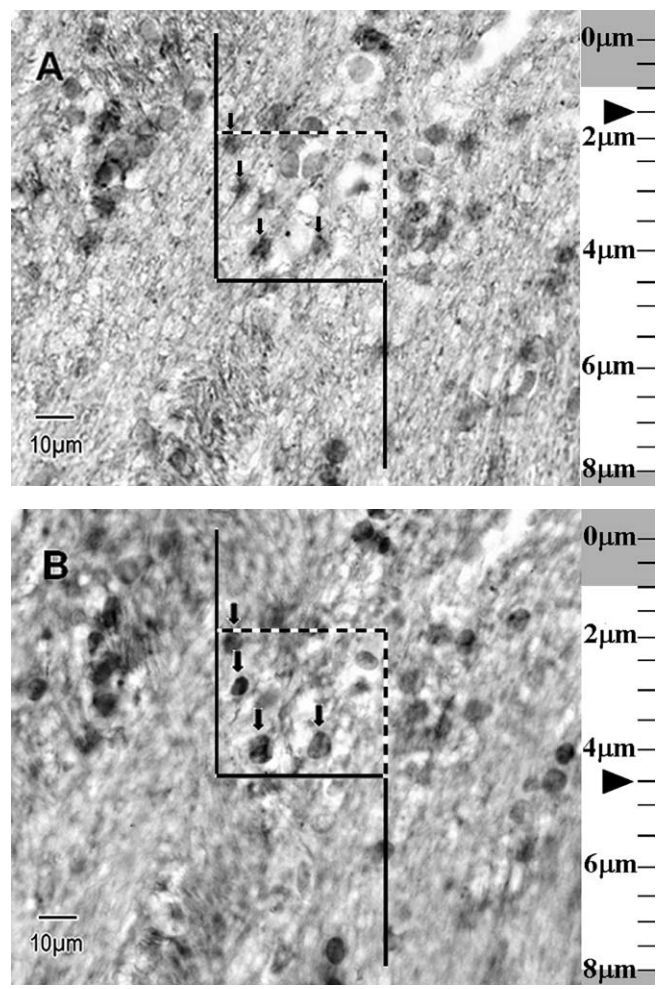

Fig. 1. Photomicrographs of CNPase-immunostained sections through the corpus callosum to illustrate how to count CNPase positive cells with optical disector. An overlay containing unbiased counting frame is shown. With the height of optical disector, CNPase positive cells are counted when their nuclei first come into focus and they are completely inside the counting frame or partly inside the counting frame but only torching the inclusion (dotted) lines. In this illustration, four CNPase positive cells are counted, as indicated by arrows. (A) represents the upper focal plane of the optical disector and (B) represents the lower focal plane of the optical disector.

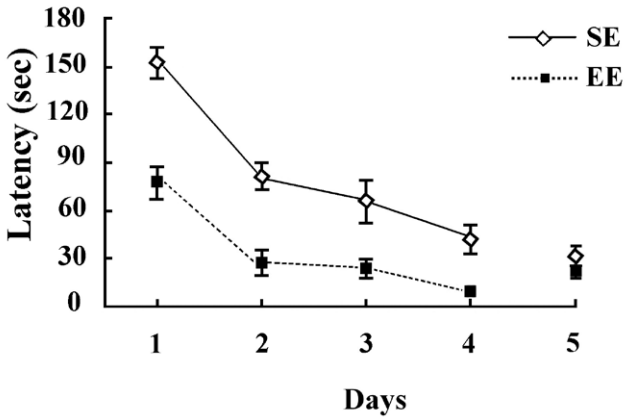

Fig. 2. Effects of enriched environment on spatial learning capacity. Escape latency in EE group from day 1 to day 4 is significantly shorter when compared to SE group, respectively. For visible-platform task on day 5, there is no significant difference between EE rats and SE rats. Each point represents the mean ± SEM (standard error of the mean) for each group during one test session. EE indicates enriched environment and SE indicates standard environment.

cantly increased by 343.6% (P<0.001), when compared to that in SE rats (Table I). The mean CE for the estimation of the total number of CNPase positive cells in the corpus callosum of SE rats and EE rats was 0.04 and 0.05, respectively (Table I).

DISCUSSION

Regarding the enrichment-induced changes of the oligodendrocyte number in the different regions of central nervous system, the previous results were inconsistent. Diamond and coauthors (1966) counted the number of cell profiles in a rectangular portion of cortex with known width but variable depth and then calculated the oligodendrocyte-to-neuron ratio in these areas. Szeligo and Leblond (1977) estimated the numerical density of the oligodendrocyte profiles in corpus callosum. Bhide and Bedi (1984) estimated the numerical density of the oligodendrocytes in cortex, that was, the number of oligodendrocytes per unit volume of visual cortex. Sirevaag and Greenough (1987) estimated the volume fraction (Vv) of glial nuclei in the upper 4 layers of occipital cortex, which was calculated as the sum of nuclei in sampled area divided by the sample area. According to what they described, they all investigated of oligodendrocyte density in brain. Biological conclusions based on density were very difficult to interpret because it would never be known if changes in density

Table I

Stereological estimates of CNPase positive cells in the corpus callosum

Group	ΣQ^-	N (10^6)	CE
SE			
1	538	1.20	0.04
2	334	0.74	0.05
3	449	1.05	0.05
4	387	0.88	0.05
5	337	0.81	0.05
Mean	409	0.94	0.05
SEM		0.08	
CV (%)		19.9	
EE			
1	813	4.44	0.04
2	1002	5.36	0.03
3	936	5.16	0.03
4	562	3.20	0.04
5	511	2.62	0.04
Mean	765	4.16	0.04
SEM		0.54	
CV (%)		29.0	

($\sum Q^-$) indicates the CNPase positive cell number counted per rat; (N) indicates the total number of CNPase positive cells in the corpus callosum; (CE) indicates coefficient of error for the stereological estimation of the CNPase positive cell number in rat corpus callosum; standard efficient of mean (SEM) and coefficient of variation (CV = SD/Mean) are given below each variable.

were due to an alteration of total quantity and/or an alteration in the reference volume (Braendgaard and Gundersen 1986). Furthermore, the exact boundaries of certain portions in the cortex or in the corpus callosum selected in the previous study were very difficult to distinct and keep identity for all groups. In addition, it was difficult to discriminate oligodendrocytes in brain if only the morphological criteria of oligodendrocyte nucleus were used. In present study, we quantitatively investigated the total number of the CNPase positive cells in the corpus callosum by means of 3-demensional unbiased stereological technique. When the optical fractionator is used to estimate the total number of the cells in the region of interest, the results will not be affected by differential tissue shrinkage (West et al. 1991) which is the case in the current study. However, it should be pointed out that the limitation of the present study was the smaller guard area. In the current study, the sections with the thickness of 14 μm were cut. However, the section processing resulted in a high shrinkage in the z-axis, leaving less than 3 μm to act as guard area. In order to overcome this limitation, future study needs to cut thicker sections so that optical guard area and disector height could be obtained.

There are four stages from oligodendrocyte progenitor cells (OPCs) to mature oligodendrocyte, including the precursor stage, pro-oligodendrocyte stage, the immature oligodendrocyte stage and the mature oligodendrocyte stage (LeVine and Goldman 1988, Agresti et al. 2005), among which the precursor and pro-oligodendrocyte stages do not produce the CNPase, while the near-mature oligodendrocyte and the mature oligodendrocyte produce CNPase (Gravel et al. 1996, Yin et al. 1997, Lyck et al. 2008). In this study, the near-mature and the mature oligodendrocytes were labeled by anti-CNPase antibody. It is well known that the major cells in the corpus callosum are mature oligodendrocyte after myelin sheath has been formed (Mori and Leblond 1970), and that there are only small amount of near-mature oligodendrocytes in the corpus callosum. Although the near-mature oligodendrocytes have not formed myelin sheath in the corpus callosum, the near-mature oligodendrocytes are developing or about to develop into mature oligodendrocytes. Therefore, near-mature oligodendrocytes can be regarded as compensatory cells for formation of myelin sheath, and they are important for the replacement of the damaged mature oligodendrocytes to protect the integrity of myelinated nerve fiber or form new myelinated nerve fibers. It was, therefore, appropriate to assess the enrichment-induced changes of the near-mature and mature oligodendrocytes together in the corpus callosum.

In the present study, we found the total number of CNPase positive cells in the corpus callosum of enriched-environment middle-aged rats was significantly increased when compared to standard rats. In the brain, the function of neurons and their long axonal processes depended upon neighbouring support cells called glia, such as oligodendrocytes. Oligodendrocytes wrapped the neurons' axons in a multilayered spiral extension of their own cell membrane to form myelin sheath. Disruption of the myelin sheath could therefore contribute to cognitive decline, such as that observed during the normal aging process (D'Hooge and De Deyn 2001, Peters and Sethares 2002). The brain had a remarkable ability to repair itself. With age, however, the efficiency of this remyelination declined or was lost completely (Nave 2003). There was evidence that adult oligodendrocyte precursor (OLPs) generated new myelinating oligodendrocytes in corpus callosum (Rivers et al. 2008). Our present results, enriched environment-induced significant increase of CNPase positive cells in the corpus callosum of middle-aged rats, suggested that the oligodendrocytes in the corpus callosum of middle-aged rats were still sensitive to the enriched environment intervention. What might be the importance of the enriched environment-induced changes of the oligodendrocytes in the corpus callosum of middle-aged rats? CNPase appeared to be involved in the migration or expansion of oligodendrocyte membranes during myelination (Gravel et al. 1996, Yin et al. 1997) and to be considered to possibly have distinct roles in subcellular compartments of myelin that serve axon–glial communication (Rasband et al. 2005). Therefore, we speculated that the increase of CNPase positive cells in corpus callosum might be related to the enriched environment-induced myelination in corpus callosum. However, the exact consequence of the enriched environment-induced changes of the oligodendrocytes in corpus callosum needs to be further studied.

We detected a significant difference in performance on the Morris water maze task between enriched-environment rats and standard rats. The finding was in accordance with reports by other groups (Leggio et al. 2005, O'Callaghan et al. 2009). The neocortex connected by corpus callosum (Innocenti 1986, Gazzaniga 2000) was the structural fundament of spatial reference memory, which can be tested using Morris water maze task (Morris 1983, Lambert et al. 2005). Furthermore, the efficiency of myelination in corpus callosum was

closely related to the cortical cognition (Bloom and Hynd 2005). Our present results suggested that the enriched environment-induced increase of CNPase positive cells in the corpus callosum might be related to the enriched environment-induced improvement of spatial learning tasks. However, a true causal link between them needs to be further studied.

CONCLUSIONS

The present study for the first time investigated the effects of the enriched environment on the CNPase positive cells in the corpus callosum of middle-aged female rats using the 3-dimensional unbiased stereological methods and immunohistochemisty. The enriched environment enhanced the spatial learning memory of middle-aged female rats and increased the total number of the CNPase positive cells in the corpus callosum of middle-aged female rats.

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