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Short communication

Effect of Environmental Enrichment on Morphology of Deep Layer III and Layer V Pyramidal Cells of Occipital Cortex in Oldest-old Rat – A Quantitative Golgi Cox Study

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A B S T R A C T

Dendrites and dendritic spine density regress extensively during aging in rats housed under standard conditions (SC), which can be ameliorated by housing in the enriched environment (EE). This event is particularly pronounced on neurons where high rates of plasticity are conceivable, such as on projection neurons of archicortical regions of dentate gyrus[1]. However, effects of EE on neocortical projection neurons are still poorly understood. Therefore, we investigated the effect of EE housing on a deep layer III (L3) and layer V pyramidal cell (L5) morphology in the associative occipital neocortex of male Sprague-Dawley rats at 24 months of age. Rats were randomly distributed in two groups and reared under either SC (n=5) or EE conditions (n=6) for 26 days. In depth quantitative analysis of dendritic tree morphology and spine density on occipital projection neurons, from Golgi-Cox stained sections, showed similar trend in both EE occipital layers L3 and L5. Significant increase was found in total number of dendritic segments (L3 – 37.5 %, L5 – 33 %) and in dendritic diameter of intermediate segments (for more than 20 %), while increase in total spine number was around the level of significance (p>0.55; L3 – 30 %, L5 – 64 %). These findings suggest an outgrowth of new dendritic segments. When compared to archicortical region of dentate gyrus, effects of aging in the associative occipital cortex were less pronounced. Taken together, these findings suggest that structures being more affected by the aging process are more susceptible to the environmental enrichment in old age.

Key words: *aging, dendritic tree, morphometry, plasticity, spines, Alzheimer disease*

Introduction

Neurodegeneration during aging affects dendrites and dendritic spines in rats that are housed in standard conditions (SC)[2–6]. This age-related neurodegeneration of dendritic tree can be ameliorated if animals are exposed to enriched environment (EE), where animals are housed in conditions providing enhanced sensory, cognitive, motor and social stimulation than in SC[1,4,5,7,8]. In this condition several animals are housed together in large cages with continuous variation of environmental stimuli, such as objects that encourage exploration and physical activity[9]. Living in EE conditions induces molecular and morphological changes in the brain that lead to

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improvements in behavioral parameters, especially in learning and memory[9,10]. These findings have provided new insights into the mechanisms of environmental-dependent plasticity of the brain and its importance for aging and neurodegenerative diseases[11–13].

According to the concept of cognitive reserve[14], the effect of neurodegeneration can be buffered with certain aspects of the brain structure and function[15]. In particular, this idea proposes that synaptic connectivity is a principal feature of the brain cognitive reserve. Importantly, the majority of the neocortical inputs are transmitted via synaptic connections that are spread across dendrites and dendritic spines of the pyramidal neurons. Therefore, an enhancement of dendritic branching and spine density might allow higher synaptic connectivity and therefore could increase the brain cognitive reserve. Significant increase in dendritic branching and spine density was induced during EE housing, thus, in depth analysis of the EE housing effects in aged animals, may improve our understandings of possible beneficial role of enrichment in the normal aging and neurodegeneration.

EE-induced changes in neuronal morphology are found in specific areas of the brains of young and aged animals although the dynamics and final patterns of changes are not necessarily the same for each neocortical region[8,10]. Occipital region is an associative neocortical brain area found to be the most responsive to the environmental influence in the initial studies that led to systematic examination of various measures induced by EE in this neurocortical area[9,10]. These studies confirmed changes in dendrites and dendritic spines after exposure to EE in distinct occipital layers of both young and adult animals[16–18]. In young rats (30 days of age), only 4 days of housing in EE resulted in an increase in length and branching of the basilar dendritic tree of layer III pyramidal neurons in the occipital cortex[19]. Prolonged housing in EE causes an increase of total dendritic length, terminal segment length and dendritic branching of the layer III and V pyramidal neurons and layer IV stellate neurons[20] and an increase in number of higher ordered dendritic segments on layer II, IV-V pyramidal neurons in the rat occipital cortex[21]. However, in the aged animals (600 days of age), housing in EE changes in the occipital cortex were seen only as an increase of the number[4] and length[22] of segments of 6th order of the basal dendrites of layer II-IV pyramidal neurons, or if the concentric circle method was used for the analysis, no effect was seen after EE[2]. In addition, the EE decreased the number of nubbin type of the spines (considered as degenerating spines whose number increases during aging process) on the basal branches of the of the layer II and III pyramidal neurons in the occipital cortex of the aged EE rats[7]. However, the precise effects of EE on the specific subpopulations of the occipital pyramidal neurons are yet to be determined. Here, we characterized the effect of EE on the morphology of the layer III and layer V pyramidal neurons of the aged rat occipital cortex. This study is a continuation of our previous work reporting strong environmental effect on the neurons of the dentate gyrus in aged animals[1].

Materials and Methods

Animals and environmental housing

The animals used were 11 male Sprague-Dawley rats, 24 months of age, obtained from a commercial breeder (Alab AB, Sollentuna, Sweden). They were housed on a 12h on/12h off illumination schedule (lights on 6.00 A.M.) with unlimited access to food and water. The animals were randomly divided into two experimental groups and exposed to differential environmental housing, enriched conditions (EE; n=6) and standard social conditions (SC; n=5), respectively, for 26 days. During the experimental period the animals were kept in the same room at the animal department so that additional stimulation from environment (handlers, noise) were the same for both groups.

The EE consisted of large wire mesh cages measuring 100x60x35 cm (eight rats *per* cage) containing ladders, shelves, tunnels and additional diverse toy objects. EE rats were exposed to three different objects (toys) that were changed daily. The control rats were housed in standard laboratory Plexiglas cages measuring 45x30x20 cm (four rats *per* cage) conditions we refer as SC. All the experimental procedures and housing conditions used in this study followed the guidelines of Swedish animal protection legislation and were approved by the Stockholm South Animal Ethics Committee.

Tissue preparations and identification of analyzed region

After the 26 days of the experimental period all rats were deeply anaesthetized intraperitonealy with pentobarbital overdose (60 mg/kg) and then decapitated. The brains were removed and divided in the midsagital direction. Both hemispheres were alternatively cut in a coronal plane into 3 blocks of tissue that were placed in Golgi-Cox solution for 3 weeks, with one change of solution after 3 days[23]. After impregnation, the tissue was dehydrated, embedded in celloidin and sectioned coronaly at 180 μm. For developing the staining, the sections were immersed in 20% ammonium hydroxide for 5 min and then transferred to a 15% ammonium hydroxide solution for 25 min. After rinsing they were further processed with 1% thiosulfate for 7 min, dehydrated in alcohol, cleared in Histoclear (National Diagnostic, Atlanta, GA) and covered with Histomount (National Diagnostic) mounting media[23].

The subjects were coded so that the investigators were not aware of the housing conditions. We have selected typical pyramidal neurons located in deep part of layer III and in layer V of the associative occipital cortex that were cut perpendicularly and were positioned in the middle third of sections thickness (z dimension starting from –60μm from the surface). In Golgi Cox slices neurons that lie in the middle third of section thickness have highest level of impregnation[23], and this criterion could reduce the number of segments cut at the section surface. Approximately 5–10 consecutive stained pyramidal neurons *per* each layer in animal were sampled for quan-

titative analysis. Since all brain tissue was processed under the same laboratory conditions, the obtained values were not corrected for the shrinkage factor[24,25].

Dendritic tree reconstruction

Quantitative morphometric analysis was performed using Neurolucida 3,18 software (Microbrightfield Inc., Colchester, VT) and automatic dendrite measuring system that provides three-dimensional data of the dendritic tree[1,26,27]. Measurements were made using a 60x air objective with actual enlargement on a screen equal to 4200x. Neurons were drawn over the live picture on the PC screen, bringing the signed point into the sharp focus when drawing. Changes in depth (z-dimension) were identified for each drawn point, and automatically corrected according to Snell's law for diffraction air correction factor (1.515). X-Y coordinates were also given to the each point in relation to the reference point. In this study we have analyzed 130 pyramidal neurons – in EE group 34 layer III neurons (5–9 neurons *per* animal) and 30 layer V neurons (5 neurons *per* animal), meanwhile in SC group we have analyzed 33 neurons in both layers (5–10 neurons *per* animal).

Morphometric measurements

The following parameters of the basal dendritic tree were analyzed: a) somatic cell surface, i.e., the area of the cell soma projected onto a two-dimensional (x-y) plane of sectioning to indicate its size, b) number of primary dendrites, c) total number of segments, d) total dendritic length *per* neuron, e) average length of intermediate and terminal segments, f) average dendritic diameter, g) base dendritic diameter, h) average and base dendritic diameter on intermedial and terminal segments, i) average spine number and spine density, j) spine density on intermedial and terminal segments.

For the analysis of segment length and spine density, each dendritic tree was divided into subgroups of inter-

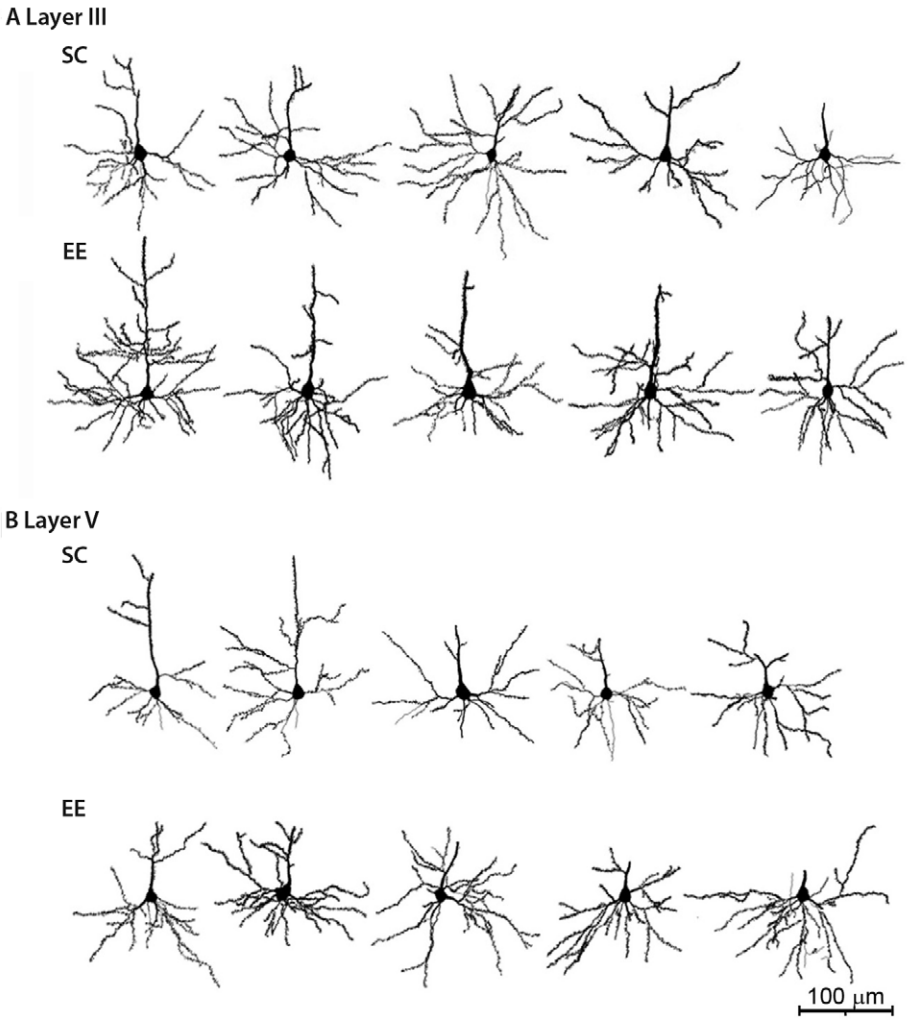

Fig. 1. Neurolucida reconstructions of Golgi Cox impregnated deep layer III (Fig. 1A) and layer V pyramidal cells (Fig. 1B) of the associate neocortex in aged rats under standard social conditions (SC) and enriched environment (EE). In control (SC) group dendritic complexity was slightly lower then in animals housed in the enriched environment conditions.

mediate and terminal segments. These subgroups were shown to have different length distributions[24,25]. Intermediate segments are the segments between the dendritic origin and the first bifurcation point, or between two consecutive bifurcation points. Terminal segments are segments between the terminal tip of dendrites and the last bifurcation point before the terminal tip. Incomplete segments refer to segments that were impossible to trace completely, since they were cut at the surface of the section or ran into a precipitation, and these were excluded from the analysis of individual segment length. Spine counts were done on whole dendritic tree of selected neurons.

Data analysis

All the parameters were calculated as mean ± standard error of mean values *per* neuron for each animal, and these data were used for the statistical analysis. We have performed both Student t-test and non-parametric Mann-Whitney U test obtaining similar results. Differences between groups were considered significant at $p<0.05$.

Results and Discussion

Here we demonstrated that housing of aged rats in EE induces moderate increase in dendritic tree complexity and dendritic diameter of layer III neurons in the occipital cortex (Figure 1A, Table 1). The majority of dendritic parameters measured are increased in EE group when compared to SC group, with the exception of the average dendritic diameter of terminal segments, and the average length of both segment types (Table 1). The strongest EE effect was observed in total number of segments *per* neuron (37.5% increase; $p=0.0008$), while the increase in total dendritic length was less pronounced (around 19%; $p=0.055$). In addition, EE induced a significant increase in base and average dendritic diameter, which may reflect an increase in the functional activity of pyramidal neurons. Even though dendritic spine density remained unchanged, the total number of dendritic spines is increased for 30 % in EE group without reaching statistical significance ($p=0.055$). This may be result of either greater interindividual variability or the formation of new dendritic spines on the newly formed segments. Collectively, these data suggest that housing of old animals in the EE induces dynamic changes in dendritic tree and denritic spines of layer III pyramidal neurons.

Similar to layer III pyramids, enriched environment induced moderate increase in dendritic tree complexity and dendritic diameter of layer V pyramidal neurons (Figure 1B, Table 2). Interestingly, we observed that the standard deviations for majority of parameters were considerably larger, which could probably due to larger morpho-functional diversity of layer V pyramidal neurons than of layer III. This high morpho-functional diversity of layer 5 pyramids caused a non significant increase of 64% ($p=0.1$) in total spine number *per* neuron, and 33 % ($p=0.1$) of increase in total number of segments *per* neurons. Most of the dendritic parameters increased between 9–64% in EE group, excluding average dendritic diameter of terminal segments, and average length of intermediate segments. However, in contrast to layer III,

TABLE 1
EFFECTS OF ENRICHED ENVIRONMENT ON BASILAR DENDRITIC TREE MORPHOLOGY OF LAYER III PYRAMIDAL NEURONS IN THE OCCIPITAL CORTEX

Patrameter/Group	SC	EE	p
	Mean±SD	Mean±SD	
Soma surface (μm²)	146.41±16.55	167.9±35.42	n.s.
Primary dendrites *per* neuron (n)	2.45±0.29	2.75±0.37	n.s.
Segments *per* neuron (n)	25.75±3.87	35.39±2.19	***
Total dendr. length (μm)	906±199	1077±82	n.s.
Aver. dendr. diameter (μm)	1.02±0.08	1.16±0.09	*
Base dendr. diameter (μm)	1.18±0.13	1.42±0.12	*
Aver. length of int. seg. (μm)	17.46±2.26	16.13±1.03	n.s.
Aver. length of ter. seg. (μm)	48.23±6.58	48.71±4.55	n.s.
Aver. dendr. diameter – int. seg. (μm)	1.21±0.12	1.46±0.11	**
Base dendr. diameter – int. seg. (μm)	1.39±0.15	1.76±0.14	**
Aver. dendr. diametar – ter. seg. (μm)	0.86±0.09	0.88±0.14	n.s.
Base dendr. diameter – ter. seg. (μm)	0.98±0.17	1.17±0.2	n.s.
Spine number (n)	320.7±81.5	418.6±61.8	n.s.
Spine density (n/μm)	0.35±0.03	0.39±0.06	n.s.

Values shown in the table are means and standard deviation of means (SD). SC, control animals; EE, animals housed in enriched environment dendr., dendritic; aver., average, int. seg., intermediate segments; ter. seg., terminal segments.; t-test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. not significant

TABLE 2
EFFECTS OF ENRICHED ENVIRONMENT ON BASILAR DENDRITIC TREE MORPHOLOGY OF LAYER V PYRAMIDAL NEURONS IN THE OCCIPITAL CORTEX

Patrameter/Group	SC	EE	p
	Mean±SD	Mean±SD	
Soma surface (μm²)	243.67±97.24	400.3±267.56	n.s.
Primary dendrites *per* neuron (n)	3.89±0.87	5.06±0.6	*
Segments *per* neuron (n)	23.06±4.4	30.64±11.77	n.s.
Total dendr. length (μm)	774.3±207.8	909.5±364.3	n.s.
Aver. dendr. diameter (μm)	0.94±0.11	1.01±0.09	n.s.
Base dendr. diameter (μm)	1.07±0.15	1.35±0.15	*
Aver. length of int. seg. (μm)	16.76±3.71	15.5±4.42	n.s.
Aver. length of ter. seg. (μm)	50.64±8.85	56.75±18.2	n.s.
Aver. dendr. diameter – int. seg. (μm)	1.14±0.24	1.38±0.23	***
Base dendr. diameter – int. seg. (μm)	1.29±0.33	1.6±0.27	***
Aver. dendr. diametar – ter. seg.(μm)	0.87±0.11	0.76±0.08	**
Base dendr. diameter – ter. seg. (μm)	1.02±0.23	1.11±0.32	n.s.
Spine number (n)	243.55±94.94	391.61±188.16	n.s.
Spine density (n/μm)	0.3±0.07	0.38±0.11	n.s.

Values shown in the table are means and standard deviation of means (SD). SC, control animals; EE, animals housed in enriched environment dendr., dendritic; aver., average, int. seg., intermediate segments; ter. seg., terminal segments.; t-test: * $p<0.05$; ** $p<0.01$; *** $p<0.001$; n.s. not significant

significant environmental effect was observed for number of primary dendrites (30% increase). We suggest that this phenomenon could be the result of observed 64% increase in the soma size of the layer V pyramids in EE group that was not statistically significant due to large standard deviation and affected only part of the population of the analyzed neurons ($p=0.1$, Table 2). This increase in neuron soma could lead to the consequent changes in the soma shape causing that primary dendrite becomes defined as a part of soma and its branches counted as new primary dendrites.

Our findings show that housing of aged rats in EE for 26 days had a modest effect on the basal dendritic tree morphology of both, layer III and layer V pyramidal neurons. We found the most profound effect in the outgrowth of new segments with formation of new spines on this newly growing part of dendritic tree. Although the increase in spine density did not reach statistical significance, the increase in dendritic diamater on intermediate segments suggests that new spines could be formed on already present dendrites[28].

In the adult rat visual cortex the effect of the enriched environment were shown on both the layer III and layer V pyramidal neurons[20,29]. Comparing our data with the values of the total dendritic length obtained in this study, we can observe that basal dendritic tree of the layer III pyramidal neurons in aged rat were similar or slightly larger than in the adult (1077 μm for aged EE in comparison with 880 μm for EE adult). In contrast, the basal dendritic tree of the layer V pyramids in the aged brain showed 60% regression of the total length in the comparison with adult values (910 μm for aged EE in comparison with 1400 μm for EE adult). Similar layer specific aging effect was described previously in the human prefrontal cortex[30].

Although the associative occipital cortex is considered as a cortical region most susceptible to modification by environmental manipulation, and changes in the morphology of pyramidal neurons have been confirmed in various studies in the adult animals[20,21,29,31,32], in the aging brain environmental effect in this region was only moderate[4,22]. In contrast, dendritic tree of the granule cells in dentate gyrus of the same animals used in this study showed marked changes after enriched housing[1], but also much stronger aging effect than we observed in the occipital cortex (300% of regression in the length of dendritic tree in the comparison with adult values). We therefore suggest that structures that are more affected by the aging process are more susceptible to the environmental enrichment in aging.

However, an interaction between the negative effects of aging and the positive plasticity processes associated with environmental enrichment can provide a counterbalance through which neuronal circuitry activity coding for specific cognitive or motor behavior could be restored. This could be the case for neuronal circuits in the cerebral cortex and hippocampus, as complex functions subserved by these brain regions have been shown to be particularly vulnerable to aging.

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UTJECAJ STIMULATIVNE OKOLINE NA MORFOLOGIJU PIRAMIDNIH NEURONA DUBOKIH SLOJEVA III I V OKCIPITALNOG KORTEKSA VRLO STARIH ŠTAKORA: KVANTITATIVNA GOLGI COX STUDIJA

SAŽETAK

Dendriti i gustoća dendritičkih trnova pokazuju značajne regresivne promjene tijekom starenja kod štakora koji borave u standardnim laboratorijskim uvijetima (engl. standard conditions, SC), a koje se mogu ublažiti boravkom u uvijetima stimulativne okoline (engl. enriched environment, EE). Taj efekat je osobito izražen na neuronima s velikim stupnjem plastičnosti kao što su projekcijski neuroni arhikortikalne regije gyrusa dentatusa. Međutim, utjecaj boravka u EE na projekcijske neurone u neokorteksu je još uvijek nedovoljno istražen. U ovoj studiji ispitali smo utjecaj boravka u stimulativnoj okolini na morfologiju piramidnih neurona dubokog sloja III (L3) i sloja V (L5) u asocijativnoom okcipitalnom neokorteksu muških Sprague-Dawley štakora starih 24 mjeseca. Štakori su nasumično raspoređeni u dvije grupe koje su boravile u SC (n=5) ili EE (n=6) kroz 26 dana. Detaljna kvantitativna analiza morfologije dendritičkog stabla i gustoće dendritičkih trnova na projekcijskim neuronima okcipitalnog korteksa prikazanih Golgi Cox metodom pokazala je sličan utjecaj boravka u EE u oba analizirana sloja – L3 i L5. Statistički značajan porast nađen je u ukupnom broju segmenata (L3 – 37,5%, L5 – 33%), te u promjeru dendrita na intermedijarnim segmentima (za više od 20%), dok je porast ukupnog broja derndritičkih trnova bio vrlo blizu statističke značajnosti ($p>0,55$; L3 – 30%, L5 – 64%). Ti podaci pokazuju da je najizraženiji efekat boravka u EE okolini bio izrastanje novih dendritičkih segmenata. S obzirom da je utjecaj starenja u okcipitalnom korteksu bio manje izražen nego u ontogenetski starijim moždanim strukturama kao sto je gryus dentatus, smatramo da strukture koje su jače zahvaćene staračkom regresijom plastičnije reagiraju na boravak u stimulativnoj okolini tijekom starenja.