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# Multiple Sessions of Entorhinal Cortex Deep Brain Stimulation in C57BL/6J Mice Increases Exploratory Behavior and Hippocampal Neurogenesis*

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*Abstract*— **Deep brain stimulation (DBS) has been a medical intervention for a variety of nervous system diseases and mental diseases. The input of DBS in the entorhinal cortex (EC) regulates the neurophysiological activities in its downstream regions, such as the dentate gyrus (DG) area. EC DBS may play a role in the treatment of diseases through hippocampal neurogenesis. This study we examined the effect of multiple sessions of EC DBS on the regulation of hippocampal neurogenesis. 4-month-old male C57BL/6J mice received bilateral multiple sessions of EC DBS (130 Hz, 90 μs, 100 μA, 1 h/d, 21 days), and the DBS parameters used are close to the high-frequency DBS parameters in clinical studies. The open field test (OFT) was used to test the exploratory behavior of mice, and hippocampal neurogenesis was detected by immunofluorescence staining with anti-doublecortin (DCX). We found that multiple sessions of EC DBS were tolerated in C57BL/6J mice, significantly increased exploratory behavior and the number of DCX-positive neurons in the DG area.**

*Clinical Relevance*— **Hippocampal neurogenesis may be part of the reason for DBS to improve memory, and the results of this study show that multiple sessions of EC DBS increases exploratory behavior and hippocampal neurogenesis, which is conducive to the application of DBS in nervous system diseases and mental diseases related to memory impairment.**

## I. Introduction

Since Aldini first reported the use of electrical stimulation to treat a major depression patient in 1804, which many medical intervention based on electrical stimulation methods has been developed [1, 2]. Deep brain stimulation (DBS) is an invasive neurosurgical technique that applies electrical stimulation to specific brain regions via implanted electrodes to improve symptoms of neurological and mental illnesses [3]. The DBS apparatus is mainly composed of stimulating electrodes, subcutaneous lead and pulse generators, which can be turned on and off at will. It is worth noting that DBS not only affects the activity of neurons in the target area where the electrodes are located, but also extends to further connections. At present, DBS is used primarily to treat movement disorders. The therapeutic benefits of Parkinson's disease have led researchers to attempt to apply DBS for other neurological diseases, such as depression [4] and Alzheimer's disease (AD) [5].

DBS is proven to be safe and can improve the memory of AD patients and animals [5]. One potential mechanism through which DBS may improve memory is via activity-dependent regulation of hippocampal neurogenesis [6]. Neurogenesis is a process of continuously adding newborn neurons to neurogenic regions such as the subgranular zone of the dentate gyrus (DG) of hippocampus in the adult mammals, which is related to learning, memory, and cognitive functions, especially short-term memory and spatial memory [7]. More and more evidences show that neurogenesis underlines the brain's capacity for new memories. Itaru imayoshi et al. reported that the ability of maintaining spatial memory requires constant renewal of neurons [8]. Inokuchi Kaoru and his colleagues found that reducing neurogenesis delayed the recovery of memory capacity in rats, while enhancement of neurogenesis through a running wheel accelerated the recovery [9]. These findings suggest that neurogenesis is the basis of maintaining brain memory capacity.

One of the targets of hippocampal neurogenesis is entorhinal cortex (EC), while EC provides the main afferent of hippocampal input through its connection with dentate gyrus (DG) area [10]. The subgranular zone in DG is a neurogenic region. Neural stem cells in this region can proliferate continuously and differentiate into neurons [11]. Once mature, new neurons can integrate into hippocampal neural circuits and play an important role in hippocampal-dependent learning and memory tasks [12]. Previous studies have shown that a single session of EC DBS can promote neurogenesis in the DG area of the hippocampus, and improve the spatial memory ability in a delayed manner [6, 13]. Most DBS animal studies usually relied on acute stimulation, but clinical practice usually uses extended treatment periods. Moreover, the effect of multiple sessions of EC DBS on neurogenesis is not yet known.

Hence, in this study, we tested the effect of multiple sessions of EC DBS on exploratory behavior by open field test (OFT) and the hippocampal neurogenesis of 4-month-old C57BL/6J mice.

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## II. Materials and Methods

### A. Animals

The protocol was approved by the Ethic Committee of Southwest Hospital (Chongqing, China). C57BL/6J mice (male, Weight: 18-28g, Chongqing Ensiweier biology science and technology co., Ltd, Chongqing, China) were bred and housed at the Army Medical University (Chongqing, China). All mice got food and water freely, and they were housed on a 12-h light/dark cycle at a constant temperature (24 ± 1℃). C57BL/6J mice were randomly divided into two groups: C57BL/6J+DBS group (DBS, n = 6), C57BL/6J group (Sham, n=9).

### B. Surgery and Stimulation

Mice were anesthetized with isoflurane. The induction dose was 1%, the concentration gradually increased to 1.5% - 3% within 5-10 minutes, and the maintenance dose was 1% - 2%. Mice were fixed in a stereotaxic apparatus and their body temperature maintained at 37℃ using a heating blanket. The unipolar electrode (0.0045″ coated diameter, 0.002″ bare diameter, tungsten wire) was implanted into bilateral entorhinal cortex (AP: -4.0, ML: ±3.12, DV: 5.0) and fixed by glass ionomer cement. Stimulation was performed after 1 week's recovery. At this time, all mice were 4-month-old. The mice were stimulated for 21 days for 1 h per day by a Master-9 pulse stimulator paired to stimulus isolators (ISO-Flex, A.M.P.I, Jerusalem, Israel) using frequency (130 Hz) and pulse width (90 μs) settings close to high-frequency DBS in clinical practice [6]. The current intensity of 100 μA is closer to the physiological spontaneous current [14], avoiding possible side effects, and may be more suitable for repeated stimulation. Mice without implanted electrodes were used as control.

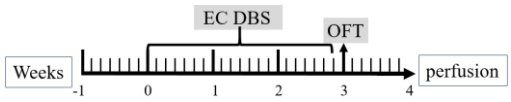


Figure 1. Experiment timeline.

### C Open Field Testing

After DBS, the OFT was completed in the Supermaze animal behavior video analysis system (XR-XZ301, Xmaze, Shanghai, China). The size of the test arena is 50 * 50cm with 40cm high walls and a white floor, and the arena was divided into a center region and a peripheral region. Animals were individually placed in the center of the arena and were allowed to move freely for 5 minutes. After each trial, the arena was cleaned with ethanol solution to eliminate the interference of odor on mice. The following open-field-dependent parameters were evaluated: distance moved, average movement speed, duration of movement, number of entries.

### D Tissue Collection

After the behavior testing, transcardiac perfusion was performed on deeply anesthetized mice with saline and 4% paraformaldehyde (PFA). The brains were removed and placed in 4% PFA overnight. Then the brains were transferred sequentially to 10%, 20% and 30% sucrose solutions in PFA. Frozen mouse brain coronal sections with a thickness of 20 μm were obtained with a freezing microtome (CM1900, Leica, Nussloch, Germany).

### E. Immunofluorescence Staining

The sections were washed in 1% PBS (3 times for 5 min each time) at first, and incubated with 10% goat serum at 37°C for 30 min in an incubator. Then, the sections were incubated at 4°C overnight with the following primary antibodies: anti-doublecortin (DCX) antibody (1:500, ab18723, Abcam, Cambridge, UK). The next day, the sections were washed three times with 1% PBS for 5 min each time, and then incubated with anti-rabbit IgG (1:1,000, 4412S, Cell Signaling Technology, Massachusetts, USA) at 37°C for 1 h. Brain sections were placed in 4',6-diamidino-2-phenylindole (D8417, Sigma-Aldrich, St. Louis, USA) to stain nuclei for 10 min at room temperature and then washed three times in 1% PBS for 10 min each time. All sections are mounted on glass slides for observation. The immunofluorescence images were acquired by an Olympus microscopy (BX60, Olympus Co. Ltd., Tokyo, Japan). The number of DCX positive cells was counted by Fiji (National Institutes of Health, Maryland, USA).

### F. Statistical Analysis

The statistical result of the data was showed as mean± standard error of the mean (SEM). Data was analyzed using IBM SPSS 25.0 by unpaired *t*-test for two groups. $P<0.05$ was considered statistically significant.

## III. Results

### A. Open Field Test

*Overall activity in the OFT:* From the overall activity within 5 minutes in OFT, there was no significant difference in the total moving distance (DBS, 2231.53±117.58 cm; Sham, 2143.46±226.14 cm; $P>0.05$) and the average movement speed (DBS, 7.92±0.51 cm/s; Sham: 6.13±0.60 cm/s; $P>0.05$) between the DBS group and Sham group (Fig. 2).

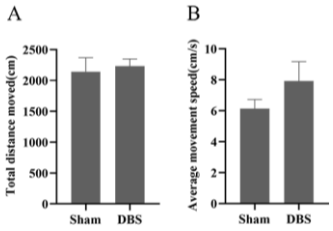


Figure 2. Activity in the OFT. (A) Total distance moved. (B) Average movement speed. Data expressed as means ± SEM.

*Activity in the peripheral region:* The duration of movement

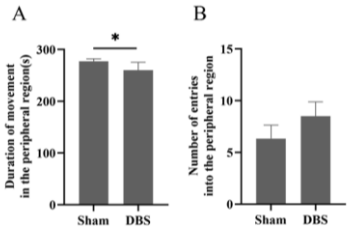


Figure 3. Activity in the peripheral region. (A) Duration of movement in the peripheral region. (B) Number of entries into the peripheral region. *$P<0.05$. Data expressed as means ± SEM.

in the peripheral region of the DBS group (260.05±6.21 s) was significantly (Fig. 3A, P<0.05) less than that of the Sham group (277.45±4.64 s). But there was no significant difference (Fig. 3B, P>0.05) in the number of entries into the peripheral region between the two groups (DBS: 8.50±1.38; Sham: 6.33±1.30).

*Activity in the center region:* There were significant differences in the duration of movement (DBS, 22.81±5.08 s; Sham, 11.74±2.33 s, P<0.05) and the distance moved (DBS, 222.23±31.88 cm; Sham, 130.07±23.95 cm, P<0.05) in the center region between DBS group and sham group (Fig. 4A, B). But there was no significant difference (Fig. 4C, P>0.05) in the number of entries into the center region between the two groups (DBS, 7.67±1.41; Sham, 6.40±1.15).

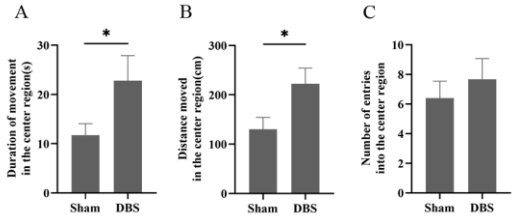


Figure 4. Activity in the center region. (A) Duration of movement in the center region. (B) Distance moved in the center region. (C) Number of entries into the center region. * P<0.05. Data expressed as means ± SEM.

*B. DCX immunofluorescence staining*

The number of DCX-positive was quantified. DCX is a microtubule-related phosphorylated protein, expressed in neuronal progenitors and immature neurons, and is a marker of adult neurogenesis [15]. Compared with the Sham group, we found that multiple sessions of EC DBS significantly increased the number of DCX-positive cells of the DBS group in the DG area (DBS, 55.75±7.77; Sham, 12.67±2.81; Fig. 4, P>0.05).

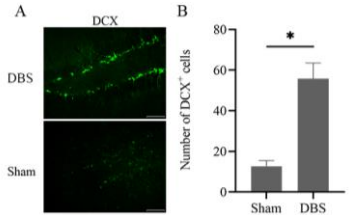


Figure 5. Effect of multiple sessions of EC DBS on the expression of DCX-positive cells in the dentate gyrus. (A) DCX immunofluorescence staining results of the DBS and Sham groups. Scale bar, 200μm. (B) Comparison of number of DCX-positive cells of the DBS and Sham groups. * P<0.05. Data expressed as means ± SEM. EC, entorhinal cortex; DBS, deep brain stimulation; DCX, doublecortin.

## IV. DISCUSSION

The aim of this study was to investigate the effects of multiple sessions of EC DBS on exploratory behavior and neurogenesis in the DG area of C57BL/6J mice. We found multiple sessions of EC DBS were tolerated for C57BL/6J mice. Compared with Sham group, multiple sessions of EC DBS significantly increased exploratory behavior in the OFT and prompted the number of DCX-positive cells in the DG area of the DBS group.

When the mice explored freely in the OFT, there was no significant difference in the total distance moved and average movement speed between the DBS and the Sham group (P>0.05, Fig. 2), indicating that DBS had no significant effect on exploratory behavior of the mice. The design principle of the OFT is based on the positive thigmotaxis of mice, which means that mice are afraid of open or potentially dangerous places, so they have the nature of "sticking to the wall" [16]. The thigmotaxis is evaluated based on the total duration the mouse spends in the peripheral region of the OFT. Obviously, in terms of the duration of movement in the peripheral region reflecting the thigmotaxis, the mice in the DBS group were significantly less than the Sham group (P<0.05, Fig. 3A), and the DBS group mice were more "adventurous" and the duration of movement in the center region increased significantly (P<0.05, Fig. 4A). This indicating multiple sessions of EC DBS does not cause anxiety-related side effects in mice, and increases their exploratory behavior.

We checked the effect of multiple sessions of EC DBS to make it consistent with current DBS cases that mainly use multiple or chronic stimulation sessions [17]. Our work showed that multiple sessions of EC DBS increased the number of DCX-positive cells in the DG area of the DBS group compared with the Sham group (Fig. 5). The multiple stimulation pattern in this study promoted hippocampal neurogenesis. In addition, other stimulation patterns in previous studies also increased neurogenesis. Stone et al., reported that 1 hour EC DBS promoted the number of BrdU-positive cells in the DG area, neurogenesis as a consequence of stimulation promoted the water maze performance, and inhibition of neurogenesis hindered spatial memory enhancement [6]. Mann et al. found chronic EC DBS increased the numbers of BrdU-/NeuN-positive double labeled neurons, and improved their impaired performance in the Morris water maze task in 4 months old 3xTg mice [18]. Ronaghi showed that a unilateral single session of EC DBS increased DCX expression on the ipsilateral side [13]. Coincidentally, a single session of DBS in anteromedial thalamic nucleus (ATN) also increased hippocampal neurogenesis [19], and multiple sessions of ATN DBS induced higher increase in hippocampal neurogenesis [20]. Similarly, we believe that multiple sessions of EC DBS will induce higher levels of neurogenesis than a single session of EC DBS.

Our result supports the potential of multiple sessions of EC DBS as a hippocampal neurogenesis strategy. But why do we attach so much importance to EC DBS? Because the EC area contains the place and grid cells, which are thought to assist navigation and spatial memory [21]. So the role of EC-hippocampal circuit in spatial information and memory processing is well understood. It is often found that direct electrical stimulation of the hippocampus proper destroys memory [7, 22-24]. However, Suthana et al. found that even if the identical stimulation in the hippocampus was not beneficial, the application of stimulation in the EC area showed improved memory performance during the spatial navigation task with a resetting of the phase of the theta rhythm [25]. This study proved that stimulating the brain area directly projected to the hippocampus may be more effective for memory improvement than stimulating the hippocampus itself. However, Jacobs et al. used different spatial memory

experiments from Suthana's study, and the results showed that both EC and hippocampus stimulation could damage memory [24]. Nevertheless, Hansen et al. found that during the noun-color associations learning task, the hippocampal event-related potentials were enhanced after entorhinal stimulation, but it had no effect on memory performance [26]. The difference between these studies may be different stimulation sites of entorhinal area, which may lead to different physiological effects on the hippocampus, or because different spatial memory tasks are used in these studies, the results are not comparable. However, combined with the results of clinical and animal studies, EC DBS has shown certain efficacy in improving memory and symptoms, and the neurogenesis induced by EC DBS may be a possible mechanism for its function.

It is noteworthy that neurogenesis severely depleted in both major depressive disorder (MDD) and AD with increasing age [27]. For neurodegenerative diseases, a potential repair approach is to add newborn neurons at early stage of neurodegeneration to compensate for neuronal loss so as to improve the symptoms [28]. Neurogenesis induced by multiple sessions of EC DBS is expected to be the target of AD and MDD treatment, and further research is needed to explore its treatment mode.

## V. Conclusion

All in all, our results suggest that multiple sessions of EC DBS increased exploratory behavior and hippocampal neurogenesis in the DG area of C57BL/6J mice. These results will serve to better explore the application of EC DBS in nervous system diseases and mental diseases. Areas worthwhile exploring are the effect of different stimulation parameters of EC DBS on neurogenesis, the influence of EC DBS in different neurodegenerative stages and the possibility of improving memory of nervous system diseases and mental diseases.

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