

Effects of Electroacupuncture at Zusanli and Baihui on Brain-Derived Neurotrophic Factor and Cyclic AMP Response Element-Binding Protein in the Hippocampal Dentate Gyrus

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ABSTRACT. Previously, we observed that electroacupuncture (EA) at ST36 (Zusanli) and GV20 (Baihui) enhanced cell proliferation and neuroblast differentiation in the rat dentate gyrus. In this study, we investigated the possible mechanisms of EA in this effect. For this, we applied EA at ST36 and GV20 of Wistar rats (13-week-old) once a day for 3 weeks. Application of EA at these acupoints significantly increased the number of phosphorylated cyclic AMP response element-binding protein (pCREB)-immunoreactive cells in the dentate gyrus. In addition, EA significantly increased the levels of brain-derived neurotrophic factor (BDNF) and pCREB protein in the dentate gyrus. The administration of K252a, an inhibitor of BDNF receptor, significantly reduced cell proliferation in the subgranular zone of dentate gyrus. These results suggest that EA significantly increased neuroblast plasticity via pCREB and BDNF activation in the dentate gyrus.

KEY WORDS: electroacupuncture, growth factor, hippocampus, neurogenesis, rat.

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Acupuncture is believed to be an effective alternative and complementary treatment in many disorders [4]. During acupuncture, fine needles are inserted in acupoints that have functional specificity and are specifically used to treat disorders [13, 20, 33]. In acupuncture with electrical stimulation (electroacupuncture, EA), the needles inserted into acupoints are attached to a trace pulse current in order to produce synthetic electric and needling stimulation. EA stimulates the release of neurotrophic factors and has various physiological effects in rodents as well as dogs [3, 6, 7, 16, 17, 24, 30, 34, 37].

It has been reported that some acupoints are closely related to brain function. In particular, ST36 (Zusanli) and GV20 (Baihui) are major acupoints that improve cognitive impairment in dementia [35, 36] and the stimulation of these sites enhances the cell proliferation and neuroblast differentiation in the dentate gyrus [11], even though these acupoints are regionally different (head point and body point, respectively).

Neurogenesis is regulated by a number of signaling pathways. One of these signaling pathways is phospho-cAMP response element-binding protein (pCREB), which plays a prominent role in the proliferation, differentiation, and survival of neuronal precursor cells [10, 18, 22]. In addition, pCREB directly regulates the expression of brain-derived neurotrophic factor (BDNF) [1, 26]. pCREB expression is restricted to the dentate gyrus in adult mice. pCREB is also

expressed in immature neurons, but its expression is very low in mature neurons [19, 27].

There are many studies about the effects of EA on neurons in various conditions such as Parkinson's disease, depression and stroke [3, 6–8, 12, 23, 24, 30, 31, 34–37]. However, there are few reports about mechanisms of EA in the dentate gyrus of normal rats. We previously observed that EA enhanced cell proliferation and neuroblast differentiation in the dentate gyrus [11]. In this study, we investigated this effect throughout the hippocampal BDNF and pCREB signaling pathways.

MATERIALS AND METHODS

Experimental animals: Male Wistar rats were purchased from Orient Bio Inc. (Seongnam, South Korea). They were housed in a conventional state under adequate temperature (23°C) and humidity (60%) control with a 12-hr light/12-hr dark cycle, and free access to food and water. The procedures for handling and caring for the animals adhered to the guidelines that are in compliance with the current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85–23, 1985, revised 1996). All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Application of EA: Male Wistar rats were randomly divided into 3 groups: control ($n=17$), sham-acupuncture (sham-ACU, at bilateral nearby nonacupoints in the hamstring muscles) ($n=17$), EA ($n=27$) groups. At 13 weeks of age, the animals were anesthetized with chloral hydrate (30

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mg/kg) and sham-ACU or EA was administered once a day for 3 weeks. The prescription of acupoints includes ST36 (5 mm below head of fibula under knee joint, and 2 mm lateral to the anterior tubercle of the tibia) and GV20 (located at the midmost point of parietal bone) at a depth of 5 mm into the skin with stainless needle measuring 0.25×20 mm of length with guide-tube for 20 min (Wujiang Shenli Medical & Health Material Co., Ltd., China). Electric stimulation was generated by an EA apparatus (Model G-6805) for 20 min and the stimulation parameter were disperse-dense waves of 5/20 Hz (28.5 ms/15 ms pulse duration) of frequency and current density of 2–4 mA.

Tissue processing for histology: For immunohistological analysis, animals in each group ($n=7$) were anesthetized with chloral hydrate at 16 weeks of ages and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB, pH 7.4). The brains were removed and post-fixed in the same fixative for 12 hr. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thereafter the frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30- μ m coronal sections, and they were then collected into six-well plates containing PBS.

Immunohistochemistry for pCREB: For immunohistochemistry, the free-floating sections were processed under the same conditions as previous mentioned method [11]. The sections were chosen with 150 μ m interval according to anatomical landmarks corresponding to Bregma $-3.00 \sim -4.08$ mm of the rat brain atlas [25]. The sections were sequentially treated with 0.3% hydrogen peroxide (H_2O_2) in PBS and 10% normal horse serum in 0.05 M PBS. They were next incubated with diluted rabbit anti-pCREB antibody (1:1,000, Upstate Biotechnology, Lake Placid, NY, U.S.A.) overnight and subsequently exposed to biotinylated rabbit anti-goat IgG (diluted 1:200, Vector, Burlingame, CA, U.S.A.) and streptavidin peroxidase complex (diluted 1:200, Vector). Then, the sections were visualized by incubating with 3,3'-diaminobenzidine tetrahydrochloride and hydrogen peroxide.

In order to establish the specificity of primary antibody, the procedure included the omission of the primary antibody, substitution of normal horse serum for the primary antibody. As a result, immunoreactivity disappeared completely in tissues (data not shown).

The measurement of the number of pCREB-immunoreactive cells in each group was performed using an image analyzing system equipped with a computer-based CCD camera (software: Optimas 6.5, CyberMetrics, Scottsdale, AZ, U.S.A.). The number of the cells was counted as a mean number in the dentate gyrus per each section.

Western blot analysis for total CREB and pCREB: To confirm changes in pCREB levels in the dentate gyrus at each group, 5 animals at control, sham-ACU and EA group were sacrificed and used for Western blot analysis. After sacrificing them and removing the brain, hippocampal dentate gyrus were then dissected with a surgical blade. The tis-

ues were homogenized in 50 mM PBS (pH 7.4) containing 0.1 mM ethylene glycol bis (2-aminoethyl Ether)-N,N,N',N' tetraacetic acid (EGTA) (pH 8.0), 0.2% Nonidet P-40, 10 mM ethylenediamine tetraacetic acid (EDTA) (pH 8.0), 15 mM sodium pyrophosphate, 100 mM β -glycerophosphate, 50 mM NaF, 150 mM NaCl, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT). After centrifugation, the protein level was determined in the supernatants using a Micro BCA protein assay kit with bovine serum albumin as the standard (Pierce Chemical, Rockford, IL, U.S.A.). Aliquots containing 20 μ g of total protein were boiled in loading buffer containing 150 mM Tris (pH 6.8), 3 mM DTT, 6% SDS, 0.3% bromophenol blue and 30% glycerol. The aliquots were then loaded onto a 10% polyacrylamide gel. After electrophoresis, the gels were transferred to nitrocellulose transfer membranes (Pall Crop, East Hills, NY, U.S.A.). To reduce background staining, the membranes were incubated with 5% non-fat dry milk in PBS containing 0.1% Tween 20 for 45 min, followed by incubation with rabbit anti-CREB (1:1,000, SantaCruz Biotechnology, Santa Cruz, CA, U.S.A.) and rabbit anti-pCREB antibody (1:1,000, Upstate Biotechnology) and anti- β -actin (1:1,000, SantaCruz Biotechnology) for loading control, peroxidase-conjugated anti-rabbit IgG (Sigma, St. Louis, MO, U.S.A.) and an ECL kit (Pierce Chemical).

The result of the Western blot analysis was scanned, and the quantification of the Western blotting was done using Scion Image software (Scion Corp., Frederick, MD, U.S.A.), which was used to count the relative optical density (ROD): A ratio of the ROD was calibrated as %.

Measurement of BDNF levels in the hippocampal dentate gyrus: To confirm changes in BDNF levels in the hippocampal dentate gyrus at each group, 5 animals at control, sham-ACU and EA group were decapitation and the hippocampus was dissected from the brain and stored in liquid nitrogen. This was measured with Promega BDNF Emax immunoassay kit (Madison, WI, U.S.A.). Samples were weighed and 300 μ l lysis buffer added to each sample. Samples were sonicated for 30 sec and centrifuged at 4°C for 20 min. The supernatant was stored at -20°C until analysis. All samples were assayed in duplicate and absorbance was read on an ELISA plate reader (Bio-Tek, Winooski, VT, U.S.A.) and the concentration of each sample was calculated by the computer by plotting the absorbance values on standard curve with known concentrations generated by the assay.

Effects of a BDNF receptor blocker, K252a [29], on cell proliferation in the EA-treated group: To inhibit the BDNF receptor in the dentate gyrus, 5 μ l of 1% dimethylsulfoxide (vehicle group) or K252a (Sigma), a blocker of BDNF receptor, was infused at a rate of 1 μ l/min into the left lateral ventricle of EA group ($n=10$). Animals were sacrificed 3 days after K252a treatment and immunohistochemistry was conducted as described in section of tissue processing for histology and immunohistochemistry. In brief, the frozen tissues were serially sectioned on a cryostat (Leica) into 30- μ m coronal sections, and immunostained with rabbit anti-

Ki67 antibody (1:1,000, Abcam, Cambridge, UK) to detect the proliferating cells during all active cell cycles except early G1 [14].

Statistical analysis: The data shown here represent the means of experiments performed for each experimental area. Differences among the means were statistically analyzed by Student *t*-test or one-way analysis of variance followed by Duncan's new multiple range method in order to elucidate differences between groups.

RESULTS

Effects of EA on pCREB immunoreactivity: In the control group, few pCREB-immunoreactive nuclei were detected in

the dentate gyrus (Fig. 1A and 1B). In the sham-ACU group, some pCREB-immunoreactive nuclei were observed in the dentate gyrus (Fig. 1C and 1D). However, pCREB-immunoreactive cells were abundant in the subgranular zone of the dentate gyrus (Fig. 1D). In the EA group, pCREB-immunoreactive cells were dramatically increased in the dentate gyrus. In this group, the number of pCREB-immunoreactive cells was increased by 7.4-fold compared to that in the sham-ACU group (Figs. 1E, 1F and 2).

In addition, CREB protein levels were similar in all groups, while pCREB protein levels were significantly increased in the EA group compared to that in the sham-ACU group (Fig. 3).

Effects of EA on BDNF levels: In the control group,

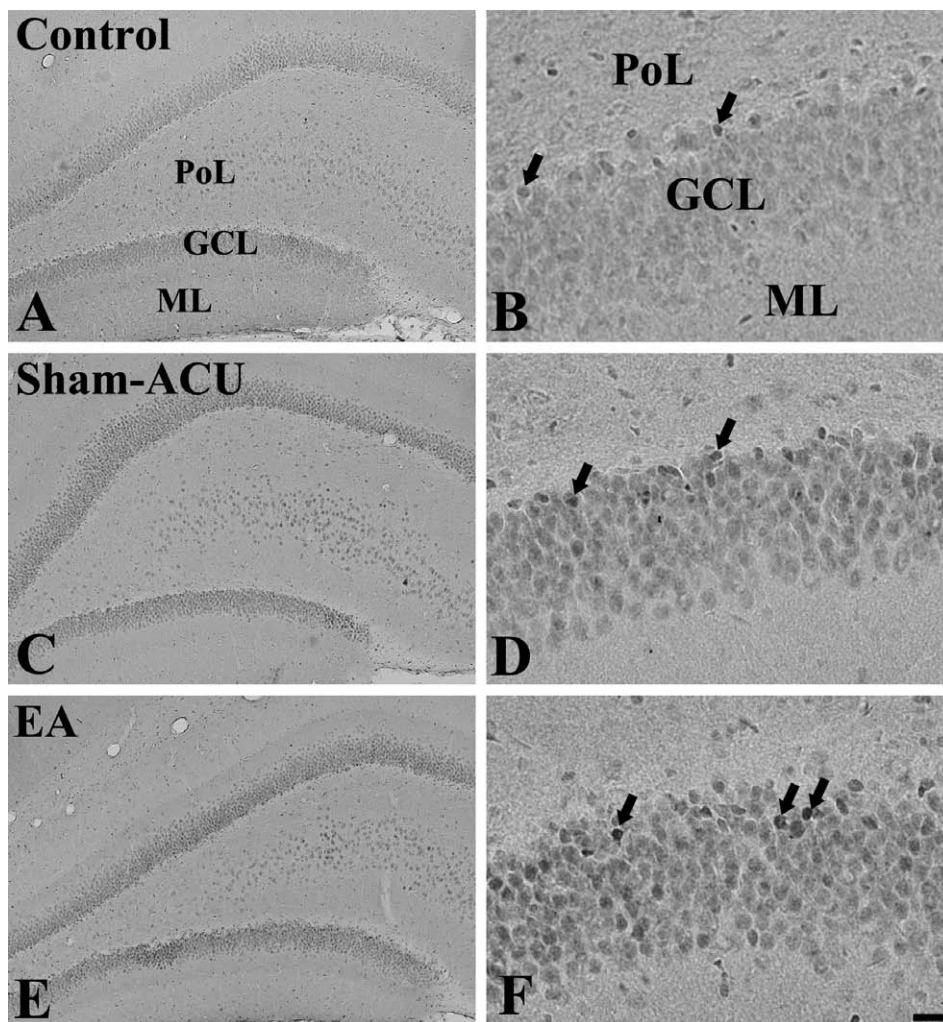


Fig. 1. Immunohistochemistry for pCREB in the dentate gyrus in control (A and B), sham-acupuncture (sham-ACU, C and D), and electroacupuncture (EA, E and F) groups. In the control group, few pCREB-immunoreactive cells are detected in the dentate gyrus. In the sham-ACU group, some pCREB-immunoreactive cells are detected in the dentate gyrus, especially in the subgranular zone of the dentate gyrus. In the EA group, pCREB-immunoreactive cells are abundant in the dentate gyrus. Arrows indicate pCREB immunoreactive cells. GCL, granule cell layer; ML, molecular layer; PoL, polymorphic layer. Bar=100 μ m (A, C and E), 12.5 μ m (B, D and F).

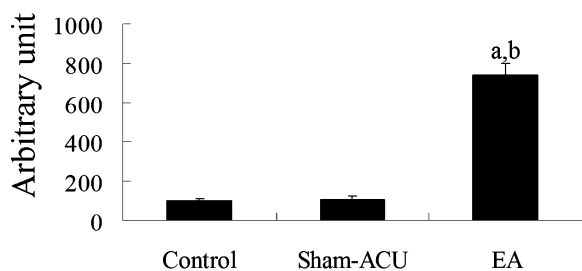


Fig. 2. Analysis of pCREB-immunoreactive cells per section in the control, sham-acupuncture (sham-ACU), and electroacupuncture (EA) groups ($n=7$ per group; ^a $P<0.05$, significantly different from control group, ^b $P<0.05$, significantly different from the sham-ACU group). The bars indicate the means \pm SEM.

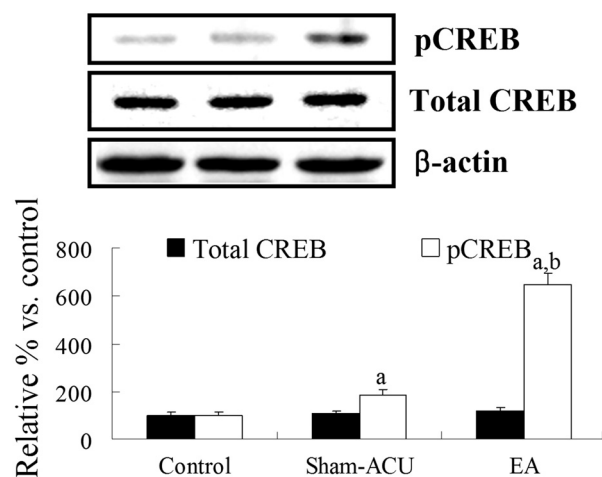


Fig. 3. Western blot analysis of CREB and pCREB in the hippocampi of control, sham-acupuncture (sham-ACU), and electroacupuncture (EA) groups. The relative optical density of immunoblot bands are shown as percent values ($n=5$ per group; ^a $P<0.05$, significantly different from control group, ^b $P<0.05$, significantly different from the sham-ACU group). The bars indicate the means \pm SE.

BDNF levels were 21,465 pg/g wet weights. In the sham-ACU group, BDNF levels were similar to that in the control group. However, BDNF protein levels were significantly increased by 142% in the EA group compared to that in the sham-ACU group (Fig. 4).

Effects of K252a on proliferating cells in EA group: In both vehicle- and K252a-treated EA groups, Ki67 positive cells were detected with clusters in the subgranular zone of dentate gyrus (Fig. 5A and 5B). However, there were significant differences in number of Ki67 positive cells. In the K252a-treated EA group, the number of Ki67 positive cells was significantly decreased to 31.2% of vehicle-treated EA group (Fig. 5C).

DISCUSSION

It has been reported that EA restores learning and mem-

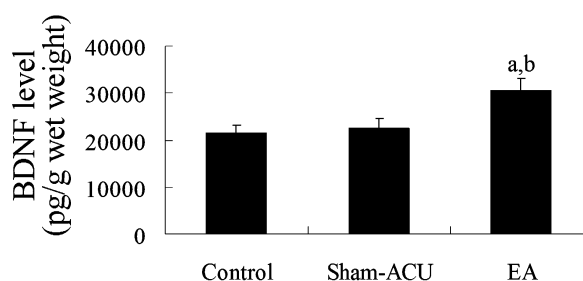


Fig. 4. BDNF levels in the hippocampi of control, sham-acupuncture (sham-ACU), and electroacupuncture (EA) groups. Note that BDNF levels are significantly increased in the EA group compared to that in the control or sham-ACU group ($n=5$ per group; ^a $P<0.05$, significantly different from control group, ^b $P<0.05$, significantly different from the sham-ACU group). The bars indicate the means \pm SE.

ory impairment induced by both diabetes mellitus and cerebral ischemia in rats [12]. In addition, EA reduces free radicals in the brain tissue of rats with vascular dementia [31]. In normal rats, EA stimulates phospho-Akt expression in the hippocampal CA1 region [32]. In our previous study, we observed that EA significantly increased cell proliferation and neuroblast differentiation in the dentate gyrus [11]. In this study, we explored the mechanism of EA in these increases of neuroblasts in the dentate gyrus using BDNF and CREB, because the CREB and CRE-mediated system is involved in memory, learning and synaptic transmission as well as in neuronal survival, differentiation and axon growth in the brain [10, 18, 22]. In addition, CREB directly regulates the expression of brain-derived neurotrophic factor (BDNF), which also enhances cell survival and differentiation of subventricular zone progenitor cells *in vitro* and increases the number of newborn cells *in vivo* [1, 26].

In this study, EA at GV20 and ST36 significantly increased the number of pCREB-immunoreactive cells in the dentate gyrus and elevated the protein levels of pCREB in the dentate gyrus homogenates. pCREB immunoreaction was confined to the subgranular zone of the dentate gyrus, a region in which neuroblasts are found [21]. This result is supported by a report that pCREB expression in the hippocampus of depressed rats increased significantly in the head point group (including ST36) + fluoxetine group, while in the body point (including GV20) + fluoxetine group, pCREB expression in the hippocampus did not increase significantly [8]. In addition, it was reported that inhibition of CREB blocked neurogenesis in the dentate gyrus [38].

Next, we observed the BDNF protein levels after EA at GV20 and ST36 because CREB is phosphorylated by BDNF at the transcription regulatory site and CREB can feed back on BDNF by regulating its gene transcription via a calcium-dependent mechanism [9]. There are some researches about correlations between BDNF/trkB and neurogenesis. Intrahippocampal infusion of BDNF increases neurogenesis [28]. Furthermore, decreasing either full-length TrkB activity or BDNF protein levels causes a reduc-

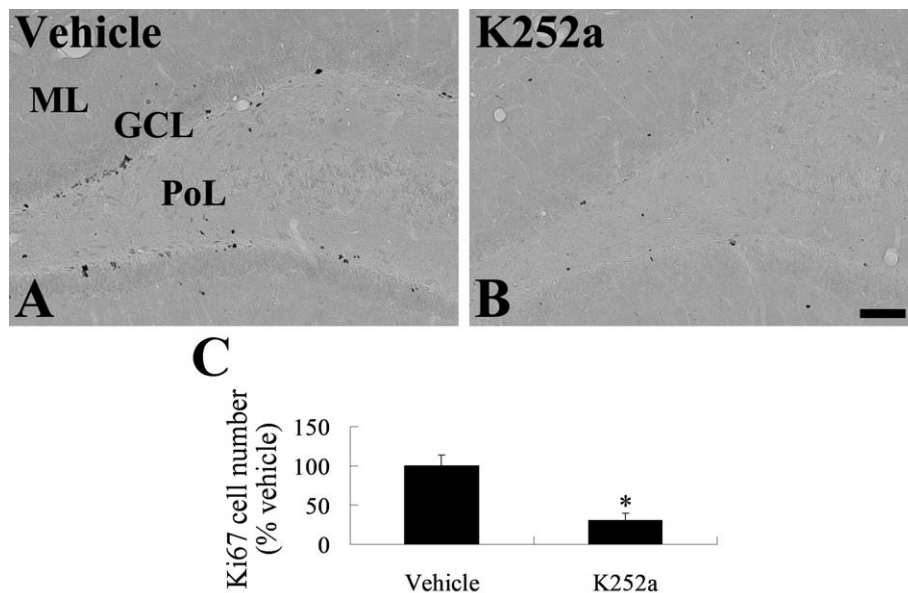


Fig. 5. Immunohistochemistry for Ki67 in the dentate gyrus in vehicle- (A) and K252a-treated (B) electroacupuncture (EA) group. In the vehicle-treated EA group, abundant Ki67-immunoreactive cells are detected in the dentate gyrus. In the K252a-treated EA group, Ki67-immunoreactive cells are less abundant in the dentate gyrus compared to that in the vehicle-treated EA group. GCL, granule cell layer; ML, molecular layer; PoL, polymorphic layer. Bar=100 μ m. C: Analysis of Ki67-immunoreactive cells per section in the vehicle- and K252a-treated EA group ($n=5$ per group; * $P<0.05$, significantly different from vehicle-treated EA group). The bars indicate the means \pm SEM.

tion in neurogenesis [26]. In addition, immature neuroblasts are more likely to express TrkB, especially as they present a more mature morphology [5].

In this study, EA significantly increased the BDNF protein levels in the dentate gyrus homogenates. In addition, we observed that the administration of K252a, a blocker of BDNF receptor, significantly reduced the cell proliferation in the subgranular zone of dentate gyrus. This result suggests that EA strongly increases the BDNF and this finally increases cell proliferation in the subgranular zone of dentate gyrus. This result is supported by a previous study in which a single bout of EA stimulation notably augmented BDNF mRNA declined by immobilization stress [37]. However, in an ischemia model of middle cerebral artery occlusion, there is no statistical significance between BDNF levels in the cerebral hemisphere of control and EA (including ST36 and GV20) groups [15]. In the dorsal ganglia of intact cats, EA does not influence BDNF mRNA levels [2]. However, in a rat model of retinitis pigmentosa, EA causes significant increases in BDNF and its major receptor (TrkB) proteins and mRNA, whereas BDNF-mRNA remains unchanged [23]. These discrepancies may be associated with specific target tissues and BDNF protein or mRNA levels.

In conclusion, EA significantly increased pCREB immunoreactivity and protein levels as well as BDNF protein levels in the rat dentate gyrus. This result explains why EA enhances cell proliferation and neuroblast differentiation in the dentate gyrus.

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