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RESEARCH ARTICLE

# Green tea epigallocatechin-3-gallate (EGCG) promotes neural progenitor cell proliferation and sonic hedgehog pathway activation during adult hippocampal neurogenesis

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**Scope:** Adult hippocampal neurogenesis is a lifelong feature of brain plasticity that appears to be critically involved in adult brain function and neurological disease. Recent studies suggest that (–)-epigallocatechin-3-gallate (EGCG), which is the main polyphenolic constituent of green tea, may be used for the prevention and treatment of various neurodegenerative diseases. We hypothesized that EGCG promotes adult neurogenesis, which may be beneficial to hippocampus-dependent learning and memory.

**Methods and results:** We show that EGCG treatment significantly increased the number of 5-bromo-2′-deoxyuridine (BrdU)-labeled cells in adult hippocampal neural progenitor cell (NPC) cultures and in the dentate gyrus of adult mice. Meanwhile, EGCG markedly improved spatial cognition in mice. These events are associated with the sonic hedgehog (Shh) signaling pathway. We observed that EGCG triggered a robust upregulation of Shh receptor (Patched) mRNA and protein expression in cultured NPCs as well as an upregulation of the downstream Shh transcriptional target Gli1. These changes were further confirmed in the hippocampus of mice administered EGCG. The blockage of the Shh signal with the pharmacological inhibitor cyclopamine attenuated EGCG-induced hippocampal neurogenesis.

**Conclusion:** Our results provide strong evidence that EGCG enhances adult hippocampal neurogenesis.

**Keywords:**
Green tea / Hippocampus / Learning and memory / Neural progenitor cell / Sonic hedgehog

## 1 Introduction

Adult hippocampal neurogenesis represents a prominent form of brain plasticity, and its potential to affect learning and memory has been increasingly recognized [1, 2]. In humans, hippocampal neurogenesis declines with age, and this decline is involved in various neurological disorders, many of which are associated with cognitive deficits [3, 4]. Similar to other forms of neural activity-induced plasticity, adult neurogenesis is modulated by numerous extrinsic factors

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**Abbreviations:** **AD**, Alzheimer's disease; **BrdU**, 5-bromo-2′-deoxyuridine; **DG**, dentate gyrus; **EGCG**, (–)-epigallocatechin-3-gallate; **GCL**, granule cell layer; **GFAP**, glial fibrillary acidic protein; **HBC**, 2-hydropropyl-β-cyclodextrin; **NPC**, neural progenitor cell; **PD**, Parkinson's disease; **Ptc**, patched; **SGZ**, subgranular zone; **Shh**, sonic hedgehog; **Smo**, Smoothened; **TuJ1**, β-III tubulin

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[5, 6]. Thus, the enhancement of neurogenesis will hopefully improve cognition in the aged population and aid in the development of new therapies for these neurological diseases.

Green tea, which is a popular beverage worldwide, has attracted scientific attention with respect to its health benefits in the prevention and treatment of cancer, cardiovascular diseases, inflammatory diseases, and diabetes [7]. (–)-Epigallocatechin-3-gallate (EGCG), which is the major polyphenol in green tea, is believed to be a critical active ingredient. EGCG can easily pass through the blood–brain barrier and reach the brain parenchyma [8]. Human epidemiological data show that green tea consumption is inversely correlated with the incidence of dementia, Alzheimer's disease (AD) and Parkinson's disease (PD) [9, 10]. EGCG has also been demonstrated to correct brain morphogenesis alterations, suggesting that it may improve the cognitive performance of Down syndrome patients [11]. Numerous animal studies also suggest that EGCG exerts neuroprotective effects against age-related cognitive decline and neurodegenerative diseases [12–14]. However, the precise mechanisms underlying these neuroprotective effects are largely unknown. Emerging evidence has revealed that, in addition to its well-established antioxidant properties, the activities of EGCG are associated with a spectrum of cellular mechanisms. Of particular interest was the finding that EGCG potently facilitates evoked glutamate release in the rat cerebral cortex [15] and interacts with $GABA_A$ receptors in mouse hippocampal neurons [16]. Recently, a series of studies has identified several neurotransmitters that are major regulators of adult neurogenesis, including monoamines, GABA, and glutamate [17]. Therefore, we hypothesized that EGCG improves cognitive function by increasing adult hippocampal neurogenesis.

Adult neurogenesis is tightly controlled by intricate molecular networks [18]. Sonic hedgehog (Shh) is known to be important in the developing nervous system, and it is also understood to be a crucial signal in adult neurogenesis [19, 20]. Specifically, Shh acts as a mitogen and increases the proliferation of adult neural progenitor cells (NPCs) [21]. The available data provide convincing support that Shh signaling plays an active role in the tissue-repair process in brain diseases [22]. In addition, some evidence from animal studies suggests that neurotransmitters regulate the expression of Shh components [17], which raises the possibility that EGCG may affect Shh signaling via neurotransmitters.

The present study sought to determine whether EGCG influences adult hippocampal neurogenesis, and if so, by what underlying mechanism. Here we report that EGCG acts directly on adult NPCs to favor proliferation both in vitro and in vivo. Notably, these effects involve the activation of the Shh signaling pathway. These findings support the continuation of research on EGCG as a potent therapeutic for neurological disorders.

## 2 Materials and methods

### 2.1 Adult hippocampal NPC culture

Neural progenitors were isolated and cultured from the hippocampi of adult male mice (C57BL/6J, 8–10 weeks, purchased from Experimental Animal Center of the Third Military Medical University, Chongqing, China) as previously described [23]. Cells were propagated in DMEM/F-12 media with 1% N2 supplement (Invitrogen, Carlsbad, CA, USA), 20 ng/mL fibroblast growth factor-basic (FGF-2; Peprotech, Rocky Hill, NJ, USA) and 20 ng/mL epidermal growth factor (EGF; Peprotech). All animal experimental procedures were approved by the Third Military Medical University Institutional Animal Care and Use Committee (IACUC) and performed in accordance with protocols IACUC 06863.

We test the self-renewal capacity and the multipotency of adult NPCs as described previously [24]. We performed immunostaining using antibodies to nestin (1:1000, BD Biosciences, San Jose, CA, USA), 5-bromo-2′-deoxyuridine (BrdU, 1:1000, Millipore, Billerica, MA, USA), neuron-specific β-III tubulin (TuJ1, 1:4000, R&D Systems, Minneapolis, MN, USA), glial fibrillary acidic protein (GFAP, 1:1000, Dako, Glostrup, Denmark), and CNPase (1:1000, Chemicon, Temecula, CA, USA). After washing with Dulbecco's phosphorylated buffered saline (DPBS), the cells were incubated with the appropriate FITC or Cy3 conjugated secondary antibodies (1:500, Invitrogen). Cells were then counterstained with the fluorescent nuclear dye DAPI and mounted with Vectashield (Vector, Burlingame, CA, USA).

EGCG (Sigma, St. Louis, MO, USA) was dissolved in sterile water to generate a 10 mM stock solution. To examine the direct effect of EGCG on NPC proliferation and differentiation, NPCs were cultured in control medium or EGCG (0–40 μM); to examine whether the Shh/Gli signaling pathway is involved in the effects of EGCG on neurogenesis, NPCs were incubated in the presence of EGCG (20 μM) with or without cyclopamine (a natural specific inhibitor of Shh signaling, 5 μM; Toronto Research Chemicals, Ontario, Canada). Cyclopamine for in vitro use was dissolved in ethanol to generate a 10 mM stock solution. All culture conditions and experimental manipulations were replicated in at least three independent experiments.

### 2.2 In vitro cell proliferation and differentiation assays

For the proliferation assay, neurospheres at passages 4–8 were dissociated and plated at a density of $1 \times 10^5$ cells/mL in 96-well plates. Cell proliferation rates were determined by the amount of incorporated BrdU using a labeling and detection kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. To assay neural differentiation, the percentages of neurons and astrocytes were counted as reported [25].

 

### 2.3 Animals and procedures

Two-month-old male C57BL/6J mice purchased from the Experimental Animal Center of the Third Military Medical University were randomly divided into four groups. Groups I and II were intraperitoneally injected with PBS (control) or EGCG (20 mg/kg) once daily for 60 days based on methods previously described [14]. Groups III and IV were pretreated with EGCG followed by 10 mg/kg/day intraperitoneal injections of cyclopamine for 10 days [26] or 2-hydropropyl-β-cyclodextrin [HBC (Sigma)] injections as a control. Cyclopamine was used at 1 mg/mL and conjugated with HBC, which was then prepared as a 45% solution in PBS.

### 2.4 BrdU labeling, detection, and stereological analysis

To evaluate cell proliferation and differentiation, BrdU (100 mg/kg of body weight dissolved in 0.9% NaCl, intraperitoneal injection) was administered to mice. Briefly, for analysis of the number of newborn cells ($BrdU^+$) and immature newborn neurons ($BrdU^+$ and $DCX^+$) in the dentate gyrus (DG), mice received a single injection of BrdU 2 h following treatment and were killed 2 h later. For analysis of the number of surviving mature neurons ($BrdU^+$ and $NeuN^+$) and mature astrocytes ($BrdU^+$ and $GFAP^+$), 4-month-old mice were given four injections of BrdU (once daily, 4 consecutive days) and killed after 4 weeks. The immunohistochemistry for BrdU and stereological analysis were performed as previously described [27].

### 2.5 Immunofluorescence and cell quantification

Immunofluorescence detection was performed as described previously [27]. The primary antibodies and final dilutions that were used are as follows: rat anti-BrdU (1:400, Abcam, Cambridge, UK), rabbit anti-DCX (1:400, Abcam), mouse anti-NeuN (1:400, Chemicon), rabbit anti-GFAP (1:500, Dako). The corresponding secondary antibodies were goat anti-mouse, anti-rat conjugated with FITC (Invitrogen) or goat anti-mouse, anti-rabbit conjugated with Cy3 (Invitrogen), respectively. To determine the frequency of the neuronal differentiation of newborn cells, every sixth section (240 μm interval) was examined using a fluorescence microscope (Leica, Nussloch, Germany). On average, 50 BrdU-labeled cells per animal were analyzed for neuronal differentiation.

### 2.6 Morris water maze

The mice ($n = 10$–12 per group) were trained to locate an escape platform in a circular pool (1.1-m diameter) of water made opaque by the addition of nontoxic paint. Around the tank, white paper stars were placed on the black surrounding curtain to serve as spatial cues. First, mice were trained for 3 days in three trials per day using a visible platform (8-cm diameter). This procedure served the purpose of habituation and also allowed us to identify any swimming deficits. After habituation, the training trials began. A 1-cm submerged platform (8 cm diameter) was placed in one quadrant of the maze. During training, the mice began from random start positions, and their latency to reach the hidden platform was measured. Mice were given three training trials per day for seven consecutive days. Twenty-four hours after the last day of training, the mice were given a 1-min probe test in which the platform was removed. Escape latencies, swim paths, swim speeds, and the amount of time spent in each quadrant were recorded with a video tracking system (Beijing, China).

### 2.7 Real-time RT-PCR

Quantitative PCR was performed using the SYBR Green real-time PCR method. Total RNA was isolated from adult NPC cultures and hippocampi using an Rneasy Mini Kit (Qiagen, Valencia, CA, USA) and Trizol Reagent (Invitrogen). Quantitative RT-PCR was performed using an ABI 7000 PCR instrument (Applied Biosystems, Foster City, CA, USA) with three-stage program parameters as follows: 2 min at 50°C, 10 min at 95°C, 40 cycles of 15 s at 95°C and 1 min at 60°C. Each sample was tested in triplicate, and the samples obtained from three independent experiments were used for the analysis of relative gene expression using the $2^{-\Delta\Delta}$CT method. The following primers were used for real-time PCR: β-actin, forward, 5′-ACTGTGTTGGCATAGAGGTCTTTA-3′; reverse, 5′-CTAGACTTCGAGCAGGAGATGG-3′; Shh, forward, 5′-CCT TTA CCC TAC AAG CAG TTT ATT GC-3′; reverse, 5′-GTA ATT GGG GGT GAG TTC CTT AAA TC-3′; Patched (Ptc), forward, 5′-TAG CGC CTT CTT CTT TTG GA-3′; reverse, 5′-GTG GAA GTT GGT GGA CGA GT-3′; Gli1, forward, 5′-TCC ACA CGC CCC CTA GTG-3′; reverse, 5′-TGG CAA CAT TTT CGG TGA TG-3′.

### 2.8 Western blot analysis

Lysates from adult NPCs and hippocampi were sonicated for 10 s and centrifuged at 14 000 × *g* for 20 min. Equal amounts of protein were loaded into a 10% SDS-polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes, and the blots were subsequently probed with the following antibodies: Shh (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Ptc (1:200, Santa Cruz Biotechnology) and Gli1 (1:3000, Abcam). For detection, horseradish peroxidase-conjugated secondary antibodies were used (1:2000, Dako), followed by enhanced chemiluminescent development (Pierce, Rockford, IL, USA). To control for small variations in the amount of protein in each well, we ran parallel Western blots using an antiactin antibody (1:5000, Sigma) as a loading control. The optical density

 

of the bands was quantified using Quantity One, which is an image processing and analysis software package (Bio-Rad, Hercules, CA, USA).

### 2.9 In situ hybridization

In situ hybridization was performed as previously described [28]. For visualization of the in situ hybridization results, we used the digoxigenin Nucleic Acid Detection kit (Roche). Finally, the slides were dried at room temperature and mounted with Crystal Mount (Biomeda, Foster City, CA, USA). The hippocampus was analyzed by outlining an equivalent area for each sample. Optical density measurements from both hemispheres of three to four individual sections from each animal were analyzed, and the mean optical density value was calculated.

### 2.10 In vivo microdialysis and glutamate assay

The microdialysis procedure used has been previously described [29]. Briefly, anesthetized mice (chloral hydrate; 400 mg/kg, i.p.) were implanted with a microdialysis guide cannula (CMA/7; CMA/Microdialysis, Solna, Sweden) aimed at the basal forebrain using standard stereotaxic techniques. The coordinates (mm) were: 1.0 posterior to the bregma, 2.2 lateral to the midline, and 3.8 below the bregma according to Franklin and Paxinos [30]. Seven days after surgery, the microdialysis probe (CMA/7; 6 kDa cutoff; 1.0 mm length, 0.24 mm diameter cuprophane membrane; CMA/Microdialysis) was inserted through the guide cannula, and the animals were returned to their home cages. Animals were transferred to the microdialysis plexiglass test chambers 24 h after probe implantation. Probes were then connected to a microinfusion pump and perfused with modified artificial CSF at a rate of 1 μL/min (CSF contained, in mM, NaCl, 145; KCl, 2.7; $CaCl_2$, 1.2; $MgCl_2$, 1; $Na_2HPO4/NaH_2PO_4$ (buffer), 2, pH 7.4). Four consecutive 20-min samples were collected to determine glutamate levels. Our definition of the term "mean" is the average of these four sample measures. Samples were stored at −80°C until glutamate levels were analyzed using HPLC [31].

### 2.11 Statistical analysis

All morphological analyses were performed on coded slides to ensure blinding. Statistical analyses were performed using GraphPad Prism. Group differences were tested using statistics including Student's *t* tests, one- or two-way ANOVAs or repeated-measures ANOVA for the water maze test. We followed our analyses with Bonferroni corrections or Tukey's post-hoc tests when necessary. $p < 0.05$ was considered to be statistically significant. Data are reported as the mean ± SEM.

## 3 Results

### 3.1 EGCG increases the proliferation of adult hippocampal NPCs in vitro

To examine the effect of EGCG on adult NPCs, we employed a neurosphere assay that has been widely used to investigate the biology of NPCs [32]. Nearly all cultured NPCs were positive for the NPC marker nestin, which suggests a relative homogeneity in these primary adult NPCs. These adult NPCs incorporate BrdU, a thymidine analogue, under proliferative conditions and produce TuJ1-positive neuronal cells, GFAP-positive astrocytes and CNPase-positive oligodendrocytes under differentiating conditions. Thus, these cells possess the same essential properties as NPCs (Supporting Information Fig. S1).

We then determined whether EGCG promotes adult NPC proliferation. Adult NPCs were cultured in growth medium containing EGCG (0, 5, 10, 20, 40 μM) for 24 h. The exposure of the adult NPCs to EGCG (10, 20, 40 μM) resulted in a significant increase in the number and size of neurospheres when compared with the control group. Furthermore, treatment with EGCG significantly ($p < 0.01$) increased the number of BrdU-positive cells (Fig. 1A), which suggests that the increases in the number and size of neurospheres resulted from an increase in adult NPCs rather than an increase in cell aggregation. Collectively, these results demonstrate that EGCG augments adult NPC proliferation.

We next determined the effect of EGCG on adult NPC differentiation. Single cells dissociated from neurospheres were reseeded on laminin-coated glass coverslips and treated with EGCG (0, 5, 10, 20, 40 μM) for 4 days in differentiation medium without growth factors. We found that the number of TuJ1- and GFAP-positive cells in the EGCG group was not significantly different from the number in the control group (Fig. 1B and C), indicating that EGCG did not selectively bias adult NPC differentiation. It should be noted that a relatively high concentration of EGCG (80 μM) increased adult NPC proliferation but inhibited differentiation (data not shown), suggesting that a modest concentration of EGCG is more capable of enhancing the total number of newborn neurons in vitro.

### 3.2 EGCG increases adult hippocampal neurogenesis by stimulating the proliferation of adult NPCs in vivo

To assess the effect of EGCG on adult hippocampal NPC proliferation in vivo, we examined the influence of chronic EGCG treatment on BrdU-positive cell numbers in the DG. Cells positive for BrdU were predominantly localized to the area of the subgranular zone (SGZ) lining the border with the granule cell layer (GCL) and hilus (Fig. 2A). Analysis of the numbers of BrdU-positive cells in the DG revealed a

 

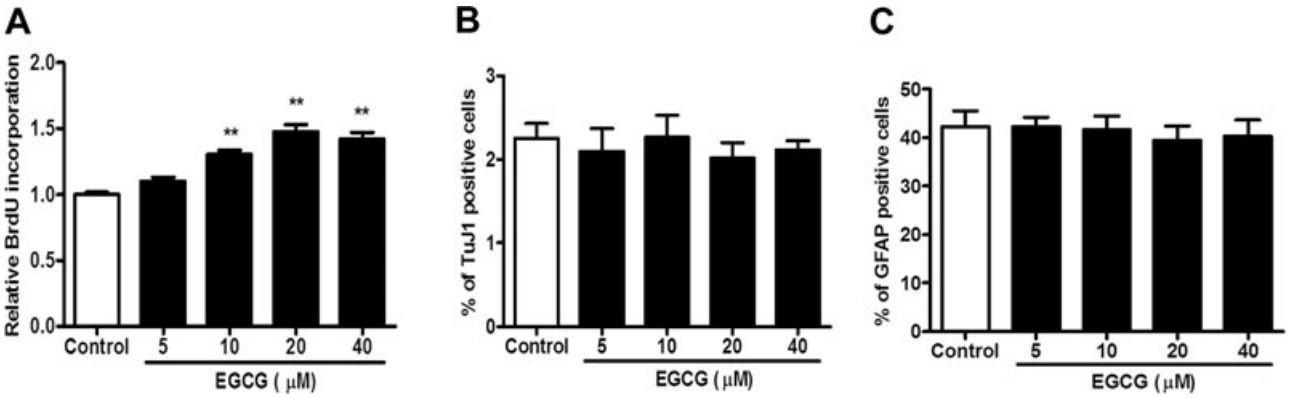


**Figure 1.** EGCG treatment leads to increased proliferation but not to differentiation of adult hippocampal NPCs in vitro. (A) Adult NPC proliferation was analyzed by BrdU incorporation. Data are represented as relative levels of BrdU incorporation after EGCG treatment relative to controls (untreated). After adult NPC cultures were treated with EGCG (0–40 μM) for 24 h, BrdU incorporation was measured. One-way ANOVAs followed by post hoc comparisons revealed significant differences between EGCG and control groups at 40, 20, and 10 μM treatment concentrations. Adult NPCs were treated with EGCG (0–40 μM) in differentiation media for 4 days. Quantitative analyses showing that, under differentiation conditions, adult NPCs treated with EGCG at 0–40 μM for 7 days exhibited equal neuronal differentiation capacities compared with controls (untreated) (B and C). Scale bar, 50 μm. $^{**}p < 0.01$ versus control. Data in each panel represent the means ± SEM from at least three independent experiments.

significant increase (40.9%) in the BrdU-positive cell number after chronic EGCG treatment (Fig. 2B). Thus, EGCG indeed stimulated the proliferation of adult NPCs in the hippocampus. No significant differences in DG volume were observed between groups (Fig. 2C). However, the effect of EGCG treatment on cell proliferation was confirmed by the calculation of the hippocampal cell density, which differed between groups ($p < 0.01$) (Fig. 2D).

To further confirm whether EGCG is anticipated to enhance the neurogenic effect, animals were killed 2 h after the BrdU injection, and brain slices were examined for DCX and $BrdU^+/DCX^+$ immunostaining (Supporting Information Fig. S2A). To evaluate the effects of EGCG on cell differentiation in vivo, proliferating cells in mice were labeled with BrdU and characterized after 4 weeks (newborn cells were allowed to differentiate for 4 weeks) by their expression of either NeuN (mature neuronal marker) or GFAP (astrocyte marker) (Supporting Information Fig. S2B and C). Double-positive cells in the GCL were counted (Fig. 3A to C). The fraction of neurons or astrocytes among BrdU-labeled cells in the GCL was similar between EGCG-treated and untreated mice (the percentage of mature neurons in control mice was 80.9 ± 2.0%, and that in EGCG mice was 84.7 ± 1.8%; the percentage of astrocytes in control mice was 4.35 ± 0.5%, and that in EGCG mice was 5.26 ± 1.2%; there were no significant differences in the number of neurons or astrocytes between EGCG and control mice). Together, these data indicate that EGCG enhances neurogenesis but does not influence the differentiation of newborn cells in the DG that were derived from adult NPCs.

### 3.3 EGCG improves the spatial learning and memory of mice

Changes in learning have been associated with changes in the level of neurogenesis in the adult DG [6]. We thus sought to determine whether this increase in neurogenesis was associated with improved spatial learning and memory performance. In the acquisition phase, we initiated training using

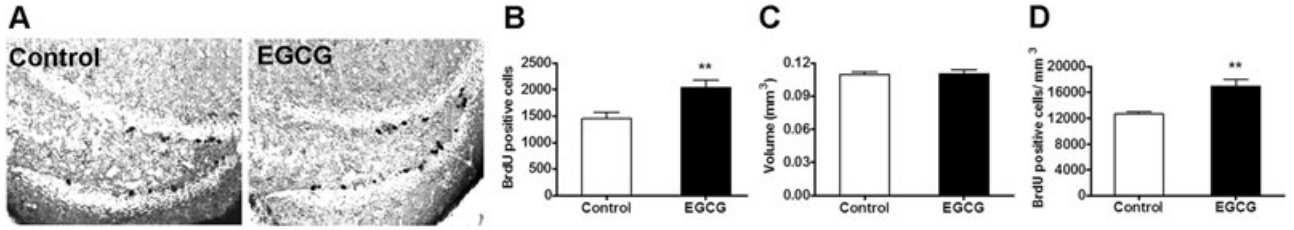


**Figure 2.** EGCG promoted the proliferation of adult hippocampal NPCs in vivo. (A) Representative immunohistochemistry images of the BrdU-positive cells in the SGZ of EGCG-treated and control PBS-treated mice. Quantitative analysis showed EGCG increased the number of newborn cells (B) and cell density of the hippocampal DG (D) compared with PBS controls. No statistical differences were observed in hippocampal volume (C) between groups. $^{**}p < 0.01$ versus control. Data represent means ± SEM from three independent experiments.

 

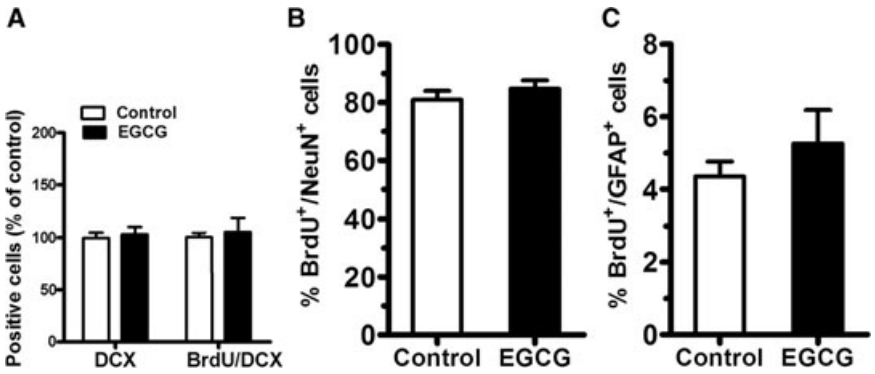


**Figure 3.** EGCG did not influence the differentiation of adult hippocampal NPCs in vivo. Numbers of immature neurons, mature neurons, and astrocytes in the SGZ of mice were determined at 2 h and 4 weeks after BrdU injection. Quantifications of double-labeled immature neurons (A), mature neurons (B), and astrocytes (C) in the SGZ showed were no statistical differences between EGCG-treated and control mice ($n = 5$–6 per group).

a visible platform to give the animals an opportunity to acclimate to test conditions and learn to find the visible platform over the course of 3 days (three trials per day). As shown in Fig. 4A, EGCG-treated mice performed similarly to controls, and the visible platform escape latency did not differ between groups. Next, the mice were trained for 7 days to find a hidden platform using spatial cues located throughout the room. Both groups showed a reduced latency to find the hidden platform across training days. The EGCG-treated mice required less time to find the hidden platform than the controls (Fig. 4B). In the probe trial phase that occurred 24 h after the last training day, mice were given a 1-min trial to measure the percentage of total time they spent in each quadrant of the maze. The EGCG-treated mice spent a significantly larger proportion of time (51.3 ± 2.7% of the time) in the target quadrant (i.e., the quadrant in which the platform was located during the hidden platform training) than did the controls (42.5 ± 2.5% of the time), $p < 0.01$ (Fig. 4C). In addition, there were no significant differences between groups in swimming speed ($p > 0.05$) or locomotor abilities ($p > 0.05$). Together, our results suggest that EGCG enhances learning and memory in 4-month-old mice as shown by improvements in object recognition and spatial memory.

### 3.4 The Shh/Gli1 signaling pathway regulates the effect of EGCG on adult hippocampal NPC proliferation in vitro

Because EGCG administration significantly increased adult NPC proliferation, we attempted to elucidate the mechanisms underlying this effect. The Shh/Gli1 signaling pathway is important in the maintenance and proliferation of NPCs in the adult rodent brain [20, 33], so we investigated whether Shh activation is responsible for the effects that occur during this process. The Shh pathway begins when the secreted Shh peptide binds to its membrane-bound receptor Ptc, thus

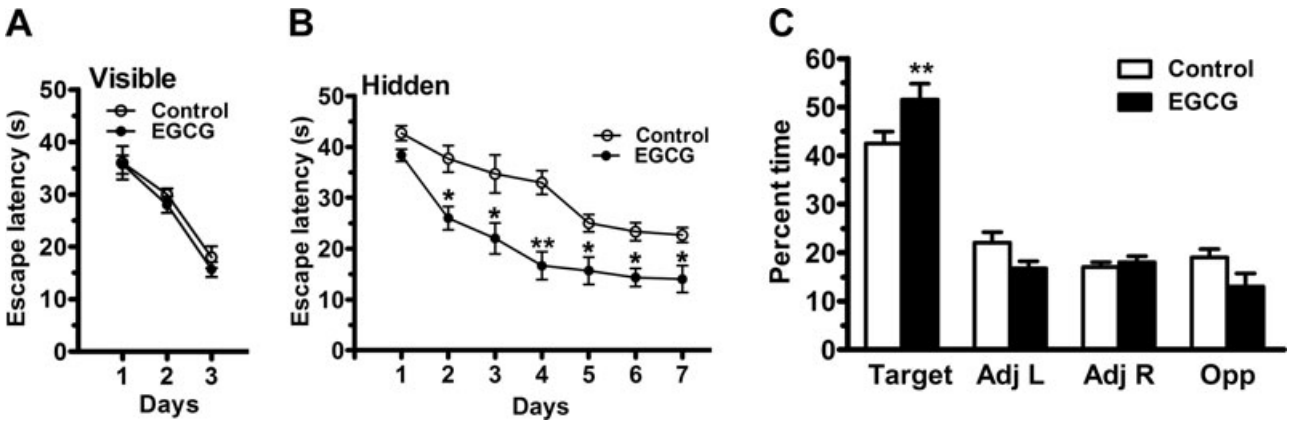


**Figure 4.** Mice treated with EGCG performed better in the Morris water maze. (A) From days 1 to 3, we measured the latency to reach the platform. The performance of EGCG-treated mice did not differ from that of PBS-treated controls. (B) From days 4 to 10, the animals were trained with a hidden platform to test their spatial learning abilities. Vehicle control mice took a significantly longer time (latency) than the EGCG-treated mice to find the hidden platform. (C) On day 11, memory was evaluated with a probe test. The graph represents the percentage of time (in 60 s) that the mouse spent in the quadrant that had previously contained the hidden platform (target quadrant). Target, adjacent (Adj) left (L) or right (R) and opposite (Opp) refer to quadrants. Both groups spent more time in the target quadrant during training. Furthermore, EGCG-treated mice spent significantly more time in the target quadrant (51.5 ± 6.6% of the time) than control mice (42.5 ± 4.8% of the time). *$p < 0.05$, **$p < 0.01$ versus control. Data are the means ± SEM.

 

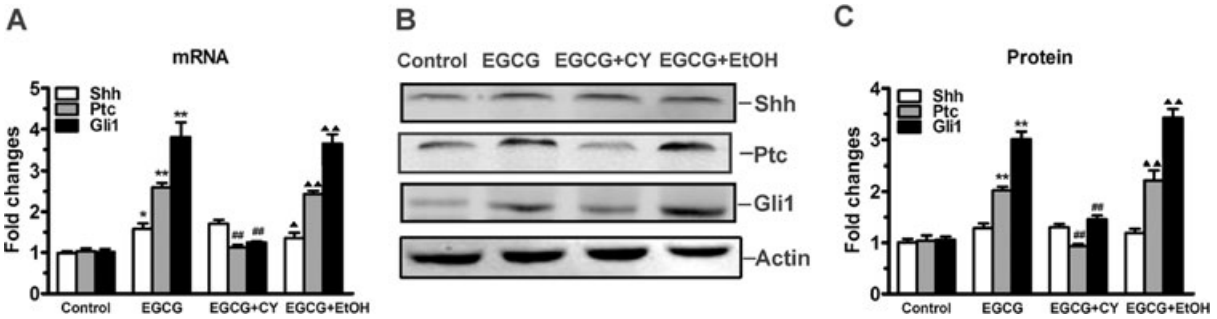


**Figure 5.** Treatment with EGCG increased Shh signaling pathway components in adult hippocampal NPCs in vitro. After adult NPC cultures in 24-well plates were treated with media alone (control), EGCG (20 μM), EGCG with the Shh antagonist cyclopamine (CY, 5 μM) or an equal dose of ethanol (EtOH) used as a carrier for 24 h. Quantitative analysis showed that the incubation of adult NPCs with EGCG significantly increased mRNA (A) levels and protein (C) levels of Ptc and Gli1, and this effect was blocked by cyclopamine. (B) Representative Western blot images. $*p < 0.05$, $**p < 0.01$ versus the control, $\#\#p < 0.01$ versus the EGCG group and $\blacktriangle p < 0.05$, $\blacktriangle\blacktriangle p < 0.01$ versus the control, respectively. Data represent the means ± SEM from five independent experiments.

relieving its inhibition of Smoothened (Smo). Next, a complex signaling cascade involving the transcription factors of the Gli family is triggered. Finally, target genes including Ptc and Gli are activated [34]. The only Gli factor to be transcriptionally induced following Shh pathway activation in the SGZ is Gli1, which is required for self-renewal of the adult NPCs [35]. Therefore, Gli1 is a principal effector of Shh signaling in adult NPCs and is classically used as a sensitive measure of pathway activation. We first observed gene expression in this signaling pathway. Real-time RT-PCR (Fig. 5A) and Western blot analyses (Fig. 5B and C) revealed that adult NPCs expressed Shh, Ptc, and Gli1, and treatment with EGCG significantly upregulated these genes. To test whether Shh signaling is necessary for EGCG to promote the proliferation of adult NPCs, we treated adult NPCs with EGCG in the presence of the Shh antagonist cyclopamine. Cyclopamine is a small-molecule plant alkaloid that selectively inhibits Shh signaling and is thought to function by directly binding to Smo [36], which is the signaling component of the Shh receptor complex. The application of cyclopamine (5 μM) abolished the ability of EGCG to induce Ptc and Gli1 expression, but it did not suppress EGCG-induced Shh expression (Fig. 5A to C). In addition, cyclopamine significantly counteracted the EGCG-induced increase in the number and size of neurospheres and the number of BrdU-positive cells (Fig. 6). Conversely, when an equal dose of ethanol was used as a carrier for in vitro culture, cyclopamine had no similar effect (Fig. 6). These data indicate that the Shh/Gli1 pathway is involved in EGCG-enhanced adult NPC proliferation.

### 3.5 EGCG upregulates hippocampal gene and protein expression in the Shh signaling pathway

We further investigated whether the Shh pathway is a candidate for the regulation of EGCG-induced adult NPC cell proliferation in vivo. Although our in situ hybridization results are consistent with previous reports that Shh transcripts are not present at detectable levels in the adult hippocampus,

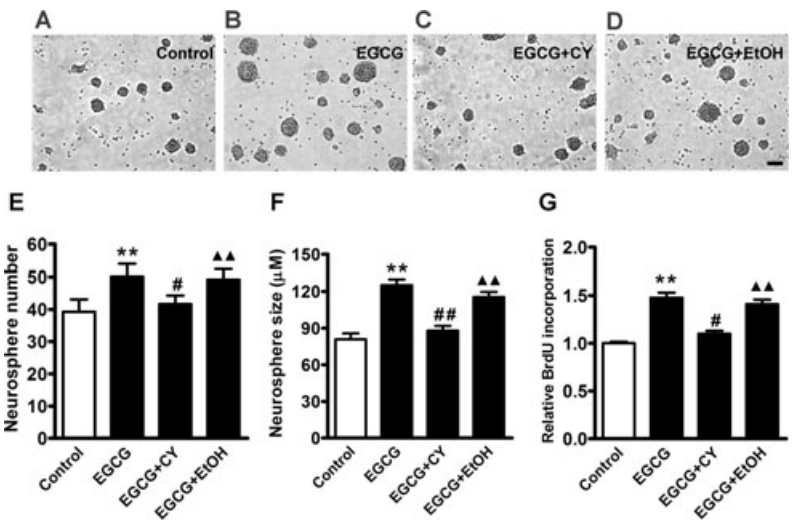


**Figure 6.** Cyclopamine inhibited the EGCG-induced proliferation of adult hippocampal NPCs in vitro. Representative neurospheres are shown in (A–D). Adult NPC proliferation was assessed by BrdU incorporation. Data are represented as relative levels of BrdU incorporation relative to the controls (untreated). Quantitative data show that cyclopamine partially inhibited EGCG-induced increases in neurosphere number (E), size (F), and BrdU-positive cells (G). Scale bar, 50 μm. $**p < 0.01$ versus controls, $\#p < 0.05$, $\#\#p < 0.01$versus the EGCG group, and $\blacktriangle\blacktriangle p < 0.01$ versus the controls, respectively. Data represent the means ± SEM from five independent experiments.

 

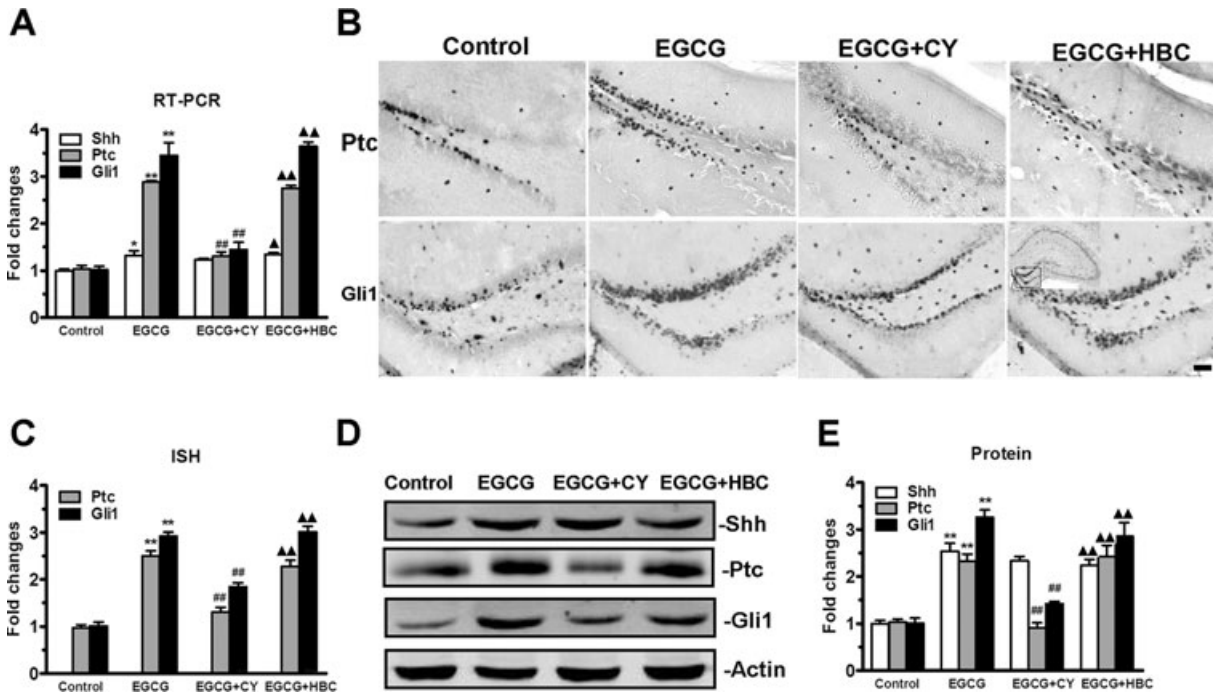


**Figure 7.** Treatment with EGCG increased the expression of Shh signaling pathway components in the adult hippocampus. Beginning at 2 months of age, male mice were intraperitoneally injected with EGCG (20 mg/kg) or PBS for 60 days, after which some mice were given intraperitoneal injections of cyclopamine at 10 mg/kg/day for 10 days or HBC alone as a control ($n = 4$–5 per group). (A) Quantitative analysis of real-time RT-PCR showed that EGCG significantly increased Ptc and Gli1 mRNA levels in the hippocampus, and this effect was blocked by cyclopamine. Representative images of the in situ hybridization (B) and quantitative analysis of these data (C) revealed that cells residing in the DG expressed Ptc and Gli1, and treatment with EGCG significantly upregulated these genes. In addition, cyclopamine significantly counteracted the EGCG-induced increase of Ptc and Gli1 transcripts, whereas an equal dose of HBC used as a carrier in vivo was not similarly affected by cyclopamine. (D) Representative images of the Western blot analysis. (E) Quantitative analysis of Western blots showed that protein levels of Shh, Ptc, and Gli1 were significantly increased in the hippocampus following EGCG treatment. The application of cyclopamine abolished the ability of EGCG to induce Ptc and Gli1 expression, but it did not suppress EGCG-induced Shh expression. Scale bar, 50 μm. $^{*}p < 0.05$, $^{**}p < 0.01$ versus controls, $\#\#p < 0.01$ versus the EGCG group, and $^{\blacktriangle}p < 0.05$, $^{\blacktriangle\blacktriangle}p < 0.01$ versus the controls, respectively. Data represent the mean ± SEM for fold changes in controls.

we detected Shh mRNA transcripts using real-time RT-PCR. In addition, if Shh signaling is active in the adult hippocampus, the Ptc receptor and target gene Gli1 should also be expressed. Therefore, we analyzed the expression patterns of Shh signaling targets. Real-time PCR revealed a 3- and 3.5-fold increase in Ptc and Gli1 mRNA levels, respectively, in the hippocampus of EGCG-treated mice compared with controls (Fig. 7A). We also used in situ hybridization to measure the gene expression in the hippocampus and observed a robust expression of Ptc mRNA in EGCG-treated mice, whereas the control mice had minimal Ptc mRNA expression. In situ hybridization also revealed that the upregulated Ptc mRNA was located within the GCL and SGZ of the DG. This upregulation of Ptc mRNA was restricted to the DG and was not observed in any other hippocampal subregion. These findings are consistent with the hypothesis that Shh-dependent transcription takes place in hippocampal adult NPCs after EGCG treatment. The expression of Gli1 mRNA was also strongly increased, which is consistent with the results from the quantitative PCR (Fig. 7B and C).

Because high expression of Ptc and Gli1 mRNA is an indicator of activity in the Shh pathway, we investigated whether EGCG treatment also influences the expression of the Shh pathway protein constituents. Western blotting confirmed that EGCG increased the levels of Shh, Ptc, and Gli1 by 2.5-, 2.3- and 3.2-fold, respectively, in whole-hippocampus homogenates (Fig. 7D and E). Furthermore, quantitative analysis also corroborated our in vitro work indicating that cyclopamine inhibits the expression of Ptc and Gli1 proteins (Fig. 7A to E). Based on these studies, we conclude that EGCG treatment causes the activation of Shh signaling in the hippocampus.

Although we detected Shh transcripts in the adult hippocampus using real-time RT-PCR, Shh is predominantly expressed in several adult basal forebrain structures that are known to project to the DG. Previous reports have demonstrated that the basal forebrain may regulate adult neurogenesis by transporting Shh to the hippocampus [21]. In addition, findings from Chou et al. [15] suggest that EGCG facilitates $Ca^{2+}$-dependent glutamate release via activation of

 

protein kinase C in the cerebral cortex. Glutamate is the major neurotransmitter regulator of neurogenesis, and recently its interaction with Shh has been further considered [17]. To understand the cause of increased Shh secretion in response to EGCG, we performed microdialysis in freely moving animals to measure the extracellular concentrations of glutamate in the mouse basal forebrain. In the EGCG-treated mice, the mean glutamate concentration was 40% higher ($2.09 \pm 0.68$ μM; $n = 4$) than in controls ($1.49 \pm 0.13$ μM; $n = 4$) ($p < 0.05$). These results introduce the intriguing possibility that EGCG-induced alterations of glutamate levels in the basal forebrain may be responsible for the observed elevation in Shh expression.

### 3.6 Inhibition of Shh signaling in vivo attenuates EGCG-induced adult NPC proliferation in the adult hippocampus

To determine whether the inhibition of Shh signaling in vivo would alter the ability of EGCG to enhance the proliferation of NPCs in the adult hippocampus, we blocked Shh pathway activity using daily intraperitoneal injections of cyclopamine or of the vehicle (HBC carrier) alone for 10 days. This method does not injure the brain, which is a common, unavoidable result of direct intracerebral injections [20]. The animals were killed 2 h after the injection of BrdU, and we then immunostained their brains to investigate the effects of cyclopamine on the proliferation of adult NPCs. We analyzed the number of BrdU-positive cells in the DG. Immunohistochemistry confirmed that cyclopamine treatment of adult mice resulted in a decrease of BrdU-positive cells within the DG compared with animals receiving EGCG. In contrast to cyclopamine-treated counterparts, animals receiving HBC exhibited a significant increase in the number of BrdU-positive cells, similar to EGCG-treated mice (Fig. 8A). The quantification of the proliferating cells in the hippocampus of cyclopamine-treated mice showed a significant reduction in cell proliferation ($p < 0.05$) (Fig. 8B). These results indicate that the proliferation of adult NPCs in the EGCG-treated mice depends at least in part on Shh signaling.

## 4 Discussion

There is growing interest in the beneficial effects of the green tea component EGCG in the aging and diseased brain [10]; however, the cellular/molecular mechanism underlying these effects has not been fully established. The major findings of this study are as follows: EGCG promotes adult NPC proliferation in vitro and in vivo, generally indicating that EGCG enhances adult neurogenesis in the hippocampus, and this effect requires activation of the Shh pathway in hippocampal adult NPCs because it could be partially blocked by a selective antagonist.

Neurodegenerative diseases represent a growing public health challenge. Current medications provide limited benefit by alleviating certain symptoms, but none halt or retard neurodegeneration. Various naturally occurring compounds have been analyzed for their safety and efficacy in the modulation of these pathological events, and one such promising compound achieving worldwide popularity is green tea. Continuing research in humans and animal models indicates that dietary supplementation with green tea has an impact on cognitive deficits in individuals of advanced age [9, 36, 37]. As a consequence, EGCG, which is the most significant active polyphenol component in green tea, is now being considered as a preventive and therapeutic agent that alters brain aging and progressive neurodegenerative disorders, such as PD and AD [13, 38].

Adult hippocampal neurogenesis is a unique form of neural circuit plasticity that is present throughout life [39, 40]. There is ample evidence of a crucial role for hippocampal neurogenesis in mediating specific cognitive functions [2, 41, 42]. Furthermore, it is becoming increasingly clear that adult neurogenesis readily responds to and is correlated with brain pathology. Decreased or abnormal hippocampal neurogenesis is strongly correlated with deficits in hippocampal structure and function in various neurological disorders including depression, epilepsy, schizophrenia, cerebral ischaemia, AD, PD, HIV infection, and drug addiction [43]. Of interest was the finding that treatments that ameliorate the behavioral alterations in animal models or humans typically enhance or normalize neurogenesis [44, 45]. Thus, adult hippocampal neurogenesis may be an essential therapeutic target for the behavioral effects of distinct neuropsychiatric diseases. Stem cells in neurogenic areas of the adult mammalian brain are able to switch between quiescence and proliferation, so it is pivotal to trigger their reactivation. Chell and Brand [46] show that, in *Drosophila*, neural stem cell reactivation is induced in response to a nutritional stimulus. In this study, we observed that EGCG enhanced adult hippocampal neurogenesis; it has also been reported that EGCG rescues brain volume in mice with accelerated senescence [47] and in mice with a deletion of the DYRK1a gene [48], which is an essential gene for normal adult neurogenesis. Thus, our findings provide critical evidence for EGCG regulation of adult hippocampal neurogenesis that may contribute to the improvement of cognitive function.

Shh is conserved and functions prominently in both the developing nervous system and the germinal zones of the adult brain. Although our in situ hybridization results showed that Shh transcripts were not present at detectable levels in the adult hippocampus, we detected Shh expression in this region using RT-PCR. A possible explanation for this discrepancy is that the latter is a more sensitive technique. This observation is consistent with a report from the Schaffer laboratory [21], which reported that the septal (i.e., fimbria) fibers projecting to the hippocampus are a source of Shh within this structure. Although Shh immunofluorescence was not evident, it seems likely that the cells express low levels of Shh. As described by Machold et al. [19], Shh staining could only be visualized by LacZ enzymatic amplification in the CA3

 

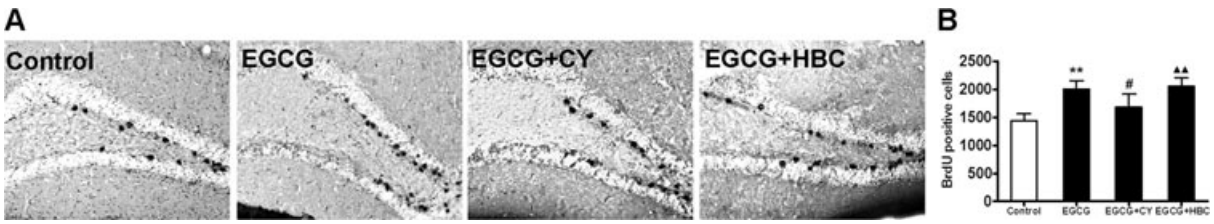


**Figure 8.** Cyclopamine blocked EGCG-induced adult hippocampal NPC proliferation in vivo. (A) Representative sections of hippocampal tissue used for BrdU immunohistochemistry are shown for each of the treated groups. Quantitative analysis showed that cyclopamine significantly reduced the number of EGCG-mediated increased BrdU-positive cells (B) in the DG. $^{**}p < 0.01$ versus control, $\#p < 0.05$ versus the EGCG group, and $^{\blacktriangle\blacktriangle}p < 0.01$ versus the control, respectively. Data represent means ± SEM.

and hilus regions of the hippocampus. The upregulation of Ptc and Gli1 mRNA in the DG was observed in the SGZ, which further supports that adult hippocampal progenitors may receive an enhanced Shh signal following EGCG treatment. Moreover, the inhibition of adult NPC proliferation in the SGZ by cyclopamine further supports the hypothesis that endogenous Shh signaling is required for adult NPC expansion. In addition, mature granule neurons also express the Shh receptor Ptc, and EGCG effects on hippocampus-dependent learning and memory could be mediated by both newborn and older neurons. Several synthetic and naturally occurring small-molecule modulators of Shh signaling pathway have been discovered, and most of them act at the level of Smo, such as cyclopamine. We chose cyclopamine because in the previous reports of Shh regulation the proliferation of adult NPCs [20, 21, 49–51], the cyclopamine is the general inhibitor, which can inhibit transcription of Gli1, Ptc, and other Shh-target genes. However, other Shh-related protein targets can also be explored in the future. Many reports have illustrated the targeting of upstream elements (robotnikinin) and downstream elements (GANT58, GANT61) of Smo [52]. Robotnikinin is a synthetic inhibitor, which binds directly to Shh. In contrast, GANT58 and GANT61 act downstream of Smo and repress Gli-mediated transcription. We may take advantage of different classes of small-molecule Shh inhibitors to gain greater mechanistic understanding of the processes of Shh regulation of adult neurogenesis.

How Shh signaling is activated by EGCG is not yet clear, but specific neurotransmitters in the neurogenic niche can be considered; for example, neurotransmitters such as glutamate and GABA are regulated by EGCG [15, 16]. Neurotransmitters have largely conserved signaling functions across phyla, and one possibility is that neurotransmitters are integrated with other well-conserved and critical molecules to activate intracellular pathways implicated in neurogenesis. A recent finding indicates that Shh is a chemoattractant for midbrain dopaminergic axons [53]. Furthermore, changes in monoamine levels through chemical depletion in vivo have been shown to regulate the expression of the Shh signaling cascade in the adult rodent brain [54]. It was also reported that electroconvulsive seizures upregulated Shh signaling pathways in the SGZ, suggesting that neurotransmitters released during seizures may impact the expression of Shh and its receptors [49]. It is thus conceivable that EGCG may regulate the expression of Shh components via neurotransmitters. In addition, Shh appears to be expressed primarily by GABAergic neurons in the basal forebrain, which raises the possibility that the activity-dependent synthesis or secretion of Shh into the hippocampus by these neurons may be a mechanism for the regulation of neurogenesis. Supporting this hypothesis, our experiments showed a significant increase in basal forebrain glutamate release after EGCG administration. Future studies will help to elucidate the involvement of glutamate with other neurotransmitters and targets upstream of Shh pathway in the regulation of Shh signal.

To date, numerous extrinsic factors have been implicated in regulating adult NPCs [43], such as growth factors, neurotrophins, and morphogens, including Wnt and Noggin [55, 56]. Additionally, intrinsic factors, such as transcription factors, comprise major components of control over the process of adult neurogenesis. There is also increasing evidence that epigenetic mechanisms, such as DNA methylation, chromatin remodeling, histone modification, and noncoding RNA expression, are closely associated with multiple aspects of adult neurogenesis [57]. Taken together, these findings highlight the complexity and specificity of regulation during adult neurogenesis. Moreover, Shh inhibitor cyclopamine could not completely abolish the proliferation effect of EGCG on adult NPCs. Hence, it is unlikely that the increase in adult hippocampal neurogenesis was specifically attributable to Shh, and the involvement of above regulatory factors should be taken into consideration in EGCG-induced adult neurogenesis in future studies. Recent studies indicate that the progression from adult NPCs to mature neurons is tightly controlled in distinct stages of adult neurogenesis, including the proliferation of NPCs, the migration of neuroblasts, and the integration of newborn neurons [58]. Here we demonstrate that EGCG acts directly on adult hippocampal NPCs to favor neuron production. However, we cannot rule out the possibility of an effect of EGCG on other stages of adult hippocampal neurogenesis. Future experiments need to target the above aspects for an integrated description of molecular mechanisms of EGCG on adult NPC regulation.

Green tea has a varied reputation, from a simple ancient beverage to a nutrient endowed with possibly benefi-

 

cial neurobiological–pharmacological actions. Based on previous safety and pharmacokinetic studies, it is likely that a daily 1500–1600 mg bolus of EGCG in humans would achieve physiological levels similar to those in the sera of EGCG-treated (20 mg/kg) mice [59]. Oral doses of similar magnitudes have been used in clinical trials, although EGCG has not been administered to humans on a regular basis. In summary, our data provide a new explanation for the numerous studies showing health benefits of green tea on cognition. These findings warrant a general recommendation to consume green tea regularly for disease prevention and provide support that EGCG may have therapeutic uses for treating neurodegenerative disorders.

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