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# Hippocampal VEGF is necessary for antidepressant-like behaviors but not sufficient for antidepressant-like effects of ketamine in rats

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ABSTRACT

We investigated the effects of ketamine on both the temporal and spatial profiles of neural precursor cells located in the hippocampus, and on antidepressant-like behaviors in rats. A single dose of ketamine resulted in a significant increase in the number of 5-bromo-2-deoxyuridine-positive ($BrdU^+$) cells in the dentate gyrus (DG) of rats at 24 h, but not at 28 days, after treatment completion. Ketamine caused antidepressant-like behaviors in the forced swim test (FST) and novelty suppressed feeding test (NSFT). Viral-mediated hippocampal knockdown of vascular endothelial growth factor (VEGF) produced depressive-like behaviors in the FST and NSFT, which were partially recovered by ketamine to the level observed in the control group. The behavioral effects of VEGF knock down were accompanied by a decrease in hippocampal neurogenesis, which was also partially recovered by ketamine.

Our results suggest that basal hippocampal VEGF expression is necessary for ketamine-induced antidepressant-like behaviors in rats, but ketamine-induced VEGF expression only partially contributes to hippocampal neurogenesis and the antidepressant-like effects of ketamine.



## 1. Introduction

Major depressive disorder (MDD) affects approximately 7% of the adult population in the U.S. in any given year, with a lifetime prevalence of approximately 20% [1]. MDD is commonly treated with monoaminergic-transmission-based antidepressants, such as serotonin selective reuptake inhibitors (SSRIs). However, between 20% and 30% of patients do not respond adequately to current antidepressant therapies. Ketamine, a noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, has shown remarkable consistency in rapidly ameliorating depressive symptoms in MDD [2]; however, the targets and actions of ketamine are still unclear at the biochemical level. It has been previously demonstrated that ketamine affects several signaling cascades in the brain; it stimulates mammalian target of rapamycin complex 1 (mTORC1) [3], brain-derived neurotrophic factor (BDNF) expression [4], and glutamate release [5]. In addition, it increases neurogenesis in the dentate gyrus (DG) of adult rodents [6]. This is a particularly attractive property, as it has been shown that adult-generated new neurons make a significant contribution to alleviating psychiatric disorders [7]. Notably, adult neurogenesis, especially that which occurs in the subgranular layer of the hippocampus [8,9], is affected by antidepressants, including SSRIs [10].

Neurotrophins, such as BDNF, are associated with mood disorders, and antidepressant drugs act, at least in part, via neurotrophic-factor-involved pathways [11,12]. Evidence also points to the additional involvement of vascular endothelial growth factor (VEGF) in the neurobiology of chronic stress [13] and MDD [14]. For example, diverse antidepressant therapies, including drugs and electroshock therapy, increase VEGF expression in the hippocampus of rodents [15], and antagonism of VEGF type 2 receptor (VEGFR2) abolishes antidepressant-like behavioral effects in preclinical models for depression [15,16]. Furthermore, VEGF stimulates adult neurogenesis in the subgranular and subventricular zones, with important implications for the pathophysiology and treatment of MDD [17].

Similar to classical antidepressants, ketamine has been shown to regulate BDNF expression [18]. However, the involvement of VEGF in mediating the antidepressant-like effects of ketamine has not been studied.

The aim of the present study was to investigate whether ketamine regulates VEGF expression in the DG of the adult rat hippocampus and the potential influence of ketamine-induced VEGF on hippocampal neurogenesis. We also wanted to determine if ketamine-induced VEGF expression affects the antidepressant-like effects of ketamine.

*Abbreviations:* BrdU, bromodeoxyuridine; DG, dentate gyrus; GCL, granule cell layer; SGZ, subgranular zone; VEGF, vascular endothelial growth factor.

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## 2. Results

### 2.1. Ketamine enhances hippocampal neurogenesis

As it is known that antidepressants increase hippocampal neurogenesis in the adult brain [19–21], we investigated whether ketamine treatment results in the proliferation and survival of neural progenitor cells in the hippocampal DG in rats (Fig. 1A, B). We quantitated the number of new cells in the subgranular zone (SGZ) of the DG (Fig. 1C). When immunohistochemical analysis was performed 24 h after treatment with ketamine (Fig. 1A), the number of 5-bromo-2-deoxyuridine-positive ($BrdU^+$) cells increased approximately two-fold relative to control animals (Fig. 1D). It takes approximately 4 weeks for newly divided cells to migrate over the granule cell layer (GCL) and function as mature granule cells [22]. However, four weeks after ketamine treatment we no longer observed a significant increase in $BrdU^+$ cell numbers in the GCL (Fig. 1C, E), indicating that the ketamine treatment did not support the survival of new DG cells. The majority of the $BrdU^+$ cells were neuronal, as detected by BrdU–neuron-specific nuclear (NeuN) colocalization (Fig. 1F). There was no significant difference in the percentage of cells double labeled for BrdU and NeuN between ketamine-treated and control animals when investigated 28 days after the injection of ketamine and BrdU (Fig. 1F, G), despite the 83% initial increase in the number of $BrdU^+$ cells in ketamine-treated rats in comparison to control rats.

These results indicate that baseline neurogenesis is increased by ketamine at early time points after treatment (24 h); however, it had no significant effect on neuronal maturation or survival.

### 2.2. Ketamine induces hippocampal VEGF

We then investigated the effect of *in vivo* administration of ketamine on VEGF expression by administering a single dose of either ketamine (10 mg/kg) or saline to young adult male rats. Because new granule neurons take 4 weeks to mature, the functional effects of an increase in granule cell numbers, including VEGF expression are likely to be observed only over a longer time course. In addition, we wanted to know whether ketamine has long-lasting effects on VEGF expression. Therefore, we investigated the effects of ketamine on VEGF expression at two time points; 24 h and 28 days after ketamine treatment. We found that the protein levels of VEGF were highly and significantly upregulated in ketamine-treated rats 24 h, but not 28 days, after ketamine injection (Fig. 2A, B: $P < 0.001$; $P > 0.05$, respectively). Consistently, the increased immunoreactivity of VEGF was observed in the DG area 24 h, but not 28 days, after the injection of ketamine, compared to the saline controls (Fig. 2C, D$P < 0.05$; $P > 0.05$, respectively). Our results indicate that the single dose of ketamine induced hippocampal VEGF expression, and that this initial increase in VEGF expression returns to basal levels within 28 days of treatment.

### 2.3. Ketamine-induced VEGF in the DG contributes partially to antidepressant-like behaviors

VEGF has been implicated in hippocampal neurogenesis in the rodent brain [15,23] and it is also known to mediate antidepressant action [15]. As ketamine induces both VEGF expression and antidepressant-like behavior in rats, we speculated that ketamine-induced VEGF contributes to the antidepressant-like effects of ketamine. To test this hypothesis, we knocked down hippocampal VEGF in rats by bilateral administration of a lentivirus expressing small hairpin RNAs (shRNAs) targeted against rat VEGF (lenti–shVEGF–enhanced green fluorescent protein [EGFP]) into DG (Fig. 3). A vector expressing only EGFP, lenti–EGFP, was used as a control. Injection of the lenti–shVEGF–EGFP vector resulted in widespread gene expression, as shown by EGFP fluorescence 4 weeks after the injection (Fig. 3A), when the lentivirus is fully

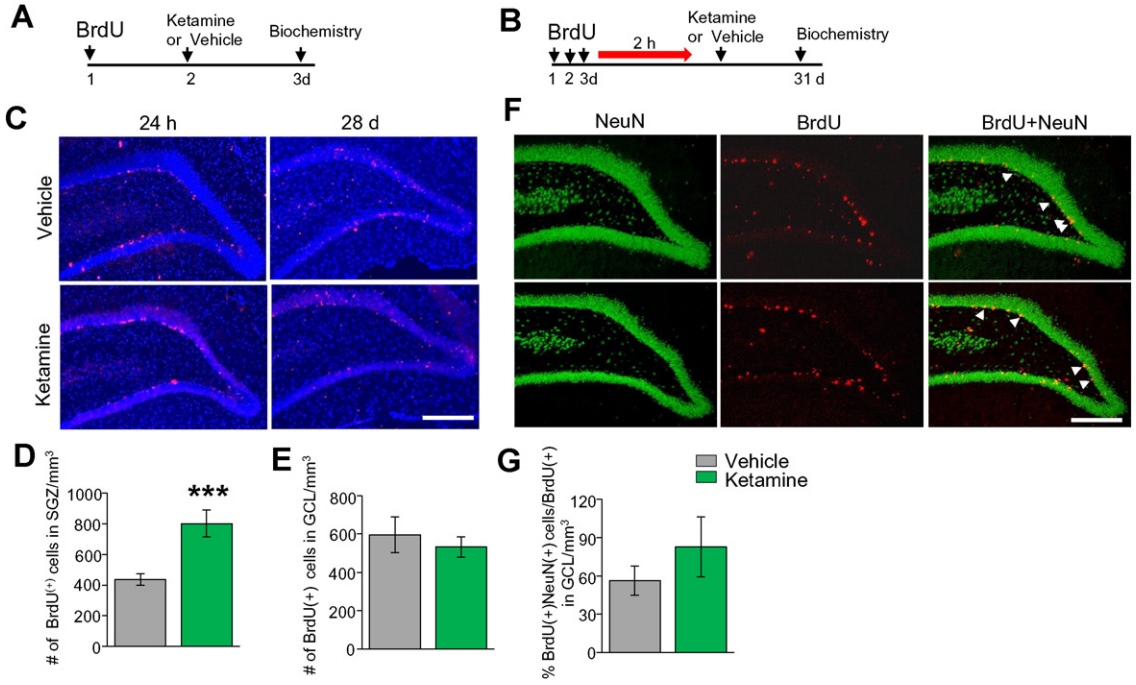


**Fig. 1.** Effects of ketamine treatment on neurogenesis in the hippocampal dentate gyrus. Experimental paradigms: (A) and (B). (C) Representative images of brain sections labeled for BrdU (red). $BrdU^+$ cells were observed in the subgranular zone (SGZ) of the DG or in the granule cell layer (GCL). (D, E) Total number of $BrdU^+$ cells per unit volume ($mm^3$) in SGZ and GCL. Stereological three-dimensional counting revealed that ketamine treatment, compared with saline treatment, produced a significant 83.7% ± 2.3% increase (saline 436 ± 38 cells/$mm^3$ vs. ketamine 801 ± 87 cells/$mm^3$, n = 6 animals in each group) in $BrdU^+$ cells in SGZ when analyzed 24 h after treatment completion (D), but not in the GCL 28 days after treatment completion (E) (saline 595 ± 93 cells/$mm^3$ vs. ketamine 532 ± 54 cells/$mm^3$, n = 9 animals in each group). (F) Representative images of brain sections double labeled (yellow; arrowhead) for NeuN (green) and BrdU (red) 28 days after treatment with ketamine. Fluorescent signals were detected using a confocal laser-scanning microscope, and separate optical images of BrdU and NeuN immunoreactivity were captured from the same optical view. (G) Twenty-eight days after treatment the percentage of $BrdU^+$ cells double-stained with NeuN was 56.3% ± 11.3% in the control rats and 82.5% ± 23.4% in the rats treated with ketamine; there was no significant difference. Data are presented as mean ± SEM. Student's *t*-test: $^{***}P < 0.001$. 24 h, 24 h after ketamine treatment; 28 days, 28 days after ketamine treatment. Arrowhead, $BrdU^+/NeuN^+$ cells. Scale bar, 100 μm.

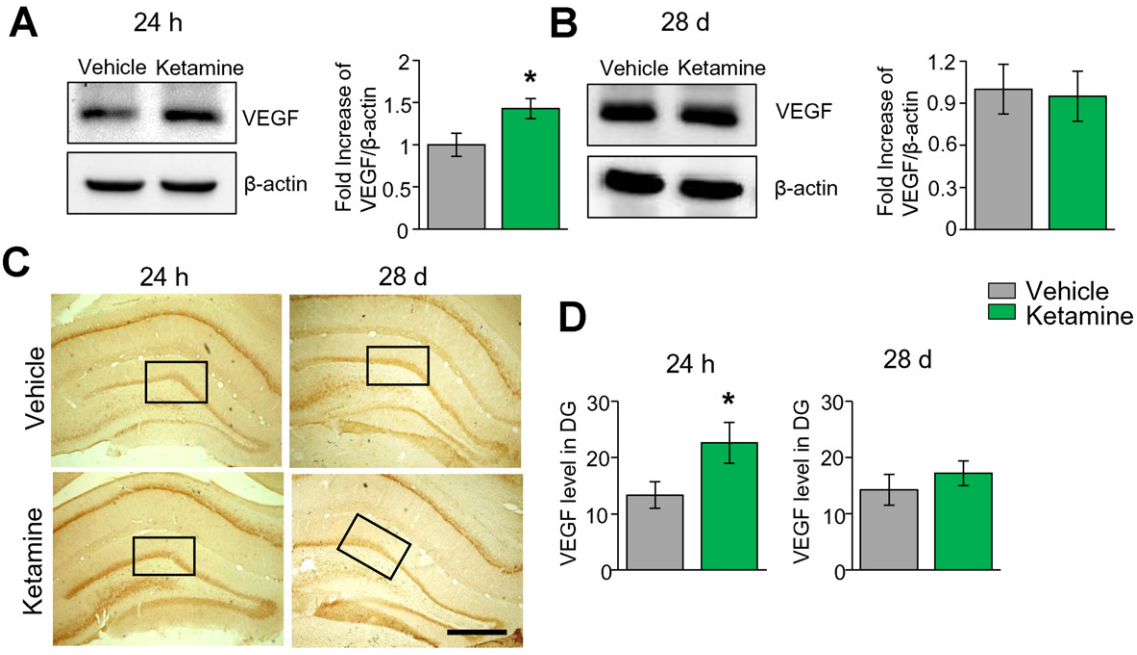


**Fig. 2.** Induction of hippocampal VEGF 24 h and 28 days after ketamine injection. VEGF levels in the hippocampi of rats (A) 24 h and (B) 28 days after ketamine injection by Western blotting. A significant effect of ketamine on VEGF expression is seen 24 h after ketamine treatment ($^{***}P < 0.001$, Student's *t*-test; $n = 6$ animals per treatment), but there is no significant difference at 28 days after ketamine treatment. Expression of VEGF was normalized to expression of β-actin, a housekeeping gene. Normalized expression values were averaged, and average fold changes were calculated. (C) VEGF levels in the hippocampi of rats 24 h and 28 days after ketamine injection by immunohistochemistry. (D) Densitometric analyses of DG granule cells in (C). A significant effect of ketamine on VEGF expression is seen 24 h after ketamine treatment ($^{*}P < 0.05$, Student's *t*-test; $n = 6$ animals per treatment), but there is no significant difference 28 days after ketamine treatment. Results are expressed as percent of saline-treated controls and are means ± SEM. 24 h, 24 h after ketamine treatment; 28 days, 28 days after ketamine treatment. $^{*}P < 0.05$. $^{***}P < 0.001$. Scale bar, 100 μm.

expressed and stable [24]. Indeed, the lenti–shVEGF–EGFP treatment repressed VEGF mRNA expression in DGs *in vivo* (Fig. 3B). Lenti–shVEGF-EGFP infusions repressed VEGF protein levels in the rat hippocampal neuron cells (Fig 3C).

The depressive status of the rats was measured 24 h after ketamine treatment to investigate whether VEGF knockdown abolishes the antidepressant-like effects of ketamine. Each rat was subjected to the locomotor activity assessment (LMA), the sucrose preference test (SPT), the novelty suppressed feeding test (NSFT), the forced swim test (FST) and the learned helplessness test (LHT) to assess depressive-like behavior. The tests were conducted sequentially, one per day, to minimize the impact of immediate behavioral testing on subsequent tests (Fig. 3D), as previously described [25]. During the NSFT, control rats injected with lenti–shVEGF–EGFP had an increased latency time to feed, and ketamine treatment of lenti–shVEGF–EGFP-infused rats resulted in a decrease in latency time compared to control rats injected with lenti–shVEGF–EGFP alone (Fig. 3E; two-way analysis of variance [ANOVA], least significant differences [LSDs] multiple comparison, $P < 0.05$). During the FST we observed similar behaviors to those observed during the NSFT; rats injected with lenti–EFGP and treated with ketamine showed antidepressant-like behaviors (Fig. 3G). Animals injected with lenti–EGFP and treated with ketamine showed a decrease in immobility time, as expected, whereas those injected with lenti–shVEGF–EGFP alone showed increased immobility time. Ketamine treatment of lenti–shVEGF–EGFP-infused rats resulted in a decrease in immobility time compared to lenti–shVEGF–EGFP alone and was similar to the behavior of rats infused with lenti–EGFP and treated with saline (two-way ANOVA, LSDs multiple comparison, $P < 0.001$). Lenti–shVEGF–EGFP infusion did not alter either the amount of sucrose consumed in the SPT (a measure of anhedonia, which is a core symptom of depression) or the number of escape failures in the LHT (Fig. 3F and H). Taken together, these results demonstrate that lenti–shVEGF–EGFP produces depressive-like effects in certain models, and this effect is reversed by ketamine to a level seen in control rats infused lenti–EGFP and treated with saline. The behavioral effects of lenti–shVEGF–EGFP were observed in the absence of any effect on food intake, fluid consumption, or total locomotor activities (Fig. 3I–3K). These results demonstrate that VEGF in the DG of the hippocampus is necessary for ketamine-induced antidepressant-like behaviors but is not sufficient for the antidepressant-like effects of ketamine.

### *2.4. Ketamine-induced VEGF in the DG contributes partially to hippocampal neurogenesis*

It has been suggested that the behavioral effects of chronic antidepressants are mediated, at least in part, by stimulation of neurogenesis in the hippocampus [26–28]. Therefore, we investigated whether ketamine-induced VEGF stimulates neurogenesis. We administered ketamine and BrdU 24 h prior to euthanasia of lentivirus-infused rats. If ketamine-induced VEGF expression in DG does mediate neurogenesis, then lenti–shVEGF–EGFP should block the effect of ketamine on neurogenesis. Ketamine treatment produced a significantly higher number of $BrdU^{+}$ cells in lenti–EGFP-infused rats than were produced in the corresponding saline-treated lenti–EGFP-infused rats (Fig. 4A, B), consistent with increased proliferation of neural progenitor cells. However, the rats injected with lenti–shVEGF–EGFP showed a significant decrease in the number of $BrdU^{+}$ cells compared with rats injected with lenti–EGFP (Fig. 4A, B). This is consistent with observations that lenti–shVEGF–EGFP decreased basal VEGF protein levels and increased depressive-like behaviors as measured by both immobility time in the FST and latency time in the NSFT. Noticeably, the decrease in the number of $BrdU^{+}$ cells in lenti–shVEGF–EGFP was recovered by ketamine to a level observed in control rats infused with lenti–EGFP and treated with saline (Fig. 4B; two-way ANOVA, LSDs multiple comparison, $P < 0.01$), similar to our observations that ketamine partially recovered antidepressant-like behaviors in lenti-shVEGF–EGFP-infused rats. These results indicate that ketamine-induced VEGF is linked to hippocampal neurogenesis, and ketamine-induced neurogenesis might be additionally mediated by pathways that do not involve VEGF.

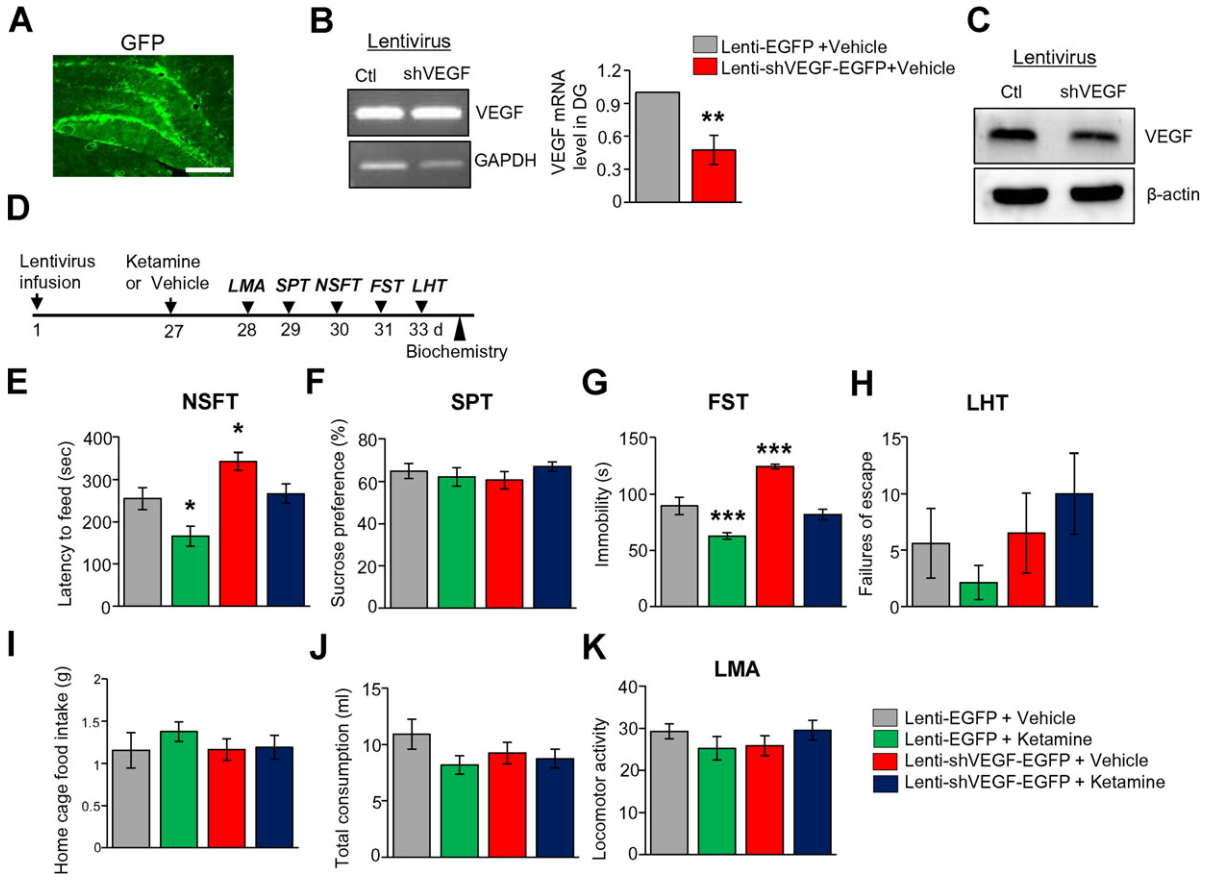


**Fig. 3.** Partial blockade of the ketamine-induced antidepressant-like effects by shRNA knockdown of VEGF in the dentate gyrus. (A) Green fluorescent protein (GFP) expression by lenti–shVEGF-EGFP in the DG. (B) RT-PCR and quantitative PCR showing lenti–shVEGF-mediated knockdown of VEGF mRNA versus lenti–EGFP (Ctl). Lenti–shVEGF–EGFP specifically reduced VEGF mRNA expression in DG ($^{**}P < 0.01$ versus lenti–EGFP, Student's *t*-test; $n = 4$ animals per group). (C) Western blot analysis of lentiviral-mediated knockdown of VEGF protein levels in the rat hippocampal neuron cells. (D) Behavioral paradigm. (E) NSFT. Main effect of ketamine: $P < 0.05$; main effect of virus: $P < 0.05$; interaction: $P > 0.05$. Ketamine administration significantly decreased the latency to feed in lenti–EGFP rats ($^{*}P < 0.05$). (F) SPT. Main effect of ketamine: $P > 0.05$; main effect of virus: $P > 0.05$; interaction: $P > 0.05$. Ketamine had no effect in both lenti–EGFP and lenti–shVEGF–EGFP rats. (G) FST. Main effect of ketamine: $P < 0.001$; main effect of virus: $P < 0.001$; interaction: $P > 0.05$. Rats treated with ketamine had a shorter immobility score (time in seconds) than rats treated with saline in both lenti–EGFP rats ($^{***}P < 0.001$) and in lenti–shVEGF–EGFP rats. Lenti–shVEGF-injected animals showed an increase in immobility compared to lenti–EGFP rats in the absence of ketamine ($^{***}P < 0.001$). (H) LHT. Main effect of ketamine: $P > 0.05$; main effect of virus: $P > 0.05$; interaction: $P > 0.05$. Ketamine had a tendency to decrease the escape failures in lenti–EGFP rats ($P > 0.05$). Lenti–shVEGF-injected rats had a similar number of escape failures compared to lenti–EGFP rats ($P > 0.05$). There was no significant difference in the home cage food intake (I), total fluid consumption (J) and total distance moved in the box (K) between groups. LMA, locomotor activity assessment; SPT, sucrose preference test; NSFT, novelty suppressed feeding test; FST, forced swim test; LHT, learned helplessness behavioral test. Data are mean ± SEM ($n = 14$–19 rats per group). Two-way ANOVA followed by LSD *post hoc* analysis. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle-treated lenti–EGFP rats. Scale bar, 100 μm.

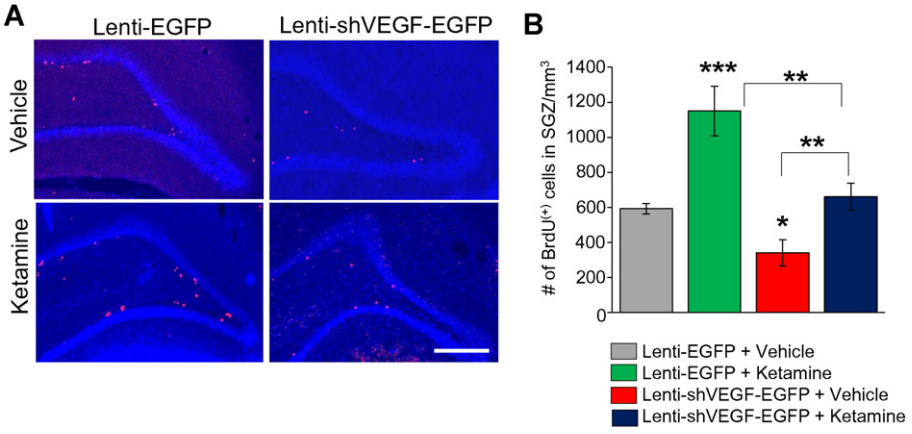


**Fig. 4.** Partial blockade of ketamine-induced neurogenesis by shRNA knockdown of VEGF in the dentate gyrus. (A) Representative images of brain sections labeled for $BrdU^{+}$ in the hippocampi of rats infused with lentivirus and treated with ketamine. (B) Lenti–shVEGF-EGFP specifically reduced the number of $BrdU^{+}$ cells in the DG ($^{*}P < 0.05$ versus lenti–EGFP, Student's *t*-test; $n = 4$ animals per group). Ketamine increased the number of $BrdU^{+}$ cells in lenti–shVEGF-EGFP rats ($^{**}P < 0.01$ versus lenti–shVEGF-EGFP and lenti–EGFP treated with ketamine; $P > 0.05$ versus lenti–EGFP. Two-way ANOVA, LSD *post hoc*; $n = 4$–6 animals per group). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared to vehicle-treated lenti–EGFP rats. Scale bar, 100 μm.

## 3. Discussion

Our aim was to determine the effects of ketamine treatment on hippocampal neurogenesis and VEGF expression. We demonstrate for the first time that a single subanesthetic dose of ketamine robustly increases hippocampal VEGF expression and hippocampal neurogenesis *in vivo* in rats 24 h after ketamine treatment. In addition, we show that VEGF knockdown results in depression-related behaviors, which are, in part, recovered by ketamine. However, the antidepressant effects of ketamine are also mediated by pathways that do not involve VEGF, because the administration of ketamine had only a partial recovery effect on the depression status produced by VEGF knockdown in rats (Fig. 3). These results suggest a contribution of ketamine-induced hippocampal VEGF to the increased hippocampal neurogenesis and antidepressant-like behaviors.

The observation that the antidepressant-like effects of ketamine were partially intact in lenti-shVEGF–EGFP-infused rats suggests that the effects of ketamine might involve signaling via non-VEGF pathways. Given that ketamine induces BDNF [4], the antidepressant-like effects of ketamine might involve BDNF signaling. However, it is noteworthy that ketamine produced similar antidepressant-like responses in both wildtype and *Bdnf*$^{-/-}$ mice, and did not increase BDNF expression in *Bdnf*$^{+/-}$ mice [29], thereby suggesting that BDNF does not have a central role in the antidepressant-like effects of ketamine.

It is possible that although we have shown that a reduction in VEGF mRNA levels to 40 % is sufficient to produce depressive-like behaviors in VEGF-knockdown animals, the remaining VEGF mRNA is able to mediate the antidepressant-like effects of ketamine. Therefore, future studies should investigate the effects of ketamine when VEGF levels are reduced even further.

In humans a single intravenous infusion of ketamine produces a rapid and robust antidepressant effect that can persist for a week or longer [30–32]. In order to model this effect in rats and investigate whether the antidepressant effect is caused by neurogenesis, we assessed the ketamine-induced neurogenesis 28 days after a single ketamine injection. Rats treated with ketamine did not show antidepressant-like behaviors 28 days after ketamine injection (data not shown). These results agree with others [33], who failed to detect a sustained effect in antidepressant-like behaviors. Moreover, neither hippocampal VEGF levels nor neurogenesis was influenced by ketamine 28 days after treatment. VEGF expression in rats infused with lenti-shVEGF–EGFP and treated with ketamine is consistent with the effects of ketamine on antidepressant-like behaviors and neurogenesis in rats infused with lenti-shVEGF–EGFP.

Interestingly, ketamine treatment stimulated the production of BrdU$^+$ cells 24 h after treatment (Figure 2D), but did not change the relative number of neurons (Fig. 2G). It is essential that we are able to distinguish whether the discontinued increase in the number of BrdU$^+$ cells is due to reduced proliferation or survival, or both. Given the chronic time frame of this study, in which BrdU was administered 24 hours before ketamine injection and four weeks prior to euthanasia, it is likely that reduced survival of BrdU$^+$ cells is the contributing factor. We used colabeling with BrdU and NeuN to estimate the degree of cell differentiation towards a neuronal phenotype. Four weeks after the injection of ketamine, the proportion of the cells labeled with both BrdU and NeuN remained constant in the ketamine group, and comparable to the control group. Thus, although ketamine appears to accelerate cell production soon after injection, it does not enhance cell differentiation or maturation into neurons. These results indicate that the ketamine-induced VEGF expression contributes to increased neurogenesis and, in part, to the upregulation of antidepressant-like behaviors 24 h after administration of ketamine.

In conclusion, the novel finding of this study is that ketamine enhances VEGF expression in the DG and induces neurogenesis in the rat; however, VEGF cannot be considered pivotal to the antidepressant-like actions of ketamine, as it is likely that other signaling pathways in the hippocampus also mediate the effects of ketamine.

## 4. Materials and methods

### 4.1. Animals and treatments

Adult male Sprague Dawley rats (Harlan Sprague Dawley, Indianapolis, IN, USA) were housed in a 12-hour light/dark cycle animal facility. After treatment with BrdU at 50 mg/kg (dissolved in 0.9% NaCl and filtered under sterile conditions at 0.2 μm; Sigma, St. Louis, MO, USA), rats received a single injection of either ketamine (10 mg/kg) or saline (control) intraperitoneally. Rats were killed at two time points after the ketamine or saline injection: either 24 h or 28 days post-injection by perfusion with phosphate-buffered saline (PBS) to prepare for immunohistochemistry. Brain samples were also collected under pentobarbital anesthesia at 24 h and 28 days after ketamine or saline injection. No significant differences in the body weights of the control and ketamine-treated rats were observed.

### 4.2. Lentiviral production

To induce VEGF knockdown, we cloned a short hairpin RNA (shRNA) sequence (5′-TCG CGG GAT TGC ACG GAA ACT-3′) against rat VEGF into pll3.7 (Addgene, Cambridge, MA, USA) and used a control non-targeting shRNA [25]. Lentivirus was produced as indicated. Typical titers for *in vivo* injections were $8 \times 10^6$ to $20 \times 10^6$.

### 4.3. Stereotaxic surgery and infusions

Adult male Sprague Dawley rats (8–10 weeks old; Charles River Laboratories, Wilmington, MA, USA) weighing 200 g to 250 g were pair housed and maintained on a 12-hour light/dark cycle with access to food and water *ad libitum*. All procedures were in strict accordance with Institutional Animal Care and Use Committee guidelines and use of laboratory animals was approved by the Hanyang University Animal Care and Use Committee (#2015-0094). Animals were randomly assigned to experimental groups. For behavioral experiments, rats were injected intraperitoneally with ketamine (10 mg/kg body weight) or saline and then analyzed 24 h after the injection or at indicated time points (Fig. 3D). Stereotaxic surgery and infusions were conducted as previously described [25]. Rats were anesthetized with xylazine (25 mg/kg) (Rompun, Bayer, Leverkusen, Germany) and Zoletil50 (50 mg/kg: Virbac Philipines, Bonifacio Global City, France). Bilateral injections were performed at coordinates −4.1 mm (anterior/posterior), ±2.4 mm (lateral), and −4.6 mm (dorsal/ventral) relative to the bregma. A total of 6 μl of purified virus was delivered at a rate of 0.1 μl/min followed by 10 min of rest. Needles were removed and the scalp incision was closed using wound clips. Inhabitants of the same cage were assigned randomly to different experimental groups for behavioral studies, and the order of testing was distributed randomly across the groups. After the behavioral testing, animals were euthanized by perfusion with PBS. The brain was kept overnight in 4% paraformaldehyde and then transferred to a 30% sucrose solution. Brain sections (30 μm thickness) were cut using a microtome for visualization of EGFP. We assessed gene-transfer efficacy with immunofluorescent staining 4–8 weeks after surgery.

### 4.4. Forced swim test

A FST was conducted as previously described [3,25]. On the test day, rats were placed in a clear cylinder with water (24 °C ± 1 °C, 45-cm depth) for 15 min. After the test, animals were dried under a lamp for 30 min. All FST experiments were filmed by a camcorder, and the first 5 min or 15 min of swim was scored offline for the duration of immobility. Immobility was defined as floating or remaining motionless without

leaning against the wall of the cylinder [34]. Behavioral tests were analyzed by an experimenter blinded to the study code.

### 4.5. Novelty suppressed feeding test

An NSFT was conducted as previously described [25,35]. Animals were deprived of food for 12 h and, on the test day, they were placed in an open field (76.5 cm × 76.5 cm × 40 cm, Plexiglas) with eight pellets of food in the center. The animals were given 8 min to approach the food and eat it. The test was stopped as soon as the animal took the first bite. The latency to eat was recorded in seconds. Home cage food intake was also measured as a control.

### 4.6. Sucrose preference test

An SPT was conducted as previously described [25]. SPT consisted of a 48-hour period of exposure to sucrose solution (1%; Sigma) for acclimation, followed by 4 h of water deprivation and 1 h of exposure to two identical bottles, one filled with sucrose solution and the other with water. Sucrose and water consumption was determined by measuring the change in volume of fluid consumed. Sucrose preference was defined as the ratio of the volume of sucrose vs. total volume of sucrose plus water consumed during the 1-hour test.

### 4.7. Locomotor test

The general locomotor activity (LMA) was conducted as previously described [36]. LMA of the rats in the open field test was measured by an ANY-maze tracking system (Stoelting Co., Wood Dale, IL, USA). Rats were placed in the central part of the square-shaped arena (76.5 cm × 76.5 cm × 40 cm) and allowed to explore it for 10 min. Total distance traveled (locomotion activity) during the 10 min was recorded.

### 4.8. Learned helplessness test

The learned helplessness procedure was performed in commercial shuttle boxes divided into two equal compartments by a central barrier (Gemini Avoidance System, San Diego Instruments, San Diego, CA, USA), as previously described [3]. A computer-operated guillotine door built into the central barrier allowed passage between compartments. On day 1, inescapable footshock (IES) was administered at one side of the shuttle box with the guillotine door closed (60 footshocks, 0.85 mA intensity, 15 s average duration, 60 s average intershock interval). Active avoidance testing consisted of 30 trials of escapable footshock (0.65 mA intensity, 35 s maximum duration, 90 s average intertribal interval) with the guillotine door open. Each trial used a fixed-ratio 1 schedule, during which one shuttle crossing by rats terminated the shock. Shock was terminated automatically if rats did not escape after 35 s. A computer automatically recorded the number of escape failures. Results are expressed as number of escape failures, that is, the number of times that the animal did not terminate the footshock.

### 4.9. Immunohistochemistry

Twenty four hours after the last BrdU injection, the rats were deeply anesthetized with pentobarbital and perfused via the ascending aorta with saline until the outflow became clear. They were then perfused with 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 20 min. The brains were removed rapidly, frozen immediately and stored at −70 °C. Serial sections (30 μm/section) were cut coronally through the entire anteroposterior extension of the hippocampi. For BrdU immunolabeling, sections were processed using the method described by Son et al. [37]. For other immunofluorescent labeling, sections were fixed by 0.1 M phosphate buffer (pH 7.4) containing 4% paraformaldehyde for 20 min, washed with PBS, permeabilized in 100% ethyl alcohol for 10 min and then incubated in 10% normal goat serum (NGS) and 0.1 % Triton X-100 for 30 min at room temperature. Sections were then incubated in 0.1 M phosphate buffer (pH 7.4) containing primary antibody and 0.1% Triton X-100 overnight at 4 °C. The next day, sections were incubated at room temperature for an additional 3–4 h with secondary antibodies conjugated to fluorescein isothiocyanate, cyanin 3 (Cy3, donkey; Jackson ImmunoResearch, West Grove, PA, USA) and Alexa488 (Invitrogen, Carlsbad, CA, USA). Secondary antibodies were used at a final dilution of 1:200 in 10% NGS. The samples were then washed with PBS, treated with 10 mg/ml 4′, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA) for 10 min and mounted with a coverslip using Vestashield Æ (Vector Laboratories). Primary antibodies generated in mouse, rat, and rabbit were used at the following concentrations: mouse anti-NeuN (1:300; Chemicon, Temecula, CA, USA), rat anti-BrdU (1:300; Abcam, Cambridge, UK), mouse anti-GFP (1:200; Roche Diagnostics GmbH, Germany). For VEGF immunoperoxidase staining, sections were processed using the method described by Son et al [24]. For immunoperoxidase staining of VEGF, free-floating sections were incubated in 5% NGS plus 0.3% Triton X-100 in PBS for 1 h and then with primary antibody in PBS plus 5% NGS overnight at 4 °C. The primary antibody used was rabbit anti-VEGF IgG (1:200; Santa Cruz, CA, USA). The sections were incubated in secondary antibody conjugated to biotin–rabbit IgG (H + L) (1:200; Vector Laboratories) for 1 h, then horseradish peroxidase streptavidin (1:400; Vector Laboratories) for 2 h, before incubating with a peroxidase substrate kit (DAB kit; Vector Laboratories) to visualize staining. Fluorescent signals were detected using a confocal laser-scanning microscope (Leica TCS, Leica Microsytem, Wetzlar, Germany), which enabled the simultaneous evaluation of up to four separate fluorophores. When it was necessary to observe nuclei in addition to four immunological markers, cells were counterstained with DAPI.

### 4.10. Double-labeling of BrdU and NeuN protein

For the double-labeling of sections with both BrdU and NeuN, sections that had been pretreated for DNA denaturation were incubated in 0.01 M PBS containing 1% NGS, 0.3% Triton X-100, mouse anti-NeuN monoclonal antibody (1:300; Chemicon) and rat anti-BrdU monoclonal antibody (1:300; Abcam) for 24 h at 4 °C.

The sections were then incubated in PBS containing Cy3-conjugated donkey anti-rat IgG (1:300; Jackson ImmunoResearch) and goat anti-mouse secondary antibody conjugated to Alexa488 (1:300; Invitrogen) for 1 h at room temperature. After several washes in PBS, the sections were mounted on gelatin-coated slides with Vectashield Æ. Confocal images were produced at a fixed laser power setting with a 40× oil-immersion objective. Separate optical images of BrdU and NeuN immunoreactivity were captured of the same optical view. The captured images were then pseudocolored green or red. A digital overlay was generated and companion images were superimposed. Colocalization was reflected by superimposing green and red pixels, thus appearing yellow. Image analysis was performed using the standard system operating software provided with the confocal microscope (Version 1.6).

### 4.11. Western blot analysis

Protein extracts were prepared as described previously [38]. The blot was probed with mouse monoclonal β-actin (1:2000, Santa Cruz) and rabbit polyclonal anti-VEGF (1:500; Santa Cruz) followed by treatment with goat anti-mouse (1:2000; Santa Cruz) or anti-rabbit IgG (1:2000; Santa Cruz) conjugated with horseradish peroxidase. Bands were visualized with an enhanced chemiluminescence (ECL) detection kit (Neruonex, Daegu, Korea). Expression of VEGF was normalized to expression of β-actin, a housekeeping gene. Normalized expression values were averaged, and average fold changes were calculated.

### 4.12. Cell counting

$BrdU^+$ cells were counted on one-in-six sections (180 μm apart) using a computer-assisted image analysis system (MetaView Imaging version 3.6, Universal Imaging Corporation, West Chester, PA, USA). The GCL and SGZ, which were defined as a zone two cell bodies wide (~10 μm) along the border of the GCL, were investigated. $BrdU^+$ cells with cell nuclei that had a complete nuclear contour were counted in the focal plane in which they appeared largest within the counting box. The GCL was traced using $DAPI^+$ cells, and the GCL reference volume was determined by summing the traced GCL area for each section (the sectional volume) and multiplying this by the distance between the sections sampled. The number of $BrdU^+$ cells per section was then related to the GCL sectional volume and multiplied by the reference volume to produce an estimate of the total number of $BrdU^+$ cells.

### 4.13. Quantitation in confocal images

Quantification of immunohistochemical images was conducted as previously described [24]. To measure the intensity of immunoreactivity in the GCL of the DG, images were acquired with a digital camera (Nikon E800, Nikon, Tokyo, Japan) and analyzed using an image analysis program (AnalySIS version 3.0, Soft Image Analysis System GmBH, Münster, Germany). In each image a region of interest (ROI) that represented the DG was determined by free-hand drawing, and the mean gray values in each ROI were measured. The results presented are the ratio of the intensity, which was computed by dividing the mean gray value of a ROI in an experimental brain by the corresponding value in a saline control. Generally, four to eight images of each section were averaged to determine a value for a section.

### 4.14. Statistical analyses

Statistical differences were determined by ANOVA (StatView 5; SAS software) followed by Fisher's LSD post hoc analysis. The F values, and group and experimental degrees of freedom are included in the figure legends. For experiments with two groups, the Student's *t*-test was used. The level of statistical significance was set at $P < 0.05$ using two-tailed tests.

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## References

[1] R.C. Kessler, P. Berglund, O. Demler, R. Jin, D. Koretz, K.R. Merikangas, A.J. Rush, E.E. Walters, P.S. Wang, R. National Comorbidity Survey, The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R), Jama 289 (2003) 3095–3105.
[2] J.W. Murrough, D.V. Iosifescu, L.C. Chang, R.K. Al Jurdi, C.E. Green, A.M. Perez, S. Iqbal, S. Pillemer, A. Foulkes, A. Shah, D.S. Charney, S.J. Mathew, Antidepressant efficacy of ketamine in treatment-resistant major depression: a two-site randomized controlled trial, The American journal of psychiatry 170 (2013) 1134–1142.
[3] N. Li, B. Lee, R.J. Liu, M. Banasr, J.M. Dwyer, M. Iwata, X.Y. Li, G. Aghajanian, R.S. Duman, mTOR-dependent synapse formation underlies the rapid antidepressant effects of NMDA antagonists, Science 329 (2010) 959–964.
[4] A.C. Chen, K.H. Shin, R.S. Duman, G. Sanacora, ECS-Induced mossy fiber sprouting and BDNF expression are attenuated by ketamine pretreatment, The journal of ECT 17 (2001) 27–32.
[5] C.G. Abdallah, G. Sanacora, R.S. Duman, J.H. Krystal, Ketamine and rapid-acting antidepressants: a window into a new neurobiology for mood disorder therapeutics, Annual review of medicine 66 (2015) 509–523.
[6] G. Keilhoff, H.G. Bernstein, A. Becker, G. Grecksch, G. Wolf, Increased neurogenesis in a rat ketamine model of schizophrenia, Biological psychiatry 56 (2004) 317–322.
[7] T.J. Schoenfeld, H.A. Cameron, Adult neurogenesis and mental illness, Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 40 (2015) 113–128.
[8] E. Gould, C.G. Gross, Neurogenesis in adult mammals: some progress and problems, The Journal of neuroscience: the official journal of the Society for Neuroscience 22 (2002) 619–623.
[9] H. van Praag, G. Kempermann, F.H. Gage, Running increases cell proliferation and neurogenesis in the adult mouse dentate gyrus, Nature neuroscience 2 (1999) 266–270.
[10] R.S. Duman, S. Nakagawa, J. Malberg, Regulation of adult neurogenesis by antidepressant treatment, Neuropsychopharmacology: official publication of the American College of Neuropsychopharmacology 25 (2001) 836–844.
[11] R.S. Duman, L.M. Monteggia, A neurotrophic model for stress-related mood disorders, Biological psychiatry 59 (2006) 1116–1127.
[12] S. Hayley, D. Litteljohn, Neuroplasticity and the next wave of antidepressant strategies, Frontiers in cellular neuroscience 7 (2013) 218.
[13] C.P. Hutton, N. Dery, E. Rosa, J.A. Lemon, C.D. Rollo, D.R. Boreham, M. Fahnestock, D. deCatanzaro, J.M. Wojtowicz, S. Becker, Synergistic effects of diet and exercise on hippocampal function in chronically stressed mice, Neuroscience 308 (2015) 180–193.
[14] M.M. Nowacka, E. Obuchowicz, Vascular endothelial growth factor (VEGF) and its role in the central nervous system: a new element in the neurotrophic hypothesis of antidepressant drug action, Neuropeptides 46 (2012) 1–10.
[15] J.L. Warner-Schmidt, R.S. Duman, VEGF is an essential mediator of the neurogenic and behavioral actions of antidepressants, Proceedings of the National Academy of Sciences of the United States of America 104 (2007) 4647–4652.
[16] J. Greene, M. Banasr, B. Lee, J. Warner-Schmidt, R.S. Duman, Vascular endothelial growth factor signaling is required for the behavioral actions of antidepressant treatment: pharmacological and cellular characterization, Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 34 (2009) 2459–2468.
[17] K. Jin, Y. Zhu, Y. Sun, X.O. Mao, L. Xie, D.A. Greenberg, Vascular endothelial growth factor (VEGF) stimulates neurogenesis in vitro and in vivo, Proceedings of the National Academy of Sciences of the United States of America 99 (2002) 11946–11950.
[18] L.S. Garcia, C.M. Comim, S.S. Valvassori, G.Z. Reus, L.M. Barbosa, A.C. Andreazza, L. Stertz, G.R. Fries, E.C. Gavioli, F. Kapczinski, J. Quevedo, Acute administration of ketamine induces antidepressant-like effects in the forced swimming test and increases BDNF levels in the rat hippocampus, Progress in neuro-psychopharmacology & biological psychiatry 32 (2008) 140–144.
[19] P. Gass, M.A. Riva, CREB, neurogenesis and depression, BioEssays: news and reviews in molecular, cellular and developmental biology 29 (2007) 957–961.
[20] S. Nakagawa, J.E. Kim, R. Lee, J. Chen, T. Fujioka, J. Malberg, S. Tsuji, R.S. Duman, Localization of phosphorylated cAMP response element-binding protein in immature neurons of adult hippocampus, The Journal of neuroscience: the official journal of the Society for Neuroscience 22 (2002) 9868–9876.
[21] S. Nakagawa, J.E. Kim, R. Lee, J.E. Malberg, J. Chen, C. Steffen, Y.J. Zhang, E.J. Nestler, R.S. Duman, Regulation of neurogenesis in adult mouse hippocampus by cAMP and the cAMP response element-binding protein, The Journal of neuroscience: the official journal of the Society for Neuroscience 22 (2002) 3673–3682.
[22] N.J. Kee, E. Preston, J.M. Wojtowicz, Enhanced neurogenesis after transient global ischemia in the dentate gyrus of the rat, Experimental brain research 136 (2001) 313–320.
[23] L. Cao, X. Jiao, D.S. Zuzga, Y. Liu, D.M. Fong, D. Young, M.J. During, VEGF links hippocampal activity with neurogenesis, learning and memory, Nature genetics 36 (2004) 827–835.
[24] J.S. Lee, D.J. Jang, N. Lee, H.G. Ko, H. Kim, Y.S. Kim, B. Kim, J. Son, S.H. Kim, H. Chung, M.Y. Lee, W.R. Kim, W. Sun, M. Zhuo, T. Abel, B.K. Kaang, H. Son, Induction of neuronal vascular endothelial growth factor expression by cAMP in the dentate gyrus of the hippocampus is required for antidepressant-like behaviors, The Journal of neuroscience: the official journal of the Society for Neuroscience 29 (2009) 8493–8505.
[25] H. Son, M. Banasr, M. Choi, S.Y. Chae, P. Licznerski, B. Lee, B. Voleti, N. Li, A. Lepack, N.M. Fournier, K.R. Lee, I.Y. Lee, J. Kim, J.H. Kim, Y.H. Kim, S.J. Jung, R.S. Duman, Neuritin produces antidepressant actions and blocks the neuronal and behavioral deficits caused by chronic stress, Proceedings of the National Academy of Sciences of the United States of America 109 (2012) 11378–11383.
[26] J.E. Malberg, A.J. Eisch, E.J. Nestler, R.S. Duman, Chronic antidepressant treatment increases neurogenesis in adult rat hippocampus, The Journal of neuroscience: the official journal of the Society for Neuroscience 20 (2000) 9104–9110.
[27] A. Sahay, R. Hen, Adult hippocampal neurogenesis in depression, Nature neuroscience 10 (2007) 1110–1115.
[28] L. Santarelli, M. Saxe, C. Gross, A. Surget, F. Battaglia, S. Dulawa, N. Weisstaub, J. Lee, R. Duman, O. Arancio, C. Belzung, R. Hen, Requirement of hippocampal neurogenesis for the behavioral effects of antidepressants, Science 301 (2003) 805–809.
[29] J.S. Lindholm, H. Autio, L. Vesa, H. Antila, L. Lindemann, M.C. Hoener, P. Skolnick, T. Rantamaki, E. Castren, The antidepressant-like effects of glutamatergic drugs ketamine and AMPA receptor potentiator LY 451646 are preserved in bdnf(+)/(−) heterozygous null mice, Neuropharmacology 62 (2012) 391–397.
[30] M. aan het Rot, K.A. Collins, J.W. Murrough, A.M. Perez, D.L. Reich, D.S. Charney, S.J. Mathew, Safety and efficacy of repeated-dose intravenous ketamine for treatment-resistant depression, Biol. Psychiatry 67 (2010) 139–145.
[31] R.M. Berman, A. Cappiello, A. Anand, D.A. Oren, G.R. Heninger, D.S. Charney, J.H. Krystal, Antidepressant effects of ketamine in depressed patients, Biological psychiatry 47 (2000) 351–354.
[32] C.A. Zarate Jr., J.B. Singh, P.J. Carlson, N.E. Brutsche, R. Ameli, D.A. Luckenbaugh, D.S. Charney, H.K. Manji, A randomized trial of an N-methyl-D-aspartate antagonist in treatment-resistant major depression, Archives of general psychiatry 63 (2006) 856–864.
[33] R.J. Liu, M. Fuchikami, J.M. Dwyer, A.E. Lepack, R.S. Duman, G.K. Aghajanian, GSK-3 inhibition potentiates the synaptogenic and antidepressant-like effects of subthreshold doses of ketamine, Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 38 (2013) 2268–2277.

[34] A.M. Pliakas, R.R. Carlson, R.L. Neve, C. Konradi, E.J. Nestler, W.A. Carlezon Jr., Altered responsiveness to cocaine and increased immobility in the forced swim test associated with elevated cAMP response element-binding protein expression in nucleus accumbens, The Journal of neuroscience: the official journal of the Society for Neuroscience 21 (2001) 7397–7403.
[35] M. Banasr, R.S. Duman, Glial loss in the prefrontal cortex is sufficient to induce depressive-like behaviors, Biological psychiatry 64 (2008) 863–870.
[36] M. Choi, S.E. Wang, S.Y. Ko, H.J. Kang, S.Y. Chae, S.H. Lee, Y.S. Kim, R.S. Duman, H. Son, Overexpression of human GATA-1 and GATA-2 interferes with spine formation and produces depressive behavior in rats, PloS one 9 (2014), e109253.
[37] H. Son, I.T. Yu, S.J. Hwang, J.S. Kim, S.H. Lee, Y.S. Lee, B.K. Kaang, Lithium enhances long-term potentiation independently of hippocampal neurogenesis in the rat dentate gyrus, Journal of neurochemistry 85 (2003) 872–881.
[38] J.S. Kim, M.Y. Chang, I.T. Yu, J.H. Kim, S.H. Lee, Y.S. Lee, H. Son, Lithium selectively increases neuronal differentiation of hippocampal neural progenitor cells both in vitro and in vivo, Journal of neurochemistry 89 (2004) 324–336.

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Corrigendum

# Corrigendum to "Hippocampal VEGF is necessary for antidepressant-like behaviors but not sufficient for antidepressant-like effects of ketamine in rats" [BBA-Mol. Basis Dis. 1862/7 (2016) 1247–1254]

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