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Ketamine induces brain-derived neurotrophic factor expression via phosphorylation of histone deacetylase 5 in rats

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ABSTRACT

Ketamine shows promise as a therapeutic agent for the treatment of depression. The increased expression of brain-derived neurotrophic factor (BDNF) has been associated with the antidepressant-like effects of ketamine, but the mechanism of BDNF induction is not well understood. In the current study, we demonstrate that the treatment of rats with ketamine results in the dose-dependent rapid upregulation of *Bdnf* promoter IV activity and expression of *Bdnf* exon IV mRNAs in rat hippocampal neurons. Transfection of histone deacetylase 5 (HDAC5) into rat hippocampal neurons similarly induces *Bdnf* mRNA expression in response to ketamine, whereas transfection of a HDAC5 phosphorylation-defective mutant (Ser259 and Ser498 replaced by Ala259 and Ala498), results in the suppression of ketamine-mediated BDNF promoter IV transcriptional activity. Viral-mediated hippocampal knockdown of HDAC5 induces *Bdnf* mRNA and protein expression, and blocks the enhancing effects of ketamine on BDNF expression in both unstressed and stressed rats, and thereby providing evidence for the role of HDAC5 in the regulation of *Bdnf* expression. Taken together, our findings implicate HDAC5 in the ketamine-induced transcriptional regulation of *Bdnf*, and suggest that the phosphorylation of HDAC5 regulates the therapeutic actions of ketamine.

1. Introduction

Ketamine, the noncompetitive *N*-Methyl-D-aspartate receptor antagonist, has shown remarkable consistency in rapidly ameliorating depressive symptoms in patients with major depressive disorder [1]. The antidepressant effects of ketamine in rodents are associated with the activation of signaling systems that include neurotrophic factors, such as brain-derived neurotrophic factor (BDNF), and therefore an understanding the mechanisms of *Bdnf* regulation by ketamine is of high importance.

The *Bdnf* gene contains at least nine differentially regulated promoters [2]. A number of *cis*-regulatory elements have been identified in *Bdnf* promoters, of which the best characterized are the elements mediating the neuronal activation of promoter IV [3]. The regulatory mechanisms of promoter IV have been studied thoroughly, and exon IV contains transcripts that are highly expressed in neurons [3,4]. Loss of promoter IV-driven *Bdnf* expression leads to depression-like behavior in mice [5], and epigenetic modification at promoter IV is observed in a rat model of depression [6].

Previous studies demonstrate that histone deacetylase 5 (HDAC5) has epigenetic control in the nucleus accumbens over behavioral adaptations to chronic emotional stimuli [7], and that overexpression of HDAC5 in the hippocampus blocks the action of antidepressants in stressed mice [8]. HDAC5 is highly expressed in the brain, with strong expression in the forebrain regions, including the hippocampus, cortex, and amygdala [9]. HDAC5 is tightly regulated by neuronal activity [10,11] and interacts with myocyte enhancer factor-2 (MEF2) to repress target gene expression [11].

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Phosphorylation of HDAC5 by HDAC5 kinases liberates nuclear MEF2 transcription factors through nuclear export of phosphorylated HDAC5 [11].

Several studies have explored the connection between BDNF and HDACs in nervous system disorders [12]. However, little is known about the contribution of individual HDAC isoforms to the regulation of *Bdnf* transcription, except for HDAC2 which binds to *Bdnf* promoters I, II and IV [13].

In the current study, we found that ketamine downregulates HDAC5 to attenuate its repressive influence on transcription of *Bdnf* in the hippocampus. Furthermore, we show that the knockdown of HDAC5 in rat hippocampus using small hairpin RNA (shRNA) blocks the behavioral actions of ketamine in unstressed rats, and is alone sufficient to produce antidepressant responses in rodents exposed to chronic stress.

2. Materials and methods

Preparation of rat hippocampal neurons. Primary rat hippocampal neurons were prepared and processed as described previously [14].

Quantitative real-time RT-PCR. Total RNA was prepared from *in vitro* rat hippocampal neurons and whole rat hippocampus using Trizol reagent (Invitrogen, Carlsbad, CA, USA). Reverse-transcription was conducted as previously described [15]. The primers used in this analysis were for *Bdnf*, *Bdnf* IV, *Hdac5*, *and Gapdh*. *Bdnf* 5′-GTGACAGTATTAGCGAGTGGG-3′ (forward), 5′-GGGTAGTTCGGCATTGC-3′ (reverse), *Bdnf* IV 5′-AAAGCGTCTTTTCCGAGGTT-3' (forward), 5′-CAGCCTACACCGCTAGGAAG-3′ (reverse), *Hdac5* 5′-ATGGGATTCTGCTTCTTCAA-3′ (forward), 5′-TGTCCTTCAACAGCATCAAA-3' (reverse), *Gapdh* 5′-ATGTATCCGTTGTGGATCTGACAT-3′ (forward), 5′-ACCTGCTTCACCACCTTCTTGA-3' (reverse).

Western blot analysis Protein extracts were prepared and Western blot analyses were performed as described previously [16], using rabbit anti-BDNF (1:500, Santa Cruz, Dallas, TX, USA), and mouse anti-β-actin (1:1000, Santa Cruz) antibodies.

Luciferase reporter assay Luciferase reporter assays of rat BDNF IV promoter activity were performed using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA). The rat BDNF IV promoter (−921 ~+11) was generated from rat genomic DNA by PCR and cloned into the luciferase reporter vector pGL3. Primary hippocampal neurons were transfected with the luciferase reporter plasmid containing the promoter sequence of rat BDNF IV in pGL3 (*firefly* luciferase vector) and pGL3-Luc (*renilla* luciferase vector, for normalization) using Lipofectamine 2000 reagent (Invitrogen). Luciferase activity was measured with a luminometer (Panomics, San Jose, USA) using a reagent kit (Promega). Transfection efficiency was normalized by the activity of *renilla* luciferase.

Immunohistochemistry Immunofluorescent labeling was performed as described previously [17].

Lentiviral vector production For HDAC5 knockdown, we cloned a shRNA sequence against HDAC5 [18] into pll3.7 (Addgene) and used a nontargeting shRNA as a control [14]. Lentivirus was produced as preciously described [15]. Typical titers for *in vivo* injections were in the range 8×10^6 to 20×10^6.

Animals, drug administration, stereotaxic surgery and infusions Male Sprague-Dawley rats (Charles River Laboratories) weighing 230–250 g were used. All procedures were in strict accordance with Institutional Animal Care and Use (IACUC) guidelines and Use of Laboratory Animals and were approved by the Hanyang University Animal Care and Use Committee. Rats were injected intraperitoneally (i.p.) with ketamine (10 mg/kg body weight) or saline. Rats were analyzed 24 h after their last injection. Stereotaxic surgery and infusions were conducted as previously described [14].

Chronic unpredictable stress (CUS) procedure The animals exposed to CUS were subjected to exactly the same sequence of 12 stressors (two per day for 28 d) described in Banasr *et al* [19]. The dose of ketamine used in this study was similar to that used in previous studies [15].

Statistical analyses Statistical differences were determined by analysis of variance (ANOVA, StatView 5) followed by Fisher's least significant difference *post hoc* analysis. For experiments comparing two groups, the Student's *t*-test was used. The level of statistical significance was set at $P < 0.05$ using two-tailed tests.

3. Results

3.1. Ketamine induces Bdnf promoter IV activity in rat hippocampal neurons

To examine the potential role of BDNF in ketamine-induced signaling and function in rat hippocampal neurons, we first examined the expression of endogenous BDNF in response to ketamine. The exposure of cultured rat hippocampal neurons to ketamine induced the expression of BDNF protein in a concentration-dependent manner, reaching peak levels at approximately 100 nM ketamine (Fig. 1A), a concentration lower than the comparable plasma concentrations required to produce anesthesia in humans (5–10 μM) [20]. This correlates with a significant increase in endogenous rat *Bdnf* mRNAs in hippocampal cultures treated with ketamine (Fig. 1B).

A lack of *Bdnf* driven by promoter IV leads to depression-like behavior in mice [5,21], and epigenetic modification at *Bdnf* promoter IV is observed in a rat model of depression [6,22]. We therefore investigated whether *Bdnf* promoter IV responds to ketamine in our primary cell model. Quantitative real-time PCR (qRT-PCR) analysis showed that expression of *Bdnf* exon IV mRNA is induced in cells exposed to ketamine, with similar kinetics and fold increases as total *Bdnf* mRNA (Fig. 1C).

The ability of ketamine to induce BDNF expression *in vitro* suggests that BDNF promoter activity is also increased in response to ketamine. Using a luciferase reporter assay to monitor BDNF promoter IV activity, we found that ketamine increases the transcriptional activity of BDNF promoter IV significantly, and in a concentration-dependent manner, with peak activity at approximately 100 nM ketamine (Fig. 1D). This is consistent with a significant increase in rat endogenous *Bdnf* exon IV mRNAs in hippocampal cultures treated with ketamine.

As ketamine is capable of inducing BDNF expression *in vitro*, we investigated whether BDNF expression is influenced by a single dose of ketamine *in vivo*. We observed that ketamine (10 mg/kg, i.p.) increased *Bdnf* mRNA levels within 30 min of injection. *Bdnf* mRNA levels peaked at 6 h and remained moderately elevated for at least 24 h, as measured by qRT-PCR (Fig. 1E), indicating that expression of *Bdnf* mRNA in response to ketamine occurs in the hippocampus. Injection with ketamine (10 mg/kg, i.p.) increased the expression of *Bdnf* exon IV mRNA; this increase was significant at 30 min after injection, maximal (approximately 1.7-fold) after 6 h and remained elevated at 24 h (Fig. 1F).

As ketamine is capable of inducing BDNF *in vivo* 24 h after treatment, we investigated whether the induction of BDNF was long lasting (Fig. 2A). We measured BDNF in the hippocampi of rats 4 weeks after treatment with ketamine (10 mg/kg i.p.) by qRT-PCR (Fig. 2B), immunohistochemistry (Fig. 2C and D) and Western blotting (Fig. 2E and F). The increased level of BDNF seen in the hippocampus 24 h after a single ketamine injection was not

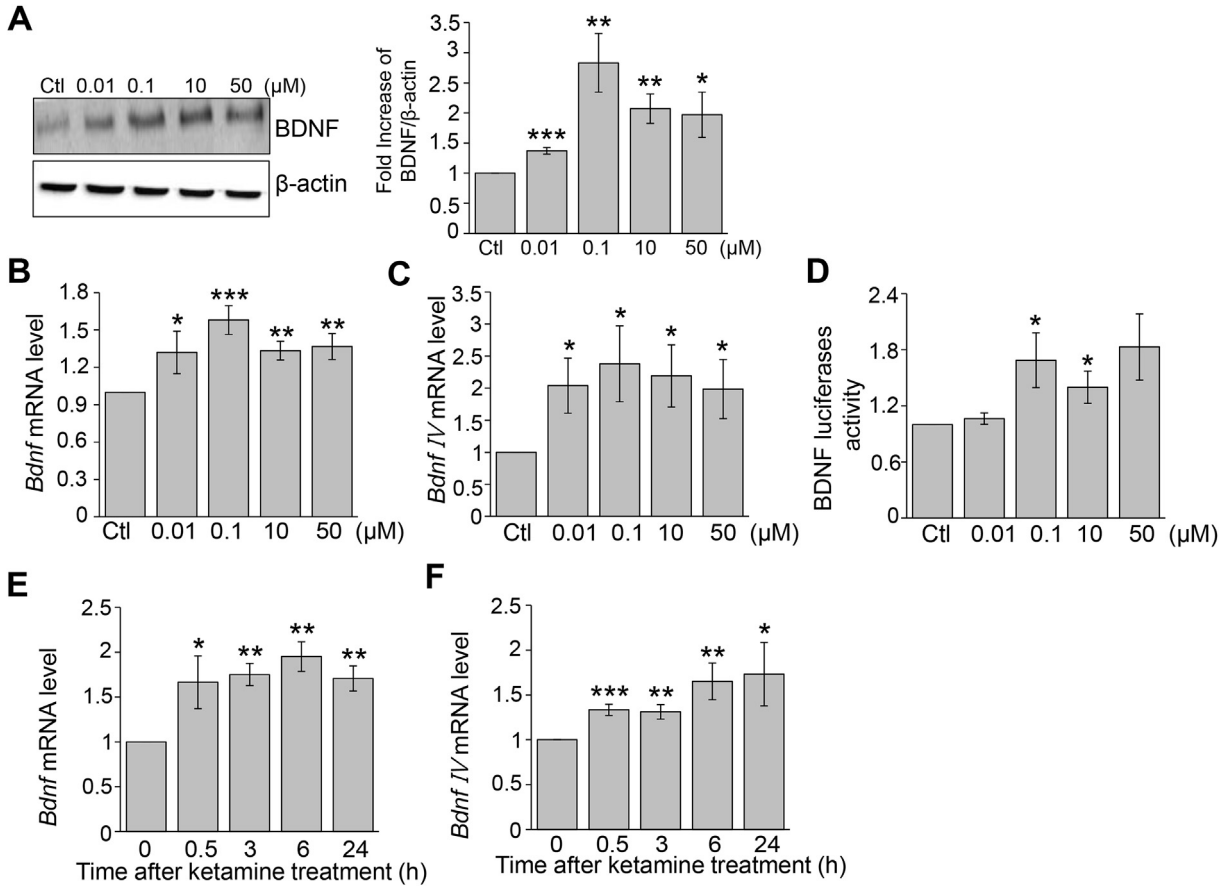

Fig. 1. Ketamine regulates BDNF expression in rat hippocampal neurons.
(A) Representative immunoblots for BDNF protein. Cultured hippocampal neurons were exposed to ketamine at various concentrations for 24 h. Quantitative data for BDNF expression are shown, normalized to the level of β-actin. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test; $n = 2$ animals per treatment). qRT-PCR analysis shows the expression of (B) *Bdnf* mRNA, and (C) *Bdnf exon IV* mRNA in rat hippocampal neurons after treatment with ketamine at various concentrations. (*$P < 0.05$, Student's *t*-test; $n = 4$–5 animals per treatment). (D) Luciferase assay. Hippocampal neurons transfected with pGL3–Luc (encoding *renilla* luciferase) and pGL3–BDNF-IV–Luc (encoding *renilla* luciferase) were treated with ketamine at various concentrations. (*$P < 0.05$, Student's *t*-test; n = 3 animals per treatment). qRT-PCR analysis shows the expression of (E) *Bdnf* mRNA, and (F) *Bdnf exon IV* mRNA in rat hippocampus after treatment with ketamine (10 mg/kg, i.p.) at various time points. The mRNA levels at each time point were normalized to the level of the vehicle-treated control and are shown as fold changes relative the value at 0 h. (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, Student's *t*-test; $n = 3$–4 animals per treatment).

apparent after 4 weeks, indicating that the effects of ketamine on BDNF are not long lasting.

It has been demonstrated previously that HDAC5 regulates BDNF expression [8,23], and we have demonstrated previously that ketamine-induced HDAC5 phosphorylation and the cytoplasmic localization of p-HDAC5 are involved [15]. As ketamine induces BDNF expression *in vivo*, we investigated whether HDAC5 is involved in the expression of BDNF in response to ketamine in rat hippocampi. We knocked down hippocampal HDAC5 by bilateral administration of a lentivirus expressing shRNAs targeted against rat HDAC5 and conjugated to enhanced green fluorescent protein (lenti–shHDAC5–EGFP) into the granular cells of the dentate gyrus (DG) (Fig. 3). Infusion of the lenti–shHDAC5–EGFP construct resulted in widespread gene expression, as shown by EGFP fluorescence 4 weeks after infusion (Fig. 3A). Consistent with the effects in naïve animals, ketamine upregulated BDNF in non-stressed animals transfected with lenti–EGFP (Fig. 3C). Lenti–shHDAC5-EGFP alone promptly repressed *Hdac*5 mRNA expression (Fig. 3B) and caused increased expression of BDNF in the hippocampus (Fig. 3D and E), indicating that HDAC5 represses BDNF expression. The enhancing effects of lenti–shHDAC5-EGFP on BDNF expression blocked the induction of BDNF by ketamine (Fig. 3D and E), suggesting that ketamine-induced BDNF expression requires the blockade of HDAC5's activity. The induction of *Bdnf* IV mRNAs was also detected in animals transfected with lenti–shHDAC5–EGFP, which have reduced induction of BDNF by ketamine (Fig. 3F). Taken together, these data indicate that the inhibition of HDAC5 induces the transcription of BDNF.

Previously, we reported that exposure to chronic unpredictable stress (CUS) induced behavioral deficits in rats, which was reversed by a single injection of ketamine. We also reported that ketamine reverses CUS-induced upregulation of HDAC5 and increases phosphorylation of HDAC5 *in vivo* [15]. As BDNF plays a role in the antidepressant action of ketamine in stressed rats [24], we investigated whether HDAC5 is also involved in ketamine-induced BDNF expression under stress. We therefore knocked down hippocampal HDAC5 by lenti–shHDAC5-EGFP in rat DG granular cells (Fig. 3G). We first confirmed that ketamine induced the expression of *Bdnf* mRNA in animals infused with lenti–EGFP and exposed to CUS (Fig. 3H). Similar to the responses in naïve, non-stressed animals, rats exposed to CUS and transfected with lenti–shHDAC5-EGFP alone produced significantly more BDNF, and the expression of BDNF in these animals was reduced in response to ketamine compared to animals transfected with lenti-EGFP and ketamine (Fig. 3H and I), indicating that lenti–shHDAC5-EGFP blocks the action of ketamine. These results are consistent with behavioral responses to transfection with lenti–shHDAC5-EGFP alone, which also produces significant antidepressant actions in the novelty

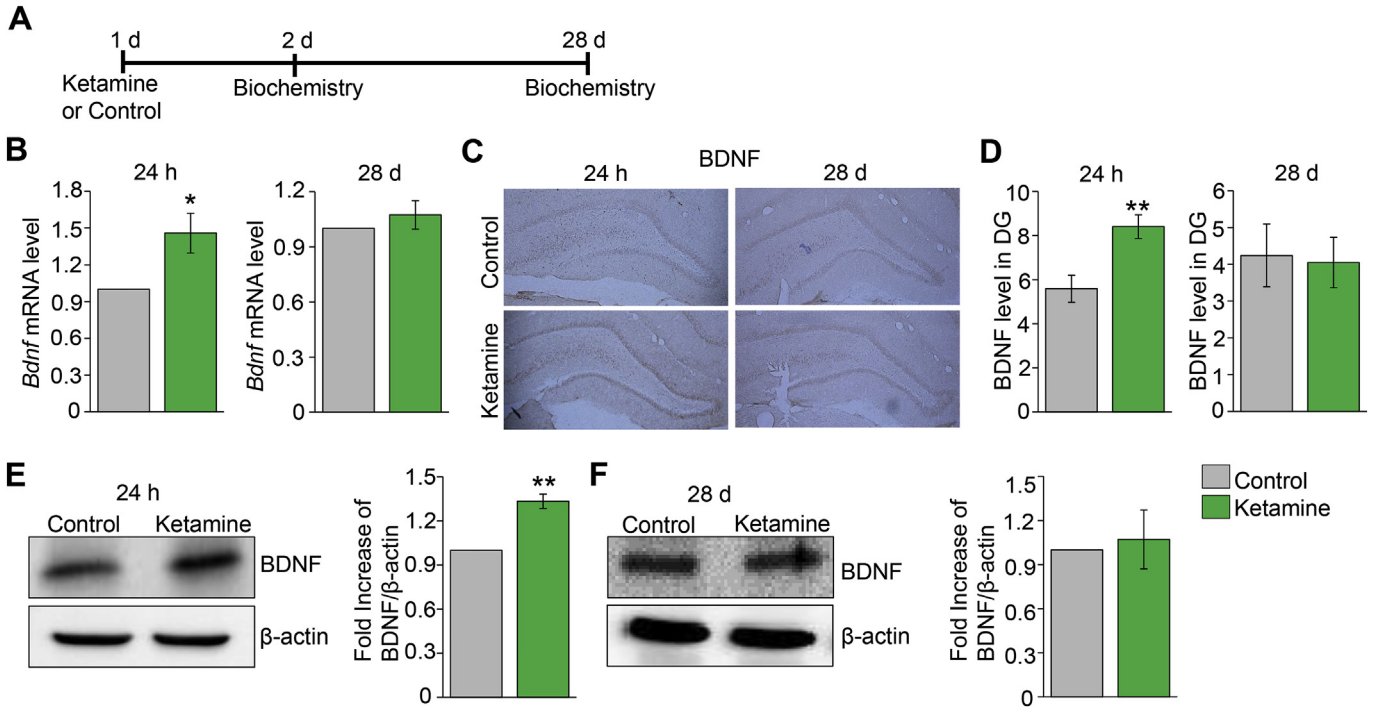

Fig. 2. Induction of rat hippocampal BDNF 24 h and 28 days after ketamine injection.
(A) Experimental paradigms. (B) *Bdnf* mRNA levels 24 h and 28 days after ketamine injection (10 mg/kg, i.p.). A significant effect of ketamine on BDNF expression is seen 24 h after ketamine treatment (*$P < 0.05$, Student's *t*-test; $n = 3$–4 animals per treatment) but there is no significant difference after 28 days. (C) Immunohistochemistry of BDNF levels in the hippocampi of rats 24 h and 28 days after ketamine injection (10 mg/kg, i.p.). (D) Densitometric analyses of the dentate gyrus granule cells in (C). A significant effect of ketamine on BDNF expression is seen 24 h after ketamine treatment (10 mg/kg, i.p.) (**$P < 0.01$, Student's *t*-test; $n = 4$ animals per treatment) but there is no significant difference after 28 days. Western blotting at (E) 24 h and (F) 28 days after ketamine injection (10 mg/kg, i.p.). A significant effect of ketamine on BDNF expression is seen 24 h after ketamine treatment (**$P < 0.01$, Student's *t*-test; $n = 2$–3 animals per treatment) but there is no significant difference at 28 days after ketamine injection.

suppressed feeding test, the sucrose preference test, the forced swim test and the learned helplessness test, and these effects were reduced in response to ketamine [15]. The expression of *Bdnf* IV mRNA was observed in a similar manner compared to *Bdnf* mRNA (Fig. 3J). These results demonstrate that ketamine increases the transcriptional regulation of both BDNF and BDNF IV *in vivo* and suggest that ketamine-induced BDNF expression is, at least in part, mediated by the inhibition of HDAC5 signaling.

We next investigated the role of HDAC5 as a regulator of BDNF IV expression, by co-transfecting rat hippocampal neurons with plasmids expressing pCI–HDAC5–WT and a luciferase reporter construct carrying 0.5 kb of the rat BDNF promoter IV sequence in front of the firefly luciferase coding sequence. Consistent with the effects observed *in vivo*, ketamine increased BDNF IV promoter activity in cells transfected with control vector (Fig. 4A). BDNF promoter IV activity was significantly increased by treatment with ketamine in cells transfected with pCI–HDAC5–WT (Fig. 4A), indicating that HDAC5 overexpression itself does not affect ketamine-induced BDNF IV activity. We demonstrated that overexpression of pCI–HDAC5–S/A, a mutant HDAC5 in which serine 259 and 498 are mutated to alanine, and which causes HDAC5 to be retained in the nucleus, downregulates gene expression in hippocampal neurons. Ketamine did not induce BDNF IV activity in cells infected with pCI–HDAC5–S/A. Taken together, these results indicate that ketamine-induced BDNF expression is mediated via phosphorylation of HDAC5 at serine 259 and 498.

4. Discussion

In the present study, we provide evidence for the regulation of BDNF expression in neurons by ketamine via the phosphorylation of HDAC5, and suggest that induction of BDNF expression by ketamine may result from the suppression of the repressor activity of HDAC5. Our results indicate that HDAC5 is critical for induction of the BDNF promoter IV. Ketamine increases BDNF expression both in cell culture and *in vivo*, making ketamine an attractive target for pharmacological interventions aimed at modulating BDNF expression [12,25]. The effects of ketamine on gene expression are commonly interpreted as direct consequences of alterations to neuronal signaling. Although this mechanism undoubtedly has an important role, other mechanisms are also likely to contribute. We previously demonstrated that ketamine alters both the phosphorylation of HDAC5 and the expression of HDAC5 target genes [15]. Taken together, it can be assumed that ketamine-induced gene expression is secondary to the activation/repression of chromatin regulators.

A comparison of *Bdnf* mRNA induction and HDAC5 phosphorylation revealed that treatment with ketamine induces *Bdnf* mRNA and phosphorylates HDAC5 over a similar time course, supporting the theory that HDAC5 participates in the transcriptional regulation of BDNF. A weak, but statistically significant, induction of *Bdnf* mRNA was observed in neurons 24 h after treatment with ketamine, but this was transient, gradually decreasing 24 h later. The mechanisms underlying such dynamics remain to be determined, but this effect could be advantageous when considering therapeutic use, because the excessive release of neurotrophic factors can lead to deleterious side effects [26]. The expression of *Bdnf* exon IV and total *Bdnf* mRNAs reached a peak following treatment with ketamine at 100 nM in cultured neurons. In addition, a time-course analysis of BDNF transcripts showed that both total *Bdnf* and *Bdnf* IV were induced to a similar degree by ketamine *in vivo*. These results suggest that ketamine induced the expression of exon IV *Bdnf*

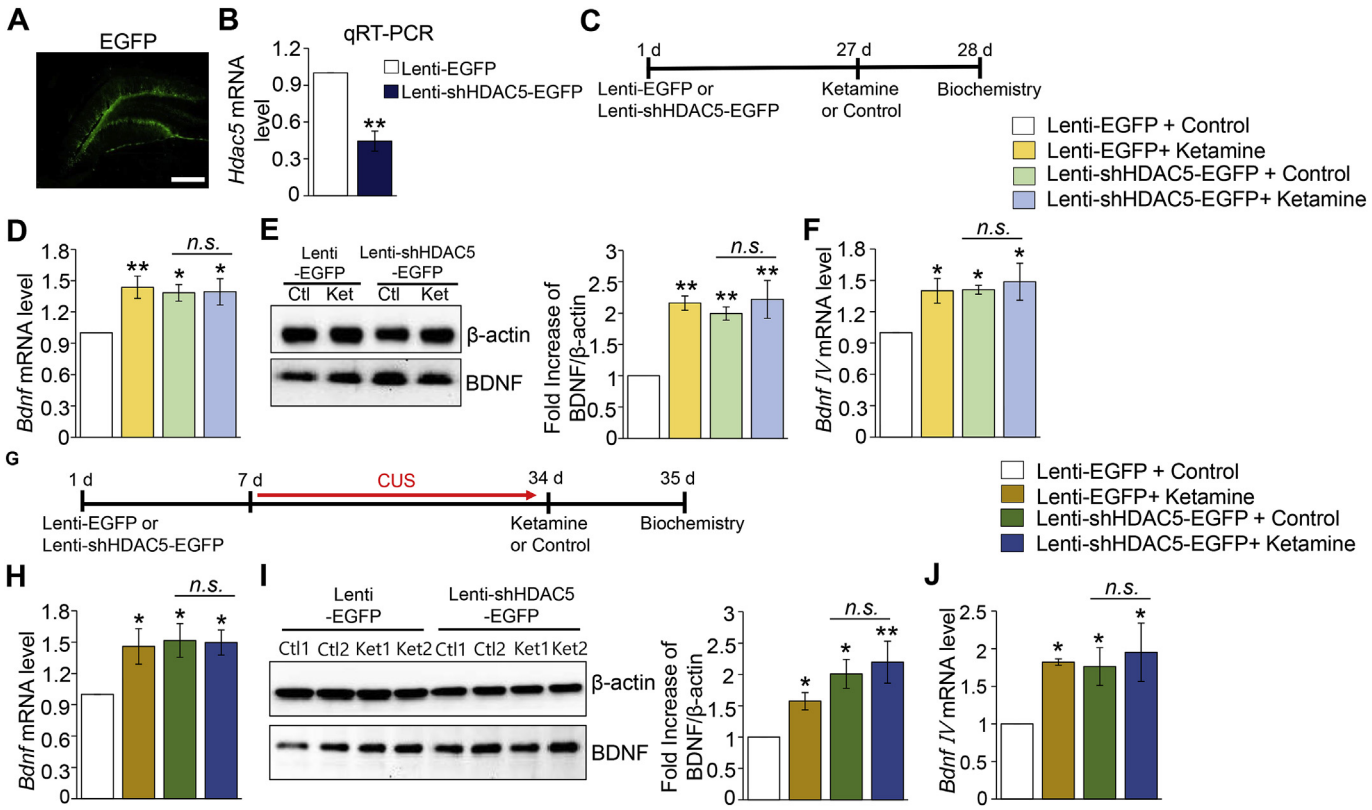

Fig. 3. Blockade of ketamine-induced BDNF by shRNA knockdown of HDAC5 in rat dentate gyrus.
(A) Enhanced green fluorescent protein (EGFP) expression by lenti–shHDAC5–EGFP-transfected rat dentate gyrus (DG). (B) qRT-PCR showing lenti–shHDAC5-mediated knockdown of *Hdac5* mRNA versus lenti–EGFP-transfected controls (Ctl). Lenti–shHDAC5–EGFP reduces *Hdac5* mRNA expression in rat DG (**$P < 0.01$ versus lenti–EGFP, Student's *t*-test; $n = 3$–4 animals per group). (C) Rats were injected with lenti–EGFP or lenti–shHDAC5–EGFP. Ketamine (10 mg/kg) was injected into half of the rats from each virus-infected group on day 27. qRT-PCR analysis of (D) *Bdnf* mRNA, and (F) *Bdnf IV* mRNA levels in DG microdissected from rat hippocampi transfected with either lenti–EGFP or lenti–shHDAC5–EGFP (*$P < 0.05$, **$P < 0.01$, $n = 3$–4 animals per treatment). (E) BDNF protein levels in DG microdissected from rat hippocampi transfected with either lenti–EGFP or lenti–shHDAC5–EGFP. Representative immunoblots and quantitative data for BDNF normalized to the level of β-actin. (**$P < 0.01$, $n = 3$–4 animals per treatment). (F) *Bdnf IV* mRNA in rat DG. (G) Rats were injected with lenti–EGFP or lenti–shHDAC5–EGFP. All virus-infected cohorts were exposed to CUS for 28 days starting on day seven after lentivirus injection. Ketamine (10 mg/kg) was injected into half of the animals from each virus-infected group on day 34. qRT-PCR analysis of (H) *Bdnf* mRNA (J) *Bdnf IV* mRNA levels in DG microdissected from rat hippocampus transfected with either lenti–EGFP or lenti–shHDAC5–EGFP (*$P < 0.05$, $n = 3$ animals per treatment). (I) BDNF expression in DG microdissected from rat hippocampus transfected with either lenti–EGFP or lenti–shHDAC5–EGFP. Representative immunoblots and quantitative data for BDNF, normalized to the level of β-actin EGFP (*$P < 0.05$, **$P < 0.01$, $n = 3$ animals per treatment). (J) *Bdnf IV* mRNA in DG. Two-way ANOVA was followed by least significant difference *post hoc* analysis. *$P < 0.05$, **$P < 0.01$ compared with vehicle-treated lenti–EGFP rats (Scale bar, 500 μm). n.s., no significance.

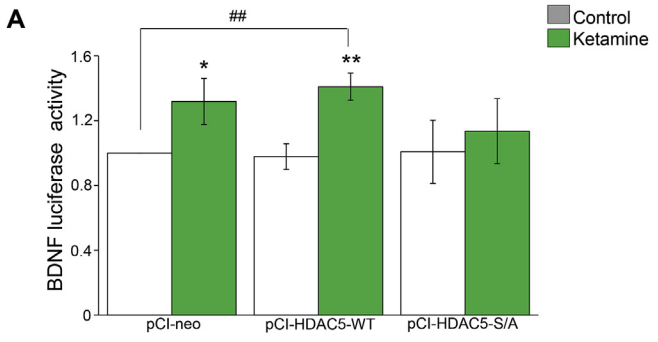

Fig. 4. Ketamine regulates BDNF IV activity via HADC5 in rat hippocampal neurons.
(A) Luciferase assay. Rat hippocampal neurons were co-transfected with BDNF IV–Luc reporter or pGL3–Luc (control), together with pCI–HDAC5–WT, pCI–HDAC5–S/A, or pCI–neo (control) for 48 h. Cell were then exposed to ketamine (10 mg/kg) for 24 h. Student's *t*-test; $n = 4$ animals per treatment, *$P < 0.05$, **$P < 0.01$, compared with vehicle treatment; ##$P < 0.01$, compared with vehicle in pCI–neo.

mRNAs, and furthermore suggests that the mechanisms of *Bdnf* mRNA induction by ketamine are also responsible for the transcriptional regulation of *Bdnf* IV.

The expression of *Bdnf* mRNA following knockdown of HDAC5 suggests that similar mechanisms are responsible for the increased activity at *Bdnf* promoter sites following HDAC5 knockdown. HDAC5 regulates gene expression through interaction with transcription factors such as MEF2 [27], and has been implicated in the regulation of gene transcription through nuclear export of its phosphorylated form to release its repressor activity in cerebellar neurons [10]. In the present study, we found that HDAC5 overexpression did not block ketamine-induced BDNF IV induction, but mutation of HDAC5 significantly blocked ketamine-induced BDNF IV induction. These results indicate that HDAC5 might be efficiently phosphorylated and translocated to cytosol in cells transfected with pCI–HDAC5, and therefore HDAC5 repressor activity is effectively removed. However, HDAC5 phosphorylation is indispensable for promoter IV activation by ketamine, because pCI–HDAC5-S/A blocks BDNF promoter IV activity efficiently. Taken together, our results indicate that HDAC5 phosphorylation is critical for the expression of *Bdnf* exon IV following exposure to ketamine and therefore class-II-selective HDAC inhibitors might have potential as therapeutic agents for psychiatric diseases.

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