Ketamine produces antidepressant-like effects through phosphorylation-dependent nuclear export of histone deacetylase 5 (HDAC5) in rats

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Ketamine produces rapid antidepressant-like effects in animal assays for depression, although the molecular mechanisms underlying these behavioral actions remain incomplete. Here, we demonstrate that ketamine rapidly stimulates histone deacetylase 5 (HDAC5) phosphorylation and nuclear export in rat hippocampal neurons through calcium/calmodulin kinase II- and protein kinase D-dependent pathways. Consequently, ketamine enhanced the transcriptional activity of myocyte enhancer factor 2 (MEF2), which leads to regulation of MEF2 target genes. Transfection of a HDAC5 phosphorylation-defective mutant (Ser259/Ser498 replaced by Ala259/Ala498, HDAC5-S/A), resulted in resistance to ketamine-induced nuclear export, suppression of ketamine-mediated MEF2 transcriptional activity, and decreased expression of MEF2 target genes. Behaviorally, viral-mediated hippocampal knockdown of HDAC5 blocked or occluded the antidepressant effects of ketamine both in unstressed and stressed animals. Taken together, our results reveal a novel role of HDAC5 in the actions of ketamine and suggest that HDAC5 could be a potential mechanism contributing to the therapeutic actions of ketamine.

ketamine | HDAC | depression | hippocampus

Depression is a multifaceted illness, characterized by somatic, cognitive, and behavioral changes. All currently available antidepressants primarily act via monoaminergic neurotransmitters, such as serotonin and/or noradrenaline (1). Currently available pharmacotherapies for depression provide some relief for patients, but these agents have significant limitations (1). In this context, new antidepressants with faster onset of action and greater efficacy are needed (2).

The noncompetitive *N*-methyl-D-aspartate (NMDA) receptor antagonist ketamine has shown remarkable consistency in rapidly ameliorating depressive symptoms in major depressive disorder (MDD) (3). Preclinical studies have demonstrated that ketamine produces rapid antidepressant responses (within hours) (4, 5). Ketamine's antidepressant effects in rodents are associated with activation of several signaling systems including the mammalian target of rapamycin complex 1 (mTORC1) (4), brain derived neurotrophic factor (BDNF) and elongation factor 2 (EF2) kinase (5). Despite these remarkable effects, the widespread use of ketamine is limited by potential side effects and abuse. Thus, studies are necessary to further elucidate mechanistic actions of ketamine at cellular and network levels.

Recent studies have generated evidence that epigenetic regulation is closely involved in the pathophysiology of depression and in the therapeutic mechanisms of typical antidepressants (6, 7). In addition, reports that sodium butyrate, a histone deacetylase (HDAC) inhibitor, has antidepressant effects indicate that HDAC inhibition is sufficient to produce an antidepressant response (8). HDACs are a family of enzymes capable of repressing gene expression by removing acetyl groups from histones to produce a less accessible chromatin structure (9).

Previous studies demonstrate that the class II HDAC, HDAC5, epigenetically controls behavioral adaptations to chronic emotional stimuli in nucleus accumbens (10) and that hippocampal overexpression of HDAC5 blocks the ability of chronic imipramine to reverse behavioral deficits caused by chronic social defeat stress (7). HDAC5 is highly enriched in the brain with strong expression in forebrain regions including the hippocampus, cortex, and amygdala (9). We focus here on HDAC5 because its subcellular localization is tightly regulated by neuronal activity (11–13). The class II HDAC family of transcriptional repressors, in particular HDAC5, interacts with myocyte enhancer factor 2 (MEF2) to repress target gene expression (13, 14). Phosphorylation of HDAC5 by HDAC5 kinases liberates nuclear MEF2 transcription factors through nuclear export of the phosphorylated HDAC5 (13).

In the present study, we found that ketamine down-regulates HDAC5 to attenuate its repressive influence on transcription in the hippocampus. We further show that HDAC5 shRNA knockdown in hippocampus blocks or occludes the behavioral actions of ketamine in unstressed rats and alone is sufficient to produce antidepressant responses in rodents exposed to chronic stress. Together, these data suggest a role for HDAC5 in the molecular machinery underlying the actions of ketamine.

Significance

The rapid antidepressant response is produced by ketamine administration. However, the molecular mechanisms underlying the antidepressant-like action of ketamine remain incomplete. Here we show for the first time to our knowledge that ketamine stimulates the phosphorylation (Ser259/Ser498) and nuclear export of histone deacetylase 5 (HDAC5). As a consequence, myocyte enhancer factor 2 (MEF2) transcriptional activity is enhanced and results in the induction of MEF2 target gene expression. We further show that ketamine down-regulates and, at the same time, phosphorylates HDAC5 to attenuate its repressive influence on transcription in the hippocampus. These studies unveil a previously unidentified role of HDAC5 in regulating neuronal function in response to ketamine, and thus provide the foundation for new approaches for the treatment of major depression.

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The authors declare no conflict of interest.

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Results

Ketamine Induces HDAC5 Phosphorylation and Nuclear Export in Hippocampal Neurons. To examine the potential role of HDAC5 in ketamine-induced signaling and function in hippocampal neurons, we first examined the phosphorylation of HDAC5 at Ser259 and Ser498 residues in response to ketamine stimulation using two phosphospecific HDAC5 antibodies. Exposure of cultured hippocampal neurons to ketamine induced HDAC5 phosphorylation in a concentration-dependent manner, which reached peak levels at ∼100 nM (Fig. 1*A*), a concentration lower than comparable plasma concentrations required to produce anesthesia in humans (5–10 μM) (15). The response to ketamine displayed an inverted U, as higher doses had no effect on HDAC5 phosphorylation. Similar inverted U dose–response curves have been demonstrated for ketamine induction of extracellular glutamate (16) and for the antidepressant behavioral actions of ketamine (4). At 100 nM ketamine, the response was time dependent, reaching peak levels at ∼3–6 h and returned to basal levels after 24 h (Fig. 1*B*). The total level of HDAC5 was unchanged during ketamine stimulation (Fig. 1 *A* and *B*). The effect of ketamine on HDAC5 phosphorylation coincides with the activation of other signaling molecules including eukaryotic initiation factor 4E binding protein 1 (4E-BP1) and cAMP-response element binding protein (CREB) (*SI Appendix*, Fig. S1), consistent with previous ketamine reports (4) as well as typical antidepressants (17).

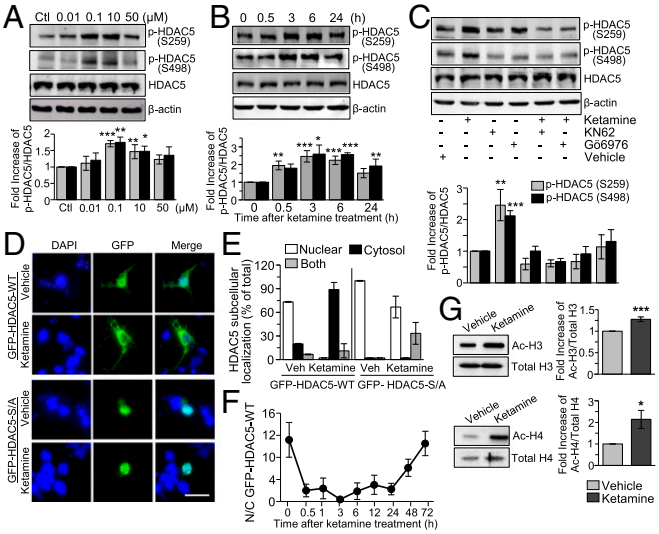

Fig. 1. Ketamine stimulates HDAC5 phosphorylation through Ca^{2+}/calmodulin-, PKD-dependent pathways and nuclear export in hippocampal neurons. Hippocampal neurons were exposed to ketamine for 30 min for various concentrations (*A*) or for various times at 100 nM (*B*). (*C*) Neurons were pretreated with KN-62 (30 μM) or Gö6976 (1 μM) for 30 min, and then exposed to ketamine (100 nM) for 6 h. (*D*) Representative fields of GFP fluorescence in saline- or ketamine-treated neurons expressing GFP-HDAC5-WT or GFP-HDAC5-S/A. Cells were counterstained with DAPI (blue). (*E*) The localization of HDAC5 was categorized as cytoplasmic, nuclear, or both, 24 h after ketamine by an experimenter blind to treatment. The percentage for each category was calculated from the total number of transfected neurons counted in each condition (n = 50–60 neurons per condition, four independent cultures). (*F*) Kinetics of nuclear export of HDAC5 in neurons expressing GFP-HDAC5-WT. Results are quantitative analysis of the GFP immunofluorescence in nucleus (N) and cytosol (C) and expressed as a ratio of vehicle-treated controls (Ctl). (*G*) Histone acetylation upon ketamine (6 h). Results of p-HDAC5 levels were normalized with the level of HDAC5. The level of p-HDAC5 is shown as fold changes relative to Ctl value (*A* and *C*). The phosphorylation levels were depicted relative to the level of Ctl at each time point and are shown as fold changes relative to the value at 0 h (*B*). Results are the mean ± SEM from four (*D*–*G*) and six (*A*–*C*) independent cultures. Student's t test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with Ctl or 0 h. (Scale bar, 25 μm.)

Previous work has shown that the phosphorylation of HDAC5 can be regulated by Ca^{2+}/calmodulin-dependent kinase II (CaMKII) activity in neurons (11) and by protein kinase D (PKD) in other cell types (18). To determine whether HDAC5 phosphorylation in response to ketamine is mediated by CaMKII and PKD in hippocampal neurons, we treated cells with the CaMKII inhibitor KN-62 and PKD-specific inhibitor Gö6976. Ketamine-induced HDAC5 phosphorylation at S259 and S498 was completely abolished by either KN-62 or Gö6976 (Fig. 1*C*). In support of these results, ketamine stimulated the phosphorylation of CaMKII and PKD (*SI Appendix*, Fig. S1). These results indicate that ketamine transiently induces HDAC5 phosphorylation via CaMKII- and PKD-dependent pathways.

Because HDAC5 is phosphorylated and exported out of the cell nucleus (12), we tested whether such cytoplasmic localization of HDAC5 might be triggered by ketamine. Western blots showed that ketamine increased p-HDAC5 levels in cytoplasm, with nuclear localization of HDAC5 (*SI Appendix*, Fig. S2). To further evaluate that ketamine-dependent phosphorylation of HDAC5 at Ser259/498 residues is required for HDAC5 nuclear export, we infected hippocampal neurons with plasmids expressing GFP-HDAC5-WT and GFP-HDAC5-S/A, a mutant construct of HDAC5 in which both serines 259 and 498 are mutated to alanine (19), and studied the subcellular localization of HDAC5. Both GFP-HDAC5-WT and GFP-HDAC5-S/A were targeted predominantly to the nucleus of hippocampal neurons under basal conditions (Fig. 1*D*). After 30 min of ketamine treatment, however, GFP-HDAC5-WT started to translocate into the cytoplasm and then returned to the nucleus within 48 h after treatment (Fig. 1*F*). In contrast, GFP-HDAC5-S/A remained in the nucleus throughout the 24-h period of ketamine treatment (Fig. 1 *D* and *E* and *SI Appendix*, Fig. S3).

Nuclear export of HDAC5 induces shifting of the chromatin state to one that favors histone acetylation (20). In support of these results, ketamine incubation increased levels of global acetylation of the core histones H3 and H4 (Fig. 1*G*). These results indicate that ketamine-induced phosphorylation of HDAC5 at Ser259/498 is required for its nuclear export and suggest that the phosphorylation could lead to derepression of gene expression that contributes to the actions of ketamine.

Ketamine Regulates MEF2-Dependent Gene Expression. The results demonstrating the ability of ketamine to catalyze the phosphorylation of HDAC5 and trigger nuclear export indicate that MEF2 activity would also be increased. Using a MEF2-luciferase reporter assay to monitor MEF2 activity, we found that ketamine significantly increased MEF2 transcriptional activity in a time-dependent manner with a peak level at ∼3–6 h of incubation (Fig. 2*A*). Given that ketamine-induced HDAC5 phosphorylation is mediated by CaMKII and PKD, we examined whether the activation of MEF2 by ketamine requires the activity of these kinases. Ketamine-induced MEF2 transcriptional activity was completely suppressed by either KN-62 or Gö6976 (Fig. 2*B*), indicating the involvement of CaMKII and PKD in the activation of MEF2-dependent transcription.

To further evaluate the role of HDAC5 in the MEF2-mediated gene expression by ketamine, we examined the regulation of the prototypical MEF2 target genes *Arc* and *Nurr77* (14). Consistent with the regulation of MEF2 activity, ketamine induced the upregulation of *Arc* and *Nurr77*, effects that were abolished after inhibition of HDAC5 phosphorylation by KN-62 or Gö6976 (Fig. 2*C*). To further define the potential role of MEF2 in ketamine-mediated gene regulation, we examined the expression of Krüppel-like factor 6 (*Klf6*), a key downstream effector of the neuronal MEF2 pathway (14, 21). We found that ketamine increased *Klf6* mRNA levels, which was abolished by KN-62 or Gö6976 (Fig. 2*C*). Overexpression of HDAC5 significantly reduced MEF2 activity and ketamine reversed this effect (Fig. 2*D*). Demonstrating the importance of HDAC5 phosphorylation, GFP-HDAC5-S/A completely

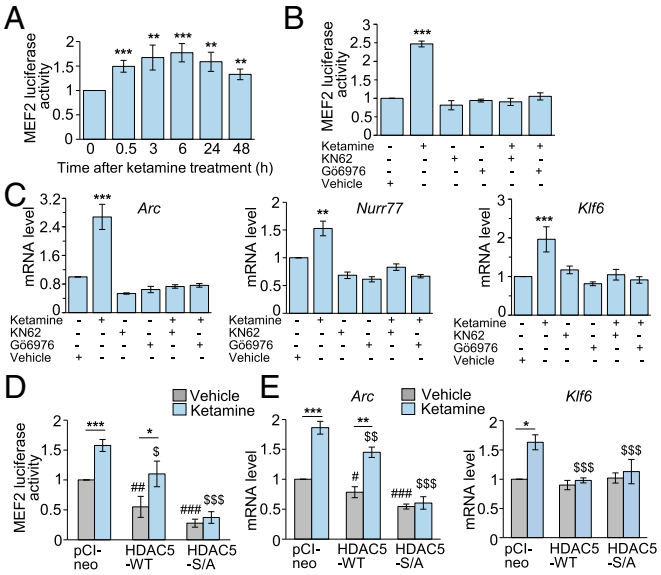

Fig. 2. Ketamine regulates MEF2 activity by phosphorylating HDAC5. (*A* and *B*) Luciferase assay. Hippocampal neurons transfected with pGL3-Luc (encoding *renilla* luciferase) and pGL3-MEF2-Luc (encoding *firefly* luciferase) were treated with ketamine for indicated times (*A*) or treated with ketamine (100 nM, 6 h) in the presence of KN-62 or Gö6976 (*B*). MEF2-luciferase activity was normalized to *renilla* luciferase activity; the MEF2-luciferase activity was depicted relative to Ctl at each time point and are expressed as fold changes relative to the value at 0 h. (*C*) The expression of mRNAs was analyzed by qRT-PCR after ketamine (6 h) in the presence of KN-62 and Gö6976. (*D* and *E*) Neurons were cotransfected with 3xMEF2-Luc reporter and pGL3-Luc, along with pCI-neo-HDAC5-WT, pCI-neo-HDAC5-S/A, or pCI-neo as control for 24 h. Cells were then exposed to ketamine (6 h). MEF2-luciferase activity is shown (*D*) and the *Arc* and *Klf6* mRNA levels were analyzed by qRT-PCR (*E*) and are expressed as fold changes relative to the vehicle-treated pCI-neo control vector value. Results are the mean ± SEM ($n = 4$ independent experiments in *A*–*E*). Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with vehicle treatment or 0 h (*A*–*C*). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared with vehicle in pCI-neo; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ compared with ketamine in pCI-neo (*D* and *E*).

blocked the ability of ketamine to activate MEF2 transcriptional activity (Fig. 2*D*). Similarly, overexpression of HDAC5 or HDAC5-S/A reduced the expression of *Arc* and *Klf6* mRNAs and blocked ketamine induction of *Arc* and *Klf6* expression (Fig. 2*E*). The regulatory regions of *Arc*, *Nurr77*, and *Klf6* have been identified to contain MEF2D-binding sites (14). We have confirmed that MEF2D is enriched in the promoter regions of *Arc*, *Nurr77*, and *Klf6* in response to ketamine in cultured hippocampal neurons (*SI Appendix*, Fig. S4). Moreover, MEF2D protein expression is increased in hippocampus after ketamine injection (*SI Appendix*, Fig. S4). Thus, ketamine may promote the transcription of a set of genes that are regulated by MEF2D. Taken together, these data demonstrate that ketamine-induced phosphorylation and nuclear export of HDAC5 derepress MEF2 and augment transcriptional activity on *Arc*, *Nurr77*, and *Klf6* target promoters.

Ketamine Induces HDAC5 Phosphorylation and MEF2 Target Gene Expression in Hippocampus. We next determined whether systemic ketamine administration at a low dose (10 mg/kg) that is reported to have antidepressant actions (4), also induces HDAC5 phosphorylation in the hippocampus, a region that contributes to antidepressant behavioral responses (22) and that is reduced in volume in patients with MDD (23). Ketamine injection transiently increased HDAC5 phosphorylation at both S259 and S498; the increase was significant at 30 min, maximal (approximately fourfold) after 6 h and still elevated at longer time points (12–24 h) (Fig. 3*A*). The level of total HDAC5 was not changed through 24 h after a ketamine injection at either the mRNA or protein levels (Fig. 3*A* and *SI Appendix*, Fig. S5).

Given that ketamine-induced HDAC5 phosphorylation activates MEF2-dependent gene transcription in vitro, we examined the mRNA levels of MEF2 target genes in hippocampus in rats after ketamine injection. Consistent with the HDAC5 phosphorylation, ketamine also led to a rapid up-regulation of *Arc* mRNA as early as 30 min, was maximal (~2.5-fold) after 6 h, and still significantly increased at 24–48 h (Fig. 3*B*). Similarly, ketamine induced the up-regulation of *Klf6* mRNA, peaking at 6 h (Fig. 3*C*), consistent with the regulation of MEF2 activity by ketamine. Ketamine administration also rapidly increased mRNA levels of the postsynaptic proteins PSD95 and GluR1, as well as the presynaptic protein synapsin I as early as 30 min, peaking at around 6 h (Fig. 3*D*), consistent with previous results (4). Ketamine induced an ~55% increase in global acetylation in H3 and H4 (Fig. 3*E*) in the hippocampus after a 6-h treatment, the time point found to maximally induce HDAC5 phosphorylation.

HDAC5 Knockdown Abolishes or Occludes Antidepressant-Like Effect of Ketamine. Having seen that ketamine is capable of phosphorylating HDAC5 in vivo, we tested whether HDAC5 knockdown influences the antidepressant behavioral responses to ketamine. We infused lentivirus expressing shRNAs targeted against rat HDAC5 (lenti-shHDAC5) into granule cells of dentate gyrus

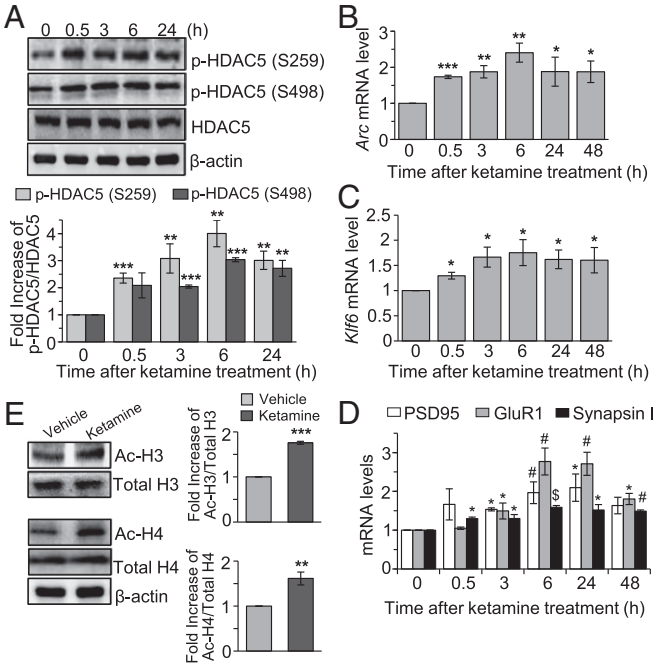

Fig. 3. Ketamine induces HDAC5 phosphorylation and MEF2 target gene expression in the hippocampus. (*A*) HDAC5 phosphorylation in hippocampus of rats exposed to ketamine (10 mg/kg) for various times. The levels of p-HDAC5 were normalized with the level of HDAC5. The phosphorylation levels were depicted relative to the level of the vehicle-treated Ctl at each time point and are shown as fold changes relative to the value at 0 h ($n = 5$ rats per time point from five independent experiments). (*B* and *C*) qRT-PCR analysis of *Arc* and *Klf6* mRNAs after ketamine. (*D*) Ketamine rapidly increases synaptic proteins *Psd95*, *GluR1*, and *Synapsin I* (Student's *t* test, $^{*}P < 0.05$, $^{\#}P < 0.01$, $^{\$}P < 0.001$ compared with 0 h). (*E*) Histone acetylation after ketamine (6 h). Results of mRNA levels were normalized with the level of GAPDH. The mRNA levels were depicted relative to the level of the vehicle-treated Ctl at each time point and are shown as fold changes relative to the value at 0 h. Results are the mean ± SEM ($n = 5$ rats per time point from five independent experiments in *B*–*D*; $n = 4$ rats per condition in *E*). Student's *t* test, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with Ctl.

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(DG), a hippocampal subregion that is reduced in volume in MDD (23), 4 wk before ketamine injection (Fig. 4 *A* and *B*). Lenti-shHDAC5 infusions repressed HDAC5 mRNA and protein levels in the DGs (Fig. 4*C*).

Consistent with the rapid antidepressant effects of ketamine in intact animals (4), ketamine produced similar behavioral responses in rats infused with lenti-GFP control virus in the novelty suppressed feeding test (NSFT) (22), the forced swim test (FST), and the learned helplessness test (LHT), assays of anxiety (NFST) and despair (FST and LHT) that are responsive to antidepressant treatments (4, 22) (Fig. 4). At this time point (5–6 d after ketamine injection), the increase in HDAC5 phosphorylation in response to ketamine was no longer detectable relative to controls (*SI Appendix*, Fig. S6 and Fig. 5*B*), consistent with its transient phosphorylation. We next investigated whether HDAC5 knockdown alters the antidepressant effects of ketamine. Rats injected with lenti-shHDAC5 showed antidepressant-like behaviors in the FST and LHT; however, they resembled lenti-GFP control rats in the NSFT and sucrose preference test (SPT), a measure of anhedonia, which is a core symptom of depression (22) (Fig. 4 *D*–*G*). Compared with its effects in lenti-GFP–infused rats, ketamine had no effects in lenti-shHDAC5–expressing rats: the latency to feed in the NSFT, the amount of sucrose consumed in the SPT, and the immobility in the FST were not altered (Fig. 4 *D*–*G*), indicating that the antidepressant effects of ketamine are blocked by lenti-shHDAC5 infusion. Ketamine also produced no further effects on the number of escape failures in the LHT in lenti-shHDAC5–expressing rats, possibly due to a floor effect of HDAC5 knockdown. Together, these results demonstrate that lenti-shHDAC5 produces modest antidepressant-like effects in certain behavioral tests and abolishes or occludes the antidepressant-like effects of ketamine in nonstressed animals.

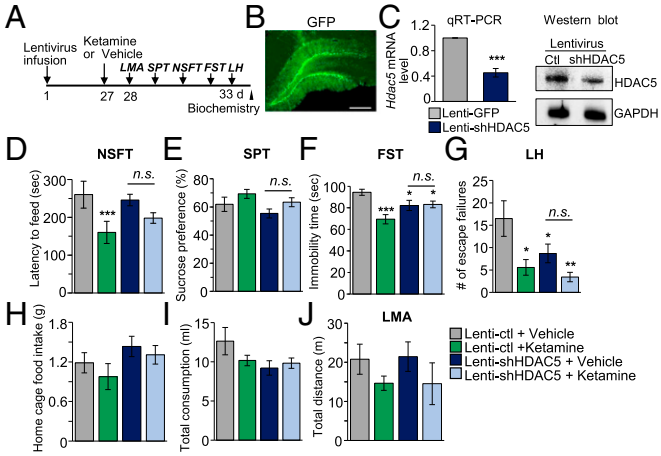

Fig. 4. Ketamine has no antidepressant effects in rats in which HDAC5 is knocked down in the hippocampus. (*A*) Behavioral paradigm. (*B*) GFP expression in DG. (*C*) Lentiviral-mediated knockdown of HDAC5 mRNA and protein (160 kDa) in the DG ($n = 4$ rats). (*D*) NSFT. Main effect of ketamine: $P < 0.01$; main effect of virus: $P > 0.05$; interaction: $P < 0.05$. A significant decrease in the latency to feed was shown by ketamine in lenti-GFP rats (***$P < 0.001$). (*E*) SPT. Main effect of ketamine: $P > 0.05$; main effect of virus: $P > 0.05$; interaction: $P > 0.05$. Ketamine had no effects in both lenti-GFP and lenti-shHDAC5 rats. (*F*) FST. Main effect of ketamine: $P < 0.001$; main effect of virus: $P > 0.05$; interaction: $P < 0.01$. Ketamine produced a shorter immobility score (time in seconds) than saline in lenti-GFP rats (***$P < 0.001$) but not in lenti-shHDAC5 rats. Lenti-shHDAC5-GFP–injected animals had a decrease in immobility compared with lenti-GFP rats (*$P < 0.05$). (*G*) LHT. Main effect of ketamine: $P < 0.05$; main effect of virus: $P < 0.05$; interaction: $P > 0.05$. Ketamine decreased the escape failures in lenti-GFP rats (*$P < 0.05$). Lenti-shHDAC5-GFP–injected rats had a decrease in the number of escape failures compared with lenti-GFP rats (*$P < 0.05$). There was no difference in the home cage food intake (*H*), total fluid consumption (*I*), or total distance moved in the box (*J*) between groups. Data are the mean ± SEM ($n = 13$–15 rats per group). Two-way ANOVA was followed by LSD post hoc analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle-treated lenti-GFP rats. (Scale bar, 500 μm.) n.s., no significance.

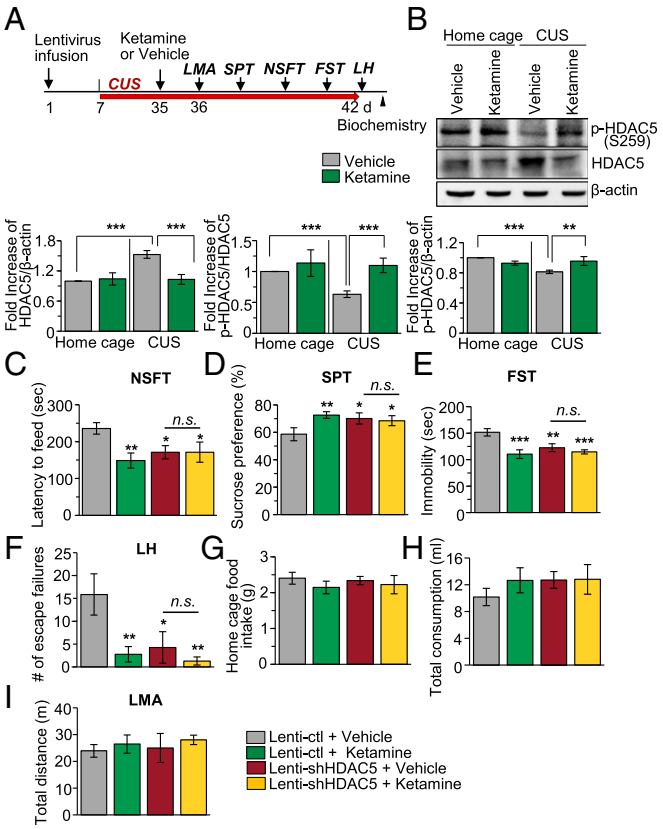

Fig. 5. HDAC5 knockdown produces antidepressant effects and occludes the actions of ketamine in chronically stressed animals. (*A*) Experimental design. Rats were injected with lenti-GFP or lenti-shHDAC5-GFP. All virus-infected cohorts were exposed to CUS for 35 d starting on day 7. Ketamine (10 mg/kg) was injected into halves of each virus-infected group on day 35. Behavioral performances were measured for 5–6 consecutive days starting on day 36. (*B*) HDAC5 expression and HDAC5 phosphorylation in hippocampus. Representative immunoblots and quantitative data of HDAC5 normalized with the level of β-actin (*Left*), HDAC5 phosphorylation normalized either with the level of HDAC5 (*Middle*), or β-actin (*Right*) are shown ($n = 4$–5 rats per group). (*C*) NSFT. Main effect of ketamine: $F_{3,43} = 4.32$, $P < 0.05$; main effect of virus: $F_{3,43} = 1.00$, $P > 0.05$; interaction $F_{3,43} = 4.322$, $P < 0.05$. Further analysis indicates that a significant decrease in the latency to feed was shown by ketamine in lenti-GFP animals. Lenti-shHDAC5-GFP animals had a decrease in latency to feed compared with lenti-GFP rats (*$P < 0.05$). (*D*) SPT. Main effect of ketamine: $F_{3,40} = 3.55$, $P < 0.05$; main effect of virus: $F_{3,40} = 1.26$, $P > 0.05$; interaction $F_{3,40} = 5.72$, $P < 0.05$. Ketamine injection into rats expressing lenti-GFP increased sucrose preference. Lenti-shHDAC5-GFP animals had an increase in sucrose preference compared with lenti-GFP rats (*$P < 0.05$). (*E*) FST. Main effect of ketamine: $F_{3,26} = 12.41$, $P < 0.01$; main effect of virus: $F_{3,26} = 3.14$, $P > 0.05$; interaction $F_{3,26} = 5.71$, $P < 0.05$. A significant decrease in immobility was shown by ketamine in lenti-GFP animals (***$P < 0.001$) but not in lenti-HDAC5-GFP rats ($P > 0.05$). Lenti-shHDAC5-GFP animals had a decrease in immobility compared with lenti-GFP rats (**$P < 0.01$ and ***$P < 0.001$). (*F*) LHT. Main effect of ketamine: $F_{3,26} = 7.31$, $P < 0.05$; main effect of virus: $F_{3,26} = 4.83$, $P < 0.05$; interaction $F_{3,26} = 2.91$, $P > 0.05$. Ketamine decreased escape failures only in lenti-GFP animals (**$P < 0.01$) but not in lenti-shHDAC5-GFP rats ($P > 0.05$). Lenti-shHDAC5-GFP rats had a decrease in the number of escape failures compared with lenti-GFP rats (*$P < 0.05$ and **$P < 0.01$). There was no difference in the home cage food intake (*G*), total fluid consumption (*H*), or total distance moved in the box (*I*) between groups. Data are the mean ± SEM ($n = 10$–13 rats per group). Two-way ANOVA was followed by LSD post hoc analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ compared with vehicle-treated lenti-GFP rats. n.s., no significance.

HDAC5 Knockdown Produces Antidepressant Effects in Chronic Stress Animals and Occludes the Actions of Ketamine. HDAC5 has been implicated in antidepressant responses in the hippocampus of mice exposed to chronic emotional stress (7). To examine whether HDAC5 is involved in the antidepressant effects of ketamine in stress conditions and to possibly circumvent a floor effect of HDAC5 knockdown, we used a chronic unpredictable stress (CUS) paradigm that causes depression-related behavioral deficits (24) (Fig. 5*A*). We first confirmed that rats exposed to CUS exhibit deficits in SPT, NSFT, LHT, and FST, and rapid reversal by administration of a single dose of ketamine (*SI Appendix*, Fig. S7). We found that exposure to CUS increases levels of HDAC5 in the DG region of the hippocampus, and that this was reversed by a single injection of ketamine (Fig. 5*B*). The fraction of p-HDAC5 relative to total HDAC5 was lowest in CUS animals and this deficit was reversed by ketamine (Fig. 5*B*), indicating that transcriptional repressor activity of HDAC5 is increased by CUS and that ketamine abolishes this repressive activity. Similar down-regulation of p-HDAC5 was observed relative to β-actin in CUS animals (Fig. 5*B*). These results demonstrate that ketamine reverses the induction of HDAC5 by CUS and coincidently increases phosphorylation of HDAC5 in vivo.

Having observed that HDAC5 is up-regulated by CUS in hippocampus, we investigated the role of HDAC5 in ketamine-induced antidepressant responses in CUS animals. Consistent with the effects in naïve animals, ketamine produced antidepressant-like effects in CUS-exposed animals (24) with lenti-GFP infusions (*SI Appendix*, Fig. S7 and Fig. 5 *C–F*). Unlike the responses in naïve, nonstressed animals, in rats exposed to CUS, infusions of shHDAC5 alone produced significant antidepressant actions in the NSFT, SPT, FST, and LHT, and these effects occluded the actions of ketamine (Fig. 5 *C–F*), indicating that lenti-shHDAC5 blocks ketamine's action. These results demonstrate that HDAC5 knockdown produces antidepressant effects in animals exposed to CUS, and that these effects are nonadditive with ketamine, suggesting that the action of ketamine, at least in part, is mediated by inhibition of HDAC5 signaling.

Discussion

Our findings reveal a previously unidentified molecular mechanism by which ketamine regulates HDAC5 nuclear export and thereby represses its activity, resulting in up-regulation of MEF2 target genes including *Arc*, *Nurr77*, *Klf6*, and *Egr1* (*SI Appendix*, Fig. S8). Our observations that ketamine induces transient phosphorylation and nuclear export of HDAC5 that contributes to the regulation of antidepressant behaviors is a previously unidentified finding. Furthermore, we found a significant regulation of HDAC5 phosphorylation and gene expression levels in response to ketamine, strongly suggesting that epigenetic regulation plays a crucial role in the actions of ketamine in vivo.

Previous studies have reported that phosphorylation in P-S259 and P-S498 levels on HDAC5 induces HDAC5 cytoplasmic localization (25). More recently, a study reported that phosphorylation of P-S279 on HDAC5 promoted cytosolic retention in neurons (26). In this regard, we found that ketamine phosphorylates HDAC5 S279 in vitro (*SI Appendix*, Fig. S9). Therefore, our results suggest that the increase in nuclear export of HDAC5 may be due, at least in hippocampal neurons, to enhanced P-S259, P-S498, and P-S279 in response to ketamine. The ketamine-induced HDAC5 phosphorylation is suggested to be CaMKII and PKD dependent. Preclinical studies demonstrate that ketamine enhances glutamatergic transmission and subsequent stimulation of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, which leads to activation of voltage-dependent Ca^{2+} channels and activity-dependent brain-derived neurotrophic factor release (27, 28). This might be a mechanistic explanation for how CaMKII and PKD are activated by ketamine. Consistent with this hypothesis, our results showed that administration of a selective AMPA receptor inhibitor, 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzolquinoxaline-2,3-dione (NBQX) before ketamine blocks ketamine induction of HDAC5 phosphorylation (*SI Appendix*, Fig. S10), indicating that ketamine-induced HDAC5 phosphorylation requires AMPA-receptor activation.

Our observations about the role and regulation of HDAC5 phosphorylation in ketamine-induced behavioral responses raise a number of interesting questions for future study: for example, What is the function of HDAC5 that is relevant to behavioral responses of ketamine? The primary function of HDAC5 in the nucleus is histone deacetylation and indirect suppression of HDAC5 target genes by inhibiting MEF2-dependent transcription in neurons (11). Our findings demonstrate that the nuclear export of HDAC5 regulates ketamine-induced MEF2 transcriptional activation. In addition, a high degree of colocalization of HDAC5-GFP with endogenous MEF2D in neurons (11, 26), consistent with the idea that MEF2 is a mediator of HDAC5 function in response to ketamine. In support of this possibility, ketamine is reported to increase the number of dendritic spines in neurons (4, 24), and many of the identified MEF2 targets are enriched in synapses (14). Together, these findings are consistent with the possibility that these synapse-related genes are important mediators of the behavioral and synaptic actions of ketamine.

Detailed identification of HDAC5 target genes after ketamine exposure may help determine how MEF2 and HDAC5 regulate fast antidepressant responses through transcriptional mechanisms in vivo. Of particular interest are time points (i.e., 30 min–6 h) when HDAC5 phosphorylation and cytoplasmic localization are observed following ketamine exposure, because the time point analyzed in the current study (i.e., 24 h) in vivo is submaximal. Indeed, after 6 h and 9 h, MC1568, a class II inhibitor including HDAC5 (29), markedly reduced the immobility of rats in FST, compared with vehicle-treated animals (*SI Appendix*, Fig. S11), consistent with the possibility that HDAC5 might be involved in rapid-acting antidepressant responses of ketamine. *Arc*, as a HDAC5-MEF2 target gene, might play a role in the dendritic morphogenesis at early time points after ketamine administration, given its fast (within 25 min) and precise localization to dendrites in an activity-dependent manner (30). In addition, the subsequent protein synthesis involving mTORC1 and EF2 (4, 5) could also be involved in the sustained antidepressant actions of ketamine that remain in place even after ketamine has been removed from the brain and metabolilzed.

We observed that ketamine down-regulates HDAC5 expression in CUS rats but not in control rats. CUS-exposed rats have enhanced reactivity to ketamine, and the effects of ketamine in CUS rats are occluded by hippocampal expression of shHDAC5 (Fig. 5). In nonstress conditions, ketamine elicits antidepressant responses via activation of signaling molecules, increased phosphorylation and nuclear export of HDAC5, and improvement of synaptic function, as HDAC5 levels are relatively low and stable. In stress conditions, ketamine acts both on HDAC5 phosphorylation and nuclear export, in addition to the blockade of de novo synthesis of HDAC5. The effects of ketamine on the expression of HDAC5 target genes may persist several days as demonstrated by MEF2 luciferase activity (Fig. 2*A*) and behavioral responses even longer (5–6 d after ketamine injection). Taken together, these findings indicate that up-regulation of endogeneous HDAC5 in the hippocampus contributes to depression-like behaviors caused by CUS exposure. Moreover, the induction of HDAC5 in response to CUS may increase the sensitivity to the chromatin remodeling actions of ketamine. This is in line with the observation that both HDAC5 knockdown and ketamine treatment in the presence of HDAC5 knockdown have antidepressant efficacy in the NSFT and SPT in CUS, but not in unstressed animals. The same manipulations in unstressed animals produced antidepressant efficacy in the FST and LHT, suggesting that a greater level of stress caused by FST and LHT could increase HDAC5 and explain why HDAC5 knockdown and ketamine are sufficient to produce an antidepressant response. However, it should be noted that because both NSFT and SPT involve feeding behavior, it is possible that the effects of ketamine and HDAC knockdown are mediated by alteration

of consummatory behavior in the context of a stressful environment. Together, these findings suggest that HDAC5 provides an essential mechanism for regulation of gene expression that supports the antidepressant actions of ketamine. As such, deficits in this process may contribute to the development of maladaptive behaviors associated with stress in humans.

Our findings reveal that ketamine regulates the transient nuclear export of HDAC5, and this likely occurs through a molecular mechanism involving CaMKII- and PKD-dependent phosphorylation of HDAC5 at two critical sites, S259 and S498. Importantly, the HDAC5 phosphorylation is critical for ketamine's ability to produce antidepressant behaviors. In this respect, the general analysis of the coordinated actions of HDAC5 within the hippocampus in response to ketamine could provide new insight into the pathophysiological mechanisms of major depression.

Materials and Methods

Primary Hippocampal Neuron Cultures. Primary hippocampal neurons were prepared from 16.5-d-old Sprague–Dawley rat embryos, as previously described (22).

HDAC5 Subcellular Localization Immunofluorescence Study. Hippocampal neurons grown on glass coverslips were transfected after 3 d in vitro with plasmids.

MEF2 Luciferase Assay. The pCI-neo-HDAC5-WT and pCI-neo-HDAC5-S/A expression plasmids were generated by subcloning the coding sequence from HDAC5-WT (Addgene, plasmid 32211) and HDAC5-S/A (Addgene, plasmid 32218) into the pCI-neo (Promega), respectively.

Lentiviral Production. For HDAC5 knockdown, we cloned a shRNA sequence against HDAC5 (31) into pll3.7 (Addgene) and used a control nontargeting shRNA (22). Lentivirus was produced as indicated in *SI Appendix*. Typical titers for in vivo injections are 8×10^6 to 20×10^6.

Behavioral Experiments.

Animals, drug administration, stereotaxic surgery, and infusions. All procedures were in strict accordance with Institutional Animal Care and Use Committee (IACUC) guidelines and approved by the Hanyang University Animal Care and Use Committee (#2015-0094). Adult male Sprague-Dawley rats (8–10 wk old; Charles River Laboratories) were used. Stereotaxic surgery and infusions were conducted as previously described (22).

CUS procedure. The CUS animals were subjected to exactly the same sequence of 12 stressors (2 per day for 35 d) described previously (32).

FST. FST was conducted as previously described (22). Rats were placed in a clear cylinder with water (24 ± 1 °C, 45-cm depth) for 15 min.

LHT. LHT procedure was performed in commercial shuttle boxes divided into two equal compartments by a central barrier (Gemini Avoidance System, San Diego Instruments), as previously described (4).

NSFT. NSFT was conducted as previously described (22). Behavioral tests were performed by an experimenter blinded to the study code.

SPT. SPT was conducted as previously described (22).

Statistical Analysis. Student's *t* tests were used for comparison of two groups, in the analysis of biochemical results. Statistical differences for behavioral experiments, consisting of four experimental groups, were determined by analysis of the variance (ANOVA; StatView 5, SAS Software) followed by least significant difference (LSD) post hoc analysis. The results were presented as mean ± SEM. The level of statistical significance was set at $P < 0.05$ using two-tailed tests. All experiments were carried out at least three times.

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