Kappa opioid receptor induces epigenetic silencing of brain derived neurotropic factor via HDAC5 in treatment-resistant depression

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Abstract

Treatment-resistant depression (TRD) occurs in almost 50% of depressed patients. Although modulation of brain-derived neurotrophic factor (BDNF) expression in major depression and antidepressant treatment is well established, the mechanisms of BDNF regulation in TRD are not clear. Previously, we have shown that chronic kappa opioid receptor (KOR) stimulation leads to TRD-like phenotype in mice, and modulation of BDNF expression in the prefrontal cortex appears to be one of the molecular determinants of the antidepressant response. Considering the role of various epigenetic pathways in BDNF expression, in this study we investigated the role of histone deacetylase (HDACs) in KOR induced BDNF downregulation in mice. First, we showed that sustained KOR activation by its agonist, U50488 resulted in selective and specific downregulation of acetylation at the 9th lysine residue of the histone H3 protein (H3K9ac) and upregulation of HDAC5 in the prefrontal cortex (PFC). However, no change was observed in H3K9ac levels in the PFC of chronic unpredictable stress-exposed mice. Further, KOR activation led to specific downregulation of *Bdnf II,* and *IV*transcripts in the PFC and primary cortical neurons. Using chromatin immune precipitation assay, we detected decreased H3K9ac levels, while increased HDAC5 binding at *Bdnf II* and *IV* promoters, which were blocked by a selective KOR antagonist, *nor*binaltorphimine. Further mechanistic studies using HDAC5 selective inhibitor LMK235 in primary cortical neurons, we confirmed the essential role of HDAC5 in KOR-mediated reduction of *Bdnf* expression in PFC. These results suggest that KOR engages multiple pathways in TRD, and may be targeted for the development of new therapeutics for treatment-resistant depression.

Introduction

As per WHO estimates (March 2023), about 3.8% of the world population is affected by depressive disorders (https://www.who.int/news-room/fact-sheets/detail/depression), and notably, almost 50% of these do not respond to the current therapeutic interventions [1]. Therefore, treatment-resistant depression is a highly unmet medical need, and the neurobiological distinction between resistant and non-resistant depression are not clear. Although esketamine was approved for treatment-resistant depression in 2019 and major depressive disorders with suicidal ideation in 2020 [2], it causes serious adverse effects such as blood pressure increase, vertigo, and dissociation [3]. Several mechanistic studies in rodent models of depression suggest that salutary effects of esketamine are mediated via regulation of brain-derived neurotrophic factor (BDNF) expression, synthesis, and release, which in turn activate synaptic plasticity genes [4, 5]. Further, decreased BDNF expression is also considered as key factors responsible for impaired synaptic plasticity observed in the rodent models of depression and in depressed patients [6]. BDNF is critical for brain development and several neuro-physiological processes, and is highly conserved among all mammalian species. The expression and function of BDNF are tightly regulated by eight exons with individual promoters that undergo tissue- and stimulus-specific alternative splicing to generate multiple *Bdnf* transcripts with distinct 5' non-coding exons as a promoter with the constitutive *exon IXa* (protein coding) gives rise to different Bdnf transcripts [7-9]. Interestingly, various stressors are known to change the levels of *Bdnf* transcripts in different brain regions, and treatment with antidepressants can restore it [10-12], but mechanisms of the regions-specific regulation of various *Bdnf* transcripts in TRD are not clear, yet.

We have earlier shown that chronic activation of the Kappa opioid receptor (KOR) induces treatment-resistant depression-like phenotype in mice, which are reversed by its antagonist, nor-binaltorphimine (norBNI), but not by selective serotonin reuptake inhibitors (SSRIs), fluoxetine and citalopram [13]. In this study, we also observed that KOR activation led to decreased prefrontal cortex BDNF expression that was blocked by

norBNI, but not by Fluoxetine or Citalopram or Imipramine. Although involvement of epigenetic modifications and histone deacetylases (HDACs) are widely known in depressive disorders [12, 14, 15], relationship between KOR signaling and HDACs in depression is not known, yet. Therefore, in the present study, we sought to investigate the effect and mechanism of chronic KOR activation on histone deacetylases (HDACs) and major *Bdnf* transcripts in the PFC and primary cortical neurons. We observed that sustained KOR activation selectively reduced the *Bdnf* transcripts *II, IV*, and *Bdnf CDS* (protein-coding *Exon IX*), which was blocked by selective KOR antagonist, norBNI. We also found significant reduction in the levels of active chromatin marker acetylation at the 9th lysine residue of the histone H3 protein (H3K9ac) in the PFC of U50488 (KOR agonist) treated mice, but no effect on levels of other modifications-H3K4me3 (tri-methylation at the 4th lysine residue of the histone H3), H3K9me3 (tri-methylation at the 9th lysine residue of the histone H3), and H3K27me3(tri-methylation at the 27th lysine residue of the histone H3).

Material and Methods

Animals

In vivo experiments and procedures were performed according to the guidelines provided by the Institutional Animal Ethics Committee (IAEC) of CSIR-Central Drug Research Institute, Lucknow, India. C57BL/6J male mice of 6-8 weeks and weight 22-25 g were used in this study. Mice were housed in a 12-h light/dark cycle under constant temperature (22-25°C) and consistent humidity (50 ± 5%) with *ad libitum* food and water supply.

Drugs

KOR agonist (−)-*trans*-(1S,2S)-U-50488 hydrochloride hydrate (Sigma Aldrich, U111) was first dissolved in Dimethyl sulfoxide (Sigma Aldrich -D8418) at a concentration of 20 mg/ml, and then diluted to 0.5 mg/ml with normal saline solution just before administration. U50488 was administered intraperitoneally (*i.p.*) at a dose of 5 mg/kg for 21 days. The KOR selective antagonist, *nor*-Binaltorphimine dihydrochloride (norBNI; Sigma Aldrich, N1771, 10mg/kg, *i.p.*) was administered only once on day 1, 30 minutes

before the U50488 treatment. Control animals were injected 0.9% sodium chloride (vehicle).

Primary neuronal culture

Primary neurons were cultured from the cerebral cortices of 0-1 day-old mouse pups as described previously [13]. Briefly, the cortices were gently dissociated in 1X Hanks′ Balanced Salt Solution (HBSS) (Sigma Aldrich-# H4641) and were digested with papain (0.1% Sigma Aldrich- #P4762) for 20 minutes at 37 °C. After digestion, tissue samples were triturated 10-15 times to get the dissociated cells, which were then suspended in neurobasal media (Gibco, #12348-017) supplemented with 1X B27 supplement (Invitrogen, #17504) and 0.5mM Glutamax (Gibco, #35050). The cell suspension was then plated onto a 12-well tissue culture plate (0.5×10^6 cells/well) coated with poly-L-lysine (PLL) (0.1mg/ ml Sigma Aldrich, # 2636P) and incubated at 37°C in a humidified atmosphere of 95% oxygen and 5% CO_2.

Reverse transcription (RT) and quantitative PCR

Mice were euthanized after 24 h of last administration of U50488 by the overdose of an anesthetic avertin (350 mg/kg). Brain tissues - prefrontal cortex (PFC), striatum, and hippocampus were rapidly micro-dissected after the decapitation of fully anesthetized mice and immediately snap-frozen in liquid nitrogen and stored at −80°C till further use. Total RNA was extracted from the brain tissues using the trizol/chloroform method. First, total RNA was treated with DNAase at 37°C for 10 min to remove genomic DNA, then reverse transcribed into cDNA (iScript™ cDNA Synthesis Kit, #1708891). This cDNA was then used for real-time PCR detection using the exon-specific primers (**Supplementary Table 1**). The condition for PCR was 95°C for 10 minutes, followed by 40 cycles of 95°C for 15s, 56°C for 30s. The housekeeping gene β-actin or GAPDH was used as a reference gene for the normalization of gene expression. The 2−ΔΔCTmethod, (i.e., delta-delta-ct analysis) was used for relative quantification (Valasek and Repa, 2005).

Western blotting

Western blotting to determine the expression of various protein were performed as described earlier [13]. Briefly, frozen brain tissues were homogenized in RIPA buffer (25

mM Tris-HCl, pH 7.6,150 mM NaCl, 0.5% NP-40,1% sodium deoxycholate,0.1% SDS). Briefly, 40-50 µg of protein samples were subjected to 8-12% SDS-PAGE and transferred to a polyvinyl difluoride (PVDF) membrane followed by blocking for two hours at room temperature by blocking buffer (5% non-fat dry milk and 3% bovine serum albumin in PBST), Specific primary antibodies (**Supplementary Table 2**) were incubated overnight at 4°C, followed by horseradish peroxidase (HRP) conjugated secondary antibodies (1:4000) at room temperature for 2 hours. Blots were developed by enhanced chemiluminescence substrate and images were acquired using Amersham™ ImageQuant™ 800 and densitometric analysis was performed by ImageJ software.

Immunohistochemistry

C57BL/6J mice were transcardially perfused with a 4% paraformaldehyde solution. The brains were removed, post-fixed overnight in 4% paraformaldehyde, and dehydrated in 30% sucrose for 24 hours. Thin coronal slices (30µm) were made using a cryostat (FSE, ThermoScientific). For staining, the sections were rinsed four times in PBS, antigen retrieval was done in 10mM sodium citrate buffer (pH-6.3), followed by permeabilization using a 0.5% TritonX 100 in 1X PBS for one hour at room temperature, and blocked with buffer (3% bovine serum albumin, 3% horse serum, 0.3% Triton X-100 in PBS) for two hours at room temperature. The sections were then incubated with mouse anti-HDAC5 (1:50) overnight at 4°C. After overnight incubation, the brain sections were washed three times with PBST (0.1% Triton X 100 in 1X PBS) followed by incubation with respective secondary antibodies conjugated with Alexa fluor 594 or 488 (1:500) and Hoechst 33258 (2 µg/ml) in blocking buffer for 1 hour at room temperature. After incubation with host specific secondary antibodies, the slices were washed thrice and were mounted onto poly-lysine-coated glass slides using Vectashield antifade mounting medium, and fluorescent images were collected with XYZ acquisition mode at 1µm step size using the Olympus BX61-FV1200-MPE microscope using a 40X oil (1.3NA) objective at 8.0µs/pixel scan speed. (Olympus, Shinjuku, Tokyo, Japan). Pixel intensity quantification for HDAC5 and Hoechst 33258 staining in anterior cingulate cortex and piriform cortex

was performed using ImageJ software (www.rcb.info.nih.gov/ij) and presented as fold change after normalization with pixel intensity of Hoechst 33258 in each region.

Chromatin immunoprecipitation (ChIP)

ChIP was performed as described earlier [16] with slight modifications. The freshly dissected PFC was crosslinked using 1% formaldehyde in PBS for 10 min at ice, followed by quenching with 125mM glycine for 10 min. Cross linked PFCs were then washed with 1X PBS and homogenized in a SDS lysis buffer (0.01% SDS, 100mM EDTA, 1mM PMSF, 50mM Tris-HCl, pH 8.1) and sonicated on ice (FB-705; Fisher Scientific) at 40% amplitude and a 10 X 10 second pulse. Sonicated samples were spun at 12,000 rcf for 10 minutes at 4°C, and supernatants were collected for dilution in the ChIP dilution buffer (167mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1) and 10% of this DNA sample was kept aside as input DNA for qPCR and fold change calculations at later stage. The supernatant containing sheared chromatin (50 microgram) was incubated with either anti H3K9ac or anti HDAC5 anitbodies 14 hours at 4°C followed by centrifugation for 2 minutes at 3000 rcfat 4°C. DNA-histone complex was collected by binding to salmon sperm DNA blocked-protein A-agarose beads for a 3-4 hours incubation at 4°C and then sequentially washed with low salt buffer (150mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1), high salt buffer (500mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1), LiCl immune complex buffer (0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris, pH 8.1) and Tris-EDTA buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). DNA-histone complexes were removed from protein A-agarose beads by incubation with elution buffer (0.1M NaHCO3, 1% SDS) for 20 minutes at room temperature. The eluted DNA-histone complexes were then digested with proteinase K at 55°C for overnight followed by DNA extraction using the phenol-chloroform-alcohol method. An equal volume of immunoprecipitated DNA and input DNA was subjected to quantitative real-time PCR using mouse *Bdnf exon* specific primers.

Results

1. Effect of chronic KOR activation on the expression of various *Bdnf* transcripts in the prefrontal cortex

In our previous study, we demonstrated that chronic treatment of U50488 (5mg/kg, *i.p*) develops a treatment-resistant depression like phenotype. We have also shown that treatment with U50488 decreased *Bdnf* expression in the prefrontal cortex (PFC) and the hippocampus of mice. Interestingly, imipramine, fluoxetine, and citalopram attenuated the KOR activation-induced decrease in *Bdnf* expression in the hippocampus, but not in the PFC, indicating a critical role of cortical BDNF signaling in treatment-resistance like phenotype observed in the chronic U50488 treated mice [13]. In the present study, we investigated underlying mechanisms of KOR-induced downregulation of BDNF protein expression in the PFC. We used previously reported U50488 administration regimen (5 mg/kg; *i.p*., 21 days) and immobility time in the forced swim test as a surrogate measure of depressive behavior [13]. As expected, we observed a significant increase in the immobility time due to chronic treatment with selective KOR agonist U50488 (**Figure S1).** We then determined if KOR activation induced decrease in BDNF protein expression in the PFC is regulated by alternative usage of five major non-coding *5'exons* (*exon I-V*) acting as promoters for generating identical protein encoded by *exon IXa,* refered as *Bdnf CDS* [7]. Indeed, we observed that chronic treatment with U50488 significantly decreased levels of *Bdnf II, IV*, and *Bdnf CDS transcripts* (**Figure 1A**), while increased the level of *Bdnf III* transcripts (**Figure S2A**) as compared to the vehicle treated animals. A single dose of selective KOR antagonist, norBNI (10 mg/kg, *i.p*.) administered 30 minutes prior to the first U50488 treatment on Day-1 blocked the effect of U50488 on all the *Bdnf* transcripts in the PFC (**Figure1A, Figure S2A**). Similar to the in vivo effects of KOR activation, we also observed U50488 (100 nM and 1000 nM, for 72hours) treatment induced reduction in the levels of Bdnf transcripts II, IV, and Bdnf CDS (**Figure 1B**) and increased the levels of Bdnf III (**Figure S2B**) in the primary cortical neurons. These results indicate that chronic KOR sctivation decreases BDNF protein expression in the PFC by inhibiting expression of Bdnf II and IV transcripts.

2.Chronic KOR activation induces epigenetic modifications in the PFC

The differential expression of *Bdnf II*, *III*, *IV* transcripts prompted us to investigate the underlying mechanisms of these changes induced by KOR activation. We first determined the effects of U50488 treatment on the PFC levels of post-translational histone modifications that are known to regulate gene expression in the central nervous system (CNS). Hence we selected two active chromatin markers, H3K9ac and H3K4me3, and two repressive chromatin markers, H3K9me3 and H3K27me3 for evaluation in the PFC tissues after various treatments. We found that treatment with U50488 selectively reduced the PFC levels of H3K9ac as compared to the vehicle treatment (**Figure 2A, B**), while no effect was observed on the levels of H3K4me3, H3K9me3 and H3K27me3 in the PFC. We further confirmed the effect of KOR stimulation in the primary cortical neurons and observed that treatment with selective KOR agonist, U50488 (100 nM for 72 hours), significantly suppressed the expression of H3K9ac (**Figure 2C,D and S3A, B**). Pre-treatment with selective KOR antagonist, norBNI blocked the U50488-induced decrease in the levels of H3K9ac (**Figure 2C, D**). Similar to the PFC tissue, we found no changes in the levels H3K9me3 and H3K27me3 in the primary cortical neurons treated with U50488 for 72 hours (**Figure S3 E, 3F, 3G, 3H**). We also observed no change in the levels of H3K4me3 after 24 hours of treatment with U50488 in the primary neurons, while there was a reduction in the levels of H3K4me3 at the 48 and 72 hour time points (**Figure S3C and D**). These results indicate that overall repressive effect of KOR activation on Histone H3 9th lysine modification that may regulate histone structure and modulate transcription factor binding. Interestingly, however, we didn't observe any significant change in the H3K9ac expression in chronic unpredictable stress (CUS) model of depression in C57BL/6J mice (**Figure S4 A, B**), indicating that change in levels of H3K9ac could be a marker of treatment-resistant depression. Therefore, further studies were persued to delineate underlying the mechanisms and the role of H3K9ac in KOR induced suppression of *Bdnf* expression in the PFC. We performed a series of chromatin Immunoprecipitation (ChIP) assays to study the levels of H3K9ac binding at different *Bdnf* exons in the PFC tissues. All assays included nonimmune immunoglobulin (IgG) and non-antibody

Immunoprecipitation as a control for the antibody specificity. We found significantly less enrichment of H3K9ac modification at *Bdnf* II, IV, and V promoters in the PFC of U50488 treated mice as compared to control mice (**Figure 2E**). These results suggest that chronic U50488 treatment induces differential epigenetic repression at the *Bdnf* promoters that correlate with the U50488-induced changes in the expression of *Bdnf* mRNA in the PFC.

3. KOR activation selectively modulates histone deacetylase 4 and 5 in the PFC.

Considering that deacetylation of lysine residues on histone tails by HDACs regulates *Bdnf* expression [17, 18] and our results showing decreased levels of H3K9ac in the PFC of chronic U50488 treated mice, we sought to investigate the expression profile of various HDACs in the PFC of U50488 treated mice. To study this, we measured the protein levels of three of the four classes of HDACs, class 1 HDACs (HDAC1, HDAC2, HDAC3), class 2 HDACs (HDAC4, HDAC5, HDAC6, HDAC7), and class 3 HDACs/Sirtuins (SIRT1-SIRT7) using western blotting. Among these HDACs, we found only a significant decrease in the expression of HDAC4 and a significant increase in the expression of HDAC5 in the PFC of mice chronically treated with U50488 (**Figure 3A-D,** and **Figure S5A, B)**. Previously, chronic treatment with the antidepressant imipramine has been shown to alleviate depression like behaviors by decreasing the expression of HDAC5 in the hippocampus, suggesting hippocampal levels of HDAC5 as a marker of antidepressant response [12]. Along that lines, we also investigated various regions of the PFC for the levels of HDAC5 expression after U50488 treatment using immunohistochemistry. Our analysis of the PFC regions revealed that expression of HDAC5 was predominately increased in the anterior cingulate (**Figure 4**) and piriform cortex regions (**Figure S6)** of chronic U50488 treated mice as compared to control mice.

4. HDAC5 regulates the expression of *Bdnf II* and *IV* transcripts.

To further understand the relationship between HDAC5 and the expression of various *Bdnf* transcripts in the PFC, we performed a ChIP assay with an anti-HDAC5 antibody and determined the enrichment of HDAC5 at various *Bdnf* promoters. We observed that HDAC5 binding was significantly increased in *Bdnf II* and *IV* promoters in chronic

U50488 treated mice as compared to control or norBNI samples, while no change in exon I, III, and V was found (**Figure 5A**). To determine the specificity of HDAC5 action on selective usage of *Bdnf II* and *IV* transcripts for BDNF synthesis, we evaluated the effect of HDAC5 specific inhibitor LMK235 (1 μM, 72 hours) on U50488 induced reduction in various *Bdnf* transcripts in the primary cortical neurons. As expected, LMK-235 completely blocked the effect of U50488, albeit increased more than vehicle (**Figure 5B**). Both HDAC4 and HDAC5 interacts with myocyte enhancer factor-2 (MEF2) and prevent the expression of its target genes, including *Bdnf* [19, 20]. It is also known that CaMKII activation dissociates MEF2C from HDAC4/5 and restores MEF2C transcriptional activity [21]. Further, CaMKII-dependent phosphorylation of nuclear HDAC5 leads to its translocation from the nucleus to cytoplasm and hence modulates the transcription repressive activity of HDAC5 [22]. In light of the above mentioned facts, we determined the expression of pCaMKII (Thr286) in the PFC of mice treated with either vehicle, U50488, or U50488+norBNI. We found significantly decreased expression of pCaMKII in U50488 group as compared to the vehicle group (Figure 5C,D). Further, treatment with a selective KOR antagonist, norBNI completely blocked the effect of U50488 on the levels of pCaMKII in the PFC. These findings indicate that increased HDAC5 expression in chronic U50488 treated mice could be due to insufficient level pCaMKII not being able to phosphorylate HDAC5 and not promoting its export from nucleus and / or degradation as reported earlier [23]. These results suggest that sustained KOR activation attenuates p-CAMKII, which in turn attenuates the nuclear export of HDAC5 and eventually represses various *Bdnf* transcripts in various brain regions. Furthermore, since MEF2C transcription factor is know to modulate neuronal depolarization induced expression of *Bdnf exon IV* [24], we determined the effect of sustained KOR activation on the expression of MEF2C in the PFC. As per our hypothesis , we observed significant inhibition of MEF2C expression in the U50488 treated mice in comparision to the vehicle treated mice, and this effect of U50488 was blocked by KOR antagonist, norBNI, suggesting KOR specific effect (**Figure 5E,F**). These results clearly suggest that sustained KOR signaling in the PFC lead to suppression of pCaMKIIα, due to which HDAC5 is retained in the nucleus to deacetylate

Histone H3 at Lysine 9 residue, and consequently inhibits MEF2C mediated transcription of *Bdnf I* and *IV* exons.

Discussion

The KOR is widely expressed in the CNS and plays an important role in regulating mood, pain, and addiction [25], The inhibitory effect of increased KOR/Dyn signaling on monoaminergic neurotransmission in various models of psychiatric disorders, including major depression, is widely known [26, 27]. Although increased BDNF expression in the PFC and hippocampus is considered a vital transducer of the antidepressant response [28], the mechanistic relationship between KOR signaling and the BDNF expression is not completely understood. In this study, we demonstrated that sustained activation of KOR leads to decrease in *Bdnf* mRNA (protein-coding transcript *exon IXa*) expression via supression of *Bdnf II* and *Bdnf IV* transcripts that act as alternative promoters. Importantly, HDAC expression profiling and post-translational H3-histone modification profiling in tissues revealed a significant increase of only HCAC5 and decreased levels of active chromatin marker H3K9ac in the PFC. ChIP analysis of the PFC tissue with anti-HDAC5 and anti-H3K9ac antibodies showed significant enrichment of HDAC5, while a decreased enrichment of H3K9ac at BDNF *II* and *IV* promoters.We also revealed that chronic KOR activation with U50488 leads to decrease in the levels of pCaMKIIα, which is known to phosphorylate HDAC5 leading to its nuclear export and ultimately facilitate BDNF transcription. We also observed descreased expression of transcription factor MEF2C that has been shown to regulate expression of *Bdnf IV* transcript.

Optimal BDNF expression is essential for a wide array of neurological functions, including neuronal survival and differentiation, synaptic neurotransmission, and synaptic consolidations [29, 30], its expression is spatiotemporally regulated by transcription of each 5'-exon controlled by a separate promoter [31] and several transcription factors [32]. Activity-dependent changes in mouse *Bdnf-I, IV*, or *IX* transcripts in the PFC and in the hippocampus have been reported [33-35]. The importance of regulation of *Bdnf* expression via changes in non-coding *Bdnf* transcripts in major depression is further highlighted by a recent study [36] that demonstrated that *Bdnf* L-3'-UTR mediates

dendritic shrinkage and depressive and anxiety-like behaviors in humans and mice. However, there is no study on the levels of other major non-coding *Bdnf* mRNA transcripts in treatment-resistant depression. In this context, our findings showing significant changes in *Bdnf II* and *IV* in the PFC of U50488-treated mice shed light on the complex regulation of BDNF expression in major depression. Essential role of *Bdnf IV* in dendritic integrity [31], and GABAergic neurotransmission in the PFC [37] has been shown. The hypomethylation of *Bdnf IV* is also associated with non-remission by antidepressants [38]. In view of these studies, our observation of KOR induced *Bdnf II* and *IV* repressions could be involved in maladaptive synaptic plasticity underlying treatment-resistant depression.

Changes in histone acetylation have been widely implicated in the pathogenesis of several neurological disorders, including major depression [39]. Earlier class II HDAC, HDAC5 has been shown to epigenetically regulate the behavioural adaptations in the nucleus accumbens in response to chronic stress [40] and to block the salutary effect of imipramine in the chronic social defeat stress model of depression [12, 41]. Further, the ketamine induced fast antidepressant response has also been shown to be mediated through HDAC5 phosphorylation by CaMKIIα, and its subsequent nuclear export, leading to the activation of several target genes (e.g., MEF2, EGR1, etc.) in the hippocampus [42]. In light of these studies, our finding of KOR-mediated increase in the HDAC5 levels in the PFC (but not in hippocampus, data not shown), and subsequent decrease in the expression of *Bdnf II* and *IV* transcripts in the PFC uncovers a new facet of KOR signaling and its role in depression. The contrasting effects of KOR activation on HDAC4 and HDAC5 could be attributed to varying cell type (GABAergic vs Glutamatergic neurons) and activity status as even spontaneous neuronal activity has been shown to decrease the HDAC4, but not of HDAC5 [43]. We didn't see any effect of KOR stimulation on the levels of the HDAC5 in the hippocampus, which is in contrast to chronic social defeat model of depression [12, 41]. These discrepencies may stem due to KOR being expressed in different neuronal populations in the PFC and the hippocampus [44]. Nevertheless, KOR induced HDAC5 and decreased H3K9ac expression in the PFC appears to be pivotal for downstream events such as decreased

expression of MEF2C, and BDNF levels. Since HDAC5 shuttling between the nucleus and the cytoplasm depends on its phosphorylation by CaMKII [41], our results revealing decreased levels of pCaMKII in the PFC, increased HDAC5 binding at *Bdnf II* and *Bdnf IV* promoters, and decreased enrichment of H3K9ac at *Bdnf II* and *IV*, are consistent with the prevailing notion of epigenetic silencing of *Bdnf* in major depression [45, 46]. Although according to the Diagnostic Statistical Manual (DSM-V) major depressive disorder (MDD) is diagnosed as a single entity, there is a huge array of symptoms that could meet the DSM-V criteria, clearly depicting the heterogeneity of etiology and pathophysiology of MDD [47]. Given that KOR modulates all three major neurotransmitters, dopamine, serotonin, and norepinephrine, in the brain [48], even subtle over-activation could lead to severe mood alteration via engaging multiple neurocircuits and signaling pathways, which could be resistant to pharmacotherapies selectively targeting one or other pathways. Furthermore, our data of KOR being a selective modulator of HDAC5 activity in the PFC further supports KOR as a druggable target to selectively modulate epigenetic signaling in CNS disorders, which is otherwise impossible to target due to a lack of tissue and cell selectivity.

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Author Contributions

Anubhav Yadav- Manuscript writing, Contribution to figure 1(A& B), 2(E), 3(A, B, C&D), 4(A, & B), 5(A, B,C, and D), S1(A,&B), S2(A,B&C), S1(A,&B), S5 and S6(A&B), Shalini Dogra- Manuscript writing, Contribution to figure 1(A& B), 2(A, B, C and D), S2(A, B&C), S3(A, B, C, D, E, F, G&H), S4(A, &B), and S5, Poonam Kumari- Analysis of ChIP data, and dissection of mice brain, Boda Arun Kumar- Optimizations of ChIP assay, Ajeet Kumar- Determining the effect of KOR agonist in a rodent model of chronic unpredictable stress, Manish Kumar Dash- Brain perfusion and immunohistochemistry, Prem Narayan Yadav- Design of experiments, data interpretation and manuscript writing

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Competing Interests

The authors declare no competing interests.

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Figure Legends

Figure1. KOR activation leads to differential regulation of *Bdnf* transcripts in the prefrontal cortex (PFC). (**A**) Chronic treatment with U50488 (5 mg/kg *i.p.*; 21 days) significantly decreased the levels of *Bdnf II, Bdnf IV*, and *Bdnf CDS (*protein coding *exon IXa)* transcripts in prefrontal cortex (PFC). A single administration of KOR antagonist, *nor*-Binaltorphimine (norBNI, *i.p.*; 10 mg/kg), blocked the effects of U50488 (U50) treatment on the expression of *Bdnf* transcripts. Data are presented as mean ± SEM of % Vehicle (Veh), One way ANOVA followed by Newman-Keul's multiple comparisons test, (*Bdnf II*: p= 0.018, $F_{(2,15)}$= 5.284; *Bdnf IV*: p= 0.014, $F_{(2,14)}$= 5.784; *Bdnf CDS:* p= 0.0087, $F_{(2,26)}$= 5.721), *p<0.05 (Veh vs U50), $^{\#}p$<0.05 (U50 vs U50+norBNI), n= 5-7 mice/group. (**B**) Representative bar graph showing reduced expression of *Bdnf CDS, Bdnf II,* and *Bdnf IV* transcripts (normalized to GAPDH transcript) in 12 days old (DIV12) primary cortical neurons treated with U50488 (100 nM and 1000 nM;72 hours). Data are presented as mean ± SEM of % Vehicle, One way ANOVA followed by Newman-Keul's multiple comparisons test (*Bdnf II*: *p= 0.009; $F_{(2,21)}$= 5.835, *Bdnf IV*: *p= 0.0014; $F_{(2,19)}$= 9.743, *Bdnf CDS:* *p<0.05; $F_{(2,18)}$= 5.125), n= 6-8 /group.

Figure 2. Chronic KOR activation causes epigenetic changes in the prefrontal cortex (PFC) of mice. (**A**) Chronic activation of KOR in the PFC of mice significantly decreased the expression of acetylation at the 9th lysine residue of the H3K9ac compared to the Vehicle-treated group. (**B**) Bar graphs showing the quantification of the immunoblots presented in panel (A) (****p<0.0001 by unpaired t-test, t= 5.057, n= 6-8 mice/group). (**C**) Expression of H3K9ac is reduced in 12 days old (DIV12) primary cortical neurons treated with U50 (100 nM, 72 hours). Pre-treatment with KOR antagonist, norBNI (1μM) significantly blocked the effects of U50 in primary cortical neurons. (**D**) Bar graph showing the quantitative analysis of immunoblots presented in panel **C** (One way ANOVA followed by Newman-Keul's multiple comparisons test, p= 0.0178, $F_{(2,10)}$= 6.192), *p<0.05 (Veh vs

U50), $^{\#}p<0.05$ (U50 vs U50+*nor*BNI), n= 4-5/group. (**E**) Chromatin immunoprecipitation (ChIP) using anti-H3K9ac antibody shows a significant reduction in the H3K9ac enrichment at *Bdnf II, IV & V* exons in the PFC. One way ANOVA followed by Newman-Keul's multiple comparisons test, *Bdnf II* (n= 9-11) $p= 0.0066$ $F_{(2,25)}= 6.184$, *Bdnf IV* (n= 7-12); $p= 0.0003$ $F_{(2,33)}= 10.68$, *Bdnf V* (n= 5-7) $p= 0.0183$ $F_{(2,15)}= 5.287$; $^{*}p<0.05$, $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$. Data are presented as mean ± SEM of fold change over input. U50= U50488, Veh= Vehicle, BNI= nor-binaltorphimine.

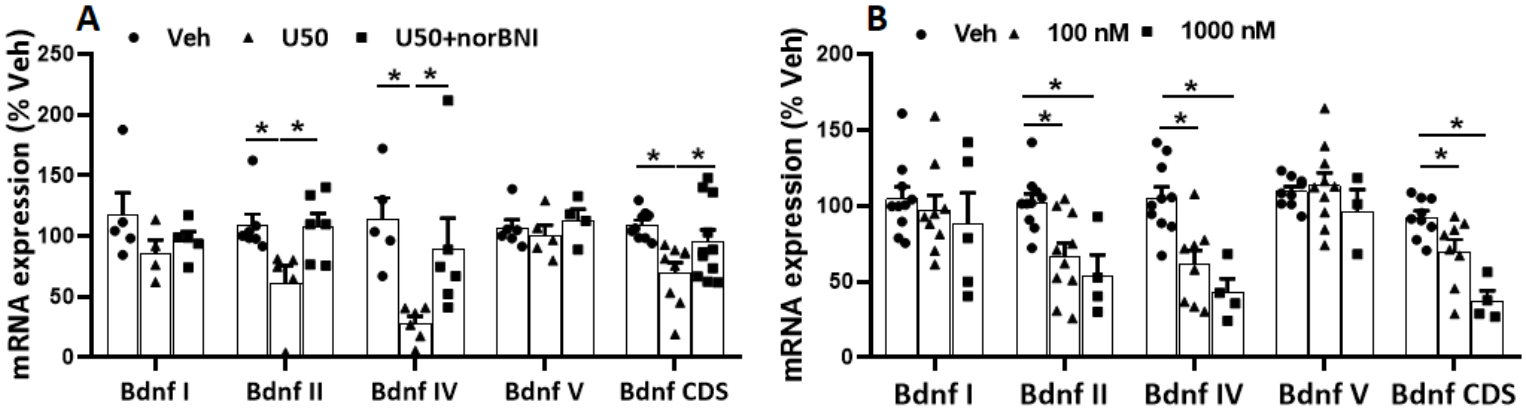
Figure 3. Effect of chronic KOR activation on histone deacetylases (HDACs) expression in the PFC.(**A**) No significant changes were observed in the expression of class I HDACs (HDAC1, HDAC2, and HDAC3). (**B**) Bar graph showing the quantification of the immunoblots, n= 6-8 mice/group. (**C**) Significant decrease in the levels of HDAC4, and increased levels of HDAC5 expression were observed in the PFC of U50 treated mice as compared to the Veh treated mice. Treatment with KOR antagonist, norBNI significantly blocked the effect of U50 on HDAC4 and HDAC5. **(D**) Bar graph depicting the quantification of immunoblots presented in panel C, $^{\#}p<0.05$, $^{**,\#\#}p<0.01$, and $^{***}p<0.001$, Unpaired t-test (HDAC 4 (n= 8-11): Veh vs U50 t= 3.045, U50 vs U50+norBNI, t= 2.911; HDAC 5 (n= 5-7): Veh vs U50 t= 3.91264, U50 vs U50+norBNI, t= 2.527).

Figure 4. Sustained activation of KOR increased the expression of HDAC5 in anterior cingulate cortex**.** (**A**) Representative immunohistochemistry images showing the expression of HDAC5 (Red) and nucleus stained with Hoechst 33258 (Blue) in the anterior cingulate cortex (ACC). (**B**) Bar graph showing the quantification of HDAC5 expression.

One way ANOVA followed by Newman-Keul's multiple comparisons test, $^{**}p$= 0.0009, $F_{(2,9)}$= 16.64, $^{**}p$<0.01, $^{\#\#\#}p$<0.001 n= 4/group, scale bar= 20 µm.

Figure 5. HDAC5 differentially regulates *Bdnf* transcripts in the PFC due to chronic KOR activation. (**A**) Chromatin immunoprecipitation (ChIP) with anti-HDAC5 shows a significant enrichment of HDAC5 at *Bdnf II* and *IV*. One way ANOVA followed by Newman-Keul's multiple comparisons test, *Bdnf II* (n= 5-7): p=0.0194, $F_{(2,15)}$= 5.814; *Bdnf IV* (n= 6-7): p= 0.0447, $F_{(2,16)}$= 3.799; $^{*,\#}p$<0.05, $^{**,\#\#}p$<0.01. Data are presented as mean ± SEM of fold change over input. (**B**) Pre-treatment of HDAC5 inhibitor LMK235 (1 µM, 72 hours) in the primary cortical neurons blocked the effect of U50 (100nM; for 72 hours),$^{*,\#,\$}p$<0.05, $^{**,\#\#,\$\$}p$<0.01, and $^{***,\#\#\#,\$\$\$}p$<0.001, $^{****,\#\#\#\#,\$\$\$\$}p$<0.001 Unpaired t-test, *Bdnf CDS:* Veh vs U50 t= 5.408; U50 vs U50+LMK235 t= 3.698; Veh vs LMK235 t= 4.687; *Bdnf II:* Veh vs U50, t= 5.139; U50 vs U50+LMK235, t= 2.308: Veh vs LMK235 t= 3.444; *Bdnf IV:* Veh vs U50, t= 6.373: U50 vs U50+LMK235, t= 2.866: Veh vs LMK235 t= 3.277; n= 7-9/group. (**C**) U50488 treatment significantly suppressed the expression of pCaMKIIα (Thr286) as compared to the Veh group. (**D**) The bar graph showing the quantification of the pCaMKIIα expression normalized to total CaMKIIα of immunoblots presented in panel D, (Unpaired t-test, $^{*}p$<0.05,$^{**}p$<0.01; pCaMKIIα: Veh vs U50 t= 3.075; U50 vs U50+norBNI t= 2.381 n= 8-9 mice/group). (**E**) U50 treatment significantly decreased the Myocyte enhancer factor 2C (MEF2C) expression in the PFC as compared to the Veh group. Treatment with KOR antagonist, norBNI significantly blocked the effect of U50. (**F**) Bar graph depicting the quantification of immunoblots presented in panel E, (Unpaired t-test, $^{***}p$<0.001, $^{\#}p$<0.05; MEF2C: Veh vs U50 t= 4.574, U50 vs U50+norBNI t= 2.637), n= 5-7 mice/group.

Figure1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Veh
U50
U50+norBNI
mRNA expression (% Veh)
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200
150
100
50
0
Bdnf I
Bdnf II
Bdnf IV
Bdnf V
Bdnf CDS
B
Veh
100 nM
1000 nM
mRNA expression (% Veh)
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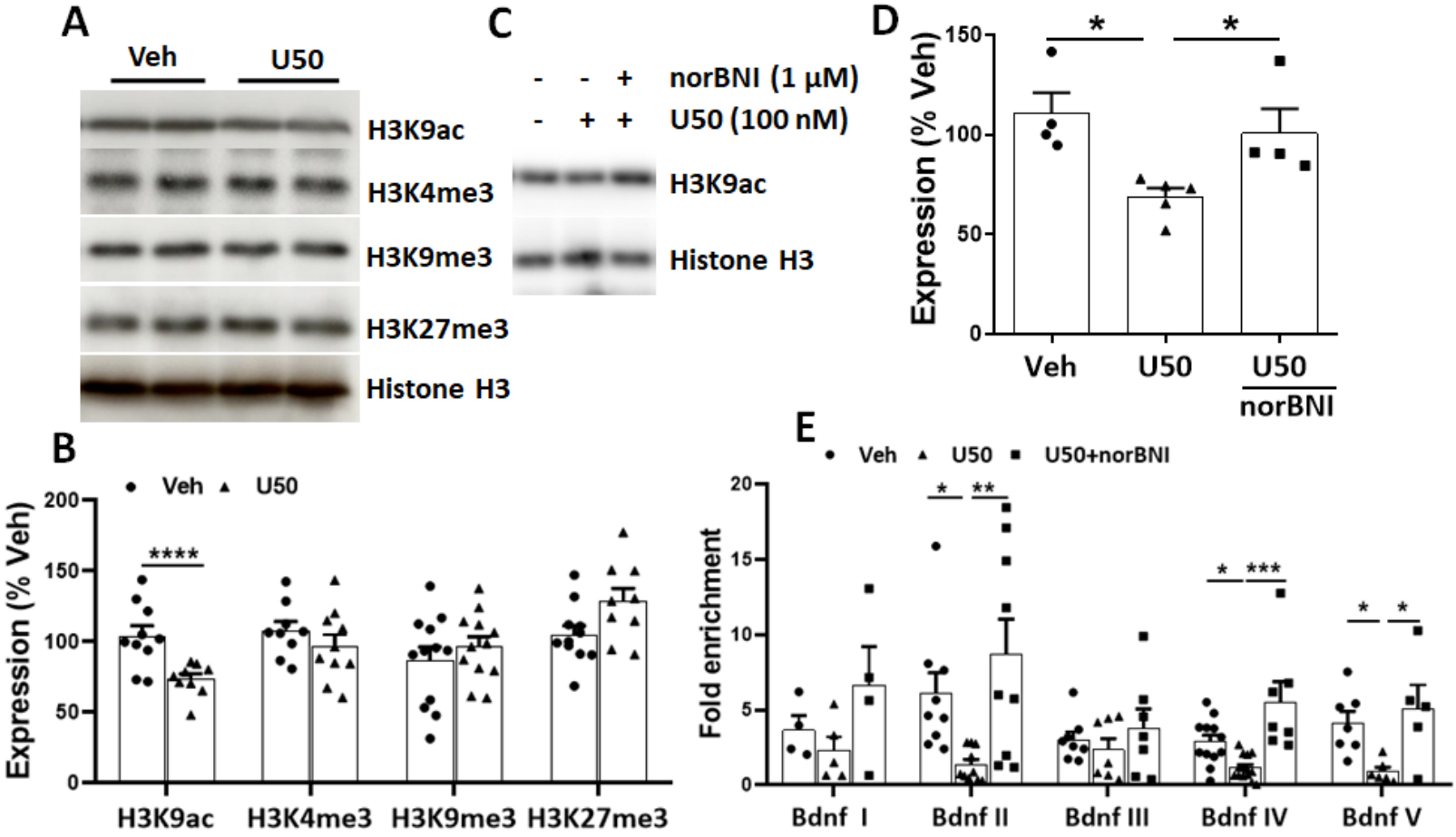
150
100
50
0
Bdnf I
Bdnf II
Bdnf IV
Bdnf V
Bdnf CDS

Figure-2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Veh
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H3K9ac
H3K4me3
H3K9me3
H3K27me3
Histone H3
C
- - + norBNI (1 μM)
- + + U50 (100 nM)
H3K9ac
Histone H3
D
Expression (% Veh)
150
100
50
0
*
*
Veh
U50
U50
norBNI
B
Veh
U50
Expression (% Veh)
200
150
100
50
0
****
H3K9ac
H3K4me3
H3K9me3
H3K27me3
E
Veh
U50
U50+norBNI
Fold enrichment
20
15
10
5
0
*
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*
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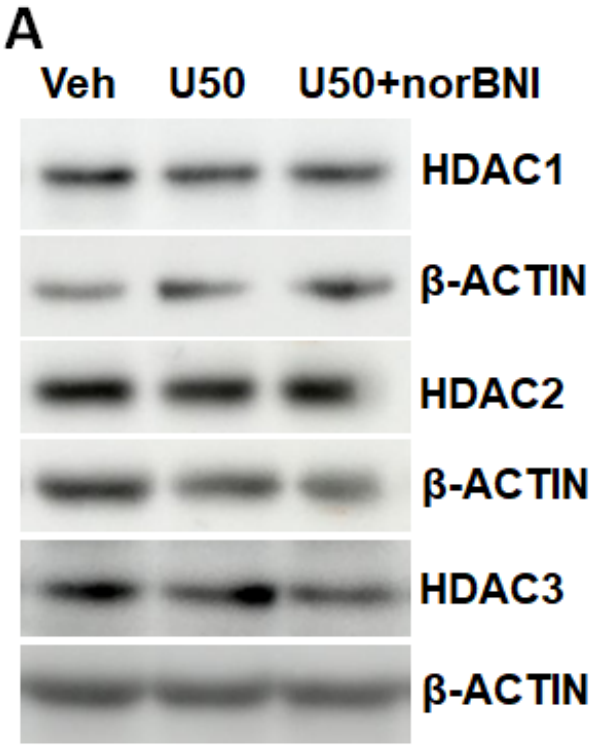
*
*
Bdnf I
Bdnf II
Bdnf III
Bdnf IV
Bdnf V

Figure 3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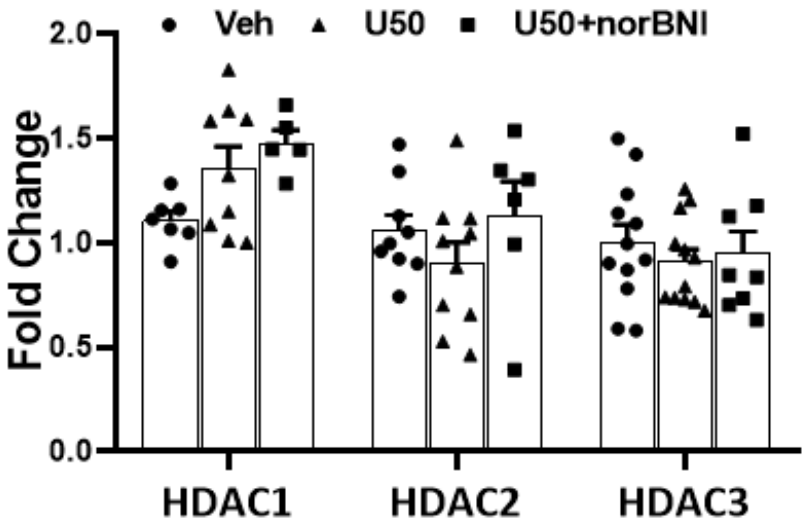
Veh
U50
U50+norBNI
HDAC1
β-ACTIN
HDAC2
β-ACTIN
HDAC3
β-ACTIN


B
Veh
U50
U50+norBNI
Fold Change
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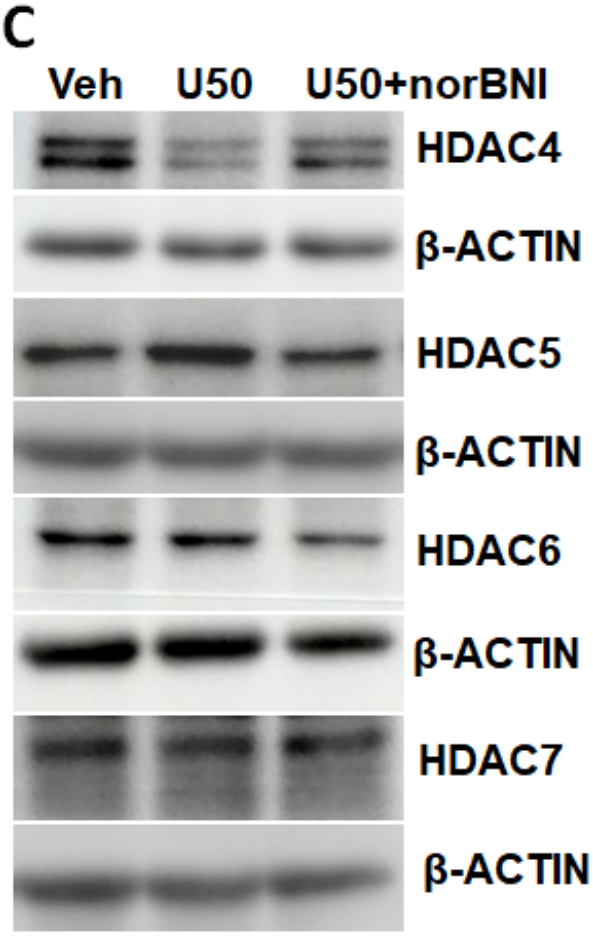
1.5
1.0
0.5
0.0
HDAC1
HDAC2
HDAC3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U50
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HDAC6
β-ACTIN
HDAC7
β-ACTIN

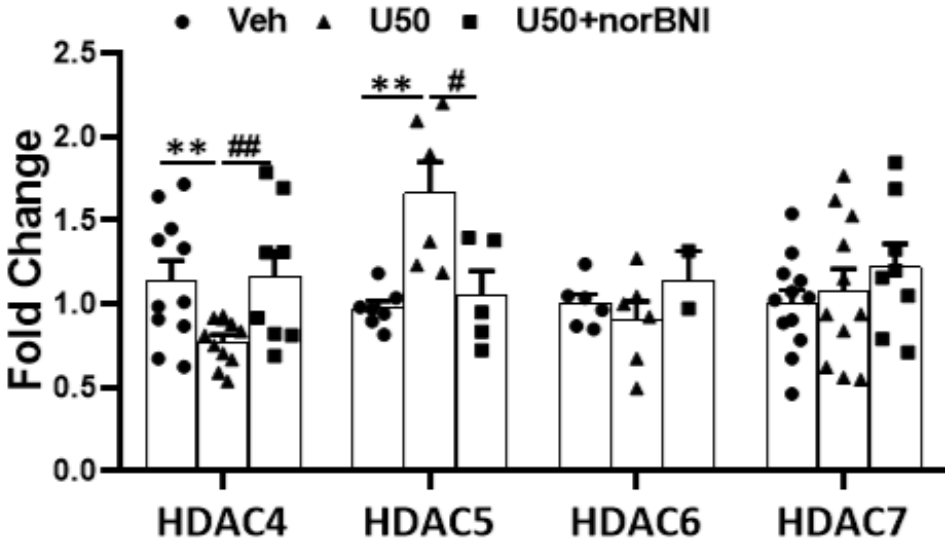
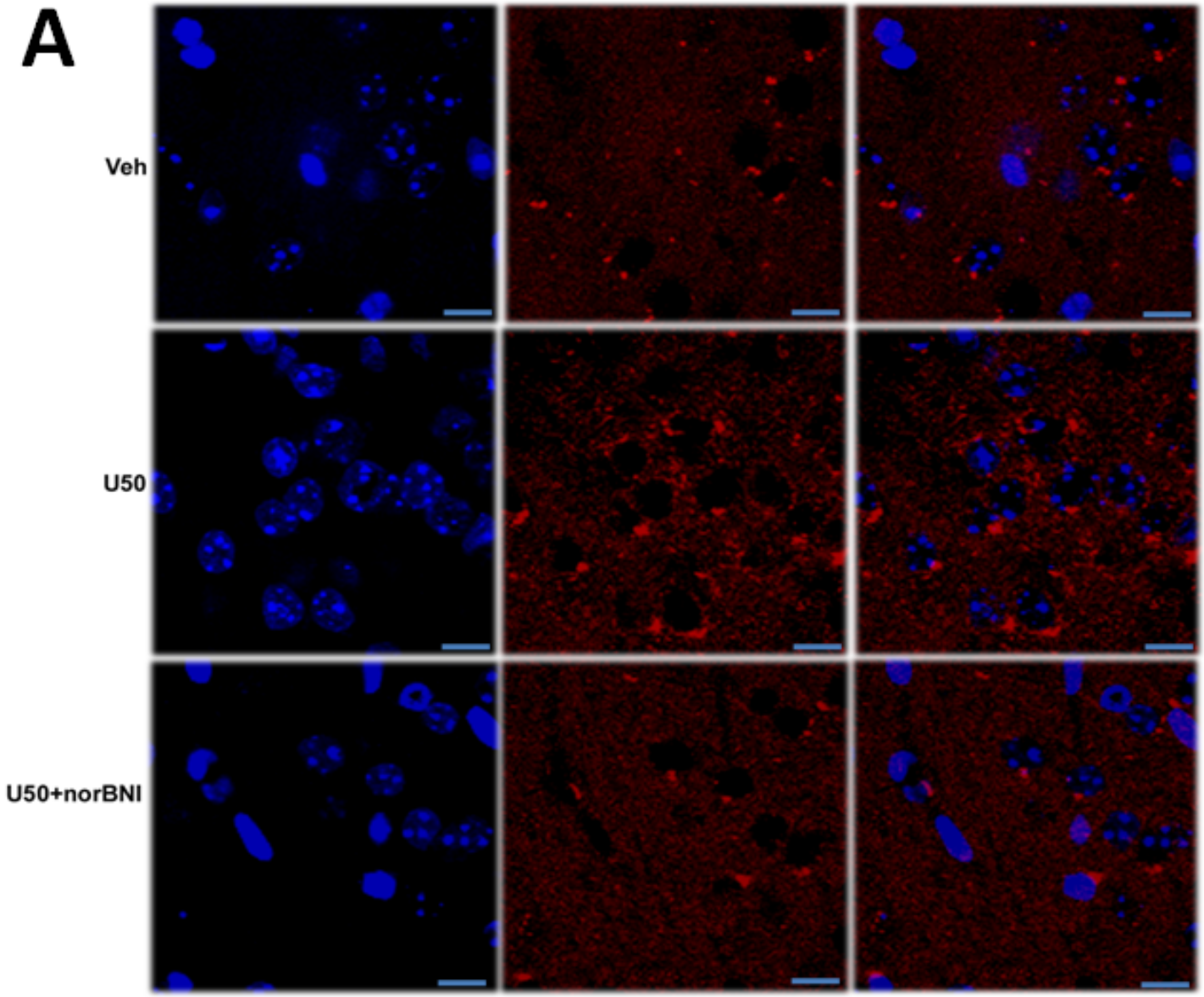

D
Veh
U50
U50+norBNI
Fold Change
2.5
2.0
1.5
1.0
0.5
0.0
**
##
**
#
HDAC4
HDAC5
HDAC6
HDAC7

Figure 4



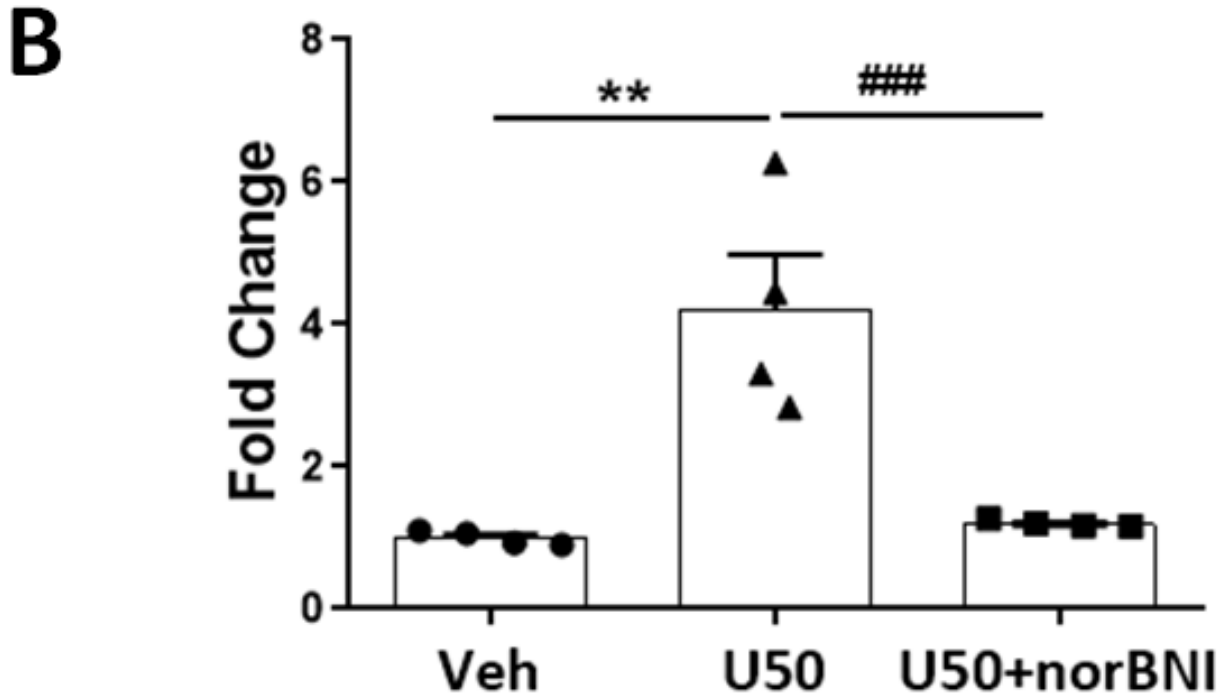
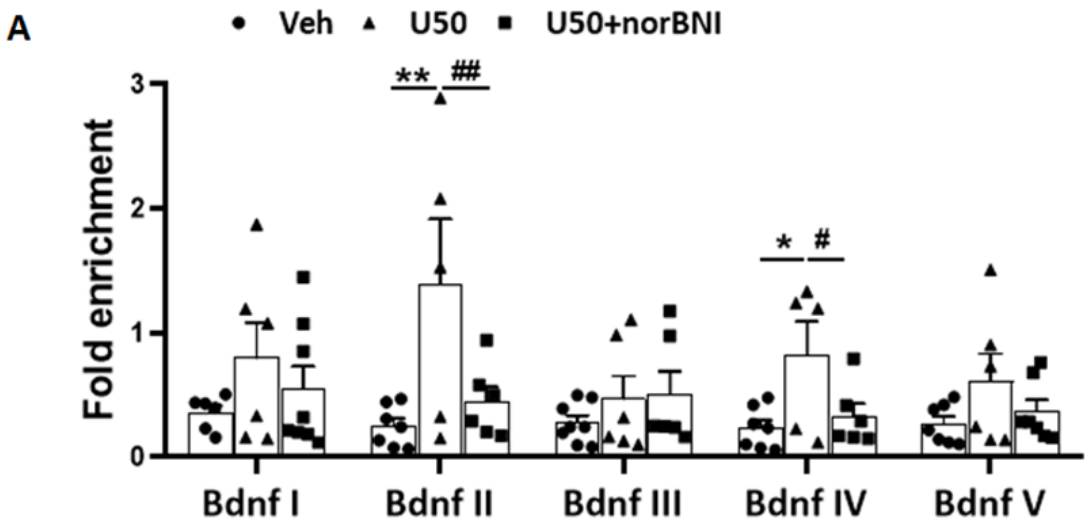


Figure 5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Fold enrichment
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Bdnf II
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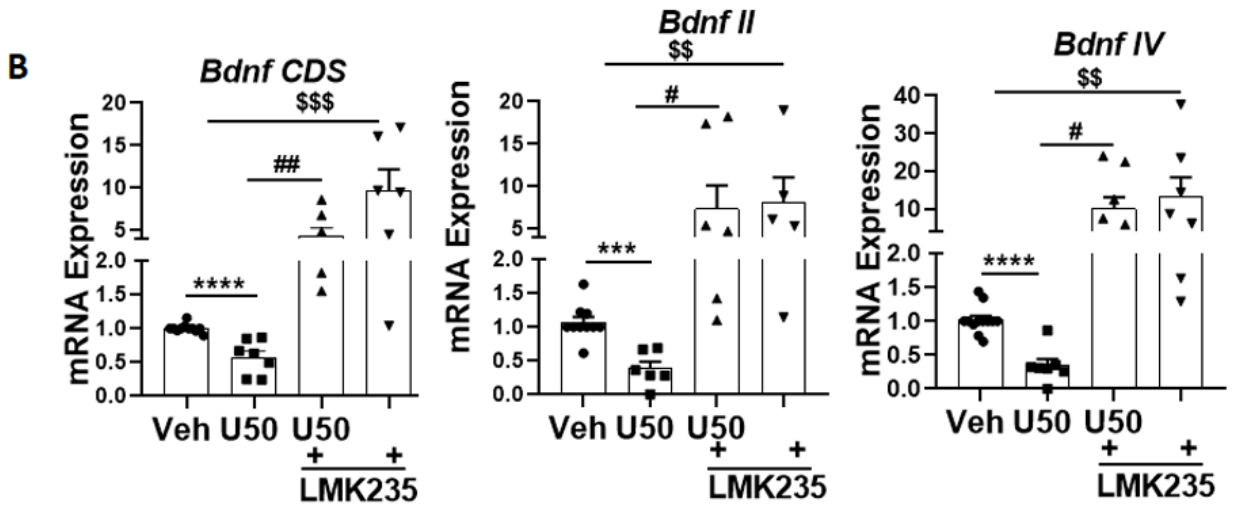
Bdnf IV
Bdnf V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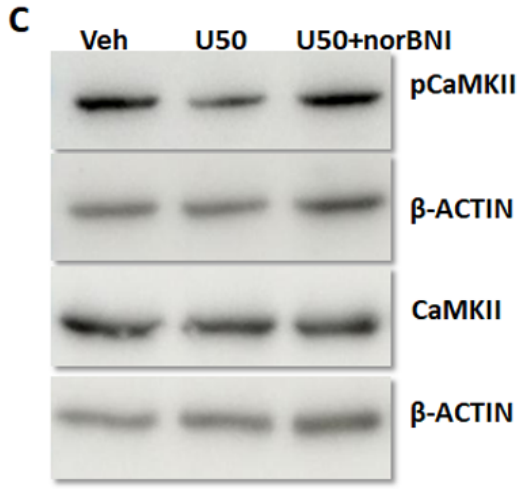
Bdnf II
Bdnf IV
mRNA Expression
Veh
U50
U50 + LMK235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pCaMKII
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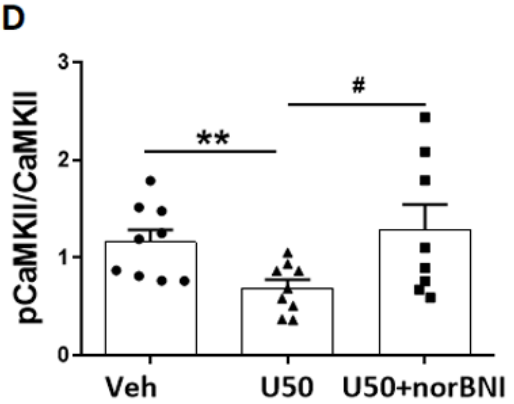
CaMKII
β-ACTIN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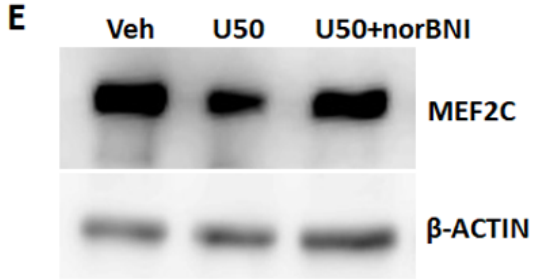
Veh
U50
U50+norBNI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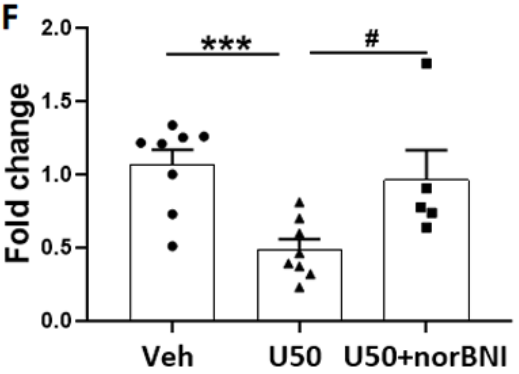
MEF2C
β-ACTIN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U50
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