

# Indispensable CBR1 in Major Depressive Disorder Therapy by Functionalized Solid Lipid Nanoparticles

Xiaolie He <sup>a,b,c</sup>, Li Yang <sup>a</sup>, Zhaojie Wang <sup>a</sup>, Guoxin Jing <sup>a</sup>, Ruiqi Huang <sup>a,b,c</sup>, Ziping Xu <sup>a</sup>, Shilong Wang <sup>a,c,\*</sup>, Rongrong Zhu <sup>a,b,c \*</sup>

<sup>a</sup> Division of Spine, Department of Orthopedics, Tongji Hospital affiliated to Tongji University School of Medicine, School of Life Science and Technology, Tongji University Shanghai, PR China

<sup>b</sup> Key Laboratory of spine and spinal cord injury repair and regeneration (Tongji University), Ministry of Education

<sup>c</sup> Research Center for Translational Medicine at East Hospital, Tongji University, Shanghai, PR China

Corresponding Author

Shilong Wang and Rongrong Zhu

Shilong Wang

Address: Division of Spine, Department of Orthopedics, Tongji Hospital affiliated to Tongji University School of Medicine, School of Life Science and Technology, Tongji University Shanghai, PR China

Research Center for Translational Medicine at East Hospital, Tongji University, Shanghai, PR China

Tel.: +86 21 65982595

E-mail: wsl@tongji.edu.cn

Rongrong Zhu:

Address: Division of Spine, Department of Orthopedics, Tongji Hospital affiliated to Tongji University School of Medicine, School of Life Science and Technology, Tongji University Shanghai, PR China

Key Laboratory of spine and spinal cord injury repair and regeneration (Tongji University), Ministry of Education

Research Center for Translational Medicine at East Hospital, Tongji University, Shanghai, PR China

Tel.: +86 21 65982595

Email: rrzhu@tongji.edu.cn

**Abstract**

Nanoparticles offer available tools for MDD research. In this assay, we applied CBR1 (cannabinoid receptor 1) knockout (CB1<sup>-/-</sup>) mice to study whether functionalized solid lipid nanoparticles loading with curcumin and dexamabinol (Cur/SLNs-HU-211) exhibited antidepressant outcomes through CBR1. Wild-type (CB1<sup>+/+</sup>) animals together with CBR1 knockout (CB1<sup>-/-</sup>) animals received daily injections of Corticosterone (CORT) for 3 weeks to obtain MDD mice model, and then the therapeutic action of Cur/SLNs-HU-211 were evaluated, respectively. Our work show that Cur/SLNs-HU-211 nanoparticles in the existence of CBR1 facilitate an efficient motor function improvement in CORT-induced MDD mice model. Cur/SLNs-HU-211 nanoparticles alleviated symptoms on CB1<sup>+/+</sup> MDD mice and resulted in dopamine and norepinephrine recovery following CORT-induced neurotoxicity. In conclusion, the possible mechanisms underlying the antidepressant effect of Cur/SLNs-HU-211 might be the induction of CB1 expression and downstream RASGEF1C and Egr1 expression, together with a significantly upregulation of neuron-specific genes in CB1<sup>+/+</sup> mice only. In conclusion, CBR1 is necessary during the process of antidepressant activities of Cur/SLNs-HU-211 nanoparticles. This study confirms that Cur/SLNs-HU-211 nanoparticles based CBR1 in vivo targeting would be a potentially feasible and safe way to motivate future therapeutic strategies of Major Depressive Disorder.

**Keywords:** major depressive disorder; functionalized solid lipid nanoparticles; CBR1

## Introduction

Major Depressive Disorder (MDD) is a common mood disturbance that is characterized primarily by a wider loss of interest for at least two weeks [1]. Selective serotonin reuptake inhibitors are frequently-used drugs, nevertheless, long-term uptake of SSRIs may induce high toxicity negating its beneficial effects [2]. Ongoing studies focus on developing new antidepressants so as to effectively improve Major Depressive Disorder.

CB1R primarily located in the peripheral and central nervous system. The endocannabinoid (eCB) system related to CB1R are involved in human emotion regulation, especially depression occurrence [3]. At the preclinical level, the misregulation in eCB pathway is associated with a depression-like phenotype [4]. It has been found that there was a significantly lower mRNA expression level of CB1 receptor in the blood of patients suffered from major depressive disorder [5]. CB1R agonists can enhance central neurotransmitters delivery and facilitate hippocampal neurogenesis, which share mechanisms with other antidepressants [6]. According to previous studies, cannabidiol, which refers to a potent CB1R antagonist, as well as the central CB1 receptor agonist HU-210 reveal an antidepressant-like behavior in the rodent forced swim models dose-dependently, similar to that of an antidepressant drug [7]. Activated CB1 receptor would prevent the depressive symptom patterns of chronic mild stress rat models on emotional learning [8]. Another research have revealed that ligands of CB1 receptor can strengthen the antidepressant effects of Bioactive metals [9].

Nanoparticles especially solid lipid nanoparticles has shown great potential in treating MDD [10,11], according to our previously research, functionalized solid lipid nanoparticles (Cur/SLNs-HU-211) could targeting CB1R and may regarded as a potential antidepressant [12]. Therefore, in this study, we applied Corticosterone (CORT) induced MDD mice model to more precisely understand the role Cur/SLNs-HU-211 played in CB1<sup>+/+</sup> and CB1<sup>-/-</sup> animal, respectively.

We performed behavioral test, neurotransmitters detection, neural marker expression analysis and transcriptome sequencing analysis to identify the function of Cur/SLNs-HU-211 in CB1<sup>+/+</sup> and CB1<sup>-/-</sup> MDD mice. According to our results we found that Cur/SLNs-HU-211 could only exhibited its antidepressant property in CB1<sup>+/+</sup> MDD mice, not CB1<sup>-/-</sup> MDD mice. We demonstrate that in wide type condition, Cur/SLNs-HU-211 could promote the induction of CB1R and activated the MEK/ERK signal pathways. The current results may provide an effective strategy in the treatment of MDD.

## Methods

### *Preparation of Cur/SLNs-HU-211 nanoparticles and CBR1 knockout mice*

Cur/SLNs-HU-211 were composited according to a method as described previously [13]. Shape of nanoparticles was pictured with a TEM.

CBR1 knockout mice were obtained from BIORAY LABORATORIES Inc (Shanghai, China) and raised in the Laboratory Animal Centre of Tongji University. These experiments were authorized by the institutional research ethics committee of Tongji University, and strictly followed the National Institutes of Health Guide for the Use and Care of mice.

Mice were randomly assigned to six groups: (1) Normal mice (CB1<sup>+/+</sup>) received saline; (2) Normal mice (CB1<sup>+/+</sup>) injected with Corticosterone (CORT) only; (3) Normal mice (CB1<sup>+/+</sup>) received CORT and Cur/SLNs-HU-211 nanoparticles (NPs); (4) CBR1 knockout mice (CB1<sup>-/-</sup>) received saline; (5) CBR1 knockout mice (CB1<sup>-/-</sup>) received with CORT only; (6) CBR1 knockout mice (CB1<sup>+/+</sup>) received CORT and Cur/SLNs-HU-211 nanoparticles.

Model of depression was obtained with injection of CORT (40 mg/kg) for 21 days [14].<sup>14,15</sup> Then NPs were given for 2 weeks at a curcumin. Mice genotype were identified by using Direct Mouse Genotyping Kit bought from APEX BIO Technology (Houston, USA), mice hippocampus and striatum of different group were used to confirm the genotype of CB1<sup>+/+</sup> and CB1<sup>-/-</sup> mice.

### *Behavioral tests*

To assess the impact on motor function, the mice were distributed to the Forced Swimming Test (FST) and the Pole test.<sup>16-18</sup> For FST, mice were well dropped into a beaker within water, all recorded. In the final 6 min of 8 min, float time were calculated. In the pole test, mice start from the top of the pole (0.8 cm in diameter; 60 cm in height) and the time of climbing down the pole was counted, as described before. All experiments were done 3 times and double blind.

### *DA and NE detection*

Mice hippocampus and striatum were collected. Samples were immersed in 400  $\mu$ L PBS and homogenized, mixing with 800  $\mu$ L of acetonitrile, and then centrifuged.<sup>19</sup> 20  $\mu$ L samples were automatically injected onto HPLC. The mobile phase used were 10% methanol and 90% 0.01 M KH<sub>2</sub>PO<sub>4</sub> (pH 3.5) at a flow velocity of 500  $\mu$ L/min. At 280 nm wavelength, the concentrations of dopamine (DA) were measured. For norepinephrine (NE), 60% methanol and 40% 0.05 M CH<sub>3</sub>COONa and a detection wavelength of 275 nm were applied. The protein concentrations in each areas of brain were quantified.

### *qRT-PCR*

qRT-PCR was performed according to the standard protocols.

### *Western blot*

Western blot was executed with a standard protocol. Antibodies specific to NeuN (ab104225, abcam), MAP2 (ab11267, abcam), Tuj1 (ab78078, abcam), CB1 (ab23703, abcam), RASGEF1C (ab139329, absin), MEK (ab32091, abcam), p-MEK (ab214445, abcam), ERK (ab184699, abcam), p-ERK (ab32538, abcam), Egr1 (ab133695, abcam) and  $\beta$ -actin (ab8227, abcam) were used.

### *Immunofluorescence*

Immunofluorescence analysis was performed using standard protocols. Antibodies specific to NeuN (1:500, ab104225, abcam), appropriate fluorescently tagged secondary antibodies and DAPI were used.

### *RNA-seq*

We collected hippocampus of six groups and carried out RNA sequencing, total RNA was extracted with TRIzol Reagent (Takara). RNA sequencing was performed by BGI., Shenzhen, China.

### *Statistical analysis*

Experimental results were presented as means  $\pm$  standard deviation (SD). One-way ANOVA was performed for each analysis, and  $P < 0.01$  was statistically significant.

## Results

### *Preparation of nanoparticles and mice*

We initially observed Cur/SLNs-HU-211 nanoparticles via a TEM (Fig. 1A), TEM analysis confirmed that our synthesis was successful and nanoparticles were in spherical shape. As shown by the schematic (Fig. 1B), normal mice (CB1<sup>+/+</sup>) and CBR1 knockout mice (CB1<sup>-/-</sup>) were given CORT and Cur/SLNs-HU-211 nanoparticles, CORT were used to model MDD, further experiments were carried out to test whether Cur/SLNs-HU-211 nanoparticles benefit CORT treated CB1<sup>+/+</sup> and CB1<sup>-/-</sup> mice, respectively. Six groups of mice hippocampus and striatum were collected and genotype of each mouse was identified, PCR amplification was performed on a thermal cycler, yielding a 804-bp fragment from homozygous CB1<sup>+/+</sup> mice and a 628-bp fragment from homozygous CB1<sup>-/-</sup> mice (Fig. 1C).

### *Cur/SLNs-HU-211 promote motor function recovery from MDD only for CB1<sup>+/+</sup> mice*

Mice behavioral tests can be a useful tool for evaluating the curative effect of drugs. In our study, we applied FST and Pole Test to evaluate mice motor function improvement. FST results (Fig. 2A) suggested that Cur/SLNs-HU-211 could greatly improve CORT treated CB1<sup>+/+</sup> mice float time in water from 36.5 sec to 46.5 sec, however no such improvement has occurred in CB1<sup>-/-</sup> mice.

In order to measure bradykinesia, we implemented the Pole Test, results (Fig. 2B) shown that the CORT group mice took longer to climb down the pole compared with the control. Treatment of the nanoparticles group (8.7 sec) reduced climbing times compared with CORT group (11.3 sec) in CB1<sup>+/+</sup> mice, which means they are much faster. For CB1<sup>-/-</sup> mice, there were no significant difference between CORT and nanoparticles group.

The results show that Cur/SLNs-HU-211 nanoparticles can just effectively reverse CORT-induced motor deficits in CB1<sup>+/+</sup> mice not CB1<sup>-/-</sup> mice.

### *Cur/SLNs-HU-211 increases DA and NE level in MDD CB1<sup>+/+</sup> mice*

Harvest mice hippocampus and striatum for HPLC analysis, DA and NE levels were detected. As shown in Fig. 3A,3B, there was a reduction in DA levels after CORT administration. For CB1<sup>-/-</sup> mice, nanoparticles treated animal exhibited no increase compared with CORT group mice in DA level. For CB1<sup>+/+</sup> mice, nanoparticles could remarkably enhance the DA release of CORT treated mice both in hippocampus and striatum.

The NE level was also measured, the results (Fig. 3C,3D), CORT induced significant decrease of NE both in hippocampus and striatum, and this decrease was reversed when given with Cur/SLNs-HU-211 in CB1<sup>+/+</sup> mice. When it comes to CB1<sup>-/-</sup> MDD mice, Cur/SLNs-HU-211 induced no significantly higher NE level.

This recovery property of Cur/SLNs-HU-211 on CB1<sup>+/+</sup> MDD mice in DA and NE levels indicated that Cur/SLNs-HU-211 could significantly elevate the levels of monoamine neurotransmitter in MDD mice, and those recovery would not happen when knock out CBR1.

### *qRT-PCR*

To study the impact of Cur/SLNs-HU-211, we analyzed the expression of neuronal-specific genes (NeuN, Map2, Tuj1). It could be seen from qRT-PCR that the mRNA levels of NeuN, Map2 and Tuj1 in hippocampus and striatum (Fig. 4) were reduced drastically in MDD mice following CORT injection compared to control group. After the administration of Cur/SLNs-HU-211, the levels of NeuN, Map2 and Tuj1 mRNA relative to the CB1<sup>+/+</sup> MDD mice increased significantly. And for CB1<sup>-/-</sup> MDD mice, Cur/SLNs-HU-211 lost this function and could not promote the increase of these mRNA levels. The results were in accordance with that of behavioral test and neurotransmitter detection. qRT-PCR results demonstrated that Cur/SLNs-HU-211 could only benefit CB1<sup>+/+</sup> MDD mice by increasing NeuN, Map2 and Tuj1 mRNA levels in hippocampus and striatum.

### **Western blot**

The expressions of NeuN, MAP2 and Tuj1 were also determined by western blot assay. Consistent with the result of qRT-PCR, protein expression levels of the mature DA neuronal marker NeuN, MAP2 and Tuj1 were significantly increased in Cur/SLNs-HU-211 administrated CB1<sup>+/+</sup> MDD mice (Fig. 5A). In Cur/SLNs-HU-211 treated CB1<sup>-/-</sup> MDD mice, there were no distinction as compare to CB1<sup>-/-</sup> MDD mice. Both the western blot bands and densitometry quantification analysis (Fig. 5B) verified that in hippocampus and striatum.

### **Immunofluorescence**

To analysis neuronal survival, we applied NeuN as a Neuronal Biomarker, as shown (Fig. 6A). Compared to the CB1<sup>+/+</sup> and CB1<sup>-/-</sup> Control group, CORT led to remarkable neuronal cell death and few NeuN positive cells were observed in the hippocampus and striatum of mice. However, there were more neurons observed in CB1<sup>+/+</sup> MDD mice following treatment of Cur/SLNs-HU-211, but less NeuN positive cells were detected in CB1<sup>-/-</sup> MDD mice. We quantify the intensity of NeuN fluorescence and found that the results (Fig. 6B) were consistent with qRT-PCR and Western blot.

### **RNA-seq**

To explore the underlying molecular mechanisms in more details, we employed transcriptome sequencing to examine the differential gene expression aiming to reveal in vivo working system.

A heat map presenting mRNA expression profiles of all samples was shown (Fig. 7A). There were 505 marked differential expressed genes between Cur/SLNs-HU-211 and MDD CB1<sup>+/+</sup> group, we listed the most relevant 17 genes (Fig. 7B), these genes are mostly neurodevelopment concerned.

2D principal component analysis (2D PCA) for all of the samples of all genes clearly separates NPs (CB1<sup>-/-</sup>) group the farthest from Control (CB1<sup>+/+</sup>) group, NPs (CB1<sup>+/+</sup>) group the closest (Fig. 7C). We recognized 8 related KEGG pathways from Cur/SLNs-HU-211 treated CB1<sup>+/+</sup> group versus MDD CB1<sup>+/+</sup> group (Fig. 7D). GO analysis revealed that NPs (CB1<sup>+/+</sup>) group compared with CORT (CB1<sup>+/+</sup>) group differ most in biological process and cellular component (Fig. 7E). KEGG results suggested that NPs (CB1<sup>+/+</sup>) group compared with CORT (CB1<sup>+/+</sup>) group may have big difference in human diseases and organismal systems.

### **Molecular mechanisms**

Among the 9 pathways with significant differences, we focused on MAPK pathway [15,16], and then we examined these genes by qRT-PCR (Fig.8A) and western blot assay (Fig.8B), quantification of western blot was also carried out (Fig.8C). The results of qRT-PCR (Fig.8A) showed that in nanoparticles treated CB1<sup>+/+</sup> mice, the CB1, Rasgef1c and Egr1 mRNA expression were increased compared with CB1<sup>+/+</sup> MDD mice, and for CB1<sup>-/-</sup> mice the expression of these genes was low and nanoparticles treated mice show no difference with MDD mice. We further investigated protein expression with western blot (Fig.8B, 8C), CB1, RASGEF1C, Egr1, together with phosphorylation of MEK and ERK were up-regulated in Cur/SLNs-HU-211 treated CB1<sup>+/+</sup> MDD mice as compared to CB1<sup>+/+</sup> MDD mice, no significantly change observed in CB1<sup>-/-</sup> mice. The total protein of MEK and ERK were almost in a same level in all group.

### **Discussion**

CORT is a commonly used inducer of depression-like symptoms in mice. CORT model could be used to explore the underlying mechanisms of MDD and to verify novel chemical compounds for their neuroprotective effects [17,18]. Our research analysed the neuroprotective and MDD rehabilitation effects of nanoparticles in CORT induced MDD CB1<sup>+/+</sup> and CB1<sup>-/-</sup> mice.

Nanoparticles can influence neuron survival, neurotransmitters release, apoptosis and behavioral response [19,20]. Although many other nanoparticles have been reported to facilitate antidepressants to cure MDD [21-23], we propose that Cur/SLNs-HU-211 may be markedly beneficial on account of its high drug load, low toxicity, ease of synthesis and controllability. We previously demonstrated that Cur/SLNs-HU-211 can benefit neuron cell and

trigger gene-transcription networks crucial for the viability of cell, and Cur/SLNs-HU-211 mainly act through targeting CBR1. These findings allowed us to investigate whether Cur/SLNs-HU-211 can only improve the MDD condition in the presence of CBR1. So, we knock out CBR1 in C57BL/6 mice and compared the function of Cur/SLNs-HU-211 in MDD CB1<sup>+/+</sup> and CB1<sup>-/-</sup> mice.

The in vivo test indicated that treatment of Cur/SLNs-HU-211 remarkably improved motor function recovery in CORT treated CB1<sup>+/+</sup> mice, however, no such effects has shown in CB1<sup>-/-</sup> mice. Neurotransmitters like DA and NE in hippocampus and striatum were also evaluated after Cur/SLNs-HU-211 treated for CB1<sup>+/+</sup> mice only. In our study, qRT-PCR and western blots assay all suggested that CORT-induced MDD mice exhibited a reduction of CB1 three weeks after receiving CORT, which indicate that CBR1 are essential for protection against CORT toxicity. In CB1<sup>+/+</sup> mice hippocampus and striatum, we found that administration of nanoparticles could increase the expression of NeuN, MAP2 and Tuj1, however, there were no significantly changes between Cur/SLNs-HU-211 and CORT administrated CB1<sup>-/-</sup> mice. Immunofluorescence analysis also verified that NeuN positive cells will increased in Cur/SLNs-HU-211 treated CB1<sup>+/+</sup> MDD mice hippocampus and striatum, these results not apply to CB1<sup>-/-</sup> mice. All of this revealed that CBR1 is a must for Cur/SLNs-HU-211 exerted its neuroprotection and antidepressants effects. To better understand the mechanism involved in, we carried out RNA-seq, results suggested that MAPK signal pathway may be a key pathway, we applied qRT-PCR and western blot to verify. We found that the MEK/ERK signaling pathway was involved in and the neuroprotective effects of Cur/SLNs-HU-211 was regulated by an enhanced expression of RASGEF1C/Egr1. Importantly, MEK/ERK signaling pathway could only activated in CB1<sup>+/+</sup> MDD mice. Dueling to the knockout of CBR1, Cur/SLNs-HU-211 could not show its neuroprotective effects in CB1<sup>-/-</sup> MDD mice for the pathway was blocked and thus there were no significantly difference compared with CORT treated CB1<sup>-/-</sup> mice.

Taken together (Fig.9), our findings suggest that CBR1 is a necessary for Cur/SLNs-HU-211 to exerted its neuroprotection and antidepressants effects, and Cur/SLNs-HU-211 nanoparticles may provide a novel medicinal strategy for MDD and other neurodegenerative diseases.

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### Disclosure Statement

No potential conflicts of interest were disclosed.



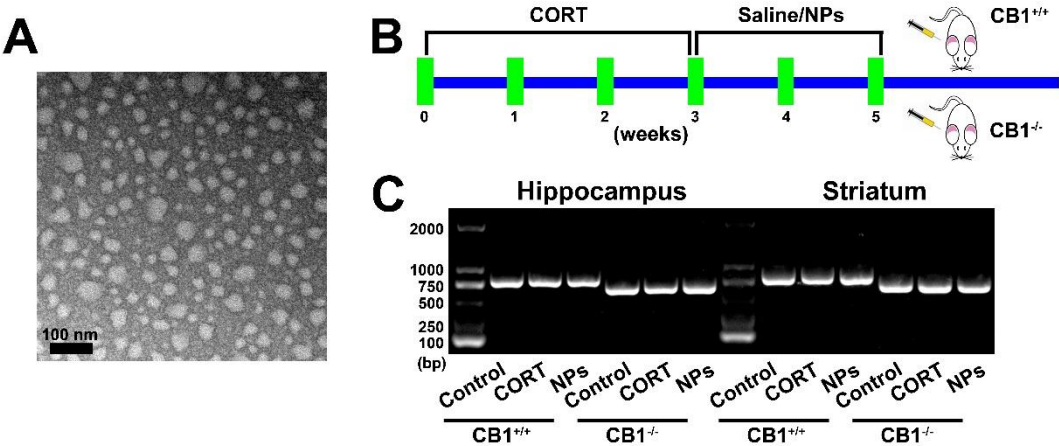
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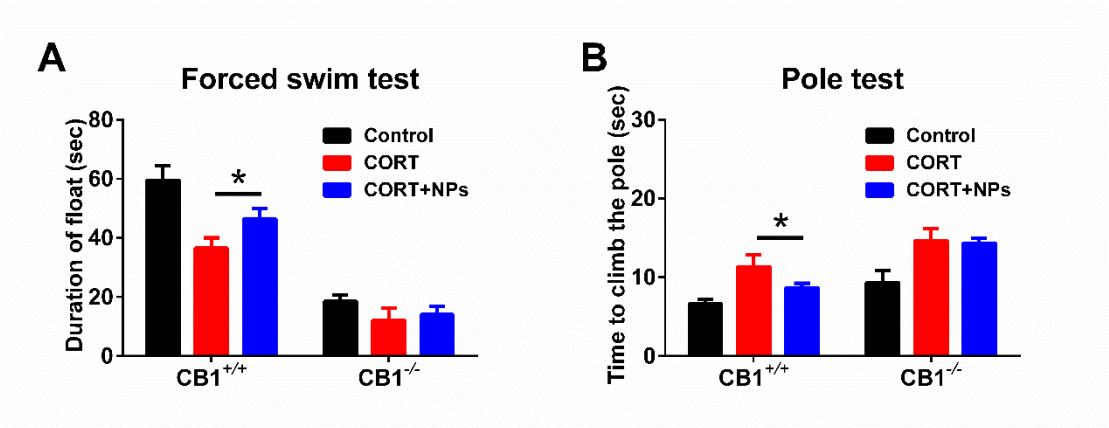


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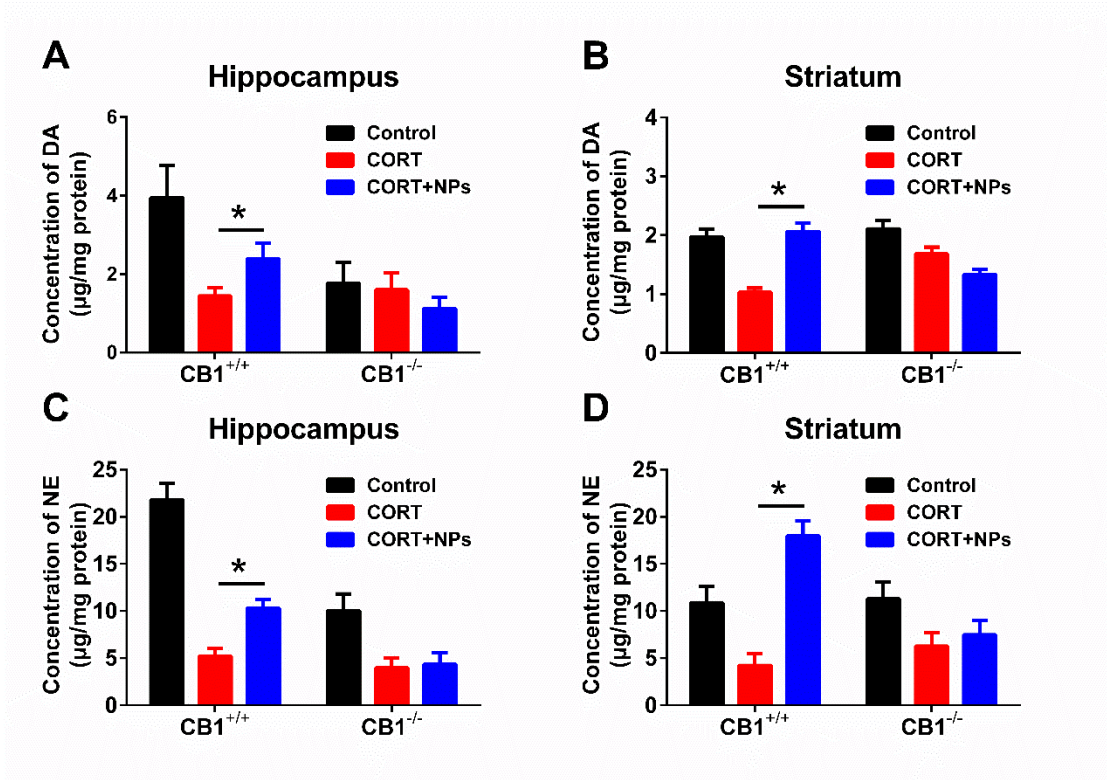
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**Fig. 1. Preparation of nanoparticles and mice.** **A**, Transmission electron microscopy images of Cur/SLN-HU-211 nanoparticles. Scale bars, 100 nm. **B**, Flow chart show the mice model of MDD and drug given. **C**, Genotype identification of  $CB1^{+/+}$  and  $CB1^{-/-}$  mice.



**Fig. 2. Cur/SLNs-HU-211 improve behavioral recovery from MDD in CB1<sup>+/+</sup> mice.** A, Time of mice float in FST. B, Time to climb the pole in the pole test. For significance, \*P<0.05 compared with CORT group.



**Fig. 3. Cur/SLN-HU-211 enhance DA and NE release in CB1<sup>+/+</sup> MDD mice hippocampus and striatum.**  
**A,** Concentration of DA in hippocampus. **B,** Concentration of DA in striatum. **C,** NE concentration in hippocampus. **D,** NE concentration in striatum. For significance, \*P<0.05 compared with CORT group.

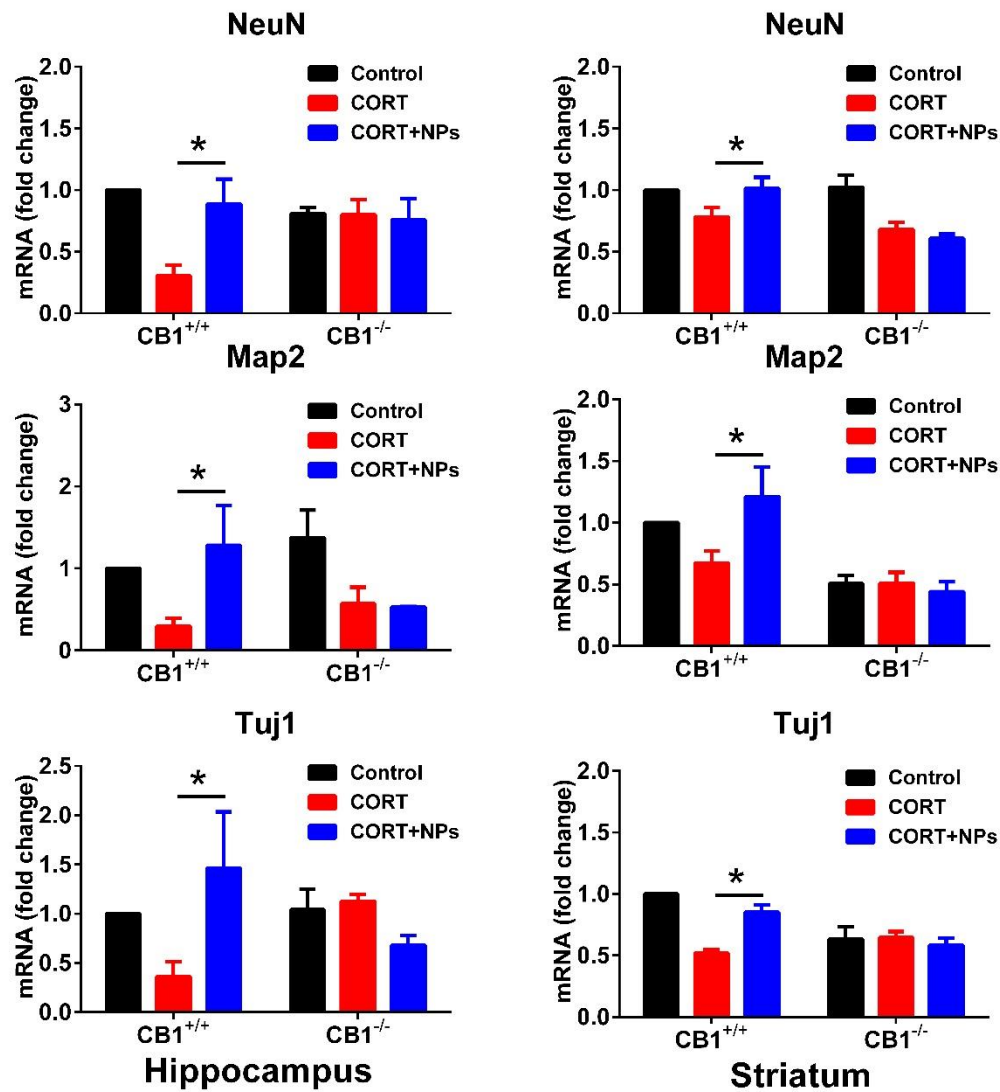


Fig. 4. qRT-PCR analysis of the neuronal markers. Expression levels were normalized to GAPDH. Data are presented as mean  $\pm$  s.e.m., ANOVA. For significance, \* $P < 0.05$  compared with CORT group.

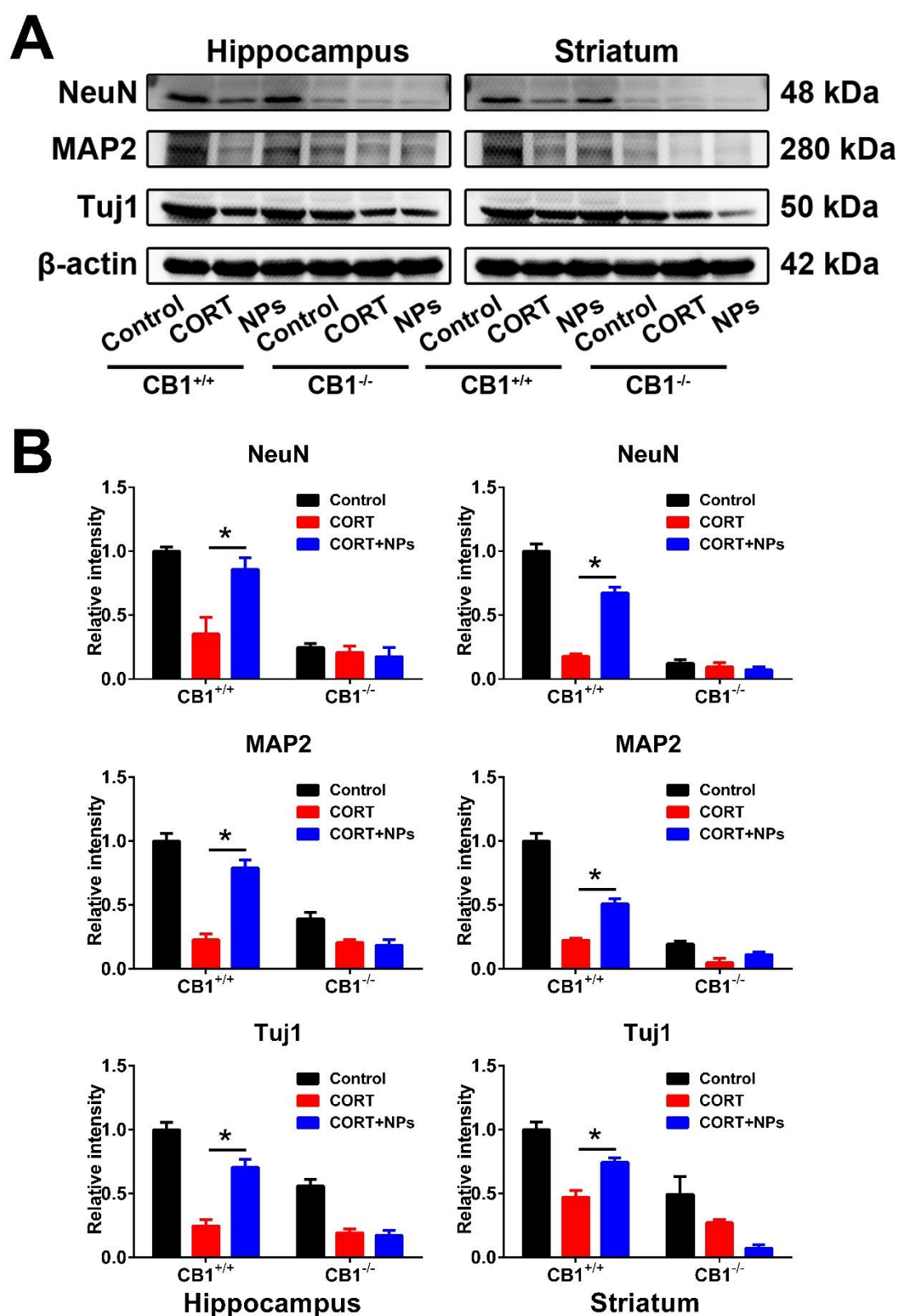
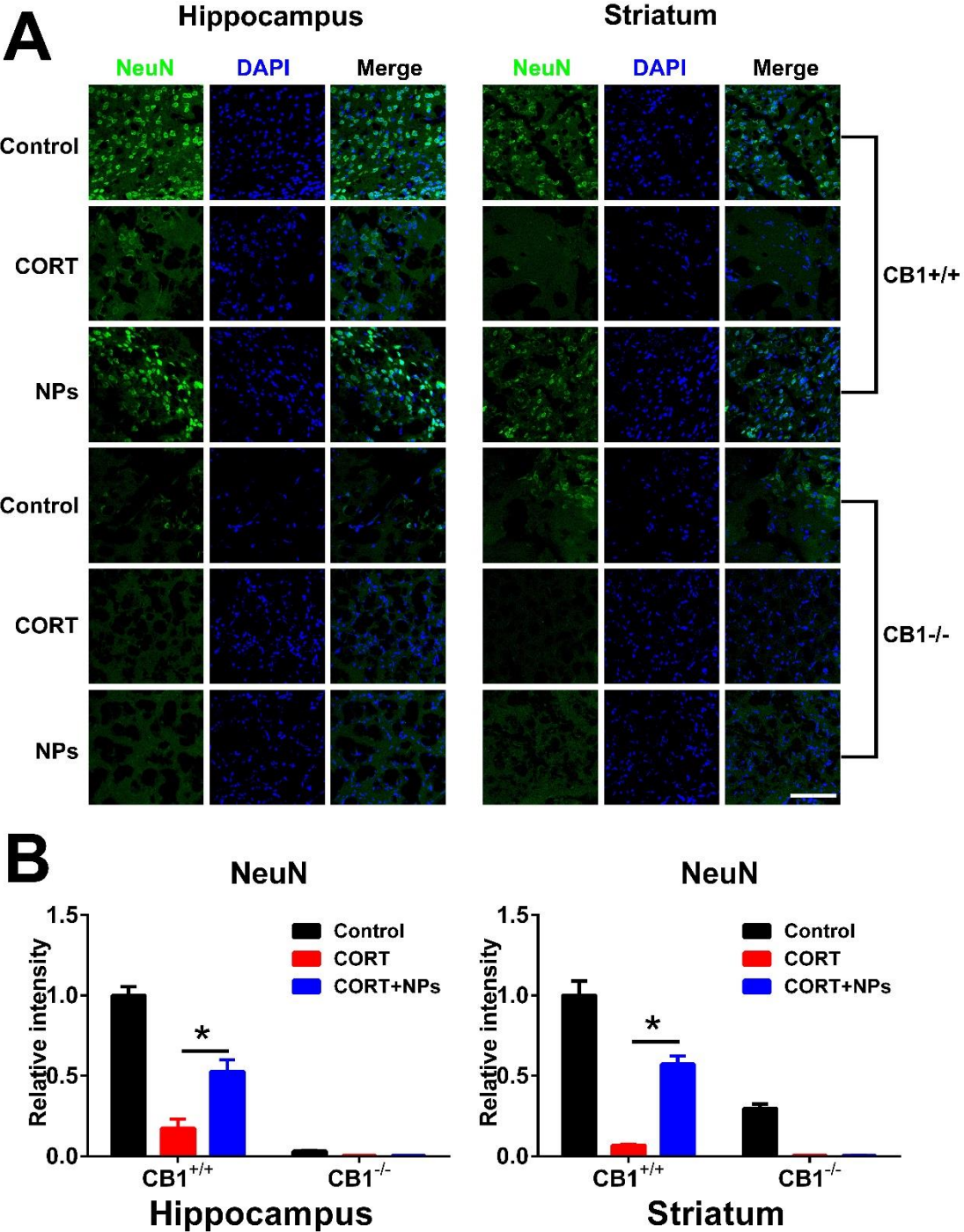
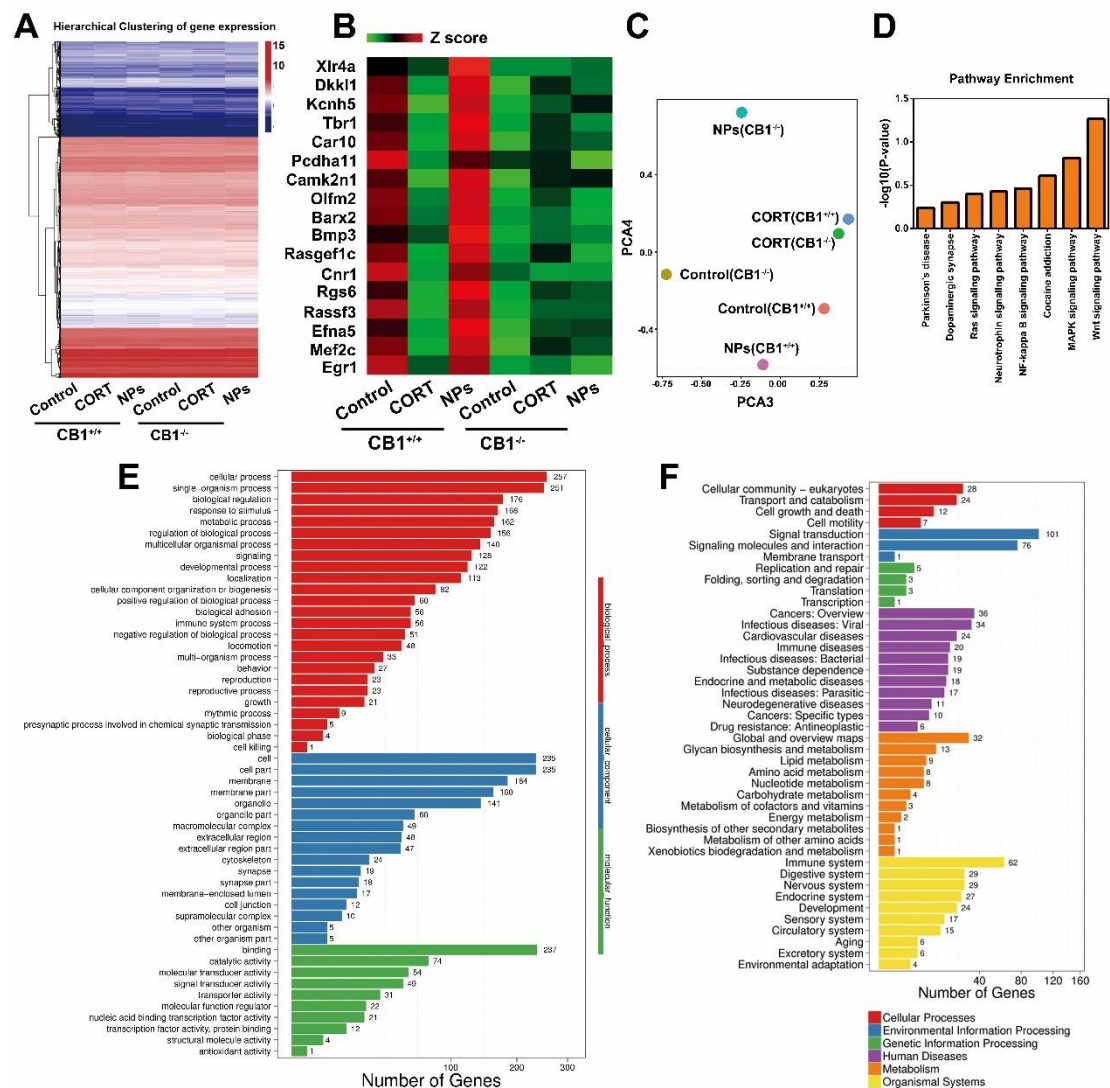


Fig. 5. Cur/SLN-HU-211 increase protein Levels of NeuN, MAP2 and Tuj1 in CB1<sup>+/+</sup> MDD mice hippocampus and striatum. **A**, Western blot images for neuronal markers. **B**, Quantification of bands, expression levels were normalized to  $\beta$ -actin. For significance, \* $P < 0.05$  compared with CORT group.

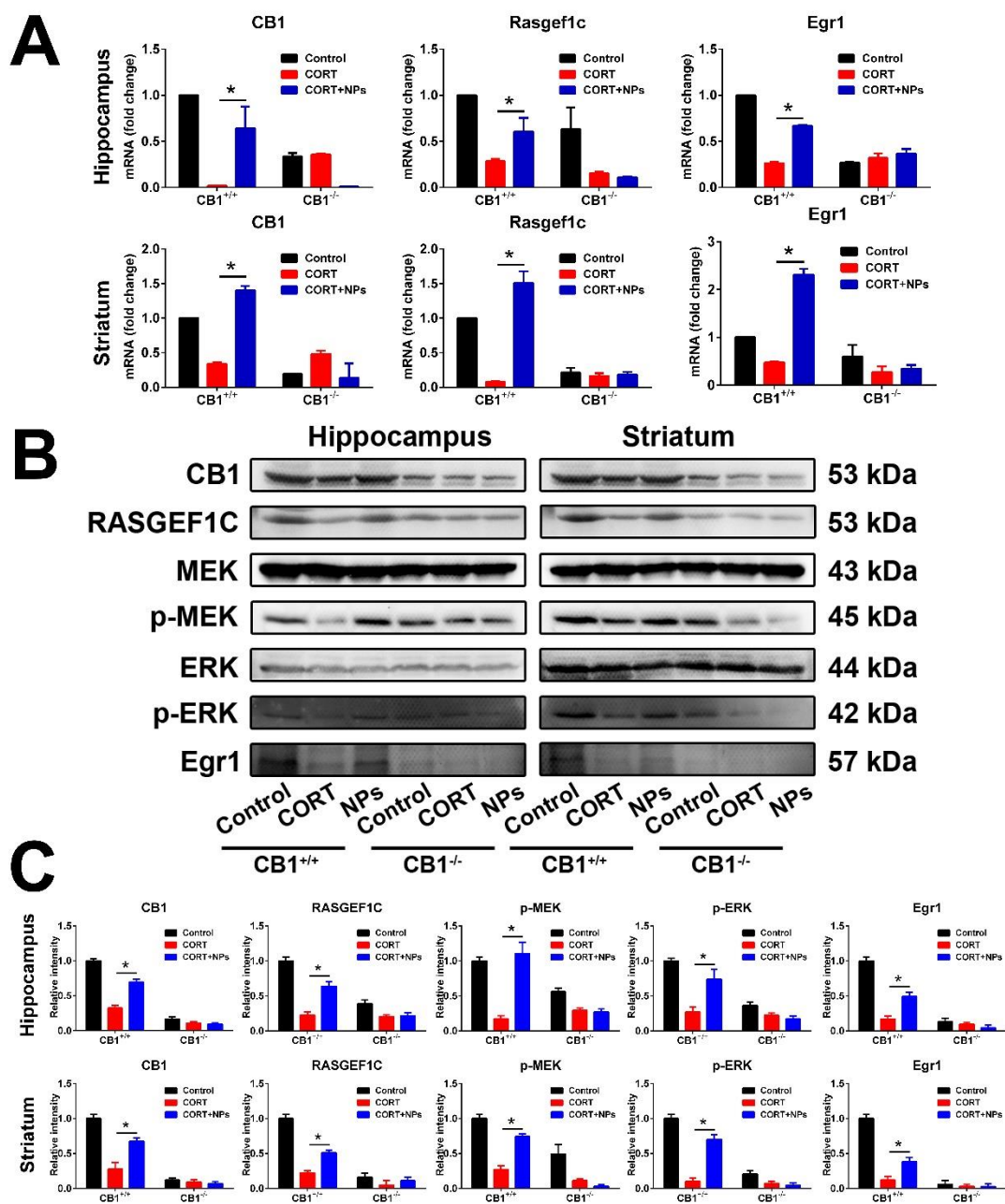


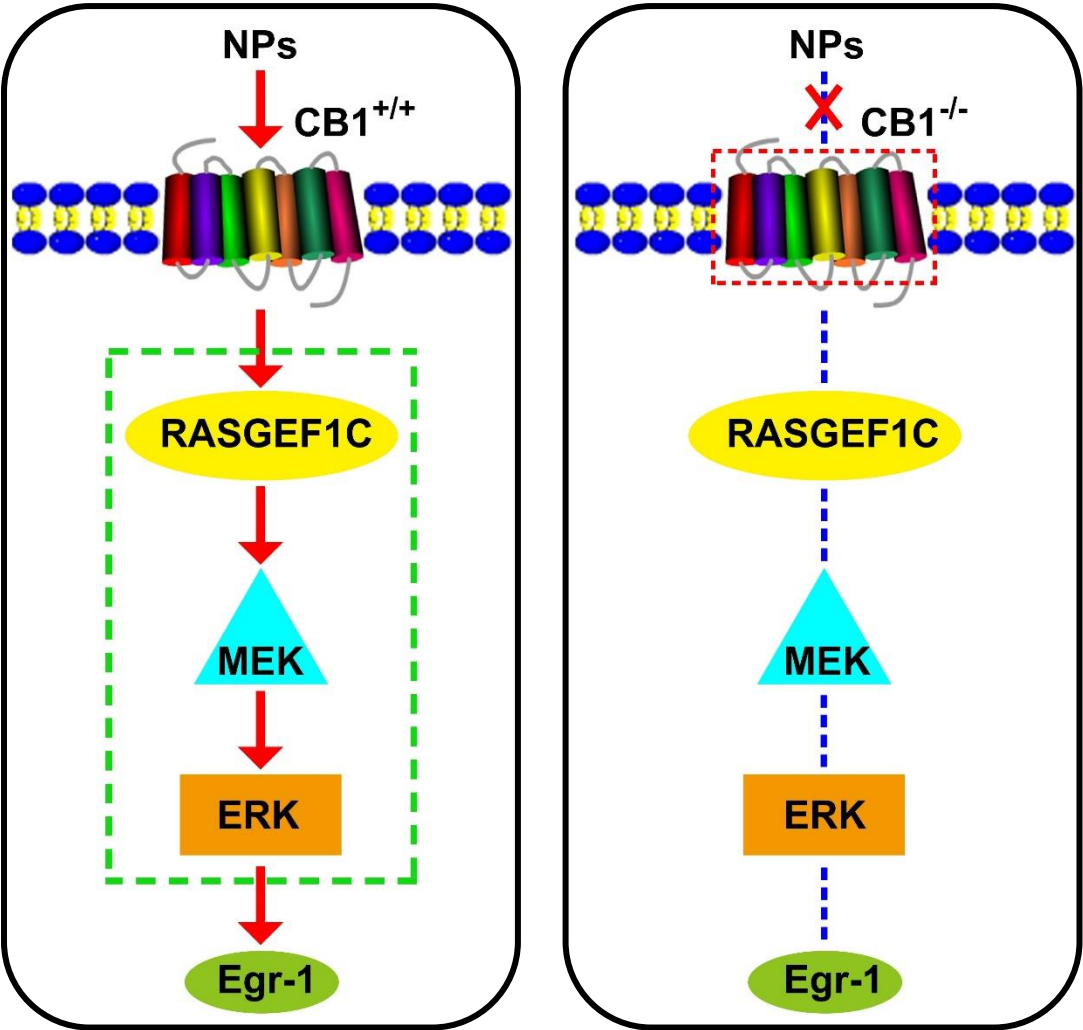


**Fig. 6. Cur/SLNs-HU-211 promote NeuN positive cells survival after CORT treatment in CB1<sup>+/+</sup> mice hippocampus and striatum.** **A**, Representative immunofluorescence images of NeuN expression in hippocampus and striatum. **B**, Quantification of fluorescence intensity. For significance, \*P<0.05 compared with CORT group. Scale bars, 100  $\mu$ m.



**Fig. 7.** RNA-seq analysis of antidepressant effects induced by Cur/SLN-HU-211 in CB1<sup>+/+</sup> MDD mice. **A**, Heat map that represents the global gene-expression pattern of transcription factors in hippocampus. **B**, Representative genes listed. **C**, 2D principal component analysis using genomewide expression data. **D**, KEGG pathway analysis. **E**, GO terms analysis. **F**, KEGG biological pathway classification and enrichment analysis.





**Fig. 9.** Schematic representation of the reason why Cur/ SLNs-HU-211 behave differently in  $CB1^{+/+}$  and  $CB1^{-/-}$  MDD mice.