

REGULAR RESEARCH ARTICLE

Farnesoid X Receptor-Mediated Cytoplasmic Translocation of CRTC2 Disrupts CREB-BDNF Signaling in Hippocampal CA1 and Leads to the Development of Depression-Like Behaviors in Mice

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Abstract

Background: We recently identified neuronal expression of farnesoid X receptor (FXR), a bile acid receptor known to impair autophagy by inhibiting cyclic adenosine monophosphate response element-binding protein (CREB), a protein whose underfunctioning is linked to neuroplasticity and depression. In this study, we hypothesize that FXR may mediate depression via a CREB-dependent mechanism.

Methods: Depression was induced in male C57BL6/J mice via chronic unpredictable stress (CUS). Subjects underwent behavioral testing to identify depression-like behaviors. A variety of molecular biology techniques, including viral-mediated gene transfer, Western blot, co-immunoprecipitation, and immunofluorescence, were used to correlate depression-like behaviors with underlying molecular and physiological events.

Results: Overexpression of FXR, whose levels were upregulated by CUS in hippocampal CA1, induced or aggravated depression-like behaviors in stress-naïve and CUS-exposed mice, while FXR short hairpin RNA (shRNA) ameliorated such symptoms in CUS-exposed mice. The behavioral effects of FXR were found to be associated with changes in CREB-brain-derived neurotrophic factor (BDNF) signaling, as FXR overexpression aggravated CUS-induced reduction in BDNF levels while the use of FXR shRNA or disruption of FXR-CREB signaling reversed the CUS-induced reduction in the phosphorylated CREB and BDNF levels. Molecular analysis revealed that FXR shRNA prevented CUS-induced cytoplasmic translocation of CREB-regulated transcription coactivator 2 (CRTC2); CRTC2 overexpression and CRTC2 shRNA abrogated the regulatory effect of FXR overexpression or FXR shRNA on CUS-induced depression-like behaviors.

Conclusions: In stress conditions, increased FXR in the CA1 inhibits CREB by targeting CREB and driving the cytoplasmic translocation of CRTC2. Uncoupling of the FXR-CREB complex may be a novel strategy for depression treatment.

Key Words: Depression, FXR, CREB, BDNF, CRTC2

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Significance Statement

Although FXR has been identified in brain neurons, its role in various brain disorders remains unclear. Recently, FXR was shown to suppress autophagy through inhibition of CREB. We report that an increase in FXR in the CA1 region, but not in the DG region or mPFC region, causes or aggravates depression-like behavior in normal or stressed mice via disrupting the CREB-BDNF signaling pathway. The disruption of the FXR-CREB complex can reverse stress-induced depression-like behavior, CREB dephosphorylation, and BDNF reduction. Uncoupling the FXR-CREB complex specifically in the CA1 region may be a novel strategy for depression therapy.

Introduction

The farnesoid X receptor (FXR) is a nuclear receptor that is highly expressed in visceral organs, including the liver (Fuchs et al., 2017) and the intestine (Massafra and van Mil, 2018). Its role in fatty acid, lipoprotein, and glucose metabolism and as a receptor for bile acids is well documented (Hylemon et al., 2017; Kim et al., 2017), and it is a potential drug target for metabolic disorders. Recently, our group identified the expression of FXR in vitro—in mouse neuronal cultures and in vivo—in mouse and human cortex and/or hippocampus (Huang et al., 2016). However, its role in neuronal physiology and brain disorders remains unclear. Cyclic adenosine monophosphate response element-binding protein (CREB) is a transcription factor that regulates the transcription of genes in many tissues. In the brain, it plays a significant role in hippocampal neuroplasticity, long-term memory formation, dendritic growth, and neurogenesis (Kida and Serita, 2014; Ortega-Martínez, 2015; Serita et al., 2017). CREB was not only found to be dysfunctional in animal models of depression and patients with depression but was also hypothesized to be involved in the onset of depression (Sulser, 2002; Yamada et al., 2003). Changes in the expression levels of brain-derived neurotrophic factor (BDNF), a molecule downstream of CREB and functioning as a stimulator for growth and survival of neurons, are demonstrated in the serum samples and the brain tissues of both experimental animals and patients with depression (Lindholm and Castrén, 2014; Björkholm and Monteggia, 2016; Youssef et al., 2018). Further, a heterozygous knockout of the BDNF gene in mice eliminated the antidepressant effects of amine-based drugs such as the tricyclic imipramine (Saarelainen et al., 2003). Therefore, it can be hypothesized that the impairment of CREB-BDNF signaling in the brain contributes to the development of depression. However, there is a paucity of data to show how this signaling pathway is affected under depressed conditions.

Activation of FXR inhibits CREB, which in turn inhibits autophagy. The inhibition of CREB depends on the cytoplasmic translocation of its transcriptional co-activator, CREB-regulated transcription coactivator 2 (CRTC2), which mediates numerous physiological processes, including gluconeogenesis and cell proliferation in the liver and pancreas (Eberhard et al., 2013; Seok et al., 2014; Li et al., 2017). In a recent study, FXR was found to impair BDNF signaling in the hippocampus and mediate chronic unpredictable stress (CUS)-induced depression-like behaviors in rats (Chen et al., 2018). This observation points toward a possible role for FXR in the pathogenesis of depression. In the present study, we show that FXR contributes to the pathogenesis of depression by driving the cytoplasmic translocation of CRTC2, impairing the hippocampal CREB-BDNF signaling pathway.

Methods

Animals

Eight- to 10-week-old C57BL/6J male mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd.

(Beijing, China) and housed 5 per cage under standard vivarium conditions (12-hour-light/-dark cycle [7:00 AM to 7:00 PM]; 23°C ± 1°C ambient temperature; 55% ± 10% relative humidity) for 1 week with free access to food and water. Each experimental group consisted of 9 to 12 mice. Intra-hippocampal injection was performed after the discontinuation of CUS, and 14 days after lentiviral vector injection behavioral tests were applied to measure the changes in depression-like behaviors in mice (supplementary Figure 1A). Three common tests that are widely used in depression study in rodents, including the tail suspension test (TST), the forced swim test (FST), and the sucrose preference test (SPT), were selected to investigate the role of FXR in CUS-induced depression-like behavior. The behavioral tests were performed in the order of TST, FST, and SPT. To investigate the role of BDNF signaling in FXR's regulation on CUS-induced depression-like behavior, the mice were injected with K252a, an inhibitor of tyrosine kinase receptor B (TrkB), a membrane-bound receptor for BDNF, at a dose used in previous studies (25 µg/kg; Liu et al., 2017) immediately after lentiviral vector injection (i.p. injection, once daily, 14 days; supplementary Figure 1B). For western blot, ELISA, co-immunoprecipitation, and immunofluorescence, the mice were sacrificed after stress exposure or behavioral tests (supplementary Figure 1). The animal studies were approved by the International Animal Care and Use Committee of Nantong University (permit no. 2110836) and conducted in accordance with the guidelines of the "Guide for the Care and Use of Laboratory Animals" (National Research Council, 1996). Every effort was made to minimize animal suffering and to reduce the number of animals used. Behavioral experiments were carried out during the light phase.

Intrahippocampal Injection of Lentiviral Vectors

The mice in different groups were anesthetized with 0.5% pentobarbital sodium and fixed in stereotaxic frames. The scalp was removed, and the skull was exposed using 75% alcohol and 1% hydrogen peroxide solution. The 5-mL microsyringes (Hamilton, Fisher Labs) were used to deliver the lentiviral vectors. After making a small drill hole in the skull above the dorsal hippocampus, the microsyringes were positioned at the following coordinates (Sekeres et al., 2012; Hübner et al., 2014; Stackman et al., 2016): anteroposterior (AP) = -2.0 mm, medialateral = +1.5 mm, ventral = -1.6 mm (CA1) (Stackman et al., 2016); AP = -2.3 mm, medialateral = +1.5 mm, ventral = -1.8 mm (dentate gyrus [DG]) (Sekeres et al., 2012); AP = +1.9 mm, medialateral = +0.3 mm, ventral = -2.1 mm (medial prefrontal cortex [mPFC]) (Hübner et al., 2014). In our initial observations, we found that a unilateral injection of lentiviral vectors (into the right CA1 of the dorsal hippocampus) that expressed the full length of FXR was enough to induce depression-like behaviors in stress-naïve mice. Thus, the injections for the lentiviral vectors that expressed either short hairpin RNA (shRNA) or the full length of FXR or CRTC2

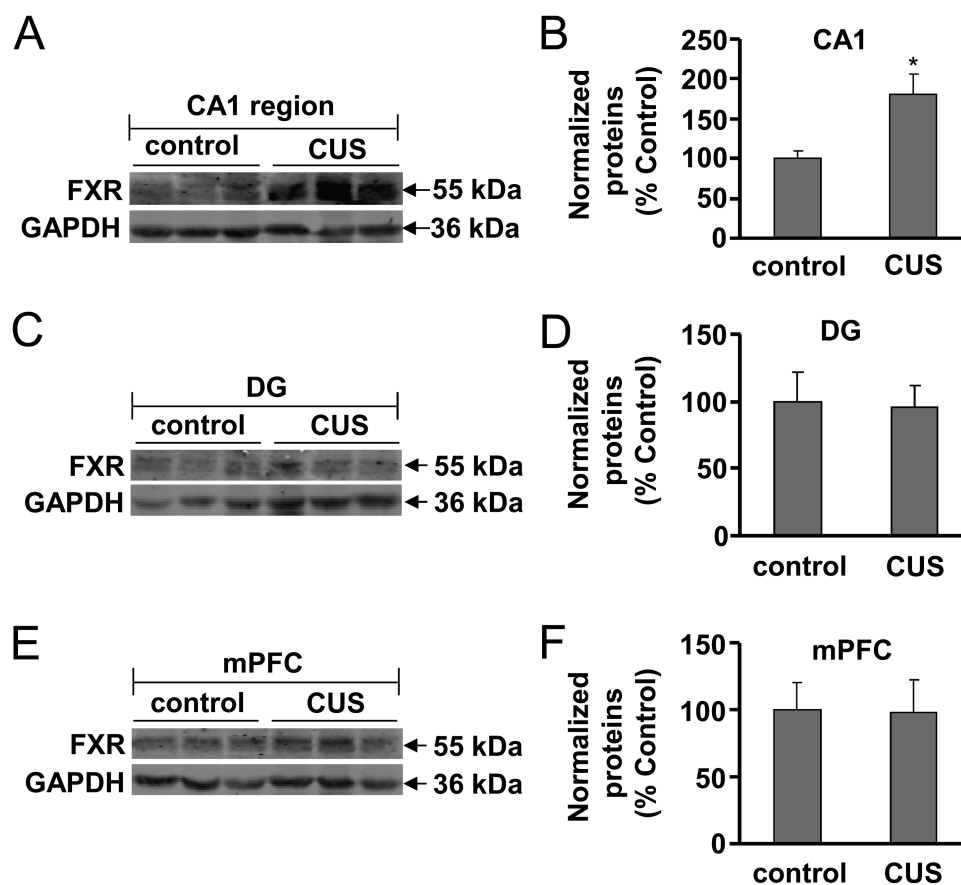


Figure 1. Effect of chronic unpredictable stress (CUS) on farnesoid X receptor (FXR) protein expression in the CA1, dentate gyrus (DG), and medial prefrontal cortex (mPFC) in mice. Representative images and quantitative analysis show the change in FXR protein expression in the CA1 (A–B, $n = 6$; $*P < .05$ vs control), DG (C–D, $n = 6$), and mPFC (E–F, $n = 6$) in stress-naïve and CUS-exposed mice. Data are shown as mean \pm SEM.

were performed unilaterally (right) in the hippocampus and the mPFC at a rate of 0.4 $\mu\text{L}/\text{min}$ (final volume, 2 $\mu\text{L}/\text{side}$) while maintaining the microsyringes in place for 4 minutes to limit reflux along the injection track. After the injection, the incisions were sutured carefully. The success rate for lentivirus injection in animals is 80%.

Additional experimental procedures are available online in the supplementary Materials.

Statistics

The statistical analyses were performed using SPSS 13.0 software (SPSS Inc.), and the data are shown as mean \pm SEM. Differences between the mean values were evaluated using the Student's t test, 1-way or 2-way ANOVA, and Bonferroni's post hoc test for isolated comparisons. $P < .05$ was considered statistically significant.

Results

Roles of FXR in Hippocampal CA1 in CUS-Induced Depression-Like Behaviors in Mice

Initially we observed induction of CUS increased the FXR protein expression levels in the CA1 (Figure 1A,B; $t_5 = 6.18$, $P < .01$) but not in the DG (Figure 1C,D; $t_5 = 0.48$, $P = .65$) or mPFC (Figure 1E,F; $t_5 = 0.20$, $P = .85$), suggesting that hippocampal FXR may be associated with CUS-induced depression-like behavior in mice. To

prove this hypothesis, a lentiviral vector containing FXR-eGFP fusion protein was constructed and injected into the hippocampus to induce FXR overexpression. We found that a 14-day of FXR overexpression in the CA1 (supplementary Figure 2A–C, $t_5 = 2.85$, $P < .05$) induced a more severe depression-like behavior in CUS-exposed mice as measured by the TST (supplementary Figure 2D) 2-way ANOVA: significance was achieved for CUS ($F_{1,34} = 7.56$, $P < .05$) and lentiviral vector treatment ($F_{1,34} = 9.90$, $P < .01$) but not for stress \times treatment interaction ($F_{1,34} = 0.02$, $P = .88$); FST (supplementary Figure 2E) 2-way ANOVA: significance was achieved for CUS ($F_{1,34} = 17.88$, $P < .001$) and lentiviral vector treatment ($F_{1,34} = 13.05$, $P < .01$) but not for stress \times treatment interaction ($F_{1,34} = 0.006$, $P = .94$); and SPT (supplementary Figure 2F) 2-way ANOVA: significance achieved for CUS ($F_{1,34} = 35.15$, $P < .001$) and lentiviral vector treatment ($F_{1,34} = 13.63$, $P < .01$) but not for stress \times treatment interaction ($F_{1,34} = 0.30$, $P = .59$). We observed that FXR overexpression in stress-naïve mice also induced depression-like behaviors as measured by the TST, FST, and SPT (supplementary Figure 2D–F). To ascertain that the role of FXR in the regulation of depression is CA1 specific, we injected lentiviral vector containing the FXR-eGFP fusion protein into the DG and mPFC and found that the FXR overexpression in these 2 regions did not alter the depression-like behaviors in CUS mice (supplementary Results; supplementary Figure 3A–L).

To further characterize the role of FXR in the regulation of depression, we utilized shRNA lentiviral particles to knockdown the FXR protein. We found that the downregulation of FXR by lentiviral injection of FXR shRNA in the CA1 (supplementary

Figure 4A–C; $t_5=11.46$, $P<.01$) ameliorated the CUS-induced depression-like behaviors as measured in the TST (supplementary Figure 4D) 2-way ANOVA: significance was achieved for shRNA treatment ($F_{1,36}=6.18$, $P<.05$) and stress×treatment interaction ($F_{1,36}=11.43$, $P<.01$) but not for CUS ($F_{1,36}=0.54$, $P=.47$); FST (supplementary Figure 4E) 2-way ANOVA: significance was achieved for shRNA treatment ($F_{1,36}=4.18$, $P<.05$) and stress×treatment interaction ($F_{1,36}=7.49$, $P<.01$) but not for CUS ($F_{1,36}=0.86$, $P=.36$); and SPT (supplementary Figure 4F) 2-way ANOVA: significance was achieved for CUS ($F_{1,36}=18.31$, $P<.001$), shRNA treatment ($F_{1,36}=6.84$, $P<.05$), and stress×treatment interaction ($F_{1,36}=8.81$, $P<.01$). Further, we did not observe any antidepressant effect of FXR shRNA injection (into CA1) in stress-naïve mice in the TST, FST, and SPT (supplementary Figure 4D–F).

CREB-BDNF Signaling Mediates the Pathogenic Effects of FXR Overexpression

We observed that the CUS exposure resulted in significant reduction in phosphorylated CREB levels within the CA1 region, an effect that was reversed by injection of FXR shRNA (Figure 2A–B) 2-way ANOVA: significance was achieved for CUS ($F_{1,16}=13.22$, $P<.01$), shRNA ($F_{1,16}=5.40$, $P<.05$), and stress×shRNA interaction ($F_{1,16}=8.18$, $P<.05$). This observation suggests that the reduction in CREB levels may result in the induction of depression-like behaviors in CUS-exposed and FXR-overexpressed mice and that the reversal of such behaviors with FXR shRNA may occur via an increase in CREB levels. To confirm this hypothesis, we injected CREB shRNA in the CA1 region of CUS-exposed/FXR shRNA-treated mice to downregulate CREB and evaluate the resulting changes in depression-like behaviors. A 14-day treatment of CREB shRNA (in the form of a lentiviral injection in CA1) (Figure 2C–E; $t_5=11.46$, $P<.01$) suppressed the antidepressant effect of FXR shRNA on CUS-induced depression-like behaviors in mice as measured by the TST (Figure 2F) 2-way ANOVA: significance was achieved for CUS ($F_{1,67}=16.27$, $P<.001$), shRNA treatment ($F_{3,67}=3.43$, $P<.05$), and stress×treatment interaction ($F_{3,67}=5.43$, $P<.01$); FST (Figure 2G) 2-way ANOVA: significance was achieved for CUS ($F_{1,67}=18.67$, $P<.001$), shRNA treatment ($F_{3,67}=4.82$, $P<.01$), and stress×treatment interaction ($F_{3,67}=4.21$, $P<.01$); and SPT (Figure 2H) 2-way ANOVA: significance was achieved for CUS ($F_{1,67}=28.10$, $P<.001$) and shRNA treatment ($F_{3,67}=4.08$, $P<.05$) but not for stress×treatment interaction ($F_{3,67}=1.91$, $P=.14$) in mice.

We also tested whether BDNF, a molecule downstream of CREB, mediates the effect of FXR on depression-like behaviors in mice. We found that FXR overexpression reduced the BDNF protein levels in the CA1 region in CUS mice (supplementary Figure 5A) 2-way ANOVA: significance was achieved for CUS ($F_{1,36}=10.61$, $P<.01$) and lentiviral vector treatment ($F_{1,36}=8.74$, $P<.01$) but not for stress×treatment interaction ($F_{1,36}=0.21$, $P=.65$) while the FXR shRNA inhibited this reduction (supplementary Figure 5B): 2-way ANOVA: significance was achieved for CUS ($F_{1,36}=12.39$, $P<.01$) and stress×treatment interaction ($F_{1,36}=11.25$, $P<.01$) but not for shRNA treatment ($F_{1,36}=3.72$, $P=.06$). Similar to CUS mice, nonstressed naïve mice also experienced a reduction in BDNF protein levels in the CA1 from FXR overexpression, but FXR shRNA did not show any inhibitory effect in this case (supplementary Figure 5A–B).

In the next sets of experiments, we used K252a to assess the role of BDNF signaling in depression-like behaviors mediated by FXR in mice. Intraperitoneal administration of 25 µg/kg K252a suppressed the antidepressant effect of FXR shRNA on CUS-induced depression-like behaviors as measured by the

TST (supplementary Figure 5C) 2-way ANOVA: significance was achieved for CUS ($F_{1,68}=20.57$, $P<.001$), drug/shRNA treatment ($F_{3,68}=4.46$, $P<.05$), and stress×treatment interaction ($F_{3,68}=5.79$, $P<.01$); FST (supplementary Figure 5D) 2-way ANOVA: significance was achieved for CUS ($F_{1,68}=11.58$, $P<.01$) and stress×treatment interaction ($F_{3,68}=4.51$, $P<.01$) but not for drug/shRNA treatment ($F_{3,68}=2.42$, $P=.07$); and SPT (supplementary Figure 5E) 2-way ANOVA: significance was achieved for CUS ($F_{1,68}=19.45$, $P<.001$) but not for drug/shRNA treatment ($F_{3,68}=2.64$, $P=.06$) and stress×treatment interaction ($F_{3,68}=2.26$, $P=.09$).

Further, we knocked down BDNF using shRNA, obtaining a marked reduction in BDNF protein levels in the CA1 after 14 days of injection (Figure 3A–B; $t_5=4.47$, $P<.01$). BDNF shRNA suppressed the antidepressant effect of FXR shRNA on CUS-induced depression-like behaviors in the TST (Figure 3C) 2-way ANOVA: significance was achieved for CUS ($F_{1,75}=18.15$, $P<.001$), shRNA treatment ($F_{3,75}=5.90$, $P<.01$), and stress×treatment interaction ($F_{3,75}=4.27$, $P<.01$); FST (Figure 3D) 2-way ANOVA: significance was achieved for CUS ($F_{1,75}=33.65$, $P<.001$), sham/shRNA treatment ($F_{3,75}=4.70$, $P<.01$), and stress×treatment interaction ($F_{3,75}=2.76$, $P<.05$); and SPT (Figure 3E) 2-way ANOVA: significance was achieved for CUS ($F_{1,75}=46.15$, $P<.001$), shRNA treatment ($F_{3,75}=4.07$, $P<.05$), and stress×treatment interaction ($F_{3,75}=5.04$, $P<.01$).

Disruption of the FXR-CREB Complex in Hippocampal CA1 Reduces CUS-Induced Depression-like Behaviors in Mice

To investigate whether FXR aggravates CUS-induced depression-like behaviors by interacting with CREB, we examined the FXR-CREB interaction in mice with or without CUS. We observed a significant increase in CREB-FXR interaction in the CA1 region of CUS-exposed mice (Figure 4A–C, $t_7=4.60$, $P<.05$). Using a lentiviral vector carrying the 245C terminus of FXR to disrupt CREB-FXR interaction in mice (Seok et al., 2014), we were able to reverse the CUS-induced depression-like behaviors as measured by the TST (Figure 4D) 2-way ANOVA: significance achieved for CUS ($F_{1,34}=8.58$, $P<.01$), lentiviral vector treatment ($F_{1,34}=30.37$, $P<.001$), and stress×treatment interaction ($F_{1,34}=35.93$, $P<.001$), FST (Figure 4E) 2-way ANOVA: significance achieved for CUS ($F_{1,34}=7.47$, $P<.01$), lentiviral vector treatment ($F_{1,34}=8.06$, $P<.01$), and stress×treatment interaction ($F_{1,34}=12.00$, $P<.01$), and SPT (Figure 4F) 2-way ANOVA: significance achieved for CUS ($F_{1,34}=14.16$, $P<.01$), lentiviral vector treatment ($F_{1,34}=6.92$, $P<.05$), and stress×treatment interaction ($F_{1,34}=9.56$, $P<.01$) in mice. Consistent with the effect of FXR shRNA, FXR-245C overexpression did not reduce the depression-like behaviors in stress-naïve mice (Figure 4D–F). After confirming from the above findings that FXR-245C exerts antidepressant effects via uncoupling of the FXR-CREB complex, we showed a reduction in the FXR-CREB interaction in CUS-exposed mice after FXR-245C lentiviral vector injection (Figure 4G and 4H) 2-way ANOVA: significance achieved for CUS ($F_{1,24}=8.91$, $P<.01$) but not for lentiviral vector treatment ($F_{2,24}=1.66$, $P=.21$) and stress×lentiviral vector interaction ($F_{2,24}=1.59$, $P=.23$). This suggests that the endogenous FXR is impaired in the presence of FXR-245C. Further, FXR-245C expression was also found to suppress the CUS-induced decrease in CREB phosphorylation (Figure 4I and 4J) 2-way ANOVA: significance achieved for CUS ($F_{1,16}=5.59$, $P<.05$) and lentiviral vector treatment ($F_{1,16}=6.02$, $P<.05$) but not for stress×lentiviral vector interaction ($F_{1,16}=3.09$, $P=.10$) and BDNF levels (Figure 4K) 2-way ANOVA: significance achieved for CUS ($F_{1,28}=11.27$, $P<.01$) and stress×lentiviral vector interaction ($F_{1,28}=4.87$, $P<.05$) but not for lentiviral vector treatment ($F_{1,28}=1.60$, $P=.22$) in the CA1 region.

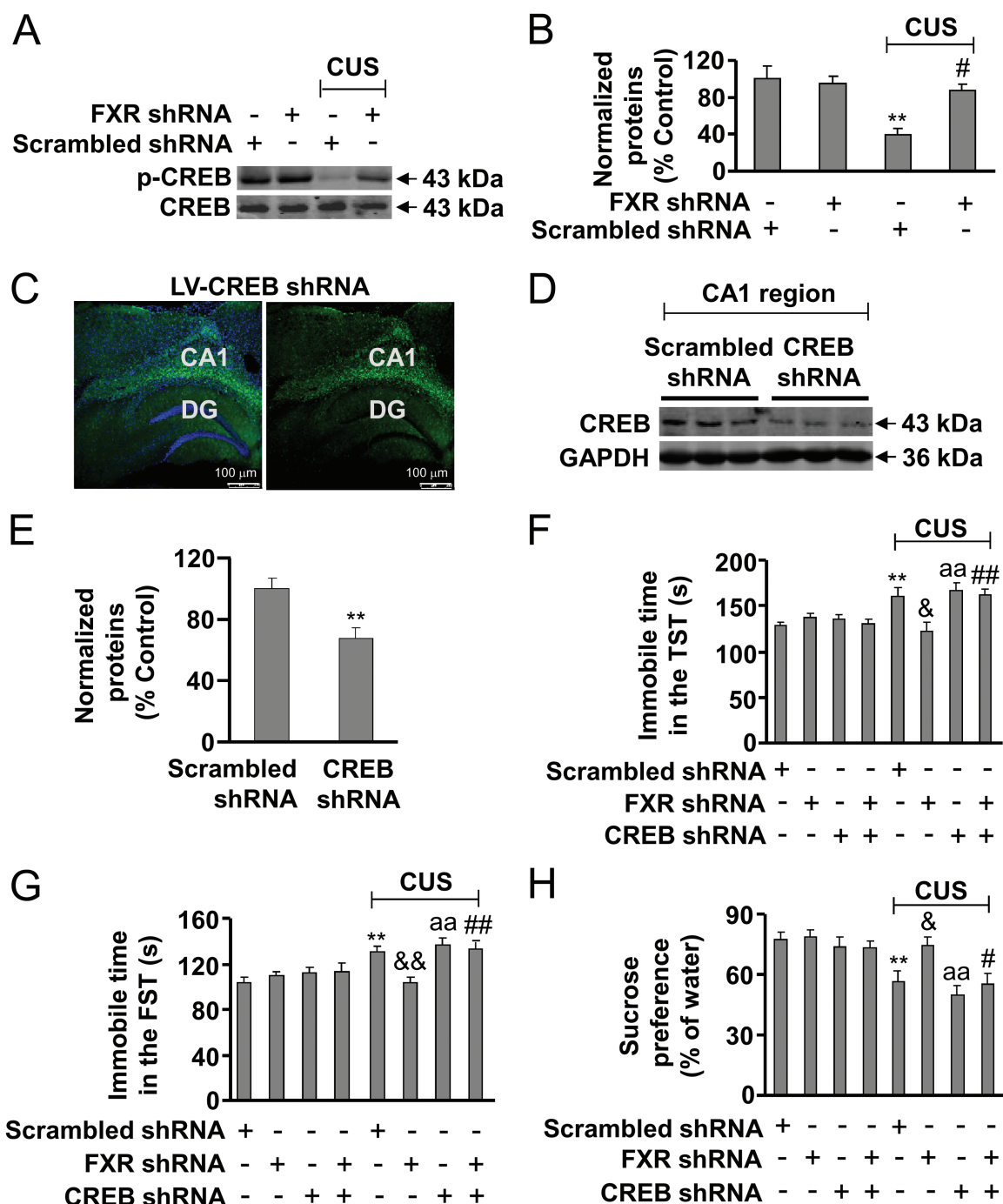


Figure 2. Cyclic adenosine monophosphate response element-binding protein (CREB) knockdown in the CA1 reduces the antidepressant effect of farnesoid X receptor (FXR) knockdown. Representative images (A) and quantitative analysis (B, $n = 6$; $**P < .01$ vs scrambled short hairpin RNA (shRNA), $\#P < .05$ vs scrambled shRNA + chronic unpredictable stress (CUS)) showing the inhibitory effect of FXR shRNA on CUS-induced decrease in CREB phosphorylation levels in the CA1. Representative images (C: immunofluorescence; D: western blot) and quantitative analysis (E, $n = 6$; $**P < .01$ vs scrambled shRNA) showing the knockdown efficiency of CREB shRNA on CREB protein in the CA1. Quantitative analysis showing the suppressive effect of CREB shRNA on FXR shRNA-induced decrease in immobility time in the tail suspension test (TST, F) and forced swimming test (FST, G) and on FXR shRNA-induced increase in sucrose preference in the sucrose preference test (SPT, H) in CUS-exposed mice ($n = 9-10$; $**P < .01$ vs scrambled shRNA, $\&P < .05$ or $\&\&P < .01$ vs scrambled shRNA + CUS, $aaP < .01$ vs CREB shRNA, $\#P < .05$ or $\#\#\#P < .01$ vs FXR shRNA + CUS). Data are shown as mean \pm SEM.

CRTC2 Mediates the Regulatory Effect of FXR on CUS-Induced Depression-Like Behaviors via Disrupting the FXR-CREB Complex

We found that the CRTC2 in normal conditions is distributed in the cytoplasm and nucleus of CA1 and DG neurons. After CUS stimulation, CRTC2 in the CA1 neurons but not in the DG neurons translocates to the cytoplasm (Figure 5A-B). Together

with the fact that the cytoplasmic translocation of CRTC2 mediates the inhibitory effect of FXR on CREB-mediated autophagy in cells in vitro (Seok et al., 2014), we hypothesized that the FXR in the CA1 impairs the CREB signaling by driving the translocation of neuronal CRTC2 translocation to cytoplasm. To this end, we examined the subcellular localization of the CRTC2 in CA1 neurons in mice with or without CUS and/or FXR shRNA treatment. Compared with the scrambled shRNA, FXR shRNA

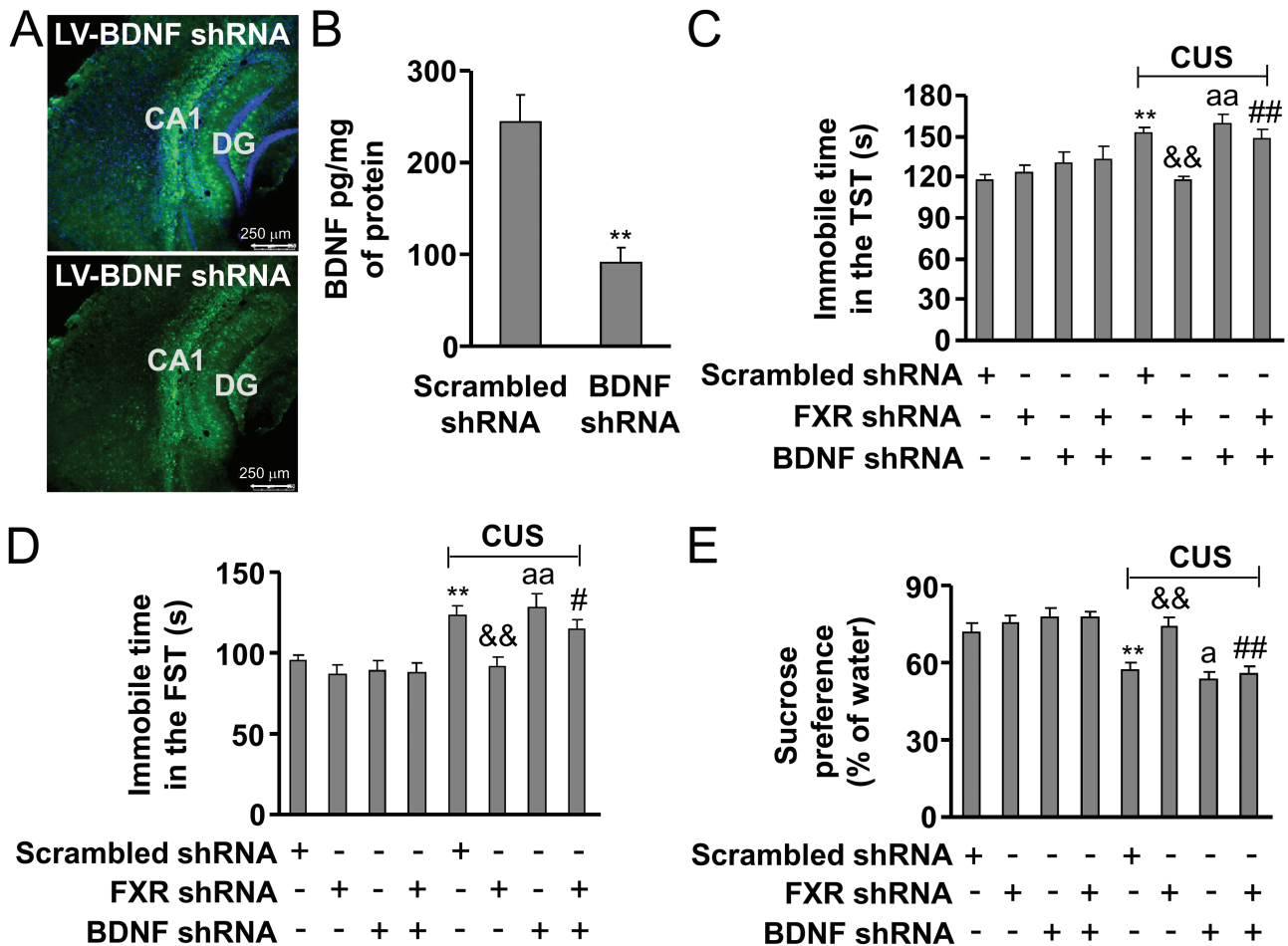


Figure 3. Brain-derived neurotrophic factor (BDNF) knockdown in the CA1 suppresses the antidepressant effect of farnesoid X receptor (FXR) knockdown. (A, B) Representative images (A) and quantitative analysis (B, $n = 6$; $**P < .01$ vs scrambled short hairpin RNA (shRNA)) showing the knockdown efficacy of BDNF shRNA on BDNF protein in the CA1. Quantitative analysis showing the suppressive effect of BDNF shRNA on FXR shRNA-induced decrease in immobility time in the tail suspension test (TST, C) and forced swimming test (FST, D) and on FXR shRNA-induced increase in sucrose preference in the sucrose preference test (SPT, E) in chronic unpredictable stress (CUS)-exposed mice ($n = 10-12$; $**P < .01$ vs scrambled shRNA, $\&\&P < .01$ vs scrambled shRNA + CUS, $aP < .05$ or $aaP < .01$ vs BDNF shRNA, $\#P < .05$ or $\#\#\#P < .01$ vs FXR shRNA + CUS). Data are shown as mean \pm SEM.

markedly suppressed the CUS-induced decrease in the nuclear CRT2 protein levels (Figure 5C–D) 2-way ANOVA: significance achieved for stress \times treatment interaction ($F_{1,16} = 4.93$, $P < .05$) but not for CUS ($F_{1,16} = 2.10$, $P = .17$) and scrambled shRNA treatment ($F_{1,16} = 2.56$, $P = .13$), and the CUS-induced increase in the cytoplasmic CRT2 protein levels (Figure 5E–F) 2-way ANOVA: significance achieved for scrambled shRNA treatment ($F_{1,16} = 6.86$, $P < .05$) and stress \times treatment interaction ($F_{1,16} = 6.00$, $P < .05$) but not for CUS ($F_{1,16} = 0.94$, $P = .35$) in the CA1.

The role of CRT2 in FXR-mediated depression-like behavior was ascertained by FXR/CRT2 co-shRNA. Delivering CRT2 shRNA in the CA1 through lentiviral particles significantly reduced the protein levels of CRT2 (Figure 6A–C; $t_5 = 2.82$, $P < .05$) and suppressed the antidepressant effect of FXR shRNA on CUS-induced depression-like behaviors as measured by the TST (Figure 6D) 2-way ANOVA: significance was achieved for CUS ($F_{1,72} = 11.24$, $P < .01$) and shRNA treatment ($F_{3,72} = 16.28$, $P < .001$) but not for stress \times treatment interaction ($F_{3,72} = 1.96$, $P = .13$); FST (Figure 6E) 2-way ANOVA: significance was achieved for CUS ($F_{1,72} = 17.66$, $P < .001$) and shRNA treatment ($F_{3,72} = 13.64$, $P < .001$) but not for stress \times treatment interaction ($F_{3,72} = 1.62$, $P = .19$); and SPT (Figure 6F) 2-way ANOVA: significance was achieved for CUS ($F_{1,72} = 7.49$, $P < .01$) and shRNA treatment ($F_{3,72} = 7.25$, $P < .001$) but not for stress \times treatment interaction ($F_{3,72} = 2.08$, $P = .11$). Further,

CRT2 shRNA suppressed FXR shRNA-induced increase in CREB phosphorylation (Figure 6G–H) 2-way ANOVA: significance was achieved for CUS ($F_{1,16} = 6.29$, $P < .05$) and shRNA treatment ($F_{1,16} = 5.31$, $P < .05$) but not for stress \times treatment interaction ($F_{1,16} = 3.42$, $P = .08$); and BDNF levels (Figure 6I) 2-way ANOVA: significance was achieved for CUS ($F_{1,28} = 12.31$, $P < .01$) and shRNA treatment ($F_{1,28} = 11.10$, $P < .01$) but not for stress \times treatment interaction ($F_{1,28} = 0.69$, $P = .41$) in the CA1 in CUS-exposed mice.

To further investigate the role of CRT2 in FXR-mediated depression-like behavior, we used a lentiviral vector expressing the CRT2-eGFP fusion protein. We observed that CRT2 overexpression (Figure 7A–C; $t_5 = 2.78$, $P < .05$) reduced the aggravation effect of FXR overexpression on depression-like behaviors in stress-naïve and CUS-exposed mice as measured by the TST (Figure 7D) 2-way ANOVA: significance was achieved for CUS ($F_{1,60} = 14.25$, $P < .001$) and lentiviral vector treatment ($F_{2,60} = 10.47$, $P < .001$) but not stress \times treatment interaction ($F_{2,60} = 1.07$, $P = .35$); FST (Figure 7E) 2-way ANOVA: significance was achieved for CUS ($F_{1,60} = 17.16$, $P < .001$) and lentiviral vector treatment ($F_{2,60} = 23.94$, $P < .001$) but not for stress \times treatment interaction ($F_{2,60} = 1.19$, $P = .31$); and SPT (Figure 7F) 2-way ANOVA: significance was achieved for CUS ($F_{1,60} = 7.94$, $P < .01$) and lentiviral vector treatment ($F_{2,60} = 4.19$, $P < .05$) but not for stress \times treatment interaction ($F_{2,60} = 1.60$, $P = .21$) in stress-naïve and CUS-exposed mice.

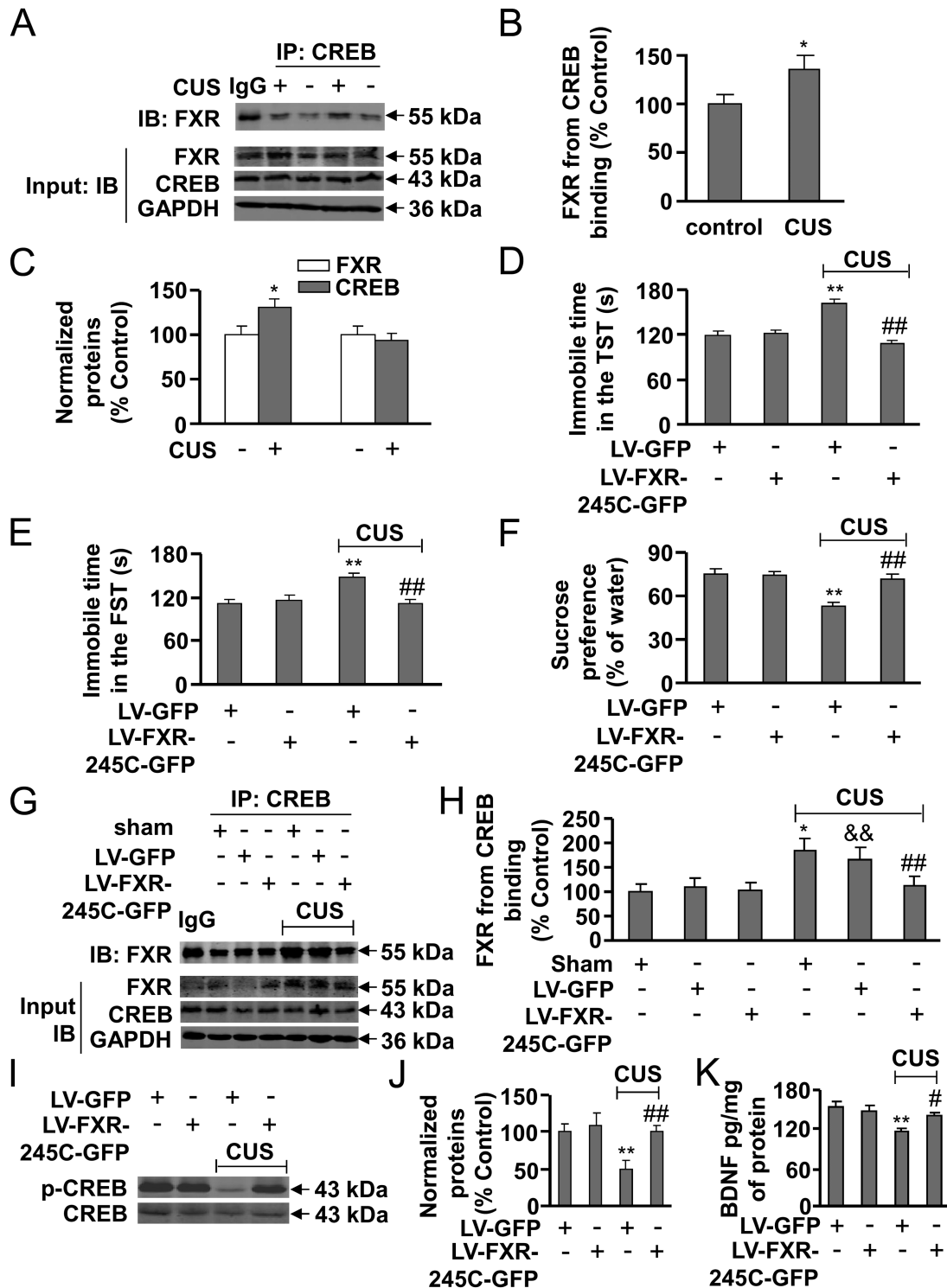


Figure 4. Disruption of the farnesoid X receptor (FXR)-cyclic adenosine monophosphate response element-binding protein (CREB) complex in the CA1 abrogates chronic unpredictable stress (CUS)-induced depression-like behaviors in mice. (A–C) Representative images (A) and quantitative analysis showing the effect of CUS on FXR-CREB interaction (B, $n = 8$, * $P < .05$ vs control) and on FXR (C, $n = 8$, * $P < .05$ vs control) and CREB (C, $n = 8$) expression levels in the CA1. (D–F) Quantitative analysis showing the suppressive effect of lentivirus-FXR-25C-green fluorescent protein (LV-FXR-25C-GFP) on CUS-induced increase in immobility time in the tail suspension test (TST, D) and forced swimming test (FST, E) and on CUS-induced decrease in sucrose preference in the sucrose preference test (SPT, F) in CUS-exposed mice ($n = 9–10$; ** $P < .01$ vs LV-GFP, ## $P < .01$ vs LV-GFP + CUS). Representative images (G) and quantitative analysis (H, $n = 5$; * $P < .05$ vs sham, && $P < .01$ vs LV-GFP, ## $P < .01$ vs LV-GFP + CUS) showing the inhibitory effect of LV-FXR-25C-GFP on CUS-induced increase in FXR-CREB interaction in the CA1. Representative images (I) and/or quantitative analysis (J–K) showing the inhibitory effect of LV-FXR-25C-GFP on CUS-induced reduction in CREB phosphorylation (J) and brain-derived neurotrophic factor (BDNF, K) levels in the CA1 in CUS-exposed mice (phospho-CREB: $n = 5$; BDNF: $n = 8$; ** $P < .01$ vs LV-GFP, # $P < .05$ or ## $P < .01$ vs LV-GFP + CUS). Data are shown as mean \pm SEM.

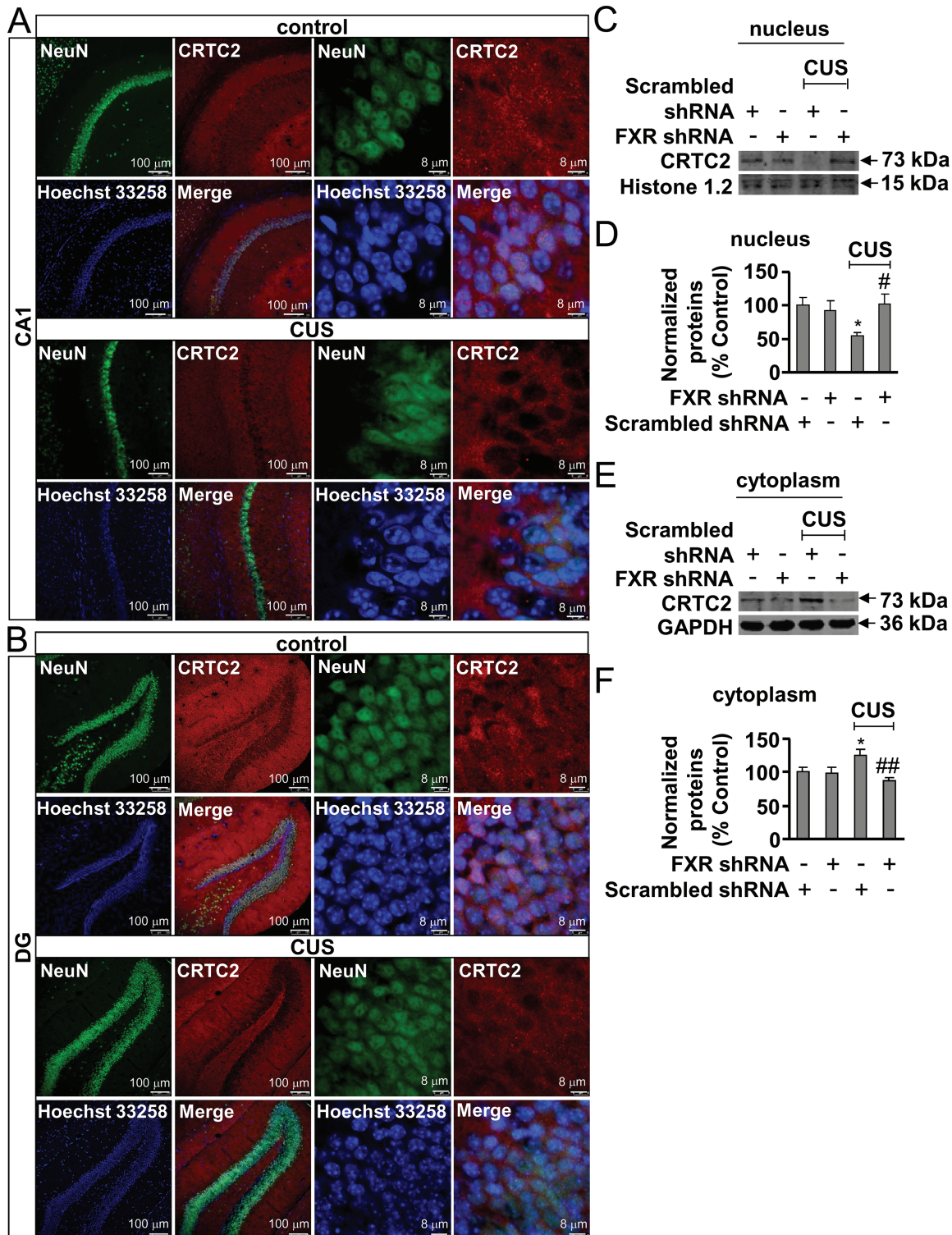


Figure 5. Effect of farnesoid X receptor (FXR) short hairpin RNA (shRNA) on cyclic adenosine monophosphate response element-binding protein-regulated transcription coactivator 2 (CRTC2) subcellular distribution in CA1 and dentate gyrus (DG) neurons in normal and chronic unpredictable stress (CUS)-exposed mice. Representative images showing the co-localization of neuronal nuclear antigen (NeuN, green), CRTC2 (red), and nucleus (blue: Hoechst 33258) in CA1 (A) and DG (B) neurons in stress-naïve and CUS-exposed mice with low (left, scale bars = 100 μ m) and high (right, scale bars = 8 μ m) power magnification view. Representative images (C, E) and quantitative analysis (D, F) showing the effect of FXR shRNA on CRTC2 nuclear (C–D) and cytoplasmic (E–F) distribution in CA1 neurons in stress-naïve and CUS-exposed mice (n = 5; *P < .05 vs scrambled shRNA, #P < .05 or ##P < 0.01 vs scrambled shRNA + CUS). Data are shown as mean \pm SEM.

Similarly, CRTC2 overexpression reduced the aggravation effect of FXR overexpression on CUS-induced decrease in CREB phosphorylation (Figure 7G–H; 1-way ANOVA: $F_{2,21} = 12.41$, $P < .01$) and BDNF levels (Figure 7I; 1-way ANOVA: $F_{2,21} = 13.82$, $P < .001$) in the CA1.

Discussion

In extension of our previous work demonstrating the expression of FXR in brain neurons (Huang et al., 2016), we identified a

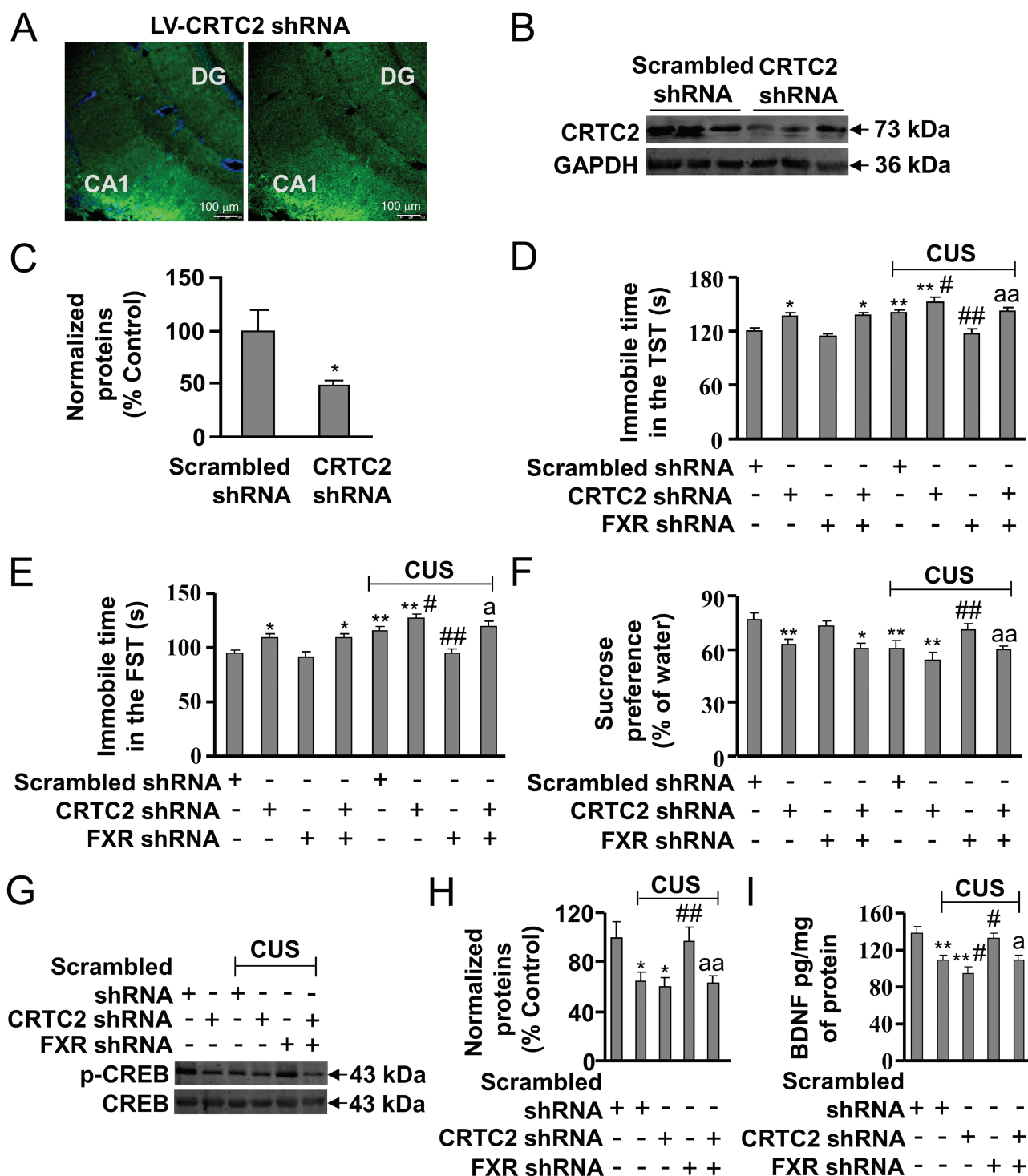


Figure 6. Role of cyclic adenosine monophosphate response element-binding protein-regulated transcription coactivator 2 (CRTC2) short hairpin RNA (shRNA) in the regulation of depression-like behavior by farnesoid X receptor (FXR) shRNA in the CA1. Representative images (A–B) and quantitative analysis (C, $n = 6$; $*P < .05$ vs scrambled shRNA) showing the knockdown efficiency of CRTC2 shRNA on CRTC2 protein in the CA1. Quantitative analysis showing the suppressive effect of CRTC2 shRNA on FXR shRNA-induced decrease in immobility time in the tail suspension test (TST, D) and forced swimming test (FST, E) as well as on FXR shRNA-induced increase in sucrose preference in the sucrose preference test (SPT, F) in chronic unpredictable stress (CUS)-exposed mice ($n = 10$; $*P < .05$ or $**P < .01$ vs scrambled shRNA, $\#P < .05$ or $\#\#P < .01$ vs scrambled shRNA + CUS, $aP < .05$ or $aaP < .01$ vs FXR shRNA + CUS). Representative images (G) and/or quantitative analysis (H–I) showing the effect of FXR shRNA and/or CRTC2 shRNA on CUS-induced reduction in cyclic adenosine monophosphate response element-binding protein (CREB) phosphorylation (H) and brain-derived neurotrophic factor (BDNF, I) levels in the CA1 in CUS-exposed mice (phospho-CREB: $n = 5$; BDNF: $n = 8$; $*P < .05$ or $**P < .01$ vs scrambled shRNA, $\#P < .05$ or $\#\#P < .01$ vs scrambled shRNA + CUS, $aP < .05$ or $aaP < .01$ vs FXR shRNA + CUS). Data are shown as mean \pm SEM.

role for FXR in CUS-induced depression-like behaviors in mice. We showed that the overexpression of FXR in CA1 aggravates CUS-induced depression-like behaviors, while the FXR shRNA

rescues from such effects. FXR overexpression in CA1 also induces depression-like behaviors in stress-naïve mice, suggesting that increased FXR levels, even in nonstressed mice, can

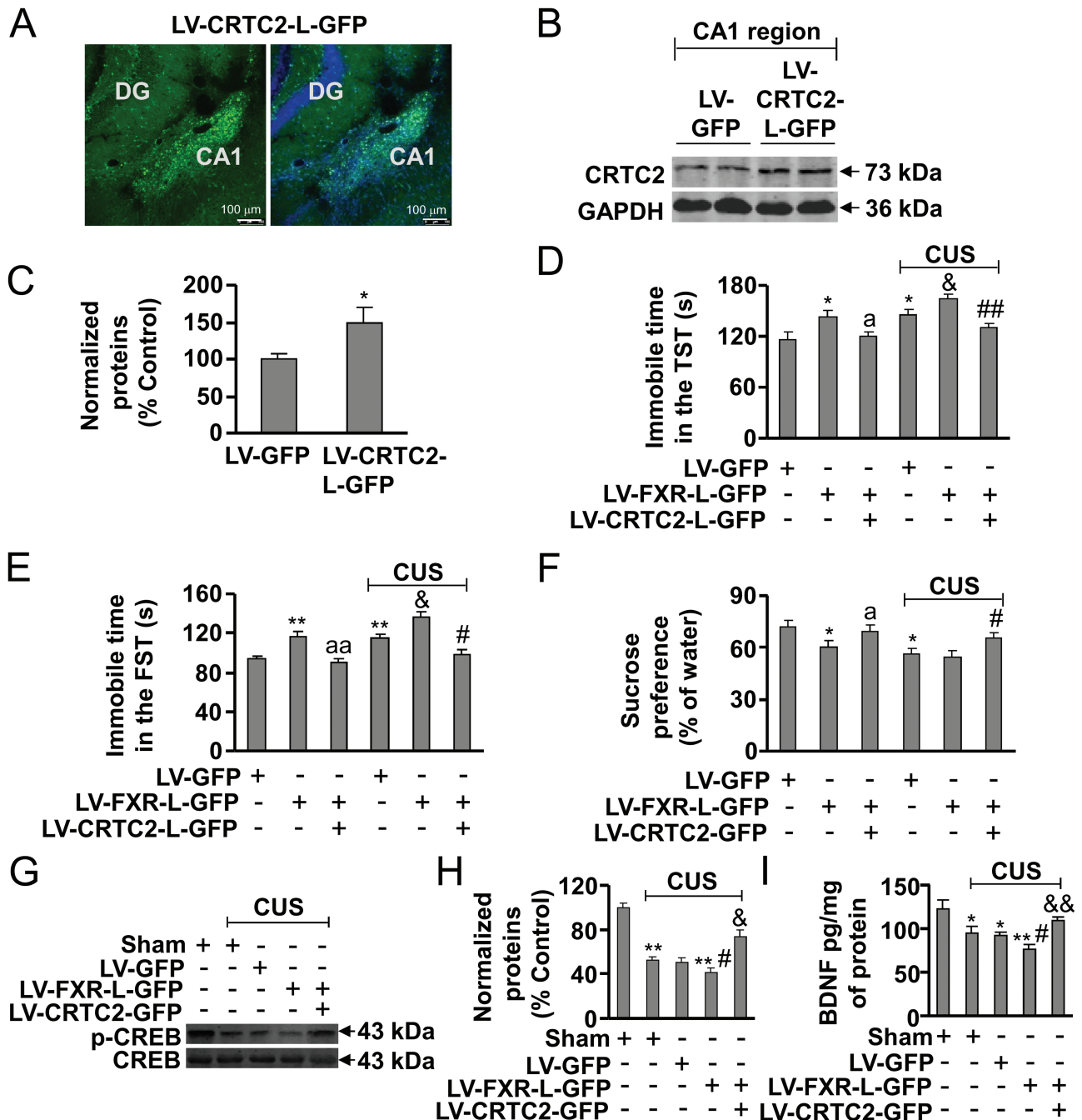


Figure 7. Role of cyclic adenosine monophosphate response element-binding protein-regulated transcription coactivator 2 (CRT2) overexpression in regulation of depression by overexpression of farnesoid X receptor (FXR) in the CA1. Representative images (A–B) and quantitative analysis (C, $n = 6$; $*P < .05$ vs lentivirus-green fluorescent protein (LV-GFP)) showing the lentiviral expression of CRT2 in the CA1. Quantitative analysis showing the suppressive effect of CRT2 overexpression on FXR overexpression-induced increase in immobility time in the tail suspension test (TST, D) and forced swimming test (FST, E) and on FXR overexpression-induced decrease in sucrose preference in the sucrose preference test (SPT, F) in stress-naïve and chronic unpredictable stress (CUS)-exposed mice ($n = 11–12$; $*P < .05$ or $**P < .01$ vs LV-GFP, $aP < .05$ or $aaP < .01$ vs LV-FXR-L-GFP, $\&P < .05$ vs LV-GFP + CUS, $\#P < .05$ or $\#\#\#P < .01$ vs LV-FXR-L-GFP + CUS). Representative images (G) and/or quantitative analysis (H–I) showing the effect of LV-FXR-L-GFP and/or LV-CRTC2-L-GFP on CUS-induced reduction in cyclic adenosine monophosphate response element-binding protein (CREB) phosphorylation (H) and brain-derived neurotrophic factor (BDNF, I) levels in the CA1 in CUS-exposed mice (phospho-CREB: $n = 5$; BDNF: $n = 8$; $*P < .05$ or $**P < .01$ vs sham, $\#P < .05$ vs LV-GFP + CUS, $\&P < .05$ or $\&\&P < .01$ vs LV-FXR-L-GFP + CUS). Data are shown as mean \pm SEM.

lead to depression. However, FXR overexpression in the DG and mPFC (areas known to be associated with depression) did not induce depression-like behaviors in mice. This suggests that the regulatory effect of FXR on depression-like behaviors is regionally specific. Additional support for this conclusion came from the observation that after CUS exposure, the expression

of FXR protein increased in CA1 but not in the DG or mPFC. These findings provide a novel mechanism for CUS-induced depression-like behaviors in mice and may facilitate the development of FXR-based antidepressants. It is worth pointing out that the experiments mentioned above were performed specifically in the right hemispheres of the brain. A major reason for

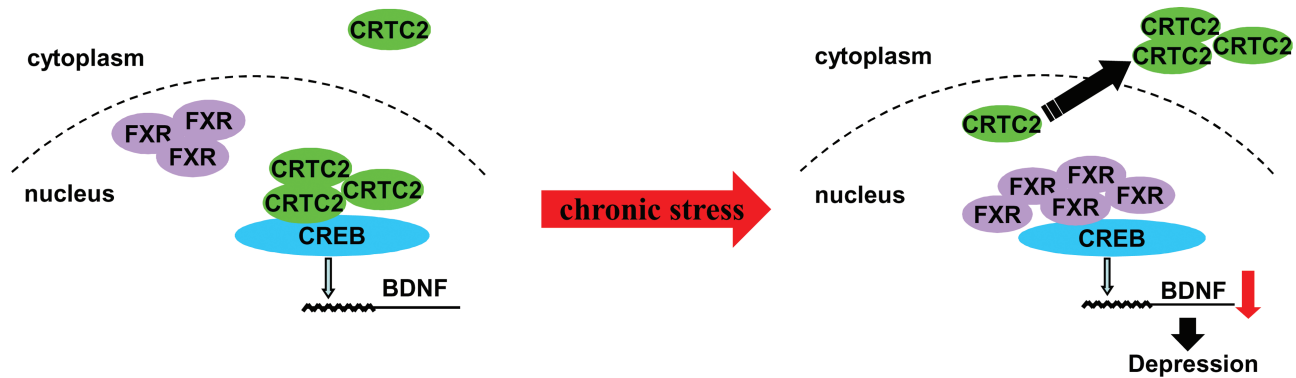


Figure 8. A proposed scheme showing the regulation of the cyclic adenosine monophosphate response element-binding protein (CREB)-brain-derived neurotrophic factor (BDNF) signaling by neuronal farnesoid X receptor (FXR): on chronic unpredictable stress (CUS) stimulation, the increased FXR levels in CA1 drives the cytoplasmic translocation of cyclic adenosine monophosphate response element-binding protein-regulated transcription coactivator 2 (CRTC2) by targeting CREB, thereby impairing the CREB-BDNF signaling pathway and inducing depression-like behaviors in mice.

this selection is that in our initial observations we found that a unilateral injection of lentiviral vectors (into the right CA1 of the dorsal hippocampus) that expressed the full length of FXR was enough to induce depression-like behaviors in stress-naïve mice. However, this does mean that the FXR in the left CA1 or the other regions of the brain is not involved in the pathogenesis of depression. In fact, a number of studies have revealed the asymmetric contribution of the left and right hemispheres of the brain, especially the hippocampus, to diverse brain function manipulations in animals (Mehta et al., 1997; Li et al., 1999; Zhou et al., 2016). Thus, more studies are needed to elucidate the roles of FXR in different regions of the brain in depression.

The impairment of the CREB-BDNF signaling pathway is implicated in the progression of depression (Sulser, 2002; Saarelainen et al., 2003; Yamada et al., 2003): the activity and expression of CREB and BDNF in brain tissues are markedly reduced, and their restoration may be responsible for the therapeutic effect of several clinical and preclinical antidepressants (Liu et al., 2017; Lian et al., 2018; Lin et al., 2018). We demonstrated the role of CREB-BDNF signaling in depression-like behavior mediated by FXR through the following observations: (1) FXR shRNA reversed the CUS-induced reduction in CREB phosphorylation in the CA1 region; (2) FXR overexpression worsened the BDNF reduction in the CA1 region in CUS-exposed mice while FXR shRNA reversed the BDNF reduction; (3) CREB knockdown in the CA1 suppressed the antidepressant effect of FXR shRNA on CUS-induced depression-like behaviors; and (4) both TrkB inhibition and BDNF knockdown suppressed the antidepressant effect of FXR shRNA on CUS-induced depression-like behaviors. Since our results are consistent with a previous study demonstrating the inhibitory effect of FXR on CREB activity in vitro (Seok et al., 2014), it gives more strength to our hypothesis that FXR in the CA1 region either causes or aggravates depression-like behaviors in mice, most likely through impairing the CREB-BDNF signaling pathway. The regional specificity observed in our results is also consistent with the role of CREB in hippocampal neuroplasticity. Our results highlight some of the molecular mechanisms underlying CUS-induced depression-like behaviors in mice and connect these mechanisms to region-specific variations. Although the role of the DG and mPFC in depression is well documented (Culig et al., 2017; Liu et al., 2017; Zhou et al., 2017; Peng et al., 2018; Zhao et al., 2018), only a few studies have focused on the hippocampal CA1. For example, impairment of hippocampal neurogenesis in the

DG has been extensively reported to mediate the development of depression-like behaviors in rodents, and the therapeutic effect of antidepressants is often achieved by an increase in hippocampal neurogenesis (Culig et al., 2017; Liu et al., 2017; Peng et al., 2018). In the PFC, increases in expression levels of γ -aminobutyric acid- and glutamate-related genes are associated with depression and suicidality (Zhao et al., 2018). Further, the dysfunction of CREB-BDNF signaling in the PFC is reported to mediate the stress-induced depression-like behaviors in mice (Zhou et al., 2017). Our results show that induction or aggravation of depression-like behaviors by FXR occurs only if FXR is injected into the CA1 region, but not if it is injected into the DG or mPFC. Inhibition of the CREB-BDNF signaling specifically in the CA1 suppressed the antidepressant effect of FXR shRNA in CUS-exposed mice. These findings highlight the importance of the CA1 region in CUS-induced depression-like behaviors in mice, though its exact role remains to be elucidated. Our findings about the regulation of FXR-mediated changes in depression-like behavior by BDNF/TrkB may help to explain the role played by CA1 in the regulation of depression through FXR due to reported findings: chronic stress impairs long-term potentiation in rodent CA1 regions (Aleisa et al., 2006a, 2006b; Alzoubi et al., 2013); high corticosterone concentrations in depressed individuals can convert long-term potentiation to long-term depression in CA1 (Sharvit et al., 2015); and decreased BDNF levels in CUS-exposed animals can decrease synaptic transmission between the hippocampal CA3-CA1 synapses (Qiao et al., 2014). In future studies, we plan to investigate whether the FXR-related depression-like behavior and change in BDNF levels in CA1 coincide with electrophysiological and morphological changes within that region.

Highlighting the mechanism underlying the FXR-mediated induction of depression-like behaviors, we showed that CUS specifically increased the FXR-CREB interaction in the CA1. This indicates that CUS impairs CREB-BDNF signaling, likely through enhancing FXR-CREB interaction, and the disruption of this interaction may mitigate depression-like behaviors. To this end, we observed that the overexpression of the ligand-binding domain in FXR (LV-GFP-C-FXR), necessary for FXR-CREB interaction, ameliorated CUS-induced depression-like behaviors in mice. Unlike the observation that the overexpression of FXR induces depression, overexpression of both FXR shRNA and LV-GFP-C-FXR (used to attenuate FXR function) did not induce antidepressant effects in nonstressed mice. The observation could be due

to a weak association between FXR and CREB at the basal level; in normal animals, the basal activity of CREB, at least in the CA1, may be primarily controlled by other factors, and upon stressful stimulation, the specific increase in FXR in CA1 neurons inhibits CREB-BDNF signaling via physical targeting of CREB, inducing depression-like behavior.

CRTC2 is a known coactivator of CREB (Eberhard et al., 2013; Li et al., 2017). Although it is expressed in neurons (Kovács et al., 2007; Lerner et al., 2009), its roles in neural physiology and disorders remain largely unknown. Seok et al. (Seok et al., 2014) reported that the FXR-mediated impairment of CREB signaling in autophagy *in vitro* is achieved through the interruption of coactivation of nuclear CREB by CRTC2. Meanwhile, CRTC2 gets translocated to the cytoplasm (Seok et al., 2014). Our results showed that (1) at basal levels neuronal CRTC2 in the CA1 region is evenly distributed in both the cytoplasm and the nucleus; (2) CUS induces the cytoplasmic translocation of CRTC2 in CA1 neurons, with the majority of CRTC2 found to be located in the cytoplasm after CUS (reversed by FXR shRNA); (3) CRTC2 overexpression reverses the effect of FXR in aggravating depression-like behaviors in CUS-exposed mice and in decreasing CREB phosphorylation and BDNF levels in the CA1 region; and (4) CRTC2 shRNA prevents the rescue effect of FXR shRNA on depression-like behaviors and the decreased CREB phosphorylation and BDNF levels in the CA1 region in CUS-exposed mice. We also found that CUS did not induce cytoplasmic translocation of CRTC2 in the DG, indicating that FXR only impacts the cellular distribution of CRTC2 in CA1 neurons. Importantly, consistent with our results related to FXR overexpression, CRTC2 shRNA alone was sufficient to induce or aggravate depression-like behaviors in stress-naïve and CUS-exposed mice, respectively. Taken together, these results demonstrate that the CRTC2 in CA1 neurons may be a critical molecule for the regulation of depression and that the increased FXR in the CA1 region mediates CUS-induced depression-like behaviors by driving the cytoplasmic translocation of CRTC2, which reduces CREB transcriptional activity and subsequent BDNF protein expression.

Chen et al. (Chen et al. 2018) reported that the FXR in the hippocampus mediates CUS-induced depression-like behaviors in rats, an observation consistent with some of our findings. However, unlike Chen et al., we observed a region-specific effect of FXR, highlighting that FXR in CA1 but not in the DG or mPFC mediates CUS-induced depression-like behaviors in mice. Similar to Chen et al., we found that overexpression of FXR results in CUS-induced depression-like behaviors by inhibiting BDNF signaling in the hippocampus, but we observed that this inhibition mainly occurs in the CA1 and not in DG. We also showed that FXR-triggered CREB inhibition is required for CUS-induced impairment in BDNF signaling and CUS-induced depression-like behaviors in mice. Our results also reveal a key role of CRTC2 in FXR's regulation on CREB activity and depression-like behavior: CRTC2 overexpression or shRNA blocks the regulatory effect of FXR overexpression or shRNA on CREB-BDNF signaling and depression-like behaviors in CUS-exposed mice. Impairment of CREB-BDNF signaling in the brain constitutes a critical aspect of the pathology of depression. Until now, the exact mechanism underlying the impairment of CREB-BDNF signaling remained unclear, but our results provide some valuable insights into this mechanism.

CRTC1 is a structural analog of CRTC2 (Breuillaud et al., 2012). Similar to the decrease in CRTC2 levels, the decrease in CRTC1 is found to induce depression-like behaviors in mice via a decrease in the expression of CREB-target genes such as BDNF (Breuillaud

et al., 2012). CRTC1-deficient mice are being used as a model to investigate not only the pathophysiological mechanisms of depression but also the mechanism of action for novel antidepressants (Meylan et al., 2016a, 2016b). Therefore, the alterations in CRTC1-CREB signaling may be an important aspect of depression pathogenesis. This hypothesis is, to some extent, supported by a recent finding that a reduced CRTC1 nuclear translocation and binding of CRTC1 to CREB in the hippocampus is shown to mediate chronic stress-induced depression-like behaviors in mice (Jiang et al., 2019). Although CRTC1 gene deficiency can induce depression-like behaviors in mice, the effect of CRTC1 gene deficiency on the CREB-BDNF signaling mainly occurs in the prefrontal cortex and not in the hippocampus (Breuillaud et al., 2012), suggesting that the differential expression of CRTC1 may be responsible for regional variation in the progression of depression. The reason for this regional variation in the function of CRTC1 in CRTC1-deficient mice and chronically stressed mice remains to be determined. In normal conditions, CRTC1 in neurons is distributed mainly in the cytoplasm, and its nuclear translocation is shown to be critical for normal CREB signaling and behavioral homeostasis (Ch'ng et al., 2012; Uchida et al., 2017). The hypothesis suggests that if the CRTC1 is involved in FXR's regulation on depression, it must redistribute into the nucleus on the silencing of the FXR gene. Our unpublished results show that the neuronal CRTC1 in the CA1 in FXR shRNA-treated mice remains in the cytoplasm, suggesting that FXR's regulation in the hippocampus on depressive behavior may be independent of CRTC1. CRTC2 and CRTC1 in the brain may exhibit divergent physiological functions via different transcriptional pathways. For example, CRTC2 and CRTC1 are shown to have overlapping but distinct patterns of expression (Lerner et al., 2009), and coactivation of CREB through CRTC2 in some conditions is unable to compensate for the loss of CRTC1 in CRTC1-deficient mice (Altarejos et al., 2008). Future studies should work toward discerning the role of CRTC1 and CRTC2 in behavior and depression.

Taken together, our results show that the FXR-CREB interaction and CRTC2 cytoplasmic translocation in the CA1 are required for CUS-induced depression-like behaviors in mice (Figure 8). These findings not only reveal a novel mechanism for the impairment of the CREB-BDNF signaling under stress conditions but also demonstrate that inhibition of FXR function or FXR-CREB interaction may be exploited for the development of novel antidepressant strategies.

Supplementary Materials

Supplementary data are available at *International Journal of Neuropsychopharmacology (IJNPPY)* online.

Acknowledgments

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Statement of Interest

None.

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