Salt-inducible kinase 1-CREB-regulated transcription coactivator 1 signalling in the paraventricular nucleus of the hypothalamus plays a role in depression by regulating the hypothalamic–pituitary–adrenal axis

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Article

Keywords: Chronic stress, Corticotropin-releasing hormone, CREB-regulated transcription co-activator 1, Depression, Paraventricular nucleus, Salt-inducible kinase 1

Posted Date: August 19th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1932234/v1

Version of Record: A version of this preprint was published at Molecular Psychiatry on November 25th, 2022. See the published version at https://doi.org/10.1038/s41380-022-01881-4.

Abstract

Elucidating the molecular mechanism underlying the hyperactivity of the hypothalamic–pituitary–adrenal axis during chronic stress is critical for understanding depression and treating depression. The secretion of corticotropin-releasing hormone (CRH) from neurons in the paraventricular nucleus (PVN) of the hypothalamus is controlled by salt-inducible kinases (SIKs) and CREB-regulated transcription co-activators (CRTCs). We hypothesized that the SIK-CRTC system in the PVN might contribute to the pathogenesis of depression. Thus, the present study employed chronic social defeat stress (CSDS) and chronic unpredictable mild stress (CUMS) models of depression, various behavioral tests, virus-mediated gene transfer, enzyme linked immunosorbent assay, western blotting, co-immunoprecipitation, quantitative real-time reverse transcription polymerase chain reaction, and immunofluorescence to investigate this connection. Our results revealed that both CSDS and CUMS induced significant changes in SIK1-CRTC1 signaling in PVN neurons. Both genetic knockdown of SIK1 and genetic overexpression of CRTC1 in the PVN simulated chronic stress, producing a depression-like phenotype in naïve mice, and the CRTC1-CREB-CRH pathway mediates the pro-depressant actions induced by SIK1 knockdown in the PVN. In contrast, both genetic overexpression of SIK1 and genetic knockdown of CRTC1 in the PVN protected against CSDS and CUMS, leading to antidepressant-like effects in mice. Moreover, stereotactic infusion of TAT-SIK1 into the PVN also produced beneficial effects against chronic stress. Furthermore, the SIK1-CRTC1 system in the PVN played a role in the antidepressant actions of fluoxetine, paroxetine, venlafaxine, and duloxetine. Collectively, SIK1 and CRTC1 in PVN neurons are closely involved in depression neurobiology, and they could be viable targets for novel antidepressants.

Introduction

As one of the leading public health problems in the 21st century, major depressive disorder (MDD) afflicts approximately 17% of the population and imposes a heavy economic burden on both families and societies (Kessler et al., 2003). Correspondingly, antidepressants (selective serotonin reuptake inhibitors (SSRIs), serotonin and norepinephrine reuptake inhibitors (SNRIs), etc.) are now among the most commonly prescribed medications but have notable limitations. For example, weeks or even months of administration are required for SSRIs and SNRIs to produce a therapeutic response, and fewer than half of MDD patients respond to their first medication prescribed (Trivedi et al., 2006). Therefore, it is now necessary and popular to explore novel pharmacological targets beyond the monoaminergic system. Much evidence indicates that MDD is mainly precipitated by stressful life events, interacting with genetic and other predisposing factors (Caspi et al., 2003; Fava and Kendler, 2000). The hypothalamic–pituitary–adrenal (HPA) axis has been well documented to be the major component of the neuroendocrine network responding to both acute and chronic stress (Leistner and Menke, 2020). For this axis, corticotropin-releasing hormone (CRH) secreted from neurons in the paraventricular nucleus (PVN) of the hypothalamus stimulates the synthesis and release of adrenocorticotropic hormone (ACTH) from the anterior pituitary, and ACTH further stimulates the synthesis and release of glucocorticoids (cortisol in humans, corticosterone in rodents) from the adrenal cortex. HPA is necessary for the maintenance of

mental and physical health, while its dysfunction leads to several mental and physical disorders, including depression (Leistner and Menke, 2020). The neuroendocrine hypothesis of depression proposes that chronic stress induces hypercortisolaemia (sustained elevation of glucocorticoid levels), which produces damage in neurons in several important brain regions (hippocampus, medial prefrontal cortex (mPFC), etc.) and then contributes to various behavioural and biological symptoms in depression. Nonetheless, as to how chronic stress leads to a hyperactive HPA axis, the precise underlying mechanism remains incompletely understood.

CRH is the main activator of the HPA axis during stress. The majority of CRH neurons are located in the PVN, and their activity shows different patterns under resting and stress conditions (Aguilera and Liu, 2012). Recently it has been demonstrated that the activation of CRH transcription depends not only on phosphorylation of cAMP response element-binding protein (CREB) but also on interaction between CREB and a co-activator, CREB-regulated transcription co-activators (CRTCs, comprising CRTC1, 2 and 3) (Aguilera and Liu, 2012; Jurek et al., 2015; Liu et al., 2010; Liu et al., 2011; Liu et al., 2012; Martín et al., 2012). CRTCs potentiate CREB-mediated gene transcription by binding to its leucine-zipper domain (Conkright et al., 2003; Takemori et al., 2007). Under basic conditions, CRTCs are localized in the cytoplasm in a phosphorylated state, and their phosphorylation is mainly mediated by members of the AMP-activated protein kinase (AMPK) family, such as salt-inducible kinases (SIKs, comprising SIK1, SIK2 and SIK3) (Katoh et al., 2006; Takemori et al., 2007; Takemori and Okamoto, 2008). Dephosphorylation of CRTCs in response to different stimuli (Ca^{2+} signaling, cAMP signaling, etc.) causes them to be translocated to the nucleus, where they can freely interact with CREB (Saura and Cardinaux, 2017). The phosphorylation sites of CRTC1 and CRTC2 are Ser151 and Ser171, respectively, but that of CRTC3 remains undetermined (Jiang et al., 2019). SIKs were originally identified from the adrenal glands of rats treated with a high-salt diet; these proteins perform important functions in glucose homeostasis by inhibiting CRTCs (Wang et al., 1999; Choi et al., 2011). To date, several studies have reported a role of the SIKs-CRTCs system in regulating CRH transcription. For example, Liu *et al*. in 2011 reported that 30 min of restraint stress (acute stress) markedly increased the transcription of CRH and nuclear translocation of CRTC2 in the PVN (Liu et al., 2011). In 2012, Liu *et al*. further reported that SIK2 mediates inactivation of CRH neurons under basal conditions, whereas induction of SIK1 limits CRH transcription (Liu et al., 2012). Moreover, Martín *et al*. found that HPA hyperactivity induced by morphine withdrawal is accompanied by enhanced CREB phosphorylation and decreased CRTC1 phosphorylation in the PVN (Martín et al., 2012). Jurek *et al*. showed that oxytocin regulates acute restraint stress–induced CRH transcription through CRTC3 (Jurek et al., 2015). Importantly, however, none of these studies involved chronic stress and depression.

Therefore, in this study, we aimed to explore whether SIKs and CRTCs in PVN neurons mediate the effects of chronic stress on the HPA, playing a role in the pathogenesis of depression. As there are currently no acknowledged in vitro cellular models of depression, a multidisciplinary approach including in vivo rodent models of depression was used to explore our assumption.

Methods And Materials

Ethical Statements

All experimental procedures involving mice and their care were carried out in compliance with the ARRIVE guidelines (Kilkenny et al., 2010; McGrath and Lilley, 2015), and approved by the Animal Welfare Committee of Nantong University. All efforts were made to minimize the suffering of the mice.

Animals

SLAC Laboratory Animal Co., Ltd. (Shanghai, China) provided the adult male C57BL/6J mice (8 weeks old, 22–24g) that served as the experimental subjects in this study. Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China) provided the retired CD1 breeders (12 months old, 30–35g) that serves as aggressors in the chronic social defeat stress (CSDS) paradigm. Before use, all C57BL/6J mice were subjected to stratified randomization according to body weight and maintained under standard conditions that have been fully described in our previous studies (Guan et al., 2021; Jiang et al., 2019; Liu et al., 2021; Song et al., 2018; Xu et al., 2018) for 1 week with water and food available *ad libitum*. All behavioural tests were carried out between 8: 00 am and 5: 00 pm, and afterwards, C57BL/6J subjects were randomly selected and sacrificed (anaesthetized by carbon dioxide first and then killed by cervical dislocation) at 9: 00 am for all in vitro studies. The sample sizes (in vivo, n = 10; in vitro, n = 5) were determined by power analysis and according to our previous studies (Guan et al., 2021; Jiang et al., 2019; Liu et al., 2021; Song et al., 2018; Xu et al., 2018).

Drugs

Fluoxetine, venlafaxine, paroxetine, and duloxetine were purchased from Target Mol (T0450L, T0472, T1636, T1471; Boston, USA), and 5-bromo-2-deoxyuridine (BrdU) was purchased from Sigma–Aldrich (B5002; St. Louis, USA). Fluoxetine, venlafaxine and BrdU were dissolved in 0.9% saline, whereas paroxetine and duloxetine were dissolved in 5% dextrose (pH 7.0) with 2.5% DMSO and 10% Cremophor EL. The doses of fluoxetine (20 mg/kg), venlafaxine (10 mg/kg), paroxetine (20 mg/kg), duloxetine (10 mg/kg) and BrdU (75 mg/kg) were determined according to previous reports (Jiang et al., 2019; Meejuru et al., 2021; Song et al., 2018; Xu et al., 2018). All these substances were injected intraperitoneally (i.p.) at a volume of 10 ml/kg.

Chronic Social Defeat Stress (CSDS)

The protocol for CSDS has been described in several of our previous studies (Guan et al., 2021; Jiang et al., 2019; Liu et al., 2021; Song et al., 2018; Xu et al., 2018). Before CSDS, a sufficient number of male CD1 aggressors were chosen. As intruders, male C57BL/6J mice were individually exposed to an unfamiliar CD1 aggressor for 7-10 min each day for a period of 10 days (d). Each day, the defeat session was stopped when the intruders showed signs of stress and subordination (immobility, crouching, trembling, fleeing and upright posture), and perforated Plexiglas dividers were immediately put in place to separate the intruders and aggressors, providing further stressful sensory cues without physical contact for the

remainder of the next 24 h. C57BL/6J mice in the non-stressed groups were housed in pairs under the same conditions as their experimental counterparts and handled daily. All groups of C57BL/6J mice were subjected to behavioural tests including the forced swim test (FST), tail suspension test (TST), sucrose preference test and social interaction test

Chronic Unpredictable Mild Stress (CUMS)

The protocol for CUMS has been described in several of our previous studies (Gao et al., 2020; Jiang et al., 2019; Liu et al., 2021; Tang et al., 2022; Xu et al., 2018; R24). Male C57BL/6J mice were singly caged and subjected to a stress protocol that lasted for a period of 8 weeks and consisted of a randomly arranged application of 8 different stressors. These stressors included food or water deprivation (23 h), damp sawdust (12 h), restraint (2 h), cage rotation (30 min), inversion of light/dark cycle, 45 °C cage tilting in empty cage (12 h) and cold (4 °C for 1 h). C57BL/6J mice in the non-stressed groups were left undisturbed and handled daily. All groups of C57BL/6J mice were subjected to behavioural tests including the forced swim test (FST), tail suspension test (TST) and sucrose preference test.

Statistical Analysis

All data are presented as the means ± standard errors of the means (S.E.M.). For statistical analysis, SPSS 26.0 software (SPSS Inc., Chicago, USA) was used to perform t tests, one-way analysis of variance (ANOVA) with Tukey's test, two-way ANONA with Bonferroni's test, and three-way ANONA with Bonferroni's test. A value of $P< 0.05$ (two-tailed) was considered statistically significant.

Additional Methods and Materials

See the Supplemental Methods and Materials for description of the forced swim test (FST), tail suspension test (TST), sucrose preference test, social interaction test, open field test (OFT), adeno-associated virus (AAV)-mediated gene transfer, western blotting, co-immunoprecipitation (Co-IP),quantitative real-time reverse transcription PCR (qRT-PCR), immunofluorescence and other details.

Results

Exposure to chronic stress led to significant changes in SIK1-CRTC1 signalling in PVN neurons

Since CSDS and CUMS are two well-validated and widely-used rodent models of depression (Antoniuk et al., 2019; Wang et al., 2021), they were applied together in this study. As shown in Figure 1A and 1B, exposure to CSDS and CUMS induced significant depression-like behaviours in C57BL/6J mice, as revealed by the FST, TST, sucrose preference test, and social interaction test. Meanwhile, exposure to CSDS and CUMS robustly enhanced the plasma levels of corticosterone and ACTH in mice (Figure 1A and 1B), representing a hyperactive HPA axis in response to chronic stress. Subsequently, western blotting was performed to detect the expression of SIK1-SIK3 in the total protein homogenates of PVN neurons. It was found that CSDS exposure downregulated SIK1 expression in the PVN by 55.4% ± 7.2%, whereas it had no influence on SIK2 or SIK3 in the PVN (Figure 1C). Similarly, CUMS exposure decreased SIK1

expression in the PVN by 58.9% ± 5.4% but induced no effects on SIK2 or SIK3 in the PVN (Figure 1D). The effects of chronic stress on the protein expression of CRTC1-CRTC3 in the PVN were also evaluated. As shown in Figure 1E and 1F, both CSDS and CUMS fully enhanced the level of nuclear CRTC1 in the PVN, but the levels of nuclear CRTC2 or CRTC3 were not affected. In contrast, chronic stress notably decreased the level of cytoplasmic phosphorylated CRTC1 (pCRTC1) in the PVN, while that of pCRTC2 was unchanged (Figure 1E and 1F). Interestingly, the total protein level of CRTC1 in the PVN in stressed mice was also significantly higher than that of control mice, whereas the levels of total CRTC2 and total CRTC3 did not increase (Figure 1E and 1F). As such, we focused on SIK1 and CRTC1 in the following studies.

Then, qRT—PCR was performed to assay the mRNA levels of SIK1 and CRTC1 in PVN neurons. Figure S1A shows that CSDS and CUMS downregulated the SIK1 mRNA in the PVN by 55.8% ± 4.7% and 62.4% ± 8.6%, respectively. Figure S1A also reveals that CSDS and CUMS upregulated the CRTC1 mRNA in the PVN by 140.9% ± 9.5% and 166.3% ± 12.3%, respectively. Next, we examined the binding level of nuclear CRTC1 and CREB by Co-IP. It was found that chronic stress strongly promoted binding between nuclear CRTC1 and CREB in the PVN (Figure S2A and S2B), in accordance with the western blotting results of nuclear CRTC1 in Figure 1E and 1F. Moreover, we examined the neuronal distribution of CRTC1 in the PVN by immunofluorescence, with NeuN and DAPI as the nuclear markers. As shown in Figure 3A and 3B, compared with the control group, both CSDS and CUMS significantly increased CRTC1 single staining, CRTC1/NeuN double staining and CRTC1/NeuN/DAPI triple staining in PVN neurons, representing notable nuclear translocation and enhanced biosynthesis of CRTC1 under the effects of chronic stress. Taken together, these findings suggest that SIK1-CRTC1 signalling in PVN neurons is implicated in the pathophysiology of depression.

Both genetic knockdown of SIK1 and genetic overexpression of CRTC1 in the PVN induced a depression-like phenotype in naïve mice

Since chronic stress downregulated SIK1 expression in the PVN, we then studied whether genetic knockdown of SIK1 in the PVN of naïve mice would simulate chronic stress, inducing depression-like behaviours. To achieve this purpose, an enhanced green fluorescent protein (EGFP)-containing AAV vector that expresses specific short hairpin RNAs (shRNAs) against SIK1 (AAV-SIK1-shRNA-EGFP) was generated. SIK1-shRNA or Control-shRNA was stereotactically and bilaterally infused into the PVN of naïve C57BL/6J mice, and after 14 d, numerous EGFP-positive neurons and notably decreased SIK1 expression were both observed in this region (Figure 2A). In behavioural tests, it was found that knockdown of SIK1 in the PVN largely enhanced the immobility of mice in both the FST and TST compared with that of the control group (Figure 2B), representing a behaviour of desperation and helplessness. To exclude the possible effects of SIK1 knockdown on mouse locomotor activity which may have led to a false-positive conclusion, the OFT was conducted. There was no significant difference in the number of squares the animals crossed in either the peripheral area or central area among all groups (Figure 2B). It was also found that mice with SIK1 knockdown in the PVN showed significantly less sucrose preference and social interaction than control mice (Figure 2B), representing behaviours of

anhedonia and social avoidance. Meanwhile, the mice infused with SIK1-shRNA had remarkably higher levels of plasma corticosterone and ACTH than those of control mice (Figure 2B), representing a hyperactive HPA axis in response to SIK1 knockdown in the PVN. In contrast, the usage of Control-shRNA induced no effects on these evaluation indices.

Furthermore, knockdown of SIK1 in the PVN not only significantly increased the expression of nuclear CRTC1 (Figure 2C and 3C), total CRTC1 (Figure 2C and 3C), and CRTC1-CREB binding (Figure S2C) but also notably decreased the expression of cytoplasmic pCRTC1 (Figure 2C) in this region. More importantly, SIK1 knockdown remarkably enhanced the expression of CRH in the PVN (Figure 2C), consistent with the corticosterone and ACTH results in Figure 2B. Depression is accompanied by some critical pathological phenomena that can be attributed to the excessive release of glucocorticoids, such as decreased hippocampal neurogenesis and a downregulated brain-derived neurotrophic factor (BDNF) signalling cascade in the hippocampus and mPFC (Jiang et al., 2021; Liu et al., 2020; Masi and Brovedani, 2011; Mahar et al., 2014; Snyder et al., 2011). Interestingly, Figure 2D shows that the mice infused with SIK1-shRNA had significantly lower protein levels of BDNF, phosphorylated tyrosine receptor kinase B (pTrkB), and phosphorylated CREB (pCREB) in the hippocampus and mPFC than those of control mice, whereas the expression of total β-actin, TrkB, and CREB among all groups remained unchanged. Similarly, Figure 2E reveals that the mice infused with SIK1-shRNA had notably fewer cells labelled with doublecortin (DCX) and fewer cells co-labelled with NeuN and BrdU in the dentate gyrus (DG) than those of control mice, representing a decrease in hippocampal neurogenesis due to SIK1 knockdown in the PVN. In contrast, infusion of Control-shRNA produced no influence on any of these biological indices. Taken together, these findings suggest that downregulated SIK1 expression in the PVN contributes to the pathogenesis of depression.

In addition, AAV-mediated selective overexpression of CRTC1 in the PVN was also adopted in this study, and the efficacy of AAV-CRTC1-EGFP is shown in Figure S3A. AAV-CRTC1 or AAV-Control was infused into the PVN of naïve C57BL/6J mice. After 14 d, it was found that compared with mice in the control group, the mice infused with AAV-CRTC1 but not AAV-Control exhibited evidently more immobility in the FST and TST as well as notably less sucrose preference and social interaction (Figure S3B). Meanwhile, CRTC1 overexpression in the PVN did not affect the locomotor activity of mice, as revealed by the OFT results (Figure S3B). Moreover, treatment with AAV-CRTC1 but not AAV-Control robustly promoted the plasma levels of corticosterone and ACTH in mice (Figure S3B). All these findings are consistent with the above SIK1-shRNA results. Therefore, enhanced CRTC1 expression in the PVN also contributes to depression.

The CRTC1-CREB-CRH pathway mediates the pro-depressant actions induced by SIK1 knockdown in the PVN

To understand whether the pro-depressant actions induced by SIK1 knockdown in the PVN required CRTC1, AAV-CRTC1-shRNA-EGFP was generated, and its efficacy was confirmed, as shown in Figure S4A. CRTC1-shRNA was infused into the PVN of naïve C57BL/6J mice, and after 14 d, SIK1-shRNA was also infused. After another 14 d, behavioural tests were performed. As shown in Figure S4B, compared with

the control group, knockdown of CRTC1 in the PVN alone did not influence mouse behaviours in the FST, TST, sucrose preference test or social interaction test. However, CRTC1-shRNA pre-infusion significantly attenuated not only the tendency of SIK1-shRNA infusion to promote immobility in mice in the FST and TST but also the tendency of SIK1-shRNA infusion to reduce sucrose preference and social interaction in mice (Figure S4B). Moreover, CRTC1-shRNA pre-infusion notably prevented the enhancing effects of SIK1-shRNA infusion on the levels of plasma corticosterone and ACTH in mice (Figure S4B). The usage of Control-shRNA had no influence on these indices. In addition, Figure S4C reveals that the silencing effects of SIK1-shRNA and CRTC1-shRNA did not interfere with each other.

Then, to investigate whether the pro-depressant actions induced by SIK1 knockdown in the PVN required CREB, AAV-CREB-shRNA-EGFP was adopted, and its efficacy was confirmed, as shown in Figure S5A. By using an experimental procedure the same as described above, naïve C57BL/6J mice received infusions of CREB-shRNA (first) and SIK1-shRNA (second) into the PVN, followed by behavioral tests. The behavioural results are summarized in Figure S5B. Compared with the control group, knockdown of CREB in the PVN alone did not influence mouse behaviours. However, CREB-shRNA pre-infusion significantly prevented the pro-depressant effects of SIK1-shRNA infusion on mouse behaviours in the FST, TST, sucrose preference test and social interaction test. Moreover, CREB-shRNA pre-infusion fully abolished the promoting actions of SIK1-shRNA infusion on HPA activity, as revealed by the ELISA results involving plasma corticosterone and ACTH (Figure S5B). In addition, Figure S5C shows that the silencing effects of SIK1-shRNA and CREB-shRNA did not interfere with each other.

Furthermore, to explore whether the pro-depressant actions induced by SIK1 knockdown in the PVN required CRH, AAV-CRH-shRNA-EGFP was used, and its efficacy was confirmed, as shown in Figure S6A. As described above, CRH-shRNA (first) and SIK1-shRNA (second) were infused into the PVN of naïve C57BL/6J mice. The usage of CRH-shRNA significantly prevented the depression-like behaviours induced by SIK1-shRNA infusion, as revealed by the FST, TST, sucrose preference test and social interaction test (Figure S6B). Our ELISA results show that the usage of CRH-shRNA also notably blocked the promoting actions of SIK1-shRNA infusion on HPA activity (Figure S6B). Knockdown of CRH in the PVN alone had no influence. Figure S6C indicates that the silencing effects of SIK1-shRNA and CRH-shRNA did not interfere with each other.

Collectively, these findings suggest that the pro-depressant actions induced by SIK1 knockdown in the PVN require the CRTC1-CREB-CRH pathway.

Both genetic overexpression of SIK1 and genetic knockdown of CRTC1 in the PVN produced antidepressant-like effects in mice

We were interested in testing whether averting chronic stress–induced SIK1 downregulation in the PVN prevents depression. To achieve this purpose, AAV-mediated selective overexpression of SIK1 in the PVN was adopted, and the efficacy of AAV-SIK1-EGFP is demonstrated in Figure 4A. The CSDS-exposed mice were subjected to PVN infusion of AAV-SIK1 or AAV-Control, and after 14 d, behavioural tests were performed. As shown in Figure 4B, the CSDS-exposed mice treated with AAV-SIK1 displayed significantly

decreased immobility in the FST and TST, increased sucrose preference and social interaction, and lower levels of plasma corticosterone and ACTH than the CSDS-exposed mice and the CSDS-exposed mice treated with AAV-Control. In parallel, the CUMS-exposed mice received PVN infusion of AAV-SIK1 or AAV-Control, followed by behavioural tests. As shown in Figure 4C, the CUMS-exposed mice treated with AAV-SIK1 also exhibited evidently less immobility in the FST and TST, higher sucrose preference, and reduced levels of plasma corticosterone and ACTH than the CUMS-exposed mice and the CUMS-exposed mice treated with AAV-Control. Treatment with AAV-Control did not influence these indices.

Then, changes in SIK1-CRTC1 signalling in PVN neurons between all groups were detected by western blotting, Co-IP, and immunofluorescence. AAV-SIK1 treatment not only fully reversed the CSDS-induced decrease in SIK1 (Figure 5A) and cytoplasmic pCRTC1 (Figure 5A) expression but also significantly prevented the CSDS-induced increase in the levels of total CRTC1 (Figure 5A and 6), nuclear CRTC1 (Figure 5A and 6), and CRTC1-CREB binding (Figure S13A). More importantly, AAV-SIK1 treatment notably antagonized the promoting effects of CSDS on CRH expression in PVN neurons (Figure 5A), consistent with the corticosterone and ACTH results in Figure 4B. Similarly, AAV-SIK1 treatment also fully reversed the CUMS-induced effects on SIK1 (Figure 5B), CRH (Figure 5B), nuclear CRTC1 (Figure 5B and 7), cytoplasmic pCRTC1 (Figure 5B), total CRTC1 (Figure 5B and 7), and CRTC1-CREB binding (Figure S13B) in PVN neurons. In addition, AAV-SIK1 treatment enhanced the expression of cytoplasmic pCRTC1 (Figure 5A and 5B) and downregulated the levels of total CRTC1 (Figure 5A, 5B, 6, and 7), nuclear CRTC1 (Figure 5A, 5B, 6, and 7), and CRTC1-CREB binding (Figure S13A and S13B) in the PVN of naïve control mice. Treatment with AAV-Control did not affect SIK1-CRTC1 signalling.

Next, the levels of the BDNF signalling cascade and neurogenesis among all groups were examined. AAV-SIK1 treatment evidently blocked both the CSDS-induced and CUMS-induced decreases in the expression of BDNF (Figure 5C and 5D), pTrkB (Figure 5C and 5D), and pCREB (Figure 5C and 5D) in the hippocampus and mPFC, whereas the expression of total β-actin, TrkB, and CREB in the two regions did not change. Similarly, AAV-SIK1 treatment markedly abolished both the CSDS-induced and CUMS-induced decrease in hippocampal neurogenesis, as revealed by Figure 8A and 8B. In addition, AAV-SIK1 treatment did not affect the levels of BDNF, pTrkB, pCREB, or neurogenesis in naïve control mice (Figure 5C, 5D, 8A, and 8B). Treatment with AAV-Control also produced no effects.

Furthermore, AAV-CRTC1-shRNA-EGFP was applied again. The CSDS-exposed mice were subjected to PVN infusion of CRTC1-shRNA or Control-shRNA, and after 14 d, behavioural tests were performed. As shown in Figure S7A, the CSDS-exposed mice treated with CRTC1-shRNA displayed significantly decreased immobility in the FST and TST, increased sucrose preference and social interaction, and lower levels of plasma corticosterone and ACTH than the CSDS-exposed mice and the CSDS-exposed mice treated with Control-shRNA. In parallel, the CUMS-exposed mice received PVN infusion of CRTC1-shRNA or Control-shRNA, followed by behavioural tests. As shown in Figure S7B, the CUMS-exposed mice treated with CRTC1-shRNA also exhibited evidently less immobility in the FST and TST, higher sucrose preference, and reduced levels of plasma corticosterone and ACTH than the CUMS-exposed mice and the CUMS-exposed mice treated with Control-shRNA. The usage of Control-shRNA had no influence.

Collectively, both genetic overexpression of SIK1 and genetic knockdown of CRTC1 in PVN neurons produced beneficial effects against chronic stress.

Stereotactic infusion of TAT-SIK1 into the PVN produced antidepressant-like effects in mice

Beyond genetic manipulation, we employed the TAT-SIK1 fusion protein (TAT-SIK1), a cell permeable peptide that can be efficiently introduced into neurons to increase the biological function of SIK1. The efficacy of TAT-SIK1 is demonstrated in Figure S8A. The CSDS-exposed mice were subjected to daily PVN infusion of TAT-SIK1 or TAT-Control (nonactive TAT-SIK1) for 14 d, followed by behavioural tests. As shown in Figure S8B, the CSDS-exposed mice treated with TAT-SIK1 displayed significantly decreased immobility in the FST and TST, increased sucrose preference and social interaction, and lower levels of plasma corticosterone and ACTH than the CSDS-exposed mice and the CSDS-exposed mice treated with TAT-Control. In parallel, the CUMS-exposed mice received daily PVN infusion of TAT-SIK1 or TAT-Control during the last 2 weeks, followed by behavioural tests. As shown in Figure S8C, the CUMS-exposed mice treated with TAT-SIK1 also exhibited evidently less immobility in the FST and TST, higher sucrose preference, and reduced levels of plasma corticosterone and ACTH than the CUMS-exposed mice and the CUMS-exposed mice treated with TAT-Control. Treatment with TAT-Control did not influence these indices.

Subsequently, changes in SIK1-CRTC1 signalling in PVN neurons among all groups were examined by western blotting, Co-IP, and immunofluorescence. TAT-SIK1 treatment not only fully reversed the CSDS-induced decrease in SIK1 (Figure S9A) and cytoplasmic pCRTC1 (Figure S9A) expression but also significantly prevented the CSDS-induced increase in the levels of total CRTC1 (Figure S9A and S11), nuclear CRTC1 (Figure S9A and S11), and CRTC1-CREB binding (Figure S13C). More importantly, TAT-SIK1 treatment notably antagonized the promoting effects of CSDS on CRH expression in PVN neurons (Figure S9A), consistent with the corticosterone and ACTH results in Figure S8B. Similarly, TAT-SIK1 treatment also fully reversed the CUMS-induced effects on SIK1 (Figure S10A), CRH (Figure S10A), nuclear CRTC1 (Figure S10A and S12), cytoplasmic pCRTC1 (Figure S10A), total CRTC1 (Figure S10A and S12), and CRTC1-CREB binding (Figure S13D) in PVN neurons. In addition, TAT-SIK1 treatment enhanced the expression of cytoplasmic pCRTC1 (Figure S9A and S10A) and downregulated the levels of total CRTC1 (Figure S9A, S10A, S11, and S12), nuclear CRTC1 (Figure S9A, S10A, S11, and S12), and CRTC1-CREB binding (Figure S13C and S13D) in the PVN of naïve control mice, further confirming the biological actions of TAT-SIK1. Treatment with TAT-Control did not affect SIK1-CRTC1 signalling.

Afterwards, the levels of the BDNF signalling cascade and neurogenesis in each group were assayed. TAT-SIK1 treatment evidently blocked both the CSDS-induced and CUMS-induced decreases in the expression of BDNF (Figure S9B and S10B), pTrkB (Figure S9B and S10B), and pCREB (Figure S9B and S10B) in the hippocampus and mPFC, whereas the expression of total β-actin, TrkB, and CREB in the two regions did not change. Similarly, TAT-SIK1 treatment markedly abolished both the CSDS-induced and CUMS-induced decreases in hippocampal neurogenesis, as revealed by Figure S14A and S14B. In addition, TAT-SIK1 treatment did not affect the levels of BDNF, pTrkB, pCREB, or neurogenesis in naïve control mice (Figure S9B, S10B, S14A, and S14B). Treatment with TAT-Control also produced no effects.

Combined with the above results involving AAV-SIK1, it can be concluded that SIK1 in the PVN is a feasible and novel antidepressant target.

Fluoxetine, paroxetine, venlafaxine, and duloxetine can modulate SIK1-CRTC1 signalling in PVN neurons

Currently, SSRIs and SNRIs are the most commonly used antidepressants in clinical practice. Fluoxetine and paroxetine are representative drugs of SSRIs. Venlafaxine and duloxetine are representative SNRI drugs. Here, we studied whether the antidepressant actions of fluoxetine, paroxetine, venlafaxine, and duloxetine involve the SIK1-CRTC1 system in the PVN.

The CSDS-exposed mice received a daily injection of fluoxetine, paroxetine, venlafaxine, or duloxetine for 14 d, and behavioural tests were performed thereafter. As shown in Figures 9A and S15A, repeated treatment with the four drugs not only significantly reversed the CSDS-induced depression-like behaviours in mice but also entirely prevented CSDS from increasing the levels of plasma corticosterone and ACTH in mice. Then, it was found that all these drugs thoroughly reversed the CSDS-induced downregulation of SIK1 and upregulation of CRH in the PVN of mice (Figure 9C and S15B). Moreover, administration of the four drugs notably blocked not only the tendency of CSDS to promote nuclear CRTC1 (Figure 9C, 10, S15B, and S17), total CRTC1 (Figure 9C, 10, S15B, and S17), and CRTC1-CREB binding (Figure S19A and S19C) but also the inhibitory effects of CSDS on cytoplasmic pCRTC1 in the PVN (Figure 9C and S15B) of mice. Furthermore, administration of the four drugs evidently increased the SIK1 mRNA level and decreased the CRTC1 mRNA level in the PVN of the CSDS-exposed mice (Figure S1B and S1C). In addition, none of these drugs affected the SIK1-CRTC1 system in the PVN of naïve control mice (Figure S1B, S1C, 9C, 10, S15B, and S17).

The CUMS-exposed mice were subjected to daily injection of fluoxetine, paroxetine, venlafaxine, or duloxetine during the last 2 weeks, and then, behavioural tests were performed. Figures 9B and S16A show that the four drugs also demonstrated notable antidepressant actions in the CUMS model. More importantly, in parallel with the CSDS results, the CUMS-induced effects on the expression of SIK1 (Figure 9D and S16B), CRH (Figure 9D and S16B), nuclear CRTC1 (Figure 9D, 11, S16B, and S18), total CRTC1 (Figure 9D, 11, S16B, and S18), cytoplasmic pCRTC1 (Figure 9D and S16B), and CRTC1-CREB binding (Figure S19B and S19D) in the PVN of mice were all thoroughly reversed by administration of these drugs, as revealed by western blotting, Co-IP, and immunofluorescence. In addition, treatment with these drugs all significantly prevented the effects of CUMS on SIK1 mRNA and CRTC1 mRNA in the PVN of mice (Figure S1B and S1C).

In summary, fluoxetine, paroxetine, venlafaxine, and duloxetine can modulate SIK1-CRTC1 signalling in PVN neurons.

The SIK1-CRTC1 system in the PVN is necessary for the antidepressant actions of fluoxetine, paroxetine, venlafaxine, and duloxetine

AAV-SIK1-shRNA-EGFP was further used to determine whether SIK1-CRTC1 signalling in PVN neurons participates in the antidepressant mechanisms of fluoxetine, paroxetine, venlafaxine, and duloxetine.

C57BL/6J mice pre-infused with SIK1-shRNA were subjected to 10 d of CSDS and then daily injection of fluoxetine, paroxetine, venlafaxine, or duloxetine for 14 d, followed by behavioural tests. As shown in Figures 12A and S20A, knockdown of SIK1 in the PVN significantly attenuated the reducing effects of all these drugs on the immobility duration of the CSDS-exposed mice in the FST and TST. Additionally, knockdown of SIK1 in the PVN also abolished the promoting effects of the four drugs on the sucrose preference and social interaction of the CSDS-exposed mice. Moreover, C57BL/6J mice pre-treated with SIK1-shRNA were exposed to 8 weeks of CUMS and daily treatment with fluoxetine, paroxetine, venlafaxine, or duloxetine during the last 2 weeks, and behavioural tests were then performed. Pre-treatment with SIK1-shRNA notably antagonized the antidepressant effects of all these drugs against CUMS in the FST, TST and sucrose preference test (Figure 12B and S20B). In contrast, the usage of Control-shRNA produced no effects on the behavioural results of mice (Figure 12A, 12B, S20A, and S20B). Combined with the above results, the SIK1-CRTC1 system in the PVN is required for the therapeutic actions of the four antidepressants used in clinical practice.

Discussion

Here, we found that chronic stress induced not only depression-like behaviours but also significant changes in SIK1-CRTC1 signalling in PVN neurons. Both genetic knockdown of SIK1 and genetic overexpression of CRTC1 in the PVN mimicked chronic stress that induced depression-like phenotypes in naïve mice, and this was mediated by promoting CRH biosynthesis and enhancing HPA activity. In contrast, both genetic overexpression of SIK1 and genetic knockdown of CRTC1 in the PVN produced antidepressant-like effects in mice exposed to chronic stress. Furthermore, pharmacological overexpression of SIK1 in the PVN by stereotactic infusion of TAT-SIK1 also protected against chronic stress in mice. In addition, the antidepressant actions of fluoxetine, paroxetine, venlafaxine, and duloxetine, four clinically used SSRIs/SNRIs, all involve the SIK1-CRTC1 system in the PVN (Figure 13).

Maintenance of body homeostasis requires continuous adaptation to internal and external stressors, and one of the most important adaptive responses is activation of the HPA axis by CRH, resulting in a rapid increase in circulating ACTH and subsequent rise in glucocorticoids (Aguilera and Liu, 2012). However, excessive release of glucocorticoids induced by chronic stress can lead to many negative changes, including psychiatric disorders such as depression. Thus, elucidating the molecular mechanism underlying CRH modulation during chronic stress is essential for understanding the pathogenesis and treatment of depression. Many previous studies have demonstrated that activation of the cAMP/protein kinase A (PKA) signalling pathway, which leads to pCREB recruitment to the cAMP response element (CRE) in the CRH promoter, is required for CRH transcription (Adler et al., 1990; Cheng et al., 2000; Guardiola-Diaz et al., 1994; Seasholtz et al., 1988). Recently, it has become evident that pCREB is necessary but not sufficient for the stimulation of CRH biosynthesis, and the presence of CRTCs is also critical (Liu et al., 2008; Liu et al., 2010; Liu et al., 2011). Moreover, it is well established that SIKs regulate

the activity of CRTCs by controlling their trafficking from the cytoplasm to the nucleus (Conkright et al., 2003; Takemori et al., 2007; Takemori and Okamoto, 2008). Therefore, it is of great significance to investigate whether SIKs and CRTCs mediate the ability of chronic stress to promote CRH biosynthesis.

In 2019, we published a report in *Biological Psychiatry* investigating the role of hippocampal SIK2 in the pathophysiology of depression (Jiang et al., 2019). In this report, in addition to hippocampal SIK2, we also found that chronic stress moderately downregulated SIK1 expression in the hypothalamus (Jiang et al., 2019). However, and importantly, the greatest shortcoming of that study is that we studied only the whole hypothalamus and not specifically the PVN, whereas CRH neurons are mainly located in the hypothalamic PVN. In the present study, as expected, our results showed that both CSDS and CUMS greatly downregulated SIK1 expression in the PVN, and the magnitude of the decrease was much greater than observed for the whole hypothalamus in our previous report (Jiang et al., 2019). Interestingly, Liu *et al*. in 2012 indicated that the mRNA level of SIK1 but not SIK2 in the PVN of rats was significantly enhanced after acute restraint stress as part of the negative feedback mechanisms controlling HPA activity by inactivating CRTCs (Liu et al., 2012). Here, our results suggested that under depressive conditions, the SIK1-mediated negative feedback mechanism disappeared as its expression changed from upregulation to downregulation. Our results further revealed that the effects of chronic stress on SIKs in the PVN were biologically selective, as both SIK2 and SIK3 in the PVN were unaffected by CSDS and CUMS. The differences between SIK1-SIK3 in our findings are interesting, suggesting the complexity of depression neurobiology.

Regarding how chronic stress affects the expression of SIK1 in the PVN, given that chronic stress significantly decreased its mRNA level, the effects likely occurred at the transcriptional level. It is possible that chronic stress induced excessive release of glucocorticoids that bound to intracellular glucocorticoid receptors in PVN neurons, resulting in protein complex translocation into the nucleus to exert biological effects on SIK1 at the genomic level. This possibility could be further verified by studying whether the DNA sequence of the SIK1 gene in PVN neurons possesses a specific negative glucocorticoid response element (nGRE) using bioinformatics and whether glucocorticoid receptors interact with SIK1-DNA in the PVN using chromatin immunoprecipitation. Alternatively, the effects may also occur at the protein level. For example, stabilization of SIK1 may contribute to its protein decrease. From previous studies we have learned that SIKs are liable proteins, and their stability is controlled by Thr182 phosphorylation (Hashimoto et al., 2008). The upstream kinase responsible for Thr182 phosphorylation of SIKs is liver kinase B1 (LKB1), and disruption of this phosphorylation by mutagenesis results in rapid protein degradation of SIKs (Darling and Cohen, 2021; Katoh et al., 2006). Therefore, in addition to genetic downregulation, it is also possible that chronic stress reduced LKB1 function and promoted SIK1 degradation in PVN neurons, which needs further investigation. Moreover, in addition to pCREB enhancement, cAMP/PKA signalling is known to inactivate SIK1 by Ser577 phosphorylation and thus prevent CRTC phosphorylation, leading to increased activity of CRTCs (Darling and Cohen, 2021; Takemori et al., 2002; Takemori et al., 2003). It has been demonstrated that CRH neurons in the PVN receive not only positive autoregulatory input from CRH but also an additional stimulating effect of pituitary adenylate cyclase activating polypeptide (PACAP) during stress (Aguilera and Liu, 2012; Jiang et

al., 2016; Luo et al., 1994; Lehmann et al., 2013; Mustafa, 2013; Rivest et al., 1995). Both CRH and PACAP are stimulants acting upon receptors (CRHR1 and PAC1) coupled to adenylate cyclase, providing a source of cAMP (Aguilera and Liu, 2012). According to all this information, we consider that the inhibitory effects of chronic stress on SIK1 in the PVN are dual, involving not only its biological synthesis but also its kinase activity.

Previous studies have demonstrated that all three subtypes of CRTC are distributed in the PVN (Watts et al., 2011). In our study, it was found that both CSDS and CUMS affected only CRTC1 and not CRTC2 or CRTC3 in the PVN, which is interesting and further suggests the complexity of the neurobiology underlying depression. We have noticed some previous studies involving CRTCs and CRH transcription. For example, Liu *et al*. in 2011 showed that acute restraint stress induced parallel changes in CRH transcription and nuclear translocation of CRTC2 in PVN neurons of Sprague–Dawley rats (Liu et al., 2011). Martín *et al*. found that morphine withdrawal in Sprague–Dawley rats caused not only HPA hyperactivity but also increased pCREB expression and decreased pCRTC1 expression in the PVN (Martín et al., 2012). Jurek *et al*. reported that the attenuating effects of oxytocin on enhanced CRH transcription in both Wistar rats and C57BL/6J mice in response to acute restraint stress were mediated through modulation of CRTC3 but not CRTC2 in the PVN (Jurek et al., 2015). Thus, our findings are consistent with those of Martín *et al*. but not with those of Liu *et al*. or Jurek *et al*. Currently, we have no persuasive explanation for these discrepancies, and it may be attributed to individual differences, species differences, or different types of external stressors. It is known that the PVN can be divided into three subdivisions: (a) The dorsomedial parvocellular division containing CRH neurons (co-expressing small amounts of vasopressin (VP)), which are responsible for the stimulation of ACTH release. (b) The dorsolateral magnocellular division, which contains VP neurons and oxytocin neurons (co-expressing small amounts of CRH). (c) Autonomic parvocellular division containing CRH neurons, which are involved in regulating the sympathoadrenal system (Aguilera and Liu, 2012; Swanson and Kuypers, 1980). Here, our immunofluorescence results involving CRTC1 in the PVN revealed that under normal conditions, the CRTC1-immunopositive neurons were mainly located in the (b) and (c) regions, while under depressive conditions, newborn CRTC1-immunopositive neurons were mainly located in the (a) region. This finding is highly consistent with the established point that CRH neurons in the dorsolateral magnocellular division of the PVN are the major regulator of HPA activity during stress (Bale and Vale, 2004; Herman et al., 2016; Leistner and Menke, 2020).

For CRTC1, another interesting finding is the stress-induced changes in nuclear CRTC1, cytoplasmic pCRTC1, and total CRTC1 in the PVN. Both CSDS and CUMS promoted nuclear translocation of CRTC1 in PVN neurons, leading to increased nuclear CRTC1 expression and decreased cytoplasmic pCRTC1 expression, while total CRTC1 expression did not remain constant but was upregulated. We speculate that, under normal conditions, the biosynthesis and biodegradation of CRTC1 in the cytoplasm (pCRTC1) maintain a finely tuned balance. However, under depressive conditions, the enhanced nuclear CRTC1 translocation in the PVN not only significantly decreases the pCRTC1 level in the cytoplasm but also markedly promotes CRTC1 biosynthesis. When the balance between CRTC1 biosynthesis (strengthened) and biodegradation (constant) is lost, the level of pCRTC1 in the cytoplasm increases, but it is still lower

than that under normal conditions. Consequently, the total level of CRTC1 in the PVN also increases. This supposition was partially supported by our qRT—PCR results, which revealed that chronic stress significantly enhanced the mRNA level of CRTC1 in the PVN. Moreover, the main neurotransmitters released in the PVN during stress are glutamate (Glu) and norepinephrine (NA) (Evanson and Herman, 2015; Flak et al., 2009; Herman et al., 1996; Palkovits et al., 1999). Although Glu and NA do not stimulate the production of cAMP, they can increase cytoplasm calcium by acting on NMDA, GluR5, and α-adrenergic receptors located in CRH neurons (Aguilera and Liu, 2012). It is known that increases in cytoplasmic calcium can contribute to CRTC activation by activating calcineurin (Liu et al., 2010). Thus, in our findings, CRTC1 activation in response to chronic stress may involve a Glu/NA-calcium-calcineurin-dependent mechanism in addition to SIK1 modulation, which needs further elucidation in the future.

Positive and negative manipulation of SIK1 and CRTC1 in the PVN via AAV-mediated overexpression and knockdown together support the possibility that they are feasible and novel antidepressant targets. The related results are highly consistent across two rodent models of depression (CSDS and CUMS), various behavioural measures, ELISA, western blotting, Co-IP, and immunofluorescence. To confirm the generality of our findings, some other rodent models of depression, such as chronic restraint stress, will be employed in further studies. We initially planned to perform more studies involving pharmacological inhibition of SIK1 in the PVN of naïve mice to supplement the results involving SIK1-shRNA; however, there are no selective SIK1 antagonists. Previous studies have indicated that SIKs regulate the cytoplasmic localization and biologic activity of not only CRTCs but also class IIa histone deacetylases (HDAC4, 5, 7, and 9), which can prevent MEF2-dependent gene transcription and activate forkhead family transcription factors (Berdeaux et al., 2007; Finsterwald et al., 2013; Haberland et al., 2009; Mihaylova et al., 2011; van der Linden et al., 2007; Wein et al., 2018). Moreover, SIK1 and SIK3 have been found to inhibit the toll-like receptor 4 (TLR4)-mediated signalling cascade, and CRTCs have been reported to associate with some transcription factors other than CREB, such as the homeobox protein MEIS1A (Goh et al., 2009; Yong Kim et al., 2013). Together, these studies suggest that the depression-like behaviours and enhanced HPA activity induced by SIK1 knockdown in the PVN of naïve mice may be due to modulation of HDACs, TLR4, or MEIS1A, and this possibility has been ruled out by the combined usage of CRTC1-shRNA, CREB-shRNA, and CRH-shRNA. These studies also indicate that it may be necessary to determine whether the antidepressant-like effects induced by SIK1 overexpression in the PVN were mediated by downstream CRTC1. To achieve this purpose, CRTC1-Ser151 mutant mice in which SIK1 is unable to phosphorylate CRTC1 can be constructed and used together with AAV-SIK1, CSDS, and CUMS in further studies.

To date, there are no selective SIK1 agonists. Instead, stereotactic infusion of TAT-SIK1 was used to pharmacologically activate SIK1-CRTC1 signalling in PVN neurons, and the related results further confirmed the effectiveness of SIK1 as an antidepressant target. To determine whether the antidepressant-like actions induced by TAT-SIK1 infusion were achieved by modulating the downstream CRTC1, a modified TAT-SIK1 peptide with its biological kinase domain (AA27-278) removed (unable to phosphorylate CRTC1) will be constructed and tested in rodent models of depression in the next study.

Moreover, according to the bZIP domain (AA285-339) of CREB which is responsible for its binding with CRTC1 (Altarejos and Montminy, 2011; Böer, et al., 2007), we plan to design a small cell-permeable peptide that can effectively disrupt the CRTC1-CREB interaction in the future and examine whether infusion of this peptide into the PVN of mice prevents chronic stress. In addition, the present study is limited in that we used only male C57BL/6J mice, while female subjects were not included.
Another limitation of this study is the exclusive use of rodent models of depression; our conclusion could be greatly strengthened if human samples were involved.

Although SSRIs and SNRIs rapidly increase the levels of synaptic 5-HT and NA, weeks of treatment are always required for these medications to produce therapeutic efficacy (Boku et al., 2018; Dale et al., 2015; Massart et al., 2012). Therefore, something downstream of or beyond 5-HT/NA-mediated signalling may be critical. It is very interesting to observe that fluoxetine, paroxetine, venlafaxine, and duloxetine all regulated the SIK1-CRTC1 system in the PVN at the transcriptional level. Regarding the molecular mechanism underlying this interesting phenomenon, one candidate is 5-HT, since the four drugs all act on this neurotransmitter, and moreover, 5-HT neurons of the raphe nuclei send projections to both the PVN proper and its surroundings (Herman et al., 2016). However, there is evidence showing that the release of 5-HT enhances HPA activity by stimulating multiple receptor subtypes (Goel et al., 2014; Jensen et al., 1999; Jongsma et al., 2005), which does not support the above assumption. Another possibility is that the four drugs all indirectly affected SIK1 expression in PVN neurons by recovering the chronic stress-damaged negative feedback mechanism arising from the hippocampus and prefrontal cortex. It is well known that the hippocampus and prefrontal cortex send glutamatergic projections to GABAergic PVN-projecting neurons in regions such as the bed nucleus of the stria terminalis (BNST) (Aguilera and Liu, 2012; Herman et al., 2003; Herman et al., 2016; Ulrich-Lai and Herman, 2009), and we will perform more in-depth molecular studies involving the BNST region in the future. The usage of SIK1-shRNA further supports the conclusion that the SIK1-CRTC1 system in the PVN participates in the antidepressant mechanism of the four drugs, extending the knowledge of their pharmacological actions. In addition to SSRIs and SNRIs, there are some other excellent and well-known antidepressants in clinical practice, such as vortioxetine (a multimodal-acting antidepressant) and mirtazapine (a noradrenergic and specific serotonergic antidepressant). Exploring the relationship between these antidepressants and the SIK1-CRTC1 system in the PVN would be of considerable interest and will be part of our next study.

In conclusion, our findings suggest that SIK1 and CRTC1 in PVN neurons are closely involved in the pathophysiology of depression by regulating HPA activity, and they can be candidates for novel antidepressant targets. The present study contributes to the neuroendocrine hypothesis of depression, provides support for the potential adoption of SIK1 promotion strategies against chronic stress-related neuropsychiatric disorders, and further extends the knowledge of the role of SIK1 and CRTC1 in the brain.

Declarations

Author Contributions

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Acknowledgments

This work was supported by four grants from the National Natural Science Foundation of China (No. 82071519, 81873795, 81900551, and 82001606).

Conflict of Interests

The authors declare no conflicts of interest for this study.

Supplemental Information

Twenty supplemental figures, additional Methods and Materials, and statistics of all results are included.

Data Availability Statement

The authors declare that all data supporting the findings of this study are available within the paper and its supplemental information files.

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Figures

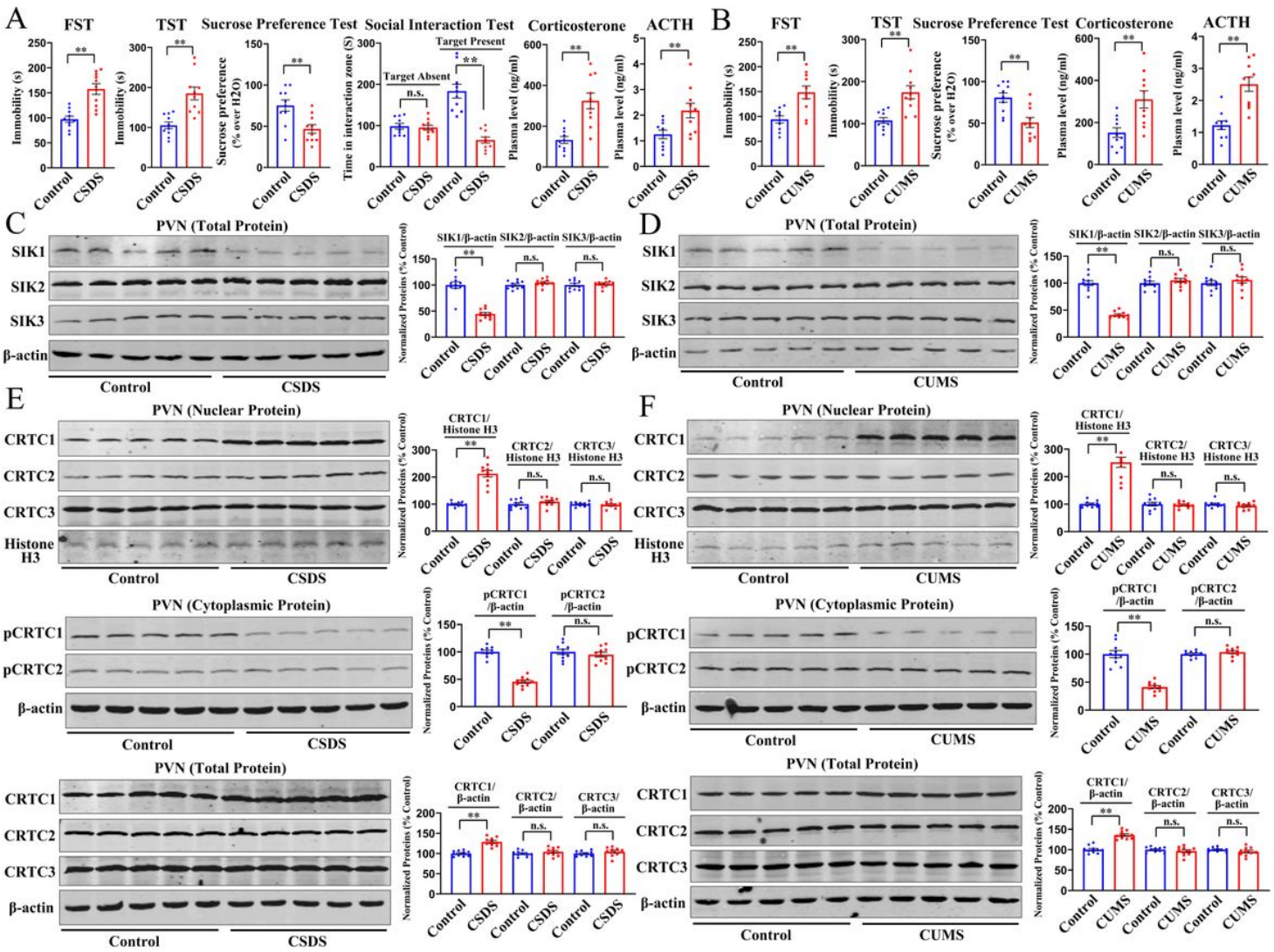

Figure 1

Exposure to chronic stress notably modulated SIK1-CRTC1 signalling in the PVN of mice. (A and B) The results of the FST, TST, sucrose preference test, social interaction test, and ELISA together reveal that both CSDS and CUMS induced not only depression-like behaviours but also HPA hyperactivity in C57BL/6J mice, which manifested as behavioural despair, anhedonia, social avoidance, and enhanced plasma levels of corticosterone and ACTH (n = 10). (C and D) Representative western blotting images and related data analyses show the effects of CSDS and CUMS on the protein expression of SIK1-SIK3 in the PVN of C57BL/6J mice (n = 10). (E and F) Representative western blotting images and related data analyses show the effects of CSDS and CUMS on the protein expression of nuclear CRTC1-CRTC3, cytoplasmic pCRTC1 and pCRTC2, and total CRTC1-CRTC3 in the PVN of C57BL/6J mice (n = 10). All results are represented as the means ± S.E.M. values; **P< 0.01; n.s., no significance. The comparisons were made using Student's t test.

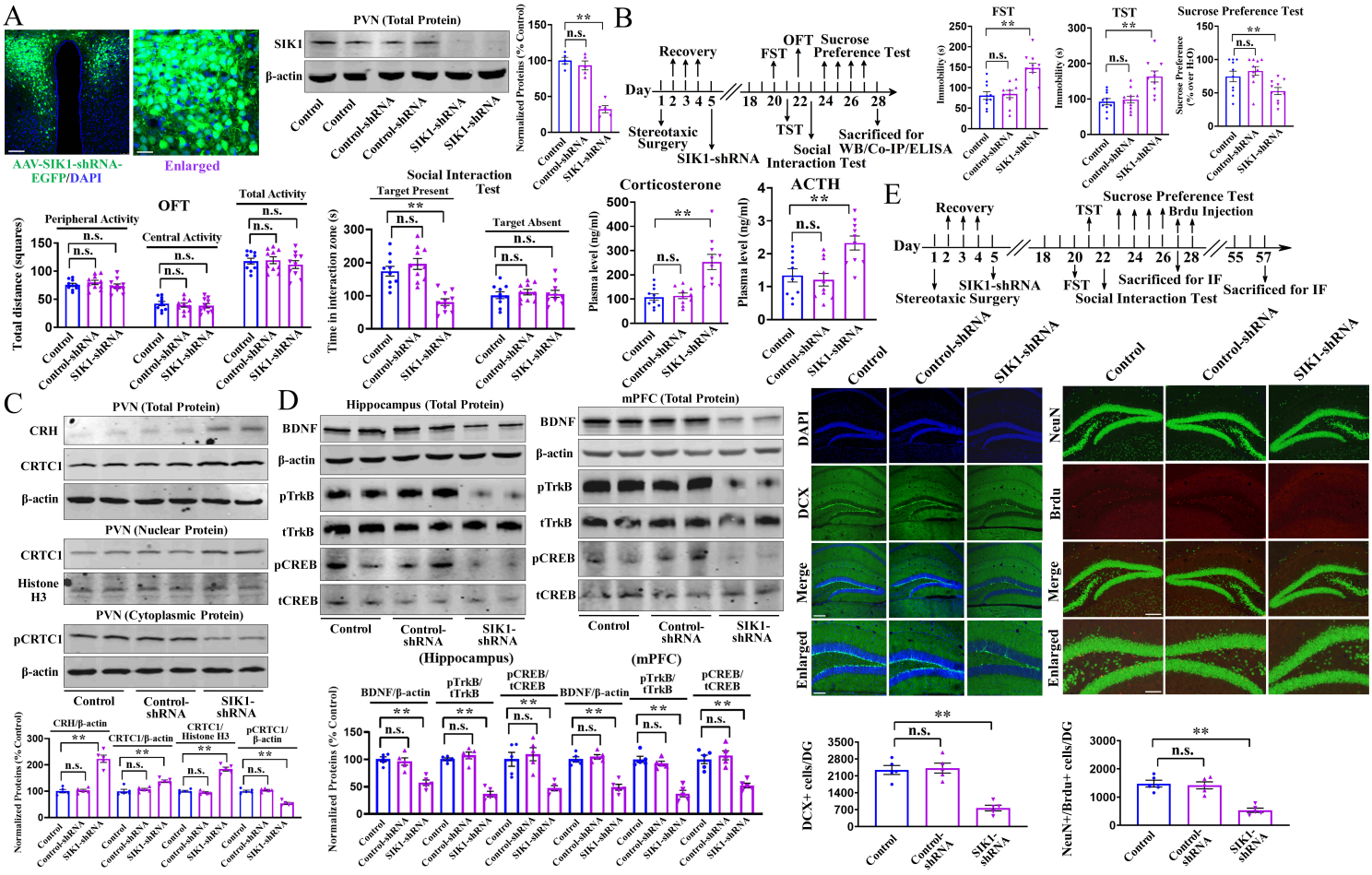

Figure 2

AAV-mediated genetic knockdown of SIK1 in the PVN of naïve mice produced various depression-like symptoms. (A) Fluorescence images of a fixed brain slice that expressed AAV-SIK1-shRNA-EGFP in the PVN 14 d after stereotactic infusion. The scale bars for the representative image and enlarged image are 100 μm and 25 μm, respectively. The western blotting (WB) results confirmed the silencing efficacy of SIK1-shRNA (n = 5). (B) Treatment with SIK1-shRNA induced both depression-like behaviours and HPA hyperactivity in naïve C57BL/6J mice without affecting their locomotor activity, as revealed by the FST, TST, OFT, sucrose preference test, social interaction test, and ELISA (n = 10). A schematic timeline of the experimental procedures is provided. (C) Representative western blotting images and related data analyses indicate that SIK1-shRNA treatment significantly enhanced the expression of CRH, nuclear CRTC1, and total CRTC1, whereas it fully downregulated the expression of cytoplasmic pCRTC1 in the PVN of naïve C57BL/6J mice (n = 5). (D) Representative western blotting images and related data analyses reveal notable decreasing effects of SIK1-shRNA treatment on the levels of BDNF, pTrkB, and pCREB in the hippocampus and mPFC of naïve C57BL/6J mice (n = 5). (E) Representative immunofluorescence (IF) images and related data analyses show an evident reducing effect of SIK1-shRNA treatment on hippocampal neurogenesis in naïve C57BL/6J mice, as revealed by DCX/DAPI staining and BrdU/NeuN staining in the DG (n = 5). The scale bars for the representative image and enlarged image are 150 μm and 75 μm, respectively. A schematic timeline of the experimental procedures

is also provided. All results are represented as the means ± S.E.M. values; **P< 0.01; n.s., no significance. The comparisons were made using one-way ANOVA followed by Tukey's test.

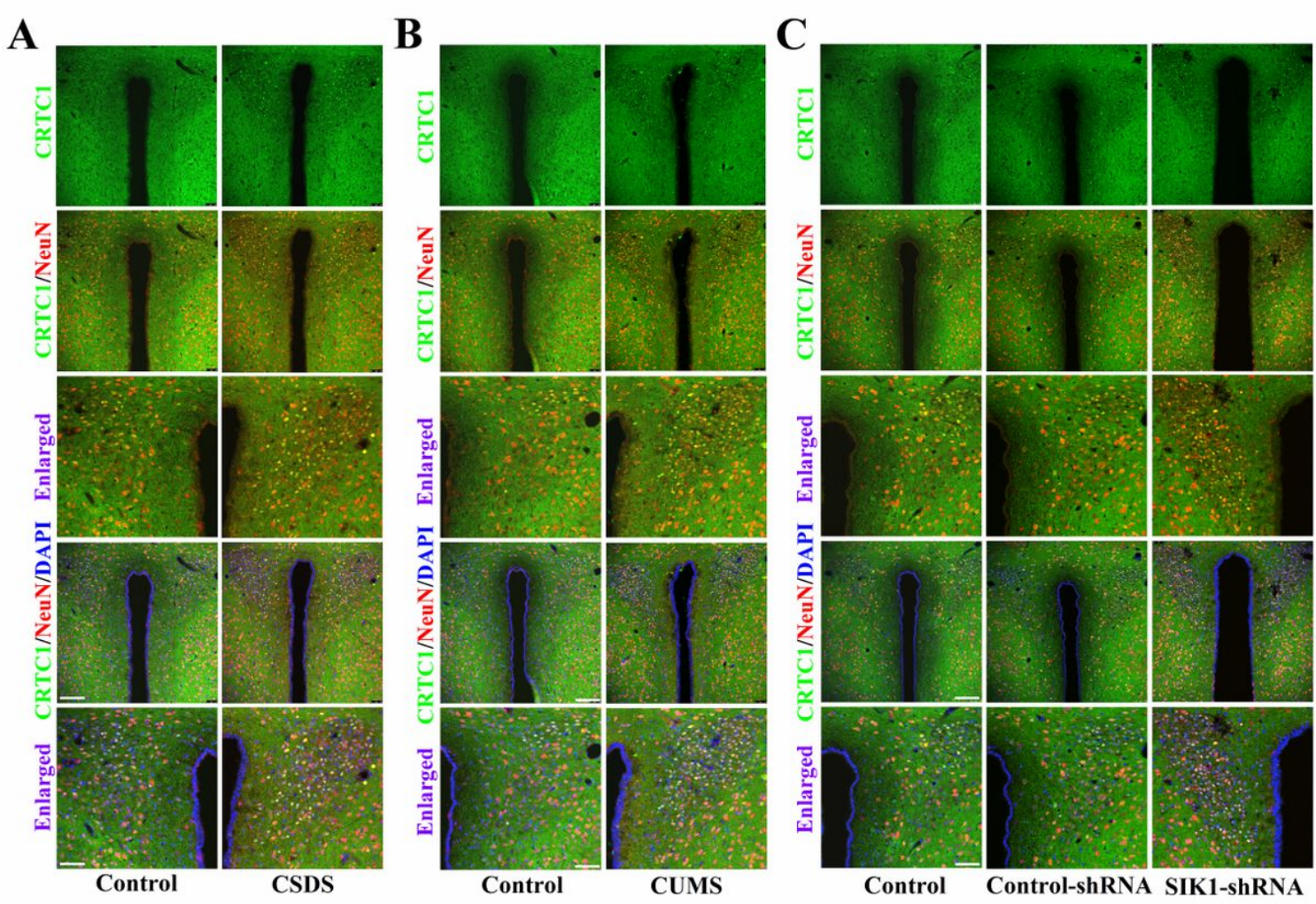

Figure 3

Representative immunofluorescence images showing the effects of CSDS (A), CUMS (B), and SIK1-shRNA infusion (C) on the neuronal distribution of CRTC1 in the PVN region of C57BL/6J mice, with NeuN and DAPI as the nuclear markers (n = 5). CSDS, CUMS, and SIK1-shRNA treatment all significantly promoted the nuclear translocation and biosynthesis of CRTC1 in PVN neurons. The scale bar is 100 μm for the representative image and 50 μm for the enlarged image.

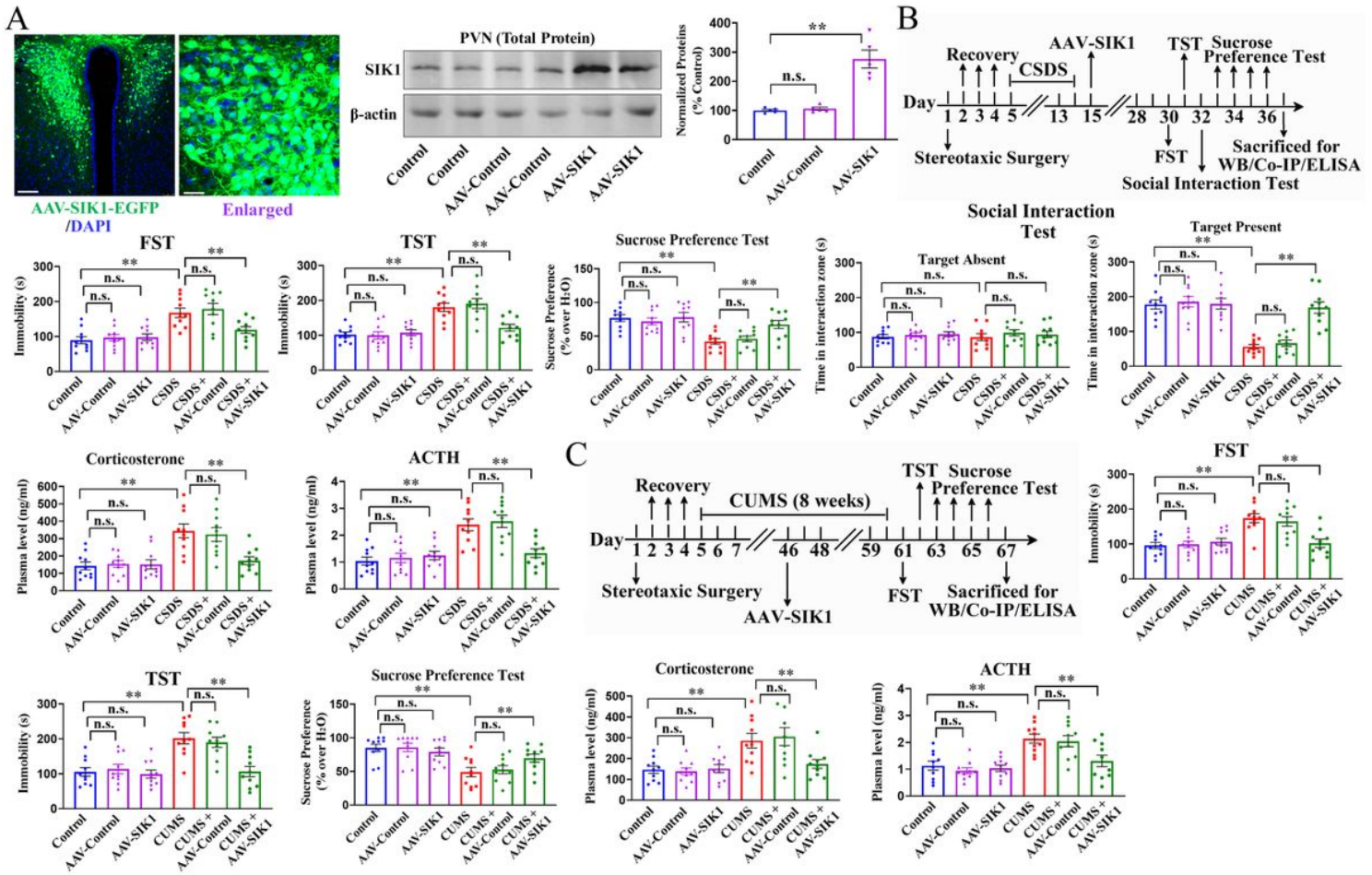

Figure 4

AAV-mediated genetic overexpression of SIK1 in the PVN exerted significant antidepressant-like effects in mice. (A) Fluorescence images of a fixed brain slice expressing AAV-SIK1-EGFP in the PVN 14 d after stereotactic infusion. The scale bars for the representative image and enlarged image are 100 μm and 25 μm, respectively. The western blotting (WB) results confirmed the overexpression efficacy of AAV-SIK1 (n = 5). (B) The results of the FST, TST, sucrose preference test, social interaction test, and ELISA together show that AAV-SIK1 treatment notably prevented CSDS-induced depression-like behaviours and HPA hyperactivity in C57BL/6J mice (n = 10). A schematic timeline of the experimental procedures is provided. (C) Treatment with AAV-SIK1 also fully reversed CUMS-induced depression-like behaviours and HPA hyperactivity in C57BL/6J mice, as revealed by the FST, TST, sucrose preference test, and ELISA (n = 10). A schematic timeline of the experimental procedures is also provided. All results are represented as the means ± S.E.M. values; ** $P < 0.01$; n.s., no significance. For panel (A), the comparisons were made using one-way ANOVA followed by Tukey's test. For panels (B) and (C), the comparisons were made using two-way ANONA followed by Bonferroni's test.

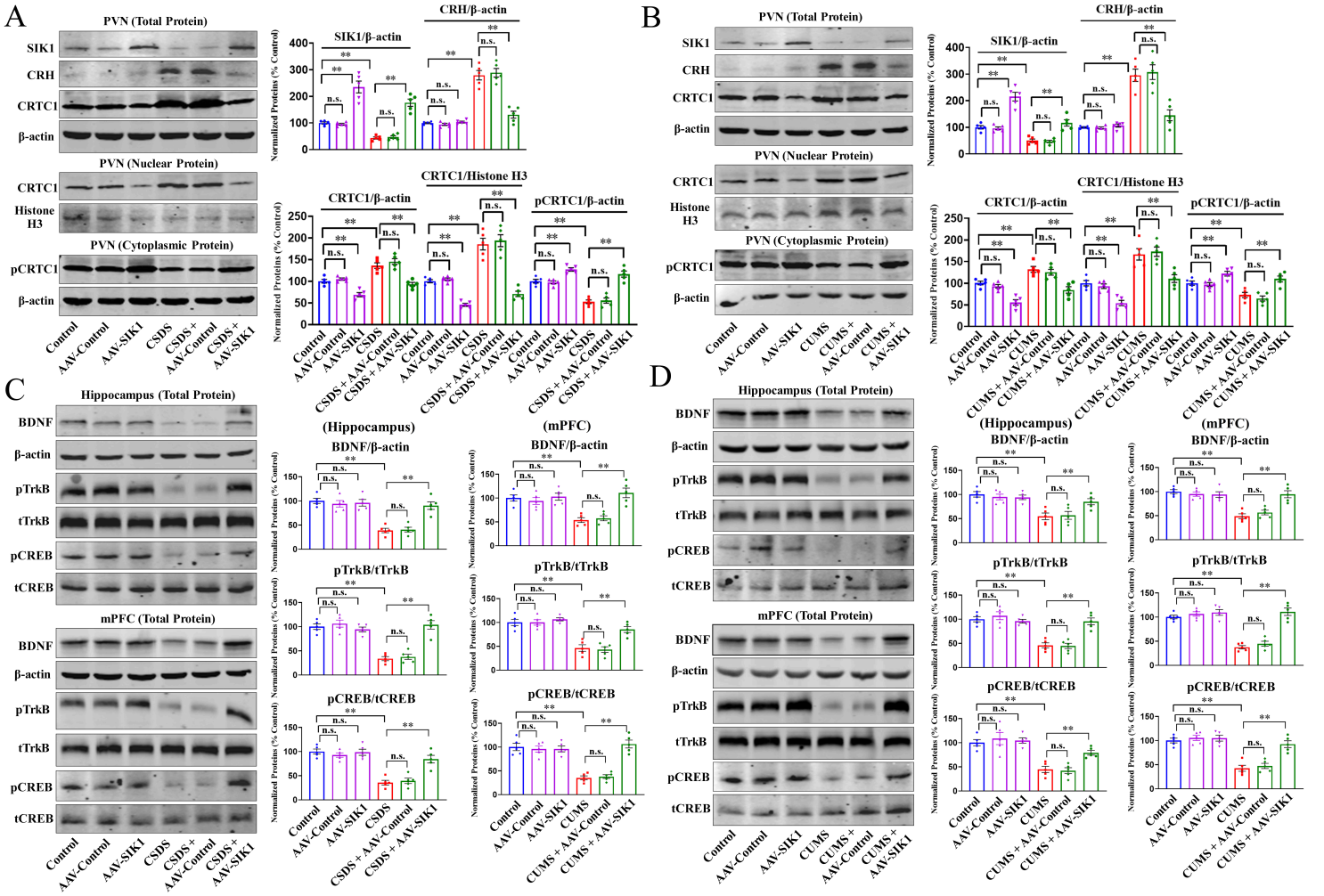

Figure 5

AAV-mediated genetic overexpression of SIK1 in the PVN exerted significant protective effects against chronic stress on the SIK1-CRTC1-CRH pathway in the PVN and the BDNF signaling cascade in the hippocampus and mPFC. (A and B) Representative western blotting images and related data analyses indicate that AAV-SIK1 treatment notably abolished both the CSDS-induced and CUMS-induced effects on the expression of SIK1, CRH, nuclear CRTC1, cytoplasmic pCRTC1, and total CRTC1 in the PVN of C57BL/6J mice (n = 5). (C and D) Representative western blotting images and related data analyses reveal that treatment with AAV-SIK1 fully blocked the down-regulatory effects of CSDS and CUMS on the levels of BDNF, pTrkB, and pCREB in the hippocampus and mPFC of C57BL/6J mice (n = 5). All results are represented as the means ± S.E.M. values; **P< 0.01; n.s., no significance. The comparisons were made using two-way ANONA followed by Bonferroni's test.

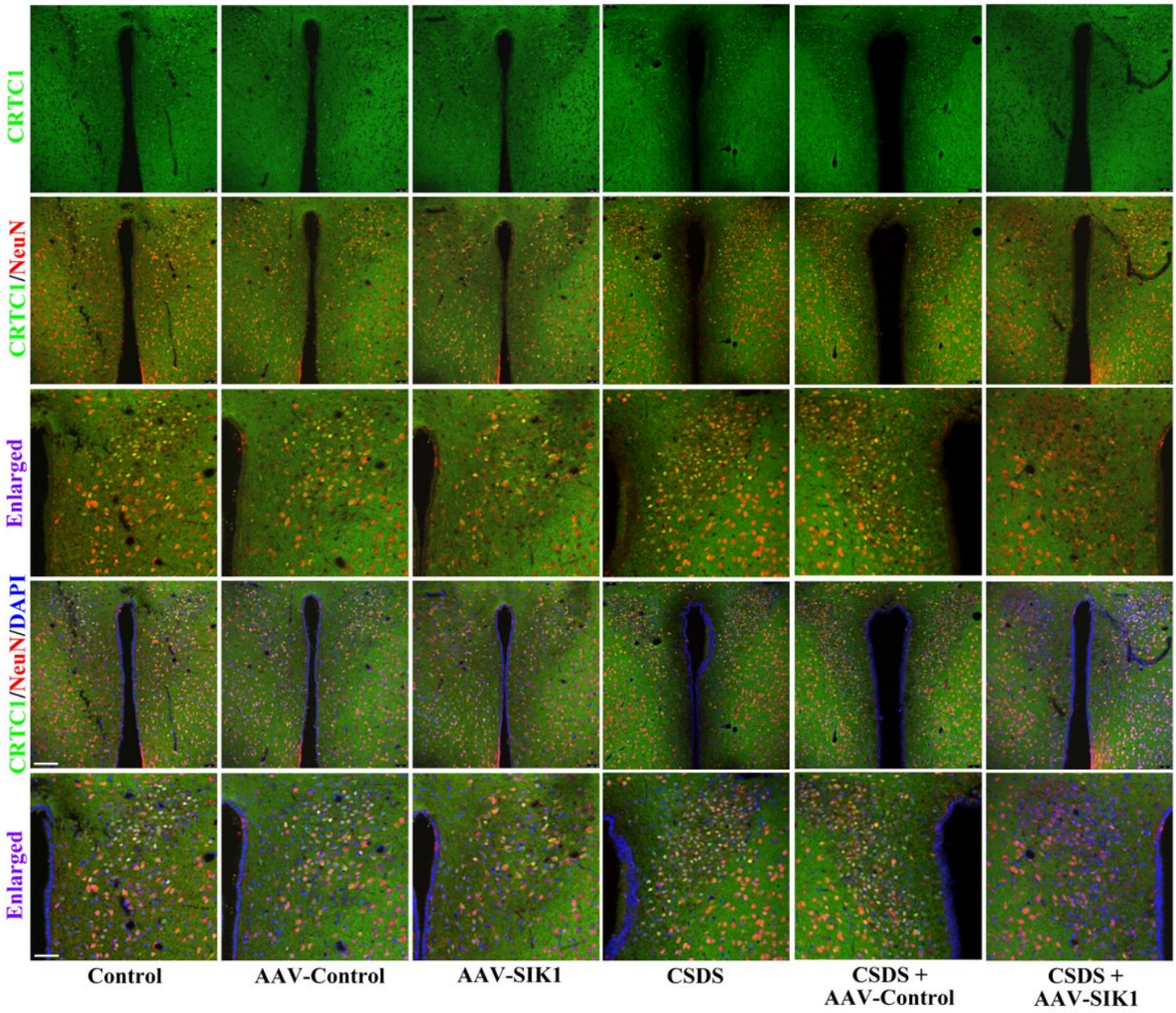

Figure 6

Representative immunofluorescence images showing the effects of CSDS and AAV-SIK1 infusion on the neuronal distribution of CRTC1 in the PVN region of C57BL/6J mice, with NeuN and DAPI as the nuclear markers (n = 5). AAV-SIK1 robustly prevented the promoting effects of CSDS on the nuclear translocation and biosynthesis of CRTC1 in PVN neurons. The scale bar is 100 μm for the representative image and 50 μm for the enlarged image.

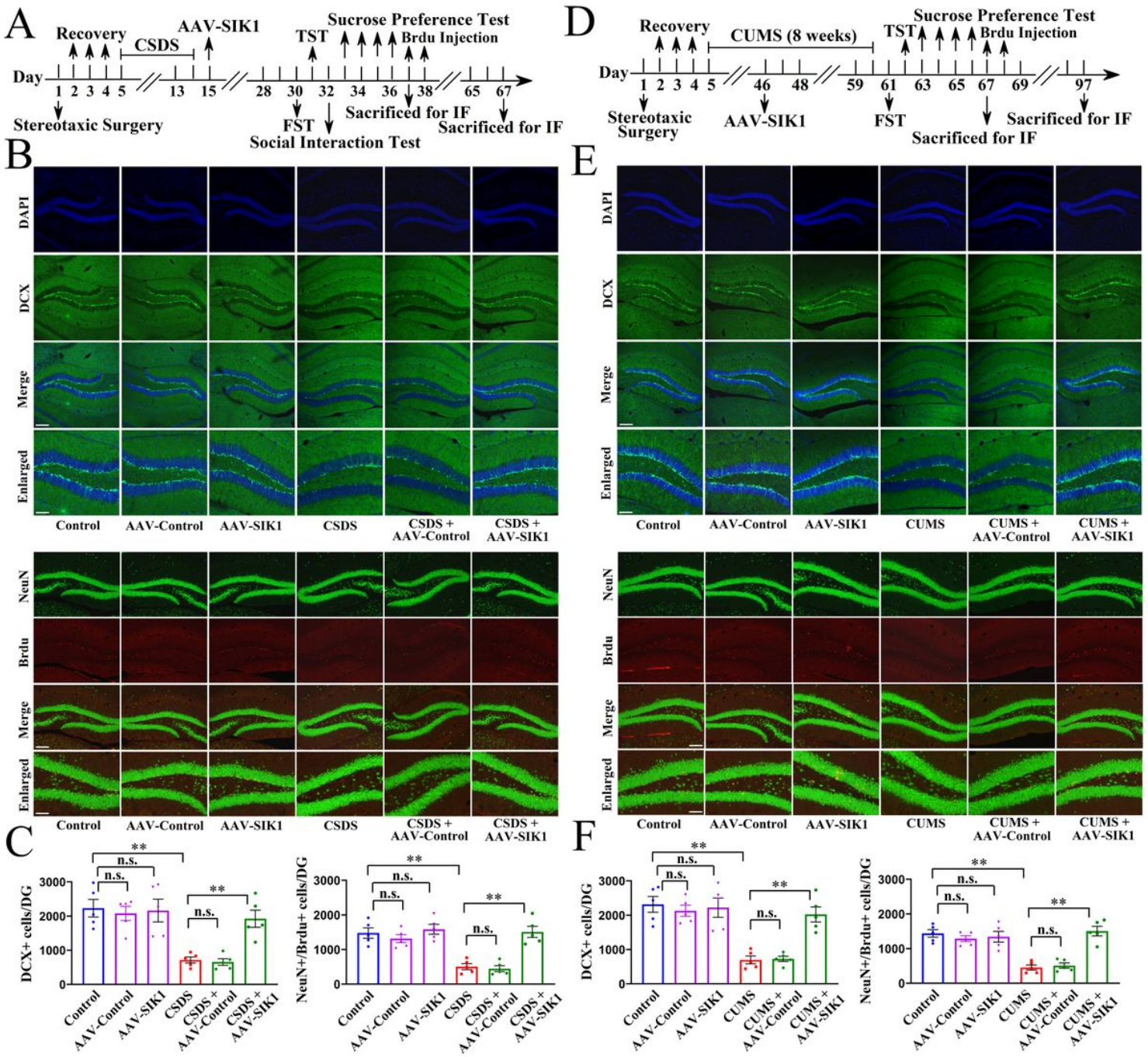

Figure 8

AAV-mediated genetic overexpression of SIK1 in the PVN fully reversed the inhibitory effects of chronic stress on hippocampal neurogenesis. (A, B, and C) Representative immunofluorescence (IF) images and related data analyses display the effects of CSDS and AAV-SIK1 infusion on hippocampal neurogenesis in C57BL/6J mice, as revealed by DCX/DAPI staining and BrdU/NeuN staining in the DG region (n = 5). The scale bars for the representative image and enlarged image are 150 μm and 75 μm, respectively. A schematic timeline of the experimental procedures is provided. (D, E, and F) Representative immunofluorescence images and related data analyses display the effects of CUMS and AAV-SIK1 infusion on hippocampal neurogenesis in C57BL/6J mice, as revealed by DCX/DAPI staining and

BrdU/NeuN staining in the DG region (n = 5). The scale bars for the representative image and enlarged image are 150 μm and 75 μm, respectively. A schematic timeline of the experimental procedures is also provided. All results are represented as the means ± S.E.M. values; **P< 0.01; n.s., no significance. The comparisons were made using two-way ANONA followed by Bonferroni's test.

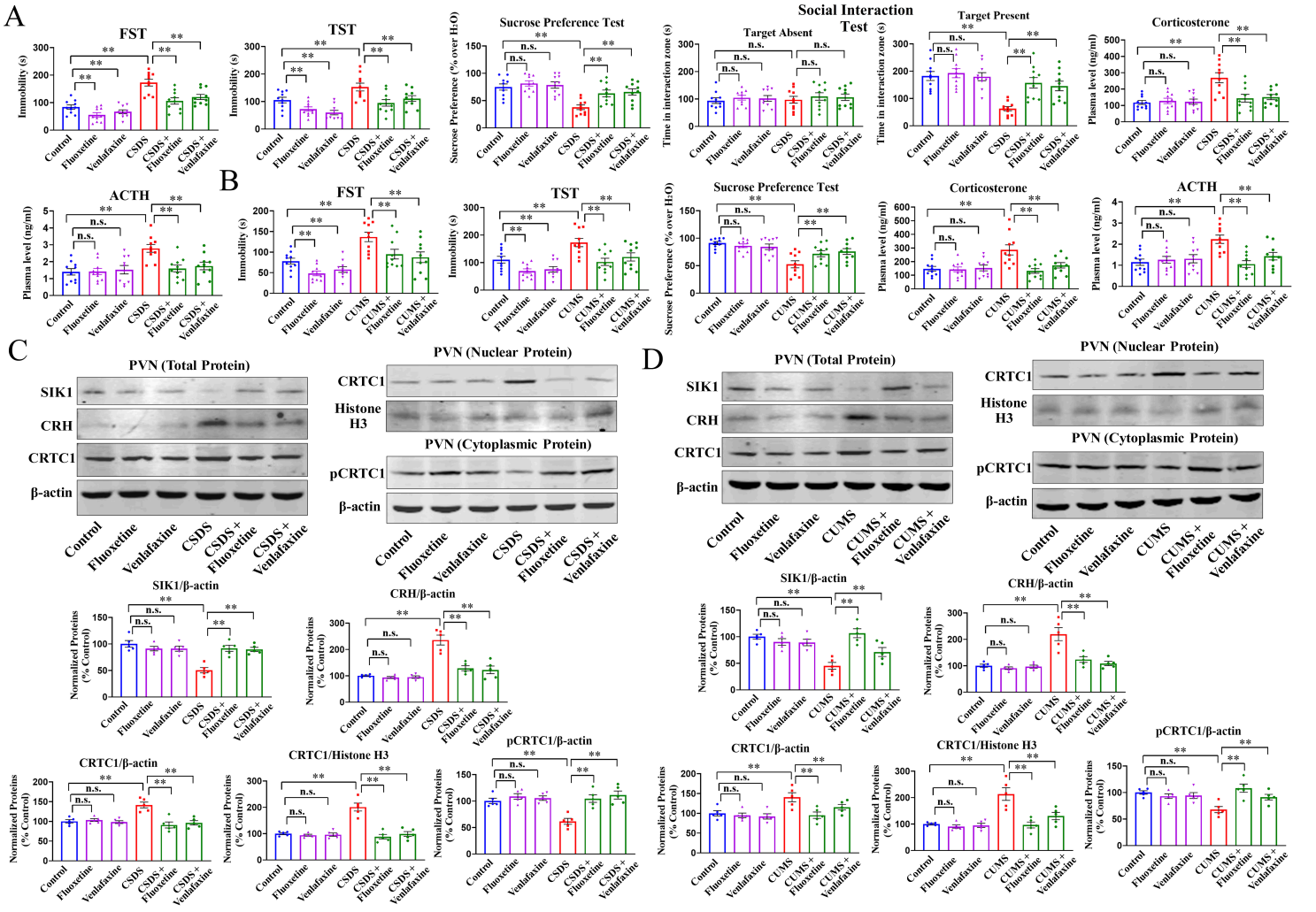

Figure 9

Repeated administration of fluoxetine and venlafaxine notably reversed the effects of chronic stress on the SIK1-CRTC1-CRH pathway in the PVN. (A and B) The results of the FST, TST, sucrose preference test, social interaction test, and ELISA together show that i.p. injection of fluoxetine and venlafaxine for 14 d produced significant reversal effects on both the CSDS-induced and CUMS-induced depression-like behaviours and HPA hyperactivity in C57BL/6J mice (n = 10). (C and D) Representative western blotting images and related data analyses suggest that repeated injection of fluoxetine and venlafaxine fully abolished the effects of both CSDS and CUMS on the expression of SIK1, CRH, nuclear CRTC1, cytoplasmic pCRTC1, and total CRTC1 in the PVN of C57BL/6J mice (n = 5). All results are represented as the means ± S.E.M. values; **P< 0.01; n.s., no significance. The comparisons were made using two-way ANONA followed by Bonferroni's test.

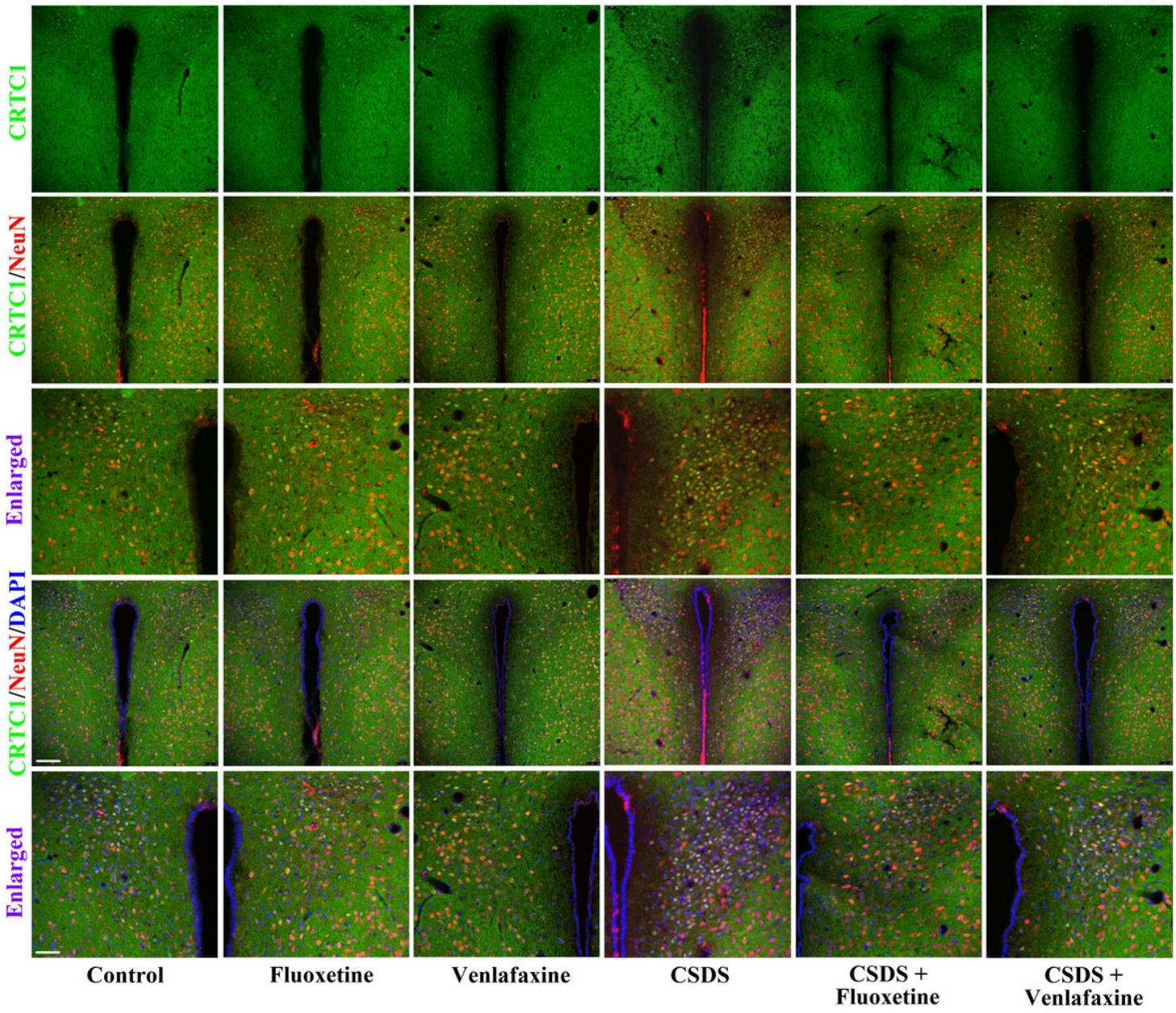

Figure 10

Representative immunofluorescence images showing the effects of CSDS, fluoxetine, and venlafaxine on the neuronal distribution of CRTC1 in the PVN region of C57BL/6J mice, with NeuN and DAPI as the nuclear markers (n = 5). Fluoxetine and venlafaxine notably attenuated the action of CSDS in promoting the nuclear translocation and biosynthesis of CRTC1 in PVN neurons. The scale bar is 100 μm for the representative image and 50 μm for the enlarged image.

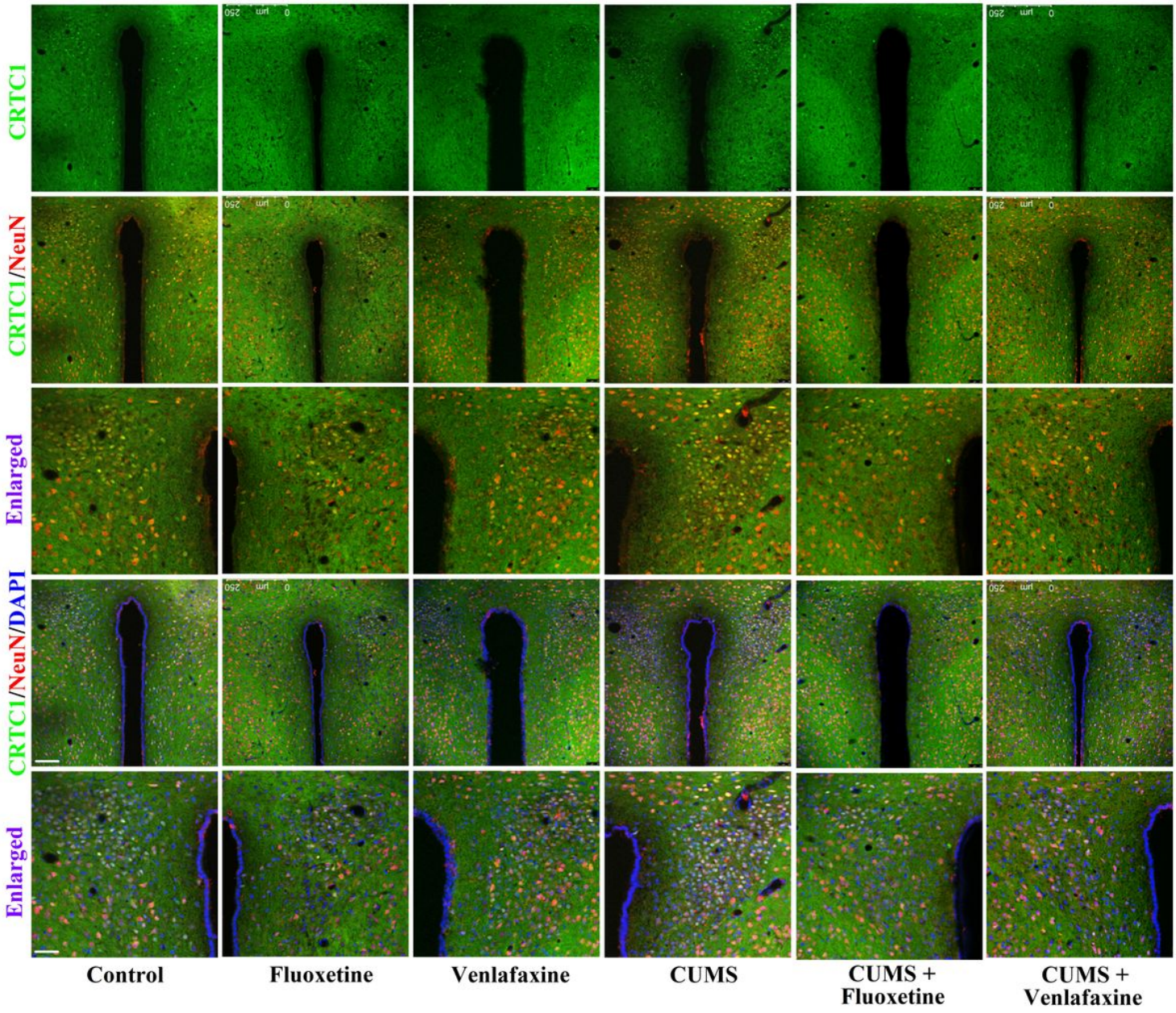

Figure 11

Representative immunofluorescence images showing the effects of CUMS, fluoxetine, and venlafaxine on the neuronal distribution of CRTC1 in the PVN region of C57BL/6J mice, with NeuN and DAPI as the nuclear markers (n = 5). Fluoxetine and venlafaxine significantly ameliorated the enhancing effects of CUMS on the nuclear translocation and biosynthesis of CRTC1 in PVN neurons. The scale bar is 100 μm for the representative image and 50 μm for the enlarged image.

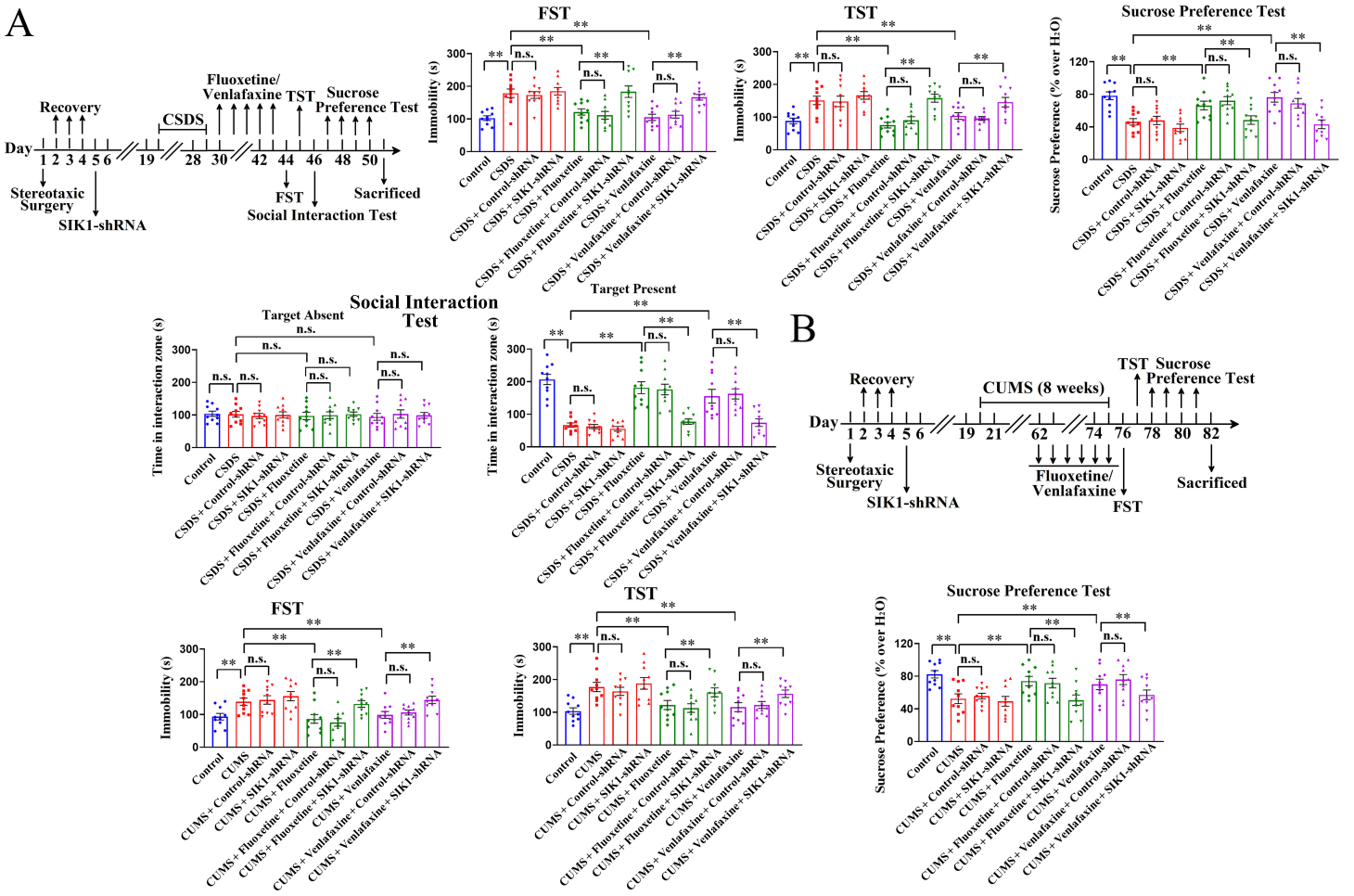

Figure 12

Knockdown of SIK1 in the PVN abrogated the antidepressant-like effects of fluoxetine and venlafaxine in mouse models of depression. (A) The results of the FST, TST, sucrose preference test, and social interaction test collectively suggest that the usage of SIK1-shRNA markedly blocked the reversal effects of fluoxetine and venlafaxine on the CSDS-induced depression-like behaviours in C57BL/6J mice (n = 10). A schematic timeline of the experimental procedures is provided. (B) The usage of SIK1-shRNA also evidently antagonized the protective effects of fluoxetine and venlafaxine against CUMS-induced depression-like behaviours in C57BL/6J mice, as shown by the FST, TST, and sucrose preference test (n = 10). A schematic timeline of the experimental procedures is also provided. All results are represented as the means ± S.E.M. values; ***P*< 0.01; n.s., no significance. The comparisons were made using three-way ANONA followed by Bonferroni's test.

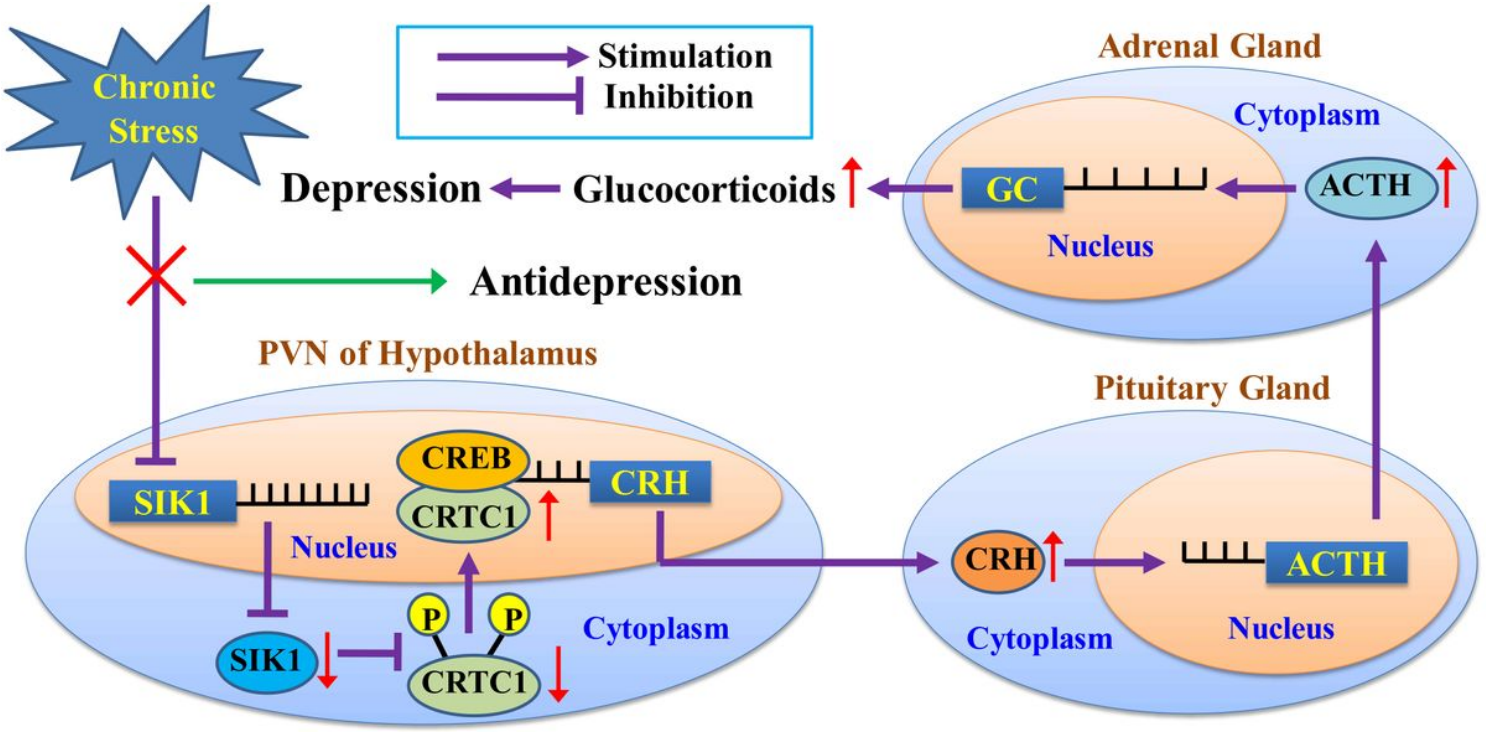

Figure 13

Schematic representation of a suggested model describing the role of SIK1-CRTC1 signalling in PVN neurons in chronic stress-induced depression is provided. Chronic stress significantly decreased the mRNA and protein expression of SIK1 in the PVN, which dephosphorylates cytoplasmic CRTC1 and promotes its nuclear translocation, leading to increased CRTC1-CREB binding in PVN neurons. Due to the enhancement in CRTC1-CREB supporting, the levels of CRH biosynthesis in the PVN, ACTH biosynthesis in the pituitary gland, and glucocorticords biosynthesis in the adrenal gland are greatly up-regulated, resulting in depression. Blockade of the chronic stress-induced effects on SIK1 and CRTC1 in PVN neurons produces antidepressant actions.

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