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Archival Report

Hippocampal Salt-Inducible Kinase 2 Plays a Role in Depression via the CREB-Regulated Transcription Coactivator 1–cAMP Response Element Binding–Brain-Derived Neurotrophic Factor Pathway

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ABSTRACT

BACKGROUND: Developing novel pharmacological targets beyond monoaminergic systems is now a popular strategy for finding new ways to treat depression. Salt-inducible kinase (SIK) is a kinase that regulates the nuclear translocation of cyclic adenosine monophosphate response element binding protein (CREB)-regulated transcription coactivator (CRTC) by phosphorylation. Here, we hypothesize that dysfunction of the central SIK-CRTC system may contribute to the pathogenesis of depression.

METHODS: Chronic social defeat stress (CSDS) and chronic unpredictable mild stress (CUMS) models of depression, various behavioral tests, viral-mediated gene transfer, Western blotting, coimmunoprecipitation, quantitative real-time reverse transcription polymerase chain reaction, and immunohistochemistry were used in this study (for in vivo studies, $n = 10$; for in vitro studies, $n = 5$).

RESULTS: Both CSDS and CUMS markedly increased the expression of hippocampal SIK2, which reduced CRTC1 nuclear translocation and binding of CRTC1 and CREB in the hippocampus. Genetic overexpression of hippocampal SIK2 in naïve mice simulated chronic stress, inducing depressive-like behaviors in the forced swim test, tail suspension test, sucrose preference test, and social interaction test, as well as decreasing the brain-derived neurotrophic factor signaling cascade and neurogenesis in the hippocampus. In contrast, genetic knockdown and knockout of hippocampal SIK2 protected against CSDS and CUMS, exerting significant antidepressant-like effects that were mediated via the downstream CRTC1-CREB–brain-derived neurotrophic factor pathway. Moreover, fluoxetine, venlafaxine, and mirtazapine all significantly restored the effects of CSDS and CUMS on the hippocampal SIK2-CRTC1 pathway, which was necessary for their antidepressant actions.

CONCLUSIONS: The hippocampal SIK2-CRTC1 pathway is involved in the pathogenesis of depression, and hippocampal SIK2 could be a novel target for the development of antidepressants.

Keywords: Brain-derived neurotrophic factor, Cyclic AMP response element binding protein, CREB-regulated transcription coactivator 1, Depression, Hippocampus, Salt-inducible kinase 2

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Depression is a common mental disorder (1), affecting approximately 17% of the general population in the United States (2). Chronic stress can precipitate depression in susceptible individuals, but the underlying molecular pathogenesis in the brain remains elusive (3,4). Similarly, although current antidepressants (e.g., selective serotonin reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors) provide relief for many individuals, their pharmacological mechanisms of actions are not completely understood (5,6). In the brain, many regions, including the hippocampus, medial prefrontal cortex (mPFC), hypothalamus, amygdala, ventral tegmental area (VTA), and nucleus accumbens (NAc), are closely implicated in the pathophysiology of depression (3,4,7–9). Among all these regions, the hippocampus is most extensively investigated (10–12). By now, a leading hypothesis of depression pathogenesis suggests that cyclic adenosine monophosphate (cAMP) response element binding protein (CREB) and brain-derived neurotrophic factor (BDNF) play important roles in the adaptation of the hippocampus to chronic stress and antidepressants (3,4,6,7). As a crucial

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transcription factor in the brain, CREB controls the biosynthesis of many pro-survival proteins, including BDNF (13). BDNF also induces the phosphorylation and activation of CREB (14–16). An increasing number of findings have demonstrated that chronic stress attenuated CREB activation and BDNF expression in the hippocampus of rodents, which could be reversed by antidepressant treatments (17–19). Deficiency of either CREB or BDNF in the hippocampus renders rodents susceptible to depression, whereas activation of the hippocampal BDNF-CREB signaling cascade shows antidepressant-like effects in models of depression (20–23). Also, depression is accompanied by increases in the levels of BDNF and phosphorylated CREB (pCREB) in the NAc, and decreases in their levels the mPFC; these levels can be restored by antidepressant treatments (24–27).

Although the phosphorylation of CREB at the serine 133 (Ser-133) site has been used as a marker for the activation of CREB-mediated gene transcription, the discovery of a family of coactivators (i.e., CREB-regulated transcription coactivators [CRTCs], with three isoforms: CRTC1, CRTC2, and CRTC3) provides new insights into CREB activation (28). The three CRTC isoforms are phosphorylated and sequestered in the cytoplasm under nonstimulated conditions and are translocated into the nucleus when they are dephosphorylated in response to calcium ion and cAMP signals (29,30). The phosphorylation sites of CRTC1 and CRTC2 are Ser-151 and Ser-171, respectively, but that of CRTC3 remains undetermined. The CRTC family proteins bind to the basic leucine zipper domain of CREB and facilitate CREB-mediated gene transcription independent of Ser-133 phosphorylation (31). CRTC1 is abundantly expressed in the brain, and it is closely implicated in hippocampal long-term potentiation, neuron survival, and neurogenesis (32,33). Salt-inducible kinase (three isoforms: SIK1, SIK2, and SIK3) is an enzyme induced by high-salt diet in the rat adrenal glands (34). It has been demonstrated that SIK maintains the phosphorylation level of CRTC (31,35,36). For example, Sasaki *et al.* (37) reported that degradation of cortical SIK2 induced by calcium ion and/or calcium/calmodulin-dependent protein kinase (CaMK) led to the dephosphorylation of CRTC1 and moved CRTC1 from the cytoplasm into the nucleus, thereby activating CREB and its downstream gene targets. Previous reports have documented the expression of SIK in the central nervous system (37–40). However, the correlation between central SIK-CRTC signaling and depression is unknown. Here, we used a multidisciplinary approach to explore the role of central SIK-CRTC signaling in the pathogenesis of depression.

METHODS AND MATERIALS

Ethical Statements

The experimental procedures involving animals and their care were conducted in compliance with the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (41,42) and approved by the Animal Welfare Committee of Nantong University.

Forced Swim Test

A forced swim test (FST) test was performed according to previously published methods (27,43–50). Mice were individually placed for 6 minutes in clear cylinders (45-cm height, 20-cm internal diameter) containing fresh water (25°C ± 1°C, 15-cm depth). The sessions were videorecorded, and the duration of immobility in the last 4 minutes was scored by an investigator unaware of animal grouping. Water was changed after each trial. The mice were considered immobile when they were floating in the water without struggling or were only slightly moving to keep the nose above the surface.

Additional Methods and Materials

See the Supplemental Methods and Materials for descriptions of the tail suspension test (TST), the sucrose preference test, the social interaction test, and the open field test, as well as other details.

RESULTS

Chronic Stress Induced Robust Changes in Hippocampal SIK2-CRTC1 Signaling

As the first step toward characterizing the association between depression and the SIK-CRTC signaling, the chronic social defeat stress (CSDS) and chronic unpredictable mild stress (CUMS) models, two well-validated models of depression (24,51), were used. Both CSDS- and CUMS-exposed mice displayed significant depressive-like behaviors in the FST, TST, sucrose preference test, and social interaction test (Figures 1A and 2A). We then detected SIK1–3 expression in the total protein homogenates of different brain regions after chronic stress exposure. CSDS exposure elevated SIK2 expression in the hippocampus and mPFC by 163.4% ± 9.1% and 31.7% ± 4.8%, respectively, whereas it decreased SIK1 expression in the hypothalamus and VTA by 25.3% ± 3.6% and 21.2% ± 5.6%, respectively (Figure 1B). Similarly, CUMS increased SIK2 expression in the hippocampus and mPFC by 180.2% ± 11.3% and 34.8% ± 5.1%, respectively, but reduced SIK1 expression in the hypothalamus and VTA by 27.8% ± 2.9% and 23.4% ± 4.5%, respectively (Figure 2B). Neither CSDS nor CUMS exposure affected SIK1–3 expression in the amygdala or NAc (Figures 1B and 2B). As such, we focused on hippocampal SIK2 in subsequent studies.

We also evaluated the effects of chronic stress on hippocampal CRTC1–3 levels. Repeated stress exposure significantly decreased the level of nuclear CRTC1 in the hippocampus, but that of nuclear CRTC2 or CRTC3 was not affected (Figure 3A, C). CSDS and CUMS exposure significantly increased the level of cytoplasmic phosphorylated CRTC1 (pCRTC1) in the hippocampus, with that of pCRTC2 unchanged (Figure 3A, C). Interestingly, the total protein level of hippocampal CRTC1 in stressed mice was also significantly lower than that of control mice, whereas neither total CRTC2 nor CRTC3 level was decreased (Figure 3B, D).

CSDS and CUMS upregulated the SIK2 messenger RNA (mRNA) level by 141.4% ± 7.8% and 165.2% ± 9.6%, respectively (Supplemental Figure S1A, B). In contrast, CSDS and CUMS exposure reduced the CRTC1 mRNA level by 49.5% ± 4.3% and 51.1% ± 3.7%, respectively

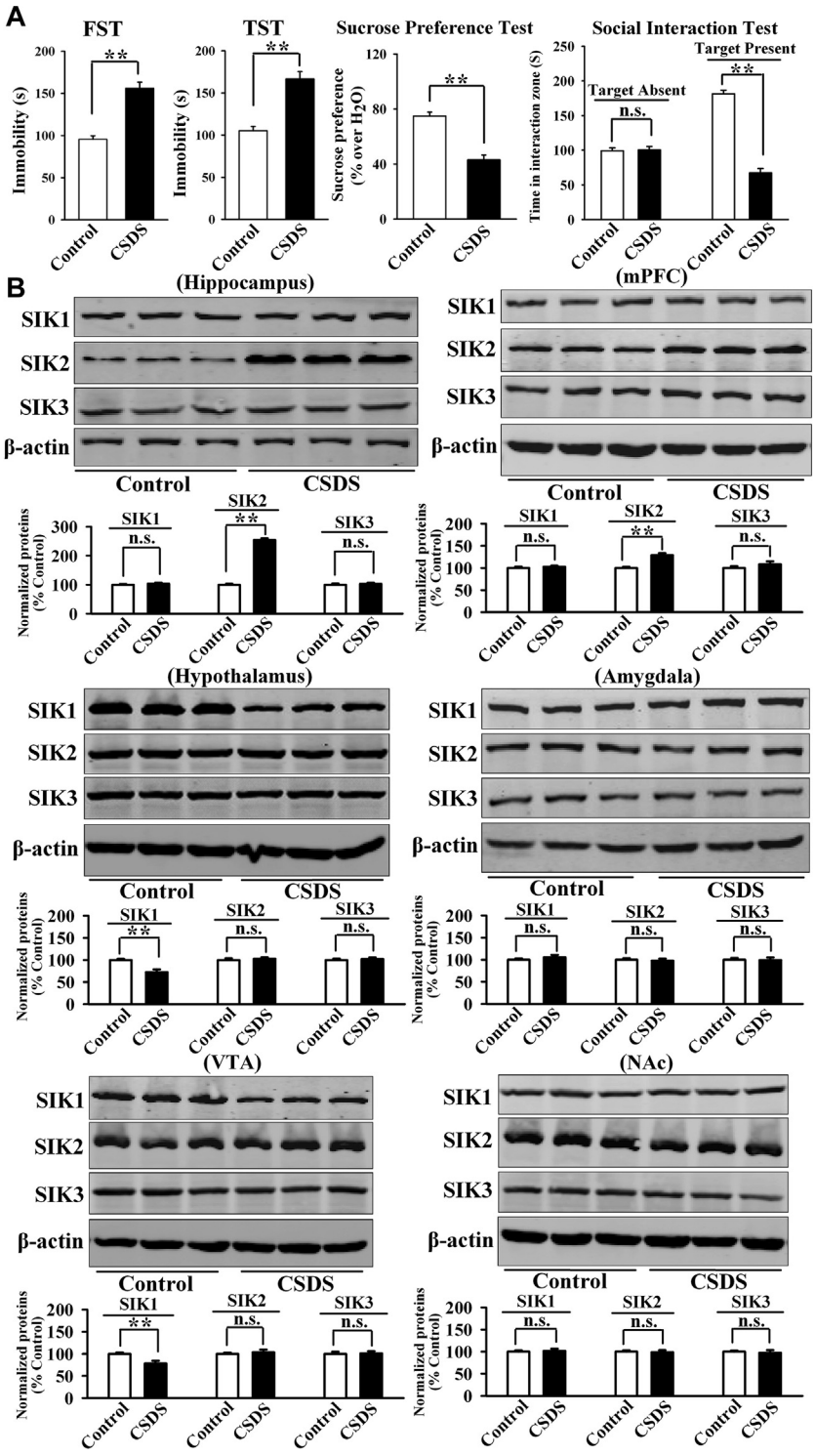

Figure 1. Chronic social defeat stress (CSDS) robustly increased salt-inducible kinase 2 (SIK2) protein expression in the hippocampus. **(A)** CSDS induced notable depressive-like behaviors in C57BL/6J mice, which manifested as helplessness, anhedonia, and social avoidance (n = 10). **(B)** Representative Western blotting images show the effects of CSDS on SIK1–SIK3 protein expression in different brain regions, including the hippocampus, medial prefrontal cortex (mPFC), hypothalamus, amygdala, ventral tegmental area (VTA), and nucleus accumbens (NAc) (n = 5). All results are represented as mean ± SEM. Comparisons were made by t test. $^{**}p < .01$. FST, forced swim test; n.s., no significance; TST, tail suspension test.

(Supplemental Figure S1A, B). We then examined the binding of nuclear CRTC1 and CREB. Supplemental Figure S1D, E shows that repeated stress fully downregulated the association between hippocampal CRTC1 and CREB, consistent with the stress-induced changes in nuclear CRTC1 level. Taken together, these findings indicate that hippocampal SIK2-CRTC1 signaling was closely involved in the pathogenesis of depression.

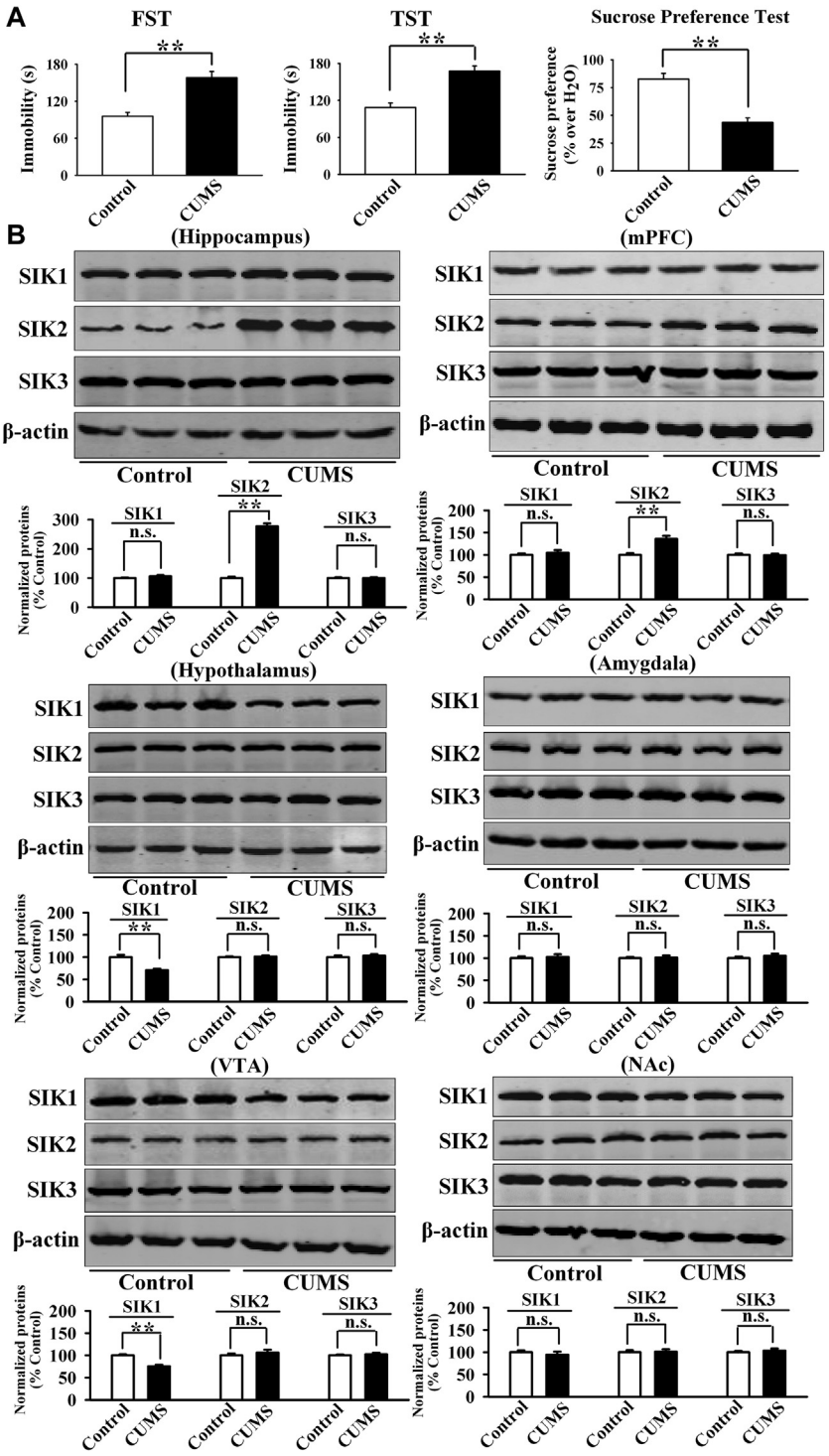

Figure 2. Chronic unpredictable mild stress (CUMS) substantially promoted salt-inducible kinase 2 (SIK2) protein expression in the hippocampus. **(A)** CUMS induced significant depressive-like behaviors in C57BL/6J mice, as revealed by the forced swim test (FST), the tail suspension test (TST), and the sucrose preference test (n = 10). **(B)** Representative Western blotting images show the effects of CUMS on SIK1–SIK3 protein expression in the hippocampus, medial prefrontal cortex (mPFC), hypothalamus, amygdala, ventral tegmental area (VTA), and nucleus accumbens (NAc) (n = 5). All results are represented as mean ± SEM. Comparisons were made by t test. $^{**}p < .01$. n.s., no significance.

Genetic Overexpression of Hippocampal SIK2 Induced Depressive-like Effects in Mice

Because chronic stress increased hippocampal SIK2 expression, we then studied whether hippocampal SIK2 overexpression in naïve mice would induce depressive-like behaviors. An adeno-associated virus (AAV) vector that selectively expresses SIK2 with enhanced green fluorescent protein (EGFP) (AAV-SIK2-EGFP) was generated. AAV-SIK2 or AAV-Control was stereotaxically infused into bilateral hippocampi of naïve mice, and after 14 days, numerous EGFP-positive cells were found and enhanced

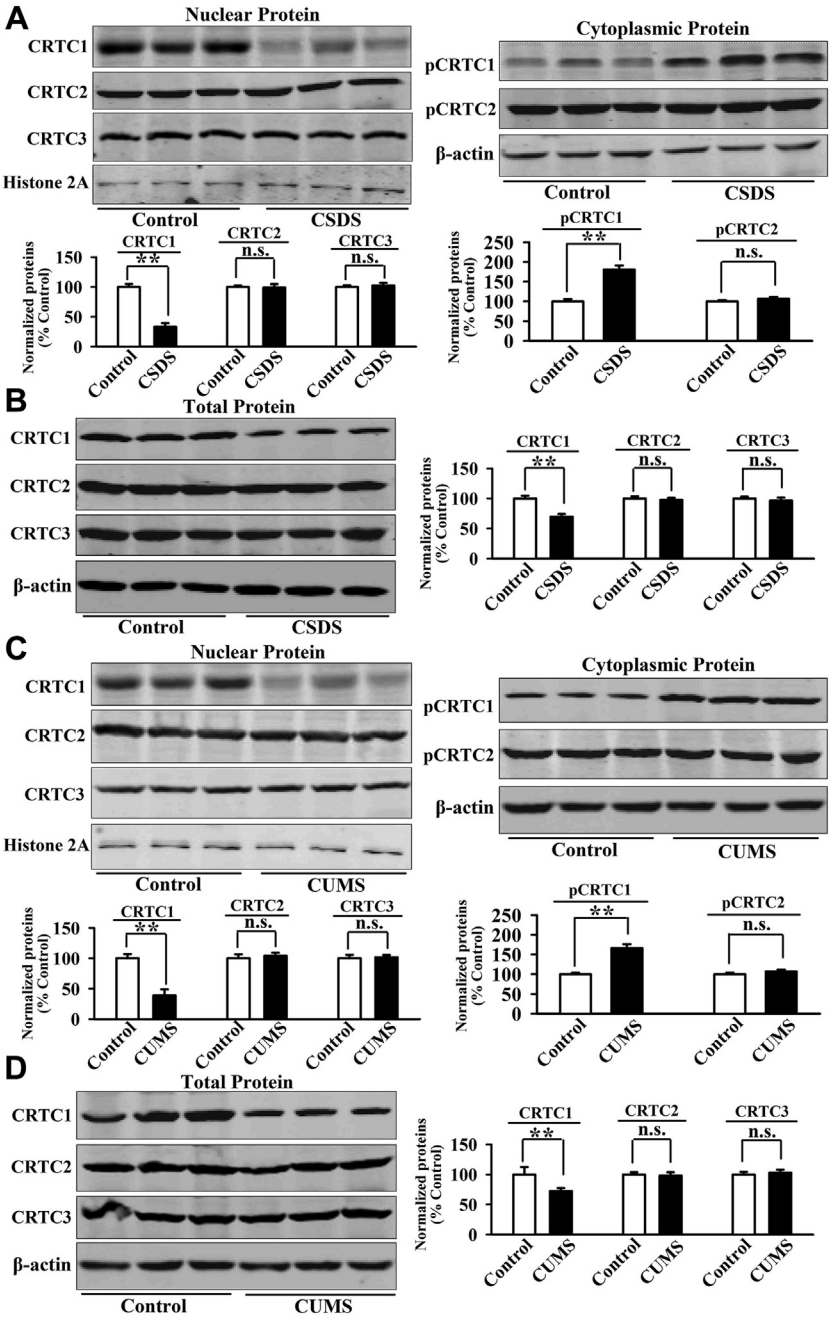

Figure 3. Chronic stress markedly decreased the levels of nuclear cyclic adenosine monophosphate response element binding protein–regulated transcription coactivator 1 (CRTC1) and total CRTC1 but increased that of cytoplasmic phosphorylated CRTC1 (pCRTC1) in the hippocampus. **(A, B)** Representative Western blotting images show the effects of chronic social defeat stress (CSDS) on nuclear CRTC1–CRTC3, cytoplasmic pCRTC1 and pCRTC2, and total CRTC1–CRTC3 expression in the hippocampus (n = 5). **(C, D)** Representative Western blotting images showed the effects of chronic unpredictable mild stress (CUMS) on nuclear CRTC1–CRTC3, cytoplasmic pCRTC1 and pCRTC2, and total CRTC1–CRTC3 expression in the hippocampus (n = 5). All results are represented as mean ± SEM. Comparisons were made by t test. $^{**}p < .01$. n.s., no significance.

SIK2 expression was observed (Figure 4A). In behavioral tests, hippocampal SIK2 overexpression significantly increased the immobility of mice in both the FST and TST compared with that of the control group, exerting a depressive-like effect (Figure 4B). To exclude the possible effects of SIK2 overexpression on spontaneous locomotor activity, which may confound the immobility results in the FST and TST, an open field test was also 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 4B). Also, mice with SIK2 overexpression had significantly less sucrose preference and social interaction than those of control mice (Figure 4B). In contrast, AAV-Control did not alter the mouse behaviors.

In addition, SIK2 overexpression not only decreased the levels of nuclear CRTC1 (Figure 4C), total CRTC1 (Figure 4D), and CRTC1-CREB binding (Figure 4E), but also increased the level of cytoplasmic pCRTC1 (Figure 4C) in the hippocampus. Depression is accompanied by an attenuated BDNF signaling cascade and decreased neurogenesis in the hippocampus (3,7,12). Interestingly, AAV-SIK2–treated mice had significantly lower protein expression of hippocampal BDNF, phosphorylated tyrosine receptor kinase B (pTrkB),

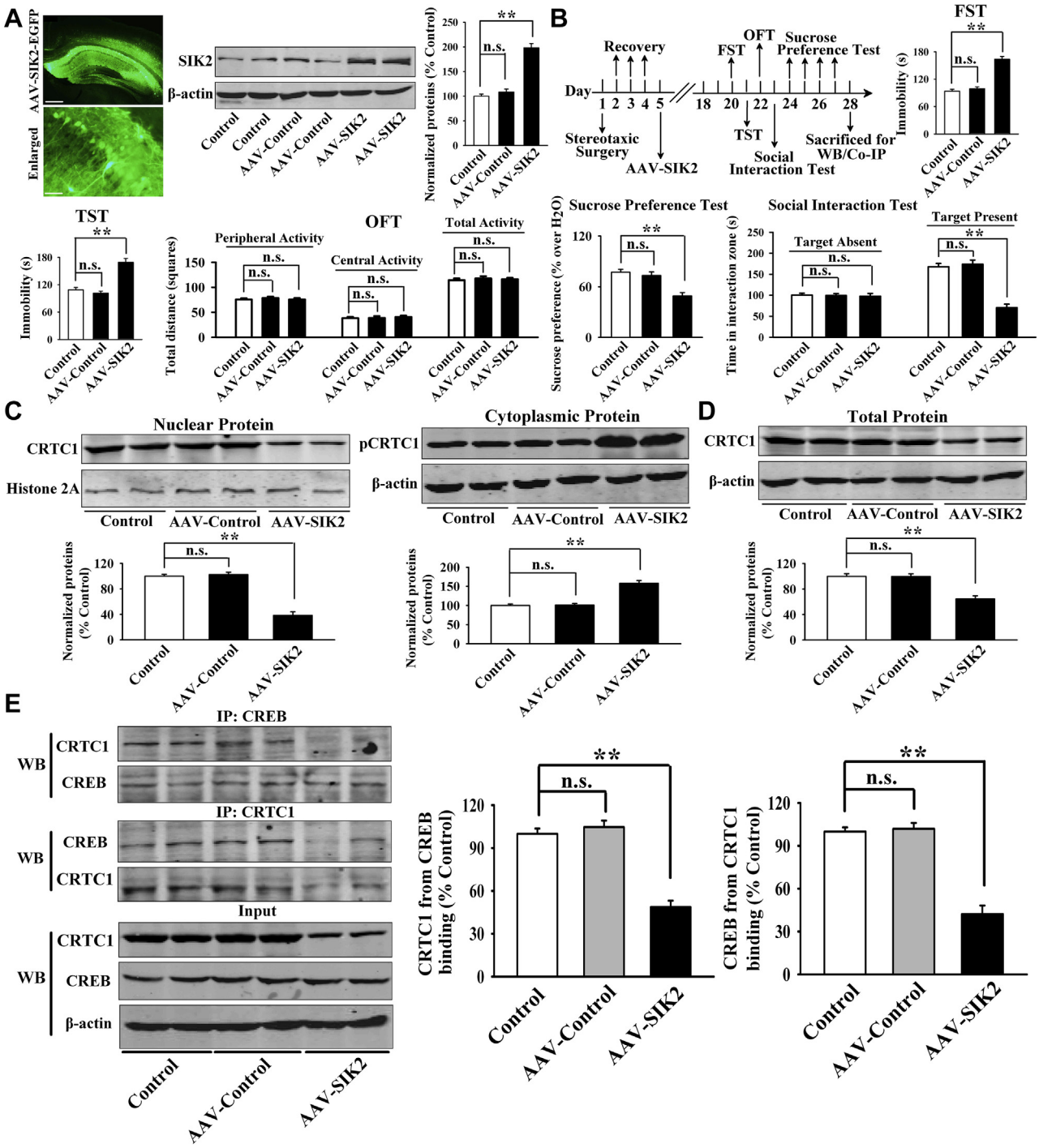

Figure 4. Hippocampal salt-inducible kinase 2 (SIK2) overexpression by an adeno-associated virus (AAV) vector that selectively expresses SIK2 with enhanced green fluorescent protein (AAV-SIK2-EGFP) induced various depressive-like symptoms in mice. **(A)** Fluorescence image of a fixed brain section that expressed AAV-SIK2-EGFP in the hippocampus 14 days after infusion. Scale bar = 400 μm for the representative image. Scale bar = 50 μm for the enlarged image. Western blotting (WB) results confirmed the overexpression efficacy of AAV-SIK2 (n = 5). (**B**) AAV-SIK2 infusion induced notable depressive-like behaviors in the forced swim test (FST), tail suspension test (TST), sucrose preference test, and social interaction test, without affecting the locomotor activity (n = 10). **(C, D)** AAV-SIK2 infusion significantly decreased the levels of nuclear cyclic adenosine monophosphate response element binding protein (CREB)–regulated transcription coactivator 1 (CRTC1) and total CRTC1, whereas it elevated that of cytoplasmic phosphorylated CRTC1 (pCRTC1) in the hippocampus (n = 5). **(E)** AAV-SIK2 infusion also fully downregulated the level of hippocampal CRTC1-CREB (n = 5). All results are represented as mean ± SEM. Comparisons were made by one-way analysis of variance followed by Tukey's test. **$p < .01$. co-IP, coimmunoprecipitation; n.s., no significance; OFT, open field test.

phosphorylated extracellular signal-regulated kinase 1 and 2 (pERK1/2), phosphorylated protein kinase B (pAKT), phosphorylated CaMK IV (pCaMKIV), and pCREB than those of control mice, while the total hippocampal β-actin, TrkB, ERK1/2, AKT, CaMKIV and CREB levels of each group were unchanged (Supplemental Figure S2). Moreover, AAV-SIK2–treated mice had significantly fewer doublecortin-labeled cells and fewer cells colabeled with $NeuN^+$ and 5-bromo-2′-deoxyuridine in the dentate gyrus than those of control mice (Supplemental Figure S3), suggesting that SIK2 overexpression fully downregulated hippocampal neurogenesis. In contrast, AAV-Control did not affect the hippocampal SIK2-CRTC1 system, BDNF signaling, or neurogenesis. Taken together, these findings suggest that hippocampal SIK2-overexpression contributed to depression.

Genetic Knockdown of Hippocampal SIK2 Produced Antidepressant-like Effects in Mice

To test whether averting chronic stress–induced high SIK2 expression in the hippocampus prevented depression, we used AAV-mediated expression of specific short hairpin RNAs (shRNAs) against SIK2. SIK2-shRNA demonstrated clear silencing efficacy (Figure 5A). Both CSDS- and CUMS-exposed mice were intrahippocampally infused with SIK2-shRNA or control-shRNA, and behavioral tests were conducted 14 days after injection. Figure 5C shows that CSDS-exposed mice treated with SIK2-shRNA had significantly increased sucrose preference and social interaction, as well as reduced immobility in the FST and TST, than those of CSDS-exposed mice and CSDS-exposed mice treated with control-shRNA. Figure 5D shows that CUMS-exposed mice treated with SIK2-shRNA also displayed evidently higher sucrose preference and less immobility in the FST and TST than those of CUMS-exposed mice and CUMS-exposed mice treated with control-shRNA. In addition, SIK2-shRNA significantly reduced the immobility of naïve mice in both the FST and TST without affecting their locomotor activities (Figure 5B), further supporting the antidepressant-like actions of hippocampal knockdown SIK2 (SIK2-KD). In contrast, control-shRNA treatment did not influence the mouse behaviors.

Next, the hippocampal SIK2-CRTC1 signaling changes of all groups were detected. SIK2-shRNA treatment not only restored the CSDS-induced increase in hippocampal SIK2 (Supplemental Figure S4A) and cytoplasmic pCRTC1 (Supplemental Figure S4B) expression but also reversed the CSDS-induced decrease in hippocampal level of total CRTC1 (Supplemental Figure S4A), nuclear CRTC1 level (Supplemental Figure S4B), and CRTC1-CREB binding level (Supplemental Figure S4C). Similarly, SIK2-shRNA treatment completely restored CUMS-induced effects on hippocampal SIK2 (Supplemental Figure S5A), total CRTC1 (Supplemental Figure S5A), nuclear CRTC1 (Supplemental Figure S5B), cytoplasmic pCRTC1 (Supplemental Figure S5B), and CRTC1-CREB binding (Supplemental Figure S5C). In addition, SIK2-shRNA treatment decreased the level of cytoplasmic pCRTC1 (Supplemental Figures S4B and S5B) and increased the levels of CRTC1 (Supplemental Figure S4A and S5A), nuclear CRTC1 (Supplemental Figures S4B and S5B), and CRTC1-CREB binding (Supplemental Figures S4C and S5C) in the hippocampus of naïve mice. Control-shRNA treatment did not affect the hippocampal SIK2-CRTC1 signaling.

The hippocampal BDNF signaling cascade and neurogenesis among all groups were also examined. SIK2-shRNA treatment not only blocked CSDS- and CUMS-induced decreases in hippocampal BDNF, pTrkB, pERK1/2, pAKT, pCaMKIV and pCREB expression but also enhanced these proteins in naïve mice (Supplemental Figure S6). However, the total hippocampal β-actin, TrkB, ERK1/2, AKT, CaMKIV, and CREB levels did not change. Similarly, SIK2-shRNA treatment markedly prevented CSDS- and CUMS-induced decreases in hippocampal neurogenesis (Supplemental Figures S7 and S8). Control-shRNA treatment did not affect the hippocampal BDNF signaling or neurogenesis. Collectively, reversing chronic stress–induced high SIK2 expression in the hippocampus produced significant antidepressant-like effects.

Genetic Knockout of SIK2 Protected Against Depressive-like Behavior in Mice

Afterwards, SIK2 knockout (SIK2-KO) mice were used, and their validity was confirmed (Supplemental Figure S9). Both wild-type (WT) and SIK2-KO mice were subjected to the FST and TST. As shown in Figure 6A, SIK2-KO mice were much less immobile than WT mice in both tests, suggesting an antidepressant phenotype. The results of an open field test showed that SIK2-KO did not affect the locomotor activity of mice (Figure 6A). Both types of mice were then subjected to CSDS, followed by behavioral tests. Unlike in WT mice with apparent depressive-like behaviors, in SIK2-KO mice CSDS-induced increase of immobility and decrease of sucrose preference and social interaction were completely abolished (Figure 6B). Also, the mice were subjected to CUMS, followed by behavioral tests. Figure 6C shows that while CUMS markedly enhanced the immobility and decreased the sucrose preference of WT mice, these behavioral changes were not observed in SIK2-KO mice.

Next, the hippocampal SIK2-CRTC1 signaling among all groups were investigated. Compared with WT mice, SIK2-KO mice showed effects that were CSDS-induced in WT mice—increase in cytoplasmic pCRTC1 level (Supplemental Figure S10A) as well as decreases in nuclear CRTC1 level (Supplemental Figure S10A), total CRTC1 level (Supplemental Figure S10B), and binding to CRTC1 and CREB (Supplemental Figure S10C) in the hippocampus—to be completely abolished. Also, similar results were observed in the CUMS model of depression (Supplemental Figure S11). Interestingly, under normal conditions, SIK2-KO mice still had significantly less cytoplasmic pCRTC1 (Supplemental Figures S12A and S13A) as well as more nuclear CRTC1, more total CRTC1, and more CRTC1-CREB binding in the hippocampus than WT mice had (Supplemental Figures S10 and S11).

Furthermore, the hippocampal BDNF signaling pathway and neurogenesis of each group were examined. Supplemental Figure S12 showed that SIK2-KO not only prevented CSDS- and CUMS-induced decreases in hippocampal BDNF, pTrkB, pERK1/2, pAKT, pCaMKIV, and pCREB expression but also augmented the hippocampal BDNF signaling in WT mice. The total hippocampal levels of β-actin, TrkB, ERK1/2, AKT, CaMKIV, and CREB remained unaltered. Similarly, compared with WT mice, SIK2-KO mice showed fully abolished

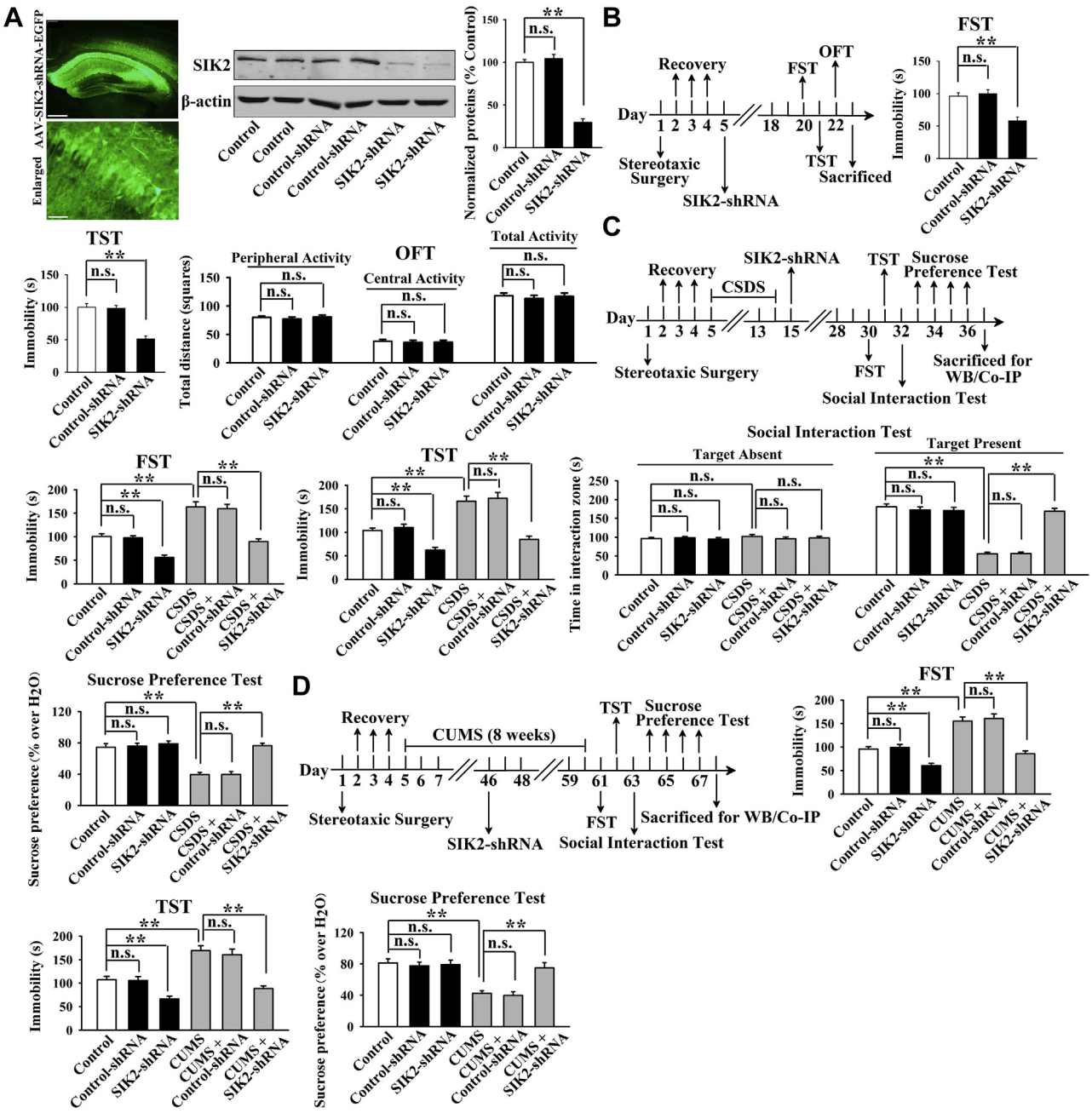

Figure 5. Hippocampal salt-inducible kinase 2 knockdown (SIK2-KD) by using an adeno-associated virus (AAV) vector that selectively expresses SIK2–short hairpin RNA with enhanced green fluorescent protein (AAV-SIK2-shRNA-EGFP) exerted significant antidepressant-like effects on mice. **(A)** Fluorescence image of a fixed brain section expressing AAV-SIK2-shRNA-EGFP in the hippocampus 14 days after infusion. Scale bar = 400 μm for the representative image. Scale bar = 50 μm for the enlarged image. Western blotting (WB) confirmed the silencing efficacy of AAV-SIK2-shRNA (n = 5). **(B)** SIK2-shRNA infusion remarkably decreased the immobility of naïve mice in the forced swim test (FST) and tail suspension test (TST) without affecting their locomotor activities (n = 10). **(C)** SIK2-shRNA infusion completely reversed chronic social defeat stress (CSDS)–induced depressive-like behaviors in the FST, TST, sucrose preference test, and social interaction test (n = 10). **(D)** SIK2-shRNA infusion also fully restored chronic unpredictable mild stress (CUMS)–induced depressive-like behaviors in the FST, TST, and sucrose preference test (n = 10). All results are represented as mean ± SEM. For panels **(A)** and **(B)**, comparisons were made by one-way analysis of variance followed by Tukey's test. For panels **(C)** and **(D)**, comparisons were made by two-way analysis of variance followed by Bonferroni's test. $^{**}p < .01$. co-IP, coimmunoprecipitation; n.s., no significance; OFT, open field test.

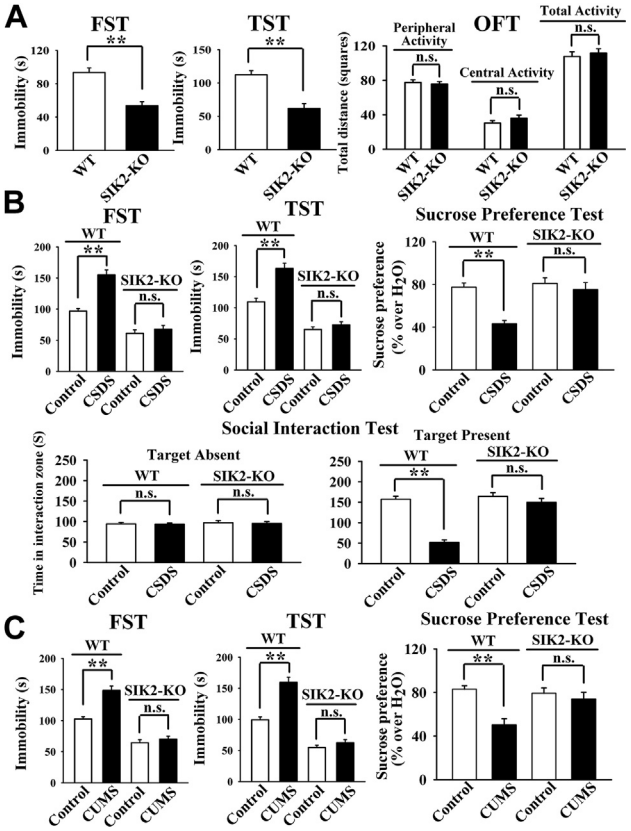

Figure 6. Salt-inducible kinase 2 knockout (SIK2-KO) led to mouse resistance against chronic stress. **(A)** SIK2-KO notably reduced the immobility of mice in the forced swim test (FST) and tail suspension test (TST) without influencing their locomotor activities (n = 10). **(B)** Wild-type (WT) mice were susceptible to chronic social defeat stress (CSDS), while SIK2-KO completely abolished CSDS-induced depressive-like behaviors in the FST, TST, sucrose preference test, and social interaction test (n = 10). **(C)** Also, WT mice were susceptible to chronic unpredictable mild stress (CUMS), whereas SIK2-KO fully prevented CUMS-induced depressive-like behaviors in the FST, TST, and sucrose preference test (n = 10). All results are represented as mean ± SEM. For panel **(A)**, comparisons were made using the t test. For panels **(B)** and **(C)**, comparisons were made by two-way analysis of variance followed by Bonferroni's test. $^{**}p < .01$. n.s., no significance; OFT, open field test.

CSDS- and CUMS-induced decrease in hippocampal neurogenesis (Supplemental Figures S13 and S14). These findings suggest that hippocampal SIK2 could be a therapeutic target for treating depression.

The CRTC1-CREB Pathway Mediates the Antidepressant-like Actions of Hippocampal SIK2-KD

To understand whether the antidepressant actions induced by hippocampal SIK2-KD required CRTC1, AAV-CRTC1-shRNA-EGFP was used, and its efficacy was confirmed (Figure 7A). First, CRTC1-shRNA was infused into the hippocampi of naïve mice, and then SIK2-shRNA was infused; these infusions were followed by the FST and TST. The silencing effects of SIK2-shRNA and CRTC1-shRNA did not interfere with each other (Figure 7C). We found that CRTC1-shRNA infusion alone increased the immobility time of naïve mice in the FST and TST (Figure 7B), consistent with the findings of Meylan *et al.* (52). More importantly, CRTC1-shRNA preinfusion fully abolished the effects of SIK2-shRNA on naïve mice in the FST and TST (Figure 7B). Next, CRTC1-shRNA–pretreated mice were subjected to CSDS and then infused with SIK2-shRNA, after which they underwent behavioral tests. Hippocampal CRTC1-KD significantly prevented the antidepressant-like effects of SIK2-shRNA on CSDS-stressed mice (Figure 7D). Finally, CRTC1-shRNA–pretreated mice were subjected to CUMS and then treated with SIK2-shRNA, after which they underwent behavioral tests. As shown in Figure 7E, hippocampal CRTC1-KD treatment fully abolished the antidepressant-like effects of SIK2-shRNA on CUMS-exposed mice.

To investigate whether the antidepressant-like actions induced by hippocampal SIK2-KD required CREB, AAV-CREB-shRNA-EGFP was used, and its efficacy was confirmed (Supplemental Figure S15A). Naïve mice received hippocampal infusions of CREB-shRNA (first) and SIK2-shRNA (second), and the FST and TST were performed after the infusions. The silencing effects of SIK2-shRNA and CREB-shRNA did not interfere with each other (Supplemental Figure S15C). Consistent with previous reports (53), we found that CREB-shRNA infusion alone increased the immobility time of naïve mice in the FST and TST (Supplemental Figure S15B). CREB-shRNA fully blocked the effects of SIK2-shRNA on naïve mice in the FST and TST (Supplemental Figure S15B). In addition, CREB-shRNA–pretreated mice were subjected to CSDS and received SIK2-shRNA infusion, after which they underwent behavioral tests. As shown in Figure S15D, hippocampal CREB-KD treatment prevents the antidepressant-like effects of SIK2-shRNA on CSDS-exposed mice. Moreover, CREB-shRNA–pretreated mice were subjected to CUMS and received SIK2-shRNA infusion, and such treatment abolished the antidepressant-like effects of SIK2-shRNA on CUMS-exposed mice (Supplemental Figure S15E). Combined, SIK2-shRNA–induced antidepressant-like effects were mediated through the downstream CRTC1-CREB system.

The BDNF Signaling Cascade Was Required for the Antidepressant-like Actions of Hippocampal SIK2-KD

Given that BDNF may underlie the role of hippocampal SIK2-CRTC1 signaling in the pathogenesis of depression, AAV-BDNF-shRNA-EGFP was used. The silencing effects of BDNF-shRNA were shown in Figure 8A. Naïve mice were first infused with BDNF-shRNA and then treated with SIK2-shRNA. SIK2-shRNA and BDNF-shRNA did not interfere with each other (Figure 8C). Although BDNF-shRNA alone increased the immobility time of mice in the FST and the TST (23), its pretreatment significantly prevented the effects of SIK2-shRNA in naïve mice (Figure 8B). Figure 8D revealed that BDNF-shRNA pretreatment fully abolished the antidepressant-like effects of SIK2-shRNA in the CSDS model of depression. Similar results were observed in the CUMS model of depression (Figure 8E).

In a parallel experiment, AAV-TrkB-shRNA-EGFP was used (its efficacy is shown in Supplemental Figure S16A).

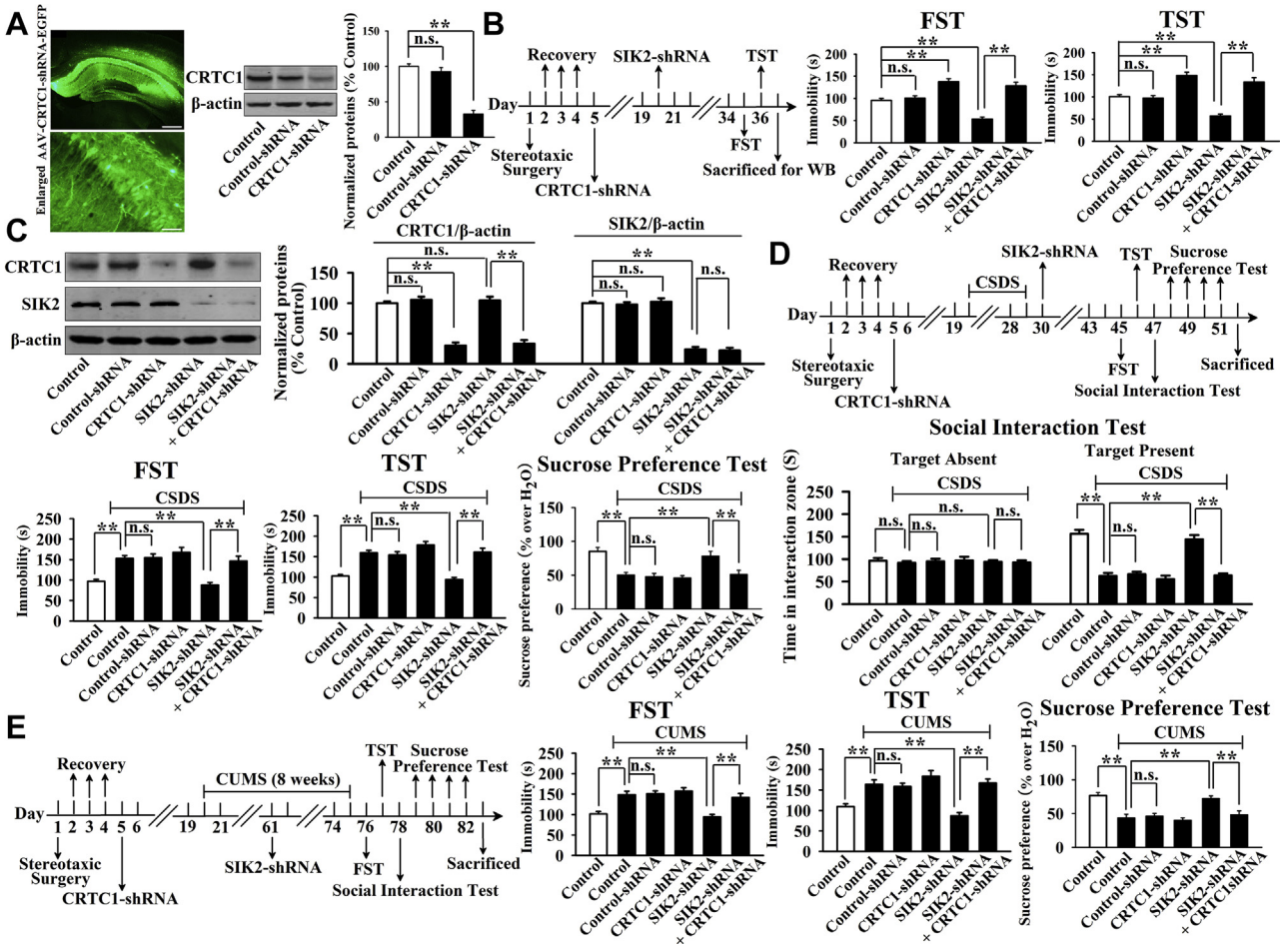

Figure 7. Hippocampal cyclic adenosine monophosphate response element binding protein–regulated transcription coactivator 1 knockdown (CRTC1-KD) by using an adeno-associated virus (AAV) vector that selectively expresses CRTC1–short hairpin RNA with enhanced green fluorescent protein (AAV-CRTC1-shRNA-EGFP) abolished the antidepressant-like effects of AAV–salt-inducible kinase 2 (SIK2)–shRNA–EGFP on mice. **(A)** Fluorescence image of a fixed brain section expressing AAV-CRTC1-shRNA-EGFP in the hippocampus 14 days after infusion. Scale bar = 400 μm for the representative image. Scale bar = 50 μm for the enlarged image. Western blotting (WB) results confirmed the knockdown efficacy of AAV-CRTC1-shRNA (n = 5). **(B)** CRTC1-shRNA preinfusion fully abolished the antidepressant-like effects of SIK2-shRNA on naïve mice in the forced swim test (FST) and tail suspension test (TST) (n = 10). **(C)** Representative WB images showed that the silencing effects of SIK2-shRNA and CRTC1-shRNA did not interrupt each other (n = 5). **(D)** CRTC1-shRNA preinfusion significantly prevented the reversal effects of SIK2-shRNA on chronic social defeat stress (CSDS)–induced depressive-like behaviors in the FST, TST, sucrose preference test, and social interaction test (n = 10). **(E)** CRTC1-shRNA preinfusion also blocked the restoring effects of SIK2-shRNA on chronic unpredictable mild stress (CUMS)–induced depressive-like behaviors in the FST, TST, and sucrose preference test (n = 10). All results are represented as mean ± SEM. For panels **(A–C)**, comparisons were made by one-way analysis of variance followed by Tukey's test. For panels **(D)** and **(E)**, comparisons were made by two-way analysis of variance followed by Bonferroni's test. $^{**}p < .01$. n.s., no significance.

SIK2-shRNA and TrkB-shRNA did not interfere with each other (Supplemental Figure S16C). Similar to the results of BDNF-shRNA treatment, TrkB-shRNA treatment alone increased mouse immobility, and its preinfusion significantly prevented the effects of SIK2-shRNA on naïve mice in the FST and the TST (Supplemental Figure S16B). TrkB-shRNA preinfusion markedly blocked the antidepressant-like actions of SIK2-shRNA in the CSDS model of depression (Supplemental Figure S16D). Similar results were observed in the CUMS model of depression (Supplemental Figure S16E). Together, these findings suggest that the hippocampal BDNF signaling cascade was necessary for SIK2-KD–mediated antidepressant-like actions.

The Antidepressant Actions of Fluoxetine, Venlafaxine, and Mirtazapine Involved the Hippocampal SIK2-CRTC1 Signaling

Currently, selective serotonin reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors, and noradrenergic and specific serotonergic antidepressants are the most used antidepressants in clinical practice. Fluoxetine, venlafaxine, and mirtazapine are representative drugs of selective serotonin reuptake inhibitors, serotonin and norepinephrine reuptake inhibitors, and noradrenergic and specific serotonergic antidepressants, respectively. Here, we studied whether the actions of fluoxetine, venlafaxine, and mirtazapine involved the hippocampal SIK2-CRTC1 system.

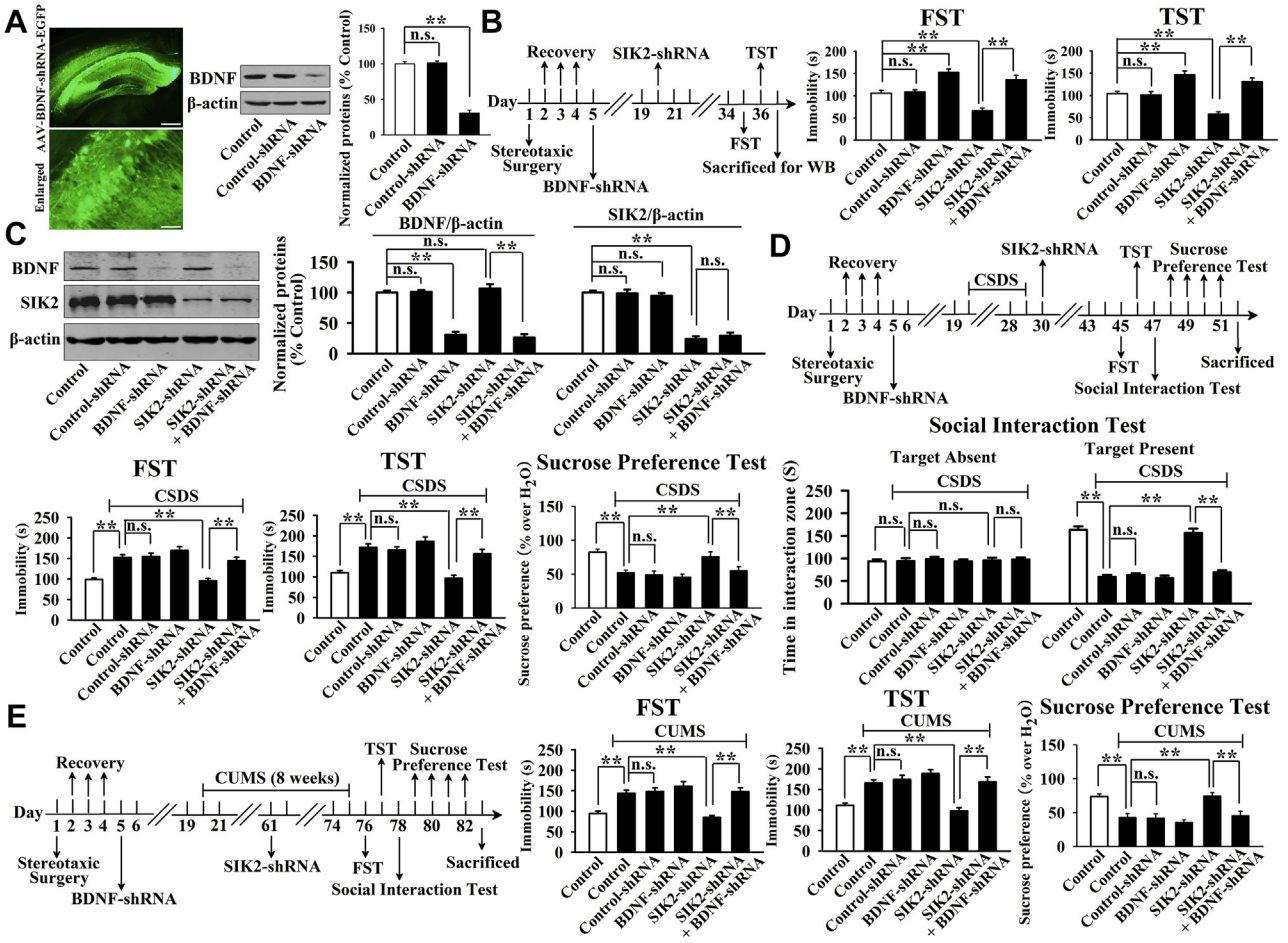

Figure 8. Hippocampal brain-derived neurotrophic factor knockdown (BDNF-KD) by using an adeno-associated virus (AAV) vector that selectively expresses BDNF–short hairpin RNA with enhanced green fluorescent protein (AAV-BDNF-shRNA-EGFP) abrogated AAV–salt-inducible kinase 2 (SIK2)–shRNA–EGFP–induced antidepressant actions in mice. **(A)** Fluorescence image of a fixed brain section that expressed AAV-BDNF-shRNA-EGFP in the hippocampus 14 days after infusion. Scale bar = 400 μm for the representative image. Scale bar = 50 μm for the enlarged image. Western blotting (WB) results confirmed the knockdown efficacy of AAV-BDNF-shRNA ($n = 5$). **(B)** BDNF-shRNA preinfusion significantly blocked the antidepressant actions of SIK2-shRNA in naïve mice in the forced swim test (FST) and tail suspension test (TST) ($n = 10$). **(C)** Representative WB images showed that the silencing effects of SIK2-shRNA and BDNF-shRNA did not interfere with each other ($n = 5$). **(D)** BDNF-shRNA preinfusion fully prevented the reversal effects of SIK2-shRNA on chronic social defeat stress (CSDS)–induced depressive-like behaviors in the forced swim test (FST), tail suspension test (TST), sucrose preference test, and social interaction test ($n = 10$). **(E)** BDNF-shRNA preinfusion also prevented the restoring effects of SIK2-shRNA on chronic unpredictable mild stress (CUMS)–induced depressive-like behaviors in the FST, TST, and sucrose preference test ($n = 10$). All results are represented as mean ± SEM. For panels **(A–C)**, comparisons were made by one-way analysis of variance followed by Tukey's test. For panels **(D)** and **(E)**, comparisons were made by two-way analysis of variance followed by Bonferroni's test. $^{**}p < .01$. n.s., no significance.

CSDS-exposed mice received daily injections of fluoxetine, venlafaxine, or mirtazapine for 14 days, and behavioral tests were performed thereafter. As shown in Figures 9A and 10A, long-term treatment with fluoxetine, venlafaxine, or mirtazapine thoroughly reversed CSDS-induced depressive-like phenotypes. Western blotting revealed that fluoxetine, venlafaxine, and mirtazapine not only restored CSDS-induced increases in hippocampal SIK2 expression but also downregulated SIK2 level in naïve mice (Figures 9B and 10B). Moreover, the three drugs all reversed the CSDS-induced increase in cytoplasmic pCRTC1 as well as the decrease in nuclear CRTC1 level and total CRTC1 level in the hippocampus (Figures 9B, C and 10B, C). Also, these drugs decreased cytoplasmic pCRTC1 level and increased nuclear CRTC1 and total CRTC1 levels in the hippocampus of naïve mice (Figures 9B, C and 10B, C). In addition, all three compounds significantly decreased SIK2 mRNA level, increased CRTC1 mRNA level, and increased CRTC1-CREB binding in the hippocampus of mice (Supplemental Figure S17).

In the CUMS model, fluoxetine, venlafaxine, and mirtazapine all demonstrated notable antidepressant-like effects (Supplemental Figures S18A and S19A). In parallel with the CSDS results, the CUMS-induced effects on SIK2, total CRTC1, nuclear CRTC1, and cytoplasmic pCRTC1 levels in the hippocampus were all thoroughly reversed by these drugs (Supplemental Figures S18B, C and S19B, C). Also, these drugs all significantly restored the effects of CUMS on SIK2 mRNA, CRTC1 mRNA, and CRTC1-CREB binding levels in the hippocampus (Supplemental Figure S20).

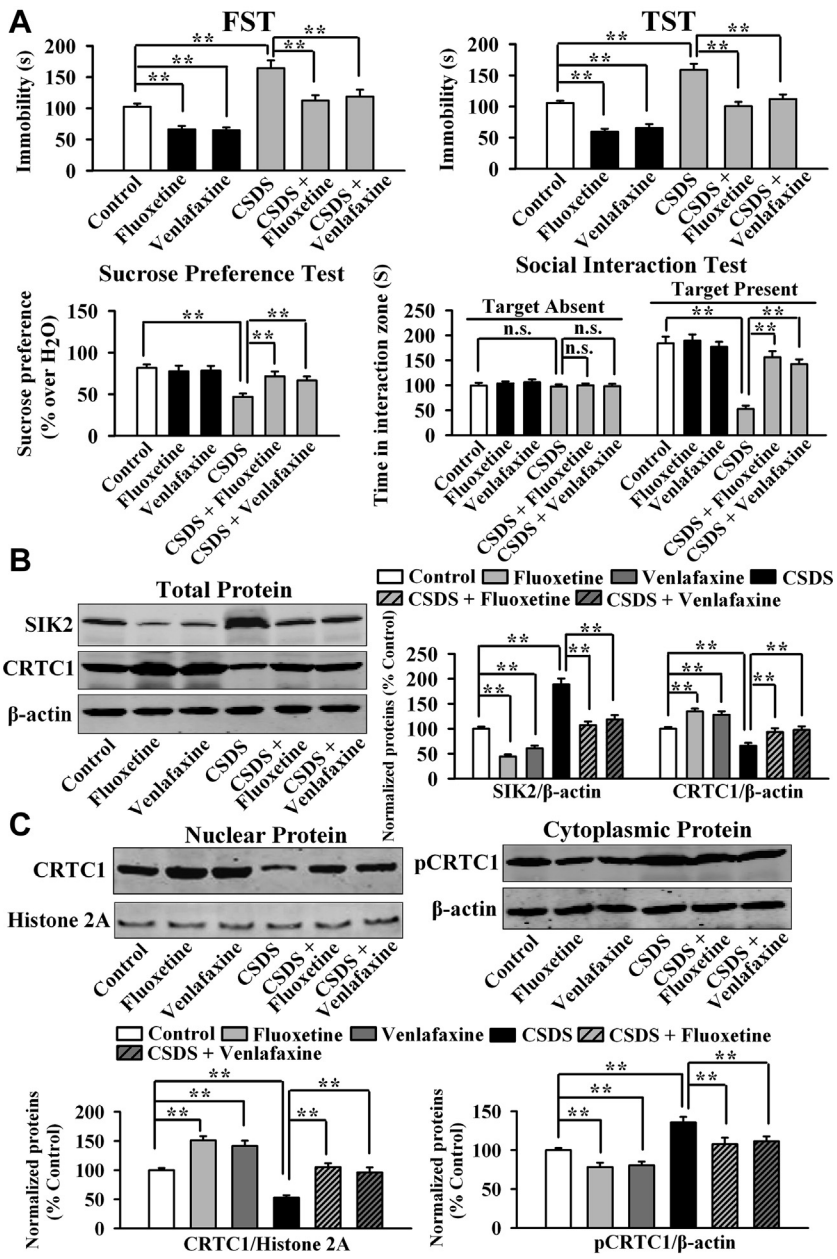

Figure 9. Fluoxetine and venlafaxine had reversal effects on the hippocampal salt-inducible kinase 2 (SIK2)–cyclic adenosine monophosphate response element binding protein–regulated transcription coactivator 1 (CRTC1) signaling in chronic social defeat stress (CSDS)–stressed mice. **(A)** Administration of fluoxetine and venlafaxine for 14 days both significantly antagonized CSDS-induced depressive-like behaviors in the forced swim test (FST), tail suspension test (TST), sucrose preference test, and social interaction test (n = 10). **(B)** Representative Western blotting images showed that both fluoxetine and venlafaxine reversed CSDS-induced changes in hippocampal SIK2 and total CRTC1 expression (n = 5). **(C)** Representative Western blotting images revealed that both fluoxetine and venlafaxine reversed the effects of CSDS on nuclear CRTC1 and cytoplasmic phosphorylated CRTC1 (pCRTC1) expression in the hippocampus (n = 5). All results are represented as mean ± SEM. Comparisons were made by two-way analysis of variance followed by Bonferroni's test. $^{**}p < .01$. n.s., no significance.

In addition, CRTC1-KD mice received a single injection of fluoxetine, venlafaxine, or mirtazapine 30 minutes before the FST and/or TST. We found that although they decreased the immobility time of WT mice, these drugs all produced no significant effects in CRTC1-KD mice in the FST and TST (Supplemental Figures S21A, S22A, and S23A). Moreover, CRTC1-KD mice were subjected to CSDS and CUMS, and then they were treated long-term with fluoxetine, venlafaxine, and mirtazapine. Behavioral tests showed that hippocampal CRTC1 silencing fully abolished the antidepressant-like effects of these three drugs in both CSDS and CUMS models of depression (Supplemental Figures S21B, C; S22B, C; and S23B, C). These results were consistent with those of Breuillaud *et al.* (54) and Meylan *et al.* (55). Collectively, the antidepressant-like effects of fluoxetine, venlafaxine, and mirtazapine all involved hippocampal SIK2-CRTC1 signaling, further supporting the idea that hippocampal SIK2 could be a potential antidepressant target.

DISCUSSION

In this study, we found that depression was accompanied by increased SIK2 expression and decreased nuclear CRTC1 translocation and CRTC1-CREB binding in the hippocampus.

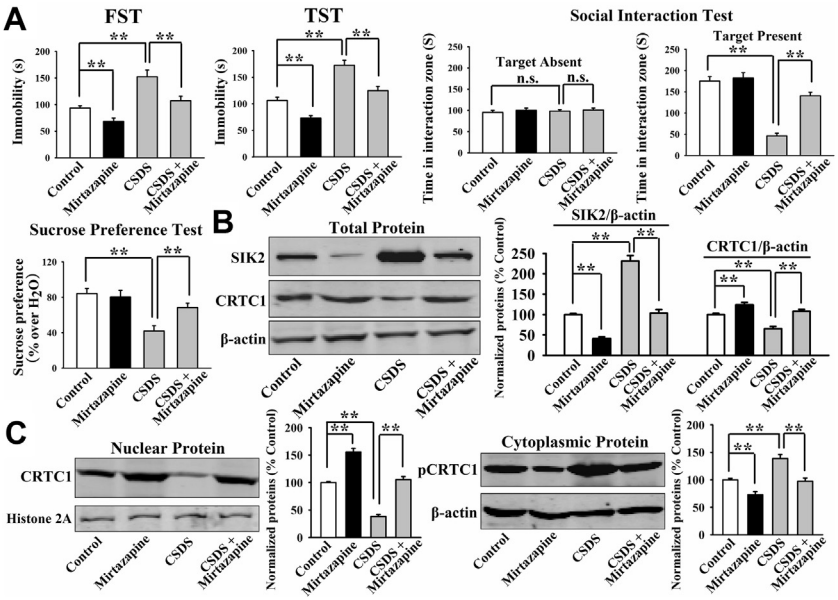

Figure 10. Mirtazapine protected against chronic social defeat stress (CSDS) on the hippocampal salt-inducible kinase 2 (SIK2)–cyclic adenosine monophosphate response element binding protein–regulated transcription coactivator 1 (CRTC1) signaling in mice. **(A)** Repeated injection of mirtazapine for 14 days significantly antagonized CSDS-induced depressive-like behaviors in the forced swim test (FST), tail suspension test (TST), sucrose preference test, and social interaction test (n = 10). **(B)** Representative Western blotting images showed that mirtazapine notably reversed CSDS-induced changes in hippocampal SIK2 and total CRTC1 expression (n = 5). (**C**) Representative Western blotting images revealed that mirtazapine evidently reversed the effects of CSDS on nuclear CRTC1 and cytoplasmic phosphorylated CRTC1 (pCRTC1) expression in the hippocampus (n = 5). All results are represented as mean ± SEM. Comparisons were made by two-way analysis of variance followed by Bonferroni's test. $^{**}p < .01$. n.s., no significance.

Hippocampal SIK2 overexpression mimicked chronic stress that produced depressive-like phenotypes in naïve mice, whereas hippocampal SIK2-KD and SIK2-KO protected against chronic stress. Furthermore, the BDNF signaling cascade mediates the role of the hippocampal SIK2-CRTC1 system in the pathogenesis of depression. The actions of fluoxetine, venlafaxine, and mirtazapine, three clinically used antidepressants, all involve the hippocampal SIK2-CRTC1 system (Figure 11).

It is well known that the transcription factor CREB plays key roles not only in gene expression but also in the pathogenesis of many neuronal disorders (56,57). Although phosphorylation at Ser-133 in the kinase-inducible domain of CREB is often equated to the transcription of CREB target genes, Ser-133 alone is insufficient to activate target genes containing cAMP response elements (56,58). Recently, CRTC1 activation and SIK repression have been reported to predominantly control Ser-133–independent CREB activation (28–31). Since the pathogenesis of depression involves CREB dysfunction in the brain (3,4,17), it is of great significance to investigate the adaptations of central SIK-CRTC signaling to chronic stress. Our results suggest that the effects of chronic stress on central SIK-CRTC signaling were biologically and regionally selective (Figures 1–3). As expected, both CSDS and CUMS induced SIK1 and SIK2 changes in the hippocampus, mPFC, hypothalamus, and VTA. Although hippocampal SIK2 was the focus of this study, SIK1 and SIK2 in the mPFC, hypothalamus, and VTA may also play important roles in depression. For example, depression-associated decrease of BDNF expression in mPFC may be attributed to promoted SIK2 expression and subsequent reduction in nuclear CRTC-CREB binding in mPFC neurons. Likewise, depression-related elevation of BDNF level in NAc may be ascribed to a reduced SIK1 level and a subsequent increase in nuclear CRTC-CREB binding in VTA dopaminergic neurons. Also, because Liu *et al.* (38) reported that SIK was involved in the regulation of corticotrophin-releasing hormone transcription in hypothalamic neurons, chronic stress–induced hyperfunction of the hypothalamic-pituitary-adrenal axis may be ascribed to downregulated SIK1 level and subsequent promotion of CRTC-CREB binding in the paraventricular nucleus of the hypothalamus (59). All these possibilities are critically important to better understanding the neurobiology of depression.

It is interesting that hippocampal CRTC2 and CRTC3 levels were not affected by chronic stress, which further suggests the complexity in the neurobiology of depression. One possible interpretation of this result is that CRTC2 and CRTC3 are mainly regulated by SIK1 and SIK3 but not SIK2 in the hippocampus, while chronic stress does not affect hippocampal SIK1 and SIK3. Another interesting finding is the stress-induced changes of nuclear CRTC1, cytoplasmic pCRTC1, and total CRTC1 levels in the hippocampus. Chronic stress significantly prevented the nuclear translocation of CRTC1, leading to decreased nuclear CRTC1 and enhanced cytoplasmic pCRTC1 levels, while the total CRTC1 level did not remain constant but was downregulated. Here, we have a supposition: under normal conditions, the biosynthesis and biodegradation of CRTC1 in the cytoplasm (pCRTC1) maintains a finely tuned balance. However, under depressive conditions, the reduced nuclear CRTC1 translocation in the hippocampus not only markedly increases the pCRTC1 level in the cytoplasm but also significantly downregulates CRTC1 biosynthesis. When the balance between CRTC1 biosynthesis and biodegradation is lost, the level of pCRTC1 in the cytoplasm decreases, but it is still higher than that under normal conditions. Consequently, the total level of CRTC1 in the hippocampus also decreases. This supposition was partially supported by the quantitative real-time reverse transcription polymerase chain reaction results showing that chronic stress significantly reduced the mRNA level of CRTC1 in the hippocampus.

Next, we examined the mechanism by which chronic stress affected the expression of hippocampal SIK2. Given that chronic stress significantly increased the mRNA level of hippocampal SIK2, the effects likely occurred at the

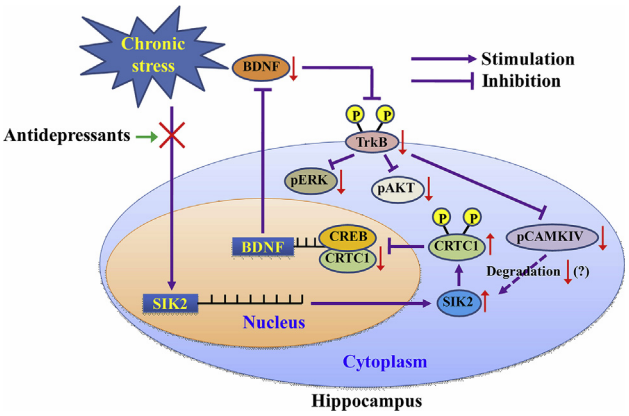

Figure 11. Schematic representation of a suggested model describing the role of hippocampal salt-inducible kinase 2 (SIK2)–cyclic adenosine monophosphate response element binding protein–regulated transcription coactivator 1 (CRTC1)–cyclic adenosine monophosphate response element binding protein (CREB)–brain-derived neurotrophic factor (BDNF) pathway in chronic stress–induced depression. Chronic stress significantly promoted the messenger RNA and protein expression of hippocampal SIK2, which phosphorylates cytoplasmic CRTC1 and prevents its nuclear translocation, leading to decreased CRTC1-CREB binding in the hippocampus. Because of the deficiency in CRTC1-CREB binding, the levels of BDNF biosynthesis as well as its downstream signaling cascades (mitogen-activated protein kinase/extracellular-signal-regulated kinase, PI3K/AKT, and calcium/calmodulin-dependent protein kinase [CaMK]/CaMKIV pathways) are fully downregulated, resulting in depression. The decrease of CaMKIV activity may lead to reduction in SIK2 degradation, which further contributes to hippocampal SIK2 level. Blockade of the chronic stress–induced effects on hippocampal SIK2 produces antidepressant actions. P, phosphorus; pAKT, phosphorylated protein kinase B; pCaMKIV, phosphorylated CaMKIV; pERK, phosphorylated extracellular-signal-regulated kinase; TrkB, tyrosine receptor kinase B.

transcriptional level. It is possible that chronic stress induced the oversecretion of glucocorticoids that bind to intracellular glucocorticoid receptors in the hippocampus, resulting in receptor–glucorticord complex translocation into the nucleus to exert biological effects on *SIK2* at the genomic level. This possibility could be further verified by studying whether the DNA sequence of the *SIK2* gene had specific glucocorticoid response elements using bioinformatics and whether glucocorticoid receptors interacted with *SIK2* DNA using chromatin immunoprecipitation. Alternatively, the effects may occur at the protein level. We found that chronic stress significantly decreased the activity of hippocampal CaMKIV, and Sasaki *et al.* (37) showed that CaMKIV was capable of phosphorylating SIK2 at the threonine 484 site, resulting in SIK2 degradation in cortical neurons (37). Therefore, the stress-induced decrease of CaMKIV activity may reduce hippocampal SIK2 degradation, further increasing its level. This possibility could be further studied using SIK2–threonine 484 mutant mice in which CaMKIV is unable to phosphorylate SIK2. Moreover, several reports have demonstrated that liver kinase B1 is an upstream kinase of SIK (36,60,61). Liver kinase B1 directly phosphorylates the activation loop of adenosine monophosphate kinase α1 and α2 and α12 other adenosine monophosphate kinase–related kinases including SIK1/2/3 (60), so it is also possible that chronic stress affects liver kinase B1.

Positive and negative manipulation of SIK2 via virally mediated overexpression, knockdown, and knockout strongly supports the possibility that SIK2 could be a drug target. The results are highly consistent across multiple animal depression models and behavioral measures. Further studies in our laboratory will employ other animal models of depression, including chronic restraint stress and learned helplessness models, to confirm the generality of the findings reported here. In this study, we did not use pharmacological approaches. Recently, Bon *et al.* (62) and Zhou *et al.* (63) reported a novel small-molecule inhibitor of SIK2, ARN-3236, that is currently under preclinical development. It would be very interesting to see whether ARN-3236 has antidepressant-like effects in CSDS and CUMS models. SIK has been demonstrated to regulate polarity protein Par3, the Hippo signaling pathway, and cytoplasmic histone deacetylase 4 besides CRTC (64–66). The CRTC1-shRNA and CREB-shRNA results reported here suggested that hippocampal SIK2 is involved in the pathophysiology of depression via the CRTC1-CREB pathway. BDNF was selected as a candidate downstream molecule relating the hippocampal SIK2-CRTC1 system with depression, given the well-known role of hippocampal BDNF in depression (3,4,18), and the fact that BDNF biosynthesis is closely controlled by CREB (13). The results using BDNF-shRNA and TrkB-shRNA convincingly demonstrated that BDNF was indeed an important mediator underlying the role of hippocampal SIK2-CRTC1 system in depression. However, given the complex nature of depression neurobiology, which involves not only BDNF but also many other factors regulated by CREB (vascular endothelial growth factor, glycogen synthase kinase 3β, mammalian target of rapamycin, etc.) (67–69), other possible mechanisms cannot be ruled out.

Determining the correlation between hippocampal SIK2 and BDNF is of great significance because BDNF is also implicated in many neuropsychiatric disorders besides depression, such as Parkinson's disease, Alzheimer's disease, bipolar disorder, and schizophrenia (70–72). As such, hippocampal SIK2 may also be involved in these disorders. Although hippocampal neurogenesis was mainly used as a biological index to evaluate the depressive-like and antidepressant-like activities in this study, the results suggested that SIK2 was able to regulate neurogenesis, extending the knowledge of its physiological functions. It is very likely that SIK2 modulates neurogenesis via the CRTC1-CREB-BDNF pathway given the well-established correlation between CREB, BDNF, and neurogenesis. It is also possible that SIK2 can modulate some well-known proneurogenic factors (sex determining region Y box 2, paired box protein 6, neuronal differentiation 1, etc.) (73), a possibility that needs further investigation.

Although fluoxetine, venlafaxine, and mirtazapine can quickly increase the concentrations of synaptic 5-hydroxytryptamine (5-HT) and noradrenaline, weeks of administration are usually required to produce clinical efficacy, indicating that the therapeutic targets may be something downstream of the monoaminergic system (5,6). These antidepressant drugs all act on the 5-HT–mediated signaling pathway. Stimulation of inhibitory G protein/other G protein–coupled 5-$HT_{1A\text{-}1F}$ and 5-$HT_{5A/5B}$ receptors can inhibit the adenylyl cyclase–cAMP–protein kinase A pathway, whereas activation of Gs protein–coupled 5-$HT_{4/6/7}$ receptors enhances the adenylyl cyclase–cAMP–protein kinase A pathway

(74,75). Also, Gq/11 protein–coupled 5-HT_{2A-2C} receptors are linked to the phospholipase 3–inositol triphosphate–calcium ion/CaMK pathway, and 5-HT_3 receptor stimulation leads to the influx of sodium ions and calcium ions (74,75). We postulated that fluoxetine, venlafaxine, and mirtazapine may act on hippocampal SIK2-CRTC1 signaling via two possible pathways: downregulating the level of SIK2 mRNA through 5-HT_{1A-1F} and 5-$HT_{5A/5B}$ receptors–mediated inhibition of adenylyl cyclase–cAMP–protein kinase A–CREB, and promoting the degradation of SIK2 protein through 5-HT_{2A-2C} and 5-HT_3 receptors–mediated calcium ion/CaMKIV activation. This postulation can be validated by using selective antagonists against these 5-HT receptors in future studies. It is also possible that fluoxetine and/or venlafaxine and/or mirtazapine directly phosphorylates SIK2. Regardless, our findings extend the knowledge of these drugs' pharmacological actions.

In conclusion, hippocampal SIK2 is involved in the pathogenesis of depression via regulating the CRTC1-CREB-BDNF pathway, and it is a feasible antidepressant target. The findings may also contribute to the neurotrophic hypothesis of depression.

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BJ and J-XL designed this study. BJ wrote the manuscript. BJ, HW, J-LW, Y-JW, LS, T-TG, YW, and C-NW performed the experiments. WZ and G-LM helped acquire supporting funds. QZ, FW, and YL helped collect and analyze the data. All the authors read and approved this manuscript.

The authors report no biomedical financial interests or potential conflicts of interest.

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