



## RESEARCH ARTICLE

# SKF83959 Produces Antidepressant Effects in a Chronic Social Defeat Stress Model of Depression through BDNF-TrkB Pathway

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## Abstract

**Background:** SKF83959 stimulates the phospholipase C $\beta$ /inositol phosphate 3 pathway, resulting in the activation of Ca<sup>2+</sup>/calmodulin-dependent kinase II $\alpha$ , which affects the synthesis of brain-derived neurotrophic factor, a neurotrophic factor critical for the pathophysiology of depression. Previous reports showed that SKF83959 elicited antidepressant activity in the forced swim test and tail suspension test as a novel triple reuptake inhibitor. However, there are no studies showing the effects of SKF83959 in a chronic stress model of depression and the role of phospholipase C/inositol phosphate 3/calmodulin-dependent kinase II $\alpha$ /brain-derived neurotrophic factor pathway in SKF83959-mediated antidepressant effects.

**Methods:** In this study, SKF83959 was firstly investigated in the chronic social defeat stress model of depression. The changes in hippocampal neurogenesis, dendrite spine density, and brain-derived neurotrophic factor signaling pathway after chronic social defeat stress and SKF83959 treatment were then investigated. Pharmacological inhibitors and small interfering RNA/short hairpin RNA methods were further used to explore the antidepressive mechanisms of SKF83959.

**Results:** We found that SKF83959 produced antidepressant effects in the chronic social defeat stress model and also restored the chronic social defeat stress-induced decrease in hippocampal brain-derived neurotrophic factor signaling pathway, dendritic spine density, and neurogenesis. By using various inhibitors and siRNA/shRNA methods, we further demonstrated that the hippocampal dopamine D<sub>5</sub> receptor, phospholipase C/inositol phosphate 3/calmodulin-dependent kinase II $\alpha$  pathway, and brain-derived neurotrophic factor system are all necessary for the SKF83959 effects.

**Conclusion:** These results suggest that SKF83959 can be developed as a novel antidepressant and produces antidepressant effects via the hippocampal D<sub>5</sub>/phospholipase C/inositol phosphate 3/calmodulin-dependent kinase II $\alpha$ /brain-derived neurotrophic factor pathway.

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**Keywords:** depression; SKF83959; brain-derived neurotrophic factor; chronic social defeat stress

#### Abbreviations:

AKT	Protein Kinase B
ANOVA	analysis of variance
BDNF	brain-derived neurotrophic factor
CaMKII $\alpha$	calcium/calmodulin-dependent kinase II $\alpha$
CMS	chronic mild stress
CREB	cAMP response element-binding protein
CSDS	chronic social defeat stress
DCX	doublecortin
DG	dentate gyrus
ERK	extracellular signal-regulated kinase
FST	forced swim test
HPA	hypothalamic-pituitary-adrenocortical
IP3	inositol phosphate 3
PCPA	P-chlorophenylalanine methyl ester
PI	phosphatidylinositol
PLC	phospholipase C
TrkB	tyrosine kinase B
TST	tail suspension test

## Introduction

Depression is a serious mood disorder that affects 17 to 20% of the population of the world and may result in major social and economic consequences (Blazer et al., 1994). For the pathophysiology of depression, the discovery that chronic antidepressant treatment increases the hippocampal brain-derived neurotrophic factor (BDNF) and phosphorylation of cAMP response element-binding protein (CREB) led to the deduction that a deficiency in neurotrophic factor synthesis and signaling could underlie depression (Nibuya et al., 1995, 1996; Shirayama et al., 2002; Saarelainen et al., 2003). The finding that antidepressants can also increase hippocampal neurogenesis, which is a kind of cellular form of neuroplasticity and necessary for the behavioral improvement, further supports this hypothesis (Santarelli et al., 2003; Dranovsky and Hen, 2006; Perera et al., 2007).

SKF83959 is one compound that belongs to benzazepine family and has been widely used in researching the dopaminergic system. SKF83959 could stimulate PI-hydrolysis via phospholipase C $\beta$  and results in the production of inositol phosphate 3 (IP3), which subsequently induces intracellular Ca<sup>2+</sup> release and the transient activation of Ca<sup>2+</sup>/calmodulin-dependent kinase II $\alpha$  (CaMKII $\alpha$ ) (Zhen et al., 2004). For the pharmacologic targets of SKF83959, however, there are different reports. Several studies identified SKF83959 as a selective D<sub>1</sub>-D<sub>2</sub> heteromer agonist (Rashid et al., 2007a, 2007b), while Sahu et al. (2009) found that the dopamine D<sub>5</sub> receptor was also required for SKF83959 to induce the PLC/IP3 pathway. Besides, SKF83959 has been demonstrated to have high affinity for the serotonergic 5-HT<sub>2C</sub> and adrenergic  $\alpha_{2C}$  receptors and is also a potent allosteric modulator of the sigma-1 receptor (Chun et al., 2013; Guo et al., 2013). The effects of SKF83959 on the central nervous system are being explored. For example, SKF83959 can be used to treat Parkinson's disease (Zhang et al., 2007) and produce neuroprotective effects on cortical neurons (Yu et al., 2008) and also modulates hippocampal long-term depression (Liu et al., 2009).

It has been demonstrated that depressive behavior is associated with the downregulation of the PLC/IP3 pathway (Dwivedi et al., 2005). Previous studies suggest that CaMKII $\alpha$  is not only involved in the synthesis of BDNF (Chen et al., 2012; Yu et al.,

2013) but is also modulated by chronic stress (Suenaga et al., 2006; Barbiero et al., 2007; Han et al., 2009). It has also been demonstrated that SKF83959 promotes both striatal and prefrontal BDNF expression through CaMKII $\alpha$  activation (Rashid et al., 2007b; Hasbi et al., 2009; Perreault et al., 2013). A more recent study showed that SKF83959 elicited antidepressant activity in the forced swim test (FST) and tail suspension test as a novel triple reuptake inhibitor (Fang et al., 2013). Considering the fact that SKF83959 could activate the PLC/IP3/Ca<sup>2+</sup>/CaMKII $\alpha$  pathway, which is linked with BDNF signaling, we thus hypothesized that SKF83959 may produce antidepressant-like effects via a mechanism other than as reuptake inhibitor. To address this issue, we first assessed the effects of SKF83959 in a chronic social defeat stress (CSDS) model of depression and then investigated the related molecular mechanisms.

## Materials and methods

### Animals

Male C57BL/6J mice (9–11 weeks old) and CD1 retired breeder mice (9–13 months old) were obtained from the Animal Center of Tongji Medical College and maintained under standard conditions with a 12-h-light/dark cycle and ad libitum access to food and water for 1 week before being used. Behavioral testing was performed from 10:00 AM to 5:00 PM concurrent with stated housing conditions and each experimental group consisted of 20 mice. The experiments were carried out in accordance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Animal Care Committee of the University.

### Materials

6-Chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF83959) was kindly provided by the National Institute of Mental Health (NIMH) synthesis program. Fluoxetine, 7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol (SCH23390), 6-chloro-2,3-dihydro-5-methyl-N-[6-[(2-methyl-3-pyridinyl)oxy]-3-pyridinyl]-1H-indole-1-carboxamide (SB242084), 1-[2-(3,4-dichlorophenyl)ethyl]-4-methylpiperazine (BD1063), 1,2-bis-(2-aminophenoxy)ethane-N,N,N,N-tetracetic acid-acetoxymethyl-acetoxymethyl ester (BAPTA-AM), 1-[6-[[[(17 $\beta$ )-3-methoxyestra-1,3,5[10]-trien-17yl] amino]hexyl]-1H-pyrrole-2,5-dione (U73122), and 2-aminoethoxy-diphenylborate (2-APB) were purchased from Sigma (St. Louis, MO). N-[4-(4-Methyl-1-piperazinyl)phenyl]-9-acridinamine (JP1302) and 3,5-dichloro-N-[[[2S]-1-ethyl-2-pyrrolidinyl]methyl]-2-hydroxy-6-methoxybenzamide (raclopride) were purchased from Tocris Bioscience (Bristol, UK). K252a was obtained from Alomone Laboratories (Jerusalem, Israel). The D<sub>5</sub> small interfering RNA (siRNA) (catalog no: 4457308) and scrambled siRNA (catalog no: 4457287) were obtained from Ambion (Austin, TX). For SKF83959 and fluoxetine, compounds were administered intraperitoneally in a volume of 10 mL/kg. For SCH23390, raclopride, SB242084, BD1063, JP1302, K252a, U73122, BAPTA-AM, and 2-APB, drugs were dissolved in artificial cerebrospinal fluid and bilaterally injected into the hippocampus of C57BL/6J mice.

The experimental procedures are available online in [supplementary information](#).

### Statistical Analysis

All analyses were performed using SPSS 13.0 software (SPSS Inc) and data are presented as mean ± SEM. Differences between mean values were evaluated using 1-way or 2-way analysis of variance (ANOVA), as appropriate. For all 1-way ANOVAs, posthoc tests were performed using Least Significant Difference test. For all 2-way ANOVAs, Bonferroni posthoc tests were used to assess isolated comparisons.  $P < .05$  was considered statistically significant.

## Results

### SKF83959 Produces Antidepressant-Like Effects in Mice

To characterize the antidepressant effects of SKF83959, we utilized the CSDS model, as we previously used (Jiang et al., 2012). As shown in Figure 1A, in the absence of an aggressor, all mice spent similar amounts of time in the interaction zone. Compared to control mice, defeated mice spent about 70±11% less time in the interaction zone when an aggressor was introduced into the cage ( $n=10$ ,  $P < .01$  vs control) (Figure 1A). Interestingly, the 14-day treatment of SKF83959 significantly increased the interaction time of defeated mice, similar to fluoxetine ( $n=10$ ,  $P < .01$  vs defeated) (Figure 1A). Over the next 4 days, mice were examined for sucrose preference. Figure 1B showed that the defeated mice displayed a significantly reduced preference for sucrose solution ( $P < .01$  vs control), and this effect was reversed by SKF83959 treatment ( $n=10$ ,  $P < .01$  vs defeated). Together, these results suggest that SKF83959 could produce antidepressant effects in the CSDS model of depression.

### SKF83959 Counteracts the CSDS-Induced Deficits in Hippocampal Neurogenesis and Dendrite Spine Density

Since adult hippocampal neurogenesis can be upregulated by chronic antidepressant treatment (Malberg et al., 2000), we thus investigated the possible effects of SKF83959 on hippocampal neurogenesis. Neurogenesis was studied by doublecortin immunohistochemistry in the dentate gyrus region, since DCX

is a microtubule-associated protein that serves as a marker of neurogenesis by virtue of transient expression in newly formed neurons between the timing of their birth and final maturation (Brown et al., 2003). Figure 2A-B shows that chronic stress resulted in a 67±3% reduction in the number of DCX+ cells when compared to that in control mice ( $n=5$ ,  $P < .01$  vs control). The decreased number of DCX+ cells in the stressed group was reversed by chronic SKF83959 treatment, especially at the dose of 1 mg/kg ( $n=5$ ,  $P < .01$  vs defeated) (Figure 2B). Correspondingly, the Western blotting results showed a significant decrease in the hippocampal DCX protein level of stressed mice ( $n=5$ ,  $P < .05$  vs control; Figure 2C), which was counteracted by SKF83959 treatment ( $n=5$ ,  $P < .01$  vs defeated; Figure 2C).

Previous studies reported that chronic stress induced neuronal atrophy and dendritic arborization of CA3 pyramidal neurons (Magarinos et al., 2011). We thus performed Golgi-Cox staining. As shown in Figure 2D, repeated stress induced a severe decrease in the dendritic spine density of CA3 pyramidal neurons ( $n=6$ ,  $P < .01$  vs control), and SKF83959 treatment reversed the reduction of spine density ( $n=6$ ,  $P < .01$  vs defeated). These results indicate that the stress-induced decrease in hippocampal neurogenesis and dendritic spine density are also rescued by SKF83959 treatment.

### Administration of SKF83959 Enhances BDNF Signaling Pathway and Neurogenesis in the Hippocampus of Adult Normal Mice

Since SKF83959 has been demonstrated to promote BDNF production in striatum and cortex (Hasbi et al., 2009; Perreault et al., 2013), we thus hypothesized that SKF83959 may also enhance hippocampal BDNF expression. As shown in supplementary Figure S1A-B, both the hippocampal BDNF mRNA and protein levels were significantly elevated by SKF83959 administration, especially at the dose of 1mg/kg ( $n=5$ ,  $P < .01$  vs control). The Western blotting results showed that SKF83959 produced a similar increase in the phosphorylated and activated forms of ERK1/2 (pERK1/2), AKT (pAKT), and CREB (pCREB), which are linked to BDNF signaling activation ( $n=5$ ,  $P < .01$  vs control) (supplementary Figure S1C) (Shaywitz and Greenberg, 1999). We also detected a significant increase in

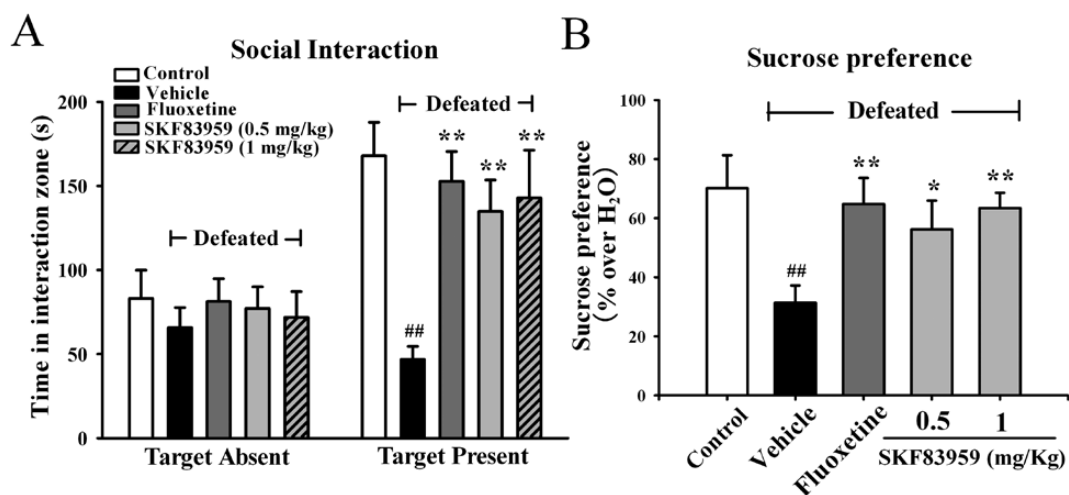
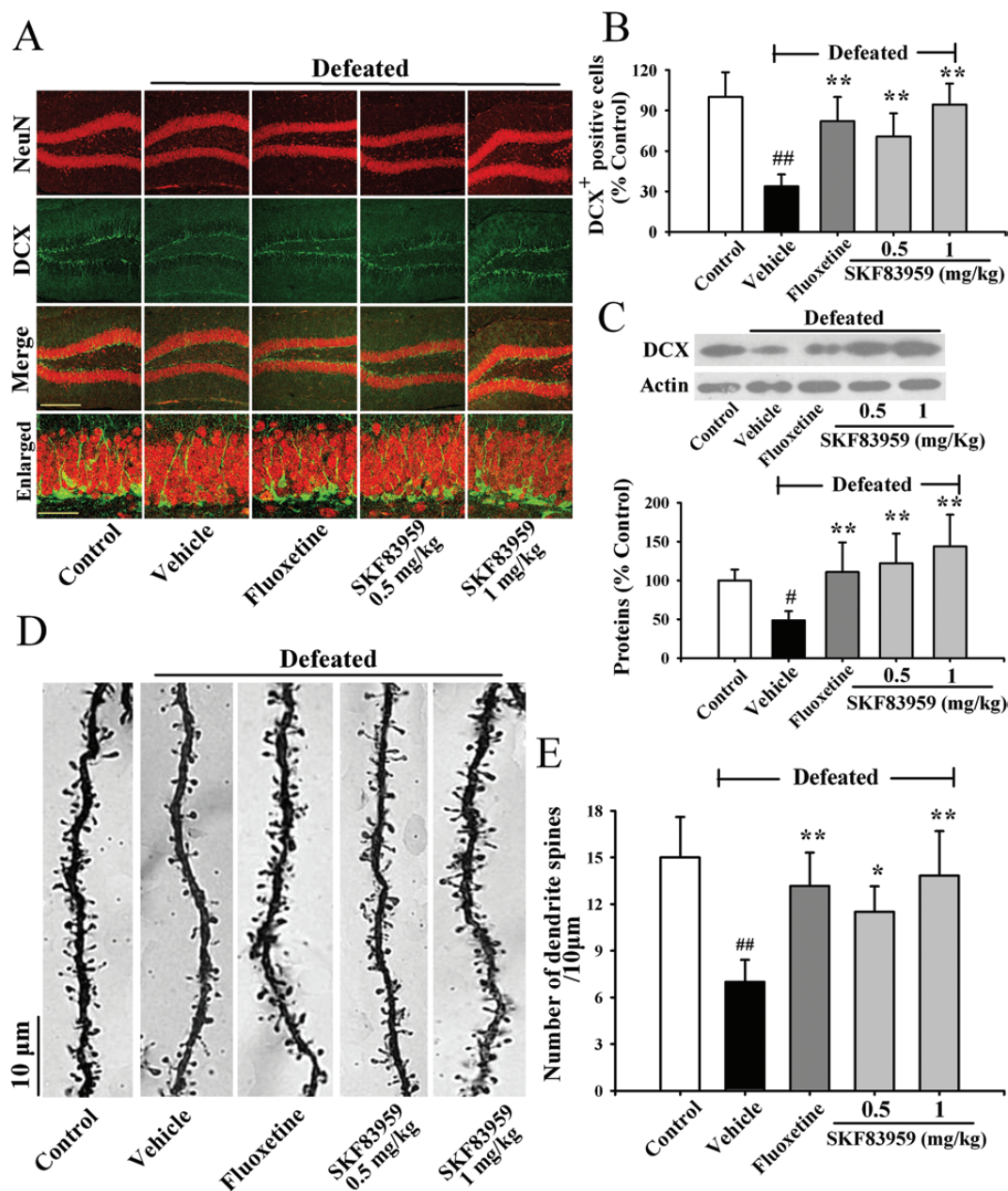


Figure 1. 6-Chloro-7, 8-dihydroxy-3-methyl- 1-(3-methylphenyl)-2,3,4,5- tetrahydro-1H-3- benzazepine (SKF83959) produces robust antidepressant effects in rodent models of depression. (A) The antidepressant effects of SKF83959 in the social interaction test. Mice were exposed to defeat stress for 10 days and received a daily injection of SKF83959 (0.5, 1mg/kg, i.p.) for another 14 days; behavioral tests were then conducted. SKF83959-treated mice spent significantly more time engaged in social interaction than vehicle-treated mice. (B) SKF83959 treatment reversed the decrease in sucrose consumption induced by chronic social defeat stress (CSDS). SKF83959-treated mice displayed higher sucrose preference than vehicle-treated mice. Data are expressed as means ± SEM ( $n=10$ ); #  $P < .05$ , ##  $P < .01$  vs control; \*  $P < .05$ , \*\*  $P < .01$  vs defeated+vehicle group. Comparison was made by 2-way analysis of variance (ANOVA) followed by posthoc Bonferroni's test.



**Figure 2.** 6-Chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2, 3,4, 5-tetrahydro-1H-3-benzazepine (SKF83959) treatment restores the decrease in hippocampal spine density and proliferation of hippocampal progenitor cells induced by defeat stress. (A) Representative confocal microscopic images showed the colocalization of NeuN (red) with doublecortin (DCX) (green) in the dentate gyrus (DG). The scale bar is 200 µm for representative images and 25 µm for enlarged images, respectively. (B) Chronic SKF83959 treatment significantly increased the number of DCX-stained cells in the DG of stressed animals (n=5). (C) Representative Western blotting of DCX showed that SKF83959 counteracted the deficiency of hippocampal DCX protein level caused by stress (n=5). (D) Representative photomicrograph of a Golgi-Cox stained pyramidal neuron of CA3 hippocampus. Scale bar=10 µm. (E) Summary data showed that the stress-induced decrease of spine density was restored by SKF83959 (n=6). Data are expressed as means ± SEM; \* P < .05, \*\* P < .01 vs control; # P < .05, ## P < .01 vs defeated + vehicle group. Comparison was made by 2-way analysis of variance (ANOVA) followed by posthoc Bonferroni's test.

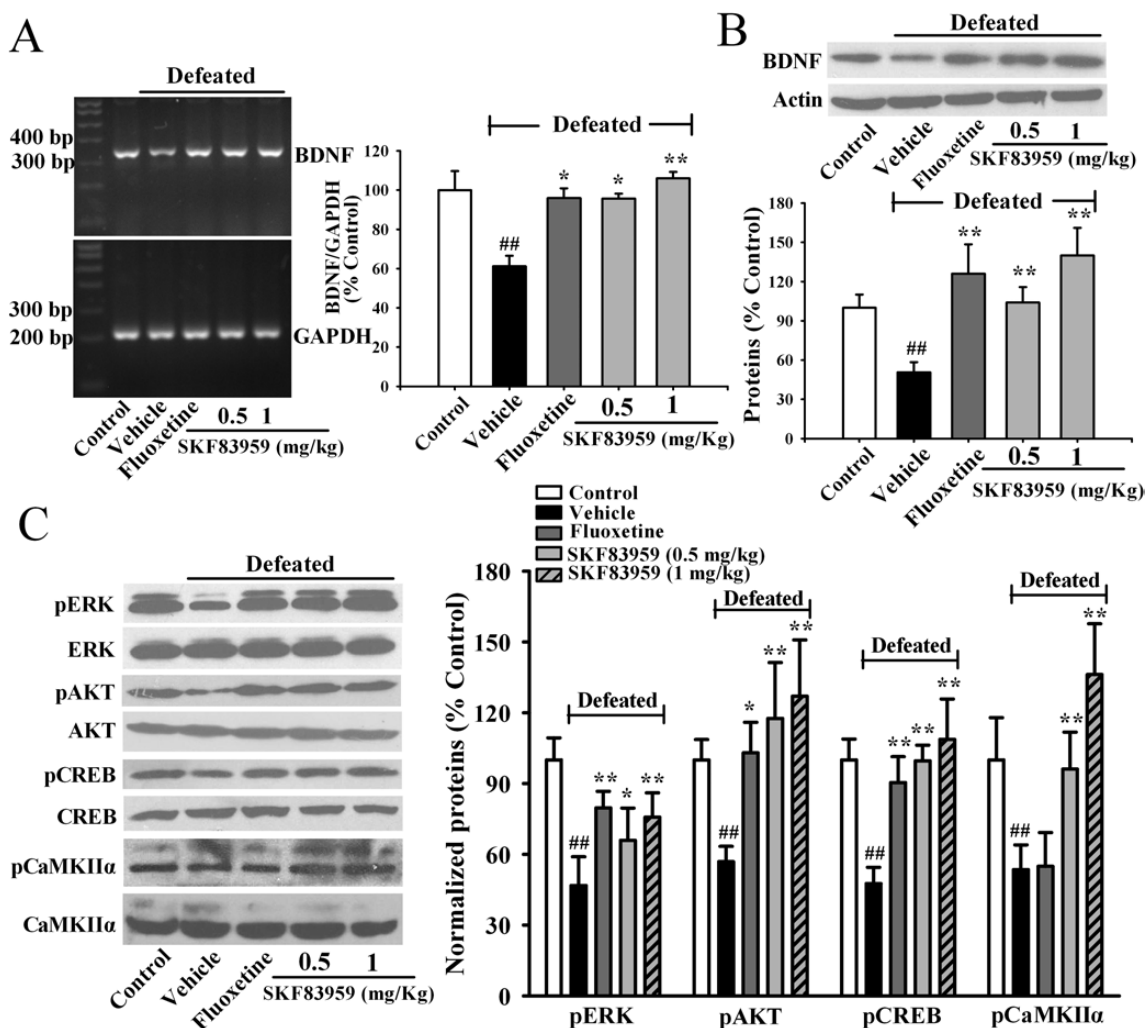
the phosphorylation of hippocampal CaMKIIα (pCaMKIIα) after SKF83959 exposure (n=5, P<.01 vs control) (supplementary Figure S1C). Correspondingly, the level of hippocampal DCX protein was increased in SKF83959-treated mice (n=5, P<.01 vs control) (supplementary Figure S1C), suggesting that SKF83959 also promotes hippocampal neurogenesis in normal mice.

### SKF83959 Treatment Restores the Stress-Induced Decrease in Hippocampal BDNF Signaling Pathway

Since the hippocampal BDNF system is involved in the pathophysiology of depression, we measured BDNF mRNA and

protein levels in the hippocampus following CSDS. As shown in Figure 3A-B, the average BDNF mRNA and protein levels were decreased in the hippocampus of mice exposed to CSDS compared with control mice (n=5, P<.01 vs control), and this was completely reversed by 1 mg/kg SKF83959 (n=5, P<.01 vs defeated).

Four members of the neurotrophic factors family have been identified: nerve growth factor (NGF), BDNF, neurotrophins-3 (NT3), and neurotrophins-4 (NT4) (Quartu et al., 2003). These proteins have similar physical characteristics and biological activities. However, we found that although CSDS indeed decreased the levels of NGF, NT3, and NT4 (n=5, P<.01 vs control)



**Figure 3.** 6-Chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF83959) treatment increases the hippocampal brain-derived neurotrophic factor (BDNF) signaling cascade of chronic social defeat stress (CSDS)-treated mice. (A) Chronic SKF83959 administration reversed the decrease in hippocampal BDNF mRNA level induced by CSDS. (B) SKF83959 treatment reversed the CSDS-induced reduction of hippocampal BDNF protein. (C) SKF83959 also restored the CSDS-induced inhibition of hippocampal phosphorylated extracellular signal-regulated kinase (pERK), Protein Kinase B (pAKT), cAMP response element-binding protein (pCREB), and calcium/calmodulin-dependent kinase IIα (pCaMKIIα). Data are expressed as means ± SEM (n=5); <sup>##</sup>P < .01 vs control; <sup>\*</sup>P < .05; <sup>\*\*</sup>P < .01 vs defeated + vehicle group. Comparison was made by 2-way analysis of variance (ANOVA) followed by posthoc Bonferroni's test.

(supplementary Figure S2A), SKF83959 treatment produced no effects on these neurotrophins, which is contrary to BDNF (n=5) (supplementary Figure S2A), indicating that SKF83959 specifically modulates the expression of BDNF in CSDS-treated animals.

We then examined the expression of hippocampal pERK1/2, pAKT, and pCREB. As shown in Figure 3C, chronic SKF83959 treatment significantly increased the hippocampal pERK1/2, pAKT, and pCREB expression of stressed mice, equivalent to fluoxetine (n=5, P < .01 vs defeated). Similarly, the inhibition of pCaMKIIα caused by stress was also restored by SKF83959 (n=5, P < .01 vs defeated). Thus, the antidepressant effects of SKF83959 may involve the promotion of hippocampal BDNF signaling.

Other brain regions, like the medial prefrontal cortex (mPFC) and nucleus accumbens (NAc), are also implicated in depression (Di Chiara et al., 1999; Krishnan et al., 2007; Li et al., 2010). As previously reported, chronic stress reduced the expression of BDNF and pCREB in the mPFC (Gourley et al., 2008; Castren and Rantamaki, 2010), while increased BDNF and pCREB levels were detected in the NAc (Newton et al., 2002; Eisch et al., 2003;

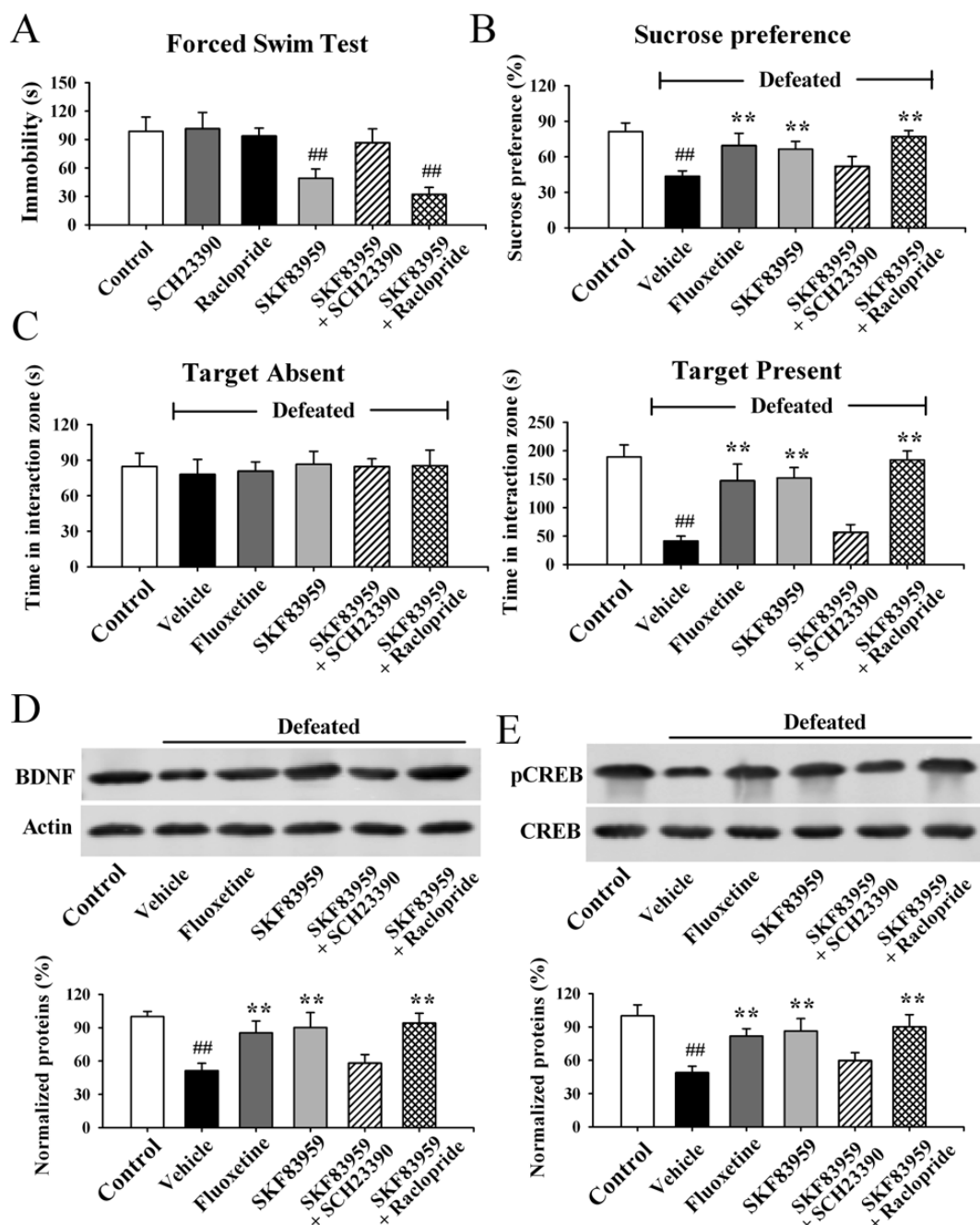
Krishnan et al., 2007). However, SKF83959 treatment did not restore the stress-induced changes of BDNF and pCREB in the 2 regions (n=5) (supplementary Figure S2B-C), suggesting that it is the hippocampus, not mPFC or NAc, that is important for the SKF83959-mediated antidepressant effects.

### SKF83959 Produces Antidepressant-Like Effects through Activation of D<sub>5</sub> Receptor in Hippocampus of Mice

It has been demonstrated that SKF83959 has a high affinity for various receptors in the brain, including the D<sub>1</sub>-D<sub>2</sub> heteromer, D<sub>5</sub> receptor, 5-HT<sub>2C</sub>, and α<sub>2C</sub> receptors (Hasbi et al., 2009; Chun et al., 2013; Guo et al., 2013). To explore which receptor mediates the antidepressant effects of SKF83959, we firstly used the selective D<sub>1</sub>-like (D<sub>1</sub>, D<sub>5</sub>) receptor antagonist SCH23390 and D<sub>2</sub>-like (D<sub>2</sub>, D<sub>3</sub>, D<sub>4</sub>) receptor antagonist raclopride. It was found that while SCH23390 (10 nmol/mouse) or raclopride (10 nmol/mouse) alone produced no influence on the immobility, SCH23390, however, not raclopride, significantly blocked

the SKF83959-induced effects in the FST ( $n=10$ ,  $P<.01$  vs control) (Figure 4A). The SKF83959-induced increase in sucrose preference (Figure 4B) and social interaction (Figure 4C) were also prevented by SCH23390, not raclopride ( $n=10$ ). The SCH23390-induced effects indicate that the antidepressant effects of SKF83959 are mediated through the  $D_1$ - $D_2$  heteromer or  $D_5$  receptor or both. The raclopride-induced effects

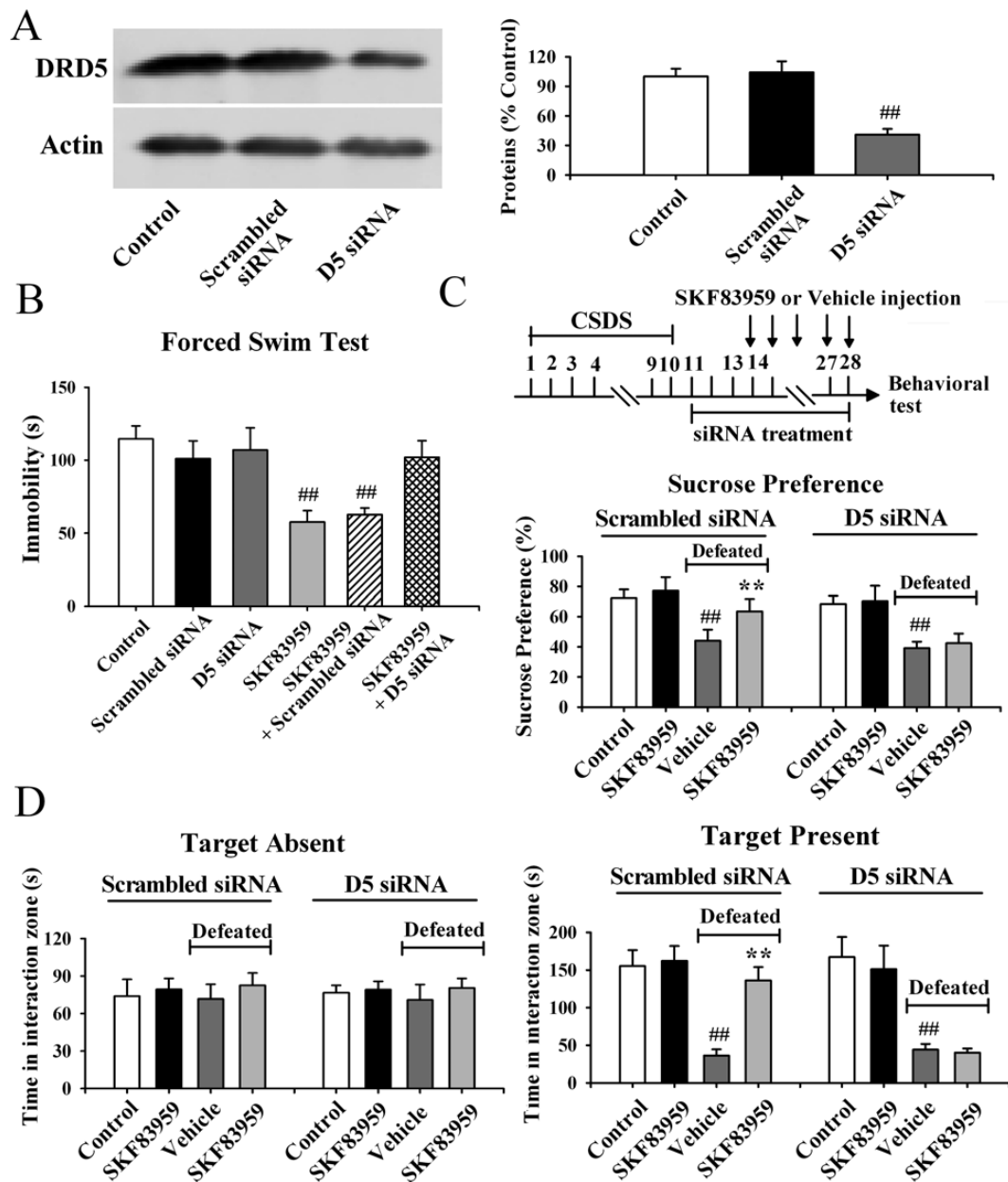
further indicate that the  $D_1$ - $D_2$  heteromer is not necessary for the SKF83959-mediated effects. Similarly, pretreatment with SCH23390, not raclopride, abolished the SKF83959 effects on hippocampal BDNF expression of normal mice ( $n=5$ ; supplementary Figure S1D), hippocampal BDNF expression ( $n=5$ ; Figure 4D), and CREB phosphorylation ( $n=5$ ; Figure 4E) of CSDS-defeated mice.



**Figure 4.** Blockade of hippocampal  $D_1$ -like receptor, not  $D_2$ -like receptor, prevents the antidepressant effects of 6-Chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF83959). (A) Mice were daily pretreated with the antagonist of  $D_1$ -like receptor (7-chloro-3-methyl-1-phenyl-1,2,4,5-tetrahydro-3-benzazepin-8-ol [SCH23390]) or  $D_2$ -like receptor (raclopride) for 3 days before SKF83959 (1 mg/kg, i.p.) administration, respectively. Pretreatment with SCH23390, not raclopride, prevented the SKF83959-induced decrease in immobility duration in the forced swim test (FST) test ( $n=10$ ). (B) CSDS-treated mice were co-injected with SKF83959 and SCH23390/raclopride for 14 days. Administration of SCH23390, not raclopride, blocked the behavioral effects of SKF83959 in the sucrose preference test ( $n=10$ ). (C) Administration of SCH23390, not raclopride, blocked the behavioral effects of SKF83959 in the social interaction test ( $n=10$ ). (D) The effects of SKF83959 on hippocampal brain-derived neurotrophic factor (BDNF) expression were blocked by SCH23390, not raclopride ( $n=5$ ). (E) The effects of SKF83959 on hippocampal pCREB level were also abolished by SCH23390, not raclopride ( $n=5$ ). Data are expressed as means  $\pm$  SEM; <sup>##</sup> $P<.01$  vs control; <sup>\*\*</sup> $P<.01$  vs defeated + vehicle group. Comparison was made by 2-way analysis of variance (ANOVA) followed by posthoc Bonferroni's test.

To further determine whether SKF83959 produces effects through the D<sub>5</sub> receptor, D<sub>5</sub> siRNA was injected into the hippocampus to interrupt the D<sub>5</sub> receptor. Figure 5A showed the effectiveness of D<sub>5</sub> siRNA (n=5, P<.01 vs control). As shown in Figure 5B, while D<sub>5</sub> or scrambled siRNA (2 nmol/mouse) alone produced no effects on the immobility, D<sub>5</sub> siRNA pretreatment prevented the antidepressant effects of SKF83959 in the FST (n=8, P < 0.01 vs control). Moreover, D<sub>5</sub> siRNA also abolished the SKF83959 effects in the sucrose preference test (n=8, Figure 5C) and social interaction test (n=8, Figure 5D).

Next, the 5-HT<sub>2c</sub> receptor antagonist SB242084 (5 nmol/mouse), α<sub>2c</sub> receptor antagonist JP1302 (5 nmol/mouse), and sigma-1 receptor antagonist BD1063 (5 nmol/mouse) were used. It was found that neither of these antagonists produced influence on the SKF83959-induced shortening of immobility in the FST (n=10) (supplementary Figure S3A). Similarly, these antagonists could not block the antidepressant effects of SKF83959 in the sucrose preference test (n=10; supplementary Figure S3B) and social interaction test (n=10; supplementary Figure S3C). Together, these results indicate that SKF83959 produces antidepressant effects through D<sub>5</sub> receptor.

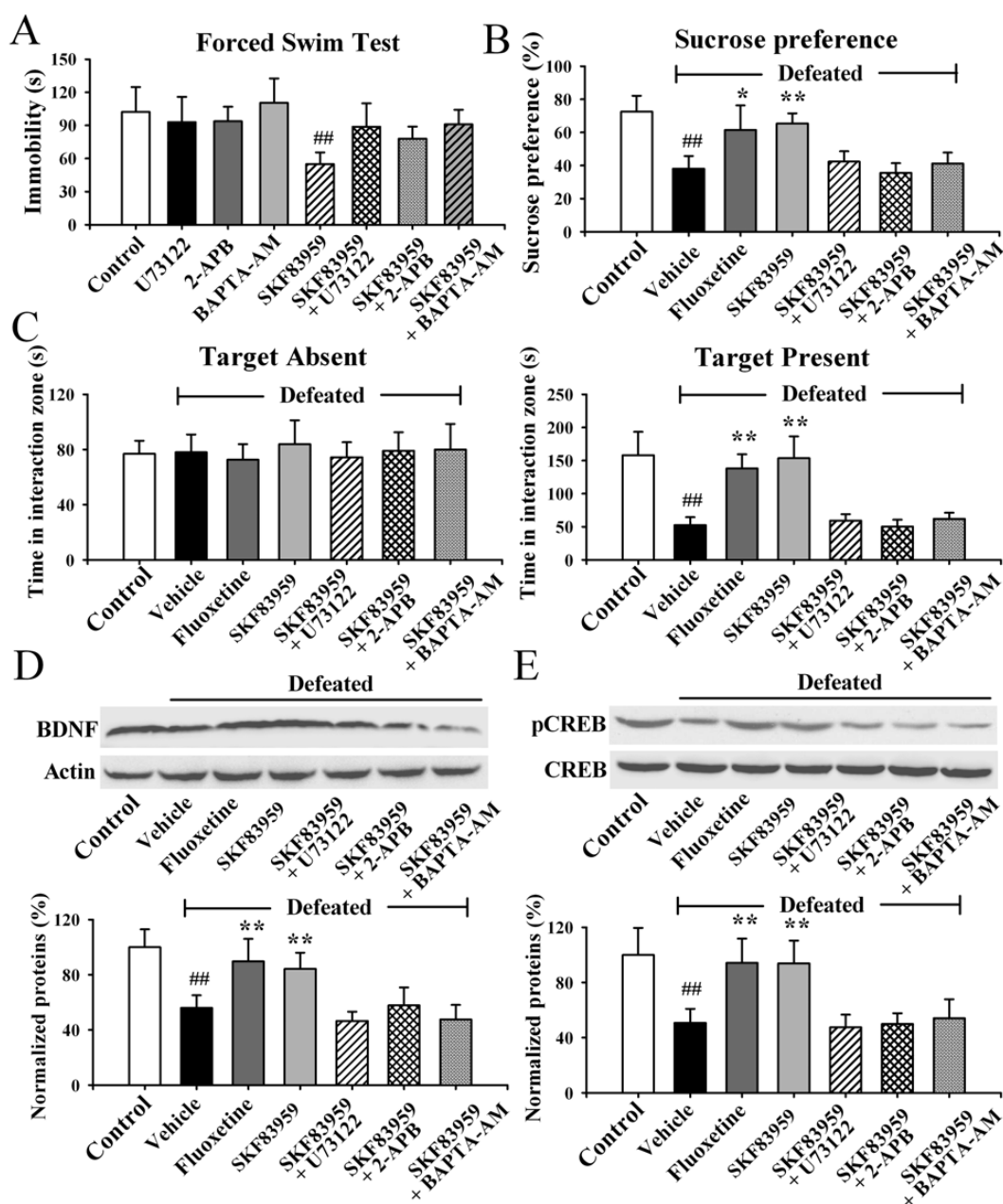


**Figure 5.** The antidepressant effects of 6-Chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF83959) require the hippocampal D<sub>5</sub> receptor. (A) Western blotting results confirmed the specificity and efficacy of D<sub>5</sub> small interfering RNA (siRNA) (n=5). (B) Mice were first treated with D<sub>5</sub> or scrambled siRNA (2 nmol/mouse, daily) for 3 days, then administrated with SKF83959 (1 mg/kg, i.p.) and followed by an forced swim test (FST). D<sub>5</sub> siRNA pretreatment dramatically prevented the SKF83959-induced decrease in immobility in the FST (n=8). (C) Chronic social defeat stress (CSDS)-treated mice were co-treated with SKF83959 and D<sub>5</sub> siRNA for 14 days, behavioral tests were then performed. Co-treatment SKF83959 with D<sub>5</sub> siRNA blocked the antidepressant effects of SKF83959 in the sucrose preference test (n=8). (D) Co-treatment SKF83959 with D<sub>5</sub> siRNA also blocked the antidepressant effect of SKF83959 in the social interaction test (n=8). Data are expressed as means ± SEM; <sup>##</sup>P < .01 vs control; <sup>\*\*</sup>P < .01 vs defeated + vehicle group. For A, comparison was made by 1-way analysis of variance (ANOVA) followed by posthoc Least Significant Difference test. For B-D, comparison was made by 2-way ANOVA followed by posthoc Bonferroni's test.

### The SKF83959-Mediated Antidepressant-Like Effects Require Activation of the PLC Signaling Pathway

SKF83959 is known to stimulate PI-hydrolysis via phospholipase C $\beta$  and results in the production of IP $_3$ , which subsequently induces intracellular calcium release (Jin et al., 2003;

Zhen et al., 2004). Then, we employed the inhibitors of the PLC signaling pathway to assess the role of the PLC/IP $_3$  pathway in SKF83959-mediated behavioral effects. As shown in Figure 6A, while PLC inhibitor U73122 (5 nmol/mouse) infusion alone had no detectable effects on immobility in the FST, it dramatically prevented the SKF83959-induced shortening of immobility time



**Figure 6.** The 6-Chloro-7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF83959)-induced antidepressant effects require the phospholipase C (PLC) signaling. (A) Mice were daily pretreated with the inhibitors of PLC (U73122), IP $_3$  (2-APB), or the intracellular Ca $^{2+}$  chelator (BAPTA-AM) for 3 days before SKF83959 (1 mg/kg, i.p.) administration, respectively. Pretreatment with these inhibitors prevented the SKF83959-induced decrease of immobility duration in the FST test (n=10). (B) CSDS-treated mice were co-injected with SKF83959 and PLC signaling inhibitors for 14 days. Administration of U73122, 2-APB, or BAPTA-AM blocked the behavioral effects of SKF83959 in the sucrose preference test (n=10). (C) Administration of PLC signaling inhibitors also blocked the antidepressant-like effects of SKF83959 in the social interaction test (n=10). (D) The effects of SKF83959 on hippocampal brain-derived neurotrophic factor (BDNF) expression were blocked by U73122, 2-APB, or BAPTA-AM, respectively (n=5). (E) The effects of SKF83959 on hippocampal phosphorylated cAMP response element-binding protein (pCREB) level were also abolished by PLC signaling inhibitors (n=5). Data are expressed as means  $\pm$  SEM; <sup>#</sup> P < .01 vs control; <sup>\*</sup> P < .05, <sup>\*\*</sup> P < .01 vs defeated + vehicle group. Comparison was made by 2-way analysis of variance (ANOVA) followed by posthoc Bonferroni's test.



( $n=10$ ,  $P < 0.01$  vs control). Moreover, the increased sucrose preference (Figure 6B) and social interaction (Figure 6C) induced by SKF83959 were also blocked by U73122 ( $n=10$ ). It was also found that IP<sub>3</sub> inhibitor 2-APB (3 nmol/mouse) or intracellular Ca<sup>2+</sup> chelator BAPTA-AM alone (5 nmol/mouse) had no effects on the duration of immobility but effectively prevented the effects of SKF83959 in the FST ( $n=10$ ; Figure 6A), sucrose preference test ( $n=10$ ; Figure 6B), and social interaction test ( $n=10$ ; Figure 6C).

A series of Western blotting assays were further performed. Supplementary Figure S1E shows that U-73122, 2-APB, and BAPTA-AM all blocked the SKF83959-induced increase in the hippocampal BDNF level of normal mice ( $n=5$ ,  $P < 0.01$  vs control), suggesting that the SKF83959-stimulated BDNF upregulation involves the activation of the PLC/IP<sub>3</sub>/Ca<sup>2+</sup> pathway. Figure 6D shows that pretreatment with 2-APB, BAPTA-AM, or U73122 prevented the SKF83959-induced effects on hippocampal BDNF expression of stressed mice. Parallel to BDNF, infusion of U73122, 2-APB, and BAPTA-AM also blocked the SKF83959 effects on hippocampal CREB phosphorylation ( $n=5$ ) (Figure 6E). Together, these results demonstrate that the PLC/IP<sub>3</sub> pathway is necessary for the SKF83959-mediated antidepressant effects.

### BDNF-TrkB System Is Necessary for the SKF83959-Mediated Antidepressant-Like Effects

To determine whether the BDNF-TrkB system is necessary for the antidepressant-like effects of SKF83959, K252a, a potent pharmacological inhibitor of the BDNF receptor TrkB (Tapley et al., 1992), was used. As shown in Figure 7A, while K252a (5 nmol/mouse) alone had no effects on the immobility, it prevented the antidepressant effects of SKF83959 in the FST ( $n=10$ ,  $P < 0.01$  vs control), sucrose preference test ( $n=10$ ; Figure 7B), and social interaction test ( $n=10$ ; Figure 7C). Moreover, the SKF83959-induced increase in hippocampal BDNF expression of defeated mice was also blocked by K252a treatment ( $n=5$ ; Figure 7D). In line with this, K252a also abolished the effects of SKF83959 on the expression of hippocampal pERK1/2, pAKT, and pCREB ( $n=5$ ; Figure 7D).

In a parallel experiment, the effects of K252a on the SKF83959-induced increase in neurogenesis were investigated. As shown in supplementary Figure S4A-C, the SKF83959-induced increase in the DCX+ cell amount ( $n=5$ ) and DCX protein level ( $n=5$ ) in the DG of defeated mice were blocked by chronic K252a injections. Next, the Golgi-Cox staining showed that K252a treatment also prevented the SKF83959-induced increase in hippocampal dendritic spine density ( $n=6$ ; supplementary Figure S4D-E) of defeated mice.

Furthermore, we used the lentiviral expression of specific short hairpin RNAs (shRNAs) against TrkB to downregulate the hippocampal BDNF-TrkB system. The expression of lentivirus-delivered shRNAs was stable at day 14 after the injection (Figure 8A), and their efficacy in downregulating the TrkB expression was further confirmed ( $n=5$ ,  $P < 0.01$  vs control) (Figure 8B). As shown in Figure 8C, while TrkB shRNA or control shRNA ( $3 \times 10^6$  TU/mouse) alone had no effects on the immobility duration, pretreatment of TrkB shRNA fully abolished the antidepressant effects of SKF83959 in the FST ( $n=9$ ,  $P < 0.01$  vs control). Also, TrkB shRNA abolished the effects of SKF83959 in the sucrose preference test ( $n=9$ ; Figure 8D) and social interaction test ( $n=9$ ; Figure 8E). Collectively, these results indicate that the BDNF-TrkB pathway is necessary for the SKF83959-mediated neurogenic and antidepressant effects.

## Discussion

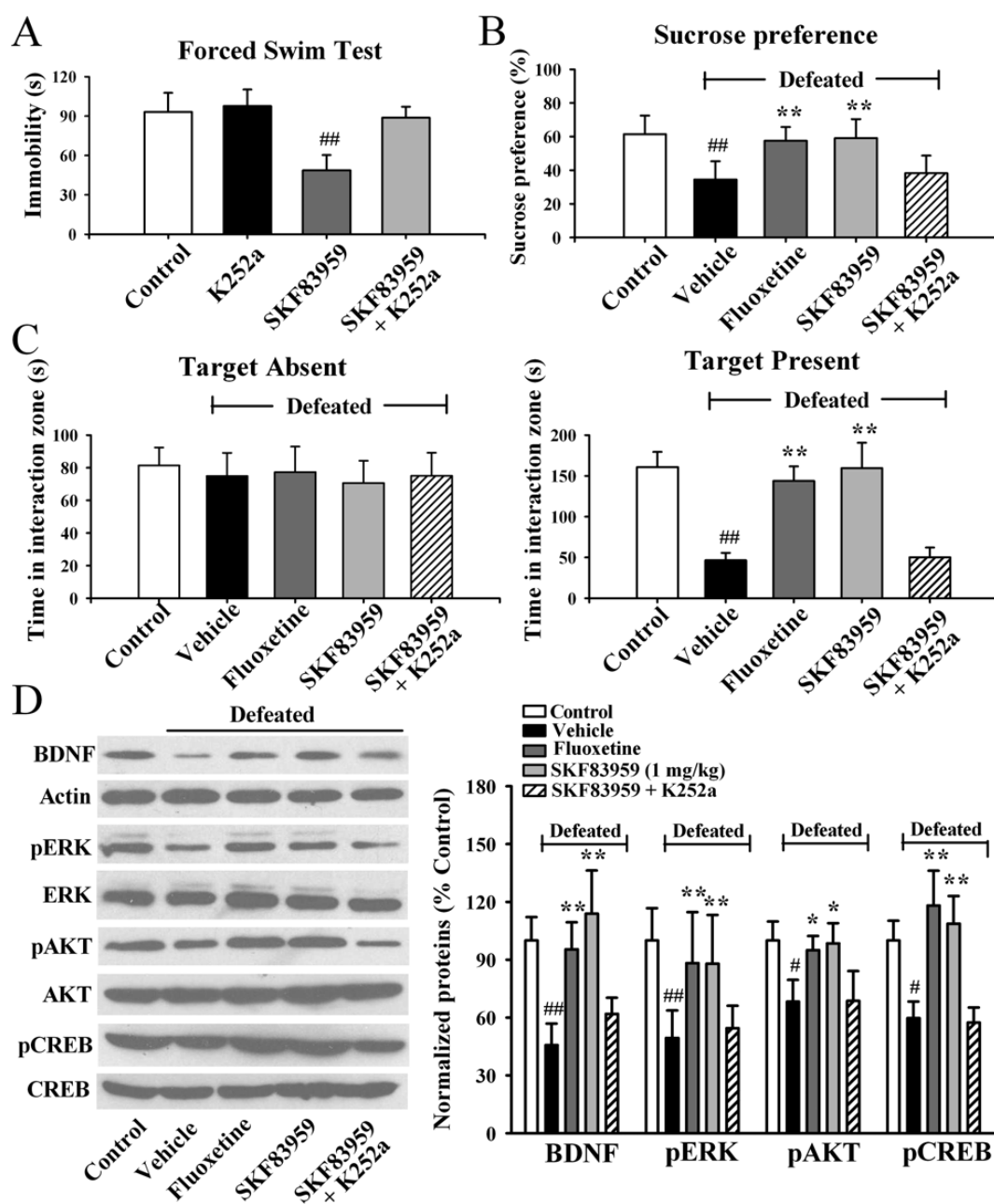
In the present study, we demonstrated that SKF83959 produced robust antidepressant effects in the CSDS model of depression.

It was found that chronic treatment of SKF83959 could reverse the CSDS-induced decrease of hippocampal neurogenesis and dendritic spine density and also restored the stress-induced decrease in hippocampal BDNF signaling cascade. By using various inhibitors and siRNA/shRNA methods, we further confirmed that these effects were mediated by activating the D<sub>5</sub> receptor, PLC/IP<sub>3</sub>/CaMKII $\alpha$  pathway, and BDNF-TrkB system. We also found that the hippocampus was important for the SKF83959-mediated effects.

The conclusion that SKF83959 has antidepressant effects in the CSDS model of depression should be reliable and believable, since Fang et al. (2013) reported that SKF83959 could reduce the immobility of mice in the FST and tail suspension test, two widely used behavioral assays for detecting potential antidepressant-like activity, and have high predictive validity for antidepressant activity. Pharmacological and biological blockade of hippocampal D<sub>5</sub> receptor abolished the antidepressant effects of SKF83959. This is very interesting and may suggest that the hippocampal D<sub>5</sub> receptor can be a target for antidepressants. SKF83959 also activates the D<sub>1</sub>-D<sub>2</sub> heteromer, and one recent paper demonstrated that uncoupling the D<sub>1</sub>-D<sub>2</sub> heteromer complex in the PFC exerted antidepressant effects (Pei et al., 2010), which is in contrast to our study. One explanation for this discrepancy may be that the hippocampal SKF83959-D<sub>5</sub> receptor activation-induced effects exceed the prefrontal SKF83959-D<sub>1</sub>-D<sub>2</sub> heteromer activation-induced effects. The behavioral data of Figure 4 may support this explanation, since the immobility time of SKF83959+raclopride co-treated group was even less than the SKF83959-treated group in the FST (SKF83959,  $49.1 \pm 9.8$  seconds; SKF83959+raclopride,  $32.1 \pm 7.3$  seconds; Figure 4A), and the sucrose consumption and social interaction of SKF83959+raclopride+CSDS group were even higher than the SKF83959+CSDS group (Figure 4B-C), indicating that blockade of the D<sub>1</sub>-D<sub>2</sub> heteromer antagonized the SKF83959-D<sub>1</sub>-D<sub>2</sub> heteromer activation-induced depressive effects.

Using the PLC/IP<sub>3</sub> pathway inhibitors, we found that SKF83959 produced antidepressant effects through this signaling pathway. This is consistent with previous reports showing that repeated stress reduced both the activity and expression of PLC in the frontal cortex and hippocampus of animals, and elevation of IP<sub>3</sub> by administering inositol reduced depressive-like behaviors in animal models of depression (Einat et al., 1999; Dwivedi et al., 2005). For the downstream pharmacological target of SKF83959, we selected BDNF in our study, as (1) SKF83959 induces the activation of CaMKII $\alpha$ , which could affect the expression of BDNF (Chen et al., 2012; Yu et al., 2013), and (2) SKF83959 promotes the striatal BDNF expression of normal mice through D<sub>1</sub>-D<sub>2</sub> heteromer (Hasbi et al., 2009) and also promotes the prefrontal BDNF expression of normal mice through D<sub>5</sub> receptor (Perreault et al., 2013). Our Western blotting data revealed that SKF83959 affected the BDNF system in the hippocampus, not PFC or NAC, of depressed mice. The nonobservance of enhanced prefrontal and striatal BDNF expression of depressed mice after SKF83959 treatment is interesting. The explanation may be that chronic stress led to decreased D<sub>1</sub>-like dopaminergic function in the PFC and also decreased D<sub>2</sub> receptor expression in the NAC (Papp et al., 1994; Mizoguchi et al., 2002), suggesting the downregulated function and response to SKF83959 of prefrontal D<sub>5</sub> receptor/striatal D<sub>1</sub>-D<sub>2</sub> heteromer under depressive conditions compared to normal conditions. Besides, it was found that SKF83959 had no influence on the NGF, NT3, and NT4, 3 other neurotrophic factors, indicating that the antidepressant role of SKF83959 is not only regionally selective but also biologically selective.

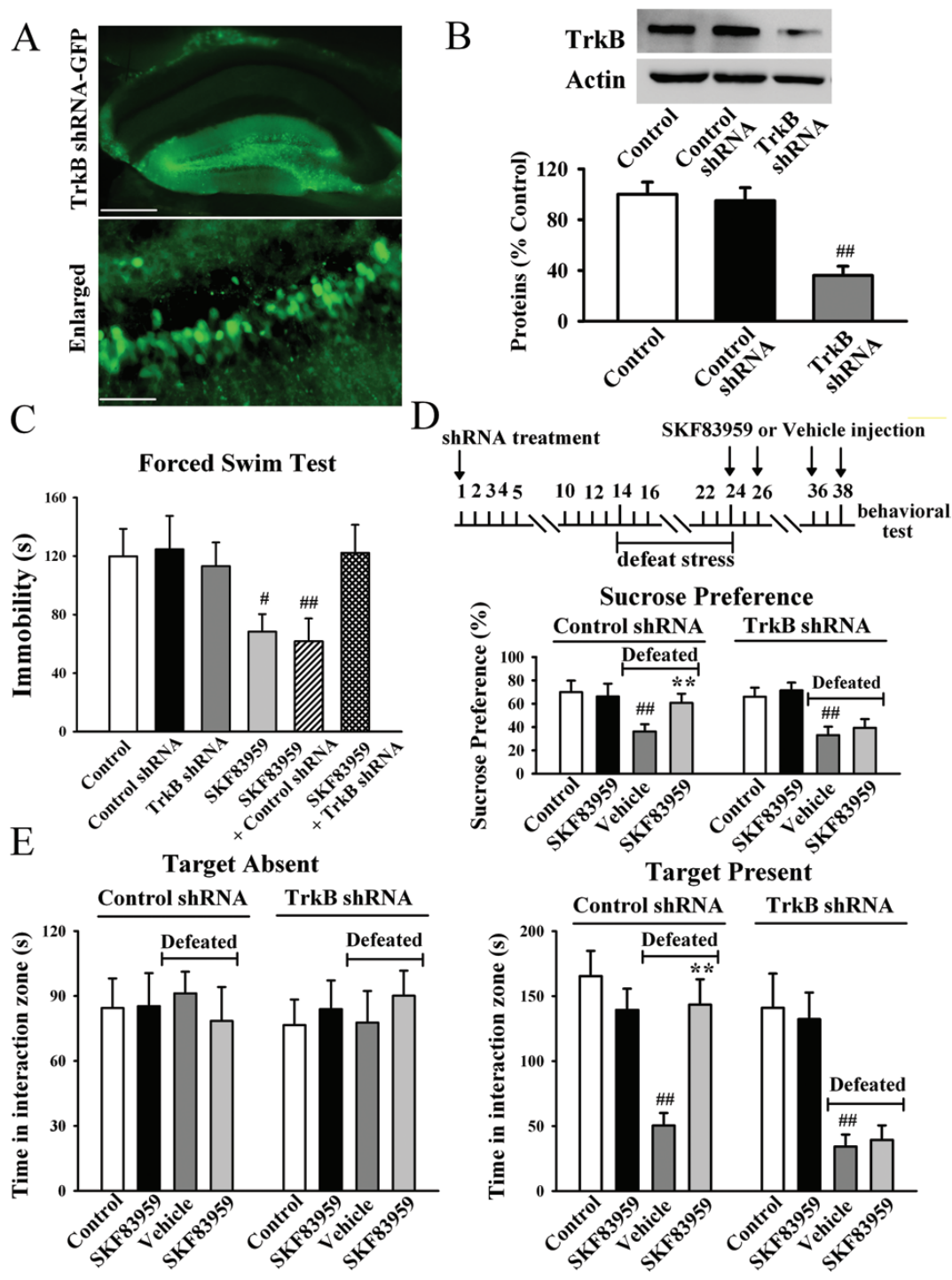
Chronic stress affects the neurogenic and neurotrophic pathways that maintain ionic homeostasis (Duman and Monteggia,



**Figure 7.** Blockade of brain-derived neurotrophic factor (BDNF)-tyrosine kinase B (TrkB) signaling by K252a abolishes the antidepressant actions of 6-Chloro- 7,8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF83959). (A) Mice were first treated with K252a (5 nmol/mouse, daily) for 3 days, then administrated with SKF83959 (1 mg/kg, i.p.) and followed by examination of forced swim test (FST). K252a pretreatment dramatically prevented the SKF83959-induced decrease of immobility in the FST (n=10). (B) Chronic social defeat stress (CSDS)-treated mice were co-injected with SKF83959 and K252a for 14 days; behavioral tests were then performed. SKF83959+K252a mice displayed lower sucrose preference than SKF83959-treated mice (n=10). (C) Co-treatment SKF83959 with K252a blocked the antidepressant effects of SKF83959 in the social interaction test (n=10). (D) K252a injection antagonized the actions of SKF83959 on hippocampal BDNF, phosphorylated extracellular signal-regulated kinase (pERK), Protein Kinase B (pAKT), and cAMP response element-binding protein (pCREB) of chronic social defeat stress (CSDS)-treated mice (n=5). Data are expressed as means ± SEM; \*P < .05, \*\*P < .01 vs control; #P < .05, ##P < .01 vs defeated + vehicle group. Comparison was made by 2-way analysis of variance (ANOVA) followed by posthoc Bonferroni's test.

2006; Razzoli et al., 2011). The immunohistochemical data revealed that SKF83959 promoted the hippocampal neurogenesis of both the depressed mice and normal mice, suggesting that SKF83959 may also be developed as a proneurogenic compound. Depression is also accompanied with hippocampal neuronal atrophy and dendritic arborization (Magarinos et al., 2011). Our results showed a significant antidepressant effect of SKF83959 by increasing the spine density of CA3 pyramidal neurons. This

observation is consistent with previous studies showing that activation of dopamine signaling and CaMKII $\alpha$  in the neurons of NAc leads to enhanced neuronal differentiation and spine density (Schmidt et al., 1996; Ciani et al., 2011). Moreover, the usage of K252a found that these SKF83959-induced neurogenic and neurotrophic effects were all mediated through the BDNF system. In addition, repeated administration of SKF83959 has been shown to result in desensitization of the behavioral and



**Figure 8.** Blockade of brain-derived neurotrophic factor (BDNF)-tyrosine kinase B (TrkB) signaling by TrkB shRNA abolishes the antidepressant actions of 6-Chloro-7, 8-dihydroxy-3-methyl-1-(3-methylphenyl)-2,3,4,5-tetrahydro-1H-3-benzazepine (SKF83959). (A) GFP fluorescence of a fixed brain slice, which expressed the GFP and TrkB shRNA in the hippocampus on 14th day after stereotaxic injection of the lentivirus vectors. The scale bar is 200  $\mu$ m for representative images and 25  $\mu$ m for enlarged images. (B) Examples of Western blots for TrkB and  $\beta$ -actin in the same lysate of noninfected hippocampal neurons (control) and hippocampal neurons infected with lentivirus containing control short hairpin RNA (shRNA) or TrkB shRNA ( $n = 5$ ). (C) TrkB shRNA pretreatment before SKF83959 (1 mg/kg, i.p.) administration dramatically prevented the SKF83959-induced decrease of immobility in the FST ( $n = 9$ ). (D) TrkB shRNA or control shRNA pretreated mice received 10 days of defeat stress and then 14 days of SKF83959 (1 mg/kg, i.p.) or vehicle injection, respectively. Behavioral tests were then performed. TrkB shRNA, not control shRNA, fully blocked the antidepressant effects of SKF83959 in the sucrose preference test ( $n = 9$ ). (E) TrkB shRNA, not control shRNA, also blocked the antidepressant effects of SKF83959 in the social interaction test ( $n = 9$ ). Data are expressed as means  $\pm$  SEM; <sup>#</sup> $P < .05$ , <sup>##</sup> $P < .01$  vs control; <sup>\*\*</sup> $P < .01$  vs defeated + vehicle group. For B, comparison was made by 1-way analysis of variance (ANOVA) followed by posthoc Least Significant Difference test. For C-E, comparison was made by 2-way ANOVA followed by posthoc Bonferroni's test.

molecular effects of the drug, but this is not likely involved in the effect of SKF83959 in the present study, because our experiments demonstrated that the antidepressant effects of SKF83959 are mediated through activating the  $D_5$  receptor. In summary,

our study reveals a new action of SKF83959 on central nervous system, which may lead to the development of new treatments for depression and other psychiatric disorders. Moreover, in addition to depression, the BDNF and dopaminergic systems are

implicated in some other neurodegenerative dysfunctions, like Alzheimer's disease (Voineskos et al., 2011), so it is possible that SKF83959 may also produce effects in these disorders.

## Supplementary Material

For supplementary material accompanying this paper, visit <http://www.ijnp.oxfordjournals.org>

## Acknowledgments

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## Statement Interest

None.

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