Role of proBDNF and BDNF in Dendritic Spine Plasticity and Depressive-like Behaviors induced by an Animal Model of Depression

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ABSTRACT

Major depressive disorder (MDD) is one of the most common psychiatric disorder, but the underlying mechanisms are largely unknown. Increasing evidence shows that brain-derived neurotrophic factor (BDNF) plays an important role in the structural plasticity induced by depression. Considering the opposite effects of BDNF and its precursor proBDNF on neural plasticity, we hypothesized that the balance of BDNF and proBDNF plays a critical role in chronic unpredicted mild stress (CUMS)-induced depressive-like behaviors and structural plasticity in the rodent hippocampus. The aims of this study were to compare the functions of BDNF and proBDNF in the CUMS-induced depressive-like behaviors, and determine the effects of BDNF and proBDNF on expressions of kalirin-7, postsynaptic density protein 95 (PSD95) and NMDA receptor subunit NR2B in the hippocampus of stressed and naïve control rats, respectively. Our results showed that CUMS induced depressive-like behaviors, caused a decrease in the ratio of BDNF/proBDNF in the hippocampus and resulted in a reduction in spine density in hippocampal CA1 pyramidal neurons; these alterations were accompanied by a decrease in the levels of kalirin-7, PSD95 and NR2B in the hippocampus. Injection of exogenous BDNF into the CA1 area of stressed rats reversed CUMS-induced depressive-like behaviors and prevented CUMS-induced spine loss and decrease in kalirin-7, NR2B and PSD95 levels. In contrast, injection of exogenous proBDNF into the CA1 region of naïve rats caused depressive-like behavior and an accompanying decrease in both spine density and the levels of kalirin-7, NR2B and PSD95. Taken together, our results suggest that the ratio of BDNF to proBDNF in the hippocampus plays a key role in CUMS-induced depressive-like behaviors and alterations of dendritic spines in hippocampal CA1 pyramidal neurons. Kalirin-7 may play an important role during this process.

KEY WORDS: BDNF; proBDNF; Depression; Stress; Kalirin; Dendritic spine

Abbreviations: Brain-derived neurotrophic factor (BDNF); Major depressive disorder (MDD); Chronic unpredicted mild stress (CUMS); NMDA receptor subtype 2B (NR2B); Postsynaptic density protein 95 (PSD95); Tropomyosin-related kinase B (TrkB).

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1. Introduction

Depression is a severe psychiatric disorder that affects up to 7% of the population in the United States (Levinstein and Samuels 2014). Although depression has been studied for several decades, the underlying mechanisms which cause depression are still not clear. Some depression patients are resistant to currently available antidepressant treatments. In order to develop more effective treatments, it is important to identify the mechanisms underlying depression. Accumulating evidence shows that structural plasticity of the neurons, particularly atrophy of dendrites and loss of dendritic spines in hippocampal neurons, may play a key role in the pathogenesis of depression (von Bohlen Und Halbach 2009, Mandela and Ma 2012, Duman and Duman 2015).

It is well documented that depression-mediated structural plasticity in the hippocampus is associated with alterations in brain-derived neurotrophic factor (BDNF) signaling (Duman and Monteggia 2006, Castren and Rantamaki 2010), however the role of its precursor (proBDNF) has not been clearly elucidated. BDNF is initially synthesized as a precursor protein (proBDNF) (Matsumoto et al. 2008). BDNF has been shown to promote neuronal survival and differentiation and increase formation of dendritic spines and synapses (Tyler and Pozzo-Miller 2003, Hiester et al. 2013) via its high-affinity receptor, tropomyosin-related kinase B (TrkB) (Huang and Reichardt 2003). However, ProBDNF causes a simplification of the dendritic arbor, decreases spine density (Zagrebelsky et al. 2005, Yang et al. 2014), and induces long-term depression (LTD) (Woo et al. 2005) through its p75 neurotrophin receptor (p75NTR) in hippocampal neurons.

Chronic unpredictable mild stress (CUMS) has been used as an established animal model of depression (Qiao et al. 2016). The mechanisms through which CUMS causes structural and functional alterations in the hippocampus are not clear. Considering the opposite functions of BDNF and proBDNF in neuronal plasticity (Martinowich et al. 2007, Je et al. 2012) and the conflicting reports about CUMS's ability to reduce BDNF levels in the hippocampus (Allaman et al. 2008, Larsen et al. 2010), we hypothesized that the balance of proBDNF and BDNF in the hippocampus plays a key role in the development of CUMS-induced depressive-like behaviors.

Kalirin-7, the most abundant kalirin isoform in the adult rodent brain, is exclusively

localized to the postsynaptic side of excitatory synapses (Ma et al. 2008, Ma et al. 2011). Kalirin-7 which interacts with many synaptic proteins including PSD95 and NR2B in the postsynaptic side of excitatory synapses plays a key role in spine morphogenesis and synaptic plasticity (Penzes et al. 2001, Ma et al. 2008, Ma et al. 2008, Kiraly et al. 2011, Mandela and Ma 2012). Hippocampal CA1 pyramidal neurons of kalirin-7 knockout (KO) mice show a decrease in spine density and deficit in long-term potentiation (LTP). Accumulating evidence demonstrates that chronic stress results in a decrease in spine density in the pyramidal neurons of the hippocampal CA3 (Magarinos et al. 1996, Sousa et al. 2000, Sandi et al. 2003, Pawlak et al. 2005, Chen et al. 2010, Qiao et al. 2014) or CA1 (Kassem et al. 2013, Qiao et al. 2014, Castaneda et al. 2015, Huang et al. 2015) or both CA1 and CA3 (Hajszan et al. 2009, Magarinos et al. 2011, Kim et al. 2013) regions. Our previous study showed that a CUMS-mediated decrease in spine density in CA1 and CA3 pyramidal neurons is accompanied by a decrease in the levels of kalirin-7 (Qiao et al. 2014). Kalirin has been identified as a downstream target of BDNF in cultured neurons *in vitro* (Yan et al. 2016). Although the roles of BDNF and kalirin-7 in spine formation are well established (Tyler and Pozzo-Miller 2003, Mandela and Ma 2012, Ma et al. 2014), it remained unknown how BDNF or proBDNF regulates kalirin-7 expression in the hippocampus after exposure to CUMS. Therefore, we hypothesized that the ratio of BDNF/proBDNF plays an important role in CUMS-induced depressive-like behaviors and CUMS-mediated decreases in spine density in hippocampal neurons. Kalirin-7 may work as a downstream signal of BDNF and proBDNF to regulate CUMS-mediated spine plasticity.

To test our hypothesis, we determined the role of the BDNF/proBDNF ratio in CUMS-induced depressive-like behaviors and spine loss and examined the expression of kalirin-7 and its binding partners, including PSD95 and NR2B, in the hippocampus after exposure to CUMS or pretreatment with proBDNF in rat. The experimental design is showed in Figure 1.

2. Results

CUMS-induced depressive-like behaviors in rats

The open field test (OFT) is one of the most widely used tests for estimating the Anxiety related behaviors of rodents, and is commonly used for measuring general locomotor activity, exploratory behavior (rearing) and self-care behavior (grooming) in rodents (Luo et al. 2008). As expected, CUMS decreased the locomotor activity (116.1 ± 17.7 in the CUMS group vs. 144.5 ± 16.5 of control, $n = 8$, $p < 0.05$, Figure 2A), the number of rearings (6.7 ± 1.5 in the CUMS group vs. 14.5 ± 2.4 in the control group, $n = 8$, $p < 0.01$, Figure 2B) and grooming behavior (4.0 ± 1.2 in the CUMS group vs. 9.5 ± 1.7 of control, $n = 8$, $p < 0.01$, Figure 2C) in the open field test. The tail suspension test (TST) is used to assess moods in rodents (Guan et al. 2015). A longer immobility time indicating a depressive-like behavior. In agreement with our previous report (Qiao et al., 2014), the tail suspension test showed that CUMS increased the duration of immobility (135.2 ± 13.2 sec in the CUMS group vs. 70.6 ± 7.1 sec in the control group, $n = 8$, $p < 0.01$, Figure 2D).

2.1 CUMS decreased the ratio of BDNF/proBDNF by decreasing BDNF expression without altering proBDNF level in the hippocampus.

Western blot analysis showed that the expression of BDNF in the hippocampus decreased in the CUMS group compared with the control group (83.3 ± 4.6 % of control, $n = 8$, $p < 0.05$, Figure 2). Though the expression of proBDNF tended to increase in the CUMS group, it did not reach a statistically significant difference compared with the control group (109.2 ± 9.9 % of control, $n = 8$, $p > 0.05$, Figure 3). The ratio of BDNF/proBDNF was significantly decreased in the CUMS group (70.1 ± 4.7 % of control, $n = 8$, $p < 0.01$, Figure 2B).

2.2 CUMS-induced depressive-like behaviors were partially attenuated by microinjection of BDNF into hippocampal CA1 region, while microinjection of proBDNF into the same area induced depressive-like behaviors in naïve control rats.

Microinjection of BDNF into the rat CA1 area in the CUMS group reversed CUMS-induced decreases in locomotor activity (140.2 ± 10.4 in the CUMS + BDNF group vs. 116.1 ± 17.7 in the CUMS group, $n = 8$, $p < 0.05$; Figure 2A), the numbers of rearings (9.0 ± 1.6 in the CUMS + BDNF group vs. 6.7 ± 1.5 in the CUMS group, $n = 8$, $p < 0.05$, Figure 2B),

and the duration of immobility (66.7 ± 10.1 sec in the CUMS + BDNF group vs. 135.3 ± 13.3 sec in the CUMS group, $n = 8$, $p < 0.01$, Figure 2D) compared with the CUMS group which received saline only. Interestingly, microinjection of proBDNF into the CA1 region of naïve control rats induced depressive-like behaviors characterized by lower locomotor activity (108.1 ± 14.0 in the proBDNF group vs. 144.5 ± 16.5 in the control group, $n = 8$, $p < 0.01$, Figure 2A), decreased rearing activity (5.2 ± 1.6 in the proBDNF group vs. 14.5 ± 2.4 in the control group, $n = 8$, $p < 0.01$, Figure 2B) and decreased grooming behavior (2.1 ± 1.0 in the proBDNF group vs. 9.5 ± 1.7 in the control group, $n = 8$, $p < 0.01$, Figure 2C) as well as an increased time of immobility in the proBDNF group compared with the control group (138.5 ± 6.7 in the proBDNF group vs. 70.6 ± 7.1 sec in the control group, $n = 8$, $p < 0.01$) (Figure 2D).

2.3 CUMS-induced decrease in spine density in CA1 pyramidal neurons was reversed by injection of BDNF into CA1 area while injection of proBDNF into the same area decreased spine density in CA1 pyramidal neurons in native control rats.

To compare the role of BDNF and proBDNF on dendritic spines, we counted the dendritic spine density of hippocampal CA1 pyramidal neurons in the control (Figure 4A-4D), CUMS (Figure 4E-4G), CUMS+BDNF (Figure 4H-4J) and proBDNF (Figure 4K-4M) groups after Golgi staining (Figure 5). The CUMS group exhibited a significant reduction in spine density in CA1 apical dendrites in comparison with the control group ($6.3 \pm 1.6/10 \mu\text{m}$ in the CUMS group vs. $10.8 \pm 2.1/10 \mu\text{m}$ in the control group, $n = 8$, $p < 0.01$, Figure 4B-4D vs. 4E-4G, Figure 5). Injection of BDNF into the hippocampal CA1 area prevented CUMS-mediated reduction in spine density in CUMS animals ($11.8 \pm 1.5/10 \mu\text{m}$ in the CUMS + BDNF group vs. $6.3 \pm 1.6/10 \mu\text{m}$ in the CUMS group, $n = 8$, $p < 0.01$, Figure 4B-4D vs. 4H-4J), Figure 5). In contrast, injection of proBDNF into the hippocampal CA1 region of naïve control rats resulted in a decrease in spine density in CA1 pyramidal neurons ($4.4 \pm 1.1/10 \mu\text{m}$ in the proBDNF group vs. $10.8 \pm 2.1/10 \mu\text{m}$ in control, $n = 8$, $p < 0.01$, Figure 4B-4D vs. 4K-4M, Figure 5).

2.4 CUMS-induced decrease in kalirin-7 expression in the hippocampus was reversed by microinjection of BDNF into the CA1 area while injection of proBDNF into the same CA1 area decreased kalirin-7 expression in the hippocampus in naïve control rats.

Kalirin-7 which interacts with several synaptic proteins in the PDZ, including PSD95 and NR2B plays a key role in spine morphogenesis and synaptic plasticity (Penzes et al. 2001, Ma et al. 2008, Ma et al. 2008). Kalirin is a down-stream target of BDNF (Yan et al. 2016). The aim of this experiment was to determine the roles of BDNF and proBDNF in CUMS-mediated alterations in kalirin-7 expression, which may help us understand the effects of BDNF and proBDNF on CUMS-induced depressive-like behaviors and dendritic spine plasticity in the hippocampus. Kalirin-7 expression was significantly decreased in the CUMS group compared to the control group (59.1 ± 6.1 % of the control group, $n = 8$, $p < 0.01$, Figure 6A-6B). Injection of BDNF into the hippocampal CA1 region of CUMS rats prevented CUMS-induced reduction in kalirin-7 levels (115.0 ± 11.2 % of control in the CUMS + BDNF group vs. 59.1 ± 6.1 % of control in the CUMS group, $p < 0.01$, Figure 6A-6B). However, microinjection of proBDNF into the hippocampal CA1 region in naïve control animals caused a decrease in the levels of kalirin-7 (62.5 ± 7.2 % of control, $p < 0.01$, Figure 6A-6B), the extent of this decrease is similar to the decrease in kalirin-7 levels induced by CUMS.

2.5 CUMS-induced decrease in PSD95 levels was reversed by microinjection of BDNF into the CA1 area in stressed rats, while injection of proBDNF in the CA1 area of naïve control rats decreased the levels of both PSD95 and NR2B in the hippocampus.

Interactions of Kalirin-7 with PSD95 and NR2B play an important role in kalirin-7-mediated synapse formation (Ma et al. 2008, Ma et al. 2008, Kiraly et al. 2011). We wanted to determine whether CUMS alters PSD95 and NR2B expression. Our results showed that CUMS resulted in a decrease in the levels of PSD95 (72.5 ± 9.6 % of control, $p < 0.01$, Figure 6C, 6E), but not the levels of NR2B (102.3 ± 9.4 % of control, $p > 0.05$, Figure 6C-6D) in the hippocampus compared to control. Injection of BDNF into the CA1 region of CUMS-stressed animals reversed the CUMS-induced decrease in PSD95 levels and caused an increase in NR2B levels in the CUMS group compared to the CUMS group which received saline only (PSD95: 129.3 ± 7.8 % of control in the CUMS + BDNF group vs. 72.5 ± 9.6 % of control in the CUMS group, $p < 0.01$; NR2B: 134.8 ± 10.4 % of control in the CUMS + BDNF group vs. 102.3 ± 9.4 % of control in the CUMS group, $p < 0.01$, Figure 6C-6E).

In addition, injection of proBDNF into the hippocampal CA1 region in naïve control animals resulted in a decrease in the levels of both PSD95 (54.8 ± 5.8 % of control, $p < 0.01$)

and NR2B (51.7 ± 10.4 % of control, $p < 0.01$) compared with the control group which received saline injection only (Figure 6C-6E). The levels of PSD95 in the proBDNF group were even lower than the levels of PSD95 in the CUMS group (54.8 ± 5.8 % of control in the proBDNF group *vs.* 72.5 ± 9.6 % of control in the CUMS group, $p < 0.05$, Figure 6C-6E). Similarly, the levels of NR2B in the proBDNF group were also lower than the levels of NR2B in the CUMS group (51.7 ± 10.6 % of control in the proBDNF group *vs.* 102.3 ± 9.4 % of control in the CUMS group, $p < 0.01$, Figure 6C-6E). These results showed that the proBDNF-mediated decrease in NR2B and PSD95 expression were coincident with the proBDNF-mediated decrease in expression of kalirin-7 in native control group (Figure 6).

3. Discussion

3.1 CUMS-induced depressive-like behaviors were accompanied by a decrease in spine density in hippocampal CA1 pyramidal neurons

In this study, depressive-like behaviors were successfully induced by three-weeks of CUMS, an established animal paradigm of depression (Qiao et al. 2014, Qiao et al. 2016). As expected, rats in the CUMS group exhibited lower locomotor activity, fewer rearings, and decreased grooming activity after 21 days of CUMS compared with the control group which only received daily handling. CUMS-induced depressive-like behaviors were accompanied by a significant reduction in spine density in the apical dendrites of hippocampal CA1 pyramidal neurons; this agrees with our previous study (Qiao et al. 2014) and reports by others (Pawlak et al. 2005, Kassem et al. 2013).

3.2 The balance of BDNF and proBDNF plays a critical role in CUMS-induced depressive-like behaviors and decrease in spine density

The CUMS-induced decrease in BDNF levels, but not those of proBDNF, contributed to the decrease in the ratio of BDNF/proBDNF, which was accompanied by depressive-like behaviors and a reduction in spine density in hippocampal CA1 pyramidal neurons and. The CUMS-induced decrease in BDNF levels was accompanied by depressive-like behaviors and a decrease in spine density, which is in line with previous reports which show that the low levels of BDNF in the hippocampus may lead to some functional and structural alterations in hippocampal neurons, induce depressive-like behaviors in rodents, and ultimately contribute to the symptoms of depression in humans (Duman and Monteggia 2006, Qiao et al. 2014). Our results agree with clinical findings which show that serum levels of BDNF are decreased in patients with (Major depressive disorder) MDD (Yoshida et al. 2012, Yoshimura et al. 2014), while the serum levels of proBDNF are not changed in these patients (Yoshida et al. 2012, Yoshimura et al. 2014). These findings raised a question whether proBDNF is involved in the development of depression symptoms in patients with MDD or depressive-like behaviors in animal models. To address this question, we delivered BDNF and proBDNF separately into the rat hippocampal CA1 area.

Mature BDNF and proBDNF play opposite roles in the formation of dendritic spines through binding to their receptors, TrkB and p75NTR, respectively. BDNF increases spine

formation (Lee et al. 2001), while proBDNF preferentially decreases spine density in hippocampal neurons (Zagrebelsky et al. 2005, Yang et al. 2014). In addition, BDNF plays a critical role in LTP induction, while proBDNF facilitates LTD induction (Martinowich et al. 2007). ProBDNF knock-in mice exhibit depressed synaptic transmission, impaired LTP, and enhanced LTD in the CA1 region of the hippocampus (Yang et al. 2014). Interestingly, the serum levels of mature BDNF, and its receptor TrkB, are negatively correlated with the severity of depression symptoms, while the levels of proBDNF and p75NTR are positively correlated with the severity of depression symptoms in depression patients (Zhou et al. 2013). Correlation of proBDNF levels with the severity of depression symptoms is supported by a recent preclinical study showing that the levels of proBDNF in the prefrontal cortex were increased in animals exposed to CUMS (Pesarico et al. 2016). These studies suggest an important role for proBDNF in the development of depressive-like behaviors induced by CUMS in rodents.

To further understand the roles of BDNF and proBDNF in the development of depressive-like behaviors, exogenous BDNF and proBDNF were each microinjected into the rat hippocampal CA1 area. As expected, injection of BDNF reversed both CUMS-induced depressive-like behaviors and decreases in the spine density of hippocampal CA1 pyramidal neurons. Interestingly, injection of proBDNF into the hippocampal CA1 area of naïve control rats induced depressive-like behaviors and caused a decrease in CA1 spine density, which is similar to the effects observed after CUMS exposure. These results suggested: CUMS-mediated decrease in BDNF levels plays an important role in CUMS-induced depressive-like behaviors, and proBDNF is involved in the CUMS-induced spine loss and the development of depressive-like behaviors, although no significant alteration in proBDNF expression was observed in the CUMS group compared to the control group. These findings support our hypothesis that the balance of BDNF and proBDNF plays a critical role in both CUMS-induced depressive-like behaviors and decreases in spine density on CA1 pyramidal neurons (Qiao et al. 2014). Our previous study showed that two-weeks of CUMS neither altered spine density on hippocampal CA1 pyramidal neurons nor did it induce depressive-like behaviors in rats, although BDNF levels were significantly lower in CUMS rats than in control rats (Qiao et al. 2014). This may result from the normal ratio of

hippocampal BDNF/proBDNF in the CUMS group compared with the control group (see supplementary material). Taken together, these data showed that CUMS-mediated reduction of BDNF levels alone may not be enough to affect the spine density, the ratio of the BDNF/proBDNF is critical, and up-regulation of proBDNF without alteration of BDNF expression leading to a reduction in the ratio of BDNF/proBDNF could induce spine loss and depressive-like behaviors, suggesting that the altered ratio of BDNF/proBDNF contributes to both CUMS-induced spine loss and depressive-like behaviors.

3.3 Kalirin may play an important role in CUMS-induced decrease in dendritic spine density

Kalirin-7 may play an important role in CUMS-induced spine loss. Overexpression of kalirin-7 causes an increase in dendritic spine density, spine size, and synapse number, while reduction of endogenous kalirin-7 levels leads to a reduction in spine density, spine size, and synapse number in cultured hippocampal and cortical neurons (Penzes et al. 2001, Ma et al. 2003, Mandela and Ma 2012). Additionally, CA1 pyramidal neurons from kalirin-7 deficient mice show a decrease in spine density (Ma et al. 2008). Importantly, Kalirin is identified as a downstream signal of BDNF (Yan et al. 2016). However, the relationship between proBDNF or BDNF with kalirin-7 and their role in the regulation of spine formation during CUMS is not clear. A recent study found that exposure of cultured hippocampal neurons to proBDNF dramatically reduced dendritic spine density (Guo et al. 2016). The above findings raised the possibility that kalirin-7 may play an important role in BDNF rescuing CUMS-induced spine loss and proBDNF-mediated decrease in dendritic spine density in naïve control rats. In our current study, CUMS induced a significant reduction of kalirin-7 expression in the hippocampus, while BDNF pretreatment prevented this reduction, and reversed CUMS-mediated decrease of spine density. In contrast, microinjection of exogenous proBDNF into the CA1 area caused a decrease in the levels of kalirin-7, accompanied by a reduction in spine density and depressive-like behaviors similar to those observed after CUMS exposure. These results suggested that kalirin-7 is a key player in CUMS induced spine loss and depressive-like behaviors, which will be addressed in our future study.

3.4 Postsynaptic complex of NR2B, PSD95 and kalirin-7 may play an important role in CUMS-mediated depressive-like behavior and spine loss in hippocampal CA1 pyramidal

neurons.

In order to explore the potential mechanism underlying CUMS-mediated spine loss, we studied the binding partners of kalirin-7, including PSD95 and NR2B. PSD95, a marker for postsynaptic specialization in the excitatory synapses (Sanchez et al. 2006), is associated with postsynaptic receptors and cytoskeletal elements in the excitatory synapse. It facilitates glutamate receptor transmission and participates in the etiology of depression (Ronesi et al. 2012, Li et al. 2015). Our results showed that the change in PSD95 levels induced by CUMS is similar to the change in Kalirin-7 levels induced by CUMS. CUMS caused a decrease in the expression of PSD95, and this decrease was reversed by BDNF pretreatment. Interestingly, microinjection of proBDNF into the hippocampal CA1 region of naïve control rats also caused a decrease in PSD95 levels compared to the control group which received saline injection. This decrease is similar to the decrease observed after exposure to CUMS.

NMDA receptors are thought of as primary postsynaptic targets for the BDNF–TrkB signaling cascade. NR2B interacts with kalirin-7 at excitatory synapses (Ma et al, 2008; Kirly et al., 2012). Our results demonstrated that CUMS did not significantly alter NR2B levels in the hippocampus. This agrees with one previous report (Yilmaz et al. 2011), but not with others which show that chronic stress increases expression of NR2B in the hippocampus (Gourley et al. 2009, Xu et al. 2009, Lee and Goto 2011, Quan et al. 2011). However, similar to the effect on kalirin-7 and PSD95, pretreatment of CUMS-stressed animals with BDNF significantly reversed the CUMS-induced decrease in NR2B expression compared with vehicle-treated groups, while intra-hippocampal administration of proBDNF to naïve control rats decreased the NR2B expression. This suggested that NR2B could be the downstream target of BDNF (Nakai et al. 2014) and proBDNF.

Kalirin-7 consistently colocalizes with PSD95 and NR2B in the postsynaptic side of excitatory synapses in hippocampal neurons where kalirin-7, PSD95 and NR2B may form a synaptic complex that is a down-stream target of BDNF (Ma et al. 2008, Ma et al. 2011). Interruption of the interaction between kalirin-7 and PSD95 decreases kalirin-7-induced formation of excitatory synapses (Ma et al. 2008). Kalirin-7 plays an important role in NMDA receptor activation-mediated enlargement of existing spines in hippocampal neurons (Xie et al. 2007). Loss of kalirin-7 results in an impairment in NMDA receptor-dependent LTP

induction (Lemtiri-Chlieh et al. 2011). It is likely that a higher BDNF/proBDNF ratio could enhance NR2B expression to overcome the negative effect of CUMS on dendritic spines. These data suggest that kalirin-7 in the complex of NR2B-PSD95-kalirin-7 is the down-stream target of BDNF and proBDNF and that CUMS-mediated spine loss may be through kalirin-7. This highlights an important role for kalirin-7 in depression, which is the focus of our future direction.

4. Conclusion

The balance of BDNF and proBDNF plays a key role in CUMS-induced spine loss and depressive-like behaviors. Kalirin-7 may play an important role in BDNF- and proBDNF-mediated spine formation during CUMS.

Experimental Procedure

Animal

The animal protocol of this study was approved by national legislations of China and local guidelines. The investigation was conducted in accordance with the ethical principles of animal use and care. Adult male Sprague-Dawley rats ($n = 64$), weighing 250 - 280 g, were purchased from Shaanxi Academy of traditional Chinese Medicine. The rats were kept on a 12 h light/12 h dark schedule with free access to food and water. The ambient temperature was 22 ± 1 °C with 52 ± 2 % humidity. Before the experiment, animal were adapted to the laboratory conditions for seven days.

Cannulation and intra-CA1 microinjection of BDNF and proBDNF

Animals were anaesthetized with sodium pentobarbital (Sigma, 40 mg/kg, i.p.) and fitted into a stereotaxic apparatus (Stoelting, USA). A stainless steel guide cannula was implanted into the hippocampal CA1 area with the following stereotaxic coordinates according to the rat brain atlas by Paxinos and Watson (Paxinos 1998): anterior (AP) 3.8 mm, lateral (LAT) 2.2 mm and ventral (DV) 2.0 mm from the bregma, and then fixed to the skull with dental cement. Dummy guides were placed into the cannula to prevent blockage and to prevent debris from entering the brain. After surgery, animals received penicillin (20,000 units per day for 3 days) and were allowed to recover for at least 1 week before receiving CUMS and microinjections of vehicle, BDNF or proBDNF into the hippocampal CA1 area.

All animals were then randomly divided into four groups ($n = 16$ each group): control + saline, CUMS + saline, CUMS + BDNF and proBDNF. On the 1st, 7th, 14th and 21st after the onset of CUMS, rats in these 4 groups received intra-CA1 microinjections of 1 μ l 0.9 % physiological saline, 1 μ l BDNF (0.25 μ g/ μ l, B3795, sigma) (Kim et al. 2012), and 1 μ l proBDNF (0.5 μ g/ μ l, b-256, Alomonel lab) (Holm et al. 2009), respectively. Drugs were delivered at the speed of 0.1 μ l/min using a stainless steel injector that extended 1 mm below the guide cannula tip. The injector was stayed in the injection site for a period of 2 min after completing infusion to prevent solution backflow from the cannula.

Chronic unpredictable mild stress (CUMS)

In both CUMS and CUMS + BDNF groups, animals were subjected to daily CUMS for three weeks. The CUMS procedure followed our previously described strategy (Qiao et al.

2014), and depressive-like behaviors were induced in rats after 21 days of CUMS (Luo et al. 2008, Liu et al. 2014). In brief, the rats were kept individually (1 rat/cage) and subjected to a variety of stressors including: inversion of the light/dark cycle, swimming in 4 °C cold or 45 °C hot water for 5 min, caged with damp sawdust for 24 h, cage tilting for 24 h, shaking for 5 min, tail nipping for 1 min, fasting and water deprivation for 24 h. Rats received one of these stressors per day in the sequence shown above and the same stressor was not applied in two consecutive days.

Behavioral tests

Behavioral tests were performed as described (Qiao et al. 2014). Rats were placed in the holding room to adapt for 30 min before onset of the behavioral tests. All tests took place in a sound-proof room between 8:00 am and 1:00 pm, unless otherwise stated. After each test, rats were immediately returned to their home cages.

Open field test (OFT)

The **Open field test** was performed to measure spatial exploration behavior in rats as previously described (Luo et al., 2008). Briefly, the apparatus, consisting of a black square cage $60 \times 60 \times 40 \text{ cm}^3$, was divided into 5×5 equal squares on the floor of the arena. A single rat was placed in the center of the cage, and its locomotion activity was recorded for 5 min (Liang et al. 2015) as the total number of visits per square, using Video Mot 2 (TSE, German). In addition, the number of rears (posture sustained with hind-paws on the floor) and the incidences of grooming (including washing or mouthing of forelimbs, hind-paws, face, body and genitals) were also counted manually within this 5 min by a researcher blind to this experiment. The cage was thoroughly cleaned with 90% alcohol after each test.

Tail suspension test (TST) (Dunn and Swiergiel 2005)

The procedure is similar to the forced swim test which measures “behavioral despair” of rodents (i.e. rats stop struggling in the face of an inescapable stressor). Each rat was suspended individually by its tail using adhesive laboratory tape, to a flat metal bar connected to a strain gauge within a tail suspension chamber. The duration of the test was 6 min. Data acquisition and analysis were performed automatically, using Tail Suspension Monitor (TSE, German).

Golgi staining

After behavioral tests, eight rats in each group were deeply anesthetized with sodium pentobarbital and were perfused with 0.1 M phosphate buffer solution (PBS, pH7.4) followed by 4 % paraformaldehyde in 0.1 M PBS (Ma et al. 2002). Golgi staining and spine density analysis in the apical dendrites of hippocampal CA1 pyramidal neurons were performed as described in our previous study (Ma et al. 2008, Qiao et al. 2014). Spines were counted manually on randomly selected second-order segments from the apical dendrites. Approximately 2 -3 segments were counted per slide and at least six slides were taken from each rat.

Western blot

Rats in each group were sacrificed by decapitation (n=8 each group). Their hippocampi were collected and Western blot was performed as described (Ma et al. 2008). Protein samples (60 µg/lane) were separated using SDS–PAGE and transferred to PVDF membranes. The membranes were incubated with rabbit anti-kalirin-7 (1:1000) (Qiao et al. 2014), mouse anti-BDNF (1:5000, Sigma, B9436), mouse anti-proBDNF (1:200, sigma, P1374), anti-NR2B (1:1000, NeuroMab, 75-101) and anti-PSD95 (1:1000, NeuroMab, 75-028) overnight at 4 °C. After further incubation with horseradish peroxidase-conjugated goat anti-mouse or rabbit IgG (1:7500, Bioss) for 1 h at 20 °C, membranes were developed with ECL reagents. To normalize the protein content, blots were probed with anti-GAPDH (1:1000, TA-08, ZSBIO, PRC). The densitometric values of the target protein of each group were normalized to their corresponding GAPDH (Wang et al. 2013), and then expressed as a percentage of control group values.

Statistics

Statistical analyses were performed with SPSS 16.0 and the results were reported as mean ± SD except in the expressions of target proteins which were reported as % of control. One-way analysis of variance (ANOVA) was performed, followed by Fisher's LSD post hoc test. The level of significance was set at $p < 0.05$.

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Footnotes

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Figure legends

Figure 1. Experimental design. CUMS: 21 days of chronic unpredicted mild stress (CUMS). OFT: open field test on day 22. TST: tail suspension test on day 22. Microinjection: CUMS animals received microinjections of saline (the CUMS group) or BDNF (the CUMS+BDNF group) into the CA1 region on day 1, 7, 14 and 21 after the onset of CUMS, respectively; naïve rats in control group received microinjections of saline (control group) or proBDNF (the proBDNF group) into the CA1 region on day 1, 7, 14 and 21, respectively. Rats received the cannulation surgery 7 days before the onset of CUMS.

Figure 2. Behavioral tests. Open field test (OFT) and tail suspension test (TST) were performed in the control, CUMS, CUMS+BDNF and proBDNF groups, respectively. Control, control rats received microinjections of saline into the hippocampal CA1 region on day 1, 7, 14 and 21, respectively. CUMS: rats received 21-day CUMS and microinjections of saline into the hippocampal CA1 region on days 1, 7, 14 and 21, respectively. CUMS + BDNF: CUMS animals received microinjections of BDNF into the hippocampal CA1 region on day 1, 7, 14 and 21 after the onset of CUMS as shown in Figure 1. ProBDNF: naïve control rats received microinjections of proBDNF into the hippocampal CA1 region on day 1, 7, 14 and 21, respectively. (A) locomotor in OFT ($F_{(3, 28)} = 4.24, p < 0.01$), (B) rearing in OFT ($F_{(3, 28)} = 5.71, p < 0.01$), (C) grooming in OFT ($F_{(3, 28)} = 6.23, p < 0.01$), and (D) duration of immobility in TST ($F_{(3, 28)} = 6.36, p < 0.01$). One-way ANOVA. * $p < 0.05$, ** $p < 0.01$ vs. the control group; # $p < 0.05$, ## $p < 0.01$ vs. the CUMS group.

Figure 3. Expressions of BDNF and proBDNF in the hippocampus in the control and chronic unpredicted mild stress (CUMS) groups. (A) Microphotographs of Western blot analysis showed the levels of BDNF and proBDNF in rat hippocampus from the control ($n = 8$) and the CUMS ($n = 8$) groups. (B) Bar graphs showed the levels of BDNF and proBDNF, and the ratio of BDNF/proBDNF in the control and the CUMS groups. * $p < 0.05$, ** $p < 0.01$.

Figure 4. Dendritic spines in hippocampal CA1 pyramidal neurons. A, a represent microphotograph showed Golgi stained pyramidal neurons in the hippocampal CA1 area. B-D, images from one control rat received microinjections of saline into the hippocampal CA1 region on day 1, 7, 14 and 21, respectively. E-G, images from one rat of the CUMS group

received three-week CUMS and microinjections of saline into the CA1 region on day 1, 7, 14 and 21, respectively. **H-J**, images from one animal of BDNF + CUMS group received microinjections of BDNF into the hippocampal CA1 region on day 1, 7, 14 and 21 after the onset of CUMS as shown in Figure 1. **K-M**, images from one naïve control rat received microinjections of proBDNF into the hippocampal CA1 region on day 1, 7, 14 and 21, respectively.

Figure 5. Quantification of spine density in hippocampal CA1 pyramidal neurons.

CUMS: Rats received 21 days of CUMS and microinjections of saline into the CA1 region on day 1, 7, 14 and 21, respectively. CUMS+BDNF: CUMS animals received microinjections of BDNF into the CA1 region on day 1, 7, 14 and 21 after the onset of CUMS as shown in Figure 1. ProBDNF: naïve control rats received microinjections of proBDNF into the CA1 region on day 1, 7, 14 and 21, respectively. One way ANOVA, $F_{(3, 28)} = 41.37$, $p < 0.01$. ** $p < 0.01$ vs. the control group; ## $p < 0.01$ vs. the CUMS group.

Figure 6. Western blot analysis showed that the levels of kalirin-7, NR2B and PSD95 in the hippocampus.

Control: control animals received microinjections of saline into the hippocampal CA1 region on day 1, 7, 14 and 21, respectively. CUMS: rats received 21 days of CUMS and microinjections of saline into the CA1 region on day 1, 7, 14 and 21, respectively. CUMS+BDNF: CUMS animals received microinjections of BDNF into the CA1 region on day 1, 7, 14 and 21 after the onset of CUMS as shown in Figure 1. ProBDNF: naïve control rats received microinjections of proBDNF into the CA1 region on day 1, 7, 14 and 21, respectively. Microphotographs (A, C) and bar graphs (B, D-E) showed the levels of kalirin-7 (A-B, $F_{(3, 28)} = 61.70$, $p < 0.01$), NR2B and PSD95 (C-E) in the control, CUMS, CUMS+BDNF and proBDNF groups, respectively. GAPDH was used as an internal control. ** $p < 0.01$ vs. the control group; # $p < 0.05$, ## $p < 0.01$ vs. the CUMS group.

Figure 2

Figure 3

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Figure 5

Figure 6