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Blunted Behavioral and Molecular Responses to Chronic Mild Stress in Adult Rats with Experience of Infancy Maternal Separation

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Childhood adversity has profound and persistent effects on brain functions and has been implicated in the etiology of depression. Brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB) play critical roles during brain development to maintain neuronal function and structural integrity in adulthood. We therefore investigated the long-term effects of early life adversity on the depression-related behavior and the expression of BDNF and CREB in the hippocampus. Male Sprague-Dawley newborn rats were subjected to maternal separation for 3 h/day on postnatal days 2-14. After the postnatal day 90, rats with or without the experience of infancy maternal separation received a series of unpredictable chronic mild stress (CMS) for 21 days. Sucrose preference and spontaneous activity in the open field test were recorded, and the expression of BDNF and CREB in the hippocampus was measured by real-time RT-PCR and Western blot analyses. Before exposure to CMS, the rats with maternal separation showed the significant decreases in sucrose preference, spontaneous activity, and hippocampal expression of BDNF and CREB, compared to the animals without maternal separation. In contrast, the rats without maternal separation showed greater decreases of the above indictors after CMS, the levels of which were lower than those observed in the rats with maternal separation. Thus, early life adversity leads to long-term decreases in the capacity of enjoying sweetness, spontaneous activity, and hippocampal expression of BDNF and CREB. Moreover, childhood neglect may decrease the neurobehavioral plasticity, thereby blunting the responses to adulthood stress and increasing the susceptibility to depression.

Keywords: brain-derived neurotrophic factor; cAMP response element-binding protein; chronic mild stress; hippocampus; maternal separation
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Introduction

Clinical studies showed that patients with depression, who experienced childhood traumatic events, exhibited more severe depressive symptoms and responded poorly to commonly used antidepressants. Depression in these patients usually has an earlier onset, longer disease course and is more easily recurrent in comparison to those without childhood adversity (Tennant et al. 1981; Wiersma et al. 2009; Shamseddeen et al. 2011). In addition, animal experiments with early or/and adult stresses revealed more depression-like symptoms in animals that had experienced early stress. For example, Marais and Øines groups (Marais et al. 2008; Øines et al. 2012) induced early stress in rats by maternal separation after birth and found that these rats in adulthood consumed less fluid than the control animals (Øines et al. 2012). If a second restraint-stress was induced in these rats, the animals in the double stress group were less capable of swimming for 15 minutes on day 65 (Marais et al. 2008). These results suggest that an early life adversity leads to animals to exhibit more severe depression-like behavior in response to adulthood stress. However the mechanism responsible for this remains unclear.

Preclinical studies suggest that stress in early life may cause long-term changes in neurotransmitter systems and brain structures (Cirulli et al. 2009). In comparison with healthy controls, patients with depression are reported to exhibit abnormal hippocampal structures (i.e. reduced hippocampal volume and reduced neuron volume with decreased neuron and glial cell densities) (Sapolsky 2000; Czéh et al. 2001). These molecular and structure abnormities have been implicated in the etiology of depression

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(Cirulli et al. 2009).

Brain-derived neurotrophic factor (BDNF) is a dimeric protein found throughout the brain, with particular abundance in the hippocampus and in the cerebral cortex. The transcription factor, cAMP response element-binding protein (CREB), is an important regulator of BDNF-induced gene expression. BDNF and CREB are involved in cellular proliferation, migration and differentiation in the developing brain. They are necessary for the survival and normal physiological functions of mature neurons in the central nervous system (Shaywitz and Greenberg 1999; Nakagawa et al. 2002; Morcuende et al. 2003). Transcription of the BDNF gene is regulated by CREB (Tao et al. 1998), and BDNF signaling induces CREB phosphorylation (Ying et al. 2002). A reduction in BDNF and CREB expression negatively impacts neural plasticity and normal hippocampal functions. Stress induced down-regulation of BDNF has been implicated in the hippocampal damage observed in depression patients (Brunoni et al. 2008; Sen et al. 2008) and animal models of depression (Roceri et al. 2002; Pizarro et al. 2004; Duman and Monteggia 2006; Franklin and Perrot-Sinal 2006; Nair and Vaidya 2006; Fuchikami et al. 2009; Banerjee et al. 2014). Serum BDNF levels were negatively correlated with depression severity in patients with untreated depression (Karege et al. 2002, 2005). Injection of BDNF into the hippocampus of animals resulted in behavioral changes, which were similar to those induced by antidepressant treatment (Shirayama et al. 2002). It was also found that antidepressant treatment were ineffective in transgenic mice with a dominant negative form of the BDNF receptor (TrkB) (Saarelainen et al. 2003). D'Sa and Duman (2002) attributed the cellular and volumetric impairments observed in patients with depression to disruption of neurogenesis and specific signal transduction cascades at multiple levels, particularly the cAMP-CREB and BDNF-MAPK cascades.

Preclinical studies also suggested that stress in early life could cause long-term changes in the expression of BDNF and CREB in the hippocampus (Kuma et al. 2004; Greisen et al. 2005; Morinobu et al. 2006; Lippmann et al. 2007; Nair et al. 2007). This suggests that alterations in BDNF/CREB may contribute to generation of individual differences in stress neurocircuitry, altering vulnerability to depressive disorders. However, the causal connection among the alterations of BDNF/CREB expression, stress and the onset of depression remains unclear. Investigations into this mechanism have so far produced inconsistent results (Morinobu et al. 2006; Faure et al. 2007; Marais et al. 2008; Herpfer et al. 2012).

In this context, we hypothesized that although early adversity induced dysfunction of the hippocampal BDNF-CREB system may result in vulnerability to stress in later life, some detailed causal connection might be neglected.

Methods

Animals

All animal experiment protocols conformed to the Guide for the Care and Use of Laboratory Animals US National Institutes of Health Publication 2011 and were approved by the Institutional Animal Care Committee of Wuhan University.

Male Sprague-Dawley newborn rats (n = 48) were housed in 40 × 25 × 20 cm Plexiglas cages with wood shaving as bedding at 25 ± 1°C and 60% humidity in a 12 h light/dark cycle. Food and water were available *ad libitum*.

Experimental design

Half of the newborn rats (n = 24) were subjected to infancy maternal separation (IMS) between postnatal days 2 and 14, and the remaining pups did not (group without IMS). After growing into adulthood (the postnatal days 90), half (n = 12) from each group were subjected to chronic mild stress (CMS) from the postnatal days 91 to 112. The sucrose preference and open field tests were performed in all animals immediately before and after CMS.

Procedure of IMS

IMS was performed according to Ladd et al. (2000) with minimal modifications. Briefly, newborns were separated from their mothers each day for 180 min from 9:30 to 12:30. During IMS, the pups were housed in a clean cage with nesting materials under the conditions similar to those for the pups without IMS. After IMS, the pups were returned to their mother's cage.

Procedure of CMS

The CMS protocol has been shown to induce lower consumption of sucrose postulated to reflect anhedonia in animals (diminished capacity to experience pleasure), one of the core symptoms of depression (Willner et al. 1987). CMS involved sequential exposure to one of seven unpredictable mild stressors for 21 days, including food deprivation (24 h), water deprivation (24 h), tail clamping (1 min), 45°C atmosphere (5 min), inversion of the light/dark cycle, restraint in a 25 × 7 cm plastic tube (2 hours), ice water swimming (5 min at 4°C). Animals were exposed to each stressor singly and the stressors were never presented simultaneously.

Sucrose preference test

Sucrose preference test, measured by the percentage of sucrose intake, was used to examine the capacity to experience pleasure of sweetness. The decreased sucrose preference in animals usually represents anhedonia, a core symptom of depression (Fawcett et al. 1983). Briefly, sucrose preference tests were preceded by 24 h deprivation of food and water. Then, water and 1% sucrose were made available in standard drinking bottles with a 5-cm stainless-steel spout for 60 min. Sucrose preference was calculated according to the following formula: the sucrose preference (%) = (sucrose intake/total fluid intake) × 100. The water deprivation period preceding sucrose preference measurement may be considered as a further stress applied on top of the CMS protocol.

Open field test

The open field test was used to examine spontaneous activity in animals. A decreased spontaneous activity in animals usually represents depressive-like behavior, an important symptom of depression.

According to the procedure previously described by Blokland et al. (2002), each animal was placed in the center of a dimly illuminated rectangular cage (120 cm × 90 cm × 35 cm). Behavior was recorded using an automated video tracking system (Ethovision3.0, Noldus, the Netherlands) during a 10-min period in the open field. The frequency of rearing (standing upright on the hind legs, while forepaws are free) was registered manually. Locomotor activity (the total, the central distance moved and mean velocity) was quantified using the video tracking system. The apparatus was cleaned with 70% alcohol thoroughly between tests to prevent the influence of animal scent on subsequent test.

Real-time quantitative polymerase chain reaction (RT-qPCR)

Immediately before or after CMS, the animals were sacrificed by decapitation. The hippocampus was dissected from the brain on an ice-cold aluminium plate and stored at −80°C. For RT-qPCR, total RNA was extracted with Trizol reagent and cDNAs were synthesized according to the manufacturer's protocol with following specific primers: BDNF (5′-GACAAGGCAACTTGGCCTAC-3′ and 5′-CCTGTCACACACGCTCAGCTC-3′; the size of amplified cDNA, 356 bp); and CREB (5′-TACCCAGGGAGGAGCAATAC-3′ and 5′-GAGGCAGCTTGAACAAC-3′; 183 bp).

The reaction mixture was added to the RNA solution and incubated at 42°C for 1 h, heated at 94°C for 5 min, and chilled at 48°C. The reaction was performed with the SYBR Green PCR Master Mix Kit (TIANGNE, Beijing, China) in a Rotor-Gene 3000 (Corbett Research, Australia) with an initial denature at 94°C for 5 min, followed by 40 cycles at 94°C for 30 seconds, 58°C for 30 seconds and 72°C for 45 seconds. Data were analyzed with Rotor-Gene version 4.6 (Corbett Research, Australia). The relative expression level of the gene of interest was examined with respect to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to normalize for variation in the quality of RNA.

Western blot analysis

Total proteins extracted from hippocampus were subjected to SDS-PAGE on 12% separating gels and electrophoretically transferred onto nitrocellulose membranes. After blocking with 5% nonfat dry milk, the membranes were incubated with the primary anti-CREB (sc-186, Santa Cruz Biotechnology) or anti-BDNF (sc-20981, Santa Cruz Biotechnology) antibody at 4°C overnight, followed by a corresponding horseradish peroxidase conjugated secondary antibody (Sigma-Aldrich) for 1 h at room temperature. The reaction was visualized by TMB staining. Densitometric signals were normalized to internal standard β-actin and analyzed with Bio 1D software.

Statistical analysis

Data were expressed as mean ± S.D. and analyzed with Post hoc multiple comparisons after One-way ANOVA. A difference was considered statistically significant when $p < 0.05$.

Results

Behavioral assessment

Before CMS, the measurements of spontaneous behavior in the open field tests (including total distance, central distance, rearing counts and mean velocity) were significantly lower in rats with IMS, compared to those in the animals without IMS (Fig. 1A-D). However, a greater

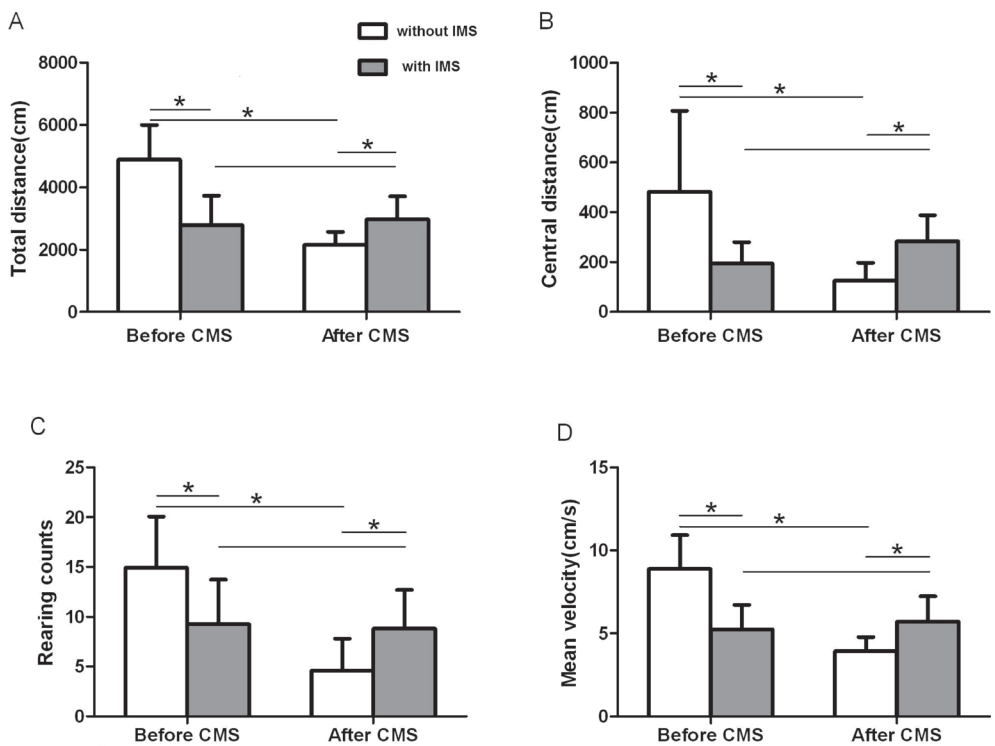

Fig. 1. Behavioral assessment of adult rats with or without IMS in response to CMS.
Open field tests were carried out before or after adulthood CMS in rats with ($n = 12$) or without ($n = 12$) IMS. Behavior of the rats was recorded by an automated video tracking system. Measurements of total distance (A), central distance (B), rearing counts (C) and mean velocity (D) are presented as mean ± S.D. *$P < 0.05$.

decrease in spontaneous activity was observed after CMS in animals without IMS, whereas CMS did not noticeably influence the spontaneous activity in animals with IMS. Thus, CMS significantly decreased spontaneous behavior in the rats without IMS, compared to the animals with IMS. Similar results were also observed in sucrose preference tests, in which the percentage of sucrose consumption was higher in the rats without IMS before CMS, but after CMS, the sucrose consumption in this group was greatly decreased to a level that was significantly lower than that of the animals with IMS (Fig. 2).

Hippocampal expression of BDNF and CREB

RT-qPCR analysis showed that before CMS, the expression levels of BDNF and CREB mRNAs were lower in the rats with IMS compared to those without IMS (Fig. 3). However, after CMS, the expression levels of BDNF and CREB mRNAs were not significantly changed in the IMS group. In contrast, their expression levels were profoundly decreased in the animals without IMS; namely, the rats without IMS showed significantly decreased hippocampal expression of BDNF and CREB mRNAs after CMS, compared to those with IMS. In parallel to the changes in mRNA levels, the expression levels of BDNF and CREB proteins were decreased in the rats with IMS, as judged by Western blot analysis (Fig. 4), but they remained unchanged even after CMS. In contrast, CMS profoundly decreased the expression levels of BDNF and CREB proteins in the rats without IMS.

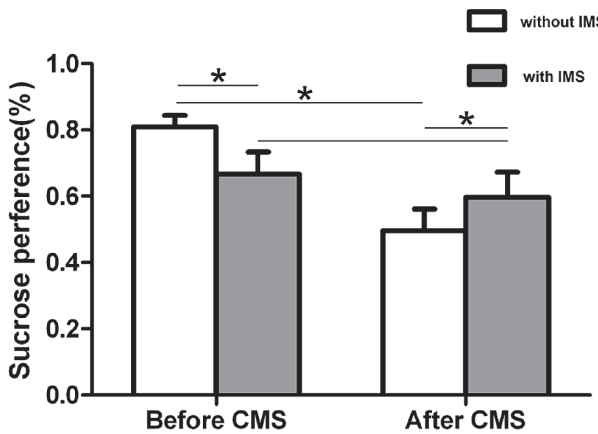

Fig. 2. Changes in sucrose preference of adult rats with or without IMS in response to CMS.
Sucrose preference tests were carried out before or after adulthood CMS in rats with (n = 12) or without (n = 12) IMS. Data are presented as the mean ± S.D. *P < 0.05.

Discussion

The present study demonstrated that childhood neglect alone caused long-lasting changes in both behavior and neurotrophic factor systems in rats in later life, including weakened sucrose preference, decreased spontaneous activity, and decreased hippocampal expression of BDNF and CREB. This is in line with numerous preclinical and clinical observations that an early life adversity may predispose an adult to psychiatric disorders, such as depression and anxiety. It is well documented that BDNF and CREB are neurotrophic factors important for both development and functions of brain, and a disruption of the cAMP-CREB and BDNF-MAPK cascades is related to the structural changes in the hippocampus observed in patients with depression (D'Sa and Duman 2002).

An unexpected finding in the present study is that after exposure to an adulthood stress (CMS), the control animals (without experience of IMS) exhibited a relatively strong response represented by obviously decreased sucrose preference, spontaneous activity and hippocampal expression of BDNF and CREB, whereas such responses to CMS were not observed in the animals with the experience of IMS. This suggests that a childhood stress may make the animal in later life to have a blunted behavioral and neurotrophic factor responses to ignore the CMS.

According to the "two-hit model", some psychiatric disorders are predisposed by genetic or environmental elements, which can be revealed by a precipitating event in adulthood. Stress may represent such an event, and the effect of stress on mood may be aggravated in individuals who experienced adversities early in life (Pani et al. 2000;

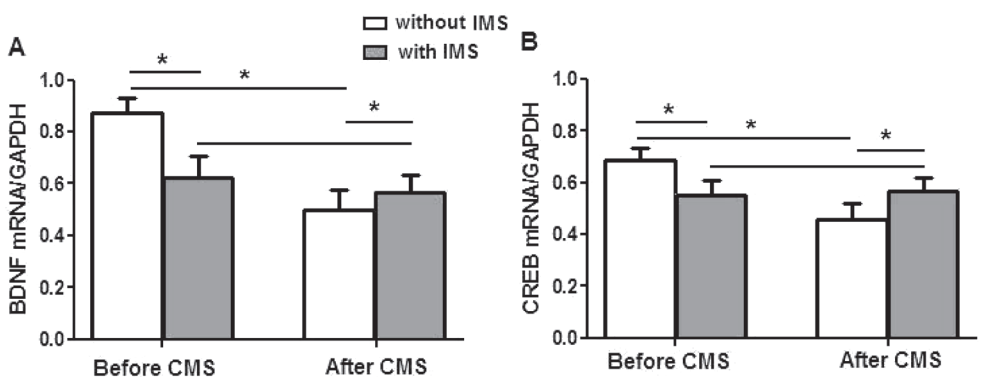

Fig. 3. Changes in expression levels of BDNF and CREB mRNAs in the rat hippocampus.
The mRNA expression of BDNF (A) and CREB (B) in hippocampus was assessed by RT-qPCR before or after adulthood CMS in rats with (n = 12) or without (n = 12) IMS. Data are presented as the mean ± S.D. *P < 0.05.

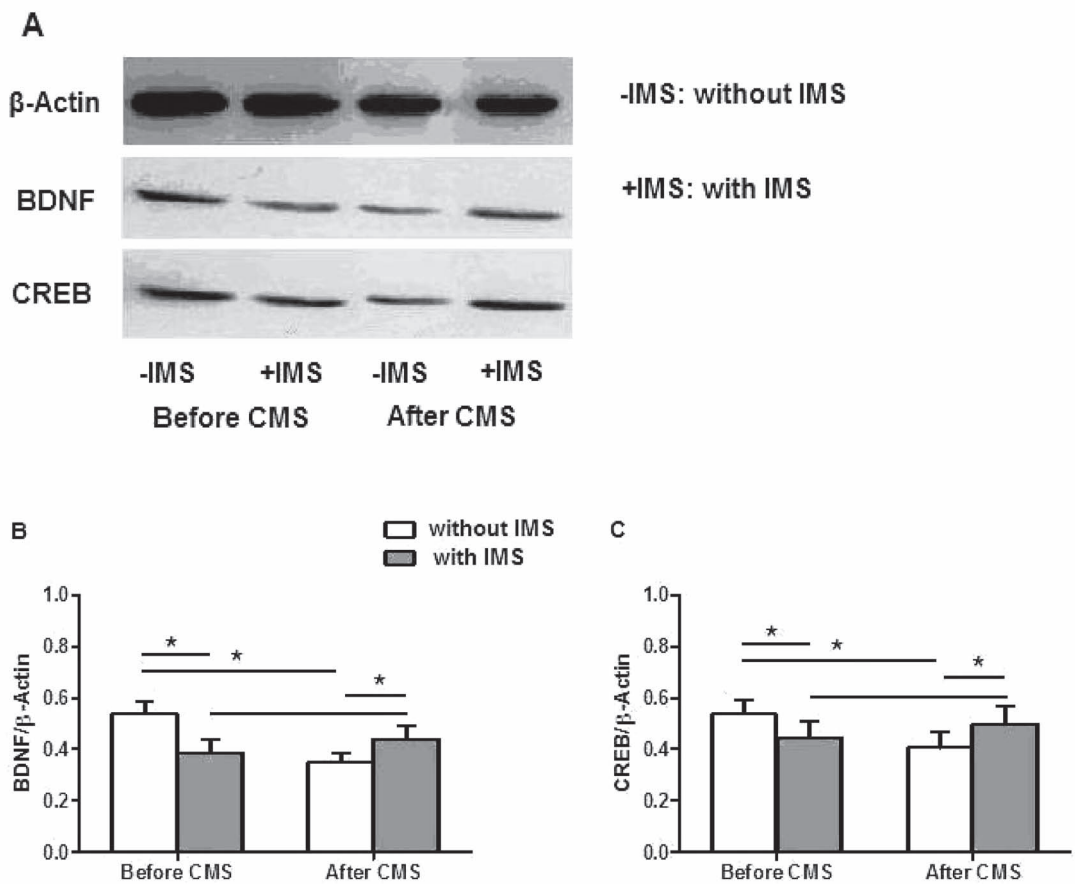

Fig. 4. Changes in expression levels of BDNF and CREB proteins in the rat hippocampus.
The expression of BDNF (B) and CREB (C) in hippocampus was assessed by Western blots before or after adulthood CMS in rats with (n = 12) or without (n = 12) IMS. Data are presented as the mean ± S.D. *P < 0.05.

Maynard et al. 2001). However, previous studies on behavioral and neurotrophic factor responses in "two-hit" animal models reported controversial results. For instance, a number of authors found that IMS made the animal to have altered hippocampal neurotrophic factor expression in adulthood, concomitant with impaired performance (Marais et al. 2008; Øines et al. 2012). However, the others suggested that IMS did not necessarily cause persistent negative changes in both behavior and hippocampal BDNF and CREB expression in response to adulthood stress. For example, Faure et al. (2007) found no significant difference in performance in the open field test and elevated plus maze test between Sprague-Dawley rats that experienced maternal separation plus adult stress and those that experienced only adult stress. However, those with maternal separation had increased levels of BDNF in the hippocampus (Faure et al. 2007). Hulshof et al. (2011) found that Wistar rats subjected to a wide variety of stressors did not show different behavioral responses if animals had experienced maternal separation. Morinobu et al. (2006) found that phosphorylation of CREB in response to adulthood stress was significantly increased in the hippocampus of rats subjected to neonatal isolation. Suri et al. (2013) found that animals with experience of maternal separation exhibited enhanced hippocampal neurogenesis, enhanced BDNF levels, and improved performance on the stress-associated Morris water maze during postnatal life and young adulthood. Strikingly, opposing changes in hippocampal neurogenesis and epigenetic regulation of BDNF expression, concomitant with impaired performance in hippocampal-dependent cognitive tasks, were observed in middle-aged (15 months) animals (Suri et al. 2013). The controversy is probably due to the differences in types, intensity, applying time or duration of adult stresses.

In present study, we employed CMS as an adulthood stress, which was designed to mimic the stimuli for human chronic stress reactions (Willner 2005). CMS is relatively mild and continuous, representing unpleasant things in daily life, rather than a strong adulthood stress or a single serious psychological "hit" as used by other authors (Morinobu et al. 2006; Faure et al. 2007; Marais et al. 2008; Hulshof et al. 2011; Øines et al. 2012; Suri et al. 2013). Thus, we speculate that the blunted behavioral and neurotrophic factor responses to CMS in the animals with IMS may represent that the childhood neglect may endow individuals withdrawn personality. Therefore, the individual becomes inattentive to mild unpleasant things in daily life and stays away from the crowd. It is a kind of personality

that may predispose the susceptibility to major depression under some serious stress.

In summary, the present study demonstrated that childhood neglect has a long-term affect on hippocampal BDNF and CREB expression, thereby likely weakening the neurobehavioral plasticity and endowing the individual withdrawn personality.

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Conflict of Interest

The authors declare no conflict of interest.

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