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Research article

Repeated maternal separation causes transient reduction in BDNF expression in the medial prefrontal cortex during early brain development, affecting inhibitory neuron development

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ABSTRACT

It is widely accepted that maternal separation (MS) induces stress in children and disrupts neural circuit formation during early brain development. Even though such disruption occurs transiently early in life, its influence persists after maturation, and could lead to various neurodevelopmental disorders. Our recent study revealed that repeated MS reduces the number of inhibitory neurons and synapses in the medial prefrontal cortex (mPFC) and causes mPFC-related social deficits after maturation. However, how MS impedes mPFC development during early brain development remains poorly understood. Here, we focused on brain-derived neurotrophic factor (BDNF) involved in the development of inhibitory neurons, and examined time-dependent BDNF expression in the mPFC during the pre-weaning period in male rats exposed to MS. Our results show that MS attenuates BDNF expression only around the end of the first postnatal week. Likewise, mRNA expression of activity-regulated cytoskeleton-associated protein (Arc), an immediate-early gene whose expression is partly regulated by BDNF, also decreased in the MS group along with the reduction in BDNF expression. On the contrary, mRNA expression of tropomyosin-related kinase B (TrkB), which is a BDNF receptor, was scarcely altered, while its protein expression decreased in the MS group only during the weaning period. In addition, MS reduced mRNA levels of glutamic acid decarboxylase (GAD) 65, a GABA synthesizing enzyme, only during the weaning period. Our results suggest that repeated MS temporarily attenuates BDNF signaling in the mPFC during early brain development. BDNF plays a crucial role in the development of inhibitory neurons; therefore, transient attenuation of BDNF signaling may cause delays in GABAergic neuron development in the mPFC.

1. Introduction

Child care environments have been reported to play an important role in the acquisition of physiological and mental functions [1, 2, 3, 4]. Stressful environments during early life, such as those characterized by child abuse and neglect, affect neural network formation and consequently cause impairments of various brain functions that persist into adulthood. In fact, previous studies have reported that childhood stress reduces the volumes of the prefrontal cortex and the hippocampus, and affects emotional expression and cognitive function in adults [5, 6].

The mother-infant interaction is one of the most important factors influencing brain development, and maternal separation (MS) in rodents, which induces stress in neonates, reportedly causes various behavioral abnormalities. One of these abnormalities is social deficit, which can be triggered by stressful environments [7, 8, 9]. Our recent study with rats revealed that MS during preweaning caused social deficits related to social recognition after maturation [10]. In addition, MS decreases the number of inhibitory neurons and synapses, and causes an excitatory and inhibitory (E/I) imbalance in the medial prefrontal cortex (mPFC), which is partly involved in the pathology of social recognition deficits [10].

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However, it still remains unclear how MS disrupts neural circuit formation in the mPFC during the developmental period.

During early brain development, the critical period is characterized by higher neuronal plasticity, and is an important period for neural circuit formation, including for mechanisms underlying axonal/dendritic arborization and synaptogenesis [11, 12]. The precise timing and duration of the critical period are crucial for the acquisition of appropriate E/I balances in each brain region, and previous studies have indicated that disruption of this period leads to various neurodevelopmental disorders [13, 14]. Maturation of inhibitory neurons during the developmental trajectory plays an important role in the onset and closure of the critical period [15]. Attenuated function of inhibitory neurons leads to delayed onset and closure of the critical period, which can result in social deficits as has been observed in patients with neurodevelopmental disorders [16, 17].

Brain derived neurotrophic factor (BDNF), which is a neurotrophic factor involved in axonal/dendritic growth and synapse production via tropomyosin-related kinase B (TrkB), regulates the critical period through the maturation of inhibitory neurons [17]. Our previous study showed that MS transiently reduced the expression of BDNF in the hippocampus during early development but not in juveniles [18]. Likewise, MS is likely to affect BDNF expression in the mPFC, which might cause a reduction in inhibitory function followed by E/I imbalances related to social recognition deficits reported in our previous study [10]. However, many earlier studies have focused on the observation that MS affects BDNF expression in the mPFC during adolescence to adulthood, while there are only a few reports about the influences of MS on BDNF during early brain development. Given that various developmental disturbances can occur in early life, we believe that understanding the possible early changes in BDNF is important.

Here, we hypothesized that MS affects BDNF expression in the mPFC during early brain development and consequently results in the formation of immature inhibitory circuits. We investigated a period in which MS affects BDNF and TrkB expression in the mPFC using rat offspring exposed to repeated MS during the preweaning period. In addition, we analyzed the expression of glutamic acid decarboxylase (GAD) 65 and 67, which are GABA synthesizing enzymes, to determine whether MS influences the development of GABAergic neurons in the mPFC early in life.

2. Materials and methods

2.1. Animals and maternal separation procedure

Pregnant Sprague–Dawley rats, which were purchased from Japan SLC (Hamamatsu, Japan), were used in this study. Animal rearing procedures have been described in our previous study [10]. Briefly, the rats were individually housed in plastic cages with a light/dark cycle of 12 h each (lights on from 0600 to 1800) in a temperature-controlled room (22 ± 2 °C), and were given food and water ad libitum. Pregnant rats were allowed to give birth and the day of birth was designated as postnatal day (PD) 0. Pups collected from at least 6 litters were randomly redistributed to the dams at PD 2 so that each dam received 8 pups (male/female = 6/2). In each dam, half the male pups were assigned to the MS group, and the other half of the male pups were assigned to the mother-reared control (MRC) group to remove differences caused by maternal care. The MS procedure has been described previously [10, 18, 19]. In brief, in the MS group, the pups were separated from their dams for 3 h, twice a day (from 0900 to 1200 and from 1300 to 1600) between PD 2 and PD 20. During the separation period, the pups were isolated individually in a plastic case without bedding at room temperature (22 ± 2 °C), and returned to their dams from 1200 to 1300 to avoid negative effects on their nutritional state. Our previous study showed that pups in the MS group do not show undernutrition, and serum corticosterone level is increased by a factor of four directly after separation [19]. Except during the separation period, pups from the MS group were returned to their home cage and reunited with the same dams. MRC groups were allowed to remain in their home cage with the dams, and were not handled except to change the cage bedding at PD 8 and 15. The analyses were conducted using male offspring to avoid the influence of the estrous cycle. All experiments were approved by the Animal Care and Use Committee for Kagawa University (approval numbers: 15135, 18621) and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Tissue sampling for real-time RT-PCR and western blot

The mPFC was acquired as previously described [10]. Briefly, pups from both groups were anesthetized with isoflurane and intracardially perfused with medical-grade physiologic saline at PDs 4, 7, 10, 14, and 21. Sampling at PD 4, 7, 10, and 14 was conducted immediately after 3 h of separation to accurately estimate changes during MS. Sampling at PD 21 was conducted 20–22 h after MS at PD20 was finished. All samples were obtained between 1200 and 1400 to minimize circadian factors. After perfusion, brains were quickly removed from the skulls and sectioned in the coronal plane to yield 1-mm-thick slices using Brain Matrix (Roboz Surgical Instrument, Maryland, USA). The mPFC was dissected from each slice under a stereoscopic microscope (Leica Geosystems, Heerbrugg, Switzerland) by referring to the rat brain atlas [20]. Six male pups from two dams were used to analyze gene expression in each group at PD 4, 7, 10, 14, and 21 and at PD 21 for western blot. A part of the 15 male pups collected from 5 dams were used for western blot in each group at PD 7 (MS, n = 12; MRC, n = 13). Samples were stored at -80 °C until required.

2.3. Real-time RT-PCR

Homogenization of the mPFC tissue and extraction of total RNA were performed with ISOSPIN Cell & Tissue RNA (Nippon Gene, Toyama, Japan) in accordance with the manufacturer's protocol. The concentration and purity of the extracted total RNA were evaluated by optical density measurements at 260 nm and 280 nm using NanoDrop 1000 (Thermo Fisher Scientific, Massachusetts, USA). ReverTra Ace qPCR RT Master Mix (TOYOBO, Osaka, Japan) was then used to synthesize cDNA with genomic DNA removal from the total sampled RNA. Gene expression was quantified using the ViiA^{TM}7 (Thermo Fisher Scientific, Massachusetts, USA) with the Fast SYBR Green Master Mix (Thermo Fisher Scientific). Primer pairs used in the current study were designed in accordance with our previous study [18]. The amounts of each mRNA were estimated by normalization to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA level in the same sample.

2.4. Western blot

Total lysates of mPFC tissue were obtained by sonication of tissue in ice-cold lysis buffer, the composition of which was as follows: 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% (v/v) Nonidet P-40, 0.5% (w/v) sodium deoxycholate, and 2% (w/v) sodium dodecyl sulfate with protease inhibitor (Sigma Aldroch, St. Louis, Mo, USA). Protein content was quantified by a BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were mixed with equal amounts of Laemmli sample buffer [100 mM Tris-HCl pH 6.8, 4% (w/v) sodium dodecyl sulfate, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue, 10% (v/v) β-mercaptoethanol], and boiled at 95 °C for 5 min. Equal amounts of protein from both groups were subjected to SDS-PAGE on Mini-PROTEAN TGX gels (Bio-Rad, California, USA) and transferred to PVDF membrane with the Trans-Blot Turbo Blotting System and Trans-Blot Turbo Transfer Pack (Bio-Rad). The membranes were blocked with 5% skim milk (Nacalai tesque) or PVDF Blocking Reagent (TOYOBO, Osaka, Japan) for 1 h at 24 °C, and then washed three times for 5 min each in Tris-buffer saline containing 0.1% (w/v) Tween 20 (TBST). The membranes were incubated with each primary antibody at 4 °C overnight. The membranes were then washed three times for 5 min each in TBST and incubated with horseradish

peroxidase-conjugated secondary antibodies (MBL, Nagoya, Japan) at 24 °C for 1.5 h. The membranes were again washed three times for 5 min each in TBST and visualized with Luminata western HRP Substrate (Millipore, Massachusetts, USA). Digital images were taken using an ImageQuant LAS 4010 biomolecular imager (GE Healthcare, California, USA) and analyzed using ImageQuant TL software (GE Healthcare). The amounts of each protein were estimated by normalization to β-actin of the same sample in the same membrane. The following antibodies were used for immunoblots: BDNF (rabbit polyclonal, 1:3000; sc-546, Santa Cruz, California, USA), TrkB (mouse monoclonal, 1:2000; 610101, BD Biosciences, New Jersey, USA), GAD65 (rabbit polyclonal, 1:5000; 3988, Cell Signaling, Massachusetts, USA), GAD67 (mouse monoclonal, 1:2000; MAB5406, Millipore), and β-actin (mouse monoclonal, 1:5000; ab8226, Abcam, Cambridge, UK).

2.5. Statistical analyses

All statistical analyses were performed with SPSS Statistics (IBM, Armonk, New York, USA). After Levene's test was performed to confirm that the variance was equal, one-way ANOVA (analysis of variance) was used to compare the change in GAPDH and BDNF mRNA expression in the MRC group during PD 4 to 21. However, when the variance was not equal, Welch's test was employed. When a significant difference was observed in the results of one-way ANOVA or Welch's test, Tukey's HSD test or Games–Howell test was additionally performed as a *post-hoc* test. On the other hand, after the variances were confirmed to be equal via *F*-test, the significant differences between the MRC and MD groups at each period were analyzed using the Student's *t*-test. When the variance was not equal, Mann-Whitney's U-test was used. Results are expressed as Mean ± SEM. Statistical significance was set at $p < 0.05$.

3. Result

3.1. MS reduces body and brain weight

Body and brain weights are shown in Table 1. Body weight of the MS group was significantly lower than that of the MRC group at PD 4 [$t(10) = 2.707$, $p = 0.034$], PD 7 [$t(10) = 5.032$, $p = 0.003$], PD 10 [$t(10) = 2.734$, $p = 0,021$], PD 14 [$t(10) = 9.582$, $p < 0.001$], and PD 21 [$t(10) = 8.339$, $p < 0.001$], respectively. Brain weight of the MS group was also significantly decreased compared to that of the MRC group at PD 7 [$t(10) = 3.515$, $p = 0.011$], PD 10 [$t(10) = 2.902$, $p = 0.016$], PD 14 [$t(10) = 5.952$, $p < 0.001$], and PD 21 [$t(10) = 5.023$, $p = 0.001$], respectively.

3.2. MS decreases BDNF expression in the mPFC during early brain development

We examined whether MS affects the expression of BDNF exon IX in the mPFC during the developmental period. First, it was confirmed that there were no significant differences in the mRNA expression of GAPDH within the same template volume at each period between the MRC and the MS groups (Figure 1A). However, GAPDH gene expression in the MRC group gradually increased during PD 4 to PD 14 [Figure 1B; $F(4, 25) = 25.832$, $p < 0.001$]. Further, from the results of the normalized gene expression [Figure 1C; $F(4, 25) = 39.786$, $p < 0.001$] and the raw data obtained from the same template volume [Figure 1D; $F(4, 10.97) = 41.349$, $p < 0.001$], it was confirmed that the expression of BDNF exon IX mRNA in the MRC group also gradually increased during PD 4 to PD 14. The expression of BDNF exon IX mRNA in the MS group was significantly reduced at PD 7 [Figure 2A; $t(10) = 2.895$, $p = 0.016$] and PD 10 [Figure 2A; $t(10) = 2.901$, $p = 0.016$] compared to the MRC group, while there were no significant differences at PD 4, PD 14, and PD 21 between these groups. The expression of activity-regulated cytoskeleton-associated protein (Arc) mRNA was also significantly lowered in the MS group than the MRC group at PD 7 [Figure 2B; $t(10) = 3.294$, $p = 0.020$], but there was no significant difference at PD 10 [Figure 2B, $t(10) = 2.161$, $p = 0.056$]. On the other hand, there were no significant differences in TrkB mRNA levels during PD 4 to 21 (Figure 2C). Based on these results, we additionally evaluated protein expression levels of BDNF and TrkB at PDs 7 and 21. Similar to the mRNA results, BDNF protein expression in the MS group was decreased at PD 7 [Figure 2D, $t(23) = 2.773$, $p = 0.011$] but not at PD 21 [Figure 2D, $t(10) = 0.261$, $p = 0.800$]. Interestingly, protein expression of TrkB at PD 21 was reduced in the MS group unlike that observed with mRNA expression [Figure 2D, $t(10) = 2.690$, $p = 0.032$]. These results suggest that MS temporarily attenuates BDNF function during early brain development.

3.3. MS decreases mRNA expression of GAD65

We investigated mRNA expression of GAD65 and GAD67 in the mPFC during the developmental period. There were no significant difference in mRNA expression of GAD65 between the two groups at PDs 4, 7, 10, and 14. However at PD 21, the mRNA expression of GAD65 was significantly lowered in the MS group than in the MRC group [Figure 3A, $t(10) = 2.257$, $p = 0.048$]. On the other hand, there was no significant difference in GAD67 mRNA expression between the two groups during PDs 4 to 21 (Figure 3B). Additionally, there were no significant differences between the MRC and the MS groups in protein expression of GAD65 and GAD67 at PD 21 (Figure 3C). Reduction of GAD65 mRNA expression at PD 21 may indicate incomplete inhibitory synaptic formation because GAD65 is located at synaptic terminals in addition to the cell body [21].

4. Discussion

In this study, repeated MS resulted in lower body and brain weights, decreased BDNF expression around PD 7, and reduced GAD65 mRNA levels during the weaning period. Our previous study reported that rat pups do not suffer from undernutrition during MS [19], suggesting that the reductions observed in the present study are not attributable to malnutrition. These results suggest that stress caused by MS simply induces a developmental delay. However, BDNF expression, which gradually increases in the mPFC during early brain development, was transiently reduced around PD 7, whereas catching up to the same level as the MRC group after PD 14. In addition, brain weight at PD 21 was approximately twice as that at PD 7, while the difference in brain weight between the MRC and the MS groups was almost unchanged after PD 7. Thus, repeated MS may result in decreased brain weight along with

Table 1. Body and brain weights of rats in the MRC and MS groups from PD 4 to 21.

		PD 4	PD 7	PD 10	PD 14	PD 21
Body weight (g)	MRC	11.38 ± 0.18	18.45 ± 1.00	19.97 ± 0.71	38.97 ± 1.03	55.85 ± 1.79
	MS	9.90 ± 0.52*	13.28 ± 0.24**	15.95 ± 0.98*	25.65 ± 0.94**	38.83 ± 0.98**
Brain weight (g)	MRC	0.49 ± 0.01	0.78 ± 0.02	1.00 ± 0.02	1.33 ± 0.02	1.57 ± 0.03
	MS	0.48 ± 0.02	0.69 ± 0.01*	0.87 ± 0.03*	1.19 ± 0.02**	1.39 ± 0.02**

Values are expressed as mean ± SEM (n = 6/group). Asterisks indicate a statistically significant difference compared to the MRC group at the same age (Student's *t*-test, *$p < 0.05$, **$p < 0.01$). MRC, mother-reared control; MS, maternal separation; PD, postnatal day.

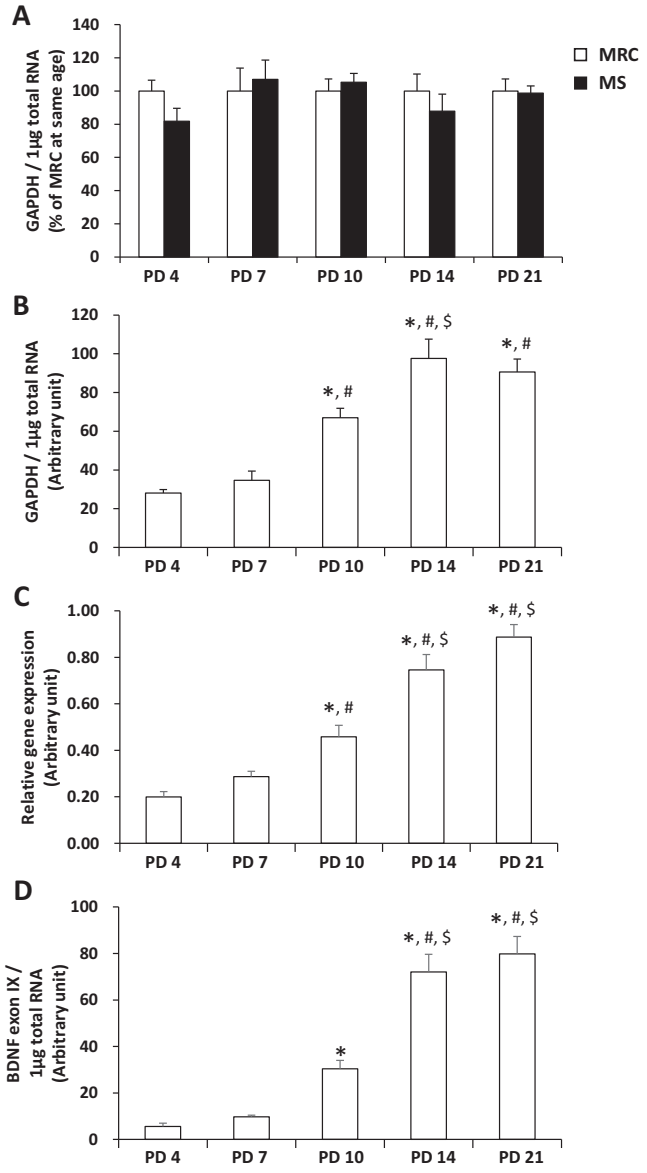

Figure 1. Gene expression of brain-derived neurotrophic factor (BDNF) gradually increased in the medial prefrontal cortex from postnatal day (PD) 4–14. (A) Effects of maternal separation on the mRNA expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) during PD 4 to PD 21 (n = 6/group at each period). (B) Change in GAPDH mRNA expression in the mother-reared control (MRC) group (n = 6/each period) during PD 4 to PD 21 (one-way ANOVA, $p < 0.001$; *post-hoc* test [Tukey's HSD test], $^{*}p < 0.05$ vs PD 4, $^{\#}p < 0.05$ vs PD 7, $^{\$}p < 0.05$ vs PD 10). (C) Change in the normalized gene expression of BDNF exon IX in the MRC group (n = 6/each period) during PD 4 to PD 21 (One-way ANOVA, $p < 0.001$; *post-hoc* test [Tukey's HSD test], $^{*}p < 0.05$ vs PD 4, $^{\#}p < 0.05$ vs PD 7, $^{\$}p < 0.05$ vs PD 10). (D) Change in the raw data of BDNF exon IX gene expression obtained from the same template volume in the MRC group (n = 6/each period) during PD 4 to PD 21 (Welch's test, $p < 0.001$; *post-hoc* test [Games-Howell test], $^{*}p < 0.05$ vs PD 4, $^{\#}p < 0.05$ vs PD 7, $^{\$}p < 0.05$ vs PD 10).

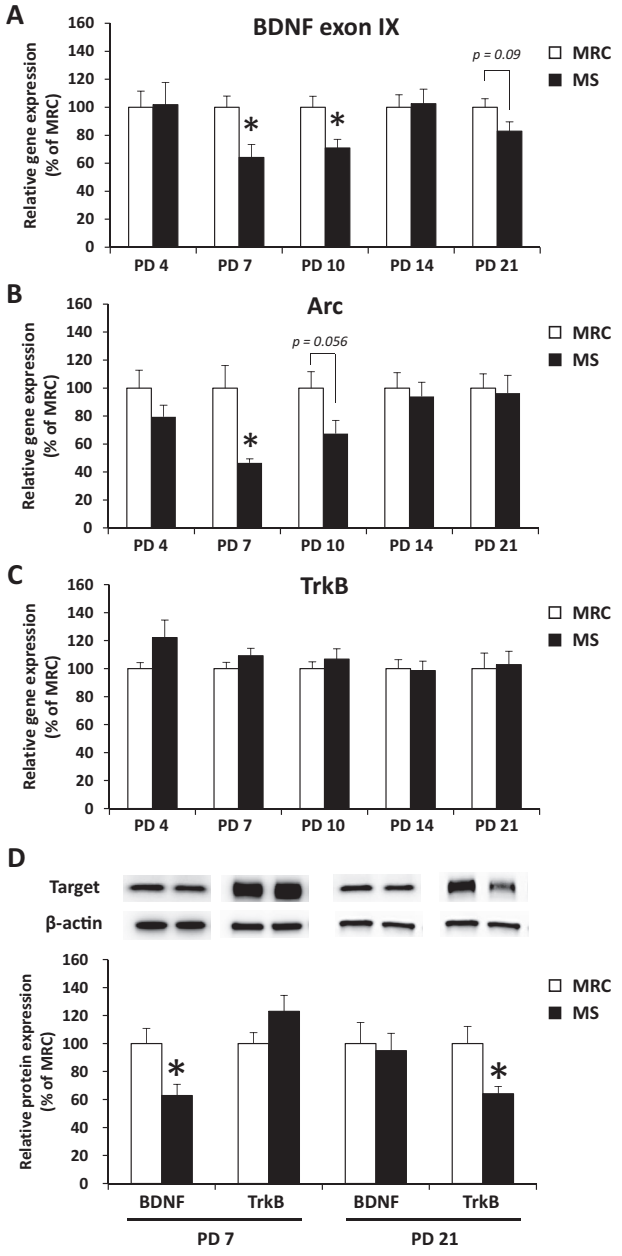

Figure 2. Maternal separation (MS) reduces brain-derived neurotrophic factor (BDNF) expression around postnatal day (PD) 7. (A) Effects of MS on the mRNA expression of BDNF exon IX during PD 4 to PD 21 (n = 6/group at each period). (B) Effects of MS on the mRNA expression of activity-regulated cytoskeleton-associated protein (Arc) during PD 4 to PD 21 (n = 6/group at each period). (C) Effects of MS on the mRNA expression of tropomyosin-related kinase B (TrkB) during PD 4 to PD 21 (n = 6/group at each period). (D) Effects of MS on the protein expression of BDNF and TrkB at PD 7 (MS, n = 12; MRC, n = 13) and PD 21 (n = 6/group). Values are expressed as mean ± SEM. Asterisks indicate a statistically significant difference from the mother-reared control (MRC) group (Student's *t*-test or Mann-Whitney's U-test, $^{*}p < 0.05$). The full, uncropped versions are shown in the supplemental data (Fig. S1: BDNF at PD 7, Fig. S2: β-actin as a loading control of BDNF at PD 7, Fig. S3: TrkB at PD 7, Fig. S4: β-actin as a loading control of TrkB at PD 7, Fig. S5: BDNF at PD 21, Fig. S6: TrkB at PD21, Fig. S7: β-actin as a loading control of BDNF and TrkB at PD 21).

insufficient dendritic outgrowth and synaptogenesis, through mechanisms involving the actions of BDNF around PD 7.

Repeated MS temporarily caused a decrease in the expression of BDNF in the mPFC during early brain development. A previous study using adult rodents reports that chronic stress, unlike acute stress, downregulates mRNA and protein expression of BDNF [22]. In addition, in the present study, we examined the expression of BDNF exon IX, which had been previously described as exon V, and which is included in all splicing variants [23]. A previous study shows that acute MS exposure for 3 h does not alter mRNA expression of BDNF exon IX at PD 7 [24]. Moreover, our findings showed that repeated MS attenuates the expression of Arc, an immediate-early gene, whose expression is partly regulated by BDNF, at PD 7. Thus, these results suggest that chronic early life stress attenuates a BDNF-related signaling pathway during early brain

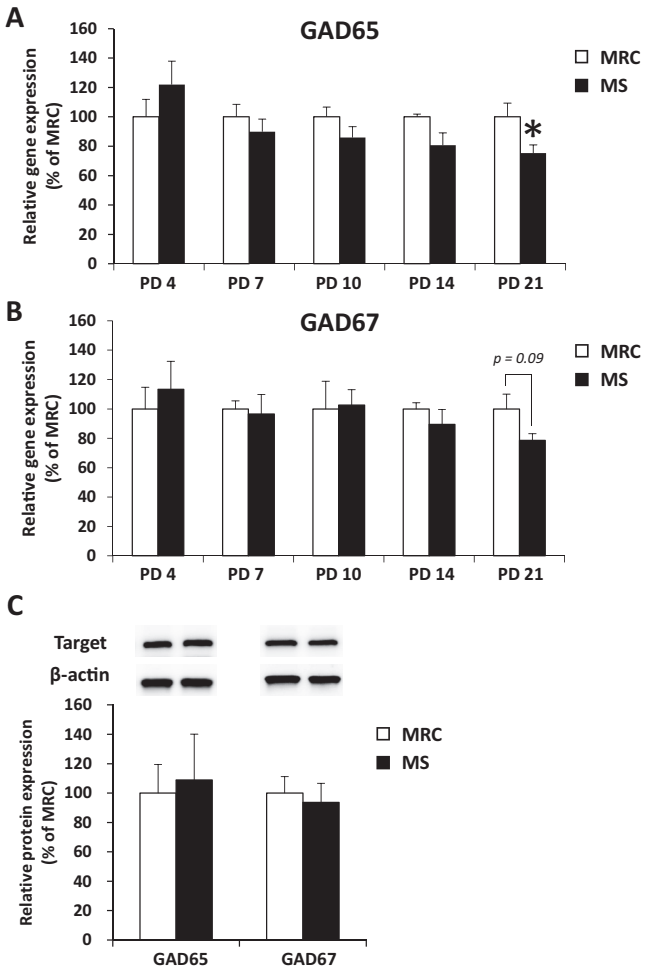

Figure 3. Maternal separation (MS) reduces the mRNA expression of glutamic acid decarboxylase (GAD) 65 at postnatal day (PD) 21. (A) Effects of MS on the mRNA expression of GAD65 during PD 4 to PD 21 (n = 6/group at each period). (B) Effects of MS on the mRNA expression of GAD67 during PD 4 to PD 21 (n = 6/group at each period). (C) Effects of MS on the protein expression of GAD65 and GAD67 at PD 21 (n = 6/group). Values are expressed as mean ± SEM. Asterisks indicate a statistically significant difference from the mother-reared control (MRC) group (Student's *t*-test $*p < 0.05$). The full, uncropped versions are shown in the supplemental data (Fig. S7: β-actin as a loading control of GAD67, Fig. S8: GAD67, Fig. S9: GAD65, Fig. S10: β-actin as a loading control of GAD65).

development. Because BDNF plays an important role in neural development, particularly by regulating neural circuit formation, a transiently decreased BDNF level around PD 7 is likely to affect neural development in the mPFC.

BDNF levels were not altered in the MS group compared to that in the MRC group after PD 14 regardless of repeated MS. Such differences may be attributed to the response to stress before and after approximately PD 9. In rodents, although there are minor differences between reported studies, the studies consistently indicate that serum corticosterone level is very low until around PD 9 [19,25,26,27]. This period is also characterized by inactivation of the hypothalamus-pituitary-adrenal (HPA) axis in response to mild stress stimulation [27, 28], and the period is referred to as the stress-hyporesponsive period (SHRP). However, repeated or severe MS can upregulate serum/plasma corticosterone levels even during the SHRP [19, 29]. In addition, early life stress during this period, including during the SHRP, disrupts dendritic morphology such as dendritic length and spine density in the mPFC and the hippocampus [30, 31]. Moreover, some studies have indicated that MS during the SHRP impairs prepulse inhibition, social behavior, cognition, and spatial learning ability after maturation [32, 33]. These results indicate that the SHRP is an important period for the protection of normal brain development from excess corticosterone exposure by activation of the HPA axis, and maternal care is one of the most important factors in the maintenance of the SHRP. Therefore, our results suggest that the SHRP is vulnerable to early life stressors such as repeated MS.

BDNF gene expression in the rat cerebral cortex [34] and the mPFC (Supplemental data 2) was still low at PD 7 and rapidly increased until PD 14. However, a previous *in vitro* study demonstrates that reactivity of downstream signaling by BDNF is highest around DIV (day *in vitro*) 7 in primary cortical neurons, in spite of a much lower gene expression of BDNF around DIV 7 than after DIV 14 [35]. In addition, this period corresponds to an important stage in the initiation of neural circuit formation, which includes dendritic outgrowth and synaptic production in the mPFC. In fact, it has been reported that a developmental peak in prefrontal cortex volume occurs from the first to the second week after birth [36], and dendritic arborization and length in the mPFC rapidly increase during this period [37, 38]. In addition, during the first postnatal week, mediodorsal thalamic projections precede neural circuit formation in the prefrontal cortex [39]. The density of mediodorsal thalamic projections to the prefrontal cortex is extremely high from birth to PD 10 in rodents, but drastically decreases after PD 13. It has been reported that this projection during the first postnatal week plays very important roles in dendritic outgrowth and synaptic production in the mPFC. In fact, lesions in the mediodorsal thalamus at PD 4 causes a reduction in the number of dendrites and in synaptic density in the prefrontal cortex [40]. Together, these results suggest that repeated MS impedes BDNF expression in the mPFC during the critical period for primary dendritic and synaptic development, and that these influences persist through subsequent brain development, possibly resulting in a decrease in synaptic density followed by abnormality of mPFC-dependent behaviors such as those that have been reported in previous studies [10, 40, 41, 42].

The present study demonstrated that GAD65 mRNA expression in the mPFC was significantly decreased in the MS group compared to the MRC group at PD 21. Conversely, GAD65 mRNA expression exhibited no change between the two groups during the separation period (PD 4, 7, 10, and 14). These results may indicate that repeated MS has a long-term effect on the development of GABAergic neurons in the mPFC and that this impact manifested at a detectable level at PD 21. Indeed, we recently showed that repeated MS from PD 2-20 decreases the number of GABAergic interneurons and synapses in the mPFC at 9 weeks of age [10], which indicates that the influence on GABAergic neuron by MS during the developmental period remains after maturation. These reductions in inhibitory neurons and synapses might be partly attributed to a decrease in BDNF expression during the early postnatal period. Previous studies have indicated that the first to the second postnatal week includes the critical period of inhibitory neural circuit formation in the somatosensory cortex and the visual cortex, and that BDNF signaling until PD 10 can promote the development of parvalbumin-expressing interneurons but not during PD 14–20 [43,44]. Likewise, BDNF knockout mice also shows delayed maturation of parvalbumin-expressing interneurons in layer 4 of somatosensory cortex around PD 12 [45]. In addition, it has been reported that maturation of inhibitory circuits is involved in the initiation and termination of the critical period, and contribute to normal formation of interlaminar (columnar) microcircuits in the visual cortex [46, 47]. Although the developmental trajectory of the mPFC is not necessarily identical to that of other cortical areas, a decrease in BDNF expression around PD 7 by repeated MS might disturb the development of inhibitory circuit formation during the first to the second postnatal week, which may cause E/I imbalances in the mPFC after maturation as reported previously. There are still very few reports about the influence of repeated MS on the mPFC during the critical period; further research is therefore needed to reveal the exact mechanisms underlying the effects of MS on the mPFC and its related behaviors.

In conclusion, we have demonstrated that repeated MS decreases BDNF expression during the early developmental period and attenuates GAD65 mRNA levels during the weaning period. Because appropriate BDNF function during specific periods plays a crucial role in proper inhibitory circuit formation, the E/I imbalance induced by repeated MS may partly results from a reduction in BDNF expression near the end of the first postnatal week even if it is transient. Our previous study reported that the same MS procedure decreased BDNF expression in the hippocampus during the similar period [18]. Repeated MS may transiently impede BDNF expression and its signaling during early brain development in broad brain regions. Given that the critical period of each brain region differs and occurs in a stepwise manner, repeated MS during early brain development may disturb the timing of the critical period, which may permanently affect higher brain function. Elucidation of this mechanism is challenging, but BDNF is likely an important player in the effects of early life stress such as MS on brain development.

Declarations

Author contribution statement

Chiaki Tenkumo, Ken-ichi Ohta, Takanori Miki: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Shingo Suzuki: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Katsuhiko Warita: Performed the experiments; Analyzed and interpreted the data.

Kanako Irie, Saki Teradaya: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Takashi Kusaka, Kenji Kanenishi, Toshiyuki Hata: Conceived and designed the experiments; Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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