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Effects of postnatal handling on adult behavior and brain mRNA expression of serotonin receptor, brain-derived neurotrophic factor and GABA-A receptor subunit

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Abstract

Development of brain and behavior is influenced by the interaction of genetic and environmental factors. Postnatal handling, a manipulation that briefly separates mouse offspring from their mother during the postnatal period, has been reported to yield beneficial effects on the behavior of adult offspring. However, brain mechanisms underlying the effects on the behavior have not been well understood. Here we first examined effects of postnatal handling on the behavior of adult male BALB/c mice. Offspring were separated for 15 min every day between postnatal day 1 (P1) and P14 and then various behaviors were tested in the adulthood. Postnatal handling reduced anxiety-like behavior in elevated plus maze and improved spatial learning and memory in Morris water maze without effects on depression-like behavior in forced swim test. Next, to elucidate mechanisms underlying the behavioral effects, we evaluated mRNA expression of the serotonin 1A (5-HT1A) receptor, brain-derived neurotrophic factor (BDNF), and GABA-A receptor subunits in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi, and dorsal raphe nucleus by quantitative RT-PCR, since these genes and brain regions have been shown to be involved in cognition and emotion. Postnatal handling up-regulated mRNA expression of the 5-HT1A receptor in the dorsal raphe nucleus and down-regulated 5-HT1A receptor mRNA expression

in the amygdala on P15 and P71. In adulthood, mRNA expression of BDNF was up-regulated in the amygdala and dorsal hippocampus and down-regulated in the dorsal raphe nucleus, whereas that of GABA-A receptor α2 subunit was down-regulated in the amygdala. Taken together, the present study suggests that postnatal handling reduced anxiety-like behavior and improved learning and memory, which were accompanied by changes in mRNA expression of 5-HT1A receptor, BDNF and GABA-A receptor α2 subunit in the amygdala, 5-HT1A receptor in the dorsal raphe nucleus and BDNF in the dorsal hippocampus.

Keywords:

Postnatal handling; 5-HT1A receptor; BDNF; GABA-A receptor; Anxiety; Cognition

1. Introduction

Formation of structure and function of the brain is genetically programmed but also modified by environmental factors during development. Maternal separation is widely used as an animal model to study the mechanism underlying the relationship between early-life environmental factors and the development of brain and behaviors. Prolonged maternal separation for 3 or more hours per day during the first two postnatal weeks has been found to produce increased anxiety and depression-like behaviors, and exaggerated hypothalamic-pituitary-adrenal (HPA)-axis response to stress in adulthood (Levine, 2000; Meaney, 2001; Pryce and Feldon, 2003). In contrast, postnatal handling (brief maternal separation) is a manipulation that briefly (3-15 min) separates rat or mouse pups from their mother daily during the postnatal period (e.g. postnatal day 1 (P1)-P10 or P1-P21) (Levine et al., 1956, 1962; Plotsky and Meaney, 1993; Fenoglio et al., 2005). Many studies have reported that postnatal handling has beneficial effects on the offspring behavior. For example, postnatal handling lowers anxiety levels in mice (Moles et al., 2004) and rats (Vallée et al., 1997; Caldji et al., 2000) and improves spatial learning and memory in mice (Zaharia et al., 1996; Anisman et al., 1998;) and rats (Vallée et al., 1999; Fenoglio et al., 2005), although the effects on anxiety-like behavior and spatial learning and memory are not necessarily consistent between mice and rats and even among different mouse strains (Zaharia et al., 1996; Millstein et al., 2007). Therefore, we first confirmed effects of the postnatal handling on the offspring behavior under our experimental paradigm.

The brain mechanisms which mediate effects of postnatal handling on adult behavior have been studied focusing on glucocorticoids in the HPA axis, but other potential mechanisms of

molecular regulation have been less investigated (Raineki et al., 2014). In the present study, to clarify the brain mechanisms, we focused on some molecules which may link postnatal handling during development and behaviors of the adult offspring.

Serotonin (5-hydroxytryptamine, 5-HT) is a monoamine with dual functions in the developing and matured brain. 5-HT regulates development of the brain as a neurotrophic factor and is involved in emotion and cognition as a neurotransmitter in adulthood (Gaspar et al., 2003; Daubert & Condron, 2010; Dayer, 2014). 5-HT neurons are located in the raphe nuclei of brainstem and project to widespread brain regions including the cerebral cortex, amygdala and hippocampus. 5-HT receptors are classified into 7 families with at least 14 different subtypes (Hoyer et al., 1994; Barnes and Sharp, 1999). It has been shown using 5-HT1A receptor knock-out (KO) mice that deletion of the 5-HT1A receptor during postnatal period increases anxiety-like behavior in adulthood (Gross et al., 2002). In addition, 5-HT1A auto-receptor in the developing raphe nucleus is required for formation of the neural circuits of adult anxiety-like behavior (Richardson-Jones et al., 2010, 2011). Furthermore, 5-HT1A receptor KO mice showed poor spatial learning and memory, suggesting that 5-HT1A receptor is also involved in cognition (Sarnyai et al., 2000).

Similar to 5-HT, brain-derived neurotrophic factor (BDNF) contributes to various functions in the developing and matured brain (Park and Poo, 2013). In addition to the formation of neural connections during brain development, BDNF has been shown to regulate neuropsychiatric-like behavioral phenotypes in adulthood. For example, pharmacological inhibition of BDNF impairs learning and memory of rodents (Bartoletti et al., 2002), and dysfunction of BDNF is related to depression (Nestler, 2002). Stress decreases the expression

of BDNF in the rat hippocampus, and antidepressants recover the stress-induced reduction of BDNF in rats (Nestler, 2002). In addition, injection of BDNF into the hippocampus has an antidepressant effect in rat experiments (Siuciak et al., 1997). Finally, BDNF and 5-HT co-regulate one another such that 5-HT stimulates the expression of BDNF, and BDNF enhances the growth, differentiation and survival of 5-HT neurons (Mattson et al., 2004; Martinowich and Lu, 2008).

Another candidate molecule which regulates anxiety is the GABA-A receptor. The GABA-A receptor is a target of anxiolytics, benzodiazepines. Benzodiazepines have acute effects in the treatment of patients with generalized anxiety disorder, social anxiety disorder, and panic disorder (Griebel and Holmes, 2013), whereas selective 5-HT reuptake inhibitors (SSRIs) show their anxiolytic effects after several weeks of the treatment (Vaswani et al., 2003). Among 19 GABA-A receptor subunits, α2 and α3 subunits modulate anxiety-like behavior. Diazepam-induced anxiolytic effect is absent in mice with the point mutation of α2 subunit, suggesting α2 subunit has anxiolytic effect in response to diazepam (Low et al., 2000).

In the present study, we first examined the effects of postnatal handling on the anxiety-like behavior, depression-like behavior and spatial learning and memory of adult offspring. Next, we examined the effects of postnatal handling on mRNA expression of the 5-HT1A receptor in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi and dorsal raphe nucleus of developing offspring, and BDNF and GABA-A receptor α2 subunit in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi of adult offspring by quantitative RT-PCR in the BALB/c mice. Finally, we correlated changes of the adult behavior with those

of the mRNA expression, and discussed the possible brain mechanisms mediating the effects of postnatal handling on the adult behavior.

2. Materials and methods

2.1. Animals

Pregnant BALB/cCrSlc mice (Japan SLC, Inc., Shizuoka, Japan) were housed under conditions of controlled lighting (lights on from 8:00 AM to 8:00 PM) and room temperature (24 °C). Animals had free access to food and water. All the experiments conformed to the guidelines issued by National Institutes of Health (USA) for Laboratory Animals, and all the procedures were approved by Animal Experiment Committee of University of Tsukuba. Efforts were made to minimize the number of animals and their suffering.

2.2. Postnatal handling

Postnatal handling was performed as previously described (Akatsu et al., 2015). The day of the pups' birth was designated as P0. In the postnatal handling group, all the offspring were moved from the dam to a new cage, separated from each other for 15 min (11:00-11:15AM) and then were returned to home-cage daily from P1 to P14. The temperature of the offspring cage was regulated at 33 ± 2 °C using an electric blanket underneath the cage. After the end of handling on P14, the offspring were group-housed with the dam and were weaned on P21. Thereafter each male mouse was singly housed to avoid aggressive behavior and only male mice were used for the following analyses.

2.3. Maternal behavior

Maternal behavior was evaluated for 120 min (11:45-13:45) on P1, P3 and P7 as previously described (Akatsu et al., 2015). The occurrence of following maternal behavior was counted each 2 min: nursing posture (arched-back nursing), pup licking and nest building. When each maternal behavior was detected in every 2 min, we calculated it as one point. Because we observed for two hours, the maximum point of total behavior is 60, and minimum point is 0.

2.4. Elevated plus maze (EPM)

On P57, anxiety-like behavior was tested by EPM (Ohara & Co., Ltd., Tokyo, Japan), under room light (530 lx). The apparatus had two opposing open arms (25 cm length x 5 cm width x 0.3 cm height) and two opposing closed arms (25 cm length x 5 cm width x 15 cm height) that were connected by the central platform (6 cm length x 6 cm width). Each animal was placed in the central platform with a nose toward the closed arm and behavior was recorded for 5 min by overhead color CCD camera. All animals were tested once between 12:00-14:00. Time spent in open and closed arms, entries into open arms and both arms were calculated, and the time spent in open arms or the number of entries into open arms were assessed as indices of anxiety-like behavior. We calculated the percentage of time spent in open and closed arms by the percentage of time out of 5 min (length of test) spent in both open and closed arms, and the percentage of open arm entries by division of total arm entries.

2.5. Morris water maze (MWM)

On P59-P65, spatial learning and memory were tested by MWM (Ohara & Co., Ltd). The

maze consisted of a circular pool (100 cm diameter and 30 cm depth) filled with water (24 °C) which was colored by white poster color. A transparent escape platform (10 cm diameter) was situated 15 cm away from the side wall and hidden 1 cm below the water surface. External spatial cues were placed around the maze. Series of tests were conducted under regular room light (530 lx). During the 5 day-course of training, a platform was placed in a stable position that was centered in one of the four quadrants of the pool. Each daily session consisted of 3 trials (11:00-15:00) in which animals were forced to swim from each of 4 random starting positions. They were allowed to search for the hidden platform for up to 90 s, and remained on the platform for 30 s after reaching it. Those animals which did not reach the platform were moved onto the platform to rest for 30 s. The latency to reach the platform was measured in the training test. On day 6, mice were subjected to a probe test in which the platform was removed from the pool, and mice were allowed to swim freely for 90 s. Both the time spent in the quadrant and numbers of crossing the quadrant where the platform had been located were measured. After the probe test, a cued test was carried out during which the platform was changed to a visible one over the water surface, and latency to reach the visible platform was measured.

2.6. Forced swim test (FST)

On P69, depression-like behavior was tested by FST (Ishikawa and Shiga, 2017). Each mouse was placed into water (23 °C) in a beaker with a diameter of 20 cm under room light (450 lx). Times of floating, swimming and climbing were measured. The behavior was analyzed during the last 4 min of the 6-min testing period.

2.7. Tissue preparation

Mice were decapitated under anesthesia with isoflurane on P15 and P71, and brains were removed. 2 mm-thick of coronal slices were made using Mouse Brain Matrix (Muromachi Kikai Co., Ltd., Tokyo, Japan), and left hemisphere was used for analysis of mRNA expression. The medial prefrontal cortex (Leuner and Shors, 2013), amygdala and dorsal raphe nucleus were punched out using Harris Micro-Punch (GE healthcare, Buckinghamshire, UK), and dorsal and ventral hippocampi were cut off by a Noyes surgical scissor (see supplemental Fig.1 in Ishikawa et al., 2017), since these brain regions have been shown to be involved in cognition and emotion (Bannerman et al., 2004). The dorsal hippocampus was defined as 50% of hippocampal volume starting at the septal pole, and the ventral hippocampus was defined as 50% of hippocampal volume starting at the temporal pole (Bannerman et al., 1999, 2004). In the present study, we dissected the dorsal and ventral hippocampi from the third and fourth 2-mm thick brain slices, respectively. Each brain region was immediately frozen in liquid nitrogen and stored at -80 ºC. To exclude effects of behavioral tests, we used adult brains for analysis from the animals that did not undergo any behavioral tests.

2.8. Quantitative analysis of 5-HT1A receptor, BDNF and GABA-A receptor α2 subunit mRNAs

Real-time reverse transcription-PCR was performed as previously described (Akatsu et al.,

2015; Ishikawa and Shiga, 2017). Each brain region was homogenized in RNA iso (Takara Bio, Shiga, Japan) on ice using sonicator (Taitec). After centrifugation at 12,600 xg at 4 ℃ for 5 min, supernatant was collected, and chloroform was added to separate RNA into aqueous layer. After re-centrifugation at 12,600 xg at 4 ℃ for 15 min, supernatant was collected and isopropanol was added to precipitate RNA. Precipitated RNA was washed with 70% ethanol and centrifuged at 12,600 xg at 4 ℃ for 5 min. Supernatant was discarded and RNA was dried out and dissolved into RNase-free water. Total RNA was diluted to 1:100 with distilled water and the concentration of total RNA was measured using spectrophotometer (Pharmacia Biotech Ultraspec 2000) to calculate 1μg of RNA. Genomic DNA was removed and cDNA was synthesized from 1 μg of total RNA using QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). For PCR amplification, cDNA was added to the reaction mixture containing SYBR Premix Ex Taq^{TM} Ⅱ (Takara Bio) and 0.2 μM of the primers. The primer sequences are listed on Table 1. PCR was carried out on Thermal Cycler Dice Real Time System (Takara TP800, Software Ver.3.00) according to the following protocol: 5 seconds at 95 ℃ and 30 seconds at 60 ℃, 50 cycles. Ct values were calculated from the crossing point of amplification curve and threshold, and relative quantitative analysis of targeted genes was carried out using calibration curve. Expression of 18S rRNA as internal control was used for correction, and the relative expression of mRNA in the experiment group was calculated when expression of mRNA in the control group was set to 1.0.

Table 1. Primer sequences for real-time reverse transcription-PCR.

Genes	Primer sequences	Tm (℃)	Length
5-HT1A-R	F: 5'-CCGTGAGAGGAAGACAGTGAAGAC-3' R: 5'-GGTTGAGCAGGGAGTTGGAGTAG-3'	60.5 62.2	176 bp
BDNF	F: 5'-GACAAGGCAACTTGGCCTAC-3' R: 5'-ACTGTCACACACGCTCAGCTC-3'	58.4 60.4	353 bp
GABA-A-R α2	F: 5'-GAGAATCGGTGCCAGCAAGAA-3' R: 5'-CAGTCCATGGCAGTGGCATAA-3'	64.9 64.2	118 bp
18S rRNA	F: 5'-ACTCAACACGGGAAACCTC-3' R: 5'-AACCAGACAAATCGCTCCAC-3'	56.1 53.9	123 bp

2.9. Statistical analysis

SPSS (IBM, Armonk, NY, USA) was used for statistical analysis. For evaluations of the maternal behavior and the training test of MWM, data were analyzed by repeated measures analysis of variance (ANOVA), with day as the within-subject factor and experiment group as between-subject factor, followed by student's t test. The probe and cued tests of MWM, EPM, FST and mRNA expression of 5-HT1A receptor, BDNF and GABA-A receptor α2 subunit were analyzed by student's t test. All data are expressed as means ± S.E.M and $p < 0.05$ was considered as statistically significant.

3. Results

3.1. Postnatal handling had no effect on maternal behavior

Effects of postnatal handling on the maternal behavior (nursing posture, pup licking, nest building) were examined on P1, P3 and P7 (Fig. 1). In the frequencies of pup licking, we found a significant main effect of day ($F(2,26) = 19.175$, $p < 0.001$) (Fig. 1B), but not the interaction between day and group. On the other hand, in the frequencies of nursing posture

(Fig. 1A) and nest building (Fig. 1C), we did not find the significant main effect of day and the interaction between day and group. These results suggest that postnatal handling did not affect maternal behavior.

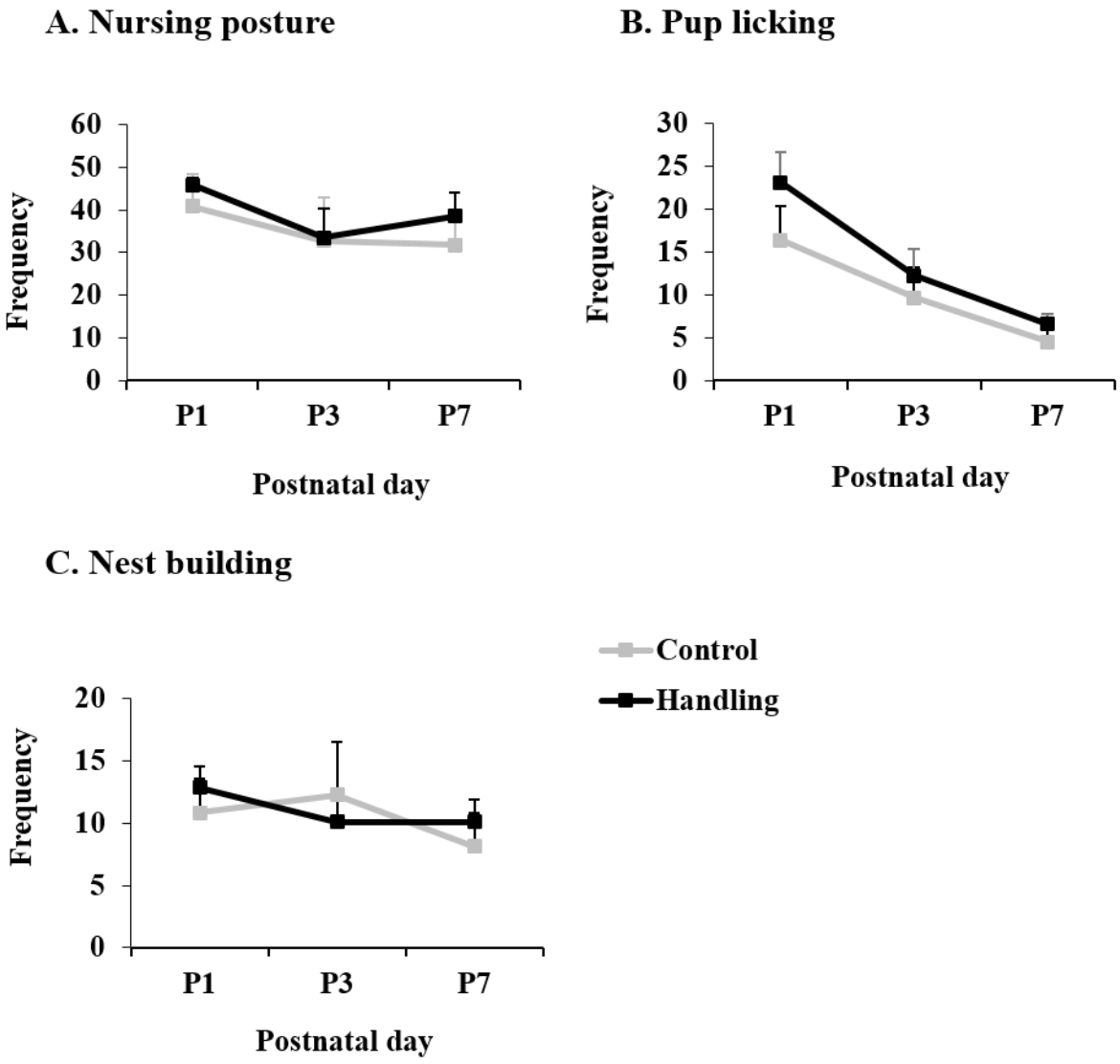


Fig. 1. Effects of postnatal handling on maternal behavior. Frequency of nursing posture (A), pup licking (B), nest building (C) on postnatal day 1, 3 and 7. Control, n=7; Handling, n=8.

3.2. Postnatal handling reduced anxiety-like behavior in the adult offspring

Effects of postnatal handling on anxiety-like behavior were tested by EPM (Fig. 2). Postnatal handling increased the time spent in open arms ($t(17) = 2.193$, $p < 0.05$) (Fig. 2A) and marginally decreased time spent in closed arms ($t(17) = 1.908$, $p = 0.073$) (Fig. 2B). However, there was no significant difference between two groups in entries into open arms (Fig. 2C) and both arms (Fig. 2D). These results suggest that postnatal handling reduced anxiety-like behavior.

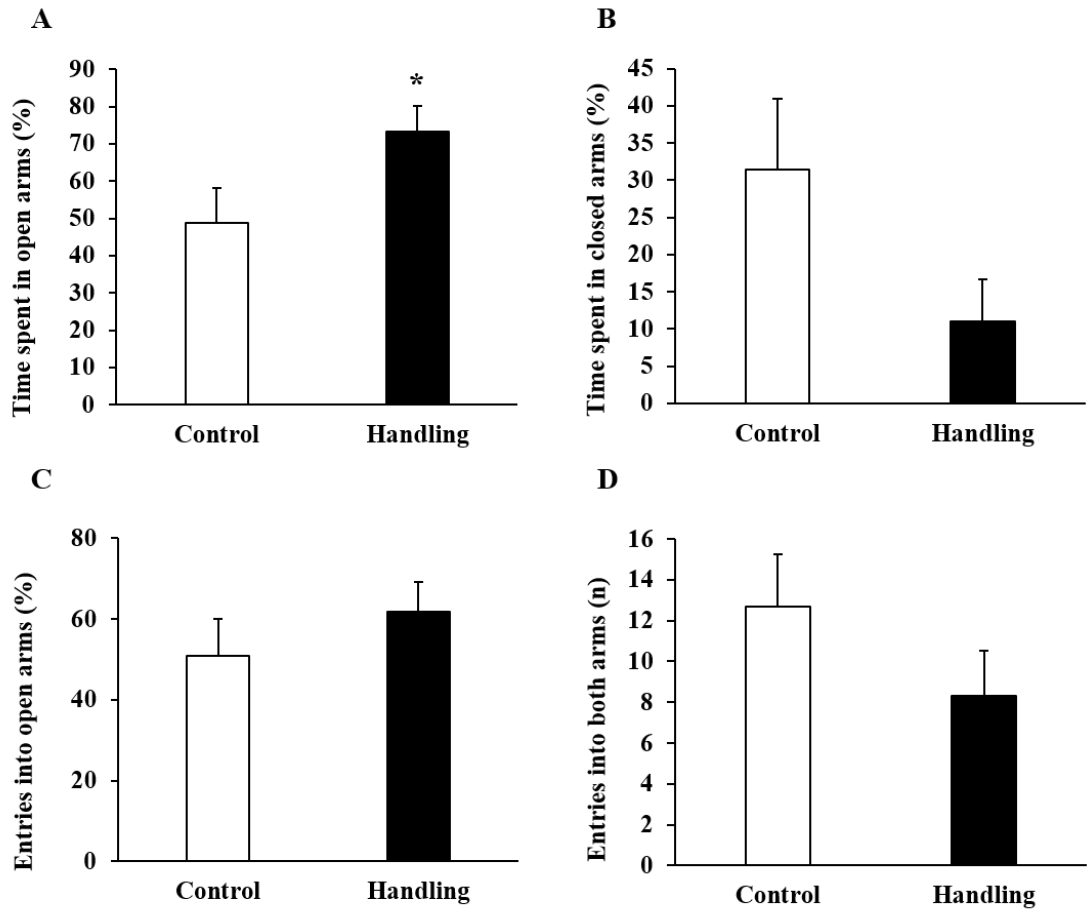


Fig. 2. Effects of postnatal handling on anxiety-like behavior in adult offspring. Percentage of the time spent in open arms (A) and in closed arms (B), percentage of the numbers of entries into open arms (C) and the numbers of entries into both arms (D) in elevated plus maze. Control, n=9; Handling, n=10. *p< 0.05.

3.3. Postnatal handling improved spatial learning and memory in the adult offspring

Effects of postnatal handling on spatial learning and memory were tested by MWM (Fig. 3). In 5-day-training, main effects of day and interaction between day and group were significant ($F(4,64) = 4.181$, $p < 0.01$; $F(4,64) = 2.818$, $p < 0.05$) (Fig. 3A), suggesting that both handling and control mice learned the place of the platform. The latency of handling group to reach the platform was decreased in training day 2 ($F(1,16) = 2.28$, $p < 0.05$) (Fig. 3A) as compared with control group. In the probe test, postnatal handling increased the time spent in the platform quadrant ($t(16) = 2.644$, $p < 0.05$) (Fig. 3B), but not in number of crossing of platform. There was no significant difference between two groups in the cued test (Fig. 3C), suggesting that postnatal handling had no effect on visual and motor functions. Taken together, these results suggest that postnatal handling improved both spatial learning and memory.

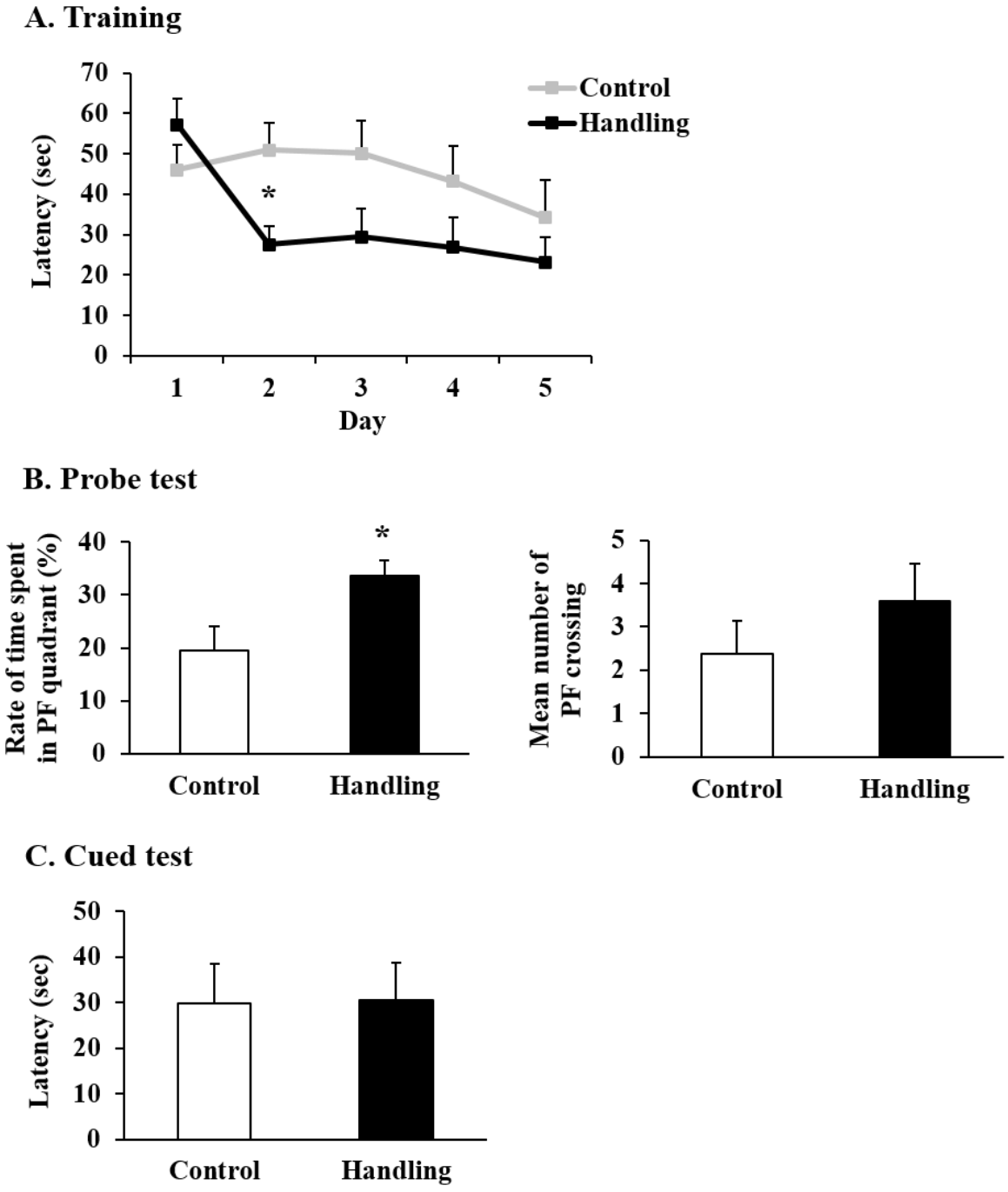


Fig. 3. Effects of postnatal handling on spatial learning and memory in adult offspring. Latency to reach the platform (PF) on training day 1-5 (A), percentage of the time spent in the quadrant of PF and mean number of PF crossing (B), latency to reach the visible PF (C) in Morris water maze. Control, n=8; Handling, n=10. *p< 0.05.

3.4. Postnatal handling did not affect depression-like behavior in the adult offspring

Effects of postnatal handling on the depression-like behavior were tested by FST (Fig. 4). There was no significant change between two groups in the time of floating (Fig. 4A), swimming (Fig. 4B) and climbing (Fig. 4C), which suggests that postnatal handling had no effect on depression-like behavior.

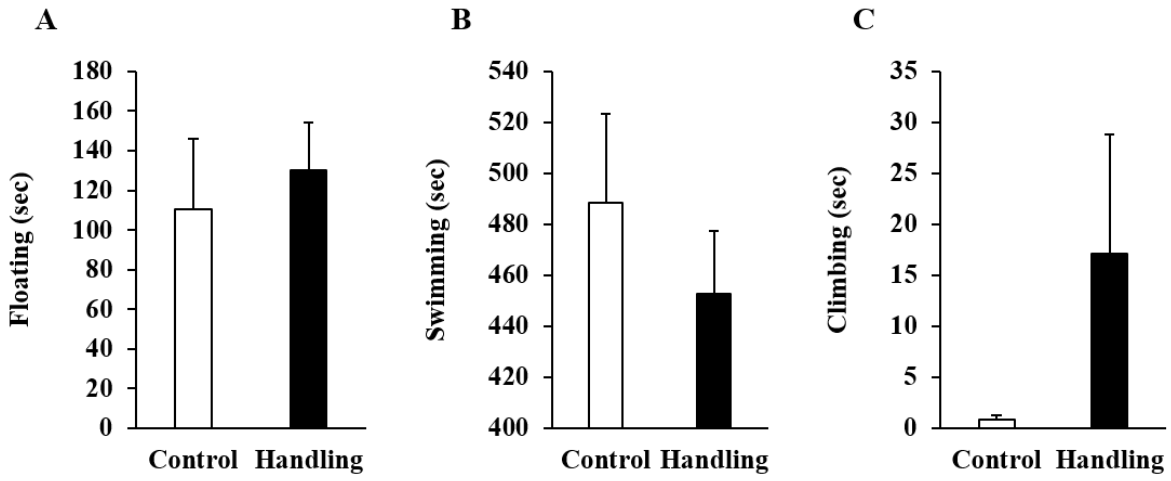


Fig. 4. Effects of postnatal handling on depression-like behavior in adult offspring. Time of floating (A) and swimming (B), climbing (C) in forced swim test. Control, n=9; Handling, n=10.

3.5. Postnatal handling changed the mRNA expression of 5-HT1A receptor in the developing and adult dorsal raphe nucleus and amygdala

We examined the effects of postnatal handling on the mRNA expression of 5-HT1A receptor in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi and dorsal raphe nucleus on P15 (Fig. 5A) and P71 (Fig. 5B). As compared with the control, postnatal handling up-regulated the mRNA expression of 5-HT1A receptor in the dorsal raphe nucleus to 281% on P15 ($t(12.644) = 3.883$, $p < 0.01$) and to 147% on P71 ($t(8) = 4.822$, $p < 0.01$). On

the other hand, postnatal handling down-regulated the mRNA expression of 5-HT1A receptor in the amygdala to 35% on P15 ($t(20) = 3.75$, $p < 0.01$) and to 65% on P71 ($t(8) = 2.383$, $p < 0.05$). Postnatal handling had no effect on the mRNA expression of 5-HT1A receptor in the medial prefrontal cortex, dorsal and ventral hippocampi on P15 and P71.

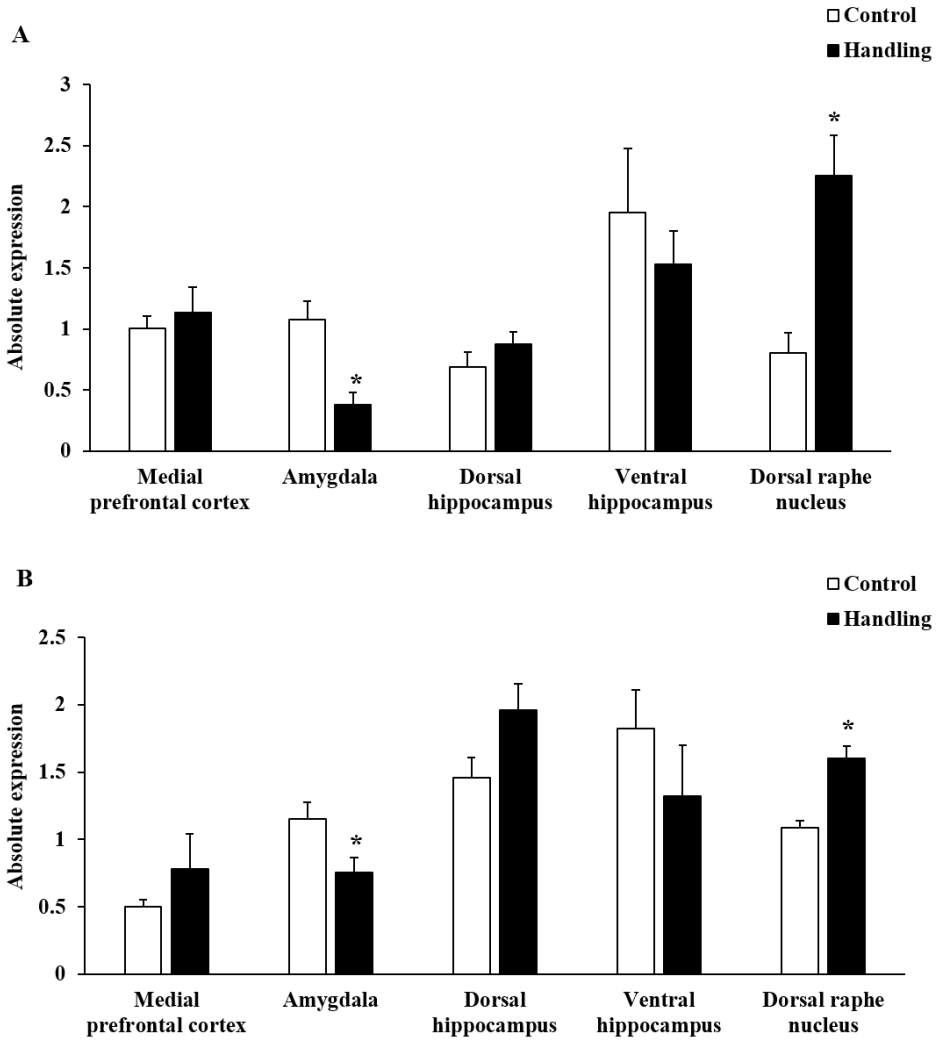


Fig. 5. Effects of postnatal handling on the mRNA expression of 5-HT1A receptor in the mouse brain on P15 and P71. Absolute expression of 5-HT1A receptor mRNA (mRNA expression of 5-HT1A receptor/18S rRNA) in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi and dorsal raphe nucleus on P15 (A) and on P71 (B). P15: Medial prefrontal cortex: n=5 (Control, Handling); Amygdala: n=12 (Control); n=10 (Handling); Dorsal hippocampus: n=5 (Control, Handling); Ventral hippocampus: n=5 (Control,

Handling); Dorsal raphe nucleus: n=6 (Control); n=10 (Handling). P71: Control, n=5; Handling, n=5. *p< 0.05.

3.6. Postnatal handling regulated mRNA expression of BDNF differently in the amygdala, dorsal hippocampus, and dorsal raphe nucleus on P71

We examined the effects of postnatal handling on the mRNA expression of BDNF in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi, dorsal raphe nucleus on P15 (Fig. 6A) and P71 (Fig. 6B). Postnatal handling up-regulated the mRNA expression of BDNF in the amygdala ($t(11) = 3.87$, $p < 0.01$) and dorsal hippocampus ($t(18) = 2.962$, $p < 0.01$), and down-regulated the mRNA expression of BDNF in the dorsal raphe nucleus ($t(4.731) = 3.452$, $p < 0.05$), but not in the medial prefrontal cortex and ventral hippocampus on P71. There was no change was found on P15 in these brain regions.

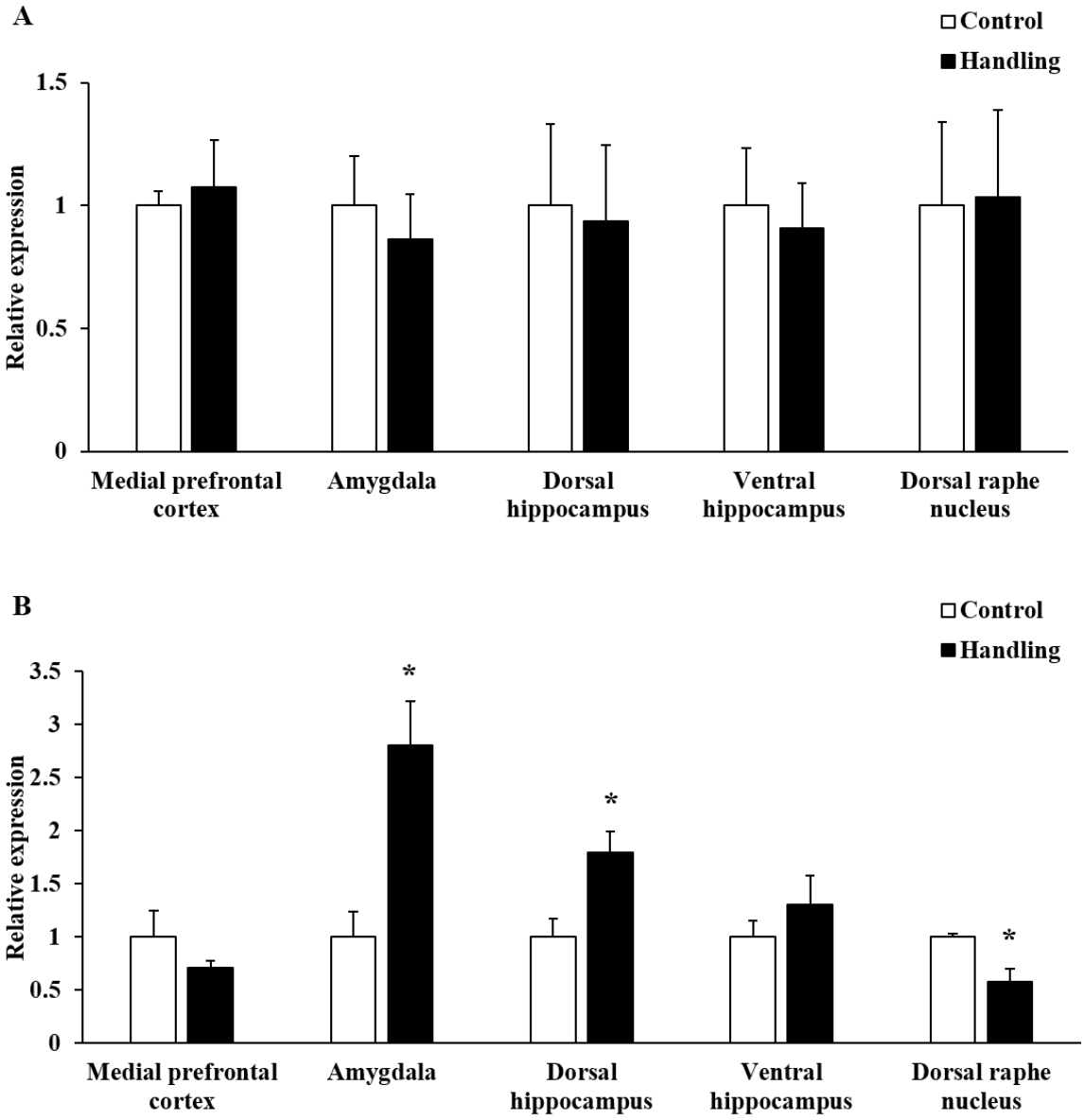
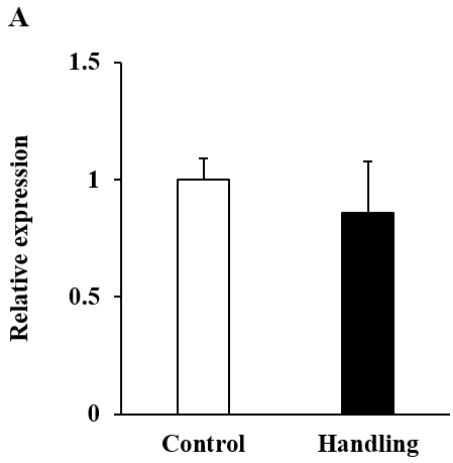


Fig. 6. Effects of postnatal handling on the mRNA expression of BDNF in the mouse brain on P15 and P71. BDNF mRNA in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi, dorsal raphe nucleus on P15 (A) and P71 (B). P15: Medial prefrontal cortex: n=5 (Control); n=4 (Handling); Amygdala: n=5 (Control, Handling); Dorsal hippocampus: n=5 (Control, Handling); Ventral hippocampus: n=5 (Control, Handling); Dorsal raphe nucleus: n=5 (Control, Handling). P71: Medial prefrontal cortex: n=7 (Control, Handling); Amygdala: n=7 (Control); n=6 (Handling); Dorsal hippocampus: n=10 (Control. Handling); Ventral hippocampus: n=8 (Control); n=7 (Handling); Dorsal raphe nucleus: n=5 (Control, Handling). *$p < 0.05$.

3.7. Postnatal handling down-regulated mRNA expression of GABA-A receptor α2 subunit in the amygdala on P71

We examined the effects of postnatal handling on the mRNA expression of GABA-A receptor α2 subunit in the amygdala on P15 (Fig. 7A) and the mRNA expression of GABA-A receptor α2 subunit in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi on P71 (Fig. 7B). Postnatal handling down-regulated the mRNA expression of GABA-A receptor α2 subunit in the amygdala ($t(5.331) = 3.432$, $p < 0.05$) on P71, but not on P15.



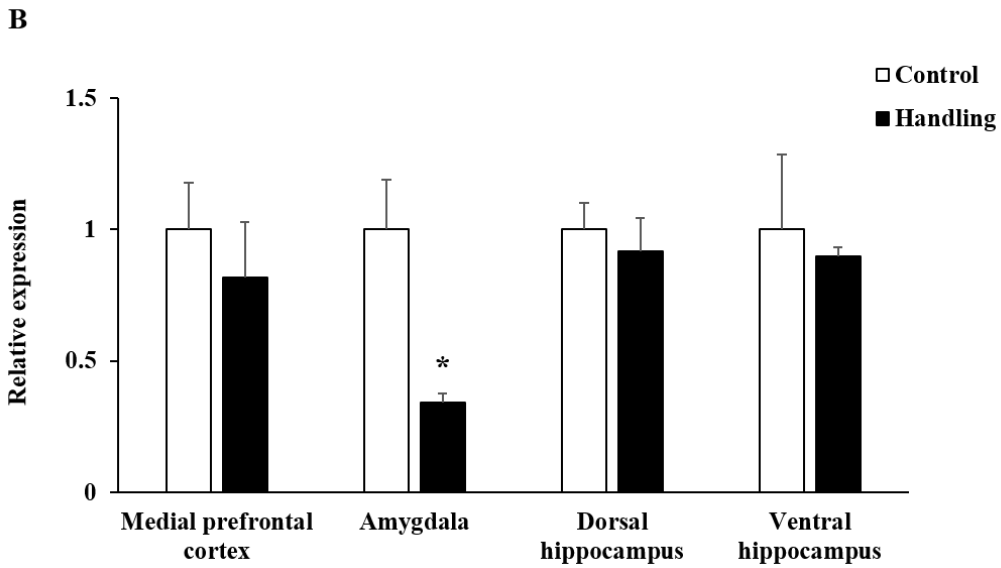


Fig. 7. Effects of postnatal handling on the mRNA expression of GABA-A receptor α2 subunit in the mouse brain on P15 and P71. GABA-A receptor α2 subunit mRNA in the amygdala on P15 (A) and GABA-A receptor α2 subunit mRNA in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi on P71 (B). P15: n=5 (Control); n=4 (Handling). P71: Medial prefrontal cortex: n=7 (Control, Handling); Amygdala: n=6 (Control, Handling); Dorsal hippocampus: n=3 (Control, Handling); Ventral hippocampus: n=4 (Control, Handling). *$p < 0.05$.

4. Discussion

In the present study, postnatal handling reduced anxiety-like behavior and improved learning and memory but had no effect on the depression-like behavior in the adult male BALB/c mice. Concomitantly, the mRNA expression of 5-HT1A receptor was up-regulated in the dorsal raphe nucleus, while down-regulated in the amygdala on P15 and P71. In the adult brain, the mRNA expression of BDNF was up-regulated in the amygdala and dorsal hippocampus and down-regulated in the dorsal raphe nucleus, while GABA-A receptor α2 subunit was down-regulated in the amygdala. Considering the functions of these molecules reported previously, the present study suggests that up-regulated expression of 5-HT1A receptor in the dorsal raphe nucleus and down-regulated expression of 5-HT1A receptor in the amygdala, and up-regulated expression of BDNF in the amygdala and dorsal hippocampus and down-regulated expression of BDNF in the dorsal raphe nucleus, and down-regulated expression of GABA-A receptor α2 subunit in the amygdala in adulthood may be correlated with reduced anxiety-like behavior and improved learning and memory.

4.1 Postnatal handling and maternal behavior

It was reported that postnatal handling increases the maternal behavior including the pup licking (e.g., Liu et al., 1997; Akatsu et al., 2015), and that maternal care affects the development of brain structure and function of offspring (Liu et al., 1997; Caldji, 1998). However, in the present study, postnatal handling had no significant effect on the maternal behavior, which suggests that maternal behavior does not mediate the effects of postnatal handling on the offspring. Roles of factors other than maternal care in mediating the effects of postnatal handling were also suggested by Macri et al. (2004). For example, both postnatal handling (brief maternal separation, 3-15 min) and maternal separation (prolonged maternal separation, more than 3 hours) increase maternal care, but postnatally handled and maternally separated rat offspring display opposite effects on the stress and fear responses (Macri et al., 2004).

4.2. Effects of postnatal handling on anxiety-like behavior

In the present EPM test, postnatal handling increased the time spent in open arms, suggesting that anxiety-like behavior of adult BALB/c mice was reduced. However, there are some discrepancies in the results using C57BL/6 mice (for review, see Millstein and Holmes, 2007). For example, in our previous study, similar postnatal handling for 15 min daily during the postnatal 2 weeks showed no effect on the anxiety-like behavior in C57BL/6N mice (Akatsu et al., 2015). Interestingly, prenatal stress elevated the anxiety-like behavior in these mice, and postnatal handling recovered the prenatal stress-induced elevation of anxiety-like behavior to the control level (Akatsu et al., 2015). Similar recovery by postnatal handling was observed in the anxiety level of prenatally-stressed Wistar rats (Bogoch et al., 2007). These

differences in the effects of postnatal handling on the anxiety-like behavior may be due to strains of mice or species of experimental animals. In particular, the postnatal handling may be effective on animals whose anxiety level is higher by prenatal stress or more vulnerable strains such as BALB/c mice (Francis et al., 2003). BALB/c mice are considered more stress sensitive than C57BL/6 mice (Belzung and Griebel, 2001), and this might explain the anti-anxiety effect of handling since their baseline anxiety may be greater.

4.3. Effects of postnatal handling on spatial learning and memory

In the present MWM test, as compared with the control, postnatal handling shortened the latency to reach the platform on day 2 of the training and increased time spent in the platform quadrant in the probe test. These results suggest that postnatal handling improved both spatial learning and memory, which is consistent with the previous report in BALB/cByJ mice (Zaharia et al., 1996). On the other hand, our previous study showed that postnatal handling improved spatial learning ability in the training of MWM test, but not spatial memory of C57BL/6N mice in the probe test (Akatsu et al., 2015). Furthermore, postnatal handling did not improve the spatial learning and memory in C57BL/6ByJ mice (Zaharia et al., 1996). It has been reported that BALB/cByJ mice display spatial learning deficits in the escape performance of Morris water maze in which many mice fail to learn the place of the platform (Upchurch and Wehner 1988; Francis et al. 1995; Zaharia et al., 1996). These differences in the effects of postnatal handling on the spatial learning and memory may be due to strains of mice or species of experimental animals. Postnatal handling may be effective on more vulnerable strains such as BALB/c mice (Francis et al., 2003).

4.4. Correlations between the effects of postnatal handling on the mRNA expression and anxiety, learning and memory

In the present study, postnatal handling up-regulated the mRNA expression of 5-HT1A receptor in the dorsal raphe nucleus and down-regulated the mRNA expression of 5-HT1A receptor in the amygdala on P15 and P71. As a result, the changes of 5-HT1A receptor mRNA in the dorsal raphe nucleus and amygdala on P15 persisted into adulthood (P71). However, based on the previous studies reporting a developmental role for the 5-HT1A receptor in the establishment of anxiety-related circuitry (Gross et al., 2002; Richardson-Jones et al., 2010; Donaldson et al., 2014), changes of 5-HT1A mRNA in the dorsal raphe nucleus and amygdala on P15 may be related to anxiety-like behavior in the present study. A previous study reported that conditional knock-down of the 5-HT1A receptor in the raphe nucleus during the postnatal stage (P14-P21) increased anxiety-like behavior in adulthood (Donaldson et al., 2014). This result suggests that 5-HT1A auto-receptor in the raphe nucleus during the postnatal stage is required to lower anxiety-like behavior in the adult mice. Based on this study, it is conceivable that postnatal handling in the present study decreased the adult anxiety behavior through up-regulation of the 5-HT1A receptor expression in the dorsal raphe nucleus. However, the results are not always consistent. Vahid-Ansari et al., 2017 reported that up-regulation of raphe 5-HT1A auto-receptors increases anxiety-like behavior. Finally, because 5-HT has neurotrophic activity through 5-HT1A receptors (Hoyer et al., 1994; Barnes and Sharp, 1999), 5-HT1A receptor may be involved in the formation of neural connections underlying the anxiety-like behavior in the present study.

In addition to the 5-HT1A receptor, the present study showed that postnatal handling up-regulated the mRNA expression of BDNF in the amygdala and dorsal hippocampus, down-regulated the mRNA expression of BDNF in the dorsal raphe nucleus in adulthood. A previous study reported that the depletion of BDNF in the adult hippocampus impaired spatial memory (Heldt et al., 2007), suggesting that hippocampal BDNF plays a critical role in the spatial memory. Considering the increase of BDNF mRNA in the adult dorsal hippocampus in the present study, it is possible that postnatal handling promotes the spatial learning and memory through up-regulating the BDNF expression in the dorsal hippocampus. BDNF is also involved in the regulation of anxiety behavior. It was reported that down-regulation of BDNF mRNA expression in the amygdala induces the increased level of anxiety in the adult rats (Pandey et al., 2006). This result suggests that up-regulated expression of BDNF in the amygdala may be involved in the postnatal handling-induced decrease of anxiety levels in the present study. As a result, BDNF mRNA expression was down-regulated by postnatal handling in the adult dorsal raphe nucleus, whereas up-regulated in the adult amygdala, showing the opposite changes of the mRNA expression of 5-HT1A receptor and BDNF between the amygdala and dorsal raphe nucleus in adulthood.

Lastly, the present study showed that postnatal handling down-regulated mRNA expression of GABA-A receptor α2 subunit in the amygdala of adult offspring. Previous studies revealed that mRNA expression of GABA-A receptor α2 subunit is higher in the amygdala of DBA/2J mice, which show higher anxiety level and less spatial learning and memory ability, as compared to C57BL/6J mice (Francis et al., 2003; Zhang et al., 2004; DuBois et al., 2006). In addition, the mRNA expression of GABA-A receptor α2 subunit was increased in the

amygdala of high-anxiety mice (Skórzewska et al., 2014). However, not all findings are consistent with our hypothesis. For example, Vollenweider et al. (2011) reported that heterozygous α2 knockout mice exhibit heightened anxiety in novelty-suppressed feeding test. Taken together, down-regulation of the mRNA expression of GABA-A receptor α2 subunit in the amygdala may be involved in the postnatal handling-induced reduction of the anxiety behavior in the present study.

The present study suggested that 5-HT1A receptor, BDNF and GABA-A receptor may be involved in mediating the effects of postnatal handling. Functional association between the 5-HT1A receptor during the postnatal development and BDNF and GABA-A receptor α2 subunit in adulthood has been suggested. Postnatal treatment with 5-HT1A receptor agonist down-regulated mRNA expression of BDNF and GABA-A receptor α2 subunit in the medial prefrontal cortex and hippocampus of adult offspring mice (Ishikawa and Shiga, 2017). In these mice, anxiety level was reduced whereas depression-like behavior was increased. In contrast, in the 5-HT1A receptor antagonist-treated mice, GABA-A-receptor α2 subunit level was up-regulated in the hippocampus (Vinkers et al., 2010). Furthermore, pharmacological 5-HT1A receptor blockade during the early postnatal period induced long-lasting effects on the anxiety and benzodiazepine sensitivity in adolescent and adult mice on a Swiss-Webster (SW) background and these phenotypes resembled those of SW 1A-KO mice (Vinkers et al., 2010). In the present study, postnatal handling up- and down-regulated mRNA expression of 5-HT1A receptor in the dorsal raphe nucleus and amygdala during the developmental stage and adult, respectively, and up-regulated BDNF in the amygdala and dorsal hippocampus and down-regulated BDNF in the dorsal raphe nucleus, and down-regulated GABA-A receptor α2

subunit in the amygdala in adulthood. Concomitantly, postnatal handling lowered anxiety levels and improved learning and memory in the present study. However, the interactions between 5-HT1A receptor, and BDNF and GABA-A receptor α2 subunit in specific brain regions need to be examined in detail.

Conclusion

The present study showed that postnatal handling reduced anxiety-like behavior and improved spatial learning and memory of adult offspring, probably via 5-HT1A receptor, BDNF and GABA-A-receptor α2 subunit in the BALB/c mice. Although the causal relationship between the gene expressions and the behaviors remain to be examined, the present study provides new information to understand the mechanisms of anxiety and learning and memory affected by postnatal handling.

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