博士論文

Effects of postnatal brief maternal separation on the development of brain and behavior in the BALB/c mice

生後発達期の短時間の母仔分離が BALB/c マウスの脳と行動の発達に与える影響

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CONTENTS

ABSTRACT

Development of brain and behavior is influenced by the interaction of genetic and environmental factors. Postnatal handling, a manipulation that briefly separates 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. In the present study, the effects of postnatal handling on the behavior of adult male BALB/c mice were examined. 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 and improved spatial learning and memory without effects on the depression. Next, to elucidate the mechanisms underlying the behavioral effects, the mRNA expression of the serotonin 1A (5-HT1A) receptor, brain-derived neurotrophic factor (BDNF), and GABA-A receptor subunits in various brain regions were evaluated by quantitative RT-PCR. Postnatal handling up-regulated mRNA expression of the 5-HT1A receptor in the dorsal raphe nucleus and down-regulated the 5-HT1A receptor in the amygdala on P15. In the adulthood, the mRNA expression of BDNF was up-regulated in the amygdala and dorsal hippocampus, whereas that of GABA-A receptor α2 subunit was down-regulated in the amygdala. Finally, to identify brain regions activated directly by postnatal handling, Fos expression was examined. Postnatal handling activated neurons in the hypothalamic paraventricular nucleus (PVN) and lateral habenula (LHb). Taken together, the present study suggests that expression changes 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, and activation of PVN and LHb, may mediate postnatal

handling-induced reduction of anxiety and improvement of learning and memory.

INTRODUCTION

Effects of early-life experience on the development of brain and behavior

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 a laboratory 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, brief maternal separation (postnatal handling) is a manipulation that briefly (3-15 min) separates rodent 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, the postnatal handling lowers anxiety level (Vallée et al., 1997; Caldji et al., 2000; Moles et al., 2004) and improves spatial learning and memory (Zaharia et al., 1996; Anisman et al., 1998; 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 among different mouse strains (Zaharia et al., 1996; Millstein et al., 2007). The brain mechanisms which mediate effects of postnatal handling on adult behavior have been well studied focusing on glucocorticoid in the hypothalamic-pituitary-adrenal (HPA) axis, but other molecular regulation is less investigated (Raineki et al., 2014). For example, recovery of stress response was faster in the postnatally

handled pups after restraint stress (Levine et al., 1967; Liu et al., 2000). Secretion of adrenocorticotropic hormone (ACTH) from anterior pituitary and secretion of corticosterone from adrenal cortex were decreased (Anisman et al., 1998; Parfitt et al., 2004). In addition, corticotropin-releasing hormone (CRH) was decreased in the hypothalamic paraventricular nucleus (PVN), amygdala central nucleus (CeA), median eminence and locus coeruleus (LC) (Plotsky & Meaney, 1993; Viau et al., 1993; Francis et al., 1999; Plotsky et al., 2005). Moreover, postnatal handling increased densities of glucocorticoid receptor (GR) in the frontal cortex and hippocampus (Meaney & Aitken, 1985; Meaney et al., 1985; Avishai-Eliner et al., 2001) and decreased the GR expression in the amygdala central nucleus (Fenoglio et al., 2004).

5-HT1A receptor

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 the adulthood. 5-HT neurons are localized 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). Among these 5-HT receptors, 5-HT1A receptor is widely expressed in the mammalian brain and has a major role in modulating traits related to mood and cognition (Ressler and Nemeroff, 2000; Akimova et al, 2009; Albert and Lemonde, 2004; Savitz et al, 2009). Within the raphe, 5-HT1A receptor acts as a presynaptic auto-receptor whose activation leads to reduced firing of serotonergic neurons and decreased serotonin levels in a variety of forebrain projection structures. In contrast, post-synaptic 5-HT1A

hetero-receptors are located on both excitatory and inhibitory neurons and modulate the activity of a number of limbic structures involved in mood and cognition, including the prefrontal cortex, amygdala and hippocampus (Barnes and Sharp, 1999; Riad et al., 2000; Santana et al., 2004). Multiple lines of evidence indicate that 5-HT1A receptor is important for mood and cognition and may be developmentally modulated. It has been shown using 5-HT1A receptor knock-out (KO) mice that blockade of 5-HT1A receptor during postnatal period increases anxiety-like behavior in the adulthood (Gross et al., 2002). In addition, 5-HT1A auto-receptor in the developing raphe nucleus formats 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).

Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) is a member of the nerve growth factor family of neurotrophic factors that is highly expressed in various brain regions and is involved in neuronal survival, differentiation and synaptic strength (Thoenen, 1995; 2000; Bibel and Barde, 2000; Poo, 2001; Carter et al., 2002; Monteggia et al., 2004). The action of BDNF is mediated by binding to tyrosine kinase B (TrkB) receptor and subsequently the activation of the MAP kinase (mitogen-activated protein kinase), phospholipase C-γ (PLC-γ) and phosphoinositide 3-kinase (PI3-kinase) signal transduction pathways (Thoenen, 1995; Impey et al., 1999; Ying et al., 2002; Pandey, 2004).

Similar to 5-HT, BDNF contributes to various functions in the developing and matured brain (Park and Poo, 2013). In addition to the formation of the neural connections during brain development, BDNF has been shown to regulate neuropsychiatric activities in the

adulthood. For example, pharmacological inhibition of BDNF impairs learning and memory in rodents (Bartoletti et al., 2002), and dysfunction of BDNF is related to depression (Nestler, 2002). Stress decreases the expression of BDNF in the hippocampus, and antidepressants recover the stress-induced reduction of BDNF (Nestler, 2002). Injection of BDNF into the hippocampus has an antidepressant effect in animal 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).

GABA-A receptor α2 subunit

Another candidate molecule which regulates anxiety is GABA-A (γ-aminobutyric acid, type A) receptor. 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 effects after several weeks of the treatment (Vaswani et al., 2003). Among 19 GABA-A receptor subunits (α1–6, β1–3, γ1–3, δ, ε, θ, π and ρ1–3) (Olsen and Sieghart, 2008; 2009), the binding sites for benzodiazepines are formed by one of the α subunits (α1, α2, α3 or α5) and a γ subunit (typically the γ2 subunit which is present in approximately 90% of GABA-A receptors) (Rudolph and Knoflach, 2011), and α2 and α3 subunits modulate anxiety-like behavior (Griebel and Holmes, 2013). 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).

Brain regions activated by postnatal handling

There are a few studies which reported activated brain regions after postnatal handling during development. The immediate-early gene c-Fos is considered as a marker of neuronal activity and has thus been widely used for functional mapping of brain areas (Sagar et al., 1988; Sharp et al., 1993). Garoflos et al. (2008) reported that c-Fos expression was increased in the rat hippocampal CA1 region, parietal and occipital cortexes on P1 following 8 hours after handling. On the other hand, Fenoglio et al. (2006) reported that c-Fos expression was increased in the thalamic paraventricular nucleus (PVT), central amygdala (ACe) and bed nucleus of stria terminalis (BnST) of P9 rat pups that were handled daily between P2 and P9 and killed 30 min after their return to the dam, as compared with the undisturbed and maternal deprived pups. In addition, c-Fos expression was induced in ACe and BnST by a single handling, but the activation of PVT neurons required recurrent handling (Fenoglio et al., 2006). Furthermore, the number of c-Fos-expressing neurons increased progressively and peaked at 30-60 min in PVT and BnST, whereas peaked at 5 min in Ace, and declined sharply in these regions by 120 min after the return of pups to the dam (Fenoglio et al., 2006). However, considering the previous studies showing that the effects of postnatal handling on anxiety-like behavior and spatial learning and memory are not necessarily consistent between mice and rats and among different mouse strains, postnatal handling may differently activate brain regions in the BALB/c mice.

BALB/c mice

BALB/c mice have a single nucleotide substitution of tryptophan hydroxylase 2 (Tph2)

and show lower 5-HT levels in the frontal cortex and striatum as compared with 129X1/SvJ mice (Zhang et al., 2004). In addition, BALB/c mice show poor learning and memory abilities and elevated anxiety-like behavior as compared with C57BL/6J mice, being used as a depression animal model (Francis et al., 2003; Uchida et al., 2011). Thus, it may be reasonable to use BALB/c mice in order to clarify the brain mechanisms underlying the effects of postnatal handling on the adult offspring behaviors, focusing on the serotonergic system.

Purpose

Formation of structure and function of the brain is genetically programmed but also modified by environmental factors, and maternal separation is a major environmental factor during the postnatal development. However, the brain mechanisms mediating the effects of the maternal separation are not well known. In the present study, I tried to elucidate the effects of the brief maternal separation (postnatal handling) on the development of the adult behavior and brain mechanisms underlying the effects on the behavior. For this purpose, I first examined the effects on the anxiety-like behavior, depression-like behavior and spatial learning and memory of adult offspring in the BALB/c mice, because effects of the postnatal handling on the development of offspring behavior are not consistent in the previous studies. Next, to elucidate the mechanisms underlying the behavioral effects, I measured the mRNA expression of 5-HT1A receptor, BDNF and GABA-A receptor by quantitative RT-PCR. In addition, to identify brain regions activated by postnatal handling, I examined the Fos expression. Finally, by comparing the changes of behaviors with those of the mRNA expression and the Fos expression induced by postnatal handling, I discussed some brain mechanisms mediating

the effects of postnatal handling on the adult offspring behaviors.

MATERIALS AND METHODS

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.

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 of both sexes were moved from the dam to a new cage and were separated from each other for 15 min (11:00-11:15AM) daily from P1 to P14. The temperature of the offspring cage was regulated at 33 ± 2 °C using a hot carpet 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 single housed and only male mice were used for the following analyses.

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 were counted each 2 min: nursing posture (arched-back nursing), pup licking and nest building. The maximum point of total behavior is 60, and minimum point is 0.

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.

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. 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.

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.

Real-time reverse transcription-PCR

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 the 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 (Fig.1), since these brain regions have been shown to be involved in cognition and emotion (Bannerman et al., 2004). Each brain regions were immediately

frozen in liquid nitrogen and stored at -80 ºC. 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 12,000 rpm at 4 °C for 5 min, supernatant was collected, and chloroform was added to separate RNA into aqueous layer. After re-centrifugation at 12,000 rpm at 4 °C for 15 min, supernatant was collected and isopropanol was added to precipitate RNA. Precipitated RNA was washed with 75% ethanol and centrifuged at 12,000 rpm at 4 °C 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 cDNA. 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™ 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 °C and 30 seconds at 60 °C, 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 compensation, 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.

Immunohistochemistry

Male pups from each group were deeply anesthetized with isoflurane 1 hour after they were returned to the dam on P14. They were then perfused with saline, followed by 4% paraformaldehyde (PFA) in 0.1M phosphate buffer (PB). Brains were removed and post-fixed in 4% PFA in PB, immersed in 10%, 20% and 30% sucrose in PB overnight at 4 °C, and stored at -80 °C. Brains were sectioned coronally at 12 μm using a cryostat. After wash with PBS for 5 min, sections were treated with 0.3% H_2O_2 in 100% MtOH for 30 min. After two washes with PBS, nonspecific binding was blocked with 5% normal goat serum (Vector Laboratories) in 0.3% Triton X-100 in PBS (blocking solution) for 1 hour. Sections were incubated with c-Fos-antibody (1:40,000; Calbiochem) in blocking solution for 24 hours at 4 °C. Subsequently, sections were washed in 0.15% Triton X-100 in PBS (TPBS) three times for 15 min and PBS once for 5 min, and incubated in biotinylated anti-rabbit IgG by goat (1:500; Vector Laboratories) in blocking solution for 1 hour. After washing in TPBS and PBS, sections were incubated in Elite ABC solution (1:100; Vector Laboratories) for 30 min and rinsed in TPBS and PBS. Slides were then stained with 3, 3'-diaminobenzidine (DAB) diluted in stable peroxide substrate buffer (1:20; Thermo Scientific) for 5 min at room temperature. Finally, they were washed, dehydrated and cleared and cover-slipped. For neuroanatomic orientation, one in every two sections were stained with Nissl stain. The image analysis program "Image J" was used for cell counting.

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. For the analyses of the probe and cued tests of MWM, EPM test, FST and mRNA expression of 5-HT1A receptor and BDNF, GABA-A receptor α2 subunit, Fos expression, data were analyzed by Student's t test. All data are expressed as mean ± S.E.M and $p < 0.05$ was considered as statistically significant.

RESULTS

Postnatal handling did not affect maternal behavior

To investigate the mechanism of postnatal handling, maternal behavior (nursing posture, pup licking, nest building) were examined on P1, P3 and P7 (Fig. 2). In the frequencies of pup licking, we found a significant main effect of day ($F(2,26) = 19.175$, $p < 0.001$) (Fig. 2B), but not the interaction between day and group. On the other hand, in the frequencies of nursing posture (Fig. 2A) and nest building (Fig. 2C), no significant main effect of day and the interaction between day and group was found. These results suggest that postnatal handling did not affect maternal behavior.

Postnatal handling reduced anxiety-like behavior in the adult offspring

Many studies have reported that postnatal handling lowers anxiety level of adult offspring (Vallée et al., 1997; Caldji et al., 2000; Moles et al., 2004). However, the effect on anxiety-like behavior is not always consistent between mice and rats or among different mouse strains (Zaharia et al., 1996; Millstein et al., 2007). To confirm the effects of postnatal handling on anxiety-like behavior, adult offspring of BALB/c mice were tested by EPM (Fig. 3). Postnatal handling increased the time spent in open arms ($t(17) = 2.193$, $p < 0.05$) (Fig. 3A) and marginally decreased time spent in closed arms ($t(17) = 1.908$, $p = 0.073$) (Fig. 3B). However, there was no significant difference between two groups in entries into open arms (Fig. 3C) and both arms (Fig. 3D). These results suggest that postnatal handling reduced anxiety-like behavior.

Postnatal handling improved spatial learning and memory in the adult offspring

Previous studies have also reported that postnatal handling improves spatial learning and memory (Zaharia et al., 1996; Anisman et al., 1998; Vallée et al., 1999; Fenoglio et al., 2005), although the effects on spatial learning and memory are not necessarily consistent between mice and rats or among different mouse strains (Zaharia et al., 1996; Millstein et al., 2007). Therefore, the effects of postnatal handling on spatial learning and memory were tested by MWM (Fig. 4). In 5-day-training, a main effect 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. 4A). The latency to reach the platform was decreased in training day 2 ($F(1,16) = 2.28$, $p < 0.05$) (Fig. 4A), but not in training day 1, 3, 4 and 5. In the probe test, postnatal handling increased the time spent in the platform quadrant ($t(16) = 2.644$, $p < 0.05$) (Fig. 4B), but not in number of crossing of platform. There was no significant difference between two groups in the cued test (Fig. 4C), 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.

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

Based on the previous studies that the postnatal handling lowers anxiety level (Vallée et al., 1997; Caldji et al., 2000; Moles et al., 2004), another emotional behavior may be changed by handling. Thus, the effects of postnatal handling on the depression-like behavior were tested by FST (Fig. 5). There was no significant change between two groups in the time of floating (Fig. 5A), swimming (Fig. 5B) and climbing (Fig. 5C), which suggests that postnatal handling had no effect on depression-like behavior.

Postnatal handling changed the mRNA expression of 5-HT1A receptor in the

developing raphe and amygdala

5-HT1A receptor is widely expressed in the mammalian brain and has a major role in modulating traits related to mood and cognition (Ressler and Nemeroff, 2000; Akimova et al, 2009; Albert and Lemonde, 2004; Savitz et al, 2009). Moreover, multiple lines of evidence indicate that 5-HT1A receptor is important for mood and cognition and may be developmentally modulated. For example, 5-HT1A receptor knock-out (KO) mice that blockade of 5-HT1A receptor during postnatal period increases anxiety-like behavior in the adulthood (Gross et al., 2002). Hence, 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 were examined on P15 (Fig. 6). Postnatal handling up-regulated the mRNA expression of 5-HT1A receptor in the dorsal raphe nucleus ($t(12.644) = 3.883$, $p < 0.01$) . On the other hand, postnatal handling down-regulated the mRNA expression of 5-HT1A receptor in the amygdala ($t(20) = 3.75$, $p < 0.01$). Postnatal handling had no effect on the mRNA expression of 5-HT1A receptor in the medial prefrontal cortex, dorsal and ventral hippocampi.

Postnatal handling up-regulated mRNA expression of BDNF in the adult amygdala and dorsal hippocampus

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). In addition to the formation of the neural connections during brain development, BDNF has been shown to regulate neuropsychiatric activities in the adulthood. For example, pharmacological inhibition of BDNF impairs learning and memory in rodents (Bartoletti et al., 2002), and dysfunction

of BDNF is related to depression (Nestler, 2002). Accordingly, the effects of postnatal handling on the mRNA expression of BDNF in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi were examined on P71 (Fig. 7). Postnatal handling up-regulated the mRNA expression of BDNF in the amygdala ($t(11) = 3.87$, $p < 0.01$) and dorsal hippocampus ($t(18) = 2.962$, $p < 0.01$), but not in the medial prefrontal cortex and ventral hippocampus.

Postnatal handling down-regulated mRNA expression of GABA-A receptor α2 subunit in the adult amygdala

Pharmacological 5-HT1A receptor blockade during the early postnatal period up-regulated adult GABA-A-receptor α2 subunit level in the hippocampus, and induced long-lasting effects on the anxiety and benzodiazepine sensitivity in adolescent and adult mice on a Swiss-Webster (SW) background (Vinkers et al., 2010). Furthermore, 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). Based on these results, the effects of postnatal handling on the mRNA expression of GABA-A receptor α2 subunit in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi were examined on P71 (Fig. 8). Postnatal handling down-regulated the mRNA expression of GABA-A receptor α2 subunit in the amygdala ($t(5.331) = 3.432$, $p < 0.05$), but not in the medial prefrontal cortex, and dorsal and ventral hippocampi.

Postnatal handling activated neurons in the PVN and LHb

Considering the previous studies showing that the effects of postnatal handling on anxiety-like behavior and spatial learning and memory are not necessarily consistent

between mice and rats or among different mouse strains, postnatal handling may differently regulate brain in the BALB/c mice. Therefore, to identify brain regions activated by postnatal handling, the effects of postnatal handling on the c-Fos expression were examined on P14. c-Fos expression were found in many brain regions, including the cortex and amygdala, hippocampus, lateral septum, thalamus, striatum in both control and handled mice. Whereas, there were significantly increased c-Fos expression in the PVN ($t(21) = 6.067$, $p < 0.001$) and the LHb ($t(25) = 6.163$, $p < 0.001$) of the handled mice (Figs. 9, 10), but no or few c-Fos expression was found in the control mice.

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. In the adult brain, the mRNA expression of BDNF was up-regulated in the amygdala and dorsal hippocampus, while GABA-A receptor α2 subunit was down-regulated in the amygdala. In addition, postnatal handling activated neurons in the PVN and LHb on P14. Considering the functions of these molecules and the activated brain regions reported previously, the present study suggests that changes of the mRNA expression of 5-HT1A receptor in the dorsal raphe nucleus and amygdala, and activated neurons in the PVN and LHb during the postnatal stage, and the mRNA expression of BDNF in the amygdala and dorsal hippocampus and the mRNA expression of GABA-A receptor α2 subunit in the amygdala in the adulthood, may underlie reduced anxiety-like behavior and improved learning and memory of adult offspring.

Postnatal handling and maternal behavior

It was reported that postnatal handling increases the maternal behavior including the pup licking (Liu et al., 1997), and that maternal care affects the development of brain structure and function of offspring (Liu et al., 1997; Caldji, 1998). Indeed, in our previous study, postnatal handling increased pup licking behavior in the C57BL/6N mice (Akatsu et al., 2015). However, in the present study, postnatal handling had no significant effect on the maternal behavior in the same experimental paradigm, which suggests that maternal

behavior does not mediate the effects of postnatal handling on the offspring in the BALB/c mice. These inconsistent results may be induced by difference in mouse strains. For example, C57BL/6 mothers lick/groom their pups more frequently than BALB/c mothers (Anisman et al, 2001). 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 and maternal separation increase maternal care, but handled and maternally separated rat offspring display opposite effects on the stress and fear responses (Macri et al., 2004). Factors mediating the postnatal handling of BALB/c mice remain to be examined.

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, the 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 the 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 addition, 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).

Effects of postnatal handling on spatial learning and memory

In the present MWM test, 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 affect the spatial learning and memory in C57BL/6ByJ mice (Zaharia et al., 1996). As was discussed above in the effects on anxiety, the inconsistent results may be dependent on strains of mice.

Correlations between the postnatal handling-induced changes of the mRNA expression and behavior

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 during the postnatal stage. A previous study reported that conditional knock-down of the 5-HT1A receptor in the raphe nucleus during the postnatal stage increased anxiety-like behavior in the 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. In addition, considering that 5-HT neurons in the raphe nucleus project to widespread brain regions including the amygdala, the up-regulated 5-HT1A receptor in the dorsal raphe nucleus may down-regulate the 5-HT1A expression in the amygdala (Hoyer et al., 1994; Barnes and Sharp, 1999). Finally, because 5-HT has neurotrophic activity through 5-HT receptors, 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 adult amygdala and dorsal hippocampus. 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.

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). 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 and improvement of the learning and memory 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 the adulthood has been suggested. Postnatal treatment with 5-HT1A receptor agonist down-regulated the 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, respectively, and up-regulated BDNF in the amygdala and dorsal hippocampus, and down-regulated adult GABA-A receptor α2 subunit in the amygdala in the adulthood. Concomitantly, postnatal handling lowered anxiety levels and improved learning and memory in the present study. Taken together, these results suggest that 5-HT1A receptor in the

developmental stage, differently regulates adult BDNF and GABA-A receptor α2 subunit to modulate emotion and cognition in the adulthood. 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.

In the present study, 5-HT1A receptor, BDNF and GABA-A receptor α2 subunit in the medial prefrontal cortex and hippocampus were not changed, except for the increased BDNF in the dorsal hippocampus. Considering that cortex and hippocampus are closely related to the emotion and cognition, other molecules in these brain regions may be involved in the handling-induced behavioral changes. For example, in our previous study, mRNA expression of 5-HT2A receptor and 5-HT2C receptor was changed in the ~~pre~~frontal cortex and hippocampus by postnatal handling in the C57BL/6N mice (Akatsu et al., 2015). These 5-HT receptors in the frontal cortex and hippocampus may regulate the postnatal handling-induced behavioral changes, although further studies are needed to confirm the functional roles.

Correlations between the postnatal handling-induced Fos expression, the mRNA expression and behavior

In the present study, postnatal handling activated neurons in the PVN and LHb. The PVN synthesizes CRH, integrates stress-relevant signals from multiple brain regions and induces the neuroendocrine response to stress (Swanson and Sawchenko, 1980; Bruhn et al., 1984; Makara et al., 1986; Denver, 2009; Bains et al., 2015). Neonatal corticosterone plays an important role in various behaviors in the adulthood, depending on the concentration. A previous study reported that low levels of neonatal corticosterone through supplementation in the maternal drinking water improved cognitive abilities and

increased natural autoantibodies levels directed to 5-HT transporter, whereas high doses of neonatal corticosterone reduced hippocampal BDNF levels in adult offspring (Macri et al., 2009). These results support the view that both adaptive plasticity and pathological outcomes in adulthood may depend on circulating neonatal corticosterone levels (Macri et al., 2011, review). On the other hand, Macri et al. (2004) reported that both postnatal brief (15 min) and prolonged (4 hour) daily maternal separation produced similar increases in maternal behavior. However, brief maternal separation resulted in reduced HPA and fear responses as compared with prolonged daily maternal separation in the adult offspring (Macri et al., 2004).

Taken together, considering the present results showing that postnatal handling did not change maternal behavior and activated neurons in the PVN of developing offspring, moderate levels of postnatal corticosterone directly through handling or indirectly through maternal milk may be involved in the reduced anxiety levels and improved learning and memory in adulthood.

Similar to the PVN, the LHb neurons have been shown to be activated by the stress-inducing or the negative value of stimuli (Dafny et al., 1990; Matsumoto et al., 1994; Shumake and Gonzalez-Lima, 2003; Matsumoto and Hikosaka, 2009; Hikosaka, 2010). The LHb neurons project directly to the raphe nucleus and regulate activity of 5-HT neurons (Wang and Aghajanian, 1977; Ferraro et al., 1996; Bernard and Veh, 2012; Sego et al., 2014). In addition, LHb activation inhibited 5-HT neurons in the raphe nucleus (Wang and Aghajanian, 1977; Stern et al., 1979; Lecourtier and Kelly, 2007, review), and inhibited 5-HT release (Reisine et al., 1982; Nishikawa and Scatton, 1985).

A previous study reported that inactivation of the LHb with muscimol, an agonist of the GABA-A receptor, increased anxiety levels in the elevated plus maze (Mathis et al., 2015).

In addition to the anxiety, blockade of excitatory inputs with 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and an antagonist of the glutamatergic AMPA receptor, and LHb inactivation with muscimol, prevented encoding and retrieval of spatial information (Mathis et al., 2015). Furthermore, LHb inactivation led to marked deficits in a hippocampus-dependent spatial recognition task (Galani et al., 1998), and a decreased metabolism in the LHb during a water-maze task in aged memory-impaired rats compared with young unimpaired animals has been reported (Villarreal et al., 2002), and LHb functionally interacts with the dorsal hippocampus and is involved in hippocampus-dependent spatial information processing (Goutagny et al., 2013).

Considering the present study showing that the postnatal handling activated neurons in the LHb, it was suggested that the activation of the LHb may mediate the effects of postnatal handling on the anxiety and learning and memory of adult offspring.

Furthermore, according to the previous study, C57BL/6N congenic mice that backcrossed to Tph2 SNP DBA/2N mice showed desensitization of 5-HT1A auto-receptors and increased anxiety-like behavior (Berger et al., 2012). Serotonin transporter (5-HTT, Slc6A4) knockout mice on a C57BL/6 background displayed elevated anxiety without depression-like alterations (Kalueff et al., 2010), and showed a desensitization of 5-HT1A auto-receptors due to a reduction of its expression (Fabre et al, 2000). Richardson-Jones et al. (2011) reported that the reduction of 5-HT1A auto-receptors expression during development led to an elevation of extracellular 5-HT concentration and to increased anxiety in adult animals, but not to depression-like behavior.

Interestingly, in the BALB/c mice with Tph2 SNP of the present study, postnatal handling up-regulated 5-HT1A receptor in the dorsal raphe nucleus during development and reduced anxiety in adult offspring, but not to depression-like behavior. In addition,

postnatal handling down-regulated 5-HT1A receptor in the amygdala during development and up-regulated BDNF in the amygdala and dorsal hippocampus, down-regulated GABA-A receptor α2 subunit in the amygdala during adulthood, and improved learning and memory of adult offspring in the present study.

In summary, the present results indicate that postnatal handling activates neurons in the PVN and then LHb, suppresses 5-HT neurons in the dorsal raphe nucleus, and may inhibit the elevation of extracellular 5-HT concentration induced by the desensitization of 5-HT1A auto-receptors in the Tph2 SNP BALB/c mice through increasing 5-HT1A receptor in the dorsal raphe nucleus. Ultimately, postnatal handling changed 5-HT1A, BDNF and GABA-A receptor α2 subunit in the amygdala and dorsal hippocampus during development and adulthood, and reduced anxiety-like behavior and improved learning and memory of adult offspring in the present study (Fig. 11).

CONCLUSION

The present study showed that postnatal handling activated stress-related brain regions, PVN and LHb, and reduced anxiety-like behavior and improved spatial learning and memory of adult offspring. Moreover, 5-HT neurons in the dorsal raphe nucleus, which receive direct input from the LHb, may be suppressed to inhibit 5-HT overflow in the Tph2 SNP BALB/c mice. Consequently, these mechanisms may contribute to the relief of high anxiety level and poor spatial learning and memory ability. Indeed, postnatal handling up-regulated 5-HT1A receptor in the dorsal raphe nucleus, and it may be involved in the modification of the desensitization of 5-HT1A auto-receptors and the reduction of anxiety level. Additionally, changes of 5-HT1A receptor and BDNF, GABA-A receptor α2 subunit in the amygdala, and of BDNF in the dorsal hippocampus, may regulate the anxiety-like behavior and spatial learning and memory, respectively. Although the causal relationship between the Fos expressions, mRNA expression and the behavior remain to be examined, the present study provides some information to understand the mechanisms of anxiety and learning and memory affected by postnatal handling.

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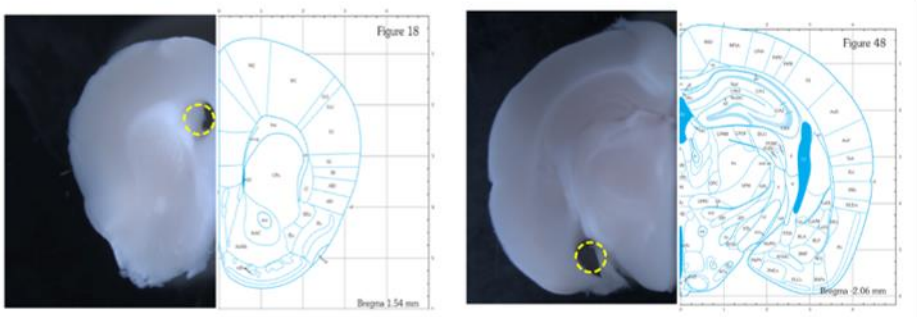
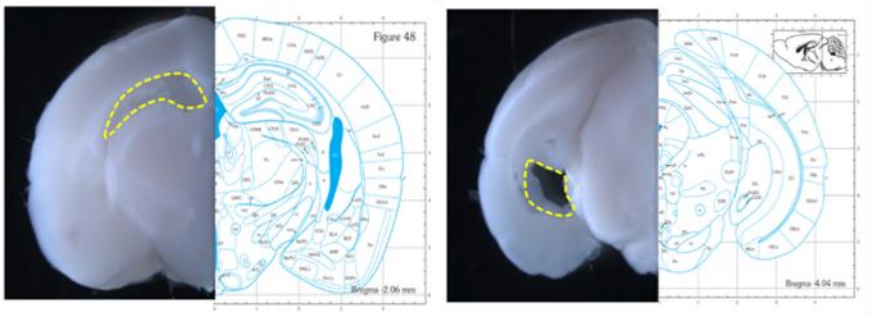
A. Medial prefrontal cortex **B. Amygdala**



C. Dorsal hippocampus **D. Ventral hippocampus**



E. Dorsal raphe nucleus

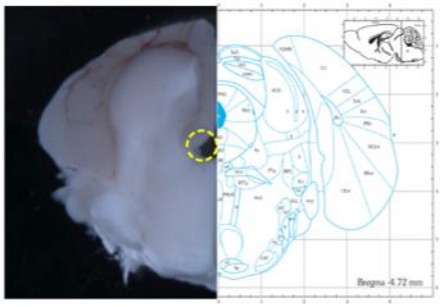


Fig. 1. Analyzed brain regions.
The photographs of coronal brain slices (left side) and corresponding brain atlas (right side). Medial prefrontal cortex (A), amygdala (B), dorsal hippocampus (C), ventral hippocampus (D), dorsal raphe nucleus (E). Areas surrounded by dotted lines showed the analyzed brain regions. The brain atlas is from The Mouse Brain in Stereotaxic Coordinates 3rd Edition Franklin & Paxinos.

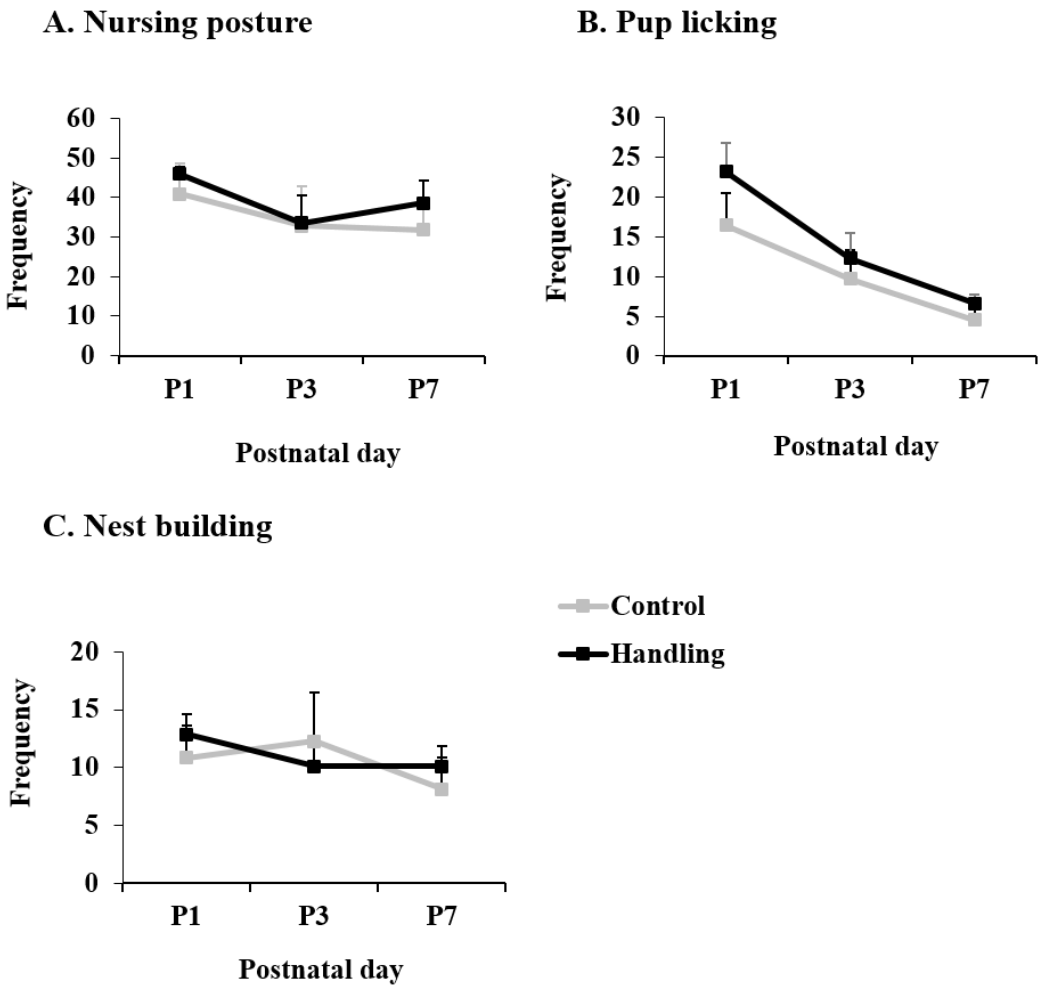


Fig. 2. Effects of postnatal handling on maternal behavior.
Frequency of nursing posture (A), pup licking (B), nest building (C) on P1, 3 and 7. Control, n=7; Handling, n=8.

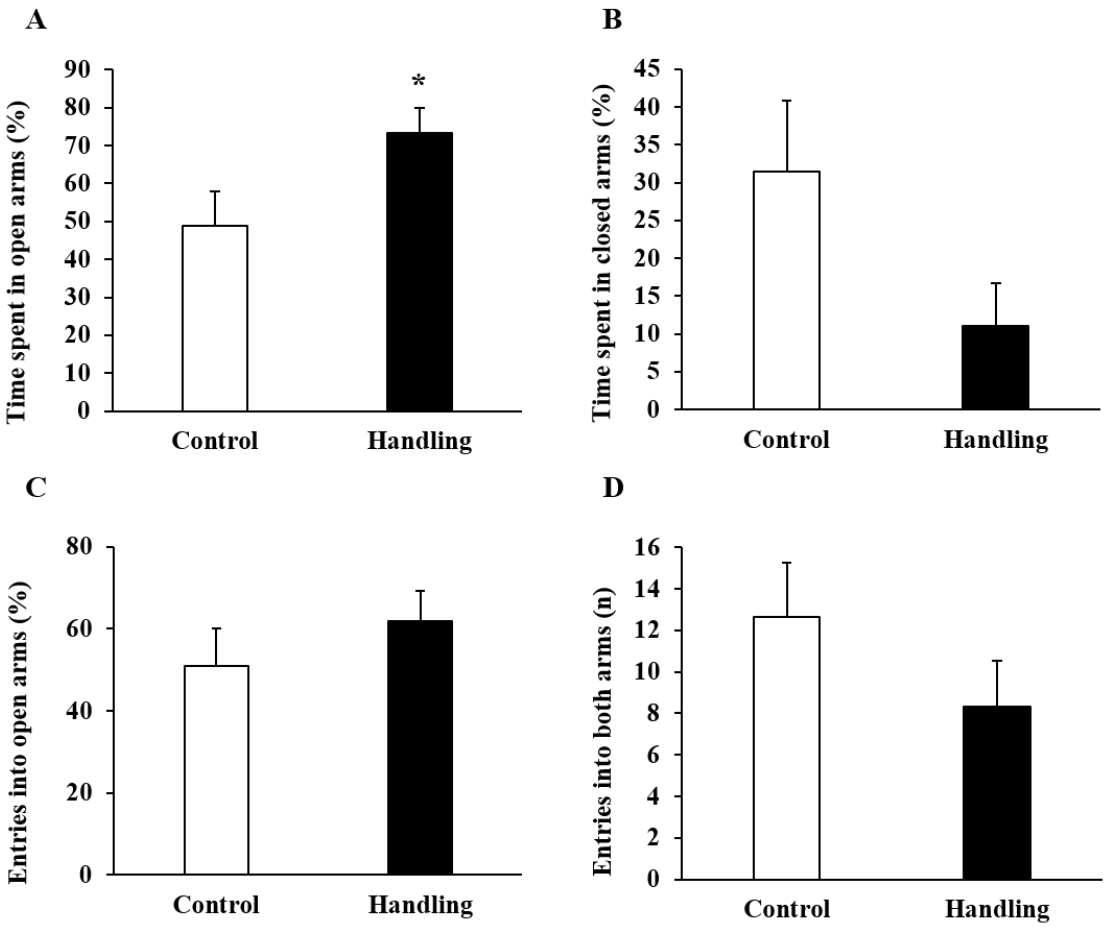


Fig. 3. Effects of postnatal handling on anxiety-like behavior.
Percentage of the time spent in open arms (A) and in closed arms (B), percentage of the numbers of entries into open arms (C) and the numbers of entries into both arms (D) in elevated plus maze on P57. Control, n=9; Handling, n=10. *p< 0.05.

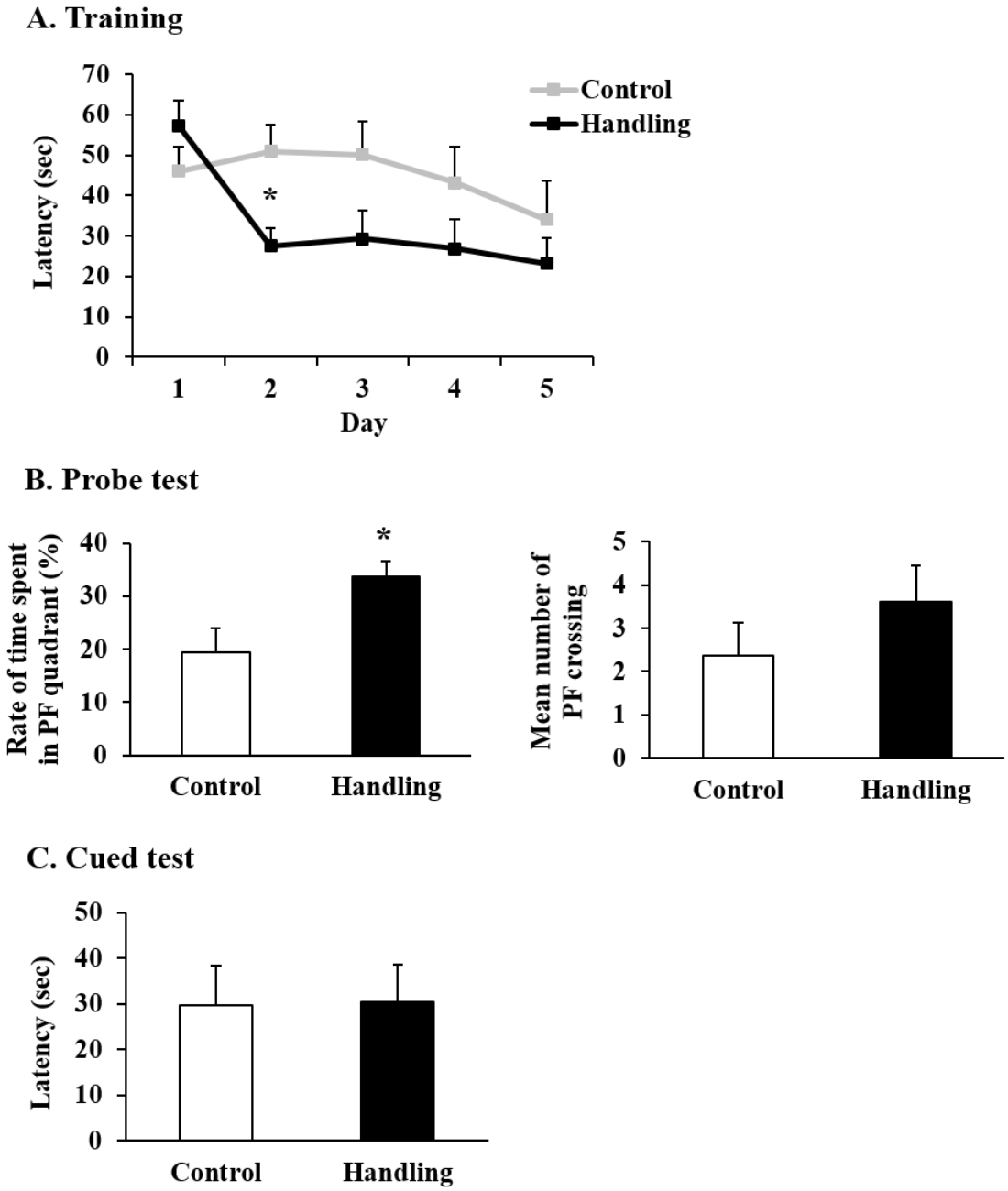


Fig. 4. Effects of postnatal handling on spatial learning and memory.
Latency to reach the platform (PF) on training day 1-5 (A), percentage of the time spent in the quadrant of PF and mean number of PF crossing (B), latency to reach the visible PF (C) in Morris water maze on P59~65. Control, n=8; Handling, n=10. *p< 0.05.

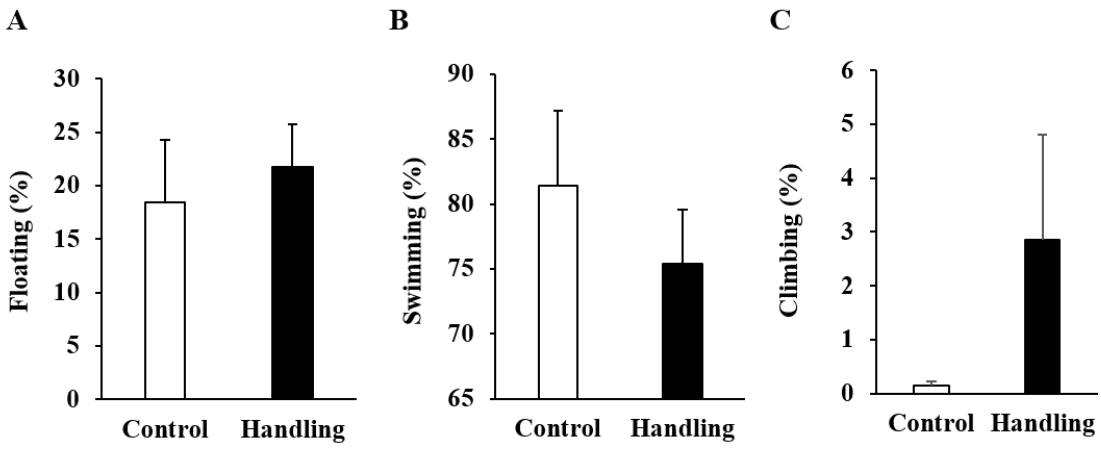


Fig. 5. Effects of postnatal handling on depressive-like behavior.
Percentage of the time of floating (A) and swimming (B), climbing (C) in forced swim test on P69. Control, n=9; Handling, n=10.

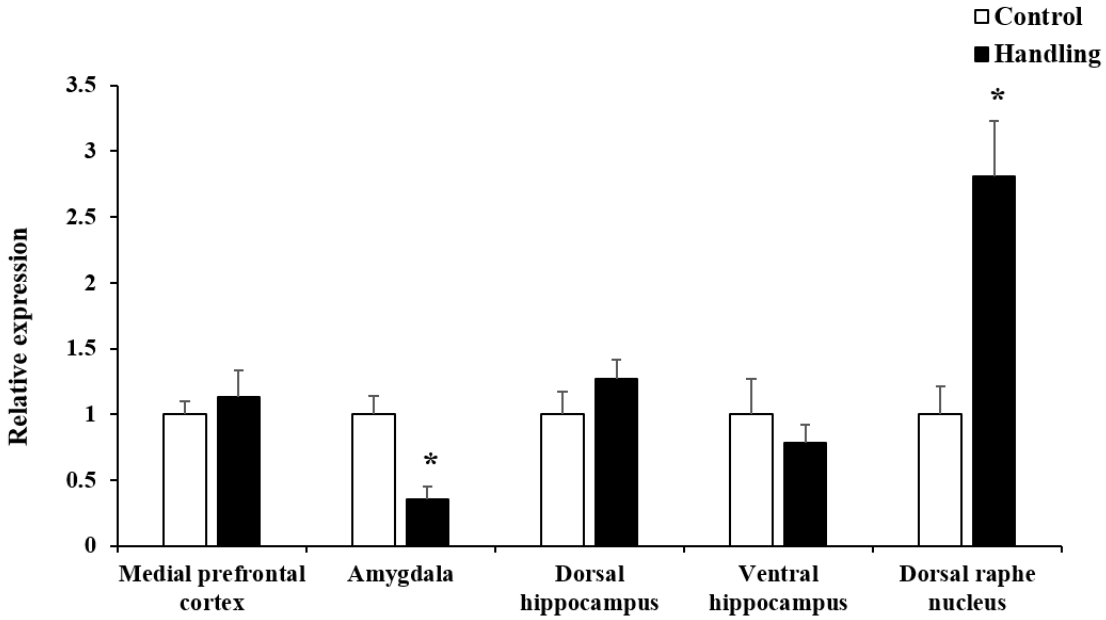


Fig. 6. Effects of postnatal handling on 5-HT1A receptor.
5-HT1A receptor mRNA in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi and dorsal raphe nucleus on P15. Control, n=5~12; Handling, n=5~10. *p< 0.01.

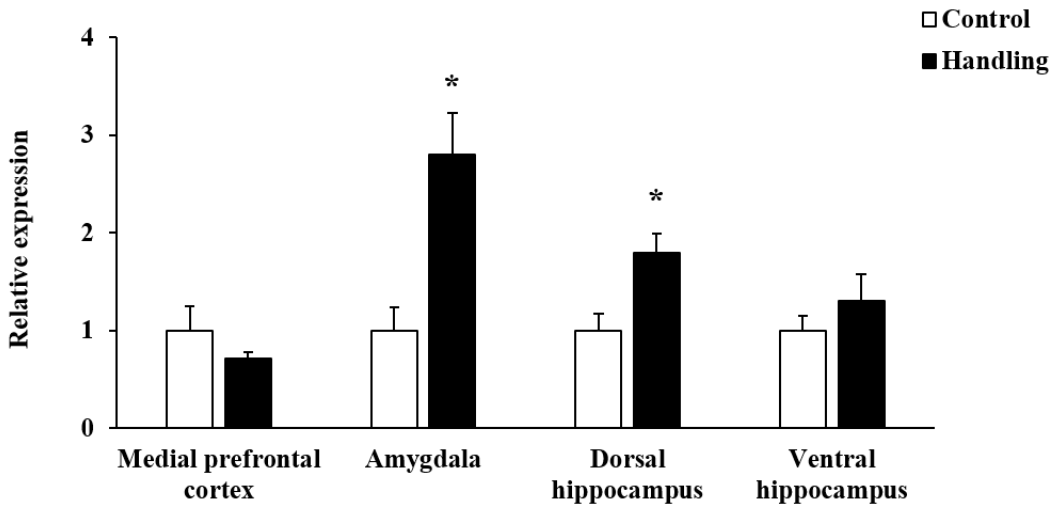


Fig. 7. Effects of postnatal handling on BDNF.
BDNF mRNA in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi on P71. Control, n=7~10; Handling, n=6~10. *p< 0.01.

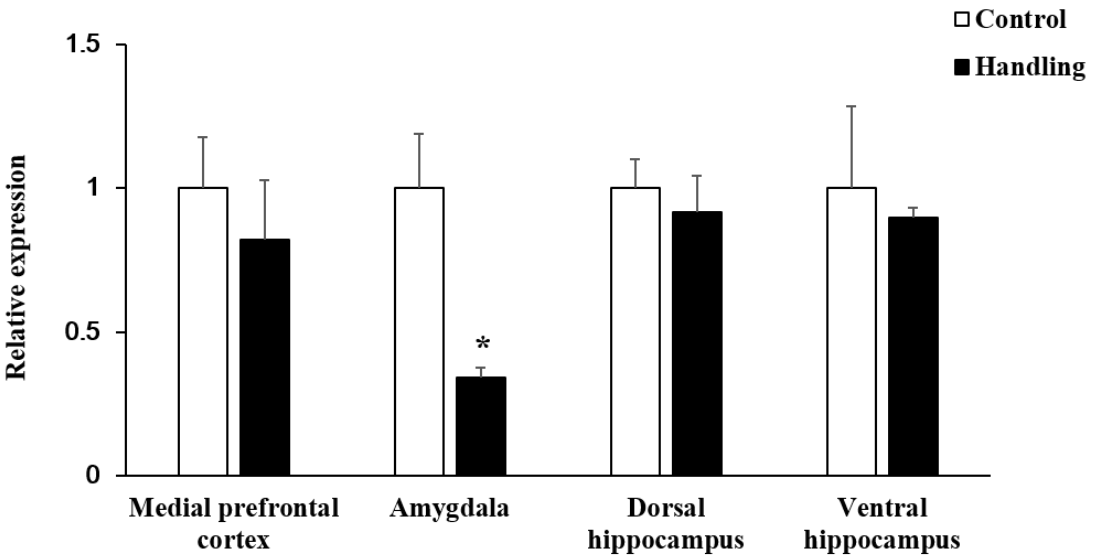


Fig. 8. Effects of postnatal handling on GABA-A receptor α2 subunit.
GABA-A receptor α2 subunit mRNA in the medial prefrontal cortex, amygdala, dorsal and ventral hippocampi on P71. Control, n=3~7; Handling, n=3~7. * $p < 0.05$.

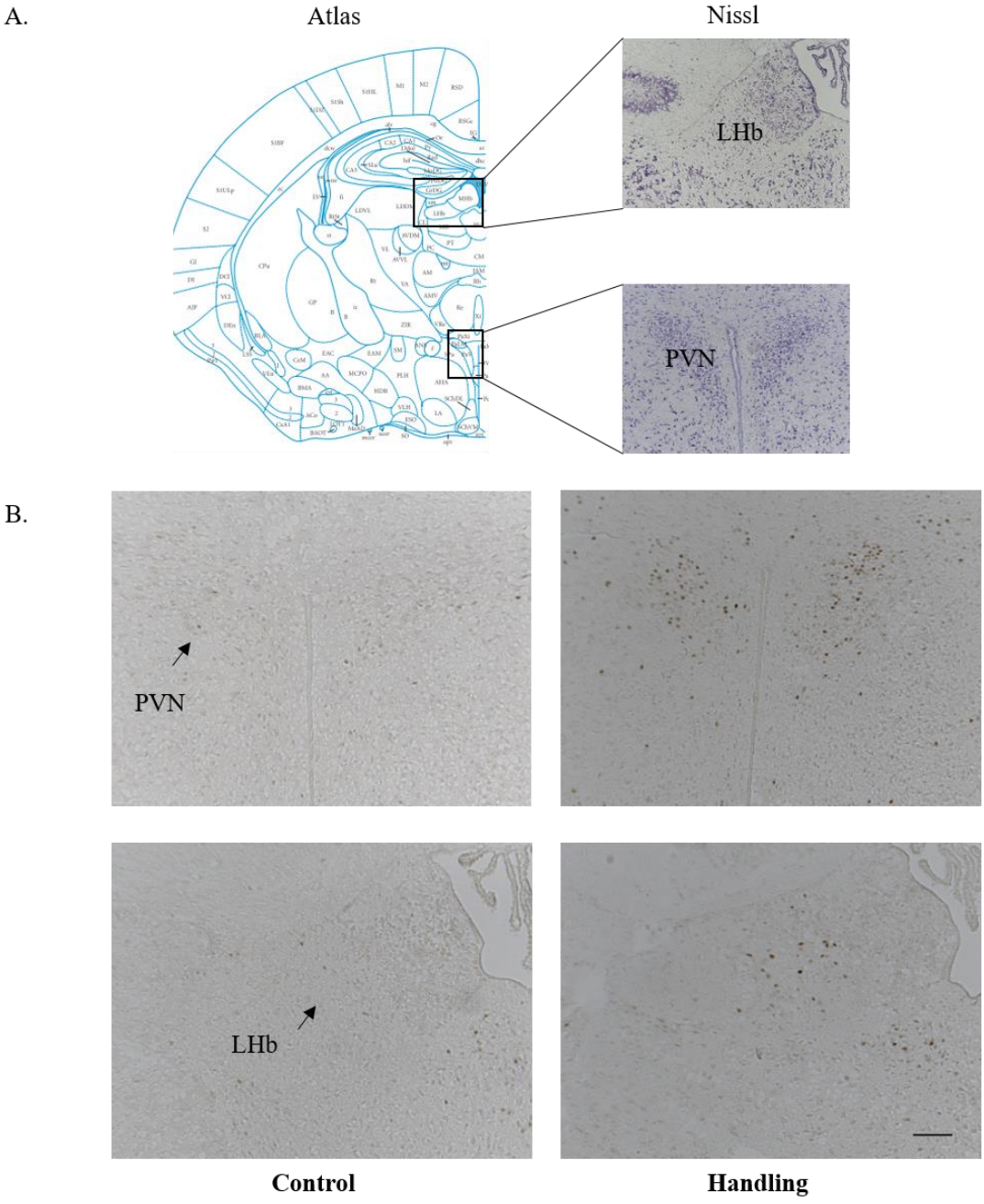


Fig. 9. Immunohistochemical images of c-Fos expression after postnatal handling.
Atlas of the developing mouse brain and Nissl stain of the lateral habenula (LHb) and hypothalamic paraventricular nucleus (PVN) (A). Representative Immunohistochemical images of c-Fos expression in the PVN and LHb of control and handling on P14 (B).
Scale bars = 100 μm.

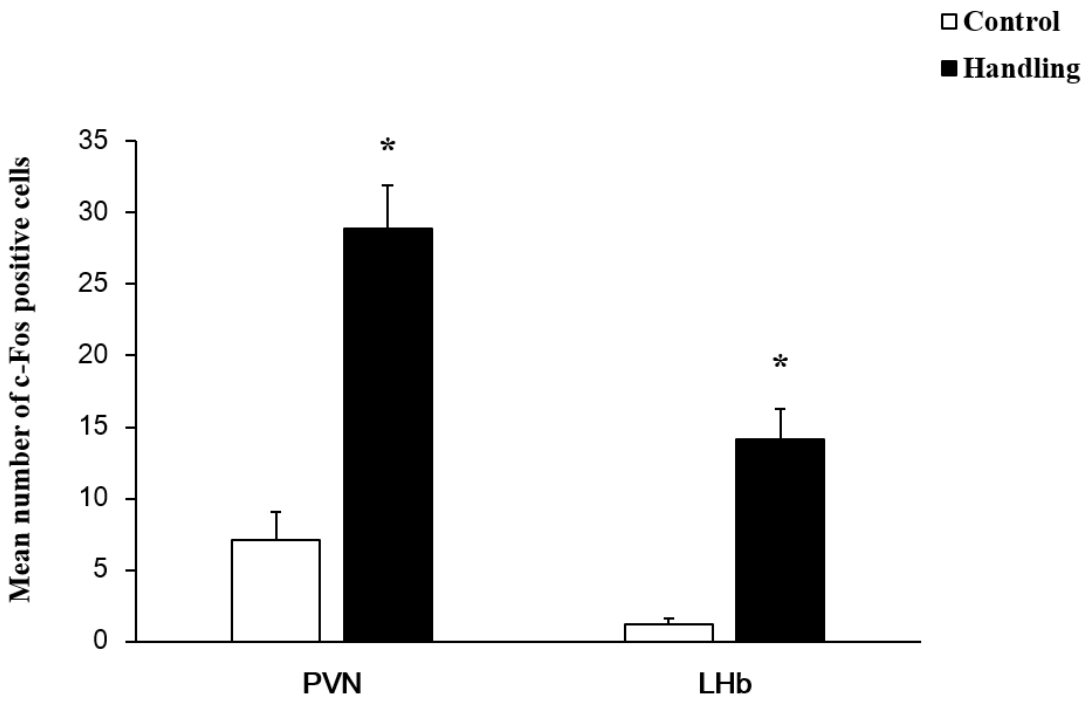


Fig. 10. c-Fos expression after postnatal handling.
c-Fos expression in the hypothalamic paraventricular nucleus (PVN) and the lateral habenula (LHb) on P14. Control, n=4~5; Handling, n=4. * $p < 0.001$.

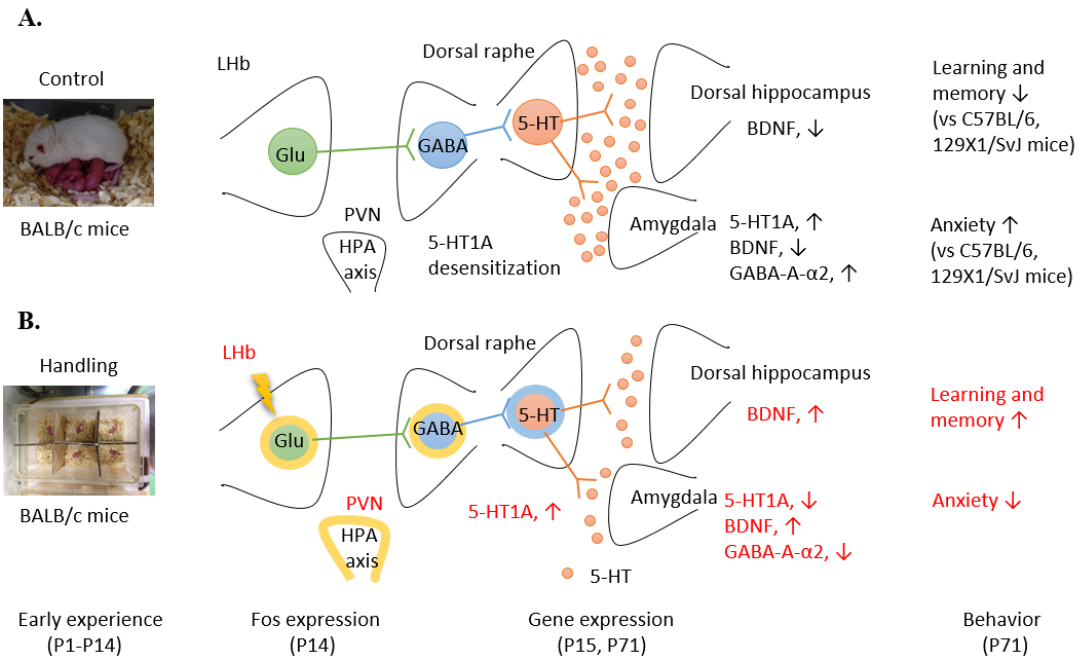


Fig. 11. Correlations between the effects of postnatal handling on brain and behavior.

Correlations between the effects of postnatal handling on Fos, gene expression and behavior during development and adulthood in the BALB/c mice. Control (A), Handling (B).

For the anatomical orientation of LHb and PVN, dorsal raphe, dorsal hippocampus and amygdala, please refer to Fig. 1 and Fig. 9.

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

Genes	Primer sequences	Tm (°C)	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

Table 2. Effects of postnatal handling on the behavior.

	Postnatal handling
Maternal behavior	=
Anxiety-like behavior	↓
Depression-like behavior	=
Spatial learning and memory	↑

↓, decreased, ↑, increased, =, no change compared to control

Table 3. Effects of postnatal handling on the brain gene expression.

	Postnatal handling		
	5-HT1A receptor (P15)	**BDNF (P71)**	**GABA-A receptor α2 (P71)**
Medial prefrontal cortex	=	=	=
Amygdala	↓	↑	↓
Dorsal hippocampus	=	↑	=
Ventral hippocampus	=	=	=
Dorsal raphe nucleus	↑		

↓, decreased, ↑, increased, =, no change compared to control

Table 4. Effects of postnatal handling on the brain fos expression.

	Postnatal handling
PVN	↑
LHb	↑

↑, increased compared to control