Regular research articles

Title: Maternal postpartum learned helplessness (LH) affects maternal care by dams and responses to the LH test in adolescent offspring

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Abstract

It is known that the early environment affects the mental development of rodent and human offspring. However, it is not known specifically whether a postpartum depressive state influences the depressive state in offspring. Using learned helplessness (LH) in rats as an animal model of depression, we examined the influence of maternal postpartum LH on responses to the LH test of offspring. Dam rats were judged as LH or non-helpless (nLH) on postnatal day (PN) 2 - 3, and maternal behavior was recorded during PN2 - 14. On PN 45 - 46, offspring were subjected to the LH test. Plasma corticosterone (CORT) levels, hippocampal levels of glucocorticoid receptor (GR) and brain-derived neurotrophic factor (BDNF) mRNA were measured before and after the LH test in offspring. Active nursing in LH dams was significantly lower than that in nLH dams. Susceptibility to LH in the offspring of LH dams was significantly higher than in those of nLH dams, and was negatively correlated with active nursing by LH dams. The GR mRNA levels before and after the LH test were lower in the offspring of LH dams than in those of nLH dams, and the reduced basal GR mRNA and protein might have resulted in the higher CORT response after the LH test. There was no significant difference in BDNF mRNA in the offspring of LH and nLH dams. These findings suggest that early postpartum LH decreased active nursing and increased depression-like behavior in the adolescent offspring via dysfunction of the hypothalamic-pituitary-adrenal axis.

Key words: learned helplessness; early postpartum period; maternal behavior; hypothalamic-pituitary- adrenal (HPA) axis; glucocorticoid receptor; corticosterone

Introduction

Numerous epidemiological studies demonstrated that childhood adversity, such as negative mother-child relationship, increased an individual risk of stress-related psychiatric disorders in later lives of the children (Canetti et al., 1997; McCauley et al., 1997; Repetti et al., 2002; Widom et al., 2007). Maternal mental disorders are considered to greatly influence mother-child relationships, and several human studies demonstrated that postpartum depression in mothers affected the mental development of their children (Lovejoy et al., 2000; Paulson et al., 2006; Righetti-Veltema et al., 2003). However, to our knowledge, there is only one series of studies demonstrating that maternal postpartum depression and subsequent maternal depression was associated with higher depressive symptoms and higher cortisol levels in adolescent offspring (Halligan et al., 2007a; Halligan et al., 2007b). In this context, although it is hypothesized that postpartum depression may enhance the susceptibility for depression in offspring via dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, the precise mechanism remains unclear. In rodents, there are very few studies examining whether the postpartum depressive state of mothers affects stress vulnerability of adolescent or adult offspring (Brummelte et al., 2006).

On the other hand, although the precise mechanisms of depression are not fully elucidated, neuroendocrinological studies postulate that the hyperactivity and inhibited negative feedback of the HPA axis are strongly involved in the pathophysiology of depression (Gold et al., 1984; Gormley et al., 1985; Nemeroff et al., 1984; Nestler et al., 2002a). Feedback regulation of the HPA axis in response to stress is mainly mediated through glucocorticoid receptors (GR) (De Kloet et al., 1998; Pariante and Miller, 2001), and it is thought that GR dysfunction in the central nervous system leads to the impaired negative feedback in depression (Manji et al., 2003; Neigh and Nemeroff, 2006; Nestler et al., 2002a). While GRs are widely distributed in the brain, they are also richly localized in the hippocampus (Joels, 2008; McEwen et al., 1986; Reul and de

Kloet, 1985), and it is postulated that hippocampal GR play an important role in the negative feedback regulation of the HPA axis (Herman et al., 2003; Jacobson and Sapolsky, 1991). In addition to the HPA axis, decreased levels of brain-derived neurotrophic factor (BDNF) in the hippocampus and frontal cortex also play an important role in the pathophysiology of depression (Altar, 1999; Chen et al., 2001; Duman and Monteggia, 2006; Nestler et al., 2002a; Nibuya et al., 1995; Shirayama et al., 2002).

It is also well known that early life experiences derived from the maternal environment induce long-lasting effects on neuroendocrinological and psychophysiological functions in rodent offspring. Several previous studies demonstrated that early adverse maternal environments, such as maternal separation or low levels of active maternal care, increased anxiety- or depression-like behavior, and diminished the negative feedback of the HPA axis (Caldji et al., 2000; Finamore and Port, 2000; Lippmann et al., 2007; Liu et al., 1997; Newport et al., 2002). Similarly, our laboratory showed that a decrease in active nursing in response to a prolonged dark phase increased anxiety-like behavior, and inhibited corticosterone (CORT) negative feedback in offspring later in life (Toki et al., 2007).

In this context, we used learned helplessness (LH) as an animal model of depression, and examined the influence of early postpartum maternal LH on the subsequent maternal behavior and on responses to the LH test in the adolescent offspring. LH was originally proposed by Overmier and Seligman (Overmier and Seligman, 1967), and its validity as an animal model of depression has been established (Malberg and Duman, 2003; Nestler et al., 2002b; Willner and Mitchell, 2002). We first grouped dam rats into learned helpless (LH) and non-learned helpless (nLH) dams, observed their maternal behavior, and examined the prevalence of LH in the adolescent offspring of LH and nLH dams. We then examined the levels of hippocampal GR and BDNF mRNAs, and plasma CORT before (baseline) and after the LH test in the adolescent

offspring of LH and nLH dams.

Materials and Methods

Animals

Pregnant female Sprague-Dawley rats were purchased from Charles River Japan (Yokohama, Japan). The pregnant rats were housed individually in standard polycarbonate cages (38 × 23 × 20 cm, SEOBiT, Japan) with sawdust bedding. The day of birth was counted as postnatal day 1 (PN1). Only litters with 11-15 pups were used, and there were no significant differences in mean litter size between nLH dams and LH dams. We adopted eye opening and weaning weight as parameters of physical growth (Toki et al., 2007). We observed the day of eye opening without touching the pups to minimize the effect of manipulation. Pups were weaned and weighed on PN21. After weaning, the male rats were housed in same litters 2-3 animals per cage (38 × 23 × 20 cm standard rat bracket cage, SEOBiT, Japan). All rats were provided with water and food *ad libitum*, and were maintained on a 12h/12h light-dark cycle (lights on at 08:00h) with constant room temperature (23 ± 2 °C) and humidity (60%). All animal procedures were conducted according to the Hiroshima University School of Medicine Animal care Committee Guiding Principles on Animal Experimentations in Research Facilities for Laboratory Animal Science, and in accordance with the 'Guidelines for Proper Conduct of Animal Experiments' given by the Science Council of Japan, and the 'Occupational Health and Safety in Care and Use of Research Animals' given by the U.S. National Research Council. All experimental protocols were approved by the Hiroshima University Animal Care and Use Committee.

Experimental schedule

We first examined the influence of LH in dams on their maternal behavior, and on the

anxiety-like and depression-like behaviors in their adolescent offspring (Experiment 1). We then measured the HPA axis function and the levels of hippocampal BDNF mRNA of the adolescent offspring (Experiment 2). All behavioral experiments were performed between 9:00 and 12:00. Less than three rats from the same litter were used for each experiment to exclude litter effects.

In Experiment 1, 79 dams were subjected to the LH test on PN3 and PN4, and grouped into 30 nLH, 14 LH and 35 intermediate dams according to their responses. Intermediate dams were not used. We randomly selected dams from each group (nLH dams n = 8; LH dams n = 8), and video recorded their maternal behavior from PN2 to PN14. The 240 male offspring were grouped into 158 offspring from nLH dams (nLHd-o) and 82 from LH dams (LHd-o) by their dam's response to the LH test. Offspring were randomly selected from each group and subjected to the open field test (nLHd-o; n = 8 , LHd-o; n = 8) and elevated plus maze (nLHd-o; n = 8 , LHd-o; n = 8) on PN45, and to the LH test (nLHd-o; n = 24 , LHd-o; n = 24) on PN45 - 46. Different sets of rats were used for these behavioral experiments. Those subjected to the LH test were the offspring of dams whose maternal behavior had been observed.

In Experiment 2, 63 dams were subjected to the LH test on PN3 and PN4, and grouped into 22 nLH dams, 15 LH dams, and 26 intermediate dams as in Experiment1. Intermediate dams were not used. The 199 male offspring were grouped into 108 nLH-o and 91 LHd-o as in Experiment 1. In the first part of Experiment 2 (Experiment 2A), offspring were randomly selected from each group and decapitated without behavioral experiments on PN45. Either blood samples or hippocampi were collected from each rat. Different sets of rats were used for the measurement of plasma CORT levels (n = 8 per group), real-time quantitative polymerase chain reaction (RT-PCR) (n = 8 per group), and western blotting (n = 5 per group).

In the second part of Experiment 2 (Experiment 2B), 83 nLHd-o and 67 LHd-o offspring were subjected to the LH test on PN45 and PN46, and divided into four groups according to their

results on the LH test, and those of their respective dam: 51 non-learned helpless offspring of non-learned helpless dams (nLHd-nLHo); 19 learned helpless offspring of non-learned helpless dams (nLHd-LHo); 15 non-learned helpless offspring of learned helpless dams (LHd-nLHo); 41 learned helpless offspring of learned helpless dams (LHd-LHo). The 24 offspring determined to be intermediate according to their response to the LH test were not used. The offspring randomly selected from each group were decapitated on PN46 immediately after the LH test. Blood samples and dissected hippocampi were used for the measurement of plasma CORT levels (n = 6 per group) and RT-PCR (n = 6 per group). Different sets of rats were used for the measurement of plasma CORT levels and RT-PCR.

Observation of maternal behavior

We observed maternal behavior using the procedure described by Myers *et al* (Myers et al., 1989) as in our previous study (Toki et al., 2007). The colonies were continuously video-recorded from PN2 to PN14. Small infrared cameras (1 per cage) with adjustable lenses were mounted on the standard laboratory rack to face the cages. Maternal behavior was observed during eight periods/day for 1 h every 3 h, starting at 9:00 (9:00, 12:00, 15:00, 18:00, 21:00, 24:00, 3:00, and 6:00). On days PN3 and PN4, the first point of maternal behavior observation for those dams undergoing the LH test, was set between 9:30 and 10:00, after the LH test; other observational points were set as usual. The behavior was scored every 3-min for 1 h (20 observations / h), giving a total of 20 × 8 = 160 observations/day per dam. All scoring was performed blindly by well-trained individuals. The following behaviors were scored: 1) off pups, 2) licking/grooming (LG), 3) arched-back posture nursing (ABN), 4) "blanket" posture nursing, in which the mother lays over the pups, 5) passive posture nursing, in which the mother is lying either on her back or side while the pups nurse. The data were analyzed as the percentage of observations in which

dams were engaged in each behavior. “LG” and “ABN” were combined into a single category “licking/grooming and arched-back nursing (LG-ABN)” as the parameter of active nursing as in a previous study (Champagne et al., 2003). We calculated the percentage of the daily scores of LG-ABN (%LG-ABN) to total daily observations.

LH test

The LH test was performed as previously described by Edwards *et al* (Edwards et al., 1986) over two days (dams on PN3 - 4 and the offspring on PN45 - 46). While the dams underwent the test, the pups remained in their home cages, and they were reunited after each session.

The test consisted of an inescapable shock session (IS-session) on day 1 and an avoidant test session (AT-session) on day 2. The experimental room was dimly lit, and the rats were habituated to the room for at least 20 min prior to each session. The test apparatus consisted of the experimental chamber (50 × 28 × 32.5 cm) with a stainless grid floor connected to a shock generator-scrambler (SGS-003,: Muromachi, Tokyo, Japan). During the AT-session, a lever was mounted 5 cm above the grid floor on one wall of each chamber. In the IS-session on day 1, each rat was placed in the chamber and exposed to 80 inescapable foot-shocks (single shock intensity: 0.8 mA, each duration: 15 s, without a light signal, interval: 10-20 s, total time: 40 min) from the electrified grid floor. After the IS-session, rats were returned to their home cage. The AT-session was performed 24 h later, on day 2. In the AT-session, each rat was placed in the same chamber as during the IS-session, and exposed to 15 foot-shock trials (single shock intensity: 0.8 mA, each duration: 60 s, inter trial time: 24 s). The current was accompanied by a light signal placed above the lever as a clue for detecting the lever and for discriminating the AT-session from the IS-session (Edwards et al., 1986; Vollmayr and Henn, 2001). Each foot-shock trial could be terminated by pressing the lever. If the escape latency, from foot-shock to lever press, was less

than 20 s, the trial was considered a ‘success’. If the escape latency was from 20 s to 60 s, the trial was considered a ‘failure’. Escape latencies were recorded automatically. Rats with more than 11 failures were classified as LH, and rats with less than 4 failures were classified as nLH. Rats with 5-10 failures were classified as ‘intermediate’.

Open field locomotor test

The open field locomotor test was conducted as previously described (Imanaka et al., 2008; Toki et al., 2007). The experimental room was dimly-lit, and the rats were habituated to the testing room for at least 20 min prior to the testing session. At the beginning of the open field test, each rat was placed in the center of the floor of cubic chamber (48 ×48 × 48 cm). The horizontal movements of each rat were measured from 0 to 5 min (locomotor activity in a novel environment), and from 5 to 10 min (locomotor activity after habituation) by automatic actography (SCANET MV-10; Melquest, Toyama, Japan). Photo beam sensors were placed in the chamber at intervals of 6 mm, and the horizontal movements were estimated as the number of interruptions of the near infrared rays. After each session, the chamber was cleaned with 70% alcohol.

Elevated plus maze

The elevated plus maze test was conducted as previously described (Imanaka et al., 2008; Toki et al., 2007) . The plus maze consisted of two open arms without walls (50 × 10 cm) and two closed arms with high plastic walls (50 ×10 × 38 cm). Each arm was arranged perpendicularly to its neighbor and placed 73 cm above the floor. The experimental room was dimly-lit, and the rats were habituated to the testing room for at least 20 min prior to testing. At the beginning of each trial, the rat was placed in the center of the apparatus facing a closed arm. During the 5 min

exposure to the maze, the number of entries and the time spent in each arm were video recorded, and the recordings were analyzed blindly by well-trained individuals. An entry was defined as the placing of two forepaws into the arm. The percentage of time spent on the open arms verses the total time spent in all arms (% open arm time) and the percentage of open arm entries to total arm entries (% open arm entries) were calculated for examining anxiety, and mean closed arm entries were calculated for examining activity as previously described (Rodgers and Dalvi, 1997; Stern et al., 2008; Toki et al., 2007).

Measurement of plasma CORT levels

The offspring without behavioral tests (Experiment 2A) were decapitated on PN45 and those submitted to the LH test on PN46 (Experiment 2B) were decapitated immediately after the test. Blood samples were collected from their cervical vein between 10:00 and 12:00 am. After centrifugation (500 × g at 4°C, for 30 min), plasma samples were frozen and stored at -70°C. The plasma CORT levels were determined using the rat corticosterone [^{125}I] assay system (Amersham Pharmacia Biotech).

Real-Time Quantitative Polymerase Chain Reaction (RT-PCR)

To determine whether the HPA axis and BDNF expression were involved in the susceptibility of the offspring to LH, we used RT-PCR to amplify and quantify relative levels of GR and BDNF mRNAs as previously described (Yamamoto et al., 2007). Hippocampus was removed from each brain and quickly frozen using liquid nitrogen, and stored at -80°C. Total RNA was extracted using RNAqueous™ Total RNA Isolation kits (Ambion, Austin, TX) according to the manufacturer's instructions, and then single-stranded cDNA was synthesized using the QuantiTect Reverse Transcription Kit (QIAGEN, Hilden, Germany), which provided a

procedure for genomic DNA elimination and reverse transcription. The primers and TaqMan hybridization probes were designed using Primer Express software (PE Applied Biosystems). Table 1 shows the sequences and fluorescent dyes of the PCR primers and TaqMan probes for each molecule. PCR was carried out with TaqMan Universal PCR Master Mix (PE Applied Biosystems). All standards and samples were assayed in triplicate. Thermal cycling was initiated with an initial denaturation at 50°C for 2 min and 95°C for 10 min. After this initial step, 40 cycles of PCR were performed. Each PCR cycle consisted of heating at 95°C for 15 s for melting and at 60°C for 1 min for annealing and extension. The PCR assay for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed for normalization using the TaqMan Rodent GAPDH Control Reagents kit (PE Applied Biosystems). The mRNA levels of GR and BDNF were detected by RT-PCR (ABI PRISM 7700 sequence detection system; PE Applied Biosystems, Foster City, CA), and the ratio of the concentration of the target molecule to that of GAPDH (target molecule/GAPDH) in unknown samples was calculated.

Western blotting

Immunoblot analyses for GR were performed as previously described (Aisa et al., 2007; Mantsch et al., 2007) with minor modification. Hippocampi were homogenized in homogenization buffer (20 mM Tris-HCl pH7.5, 5 mM EDTA, 2 mM DTT, 150 mM NaCl, 0.5 % Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 100 μM phenylmethylsulphonyl fluoride). Equal amounts of protein (50μg) for each rat were fractionated on 10% sodium dodecyl sulphate (SDS) gels (Atto, Tokyo, Japan), and transferred to a PVDF membrane using a semi-dry blotting apparatus (Bio-Rad). The blotted membrane was blocked at room temperature for 1h in TBS containing 8% non-fat dry milk and 0.05% Tween-20 (TBST- MLK), and then incubated with polyclonal antibody against GR (M-20, Santa Cruz Biotechnology, Inc., Santa Cruz, CA,

USA., 1:200) in TBST-MLK overnight at 4°C. The membrane was washed three times in TBST, and then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (Zymed, San Francisco, CA, USA, 1:2000) in TBST- MLK for 2h at room temperature. After washing in TBST, the blots were detected using the chromogenic detection kit of HRP (4CN PLUS, PerkinElmer, Wellesley, MA, USA). The blots were reprobed with anti-β-actin antibody (Sigma Chemical Co., St. Louis, MO, USA) to ensure equal protein loading. The densities of the immunoreactive bands were quantified with Atto Image analysis software (version 4.0 for Machintosh; Atto).

Statistical Analysis

All values represent means ± SEM. In Experiment 1, maternal behavior was analyzed by two-way analysis of variance (ANOVA: maternal LH, day) for repeated measures (day). Furthermore, we used the unpaired Student's *t*-test to evaluate the influence of maternal LH on each observational day. Physical growth and the results of behavioral experiments of the offspring from each group were compared by the unpaired Student's *t*-test. We used the Mann-Whitney U- test to examine whether maternal LH affected the response of the offspring to the LH test. The correlation between maternal behavior and the results of the LH test in the offspring was evaluated using Spearman's correlation-coefficient by rank test. In Experiment 2A, data for the levels of mRNA and plasma CORT were analyzed by the unpaired Student's *t*-test. In Experiment 2B, data for the levels of mRNA and plasma CORT were analyzed by two-way ANOVA (maternal LH, offspring's LH). The results were considered statistically significant at $p < 0.05$.

Results

Influence of postpartum LH in dams on their maternal behavior

Figure 1 depicts the mean daily %LG-ABN from PN2 - 14 for nLH and LH dam groups. Two-way repeated measures ANOVA showed a significant main effect of maternal LH ($F[1,14] = 16.00$, $p < 0.01$), day ($F[12,168] = 40.75$, $p < 0.01$), and a significant interaction between the maternal LH and day ($F[12,168] = 2.35$, $p < 0.01$). This finding indicated that whereas %LG-ABN gradually decreased over time in both groups, active nursing behavior was markedly affected in the LH dam group compared with that in the nLH dam group. On PN2, before the IS-session, there was no difference in %LG-ABN between the nLH and LH dam groups ($t[14] = -0.68$, $p = 0.51$, unpaired Student's *t*-test), indicating that basal active nursing did not differ between the two groups. After the IS-session on PN3, however, the LH dam group exhibited significantly lower %LG-ABN than did the nLH dam group; this significant difference persisted until PN12 with the exception of PN11(PN3: $t[14] = -3.14$, $p < 0.01$, PN4: $t[14] = -2.50$, $p < 0.05$, PN5: $t[14] = -3.91$; $p < 0.01$, PN6: $t[14] = -5.11$; $p < 0.01$, PN7: $t[14] = -4.88$; $p < 0.01$, PN8: $t[14] = -2.73$; $p < 0.05$, PN9: $t[14] = -2.68$; $p < 0.05$, PN10: $t[14] = -3.60$; $p < 0.01$, PN11: $t[14] = -1.50$; $p = 0.16$, PN12: $t[14] = -2.60$; $p < 0.05$, PN13: $t[14] = -2.16$; $p = 0.05$, PN14: $t[14] = -1.12$; $p = 0.28$).

Physical and neural development of offspring

The mean body weight of offspring of the nLH dams (nLHd-o) was 42.29 ± 0.99 g, and those of the LH dams (LHd-o) was 42.04 ± 0.94 g on PN21 (n = 24 per group). The mean eye opening day of the nLHd-o group was 15.47 ± 0.26 days, and that of the LHd-o group was 15.0 ± 0.24 days (n = 15, 5 litters per group). The unpaired Student's *t*-test showed no significant difference in either body weight ($t[46] = -0.18$, $p = 0.86$) or eye opening day ($t[28] = -1.33$, $p = 0.19$) between the two groups. These findings suggest that maternal postpartum LH did not affect the gross development of the offspring.

Influence of maternal postpartum LH on the behavior of the adolescent offspring

To examine basal locomotor activity in the nLHd-o and LHd-o groups, we investigated the levels of spontaneous locomotor activity using the open field test (n = 8 per group, Table 2). The unpaired Student's *t*-test showed no significant difference in the mean levels of basal locomotor activity between the nLHd-o and LHd-o groups during the first five minutes when the environment was novel (t[14] = 0.01, $p = 0.99$) or the during second five minutes when the rats had habituated to the novel environment (t[14] = 0.03, $p = 0.97$).

To examine anxiety-like behavior in a novel environment in the nLHd-o and LHd-o groups, we used the elevated plus maze to investigate the percentage of time spent in the open arms, the percentage of entries into open arms, and the mean numbers of entries into the closed arms (Table 2). Unpaired Student's *t*-tests showed no significant differences in any of these parameters between the two groups (% open arm time: t[14] = - 0.82; $p = 0.43$, % open arm entries: t[14] = - 0.70; $p = 0.50$, closed arm entries: t[14] = 1.38, $p = 0.19$). Together, these findings indicate that maternal postpartum LH did not affect basal locomotor activity or anxiety in the adolescent offspring.

Influence of maternal postpartum LH on the depression-like behavior (LH) of the adolescent offspring

To examine depression-like behavior in response to stress in the nLHd-o and LHd-o offsprings, we measured the number of escape failures from foot-shocks in the LH test. The forty-eight rats randomly selected from each group, whose dams had already been examined for maternal behavior, were subjected to the LH test. Figure 2A shows bar graphs of the numbers of failures made by offspring in the LH test. In the nLHd-o group, 15 offspring (62.5%) were

determined to be nLH, 5 (20.8%) were intermediate, and 4 (16.7%) were LH. In the LHd-o group, 4 offspring (16.7%) were determined to be nLH, 4 (16.7%) were intermediate, and 16 (66.7%) were LH. We compared the responses to the LH test (nLH, intermediate, LH) between the two groups, and the Mann-Whitney U- test showed a significant difference in the responses to the LH test between the nLHd-o and LHd-o groups ($p < 0.01$). In a comparison of the mean number of failures in each group (Figure 2B), an unpaired Student's *t*-test revealed that the LHd-o group exhibited significantly higher numbers of failures than the nLHd-o group ($t[46] = 4.50$, $p < 0.01$).

Furthermore, Figure 3 plots the relationship between the mean frequency of maternal active nursing from PN2 to PN14 and the number of failures in the LH test made by the offspring. In the LHd-o group (Figure 3A), the number of failures in the LH test was negatively correlated with the level of maternal active nursing by their LH dams (Spearman's rho = - 0.52, $p < 0.05$). However, in the nLHd-o group, such a significant correlation was not found (Spearman's rho = - 0.20; $p = 0.35$) (Figure 3B).

Influence of maternal postpartum LH on the HPA axis function in the adolescent offspring

To examine the influence of maternal postpartum LH on the HPA axis in the adolescent offspring, we investigated the levels of plasma CORT and hippocampal GR mRNA before (baseline) and after the LH test (Figures 4-5). The mean level of basal plasma CORT was 60.61 ± 8.25 ng/ml in the nLHd-o group, and 75.30 ±10.20 ng/ml in the LHd-o group (n = 8 per group). There was no significant difference between these values ($t[14] = 1.12$, $p = 0.28$, unpaired Student's *t*-test) (Figure 4 A). After the LH test, the mean level of plasma CORT was 340.05 ± 23.06 ng/ml in the nLHd-nLHo group, 337.03 ± 17.07 ng/ml in the nLHd-LHo, 389.67 ± 32.98 ng/ml in the LHd-nLHo, 451.88 ± 11.83 ng/ml in the LHd-LHo. (n = 6 per group). Two-way ANOVA demonstrated no significant interaction between maternal LH and

offspring LH on the levels of plasma CORT ($F[1,20] = 2.07$, $p = 0.17$) and no significant effect of offspring LH ($F[1,20] = 1.71$, $p = 0.21$). Meanwhile, there was a significant effect of maternal LH on plasma CORT levels ($F[1,20] = 13.18$, $p < 0.01$); the LHd-o group showed higher plasma CORT levels after the LH test than did the nLHd-o group (Figure 4 B).

The basal levels of hippocampal GR mRNA in the LHd-o group were significantly lower than in the nLHd-o group ($t[14] = -4.79$; $p < 0.01$, unpaired Student's *t*-test) (Figure 5A). After the LH test, two-way ANOVA demonstrated no significant interaction between maternal LH and offspring LH on the levels of hippocampal GR mRNA ($F[1,20] = 0.32$, $p = 0.58$), but there were significant effects of both maternal LH ($F[1,20] = 18.15$, $p < 0.01$) and offspring LH ($F[1,20] = 11.86$, $p < 0.01$) (Figure 5 B).

Because basal levels of GR mRNA were lower in the offspring from LH dams, we also examined whether maternal LH also affected the basal levels of hippocampal GR protein by performing anti-GR immunoblotting on hippocampus of adolescent offspring. Quantitative imaging of the western blots showed that, as with mRNA, the basal levels of hippocampal GR protein in the LHd-o group were also significantly lower than those in the nLHd-o group ($t[8] = -4.39$, $p < 0.01$, unpaired Student's *t*-test) (Figures 6 A - B).

Influence of maternal postpartum LH on BDNF mRNA expression in the adolescent offspring

To examine whether maternal postpartum LH affect BDNF in the offspring, we investigated the levels of hippocampal BDNF mRNA in the offspring of nLH and LH dams before (baseline, Figure 7A) and after the LH test (Figure 7B). There was no significant difference in the basal levels of hippocampal BDNF mRNA between the nLHd-o and LHd-o groups ($t[14] = -0.49$; $p = 0.63$, unpaired Student's *t*-test). After the LH test, two-way ANOVA demonstrated no significant

interaction between maternal LH and offspring LH on the levels of hippocampal BDNF mRNA ($F[1,20] = 0.03$, $p = 0.87$), and no significant effect of maternal LH ($F[1,20] = 0.41$, $p = 0.53$). There was, however, a significant effect of offspring LH ($F[1,20] = 8.29$, $p < 0.01$).

Discussion

Using LH in rats as an animal model of depression, we investigated the maternal behavior by LH dams and the influence of early postpartum maternal LH on the responses to the LH test, HPA axis function, and hippocampal BDNF expression in the adolescent offspring. We had four principal findings; (1) The LH dam group showed decreased active nursing compared to the nLH dam group. (2) In the adolescent offspring of LH dams, susceptibility to the LH test was enhanced compared to the offspring of the nLH dams, and there were negative correlations between the responses to the LH test in the offspring and the active nursing by their LH dams. (3) Maternal postpartum LH decreased the basal levels of hippocampal GR mRNA and GR protein in the adolescent offspring, but did not affect basal levels of plasma CORT or hippocampal BDNF mRNA. (4) Following the LH test in the adolescent offspring, maternal postpartum LH affected their levels of plasma CORT and hippocampal GR mRNA. Moreover, hippocampal GR mRNA levels were also affected by offspring LH.

Influence of maternal postpartum LH on maternal behavior and the response to the LH test in the adolescent offspring

Whereas there was no difference in the basal active nursing levels of the nLH and LH dams on PN2 before the IS-session of the LH test, the LH dams exhibited significantly decreased levels of active nursing than the nLH dams after the IS-session on PN3 that persisted for about ten days. Because it was reported that LH in the rat lasts for more than seven days after the IS-session

(Vollmayr et al., 2003), we speculated that maternal LH would last at least until PN10. Thus, it is conceivable that maternal LH led to the decreased maternal active nursing.

If one then looks at the adolescent offspring, we demonstrated that the susceptibility to LH test was significantly higher in offspring from LH dams than in those from nLH dams. Moreover, this difference did not derive from differences in physical and neural development, or in locomotor activity. It has also been reported that LH rats exhibited normal memory acquisition and retrieval (Vollmayr et al., 2004), thus, we might also rule out possible effects of learning deficits being involved in the increased susceptibility to LH in the LHd-o. Therefore, we consider postpartum maternal LH to be the major factor influencing the high prevalence of LH in the LHd-o. The results of our study also suggested that the effects of early postpartum maternal LH on the offspring were sustained at least until adolescence. These findings are consistent with those of previous studies, showing that early maternal environment, especially until PN14, affects the stress reactivity of offspring, which persists until adolescence or adulthood (Newport et al., 2002; Pryce and Feldon, 2003). In this context, we suggest that maternal LH especially during the early postpartum period was an important risk factor for LH in the adolescent offspring.

We also demonstrated negative correlation between maternal active nursing by the LH dams and the susceptibility to LH of the LHd-o. Several previous studies also reported that the levels of maternal active nursing were negatively correlated with stress reactivity in the offspring(Caldji et al., 2003; Cameron et al., 2005; Liu et al., 1997), and cross-fostering studies demonstrated that there was nongenomic transmission of stress reactivity induced by maternal behavior (Francis et al., 1999; Szyf et al., 2005), even in the offspring of a genetic stress-vulnerable strain (Friedman et al., 2006; Priebe et al., 2005). Accordingly, although it cannot be ruled out that congenital factors were involved in the increased LH susceptibility in the LHd-o group to some extent, the present findings indicate the possibility that an adverse effect of the maternal postpartum LH,

such as less active maternal care, plays a role in the increased prevalence of LH in these adolescent offspring.

Influence of maternal postpartum LH on the HPA axis function in the adolescent offspring

The LHd-o group showed significantly lower levels of basal hippocampal GR mRNA and GR protein than the nLHd-o group, and no differences in the basal levels of plasma CORT. However, following the LH test, the adolescent offspring of LH dams showed higher levels of plasma CORT along with the significantly decreased levels of hippocampal GR mRNA, compared to the offspring of nLH dams. Thus, it is conceivable that maternal postpartum LH reduces basal hippocampal GR levels in the adolescent offspring, and subsequently leads to a diminished negative feedback of the HPA axis in response to stress.

A previous study demonstrated that chronic antagonism of hippocampal GR increased vulnerability to LH (Papolos et al., 1993). Another study reported that GR heterozygous mutant mice showed increased levels of plasma CORT after stress exposure, and high susceptibility to the LH test (Ridder et al., 2005). Thus, it is likely that this dysregulation of the negative feedback of the HPA axis is associated with the high prevalence of LH in rats. Taking our results with together these previous findings, we postulate that maternal postpartum LH led to the dysregulation of the negative feedback of the HPA axis through basal hippocampal GR expression, and this dysregulation was subsequently involved in the increased susceptibility to the LH test in the adolescent offspring.

In agreement with this, several rodent studies demonstrated that an early adverse maternal environment, such as maternal separation or low levels of maternal active nursing, leads to increased stress vulnerability in adult offspring along with dysfunction of the negative feedback of the HPA axis (Francis et al., 1999; Liu et al., 1997; Macri et al., 2004). It is noteworthy that

the maternal influence on the HPA axis in the offspring persists throughout their life with epigenetic changes at the hippocampal GR gene, such as the enhancement of DNA methylation or the reduction in histone acetylation (Fish et al., 2004; Weaver et al., 2004). We did not examine the distinct mechanism by which maternal postpartum LH dysregulated the HPA axis function in offspring. However, as the results of our study showed that maternal LH decreased maternal active nursing, which was negatively correlated to LH in the offspring, it is plausible that this decreased maternal active nursing may, at least in part, contribute to the HPA axis disturbance in adolescent offspring.

Influence of maternal postpartum LH on hippocampal BDNF mRNA expression in the adolescent offspring

Although hippocampal BDNF has been thought to be associated with the pathophysiology of depression, maternal LH did not affect the basal levels of hippocampal BDNF mRNA in the adolescent offspring. However, LH in the offspring themselves significantly decreased the hippocampal levels of BDNF mRNA, regardless of maternal LH. This finding is consistent with previous studies, in which hippocampal BDNF was decreased in LH animals (Itoh et al., 2004; Shirayama et al., 2002). Taken together, these results suggest that BDNF might not be closely involved in the mechanism by which postpartum maternal LH increased the prevalence of LH in the offspring.

In summary, the results of the present study demonstrated that maternal LH induced during early postpartum period increased the susceptibility to LH in the adolescent offspring that was negatively correlated to maternal active nursing. Additionally, our results demonstrated that the adolescent offspring of LH dams exhibited a dysfunction of the negative feedback system in the

HPA axis, suggesting that this dysfunction was, in part, involved in their increased susceptibility to LH. Hence, maternal postpartum LH might be one of the important adverse factors leading to dysfunction of the negative feedback of the HPA axis and thereby, enhanced susceptibility to LH in the adolescent offspring. Further studies examining the direct influence of maternal postpartum LH on responses to the LH test in offspring, such as a cross-fostering study, are needed for better understanding of how maternal LH leads to HPA axis dysfunction and a high prevalence of LH in the offspring.

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Figure legend

Figure 1. Influence of maternal postpartum LH on maternal behavior. The daily percentages of observations containing active nursing (% LG-ABN) by the non-learned helpless dams (nLH dam, n=8) and learned helpless dams (LH dam, n=8) are shown. Data are expressed as means ± SEM. The inescapable shock session (IS) occurred on PN3, the first day of the LH test. On days PN3 and PN4, the first point of maternal behavior observation for those dams undergoing the LH test, was set between 9:30 and 10:00, after the LH test; other observational points were set as usual.

* $p < 0.05$, ** $p < 0.01$; Unpaired Student's *t*-test.

Figure 2. Influence of maternal postpartum LH on the susceptibility to the LH test in the adolescent offspring. The LH test was performed in the offspring of dams whose maternal behavior had been observed. **(A)** A bar graph of 'failures' made in the LH test by the offspring of the non-learned helpless dams (nLHd-o, opened bar, n=24) and the offspring of the learned helpless dams (LHd-o, closed bar, n=24). Less than four failures are considered to non-learned helpless (nLH) and more than 11 to represent learned helpless (LH) rats. There was a significant difference in the responses to the LH test (i.e., whether they were nLH, intermediate, or LH) between the offspring in the nLHd-o and LHd-o groups. There was a higher prevalence of LH in the LHd-o group than in the nLHd-o group (Mann- Whitney U- test, $p < 0.01$) **(B)** A bar graph of

the mean number of failures made in the LH test by the nLHd-o and LHd-o groups (n = 24 per group). Data are expressed as means ± SEM. The LHd-o group exhibited significantly higher numbers of failures than the nLHd-o group. ** $p < 0.01$; Unpaired Student's *t*-test.

Figure 3. Influence of maternal behavior on LH in the adolescent offspring. **(A-B)** Scattergrams of the frequency of maternal active nursing (% LG-ABN) during PN2 to PN14 versus failures made in the LH test by the LHd-o (A) and the nLHd-o (B) groups of offspring (n = 24 per group). * $p < 0.05$; Spearman's correlation coefficient by rank test

Figure 4. Influence of maternal postpartum LH on the levels of plasma corticosterone (CORT) in the adolescent offspring. Data are expressed as means ± SEM. **(A)** Bar graph showing the basal levels of plasma CORT in the nLHd-o and LHd-o groups (n = 8 per group). **(B)** Levels of plasma CORT in the adolescent offspring group after the LH test. Data are from the non-learned helpless offspring of non-learned helpless dams (nLHd-nLHo), learned helpless offspring of non-learned helpless dams (nLHd-LHo), non-learned helpless offspring of learned helpless dams (LHd-nLHo), and learned helpless offspring of learned helpless dams (LHd-LHo) groups (n = 6 per group). Two-way ANOVA showed a significant effect of maternal LH on plasma CORT levels (1). ** $p < 0.01$; Two-way ANOVA.

Figure 5. Influence of maternal postpartum LH on the levels of hippocampal GR mRNA expression in the adolescent offspring. Data represent the ratios of GR mRNA to that of GAPDH and are expressed as means ± SEM. **(A)** Basal levels of hippocampal GR mRNA in the LHd-o and nLHd-o groups (n = 8 per group). ** $p < 0.01$; Unpaired Student's *t*-test. **(B)** Levels of hippocampal GR mRNA in the adolescent offspring after the LH test. Data are from nLHd-nLHo, nLHd-LHo, LHd-nLHo, and LHd-LHo groups same as Figure 4 B (n = 6 per group). Two-way ANOVA showed significant effects of maternal LH (1) and offspring LH (2) on the levels of hippocampal GR mRNA. ** $p < 0.01$; Two-way ANOVA.

Figure 6. Influence of maternal postpartum LH on the basal levels of hippocampal GR protein in the adolescent offspring. **(A)** Representative immunoblots for hippocampal GR and β-actin in the nLHd-o and LHd-o groups. **(B)** A quantitative comparison of the densities of basal hippocampal GR protein between the nLHd-o and LHd-o groups (n = 5 per group). Data represent the means ± SEM of densities of GR immunoreactive bands normalized by β-actin as the percentage of the nLHd-o groups. ** $p < 0.01$; Unpaired Student's *t*-test.

Figure 7. Influence of postpartum maternal LH on the levels of hippocampal BDNF mRNA

expression in the adolescent offspring. Data represent the ratio of BDNF mRNA to that of GAPDH, and are expressed as means ± SEM. **(A)** The basal levels of hippocampal BDNF mRNA in the nLHd-o and LHd-o groups (n = 8 per group). **(B)** The levels of hippocampal BDNF mRNA after the LH test in the offspring of nLH (nLHd-o) and LH (LHd-o) dams, grouped further according to their performance on the LH test similarly as Figure 4 B (n = 6 per group). Two-way ANOVA showed that there were significant effects of offspring LH (2), but no effect of maternal LH, on the levels of BDNF mRNA in the offspring. ** $p < 0.01$; Two-way ANOVA.

Table 1. primer and probe sequence for RT-PCR

Gene	Gene Bank and EMBL accession No.	sequences	
GR	Y 12264	Forward primer	5'- TTCGAAGGAAAAACTGCCCAG -3'
		Reverse primer	5'- CGAGCTTCAAGGTTCATTCCA -3'
		Taqman probe	5'- TGCCGCTATCGGAAATGTCTTCAGG -3'
BDNF	NM 012513	Forward primer	5'- CCATAAGGACGCGGACTTGT -3'
		Reverse primer	5'- GAGGCTCCAAAGGCACTTGA -3'
		Taqman probe	5'- CACTTCCCGGGTGATGCTCAGCA -3'

FAM was used as quencher dye, and TAMRA was used as reporter dye.
GR : glucocorticoid receptor , BDNF : brain-derived neurotrophic factor

Table 2. Results of open field test and elevated plus maze

		nLHd-o	LHd-o
OF	0 - 5min	2144.88±176.50	2147.38±230.50
	5 - 10min	800.75±232.04	812.25±265.29
EPM	Time in open arms (%)	26.37±3.58	22.13±3.72
	Open arm entries (%)	46.05±1.78	43.67±2.91
	Closed arm entries	7.50±1.15	9.50±0.89

OF: open field test, EPM: elevated plus maze test

Figure 1.

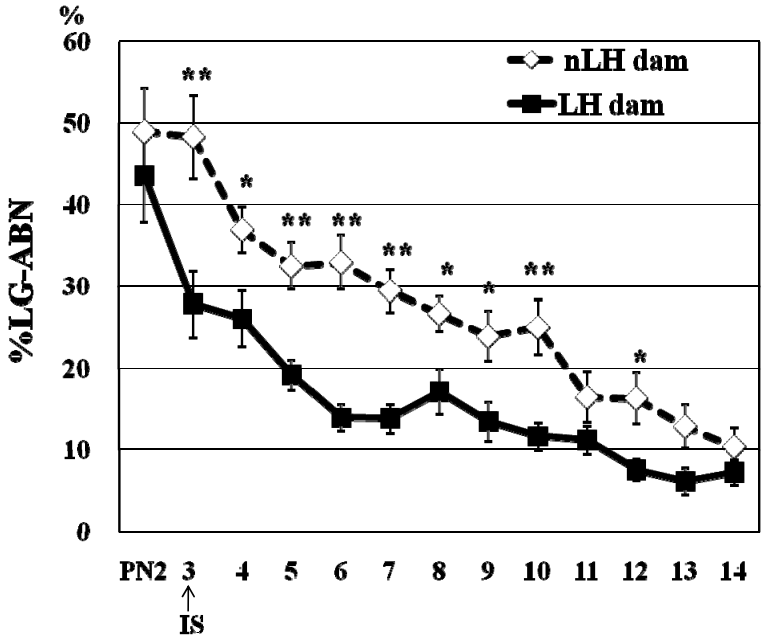

%
60
50
40
30
20
10
0
%LG-ABN
nLH dam
LH dam
**
*
**
**
**
*
*
**
*
PN2
3
4
5
6
7
8
9
10
11
12
13
14
IS

Figure 2.

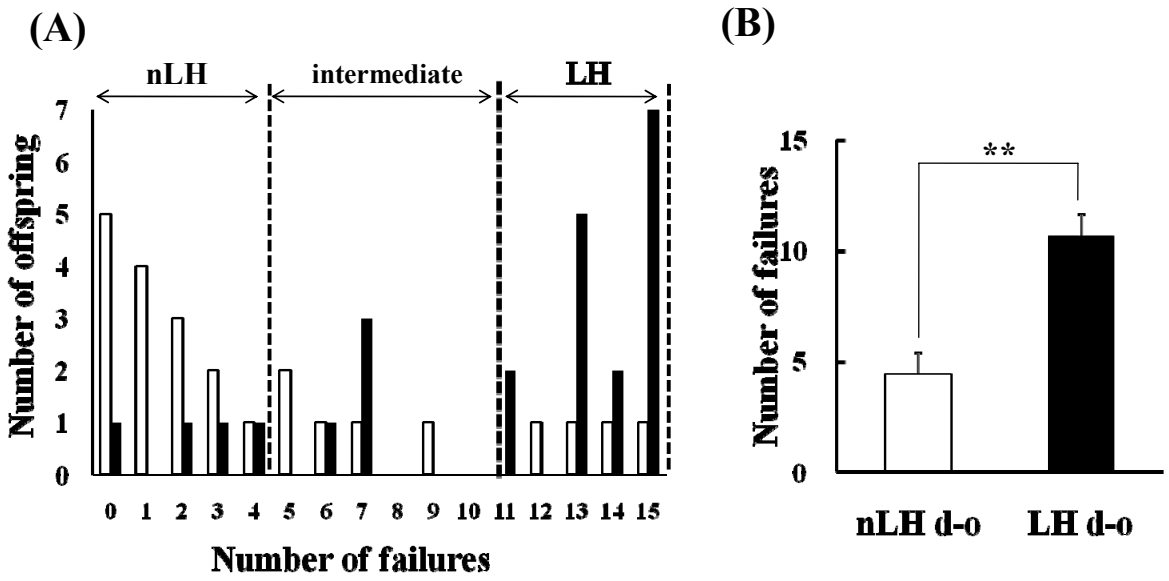

(A)
nLH
intermediate
LH
Number of offspring
0
1
2
3
4
5
6
7
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Number of failures
(B)
Number of failures
0
5
10
15
**
nLH d-o
LH d-o

Figure 3.

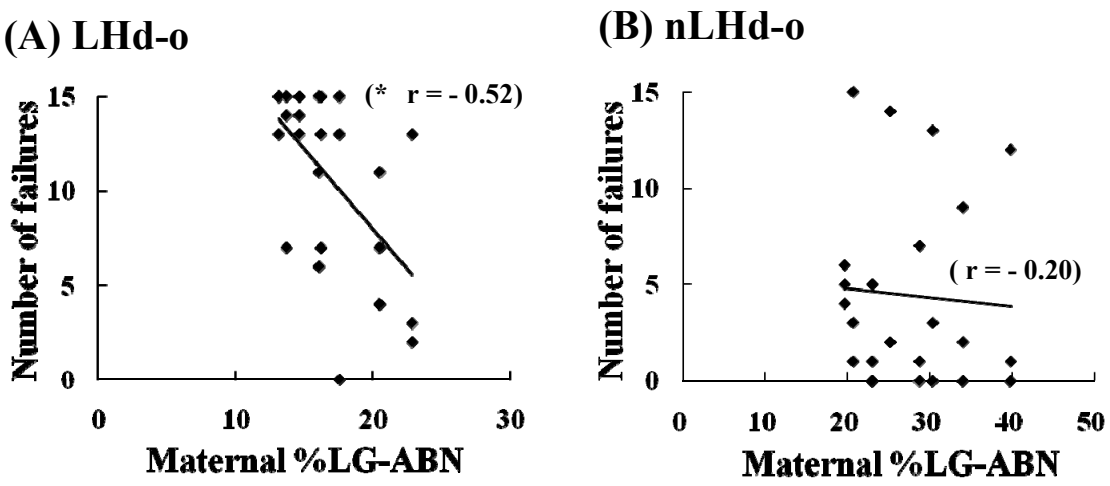

(A) LHd-o
(* r = - 0.52)
Number of failures
15
10
5
0
0
10
20
30
Maternal %LG-ABN
(B) nLHd-o
(r = - 0.20)
Number of failures
15
10
5
0
0
10
20
30
40
50
Maternal %LG-ABN

Figure 4.

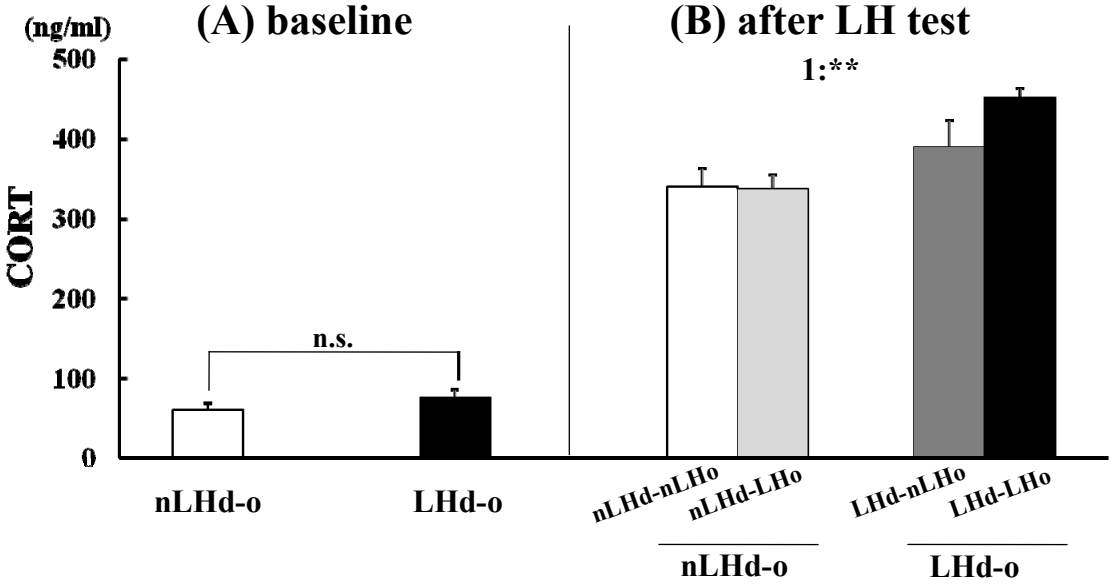

(ng/ml)
(A) baseline
(B) after LH test
1:**
500
400
300
200
100
0
CORT
n.s.
nLHd-o
LHd-o
nLHd-nLHo
nLHd-LHo
LHd-nLHo
LHd-LHo
nLHd-o
LHd-o

Figure 5.

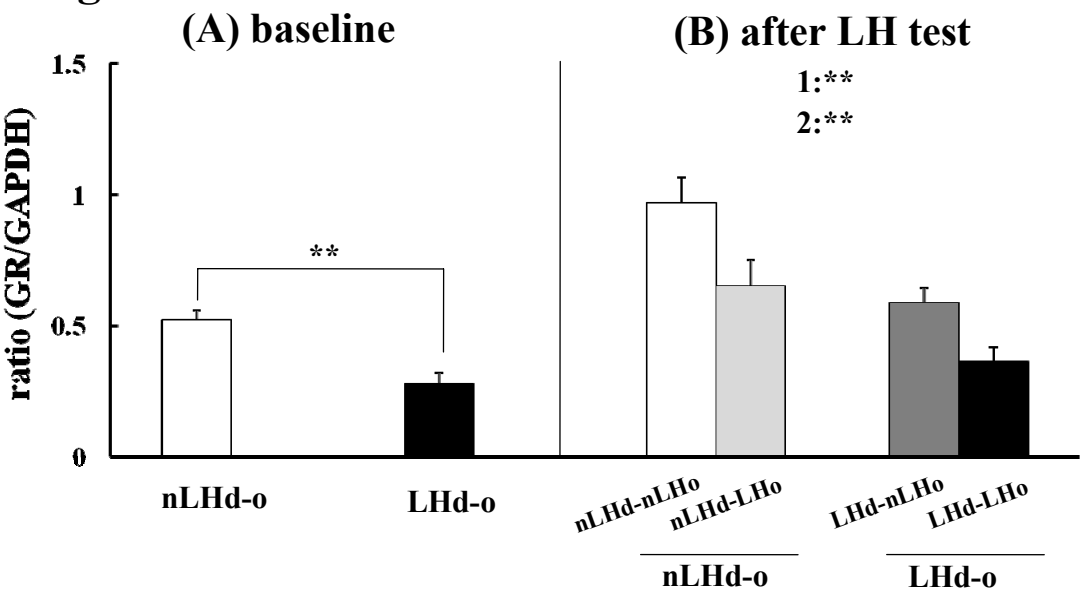

(A) baseline
(B) after LH test
1:**
2:**
ratio (GR/GAPDH)
1.5
1
0.5
0
**
nLHd-o
LHd-o
nLHd-nLHo
nLHd-LHo
LHd-nLHo
LHd-LHo
nLHd-o
LHd-o

Figure 6.

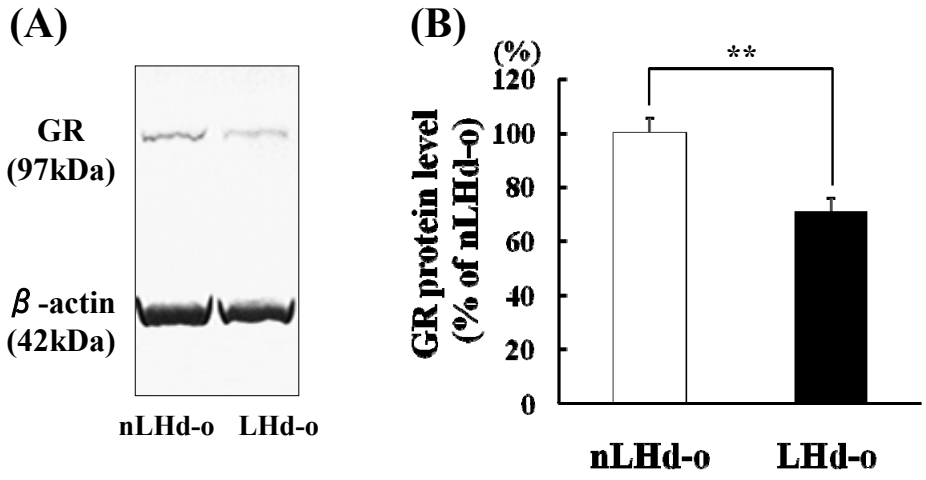
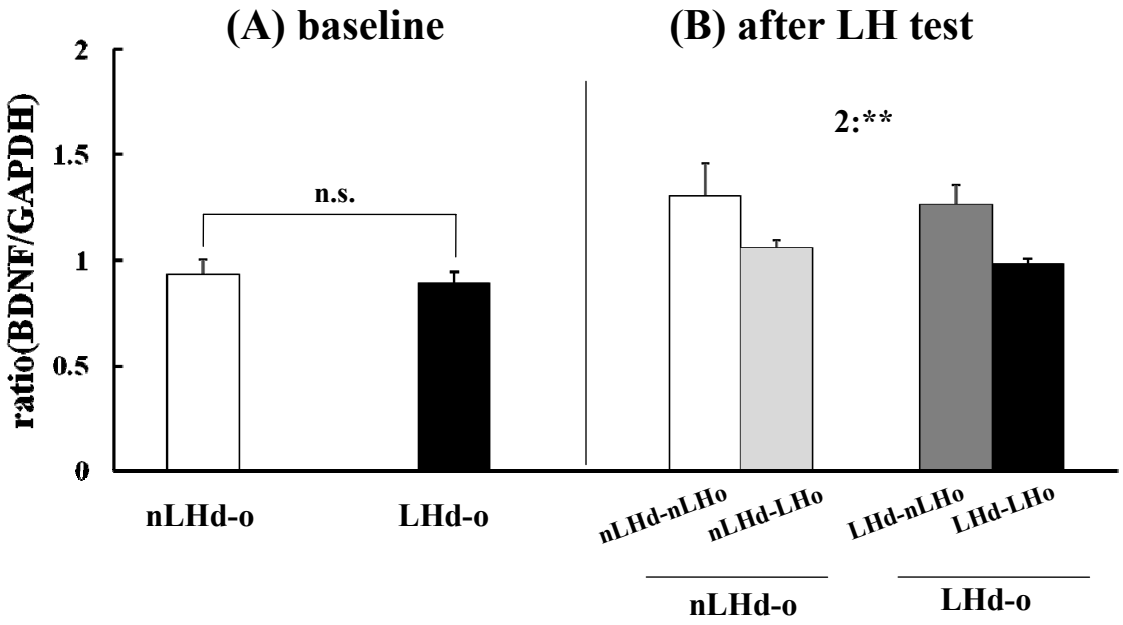

(A)
GR
(97kDa)
β -actin
(42kDa)
nLHd-o
LHd-o
(B)
(%)
120
100
80
60
40
20
0
**
GR protein level
(% of nLHd-o)
nLHd-o
LHd-o

Figure 7.


(A) baseline
(B) after LH test
ratio(BDNF/GAPDH)
2
1.5
1
0.5
0
n.s.
2:**
nLHd-o
LHd-o
nLHd-nLHo
nLHd-LHo
LHd-nLHo
LHd-LHo
nLHd-o
LHd-o