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Behavioral, hormonal, and neurochemical outcomes of neonatal repeated shaking brain injury in male adult rats

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ABSTRACT

It is well known that an abusive environment in childhood is related to individual anxiety behavior in adulthood. Though an imbalance of adrenocorticosteroid receptors and a dysfunction of monoaminergic neuron systems have been proposed, the underlying mechanisms are not fully understood. To address these problems, we recently developed a new model of shaking brain injury (SBI) in neonatal rats. These model rats showed transient microhemorrhages in the gray matter of the cerebral cortex and hippocampus. Using this model, we assessed the effects of neonatal repeated mild SBI on subsequent behavior and the stress response, and we further examined the possible contribution of adrenocorticosteroid receptors in the hippocampus and central monoaminergic neuron systems mediating such abnormalities. Behavioral screening examination with a novel open-field test showed that the rats with postnatal day (P) 3-7 shaking had significantly reduced locomotor activity and exploration behaviors than those with late (P8-14) shaking periods, indicating a critical period for neonatal SBI. In the elevated plus maze (EPM) and the light/dark transition (L/D) tests, the model rats spent less time in the open arm of the EPM and the light box of the L/D test, indicating anxiety-like behavior as adults. In adults, the novel EPM-induced adrenocorticotrophic hormone (ACTH) and corticosterone (CORT) responses were significantly increased by neonatal SBI. Further experiments showed that the expression of mineralocorticoid receptor (MR), but not glucocorticoid receptor (GR), was significantly downregulated in the hippocampus of this model rat. These results suggest that neonatal SBI-induced downregulation of MRs in the hippocampus attenuates negative feedback of the hypothalamic-pituitary-adrenal (HPA) axis, which results in abnormal secretion of ACTH and CORT. Furthermore, the neurochemical analysis showed that shaken rats

had higher dopamine (DA), serotonin (5-HT), 5-hydroxyindolacetic acid (5-HIAA), and noradrenaline (NA) levels in the dorsal part of the medial prefrontal cortex (dmPFC). In the amygdala, higher 5-HIAA and lower NA levels were observed. Both areas are known to be anxiety and stress-related. Taken together, the effects of neonatal SBI on the monoaminergic systems may also be involved in the changes of behavioral and hormonal responses in this model.

Keywords: Shaking brain injury, anxiety-like behavior, corticosterone, ACTH, adrenocorticosteroid receptors, monoamines

1. Introduction

Abusive experiences during childhood may be a major contributory factor to psychiatric disorders late in life [1-3]. Abusive head trauma (AHT), known as "shaken baby syndrome (SBS)", is characterized by an acute brain injury with focal or multifocal subdural, subarachnoid, and/or intracerebral hemorrhages associated with retinal hemorrhages and skull or other fractures of the skeletal bones [4]. Although AHT is the leading cause of fatal head injury in children under 2 years of age [5], the survivors show several physical disabilities and psychological problems, such as anxiety, depression, and posttraumatic stress disorder (PTSD) [3, 6]. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and dysfunction of monoaminergic neuron systems have been considered to be associated with these psychological changes [1, 2, 7-9]. The hippocampal glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) are known to play an important role in HPA axis regulation via their effects on glucocorticoid negative feedback [1, 2, 9]. Preclinical studies on AHT and SBS have been carried out extensively in several animal models [10, 11], among which rodent models provide useful information on the molecular and pathophysiological mechanisms underlying psychological changes following the insults [12]. As a new model of SBS, we recently made a device to induce shaking brain injury (SBI) in neonatal rats. Using this device, we have shown that neonatal repeated SBI leads to transient microhemorrhages (MHs) in the gray matter of the cerebral cortex and hippocampus. Furthermore, these rats are predisposed to anxiety as adults. [13].

The present study investigated the consequences of neonatal repeated mild SBI on behavioral, hormonal, and neurochemical changes in adult rats. First, to establish the appropriate mild SBI methods, neonatal rat pups were exposed to several shaking

conditions, changing in strength and frequency of shake and the period of exposure. At the adult stage (8 to 10 weeks of age, P8W-10W), these rats were screened by behavioral challenge based on spontaneous exploration of a novel environment using an open-field. Second, using the established protocols, the long-term effects of neonatal repeated mild SBI on adult rats were assessed. The adult rats were tested by elevated plus maze (EPM) and light/dark transition (L/D) tests, and plasma levels of adrenocorticotrophin (ACTH) and glucocorticoid (CORT) in response to novel EPM exposure were measured. Finally, to investigate the possible mechanisms of this SBI model, the expressions of GR and MR mRNAs in the hippocampus and monoamine concentrations in the related brain regions were examined by real time-polymerase chain reaction (RT-PCR) and high-performance liquid chromatography (HPLC), respectively.

2. Materials and methods

2.1. Animals

Timed pregnant female Sprague-Dawley (SD) rats were purchased from Japan Charles River Lab. Inc. (Tsukuba, Japan) and housed under controlled conditions of temperature (22 ± 2 ºC) and humidity (50-60%) and a regular 12-h light-dark cycle with ad libitum access to food and water. The date of birth was considered postnatal day 0 (P0). All experimental procedures received prior approval by the Animal Welfare Committee of Dokkyo Medical University School of Medicine and were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Throughout the experiments, the animals were exposed to only one behavioral test.

2.2. Screening experiment and histochemistry

The shaking apparatus has been described in our previous paper [13]. At the time of shaking, pups were anesthetized with isoflurane similar to another animal model of SBS and traumatic brain injury (TBI) [14].

In the first experiment, various shaking conditions were screened, as shown in Table 1. The first group (G1) pups were shaken for 180 seconds (s), then rested for 60 s, and this was repeated 5 times. G2 pups were shaken for 120 s, then rested for 60 s, and this was repeated 5 times. G3 pups were shaken for 60 s, then rested for 60 s, and this was repeated 5 times. G4 pups were shaken for 30 s, then rested for 60 s, and then repeated 5 times. G5 pups were shaken for 15 s, then rested for 60 s, and then repeated 5 times. Control groups (C) were only rested throughout the experimental period, C1, C2, C3, C4, and C5 corresponding to G1, G2, G3, G4, and G5, respectively. These shaking procedures were performed once a day through P3 to P14.

To evaluate the shaking periods and determine the critical periods, the different shaking periods, the first half (shaking period P3 to P7; S1) and the latter half (P8 to P14; S2), were further examined. After shaking, all pups were subsequently returned to their mothers during the lactation period, and then housed in cages of 2 to 3 animals after weaning until 8 to 10 weeks of age when they were analyzed.

For the initial evaluation, the novel open-field test was used for behavioral outcomes. The open-field apparatus and evaluation methods have been described elsewhere [15]. Briefly, P10W male rats from each group were exposed to the novel open-field for 15 min. The rats were placed at the center of the arena, and horizontal locomotion (number of total squares crossed; an indicator of locomotor activity) and the frequencies of

rearing (defined as the rat standing upright on its hind legs; an indicator of exploratory behavior) were recorded [16]. The experiments were performed from 9 am -11 am.

To confirm the presence of MHs, P3 and P7 rats of the S and C groups were deeply anesthetized with sodium pentobarbital and then transcardially perfused with physiological saline, followed by 4% paraformaldehyde in 0.1% phosphate-buffer (PB, pH 7.4). The sectioning and histochemical staining procedures have been reported previously [13].

2.3. Elevated plus maze (EPM) test

The EPM test assessed anxiety-like behaviors in the animals on P8W-10W of the G3 and C3 groups. The EPM was made of black-painted wood and consisted of two identical open arms (50 cm x 10 cm) and two identical closed arms (50 cm x 10 cm) with 70-cm high walls connected to an open center area (10 cm^2). The maze was elevated 70 cm above the floor. The rats were situated at the center of the maze facing one of the open arms, and they were allowed free exploration for 15 min. Their behaviors were monitored by a digital video camera above the maze for 15 min. As parameters of anxiety-like behavior, the time spent in the open and closed arms and transitions to each arm were calculated [17, 18]. The experiments were performed from 9 am -11 am.

2.4. Light/Dark transition (L/D) test

The apparatus consisted of two chambers (26 cm x 22 cm x 27 cm) joined together. One chamber was made with a black acrylic plate (dark chamber), and the other chamber was made with a transparent acrylic plate (light chamber), and between the

two chambers was a 10 cm x 27 cm gate that the rat could use to move between chambers (MELQUEST, Toyama, Japan). The light chamber was brightly illuminated by white diodes (390 lux), whereas the other chamber was dark (2 lux) [19]. On P8W-10W, the animals of the G3 (shaking) and C3 (control) groups were placed in the middle of the light chamber, facing 180 degrees away from the gate connecting the two chambers, and they were allowed to freely explore both chambers for 5 min. Their behaviors were monitored by a digital video camera above the chambers. As parameters of anxiety-like behavior, the total amount of time spent in the light chamber and transitions between two chambers were calculated [20]. The experiments were performed from 9 am -11 am.

2.5. Hormonal measurements

To examine the changes of ACTH and CORT levels with exposure to the EPM, 70 animals (experimental group n=35, control group n=35) were used. To examine the basal plasma ACTH and CORT levels, P10W rats from the shaken and control groups were decapitated without anesthesia before exposure to the novel EPM. Trunk blood was collected in a tube with heparin and centrifuged for the determination of plasma ACTH and CORT levels [21]. Animals were stressed by EPM for 15 min, as described previously. Animals were sacrificed by decapitation 5, 15, 60, and 180 min after starting EPM. ACTH and CORT were each measured by double-antibody RIAs using commercial kits of ^{125}I-labeled radioligands (ACTH kit (RK-001-21), Phoenix Pharmaceuticals, Burlingame, CA; and CORT kit (RPA 548), GE Healthcare Japan, Tokyo, Japan). The experiments were performed from 11 am -3 pm.

2.6. RNA extraction and real time-PCR assay

P10W shaken rats and age-matched control rats (n = 5-6 each) were used for the expression analyses of mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) mRNAs. Each animal was decapitated, and the hippocampus was dissected immediately. Tissues were snap-frozen and stored at -80°C until RNA extraction. Total RNA was isolated using TRIzol extraction (Life Technologies, Carlsbad, CA). Samples were treated with recombinant DNaseI (Takara Bio, Shiga, Japan) to remove any contaminating genomic DNA before reverse transcription into cDNA with ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan), according to the manufacturer's directions. Sequences of primers were as follows: *MR*, forward 5'-cctggcagcgaaacagat-3' and reverse 5'-tcctcgagaggcaagttttt-3' (78 bp; M36074.1); *GR*, forward 5'- ggccggtcagtgttttctaa-3' and reverse 5'- aggcagagcttgggaggt-3' (110 bp; M14053.1); and *GAPDH*, forward 5'-gccagcctcgtctcatagaca-3' and reverse 5'-tggtaaccaggcgtccgata-3' (75 bp; NM_017008). Quantitative PCR (qPCR) was performed using the ABI PRISM 7000 analyzer (Applied Biosystems, Tokyo, Japan) with THUNDERBIRD SYBR qPCR Mix (TOYOBO). The signal detection and analysis were run on each target sequence amplified during 40 cycles of PCR (denaturation at 95°C for 5 sec and annealing/extension at 58°C for 31 sec) according to the instruction manual. For relative quantification of gene expression, mRNA expressions were normalized to *GAPDH* mRNA expressions and compared with control rats using the comparative threshold cycle (delta-delta Ct) method. Relative quantification was calculated using the formula $2^{(-\text{delta-delta Ct})}$.

2.7. Monoamine measurements

Tissue preparation and monoamine measurement methods using high-performance liquid chromatography (HPLC) were also described previously [22]. Briefly, brains from shaken (n=7) and control (n=8) rats at age 10 weeks were immediately removed from the skull, washed with ice-cold 0.1 M PBS, and then sliced using a brain slicer at 2-mm thickness (Neuroscience Co., Tokyo, Japan). The slices containing areas of interest were collected. The dorsal part of the medial prefrontal cortex (dmPFC) including cingulate cortex area 1 and the dorsal half of the prelimbic cortex, the ventral part of the medial prefrontal cortex (vmPFC) including the ventral half of the prelimbic cortex and the infralimbic cortex (Bregma 4.5 - 2.5 mm), the amygdaloid complex (AMY) including the central nucleus, the basolateral amygdaloid nucleus, and the basomedial amygdaloid nucleus (Bregma -1.5 - -3.5 mm), and the whole hippocampus (HIP) including the dorsal and ventral hippocampus were dissected by punch-out apparatus [22] and scalpels (the Bregma level was according to the brain atlas) [23]. Tissue samples were rapidly frozen in liquid nitrogen, weighed and homogenized in 0.1 M perchloric acid containing 0.1 mM Na2-ethylenediamine tetraacetate, and then filtered. The homogenate was centrifuged at 15,000 rpm for 20 min. The supernatant was analyzed for serotonin (5-HT), 5-hydroxyindolacetic acid (5-HIAA), noradrenaline (NA), dopamine (DA), and dehydroxyphenylacetic acid (DOPAC) by HPLC with electrochemical detection (Shimaz Co., Kyoto, Japan).

2.8. Statistical analysis

Data are presented as means ± standard error of the mean (SEM). All statistical measurements were carried out using Stat View (Abacus Concepts, Inc., Berkeley, CA) or GraphPad Prism (GraphPad Software, Inc. La Jolla, CA). For the open-field test,

differences between groups were evaluated using analysis of variance (ANOVA) followed by the Bonferroni/Dunn test. For the EPM and L/D tests and the PCR and HPLC analyses, significance was determined by Student's *t*-test. For the hormone measurements, significance was determined by two-way ANOVA followed by Tukey's post hoc test. A value of $p<0.05$ was considered significant.

3. Results

3.1. Screening experiment and histochemical results

The effects of each shaking procedure (G1-5 and C1-5) on the activities of 10-week-old rats were studied in the novel open-field. Two-way ANOVA showed a significant group effect (shaken vs. control) in both line crossing ($F_{(1, 79)} = 440.8$, $P < 0.0001$) and rearing ($F_{(1, 79)} = 72.0$, $P < 0.0001$). Neonatal mild SBI significantly decreased open-field activity compare to without shaking (control). There was no significant time effect in line crossing ($F_{(4, 79)} = 1.29$, $P = 0.22$) and rearing ($F_{(4, 79)} = 0.78$, $P = 0.54$), and no significant interaction (group x time) effect in line crossing ($F_{(4, 79)} = 0.6$, $P = 0.66$) and rearing ($F_{(4, 79)} = 0.8$, $P = 0.53$). The results of activity in the novel open-field are shown in Fig. 1A, B. The procedure of G3 (shaking 1 min., resting 1 min., 5 times) was selected as an appropriate experimental protocol, and the C3 procedure was the control [13].

Next, the different shaking periods, early shaking period (S1) and late shaking period (S2), were examined, and C3 and G3 were compared. One-way ANOVA showed significant group differences in line crossing ($F_{(3, 62)} = 134.5$, $P < 0.0001$) and rearing ($F_{(3, 62)} = 36.9$, $P < 0.0001$). The differences between the groups are shown in Fig. 1C, D. These data demonstrated that the early shaking period (P3-7) with the G3 protocol was

enough to change adult behavior. After this, all experimental procedures used G3 (shaken group) and C3 (control group).

Similar to our previous report [13], MHs were observed in the gray matter of the hippocampus (Fig. 2A-C) and the prefrontal (Fig. 2D), parietal, temporal cingulate, and insular cortices of P3 - P7 in S group rats. However, there were no MHs in the amygdaloid complex of S group rats (Fig. 2E). In control rats, MHs were not detected.

3.2. EPM test

The results of the EPM test are presented in Fig. 3. Time spent in the closed-arm was significantly higher in the shaken group (766.1 ± 15.2 s) than in the control group (639.4 ± 29.7 s) ($t_{(23)}$=4.2, P < 0.001, Fig. 3A), whereas time spent in the open-arm (duration) and the ratio of open-arm time (% of total time) were significantly reduced in the shaken group (133.9 ± 15.2 s; 14.9% ± 1.7%, respectively) compared with the control group (260.6 ± 29.7 s; 30.0% ± 3.3%, respectively) ($t_{(23)}$=4.2, P < 0.001, $t_{(23)}$=4.2, P < 0.001, respectively) (Fig. 3B,C). Significantly fewer open-arm entries and a significantly lower ratio of open-arm entries were observed in the shaken group (8.7 ± 0.9, 31.1%± 0.2%, respectively) than in the control group (15.9 ± 0.9, 44.6% ± 2.3%, respectively) ($t_{(23)}$=4.5, P < 0.001, $t_{(23)}$=2.9, P < 0.001, respectively) (Fig. 3E, F). No significant difference was observed in closed-arm entries between the shaken (17.8 ± 1.2) and control (19.3 ±1.3) ($t_{(23)}$=0.7, P = 0.46) groups (Fig.3D).

3.3. L/D test

The results of the L/D test are presented in Fig. 4. The shaken group (17.4% ± 2.3%) spent less time in the light box than the control group (27.5% ± 2.1%) ($t_{(23)}$=3.15 ,

$P < 0.01$). Additionally, the number of times (frequencies) the shaken group (5.8 ± 0.9) transitioned between the two sides of the light-dark box was significantly less than that of the control group (10.5 ± 1.0) ($t_{(23)}$=3.3, $P < 0.01$).

3.4. Hormonal analysis

Plasma ACTH and CORT responses during the 3-hour exposure to 15 min EPM in shaken group rats and control group rats are shown in Fig. 5. There was no significant difference in the basal level (0 min in Fig. 5) of both ACTH (control group: 61.9 ± 17.5 pg/ml, shaken group: 80.0 ± 12.0 pg/ml, $p = 0.999$) and CORT (control group: 48.1 ± 4.7 ng/ml, shaken group: 56.5 ± 5.0 ng/ml, $p = 0.792$) between experimental and control group rats. Two-way ANOVA of ACTH data showed a significant time effect ($F_{(4, 60)}$ = 293.3, $P < 0.0001$), group effect ($F_{(1, 60)} = 80.31$, $P < 0.0001$), and interaction between time and group ($F_{(4, 60)} = 7.393$, $P < 0.0001$). *Post hoc* analysis showed that EPM exposure of shaken group rats significantly increased plasma ACTH at 5 min (control group: 364.4 ± 24.4 pg/ml, shaken group: 480.9 ± 40.1 pg/ml, $p = 0.0012$), 15 min (control group: 547.1 ± 84.2 pg/ml, shaken group: 733.8 ± 80.3 pg/ml, $P < 0.0001$), and 60 min (control group: 268.5 ± 38.6 pg/ml, shaken group: 419.1 ± 11.7 pg/ml, $P < 0.0001$). No significant difference was observed at 180 min between the two groups (control group: 151.1 ± 28.5 pg/ml, shaken group: 197.9 ± 38.2 pg/ml, $p = 0.7273$).

Two-way ANOVA of CORT data showed a significant time effect ($F_{(4, 60)} = 435.6$, $P < 0.0001$), group effect ($F_{(1, 60)} = 31.63$, $P < 0.0001$), and interaction between time and group ($F_{(4, 60)} = 4.675$, $p = 0.0024$). *Post hoc* analysis showed that EPM exposure of experimental group rats significantly increased plasma CORT at 60 min (control group: 163.8 ± 14.3 ng/ml, shaken group: 191.7 ± 14.2 ng/ml, $P < 0.0001$) and 180 min

(control group: 122.2 ± 11.6 pg/ml, shaken group: 140.8 ± 8.0 ng/ml, p = 0.012). No significant difference was observed at 5 min (control group: 64.8 ± 5.1 ng/ml, shaken group: 69.8 ± 3.0 ng/ml, p = 0.9905) and 15 min (control group: 81.5 ± 4.7 ng/ml, shaken group: 83.7 ± 4.9 ng/ml, P > 0.9999) between the two groups.

3.5. PCR analysis

The levels of GR mRNA and MR mRNA in the P10W shaken rats tended to decrease compared to age-matched control rats (Fig. 6). The level of MR mRNA in the hippocampus was significantly lower in shaken rats than in control rats (p = 0.04,), whereas no significant difference was observed in the level of GR mRNA (Fig. 6A, B).

3.6. HPLC analysis

The HPLC analysis of 10-week-old shaken rats showed regional changes in the levels of DA, 5-HT, and NA and their metabolites DOPAC and 5-HIAA (Table 2). A significant difference was observed in the dmPFC and amygdala between shaken and control groups. In the dmPFC of the shaken group, levels of DA, 5-HT, 5-HIAA, and NA were significantly increased by 57%, 61%, 43%, and 50%, respectively, compared to the control group. However, there were no significant differences in the DOPAC/DA ratio and the 5-HIAA/5-HT ratio in dmPFC (p = 0.146, p = 0.263, respectively). In the amygdala, the 5-HIAA level was significantly higher in the shaken group (28%) than in the control group, but the 5-HIAA/5-HT ratio showed no significant difference (p = 0.193). The level of NA in the amygdala was significantly lower in the shaken group (33%) than in the control group. No significant differences were seen in the vmPFC and hippocampus.

4. Discussion

In the present study, the efficacy of a new device for repeated mild SBI in developing rats was assessed. Using this device, the consequences of neonatal repeated mild SBI, which led to abnormalities in behavior, hormonal responses, and neurochemistry in adults, were demonstrated. Histochemical staining confirmed our previous findings of MHs [13].

4.1 Repeated mild SBI in neonatal rats induced anxiety-like behavior in adulthood

In the screening experiment, all shaken groups (G1-G5) had decreased horizontal and vertical activities in the novel open-field test compared to the control groups (C1-C5). Thus, the results of the previous study that showed that these model rats show anxiety-like behavior as adults were confirmed [13]. The present results further demonstrated the different behavioral outcomes between the rats with shaking in the early (P3-P7) and late (P8-P14) periods. The adult offspring subjected to the early shaking showed anxiety-like behavior in the open-field test, whereas these was no difference between the control rats and the rats subjected to late shaking, suggesting the presence of a vulnerability period for this SBI. The windows of vulnerability during different periods of development have also been reported functionally and morphologically in several brain lesion models [24-26]. Furthermore, in the present model, transient MHs have been demonstrated in the gray matter of the hippocampus and mPFC [13], both areas involved in neuronal circuits for anxiety [27]. The incidence of MHs in both areas of this model increased up to P7 and then decreased [13]. Thus, the presence of MHs in the early neonatal period (P3-7) in the hippocampus and/or

mPFC may be related to the anxiety-like behavior in adults.

In the present study, other behavioral paradigms for assessing emotionality were examined. The EPM and L/D tests are based on the spontaneous behavior of conflict between the innate desire to explore novel environments and the innate fear to aversive environments (bright, high, and open: known as psychological stressors) [15, 20, 28]. The decreased duration and entry to the open-arm of the EPM test and the tendency to avoid the bright chamber in the L/D test also confirm the greater anxiety of the shaken rats, reflecting avoidance of an aversive (anxiogenic) environment. Although little is known about behavioral changes in the rodent SBS models, similar anxiety-like behaviors have been reported in various traumatic brain injury (TBI) models, including contusion injury, blast-induced injury, and weight-drop injury [29-32]. In the present study, the previous results were confirmed with different emotional assessments.

4.2 Repeated mild SBI in neonatal rats altered response to stress in adulthood

The EPM is widely used to study the hormonal changes and to define the brain regions and mechanisms underlying anxiety behaviors. In agreement with previous studies [33-35], exposure to the EPM in this model control (C group) produced an increase in plasma ACTH and CORT levels. Furthermore, in the present study, S group rats further showed enhanced secretion of these stress hormones compared with C group rats, and during the post-EPM recovery period, both hormones remained elevated in S group rats, suggesting escalated stress conditions and impaired negative feedback to stress in this model. Alterations in the HPA axis following TBI have been demonstrated in several adult rodent models [36-38]. However, there have been no reports of the response of the HPA axis to stressors in adults following neonatal TBI and SBI. Thus,

this study is first to report the long-term consequences of the HPA response following neonatal mild SBI.

In the present model, the pups were daily removed from the mother and home cage for 10-min sessions from P3 until P14, and they were shaken under isoflurane anesthesia. During this period in rodents, which is known as a stress-hyposensitive period, CORT levels are low and are only minimally increased by exposure to several stressors [2]. Rats in this period have been used as an early-life stress model (maternal separation and neonatal handling) [2, 9]. Maternal separation rats (3 to 6-h daily separation of pups and dam) show an increased HPA response to stress as adults, whereas neonatal handling rats (3 to 15-min daily separation of pups and dam) have decreased adult HPA responses to stress and reduced anxiety-like behavior [39, 40]. In the present SBI model, the experimental age (P3-P14) and separation time (10 min) were similar to the neonatal handling models. However, the behavioral and hormonal responses to stress in the adult offspring were different from the results of the neonatal handling models. Maternal care, such as nursing, contacting, and tactile stimulation, has an influence on the behavioral and HPA responses of adult offspring [41]. Further, repeated isoflurane anesthesia during the neonatal period also affects behaviors of adult rodents [42]. Although the maternal behavior was not minutely assessed in the present study, shaken rats were compared with control rats that were placed in a holding chamber for the same daily 10-min sessions without shaking from P3 until P14 under isoflurane anesthesia, so that the shaken rats had the same neonatal handling conditions. Thus, the behavioral and hormonal differences between the shaken and the control rats could represent the presence of transient MHs.

4.3 Contribution of the decreased expression of MR in the hippocampus to the behavioral and hormonal changes

To understand the possible mechanisms underlying the different roles of glucocorticoids in the hippocampus in regulating HPA axis activity, GR mRNA and MR mRNA expressions in the hippocampus were compared between shaken and control rats using qPCR. The expression of MR mRNA in the hippocampus was significantly lower in the shaken rats than in the control rats. Although there was no significant difference, GR mRNA in the hippocampus tended to be lower in the shaken rats than in the control rats. Corticosteroid binds to MR and GR in the brain. MRs are abundantly expressed in the limbic system, especially the hippocampus, whereas GRs are ubiquitous in neurons and glial cells in high abundance in the hypothalamus, hippocampus, and amygdala [9, 43]. These two receptors operate in a complementary fashion to regulate HPA axis activity, and MRs show a tenfold higher affinity for CORT than GRs [43]. Based on these differences, the MR is activated by a low physiological level of CORT, and it is thought to have a regulatory function and determines HPA-axis sensitivity, the threshold for stress reactivity, stress resilience, and anxiety-like behavior [44, 45]. In contrast, the GR is activated by a high level of CORT [43]. Both the MR and the GR play important roles in CORT-mediated negative feedback in the HPA axis following stress [43-46]. If the reduction of MR mRNA in this model is accompanied by a reduction of protein levels, the present results suggest that the downregulated hippocampal MR may decrease the threshold for stress reactivity and reduce resilience to a novel acute stress, indicating an enhanced and prolonged HPA axis response. Thus, it seems likely that the decreased MR expression in the hippocampus leads to the dysregulation of the HPA axis and anxiety-like behavior

following psychological stress in this model. Further *in situ* hybridization and immunohistochemical studies combined with several stressors are required to reveal the mechanisms underlying MR-induced HPA dysfunction of this model.

In our previous study, modified Perl's staining, a highly sensitive iron histochemical test [47], demonstrated leakage of free iron and iron-uptake cells surrounding MHs [13]. Increased intensity of iron staining in the hippocampus and mPFC in this model suggests increased levels of free iron in these regions. Iron overloading has long-lasting adverse effects [48, 49]. In particular, iron overloading in the neonatal period represents increased superoxide production and mitochondrial dysfunction in the brain [50]. Thus, it is likely that MHs in the hippocampus and mPFC of this model cause increased formation of iron-induced superoxide production and mitochondrial dysfunction, which leads to long lasting dysfunction of these regions. Zhao et al [49] using a single prolonged stress model and reported that rats with neonatal prolonged stress showed iron accumulation in the hippocampus, PFC, and striatum, iron-induced mitochondrial damage and apoptosis in the hippocampal neurons, anxiety-like behavior, and increased GR immunoreactivity in the hippocampal pyramidal neurons as adults. The difference between the findings of Zhao et al [49] and the present results may be due to the extent of iron-accumulation in the hippocampus and PFC. The present results indicate focal iron deposition [13].

4.4 Repeated mild SBI in neonates altered basal monoamine levels in adulthood

In addition to the production of superoxide species, iron also affects monoamine turnover and glutamate and γ-aminobutyric acid (GABA) homeostasis [48, 51]. Impairment of these neurotransmitters has been known to induce emotional behavior,

including anxiety. There are a number of studies dealing with the effects of neonatal TBI and/or stress on the adult monoaminergic neuron systems [52-55]. However, the majority of these studies compared before and after injury or stress exposure. The present neurochemical analyses in the mPFC (dmPFC and vmPFC), hippocampus, and AMY of adult offspring that were not submitted to the behavioral test (open-field, EPM and L/D tests) showed the basal monoamine concentrations of adults. These examined areas are proposed to be anxiety- and stress-related areas in the brain [1, 2, 9]. In some areas, a significant difference in monoamine concentrations was observed between shaken and control rats. In the dmPFC, DA, 5-HT, and NA concentrations were significantly higher in shaken rats than in control rats. However, both DOPAC/DA and 5-HIAA/5-HT levels were no different, indicating normal DA and 5-HT turnover or metabolism and the possibility of increased synthesis of these monoamines. Kawa et al [56] reported that the expressions of tyrosine hydroxylase (TH) mRNA and tryptophan hydroxylase 2 (TPH2) mRNA, synthetic rate limiting enzymes for catecholamines (DA and NA) and 5-HT, respectively, increased in the brainstem after adult mild TBI, and concentrations of monoamines also transiently increased in the forebrain, including the PFC. Interestingly, these rats showed transient anxiety-like behavior [56]. Central monoaminergic neurons, especially DA neurons in the ventral tegmental area, NA neurons in the locus coeruleus, and 5-HT neurons in the dorsal and medial raphe nuclei, show extensive projection systems to forebrain areas including the PFC, hippocampus, and AMY [57-60]. There was conflicting evidence that the 5-HT levels of AMY decreased in shaken rats. Further, no significant differences were observed in the hippocampus and vmPFC. Although the activities and expressions of TH and TPH2 mRNAs of the monoaminergic neurons could not be examined in the present study, the

release and concentration of monoamines in the presynaptic terminals seem to depend on the microenvironment of the projected area, such as postsynaptic receptors and intrinsic neuronal circuits [59]. The iron histochemical staining of this model showed focal iron leakages in these areas [13]. Thus, the different monoamine concentrations between these areas of shaken and control rats may reflect the difference between their focal microenvironments. Further studies are needed to clarify these problems.

5. Conclusions

The present findings complement and extend our previous results of a new rat SBS model. Shaken offspring showed high anxiety-like behavior and abnormal hormonal responses to the psychological stress compared to control rats. Furthermore, lower expression of MR mRNA in the hippocampus and altered monoamine concentrations in the dmPFC and AMY were observed in the shaken rats. These different behavioral, hormonal, and neurochemical profiles might be due to the presence of focal leakage of free iron from MHs following neonatal shaking insults.

Conflict of interests

The authors declare no conflicts of interest.

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Figure Legends

Figure 1. (A and B) Novel open-field test. Shaken rats of different protocols (G1–G5) show significantly reduced exploration in horizontal (line crossing, A) and vertical (rearing, B) activities than control rats (C1-C5). * $P < 0.0001$. (C and D) Rats shaken in the P3-7 period (S1) show significantly reduced line crossing (C) and rearing (D) activities than rats shaken in the P8-14 period (S2). * $P < 0.0001$ versus C3. ** $P < 0.0001$ versus S2.

Figure 2. Histochemical staining of coronal sections through the hippocampus (A-C), prefrontal cortex (D), and amygdaloid complex (E) of shaken P3 (A) and P7 (B-E) rats. Arrows indicate microhemorrhages (MHs). (C) High magnification view of the focal MHs in B. fmi, forceps minor of the corpus callosum. Scale bar = 500μm (A, B, D, E), and 100μm (C).

Figure 3. Elevated plus maze test. Repetitive mild shaking brain injury results in increased anxiety-like behaviors. (A) The shaken group spends much more time in the closed arm than the control group. (B and C) The duration (B) and ratio (C) of open-arm time are decreased in the shaken group. (D) There is no difference in the number of entries into the closed-arm between the shaken and control groups. (E) The shaken group shows a lower number of entries into the open-arm than the control group. (F) The ratios of open-arm entries to total arm entries is decreased in the shaken group. * $P < 0.001$

Figure 4. Light/dark transition test. (A) The shaken group spends less time in the light chamber than the control group. (B) The shaken group shows a lower number of light/dark transitions than the control group. *P < *0.01*

Figure 5. Plasma ACTH (A) and corticosterone (B) levels (mean ± SEM) in P10W shaken (open circle) and age-matched control (closed circle) rats. The base level is at 0 min. Fifteen-min exposure to elevated plus maze (EPX)

Figure 6. The levels of GR mRNA (A) and MR mRNA (B) in the hippocampus of 10-week-old shaken (closed column) and control (open column) rats. The mRNA levels are presented as fold changes (mean ± SEM). * P<*0.05* compared to control.