Behavioural Brain Research 217 (2010) 142–147

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr

Research report

Retardation of neurobehavioral development and reelin down-regulation regulated by further DNA methylation in the hippocampus of the rat pups are associated with maternal deprivation

Lina Qin[a], Wenqiang Tu[a], Xiumin Sun[b], Jianlong Zhang[a], Yuchuan Chen[a,*], Hu Zhao[a,*]

[a] Department of Forensic Medicine, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou 510080, China
[b] Department of Forensic Medicine, Medical College Shantou University, Shantou 515041, China

ARTICLE INFO

Article history:
Received 18 August 2010
Received in revised form 12 October 2010
Accepted 13 October 2010

Keywords:
Early life stress
Neurobehavioral development
Reelin
DNA methylation

ABSTRACT

It is known that early life stress has profound effects in early developing hippocampus. Reelin is a large protein that regulates neuronal migration during embryonic development. The expression of reelin persists in brain, but its function is little known. The aim of the present study was to investigate the effects of maternal deprivation (MD) on early neurobehavioral development of rats, and the role of reelin and the potential mechanism underlying regulation of its expression in hippocampus. Rat pups were removed from mothers during the postnatal day (PND) 2–15 for 3 h a day. Reflex developments including grasping, gait, righting, cliff avoidance, auditory startle, hot-plate test and negative geotaxis, were tested during the first 3 weeks. The level of reelin mRNA and reelin gene methylation in the hippocampal formation were determined using real-time PCR analysis. As expected, some differences appeared in the measure of neurobehavior and expression of reelin in rat pups. Several significant deficiencies were observed in bodyweight, auditory startle and grasping reflex while a great enhancement in hot-plate test in rat pups suffering from MD. On PND 22, the expression of reelin mRNA reduced in the hippocampus followed by MD. Meanwhile, the changes of DNA methylation showed an opposite trend compared with the reelin expression. The results suggest that MD in early life has harmful effects on neurobehavioral development, and causes the down-regulation of reelin mRNA by further DNA methylation in postnatal hippocampus.

1. Introduction

Early-life stress is well-known to cause various short and long-term disturbances in cognitive, emotional and other behavioral performances [1]. Animal models of early-life stress, such as periodic infant–mother separation during early postnatal life, might influence neurobehavioral development during the infancy [2]. Maternal deprivation (MD) has been accepted to be a stressful status in rodents, presumably resulting in disturbance in normal brain development. Moreover, the exposure to stressor during the period of neurodevelopment has harmful effects on the quality of physical and mental health [3,4]. Both human and animal studies also suggest that early-life stress has profound effects on neuronal function and emotion. The hippocampus is known as one of the regions of brain which is vulnerable to stress [5]. Exposure to various forms of repeated stress in laboratory animals has deleterious effects on the hippocampus, such as impaired neurogenesis [6] and long-term potentiation [7], decreased dendritic complexity within CA3 pyramidal cells and dentate granule cells [8,9], and altered gene expression [10–12]. Other investigations indicate that MD during the critical periods of brain development can lead to disruption of hippocampus cytoarchitecture, contributing to learning disability and behavioral abnormalities [13,14].

There are notable interactions between the hippocampal processes affected by exposure to repeated stress and the biological activities of the extracellular matrix protein reelin. Reelin is synthesized and released by specific GABAergic interneurons in the adult hippocampus, where it enhances cell migration and integration, synaptogenesis, dendritic arborization and spinedensity, and synaptic plasticity [15–17]. It is also critically important for the induction and maintenance of long-term potentiation [18]. So, the deficits in reelin signaling pathway have negative consequences for hippocampal functioning. For example, heterozygous reeler mice with approximately 50% of normal levels of reelin show impaired hippocampal-dependent learning and hippocampal plasticity [19]. Interestingly, postmortem analysis indicates that patients with depression have general decreases in reelin in hippocampal tis-

Abbreviations: ELS, early life stress; MD, maternal deprivation; PND, postnatal day; RT, reverse transcription; MI, methylation index.

* Corresponding authors. Tel.: +86 20 87335987; fax: +86 20 87334353.
E-mail addresses: westiceman@163.com, cyc2800@163.com (Y. Chen), hzhao@163.com (H. Zhao).

0166-4328/$ – see front matter © 2010 Published by Elsevier B.V.
doi:10.1016/j.bbr.2010.10.018

sue [20,21]. Therefore, hippocampal reelin expression could be down-regulated by exposure to MD and this might be related to anxiety, depression and memory impairment. To our knowledge, the impact of MD on neurobehavioral development or reelin during the infancy have not been extensively studied, although some researches have been done about the role of reelin in reeler mice [22,23]. Epigenetic regulation of gene expression allows the integration of intrinsic and environmental signals in the genome [24]. Some environmental insults may result in pathological methylation with corresponding alteration of gene expression patterns [25]. As mentioned above, exposure to stress during the period of neurodevelopment has harmful effects on the quality of physical and mental health [3,4]. However, the understanding about the delayed neurobehavioral development or the induction of reelin expression in the hippocampus induced by early life stress is relatively poor.

In this study, the impact of MD on the neurobehavioral development of young rats was examined. Firstly, it's hypothesized that MD, as an early life stressor, would lead to the retardation of neurobehavioral development given prior association of MD and neurobehavioral development. Secondly, it's speculated that down-regulation of reelin gene expression by MD is probably regulated by up-regulation of reelin DNA methylation. Therefore, the methylation status of the reelin gene and the expression of reelin mRNA in the hippocampus of rat pups with MD can be investigated.

2. Experimental procedures

2.1. Animals and maternal deprivation

Primiparous pregnant female Wistar rats were individually housed in standard polypropylene cages containing 2.5 cm of wood chip bedding material. They were kept at a constant temperature room (23 ± 1 °C) and maintained on a 12/12 h light/dark cycle (lights on at 8:00). Food and water were available at will.

Birth was designated as postnatal day 1. On PND2, pups were culled to six males and two females to ensure the presence of both sexes in the litters [26]. Only the male offspring was used. Whole litters were randomly assigned to control group and MD group.

According to the previously described method [27,28], MD mission was performed daily for three consecutive hours (from 9:00 to 12:00), during which rat pups were removed from their home cage and kept alone in temperature-controlled cages at 28 ± 1 °C, where bedding was renewed every day. This procedure was applied between postnatal days 2 and 15. All pups of the control group were left undisturbed with their mother until weaning. Litter was not cleaned although some clean sawdust was added from the top of the cage.

Groups of rats were weaned and sacrificed on PND22. All experimental protocols described in this study were approved by the Local Institutional Animal Care and Use Committee. These experiments were designed to minimize the number, and discomfort, of the animals used.

2.2. Examination of neurobehavioral development

Examinations of neurobehavioral development started on PND 4 and were carried out once every other day between 9 am and 12 pm until PND 22. Neurobehavioral testing was performed in a blinded fashion. Neurobehavioral development was tested based on earlier descriptions [29–34]. Body weight was recorded once every other day until PND22. Rat pups were tested for the following neurobehavioral development reflexes once every other day until PND20: (1) *Surface righting reflex*: the time in seconds to turn over to prone position and place all four paws in contact with the surface after rats were placed in supine position, was recorded from PND4 to PND12. (2) *Air righting reflex*: the time of standing on four feet after subjects were dropped head down onto a bed of shavings from a height of 50 cm three times with the ground full of the bedding of sawdust, were recorded from PND12 to PND20. (3) *Negative geotaxis*: animals were placed head down on an inclined grid (45°) of 30 cm. The hind limbs of the rat pups were placed in the middle of the grid. The time in seconds they began to turn around and climb up the board with their forelimbs reaching the upper rim, was recorded. In cases the animal did not turn around and climb up the board within the observed 30 s, the test was considered to be negative. (4) *Fore limbs grasp*: the fore limbs were touched with a thin rod, and the time in seconds to grasp onto the rod was recorded. (5) *Gait*: the animals were placed in the center of a white paper circle of 13 cm in diameter, and the time in seconds to move off the circle with both forelimbs was recorded. In cases the animal did not leave the circle within 30 s, the test was considered to be negative. Within the time 10 s was rated as 3 scores, the time 10–20 s was 2 scores, and the time 20–30 s was one score. (6) *Auditory startle*: the noise of whistle was given to the rats three times, the times of the startle response to a whistle sound was observed and recorded. (7) *Hot-plate test*: the rats were placed in a beaker, then put the beaker in 52 ± 1 °C water-bath pot. The time in seconds of the hind foot response to the high temperature was observed. (8) *Cliff avoidance*: animals were placed head and forelimbs down on a high board with a height of 30 cm from the ground full of the bedding of sawdust. The forelimbs and the head of the rat pups were placed out of the edge of the board. The time they began to turn back their heads and the forelimbs was observed. In cases the animal did not turn back from the edge of the board within the observed 60 s, the test was considered to be negative.

2.3. Sample collection

Animals were brought into a separate room and sacrificed on PND22 by decapitation, the brain was rapidly removed and hippocampus were immediately dissected from the brain, then frozen in −70 °C. The hippocampus was used for the following experiments.

2.4. Real-time reverse transcription quantitative PCR

RNA was isolated from hippocampus tissue and purified using RNAqueous Kit (Part Number AM1912, Ambion). RT-PCR was performed by commercially available reagents (High-Capacity cDNA Reverse Transcription Kits, Applied Biosystems). For a 20 μl reaction mixture, the following reagents were used: 2 μl of 10× RT buffer, 0.8 μl of 100 mM 25× dNTP Mix, 1 μl of MultiScribe™ Reverse Transcriptase. 1 μl of RNase inhibitor (40 unit/μl), 2 μl 10× RT Random Primers, 3.2 μl of RNase-free water and 10 μl of sample RNA. Reverse transcription was carried out at 25 °C for 10 min, followed by 37 °C for 120 min and 85 °C for 5 min, then stored at −80 °C. Real-time quantitative PCR was performed using commercially available reagents Power SYBR® Green PCR Master Mix (Applied Biosystems) for reelin. All primers were designed to span exon boundaries, ensuring amplification of only mRNA. The following forward and reverse primers were used in the present study. For reelin forward (5′-GAGTCCACTATACAACCA GA-3′) and reverse (5′-TGATCGAAAGCAGAGAC GTC-3′). Product purity was confirmed by melting curve analysis and agarose gel electrophoresis in the presence of ethidium bromide. Relative gene expression comparisons were made using 18S RNA as an invariant endogenous control. The calculations of the initial mRNA copy numbers in each sample were performed according to the $^{\Delta\Delta}C_T$ method [35,36]. The data are expressed as C_T values and used to determine $^{\Delta}C_T$ values ($^{\Delta}C_T = C_T$ of the target gene $- C_T$ of the housekeeping gene). This value is calculated for each sample. The control sample was used as the baseline for each comparison to be made. The last step in quantitation is to transform these values to absolute values. The formula for fold changes in gene expression is $2^{-\Delta\Delta}C_T$. Statistical analysis was performed with $^{\Delta}C_T$ values. Reactions were carried out in a 20 μl volume with 5 μl of the cDNA, 10 μl of SYBR Green Supermix (Applied Biosystems), 1 μl of primer, and 4 μl of Depc H_2O. The amplification protocol consisted of one cycle at 95 °C for 10 min followed by 35 cycles at 95 °C for 15 s, 60 °C for 15 s, 72 °C for 20 s, and 90 °C for 2 s. Detection of the fluorescent products was carried out at the end of the 90 °C extension period. All qRT-PCR reactions were performed in triplicate.

2.5. DNA methylation assay

DNA purification (DNeasy Blood & Tissue Kit, Qiagen) was performed on hippocampus tissue from animals. Purified DNA was then processed for bisulfite modification (EZ DNA Methylation-Gold™ Kit, Zymo Research Corp). Quantitative real-time PCR was used to determine the DNA methylation status of reelin gene. Methylation-specific PCR primers were designed

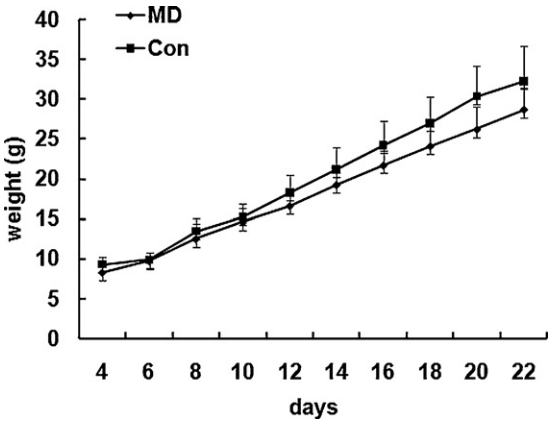

Fig. 1. The changes of body weight in control and rat pups with MD. The rat pups were weighed, respectively on PND22. There are significant differences found between control and rat pups with MD versus control group. *$P < 0.05$. All results are expressed as mean ± S.E.M.

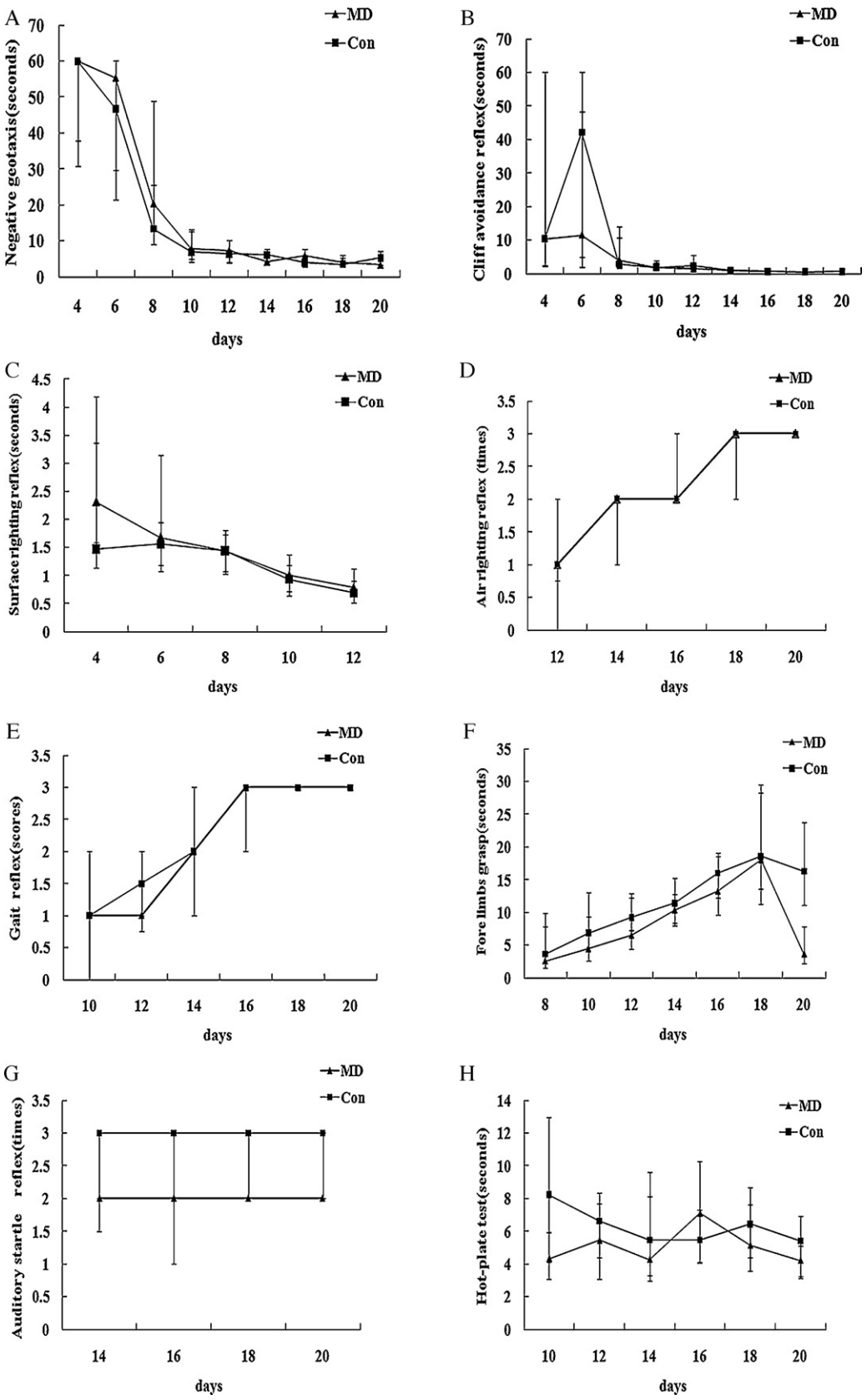

Fig. 2. The differences of neurobehavioral development in control and rat pups with MD. (A) The negative geotaxis reflex in control and rat pups with MD. $P > 0.05$ versus control group. (B) The cliff avoidance reflex in control and rat pups with MD. $P > 0.05$ versus control group. (C) The surface righting reflex in control and rat pups with MD. $P > 0.05$ versus control group. (D) The air righting reflex in control and pups with MD. $P > 0.05$ versus control group. (E) The gait reflex in control and rat pups with MD. $P > 0.05$ versus control group. (F) The fore limbs grasp reflex in control and rat pups with MD. $^{*}P < 0.05$ versus control group. (G) The auditory startle reflex in control and rat pups with MD. $^{*}P < 0.001$ versus control group. (H) The hot-plate test in control and rat pups with MD. $^{*}P < 0.001$ versus control group. All the data of neurobehavioral development are expressed as median P25, P75.

using Methprimer software (available at www.urogene.org/methprimer/). Detection of unmethylated reelin DNA was performed using the following primer: forward (5′-AAGGTGGGAGTATTTGTATAGAGTGA-3′) and reverse (5′-CCAAAACAACATAAAAAAAACAAT-3′). Detection of methylated reelin DNA was performed using the following primer: forward (5′-GCGGGAGTATTTGTAATG-AGTCG-3′) and reverse (5′-CCAAAACAAACAATAAAAAAAGCTA-5′). PCRs were performed in a total volume of 20 μl, consisting of 5 μl of bisulfite-modified DNA, 10 μl of SYBR Green Supermix (Applied Biosystems), 1 μl of primer, and 4 μl of Depc H_2O. Reactions were performed in a Cycler real-time PCR system (Applied Biosystems). Quantitative PCR was run three times with each sample. The amplification protocol consisted of one cycle at 95 °C for 10 min followed by 35 cycles at 95 °C for 15 s, 58 °C for 15 s, 72 °C for 20 s, and 90 °C for 2 s. Detection of the fluorescent products was carried out at the end of the 90 °C extension period. The reelin methylation was assessed by the comparative threshold cycle (MI) method [37–39]. The methylation index (MI; percentage) in a sample was calculated according to the equation: (concentration of methylated reelin sequence/concentrations of methylated plus unmethylated reelin sequence) × 100.

2.6. Statistical analysis

The data are expressed as mean ± S.E.M. while the data of neurological reflexes as median P25, P75. Greenhouse–Geisser test were used in body weight and neurological reflexes. The tests were also used to assess reelin mRNA levels, as well as changes in the methylation state of reelin. Significance was set at $P < 0.05$ for all tests. The statistical analysis was done using the SPSS Statistical Software version 16.0.

3. Results

3.1. Somatic development

Rat pups were weighed, respectively once every other day from PND4 to PND22. The average body weight of MD rat pups was found lower than that of control animals. There were significant differences between control and MD rat pups ($t = 4.272$, $P < 0.05$) (Fig. 1).

3.2. Neurological reflexes

The effects of MD on neurological development may be presented by different neurological reflexes. As a result, MD group showed a significant delay of forelimb grasp reflex ($F = 4.317$, $P < 0.05$) (Fig. 2F) and auditory startle reflex ($F = 57.308$, $P < 0.001$) (Fig. 2G). Hot-plate test were observed significantly different between groups MD and control ($F = 15.886$, $P < 0.001$) (Fig. 2H). A tendency of spending more time in the hot-plate could be observed in control rats rather than MD groups. There is not significant difference of negative geotaxis reflex ($F = 0.122$, $P > 0.05$) (Fig. 2A), cliff avoidance reflex ($F = 2.357$, $P > 0.05$) (Fig. 2B), surface righting reflex ($F = 0.142$, $P > 0.05$), air righting reflex ($F = 3.158$, $P > 0.05$) and gait reflex ($F = 1.858$, $P > 0.05$) (Fig. 2C–E) between the control and MD group.

3.3. Variation of reelin mRNA expression in hippocampus

To evaluate the impact of reelin on early neurobehavioral development, total reelin gene expression in rat hippocampus was performed using quantitative real-time PCR. The reelin gene expression values are expressed as the comparative threshold cycle ($2^{-\Delta\Delta}C_T$). Thus, the smaller the Ct is, the greater the gene expression is. That is to say, the gene expression is consistent with $2^{-\Delta\Delta}C_T$ and the greater the $2^{-\Delta\Delta}C_T$, the greater the gene expression. The results of the $2^{-\Delta\Delta}C_T$ of reelin are shown in Fig. 3. There was a significant reduction in MD group compared to control rat pups ($t = -2.662$, $P < 0.05$).

3.4. Changes of reelin gene DNA methylation

The reelin methylation was assessed by the comparative threshold cycle (MI) method. The methylation index (MI; percentage) in a sample was calculated according to the equation: (concentration of methylated reelin sequence/concentrations of methylated plus unmethylated reelin sequence) × 100. The results of MI of reelin are shown in Fig. 4. The high methylation was seen in the MD group. In addition, there was a significant difference between MD and control group ($t = 4.542$, $P < 0.01$). An interesting finding from these experiments is that changes of reelin gene DNA methylation showed an opposite trend to the level of reelin mRNA.

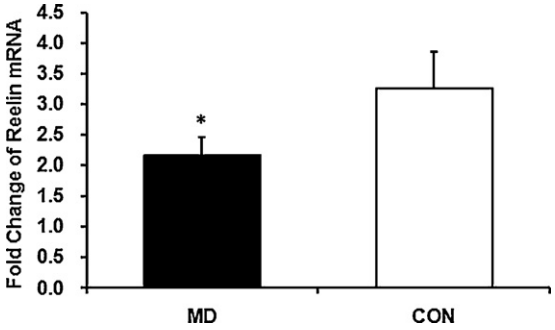

Fig. 3. The expression of reelin mRNA in the hippocampus of rat pups. The value for reelin mRNA was normalized by that for the internal standard 18S mRNA. Each column represents the mean ± S.E.M. ($n = 6$–8 rats per group). *$P < 0.05$ versus the control group.

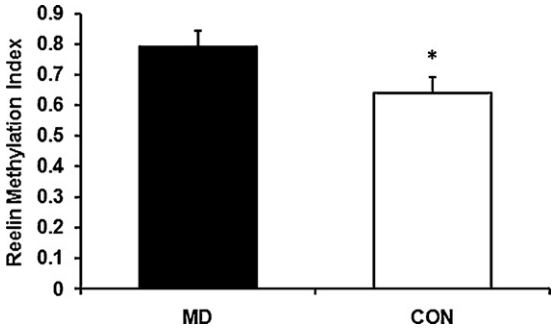

Fig. 4. Altered reelin DNA methylation with maternal deprivation. There are significant changes in methylation between MD and control groups. Data are presented as means ± S.E.M. ($n = 6$–8 rats per group). *$P < 0.01$. versus the control group.

4. Discussion

The early neurobehavioral effects of MD have been studied previously by others. A single 24-h-period paradigm showed delayed eye opening, reduced body weight, delayed emergence of walking and rearing, increased latency in negative geotaxis and retarded emergence of the behavioral response to amphetamine [40]. However, a recent study presented that a 3-h-long daily maternal separation did not lead to remarkably delay or enhancement in reflex development and motor coordination [34]. The present study carried out the further investigation on the impact of repeated episodes of MD on neurobehavioral development in rats. The results revealed that a 3-h-long daily maternal separation led to some changes in neurobehavioral development. MD rats displayed a notable reduction of body weight and a significant delay in forelimb grasp and auditory startle reflexes, and a slightly delayed emergence of walking and retarded emergence of the behavioral response to early-life stress. However, the rat pups of MD performed with a relatively increasing reaction in hot-plate test. These results suggest that MD has harmful effects on the early neurobehavioral development of rat pups, and the phenomenon is consistent with Weaver's work [41]. The results in the present study were partially in accordance with previous studies. In fact, so far

there have been many ambivalent conclusions about the impacts of maternal separation on the neurobehavioral development of young rats because of different protocols used by the different investigators in many researches. Animal studies have proven that the effects of MD depend on both separation conditions, such as time of day, length of separation, genetic background and gender of rat pups, and environmental conditions such as ambient temperature [34,42–45].

One relevant question raised by our observation is whether reelin expression is associated to MD. Reelin has been shown to contribute to normal brain development and neuronal activity-dependent processes [46,47]. Reduced levels of reelin expression may be deleterious for human brain development. For example, reelin expression is down-regulated in inhibitory cortical neurons of patients with schizophrenia and psychotic bipolar disorder [20,48], suggesting that a dysfunction in reelin-regulated neuronal maturation and synaptic activity in the postnatal brain may contribute to these disorders. Our previous study demonstrated that repeated predatory stresses might result in a pattern of intensive behavioral and endocrinal responses, and lead to the neuronal cell loss in hippocampus [49]. However, other studies indicated that rats separated from mothers for 3 h a day during PND 10–15 showed no significant changes in the total number of neurons in the hippocampus [50,51]. In the present study, young rats with MD had significant reductions of level of reelin mRNA in the hippocampus. The results suggest that the down-regulation of reelin mRNA in postnatal hippocampus of young rats is associated with MD. The majority of mRNA transcripts that showed group differences in expression were for those that encoded signal transduction proteins involved in the pathways that regulate brain formation and function [52]. Therefore, reelin may regulate developmental processes such as synaptogenesis and axon pruning as well as synaptic plasticity through life.

In addition, it is well known that epigenetic regulation of gene expression allows the integration of intrinsic and environmental signals in the genome [24]. DNA methylation may lead to marked changes in the structure of chromatin that ultimately result in significant down-regulation of transcription, and directly interfere with the ability of transcription factors to bind to regulatory elements. Treatment of NT2 cells with DNA methyltransferases (DNMT) inhibitors prior to differentiation or induction of differentiation with RA resulted in significant increases in reelin mRNA expression and loss of methylation at the reelin promoter, consistent with the hypothesis that DNA methylation plays a significant role in the regulation of reelin expression [53]. Other studies indicated that the lower expression of reelin mRNA in MD group might be responsible for an epigenetic methylation of reelin gene [54–59]. It is suggested that epigenetic regulation through DNA methylation is responsible for reelin down-regulation in the brain. Interestingly, with the reductions of reelin mRNA, methylation levels of the reelin gene in hippocampus tended to increase in MD rats as well. Thus, based on our results, it has been proven that MD, as a special environmental factor, may cause the reductions of reelin mRNA through the epigenetic mechanism of further DNA methylation of reelin promoter regions in the hippocampus.

5. Conclusion

The effects of MD on the neurobehavioral development of young rats, and the role of reelin and the potential mechanism underlying regulation of its expression in hippocampus were investigated by neurobehavioral observation and real-time PCR. The results indicate that the delayed neurobehavioral development and the down-regulation of reelin mRNA in postnatal hippocampus of young rats are associated to MD. Meanwhile, it has been proven that MD could cause the down-regulation of reelin mRNA through further DNA methylation.

Acknowledgment

This work was supported by 100 Talents Program of Sun Yat-sen University, China (5000-3171314).

References

[1] Koehl M, Lemaire V, Vallee M, Abrous N, Piazza PV, Mayo W, et al. Long term neurodevelopmental and behavioral effects of perinatal life events in rats. Neurotox Res 2001;3(January (1)):65–83.
[2] Rice D, Barone Jr S. Critical periods of vulnerability for the developing nervous system: evidence from humans and animal models. Environ Health Perspect 2000;108(June (Suppl 3)):511–33.
[3] Fumagalli F, Molteni R, Racagni G, Riva MA. Stress during development: impact on neuroplasticity and relevance to psychopathology. Prog Neurobiol 2007;81(March (4)):197–217.
[4] Gluckman PD, Hanson MA, Cooper C, Thornburg KL. Effect of in utero and early-life conditions on adult health and disease. N Engl J Med 2008;359(July (1)):61–73.
[5] Vermetten E, Bremner JD. Circuits and systems in stress. I. Preclinical studies. Depress Anxiety 2002;15(3):126–47.
[6] Gould E, Tanapat P, McEwen BS, Flugge G, Fuchs E. Proliferation of granule cell precursors in the dentate gyrus of adult monkeys is diminished by stress. Proc Natl Acad Sci U S A 1998;95(March (6)):3168–71.
[7] Artola A, von Frijtag JC, Fermont PC, Gispen WH, Schrama LH, Kamal A, et al. Long-lasting modulation of the induction of LTD and LTP in rat hippocampal CA1 by behavioural stress and environmental enrichment. Eur J Neurosci 2006;23(January (1)):261–72.
[8] Vyas A, Mitra R, Shankaranarayana Rao BS, Chattarji S. Chronic stress induces contrasting patterns of dendritic remodeling in hippocampal and amygdaloid neurons. J Neurosci 2002;22(August (15)):6810–8.
[9] Bessa JM, Ferreira D, Melo I, Marques F, Cerqueira JJ, Palha JA, et al. The mood-improving actions of antidepressants do not depend on neurogenesis but are associated with neuronal remodeling. Mol Psychiatry 2009;14(August (8)):764–73, 739.
[10] Alfonso J, Fernandez ME, Cooper B, Flugge G, Frasch AC. The stress-regulated protein M6a is a key modulator for neurite outgrowth and filopodium/spine formation. Proc Natl Acad Sci U S A 2005;102(November (47)):17196–201.
[11] Hunter RG, Bellani R, Bloss E, Costa A, McCarthy K, McEwen BS. Regulation of kainate receptor subunit mRNA by stress and corticosteroids in the rat hippocampus. PLoS ONE 2009;4(1):e4328.
[12] Joels M, Karst H, Alfarez D, Heine VM, Qin Y, van Riel E, et al. Effects of chronic stress on structure and cell function in rat hippocampus and hypothalamus. Stress 2004;7(December (4)):221–31.
[13] Huot RL, Plotsky PM, Lenox RH, McNamara RK. Neonatal maternal separation reduces hippocampal mossy fiber density in adult long evans rats. Brain Res 2002;950(September (1–2)):52–63.
[14] Matthews K, Wilkinson LS, Robbins TW. Repeated maternal separation of preweanling rats attenuates behavioral responses to primary and conditioned incentives in adulthood. Physiol Behav 1996;59(January (1)):99–107.
[15] Forster E, Jossin Y, Zhao S, Chai X, Frotscher M, Goffinet AM. Recent progress in understanding the role of reelin in radial neuronal migration, with specific emphasis on the dentate gyrus. Eur J Neurosci 2006;23(February (4)):901–9.
[16] Gong C, Wang TW, Huang HS, Parent JM. Reelin regulates neuronal progenitor migration in intact and epileptic hippocampus. J Neurosci 2007;27(February (8)):1803–11.
[17] Niu S, Yabut O, D'Arcangelo G. The reelin signaling pathway promotes dendritic spine development in hippocampal neurons. J Neurosci 2008;28(October (41)):10339–48.
[18] Weeber EJ, Beffert U, Jones C, Christian JM, Forster E, Sweatt JD, et al. Reelin and ApoE receptors cooperate to enhance hippocampal synaptic plasticity and learning. J Biol Chem 2002;277(October (42)):39944–52.
[19] Qiu S, Korwek KM, Pratt-Davis AR, Peters M, Bergman MY, Weeber EJ. Cognitive disruption and altered hippocampus synaptic function in reelin haploinsufficient mice. Neurobiol Learn Mem 2006;85(May (3)):228–42.
[20] Fatemi SH, Earle JA, McMenomy T. Reduction in reelin immunoreactivity in hippocampus of subjects with schizophrenia, bipolar disorder and major depression. Mol Psychiatry 2000;5(November (6)):654–63, 571.
[21] Knable MB, Barci BM, Webster MJ, Meador-Woodruff J, Torrey EF. Molecular abnormalities of the hippocampus in severe psychiatric illness: postmortem findings from the Stanley Neuropathology Consortium. Mol Psychiatry 2004;9(June (6)):609–20, 544.
[22] Laviola G, Ognibene E, Romano E, Adriani W, Keller F. Gene–environment interaction during early development in the heterozygous reeler mouse: clues for modelling of major neurobehavioral syndromes. Neurosci Biobehav Rev 2009;33(April (4)):560–72.
[23] Ognibene E, Adriani W, Macri S, Laviola G. Neurobehavioural disorders in the infant reeler mouse model: interaction of genetic vulnerability and consequences of maternal separation. Behav Brain Res 2007;177(February (1)):142–9.

[24] Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 2003;33(March (Suppl)):245–54.
[25] Abdolmaleky HM, Cheng KH, Russo A, Smith CL, Faraone SV, Wilcox M, et al. Hypermethylation of the reelin (RELN) promoter in the brain of schizophrenic patients: a preliminary report. Am J Med Genet B: Neuropsychiatr Genet 2005;134B(April (1)):60–6.
[26] Cirulli F, Adriani W, Laviola G. Sexual segregation in infant mice: behavioural and neuroendocrine responses to D-amphetamine administration. Psychopharmacology (Berl) 1997;134(November (2)):140–52.
[27] Rosztoczy A, Fioramonti J, Jarmay K, Barreau F, Wittmann T, Bueno L. Influence of sex and experimental protocol on the effect of maternal deprivation on rectal sensitivity to distension in the adult rat. Neurogastroenterol Motil 2003;15(December (6)):679–86.
[28] Barreau F, Ferrier L, Fioramonti J, Bueno L. Neonatal maternal deprivation triggers long term alterations in colonic epithelial barrier and mucosal immunity in rats. Gut 2004;53(April (4)):501–6.
[29] Smart JL, Dobbing J. Vulnerability of developing brain. VI. Relative effects of foetal and early postnatal undernutrition on reflex ontogeny and development of behaviour in the rat. Brain Res 1971;33(October (2)):303–14.
[30] Smart JL, Dobbing J. Vulnerability of developing brain. II. Effects of early nutritional deprivation on reflex ontogeny and development of behaviour in the rat. Brain Res 1971;28(April (1)):85–95.
[31] Altman J, Sudarshan K. Postnatal development of locomotion in the laboratory rat. Anim Behav 1975;23(November (4)):896–920.
[32] Hill JM, Gozes I, Hill JL, Fridkin M, Brenneman DE. Vasoactive intestinal peptide antagonist retards the development of neonatal behaviors in the rat. Peptides 1991;12(January–February (1)):187–92.
[33] Reglodi D, Kiss P, Tamas A, Lengvari I. The effects of PACAP and PACAP antagonist on the neurobehavioral development of newborn rats. Behav Brain Res 2003;140(March (1–2)):131–9.
[34] Farkas J, Reglodi D, Gaszner B, Szogyi D, Horvath G, Lubics A, et al. Effects of maternal separation on the neurobehavioral development of newborn Wistar rats. Brain Res Bull 2009;79(May (3–4)):208–14.
[35] Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 2001;25(December (4)):402–8.
[36] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29(May (9)):e45.
[37] Lo YM, Wong IH, Zhang J, Tein MS, Ng MH, Hjelm NM. Quantitative analysis of aberrant p16 methylation using real-time quantitative methylation-specific polymerase chain reaction. Cancer Res 1999;59(August (16)):3899–903.
[38] Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H. Hypermethylation of let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth factor-II expression and favorable prognosis. Cancer Res 2007;67(November (21)):10117–22.
[39] Mitomi H, Fukui N, Tanaka N, Kanazawa H, Saito T, Matsuoka T, et al. Aberrant p16((INK4a)) methylation is a frequent event in colorectal cancers: prognostic value and relation to mRNA expression and immunoreactivity. J Cancer Res Clin Oncol 2010;136(February (2)):323–31.
[40] Ellenbroek BA, Derks N, Park HJ. Early maternal deprivation retards neurodevelopment in Wistar rats. Stress 2005;8(December (4)):247–57.
[41] Weaver SADJ, Meaney MJ. Maternal separation leads to persistent reductions in pain sensitivity in female rats. J Pain 2007;8(12):962–9.
[42] Ellenbroek BA, Cools AR. The long-term effects of maternal deprivation depend on the genetic background. Neuropsychopharmacology 2000;23(July (1)):99–106.
[43] McIntosh J, Anisman H, Merali Z. Short- and long-periods of neonatal maternal separation differentially affect anxiety and feeding in adult rats: gender-dependent effects. Brain Res Dev Brain Res 1999;113(March (1–2)): 97–106.
[44] Slotten HA, Kalinichev M, Hagan JJ, Marsden CA, Fone KC. Long-lasting changes in behavioural and neuroendocrine indices in the rat following neonatal maternal separation: gender-dependent effects. Brain Res 2006;1097(June (1)):123–32.
[45] Yamazaki A, Ohtsuki Y, Yoshihara T, Honma S, Honma K. Maternal deprivation in neonatal rats of different conditions affects growth rate, circadian clock, and stress responsiveness differentially. Physiol Behav 2005;86(September (1–2)):136–44.
[46] D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T. A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. Nature 1995;374(April (6524)):719–23.
[47] Quattrocchi CC, Wannenes F, Persico AM, Ciafre SA, D'Arcangelo G, Farace MG, et al. Reelin is a serine protease of the extracellular matrix. J Biol Chem 2002;277(January (1)):303–9.
[48] Guidotti A, Auta J, Davis JM, Di-Giorgi-Gerevini V, Dwivedi Y, Grayson DR, et al. Decrease in reelin and glutamic acid decarboxylase67 (GAD67) expression in schizophrenia and bipolar disorder: a postmortem brain study. Arch Gen Psychiatry 2000;57(November (11)):1061–9.
[49] Zhao H, Xu H, Xu X, Young D. Predatory stress induces hippocampal cell death by apoptosis in rats. Neurosci Lett 2007;421(June (2)):115–20.
[50] Miki T, Harris SJ, Wilce PA, Takeuchi Y, Bedi KS. Effects of alcohol exposure during early life on neuron numbers in the rat hippocampus. I. Hilus neurons and granule cells. Hippocampus 2003;13(3):388–98.
[51] Miki T, Harris SJ, Wilce PA, Takeuchi Y, Bedi KS. Effects of age and alcohol exposure during early life on pyramidal cell numbers in the CA1–CA3 region of the rat hippocampus. Hippocampus 2004;14(1):124–34.
[52] Weaver IC, Meaney MJ, Szyf M. Maternal care effects on the hippocampal transcriptome and anxiety-mediated behaviors in the offspring that are reversible in adulthood. Proc Natl Acad Sci U S A 2006;103(February (9)):3480–5.
[53] Chen Y, Sharma RP, Costa RH, Costa E, Grayson DR. On the epigenetic regulation of the human reelin promoter. Nucleic Acids Res 2002;30(July (13)): 2930–9.
[54] Costa E, Dong E, Grayson DR, Guidotti A, Ruzicka W, Veldic M. Reviewing the role of DNA (cytosine-5) methyltransferase overexpression in the cortical GABAergic dysfunction associated with psychosis vulnerability. Epigenetics 2007;2(January–March (1)):29–36.
[55] Miller CA, Sweatt JD. Covalent modification of DNA regulates memory formation. Neuron 2007;53(March (6)):857–69.
[56] Ruzicka WB, Zhubi A, Veldic M, Grayson DR, Costa E, Guidotti A. Selective epigenetic alteration of layer I GABAergic neurons isolated from prefrontal cortex of schizophrenia patients using laser-assisted microdissection. Mol Psychiatry 2007;12(April (4)):385–97.
[57] Veldic M, Kadriu B, Maloku E, Agis-Balboa RC, Guidotti A, Davis JM, et al. Epigenetic mechanisms expressed in basal ganglia GABAergic neurons differentiate schizophrenia from bipolar disorder. Schizophr Res 2007;91(March (1–3)):51–61.
[58] Mill J, Tang T, Kaminsky Z, Khare T, Yazdanpanah S, Bouchard L, et al. Epigenomic profiling reveals DNA-methylation changes associated with major psychosis. Am J Hum Genet 2008;82(March (3)):696–711.
[59] Miller CA, Campbell SL, Sweatt JD. DNA methylation and histone acetylation work in concert to regulate memory formation and synaptic plasticity. Neurobiol Learn Mem 2008;89(May (4)):599–603.