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Low maternal licking/grooming stimulation increases pain sensitivity in male mouse offspring

Takashi SAKAMOTO, Yukino ISHIO, Yuiko ISHIDA, Kazutaka MOGI and Takefumi KIKUSUI

Department of Animal Science and Biotechnology, Azabu University, 1-17-71 Fuchinobe, Sagamihara, Kanagawa 252-5201, Japan

Abstract: Deprivation of maternal care has been associated with higher pain sensitivity in offspring. In the present study, we hypothesized that the maternal licking/grooming behavior was an important factor for the development of the pain regulatory system. To test this hypothesis, we used male F2 offspring of early-weaned (EW) F1 mother mice that exhibit lower frequency of licking/grooming behavior. The formalin test revealed that F2 offspring of EW F1 dams showed significantly higher pain behavior than F2 offspring of normally-weaned (NW) F1 dams. We found that the mRNA levels of transient receptor potential vanilloid 1 (TRPV1), a nociceptor, were higher in the lumbosacral dorsal root ganglion (DRG) of F2 offspring of EW F1 dams than those of F2 offspring of NW F1 dams, suggesting that the higher pain sensitivity may be attributed to low licking/grooming, which may result in developmental changes in nociceptive neurons. In the DRG, mRNA levels of Mas-related G-protein coupled receptor B4 (MrgprB4), a marker of sensory neurons that detect gentle stroking, was also up-regulated in the F2 offspring of EW F1 dams. Considering that gentle touch alleviates pain, *Mrgprb4* up-regulation may reflect a compensatory change. The present findings indicate important implications of maternal licking/grooming behavior in the development of the pain regulatory system.
Key words: dorsal root ganglion, early weaning, mas-related G-protein coupled receptor B4 (MrgprB4), pain, transient receptor potential vanilloid 1 (TRPV1)

Introduction

Pain sensitivity in adulthood has been thought to be regulated by early-life environments [1–3]. Children who experience early-life stress, including physically traumatic events or socially and/or psychologically poor environments, have an increased risk of chronic pain in adulthood [4–7]. In support of these clinical findings, animal studies have shown that maternal separation throughout the pre-weaning period induces abnormal behavioral changes against nociceptive stimuli to the skin in adulthood [8–10]. Furthermore, it has also been shown that maternal separation, depending on the timing, duration, and number of maternal separation episodes, results in variable developmental outcomes [11]. Longer periods (3–6 h during postnatal days 2–14) of maternal separation exaggerated the hypothalamic-pituitary-adrenal axis response to a stressor, whereas brief periods (~15 min) decreased the adrenal reactivity in adult offspring [12–14]. However, specific factors in maternal care that are responsible for the appropriate development of pain sensitivity in the offspring remain unknown.

Maternal care is important for the physical and mental development of offspring [14–16]. Maternal care provides multiple sensory inputs to offspring through the somatosensory, gustatory, olfactory, auditory, and visual system inputs [17–20]. For example, maternal touch in children supports neurodevelopmental outcomes [21]. In rodents, the mother provides her pups with licking and grooming (LG) stimulation as a somatosensory input, not only to keep clean but also to help urinate/defecate and regulate body temperature [22]. LG stimulation

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Corresponding author: K. Mogi. e-mail: mogik@azabu-u.ac.jp

completely or partially restores the central nervous system (CNS) dysfunction caused by maternal separation [23–26]. On the other hand, little is known about the developmental effects of LG stimulation in maternal care on the peripheral sensory neurons such as the nociceptive neurons in the dorsal root ganglia (DRGs).

Nociceptive neurons, which have a peripheral branch that innervates the skin and a central branch that carries the somatosensory information to the spinal cord, respond to intense mechanical, thermal, and noxious chemical stimuli, and express specialized molecular receptors in their peripheral terminals capable of pain transduction [27–31]. The central branch synapses with secondary sensory neurons, and the nerve terminals release neurotransmitters causing local neurogenic inflammation and activation of postsynaptic receptors associated with pain perception located in the spinothalamic tract neurons [32]. Cutaneous sensory neurons perceiving tactile stimulation have recently been suggested to be involved in pain perception [33, 34]. C-low-threshold mechanoreceptive (C-LTMR) neurons, which are not involved in direct pain response in non-pathological conditions, perceive somatosensory inputs including LG stimulation [35–37]. Activation of the C-LTMR neurons inhibits nociceptive signaling through synaptic integration in the spinal dorsal horn [33, 38–40].

Here, we hypothesized that maternal LG stimulation might be an important factor for the appropriate development of the pain sensitivity in offspring. To test this hypothesis, we utilized F2 male offspring of early-weaned (EW) F1 mother mice that show lower LG behavior [41]. A comparison between F2 offspring of normally-weaned F1 mother mice (NW-F2 offspring) and offspring of early-weaned F1 mother mice (EW-F2 offspring), who themselves were normally weaned, is a good model to examine the effect of maternal LG stimulation in the development. We compared the pain-related behavior evoked by formalin injection into the hind paw skin between NW- and EW-F2 offspring. Previously, we have observed higher gene expression levels of MAS-related G-protein coupled receptor B4 (*Mrgprb4*), a marker of C-LTMR neurons in the thoracolumbar DRGs innervating the trunk region in the EW-F2 offspring [42]. In addition to the pain-related behavior, we also hypothesized that maternal LG stimulation might induce changes in expression of nociceptive molecules in the DRGs projecting to the hindlimb region. To test this, we investigated gene expression changes in the nociceptor channels transient receptor potential vanilloid 1 (*Trpv1*) and transient receptor potential ankyrin 1 (*Trpa1*) [27–31, 43, 44], and in the nociceptive neurotransmitters substance P (*SP*) and calcitonin gene-related peptide (*CGRP*) [32] in the DRGs. We also analyzed the gene expression of *Mrgprb4*.

Material and Methods

Animal preparation and procedures

C57BL/6J mice obtained from Japan Clea Co., Ltd. (Yokohama, Japan) were used for the experiments. Male and female mice were pair-housed in cages (175 × 245 × 125 mm) for breeding, and pups were reared by both parents until weaning. Food and water were supplied *ad libitum*, and the environment was maintained at a constant temperature (24 ± 1°C) and humidity (50 ± 5%) under a 12 h light-dark cycle (lights on at 6 a.m.). All animal experiments were approved by the Ethical Committee of Azabu University (#180316-6).

The procedure to obtain EW mice (F1) was similar to our previous study [45]. Briefly, pregnant female mice were checked daily every morning until parturition. For each litter, the date of birth was designated as postnatal day 0 (PD0). On PD16, half of the litter was separated from each dam and assigned to the EW group. The remaining pups were assigned to the NW group, cared for with standard procedures, and weaned on PD28. The EW mice were fed powdered pellets until PD28. Thereafter, they were fed regular pellets, as were the NW mice after weaning. After weaning, 2 or 3 pups were kept together in cages according to their original group and sex. When both the EW and NW F1 female mice (EW-F1 and NW-F1, respectively) were 8 weeks old, each female was paired with a NW male mouse. All of the F2 litters were NW on PD28 and housed as described above. In the present study, F2 males from EW and NW F1 dams (EW-F2 and NW-F2, respectively) were studied in adulthood, because sex differences in nociception have been reported [46–48]. In addition, we utilized 4–8-month old mice in this study, based on the report indicating that there was no significant effect of age in adult mice (2–12-month-old) on pain sensitivity [49].

We first performed the formalin test using NW- and EW-F2 male offspring (34–39-week-old, NW-F2 offspring: n=9; EW-F2 offspring: n=7). Second, we examined gene expression levels in the lumbosacral and thoracolumbar DRGs of NW- and EW-F2 male offspring (22-week-old, NW-F2 offspring: n=6; EW-F2 offspring: n=3) which had not used in the formalin test. The mice were euthanized by cervical dislocation, and the lumbosacral (L3-S1) DRGs were harvested for gene expression studies.

Maternal behavior observations

To confirm the lower LG behavior of EW-F1 dams,

maternal behaviors of all F1 dams used in the present study (EW-F1: n=3; NW-F1: n=3) were digitally videotaped during six observation periods (60 min each) on the 7 day after postpartum. In addition to EW mice, the lower LG behavior until the first 10 days postpartum has been confirmed in the rat model of nongenomic transmission across the generation of the low maternal behavior [50]. All observation periods were performed during the light cycle (6 a.m.–7 a.m., 8 a.m.–9 a.m., 10 a.m.–11 a.m., 12 p.m.–1 p.m., 2 p.m.–3 p.m., 4 p.m.–5 p.m.). Within each observation period, the presence or absence of each behavior in 3 min segments were scored by a well-trained observer (20 observations/period × six periods=120 observations per dam). The observed maternal behaviors were LG, hovering and nursing, and no interaction with pups.

Formalin test

Adult male NW- and EW-F2 offspring were habituated for 60 min in an individual transparent Plexiglas cylinder. Thereafter, 2% formalin was subcutaneously injected into the plantar left hind paw (10 μl volume) of each mouse, and the behavior of each mouse was digitally videotaped for 30 min. The presence or absence of left hind paw licking/biting and self-grooming during periods of 5-s at 1-min intervals was scored using the Observer software (Noldus, Leesburg, VA). The early (acute) phase of the formalin test was defined as 0–5 min post-injection, and the late (tonic) phase as 10–30 min post-injection. The early phase of the formalin response is thought to be due to direct effects of formalin on nociceptive fibers, and the late phase is ascribed to inflammation [51, 52]. Data are presented as the percentage of licking/biting behavior exhibited in each phase by each mouse.

Analysis of mRNA levels in the trunk skin and DRGs

The lumbosacral (L3-S1) and thoracolumbar (T2-L2) DRGs were dissected for analysis of gene expression levels. The lumbosacral DRGs innervate hindlimb regions, and thoracolumbar DRGs innervate trunk regions [53]. Total RNA was isolated with the RNeasy Plus Micro Kit (QIAGEN, Venlo, Netherlands). Concentration and purity were assessed with a NanoDrop-1000 (Thermo Scientific Inc., MA, USA). Total RNA was reverse-transcribed and amplified by using a High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific Inc., MA, USA). The amount of messenger ribonucleic acid (mRNA) was quantitatively analyzed with the TaqMan gene expression assays, TaqMan Master Mix, and the 7500 Fast Real-time PCR system (Thermo Fisher Scientific Inc.). The TaqMan gene expression assays Mm00725448_s1 (Ribosomal protein P0, *Rplp0*), Mm01246302_m1 (Transient receptor potential vanilloid 1, *Trpv1*), Mm01227437_m1 (Transient receptor potential ankyrin 1, *Trpa1*), Mm01166996_m1 (Substance P, *SP*), Mm00801463_g1 (Calcitonin gene-related peptide, *CGRP*), and Mm01701887_g1 (Mas-related G-protein coupled receptor B4, *Mrgprb4*) were used. The amount of mRNA was normalized with that of *Rplp0* in individual samples [54–56]. For comparison between the NW- and EW-F2 offspring, data are shown as ratios relative to NW-F2 offspring values.

Statistical analysis

Results are expressed as means ± SE. All statistical analyses were performed using GraphPad Prism version 6.0 (GraphPad Software Inc., CA, USA). A two-way repeated measures analysis of variance (ANOVA) with Bonferroni post hoc test was performed to compare multiple groups. An unpaired *t*-test with two-tailed distribution was used to assess statistical significance where two group was compared. Spearman's correlation coefficient was used to assess associations. A P value <0.05 was considered statistically significant.

Results

Maternal behavior of normally-weaned or early-weaned F1 dams

We analyzed the maternal behavior of NW- and EW-F1 dams. As shown in Fig. 1, the percentage of LG behavior of EW-F1 dams was significantly lower than that of NW-F1 dams (P<0.05, EW-F1 dam: 18.07 ± 2.00%; NW-F1 dam: 25.28 ± 1.21%). However, there were no significant differences in the percentage of hovering and

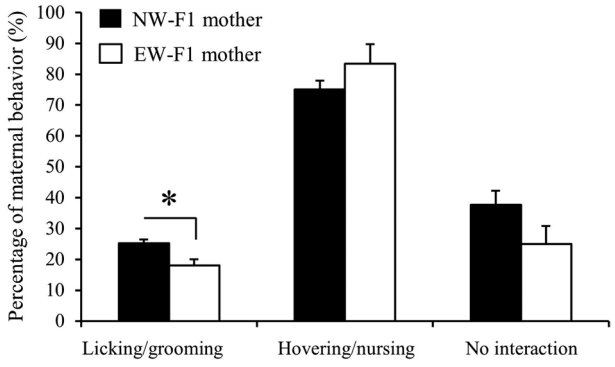

Fig. 1. Maternal behavior in normally-weaned (NW)- or early-weaned (EW)-F1 dams. The percentage of behavior of licking/grooming, hovering and nursing, and no interaction with pups in NW- (black) or EW-F1 dams (white). Data are presented as the mean ± SE (n=3) and were analyzed using an unpaired *t*-test with two-tailed distribution (*: P<0.05).

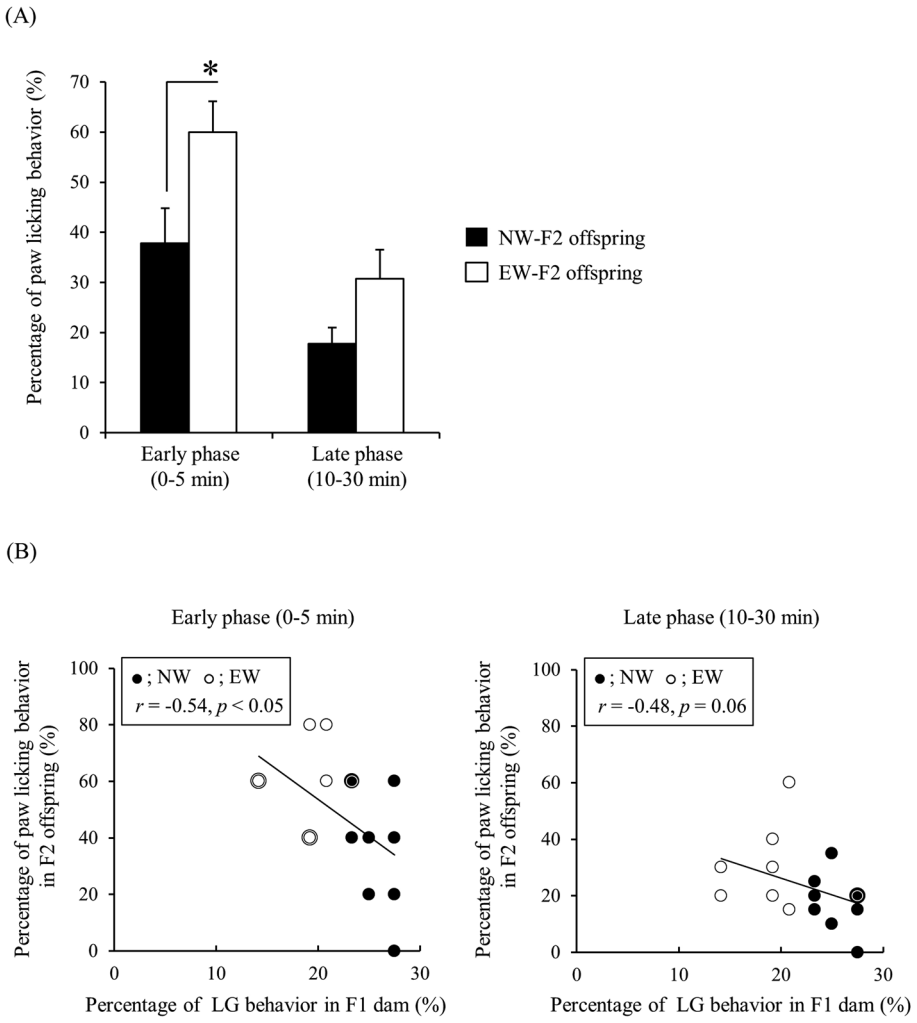

Fig. 2. Paw licking behavior of normally-weaned (NW)- or early-weaned (EW)-F2 offspring in the formalin test. (A) The percentage of paw licking behavior of NW- (black) or EW-F2 offspring (white) in the early and late post-experimental phases. Data are presented as the mean ± SE (n=7–9) and were analyzed using a two-way repeated measures ANOVA with post hoc Bonferroni analysis (*: $P<0.05$). (B) The correlation between percentage of licking and grooming (LG) behavior in F1 dams and percentage of paw licking behavior in F2 offspring. Black double circles indicate the data of overlapped NW-F2 offspring, and white double circles indicate the data of overlapped EW-F2 offspring.

nursing behavior (P=0.32, EW-F1 dam: 83.46 ± 6.35%; NW-F1 dam: 75.01 ± 2.92%) and no interaction with pups (P=0.16, EW-F1 dam: 25.00 ± 5.77%; NW-F1 dam: 37.79 ± 4.48%) between the two groups. The F2 male offspring of these F1 dams were used in the subsequent experiments.

Paw licking behavior evoked by formalin injection in adult F2 offspring of normally or early-weaned F1 dams

NW- and EW-F2 male offspring showed biphasic pain behavior evoked by formalin injection in the hind paw in agreement with previously published results [57]. A two-way repeated measures ANOVA detected a significant main effect of maternal care and phase (maternal care: F (1, 14)=7.372, $P<0.05$; phase: F (1, 14)=24.70, $P<0.05$), but there was no significant interaction between maternal care and phase (F (1, 14)=0.8767, P=0.37). Bonferroni post hoc analysis revealed that the percentage of paw licking behavior in EW-F2 offspring was significantly higher than that in NW-F2 offspring in the early phase ($P<0.05$, EW-F2 offspring: 60.00 ± 6.17%; NW-F2 offspring: 37.78 ± 7.03%, Fig. 2A). However, the difference between groups was not significant in the late phase (P=0.25, EW-F2 offspring: 30.71 ± 5.82%; NW-F2 offspring: 17.78 ± 3.24%, Fig. 2A). The correlation between percentage of LG behavior in F1 dam and percentage of paw licking behavior in F2 offspring was significant in the early phase (r=−0.54, $P<0.05$), but approached significance in the late phase (r=−0.48, P=0.06, Fig. 2B).

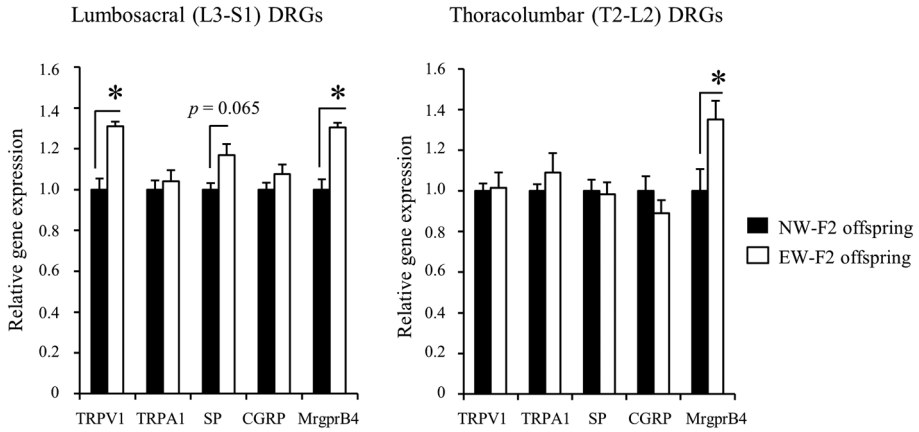

Fig. 3. Gene expression in the lumbosacral and thoracolumbar dorsal root ganglia (DRGs) of normally-weaned (NW)- or early-weaned (EW)-F2 offspring. Gene expression in the lumbosacral (L3-S1) and thoracolumbar (T2-L2) DRGs in NW- (black) or EW-F2 offspring (white). Gene expression data are shown as ratios relative to the mRNA levels in the NW-F2 offspring. Data are presented as the mean ± SE (n=3–6) and were analyzed using an unpaired *t*-test with two-tailed distribution (*: $P<0.05$).

Expression of mRNA in the lumbosacral and thoracolumbar DRGs in adult male F2 offspring of normally-weaned or early-weaned F1 dams

We quantified mRNA levels of genes involved in pain perception in the lumbosacral (L3-S1) DRGs projecting to the hindlimb skin, and thoracolumbar (T2-L2) DRGs innervating to the trunk skin. TRPV1 and TRPA1are major nociceptors of sensory neurons [27–31, 43, 44]. SP and CGRP are released from sensory neurons via activation of nociceptors, and induce neurogenic inflammation and central sensitization [32]. In the lumbosacral DRGs (Fig. 3), *Trpv1* mRNA levels in EW-F2 offspring were significantly higher than those in NW-F2 offspring ($P<0.05$, EW-F2 offspring: 1.31 ± 0.02; NW-F2 offspring: 1.00 ± 0.05). The *SP* mRNA levels were also higher in EW-F2 offspring than those in NW-F2 offspring, although the difference did not reach levels of significance ($P=0.065$, EW-F2 offspring: 1.17 ± 0.05; NW-F2 offspring: 1.00 ± 0.03). No significant difference was observed in *Trpa1* and *CGRP* mRNA levels between EW- and NW-F2 offspring (*Trpa1* [EW-F2 offspring: 1.04 ± 0.06; NW-F2 offspring: 1.00 ± 0.05], *CGRP* [EW-F2 offspring: 1.08 ± 0.05; NW-F2 offspring: 1.00 ± 0.03]). We also examined mRNA levels of the sensory neuron marker MrgprB4 in C-LTMR neurons [36] and found that *Mrgprb4* mRNA levels in EW-F2 offspring were significantly higher than those in NW-F2 offspring ($P<0.05$, EW-F2 offspring: 1.30 ± 0.02; NW-F2 offspring: 1.00 ± 0.05). In the thoracolumbar DRGs projecting to the trunk region (Fig. 3), there were no significant changes in *Trpv1*, *Trpa1*, *SP* and *CGRP* mRNA levels between the two groups (*Trpv1* [EW-F2 offspring: 1.02 ± 0.07; NW-F2 offspring: 1.00 ± 0.04], *Trpa1* [EW-F2 offspring: 1.09 ± 0.10; NW-F2 offspring: 1.00 ± 0.03], *SP* [EW-F2 offspring: 1.13 ± 0.07; NW-F2 offspring: 1.00 ± 0.05], *CGRP* [EW-F2 offspring: 0.89 ± 0.06; NW-F2 offspring: 1.00 ± 0.07]). In contrast, *Mrgprb4* mRNA levels in EW-F2 offspring were significantly higher than those in the NW-F2 offspring ($P<0.05$, EW-F2 offspring: 1.35 ± 0.09; NW-F2 offspring: 1.00 ± 0.11).

Discussion

In this study, we found that higher pain behavior, reflected by an increase in the paw licking behavior in formalin-injected adult male mice, is evoked in F2 offspring of EW-F1 dams, which show lower LG behavior than NW-F1 dams. This suggests that LG stimulation during the pre-weaning period plays an important role in the development of pain responses in male mice. In addition to the behavioral changes, the mRNA levels of TRPV1, a nociceptor in primary sensory neurons in the lumbosacral DRGs, were significantly higher in the offspring of EW-F1 dams. Similarly, mRNA levels of MrgprB4, which is expressed in the C-LTMR neurons perceiving the somatosensory inputs like LG stimuli, were significantly higher in the lumbosacral DRGs of F2 offspring of EW-F1 dams. Although it is not completely deniable that the early weaned-experience in the F1 dams had caused the epigenetic influences of their offspring's neurons in the DRG during the prenatal period, our results strongly suggest that postnatal LG stimulation has a developmental effect on an offspring's pain-related behavior and gene expression in the primary sensory neurons.

In the present study, EW-F1 dams showed lower LG

behavior than NW-F1 dams, although behavior of hovering and nursing and no interaction with pups was not different between them. This indicates that F2 offspring of EW-F1 dams are an appropriate model to examine the developmental effect of LG stimulation. Nociceptive behavior evoked by formalin injection into the hind paw in EW-F2 offspring was significantly higher than that in NW-F2 offspring in the post-injection early phase ($P<0.05$), but not in the late phase ($P=0.25$). Additionally, the correlation between percentage of LG behavior in the F1 dams and percentage of paw licking behavior in the early phase in F2 offspring was significant ($r=-0.54$, $P<0.05$). These results suggest that lower LG stimulation in the pre-weaning period induced higher pain responses in adult male mice. It has been demonstrated that naturally-occurring lower LG stimulation induces decreased withdrawal latencies to nociceptive stimulation in rat offspring [58]. These suggest that lower LG stimulation significantly impacts the development of pain sensitivity in rodents. In the formalin test, the early response phase is due to direct effects on nociceptive fibers, and the late phase is ascribed to inflammation [51, 52]. The result of significantly higher pain behavior in EW-F2 than in NW-F2 offspring in the early, but not in the late formalin post-injection phase suggests that lower LG stimulation might induce developmental changes in nociceptive sensory neurons.

Based on previous studies indicating that gene expression changes in nociceptive molecules in the peripheral sensory neurons are involved in altered behavioral nociceptive responses [59, 60], we examined the differences of gene expression in the DRGs between NW- and EW-F2 offspring. The significantly higher expression of *Trpv1* ($P<0.05$) in the lumbosacral DRGs projecting to the hindlimb skin and the higher expression of *SP* ($P=0.065$) in the EW-F2 offspring than those in NW-F2 offspring, suggest that LG stimulation may have a developmental impact on the expression of certain subsets of nociceptive molecules in the sensory neurons; the observed changes in gene expression may contribute to the pain hypersensitivity of EW-F2 offspring. In the thoracolumbar DRGs, mRNA levels of *Trpv1*, *SP*, *Trpa1*, and *CGRP* were not different between the NW-F2 and EW-F2 offspring. This difference in gene expression between lumbosacral and thoracolumbar DRGs could be because the molecules in the lumbosacral DRGs may have a predominant role in transmitting the internal or external harmful stimulation on the lower abdominal or hindlimb region. Interestingly, in the DRG sensory neurons innervating the urinary bladder, differential activations of TRPV1 among DRGs have been suggested based on the observation that the response of bladder lumbosacral DRG neurons to capsaicin was higher than that of the bladder thoracolumbar DRG neurons [61].

The present findings also raise the question of how LG stimulation during the pre-weaning period affects the development of pain signaling. It is thought that gentle stroking, as in LG stimulation, is perceived by peripheral receptors of C-LTMR neurons in the DRG [36, 37]. We currently do not know the precise mechanism for this and hypothesize that the activity of C-LTMR neurons during the pre-weaning period is involved. It has been suggested that in adult mice, increased C-LTMR activity results in elevated release of the chemokine-like secretion protein, TAFA-4, from central nerve terminals of the spinal dorsal horn that suppresses the activity of nociceptive neurons [38–40, 62]. It is possible that the activity of nociceptive neurons is higher in EW-F2 than in NW-F2 offspring during the pre-weaning period because of the lower C-LTMR activity produced by lower LG stimulation. An unknown epigenetic mechanism might underlie the frequent activation of nociceptive neurons during the pre-weaning period, which could lead to an increased expression of nociceptive molecules, such as TRPV1, in adulthood, and a subsequent induction of higher pain sensitivity. However, additional investigation is needed to test this hypothesis.

Here we observed that *Mrgprb4* expression was higher in the lumbosacral DRGs in EW-F2 offspring than that in NW-F2 offspring. Considering that the activation of C-LTMR neurons suppresses the activity of nociceptive neurons [38–40, 62], it is possible that the up-regulation of *Mrgprb4* levels in the lumbosacral DRGs of EW-F2 offspring acts as a compensatory change against the higher pain sensitivity. It has been previously shown that in the CNS, the descending pain inhibitory system from specific brain regions to the spinal cord is activated during peripheral pain inflammation [63, 64]. In addition to the CNS, the induction of a pain inhibitory system is also suggested by several reports showing that development of pain during cancer induces increased expression of Kv4.3, a voltage-activated A-type potassium ion channel, which have an inhibitory effect on pain sensitivity in the DRGs [65–67]. Although our gene expression data including nociceptive molecules has a potential limitation of a small sample size, *Mrgprb4* gene expression levels in the thoracolumbar DRGs were higher in EW-F2 offspring, which is consistent with previous results [42]. Further studies are needed to clarify the reason(s) and mechanism(s) responsible for the *Mrgprb4*+ neuron increase in EW-F2 offspring. In addition, it has been suggested that many other factors except for MrgprB4 are involved in the perception of tactile stimulation in the

DRG neurons [68–70]. In future, a detailed analysis of the transcriptome in the DRG in this animal model may delineate new insights into the developing tactile perception.

In summary, we revealed that lower maternal LG stimulation induces higher pain responses and up-regulates the expression levels of pain-related genes in the lumbosacral DRGs. This study has important implications for maternal LG behavior, suggesting an essential role of this behavior in the development of pain sensitivity.

Conflict of Interest

TS is an employee of the Kao Corporation.

Acknowledgments

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