Maternal prolactin during late pregnancy is important in generating nurturing behavior in the offspring

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Although maternal nurturing behavior is extremely important for the preservation of a species, our knowledge of the biological underpinnings of these behaviors is insufficient. Here we show that the degree of a mother's nurturing behavior is regulated by factors present during her own fetal development. We found that Cin85-deficient (*Cin85*$^{-/-}$) mother mice had reduced pituitary hormone prolactin (PRL) secretion as a result of excessive dopamine signaling in the brain. Their offspring matured normally and produced their own pups; however, nurturing behaviors such as pup retrieval and nursing were strongly inhibited. Surprisingly, when WT embryos were transplanted into the fallopian tubes of *Cin85*$^{-/-}$ mice, they also exhibited inhibited nurturing behavior as adults. Conversely, when *Cin85*$^{-/-}$ embryos were transplanted into the fallopian tubes of WT mice, the resultant pups exhibited normal nurturing behaviors as adults. When PRL was administered to *Cin85*$^{-/-}$ mice during late pregnancy, a higher proportion of the resultant pups exhibited nurturing behaviors as adults. This correlates with our findings that neural circuitry associated with nurturing behaviors was less active in pups born to *Cin85*$^{-/-}$ mothers, but PRL administration to mothers restored neural activity to normal levels. These results suggest that the prenatal period is extremely important in determining the expression of nurturing behaviors in the subsequent generation, and that maternal PRL is one of the critical factors for expression. In conclusion, perinatally secreted maternal PRL affects the expression of nurturing behaviors not only in a mother, but also in her pups when they have reached adulthood.

nurturing behavior | neglect | prenatal environment | prolactin | fetal brain

Nurturing behaviors are basic but important strategies for the continued survival of mammals. In rodents, nurturing behaviors are composed of a variety of complex behavioral patterns, including nesting, retrieval, licking, grooming, nursing, and crouching over pups to keep them warm (1). Prior research showed that the expression of nurturing behaviors is regulated by the dynamic changes in hormonal secretion during gestation and the perinatal period (2, 3). More recent research has shown that the nurturing environment provided by a mother can affect subsequent generations, impacting the expression of these behaviors in offspring (4). Because the expression of these behaviors depends on multiple factors operating over serial life cycles, elucidating underlying mechanisms is very challenging. Despite the growing number and rate of child maltreatment cases and the negative social impact (5), our understanding of the principles that govern nurturing behavior is inadequate. Further understanding the underlying biologic processes may help future prevention and interventions of child neglect (6).

Analysis of the dysfunction in nurturing behaviors in mutant or genetically modified organisms could help elucidate the mechanistic basis of these behaviors, with applicability to humans (7, 8). We previously generated a Cbl-interacting protein of 85 kDa (*Cin85*) KO mouse, which is defective in regulation of membrane receptor internalization (9). The *Cin85*-KO (*Cin85*$^{-/-}$) mice exhibited hyperactivity and neglect-like behavior. Hyperactivity is thought to be caused by excessive dopamine (DA) signaling as a result of defects in internalization of the D2 DA receptor (D2DR) in the striatum. However, we are not aware of any findings so far on the regulation of nurturing behaviors via endocrine or nervous system by CIN85. In this study, we investigated the expression of nurturing behaviors by analyzing the behavioral patterns in the offspring of *Cin85*$^{-/-}$ mice. As a result, we found that the expression of neglect behavior of *Cin85*$^{-/-}$ mice may be caused by a different mechanism than the excessive DA signaling thought to cause hyperactivity.

Results

Neglect-Like Behavior of *Cin85*$^{-/-}$ Mice Is Related to the Prenatal Environment. Litter size in *Cin85*$^{-/-}$ mice was similar than in WT; however, most *Cin85*$^{-/-}$ pups died by postnatal day (P) 2. Maternal mammary glands and milk production were found to be normal (Fig. S1), but no milk was found in stomachs of the pups. When the *Cin85*$^{-/-}$ pups were raised by WT foster mothers, they developed normally (Fig. S2). Together, these observations suggested that starvation and hypothermia were the

Significance

Maternal child neglect is an increasingly prevalent public health issue, but the underlying biologic processes causing this phenomenon are still largely unknown. This study examines the prenatal environment of mice that later exhibit neglectful behavior. We have found that, in mice, factors determining the neglect phenotype in a mother are present during her own fetal period. We present evidence that maternal prolactin (PRL) could be a key factor for generating nurturing behavior in offspring by activating neural circuits required for the expression of nurturing behaviors. Although PRL is known as a trigger for maternal behavior in dams, this is a function of PRL that has a transgenerational impact.

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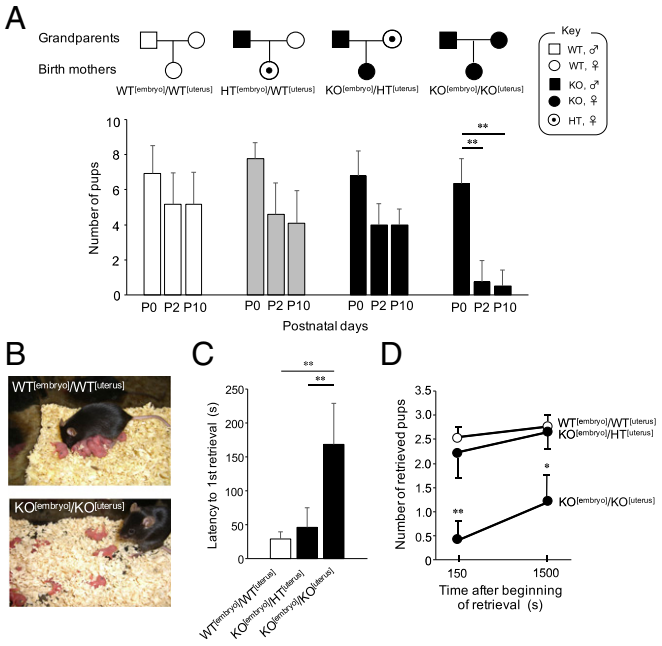

Fig. 1. Nurturing behavior is impaired in *Cin85*$^{-/-}$ mothers. (*A*) Number of surviving pups at P0, P2, and P10 born to mothers descended from parents with the indicated combinations of genotypes. WT (○, *Cin85*$^{+/+}$, WT$^{[embryo]}$/WT$^{[uterus]}$), HT (⊙, *Cin85*$^{+/-}$, HT$^{[embryo]}$/WT$^{[uterus]}$), and homozygous KO mice born to HT mice (●, KO$^{[embryo]}$/HT$^{[uterus]}$) exhibited nurturing behaviors, whereas homozygous KO mice born to homozygous KO mice (●, KO$^{[embryo]}$/KO$^{[uterus]}$) did not. Values presented as means ± SD (**$P < 0.01$ vs. P0, n = 14–17 mice). (*B*) Nurturing behaviors in WT (*Upper*) and KO (KO$^{[embryo]}$/KO$^{[uterus]}$, *Lower*) mice toward P1 pups. WT mice retrieved pups, kept them warm through crouching, and assumed an arched-back nursing position. KO mice exhibited none of these behaviors. (*C*) Time to retrieve the first of three pups in the retrieval test (**$P < 0.01$ vs. WT$^{[embryo]}$/WT$^{[uterus]}$ and KO$^{[embryo]}$/HT$^{[uterus]}$ mice, n = 10). (*D*) Numbers of pups retrieved at 150 s and 1,500 s in the retrieval test In WT$^{[embryo]}$/WT$^{[uterus]}$ mice (○), KO$^{[embryo]}$/HT$^{[uterus]}$ mice (●), and KO$^{[embryo]}$/KO$^{[uterus]}$ mice (●). Values presented as means ± SD (*$P < 0.05$ and **$P < 0.01$ vs. WT$^{[embryo]}$/WT$^{[uterus]}$ mice, n = 10).

cause of death, and that lack of maternal behaviors such as nursing and crouching to keep offspring warm were responsible for this result. When we compared WT (*Cin85*$^{+/+}$), heterozygous (HT; *Cin85*$^{+/-}$), and homozygous KO (*Cin85*$^{-/-}$) mice, we found that all WT and HT mothers, but only some KO mothers, exhibited nurturing behaviors. The behavior of a KO mouse could be attributed to its own mother's genotype (Fig. 1*A*). Homozygous KO birth mothers born to HT mothers (KO$^{[embryo]}$/HT$^{[uterus]}$) exhibited the same nurturing behaviors as adults as WT birth mothers (WT$^{[embryo]}$/WT$^{[uterus]}$). By contrast, homozygous KO birth mothers born to homozygous KO mothers (KO$^{[embryo]}$/KO$^{[uterus]}$) did not exhibit nurturing behaviors. Fig. 1*A* shows the survival rate of P10 pups as a function of the genotypes of their mother and grandmother: WT$^{[embryo]}$/WT$^{[uterus]}$, 74.7%; HT$^{[embryo]}$/WT$^{[uterus]}$, 52.8%; KO$^{[embryo]}$/HT$^{[uterus]}$, 58.8%; and KO$^{[embryo]}$/KO$^{[uterus]}$, 8.34%. Although KO$^{[embryo]}$/KO$^{[uterus]}$ mice did not exhibit cannibalistic behaviors, they failed to exhibit postnatal nurturing behaviors such as retrieving, crouching, and nursing, leading to the death of their pups (Fig. 1*B*). In a retrieval test, KO$^{[embryo]}$/KO$^{[uterus]}$ mice took 5.8 times longer than WT$^{[embryo]}$/WT$^{[uterus]}$ mice to retrieve the first pup back to the nest (Fig. 1*C*). WT$^{[embryo]}$/WT$^{[uterus]}$ and KO$^{[embryo]}$/HT$^{[uterus]}$ mothers could return almost all pups to their nest within 150 s, whereas KO$^{[embryo]}$/KO$^{[uterus]}$ mothers were not able to complete the task even after 1,500 s (1.2 ± 0.6 pups; Fig. 1*D*). These results suggest that the nurturing behavior of a birth mother mouse depends on whether her own mother had a functional copy of *Cin85*, regardless of her genotype or the postnatal environment. Therefore, we surmised that the prenatal environment is responsible for this phenotype.

To directly evaluate the influence of the prenatal environment, we performed embryo transplantation experiments (Fig. 2*A*). KO embryos were transplanted into the fallopian tubes of WT surrogate mothers, and, conversely, WT embryos were transplanted into the fallopian tubes of KO surrogate mothers. To standardize conditions unrelated to gestation, experienced WT foster parents raised all pups. Nurturing behaviors in 12-wk-old female mice born through the embryo transplant were observed after giving birth to their own litter. We found that, despite the WT genotype of the embryos, offspring born to KO surrogate mothers (WT$^{[embryo]}$/KO$^{[uterus]}$) had a nurturing rate (i.e., the proportion of mothers that exhibited nurturing behavior) of 22.2%, with a P10 survival rate of 16.1% (Fig. 2*B*). Conversely, KO pups born from WT surrogate mothers (KO$^{[embryo]}$/WT$^{[uterus]}$) had a nurturing rate of 100% and a survival rate of 76.6% (Fig. 2*C*). A plot of the survival of pups from P0 to P10 suggests that nurturing behavior is dependent on the

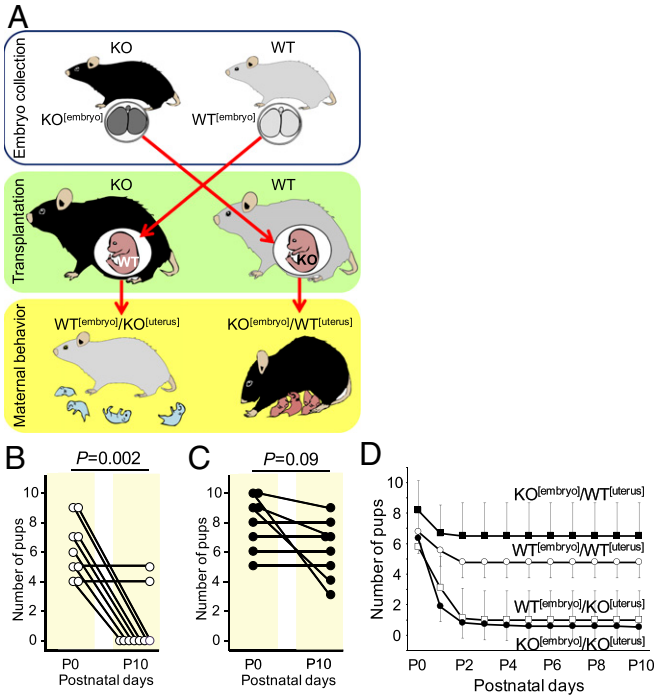

Fig. 2. Intergenerational effect of the prenatal environment using embryo transfer. (*A*) Summary of embryo transfer. Female KO or WT mice were prepared as embryo donors by i.p. injection with CGs. After mating, embryos were retrieved from these females. Surrogate WT or KO mice were mated to sterilized WT males. Retrieved KO embryos were inserted into the fallopian tubes of WT surrogate mothers; similarly, retrieved WT embryos were inserted into KO surrogate mothers. Twelve-week-old females born via transplantation were mated to males of the same genotype, and nurturing behaviors were observed. (*B*) Of the nine WT$^{[embryo]}$/KO$^{[uterus]}$ mice, only two exhibited nurturing behaviors. The number of pups significantly decreased from P0 to P10 (means ± SD; ○, **$P = 0.002$, n = 9). (*C*) Of the eight KO$^{[embryo]}$/WT$^{[uterus]}$ mice, eight females exhibited nurturing behaviors. There was no significant difference in the number of surviving pups between P0 and P10 (means ± SD; ●, P = 0.09, n = 8). (*D*) Exchanging the uterine environment drastically altered the nurturing behaviors of WT$^{[embryo]}$/WT$^{[uterus]}$ and KO$^{[embryo]}$/KO$^{[uterus]}$. When the numbers of surviving pups were compared between P0 and P10, WT$^{[embryo]}$/WT$^{[uterus]}$ and KO$^{[embryo]}$/WT$^{[uterus]}$ followed similar curves. On the contrary, WT$^{[embryo]}$/KO$^{[uterus]}$ and KO$^{[embryo]}$/KO$^{[uterus]}$ exhibited similar defective nurturing behaviors. The number of pups decreased from P0 to P2 regardless of phenotype; however, pups seldom died after P3.

prenatal environment (Fig. 2*D*). These results suggest that the prenatal environment is extremely important for the transgenerational expression of nurturing behaviors.

Impairment of Prolactin Secretion in *Cin85*$^{-/-}$ Mice During Late Pregnancy. What is the underlying factor responsible for the differences in the prenatal environment between WT and KO mice? We examined the possibilities for several molecules involved in the expression of nurturing behaviors, such as prolactin (PRL) and placental lactogen (PL). PRL is known to affect maternal behavior, and is regulated via D2DR signals (10). Thus, we first compared plasma PRL concentrations in WT, HT, and KO at 3 mo of age (virgin, diestrus), on the day before parturition [day 19 of gestation (G19)] and on the day of parturition (i.e., P0; Fig. 3*A*). PRL concentrations did not differ significantly among the three groups of virgin mice. However, on G19 and P0, we did not observe the normal increase in PRL concentrations in KO mice that was seen in WT and HT mice (17.2% and 19.4% of the WT concentration, respectively). Based on these results, we hypothesized that maternal PRL levels during late pregnancy affect the expression of nurturing behaviors in offspring. We then measured the pituitary contents of DA and PRL on G19 (Fig. 3*B*). The pituitary DA content in KO mice was 160% of that of WT mice (*Left*). By contrast, the pituitary PRL content in KO mice was 14.3% of that of WT mice (*Right*). The results suggest that chronic and excessive DA signals in the pituitary of KO mice suppressed PRL production.

PL secreted from the placenta has a high affinity for PRL receptors (PRL-R) (11) and could mimic the actions of PRL, including priming the pregnant female's brain at the end of gestation (12). To investigate whether this could be interfering with our results, we measured plasma PL levels from parturient mothers of WT and KO (G19). Levels of PL were 197.9 ± 30.3 ng/mL and 165.9 ± 29.7 ng/mL, respectively; there was no significant difference between them (Fig. S3). This suggests that the loss of CIN85 does not affect PL levels, and it did not contribute to neglect behavior of CIN85 KO mice.

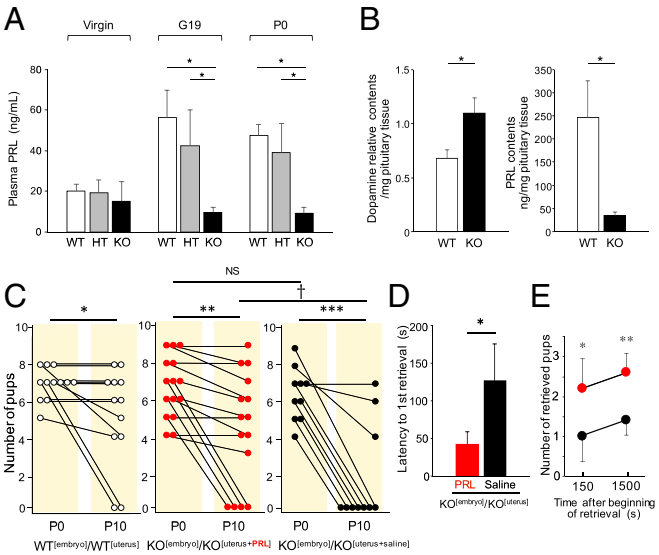

Fig. 3. Administration of PRL to the mother rescues nurturing behaviors in the offspring. (*A*) Plasma PRL concentrations were measured by ELISA in virgin female mice (12 wk old, at 12:00 PM in diestrus, virgin), on the day before parturition (G19) and on the day of parturition (P0): WT mice (□, $n = 5$–8), HT mice (gray □, $n = 3$–7), and KO mice (■, $n = 6$–7). Values presented as means ± SE (*$P < 0.05$). (*B*) Measurement of DA and PRL contents in the pituitary of G19 mice. Pituitary DA (*Left*) and PRL (*Right*) were assayed by HPLC and ELISA, respectively: WT mice (□, $n = 4$) and KO mice (■, $n = 4$). Values presented as means ± SE (*$P < 0.05$). (*C*) Changes in the number of surviving pups born to WT$^{[embryo]}$/WT$^{[uterus]}$ (○, $n = 12$), KO$^{[embryo]}$/KO$^{[uterus+PRL]}$ (red ●, $n = 13$), and KO$^{[embryo]}$/KO$^{[uterus+saline]}$ (●, $n = 10$) mice at P0 (paired t test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$; Mann–Whitney test, $^{\dagger}P < 0.05$; NS, not significant). (*D*) Time to retrieve the first of three pups to the nest in the retrieval test. The time to retrieve the first pup at P0 was reduced by 33.9% in KO$^{[embryo]}$/KO$^{[uterus+PRL]}$ mice (red □) relative to KO$^{[embryo]}$/KO$^{[uterus+saline]}$ (■) mice. Values presented as means ± SD (*$P < 0.05$, $n = 6$). (*E*) Numbers of pups retrieved at 150 s and 1,500 s. KO$^{[embryo]}$/KO$^{[uterus+saline]}$ mice (●) did not retrieve their pups at 150 s (1.0 ± 0.6 pups) or 1,500 s (1.4 ± 0.4 pups); however, KO$^{[embryo]}$/KO$^{[uterus+PRL]}$ mice (red ●) retrieved their pups at 150 s (2.2 ± 0.7 pups) and 1,500 s (2.6 ± 0.5 pups). Values presented as means ± SD (*$P < 0.05$, $n = 5$).

Administration of PRL Rescues Maternal Behavior and the Neural Activation in Offspring. In mice, PRL concentration surges rhythmically during the first half of pregnancy. It then remains at a basal level until it drastically increases in the last 2–3 d of pregnancy (3, 13). To compensate for the late-pregnancy surge, we administered 70 ng/g body weight of PRL s.c. twice daily (at 9:00 AM and 5:00 PM) to pregnant KO mice from G15 until delivery at approximately G20. This treatment increased plasma concentrations of PRL to the normal WT range (Fig. 3*A* and Fig. S4). Experienced WT foster parents raised the pups born to these mothers. We then observed nurturing behaviors in 12-wk-old female offspring that were mated to KO males and subsequently had pups. The nurturing rate of KO$^{[embryo]}$/KO$^{[uterus+PRL]}$ mice born to PRL-administered KO mice was 73.3%, with a P10 survival rate of 65.0% (Fig. 3*C*, *Middle*). By contrast, KO mice administered a saline solution as a control (KO$^{[embryo]}$/KO$^{[uterus+saline]}$) had a nurturing rate of 30.0% and a P10 survival rate of 26.6% (Fig. 3*C*, *Right*). The number of offspring born to KO$^{[embryo]}$/KO$^{[uterus+PRL]}$ mice did not differ significantly from KO$^{[embryo]}$/KO$^{[uterus+saline]}$ mice at P0. However, at P10, the number of surviving offspring born to KO$^{[embryo]}$/KO$^{[uterus+PRL]}$ mice was significantly higher than in KO$^{[embryo]}$/KO$^{[uterus+saline]}$ mice (Mann–Whitney test, $P = 0.044$). In the retrieval test, the time required to return the first pup to the nest was significantly shorter in KO$^{[embryo]}$/KO$^{[uterus+PRL]}$ mice than in KO$^{[embryo]}$/KO$^{[uterus+saline]}$ mice (Fig. 3*D*). Furthermore, KO$^{[embryo]}$/KO$^{[uterus+PRL]}$ mice retrieved more pups at 150 s and 1,500 s than KO$^{[embryo]}$/KO$^{[uterus+saline]}$ mice (Fig. 3*E*). Together, these results strongly suggest that maternal PRL in the prenatal environment is an important factor in the transgenerational expression of nurturing behaviors.

Additionally, we investigated whether maternal PRL during the fetal period affects the activity of neural circuits required for the expression of nurturing behaviors in pups. Accumulating evidence suggests that the medial preoptic area (MPOA)–ventral tegmental area (VTA)–nucleus accumbens (NA)–ventral pallidum (VP) neural circuit is vital to nurturing behaviors in mice and rats (2, 14) (Fig. 4*A*). In this study, we focused on the MPOA–NA–VP axis of this circuitry. The MPOA contains a variety of hormone receptors, including PRL-Rs that receive hormonal input in addition to sensory cues from pups via the senses of smell, hearing, and touch. The NA contains D1 and D2DR and receives projections from mesolimbic dopaminergic neurons in the VTA. The VP is responsible for output of nurturing behaviors (14) (Fig. 4*A*). In mice, the rate of newborn mortality decreased after P2 regardless of phenotype (Fig. 2*D*). We surmised that P2 is a key period that determines the success or failure of nurturing. Therefore, we temporarily separated pups from mothers and used c-Fos expression as an indicator of neural activity of the MPOA–NA–VP axis upon reuniting pups with mothers on P2. We observed a significant decrease in the number of c-Fos–positive cells in MPOA–NA–VP axis neurons in KO (KO$^{[embryo]}$/KO$^{[uterus]}$) mice compared with WT (WT$^{[embryo]}$/WT$^{[uterus]}$) mice (Fig. 4 *B* and *C*). By contrast, KO mice born to KO mothers injected with PRL (KO$^{[embryo]}$/KO$^{[uterus+PRL]}$)

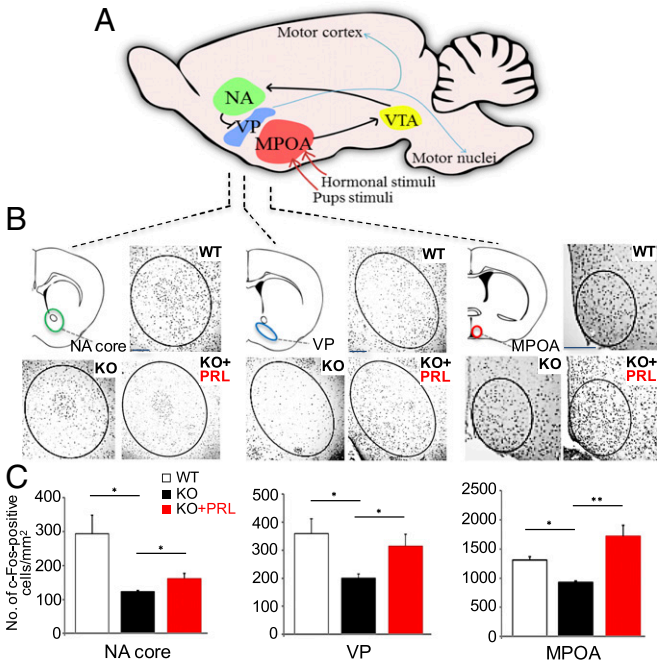

Fig. 4. Maternal PRL affects the fetal brain and activates the neural circuitry required for nurturing behaviors in adulthood. (*A*) Summary of the MPOA–VTA–NA–VP circuit in rodents. Activation of the circuit starts with detection of hormone and pup stimuli by the MPOA, and proceeds to output (i.e., nurturing behavior) from the VP. (*B*) Detection of c-Fos–positive cells by immunohistochemistry. After exposure to pups for 20 min, mothers were perfused, and coronal sections were made of the brain, including NA (*Left*), VP (*Middle*), and MPOA (*Right*). Distances from bregma to NA, VP, and MPOA were 1.09–1.33, 0.61–0.73, and −0.11 to 0.1 mm, respectively. Schematic map of the coronal section corresponding to each nucleus (green, NA core; blue, VP; red, MPOA) is shown in the top left corner of each panel. Ellipses were drawn in each section (modified from ref. 47), and the number of c-Fos–positive cells in the area were counted. (Scale bar: 200 μm.) (*C*) Graph shows the density of c-Fos–positive cells per square millimeter in each slice. The number of c-Fos–positive cells was lower in KO mice than in WT mice. However, PRL injection during pregnancy increased this number to the normal range in all three nuclei (NA, VP, and MPOA; $*P < 0.05$ and $**P < 0.01$ by Student's *t* test, $n = 4$).

exhibited a significant increase in the number of c-Fos–positive cells compared with those born to KO mothers that did not receive PRL injections. These observations suggest that neglect-like behavior in $KO^{[embryo]}/KO^{[uterus]}$ mice is associated with reduced activity in the MPOA–NA–VP axis, and that maternal PRL activates the neural circuitry in fetal brains that is associated with nurturing behavior in mature adults.

Maternal PRL Reaches the Fetal Brain. It is possible that the administered PRL had direct effects on the fetal brain, although we are not aware of any previous studies that demonstrate that maternal PRL crosses the placenta. To determine whether exogenous PRL was present in the brains of fetuses, we traced radioactively labeled [^{125}I]PRL administered intraperitoneally to mice during late pregnancy. To negate the possibility that [^{125}I]PRL would be maternally metabolized and lead to accumulation of only ^{125}I in fetal brains, to avoid detection of free ^{125}I, we performed autoradiography of SDS/PAGE gels of fetal brain extracts and inferred that a 23-kDa protein corresponding to the molecular weight of [^{125}I]PRL was transferred from the mother. The autoradiographs revealed that [^{125}I]PRL indeed accumulated in the brains of fetuses: 0.90% and 0.24% of the input amount was detected in placenta and fetal brain, respectively (Fig. S5).

Discussion

There are two major findings in this study. First, the discovery of an in utero maternal process that suppressed offspring nurturing behaviors reaffirms that the fetal stage is a crucial period for behavior development. This also suggested the existence of important mediators required for the offspring to develop a maternal brain, which lead to our second finding. PRL administration during late pregnancy rescued nurturing behavior in offspring and was found to activate behavior-related neural circuitry. These results imply that maternal PRL is an important factor that governs the transgenerational expression of nurturing behaviors.

The transgenerational effect of the prenatal environment on brain function and behavior was first described in the 1970s (15) and has been studied since then. However, the literature on the relationship between the prenatal environment and future nurturing behaviors is limited. For example, administration of benzodiazepines (16) or lipopolysaccharides (17) or stress loads (18–20) in rodents during gestation suppresses nurturing behavior in offspring. Although these studies showed interesting findings, there were limitations. For example, in some studies, the influence of gestational stressors on the fetuses were not separated from their influence on mothers. Perinatal stress can suppress the mother's nurturing behavior and indirectly affect the infant brain development (21). Therefore, to evaluate the function of the uterine environment as a generator of nurturing behavior in offspring, a method other than applying prenatal stressors was needed (22). Embryonic transplantation between inbred strains with different phenotypes serves as a powerful tool for analyzing the effects of the prenatal environment (22, 23). This approach has been employed to study the effect of the prenatal environment on postnatal brain functions such as anxiety control (24, 25). We used this technique to study the maternal effect of the prenatal environment on nurturing behavior in mice. Our results clearly demonstrated that the fetal stage is important in determining the expression of nurturing behaviors in offspring; in particular, WT uteruses enabled the expression of normal nurturing behaviors in *Cin85*$^{-/-}$ offspring with defects in PRL secretion (Fig. 2 *C* and *D*). Additionally, we showed that changes in the prenatal environment alone were sufficient to impair subsequent nurturing behaviors (Fig. 2 *B* and *D*). These observations indicate that further analyses of fetal stages may allow us to elucidate the mechanisms causing dysfunctions in nurturing behavior, including neglect-like behavior.

In the aforementioned previous study (9), we concluded that the cause of hyperactivity phenotype in CIN85-KO mice is an excess of the striatal DA signal as a result of impaired endocytosis of the DA receptor. Initially, we thought that there may be a common molecular basis for hyperactivity and neglect behavior, and we focused on DA as a potential key factor in determining the prenatal environment. There are four main dopaminergic pathways: the nigrostriatal, the tuberoinfundibular, the mesolimbic, and the mesocortical pathways (26). The nigrostriatal pathway is implicated in movement because degeneration of these neurons has been shown to cause Parkinson's disease (27). This region is also important in feeding behaviors (28). The tuberoinfundibular pathway (i.e., TIDA neurons) is projected from the hypothalamus to the median eminence and controls PRL secretion from the anterior pituitary gland (29). The mesolimbic pathway is implicated in reward and pleasure. The mesocortical pathway is involved in cognition and emotion. It is worth examining each dopaminergic pathway, but, of the four, we chose to study the tuberoinfundibular pathway because it seems most involved in the neglect-like behavior phenotype of CIN85-KO mice. Then, we focused on PRL, a lactogenic hormone regulated by DA downstream of this pathway, known to be directly involved in nurturing behavior. Certainly, plasma and pituitary PRL levels are low in CIN85-KO mice (Fig. 3 *A* and *B*, *Right*). Thus, in this instance, the DA content of the pituitary should also have been low, but our assay found significantly

higher DA content (Fig. 3*B*, *Left*). Taken together, it could be hypothesized that the neglect-like behavior in this study was caused by another mechanism that is compatible with decrease of PRL secretion and the increase of DA signal, rather than the logical DA–PRL signaling pathway we initially considered. To determine the details of this discrepancy, it will be necessary to clarify DA secretion from TIDA neurons and the dynamics of DA receptors in pituitary PRL-producing cells (i.e., lactotrophs).

We were able to pinpoint maternal PRL as a key factor involved in determining the prenatal environment. Bridges et al. identified a role for PRL in the induction of maternal behavior in rats (30) and demonstrated that PRL acted in the MPOA to stimulate this behavior by using steroid-treated nulliparous rats (31). It is also known that PRL surges early in pregnancy and promotes neurogenesis in the forebrain subventricular zone, which is involved in nurturing behaviors in mothers (10, 32). Our results, combined with those from previous studies, demonstrate that maternal PRL can affect the nurturing behaviors of mothers and their female offspring.

Although the results of the present study suggest a novel function of PRL, there are limitations because detailed physiological mechanisms of mother–offspring PRL are still unknown. Although our method of giving PRL to CIN85-KO mice adequately compensated for the lack of a prepartum PRL surge, the 5-d injection period might have exceeded the normal window of secretion (3). If this was the case, it is possible that the prolonged PRL treatment itself rescued the neglect-like behavior phenotype. Second, it is known that the dominant ligand for PRL-R during late pregnancy is PL (13). Little is known about the physiologic role of preparturition PRL, as PRL is overwhelmed by the high concentration of PL until the placenta is expelled from the uterus. However, because the preparturition PL level in CIN85-KO mice was normal (Fig. S3), we suspect that maternal behavior in the offspring is actually affected by PRL. We hypothesized that a "low-PRL window"—in other words, a lack of a preparturition PRL spurt—causes a deficit in nurturing behavior in the offspring. Future studies related to preparturition PRL will help us understand the unique functions of PRL.

Alongside why preparturition PRL is important, how PRL alters the fetal brain is also a crucial question. The present study shows that maternally injected PRL promotes the activation of neural circuitry associated with nurturing behaviors in adulthood (Fig. 4 *B* and *C*). There are two potential mechanisms: direct PRL-R stimulation in the fetal brain and/or an indirect PRL signaling pathway that influences neural development. Indirect PRL pathways are broadly possible because lactogenic hormones modulate many pregnancy-dependent changes, from metabolism to stress responses (10, 33). PRL-R agonists interact with other important hormone systems that can influence a developing brain (13, 33). To support the direct PRL-R stimulation hypothesis, Fig. S5 shows that a small amount of maternally injected PRL was detected in the fetal brain. However, we are not certain such a small quantity of PRL is enough to activate PRL-R in a fetal brain. Second, although there are PRL-Rs expressed in fetal brains (34), expression is scarce from E18.5 to early postpartum mouse brains according to the Allen Mouse Brain Atlas (35). Third, little is known how PRL and PL access entry into the brain. It has been understood that PRL and PL bind to the PRL-R of the choroid plexus and are transported into the brain through cerebrospinal fluid (36). Recently, however, Brown et al. (37), by using PRL-R–deficient (PRL-R$^{-/-}$) mice and [^{125}I]PRL, reported that the PRL-R is not required for transport of PRL into the brain. Although the odds for the direct theory seem low, a study on PRL-deficient (PRL$^{-/-}$) (38) and PRL-R$^{-/-}$ mice (39) support the idea. Both of these strains of mice are sterile as a result of dysfunctional implantation to the uterus; however, in maternal induction tests of nulliparous mice, PRL$^{-/-}$ mice exhibit completely normal behavior, whereas PRL-R$^{-/-}$ mice do not. These phenotypic differences are attributed to the fact that PRL$^{-/-}$ mice can receive maternal PRL or PL during the fetal stage to compensate for their deficiency, whereas PRL-R$^{-/-}$ mice cannot receive lactogenic hormones from any source (1). This evidence strongly implies that PRL-Rs are necessary to develop maternal behavior and also suggest that maternal lactogenic hormones directly binding to fetal PRL-R is important.

Many previous studies reported the involvement of the MPOA–VTA–NA–VP neural circuitry in nurturing behaviors (14). Our results with c-Fos not only support our conclusions deatiled here earlier, but also show that maternal PRL contributes to the activation of this pathway. In mice, nurturing behavior such as retrieving can be displayed by males, but not by all males. This paternal behavior is reported to be controlled by the same neural circuitry; therefore, it is possible that prenatal PRL also determines a father's parenting behaviors (40). Because the PRL-R gene is intensely expressed in the rat embryonic olfactory bulb (41), there might be a relationship between neglect and the in utero development of the olfactory bulb system by PRL.

Similar to our *Cin85*$^{-/-}$ mice, DA transporter-deficient mice (DAT$^{-/-}$) also exhibit nurturing behavior dysfunctions (42, 43); however, these studies did not discuss the maternal effect of nurturing behavior in offspring. Although DAT$^{-/-}$ mice are phenotypically different from *Cin85*$^{-/-}$ mice, both mutants exhibit hyperactivity and reduced PRL secretion, suggesting that dysfunctions of nurturing behavior in DAT$^{-/-}$ and *Cin85*$^{-/-}$ mice occur via similar mechanisms. Unnatural DA signaling in multiple dopaminergic pathways may cause other transgenerational behavior defects aside from the DA–PRL axis. DA signals in the brain are diverse and intricately linked to other molecules such as PRL. Future studies of nurturing behavior need to analyze the transgenerational effects of DA.

The mechanisms controlling expression of nurturing behaviors are inarguably complex, and a complete understanding requires assembly of all of the pieces of a large puzzle. Our finding that maternal PRL regulates the expression of nurturing behaviors in offspring should facilitate elucidation of novel factors and mechanisms involved in the expression of these behaviors.

Materials and Methods

Detailed materials and methods are described in *SI Materials and Methods*.

Animals. Generation of *Cin85*$^{-/-}$ mice and genotyping by PCR are described in detail in our previous report (9). The animal experimentation protocol used for this study was approved by the animal care and experimentation committee of Gunma University Showa Campus. Mice were kept in animal facilities under standard laboratory conditions.

Behavioral Observation. To evaluate nurturing behavior toward pups, analysis of arched-back nursing was performed from P0 to P10. Pup-retrieving behavior by mothers was measured on P1. The mother was moved to another cage for 10 min, and then three pups were placed in three corners of the home cage. The test began by returning the mother to the empty corner of the home cage. The times of collection of pups were monitored during a 1,500-s observation period. To determine whether PRL results in improvements in maternal behavior, pregnant *Cin85*$^{-/-}$ (KO$^{[embryo]}$/KO$^{[uterus]}$) mice were s.c. injected with recombinant mouse PRL (70 ng PRL per gram body weight, 200 μL per injection) or an equivalent volume of saline solution twice daily (9:00 AM/5:00 PM) from day 15 of pregnancy to P0. Female offspring born to PRL-injected mothers (KO$^{[embryo]}$/KO$^{[uterus+PRL]}$) were mated at 12 wk of age. Nurturing behaviors were observed from P0 to P10, and pup-retrieval tests were performed on P1.

Reciprocal Embryo Transfer. Fertilized embryos from superovulated female mice of both strains (WT and KO) were induced by i.p. injection of a donor female with equine chorionic gonadotropin (CG). Two days later, human CG was injected (i.p.), and each female was paired with a male of the same genotype. When a vaginal plug had been confirmed, the embryos were harvested from the oviduct on the following day. Twenty embryos from KO mice were transferred into WT surrogate mothers via a laparotomy incision through the opening of the oviduct. Similarly, embryos from WT were transferred into KO surrogate mothers. Surrogate mothers were prepared by naturally mating them with vasectomized WT males. Two types of offspring were obtained in this experiments: WT$^{[embryo]}$/KO$^{[uterus]}$ and KO$^{[embryo]}$/WT$^{[uterus]}$. Twelve weeks later, female offspring were mated with a male of the same genotype and impregnated. After delivery, the numbers of living offspring were counted on P0 and P10.

Measurement of DA. Dissected pituitaries of pregnant mice (G19) of both genotypes (WT and CIN85-KO) were homogenized with perchloric acid. DA levels of homogenate were determined by using HPLC with a reverse-phase analytical column. DA was detected with a graphite carbon detector electrode.

Measurement of PRL and PL. For measurement of plasma PRL and PL levels, blood was obtained from the tails of WT, HT, and KO mice at 3 mo of age (virgin, at 12:00 PM in diestrus) on the day before parturition (i.e., G19) and on P0. To determine the PRL contents of the pituitary gland, PRL was extracted from the pituitary gland of G19 mice by using 1 M urea/PBS solution. PRL and PL levels were measured by using a commercially available ELISA kit.

Detection of [^{125}I]PRL in the Fetal Brain. Pregnant WT and KO mice received i.p. injection of [^{125}I]PRL (74 kBq, 50 ng PRL, 200 μL per injection) twice per day (9:00 AM/5:00 PM) from G15 to G18. On G19, placenta and fetal brain were removed and homogenized. The supernatant of homogenate was loaded onto a SDS/polyacrylamide gel and electrophoresed. Separated proteins were autoradiographed.

Immunohistochemical Staining. Before immunohistochemical study, each mother was separated from her pups for 18 h on the day after delivery. After exposure to pups for 20 min, the mother was killed, and the brain was fixed. Paraffin sections were generated, incubated with anti–c-Fos antibody, and then incubated with streptavidin–biotin–peroxidase solution. The immunoreaction was visualized by peroxidase–diaminobenzidine reaction. The resultant images were analyzed by using ImageJ software [National Institutes of Health (NIH)].

Mammary Whole-Mount Preparation. Skin with mammary glands was collected from female mice at 3 mo of age (virgin) and at P0 and fixed in 10% neutral buffered formalin. The mammary glands were then dissected from the skin and processed as a whole mount. After defatting, the mammary glands were stained with hematoxylin and mounted.

Measurement of β-Casein. For measurement of β-casein mRNA levels, RNA was extracted from the mammary glands of WT and KO mice at 3 mo of age (virgin) and on P0. Specific primers used for real-time PCR of β-casein were as follows: sense, 5′-ggcccaagagatggcacca-3′; antisense, 5′-tcactccagcatccagtcaca-3′. To detect β-casein protein, mammary glands were homogenized and separated by SDS/PAGE. Immunoblotting was performed with anti–β-casein antibody and the HRP chemiluminescence system.

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