

## Comparison of Parental Behavior and Offspring's Anxiety Behavior Using a Reciprocal F1 Hybrid Model

Makiko OZAWA<sup>1)</sup>, Takefumi KIKUSUI<sup>1,2)\*</sup>, Yukari TAKEUCHI<sup>1)</sup> and Yuji MORI<sup>1)</sup>

<sup>1)</sup>Laboratory of Veterinary Ethology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657 and <sup>2)</sup>Companion Animal Research, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagami-hara, Kanagawa 229-8501, Japan

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**ABSTRACT.** Emotional behavior can be characterized by interactions between genes and environments, especially in the developmental period. However, in mice, the effects of paternal care on offspring's emotional development are not well understood even in cases in which the male mice show stable paternal care to the pups. In this study, we used a reciprocal hybrid model of C57BL/6 and BALB/c mice, which show different parental behaviors and emotional responses, and compared the emotional responses in pups in their adulthood by using the elevated plus-maze test. It was found that inbred male mice showed intensive parental behavior toward their pups, especially as parental contact behavior, and that the licking/grooming behavior of males was dependent on the strain of the female they were paired with. Also, BALB/c pairs showed less parental contact behavior than C57BL/6 pairs. In the elevated plus-maze test, BALB/c males and females showed higher anxiety behavior, and reciprocal hybrid offspring showed intermediate values between C57BL/6 and BALB/c mice. Although no significant difference was found in the parental care that offspring received and anxiety-related behavior between the two reciprocal hybrid strains, BALB/c pairs showed less parental contact behavior toward the pups as compared to C57BL/6 pairs, which would be associated with the higher anxiety-related behavior exhibited in the adulthood of BALB/c strain.

**KEY WORDS:** anxiety, maternal care, mice, paternal care, reciprocal F1 hybrid.

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Social environments during the early stages of life, in addition to genetic variations, have long-lasting influences on the behavioral development of individuals. The mother–pup interaction has especially strong influences on the behavioral characteristics of rodent offspring in their adulthood [6, 26]. For example, anxiety and maternal behavior are associated with maternal care offspring received in their infant period [8, 27]. Another thing is that maternally deprived pups show persistent stress-endocrine response, suggesting deficiencies in their negative feedback system [32]. We have shown that early weaned mice and rats deprived of maternal care in the neonatal period as well as in the postnatal period of day 15 to day 21 display reduced maternal behavior [23] and increased anxiety-related behavior [20, 21]. Classic studies in mice kept in semi-natural environments showed bi-parental care and monogamous breeding colonies [9, 31], indicating that not only maternal care, but also paternal care comprises important social stimuli for the developing pups. It is unfortunate that the effects of paternal care on the behavioral development of offspring have not been well documented.

The genetic influences on behavioral development have been well demonstrated. Some studies have assessed genomic influences on behavior using hybrid F1 mice. For example, offspring from different genetic strains showed different levels of anxiety even though they received mothering from a mother of the same strain [19]. Anxiety-related genes have begun to be identified [1, 10]. C57BL/6(B6) and

BALB/c strains, which are very different in behavioral phenotypes, have also been studied in attempts to identify anxiety-related genes [2]. Interestingly, Francis reported that differences in behavioral response in the elevated plus-maze test were epigenetically modulated in B6 and BALB/c mice, that is, B6 mice that were embryo-transferred and raised by BALB/c mothers showed BALB/c-like anxiety behavior [13]. In other experiments, for example, monozygotic twin mice (genetically identical mice that are generated from a single zygote by when the blastocyst divides at the eight-cell stage to produce two viable embryos) are significantly more alike for multiple complex traits than are dizygotic twin mice (genetically identical mice obtained from different zygotes) [14]. Thus, both inherited genes and environmental factors, especially mother–pup relations, produce epigenetic modifications that, in turn, influence gene expression and behavioral differences.

To address these issues, one research model uses the F1 hybrid. Two types of B6 and BALB/c hybrids, that is, B6 father and BALB/c mother or B6 mother and BALB/c father, share the same genes except for the Y chromosome in males and imprinting genes. Therefore, comparing these two hybrids can reproduce the results obtained by Francis [13], in which the same embryo developed in and raised by B6 and BALB/c females can show the behavioral patterns of B6 and BALB/c mice in the elevated plus-maze test. Another aim of this study was to examine the effects of paternal care on offspring's development. BALB/c females show lower levels of maternal care to the pups as compared to B6 mothers [3, 5]; however, father mice show intensive parental care to the pups [24, 28] and can compensate for the differences in mothers. For example, offspring from BALB/c females

\* CORRESPONDENCE TO: KIKUSUI, T., Companion Animal Research, Azabu University, 1-17-71 Fuchinobe, Chuo-ku, Sagami-hara, Kanagawa 229-8501, Japan.  
e-mail: Kikusui@azabu-u.ac.jp

receive less maternal care from their mothers but more paternal care from their B6 fathers when these two strains are paired. Therefore, we simultaneously evaluated the maternal and paternal care to the offspring, and compared the anxiety behavior in offspring that received care from both mothers and fathers.

## MATERIALS AND METHODS

**Animals:** The parental strains of BALB/cAJcl (BALB/c) and C57BL/6Jcl (B6) mice were originally obtained from Japan Clea Co., Ltd. (Yokohama, Japan). We bred them in four types of pairs; B6 female and B6 male (B6B6; mother's strain listed first), BALB/c female and BALB/c male (CC), B6 female and BALB/c male (B6C), and BALB/c female and B6 male (CB6). To obtain a standard control, two inbred strains and two reciprocal F1, B6C and CB6, were included in the study. Food and water were given *ad libitum*, and all the animals were kept at a constant temperature ( $23 \pm 1^\circ\text{C}$ ) and humidity ( $40 \pm 5\%$ ) under a 12 hr light:12 hr dark cycle (light on at 08:00 hr). All of the experiments were conducted in accordance with the guidelines in "Policies Governing the Use of Live Vertebrate Animals" of the University of Tokyo.

**Pairing:** A male and a female mouse were pair-housed in a cage ( $17.5 \times 24.5 \times 12.5$  cm) for breeding. When the female was pregnant, birth was checked for every morning. For each litter, the day of birth was assigned as the postnatal day 0 (PD0). On PD0, the litter was culled to between six and eight, with the number of males and females roughly equal to minimize the effects of variations of number and sex of pups. The pups were housed together with their mother and father until the day of weaning (PD21). After PD21, the littermates were housed together in same-sex groups of two to four mice in a  $17.5 \times 24.5 \times 12.5$ -cm cage or two or three mice in a  $12.5 \times 20.0 \times 11.0$ -cm cage until the elevated plus-maze test.

**Parental behavior:** The parental behavior of multiparous BALB/c  $\times$  BALB/c pairs (CC,  $n=5$ ), B6  $\times$  BALB/c pairs (B6C,  $n=7$ ), BALB/c  $\times$  B6 pairs (CB6,  $n=7$ ) and B6  $\times$  B6 pairs (B6B6,  $n=5$ ) was videotaped and later analyzed. Since parental behavior of mice has been reported to be more frequent in the light phase during the first 10 days after delivery [18, 33], behavior was recorded for six 1-hr observation periods in the light phase, at 08:00, 10:00, 12:00, 14:00, 16:00 and 18:00 hr on PD1, 3, 5, 7, and 9.

A small CCD camera was focused obliquely upward on the cages, and behavior was video recorded for the first 12 seconds of each minute. The following three components of parental behaviors were monitored; 1) parent passively contacting their pups (parental contact behavior); 2) active nursing behavior, namely licking and grooming the pups (licking/grooming); and 3) parent was away from the pups (parent off pups). These three elements were scored in the same way as in previous studies [18, 22]; that is, each component was scored positive if the parent exhibited the behavior during a period of three minutes (36 sec in total), such

that a score of 20 was the highest over the course of 1-hr, and the score of 120 was the highest for one day. The data are presented as percentile ratio of the frequency of the behavior occurring in one day.

**Elevated plus-maze test:** The elevated plus-maze test was conducted at the age of 8–10 weeks. More than two hours before the test, females were verified to be in their estrus stage by vaginal smear. Females in the estrus stage were not tested in order to avoid estrus cycle effects. Male mice (B6B6,  $n=15$ ; B6C,  $n=11$ ; CB6,  $n=17$ ; CC,  $n=14$ ) and female mice (B6B6,  $n=13$ ; B6C,  $n=17$ ; CB6,  $n=20$ ; CC,  $n=12$ ) were tested in the light period of the L/D cycle under a dim red light (less than 0.1 lux) using an elevated plus-maze apparatus ( $25 \times 5$ -cm closed arms, with a wall 5 cm in height) kept 20 cm above the floor [18, 22]. The color of the apparatus was gray, and the height of the lip on the open arm was 0.5 cm. The animal was placed in the neutral zone facing the opened arm, and the behavior was video-recorded for 15 min. The frequency of entry into the arms and duration on the maze were measured. In addition, the distance traveled on the maze was analyzed using a computer tracking system (Ethovision, version 3.0; Noldus Co., Ltd., Wageningen, Netherlands).

**Statistical analysis:** Statistical analysis was performed using StatView + Graphics 4.1J software (no longer available; Abacus Concepts, Inc., Berkeley, CA, U.S.A.). Differences were considered statistically significant at  $P < 0.05$ . For parental behavior, all the parameters were analyzed by four-way multivariate analysis of variance (MANOVA), with the factors of sex, strain, strain of the dyad partner, and day of postpartum as repeated measures, followed by three-way analysis of variance (ANOVA) with factors of sex, strain, and day of postpartum as repeated measures. Tukey's post-hoc test followed in when significant differences were detected. Also, the average of each behavioral parameter from PD1 to PD9 was calculated for each mouse and used to compare among groups by three-way MANOVA with main factors of sex, strain, and strain of the dyad partner, followed by Tukey's post-hoc test. Also, the parental care that each group of pups received from the mother and father was calculated and compared by one-way MANOVA with main factor of group, followed by Tukey's post-hoc test. For the elevated plus-maze test, a three-way MANOVA with the factors of sex, strain of father, and strain of mother with Hotelling's trace was conducted for the group and sex comparisons and followed by the three-way ANOVA. Tukey's post-hoc test followed in case a significant difference was detected.

## RESULTS

**Parental behavior:** Not only mothers, but also fathers showed parental behavior. The MANOVA revealed sex, strain, strain of dyad partner, and day differences for three dependent variables, namely, parental contact behavior, licking/grooming, and parent off pups [sex:  $F(3,198)=52.6$ ,  $P < 0.001$ ; strain:  $F(3,198)=15.6$ ,  $P < 0.0001$ ; strain of dyad

partner:  $F(3,198)=2.59$ ,  $P<0.05$ ; day:  $F(12,590)=14.3$ ,  $P<0.0001$ ]. We also found significant interactions [(strain  $\times$  sex:  $F(3,198)=2.57$ ,  $P<0.05$ ; strain  $\times$  partner:  $F(3,198)=12.7$ ,  $P<0.0001$ ; and sex  $\times$  day:  $F(12,590)=6.64$ ,  $P<0.0001$ ].

A repeated three-way ANOVA for each parameter disclosed significant differences in parental contact behavior according to strain and sex [strain:  $F(1,40)=11.490$ ,  $P<0.05$ ; sex:  $F(1,40)=25.921$ ,  $P<0.0001$ ], licking/grooming behavior [strain:  $F(1,40)=10.942$ ,  $P<0.01$ ; sex:  $F(1,40)=45.612$ ,  $P<0.0001$ ], and parent off pups [strain:  $F(1,40)=5.421$ ,  $P<0.05$ ; sex:  $F(1,40)=37.002$ ,  $P<0.0001$ ]. The post-hoc Tukey's test revealed a strain difference in the parental behavior, that is, B6 spent a significantly longer time on licking/grooming and parental contact behavior, and a shorter time on parent off pups (Fig. 1). Males showed significantly longer times spent on parental contact behavior than females, and females showed significantly longer times spent on licking/grooming and parent off pups (Fig. 1). Licking/grooming behavior gradually decreased over time, especially in females, whereas parent off pups in females and parental contact behavior in males increased with days.

The total average of three dependent variables during the observation period was calculated, and the three-way MANOVA with the main factors of sex, strain, and strain of dyad partner also revealed sex and strain differences [sex:  $F(3,38)=28.4$ ,  $P<0.0001$ ; strain:  $F(3,38)=12.8$ ,  $P<0.0001$ ], as well as a significant interaction between strain and partner [strain  $\times$  partner:  $F(3,38)=10.7$ ,  $P<0.0001$ ]. Post-hoc analysis for each sex revealed significant interactions between strain and partner. In males, parental contact behavior was higher in B6 paired with B6 females as compared to BALB/c paired with BALB/c females ( $P<0.05$ , Fig. 2). Interestingly, licking/grooming behavior in males was dependent on the strain of the partner, that is, B6 males showed higher licking/grooming when paired with a female from the same strain as compared to that with a female from a different strain (BALB/c). Similarly, BALB/c males showed higher licking/grooming when paired with a female from the same strain ( $P<0.05$ , Fig. 2). Parental contact behavior in B6 females paired with B6 males was higher than that in BALB/c females ( $P<0.05$ ). Also, licking/grooming behavior in BALB/c females with B6 males was lower than that in B6 females ( $P<0.05$ ).

Pups can receive parental behavior from both mother and father; therefore we added the scores of maternal and paternal care of each group and analyzed them using MANOVA, which demonstrated significant difference in three parameters [ $F(9,50)=4.26$ ,  $P<0.0001$ ]. Post-hoc analysis revealed that parental contact behavior in B6B6 pairs was higher than that for CC pairs ( $P<0.05$ , Fig. 3), whereas B6B6 pairs had lower parent off pups time compared to CC pairs ( $P<0.05$ , Fig. 3). CB6 pairs had lower licking/grooming behavior compared to B6B6 pairs and CC pairs ( $P<0.05$ , Fig. 3).

**The elevated plus-maze test:** The three-way MANOVA with main factors of sex, strain of father, and strain of mother revealed that sex, strain of father, and strain of

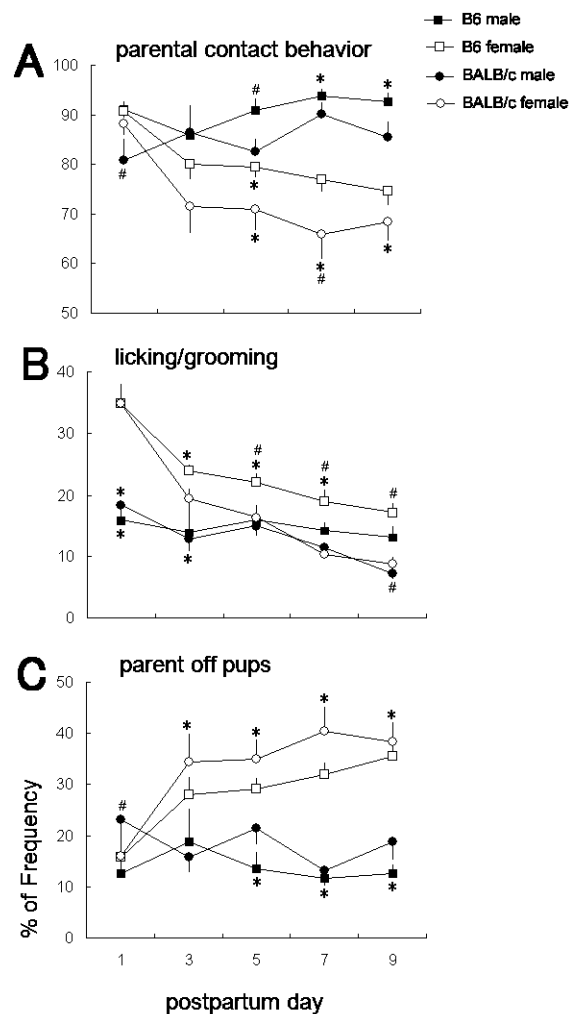


Fig. 1. Parental behavior of C57BL/6 and BALB/c mice. Both male and female mice showed steady parental behavior toward the pups. Overall analysis demonstrated that B6 was more active parent as compared to BALB/c, and males showed higher contact behavior to pups than females, and female showed more parent off pups than males. (\*  $P<0.05$  between sex, #  $P<0.05$  between strain; 3-way ANOVA and Tukey's post-hoc test)

mother showed differences for seven dependent variables: frequency of entering open arms, closed arms, and neutral zone; duration stayed in open arms, closed arms, and neutral zone; and distance moved [sex:  $F(7,106)=2.1$ ,  $P=0.05$ ; father strain:  $F(7,106)=11.8$ ,  $P<0.0001$ ; mother strain:  $F(7,106)=11.38$ ,  $P<0.0001$ ]. Significant interactions were also found [sex  $\times$  father strain:  $F(7,106)=2.84$ ,  $P<0.01$ ; mother strain  $\times$  father strain:  $F(7,106)=7.14$ ,  $P<0.0001$ ; sex  $\times$  mother strain  $\times$  father strain,  $F(7,106)=2.3$ ,  $P<0.05$ ]. A repeated-measure three-way ANOVA for each parameter revealed significant differences [Table 1; duration, open arms, mother's strain,  $F(1,112)=31.641$ ,  $P<0.0001$ ; father's strain,  $F(1,112)=48.308$ ,  $P<0.0001$ ; closed arms, mother's

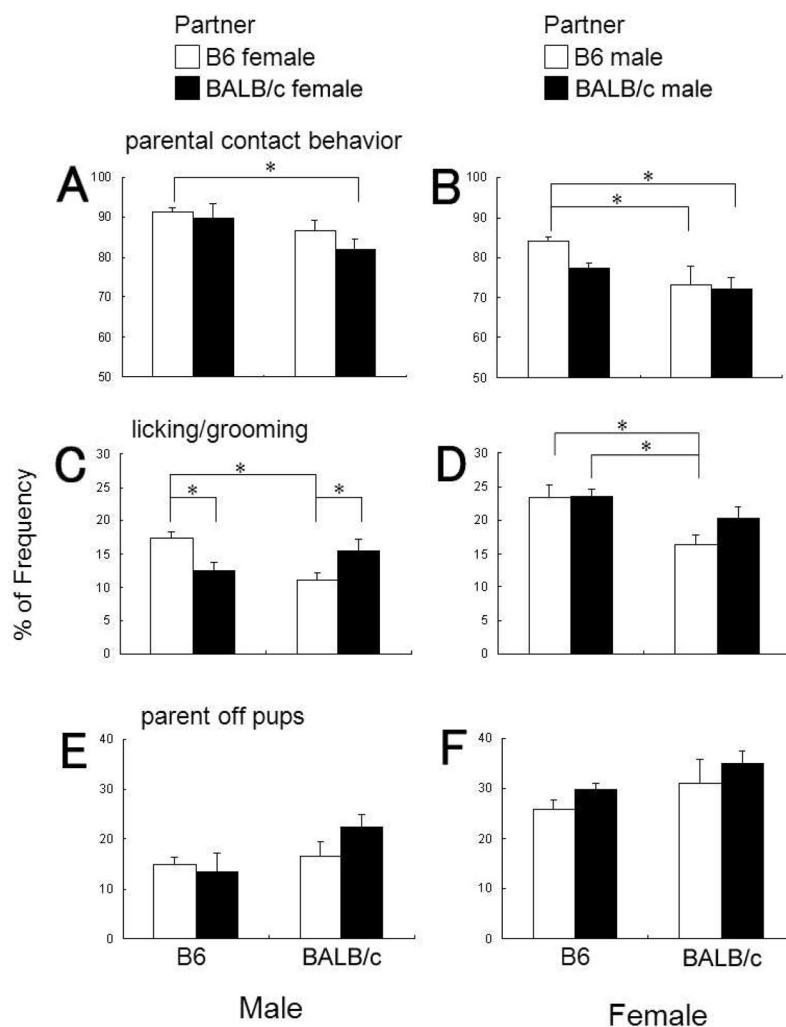


Fig. 2. Parental behavior of C57BL/6 and BALB/c mice, comparing with same strain/sex with different pairing partner. B6 and BALB/c males showed more locking/grooming behavior toward the pups when paired with same strain female, as compared to those of pairing with different strain female (\*  $P < 0.05$  3-way MANOVA and Tukey's post-hoc test).

strain,  $F(1,112)=23.135$ ,  $P < 0.0001$ ; father's strain,  $F(1,112)=33.557$ ,  $P < 0.0001$ . Open/total arms: mother's strain,  $F(1,112)=30.485$ ,  $P < 0.0001$ ; father's strain,  $F(1,112)=45.678$ ,  $pP < 0.0001$ . Frequency: open arms, mother's strain,  $F(1,112)=25.411$ ,  $P < 0.0001$ ; father's strain,  $F(1,112)=44.853$ ,  $P < 0.0001$ ; mother's strain  $\times$  father's strain,  $F(1,112)=6.636$ ,  $P < 0.05$ ; closed arms, father's strain,  $F(1,112)=18.839$ ,  $P < 0.0001$ ; mother's strain  $\times$  father's strain,  $F(1,112)=8.481$ ,  $P < 0.005$ ; open/total; mother's strain,  $F(1,112)=62.851$ ,  $P < 0.0001$ ; father's strain,  $F(1,112)=55.250$ ,  $P < 0.0001$ ; Distance moved: sex,  $F(1,112)=10.098$ ,  $P < 0.005$ ; mother's strain,  $F(1,112)=12.248$ ,  $P < 0.001$ ; mother's strain  $\times$  father's strain,  $F(1,112)=9.438$ ,  $P < 0.005$ .

The post-hoc Tukey's test for each sex revealed that both male and female CC showed lower frequencies of and

shorter durations in open arms as compared to the other groups ( $P < 0.005$ , Fig. 4). No difference was found in open arm frequency and duration between B6C and CB6 or between B6B6 and CB6 pairs. Open arm duration in B6C males and females was shorter than for B6B6 pairs ( $P < 0.005$ , Fig. 4). For closed arm duration, CC males had longer durations than did B6B6 males ( $P < 0.005$ , Table 1). CC females showed longer closed arm duration compared to the other three groups ( $P < 0.005$ , Table 1), and B6C females had longer closed arm duration compared to B6B6 females ( $P < 0.005$ , Table 1). As for closed arm frequencies, CB6 females showed higher closed arm frequencies as compared to B6C and CC females ( $P < 0.005$ , Table 1), whereas no group difference was found for males. CC male moved significantly shorter distance compared to the other strains, whereas no group difference was found for females. When

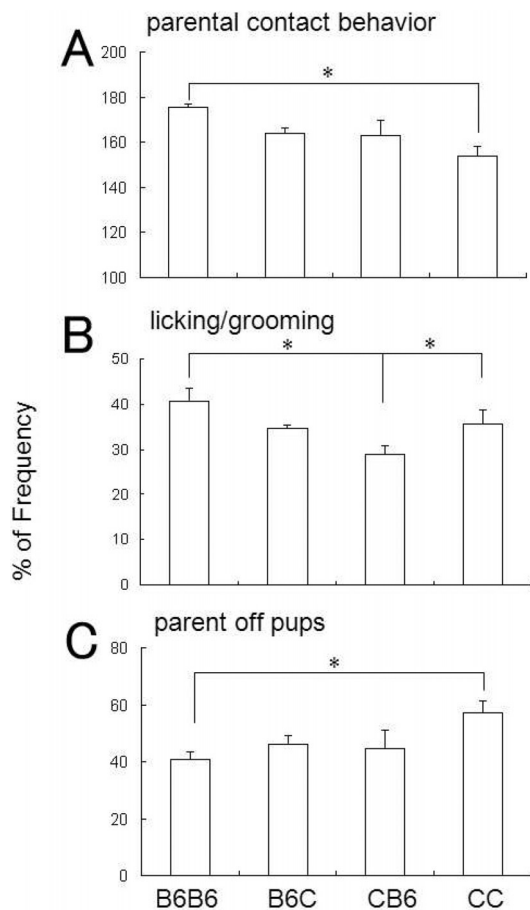


Fig. 3. Total frequency of parental behavior toward pups, from both male and female mice. CC pair showed less Contact Behavior than B6B6 pair, and CB6 pair showed less licking/grooming behavior than B6B6 pair and CC pair. (B6B6, pair of C57BL/6; B6C, pair of C57BL/6 female and BALB/c male; CB6, pair of BALB/c female and C57BL/6 male; CC, pair of BALB/c. \*  $P < 0.05$ , 1-way MANOVA and Tukey's post-hoc test).

the ratio of the open arm frequency/duration to total frequency/duration was calculated, male and female CC mice showed significantly lower ratios of entries into and duration on the open arm compared to the other strains, and no significant difference was found between B6C and CB6 mice (Fig. 4,  $P < 0.05$ ).

## DISCUSSION

In the present study, it was found that male B6 and BALB/c mice showed very similar patterns and amounts of parental care to pups during the first nine days after delivery. Although licking/grooming behavior in the first three to four days was lower in comparison to females, a similar amount of licking/grooming was observed from PD5 to PD9. In addition, parental contact behavior was higher in males than females, especially during PD3 to PD9, and par-

ent off pups was lower from PD3 to PD9, indicating that father mice can nurse the pups to an extent equivalent to females except for suckling. When the dam has contact with her pups, she must be suckled, and the amount of suckling required by the pups increases day by day. So females may stay with their pups for a minimal time to conserve their energy, while males may stay with pups in a compensatory manner.

Several studies have reported paternal behavior in laboratory mice. In monogamous rodent species such as prairie voles and California mice, males help the dam with very intensive pup-care [4]. For example, virgin male California mice tend to behave nonparentally towards pups and even engage in infanticide, whereas male parents exhibit parental behavior following the birth of their own young [11, 16]. The social experience of sexual copulation and living together with a pregnant female can induce parental behavior in male California mice [15]. Regarding white-footed mice, Hartung and Dewsbury found that during the first week after parturition, males stayed in nests, licked pups and manipulated nesting materials [17]. Similar observations were reported by studies conducted in other laboratory settings [24, 28]. Moreover, in semi-natural settings, white-footed male mice were observed in the nest with pups, usually forming a male-female monogamous pair [9, 31]. Our results support these findings and suggest that inbred laboratory mice such as B6 and BALB/c mice also show higher levels of paternal care to their pups.

From the results of the parental behavior observation, BALB/c females showed less parental contact behavior and licking/grooming than B6 females, which was consistent with previous reports [3, 5, 25]. However, in males, parental contact behavior and licking/grooming behavior was almost equal between B6 and BALB/c mice. These results were interesting, because usually maternal behavior is associated with anxiety; that is, higher anxiety is correlated with lower maternal behavior in females [12, 22]. For example, female rats immediately after delivery showed avoidance behavior toward their newborn pups, which can be reduced by the anxiolytic effects of oxytocin in the olfactory bulb and other brain regions [36]. This suggests that maternal responsiveness at parturition is regulated by actions of experience and hormones on the brain regions to inhibit avoidance and anxiety responses toward pups, accompanied by a stimulation of neural circuits regulating approach and acceptance responses [29]. BALB/c males also showed higher anxiety-related behavior compared to B6 males, but no clear difference in paternal behavior was observed. Little is known about the relationship in male mice between anxiety/avoidance behavior and paternal behavior; further studies are needed.

Another interesting finding was that in males, licking/grooming behavior was dependent on the strain of the dyad partner, that is, males showed more licking/grooming behavior when paired with a female from the same strain of mice, both in B6 and BALB/c mice. At present, there is no scientific evidence to explain this, but one possibility is that

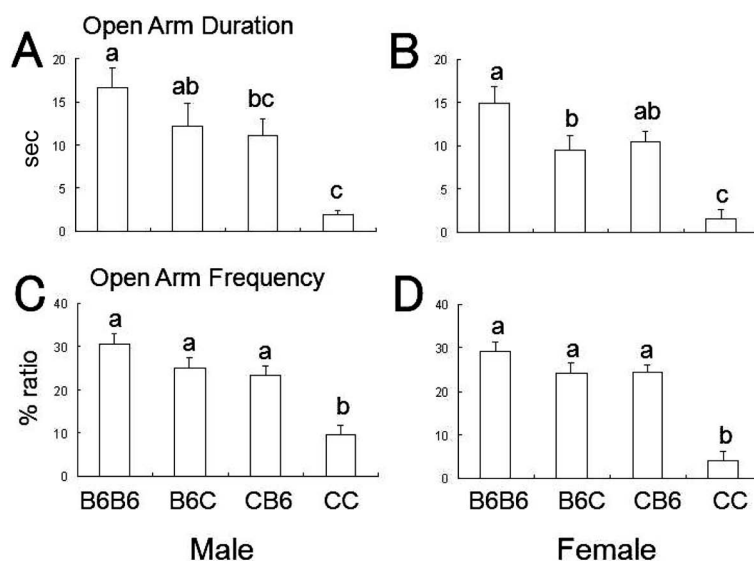


Fig. 4. Results of the elevated plus maze test. The pups from CC pair showed lower ratio of the frequency of the Open arm entry and Open arm duration as compared to the pups from B6B6 pair. Reciprocal hybrid pups showed intermediate values between B6B6 and CC (B6B6, pair of C57BL/6; B6C, pair of C57BL/6 female and BALB/c male; CB6, pair of BALB/c female and C57BL/6 male; CC, pair of BALB/c. Different letters (a, b, c) on the top of the bar,  $P < 0.05$ , 2-way ANOVA and Tukey's post-hoc test).

Table 1. Behavioral differences in the elevated plus maze test in four F1 groups

		Open Arm		Closed Arm		Distance Moved
		Frequency	Duration	Frequency	Duration	
Male	B6B6	11.8 ± 1.7 <sup>a</sup>	134.9 ± 17.3 <sup>a</sup>	25.5 ± 2.0	677.8 ± 22.6 <sup>a</sup>	3,426 ± 213 <sup>a</sup>
	B6C	8.1 ± 1.2 <sup>a</sup>	73.1 ± 12.1 <sup>b,c</sup>	22.7 ± 2.1	737.8 ± 19.9 <sup>a,b</sup>	3,744 ± 217 <sup>a</sup>
	CB6	8.4 ± 1.3 <sup>a</sup>	88.5 ± 15.6 <sup>a,b</sup>	25.5 ± 1.3	726.1 ± 20.6 <sup>a,b</sup>	3,415 ± 115 <sup>a</sup>
	CC	2.0 ± 0.5 <sup>b</sup>	15.4 ± 4.0 <sup>c</sup>	16.9 ± 3.0	806.7 ± 17.6 <sup>a</sup>	2,579 ± 168 <sup>b</sup>
Female	B6B6	9.7 ± 1.2 <sup>a</sup>	116.9 ± 14.6 <sup>a</sup>	22.4 ± 1.4 <sup>a,b</sup>	675.9 ± 20.9 <sup>c</sup>	2,951 ± 116
	B6C	7.1 ± 1.0 <sup>a</sup>	77.2 ± 9.3 <sup>b</sup>	21.2 ± 2.3 <sup>b</sup>	749.1 ± 16.1 <sup>b</sup>	3,117 ± 237
	CB6	9.2 ± 0.7 <sup>a</sup>	85.2 ± 12.8 <sup>a,b</sup>	28.7 ± 1.0 <sup>a</sup>	734 ± 19.4 <sup>b,c</sup>	2,863 ± 120
	CC	1.2 ± 0.7 <sup>b</sup>	12.7 ± 8.1 <sup>c</sup>	16.7 ± 3.3 <sup>b</sup>	839.6 ± 17.2 <sup>a</sup>	2,771 ± 193

Values: Mean ± SEM. Different letters (a, b, c) mean significant difference between groups. ( $P < 0.05$ , three-way ANOVA and Tukey's post-hoc test).

male mice can discriminate the genetic background of the pups. Hybrid F1 mice (B6C and CB6) shared 50% of genes, whereas inbred F1 mice (B6B6 for B6 father, CC for BALB/c father) shared 100% of genes, which would lead to differences in paternal but not in maternal care. Female mice parents can distinguish the odor of their own pups according to the differences in genes related to the major histocompatibility complex [35]. Other possibilities that create differences in male-female relationships, and in pups' attachment behavior that is so key in inducing parental behavior, should be also taken into consideration.

These results indicated that pups from each kind of pair received different degrees of maternal care. When the total frequency of parental care from mother and father was

summed up, a significant group difference in parental contact behavior, licking/grooming behavior, and parent off pups behavior was found. Pups from B6B6 pairs received the highest levels of parental contact behavior and licking/grooming, whereas pups from CB6 pairs received the lowest frequency of licking/grooming.

We first hypothesized that the amount of parental care from each pair would explain the difference in anxiety level in their offspring in adulthood, especially in B6C and CB6 mice, which are genetically identical except for the Y chromosome in males and imprinting genes. Therefore, differences in anxiety level between these two groups would reflect parental environment in the earliest stage of life. Therefore, we compared the anxiety level of the four groups

using the elevated plus-maze test.

In the elevated plus-maze test, CC mice showed more anxiety-related behavior than did B6B6 mice (Table 1 and Fig. 4) as previously reported [5, 13, 25]. Hybrid B6C and CB6 mice showed equivalent behavior in the test, which was intermediate between the data from B6B6 and CC mice. Results from the parental behavior observation show that BALB/c females showed less parental contact behavior and licking/grooming behavior than B6 females, which implies that parental contact and licking/grooming are regulatory factors for the development of anxiety behavior in mice, as proposed elsewhere [7, 13]. However, behavioral differences between B6B6 and CC mice were also due to genetic differences, so it is indispensable to compare the anxiety-related behavior between two other groups that share 100% of genes, namely B6C and CB6 mice. In the elevated plus-maze test, however, these two groups showed very similar results, even though the CB6 pups received the lowest levels of licking/grooming behavior from their parents. Interestingly, CC pups received higher levels of licking/grooming behavior than did CB6 mice, implying that licking/grooming is not a main regulatory stimulus from the dam to the pups for the development of anxiety behavior, as shown in the rat study [27]. Parental contact behavior was more closely correlated with anxiety-related behavioral differences in these four groups; that is, B6B6 was the highest, B6C and CB6 were intermediate, and CC was the lowest. Moreover, parental contact behavior of CC pairs was significantly lower than the other three groups on PD1 (B6B6,  $188 \pm 2.4$ ; B6C,  $180 \pm 4.3$ ; CB6,  $179 \pm 4.0$ ; CC,  $149 \pm 5.1$ ). The intensiveness of maternal licking and grooming has been shown to modulate the anxiety-related behavior and neuroendocrine function in the rat offspring [27], including the hippocampal glucocorticoid receptor expression level, which is the gate of the negative feedback loop of the adrenal glucocorticoids. However, such modulatory effects of maternal behavior on the epigenetic regulation of glucocorticoid receptor is restricted to the first few days after birth [34], which corresponds to the large difference in parental contact behavior in the present study. This suggests that parental contact behavior, especially in the first few postnatal days, is a social factor that regulates anxiety related behavior in mice. In rats, licking/grooming is demonstrated to have a significant role in the development of emotional reactivity [6]. From our results, not only licking/grooming, but also parental contact behavior has an important role in anxiety behavior. The parental contact behavior consists of warmth, the scent of the mother, and physical contact stimuli. The importance of these social stimuli in mice has not yet been demonstrated, but this should be clarified.

Some other social factors can influence the development of anxiety behavior. For example, parental behavior was not observed after PD9 in the present study. We previously demonstrated that the deprivation of parental care in the late lactating period through early weaning increased anxiety and aggressive behavior in adulthood [20, 23, 30]. Licking/grooming decreased more sharply in BALB/c mothers than

in B6 mothers. Therefore, after PD10, the amount of licking/grooming received could be less for CC pups compared to the other three groups, which can increase anxiety behavior in their adulthood.

Contrary to the result of previous study by Francis *et al.* [13], CB6 mice raised by BALB/c mother did not show BALB/c-like higher anxiety behavior. This inconsistency might be attributed to the different raising condition of the two studies, e.g., father mice attended in the present study but did not in the previous one.

Kristianne *et al.* reported no significant difference between B6 mice reared by B6 dams and B6 mice reared by BALB/c mice as measured by the elevated plus-maze test, but that there was a difference as measured by the open field test [25]. Thus, the influence of maternal care might be found in the reciprocal F1 mice (CB6 and B6C) of this experiment through the application of other behavioral tests.

In the present study, it was found that inbred male mice showed intensive parental behavior toward their pups, especially in the form of parental contact behavior. Also, the males exhibited licking/grooming behavior dependent on the strain of the female they were paired with. These results imply the influence of parental care on the offspring's emotional reactivity. However, additional experiments are needed to examine the impacts of paternal care on the anxiety behavior, such as comparing the offspring received biparental care to those raised by mother solely. The pups raised by parents of different strains were tested for anxiety-related behavior to investigate the relationship between parental care received in the neonatal period and later anxiety levels. Although no significant difference was found in the parental care that offspring received or in anxiety-related behavior between the reciprocal hybrid strains of B6C and CB6 mice, the comparison of B6 and BALB/c mice suggests that parental contact behavior is associated with anxiety-related behavior in adulthood. Additional experiments, such as fostering F1 hybrid offspring by B6B6 or CC parents, may reveal more precisely the influence of parental care on the development of anxiety behavior. These reciprocal hybrid studies offer a productive model by which to understand the genetic-epigenetic regulation of behavioral phenotypes.

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