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Neuromolecular basis of parental behavior in laboratory mice and rats: With special emphasis on technical issues of using mouse genetics

Kumi O. Kuroda [a,*], Kashiko Tachikawa [a], Sachine Yoshida [a], Yousuke Tsuneoka [a], Michael Numan [b]

[a] Unit for Affiliative Social Behavior, RIKEN Brain Science Institute, Saitama 351–0198, Japan
[b] Department of Psychology, Boston College, 140 Commonwealth Avenue, Chesnut Hill, MA 02467, USA

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ABSTRACT

To support the well-being of the parent–infant relationship, the neuromolecular mechanisms of parental behaviors should be clarified. From neuroanatomical analyses in laboratory rats, the medial preoptic area (MPOA) has been shown to be of critical importance in parental retrieving behavior. More recently, various gene-targeted mouse strains have been found to be defective in different aspects of parental behaviors, contributing to the identification of molecules and signaling pathways required for the behavior. Therefore, the neuromolecular basis of "mother love" is now a fully approachable research field in modern molecular neuroscience. In this review, we will provide a summary of the required brain areas and gene for parental behavior in laboratory mice (*Mus musculus*) and rats (*Rattus norvegicus*). Basic protocols and technical considerations on studying the mechanism of parental behavior using genetically-engineered mouse strains will also be presented.

1. Introduction

Inappropriate rearing environments impact a child's future stress reactivity and developing personality, and also shape his or her own parenting style (Bowlby, 1951; Harlow, 1979; Meaney, 2001; Rutter, 1972). To support the well-being of the parent–infant relationship and subsequent development of the infant, the neural and molecular mechanisms of each component of parental behaviors should be clarified. For all mammalian infants, maternal care such as nursing is essential for survival. Therefore the neural mechanisms supporting maternal behaviors should be conserved throughout mammalian evolution at least in their basic parts. We can plausibly contribute to the understanding of the human mother–infant relationship in the future by studying the neural mechanisms using non-human mammalian models.

Maternal behavior is defined as the collection of behaviors by the mother that can increase offspring survival (Krasnegor and Bridges, 1990; Numan and Insel, 2003). Similar nurturing behaviors as maternal behaviors, called as *paternal behavior* by fathers and *alloparental behavior* by older conspecifics, are widely seen in mammals. In this review, we will collectively refer to these maternal, paternal and alloparental behaviors as "*parental behavior*".

Among various mammalian species, mechanisms of maternal behaviors have been studied most extensively using laboratory Norway rats (*Rattus norvegicus*). More recently, genetic knockout technology using laboratory mice (*Mus musculus*) has provided new insights into the molecular basis of maternal behaviors. Qualitative similarities of maternal behaviors in laboratory rats and mice enable us to discuss and integrate the existing complimentary findings in these species. This article aims to review rather basic information required to study parental behavior in mice, especially technical considerations for studies using genetically-engineered mouse strains. In addition, we have tried to contrast several differences in parental behavior between rats and mice, and among various strains of the same species. Such variations are more prominent in alloparental and paternal caretaking than in postpartum maternal behaviors. For more detailed information on parental behaviors in rats and other mammalian species, please refer the previous literature (Elwood, 1983; Krasnegor and Bridges, 1990; Numan and Insel, 2003; Rosenblatt and Snowdon, 1996; Sluckin and Herbert, 1986).

2. Overview of parental behaviors in laboratory rats and mice

2.1. Maternal behavior

Laboratory rats and mice are born "altricial" (immobile at birth): newborn pups are hairless, incapable of temperature regulation, with their eyelids and ear holes sealed, and with underdeveloped motor skills.

Abbreviations: AH, the anterior nucleus; BLA, the basolateral amygdala; BMA, the basomedial amygdala; DOPS, L-threo-dihydroxyphenylserin; GABA, γ-amino butyric acid; KO, knockout; MGI, the Mouse Genome Informatics; MPOA, the medial preoptic area; NA, the nucleus accumbens; POA, the preoptic area; RTK, the receptor tyrosine kinase; VMH, the ventromedial nucleus; VNO, the vomeronasal organ; VTA, the ventral tegmental area.

* Corresponding author at: Unit for Affiliative Social Behavior, RIKEN Brain Science Institute, Hirosawa 2-1, Wakoshi, Saitama 351–0198, Japan.

E-mail address: oyako@brain.riken.jp (K.O. Kuroda).

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Therefore they are dependent upon the mother (dam) for thermoregulation, nutrition and protection from harm until weaning at around three weeks after delivery. Various components of maternal behaviors are summarized in Table 1 (based on Bridges, 1996). Peripartum maternal behaviors show qualitative similarities in mice and rats, so that they are described collectively, with some remarks about minor differences. An example of postpartum maternal behavior testing in the few hours after the parturition is described below (Section 4.3).

2.1.1. Behavior before parturition

2.1.1.1. Nest building. Non-pregnant rats and mice produces a flat "sleeping nest", while females at late pregnancy make a bigger and more complex nest, termed a "brood nest" (for mice, (Koller, 1952); rats, (Denenberg et al., 1969; Rosenblatt and Lehrman, 1963). Brood nest building starts from one to a few days before parturition (earlier in mice than in rats (Weber and Olsson, 2008)), continues for the first two weeks of lactation and declines. A protocol for rating the nest will be described below (Section 4.3.2.2).

2.1.2. Parturition

2.1.2.1. Placentophagia. Depending on strains and studies, laboratory rat or mouse dams typically deliver from 10 to 14 or 4 to 8 pups, respectively (Holloway et al., 1980; Nagasawa et al., 1973). The mean birth interval is about 10–20 min (Dollinger et al., 1980). Upon each pup's delivery, the dam licks the pup's body extensively to eliminate the amniotic membrane and fluid, and consumes the placenta and attached umbilical cord. This behavior is called as "Placentophagia" (Kristal, 1980). The function of placentophagia is to make the pup's body dry and clean; if placentophagia has not been properly performed, the pups' skin sticks to other particles and objects (see Fig. 2), and the pups can become entangled among themselves by remaining parts of the umbilical cords and placentas. Maternal licking also stimulates respiration of neonates.

2.1.3. Behavior after parturition

Once the parturition ends, the dam retrieves all the pups into the nest, rebuilds the nest, which tends to be broken down by the activities of parturition, and stays with the pups in the nest to lick, nurse and warm them.

2.1.3.1. Pup-retrieval behavior. If a pup becomes displaced from the nest or if the dam moves the location of her nest, *pup-retrieval behavior* occurs: the dam orients and moves toward the pup, often sniffing the pup before gently picking it up with the incisors, carrying it to the nest site, and finally depositing it there (Lonstein and Fleming, 2002). This behavior is optimally evoked by hairless pups within the first week of age. Quantification of this behavior will be introduced in more detail below (Sections 4.3 and 4.4).

2.1.3.2. Grouping. Grouping refers to gathering all pups together into one quadrant. As a result, each pup remains in contact with other pups (Fahrbach et al., 1984). If grouping is not properly performed, the pups may become scattered in the nest and are not huddled together, one or more pups may fall out of the nest, or the pups may be located in multiple (separate) nests. A large litter may be split into two nests in rats (Galler and Turkewitz, 1975). Wild-type C57BL/6J mouse dams seldom make multiple nests, even with large litters of 10 pups, while wild-type dams of 129 strains sometimes make two nests, even when the number of pups is less than 10 (our unpublished observation). This might reflect the inferior spatial cognition in some 129 strains compared with C57BL/6J (Crawley, 2007).

2.1.3.3. Pup licking. Pup licking, during which the dam moves her tongue rapidly over the body of the pup, is subdivided into anogenital licking and body (general) licking. *Anogenital licking* of rat and mouse pups during the first two postnatal weeks is important for induction of urination and defecation, like in many other mammalian species (Ewer, 1968). The pups' urine, ingested by the dam, contributes significantly to the dam's own increasing water needs that result from lactation (Baverstock and Green, 1975; Gubernick and Alberts, 1985). *Body licking* includes licking and snout contact with the general body surface except for the perineum of pups (Moore, 1992). Pup licking, together with other forms of body contact, provides pups with *tactile stimulation*, which affects pup growth through growth hormone and corticosteroid secretion (Schanberg and Field, 1987) (Levine, 2002). "Contact comfort" is another aspect of influence of body contact (Harlow, 1958). Short- and long-term effects of body contact on pup behavior and growth are difficult to discriminate

Table 1
Components of maternal Behaviors in laboratory mice and rats.

	Description	General references	References for maternal-like behavior by non-mothers
Pup-directed behaviors			
Nursing	Crouching over the pups to provide the opportunity to suckle in various nursing postures	Stern and Lonstein (2001)	Rat female, Lonstein et al. (1999)
Retrieval	Picking up the pup gently by a part of the body (most commonly, the dorsal skin) with the incisors and carries it to the nest site.	Noirot (1972), Wiesner and Sheard (1933)	Rat female, Fleming and Rosenblatt (1974a), Rosenblatt (1967); male, Rosenblatt (1967); juvenile Bridges et al. (1974). Mouse female, Leblond and Nelson, (1937); male, vom Saal and Howard (1982); juvenile, Noirot (1972).
Grouping	Gathering the pups together into one quadrant so that they touched one another	Fahrbach et al. (1984), Kinsley and Bridges (1988)	
Anogenital licking	Licking the anogenital region of the pup	Moore (1992)	Rat female, Gubernick and Alberts (1985).
Body licking	Licking the pup's body generically except for the anogenital region		
Tactile stimulation	Any contact with pups, such as stepping on pups or resting in contact with pups	Ambrose (1969), Brown et al. (1999)	Mouse pups with rat aunt, Rosenberg et al. (1970)
Non-pup directed behaviors			
Placentophagia	Ingestion of placenta, umbilical cords, amniotic membrane and fluids	Kristal (1980), Kristal, (1991)	Rat female, Kristal and Graber (1976). Mouse female, Kristal and Eleftheriou (1975).
Nest building	Transporting the nesting materials toward the nest or manipulating the material to shape the enclosed nest edge	Denenberg et al. (1969), Koller (1952)	Mouse female, Gandelman (1973).
Defense of the young	Protection of the pups from intruders, predators and environmental hazards. It is called "maternal aggression", if the target of maternal defense is unfamiliar conspecifics.	Gammie (2005), Lonstein and Gammie (2002)	Mouse female, Svare and Gandelman (1976).

from other aspects of maternal care, and can be inversely visible by depriving maternal and/or sibling's body contact, such as artificial rearing with or without tactile stimulation (Gonzalez and Fleming, 2002; Thoman and Arnold, 1968) (Kaufman and Rosenblum, 1967).

2.1.3.4. Nursing. Nursing is the behavior that provides maternal milk to pups, during which the dam is rather quiescent (immobile) and exposing the nipples to pups. Various nursing postures of rat dams are described in detail (Stern and Lonstein, 2001); high crouching (kyphosis) is characterized by rigid limb support and ventroflexion, results in a dorsal arch; low crouching, in which the maternal body mass is supported by four limbs without the arched back; passive or prone position, in which the dam lies over the pups with little or no limb support. Kyphosis is most frequent in the first week of lactation, while nursing in the passive position is seen mainly after the second week of lactation.

2.1.3.5. Defense of the young. Defense of the young is protection of the pups from intruders and environmental hazards. It is called "*maternal aggression*" if the target of maternal defense is an unfamiliar conspecific (Gandelman, 1972; Lonstein and Gammie, 2002). The function of maternal aggression has been suggested to protect pups from infanticide of non-parental conspecifics. Under laboratory conditions, however, maternal aggression is not always successful in preventing intruders from killing pups. It is possible that the protection of offspring is a byproduct of heightened territorial defense in the lactating females (Lonstein and Gammie, 2002).

2.1.3.6. Recognition of the biological offspring. It should be noted that laboratory rats and mice do not selectively care their own young, but also retrieve alien young, provided their age is comparable. At least for mice, this non-selective caring may be related to the trait of communal nesting and nursing in feral *Mus musculus* species (Manning et al., 1992). Nevertheless, when given a choice, rat and mouse dams may retrieve their own young faster than alien pups, a preference abolished by olfactory bulbectomy (Beach and Jaynes, 1956) or by masking pup odors by using perfume (Chantrey and Jenkins, 1982).

2.1.4. After weaning

If dams experience an extensive period of maternal caregiving toward their pups during lactation, their future maternal responsiveness toward pups remains high even after the weaning of their own pups for several months. Therefore, maternal experience can modify the parental brain in a long-lasting manner through a process that has been referred to as "*maternal memory*" (Bridges, 1975, 1996; Fleming et al., 1996).

2.2. Parental behavior of non-lactating adults

All components of maternal behaviors listed in the Table 1 have been observed in non-lactating animals (alloparental or paternal behaviors) at least to some extent and under specific conditions (references are summarized in the Table 1). Describing the details of each study is out of the scope of this article, therefore only one prominent example will be discussed here; maternally-sensitized (see Section 2.1.1) virgin female rats showed kyphosis (high-crouching nursing posture) with strikingly similar latency and duration as that of lactating dams (Lonstein et al., 1999). The same author also showed that the male prairie voles (*Microtus ochrogaster*), a biparental rodent species, exhibited kyphosis in response to ventral somatosensory stimulation by moving pups (Lonstein and De Vries, 1999). Kyphosis has been regarded as specific for nursing, since this posture fully presents all the nipples in the axillary and inguinal cavities, and since it is not effective for warming the pups as little skin contact with pups occurs. In mice also, though less quantitatively, parentally-behaving virgin male and female mice were reported to display a "lactation-position" (the subject covered the young with its body and did not show any other activity) (Noirot, 1969). Without developed nipples and the production of milk, maternal virgins and paternal males cannot display true nursing. Their display of the "nursing" posture during parental care suggests that paternal and alloparental behaviors may utilize the mechanisms of postpartum maternal behaviors, and that the "nursing" posture is elicited as a byproduct of the activation of "maternal" neural circuits in non-lactating females and males.

Nevertheless, compared with the typical immediate and intense maternal care seen in postpartum dams, there is large variation in the nurturing responsiveness of non-lactating animals. Not only the quantitative differences between postpartum dams and non-lactating animals, as evidenced by pup-retrieval under anxiety-evoking environment (Bridges et al., 1972; Gandelman et al., 1970; Stern and Mackinnon, 1976) or by conditioning by pup-associated cues (Hauser and Gandelman, 1985; Lee et al., 2000), non-lactating animals may exhibit qualitatively different responses such as infanticide, depending on species and social context (Brown, 1993; Dewsbury, 1985; Jakubowski and Terkel, 1985a). Such profound variation can be a source of discrepancies between the results of studies dealing with parental behavior in non-lactating animals. Here the findings in rats and mice are discussed separately below.

2.2.1. Rats

Adult virgin female and male laboratory rats initially avoid unfamiliar pups. However, after 5–8 days of continuous cohabitation with donor pups, they first become tolerant of proximate contact with pups, and then they start caring for the pups, which includes licking, retrieving, crouching, and nest building ("*pup sensitization*"), although lactation, of course, does not occur (Rosenblatt, 1967; Wiesner and Sheard, 1933). A small proportion of virgin females, and a larger proportion of virgin male rats commit *infanticide* (pup biting/killing, often but not necessarily combined with cannibalism) upon initial pup exposure (Jakubowski and Terkel, 1985a), but this behavior disappears with successive presentations of pups (Jakubowski and Terkel, 1985b). Once becoming fully parental, virgin female rats can be separated from pups and "*parental memory*" will maintain high levels of parental responsiveness for at least several weeks (Bridges and Scanlan, 2005). Therefore, nurturing experience can modify alloparental responsiveness in a long-lasting manner.

Postweaning-prepubertal male and female rats display qualitatively similar "sensitized" parental behaviors toward pups as do adults, except that their response latencies are with 1–2 days instead of 5–8 days (Bridges, 1996). By 30 days of age, the latency increases to 5–7 days and is retained until the female becomes pregnant, suggesting the development of mechanisms required for inhibition of parental behaviors before puberty. Relevant to this notion, disruption of neural activity in the amygdala or stria terminalis (the major output bundle of the amygdala) or peripheral anosmia induced by nasal infusion of zinc sulfate can facilitate the maternal responsiveness of virgin female rats (Fleming and Rosenblatt, 1974c) (see Sections 3.1 and 3.2.3 for more details). Therefore a neural circuit through the olfactory system to the amygdala may somehow mediate avoidant/repellent responses to pups in virgin adult rats, and this circuit may become functional only after 30 days of age.

Alloparental and paternal behaviors in both male and female non-lactating rats are not inhibited greatly by hypophysectomy and gonadectomy (Rosenblatt, 1967), although quantitatively the ovariectomized female rat may show some reduction of parental nest building and retrieving (Mayer and Rosenblatt, 1979). This finding suggests that a basal level of parental responsiveness can be induced by pup exposure and is independent of hormonal stimulation. On the other hand, the immediate onset of postpartum maternal behavior in primiparous (first-time) rat dams without prior experience with pups is dependent on the hormonal changes at the end of pregnancy. It had been shown that the transfusion of blood from a parturient rat to a virgin (Terkel and

Rosenblatt, 1968; Terkel and Rosenblatt, 1972) can induce parental behavior in the virgin female. Furthermore, pregnancy termination through hysterectomy (removal of the uterus, fetuses, and placenta) on days 15–17 of pregnancy (Bridges et al., 1978a,b; Rosenblatt and Siegel, 1975) also facilitates the onset of maternal behavior. Further analyses have shown that the decline of plasma progesterone and simultaneous rise of estradiol that occur at parturition in the rat and other species are among the factors involved in the induction of short-latency maternal behavior. It should be noted, however, that the endocrine basis of pregnancy and parturition can vary greatly between mammalian species, raising the possibility that the hormonal basis of the onset of parental behavior may also differ. For example, in the hamster, a dramatic decline in the progesterone-to-estrogen ratio near term does not occur, but both hormones decline together before parturition (see Numan, 1985 for more discussion).

2.2.2. Mice

Virgin females of most laboratory mouse strains may initiate parental care only after several to 30 min of cohabitation with pups (Lonstein and De Vries, 2000b; Noirot, 1972). This period is much shorter than that of the virgin female rats (a few days as described above), so that the virgin female mice are often said to be "spontaneously parental" like postpartum dams. However, Noirot has pointed that the virgin female mice undergo a change, apparently analogous to sensitization in the rat, during their initial exposure to donor pups; they first sniff the pup from a distance with closed eyes, then "nose" it (i.e., sniff while touching the pup with the snout) (Noirot, 1972). In between these bouts of investigation, the virgin runs between the pup and the nest, apparently in an approach–avoidance conflict. In addition, their retrieving latency decreases by repetitive presentation of donor pups, suggesting that the previous parenting experience can enhance parental responses even in laboratory mouse females. Infanticide upon initial pup exposure is rare in virgin female laboratory mice, and once they become parental by repeated pup exposure, they seldom revert back to rejecting pups, at least during successive daily pup exposures. As in rats, alloparental behaviors of virgin female mice are independent of hypophyseal hormones (Leblond, 1940; Leblond and Nelson, 1937).

Behavioral responses toward donor pups of virgin (naïve) male mice may differ drastically by strains and experimental conditions, ranging from immediate infanticide to intense alloparental care (for example, Kennedy and Elwood, 1988; Kuroda et al., 2008; Parmigiani et al., 1999; Wright and Brown, 2000). In many strains including C57BL/6J, a standard inbred strain with high sociability, the majority of virgin males commit infanticide (70% in C57BL/6J; vom Saal, 1985) even after repeated pup exposure (Jakubowski and Terkel, 1982). Once a male has mated with a female and cohabitates with the pregnant mate, however, he eventually stops infanticide by the time of delivery of his biological offspring. And at this time, the father will also perform paternal care toward a non-biological offspring (Priestnall and Young, 1978; vom Saal and Howard, 1982). Paternal behavior in male mice is functional, since the presence of the father facilitates pup survival (Barnett and Dickson, 1985; Wright and Brown, 2000).

Spontaneous infanticide of non-biological offspring by males is not caused by starvation or high-stress conditions (vom Saal and Howard, 1982), and has often been regarded as abnormal or pathological (e.g. Calhoun, 1963). However, evidence suggests that such infanticide seen in many wild mammalian species is adaptive in terms of *inclusive fitness*; that is, it is beneficial for the survival of their own biological offspring at the expense of non-biological offspring that are potential competitors for environmental resources (Trivers, 1972). Especially for species forming a harem (polygyny) of a small number of males with multiple females such as langurs, lions and mice, a new male tends to kill all the existing young upon take over a harem; such infanticide brings the females' lactation to an end, which hastens the occurrence of ovulation (Hrdy, 1974). The new alpha male stops infanticide by the time that its biological offspring are born. This timing is clearly correlated with the gestation period of the particular species (Parmigiani and vom Saal, 1990). These findings support the idea that infanticide of non-offspring young is a valid and adaptive reproductive strategy selected through evolution. One should not draw the conclusion that male mice are always more aggressive toward infants than are females; in feral house mice, which are more territorial and aggressive than their laboratory counterparts, adult females as well as males are infanticidal. During pregnancy, the females are even more infanticidal than males (McCarthy and vom Saal, 1985; Soroker and Terkel, 1988). During the lactation period, the females care their biological offspring, and may also adopt alien pups. A month after the weaning of the offspring, females start infanticide again. Such infanticide of feral female mice can also be explained as a reproductive strategy to increase the possibility of survival of their own offspring at the expense of the alien pups.

3. The neural systems involved in detection of pup sensory cues and in expression of the parental retrieving response

Here the neural processes that underpin the parental retrieval response, beginning with the encounter of a displaced pup, which then leads to pup retrieval, will be subdivided into three stages; 1) detection of pups through sensory cues, 2) selection of parental rather than non-parental responses to the pup, and 3) organization of behavioral responses over time and space. Disturbance of any of these steps may cause deficits in parental behavior. The relevant brain areas and genetic factors in each step will be then discussed.

3.1. Detection of pups through sensory cues

Sensory stimuli (olfactory, auditory and somatosensory) from pups should be integrated and bring about pup recognition; i.e., there is a conspecific young animal, but not another object or food. In rats, Beach and Jaynes (1956) showed that the surgical elimination of either vision, olfaction, or tactile sensitivity of the snout and the lip region leaves maternal retrieving intact in postpartum rats. Elimination of any two, or all three of these sensory systems did not completely abolish the behavior, although some deficits in maternal retrieving were observed. Herrenkohl and Rosenberg (1972) tested the effects of prior sensory desensitization performed during pregnancy on the subsequent behavior of primiparous (first-time) rat dams. Neither induced deafness by destruction of the basilar membrane, anosmia by olfactory bulbectomy, nor blinding by orbital enucleation caused a major disruption of maternal behaviors in most of the rats. These studies supported the concept of a *multisensory control* of maternal behavior, in that no one sensory modality was found to be essential for either the onset or the maintenance of maternal behavior in rats.

Olfaction deserves detailed description compared with other senses, because mice and rats are macrosmic mammals. In rats, peripherally-induced anosmia has been shown to decrease anogenital licking behavior (Moore, 1984) and maternal aggression (Ferreira et al., 1987; Mayer and Rosenblatt, 1993), but results in only minor deficits in retrieving (Benuck and Rowe, 1975). Removal of the *vomeronasal organ* (VNO) or vomeronasal nerve cuts do not result in maternal behavior deficits (Fleming et al., 1992; Jirik-Babb et al., 1984; Kolunie and Stern, 1995). More importantly, as in other mammalian species such as hamsters, rabbits and sheep, olfactory information from pups is necessary for the typical pup avoidant reaction that occurs in non-maternal virgin female rats (see Levy and Keller, 2009). Olfactory bulbectomy, complete vomeronasal nerve cuts, pharmacological blockade of transmission in the accessory olfactory bulb, or destruction of the olfactory epithelium by intranasal application of zinc sulfate, all reduce the latency to the onset of parental responsiveness in virgin rats

(Carretero et al., 2003; Fleming and Rosenblatt, 1974a,b,c). Therefore the olfactory cues from pups have bidirectional effects on rat parental behavior, to enhance avoidant reaction to pups in non-sensitized virgin females, and to help maternal identification of pups in parental rats as well as to stimulate anogenital licking (see Levy and Keller, 2009; Numan, 1985 for more discussion).

In mice, olfactory dysfunction causes unidirectional inhibition of parental behaviors in both virgin and postpartum females (see Levy and Keller, 2009). The original studies using Rockland–Swiss albino mice showed that olfactory bulbectomy not only eliminated maternal behavior but also caused cannibalism of the whole litter within 36 h in nearly all cases of both virgin and multiparous postpartum female mice (Gandelman et al., 1972, 1971); it should be noted, however, that this surgical procedure may disturb non-olfactory functions of the olfactory bulb and adjacent frontal brain areas, and consequently depression-like emotional symptoms (Kelly et al., 1997). Indeed, peripheral anosmia induced by zinc sulfate near the end of pregnancy resulted in pup-killing in 13 of 15 primiparous litters, but only 2 of 15 multiparous dams were pup-killers (Seegal and Denenberg, 1974). Recent studies in ddY mice reported milder deficits in primiparous bulbectomized mouse dams; such mice performed decreased arched-back nursing and licking/grooming of pups on postnatal day 0, but did not differ with the control mice on postnatal day 4 (Sato et al., 2010a,b) (interestingly, these deficits could be ameliorated by administration of a dopamine receptor agonist apomorphine). As a result of this deficit, the pup survival for the bulbectomized dams was less than half that of sham dams, but no cannibalism has been noted. In support of these Sato's studies, genetic mutant strains of congenital anosmia have been reported to exhibit significantly impaired postpartum maternal behavior and parental behavior by virgin females, without causing infanticidal tendency (see Section 5.5.1).

VNO removal does not affect mouse maternal retrieving, nursing or nest building, but significantly reduces maternal aggression as well as male aggression toward an unfamiliar male intruder (Bean and Wysocki, 1989). Similar findings have been reported in two genetically-engineered mouse strains (Section 5.5.2).

In summary, 1) mouse parental retrieving behavior is more dependent on the main olfaction system than that of rats, and 2) the accessory olfactory system does not positively regulate rat, or mouse parental retrieval, while it is necessary for expression of maternal aggression in mice and for aversive responses toward pups in rats.

3.2. Selection of parental versus non-parental responses to pups

3.2.1. Non-parental responses, and relevant brain areas and molecules

After pup recognition through the sensory cues, the adult mouse does not necessarily initiate parental care. For example, an adult male mouse may choose either infanticide or paternal behavior, according to his previous mating experience. If this male did not have mating experience in the appropriate time interval, it is highly unlikely that these pups are his own offspring. In such a case, infanticide is selected as the most adaptive behavior in terms of inclusive fitness, as described in Section 2.2. It has been shown that the VNO ablation decreases infanticide and induces paternal behavior in male rats (Mennella and Moltz, 1988).

Even in postpartum dams, a gradual or abrupt termination of parental behaviors occurs at weaning. In some feral mammals, this weaning process is accompanied by the aggressive forced dispersal of juveniles by parents. The ultimate causation of this phenomenon is parent–offspring conflict (Bekoff, 1977). The proximate causation for offspring dispersal may be food competition in the territory (an external cue), or seasonal changes in the hormonal milieu of the parents, which favors sexual behavior over parental behavior (internal cue). Although the sensory cues from the juveniles do not differ much before and after the start of the parental rejection of juveniles, a mechanism must exist in the parents which allows them to determine their type of reaction, according to its reproductive stage, social context, and previous experience. Although there is scarce information about the brain areas affecting such behavioral choices according to external and internal conditions, the *anterior nucleus* (AH) and the *ventromedial nucleus* (VMH) of the hypothalamus are good candidates for two reasons; first, lesions of the AH or VMH can enhance parental retrieval in virgin female rats (Bridges et al., 1999; Sheehan et al., 2001); and second, the AH or VMH influence feeding, defensiveness, and female reproductive behavior. So it is reasonable that if either the AH or VMH is activated by food scarcity or by the female sexual drive, these areas may inhibit parental responses, as continued parenting of older offspring may not be compatible with feeding or sexual behavior.

3.2.2. The medial preoptic area: the critical brain area for parenting

The *medial preoptic area* (MPOA) has been proposed as the most critical brain region for the expression of parental retrieving behavior (Morgan et al., 1999; Numan, 1994). The evidence is: 1) receptors of female reproductive hormones such as prolactin and estrogen are expressed in the MPOA, and application of these hormones can enhance parental behavior in female rats (Bridges et al., 1990, 1997; Fisher, 1956; Numan and Insel, 2003; Numan et al., 1977); 2) MPOA lesions, especially in the dorsolateral part of the MPOA specifically inhibit pup retrieval in both postpartum and pup-sensitized virgin female rats, without affecting feeding, general locomotion, female reproductive functions, or female sexual behaviors (Numan, 1974; Terkel et al., 1979, see also Kalinichev et al., 2000a; Lee and Brown, 2002) [we have confirmed similar effects of a dorsal MPOA lesion on parental retrieving in laboratory mice (Tsuneoka et al., 2010)]; and 3) when a rat or mouse takes care of pups, c-Fos and FosB, molecular markers of transcriptionally-activated neurons (Herdegen and Leah, 1998), are induced in MPOA neurons (Calamandrei and Keverne, 1994; Li et al., 1999a; Numan and Numan, 1994). No other brain area has been reported to consistently and specifically fulfill these conditions as the MPOA does.

Interestingly, the preoptic area (POA) has been implicated in avian parental behavior (see Buntin, 1996 for details); POA lesions prevented the onset of incubation and the concomitant increase in plasma prolactin levels in turkey hens (Youngren et al., 1989). In addition, axon-sparing lesions of the POA profoundly disrupted parental regurgitation feeding behavior in ring doves, as well as related activities induced by subcutaneous prolactin administration in nonbreeding doves with previous breeding experience (Slawski and Buntin, 1995). Thus, the POA may have played a key role in parental behavior even before the emergence of mammals.

Nevertheless, the MPOA does not necessarily serve a unitary function with respect to the stimulation of all kinds of parental behaviors. First, the MPOA is less responsible for parental nursing/crouching behavior than for parental retrieving (Numan, 1990). Second, like any brain region, the MPOA contains a heterogeneous population of neurons, and only some of these might influence certain aspects of parental behavior. For example, c-Fos expression in the MPOA is mildly elevated by pup exposure in infanticidal male mice (Kuroda et al., 2007), suggesting these c-Fos positive neurons may be involved in pup recognition. Third, the MPOA may even restrict parental responses in certain instances, as one recent study showed that transient MPOA inactivation disrupted rat maternal behavior at postpartum day 7–8 as expected, but surprisingly facilitated maternal behavior at postpartum day 13–14 (Pereira and Morrell, 2009). These studies suggest that different subregions or neuron populations within the MPOA may have differential roles on an individual's responsiveness to pup cues. In this sense, more detailed chemical and neuroanatomical studies within the MPOA will be required to fully understand the role of the MPOA in parental behaviors. The dorsolateral part of the MPOA has been suggested to be more important for pup retrieval behavior in both rats and in mice, compared with the ventral part of the MPOA (see (Kalinichev et al., 2000a; Numan

et al., 1990; Terkel et al., 1979) (Tsuneoka and Kuroda, manuscript in preparation), but (Lee and Brown, 2002)). However, this subregion of the MPOA has not been clearly delineated in either the rat or mouse brain atlas. There is an inherent problem with the fact that the boundary between the dorsal part of MPOA and the adjacent bed nucleus of stria terminalis cannot be clearly drawn by cytoarchitechtonic analyses, either in rats (Ju and Swanson, 1989) or in mice (Broadwell and Bleier, 1976).

Furthermore, the exact properties of c-Fos positive MPOA neurons during parental retrieving, such as their afferent and efferent connections and neurotransmitters, require further investigations. It should be noted that the c-Fos positive MPOA neurons are not homogeneous: about 25–45% of them are estrogen receptor alpha positive, and about 53% of them are GABA (γ-amino butyric acid)-ergic in postpartum rats (Lonstein and De Vries, 2000a; Lonstein et al., 2000). There might be a subpopulation of MPOA neurons that can be activated simply by pup recognition, while others may be more directly relevant to actual parenting performance, as evidenced (Li et al., 1999a; Mattson and Morrell, 2005; Numan and Numan, 1995).

At the level of the neural circuitry, important connections of the MPOA neurons with other brain areas have been reported; firstly, Numan and Numan (1997) showed that MPOA neurons that express c-Fos project to VMH and the periaqueductal gray and they suggested that such projections might serve to depress the avoidance pathway which opposes maternal responsiveness. Secondly, MPOA interactions with the mesolimbic dopamine system, which includes dopaminergic projections from the ventral tegmental area (VTA) to the nucleus accumbens (NA), plays a positive role in regulating the attraction to pup-related stimuli (Numan and Stolzenberg, 2009). This notion is evidenced by the facts that the MPOA neurons which express Fos during maternal behavior project to the VTA (Numan and Numan, 1997), that the dopamine D1 receptor agonist injection into NA reduces the onset of parental retrieving behavior in nulliparous female rats (Stolzenberg et al., 2007), and that disconnection of the MPOA from either the VTA (Numan and Smith, 1984) or from the ventral pallidum (a projection site of NA efferents) disrupts maternal retrieving behavior (Numan et al., 2005). Therefore, neural models have been proposed which suggest dual outputs from the MPOA that foster maternal responsiveness; one output may depress the aversion system and defensive avoidance responses; second output from MPOA acts to stimulate the mesolimbic dopamine system, which then increases the female's maternal responsiveness to pups.

3.2.3. Role of the amygdalar complex in regulation of parental behavior

Involvement of the *amygdalar complex* in the regulation of parental behavior appears to be complicated and deserves detailed discussion at a broader scope. Originally it had been believed that the amygdala does not play a critical role in the maternal behavior. Slotnick and colleagues found that the electrical lesions in the amygdala caused little or no deficits in maternal behavior of both postpartum rats and mice (Slotnick and Nigrosh, 1975). Moreover, electrical and excitotoxic amino acid lesions of the medial amygdala actually facilitated pup retrieval in virgin female rats (Fleming et al., 1980; Numan et al., 1993). Virgin female rats with electrical lesions of the stria terminalis, a major output from the amygdalar complex, became maternal more rapidly than did control animals (Fleming et al., 1980). Of relevance, human females with complete bilateral lesions of the amygdala, including a famous female patient SM with Urbach–Wiethe disease [of which more than half of the patients have bilaterally symmetrical damage in the amygdaloid region], were impaired in detecting negative emotions in facial expressions, but were fully capable of effectively rearing their own children (Adolphs et al., 1994; Amaral et al., 2003b; Hurlemann et al., 2007). These amygdala lesion studies suggested that the major function of the amygdalar complex was the detection of a potential threat in the environment and production of fear responses, rather than social affiliation *per se*. Fear reduction, in rats at least, may facilitate maternal responsiveness by decreasing the virgin female's defensive responses toward novel and unfamiliar infant stimuli that are processed by the cortical and medial amygdala, which then allows for shorter sensitization latencies: the females show maternal behavior sooner after pup exposure (Fleming et al., 1980).

Recent studies have suggested that the function of the amygdala is more complex than simply detecting threats and also involves the detection of reward, saliency, and biological relevance that are required for diverse emotional and social behaviors (Adolphs, 2010; Murray, 2007). Amaral and colleagues investigated the effects of neonatal (at two-weeks of age) lesions of amygdala, made by bilateral injections of ibotenic acid, on social behaviors in rhesus monkeys, compared with the sham or hippocampal lesions. These neonatally amygdala-lesioned monkeys showed reductions in innate fear responses toward inanimate objects and paradoxical fearfulness during social encounters, while leaving the fundamental aspects of age-appropriate social behaviors intact, such as physical contact with their mothers and suckling (Bauman et al., 2004; Prather et al., 2001; Amaral et al., 2003a). Immediately after the permanent separation from their mothers, the amygdala-lesioned animals did not preferentially seek proximity to their mother, nor did they produce distress vocalizations (Bauman et al., 2004), a phenomenon which could be attributed to their impaired ability to perceive potential danger rather than to a disruption of the infant's attachment to its mother. When tested as adults, these amygdala lesioned females showed decreased affiliative vocalizations toward infants (Toscano et al., 2009), although affiliative vocalization does not necessarily correlate with affiliative behavioral actions toward infants (Maestripieri, 1999). Therefore, a reasonable interpretation is that these amygdala-lesioned female monkeys were less aroused or interested by the presence of infants, and less likely to produce the species-typical vocalizations that are normally evoked in the presence of infants.

In laboratory rats, the electrolytic lesions of the basolateral amygdala (BLA) cause mild-to-moderate deficits in pup retrieval behavior (Lee et al., 2000, 1999), but more significant deficits in operant bar-pressing responses when pups are used as the reinforcing stimulus (Lee et al., 2000). A more recent study investigated the effects of the transient suppression of the basolateral and basomedial nuclei of amygdala (BLA/BMA) by muscimol on pup retrieval in postpartum rats (Numan et al., 2010). It showed that 100 and 200 ng/side of muscimol injections into BLA/BMA caused major deficits in retrieval behavior and minor deficits in nursing behavior, while muscimol injections into medial amygdala did not have disruptive effects. Therefore, the muscimol suppression of BLA/BMA may paradoxically cause larger effects on maternal behavior than permanent BLA lesions by electric damage (Lee et al., 2000, 1999) or ibotenic acid lesions (Martel et al., 2008). It is possible that reversible disruption of the BLA/BMA, where a brain region is taken offline for a short period of time, causes more severe effects than do permanent lesions.

"(The amygdala) clearly contributes to processing emotionally and socially relevant information, yet a unifying description and computational account have been lacking. The difficulty of tying together the various studies stems in part from the sheer diversity of approaches and species studied, in part from the amygdalar inherent heterogeneity in terms of its component nuclei, and in part because different investigators have simply been interested in different topics." (Adolphs, 2010). More work will be needed to determine whether amygdala lesions directly affect maternal responses, or indirectly influence the behavior through the well-established role of amygdala function in the detection and avoidance of environmental dangers. In doing so, subregions of the amygdala should be separately examined. In particular, BLA/BMA connections to the NA and adjoining ventral pallidum, a part of the mesolimbic dopamine circuitry as NA, may be relevant for maternal responses (Numan et al., 2010) and warrant further investigations.

3.3. The organization of parental responses over time and space

Once a behavioral choice has been made in favor of parenting, brain mechanisms need to decide how to do it according to the needs of pups. Upon experimental pup exposure through the introduction of donor pups to a singly-housed parous female mouse or rat with previous maternal experience, the following sequence of behaviors are observed: first, the female goes around the cage and orally retrieves pups one by one to the nest site. [If the nest has been disturbed by the experimental procedure, she first briefly establishes the new nest site and then retrieve the pups from the original nest to the new site, the behavior termed as "a corollary of the retrieving activity" (Wiesner and Sheard, 1933)]. After the last pup is retrieved to the nest, the female goes all around the cage once again to make sure that there are no pups left outside the nest ("returning"; Wiesner and Sheard, 1933). Then the female goes back to the nest and licks and grooms the pups, crouches over the pups to make them warm and nurses if possible, and then continues nest building to finally achieve a brooding nest much bigger than the normal nest for a single mouse. In this way, experienced dams follow a certain sequence of behaviors to efficiently and smoothly fulfill the pups' needs. Virgin mice have been reported to follow a similar serial order of behavior, in which pup retrieval normally precedes pup licking and nest building, and the nursing-like crouching tends to be the last of the sequence (Noirot, 1969).

In both mice and rats, the cingulate cortex, septum, fimbria and hippocampus are involved in this organization process, as surgical lesions of either of these brain areas causes inefficient parental behavior; the lesioned animal makes many small nests for each pup instead of retrieving all pups into one nest, or carries pups around and places them outside of the nest (Fleischer and Slotnick, 1978; Slotnick, 1967; Slotnick and Nigrosh, 1975; Terlecki and Sainsbury, 1978 also refer Lorberbaum et al., 2002; Murphy et al., 1981). It was suggested that maternal responsivity was not disrupted in these females, but the integration and spatial organization of the various maternal responses into an effective temporal sequence was disrupted, leading to inefficient pup care (Slotnick, 1967; Terlecki and Sainsbury, 1978). A similar pattern of retrieval failure that is seen in *GluR-BΔHS* and *Stmn1* gene mutant mice (see the Sections 5.2.4 and 5.6.2, respectively for details) such as atypical site selection for the nest building, suggested that the female mutant mice had problems in spatial and temporal organization of pup retrieval and nest building.

In summary, we may be able to safely say that parental behavior includes many stages, and there are several brain regions and molecules implicated in each stage.

4. Identification of parental care defects in genetically-engineered mouse strains

Recently, a growing number of gene knockout mouse strains have been reported to be defective in parental behavior (for review, see Gammie, 2005; Leckman and Herman, 2002; Numan et al., 2006). In the *Mouse Genome Informatics* (MGI: http://www.informatics.jax.org/), the most comprehensive and reliable database in mouse genetics, 164 genotypes are registered for abnormal parental behavior, including 20 that are abnormal in pup retrieval, 18 for pup cannibalism, and also 50 genotypes that are abnormal in nursing ability, among a total of 43,193 genotypes. Taking a closer look at each notation with the referenced publication, however, one finds that this list includes some cases that may not be appropriately classified with respect to maternal phenotype.

The popularity of reverse genetics in laboratory mice has increased the incidence of detecting unexpected and unfamiliar phenotypes in newly-developed gene-targeted mice. Poor reproductive outcomes can be easily identified, as it can be observed by just leaving knockout females and males together, and isolating the pregnant females to measure pup survival, without special equipment or time-demanding behavioral testing. When pups are born but do not survive to weaning, this poor survival tends to be attributed to poor maternal behavior. However there are many factors affecting the pup survival, each of which require careful examination before a maternal deficit can be established, as generally pointed out for behavioral phenotyping of genetic mutant mice (Bailey et al., 2006).

First several technical and methodological comparisons between studies using rats and mice will be described in Section 4.1. Next the various factors affecting survival of genetically-engineered mice will be discussed in Section 4.2. Then a protocol for screening of these factors in postpartum maternal behaviors will be proposed (Section 4.3). A similar protocol extended for general parental retrieval assay of non-maternal mice will be presented as well (Section 4.4).

4.1. Technical considerations for assessment of parental behaviors using mouse genetics

4.1.1. Components of parental behaviors: comparison between rats and mice

All parental behavior components listed in Table 1 can be quantified in laboratory rats and mice (Capone et al., 2005; Lonstein and Fleming, 2002). Among these, pup-retrieval behavior is widely used as an index of parental responsiveness in both rats and mice (e.g. Brown et al., 1996; Lucas et al., 1998; Numan et al., 1990; Rosenblatt, 1967). Especially the latency to retrieve each pup is easily and unambiguously measurable, and can be assessed not only in postpartum dams but also in non-lactating females and males. The latter point is a great advantage in studies of genetically engineered mouse strains, because it is testable even when the mutant female mice are infertile and therefore cannot be tested for postpartum maternal behavior. In addition, if the parental behaviors in both postpartum and virgin female mice are compromised, it is unlikely that the given gene mutation affects the parental behavior indirectly through physical changes associated with pregnancy and parturition.

The amount of pup licking/grooming (Champagne et al., 2001; Liu et al., 1997) is also extensively used as the index of maternal care in rat dams. This behavior, however, is technically more difficult to be precisely distinguished from pup sniffing or from maternal self grooming in mice because of their smaller body size and the consequent high speed of this behavior. Duration of various nursing postures as well has been extensively studied in rats (Lonstein et al., 1999), while in mice the kyphosis (high crouch) posture may be rather rare, at least in some strains including C57BL/6 (Capone et al., 2005; Shoji and Kato, 2006). On the other hand, assessment of the nest quality is a preferred measure of parental behavior in mice, as in many laboratory mouse strains such as C57BL/6, adult mice tend to construct a relatively complex nest (Hess et al., 2008).

Since a major aspect of this review is the examination of parental behavior using genetically-engineered mice, the mechanisms of parental behavior measured mainly by pup retrieval will be emphasized and discussed in depth in Section 4-3 and 4-4. Especially, the alloparental pup retrieval assay of virgin female mice (Section 4-4) is very cost-effective and is highly recommended, since virgin female mice do not require lengthy pup sensitization process to start pup retrieval.

4.1.2. Housing and environmental factors

Compared with wild-type animals often housed under "conventional" animal husbandry with relaxed access rules, recombinant mouse strains are usually reared in fairly-standardized housing conditions according to published guidelines, which indicate requirements on ventilation (8–20 air changes/hour), temperature (20–24 °C), humidity ($50 \pm 10\%$), lighting (60–400 lx), photoperiod (12/12 or 14/10), noise levels ($\leq$80 dB), health status, feeding, water supply, and animal enclosures (Hedrich, 2004). In the *specific pathogen free* (SPF) units, the respective animals are regularly monitored as being free from the

specified pathogens (Nicklas et al., 2002). Such regulations ensure an appropriately sterile environment, though not completely germ free, for behavioral examinations of congenic and/or genetically engineered mouse strains, which may be compromised in general health in comparison to the wild-type mice. On the other hand, such regulations inevitably limit flexibility with respect to experimental manipulations of housing conditions, such as environmental enrichment, use of large or complex test cages, and group housing, as well as the comfort of the animals (Hedrich, 2004). In addition, the costs for strict control of the environment often restrict cage space and the number of available animals for each experimenter.

Circadian rhythm and parental behavior. Rats and mice are nocturnal animals, so that the dark phase is active and the light phase is inactive for most of behaviors including social ones. Under natural conditions, however, the dam stays inside the burrow making contact with the pups during the day, foraging at night. Under laboratory conditions, rat dams in mid-lactation spend almost twice as much time near the pups when the lights are on than when the lights are off (Grota and Ader, 1969). Thus maternal behavior in rodents exhibits diurnal variations opposite in directions from many other behaviors. Yang et al. pointed out practical difficulties of dark-phase testing such as reversing the light/dark cycles, light-proof animal transportation between rooms, and decreased visual acuity for scoring behaviors and identifying animals under the red light (Yang et al., 2008). They showed that remarkably similar social scores were obtained from inbred mice tested in the light or the dark phase, providing evidence that light phase testing could yield reliable and consistent results with dark phase testing. Indeed, in both rats and mice, social behavior tasks have been performed in both light and dark phases in different laboratories (e.g., for mouse maternal behavior, in the light phase (Lucas et al., 1998); in the dark phase (Lefebvre et al., 1998)). Researchers should equalize, of course, the circadian timing of behavioral tests between the control (wild-type) and experimental (mutant) groups of animals.

4.1.3. Strategies of genetic engineering

To study the molecular basis of behavior, targeted disruption or "*knockout* (KO)" of single genes has several advantages: 1) disabling a gene is often a very precise and total ablation of the molecule; 2) phenotypes of null mutation often reflect the role of the respective gene product more clearly than do phenotypes caused by over-expression of the same gene; 3) genetic manipulation may be the only available way to interfere with the molecular function of many endogenous proteins and in a very early postnatal period (Bucan and Abel, 2002). As with many other experimental methods, however, the interpretation of behavioral data obtained from this technique may be limited because the indirect effects of the missing gene, rather than its direct effects, may affect behavior under study. Furthermore, the missing gene might affect many developmental processes throughout ontogeny and compensatory mechanisms may be activated in knockouts, as extensively discussed previously (Nelson and Young, 1998).

The precise control of gene expression, both temporally and spatially, is ideal for identification of the role of the respective gene product on a certain behavior. Various emerging techniques, utilizing the site-specific DNA recombinases Cre and FLP, the tetracyclin- and other prokaryotic/yeast regulatory mechanisms, and light-activated channels and enzymes such as channelrhodopsin-2 (optogenetics), will eventually enable reversibly turning single gene expression on and off in a tissue- or cell-type specific manner (Havekes and Abel, 2009; Luo et al., 2008; Mallo, 2006; Zhang et al., 2010). Currently, however, there are still many difficulties and shortcomings inherent in the available techniques, so that the data obtained through any technique should be interpreted with caution and should be integrated with data obtained through other methods. In addition, to be able to utilize these versatile modern genetic technologies, the precise anatomical definition and cytochemical markers must be determined at the target brain areas.

4.1.4. Genetic background

The confounding effects of *genetic background* on behavioral phenotypes have been acknowledged (Doetschman, 2009), complicating the interpretation of findings. Most of the embryonic stem cells initially used for mouse genetic engineering are derived from 129 substrains, which exhibit varying degrees of hypomorphic corpus callosum and an inferior learning ability (Balogh et al., 1999). Therefore it is preferred to *backcross* the mutant strain into the other genetic background, such as C57BL/6, a standard congenic strain, for neuroscience research. Backcrossing is usually performed by mating a heterozygous female with a C57BL/6 male, and the heterozygous female offspring is selected to mate with a C57BL/6 male again. This method is popular because female sexual maturity is achieved earlier than that of males. However if the heterozygous females have any problem in overall reproductive performance, the heterozygous males can be selected for backcrossing with the C57BL/6 female to yield the same result. After five generations of backcross, 96.875% of the whole genome is of the C57BL/6 background.

Even after the mutation has been transferred into a standard genetic background, there are, genetic complications due to the *flanking-gene effect*; that is, the closely linked genes surrounding the targeted locus tend to remain even after 20 generations of backcrossing, and affects the apparent phenotypic differences between the mutant (the flanking genes are of the original background) and the wild-type (the flanking genes are of C57BL/6 derived). This problem can be properly addressed by the specific breeding strategies proposed previously (Wolfer et al., 2002). In addition, *epistasis* (interactions between different genetic loci) is the phenomenon where the effects of one gene are modified by one or several other genes, which are sometimes called modifier genes (Crusio, 2004). Care should be taken for various functional types of genetic interactions, such as "genetic suppression" (the double mutant has a less severe phenotype than either single mutant), "intragenic (allelic) complementation" (two mutations map to the same locus, yet the two alleles complement one another in the heteroallelic diploid), or "unlinked non-complementation" (two mutations fail to complement and yet do not map to the same locus), which are extensively described in classic model organisms of genetics, such as fruit fly and yeast. These genetic interactions also provide valuable information about functional relationships of the relevant genes.

4.1.5. Breeding techniques

For production of experimental mice, in general, the subject homozygous *(−/−)*, heterozygous *(+/−)* and control *(+/+)* mice should be littermates (reared by the same *(+/−)* dams) (Crawley, 2007), in order to minimize the potential confounding influences of background genes from breeder parents and/or rearing environmental factors. Although this procedure cannot rule out the possibility that dams may treat different pup genotypes differently, at least this procedure equalizes general maternal care and cage conditions. Breeding methods in which *(−/−)* animals are from a *(−/−)* cohort and *(+/+)* animals are from a *(+/+)* cohort separately should be avoided, particularly for studying maternal behaviors, since maternal behavior is known to transmit across generations nongenomically (future maternal behavior is affected by the maternal behavior received as neonates) (Francis et al., 1999), as well as general genetic concerns (Crawley, 2007). Also, care should be taken for breeding and maintenance of the mutant strain, when a given genetic mutation causes harmful effects on survival or reproductive success. In such cases, it is important to minimize manifestation of this phenotype during breeding by environmental support or by specific breeding methods. Otherwise, the selection pressure would concentrate genetic variations and spontaneous mutations into a direction which ameliorate the harmful phenotype, so that the apparent phenotype becomes weak after multiple generations. If the harmful phenotype cannot be avoided

during breeding process (e.g. transgenics, dominant mutations), the mutant germ cells should be cryopreserved at an earlier generation, and should be periodically reanimated for use in limiting the generation after construction.

4.2. "What's wrong with my mouse mother–infant dyad?"

Here we subdivide the cause of a poor outcome in mother–infant (dam–pup) interactions within genetic mutant mice, that ultimately affects pup growth and survival, into three aspects: deficits caused by the pups' genotype (pup factors), deficits caused by the maternal genotype (maternal factors), and factors caused by the gene–environment interactions. These factors should be dissected from each other in the apparently complex dam–pup interactions, as suggested (Bailey et al., 2006).

4.2.1. Pup factors

4.2.1.1. General health. Pups with any kind of *unhealthy* signs (e.g., reduced activity, smaller body size, congenital malformations, low body temperature, bleeding, respiratory distress) can be distinguished by the dam and may be rejected, neglected, or cannibalized. This is an adaptive part of the normal repertoire of mouse maternal behaviors. This maternal selection tends to be stringent if the litter size is large, or if the contrast between healthy and unhealthy pups is large. In MGI, several strains classified as showing "abnormal parental behavior" ("abnormal maternal nurturing" or "pup cannibalism"), including *sftpb* (encoding surfactant associated protein B) (Clark et al., 1995) and *tcfap2* (Kohlbecker et al., 2002), are actually strains that contain unhealthy pups, so that the dam–pup dyad problem should be attributed to mutant pups rather than to the mutant dams. If the pups of the subject dam are all unhealthy and the experimenter still wants to test the maternal behavior of the dam, these unhealthy pups should be removed and replaced with healthy non-offspring pups of the same age, to observe whether retrieving and other types of maternal care will occur or not (Section 4.3.2.4.).

4.2.1.2. Litter size. If the number of pups delivered (*litter size*) is unusually small, even the wild-type dam may lose maternal responsivity, and may abandon the whole litter (Stern and Johnson, 1990). The *Ambp* (encoding alpha 1 microglobulin/bikunin) mutant strain (Zhuo et al., 2001), which has been classified as "abnormal maternal nurturing behavior" in MGI, might actually be suspected to fall into this case. This mutant strain showed impaired implantation, so that even when knockout females became pregnant, they delivered only one or two pups in one litter. These pups could be saved by fostering to a non-mutant dam with a larger litter, but would otherwise die within 2 days if they remained with their biological dam. In this case, the pups' death might be attributed to the abnormally small litter size, although maternal behavior defects in knockout dams cannot be ruled out. In such cases, the testing of maternal behavior by giving several healthy donor pups to these knockout dams, or the examination of allomaternal behavior in virgin female mutants, could be used to detect actual deficits in parental behavior.

4.2.1.3. Suckling. Even if the pups appear normal right after the birth, the mutant pups may have abnormalities in *suckling* maternal milk. Suckling behavior can be divided into four steps: nipple location, oral grasping of the nipple, rhythmic oral movement for suction, and swallowing. Olfaction is crucial for suckling in neonatal mice in that pheromones and other olfactants from the nipple are the main sensory cues used in the nipple's location by pups (Blass and Teicher, 1980; Distel and Hudson, 1985) [this should be separated with the issue of maternal anosmia, as discussed in the Section 5.5.1]. Tactile sensation is also important for suckling behavior, because a rooting reflex is activated, in part, by touching a nipple, a stimulus that initiates the rhythmic mouth movement and swallowing. Indeed, impaired suckling behavior is a typical abnormal phenotype in neonates with mutations in genes required for olfaction or tactile sensation. In this connection, the vomeronasal organ does not seem crucial for suckling, as suckling is normal in VNO-removed neonatal rabbits (Hudson and Distel, 1986) and in mutant mice with congenital dysfunction of the VNO-accessory olfactory system (Kimchi et al., 2007). Obviously, malformations of oral cavity, oromotor problems, and any other general problems may disturb suckling behavior (e.g., *Cnr1* gene mutants, lacking cannabinoid receptor 1 CB-1) (Fride et al., 2003).

4.2.2. Maternal factors

4.2.2.1. Parturition and general peripartum health condition. Obviously, impaired maternal *general health* can affect maternal behaviors as well as milk production. Any kind of abnormal parturition, such as delayed delivery because of defective prostaglandin metabolism, or by pups' malformation and/or intrauterine death, may significantly interfere with the postpartum maternal behavior. Even if parturition itself is normal, labor is stressful and energy-consuming, and may enhance any subtle health problems that are otherwise not quite noticeable. In the $Apc^{tm2.1Rak}/Apc+$ strain (Kuraguchi et al., 2006), which was classified as "abnormal maternal nursing", the mutant females were reported as too unhealthy to successfully nurse their infants. Under such conditions, the dam appears hypoactive, undernourished, may show a hunched posture, and her fur may look ungroomed. If any health problem with the dam is suspected, observational screens should be applied as described (Crawley, 2007).

Not only *hypoactivity*, but also *hyperactivity* and *hypersensitivity* (stress-induced hyperactivity and irritability) affect the quality of maternal care. Restless circling in the home cage is the behavior often seen in many genetically-engineered (targeted or transgenic) mouse strains, which may non-specifically cause insufficient nursing or pup biting (for example, Bond et al., 2003; Lee et al., 2001).

4.2.2.2. Lactation. Insufficient *milk production* is also a frequent phenotype in genetically engineered mice, and can be mistaken for maternal behavior deficits. Not only by genetic mutations specifically implicated to this process (e.g. prolactin and prolactin receptor KOs, Section 5.1.2), many transgenes cause this phenotype apparently non-specifically, through impaired general health or unknown mechanisms. When the dam's milk production was severely impaired, a half day after delivery, the pups starved, chilled, got pale and inactive. Partial impairment may manifest as the growth retardation of all or some pups of the litter. If the dam finally abandons or starts eating such weak and dying pups, again this is a normal behavior and should not be classified to as cannibalism or infanticide. Milk-production deficit can be exaggerated by changing the genetic background of the original mutant strain. For example, in the *FosB* mutant strain constructed using 129-derived embryonic stem cells and maintained as mixed background with C57BL/6J, the heterozygous dams did not differ from the wild-type dams in nursing and maternal behavior (Brown et al., 1996; Kuroda et al., 2007). When they are backcrossed more than 5 times into the C57BL/6J background, 60% (63 cases out of 105 deliveries) of the heterozygous dams failed to have surviving pups by two days after delivery (Kuroda et al., 2007). Almost all the heterozygous dams made good nests and retrieved cleaned pups to the nest. The dams' nipples showed signs of vigorous suckling (elongation of the nipple, and sometimes bleeding from the tip of the nipple), but the pups' stomachs were empty. Therefore, it was speculated that milk production or letdown was compromised in the postpartum female mice of *FosB (+/−)* females on the BL6 background, but not in the original 129-based background. Background- and age-dependent milk production deficit was also observed in the prolactin-receptor mutant mice (Ormandy et al.,

1997b); in the 129 or 129×C57BL/6 mixed background, the heterozygous primiparous females that mated at 6–8 weeks of age exhibited impaired mammary gland development, and subsequent loss of their pups. At 20 weeks of age, and/or after the second lactation, their lactational performance increased to support pup survival as the wild-type females. After 10 backcrosses into C57BL/6J, on the other hand, all the heterozygous dams had severe lactation deficits even after multiple pregnancies, so that the entire litters died within two days without exception (N>20, our unpublished observation). It was also noted that these heterozygous and, surprisingly also homozygous mutant dams made excellent nests, cleaned pups, retrieved all the pups to the nest and crouched over the pups to nurse, and their nipples were elongated by vigorous suckling.

The *milk ejection reflex*, induced by oxytocin secretion and resultant contraction of mammary myoepithelial cells (Wakerley, 2005), is also required for milk transfer to the pup. Deficits in this reflex are observed in oxytocin and oxytocin-receptor mutant mice (Section 5.1.3) (Gross et al., 1998; Nishimori et al., 1996; Takayanagi et al., 2005; Young et al., 1996). Also, mutant mice lacking a winged helix gene *Foxb1/Fkh5/Mf3* are defective in the milk-ejection reflex in inbred strains (Kloetzli et al., 2001; Labosky et al., 1997) (see Section 5.3.4 for details). Both a deficit in milk ejection and in milk production result in the absence of milk in the stomach of the pups even after vigorous suckling. Histological analyses of mammary tissue are required to distinguish between milk production versus milk-ejection failures (for example, Nishimori et al., 1996).

If the above mentioned confounding factors are unlikely while clear signs of insufficient *maternal care* are observed, then we should segregate what aspects of maternal behavior are compromised. In the Section 4.3, we will introduce the protocol for examining maternal behavior deficits. Before going into detail, it should be noted that maternal *age* and *parity* also affect the quality of maternal behavior. It is better to wait until female mice are 10 to 12 weeks of age to mate and test their maternal behavior, even though females may be able to get pregnant at earlier ages. Also depending on the mouse strain, the maternal behavior at the 1st parturition might be inferior compared to the 2nd or later parturition.

4.2.3. Gene–environmental interactions

Even though extensive efforts have been made, complete standardization of animal husbandry conditions in different institutions remains difficult (Crabbe et al., 1999; Wahlsten et al., 2003). Gene–environment interaction effects may further complicate the interpretation of behavioral data. Behaviors toward pups are highly dependent on the environment. For example, mutant dams lacking *Nmbr* gene, encoding Neuromedin B receptor, exhibit maternal behavior defects only after 30-min restrain stress (Yamada et al., 2002). The effects of environment may be more obvious in the parental behaviors shown by virgin mice than in robust postpartum maternal behaviors (Brown, 1993; Kuroda et al., 2008). Fig 1 shows the effects of different environments on the parental behavior of virgin *FosB (+/)* and *(−/−)* animals of both sexes (Kuroda et al., 2008). In this particular case, the pup directed behaviors of the control *(+/)* animals were even more dependent on the environmental factors, such as facilities (MTL or RIKEN) and the cage bedding (alpha-dri (paper) or beta-chip (wood chip)) than the behaviors of the *FosB (−/−)* animals. The frequency of infanticide was the characteristic most different among facilities. Previous reports have shown that prenatal stress and lower social rank strongly inhibit infanticide in virgin males (vom Saal, 1983; vom Saal and Howard, 1982). We also experienced that C57BL/6 males purchased from an animal vendor company performed much less infanticide than the males of the same strain that were bred and weaned in our institute (our unpublished observation). These findings may reflect the fact that each animal facility may provide a significantly different level of environmental stress to mice. [It is not surprising that pup-directed behaviors by virgin mice, including both parenting and infanticide, may be more variable than postpartum parenting, depending on environment and genetic background, as seen in Kuroda et al. (2008) and Parmigiani and vom Saal (1990). Domestication and selection processes of mouse strains under laboratory conditions probably exert the most selection pressure on the robustness of postpartum maternal behavior, but not much on the pup-directed behaviors of virgin mice.]

The effects of *cage bedding* warrant special attention. The postpartum and virgin female mice lacking *Fyn* tyrosine kinase displayed reduced pup retrieval and frequent nest position changes (Hamaguchi-Hamada et al., 2004b; Yagi et al., 1993). In addition, fyn *(−/−)* dams showed defects in nursing behavior and subsequent pups' lethality, only if they were housed with autoclaved wood chips as cage bedding. By gas chromatography and mass spectrometry, the authors identified the responsible compound in the bedding as hexanal, a volatile substance contained in plants and causing a grassy odor (Hamaguchi-Hamada et al., 2004b). Addition of hexanal into the cage bedding reduced the crouching behavior in fyn *(−/−)* virgin females, but not in fyn *(+/+)* virgin females, during a 30-min pup exposure and behavioral observation session. Furthermore, the authors found that exposure of fyn *(−/−)* virgin female mice, but not of fyn *(+/+)* mice, with hexanal

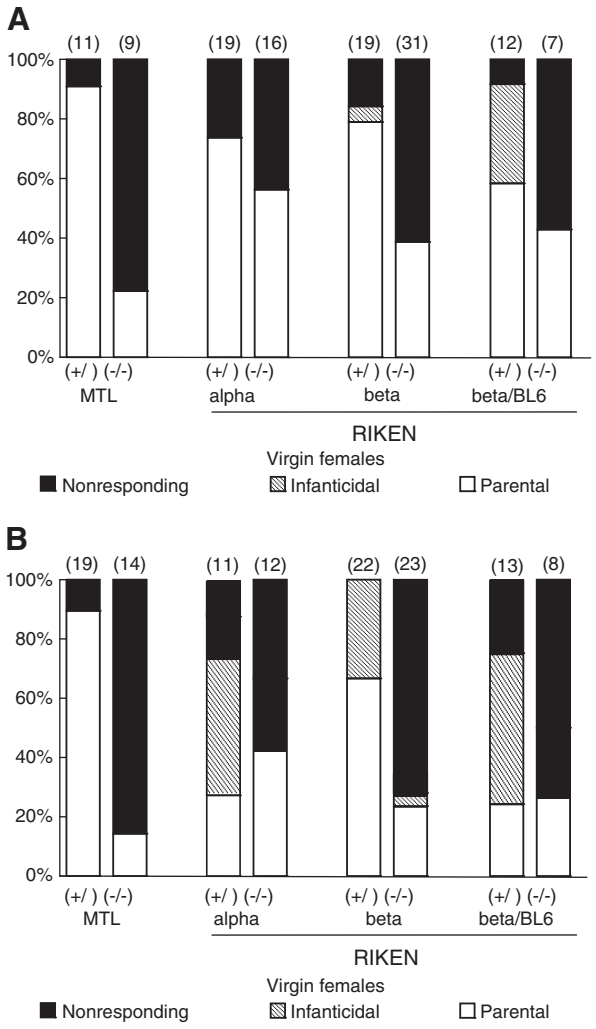

Fig. 1. Results of pup retrieval assays in *FosB* mutant virgin female (A) and male (B) mice, in two different institutions (MTL or RIKEN), with different cage beddings (alpha, paper chips; beta, wood chips), and in two different genetic backgrounds (129 Sv-C57BL/6 mixed original background; or BL6, backcrossed into C57BL/6J for more than five times), performed by the same experimenter (K.O.K.). Numbers in parentheses indicate the number of mice studied. Retrieved all 3 pups within 30 min (parental, open area), committed infanticide within 30 min (infanticidal, shaded area), or neither of them (nonresponding, solid area). The results of the second day are shown for male mice. *(+/)* means wild-type *(+/+)* or heterozygous *(+/−)*, whereas *(−/−)* means homozygous *FosB* mutant mice (data from Kuroda et al., 2008).

odor induced c-Fos immunoreactivity in the medial preoptic area and the basolateral and posteriomedial cortical amygdala, which are known to be involved in the control of maternal and emotional behavior (Hamaguchi-Hamada et al., 2004a).

Similar bedding-dependent effects on parental behavior have been observed in FosB (−/−) mice. In the same institution and under condition where other variables were identical, FosB (−/−) virgin males and females were significantly less parental than the wild-type littermates on wood chips (beta-chip), but not on paper chips (alpha-dri) (Fig. 1). There was also a tendency for the retrieving behavior and pup-cleaning behavior of postpartum dams to be more perturbed on wood-chip bedding, compared with those on purified pulp bedding (Kuroda et al., 2008). This difference was not, however, caused by hexanal in beta-chip, because the addition of hexanal to paper chips did not change the behavior of FosB wild-type or knockout animals.

Contents of chemicals such as hexanal can vary between the types of wood used for wood chips, and between the treatment of chips (autoclaving, addition of pesticides and so on). The woods used to make wood-chip bedding are variable by season and location. Therefore to avoid any unexpected complications, paper chips made from purified pulp may be preferable for behavioral testing.

4.3. Assessing maternal behavior on the first morning after delivery of genetically-engineered mouse strains

To address the frequently asked question of "What's wrong with my mouse dam–pup relationship?", the basic protocols used for assessing postpartum maternal behaviors and parental retrieval using genetically engineered mice will be introduced, based on previous publications (Brown et al., 1996; Kuroda et al., 2007, 2008; Lucas et al., 1998; Ogawa et al., 1998a; Thomas and Palmiter, 1997).

There are many other protocols for precise measurements of specific behavioral component (Bridges and Ronsheim, 1990; Capone et al., 2005; Lonstein et al., 1999; Numan et al., 1985; Rosenblatt et al., 1994). For example, the use of dividers or floor partitions in the home cage of the subject rats can inhibit spontaneous crawling of pups to the nest, resulting in more precise tests of pup retrieval (Champagne et al., 2001). Also, the use of larger cages for behavioral testing is more suitable for detecting small differences between experimental groups (Lefebvre et al., 1998). Other interesting and rigorous methods are to measure the motivation (appetitive phase) of maternal behavior, rather than its consummatory aspects (Lee et al., 2000; Lonstein and Fleming, 2002; Mattson et al., 2003). The protocol presented here is rather basic and less sensitive to small differences in parental behavior, but is compatible with the common practices of mutant mouse husbandry, such as the use of standard shoebox ventilated cages (approx. size of 265 mm × 205 mm, 140 mm high), so that it may be useful for the initial screening of parental responsiveness in mutant mouse strains.

4.3.1. Preparation of subjects

4.3.1.1. Breeding strategy for production of subject dams. The breeding strategy for production of experimental subject animals has been described in Section 4.1.5. It should be carefully distinguished from the breeding strategy for postpartum maternal behavior tests (see next section 4.3.1.2).

4.3.1.2. Breeding strategy for postpartum maternal behavior tests. To compare the effects of maternal genotype on maternal behaviors, crosses between (+/+) males with (−/−) subject females and (−/−) males with (+/+) subject females should be conducted, resulting in all the pups having the same genotype (+/−). If this is not possible because of the infertility of (−/−) males, or if one wants to compare (−/−) dams versus (+/−) for reasons related to time/cost savings, provided that preliminary analyses confirms that (+/−) dams and pups are essentially not different from (+/+), then crosses of (−/−) and (+/−) females with (+/+) males can be conducted. The principle is to avoid the use of (−/−) pups as stimuli for testing maternal behavior in their dams, while also trying to equalize the pups' genotype for (−/−) and the control (+/) (either (+/+) or (+/-)) subject dams, which avoids the pup factors described above. Mating should preferably start with 10–12 weeks of age for maternal behavior testing.

4.3.1.3. Sample size and other remarks. N = 10–20 mice per genotype are commonly needed to detect moderate behavioral differences using standard multivariate statistical analyses such as multiple and repeated-measures analysis of variance, followed by appropriate post hoc tests (Crawley, 2008). It is acceptable to start with about N = 4–6 animals/group for the initial screening, and then repeat at the same size or increased size for replications. These data can be combined, as long as the wild-type controls are not significantly different across cohorts. As in any other research area, confirmation of the initially-found behavioral phenotype across independent cohorts of mice provides compelling evidence for the functional outcome of the mutation (Crawley, 2008).

Measuring the body weight before mating would be informative. Also checking the female for a vaginal plug early in the morning following mating provides information about the possible delivery date and also the sexual behavior of this female, although this is not essential for maternal behavior assessment itself.

Once the female gets pregnant, it is isolated in a new cage containing paper bedding (see Section 4.2.3) with a cotton square (e.g. Nestlet, Ancare, Bellmore, NY) as nest material. The addition of nest material makes it easy to identify the location and quality of the nest (Deacon, 2006). Normal cotton pads or balls can be used, but the Nestlet is preferable because it is packed into a flat form. Adult mice normally bite and tear this pressed cotton sheet extensively into fluffier pieces to make their nest within a couple of days. If this square piece of Nestlet has not been torn and remains in its original form, it is suspected that the subject mouse may have some sort of health problem, or has serious defects with nest building behavior.

After the female has been moved into this cage, the bedding should not be changed during the first week of lactation, so as not to disturb the dam and pups. In case the bedding gets too dirty during this first week, one can remove the dirty part of the bedding manually, and add the same amount of new bedding, but one should avoid doing this during the peripartum period.

4.3.2. On the first morning after the delivery

The postpartum examinations should be done in this order, to minimize the stress of the dam. Throughout these observations, we try not to take mouse dams out of the cage (see also Lonstein and Fleming, 2002). To collect pups for retrieval test or for pup examination, if she is in the nest over pups, we wait for her to move away or gently push her away. Nor do we suspend her by the tail completely in the air, but let her forelimbs attached on the ground or the cage cover. Suspending a peripartum female by the tail may induce a stress reaction, which sometimes result in the dam destroying her nest or attacking her pups.

4.3.2.1. Parturition check. The pregnant mouse dams deliver pups most typically during the dark phase of 20th day after copulation, with some variations (typically a half or one day delay) (Hedrich, 2004) (see also "Biology of the laboratory mouse", The Jackson Laboratory, adapted for the Web at http://www.informatics.jax.org/greenbook/index.shtml). Around the estimated day of delivery, the cage should be checked for parturition every morning. With our animal husbandry of a 12:12-h light/dark cycle with lights on from 08:00 to 20:00, we check for delivery between 9–10 am every morning. This is because, after this time, the pups born in the previous dark phase will get weaker if the milk intake is not sufficient by any reason. If the pups get too weak and hypothermic, the maternal responses will decrease and sometimes the dam may cannibalize the dying pups, obscuring

the cause of pups' death. For the same reason, if one wants to cross foster the pups to rescue them, the success rate decreases after this time. In the afternoon it would be difficult to make a foster dam accept these weakened and chilled pups.

On the other hand, if the dam is still in delivery, one should not disturb the cage. It is normal for the nest she prepared at the end of pregnancy to get destroyed during delivery, as the dam circulates and moves restlessly in the small cage during delivery of each pup. It is better to wait for about an hour after the delivery of the last pup, until the dam settles down and finishes placentophagia, rebuilding the nest, and pup retrieval, before starting the ratings described below.

If the dam has been settled down after parturition, the nest quality and pup grouping are evaluated from outside, so as not to disturb the dam.

4.3.2.2. Nest grading. [The method described here is according to the previous publications (Kuroda et al., 2008; Hess et al., 2008), with some simplifications to be compatible to other measures of maternal behavior assessments in mice. See also Deacon (2006), Gandelman (1973), Mann (1993) Numan and Callahan (1980), Slotnick and Nigrosh (1975).] The nest is rated as 0 when there is *no nest*, or the nest location is unclear because the nest material is distributed randomly in the cage. The nest is rated as 1 when the nest is *flat* and not well focused. Still the grade-1 nest should be able to be identified in the cage. The nest is 2 when the nest is similar to a *shallow soup bowl* (Hess et al., 2008). The nest is graded as 3 when the nest is shaped into a hollow surrounded by a continuous bank (designated as incomplete dome or *half of a sphere* in Hess et al., 2008). With the appropriate nest material such as fluffy paper strips, a completely enclosed nest, rated as 4 can be achieved (*full dome*, Hess et al., 2008). In such nest, the pups are scarcely visible from any direction. The bedding is gathered toward the corner of the nest site, so that the nest floor is higher than the floor of the dirty corner of the cage, which the dam uses as an area for defecation and urination. With about 250 ml (one cup) of the paper-chip bedding and one piece of 5 cm × 5 cm Nestlet per a shoebox mouse cage as described here, however, this grade-4 nest is hardly constructed. Increasing the amount of nest material may cause mechanical troubles of automated water-supply and individual cage ventilation systems. If there are two clear nests in one cage, the grading can be made separately for each nest.

4.3.2.3. Pup grouping. At this moment, each pup's location in relation to the nest and other pups (= if they are in body contact with other pups) should be briefly recorded, by examining them from outside of the cage. Ideally all the pups should be in the nest and tightly grouped (huddled) with each other. When any of the pups are out of nest or buried in the bottom or the bank of the nest (better observable from the bottom of the clear cage), the pups' condition (e.g. healthy and reddish, pale, or dead) should be noted and examined thoroughly later as in the "*pup examination*" paragraph. Again, it is not abnormal maternal behavior to leave dead or dying pups out of the nest, and/or cannibalize them.

Pup grouping does often, but not always, correlate with the nest quality. For example, when the pups are not well grouped in the nest, the nest inevitably gets wide and flat. Still it is better to rate them separately, as some mice (e.g. juveniles) retrieve and group the pups but do not make clear nests.

4.3.2.4. Pup retrieval observation. To further quantify pup retrieval, remove the cage top, and gently but quickly take three healthy pups from the nest, and place one pup in each corner of the cage outside the nest. Then return the cage cover. The female and pups are observed continuously for 10 min and the following measures were recorded: latency to sniff a pup for the first time, to retrieve each pup into the nest, group all pups, and crouch over the pups continuously for >1 min. Pup carrying to the other place than the nest should be recorded separately. When the dam has finished retrieval and grouping, and has crouched over all the pups in the nest for more than 1 min, it is called as "*full maternal behavior*" (note that the criteria are slightly different in each literature (Bridges et al., 1985; Moltz et al., 1970; Pedersen and Prange, 1979). The latency to show nest building or pup licking can also be recorded, but mouse nest building or pup licking is not always clearly dissociated from pup regrouping or self-grooming, respectively.

If most of the pups are found in the nest at the initial check from outside, one can assume that the dam has already exhibited pup retrieval, because newborn pups are not able to group themselves together. When the pups are found grouped but the dam does not retrieve any pup during the pup retrieval observation, it is suspected that the dam is stressed by the current experimental maneuver. In that case, gently place the cage back to the husbandry shelf, where it tends to be darker, and observe another 15 min for retrieval and full maternal behavior. If the dam shows retrieval, it is suggested that the delay of retrieval may have been caused by stress hypersensitivity rather than by lack of maternal responsivity.

In the case where pups are visibly unhealthy because of inappropriate placentophagia, lack of milk intake, low body temperature or bleeding, these pups cannot be used for the stimulus pups in the pup retrieval test. In this case, the test can be made using the healthy pups (properly cleaned, milk in stomach and warm, preferably of the same genetic background) within a few days of age from another dam. It is often seen that the mutant dam with insufficient milk secretion appears to abandon her weakened pups, but when fresh healthy donor pups are introduced, the same dam shows a quick retrieval response. Formally in this case, the control dams should also be exposed with donor pups for retrieval scoring, to equalize the experimental conditions between mutant and control.

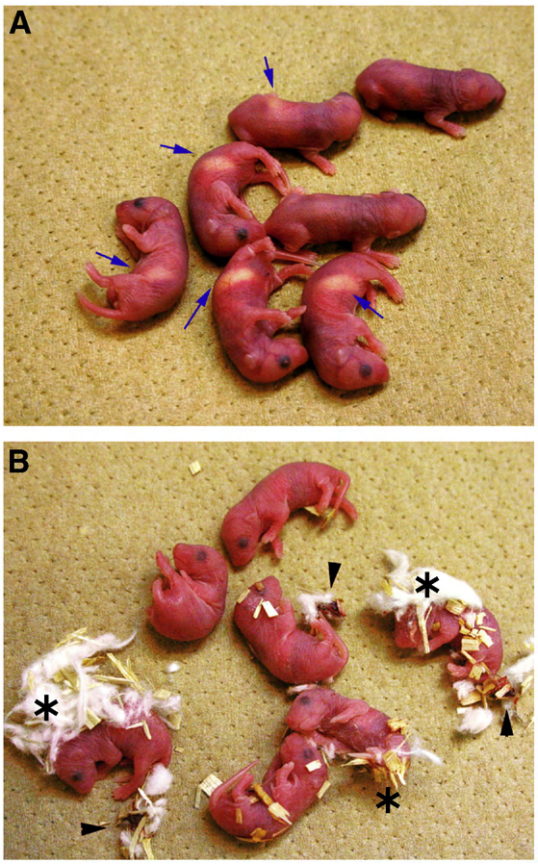

Fig. 2. The pups' appearance on the first morning after the delivery. A) Litter of a FosB (+/+) dam, B), litter of a FosB (−/−) dam.

4.3.2.5. Pup examination. Finally, all live or dead pups are taken out of the cage, and are investigated and classified either as "alive with milk in the stomach" (Fig. 2, blue arrows), "alive without milk" (e.g., the top-most pup in the bottom panel of Fig. 2), or "dead." In addition, it is recorded whether there are any remaining amniotic membranes, umbilical cord, or placenta (black arrowheads of the bottom panel of Fig. 2) attached to the body. When such sticky fetal tissue is remaining, the pup's skin may be covered by bedding materials (asterisks of the bottom panel of Fig. 2). Also the body of the pups should be briefly observed for possible bite marks or injuries. If cannibalization has occurred, any remaining pup bodies are carefully sought for in the bedding and are recorded.

4.3.2.6. Maternal body examination. If the pups are without milk, the *maternal nipple* should be examined briefly by holding its tail and lifting its hindlimbs slightly in the air, with forelimbs still on the floor. At the same time the vaginal opening is checked for bleeding and any obstruction that could be caused by a dead fetus or other remaining tissue, which are the signs of parturition problems. If the dam has nursed the young and the pups can properly suckle, the nipples should be somewhat protruded from the ventral skin surface covered by short hair (easily identified by comparing the ventral surface of a lactating female with another female that is definitely not nursing). If the pups have been suckling vigorously because milk letdown is limited, the tip of the nipple is overly elongated and may show bleeding. Together with observations of maternal crouching over the pups in the nest, these nipple observations help to confirm that maternal nursing behavior has been performed.

4.4. Parental retrieval behavior and infanticide toward donor pups by non-maternal mice

As described above, postpartum maternal behavior may be affected by several issues along with parturition, including drastic changes of hormonal milieu, physical stress and any complications inherent to delivery. Also it cannot be measured if the mutant females are infertile. Therefore it may be preferable to study alloparental behavior in virgin females and paternal behavior in fathers, both of which are devoid of such drawbacks. Additionally the pup retrieval in virgin female mice can be tested much easily and quickly than that of postpartum dams in mice or of virgin female rats, since the virgin female mice are parental within half an hour at the first pup exposure. It is a minimally two-day procedure from single housing to testing for 30 min the next day, if the donor pups can be provided from breeding colony.

To quantify parental responsiveness of non-parturient animals, male or female subject mice are individually housed for at least 1 day prior to an experiment, in a new cage containing paper bedding with a cotton square as nest material as described above. Because one-month of social isolation has been shown to be stressful and may increase aggressive behavior in male mice (Crawley, 2007; Valzelli et al., 1974), they are group housed prior to the experiment, and they are not isolated for more than two weeks.

On the test day, the nest site and quality should be recorded as described above. Then each animal is exposed to three 1- to 6-day-old donor pups. One pup is placed in each corner of the cage distant from the nest. If any of the pups is attacked during the test, which is mostly observed during the first 5 min after the pup exposure, all the pups are immediately removed and the wounded pup is euthanized as described previously (Perrigo et al., 1993). This subject is deemed as *infanticidal*. Otherwise, pups are left in the cage for 30 min. The cages are continually observed for the next 30 min and the following measures are recorded: latency to sniff a pup for the first time, to retrieve each pup into the nest, and to crouch over all the pups continuously for more than 1 min. If the subject mouse has performed all of these behaviors within 30 min, it is labeled as *fully parental*. If only part of these behaviors are seen by the end of the 30-min session, for example only one of the three pups is retrieved to the nest, or the subject does not retrieve at all but instead collects nest material to one of the pups and crouches over it, the subject is designated as *partially parental*. If the subject only sniffs the pups but does not show any retrieving or crouching responses throughout, it is labeled as *nonresponding*. If the subject does not approach and sniff any of the pups, it should be examined whether some kind of health problem or sensory dysfunction exists. After 30 min of observation, the pups are removed. If many of the subjects are nonresponding or partially parental, the same 30-min pup exposure session can be repeated for several days to examine whether changes in response subsequently develop by pup sensitization (Brown et al., 1996; Wang and Storm, 2010). On the other hand, once an animal becomes fully parental for two successive days, it seldom goes back to partially parental or infanticidal.

In case that infanticide is expected as dominant response, there is a technique to minimize harming the stimulus pups (Perrigo et al., 1990, 1989); in essence, instead of placing pups into the subject's home cage directly, first place a pup contained in a wire-mesh tube or container. If the subject mouse is observed to start biting the container with eyes squinted and tail rattled, it is highly probable to be infanticidal. If the subject mouse does not show any signs of attack, the wire-covered pup is taken away and there naked pups are introduced in the cage as described above to further test pup retrieval behavior.

5. Parental behaviors in genetically-engineered mice

Gene-targeted mouse strains that have so far been identified as exhibiting defective pup retrieval, with this phenotype not likely to be secondary to lactation or general health problems, are listed and briefly described in this section and the Table 2. Some mutants which are relevant to the mechanisms of maternal behavior but do not display robust retrieval defects are also included (e.g. oxytocin, prolactin KOs). Further, several mutant lines without quantitative investigation of lactation or virgin pup retrieval are discussed if maternal behavior defects have been explicitly reported (e.g. *Dat1*, $GABA_A$ receptors, *Pet-1*, *Nr2c2/TR4*, *Tph2*). In all cases, poor pup survival due to pup factors (Section 4.2.1) are excluded at least. Please refer also the previous publications on this topic (Leckman and Herman, 2002; Numan et al., 2006), and on maternal aggression (Gammie, 2005; Lonstein and Gammie, 2002).

5.1. Hormones implicated in female reproduction and their related molecules

5.1.1. Steroid hormones for female reproduction

Targeted mutation of *Esr1* encoding the estrogen receptor α, caused morphological and functional abnormalities in gonads, decreased sexual behavior and infertility in both males and females (Couse and Korach, 1999). Furthermore, the homozygous mutant males and females exhibited an increase in infanticide (Ogawa et al., 1998a,b). Female but not male *Esr1(−/−)* showed a mild decrease in retrieval behavior, too. Targeted mutation of *Cyp19*, encoding the aromatase enzyme (which converts testosterone to estradiol) results in diminished serum estradiol and increased testosterone levels (Fisher et al., 1998). *Cyp19 (−/−)* females showed essentially normal pup retrieval behavior (Stolzenberg and Rissman, 2011). [Another study reported that *Cyp19 (−/−)* male mice exhibited increased infanticide compared with the wild-type littermates (Matsumoto et al., 2003), but these subject mice were tested for male sexual behavior prior to the parental behavioral analysis. The mutant males were defective in sexual behavior and did not experience ejaculation during tests, while their wild-type littermates experienced multiple ejaculations. As described in Section 2.2, males' behavior toward pups is highly dependent on the mating experience. Therefore the paternal behavior of *Cyp (−/−)* mice should be re-examined under equal conditions with the control wild-type males.] Interestingly, in both

Table 2
Genotypes described in the text for parental behaviors.

Gene (allele)	Gene product	Type of mutation	Description in the text	Pup survival	Alloparental retrieval	Comment	MGI annotation for parental behavior	Reference
Adcy3	Adenylate cyclase 3	KO	5-5-1	0	0~↓↓	Anosmia, placentophagia → Maternal aggression ↓	-	Wang and Storm (2010)
CD38	CD38 antigen	KO	5-1-3	83% (WT: 96%) at weaning	ND		–	Jin et al. (2007)
Creb1 (*Creb1*$^{\alpha\Delta}$)	cAMP responsive element binding protein 1	Knock-down	5-3-2	↓	↓		–	Jin et al. (2005)
Cyp19a1	Cytochrome P450, family 19, subfamily a, polypeptide 1 (Aromatase)	KO	5-1-1	NA	↓(male, see text for caveats)	Infanticide ↑ (male, see text)	p	Matsumoto et al. (2003)
					→(ovariectomized female)			Stolzenberg and Rissman (2011)
Dbh	Dopamine-β-hydroxylase	KO	5-2-1	↓↓	↓	Placentophagia ↓	–	Thomas and Palmiter (1997)
(*Del(6)1*)	Sixteen V1r vomeronasal receptors, spanning from V1ra9 through V1rb7	Chromosomal deletion	5-5-2	→	→	Maternal aggression ↓	can	Del Punta et al. (2002)
Esr1	Estrogen receptor α	KO	5-1-1	NA	↓(female)→(male)	Infanticide ↑	r, can	Ogawa et al. (1998a,b)
FosB	AP-1 transcription factor FosB	KO	5.3.1 (4.2.2.2)	↓	↓		ns, r	Brown et al. (1996)
						Infanticide ↓ (virgin male)	can	Kuroda et al. (2007), Kuroda et al. (2008), Gruda et al. (1996)
Foxb1	Winged helix transcription factor Foxb1/Fkh5/ Mf3	KO	5-3-4	0	ND		mn	Wehr et al. (1997)
				0 (deficits in lactation in inbred strains)			ns	Labosky et al. (1997), Kloetzli et al. (2001)
Fyn	Fyn tyrosine kinase	KO	4-2-3	→ (↓↓ with KO pups and with hexanal)	↓		mn, r	Hamaguchi-Hamada et al. (2004a,b)
Gabra1	$GABA_A$ receptor α1 subunit	Knock-in, gain-of-function	5-2-5	0	ND	ND for lactation	mn	Homanics et al. (2005)
Gabrb3	$GABA_A$ receptor, β3 subunit	KO	5-2-5	↓↓	ND	ND for lactation	can	Homanics et al. (1997)
		Conditional KO (pan-neuronal)		→	ND			Ferguson et al. (2007)
		Conditional KO (forebrain selective)		↓↓?	ND	ND for lactation	mn	Ferguson et al. (2007)
Gabrd	$GABA_A$ receptor, subunit δ	KO	5-2-5	↓~↓↓	ND	ND for lactation	mn, r, can	Mihalek et al. (1999), Maguire and Mody (2008)
Gnal	Guanine nucleotide binding protein, α stimulating, olfactory type (Golf)	KO	5-5-1	0	ND	Anosmia, hyperactive	mn, r, cr	Belluscio et al. (1998)
Gnaq, *Gna11*	Heterotrimeric G protein, α subunit, $G_{q/11}$	Conditional KO (Gnaq) and KO (Gna11)	5-3-3	0~↓↓	0~↓↓	Placentophagia →	–	Wettschureck et al. (2004)
Gria2 (*GluR-BΔHS*)	Ionotrophic glutamate receptor AMPA2 (α2)	Conditional KO	5-2-4	→	ND	Postpartum maternal aggression and pup retrieval ↓↓	r	Shimshek et al. (2006)

Grin1 (*NR1Q*)	Ionotrophic glutamate receptor NMDA1 (ζ1)	Targeted point mutation, gain-of-function	5-2-4	↓↓	ND	Hyperactive, pup biting ↑	gr, r, can	Single et al. (2000)
Htr1b	5-HT receptor 1B	KO	5-2-2	→	ND	Hyperactive	–	Brunner et al. (1999)
Mbd2	Methyl-CpG binding domain protein 2	KO	5-4-2	↓	ND	Postpartum pup retrieval slightly slow ND for lactation	mn, r	Hendrich et al. (2001)
Mest	MEST(mesoderm-specific transcript)/PEG1		5-4-1	↓	↓	Placentophagia ↓	mn, r	Lefebvre et al. (1998)
Mtap6	Mtap6/STOP, microtubule stabilizing protein	KO	5-6-1	0	↓		r	Andrieux et al. (2002)
Nmbr	Neuromedin B receptor	KO	4-2-3	(↓not quantified)	→ (w or W/o stress, vs. (+/+))	Crouching and nest building ↓ after stress	–	Yamada et al. (2002)
Nos1	Neuronal nitric oxide synthase (nNos)	KO	5-5-2	→	ND	Maternal aggression ↓ Postpartum retrieval →	m	Gammie and Nelson (1999)
Nr2c2	Nuclear receptor Nr2c2/TR4	KO	5-6-3	↓↓	ND	Milk ejection ↓ suspected	–	Collins et al. (2004)
Oca2 (*rjs*)	Arsenical pump membrane protein Oca2	Radiation-induced, recessive	5-6-6	0	ND	Maternal nest building and retrieval ↓	mn	Hollander et al. (1960) Lehman et al. (1998)
Oxt	Oxytocin	KO	5-1-3	0 (deficits in milk ejection)	→		ns	Gross et al. (1998), Nishimori et al. (1996), Young et al. (1996)
					(↓)			Pedersen et al. (2006)
Oxtr	Oxytocin receptor	KO	5-1-3	0 (deficits in milk ejection)	↓		r	Takayanagi et al. (2005)
					(→)		cr	Macbeth et al. (2010)
Rora (*Staggerer*)	Nuclear steroid hormone receptor RORα	Spontaneous mutation	5-6-5	0 (Rescued by special husbandry condition)	ND	Severe motor deficits because of cerebellar dysgenesis	mn	Guastavino (1983)
Peg3	Paternally expressed gene PEG3/PW1	KO	5-4-1	↓ (better in multiparous)	↓	Milk ejection deficits	r	Li et al. (1999a,b)
				↓	→	Licking/grooming ↓	cr	Champagne et al. (2009)
Pet-1	ETS transcription factor Pet-1/Fev	KO	5-2-2	0	ND	Placentophagia →	–	Lerch-Haner et al. (2008)
Pgr	Progesterone receptor	KO	5-1-1	NA	↑ (Virgin male)	Male infanticide ↓	–	Schneider et al. (2003)
Prl	Prolactin	KO	5-1-2	NA	→		–	Horseman et al. (1997)
Prlr	Prolactin receptor		5-1-2	0 (deficits in milk production)	↓		mn, r, m	Ormandy et al. (1997a,b)
		KO	(4.2.2.2)				(ns)	Lucas et al. (1998)
Pten	Phosphatase, tensin-type, PTEN	Conditional KO (*Nse-cre*)	5-6-4	↓↓	?	Sleep nest building ↓, social interaction ↓ ND for lactation	–	Kwon et al. (2006)
Slc6a3/Dat1	Dopamine transporter	KO	5-2-3	→ (in DBA or mixed background), ↓ (in C57 background because of milk production defect)	ND	Hyperlocomotion	mn	Spielewoy et al. (2000), Morice et al. (2004)
Stmn1	Stathmin/prosolin	KO	5-6-2	↓	↓		–	Shumyatsky et al. (2005)
Tph2	Tryptophan hydroxylase 2	KO	5-2-2	↓	ND	Infanticide, aggression ↑	r can	Alenina et al. (2009)
Trpc2	Cation channel in VNO	KO	5-5-2	→	ND	Maternal aggression ↓	–	Leypold et al. (2002)

NA, not applicable because of female infertility; ND, not determined.
Abbreviations for MGI annotation; p, abnormal parental behavior; m, abnormal maternal behavior; mn, abnormal maternal nurturing; ns, abnormal nursing; r, abnormal pup retrieval; can, pup cannibalism; cr, abnormal maternal crouching; gr, abnormal maternal grooming; ag, abnormal maternal aggression.

Esr1 and *Cyp19* homozygous mutants, intermale aggression was reduced, suggesting the existence of distinct mechanisms for infanticide (aggression toward pups) and for aggression toward intruder males.

In contrast, the male mice lacking the progesterone receptor gene *Pgr* were reported to show less infanticide and more paternal behavior compared with the wild-type males (Schneider et al., 2003) [*Pgr* mutant females are sterile and are defective in uterine and mammary development].

These studies suggest that these steroid signaling mechanisms are involved in the behavioral choice between infanticide and parenting. How these steroids act in the central nervous system to influence this behavioral choice is, however, unclear, since gonadectomy does not change the parental responses as discussed in the Section 2.2. For steroid receptors, the ligand-independent activation through phosphorylation by protein kinase A, C or by MAP kinases has been reported (Weigel and Zhang, 1998). Developmental effects should also be considered, in which these steroids facilitate the maturation of certain brain circuits, which in turn regulate pup-directed behaviors in later life.

5.1.2. Prolactin

Prolactin is a peptide hormone required for mammary gland development and milk production (Freeman et al., 2000; Kelly et al., 2002). Male mice lacking the prolactin gene *Prl* (Horseman et al., 1997) or the prolactin receptor gene *Prlr* (Clement-Lacroix et al., 1999; Ormandy et al., 1997a,b) were generally healthy and fertile, while homozygous females of either mutant displayed irregular estrous cycle, failure of implantation and were infertile. Pup retrieval and crouching behaviors were impaired in virgin *Prlr (−/−)* females, and mildly in virgin and postpartum *Prlr (+/−)* females (Lucas et al., 1998; Ormandy et al., 1997a) (on 129Sv or 129Sv×C57BL/6 mixed background, see also Section 4.2.2.2). On the other hand, parental responsiveness were normal in virgin *Prl (−/−)* females (Horseman et al., 1997). This discrepancy may be caused by the prenatal action of placental lactogens, placental hormones that functionally mimic prolactin; that is, while *Prlr (−/−)* fetuses do not receive effects from plasma-borne placental lactogen, *Prl (−/−)* fetus receive it, and this effect may be necessary for proper development of parental behavior neural circuitry (Tanaka et al., 2000). It is also possible that a ligand other than prolactin might activate the prolactin receptor in virgin mice to stimulate maternal behavior.

Recently, prolactin has been positively implicated in adult neurogenesis during pregnancy and lactation (Shingo et al., 2003); they reported that at gestation day 7 and postpartum day 7, but not at postpartum day 0, mated female mice exhibit proliferation of neuronal progenitor cells in the forebrain subventricular zone. The newly generated neurons migrated into the olfactory bulb and became interneurons. This pregnancy-induced neurogenesis could be mimicked either by subcutaneous or intracerebroventricular infusions of prolactin into female or male mice. Furthermore, the pregnancy-induced neurogenesis was reduced by half in the *Prlr (+/−)* mutant females. The same group showed that neurogenesis in the olfactory bulb and hippocampus was induced in the father's brain by cohabitation with its offspring, which was related to the decreased olfactory investigation of their offspring at 6 weeks of age when compared to the investigation of age-matched non-offspring (Mak and Weiss, 2010). This neurogenesis-dependent phenomenon was diminished in *Prlr (−/−)* animals. Additionally Larsen and Grattan (2010) showed that bromocriptine-induced reduction in prolactin secretion prevented the normal increase in the generation of neural progenitors in the subventricular zone of the maternal brain, and this was associated with the occurrence of increased postpartum anxiety and impaired maternal behavior when the maternal retrieval was tested outside the home cage in a novel environment. These data suggest that adult neurogenesis plays a role in postpartum maternal behaviors possibly by affecting the recognition of olfactory cues, which is important for maternal behavior in mice. Prolactin may also exert anxiolytic effects which influence maternal behavior.

5.1.3. Oxytocin

Oxytocin is a nanopeptide hormone required for milk ejection reflex (Wakerley, 2005), and has been implicated in various affiliative social behaviors such as rat parental behaviors, avian flocking behavior, rodent pair bond, human sexual behavior and interpersonal trust (Champagne et al., 2001; Goodson et al., 2009; Kosfeld et al., 2005; Pedersen and Prange, 1979; Young et al., 1998). Three laboratories independently established mouse strains lacking the *Oxt* gene, encoding the oxytocin-neurophysin I preprohormone. Surprisingly but consistently, all of these mutant females displayed normal parturition, but the milk ejection reflex was abolished (Gross et al., 1998; Nishimori et al., 1996; Young et al., 1996) (see also Insel et al., 2001). In addition, these homozygous mutant females showed normal parental retrieval, although Pedersen et al. (2006) reported mild deficits in pup licking and retrieval when such virgins were tested in a novel environment. Social recognition memory, which discriminates familiar from unfamiliar conspecifics using olfactory memory, has been reported to be impaired in the *Oxt* homozygous mutant mice (Choleris et al., 2003, 2006; Ferguson et al., 2000; Macbeth et al., 2009). One study showed, however, that a normal preference for social novelty, measured as time spent with a second novel stranger as compared to time spent with a more familiar mouse, was seen in both the NIMH and the Baylor/Emory lines of *Oxt (−/−)* mice (Crawley et al., 2007).

Postpartum and virgin *Oxtr (−/−)* females, which lacked functional oxytocin receptor, were reported to display mildly increased pup retrieval latencies (Takayanagi et al., 2005), as well as no milk ejection and normal parturition. The same study reported a reduction in ultrasonic vocalizations in *Oxtr (−/−)* pups upon social isolation, and diminished social discrimination in *Oxtr (−/−)* adult males. In contrast, in a recently constructed mutant strain, *Oxtr (−/−)* virgin females were not overtly different from the wild-type littermates in pup retrieval (Macbeth et al., 2010), while these mutant males were reproductively defective in social recognition of familiar versus novel females of the same mouse substrains (Macbeth et al., 2009).

In this connection, Jin et al. (2007) reported on the maternal behavior of the mutant strain of the *CD38* gene, which encodes a transmembrane glycoprotein with ADP-ribosyl cyclase activity and is required for oxytocin secretion from the pituitary. The mutant males and females demonstrated deficits in social recognition and in postpartum maternal behavior, respectively, with about a 50% decrease in plasma oxytocin levels. They also reported that the mutants' defective maternal retrieval and crouching behaviors could be rescued by subcutaneous injection of oxytocin. It should be noted that the mutant mice exhibited heightened locomotor activity. In addition, their mice used in this study were bred and maintained separately in *CD38 (+/+)* and *CD38 (−/−)* cohorts (Higashida, 2007), which means that a *CD38 (−/−)* female had enough oxytocin release to support milk ejection and fed the litter sufficiently well.

Several discrepancies among studies were found in the parental behavior phenotypes resulting from the genetic targeting of oxytocin and the oxytocin receptor. Besides the differences in construction of the targeting vector and in genetic background, the anti-stress and anxiolytic effects of oxytocin may be implicated (Brunton and Russell, 2008; Neumann, 2008); the effects of oxytocin loss on stress or anxiety level may cause alterations in parental and social behaviors depending on the level of stress within each experiment or at each research institution, through gene–environment interactions as discussed in the Section 4.2.3. Interestingly, Yoshida et al. (2009) generated an oxytocin receptor-reporter mouse in which the exon 3 of the oxytocin receptor gene was replaced with Venus cDNA (a variant of yellow

fluorescent protein). Examination of the Venus expression revealed that, in the raphe nuclei, about one-half of tryptophan hydroxylase-immunoreactive neurons were positive for Venus. Infusion of a serotonin 2A/2C receptor antagonist blocked the anxiolytic effect of oxytocin, suggesting that oxytocin receptor activation in serotonergic neurons mediates the anxiolytic effects of oxytocin.

Considering the extensive implications of brain oxytocin system in various social behaviors among different mammalian species (Insel, 2010), the relative paucity of parental behavior deficits in either oxytocin or oxytocin-receptor mutant mice is puzzling. One possibility is the low level of pup aversion and high level of pup attraction in virgin female laboratory mice. Feral mice are highly aggressive even toward pups, and engage maternal care only after parturition, as described (Section 2.2). Oxytocin may be required for the inhibition of aggression toward pups during the peripartum period in feral mice. Laboratory mouse strains may have lost this aggressive tendency through domestication. Another possibility is the redundancy with the brain vasopressin system. Vasopressin is an ortholog of oxytocin and has been implicated in various social behaviors including rat maternal behavior, social recognition, ultrasonic vocal communications, pair bond, and the autism-spectrum disorders (Young et al., 1999; Bosch and Neumann, 2008; Scattoni et al., 2008; Tobin et al., 2010; but also see Yang et al., 2008). As oxytocin and vasopressin may cross react with each other's receptor (Schorscher-Petcu et al., 2010), double or triple mutant mice of oxytocin and vasopressin receptors would be of great interest. It also should be noted that the promoter region of mouse oxytocin receptor gene contains putative estrogen responsive elements and AP-1 recognition sequences (Kubota et al., 1996).

5.2. Neurotransmitters and their related molecules

5.2.1. Norepinephrine

Dopamine-β-hydroxylase (DBH) is an enzyme that catalyzes the conversion of dopamine to norepinephrine. Deletion of *Dbh* gene causes depletion of norepinephrine and epinephrine, and a concomitant increase in dopamine and l-Dopa. *Dbh (−/−)* fetuses were mostly embryonic lethal because of abnormal cardiovascular development (Thomas et al., 1995). Mutant fetuses could be rescued to survive until term by administering DOPS (L-threo-dihydroxyphenylserin), which is converted to norepinephrine by aromatic amino acid decarboxylase, thus bypassing DBH, during gestation through the dam's drinking water. Postnatally the *Dbh (−/−)* mice could survive without further support of DOPS, and displayed only mild deficits such as growth retardation and alteration in sympathetic functions. They were normal in olfactory discrimination tasks. The *Dbh (−/−)* females were fertile but defective in placentophagia and also mildly in pup retrieval, so that 80% of the pups died mostly before postnatal day 3 [all-or-none fashion by litter] (Thomas and Palmiter, 1997). These mutant dams could rear donor pups to weaning if the pups were first cleaned and fed by normal dams, or if the mutant dams were supplied DOPS in the peripartum period. And if the mutant dams reared litters through weaning, then they could rear the next litter without further support. *Dbh (−/−)* virgin females and males were also impaired in pup retrieval, but this phenotype could be rescued only poorly by DOPS administration.

Dickinson and colleagues reported that the lesions to the central noradrenergic projection to the olfactory bulbs prior to parturition resulted in cannibalism at parturition in primiparous mice, without producing a gross impairment of later maternal behavior or general anosmia in mice (Dickinson and Keverne, 1988). Similar lesions made after parturition and maternal experience were completely without effect. Together with the previous findings on peripheral anosmia in mice discussed in the Section 3.1, it may be hypothesized that the onset of placentophagia (eating placenta and amniotic membrane, but not the pups) for the first time may require modification of olfactory input from pups by norepinephrine input to the olfactory bulb that originates from the brainstem; once maternal behavior is established by maternal experience, this norepinephrinergic influence on olfactory bulb function may no longer be necessary.

5.2.2. Serotonin (5-hydroxytryptamine, 5HT)

The *Tph2* (tryptophan hydroxylases 2) gene product is an essential serotonin-synthesizing enzyme in the brain. *Tph2 (−/−)* mice lack serotonin in the central nervous system, and exhibit growth retardation (smaller body size and two-week delay of weaning, but normal size at 4 months of age) and 10–50% lethality (genetic-background dependent) in the first 4 weeks of postnatal life (Alenina et al., 2009). Autonomic control of cardiovascular and respiratory functions is mildly impaired. *Tph2 (−/−)* mice exhibited heightened aggression, including female–female aggression. *Tph2 (−/−)* females showed normal olfactory recognition of hidden cookies and were fertile. On the day of delivery, *Tph2 (−/−)* dams organized the nest and showed lactation as the pups had milk in the stomach, although these observations were not quantitative. However, during the following days the pups of *Tph2 (−/−)* dams were neglected and often cannibalized, leading to 50% lethality and 30% litter loss by postpartum day 5, irrespective of the pups' genotype. The pup retrieval test showed that only 1 out of 9 *Tph2 (−/−)* dams retrieved scattered pups within 30 min on the postpartum day 1, whereas the control dams (N = 6) did so by 3.9 ± 0.7 min. The maternal deficit phenotype of *Tph2 (−/−)* dams described in this study is unique and appears to be caused by heightened aggression due to the lack of brain serotonin.

Pet-1 (plasmacytoma-expressed transcript 1) gene encodes an ETS transcription factor and is restricted to 5HT neurons in the brain. A *Pet-1 (−/−)* mutation caused arrest of 5HT neuron development and 70% loss of serotonin immunoreactive cell bodies in adult brains (Hendricks et al., 2003). *Pet-1 (−/−)* mice exhibited increased anxiety levels in the open field and the elevated plus maze, and increased aggressive behavior, but no olfactory deficits were found. *Pet-1 (−/−)* females displayed normal sexual behavior and parturition, but all the pups died within four days after birth, irrespective of the pups' genotype (Lerch-Haner et al., 2008). These pups were found dead without placentas and were not cannibalized for a few days after birth. These pups could be rescued by fostering to the wild-type dams. *Pet-1 (−/−)* dams also exhibited reduced crouching behavior (53 ± 8.7%, compared to 73 ± 10.1% for the wild-type), nest building and grouping behaviors. In a ten-minute retrieval test, *Pet-1 (−/−)* dams retrieved an average of 4 pups, while *Pet-1 (+/+)* dams retrieved all 6 pups, and this difference was statistically significant. If the cage bedding was exchanged partly at the retrieval test, *Pet-1 (−/−)* dams retrieved only about one pup during 10 min. The authors claimed, though without quantification, that pups born to *Pet-1 (−/−)* dams were consistently observed to have milk in their stomachs on each postnatal day before death, suggesting that normal lactation was occurring; and that *Pet-1 (−/−)* dams did not appear hyperactive. [However in explaining their pup retrieval assay, the authors observed that "*Pet-1 (−/−)* dams alternated between frequent digging behavior and active traversal of the cage", which may suggest hyperactivity or hypersensitivity (see also the Supplementary Video 1).]

Mouse dams lacking the *Htr1b* gene, encoding the 5-hydroxytryptamine (serotonin) receptor 1B, are hyperactive and spend 20% more time out of the nest during the dam–pup observation period (the home cage containing the pups and dams was taken to a novel testing room and observations began 20 min later). The *Htr1b (−/−)* dams, however, showed normal retrieval behavior, suggesting that hyperactivity does not always disturb pup retrieval response (Brunner et al., 1999).

These studies on the mutants affecting serotonin signaling are devoid of specific pup retrieval assays using virgin females. Such an examination in the future would add valuable information about the role of serotonin in mouse parental behaviors.

5.2.3. Dopamine

Deletion of the dopamine transporter (DAT) gene *Slc6a3* results in increased dopaminergic tone, hyperlocomotion, and anterior pituitary hypoplasia with reduced lactotrophs and somatotrophs (Bosse et al., 1997; Giros et al., 1996). As a result, *DAT (−/−)* mice exhibited dwarfism and inability of milk production, the severity of which were dependent on genetic background (Morice et al., 2004). *DAT (−/−)* females were fertile and gave birth to normal sized litters. In C57BL/6 genetic background, however, all the pups of *DAT (−/−)* dams died within 24 h. In DBA/2J or in mixed background, pups were alive and healthy, suggesting the mutant dams could maintain lactation and maternal behaviors enough to support pup survival in these backgrounds. Mutant dams showed mild reduction of pup retrieval and nesting behaviors (Morice et al., 2004; Spielewoy et al., 2000), although these deficits may be secondary to the remarkable hyperlocomotion of this strain. The mutants showed normal intermale aggression and duration of social interaction.

5.2.4. Glutamate signaling

Grin1/NR1 gene encodes NR1 protein, the principal subunit of NMDA receptor (glutamate-gated ion channel). Targeting mutagenesis substituted asparagine in position 598 of NR1 to glutamine (Q) (Single et al., 2000) The heterozygous mutant mice of this mutant allele, $Grin1^{tm1.1Phs}$/$Grin1^{+}$ ($NR1^{+/Q}$) exhibited increased mortality (90% death by 9 months of age), but normal long-term potentiation at hippocampal CA3/CA1 synapses. $NR1^{+/Q}$ females were often hyperactive before delivery, and performed poorly on maternal behaviors, such as nest building, placentophagia, pup retrieval and crouching. Moreover the mutant dams were aggressive toward the pups, which lay scattered in the cage and had bruises and bite marks on their bodies, and the pups were sometimes cannibalized. Typically litters died or were killed within two days. Maternal performance did not improve with multiple pregnancies. Litters were occasionally raised successfully by these mutant dams if the dams were helped by the experimenter with respect to nest building, collecting the pups, and placing the dams repeatedly over the litter. As this study lacked examination of parental behaviors in virgin animals, the possibility remains that the presented defects in postpartum maternal behaviors may have been indirectly caused by peripartum stress or by hyperactivity.

$Gria2^{tm3Rsp}$/$Gria2^{tm3Rsp}$ $Tg(Gnrh1\text{-}cre)^{1Rsp}$/*0* mutant mice (*GluR-BΔHS*) lack AMPA2 ionotrophic glutamate receptor only from neurons expressing the GnRH promoter during development (this does not necessarily involve only GnRH neurons, but also includes wider brain areas such as the septum and hypothalamus) (Shimshek et al., 2006). Mutant males exhibit impaired spermatogenesis and defective sexual and aggressive behaviors. The mutant females are fertile but show decreased pup retrieval behavior and maternal aggression. However, female *GluR-BΔHS* mice were observed to build a new nest in the corner of the cage where the pups were placed by the experimenter for the retrieval test, rather than to retrieve the pups to the previous nest site. This observation suggested that the female *GluR-BΔHS* mice had problems associating the pups with their original nest, although they retain at least some maternal responsivity. This deficit may be due to abnormal spatial cognition, and consistently this strain has been shown to have impaired spatial memory. GluR-B receptor deficiency in the septum may be responsible for this phenotype. An alternative interpretation is that this *GluR-BΔHS* mutation causes GluR-receptor deficiency in MPOA neurons to mimic surgical MPOA lesions in rats; that is, a selective interference with retrieval behavior, while leaving nursing/crouching behavior relatively intact (in the cases of MPOA lesions, however, nest building was basically absent) (Numan, 1990; Numan and Callahan, 1980). We have observed a similar behavior (i.e., making a new nest at the pup's location rather than retrieving the pup into the nest) in some virgin female mice with incomplete MPOA excitotoxic lesions (Tsuneoka and Kuroda, unpublished observation).

5.2.5. GABA signaling

Gabrd encodes $GABA_A$ receptor δ subunit, which is highly expressed in the dentate gyrus of the hippocampus. The original study of *Gabrd (−/−)* mice reported that the mutants exhibited mildly reduced viability until weaning but fertile, and the (−/−) breeding pairs produced slightly reduced number of pups/litter ((−/−) mating, 6.3 pups/litter; (+/+) mating, 7.6) (Mihalek et al., 1999). Later Maguire and Mody (2008) reported that both *Gabrd (+/−)* and *(−/−)* females showed severe pup mortality of 40%, although the mutants' milk production/ejection was not assessed. They also showed that *Gabrd (−/−)* dams displayed decreased pup-grouping, as well as postpartum-specific depression-like behaviors such as increased immobility during a forced-swimming test and decreased sucrose preference. The authors suggested these behavioral "postpartum-depression" of *Gabrd (−/−)* females were caused by the altered $GABA_A$ receptor plasticity during pregnancy and lactation; during pregnancy, serum progesterone-derived neurosteroids, such as allopregnanolone, increase drastically and enhance $GABA_A$ receptor function as allosteric modulators. In the wild-type female mice, this fluctuation in GABAergic tone is balanced by a decreased expression level of $GABA_A$ receptor δ and γ2 (Maguire and Mody, 2008), but not in the *Gabrd* mutant mice. The precise function of $GABA_A$ receptor δ subunit in the postpartum maternal behavior and physiology should be, however, determined in the future studies.

Conventional global knockout mice lacking *Gabrb3*, which encodes $GABA_A$ receptor β3 subunit, exhibit 90% neonatal mortality and cleft palate (Homanics et al., 1997). The surviving *Gabrb3 (−/−)* females were fertile but failed to display appropriate maternal behavior irrespective of the pups' genotype. In contrast, pan-neuronal conditional knockout of *Gabrb3* (constructed by crossing floxed *Gabrb3* mice to Synapsin I-cre transgenic mice), showed normal palate development, 61% neonatal lethality, and normal maternal behaviors (Ferguson et al., 2007). Furthermore, the forebrain-specific conditional knockout of *Gabrb3* (constructed by crossing floxed *Gabrb3* mice to CamKII-cre transgenic mice) exhibited 30% preweaning lethality, hyperactivity, normal palate development, but reduced reproductive fitness ("four of six mutant females did not produce any offspring or produced litters infrequently and usually failed to care for pups") (Ferguson et al., 2007). Before concluding that the forebrain-selective deletion of *Gabrb3* could cause more deleterious effects on maternal behavior than the pan-neuronal KO, however, future systematic analysis will be required.

Mice harboring a gain-of-function mutation in the *Gabra1*, encoding $GABA_A$ receptor α1 ($Gabra1^{tm2.1Geh}$/$Gabra1^{+}$) exhibited 40% preweaning mortality, abnormal motor coordination, hunched posture, and hypoactivity. The heterozygous mutants were fertile and produced normal sized litters, but dams failed to rear the offspring beyond the first few days. This study, again, did not provide any further analysis on mutants' lactation ability or virgin retrieval behaviors (Homanics et al., 2005).

Because the GABA signaling has crucial importance in almost all brain mechanisms, more spatiotemporary focused gene manipulation will be required for assessing the exact role of the GABAergic transmission in regulation of maternal behaviors.

5.3. Intracellular signaling

5.3.1. FosB (FBJ osteosarcoma oncogene B)

FosB is an immediate early gene homologous to *c-Fos*, and is induced in a pattern similar to that of c-Fos in MPOA neurons during parenting (Brown et al., 1996; Kalinichev et al., 2000b; Numan et al., 1998). *FosB (−/−)* mice were about 10% smaller than the wild-type littermates, but were generally healthy and fertile (Brown et al., 1996). Brown and colleagues reported that *FosB* −/− females delivered healthy pups, but they often left the pups scattered in the cage and showed poor maternal caretaking, so that most of the pups of any genotype died before weaning. Virgin *FosB (−/−)* females and

males also showed a deficit in retrieving behavior. These *FosB (−/−)* mice, however, showed no abnormalities in olfactory discrimination. These phenotypes in parental behaviors were essentially confirmed by other studies, although the severity of the phenotype was highly sensitive to differences in genetic-background and environment (Gruda et al., 1996; Kuroda et al., 2008). Of note, *FosB (−/−)* virgin females and males were not only less parental but were also less infanticidal toward donor pups than *(+/)* littermates under all conditions (Fig. 1) (Kuroda et al., 2008). One possible interpretation of this nonresponsiveness in *FosB (−/−) mice* may be that the mutants' ability to recognize pups (discussed in the Section 3.1) is decreased. However, c-Fos expression in the MPOA of nonresponsive *FosB (−/−)* mice during pup exposure was not significantly different compared to that of parental *FosB (+/)* littermates. It has been reported that c-Fos expression in the MPOA can be induced by exteroceptive sensory stimuli (olfactory, auditory, visual) associated with pup exposure alone (Li et al., 1999a). Therefore, the defective behavioral output of *FosB (−/−) mice* might be attributed to downstream parts of the information processing pathway, such as behavioral choice (Section 3.2) or behavioral organization problems (Section 3.3).

Subsequent studies reported that *FosB (−/−)* mice were found to show broader phenotypes, including gliosis throughout the forebrain (Kuroda et al., 2008), and exaggerated responses to psychostimulants (Hiroi et al., 1997; Kuroda et al., 2010). The same intracellular ERK-fos-Sprouty/RGK signaling pathway was activated in distinct brain regions by different stimuli, in striatum and cerebral cortex by amphetamine administration and in the MPOA by pup exposure. Sprouty and RGK-type small GTP binding protein families are the known feedback regulators of the receptor tyrosine kinase (RTK) and calcium influx, respectively (Kelly, 2005; Mason et al., 2006). Both RTK and calcium influx mediate neuronal activation and in turn facilitate ERK phosphorylation. The calcium/RTK-ERK-fos-Sprouty/RGK signaling pathway seems a general feedback regulator of activated neurons. Moreover, the ERK-fos signaling has been generally implicated in adaptive neuronal changes such as the learning/memory process in the hippocampus and amygdala. Therefore it was suggested that the ERK-fos signaling in MPOA neurons upon pup exposure may be involved in formation of maternal memory. As described in the next section, ERK and CREB may work independently to induce fos family proteins, providing intracellular basis of parental responses.

5.3.2. Creb1 (cAMP responsive element binding protein 1)

Creb1 encodes one of the basic-leucine zipper (bZIP) transcription factor family proteins, and binds to the DNA sequence called CRE (cAMP response element) to mediate various cellular transcriptional responses evoked by external stimuli. *Creb1* null mutation is an embryonic lethal mutation. The *Creb1$^{\alpha\Delta}$ (−/−)* insertion mutation which abolishes expression of two isoforms of Creb1 α and Δ but sparing Creb1 β isoform, causes 90% reduction of Creb1 expression (Jin et al., 2005). *Creb1$^{\alpha\Delta}$ (−/−)* mutant mice are generally healthy and fertile. However, 40% of pups born to *Creb1$^{\alpha\Delta}$ (−/−)* females died by postnatal day 3, while 95% of them could survive by cross-fostering to the wild-type dams. *Creb1$^{\alpha\Delta}$ (−/−)* virgin females also exhibited delayed pup retrieval and poor nest building, but normal investigation of pups and of novel objects. Jin and colleagues also showed that the number of cells immunostaining for phospho-CREB (the active form) in the MPOA increased nearly three-fold in wild-type mice following exposure to pups but not to novel objects. On the other hand, basal expression and induction of FosB in response to pup exposure appeared to be independent of CREB because FosB expression levels in MPOA were equivalent between *Creb1$^{\alpha\Delta}$ (+/+)* and *(−/−)* mice. CREB is a known upstream regulator of c-Fos, and inhibition of ERK activation reduced FosB but not c-Fos expression level (Kuroda et al., 2007). These findings suggest differential contributions of ERK and CREB for fos family proteins. [The MPOA neurons expressing phospho-CREB and FosB may be distinct populations, as appears to be the case from an examination of Figure 6 of this study of Jin et al., 2005].

Two interesting similarities of *Creb1$^{\alpha\Delta}$ (−/−)* and *Dbh (−/−)* maternal behaviors should be noted; first, mutant dams would accept and care the foster pups, if such pups had first been cleaned and fed by the wild-type dams; second, once mutant dams successfully reared a litter, they would continue to do so with future litters.

5.3.3. Heterotrimeric G proteins of the $G_{q/11}$ family

Gnaq and *Gna11* genes encode the alpha-subunits of the two main members of the $G_{q/11}$ family, $G\alpha_q$ and $G\alpha_{11}$, respectively. Wettschureck and colleagues constructed a double mutant mouse using the conventional knockout for $G\alpha_{11}$ and the forebrain-selective conditional knockout for $G\alpha_q$ with a CamKIIα promoter (*Gna11^{tm1Soff}/Gna11^{tm1Soff}*, *Gnaq^{tm2Soff}/Gnaq^{tm2Soff}*, *Tg(Camk2a-cre)1Gsc/0*) (Wettschureck et al., 2004). These forebrain $G\alpha_{q/11}$-deficient mice were generally healthy, while exhibiting myoclonic or clonic–tonic seizures after 3 months of age, and a mild decrease of survival rate (Wettschureck et al., 2006). The forebrain $G\alpha_{q/11}$-deficient females delivered normally and exhibited placentophagia immediately after parturition, but nest building, pup retrieving, and active crouching over pups did not occur. Accordingly, pups were scattered and unattended and died within 48 h postpartum, irrespective of their genotype. Repetitive pregnancies did not ameliorate this phenotype. Interestingly however, if these mutant dams were housed during late gestation with a nursing wild-type female, 80% of their litters survived to postpartum day 2, and 7% to postpartum day 21. This observation, together with normal development of the mutant mammary gland, indicated normal lactation in the mutant dams. Forebrain $G\alpha_{q/11}$-deficient virgin females also exhibited severe defects in pup retrieval. c-Fos expression induced by pup exposure was significantly reduced in the MPOA, the bed nucleus of stria terminalis and lateral septum of the postpartum and virgin mutant females compared with the wild-type females. The mutants showed normal pup sniffing, olfaction, and motor behavior during the open field and rotarod tests (Wettschureck et al., 2004).

$G_{q/11}$ are the second messengers coupled with oxytocin receptor and vasopressin receptor 1a/1b. Therefore it was expected that the forebrain $G\alpha_{q/11}$-deficiency caused blockade of all the intracellular signaling of OTR and AVP1a/1b and thus brought about maternal behavior deficits. Pup exposure induced c-Fos expression in the oxytocin receptor-immunopositive cells, however, did not differ in the hypothalamus or olfactory related brain areas when the mutant female was compared to the wild type female.

5.3.4. Foxb1 (forkhead box B1)

Mutant mice lacking a winged helix gene *Foxb1/Fkh5/Mf3*, exhibit 30% preweaning lethality and are smaller, but most of survivors are fertile (Wehr et al., 1997; Labosky et al., 1997). Foxb1 protein is expressed in interneurons of the spinal cord, cells in the mammillary region of the hypothalamus, thalamus, midbrain colliculi, developing mammary gland and epithelial cells of the adult mammary ducts. *Foxb1 (−/−)* dams in inbred strains deliver litters of normal size, but failed to have any surviving pups. One study reported that the *Foxb1 (−/−)* dams in 129Sv×C57BL/6 genetic background had normally developed mammary glands (data not shown) but did not retrieve pups or build a nest (Wehr et al., 1997). In another study, however, independently constructed *Foxb1 (−/−)* dams in 129Sv×Black Swiss genetic background made nests and suckled their pups, and their nipples appeared red and distended, but the milk was never observed in the pups' stomachs (Labosky et al., 1997). Labosky and colleagues later found that *Foxb1 (−/−)* dams on an outbred Black Swiss or CD-1 genetic background could feed their pups (Kloetzli et al., 2001; Labosky et al., 1997). By comparison of brain structure abnormalities in these different genetic background (although not quantitatively), they suggested that morphological defects in the inferior colliculi might correlate with the inability of lactation. Consistently, the inferior

colliculus, especially its external nucleus, has been shown to be essential for milk ejection reflex, possibly as a somatosensory pathway rather than auditory (Dubois-Dauphin et al., 1985; see also Wakerley, 2005). To determine the impact of Foxb1 in mouse parental behaviors, specific pup retrieval assays will be needed.

5.4. Genomic imprinting and DNA methylation

5.4.1. Paternally imprinted genes Mest/PEG1 and PEG3/Pw1

Genomic imprinting is a mechanism which involves DNA methylation and histone acetylation, in order to achieve monoallelic gene expression epigenetically (without altering the genetic sequence) (Horsthemke et al., 1999). Paternally-expressed genes (PEG) are expressed from the paternal allele only.

Peg3/Pw1 heterozygous *(+/−)* mice which inherited the mutation from paternal germ line did not express Peg3 protein, were smaller but otherwise healthy and fertile (Li et al., 1999b) (in 129Sv background). Li and colleagues showed that the mutant dams showed about a 25% decrease in the number of oxytocin neurons in the hypothalamus and a consequent decrease in milk ejection. Only 8% of the litters of primiparous mutant dams survived to weaning. Nevertheless, by the third parturition, 70% of mutant dams cared for their young through weaning. Also in this study, mutant dams and virgin females exhibited an increased latency for pup retrieval and nest building, but normal sniffing latency. Quite distinct phenotypes were reported, however, of the same mutant mice later (Champagne et al., 2009), such as no retrieval deficits, better first-litter survival (71%, compared to 8% in the original study), but increased pup-sniffing latency (which was not significantly different with the wild-type control in the original study). The authors argued that such inconsistencies might be due to the selection pressure that occurred during 32 generations from the original study to their study. This may be relevant to the breeding strategy used, in which this Peg3 mutant mouse line had been kept and backcrossed by crossing heterozygote males with the wild-type females (Champagne et al., 2009). Champagne and colleagues additionally reported that the *Peg3* mutant dams in 129Sv and C57BL/6J background exhibited commonly reduced licking/grooming and nursing, and different behaviors in the open-field tests. Collectively, these behavioral phenotypes in the *Peg3* mutants were sensitive to different genetic backgrounds and experimental environments.

The paternally-transmitted heterozygous mutation of *Mest (mesoderm specific transcript)/Peg1* gene (Kaneko-Ishino et al., 1995) resulted in a more severe phenotype, including decreased body size both prenatally and postnatally (about 70% of body weight compared to wild-type mice) and increased perinatal lethality (Lefebvre et al., 1998). The mutant females were fertile and delivered a normal size of litters, but displayed defective placentophagia, and only about a half of their pups survived until weaning irrespective of pups' genotype. Nulliparous mutant females also exhibited severe defects in nest building and pup retrieval.

A widely accepted hypothesis for the evolution of genomic imprinting is the *parental conflict hypothesis*, based on kinship theory (Moore and Haig, 1991). This theory proposes that paternally-derived alleles act to maximize the extraction of maternal resources for the offspring that express the allele, while maternally-derived alleles counteract this effect and equalize the maternal investment to all progeny over the mother's entire reproductive lifespan. The initially identified functions of several imprinted genes provided striking support for this conflict hypothesis, as paternally-expressed *Igf2* (Barlow et al., 1991) facilitated fetal growth, maternally-expressed *Igf2r* and *H19* counteracted the effects of *Igf2* (Bartolomei et al., 1991), paternally expressed *Gnasxl* required for suckling (Plagge et al., 2004), along with above-mentioned *Peg1* and *3* which enhance maternal care. There are, however, some arguments against this theory, as the function of some imprinted genes requires a different type of explanation (Kaneko-Ishino and Ishino, 2010; Renfree et al., 2009; see also Wilkins and Haig, 2003).

5.4.2. MBD2 (methyl-CpG binding domain protein 2)

MBD2 is a transcriptional repressor that specifically binds to methylated DNA and is a component of the MeCP1 (methyl-CpG binding protein 1) complex. *Mbd2 (−/−)* mice were apparently healthy and fertile (Hendrich et al., 2001). Litter size of *Mbd2 (−/−)* dams was about half that of wild dams at weaning, although this study did not clarify whether the number of pups in the litter was small from delivery or whether pups were lost during lactation. Pup retrieval tests showed moderately increased latencies in *Mbd2 (−/−)* postpartum dams, half way between that of wild-type and the *Peg3 (−/−)* dams as described above. The weight gain of the pups during 24 h was also decreased in *Mbd2 (−/−)* dams to an intermediate degree between the wild type and the *Peg3* mutant. As pups appeared to be suckling equally in the *Mbd2 (−/−)* and *(+/+)* dams, milk production or ejection defects was suspected in *Mbd2 (−/−)* dams but no further analysis was done in this context. *Mbd2 (−/−)* mice lack a component of MeCP1, but dysregulation of endogenous imprinted genes such as *PEG1* or *3* was not detected.

5.5. Molecules involved in olfactory system functions

5.5.1. Molecules required for main olfactory system functions

Here we discuss on two genes, G_{olf} and *Adcy3*, where mutations cause congenital anosmia and maternal behavior defects. Before describing each mutant in detail, it should be noted that anosmic neonatal pups show defects in nipple finding and subsequent suckling, resulting in neonatal lethality (Tasaki et al., 2007; Wong et al., 2000) [characteristically, this neonatal lethality can be ameliorated by culling normal littermates to reduce competition]. This suckling defect in anosmic pups is purely caused by the pups' genotype, since this phenotype has been confirmed in *(−/−)* pups with heterozygous (+/−) dams with normal olfaction. On the other hand, to assess parental behaviors, homozygous *(−/−)* dams should be used with *(+/−)* pups as stimulus. Therefore the proper breeding strategies described in Sections 4.1.5 and 4.3.1.2 are needed to segregate pup factors from maternal factors: that is, the observed dam–pup dyad should contain the homozygous mutant only in one side, either in the dam or in pups. The studies described below have been performed pointing this proper manner.

The Adcy3 gene encodes the type III adenylyl cyclase, which coupled with G_{olf} is required for sensory transduction of the main olfactory epithelium. *Adcy3 (−/−)* mice were anosmic, although some odorants could be detected through the VNO (Wong et al., 2000). *Adcy3 (−/−)* mutants were initially smaller than wild-type littermates but caught up after 3 months of age. The mutant males exhibit reduced fertility and abnormal spermatid functions, while mutant females were fertile. *Adcy3 (−/−)* dams showed normal placentophagia, but severe deficits in pup retrieval and nest building (Wang and Storm, 2010). *Adcy3 (−/−)* virgin females as well were almost devoid of a pup retrieval response.

The G_{olf} gene (*Gnal*) encodes the olfactory-type guanine nucleotide binding protein, α subunit (Belluscio et al., 1998). Homozygous mutant mice which survived the postnatal period are anosmic and smaller but reach sexual maturity and mate. Homozygous mutant dams fail to retrieve pups and to crouch over their pups after parturition. As a result, all pups of four litters from three different dams died without milk in their stomachs by postnatal day 2. These anosmic G_{olf} mutants, however, also exhibit hyperactivity in an open field test, which may generally cause defective maternal care.

These findings as well as data from surgically-induced anosmia (Section 3.1) support the notion that the main olfactory function is of primary importance for parental behavior in mice, and its absence does not necessarily cause infanticide.

5.5.2. Molecules required for accessory olfactory system functions

The $Del(6)1^{Mom}$ mutant strain lacked a cluster of *V1r* (vomeronasal pheromone receptor family 1) genes by engineered chromosomal deletion (Del Punta et al., 2002). The *Trpc2* (transient receptor

potential cation channel, subfamily C, member 2) gene encodes an ion channel specifically expressed in VNO neurons (Leypold et al., 2002). Both of these mutants were generally healthy and fertile, but are unable to detect sensory information through the vomeronasal system, and exhibit decreased maternal aggression. In addition *Del (6)1*^{Mom} mutants display normal intermale aggression and maternal retrieval but reduced male sexual behavior. *Trpc2* mutants display normal male-to-female sexual behavior but abnormal male-to-male sexual behavior, reduced intermale aggression and maternal nesting duration.

In connection with these, *Nos1* mutant mice, lacking neuronal nitric oxide synthase, which is enriched in the accessory olfactory system, exhibited normal pup-directed maternal behaviors but reduced maternal aggression (Gammie and Nelson, 1999) and background-sensitive heightened or normal intermale aggression (Chiavegatto et al., 2001; Le Roy et al., 2000).

These phenotypes are roughly in concordance with the effects observed after the surgical removal of VNO, which showed that VNO removal does not affect mouse maternal retrieving, nursing or nest building, but significantly reduces maternal aggression as well as intermale aggression (Bean and Wysocki, 1989). Furthermore, in rats, VNO surgical ablation decreases infanticide by males and facilitates parental behavior (Fleming et al., 1992; Mennella and Moltz, 1988).

5.6. Others

5.6.1. MTAP6 (microtubule-associated protein 6)/STOP (stable tubule only peptide)

Mtap6/STOP encodes a microtubule stabilizing protein under cold conditions (Andrieux et al., 2002). *STOP (−/−)* mice were viable and had no detectable defects in brain anatomy but showed synaptic defects, with depleted synaptic vesicle pools and impaired synaptic plasticity. *STOP (−/−)* mice exhibited a variety of behavioral abnormalities reminiscent of schizophrenia, including hyperactivity in the dark phase without apparent goal orientation, freezing behavior while awake, decreased sleeping, hypersensitivity to mild stress, anxiety-like behavior evaluated by the light–dark crossing task, reduced initial investigation in resident–intruder test, reduced aggression toward intruder and deficits in social recognition (less habituation of repetitive encounter with the same conspecific). On the other hand, they appeared to have normal olfaction as detected by hidden food retrieving test (Begou et al., 2008). *STOP (−/−)* dams failed to rear their pup to weaning (none survived among 161 pups from 20 mutant dams), irrespective of the pups' genotype (Andrieux et al., 2002). Dead pups were never cannibalized. When *STOP (−/−)* dams were placed over pups repetitively by human intervention, pups showed suckling and milk could be observed in the pup's stomach, suggesting normal lactating ability of *STOP (−/−)* dams. *STOP (−/−)* dams were never aggressive toward pups, but were severely defective in nest building and pup retrieval in the home cage, and did not improve with multiple pregnancies. *STOP (−/−)* virgin females and males also exhibited pup retrieval defects compared with the wild-type mice. Most strikingly, the various deficits of *STOP (−/−)* postpartum maternal behaviors could be ameliorated by chronic, but not acute, treatment of chlorpromazine and haloperidol: 4 months treatment after weaning rescued the pup survival of *STOP (−/−)* dams from 0% to about 50%. Although the maternal behavioral defects in this mutant seemed secondary to the other behavioral disruptions described above, further analysis on the mechanism of alleviation of maternal behavior defects by neuroleptics may be useful for the understanding of the mechanistic aspects of parental behavior regulation.

5.6.2. Stathmin

The mutant mice lacking *Stmn1* gene, encoding stathmin/oncoprotein 18, an inhibitor of microtubule stabilization, were reported to have a deficiency in postpartum maternal retrieving behavior (Martel et al., 2008) along with defective amygdala-dependent fear conditioning. Stathmin protein is enriched in the lateral amygdala, but is also expressed widely in the cerebral cortex, thalamus and hypothalamus (Shumyatsky et al., 2005). *Stmn1 (−/−)* virgin female mice showed a mild deficit in pup retrieval, and in the proper selection of a nest site in an open field situation. Therefore, their defects may be related to deficits in the spatial organization of pup retrieval behavior (Section 3.3) with intact parental responsivity *per se*. In addition, stathmin knockout mice showed late-onset pan-neuronal axonopathy, gliosis and myopathy (Liedtke et al., 2002). This aging-related degeneration, though mild, may cause the atypically larger deficits in retrieving and pup survival that were observed in the second litter of these knockout females, compared with the first litter [normally, parental behavior gets better with experience].

5.6.3. Testicular orphan nuclear receptor 4 (TR4)/nuclear receptor subfamily 2, group C, member 2 (Nr2c2)

The Nr2c2/TR4 gene product is a member of the nuclear receptor superfamily for which a ligand has not yet been found. *TR4 (−/−)* mice demonstrate high rates of early postnatal mortality, significant growth retardation, low body weight (between 20 to 56% of the wild-type), impaired motor coordination and reduced fertility with abnormal gonadal morphology (Collins et al., 2004). *TR4 (−/−)* females show defects in maternal behaviors including nest building and retrieving, so that the pups of *TR4 (−/−)* dams are scattered in the cage and die within 36 h after birth with no indication of milk intake. Histology of mammary gland tissue from the mutant dam on postpartum day 1 demonstrated copious milk in the mammary ducts and normal mammary gland histology. The authors claimed that oxytocin expression was reduced in *TR4 (−/−)* mice in their preliminary analysis (no data shown), so that the milk ejection might be disturbed, or the milk might be accumulated because of reduced nursing behavior.

5.6.4. (phosphatase and tensin homolog on chromosome ten)

Pten is a tumor suppressor gene, and its mutation causes tumor-prone phenotype and brain disorders, including macrocephaly, seizure, and mental retardation (Planchon et al., 2008). *Pten* mutations also have been reported in autistic individuals with macrocephaly. *Pten* null mice is embryonic lethal. A neuron-specific-enolase (*Nse*) promoter-driven *cre* transgenic mouse was used to delete *Pten* in limited differentiated neuronal populations in the cerebral cortex and hippocampus of mice. Resulting mutant mice showed reduced social interaction, exaggerated responses to sensory stimuli, postweaning progressive macrocephaly with increased synapses. The mutant females produced normal sized litters but average with only one pup surviving after the postnatal day 5 (Kwon et al., 2006). The mutant mice also showed reduced male sexual behavior and sleep–nest building, but normal in detection of hidden treat after overnight food deprivation and in general interest in novelty.

5.6.5. Rora (RAR-related orphan receptor α)

Homozygous spontaneous mutation *Staggerer*, which later turned out to be the null allele of *Rora*, causes dysgenesis in the cerebellum and olfactory bulb, and consequent ataxia, tremor and hyposmia (Deiss and Baudoin, 1997; Sidman et al., 1962). The homozygous *Staggerer* mutant females could successfully mate and deliver pups, but were unable to reach her own genital parts at delivery (Guastavino, 1983). The mutant dams were also seriously defective in placentophagia and pup retrieval, most probably due to poor body balance. Consequently the pups typically die at birth by choking or within 2 days by starvation, under standard housing conditions. Interestingly, Guastavino succeeded to restore maternal behaviors and increase the pup survival to 67%, by enforcing the mutant dams to

stay in close physical contact with their pups using a cylindrical constraint chamber.

5.6.6. Oca2 (oculocutaneous albinism II)

Homozygous radiation-induced mutation *rjs* (runty, jerky, sterile) which is a recessive mutant allele of *Oca2* (*Oca2*$^{p-s}$), caused reduced growth, jerky gait, male sterility, female semisterility and maternal behavior defects (Hollander et al., 1960). Although the mutant dams were not completely indifferent to pups or killing the pups, they did not typically make a nest or keep the pups together (Lehman et al., 1998). Mammary tissue of the mutant dams appeared histologically normal, and milk were found in the stomachs of some pups. The pups born to the mutant dams almost never survived more than 24 h, but were successfully fostered to and reared by the wild-type mothers.

6. Concluding remarks

Mouse genetics now provides a variety of powerful strategies to investigate the neuromolecular mechanisms of mammalian parental behavior. Like all techniques, however, such powerful genetic tools may cause misleading interpretations, if applied without careful methodological considerations (Bailey et al., 2006). This review tries to provide basic information for testing parental behavior, outline the technical issues in using genetically-engineered mice, and also briefly summarize the wealth of knowledge that has accumulated with respect to the neurobiology of parental behavior.

The Section 5 of this review highlights recent findings on molecular basis of parental behaviors obtained through mouse reverse genetics. Each component of parental behaviors, such as retrieving, placentophagia and maternal aggression, is differentially disturbed in each mutant strain. Together with the existing neuroanatomical literature (Gammie, 2005; Leckman and Herman, 2002), these data suggest the unique neuromolecular basis for each behavior component. Future studies of genetic and environmental influences on parental behavior have the potential to elucidate each mechanism.

Some findings obtained using mouse genetics are consistent with the previous studies utilizing other methodology or different species (e.g. the critical role of VNO in maternal aggression), but others are less consistent (e.g. the role of oxytocin in maternal responsiveness). Such discrepancies may be resolved by technical advances, including a more precise control of gene expression both spatially and temporally, or may be inherent to each experimental model. Although parental behaviors and their mechanisms in mice and rats differ in many aspects, their commonalities rather than differences are of most interest, for elucidation of the neuromolecular regulation of parental behavior across mammals. In addition, further expansion and maintenance of databases in both genetics (e.g. MGI) and neuroanatomy (e.g. the Allen brain atlas, http://www.brain-map.org/) would contribute to dissemination of previous findings and produce new insights. Integration of various techniques ranging from anatomy, endocrinology, genetics to informatics, will be necessary to unveil the neural circuitry of mammalian parental behaviors, and to ultimately support the well-being of human parent–infant relationship.

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