Methods in molecular biology "Pheromone signaling"

Assessing postpartum maternal care, alloparental behavior and infanticide in mice: with notes on chemosensory influences

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Summary

Chemosensory signaling influences maternal care and other innate behaviors toward conspecific young animals in rodents. In this chapter, we describe basic protocols for assessment of postpartum maternal behavior and other pup-directed behaviors in laboratory mice. The specific aim of this protocol is to screen out the abnormal phenotypes in parenting of genetic mutant mice under the standard housing condition. The possible underlying mechanisms for a given abnormality in the mother-young interaction are briefly suggested as well.

Key words

Parental care, Paternal behavior, Infanticide, Mus musculus, Olfaction, Pup retrieval assay, Nest building

1. Introduction

1.1. Parental behavior and related pup-directed behaviors in mice.

Maternal behavior is defined as the collection of behaviors by the mother that can increase offspring survival [1,2]. 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**". In addition to these nurturing behaviors, mice also perform negative pup-directed behaviors, such as **nonresponding** (the subject mouse sniffs the pup at first, but stay away for most of the time), **avoidance** (moving away from the pup or burying the pup in the bedding) and **infanticide** (pup biting/killing, often but not necessarily combined with cannibalism). Infanticide is most frequent in virgin (sexually-naïve) male mice. In C57BL/6J, a standard inbred strain with high sociability, the 70-80 % of virgin males commit infanticide even after repeated pup exposure (*see Note* 1). Once the same 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 mice provide paternal care even toward non-biological offspring [4,5] (*see Note* 2).

1.2. Components of mouse parental behaviors

Among the components of rodent parental behavior listed in **Table 1**, **pup-retrieval** behavior is widely used as an index of parental responsiveness in both rats and mice (eg. [9-11]). The latency to retrieve each pup is easily and unambiguously measurable, and can be assessed not only in postpartum mothers but also in non-lactating females and males, as discussed in (Section 3.2). Assessment of the **nest quality** is also a preferred measure in mice. For postpartum mothers, their ability of **placentophagia** and **provision of maternal milk** should be assessed as well. Therefore in this chapter, the basic protocols for evaluation of postpartum maternal behaviors and pup-retrieval behavior of mon-mothers are introduced. The presented protocol is compatible with the common practices of mutant mouse husbandry in the SPF condition, so that it may be useful for the initial screening of parental responsiveness in mutant mouse strains. For more detailed information on parental behaviors in rats and other mammalian species, please refer the previous literature [12-16], and for further discussions and for listing gene mutant strains implicated in this topic, refer [17].

1.3. Effects of chemosensory signaling on the pup-directed behaviors

Pup-directed behaviors described above are largely dependent on olfaction, in many mammalian species including rodents [18]. In particular, the mouse parental behavior is totally dependent on the main olfactory function (*see Note* 3) (*see Note* 4). Anosmia caused either by postnatal surgery or by genetic mutation strongly inhibits most of parental behaviors in both virgin and postpartum mother mice. For the best studied example, t*he* ***Adcy3*** gene encodes the type III adenylyl cyclase, which coupled with $\boldsymbol{G_{olf}}$, both of which are required for sensory transduction of the main olfactory epithelium. *Adcy3* homozygous mutant *(-/-)* mice were anosmic, although some odorants could be detected through the VNO [21]. *Adcy3 (-/-)* mutants are initially smaller than the wild-type littermates but catch up after 3 months of age. *Adcy3 (-/-)* females are fertile and show normal placentophagia after parturition, but also exhibit severe deficits in pup retrieval and nest building, causing the majority of their pups' death within two days [24]. Maternal aggression of *Adcy3 (-/-)* mothers is severely disturbed too. Furthermore, *Adcy3 (-/-)* virgin females as well are almost devoid of a pup retrieval response. Consistently, the anosmic G_{olf} homozygous mutant mothers fail to retrieve pups and to crouch over the pups, so that all pups of four litters died without milk in their stomachs by postnatal day 2 [25]. The authors did not report the infanticide in these studies.

The accessory olfactory function is not required for pup retrieval behavior or nest building, but is required for maternal aggression against intruders as shown by surgical VNO removal in mice [26]. This finding is supported by the reports of two genetic mutant mouse strains, ***Trpc2*** (transient receptor potential cation channel, subfamily C, member 2) mutant [27] and $\boldsymbol{Del(6)1^{Mom}}$ mutant (lacked a cluster of vomeronasal pheromone V1 receptor family) [28]; these mutants were fertile and showed normal pup retrieval behavior, but exhibited less maternal aggression. In addition, the VNO dysfunction abolished the infanticide in virgin male mice (Tachikawa, manuscript in preparation), as well as the aversive responses toward pups in rats [29,30]. Surgical ablation of VNO and the *Trpc2* mutant mice also caused the reduction of inter-male aggression as well, although $Del(6)1^{Mom}$ mutants display normal inter-male aggression, suggesting the possible correlation of maternal aggression and infanticide (pup-directed aggression) with the inter-male

aggression. Clearly, all of these aggressive behaviors toward conspecific animals are largely dependent on the pheromone signaling.

2. Materials

2.1. Cage system, bedding and nest materials

The standard shoebox breeding cages (approx. size of 265mm x 205mm, 140mm high) with automated ventilation and water-supply in the SPF condition can be used for the behavioral assay. For breeding the subject mice, normal cage bedding materials can be used. For the assay of the pup-directed behaviors, however, we use paper chips made from purified pulp paper-pulp (eg. alpha-dri, Shepherd) as cage beddings. Wood-chip bedding may affect the outcome of parental behavior, as reported for the mice lacking Fyn tyrosine kinase [31,32] and FosB transcription factor [3]. Hexanal, a volatile substance contained in plants and causing a grassy odor was the responsible chemical component for this effect at least in fyn *(-/-)* [32]. The woods used to make the same wood-chip bedding product often vary by season. Contents of chemicals such as hexanal significantly vary between the types of wood used, and between the treatment of chips (autoclaving, addition of pesticides and so on). As mouse pup-directed behaviors are very sensitive to chemosensory signals, the wood chips are less suitable than quality-controlled, purified paper chips.

The addition of nest material makes it easy to identify the location and quality of the nest [33]. Normal cotton pads, balls, or thin paper strips can be used, but the compressed cotton piece (eg. Nestlet, Ancare, Bellmore, NY) is ideal. Adult mice normally bite and tear this densely-packed 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 highly suspected that the subject mouse should have some sort of health problems, or has serious defects with nest building behavior.

2.2. Genetic background

The confounding effects of **genetic background** on behavioral phenotypes have been acknowledged [34], complicating the interpretation of findings. Also for maternal behavior, strain

difference among different substrains has been reported [35] [36]. If the original mutant strain was constructed using 129Sv-derived embryonic stem cells, it is often preferable to **backcross** the mutant strain into C57BL/6, a standard congenic strain for neuroscience research (*see Note* 5). 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. After five generations of backcross, the 96.875 % of the whole genome is C57BL/6 background. However, genetic complications due 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 [37].

2.3. Production of subject animals

Here we describe the procedure for detecting the autosomal recessive phenotypes in adult parental behaviors of conventional targeted disruption (knockout, KO) of single genes as an example. The protocol should be appropriately modified for mutations that follow the other patterns of inheritance, such as X-linked or dominant mutations, conditional KOs, or parent-of-origin specific gene expression (genomic imprinting).

1. Cohabitate one to three heterozygous *(+/-)* female mice of 10-12 weeks old with one *(+/-)* male of 12-24 weeks old in one breeding cage (*see Note* 6).

2. According to the reproductive success, make a few to several breeding cages at a time, aiming at production of 6~8 homozygous *(-/-)* and the wild-type *(+/-)* subject females of similar ages (*see Note* 7), to be tested at a time (*see Note* 8).

3. Remove visibly pregnant females into a separate cage and check for delivery every morning.

4. The pups should be weaned at a determined time window after the birth (*see Note* 9) and

group-housed until the behavioral experiments.

5a. To test adult virgin mice for their pup-directed behaviors, single-house subject females and males at 10 and 12 weeks of age, respectively, in a new cage containing paper bedding with a cotton square as nest material described in the Section 2.1. Measure the body weight of each animal at this point would be informative.

5b. To test postpartum maternal behaviors, follow the next section 2.4. to make the females pregnant, and single-house the pregnant females as above.

2.4. Breeding strategy for postpartum maternal behavior tests

1. To compare the effects of maternal genotype on maternal behaviors, crosses between *(+/+)* males with *(-/-)* subject females and between *(-/-)* 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, then crosses between *(+/+)* females with *(+/-)* males can be conducted. The principle is to avoid the use of *(-/-)* pups as stimuli for testing maternal behavior, and also trying to equalize the pups' genotype for *(-/-)* and the control *(+/+)* subject mothers. Mating should preferably start with the 10-12 weeks of age for primiparous maternal behavior testing. Checking the females 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.

2. Once the female gets visibly pregnant, it is isolated in a new cage containing paper bedding with a cotton square as described above.

3. 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 mother 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.

3. Methods

3.1. Assessing postpartum maternal behavior on the first morning after delivery

The postpartum examinations should be done in this presented order, to minimize the stress of the mother. Throughout these observations, we try not to take mouse mothers out of the cage (see also [15]). 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 mother destroying her nest or attacking her pups.

1. *Parturition check*: Around the estimated day of delivery, the cage should be checked for parturition every morning (*see Note* 10). 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 mother 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 mother accept these weakened and chilled pups.

On the other hand, if the mother is still in delivery, one should not disturb the cage. It is normal for the nest to get destroyed during delivery, as the mother 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 mother settles down and finishes placentophagia, rebuilding the nest, pup retrieval, and stays with the pups in the nest to nurse them, before starting the ratings described below.

2. *Nest grading*:(*see Note* 11) If the parturition has been ended, first evaluate the nest quality from outside of the cage, so as not to disturb the mother. 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 [42]. 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 [42]). With the optimal nest material such as fluffy paper strips, a completely enclosed nest, rated as **4** can be achieved (full dome, [42]). In such nest, the pups are scarcely visible from any directions. 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 mother 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 x 5cm Nestlet per a shoebox mouse cage as described here, however, this grade-4 nest is rather rare. 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.

3. *Pup grouping evaluation*: Pups' 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 (e.g. "three alive pups in the nest and grouped, two dead? pups buried under the bank of the nest, one alive pup outside of the nest") (status of each pup will be examined later in more detail, so that a brief note is sufficient at this moment). Ideally all the pups should be in the nest and tightly grouped (huddled) with each other. When any 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 (eg. healthy and pinky, pale, or dead) should be 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.

4 *Pup retrieval observation*: To quantify pup retrieval latency, remove the cage top, and gently take three healthy pups from the nest, and place one pup in each corner of the cage outside the nest (Fig.

1A). 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 (*see Note* 12), 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 mother 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 [47-49]). The latency to show nest building or pup licking can also be recorded, although because of the small size, these behaviors in mice are more difficult to be distinguished from pup regrouping, pup sniffing or self-grooming compared with those in rats.

If most of the pups are found in the nest at the initial check from outside, one can assume that the mother has already exhibited pup retrieval, because newborn pups are not able to group themselves together. When the pup are found grouped but the mother does not retrieve any pup during the pup retrieval observation, it is suspected that the mother is stressed by the current experimental maneuver. In that case, gently placing 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 mother shows retrieval, the delay of retrieval may have been caused by stress hypersensitivity rather than by lack of maternal responsivity itself.

In 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) of the same age from another mother (*see Note* 13). It is often seen that the mutant mother with insufficient milk secretion appears to abandon her weakened pups, but when fresh healthy donor pups are introduced, the same mother shows a quick retrieval response. Formally in this case, the control mothers should also be exposed with donor pups for retrieval scoring, to equalize the experimental conditions between mutant and control.

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. 1B), "alive without milk", or "dead."

In addition, it is recorded whether there are any remaining amniotic membranes, umbilical cord, or placenta attached to the body. When such sticky fetal tissue is remaining, the pup's skin may be covered by bedding materials (see [17]). 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.

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 cage top. 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 mother 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.

7. Interpretation of the results. Interpret the above observatory data using the flow chart presented in Fig. 2. Analyze the latencies of each behavior during pup retrieval observations, and results from pup and maternal body examinations.

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

The pup retrieval in virgin female mice can be tested much more easily and quickly than that of postpartum mothers in mice or that of virgin female rats, since the virgin female mice are parental within half an hour at the first pup exposure [51,52]. It is minimally two-day procedure from single housing to testing for 30 min next day, if the donor pups can be provided from breeding colony. In addition the pup retrieval in virgin females is free of any physical or endocrine

complications by parturition. It should also be noted that the virgin females' oparental behaviors are independent of hypophyseal hormones both in mice [53,54] and in rats [9].

1. To quantify parental responsiveness of non-parturient animals, male or female subject mice are individually housed for two days (at least 24 hour) prior of an experiment, in a new cage containing paper bedding with a cotton square as nest material as described above (*see Note* 14).

2. On the test day, the nest site and quality should be recorded as described above.

3. Then each animal is exposed to three 1- to 6-day-old donor pups (*see Note* 15). One pup is placed in each corner of the cage distant from the nest.

4. 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 (*see Note* 16). 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. If any of the pups is attacked during the test, which is normally observed during the first few minutes after the pup exposure, stop the observation and rescue all the pups immediately. The severely wounded pup is euthanized as described previously [57]. This subject is deemed as **infanticidal** (Note 17). Otherwise, pups are left in the cage for 30 min.

5. After 30 min of observation, the pups are removed and, if suspected, examined for any bite marks.

6. 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 [11,24]. On the other hand, once an animal becomes fully parental for two successive days, it seldom goes back to partially parental or infanticidal.

4. Notes

1. We found that the proportion of infanticidal virgin males is highly dependent on the rearing environment of the males as well as the testing conditions [3]. For example, the C57BL/6 males weaned at 21 days rather than 28 days are less infanticidal in our experimental conditions.

2. 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 [6]. 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 [7]. 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 [8]. These findings support the idea that infanticide of non-offspring young is a valid and adaptive reproductive strategy selected through evolution.

3. Care should be taken for the species differences of the olfactory function in parental behaviors. Unlike in mice, maternal behavior in rats is under multisensory control, in terms that the maternal behavior is not dependent on any single sensory modalities (olfactory, visual, auditory and somatosensory) [19,20]. 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. 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 virgin female rats (see [18]). 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 female rats. Therefore the high dependence of the mouse parental behaviors on olfaction may be rather atypical in mammalian species.

4. It should be noted that anosmic neonatal pups show defects in nipple finding and subsequent suckling, resulting in neonatal lethality [21,22]. This means that the mother-pup interactions can be disturbed bidirectionally by anosmia. Therefore the proper breeding strategies are needed to segregate pup factors from maternal factors: that is, the observed mother-pup dyad should contain the homozygous mutant only in one side, either in the mother or in pups. Of additional note, the vomeronasal organ does not seem required for suckling in pups [23].

5. Milk-production deficit can be exaggerated by backcrossing the mutant lines from 129Sv into C57BL/6 as discussed [17].

6. Whenever appropriate, the subject experimental mice and the control mice should be the littermates (reared by the same mother) in general. That is, for production of experimental homozygous (-/-), heterozygous (+/-) and control (+/+) mice should be produced by crossing (+/-) females and (+/-) males, in order to minimize the potential confounding influences of background genes from breeder parents and rearing environmental factors [38]. Moreover, maternal behavior is known to transmit across generations nongenomically (future maternal behavior is affected by the maternal behavior received as neonates) [39].

7. If the preliminary analyses confirms that the given genetic mutation is complete recessive (i.e., (+/-) mothers and pups are essentially not different from (+/+)), use of the control females of (+/-) versus (-/-) subject females can be conducted for time/cost savings. In this case, produce (-/-) and (+/-) littermate females by crossing a (-/-) male with (+/-) females.

8. 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 [40]. 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 data 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 [40].

9. The wild type and mutants on C57BL/6J background are weaned at four weeks of age in our laboratory, as well as in the Jackson Laboratory (see "Breeding strategies for maintaining colonies of laboratory mice", The Jackson Laboratory Resource Manual, 2009). This is because at three weeks of age, C57BL/6J pups appears still small.

10. The pregnant mouse mothers deliver pups most typically during the dark phase of 20 day after copulation, some variations (typically a half or one day delay) may occur [41] (see also "Biology of the laboratory mouse", The Jackson Laboratory, Adapted for the Web at http://www.informatics.jax.org/greenbook/index.shtml).

11. The method described here is according to the previous publications [42], with some simplifications to be compatible to other measures of maternal behavior assessments in mice [17]. See also [43-46,33].

12. This measure of sniffing latency is useful to show the possible insensitivity or retardation of reaction to the pup introduction in the experimental animals.

13. It should be noted that laboratory mice do not selectively care their own young, but also retrieve alien young, provided their age is comparable. This non-selective caring may be related

to the trait of communal nesting and nursing in feral Mus musculus species [50].

14. Because one-month of social isolation has been shown to be stressful and may increase aggressive behavior in male mice [55,38], they are group housed prior to the experiment, and they are not isolated for more than two weeks. The cage bedding should not be changed before the testing.

15. The pups with fur are not optimal stimuli for elicitation of retrieving response.

16. If one wishes to gain more information of other components of parental behavior than retrieval, the observer can simultaneously score the behavior of each mouse once every 15 seconds during the 30 min test for a total 120 observations, as either crouching, sniffing and/or licking, nest building, or other behaviors. Similar methods were described in [56].

17. In case that infanticide is expected as dominant response, there is a technique to minimize harming the stimulus pups [58,59]; in essence, instead of placing pups into the subject's home cage directly, first place a pup contained in a wire-mesh tube or a small metal tea strainer. If the subject mouse is observed to start biting the container aggressively 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.

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