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Maternal Behavior is Impaired in Female Mice Lacking Type 3 Adenylyl Cyclase

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Abstract

Although chemosensory signals generated by mouse pups may trigger maternal behavior of females, the mechanism for detection of these signals has not been fully defined. Since some odorant receptors are coupled to the type 3 adenylyl cyclase (AC3), we evaluated the role of AC3 for maternal behavior using $AC3^{-/-}$ female mice. Here, we report that maternal behavior is impaired in virgin and postpartum $AC3^{-/-}$ mice. Female $AC3^{-/-}$ mice failed the pup retrieval assay, did not construct well-defined nests, and did not exhibit maternal aggression. Furthermore, $AC3^{-/-}$ females could not detect odorants or pup urine in the odorant habituation test and were unable to detect pups by chemoreception. In contrast to wild-type mice, adenylyl cyclase activity in main olfactory epithelium (MOE) preparations from $AC3^{-/-}$ female mice was not stimulated by odorants or pheromones. Moreover, odorants and pheromones did not evoke electro-olfactogram (EOG) responses in the MOE of $AC3^{-/-}$ female mice. We hypothesize that the detection of chemical signals that trigger maternal behavior in female mice depends upon AC3 in the MOE.

Keywords

Maternal behavior; type 3 adenylyl cyclase; cAMP

INTRODUCTION

Maternal behavior is the pattern of care given by mothers to their offspring. It increases the probability that offspring will reach maturity and is essential for the survival of mammalian newborns (Numan, 1988). Since rodents are born deaf, blind, and immobile, maternal behaviors including nest building, gathering pups together in a nest, and keeping them warm are critical for survival. Interestingly, when exposed to newborns, virgin mice (Noirot, 1969b), virgin hamsters (Rowell, 1961), and virgin monkeys (Rowell et al., 1964) exhibit spontaneous maternal behaviors comparable to postpartum females. This indicates that there is a basic maternal responsiveness which is not dependent upon hormones or sex for its arousal (Rosenblatt, 1967).

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DICLOSURE/CONFLICTS OF INTEREST

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There is considerable evidence that maternal behavior in mice depends upon the detection of odorants and/or pheromones emanating from the pups (Noirot, 1969a). For example, female mice exhibit similar maternal behavior toward live and dead pups in the T-maze retrieval test, suggesting that body movements and vocalizations of the pups are not necessary to elicit retrieving behavior (Gandelman et al., 1970). Furthermore, in a modified Y-maze test, postpartum female mice retrieve young pups by chemosensory cues from the pups without the use of visual or auditory clues (Smotherman et al., 1974). Moreover, in the pup retrieval assay, female mice prefer fetuses treated with pup urine (Londei et al., 1989). The importance of chemoreception for maternal behaviors is also supported by studies showing that olfactory bulbectomy eliminates maternal behaviors (Gandelman et al., 1971; Zarrow et al., 1971; Gandelman et al., 1972). However, the underlying chemosensory mechanisms that mediate maternal behavior are not known.

Olfactory and pheromone signals in mammals are detected by sensory neurons at two locations: the MOE in the nasal cavity and the neuroepithelium of the vomeronasal organ (VNO). It was originally thought that volatile odorants are detected exclusively by the MOE, while pheromones are detected through the VNO. Accordingly, behaviors affected by pheromone signaling such as inter-male aggression, male sexual preference, puberty acceleration, maternal aggression, and pregnancy block were typically attributed to VNO control (Del Punta et al., 2002; Leypold et al., 2002; Stowers et al., 2002; Halpern and Martinez-Marcos, 2003; Norlin et al., 2003). However, recent evidence indicates that some pheromones may be detected through the MOE in mice (Mandiyan et al., 2005; Liberles and Buck, 2006; Wang et al., 2006).

Olfactory signal transduction in the MOE is mediated by second messenger cascades including the cGMP (Juilfs et al., 1997) as well as the cAMP signal transduction pathways (Ache and Zhainazarov, 1995; Ronnett and Payne, 1995; Restrepo et al., 1996) and is initiated by interactions of odorants with receptors encoded by a multigene family (Buck and Axel, 1991; Chess et al., 1992). The discovery of a unique G protein, G_{olf} (Jones and Reed, 1989), AC3 (Bakalyar and Reed, 1990), and cyclic nucleotide gated (CNG) cation channels (Nakamura and Gold, 1987), all enriched in the olfactory cilia suggests an important role for cAMP in olfactory signaling. Furthermore, male $AC3^{-/-}$ mice cannot detect a number of odorants and show no EOG responses to odorants in the MOE, identifying cAMP as one of the major second messengers for signaling in the MOE of male mice (Wong et al., 2000). AC3 and other components of the receptor/cAMP signaling pathway including G_{olf} and CNG are expressed in the MOE and not the VNO (Berghard et al., 1996).

To evaluate the role of AC3 and cAMP signaling for the detection of chemosensory signals mediating maternal behavior, we examined maternal behavior of female $AC3^{-/-}$ mice. Our data indicate that AC3 is required for maternal behavior and suggest that some chemosensory receptors contributing to maternal behavior may be coupled to AC3 in the MOE.

MATERIALS AND METHODS

Mice

AC3$^{+/+}$and AC3$^{-/-}$ female mice were bred from heterozygotes, and genotyped as previously reported (Wong et al., 2000). In the postpartum experiments AC3$^{-/-}$ females were bred to AC3 $^{+/+}$ males yielding litters which were 100% AC3$^{+/-}$. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Washington. Adult mice used in this study were 3 to 6 months old unless indicated, and all the pups used were 1 to 3 days old unless indicated. Mice were maintained on a 12 hr light/dark cycle and had access to food and water *ad libitum*. For olfactory behavioral tests, the animals were housed individually and handled daily for at least one week prior to experiments to minimize stress.

Maternal behaviors of virgin female mice

Virgin wild-type mice spontaneously exhibit full maternal behaviors when exposed to foster mouse pups (Noirot, 1969a), a behavior which is triggered by pup odorants and/or pheromones. Virgin female mice were individually housed and were not exposed to young pups prior to testing, except for their own littermates. Maternal behaviors were assayed as described previously (Thomas and Palmiter, 1997) and a cotton nestlet was provided as nest material for each animal. On the test day, three 3-day old foster pups born to an AC3$^{+/+}$ mother were placed into the 3 corners of the cage that did not contain the nest. Typical maternal behaviors of female mice toward pups includes pup retrieval, licking of pups, nest building, and crouching over the gathered pups in the nest in a “lactation-position” (Noirot, 1969b). Therefore, latency to retrieve the 1st, 2nd, and 3rd pups, duration of licking and crouching over pups, and duration of nest building behavior were observed during a 10 min period. Only pups brought into the nest completely were counted as retrieved. Full maternal behaviors were defined as retrieval of all the three pups into the nest and crouching over them during the 10 min test period. If an animal did not complete this behavior within 10 min the test was terminated, resulting in a latency of 600 s for any behaviors not yet observed. The latency to approach and explore the pups and the duration were also recorded during this period. All the pups were returned to their donor mothers after the 10-min test was completed. To test whether consecutive pup exposure improves maternal behavior of AC3$^{-/-}$ female mice, the pup retrieval test was performed daily on four consecutive days with AC3$^{-/-}$ virgin female mice and their AC3$^{+/+}$ virgin littermates. We examined nest building behavior by placing a piece of cotton in the cage of a female AC3$^{+/+}$ or AC3$^{-/-}$ mice and examining the nest, or lack thereof 24 hr later. The number of animals used was 10 AC3$^{+/+}$ and 10 AC3$^{-/-}$ mice.

Maternal behavior of postpartum female mice

Maternal behavior deficits caused by olfactory sensory deprivation can be modified by maternal experience in primiparous dams (Seegal and Denenberg, 1974). To test whether maternal experience, as well as the physiological changes accompanying pregnancy or parturition, improve maternal behavior of female AC3$^{-/-}$ mice, maternal behaviors were assayed daily in recently postpartum AC3$^{-/-}$ females four consecutive days after parturition as described previously with some modifications (Thomas and Palmiter, 1997; Jin et al.,

2005). All female mice were separated from males and individually housed once visibly pregnant, and monitored each morning for pup birth. The date of birth was considered postpartum day zero. The retrieval assay was performed in the female's home cage during a 10 min period. On the test day, pups were separated from their mother for 1 hr and kept warm. Pup retrieval was performed as described for virgin female mice. The mother was briefly removed from her home cage, and three of her own pups were placed in each corner of the cage except for the nest. The mother was then reintroduced into her cage facing the wall. The behavior of the female mice was recorded during a 10 min period as described for virgin females. Identical sessions were assayed daily for four consecutive days after parturition (P0 to P3). Since a majority of the $AC3^{+/-}$ pups born from $AC3^{-/-}$ dams were dead before postnatal day 2, pups born to $AC3^{+/+}$ dams of the same age were provided for fostering by $AC3^{-/-}$ dams. Retrieval assays were performed with these fostered pups. The fact that postpartum $AC3^{-/-}$ females did not have their own pups during the pup retrieval assay while the $AC3^{+/+}$ mice did should not seriously impact interpretation of the data because intrastrain cross-fostering has been reported to have little affect on maternal care (van der Veen et al., 2007). Although starting testing for maternal behavior on postpartum day zero may be stressful to the mothers, $AC3^{+/+}$ and $AC3^{-/-}$ mice were subjected to the same protocol. The number of mice assayed was 11 $AC3^{+/+}$ and 9 $AC3^{-/-}$ mice.

Maternal aggression test

Another indicator of mouse maternal behavior is maternal aggression, a behavior that protects young mice from infanticide (Wolff, 1985; Parmigiani, 1986; Paul, 1986). Female mice are usually not aggressive toward intruders. However, postpartum female mice display vigorous aggressive behaviors toward intruders, especially against intruders bearing novel scents compared to intruders soaked in either water or the resident females' urine (Lynds, 1976). This is taken as evidence that the chemosensory system of postpartum females plays an important role in triggering maternal aggression. The aggressive behaviors of postpartum female mice were assayed as described previously (Norlin et al., 2003). At postpartum day 0 (P0; day of birth of litter), litter size was culled to a maximum of six pups, with at least two pups of each gender to decrease variability in maternal aggression (Maestripieri, 1990). Aggressive behaviors were observed at P4, P6, P8, and P10 daily during a 10 min period by introduction of an unfamiliar sexually naïve group-housed $AC3^{+/+}$ male into the postpartum female's home cage. The females were tested on multiple days to maximize the chance that aggressive behaviors would be observed. The pups were removed from their cage three min before the male was introduced to avoid injury. Removal of the pups does not alter aggressive behavior of postpartum females (Svare et al., 1981). The latency to first attack, the number of aggressive bouts, and the duration of aggressive behavior were recorded during the 10 min period. Tail rattling, biting, chasing, and wrestling were considered aggressive behavior, and aggressive behavior separated by less than 5 s was considered as one bout. Since the majority of pups born from $AC3^{-/-}$ dams were dead before postnatal day two, pups born to $AC3^{+/+}$ dams (> 5 days old) were provided for fostering by $AC3^{-/-}$ dams once their own pups were found dead. For a given animal, data from the day that the animal showed peak aggression were used for group comparison and statistical analysis. The number of animals assayed was 11 $AC3^{+/+}$ and 9 $AC3^{-/-}$ mice.

Olfactory behavioral test

In this assay, a cotton swab laced with 50 μl of water was introduced into the home cage of a virgin female mouse (Trinh and Storm, 2003; Wang et al., 2006). The number of times the mouse sniffed the target was recorded during a 2 min period. This trial was repeated several times with one min intervals until the animal was no longer interested in the object. Once the animal habituated to the object, a cotton swab laced with 50 μl of odorant or urine was introduced. We tested pup urine because mouse fetuses laced with pup urine stimulate greater frequency of maternal behaviors than fetuses treated with water, indicating that mouse pup urine contains odorants or pheromones that elicit maternal behavior (Londei et al., 1989). When a female mouse detected the new odorant, she sniffed the cotton swab a greater number of times than she did when the swab only contained water. The test was assayed in the female's home cage. The data are presented as a ratio of the number of times the mouse sniffed an odorant-laced cotton swab compared with the number of times it sniffed a water-laced cotton swab on the initial exposure. This is used as an indication of the ability of the female to detect a specific odorant. Adult male and pup urines were collected into containers by holding the animals by the scruff of the neck. Pup urines were collected from male and female at age of 5 to 6 days. Urine was pooled and stored in aliquots at −80°C until use. All chemical odorants were diluted in water. The concentrations of citralva and 2-heptanone were 10 μM and 50 μM, respectively. The odorant habituation test was repeated three times on different days. The number of animals assayed was 10 $AC3^{+/+}$ and 10 $AC3^{-/-}$ mice.

The pup odorant/pheromone preference test was performed as described previously with some modifications (Mak et al., 2007). A test chamber constructed with removable dividers to allow for tertiary compartmentalization was used in this assay. Dividers consisted of two perforated panels separated by 0.5 inches and offset so when in place, physical and visual contact could not be established, only odors or volatile pheromones could be transferred between compartments. During habituation pre-training, a female mouse was placed in the central compartment of the test chamber and the sniffing duration of the animal on each side of the compartment was recorded during a 10 min period. The mouse could not see the contents of the two compartments adjacent to her. Identical sessions were repeated daily for four days to allow the animal to habituate to the test chamber. On the test day, three pups 5 to 6 days old were randomly placed on either side compartment with the other side empty. The pups were kept silent by injection of ketamine to eliminate the influence of ultrasounds produced by the pups. The female's sniffing of each side was recorded during the test time. A preference was defined by a statistically greater amount of time spent sniffing one side of the compartment versus the other. The number of mice assayed was 8 $AC3^{+/+}$ and 8 $AC3^{-/-}$ mice.

Adenylyl cyclase activity assay

MOE membranes from virgin female mice were collected, homogenized, and processed as described previously (Wang et al., 2006). Protein concentrations were determined by the BCA assay kit (Pierce, Rockford, IL) according to manufacturer's instructions. Adenylyl cyclase activity was measured in a buffer consisting of 1mM cyclic AMP, 10 mM phosphocreatine, 0.5 unit of creatine phosphokinase, 5 μM GTP, 5 mM $MgCl_2$, 0.2 mM

EDTA, 50 mM Tris/HCl (pH 7.5), with [α-^{32}P] ATP to 5×10^{6} cpm/reaction at 30°C for 15 min. The concentration of citralva and 2-heptanone, which was used as stimulus, was 100 μM. Adenylyl cyclase assays were carried out in triplicate in a final volume of 250 μl. The number of mice assayed was 4 AC3$^{+/+}$ and 4 AC3$^{-/-}$ mice.

EOG recording

EOG recording of the MOE of virgin female mice was performed as described previously (Wang et al., 2006). The MOE was dissected through the septum, and the EOG was recorded with an agar- and saline-filled glass microelectrode in contact with the apical surface of the MOE in the open circuit configuration. Odorant or pheromone solutions, which were diluted in 1× ringer buffer, were puffed onto the exposed MOE for 2 s followed by a stream of moisturized oxygen. Traces were captured and digitized using a Digidata 1200A (Molecular Devices, Union City, CA) connected to a PC computer, low pass filtered at 30 Hz, and sampled at 100 Hz. The concentration of 2-heptanone and citralva for EOG recording was 50 μM. The concentration of farnesene used for EOG recording was 500 μM. All odorant and pheromone were diluted in 1× ringer buffer. The number of animals assayed was 6 AC3$^{+/+}$ and 5 AC3$^{-/-}$ mice.

Statistical analysis

All data were presented as mean ± SEM, unless otherwise indicated. We analyzed the data by the Wilcoxon test for two-samples comparison and Friedman test for multiple-sample comparisons, with $p < 0.05$ considered as statistically significant. In all behavioral tests, the investigator was blind to the genotype of the animal.

RESULTS

Pup retrieval is impaired in virgin AC3$^{-/-}$ mice

To evaluate the role of AC3 for maternal behavior, we examined virgin female AC3$^{-/-}$ mice and their wild-type littermates in the pup retrieval test as described in Methods. Foster pups were used with AC3$^{+/+}$ and AC3$^{-/-}$ females. As expected, after approaching and investigating the foster pups, AC3$^{+/+}$ female mice collected all 3 foster pups to their nests within five min (Figure 1a). Once the pups were brought to the nest, AC3$^{+/+}$ female mice exhibited a number of additional maternal activities including licking of the pups, continued nest building and crouching over the pups. With the exception of one AC3$^{-/-}$ female, which retrieved one foster pup during the 10 minute test period, all of the other AC3$^{-/-}$ females failed to retrieve any pups within the testing period (Figure 1a). In addition, the total amount of time that female mice were engaged in maternal activities including anogenital licking, nursing or nest building during the 10 min test period was monitored (Supplementary Fig. 1a). Virgin AC3$^{+/+}$ females (n=10) spent significantly more time engaged in maternal activities than AC3$^{-/-}$ females (n=10), $p < 0.01$.

To test whether consecutive pup exposure improves maternal behavior of AC3$^{-/-}$ female mice, the pup retrieval test was performed daily on four consecutive days with AC3$^{-/-}$ virgin female mice and their AC3$^{+/+}$ virgin female littermates as described in Methods. Exposure of AC3$^{+/+}$ female mice to pups over four consecutive days significantly decreased

the time for retrieval of all three pups (Figure 1b). In contrast, none of the $AC3^{-/-}$ virgin female mice retrieved pups even after four days of testing (Figure 1b). We conclude that $AC3^{-/-}$ mice lack pup retrieval behavior and that even consecutive exposure to pups over several days does not stimulate pup retrieval behavior. We also examined nest building behavior by placing a piece of cotton in the cage of a female $AC3^{+/+}$ or $AC3^{-/-}$ mice and examining the nest 24 hr later. Although most of the $AC3^{+/+}$ female mice (8/10) constructed focused, circular nests within the first 24 hr (Figure 2a), only two out of 10 $AC3^{-/-}$ females made well-defined nests (Figure 2b).

Pup retrieval is impaired in postpartum female $AC3^{-/-}$ mice

Virgin female $AC3^{+/+}$ or $AC3^{-/-}$ mice were housed with $AC3^{+/+}$ males until pregnancy, after which time the females was housed individually. All of the $AC3^{+/+}$ virgin female mice housed with $AC3^{+/+}$ males (10 out of 10 females) and 9 out of 11 $AC3^{-/-}$ mice became pregnant, indicating that female $AC3^{-/-}$ mice are fertile. The pregnant female $AC3^{-/-}$ mice all delivered pups. The litter size and physical appearance of pups born to $AC3^{-/-}$ female at birth were indistinguishable from those of $AC3^{+/+}$ mice. In addition, all of the pups born to $AC3^{-/-}$ female mice were cleaned by the mother indicating that $AC3^{-/-}$ females exhibit normal placentophagis. Since pups born to $AC3^{-/-}$ female mice were scattered throughout the mother's cage (Figure 2a and b), we suspected that maternal behaviors of postpartum $AC3^{-/-}$ mice might also be impaired.

To directly examine the maternal behaviors of postpartum $AC3^{-/-}$ females, the pup retrieval behavior of postpartum $AC3^{+/+}$ and $AC3^{-/-}$ mice were assayed daily for four consecutive days. $AC3^{+/+}$ recently postpartum females retrieved all three pups much faster than virgin female $AC3^{+/+}$ mice (Figure 1a and 2c). Once the pups were retrieved into the nest, $AC3^{+/+}$ females licked the pups, crouched over the pups and continued nest building. In contrast, only one out of nine postpartum $AC3^{-/-}$ females retrieved all 3 pups into her nest on the first test day. However, this mouse did not retrieve the pups on the third and fourth test days. Postpartum $AC3^{-/-}$ female mice did not lick pups, crouch over the pups or engage in nest building (Figure 2b). Moreover, there was no improvement in pup retrieval time of $AC3^{-/-}$ females over four days of testing (Figure 2d). The total amount of time that postpartum female mice were engaged in maternal activities including anogenital licking, nursing or nest building during the 10 min test period was monitored (Supplementary Fig. 1b). Recently postpartum $AC3^{+/+}$ females (n=11) spent significantly more time engaged in maternal activities than recently postpartum $AC3^{-/-}$ females (n=8, $p < 0.01$). Although, mother-pup interactions may be affected by starting testing at postpartum day zero, both $AC3^{-/-}$ and $AC3^{+/+}$ mice were separated from pups on day zero, controlling for any stress associated with separation from pups. Collectively, these data indicates that pup retrieval is disrupted in both virgin and postpartum $AC3^{-/-}$ female mice.

Maternal aggression is impaired in recently postpartum female $AC3^{-/-}$ mice

To assay maternal aggression, recently postpartum females were individually housed with their pups. The pups were removed 3 min before the introduction of a sexually naïve $AC3^{+/+}$ male intruder into the home cage. The number of attacks, duration of aggression, and the percentage of aggressive animals were recorded during a 10 min period on postpartum days

4, 6, 8, and 10. Since pups born to $AC3^{-/-}$ mothers died because of impaired maternal care, postpartum $AC3^{-/-}$ females were provided with foster pups. The use of foster pups with postpartum $AC3^{-/-}$ mice is appropriate because wild-type mice exhibit maternal aggression with their own pups or foster pups in their home cage (Ostermeyer and Elwood, 1983). All $AC3^{+/+}$ postpartum female mice showed intense aggressive behaviors towards the intruder on at least one trial during the 4 trials (Figure 3). Postpartum female $AC3^{-/-}$ mice displayed little or no maternal aggression. The percentage of $AC3^{-/-}$ postpartum females displaying aggression, the average number of attacks against the intruder, and the total time spent attacking the intruder were significantly lower than $AC3^{+/+}$ postpartum females (Figure 3). We conclude that AC3 $^{-/-}$ postpartum female mice do not display maternal aggression, possibly because of defects in their chemosensory mechanisms.

Detection of mouse urine and odorants is impaired in female $AC3^{-/-}$ mice

All of the virgin female $AC3^{+/+}$ mice and none of the virgin $AC3^{-/-}$ females detected citralva, 2-heptanone, male mouse urine, or pup urine during the odorant habituation test (Figure 4a). This indicates that $AC3^{-/-}$ mice are unable to detect chemical signals present in adult male or pup urine, odorants or mouse pheromones.

Because maternal behaviors may depend upon the ability of the female mouse to detect chemical signals or combinations thereof emanating from mouse pups, we examined the ability of $AC3^{-/-}$ mice to detect pups solely on the basis of chemoreception with no visual or auditory cues as described in Methods. During habituation when the adjacent compartments were empty, $AC3^{-/-}$ and $AC3^{+/+}$ mice both sniffed the two sides equally, indicating no preference for either side (Figure 4b). During testing, three anesthesized pups were placed in one adjacent compartment; the other side was empty. As anticipated, $AC3^{+/+}$ female mice spent more time sniffing the compartment containing pups than the opposite empty side (Figure 4c). However, $AC3^{-/-}$ female mice showed no preference for the compartment containing pups (Figure 4c). Similar results were obtained when dead pups were substituted for anesthesized pups (data not shown). These data confirm that $AC3^{+/+}$ female mice can detect pups by chemoreception independent of visual or auditory cues while $AC3^{-/-}$ females cannot detect these signals.

Odorants do not stimulate adenylyl cyclase activity in MOE preparations from female $AC3^{-/-}$ mice

The MOE expresses several adenylyl cyclases including the type 2, 3 and 4 adenylyl cyclases (Wong et al., 2000) and adenylyl cyclase activity in mouse MOE preparations is stimulated by odorants (Pace et al., 1985; Wang et al., 2006). To determine if biochemical signaling in the MOE of virgin $AC3^{-/-}$ females is impaired, we examined odorant stimulation of adenylyl cyclase activity in MOE preparations from female $AC3^{+/+}$ and $AC3^{-/-}$ mice. The adenylyl cyclase activity of MOE preparations from virgin $AC3^{+/+}$ female mice but not $AC3^{-/-}$ mice was significantly stimulated by citralva and 2-heptanone (Figure 5). These data are consistent with the observation that $AC3^{-/-}$ females cannot detect odorants and mouse urine.

EOG response to pheromones and odorants is impaired in female AC3$^{-/-}$ mice

We monitored the EOG responses in the MOE of virgin AC3−/− and AC3+/+ mice to two pheromones, 2-hepatone and farnesene, as well as the odorant citralva (Figure 6). Farnesene, 2-heptanone, and citralva evoked strong EOG responses in the MOE of AC3$^{+/+}$ female mice (6.9 ± 1.1, 5.3 ± 0.2, and 4.3 ± 0.2 mV, respectively). In contrast, the EOG responses evoked by these agents in the MOE from AC3$^{-/-}$ female mice was more than 20 fold lower than wild-type mice (0.3 ± 0.1, 0.2 ± 0.1, and 0.2 ± 0.1 mV, respectively). These data are consistent with the hypothesis that AC3$^{-/-}$ female mice do not exhibit maternal behaviors because they cannot detect chemical signals that elicit these behaviors.

DISCUSSION

Common maternal behaviors exhibited by female mice include pup retrieval, licking of pups, nest building, crouching over grouped pups in a well-defined nest and maternal aggression (Noirot, 1969b). Pup retrieval is not modified by deafness, and visual cues seem to play little or no role in pup retrieval (Herrenkohl and Rosenberg, 1972; Herrenkohl and Sachs, 1972). However, accumulating evidence indicates that chemosensory cues from pups trigger female maternal behaviors (Fleming and Rosenblatt, 1974; Fleming et al., 1992). However, very little is know about the chemosensory mechanisms that mediate maternal behavior or the role of cAMP signaling and specific adenylyl cyclases in the detection of signals that evoke maternal behaviors. To evaluate the role of cAMP signaling in maternal behavior, we examined maternal behavior of female AC3$^{-/-}$ mice.

We discovered that postpartum and virgin AC3$^{-/-}$ females fail to exhibit several maternal behaviors including pup retrieval, licking of pups, crouching over pups in a nest, maternal aggression and nest building. Nest building is evolutionary conserved throughout the animal kingdom and is an important indicator of maternal care. In rodents, the nest insulates the altricial young and keeps the pups warm in the mother's absence (Numann et al., 1991; Deacon, 2006). Nest building is impaired in female mice with olfactory bulb removal (Zarrow et al., 1971), indicating that nest building may depend on chemosensory mechanisms, a hypothesis that is supported by the fact that anosmic AC3$^{-/-}$ female mice showed impaired nest building.

Naïve virgin female rats do not spontaneously exhibit maternal responsiveness to young pups. However, they show maternal behaviors comparable to postpartum female rats following repeated exposure to newborns for several days (Rosenblatt, 1967). This induction of maternal behavior is not dependent upon hormones or sex, because ovariectomized and hypophysectomized virgin females also exhibit maternal behaviors after repeated exposure to young pups. It has also been reported that the latency to express full maternal behavior by female mice is reduced following two consecutive days of the pup retrieval test (Noirot, 1969a; Larsen et al., 2008). Nevertheless, exposure of AC3$^{-/-}$ females to pups repeatedly over a period of days did not induce pup retrieval behavior. These defects are most likely attributable to chemosensory defects since AC3$^{-/-}$ females were unable to detect pups, pup urine, urine from adult male mice, pheromones or odorants. In contrast to wild-type mice, adenylyl cyclase activity in MOE preparations from AC3$^{-/-}$ females was not stimulated by pheromones or odorants and EOG responses to odorant and pheromones were also non-

existent. These data support the hypothesis that chemical signals from mouse pups trigger maternal behavior by activating receptors coupled to AC3 in the MOE of female mice.

Maternal behavior can be affected by behavior of the mother and/or the pups used in the assay. This study only addressed the contribution of the female $AC3^{-/-}$ mice to maternal behavior and not the $AC3^{-/-}$ pups since foster pups were used for both virgin $AC3^{+/+}$ and $AC3^{-/-}$ female mice in the pup retrieval assay. In contrast, pups born to postpartum $AC3^{+/+}$ females were used in the pup retrieval assay while $AC3^{-/-}$ females were tested with their own pups on day one followed by foster pups in subsequent days. However, the use of foster pups should not seriously compromise the pup retrieval assays because intrastrain cross-fostering has been reported to have minimal effects on maternal behavior (van der Veen et al., 2007). For example, heterozygote pups born to $AC3^{-/-}$ females survive when they are cross-fostered to $AC3^{+/+}$ female mice (data not shown).

Because AC3 and other components of the receptor/cAMP signaling pathway including G_{olf} and cyclic nucleotide gated ion channels (CNG) are expressed in the MOE and not the VNO (Berghard et al., 1996), our data support the general hypothesis that the MOE of mice is important for the detection of chemical signals that initiate maternal behavior. Since the MOE can detect both odorants and pheromones (Mandiyan et al., 2005; Liberles and Buck, 2006; Wang et al., 2006), we cannot readily define the nature of the chemical signals that trigger pup retrieval. Although we cannot rule out a role for the VNO in maternal behavior, it is interesting that disruption of genes specifically in the VNO, including V1r cluster (Del Punta et al., 2002), and $G\alpha i_2$ (Norlin et al., 2003) does not compromise pup retrieval behavior. On the other hand, transgenic mice lacking TrpC2, a channel specifically expressed in the VNO, exhibit deficits in nest building and time on the nest suggesting that the VNO does play a role in these maternal behaviors (Hasen and Gammie, 2009; Kimchi and Dulac. 2007).

In contrast to pup retrieval behavior of female mice, maternal aggression depends on the function of the VNO and is disrupted by removal of the VNO (Bean and Wysocki, 1989). Furthermore, disruption of genes only expressed in the VNO such as TrpC2 (Leypold et al., 2002; Hasen and Gammie, 2009), Gαi2 (Norlin et al., 2003), and V1r cluster (Del Punta et al., 2002) blocks maternal aggression of postpartum females. Since $AC3^{-/-}$ females also lack maternal aggression and AC3 is expressed in the MOE and not the VNO, this suggests that maternal aggression also depends on signaling through the MOE. We conclude that chemosensory signaling through AC3 in the MOE is necessary but not sufficient for eliciting maternal aggression and that maternal aggression may depend upon the MOE and VNO.

AC3 is not specific to the olfactory epithelium and according to the Allen Brain Atlas is expressed in several areas of brain including the hippocampus, cortex, amygdala, medial preoptic nucleus and suprachiasmatic nucleus. Since the $AC3^{-/-}$ mouse strain used in this study was a global knockout, we cannot conclude with certainty that the defects in maternal behavior shown by the $AC3^{-/-}$ are due solely to the absence of the enzyme in the olfactory epithelium. For example, the olfactory and vomeronasal chemosensory inputs are integrated in the medial amygdala, the bed nucleus of the stria terminalis, and hypothalamus (Keller et al., 2009; Kang, et al 2009; Martel and Baum, 2009; Baum and Kelliher, 2009), raising the

possibility that the deficits of maternal behaviors exhibited by $AC3^{-/-}$ mice might be caused by amygdala or other integrative areas of brain. Nevertheless, the EOG data illustrating defective signaling through the MOE, the loss of odorant-stimulated adenylyl cyclase activity in the MOE, and the behavioral data showing that $AC3^{-/-}$ females cannot detect pups by chemosensory signals support the hypothesis that disruption of signaling in the MOE caused by ablation of AC3 contributes to the defects in maternal behavior.

In conclusion, the maternal behavior of mice depends upon AC3 activity. These data support the hypothesis that chemical signals emanating from pups activate receptors in the MOE coupled to AC3 through Golf and are consistent with data showing that $Golf^{-/-}$ mice also fail to exhibit maternal behavior (Belluscio et al, 1998). This suggests that cAMP generated by AC3 is a major second messenger used in the detection of chemical signals that trigger maternal behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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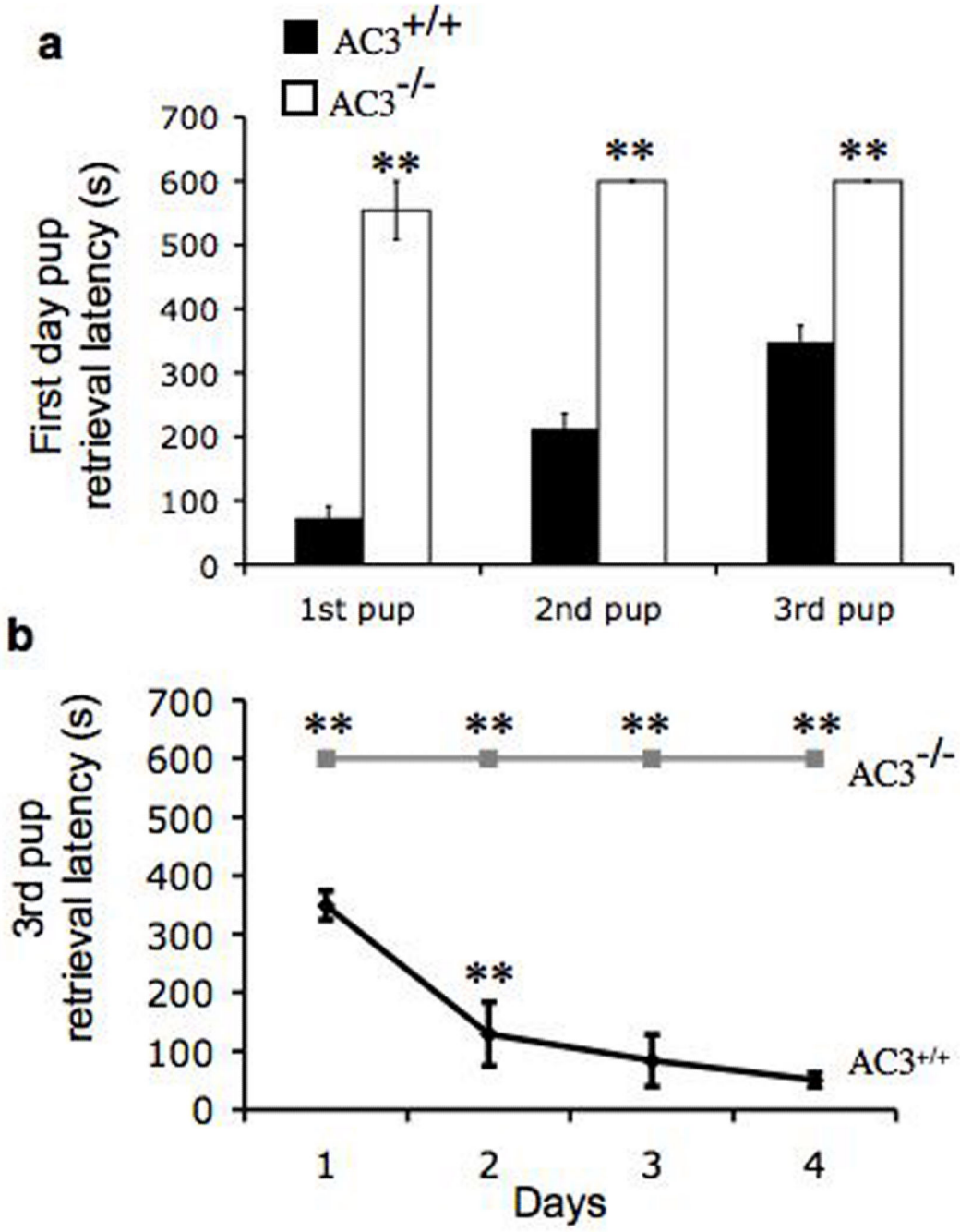

Figure 1.

Pup retrieval is impaired in virgin $AC3^{-/-}$ mice.

(a) The retrieval latencies of three pups on the first test day of virgin female $AC3^{+/+}$ and $AC3^{-/-}$ mice are shown. There were significant differences between $AC3^{+/+}$ (n=10) and $AC3^{-/-}$ (n=10) mice in the retrieval latency of the first, second, and third pups ($p < 0.001$ for each pair). Data are represented as means ± SEM. (b) The third pup retrieval latency for virgin female $AC3^{+/+}$ and $AC3^{-/-}$ mice on four consecutive days is reported. The retrieval latency for the third pup for each day ($p < 0.001$ for each pair) was significantly different

between $AC3^{+/+}$ (n=10) and $AC3^{-/-}$ females (n=10). The retrieval latency of $AC3^{+/+}$ mice, but not $AC3^{-/-}$ mice decreased with repeated tests. Data are represented as means ± SEM. **, $p < 0.001$. If an animal did not complete this behavior within 10 min the test was terminated, resulting in a latency of 600 s for any behaviors not yet observed.

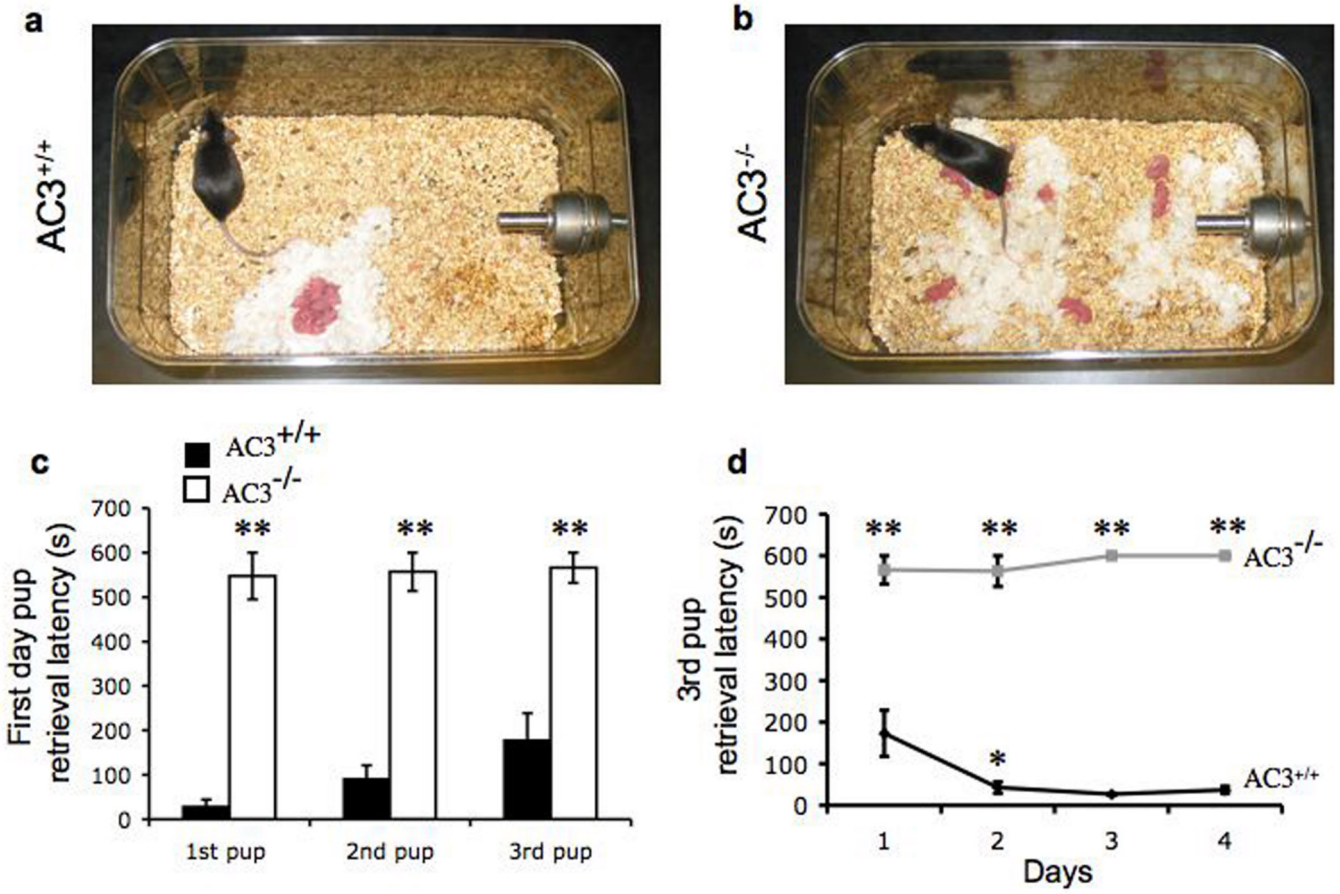

Figure 2.

Nest building and pup retrieval are impaired in recently postpartum $AC3^{-/-}$ females. (a and b) Representative nests and pup distribution for $AC3^{+/+}$ and $AC3^{-/-}$ dams. Pictures were photographed ~ 6 hr following parturition. (c) The pup retrieval latencies of the first day following partition (P0) in $AC3^{+/+}$ and $AC3^{-/-}$ dams are reported. There were significant differences between $AC3^{+/+}$ (n=11) and $AC3^{-/-}$ (n=9) mice in the retrieval latency for the first, second, and third pups ($p < 0.001$ for each pairs). Data are represented as means ± SEM. (d) The retrieval latency of the third pup for $AC3^{+/+}$ and $AC3^{-/-}$ dams on the four consecutive days following partition are shown. There were significant differences between $AC3^{+/+}$ (n=11) and $AC3^{-/-}$ (n=9) dams for the retrieval latency of the third pup on each day ($p < 0.001$ for each pairs). Data are represented as means ± SEM. **, $p < 0.001$. If an animal did not complete this behavior within 10 min the test was terminated, resulting in a latency of 600 s for any behaviors not yet observed.

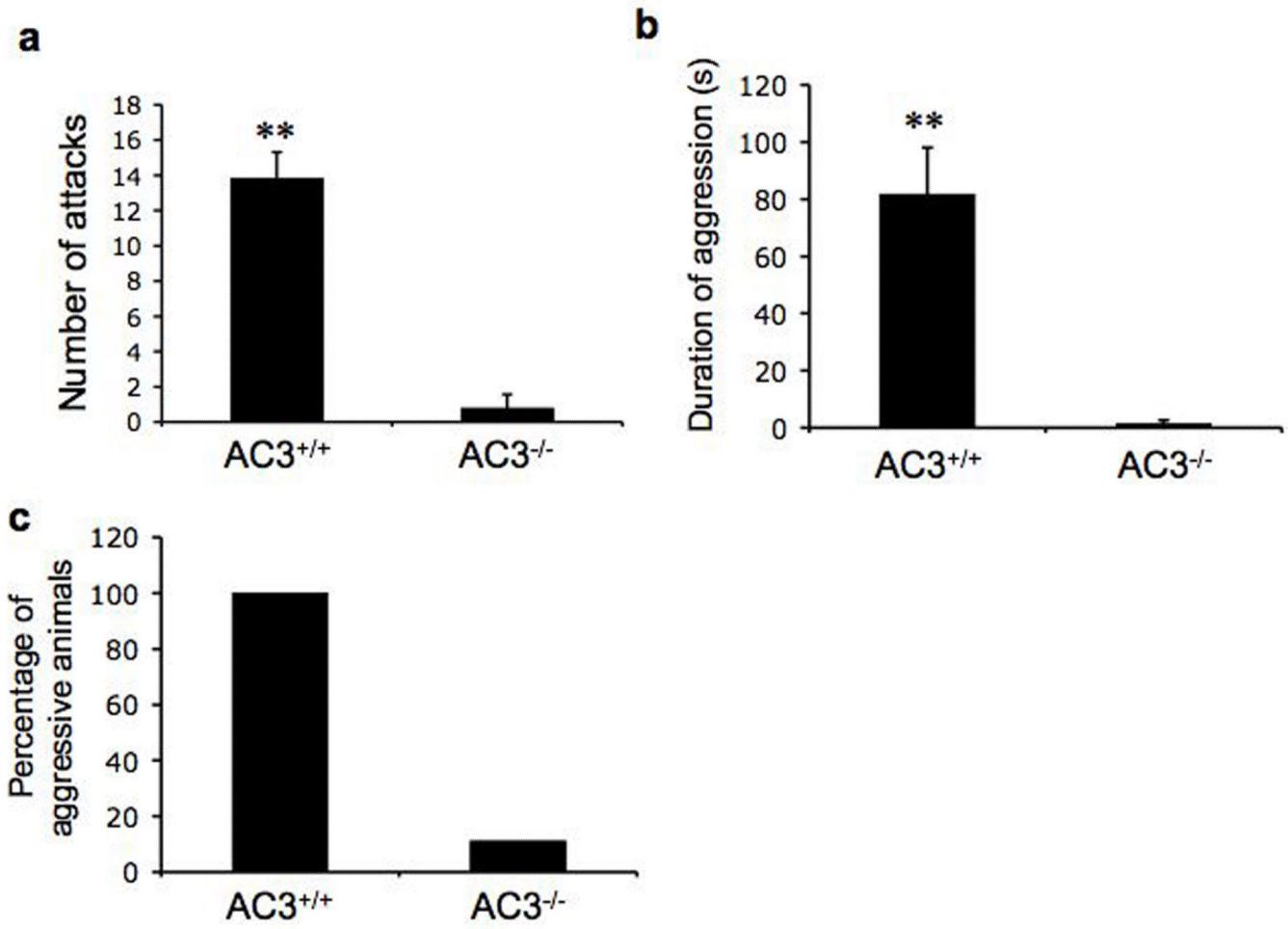

Figure 3.

Maternal aggressive behaviors are impaired in recently postpartum $AC3^{-/-}$ mice. Maternal aggressive behaviors were assayed by introducing an unfamiliar male into the home cage of each female mouse during a 10-min period. (a) There were significant differences between postpartum $AC3^{+/+}$ (n=11) and postpartum $AC3^{-/-}$ (n=9) dams in number of attacks ($p< 0.001$), (b) duration of aggression ($p< 0.001$) and (c) the percentage of dams that displayed aggressive behavior. Data are represented as means ± SEM. **, $p < 0.001$.

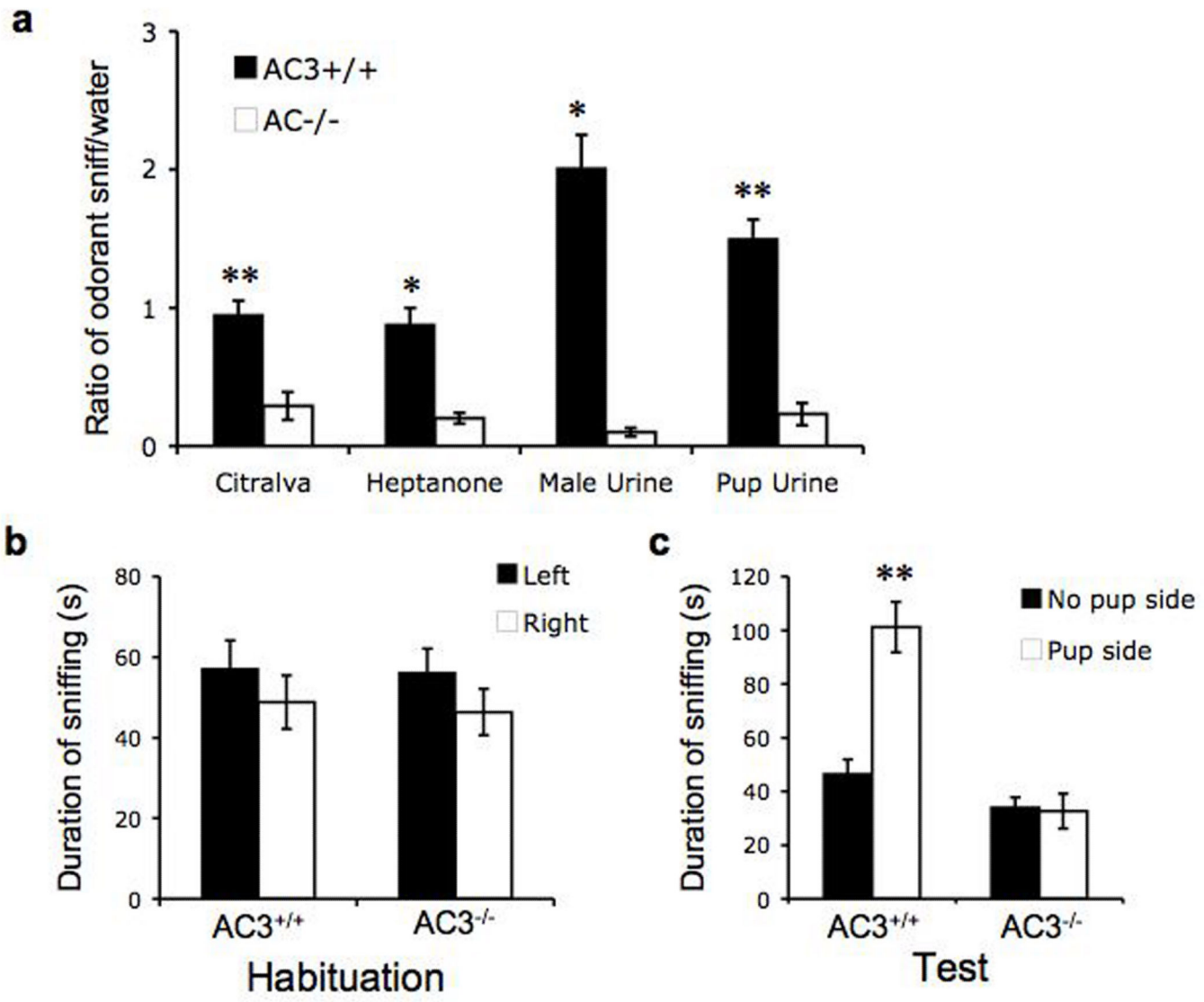

Figure 4.

Virgin AC3$^{-/-}$ female mice fail to detect odorants and pup urine. (a) Odorant habituation data for AC3$^{+/+}$ and AC3$^{-/-}$ virgin female AC3$^{-/-}$ mice are reported. Cotton swabs were laced with 50 µl of citralva (10 µM), 2-heptanone (50 µM), male urine (20-fold diluted), or pup urine (20-fold diluted). There were significant differences in the ability of AC3$^{+/+}$ (n=10) and AC3$^{-/-}$ (n=10) mice to detect citralva ($p < 0.001$), 2-heptanone ($p < 0.01$), male urine ($p < 0.01$), and pup urine ($p < 0.001$). Data are represented as means ± SEM. (b and c) Virgin female AC3$^{+/+}$ mice but not virgin AC3$^{-/-}$ females detected anesthesized pups. Non-visual pup detection was assayed as described in Materials and Methods using anesthesized pups. (b) During context habituation, AC3$^{+/+}$ (n=8) and AC3$^{-/-}$ (n=8) female mice sniffed each side chamber equally. However, during testing (c) AC3$^{+/+}$ female mice, but not AC3$^{-/-}$ females, showed a strong preference for the side chamber containing the anesthesized pup ($p < 0.001$). Data are presented as means ± SEM. *, $p < 0.01$; **, $p < 0.001$.

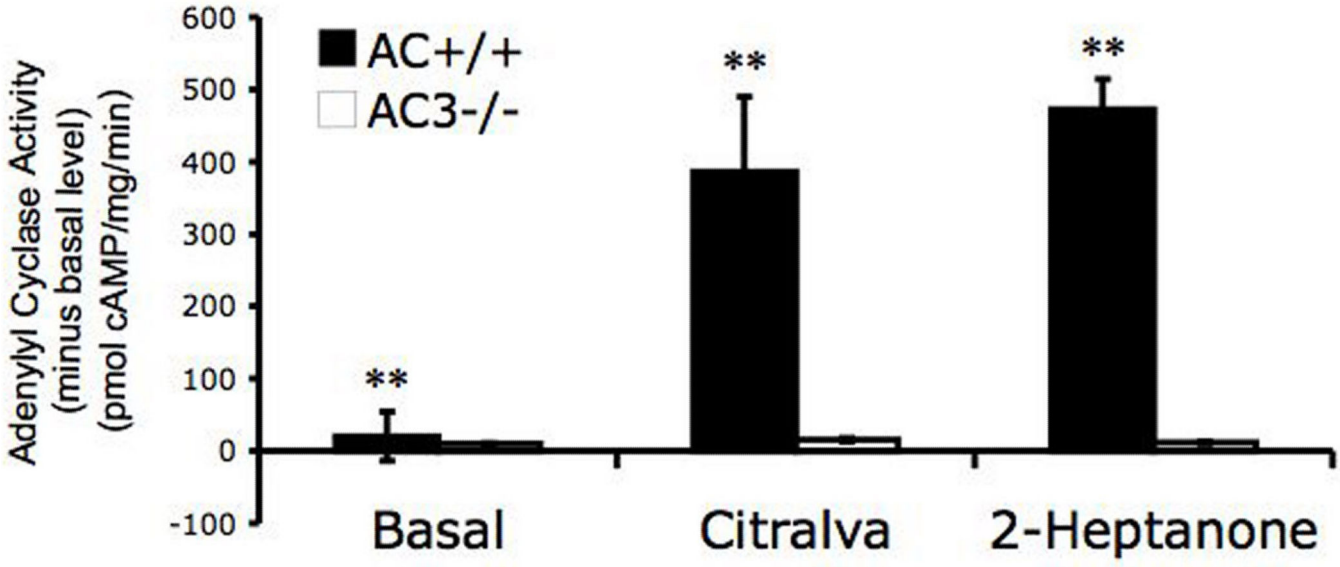

Figure 5.

Adenylyl cyclase activity in the MOE of female $AC3^{+/+}$, but not $AC3^{-/-}$ mice is stimulated by odorants and pheromones. Citralva (100 μM, $p < 0.001$) and 100 μM 2-heptanone ($p < 0.001$) stimulated adenylyl cyclase activity in membranes from virgin $AC3^{+/+}$ mice (n=4) but not virgin $AC3^{-/-}$ mice (n=4). Adenylyl cyclase activity in membrane preparations from the MOE was assayed in the absence or presence of citralva or 2-heptone as described in Methods. Data are represented as means ± SEM. **, $p < 0.001$.

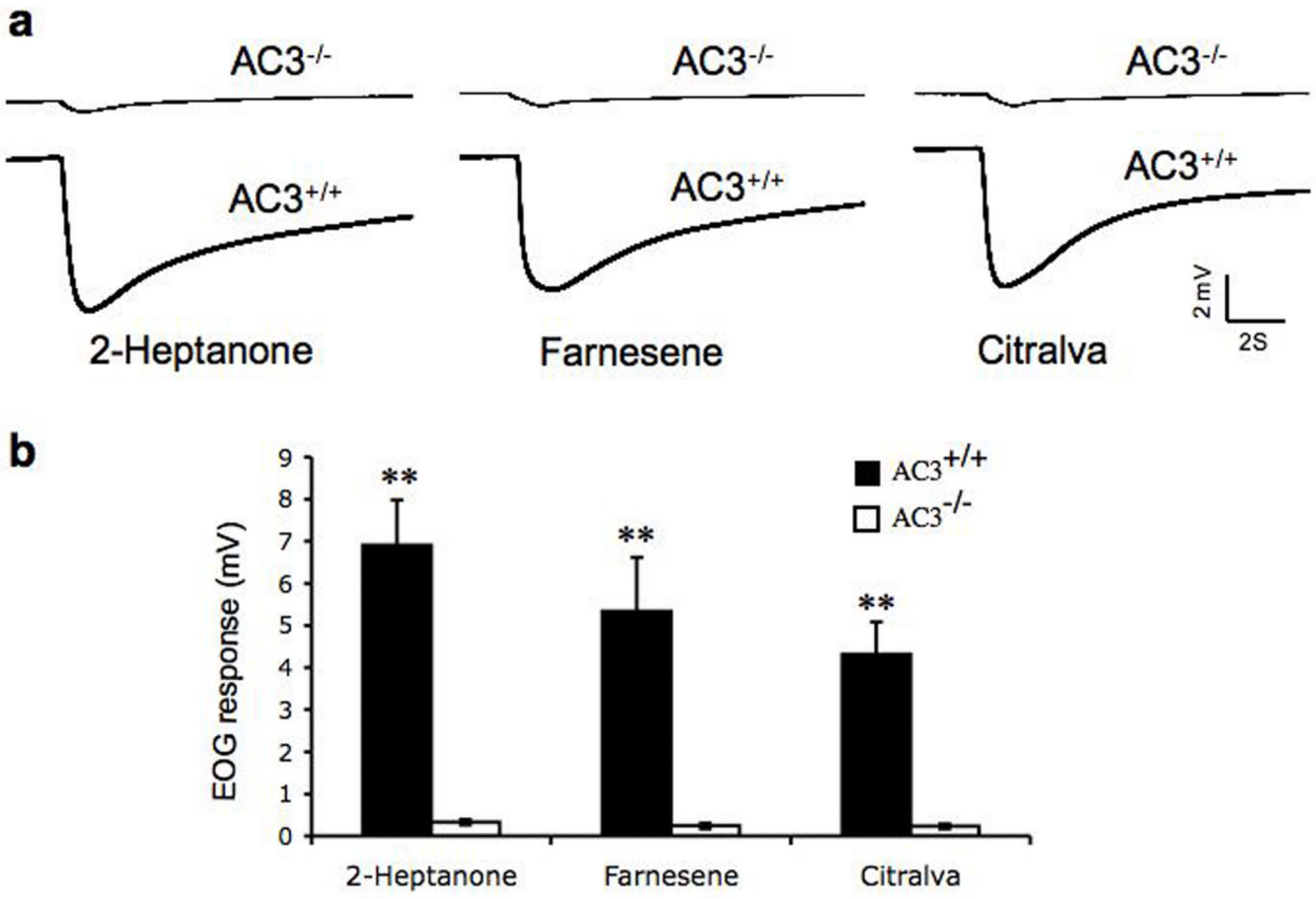

Figure 6.

EOG responses evoked by odorant and pheromone are ablated in the MOE of $AC3^{-/-}$ females. (a) Representative EOG responses evoked by 2-heptanone (50 μM), farnesene (500 μM), and citralva (50 μM) respectively, from the MOE of virgin $AC3^{+/+}$ and $AC3^{-/-}$ females are shown. All agents were diluted in 1× ringer buffer. (b) Summary of the mean EOG amplitudes in response to odorants and pheromones. Significantly greater EOG responses to all agents were exhibited in $AC3^{+/+}$ (n=6) compared to $AC3^{-/-}$ (n=5) females (all agents $p < 0.001$). Data are presented as mean ± SEM. **, $p < 0.001$.