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Maternal Care Can Rapidly Induce an Odor-Guided Huddling Preference in Rat Pups

ABSTRACT: *Olfactory-guided huddling is learned and expressed by postnatal day (PND) 15, when rat pups huddle preferentially with conspecifics or with targets bearing an odor previously associated with maternal care. Experiment 1 replicated this induction of an odor-guided huddling preference with a truncated regime of conditioning with a scented foster dam. Pups exposed to an odor in association with foster maternal care during five daily 2-hr sessions on PNDs 1–5, 5–9, or 10–14, but not pups merely exposed to the odor, displayed a huddling preference for the conditioned odor, but only when conditioning commenced after PND5. Experiment 2 demonstrated that a single, 2-hr exposure to a scented foster dam can induce a huddling preference in pups. Analysis of maternal behavior during the 2-hr conditioning sessions on PND14 revealed that frequency of maternal hovering over pups, but not licking/grooming or duration of contact, was associated with induction of the odor preference. © 2008 Wiley Periodicals, Inc. Dev Psychobiol 51: 95–105, 2009.*

Keywords: attachment; odor preference; early experience; huddling; mother–infant relations; maternal odor; olfactory learning; rat pup

INTRODUCTION

Affiliative behavior can be described and quantified in terms of preferential proximity to individuals that display particular cues. In Norway rats (*Rattus norvegicus*), social affiliation to conspecifics is evidenced by huddling behavior (Barnett, 1963). Initially, huddling among infant rats is guided and maintained most powerfully by thermal stimuli (Alberts, 1978a,b; Alberts & Brunjes, 1978; Sokoloff & Blumberg, 2001; Sokoloff, Blumberg, & Adams, 2000). By postnatal day (PND) 15, however, pups utilize olfactory cues to huddle preferentially with conspecifics rather than with other animate or inanimate sources of heat (Alberts & Brunjes, 1978; Brunjes & Alberts, 1979). When rats show preferential contact with conspecifics, the behavior is called “filial huddling,” in

contrast to heat-guided, or “physiological huddling” seen earlier in the postnatal period (Alberts & Brunjes, 1978).

The rat pups’ preference for conspecific odors appears learned. A pup’s filial preference can be assigned to an arbitrary odor by associating that odor with maternal care, suggesting that pups acquire their filial huddling to conspecifics through their experiences with the mother in the nest during the first two postnatal weeks (Alberts & May, 1984; Brunjes & Alberts, 1979). During the first two postnatal weeks or so, the infants’ motoric immaturity confines them to the nest and the mother initiates all contact with the pups (Rosenblatt & Lehrman, 1963). When the mother is present, the immature pups actively orient to her and seek contact. Maternal contact provides the pups with pairings of maternal odor with other maternal stimuli such as warmth, cutaneous contact, perioral stimulation from suckling, receipt of milk, as well as the mechanical stimulation from licking and handling. In the parlance of classical conditioning, components of maternal care may serve as an unconditioned stimulus (UCS) for pups to learn maternal odor as a conditioned stimulus (CS).

Sullivan and her associates proposed a general “mammalian imprinting model” that features a sensitive

Received 22 June 2008; Accepted 16 September 2008

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Contract grant sponsor: NIH

Contract grant number: MH-28355

Published online 21 October 2008 in Wiley InterScience
(www.interscience.wiley.com). DOI 10.1002/dev.20349

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period (PNDs 1–9) during which infant rats form attachments, with the mother's behavior serving as the source of reward (Moriceau & Sullivan, 2005). Specifically, stroking with a brush was used to mimic maternal licking and grooming. Rat pups, up to 8 days of age, that received such stroking in the presence of an olfactory stimulus acquired a preference for the odor CS based on the association of odor (CS) + stroking (UCS) (Roth & Sullivan, 2006; Sullivan, Brake, Hofer, & Williams, 1986; Sullivan & Hall, 1988; Sullivan, Hofer, & Brake, 1986; Sullivan & Wilson, 1994; Woo & Leon, 1987). After around PND10, pairing odor and stroking becomes ineffective for conditioning (Moriceau & Sullivan, 2005; Sullivan and Wilson, 1994; Woo & Leon, 1987), although other forms of associative learning remain present or emerge (e.g., Sullivan, Landers, Yeaman, & Wilson, 2000). The empirical basis for this proposed sensitive period is the diminished conditioning with tactile stimulation (i.e., stroking) that were previously effective (Moriceau & Sullivan, 2005).

According to this imprinting model, the young rat, while still in the nest, is prepared to learn about odors and to use olfactory cues for crucial behaviors such as approaching the mother and attaching to a nipple. The literature on early olfactory learning provides additional examples in which different tasks and tasks that depend on different cues can be learned at various early ages (e.g., Martin & Alberts, 1979; Pedersen & Blass, 1982; Pedersen, Williams, & Blass, 1982; Stickrod, Kimble, & Smotherman, 1982). Alberts and May (1984) examined separately various components of the maternal stimuli experienced by rat pups during the acquisition of filial huddling preferences. They concluded that suckling stimulation and milk reward did not contribute to huddling preferences, but that thermotactile stimuli were sufficient. Interestingly, their studies did not test the efficacy of stroking.

Although it is well established that rat pups acquire filial preferences during the first two postnatal weeks (cf., Alberts, 2007), there remain many questions about this learning, such as the specific elements of maternal behavior that induce odor-guided huddling preferences. The present study was designed to enable and then to make an initial, detailed examination of the maternal induction of odor-guided huddling preferences. To accomplish this, we sought to define a brief but effective induction regime to make it practical to monitor mother–pup interactions during the induction process.

We know of no efforts to discover if odor-guided huddling preferences can be acquired during portions of the first two postnatal weeks and, if so, whether there are periods of differential sensitivity for such learning. The primary purpose of the present study was to refine our knowledge about the genesis of odor-guided huddling;

some of the results, in addition, pertain to testing the generality of the mammalian imprinting model (Moriceau & Sullivan, 2005). Because odor learning is prevalent in many aspects of a rat's early life, it is instructive to evaluate the applicability of general rules relevant to such experience-based processes.

The forthcoming experiments applied two general frameworks, viz., filial imprinting and classical conditioning. As used here, the two frameworks were not mutually exclusive. They are applicable and useful, because each framework provides some parameters and constructs that help to structure and interpret the experiments. Specifically, we sought to test the ubiquity of a proposed sensitive period for olfactory learning: Does it apply to huddling preferences? By viewing maternal induction of an odor preference within a Pavlovian framework, we could ask about the features of rat maternal behavior that might function as the unconditioned stimulus (UCS) to alter the value of a previously neutral odor, that is, the conditioned stimulus (CS).

EXPERIMENT 1: A TRUNCATED PROCEDURE FOR MATERNAL INDUCTION OF ODOR-GUIDED HUDDLING

Experiment 1 was designed to examine the formation of odor-guided huddling preferences following a truncated (5-day-long) regime of conditional experiences of an odor paired with foster maternal care. We adopted the temporal hypothesis of a sensitive period ending by PND9 (Moriceau & Sullivan, 2005), and used rat pups at different early ages to test the generality of the age-related formation of attachments.

A conditioning procedure was used in which rat pups received 2 hr of maternal care (UCS) from a lactating foster mother. Paired with these bouts of maternal care was an “arbitrary” odor, either lemon or orange (CS), which was applied before each conditioning session to the ventral fur of the dam. Pups received a series of five, daily pairings, referred to as maternal conditioning.

To test our hypothesis, we applied a maternal conditioning regime to each of three age-ranges of early development, PND1–5, PND5–9, and PND10–14. If successful, learning in these situations would represent a significant truncation of preference induction. The mammalian imprinting model, in its purest form, would predict the acquisition of odor-guided huddling preferences in one or both of the younger groups (PND1–5, PND5–9) but not in the PND10–14 pups. We tested subjects on PND15, because pups do not reliably express an odor-guided, filial huddling preference until PND15 (Alberts & Brunjes, 1978; Brunjes & Alberts, 1979).

Methods

Subjects. A total of 96 rat pups were subjects. In addition, 24 lactating foster dams were used. All animals were derived from Sprague-Dawley stock originally purchased from Taconic Farms (Germantown, NY) and bred in Indiana University's Animal Behavior Laboratory colony. Litters were born and reared in standard maternity tubs (48 cm × 20 cm × 26 cm) with food and water available ad libitum through the stainless steel cage lid. The vivarium was maintained on 12:12 hr light/dark cycle (lights on at 0700 hr) at $22.0 \pm 2^\circ\text{C}$. Litters were reduced to eight pups (four males and four females) on PND1 (day of birth = PND0). On PND1, a needle-punch (30 gauge) implement (Ketchum Manufacturing Inc., Ottawa, Canada) was used to tattoo the ventral aspect of the paw(s) for individual identification. Animal care and experimentation were conducted in accordance with the guidelines of the Indiana University Institutional Animal Care and Use Committee, the NRC Guide for the Care and Use of Laboratory Animal (National Academy of Science, 1996), and the International Society for Developmental Psychobiology.

Procedures. Two treatment groups were established: (1) Maternal Odor conditioning (MO) in which pups were housed with a lactating foster dam bearing an artificial odor, and (2) Odor-Only conditioning (OO) in which pups were exposed to an artificial odor on a schedule matched to that of the MO group, thus controlling for familiarity with an assigned scent. Regimens of 2-hr conditioning were initiated either on PND1, 5, or 10 and conducted daily for 5 days, creating a total of 6 groups (2 treatments × 3 developmental periods: MO1–5, 5–9, 10–14 and OO1–5, 5–9, 10–14). Each conditioning group was composed of eight littermate pairs from different litters (one male and one female, $n = 16/\text{group}$). Conditioned odors (lemon and orange extracts: McCormick & Company, Inc., Sparks, MD) were counterbalanced across litters. All odor conditioning was conducted during the light phase. The conditioning and test arenas were sanitized in a cage washer after each session.

One hour prior to each daily conditioning session, litters were removed from their respective home cages and placed as a group in a standard mouse cage (30 cm × 13 cm × 19 cm) with fresh bedding. After this 1-hr separation from the mother, pups were moved to a conditioning arena for their assigned treatment. On PND15, subject pups in all treatment groups received a huddling preference test (see below).

Maternal conditioning (MO group). A foster dam, matched by postpartum age to the subject pups' dam, was removed from its respective home cage 1 hr prior to conditioning and placed in the conditioning arena, which

was a standard maternity cage (48 cm × 20 cm × 26 cm) with fresh bedding and water. Prior to the 2-hr conditioning session, 400 μl of test odor (either lemon or orange) was applied with a pipette the ventral midline of the dam. The odor application was repeated for each conditioning session. Immediately prior to a conditioning session, pups were voided, individually weighed, and placed with the foster dam for 2 hr. After each conditioning session, pups were re-weighed and returned to their home cage.

Odor-only conditioning (OO group). A cotton gauze pad (5 cm × 5 cm) scented with 400 μl of the test odor (either lemon or orange) was attached to the wall of a container, which was a plastic cylinder (15 cm diameter; 20 cm high) with fresh bedding on the floor, for the 2 hr conditioning period. The odor application was made for each session. Immediately prior to a conditioning session, pups were voided and individually weighed. After the pups were voided and weighed, they were placed in the container at $22.5 \pm 1^\circ\text{C}$ for 2 hr. The pups were re-weighed after each conditioning session and returned to their home cage.

Stimulus control. Selection of the olfactants used here was based on pilot studies demonstrating that PND15 pups maintained contact with a swatch of acrylic fur scented with either the orange or lemon extract presented in a test arena (see Huddling Preference Test), even when the odor was unfamiliar to them, and that PND15 pups responded equivalently to both odorants when presented simultaneously on separate huddling targets in the arena (Kojima and Alberts, unpublished work). We also made efforts to control quantity of odorant applied to an animal's fur or to the stimulus swatch. With these controls and pilot data, we decided that it was unnecessary to test naïve pups or to treat a foster mother with vehicle alone as control in the present study. The present experiments were designed so that odorants were counterbalanced across trials; orange and lemon were used equal numbers of times as CS and as novel odor.

Huddling preference test. A test arena, similar to that used in previous studies (see Alberts & May, 1984), was a plastic cylinder (15 cm diameter, 12.5 cm high) with two rectangular windows 180° apart at the base of the container. Through the window, a swatch of acrylic fur wrapped around a plastic pipe (2 cm diameter, 10 cm long) was accessible to the pups, held in place by springs from the outside of the test chamber. Just prior to the test, 200 μl of a conditioned odor (e.g., lemon) was sprayed on the fur in one side, and 200 μl of a novel odor (e.g., orange) was applied on the fur of the other side. Tests were conducted at room temperature ($22.0 \pm 2^\circ\text{C}$) for 4 hr after 25 min-acclimation period, during which pups generally sample

the test odors by sniffing. A video camera (DXC-151A, SONY) was positioned above the test arena and connected to a time-lapse recorder (TLC1800, GYYR) to capture behaviors for later analysis (12:1 record:playback ratio was used).

Data Analysis. Huddling was defined as physical contact lasting at least 30 s between the pup's body (tail excepted) and the scented fur. This operational definition was intended to exclude briefer instances of physical contact, for example, sniffing investigation. Odor preference was measured as time (min) spent in contact with the fur target bearing the odor CS – time spent in contact with the fur target bearing the novel odor. Huddling was scored by experienced evaluators using custom programs written in HyperCard for Macintosh. Interrater and intrarater reliabilities were assessed using Pearson's correlation coefficient on randomly chosen segments of behavioral data. All evaluators were trained until interrater and intrarater reliabilities reach $r_s \geq .90$ and $.95$, respectively.

Huddling preference was analyzed using the Wilcoxon signed rank test to accommodate the two related variables (time with the conditioned odor vs. time with the novel odor for each subject). Kruskal–Wallis test was conducted to examine effects of timing and type of conditioning on contact time with the test odor. Body weight change was analyzed by using two-way ANOVA in a 3×2 design (type of conditioning: PND1–5, PND5–9, and PND10–14 \times type of conditioning: MO and OO).

Results

Total Huddling Time. Pups spent most (55%) of the 4-hr test session huddling, thus providing a good sample of their contact behavior and preferences. Median total times spent huddling by pups in the PND1–5 group was 131.7 and 116.7 min for the MO and OO treatments, respectively. In the PND5–9 group, total time spent huddling was 166.6 min by MO pups and 136.1 min by OO littermates. In the PND10–14 group, the MO pups' total huddling time was 108.1 min and the OO cohort spent 122.1 min huddling with the targets. Statistically, total huddling time was unaffected by the type (MO vs. OO) or the timing (PNDs 1–5, 5–9, or 10–14) of conditioning experience ($\chi^2(1, N=96) = .72, p = .395$, and $\chi^2(2, N=96) = 3.27, p = .195$, respectively).

Huddling Preferences. Figure 1 displayed the huddling preferences. MO pups, after spending 2 hr/day with a scented, lactating foster dam on PNDs 10–14 or on PNDs 5–9, but not on PNDs 1–5, preferred the scent associated with the foster dam versus a novel odor. All OO groups lacked a huddling preference for a familiar odor versus a novel scent on PND15. There were no significant differ-

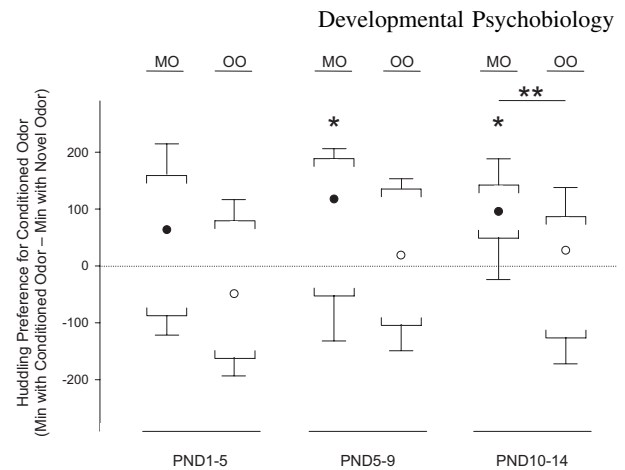


FIGURE 1 Huddling preference for conditioned odors during the 4-hr-long test. Data points show median differences within box plots of contact time between the conditioned (MO (filled circle) or OO (open circle)) and the novel odor, with quartiles depicted by the brackets. Scores significantly above or below 0 (no difference) in preference or aversion, respectively. Lack of difference in contact time between test odors indicates no odor bias. Asterisk (*) indicates that a huddling preference was expressed at $p < .05$. Double asterisk (**) indicates a statistically significant difference between the huddling preferences of the MO and OO pups ($p < .05$).

ences among the three MO groups or among the three OO groups ($\chi^2(2, N=48) = .77, p = .681$ and $\chi^2(2, N=48) = 1.70, p = .428$, respectively).

More specifically, pups in the MO1–5 group showed no preference for the conditioned odor (+62.9 min, $z = -1.34, p = .179$) in the PND15 test. Their littermate controls, receiving odor-only conditioning (OO1–5), also lacked a preference (–49.5 min, $z = -1.09, p = .278$). The leftmost panel of Figure 1 illustrates the equivalence in the contact preference for the youngest groups.

The central pair of data points in Figure 1 shows that MO5–9 experience produced a significant preference (+117.5 min) for the odor associated with a lactating foster dam ($z = -2.02, p = .044$). No such bias was displayed by littermates with the same amount of odor experience (OO5–9: +18.2 min, $z = -.57, p = .569$).

In the rightmost pair of medians in Figure 1 (filled circle), it can be seen that MO10–14 pups huddled 95.3 min more with the mother-paired odor than with the novel alternative ($z = -3.26, p = .001$). The open circle in the same pair in Figure 1 shows the +26.8 min score for the OO10–14 group. Huddling preferences exhibited by MO pups and the OO controls differed significantly ($\chi^2(1, N=32) = 6.28, p = .012$). Littermates in the OO10–14 group did not demonstrate a preference for the test odor relative to the novel stimulus ($z = -.36, p = .717$).

Table 1. Total Body Weight Change Measured in Grams (\pm SEM) During Five 2-hr-Long Exposure Sessions

Conditioning	Conditioning Period		
	PND1–5	PND5–9	PND10–14
MO	–.69 (.33)	–1.31 (.42)	–1.76 (.35)
OO	–.19 (.18)	–.46 (.16)	–.95 (.18)

Body Weight. Average body weight on the test day (PND15) ranged from 34.7 to 37.9 g across treatment groups with no significant group differences. Table 1 summarizes the average changes in body weight during the five conditioning sessions of the exposure for each of the MO groups and for the OO controls. All groups lost weight during the 2-hr sessions and there were expected, age-related differences in weight loss ($F(2, 90) = 80.27$, $p < .001$). Pups in the MO groups lost weight more than those in the OO groups (Means: MO = -1.26 g and OO = $-.90$ g, $F(1, 90) = 149.83$, $p < .001$). An interaction between the timing (PND5–7, 5–9, and 10–14) and the type (MO and OO) was significant ($F(2, 90) = 3.62$, $p = .031$). The interaction was due mainly to the smaller difference between the MO and OO groups in the PND1–5 group compared to those in the PND5–9 and PND10–14 groups, as evidenced by a fact that the interaction disappeared in the analysis in which only PND5–9 and 10–14 were included ($F(1, 60) = .03$, $p = .869$).

Discussion

The results of Experiment 1 demonstrate that five 2-hr-long experiences with a scented foster dam are sufficient to induce an odor-guided huddling preference for an odor borne by the foster caregiver. Despite the lack of thermal incentive and the use of more a stringent criterion for encoding contact behavior than that used in previous studies (Alberts & May, 1984), rat pups huddled with the inanimate, unheated, scented target stimuli for more than half of the 4-hr-long test session. Levels of this nonthermal, olfactory-guided huddling were robust.

Induction of Huddling Preference After PND10. In contrast to the hypothesized sensitive period for olfactory learning, pups in the MO10–14 group displayed a huddling preference for the conditioned odor, whereas pups in the MO1–5 group did not (Fig. 1). The preference of the MO groups cannot be attributed to the effects of familiarity, because littermates in the OO groups that had the same amount of exposure to the test odor did not demonstrate a preference for it relative to the novel stimulus. Thus, the developmental parameters for acquiring a huddling preference for maternally paired

odor do not conform to those of the proposed sensitive period for infant attachment (Moriceau & Sullivan, 2005). In Experiment 1 testing was conducted at a single, common age (PND15). Because training age varied and test age did not, the experimental design imposed different retention intervals for each age. Thus, it is possible that pups in the younger group learned a preference but did not retain it. Nevertheless, the results of Experiment 1 indicate that rat pups aged 10-day old and older are capable of forming a preference for maternal odor.

Maternal Stimuli Significant for the Establishment of Odor-Guided Huddling. In addition to testing the generality of the hypothesized sensitive period for infant attachment, the present experiment addressed the maternal stimuli that might function as a UCS for the pups' early learning. Changes in body weight during the conditioning sessions (Tab. 1) revealed modest, but consistent weight loss. Thus, although pups were often attached to the foster dam's nipples at the end of the conditioning sessions, there was no evidence of milk transfer. This was not surprising, because maternal conditioning in this study involved only two pups, and nipple stimulation from four or more pups is usually needed to induce milk letdown (Mena & Grosvenor, 1968). Inspection of Table 1 shows greater weight loss in pups housed with the foster dam than in the Odor Only pups. This difference is likely due to receipt of maternal licking and the consequent voiding via the pups' micturition reflex (e.g., Gubernick & Alberts, 1983). In agreement with previous conclusions, milk transfer is not a necessary rewarding stimulus for the acquisition of huddling preference for a maternally paired odor (Alberts & May, 1984).

Thermotactile stimulation (conductive warmth from body-to-body contact) has been considered as the key for the establishment of odor-guided huddling (Alberts, 2007; Alberts & May, 1984). One implication is that the reward value of such warmth might vary with the pups' thermoregulatory capabilities, leading to a prediction that the youngest groups would show the strongest learning, followed by diminution in conditioning as thermoregulation improves (e.g., Hill, 1992). The present results do not support this prediction.

It is important to keep in mind that during early postnatal development, maternal stimuli impinge on the offspring as they are undergoing rapid changes in size, morphology, as well as sensory and physiological function. The same maternal stimulus can produce different experiences in the pups, depending on the pups' stage of development. In addition, memory and learning undergo profound changes during early life (Campbell & Spear, 1972; Rudy, Vogt, & Hyson, 1984; Spear & McKinzie, 1994). These developmental processes must also be considered in the interpretation of experience-based mechanisms, such as those examined here.

EXPERIMENT 2: SINGLE-SESSION INDUCTION OF AN ODOR-GUIDED HUDDLING PREFERENCE

The results of Experiment 1 provided evidence that odor-guided huddling preferences can be established with fewer and shorter exposures than used previously. Specifically, the five 2-hr-long conditioning sessions comprised an induction regime much briefer than the 2-week-long regimes used previously for odor-guided, filial huddling (Alberts & May, 1984; Brunjes & Alberts, 1979). The rapidity of such learning seemed worthy of further examination. There are practical benefits to methods for rapid induction of odor preferences. Regimes for rapidly creating preferences would make possible well-controlled manipulations and observation during the conditioning period.

Experiment 2 was conducted to evaluate the efficacy of a single, 2-hr-long maternal conditioning session for the induction of odor-guided huddling. Single-session induction was attempted with rat pups at different postnatal ages, either Day 8, 10, 12, or 14. We took advantage of the discrete and manageable duration of maternal conditioning by video recording each conditioning session of the PND14 pups, so that the behavior of the foster mother could be analyzed with the goal of gaining insight into the amount or kinds of interactions that contribute to the induction of a huddling preference for maternally paired odor. We chose PND14 to observe mother–pup interaction during the odor conditioning, because Experiment 1 demonstrated a strong huddling preference by MO pups in the PND10–14 group relative to OO littermates and because odor-guided huddling is found to reliably appear by PND15 (Alberts & Brunjes, 1978; Brunjes & Alberts, 1979).

Methods

Subjects. The animals were from the same pool as used earlier; breeding and husbandry were as described in Experiment 1. A total of 78 Sprague-Dawley rat pups from 39 litters and 39 foster dams were used to examine huddling preference by a single, 2-hr-long conditioning. Additionally, 26 pups from 13 litters and 13 foster dams were used to collect extra data for guaranteeing valid behavioral samples of maternal behaviors during conditioning on PND14 for analysis. Each litter contributed one male and one female to the study.

Procedure. The basic procedures used in Experiment 1 were implemented here, except only Maternal Odor conditioning (MO) was used. In addition, MO conditioning was conducted on one day only; testing was the next day. Pups received one, 2-hr-long session of MO

conditioning on either PND8, 10, 12, or 14. The standard huddling preference test was run the day after conditioning (i.e., PND9, 11, 13, or 15, for the four treatment ages, respectively). Testing was with the standard, 4-hr-long, two-choice huddling preference test described in Experiment 1. Videorecords were analyzed as in Experiment 1.

Coding maternal behavior during conditioning sessions. Maternal care was observed during the MO conditioning on PND14, using time-lapse videography at 12:1 record:playback. A video camera (DXC-151A, SONY) was placed above the cage and connected to a video recorder (TLC1800, GYYR) to capture maternal behaviors throughout the conditioning. The following behaviors received by each pup were scored:

- (1) *Physical contact* comprised any contact between a pup and dam that lasted 30 s or more.
- (2) *Maternal licking/grooming* included all bouts (10 s or more) of licking and grooming directed to any part of a pup's body. We applied this criterion to distinguish licking/grooming from nasal contact with pups such as sniffing.
- (3) *Hovering* was scored when the mother's body completely covered the pups' bodies (tails excepted) for 1 min or more. Hovering could and often did occur while the dam engaged in another activity, such as licking/grooming. The immobile, crouching posture, defined by Stern and Johnson (1990), also comprised some portion of the time spent hovering.

Physical contact and licking/grooming were scored by experienced evaluators using custom programs written in HyperCard for Macintosh. The videorecorder's time-stamp was used to measure hovering.

Data Analysis. Huddling preference was analyzed to examine effects of maternal conditioning on huddling preference as described in Experiment 1. To examine the relation of maternal stimulation to the acquisition of an odor-guided huddling preference, we calculated the duration, frequency, and average bout durations of each of the maternal behavior parameters.

Due to nonnormality in huddling preference scores, Spearman rank correlations were used to evaluate associations between the huddling preference exhibited by individual pups on PND15 and the specific parameters of maternal care that each pup received during conditioning with a scented foster dam on PND14. To extend the analysis, the pups' huddling preferences were grouped by a YES–NO dichotomy, in which the YES group included pups that spent at least 50% of their total time huddling with the target bearing maternal odor, whereas the NO group included the others (<50% of their total time

huddling with the target bearing maternal odor). A *t*-test was used to compare means between the YES and NO groups to assess whether pups received different amounts of maternal behavior.

In addition, stepwise discriminant analysis was conducted to identify which of the maternal behavior parameters were significant for discriminating between the YES and NO groups. Stepwise discriminant analysis enabled us to assess the relative importance of the maternal behavior parameters in classifying the huddling preference. Wilk's lambda was used as criteria for variable selection, with probabilities of *F* set at .05 for entry and .10 for exclusion. Body weight change was analyzed using *t*-test and one-way ANOVA (age: PND8, 10, 12, and 14) to examine whether pups lost weight during the single session of maternal conditioning and whether weight changes were age-dependent.

Results

Total Huddling Time. Pups huddled with both of the furred targets during the test. The medians for total time spent huddling were 159.5, 134.9, 135.0, and 137.7 min by pups in PND8, 10, 12, and 14 groups, respectively. Total huddling time was unaffected by age group ($\chi^2(3, N = 78) = 1.45, p = .695$) or by sex ($\chi^2(1, N = 78) = .12, p = .730$).

Huddling Preferences. Huddling preferences after 2-hr MO conditioning is shown in Figure 2. Median huddling

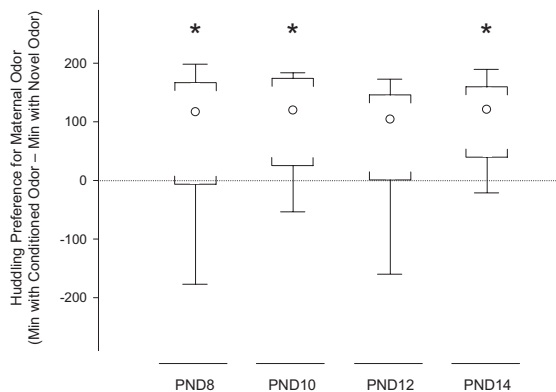


FIGURE 2 Huddling preference following a single, 2-hr-long maternal conditioning session on PND8, 10, 12, or 14. Data points show median differences within box plots of contact time between the conditioned and the novel odor, with the brackets depicting the quartiles. Scores significantly above or below 0 (no difference) reflect group differences in preference or aversion, respectively. Lack of difference in contact time between test odors indicates no odor bias. Asterisk (*) indicates preference at $p < .05$.

preference scores were +117.7, +120.1, +116.4, and +137.2 min for the PND8, 10, 12, and 14 groups, respectively, and were unaffected by age ($\chi^2(3, N = 78) = 1.59, p = .663$). Pups that received a single, 2-hr-long session of MO conditioning on PND8, 10, or 14 preferred the scent associated with the foster dam versus a novel odor ($z = -1.98, p = .048$; $z = -3.33, p = .001$; and $z = -3.45, p = .001$, respectively). Although pups in the PND12 group expressed a trend toward maternal odor, the preference did not reach statistical significance ($z = -1.90, p = .057$).

Correlations Between Maternal Behaviors and Huddling Preference. Of the 46 pups in the two-choice preference test on PND15, 32 showed a positive bias for huddling with the maternal odor target (>50% of the contact time with MO) and met the criteria for the YES group; 14 pups were categorized in the NO group. Correlations between each of the maternal behavior parameters and individual preferences were uniformly nonsignificant.

Table 2 shows mean duration, mean frequency, and mean bout duration of physical contact, licking/grooming, and hovering during the MO conditioning on PND14. Hovering frequency was only the factor different between the two groups. None of the other parameters of physical contact or licking/grooming differed between pups that had no huddling preference and those who expressed huddling preference for maternal odor.

Table 2. Mean (\pm SEM) Duration (min), Frequency, and Bout Length (min) of Physical Contact, Hovering, and Licking/Grooming Received by 14-Day-Old Pups During Maternal Conditioning

Maternal Behavior	Huddling Preference	
	Yes	No
Physical contact		
Duration	99.6 (1.85)	98.9 (3.2)
Frequency	12.6 (.95)	14.0 (1.28)
Bout	9.2 (.68)	8.4 (1.27)
Hovering		
Duration	26.1 (3.2)	16.7 (3.54)
Frequency*	4.6 (.41)	2.9 (.43)
Bout	5.4 (.44)	6.1 (1.22)
Licking/grooming		
Duration	8.8 (.88)	11.9 (4.89)
Frequency	12.5 (1.11)	11.6 (1.09)
Bout	.7 (.05)	.99 (.37)

The data are presented for pups that expressed a huddling preference for maternal odor (YES) and that did not express the preference (NO) in the huddling preference test on PND15. Asterisk (*) indicates a significant difference between YES and NO groups at $p < .05$.

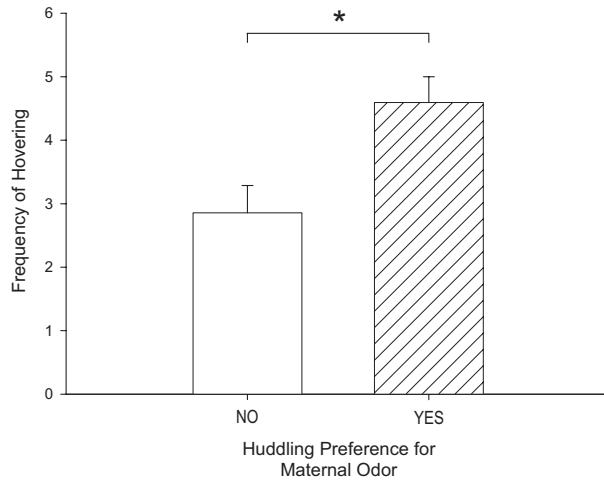


FIGURE 3 Frequency of hovering received by pups during maternal conditioning on PND14. Asterisk (*) indicates a significant difference at $p < .05$ between pups that spent at least 50% of total huddling time with maternal odor (YES) and those that spent equal to less than 50% of total contact time with the maternal odor (NO).

Pups that displayed an odor-guided huddling preference had been with a foster dam that *frequently hovered* over and fully covered the bodies of the foster pups. In contrast, pups that did not display a huddling preference for a maternally paired odor had been with a dam that *hovered less frequently* (Fig. 3: $t(44) = -2.56, p = .014$). Consistent with this difference in hovering, stepwise discriminant analysis also identified frequency of hovering as the only maternal behavioral parameter important for huddling preference (Wilk's $\lambda = .87, df = 1, p = .014$). Interestingly, total time engaged in hovering did not differ between pups that formed preferences and those that did not ($t(44) = -1.74, p = .088$).

Body Weight. Average body weight (\pm SEM) on the test day was $20.7 \pm .54$ g for pups tested on PND9, $25.6 \pm .56$ g for the PND11 group, $31.4 \pm .74$ g on PND13, and $35.5 \pm .77$ g for pups tested on PND15. Measures of changes in body weight during the single conditioning sessions of pups in MO groups for each conditioning day revealed that pups lost weight during the 2-hr sessions. Average body weight changes (g) were $-.23$ ($t(19) = -5.87, p < .001$), $-.26$ ($t(17) = -5.70, p < .001$), $-.37$ ($t(19) = -8.87, p < .001$), and $-.33$ ($t(19) = -3.46, p = .003$) on PNDs 8, 10, 12, and 14, respectively. However, age-related differences were not identified ($F(3, 74) = 1.18, p = .324$).

Discussion

A major finding of the present experiment is that odor-guided huddling can be induced with a single, 2-hr-long

session of maternal conditioning on PND8, 10, or 14. As in Experiment 1, pups 10-days old or older acquired an odor preference for huddling serving social affiliation (Alberts & Brunjes, 1978), which contradicts a hypothesized sensitive period with an upper boundary at PND9 (cf., Moriceau & Sullivan, 2005).

Among the maternal activities that were coded and quantified, hovering frequency was the only maternal behavior parameter that correlated with an acquired huddling preference. Pups that expressed the huddling preference for a maternally paired odor received more frequent hovering contact than did those that showed no preference (Fig. 3). Moreover, stepwise discriminant analysis identified frequency of hovering as a factor contributing to successful prediction of whether pups form a huddling preference.

The results of the correlations and stepwise discriminant analysis are new and intriguing. Mere amount (duration) of physical contact with the dam did not correlate with formation of the odor preference, but frequency of hovering did. Hovering was operationally defined to include only maternal postures and positions that completely occluded the pup beneath her body. Thus, we believe that this behavioral category involves continuous thermotactile exchange between the dam's ventrum and the pup's body. There are numerous other opportunities for tactile and thermal exchanges between the dam and pups, and among the pups themselves. The maternal conditioning procedure used here was unnaturally limited (e.g., only two pups for the 2-hr session), but it had the virtue of being tractable for analysis.

Total time spent hovering and average bout duration were not significant in relation to the pups' huddling preference for maternal odor. We suspect that our strict operational definition may have eliminated episodes of contact that should be recognized as important for the induction of an olfactory preference. In the present experiment, hovering was coded when the pup's whole body (except tail) was hidden by the mother's body. Mother often hovered over but not did completely cover a pup, particularly when two pups were present during conditioning. Two 14-day-old pups are relatively massive (about 70 g) and may not be fully hidden by the brooding dam. With strict adherence to our operational definition for hovering, we often excluded periods when a small part of pup's body, such as top of head and hip with hind legs, was not covered by mother's body. Given that the insignificance of hovering duration was marginal ($p = .088$), therefore, it is possible that our operational definition of hovering led us to overlook instances in which a pup received significant thermotactile stimulation.

Because of its reliable formation of odor-guided huddling as shown in Experiment 1 and previous studies

(Alberts & Brunjes, 1978; Brunjes & Alberts, 1979), we focused on events during maternal conditioning on only PND14, so videorecords of conditioning sessions on other days were not available. Although the resolution of the videos was adequate for reliably discerning the completeness of body coverage when the dams hovered over pups, it was not sufficient to support more detailed judgments, for example, body parts exposed or anogenital licking. The importance of a more detailed examination of the stimuli and the parameters that induce odor preferences in rat pups warrants further investigations.

Because pups often attach to a nipple while the dam hovers above them, it is reasonable to speculate that milk transfer and the perioral sensations of suckling also contribute to the induction of odor preference (Brake, 1981; Johanson & Hall, 1982; Johanson & Teicher, 1980). As shown in Experiment 1, however, pups lost weight during MO conditioning. Thus, it is likely that no milk transfer occurred due to the small number (2) of pups (Mena & Grosvenor, 1968). In accordance with Experiment 1, Experiment 2 supports that milk transfer is not necessary to induce odor-guided huddling, as previously suggested (Alberts & May, 1984). Because we did not directly observe nipple attachments, it remains unknown whether such perioral sensations might be associated with the formation of huddling preference for maternal odor. We doubt the importance of such perioral stimuli for odor learning by 14-day-old pups, noting that inanimate sources of warmth (i.e., a warm tube) induce huddling preference and that suckling was eliminated as a contributor in other studies (Alberts & May, 1984; Kojima & Alberts, 2006).

The results of Experiment 2 also indicated that licking/grooming during MO conditioning had no apparent relation with the formation of an odor preference in the PND14 rat. As can be seen in Table 2, pups received 9–12 min of licking or grooming during the 2-hr conditioning session. This amount and kind of tactile stimulation were not essential for odor preferences, though it is possible that our measures were not sensitive to differences that were present.

In other settings, an experimenter's stroking induced an odor preference in pups 8 days of age and younger, but this form of stimulation becomes ineffective after around PND10 (Moriceau & Sullivan, 2005; Sullivan & Wilson, 1994; Woo & Leon, 1987). It is possible that licking/grooming contributes to the induction of huddling preference in pups younger than 10-day olds. Nevertheless, the present experiment suggests that maternal licking and grooming during conditioning of 14-day olds are not essential for forming a huddling preference; other aspects of maternal stimulation appear more important. As discussed earlier in Experiment 1, the findings from

Experiment 2 may indicate that the same maternal stimulus can produce different, age-dependent experiences in pups.

GENERAL DISCUSSION

The present study demonstrates that rat pups can acquire an odor-guided huddling preference for maternal odor during a segment of the first two postnatal weeks. In Experiment 1, pups displayed an odor-guided huddling preference after five daily sessions of 2-hr odor exposure with maternal care (Fig. 1), whereas pups merely exposed to an odor on the same schedule formed no such odor preference (Fig. 1). In Experiment 2, we successfully reduced the induction procedure to a single 2-hr session with a scented foster dam, as shown in Figure 2. This dramatic truncation of a learning paradigm that was previously treated as a 2-week process is a forceful reminder of the potency of maternal stimulation on offspring development.

The single-session procedure for maternal induction of odor-guided huddling made it possible to monitor and quantify the dam's activities during the PND14 conditioning. When these data were analyzed in relation to the olfactory preferences displayed on PND15, we discovered that pups that preferred the odor CS had been with foster dams that hovered frequently, whereas pups that did not prefer the odor CS had experienced fewer bouts of hovering by the foster dam (Fig. 3). Simple physical contact and licking/grooming were not correlated with huddling preference for maternal odor. In accord with previous studies (Alberts & May, 1984; Kojima & Alberts, 2006), milk transfer seems unnecessary for the formation of huddling preference for maternal odor as evidenced by decreased body weight, probably due to the small number of pups stimulating dam (Mena & Grosvenor, 1968) in both Experiments 1 and 2.

The importance of hovering frequency for the establishment of huddling preference may reflect the significance of thermotactile stimulation for the establishment of odor-guided huddling, as previously suggested (Alberts & May, 1984; Kojima & Alberts, 2006). Yet, the duration of hovering, as defined here, did not bear a systematic relation to the preference. A couple of possibilities could account for this seeming paradox. First, it is possible that our stringent definition of hovering skewed the measurements of contact duration, thus obscuring an underlying effect. Additional trials using different camera angles could help identify aspects of maternal contact that induce preferences. Alternatively, it is possible that hovering frequency covaries with some other parameter of maternal behavior not measured here, which itself is the mechanism for inducing the learning.

We consider thermotactile stimulation as the likely critical cue in this learning, but it is possible that mothers that provided the most frequent bouts of hovering had other cues in common.

We recognize that interpretation of Experiment 1 is constrained by the different retention intervals imposed on groups trained at different ages, but tested on PND15. Generally, associations acquired during infancy are the most vulnerable to forgetting (Campbell & Spear, 1972). Lack of evidence for learning in the PND1–5 group cannot be used to conclude that they did not learn, because they were tested after ten days, whereas pups in the PND5–9 and PND10–14 groups were tested only 6- and 1-day after training, respectively. Experiment 1 demonstrated that, whether or not learning occurred in the PND1–5 group, olfactory experiences on PNDs 1–5 were not reflected by a huddling preference on PND15. Thus, for present purposes, the positive results, that is, demonstrable learning seen in the two older groups, are the relevant data. Had the youngest group also demonstrated learning, we would have expanded the emphasis of our present conclusions. Without such a demonstration, little can be concluded about the youngest group. We chose to test all groups on PND15 to ensure that they were at a developmental stage when odor-guided, filial huddling preferences are reliably expressed (Alberts & Brunjes, 1978; Brunjes & Alberts, 1979).

If the phenomenon of imprinting requires that learning occurs rapidly and within a narrowly circumscribed phase of development, then it appears unwise to apply the term to odor learning that guides the social, affiliative, and attachment behaviors of rat pups. In both experiments in the present study, rat pups acquired an odor-guided huddling preference when maternal conditioning was restricted to PNDs 10–14, which is beyond the end of the “sensitive period” reported for preference learning with an odor-stroking contingency (Moriceau & Sullivan, 2005). There are developmental phases when various stimuli, various responses, and different modes of integration are evident (Alberts & Gubernick, 1984; Lickliter, 1993; Rudy, Vogt, & Hyson, 1984). Olfactory learning appears to follow this rule of developmental flexibility.

NOTES

The authors thank Takuya Noguchi for his assistance with data analysis and Rena Fukunaga and Andrea Torotta for assistance with data collection in the preliminary study. This work was supported by NIH grant MH-28355 to JRA and a predoctoral fellowship from Center for Integrative Study of Animal Behavior, Indiana University to SK.

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