

Conditioned Activation of Fetal Behavior

WILLIAM P. SMOTHERMAN¹ AND SCOTT R. ROBINSON

*Center for Developmental Psychobiology, Department of Psychology
State University of New York at Binghamton, Binghamton, NY 13902-6000*

Received 11 December 1989

SMOTHERMAN, W. P. AND S. R. ROBINSON. *Conditioned activation of fetal behavior*. *PHYSIOL BEHAV* 50(1) 73-77, 1991.—Previous demonstrations of prenatal learning have relied upon experimental protocols involving training at one gestational age and testing at a later age, potentially confounding effects of experience and retention with developmental changes in the fetus's behavioral repertoire. In this study, a single-session paradigm, which involved the pairing of a neutral chemosensory stimulus (sucrose CS) with a second stimulus that activates behavior (lemon US), was used to assess the learning capacity of the rat fetus. Four CS-US pairings were effective in promoting behavioral activity in 20-day-old rat fetuses when they were reexposed to the sucrose CS alone. The functional expression of conditioned behavioral activation on day 20 of gestation occurs at a time when CNS structures, many of which are involved in sensory processing and learning in older animals, are still undergoing rapid differentiation in the rat fetus.

Fetal learning Classical conditioning Chemosensation

THE study of developmental learning has been promoted by adjusting experimental tasks to the sensory and motor abilities of the immature subject (10,22). Paradigms that require the subject to process chemosensory information have proven especially successful in documenting precocial sensory abilities in infant rodents. Newborn rats can detect and differentially respond to chemosensory fluids along a continuum of palatability (4, 5, 9). Moreover, the behavior expressed by infant rats can be modified through sensory experience with chemosensory stimuli. Rat pups can increase behavioral activity and perform operant responses (press against a paddle with the head) to gain access to milk reinforcements as early as postnatal day 1 (6). Three-day-old rat pups, when presented with a novel odor followed by intraoral infusion of milk, will exhibit a conditioned orientation to the odor cue (7) and express ingestive behavior when reexposed to the odor cue after conditioning (8). Conditioned activation of behavior has been viewed as a form of classical conditioning and has provided the earliest evidence for learning in the newborn rat.

Extending this general approach to the prenatal period has permitted the study of fetal learning in utero. An analogue of the conditioned aversion paradigm (1,3) has been employed to investigate learning in fetal rats. In this conditioning paradigm, a neutral chemosensory stimulus (the CS) is introduced into the amniotic sac of the fetus and is paired with an intraperitoneal injection of lithium chloride (the US). Fetuses treated in this way on day 20 of gestation are capable of retaining associations after birth and avoiding or limiting exposure to the CS as preweanlings (23) and adults (13). Fetuses that receive a CS-US pairing on day 17 of gestation exhibit a reduction in motor activity upon reexposure to the CS two days later (15), a pattern of behavior that appears to recapitulate the unconditioned response evoked

during training. Studies such as these have demonstrated that fetal rats are capable of acquiring and expressing conditioned responses in utero (16).

In the study of learning in adult animals, it is assumed that the behavioral repertoire of the subject changes little between the time of training and testing, permitting the experimenter to infer that behavioral changes elicited by reexposure to conditioned stimuli are the result of learning. Such an assumption is less tenable in very young animals in which the design of a learning experiment, especially one that incorporates a delay between training and testing, is superimposed upon rapidly developing sensory, motor and central neural processes. An experimental demonstration of an altered response of a young animal to conditioned stimuli necessarily confounds the effects of learning with other developmental changes in the animal's behavioral repertoire that occur between the times of training and testing. The most efficient approach for allaying a potential developmental confound is to employ a conditioning paradigm where training and testing can be implemented in a single session. Such a paradigm is employed in the present experiments to assess the ability of fetal rats to express conditioned activation of behavior in utero.

METHOD

Subjects

Subjects were the progeny of female Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), time-mated with Long-Evans males. Females were bred in groups of three housed in polycarbonate cages (33 × 38 × 10 cm) until the day of fetal testing. A total of 35 females contributed fetuses as subjects in this study. Subject fetuses were tested on day 20 of gestation,

¹Requests for reprints should be addressed to William P. Smotherman, Ph.D., Center for Developmental Psychobiology, Department of Psychology, P.O. Box 6000, SUNY-Binghamton, Binghamton, NY 13902-6000.

with day 0 defined as the first day in which sperm were detected in a vaginal smear. Pregnant females were maintained in a temperature- and humidity-controlled colony room under a 12 h light:12 h dark photoperiod with lights on at 0700. Observation of fetuses occurred between 1200 and 1700. Females were provided with ad lib food and water and at all times maintained in accordance with guidelines for animal care established by the National Institutes of Health (NIH publication 86-23).

Preparation for Fetal Observation

Direct observation of fetal behavior was made possible by surgical preparation of the pregnant rat and the uterine environment. A chemomyelotomy was performed by placing the pregnant rat under brief ether anesthesia and injecting 100 μ l of 100% ethanol into the spinal canal between the first and second lumbar vertebrae (14). This procedure results in irreversible spinal blockade and eliminates transmission of afferent stimuli from the posterior half of the animal, thereby permitting surgical procedures without the use of general anesthesia that suppresses fetal movements. The prepared female was placed in a Plexiglas holding apparatus, the uterus externalized through a mid-ventral incision, and the uterus and lower body immersed in a 37.5°C bath containing buffered isotonic saline. The mother and fetuses were allowed to recover from the anesthesia and acclimate to the bath for 20 min before the start of behavioral observation and testing. We have previously reported that this technique results in a robust maternal preparation that can permit direct observation of healthy fetuses in excess of 1 h (21).

Two fetuses served as experimental subjects within each pregnant rat. To ensure independence of behavioral data, fetuses from the same mother were assigned to different experimental groups. Fetal subjects were selected from near the ovarian end of a uterine horn. Immediately before observation, the subject fetus was carefully delivered from the uterus and amniotic sac into the saline bath, taking care to maintain umbilical circulation and placental-uterine attachment. All subjects exhibited pink coloration throughout the observation session, indicating adequate oxygenation. The pregnant rat and all fetuses were humanely sacrificed after observation of the second subject.

Presentation of Stimuli

Subject fetuses were exposed to control, unconditioned (US) and conditioned (CS) stimuli by delivering small volumes of liquid solutions into the fetus's mouth. To permit stimulus presentation, each subject was fitted with a dual cannula constructed from PE-10 polyethylene tubing. The cannula consisted of two independent channels, each with a flanged tip installed on the midline of the tongue in a mid-anterior position (11,17). Each channel of the cannula was connected by means of a separate length of polyethylene tubing to different microliter syringes containing one of three solutions: isotonic saline (control), sucrose (CS) or lemon (US). Neither isotonic saline nor sucrose have an appreciable effect on fetal activity (16). The CS was prepared as a 10% w/w solution of sucrose in isotonic saline. A solution prepared from lemon extract was selected as the US because intraoral infusion of lemon results in a pronounced increase in fetal activity that is associated with the moment of infusion but which is short lasting, persisting for less than one minute (18). The US was prepared as a 1:3 v/v dilution of pure lemon extract (Schilling brand) in isotonic saline (17). Stimulus presentation consisted of either 10 μ l or 20 μ l infusions of a particular solution in a 1–2-s pulse. This protocol permitted precise (± 1 μ l) delivery of stimulus solutions into the mouth of

the fetus without otherwise interrupting fetal activity.

Behavioral Observation

Fetuses were observed during 10-min observation sessions. Each instance in which a part of the fetus, including the head, mouth, forelimbs, rearlimbs, or body trunk, was observed to move was recorded as a discrete event. All events were keyed into a microcomputer, preserving a real-time record of fetal movements in different categories in temporal relation to stimulus presentation. The sum of movement events collapsed across all categories was used as an index of overall fetal activity. This method of scoring and summarizing motor activity has proven effective and reliable in previous studies of fetal behavior evoked by chemosensory stimulation (18,19). Activity scores were summarized for each minute of the 10-min session.

Data Analysis

The observation session was divided into three periods for data analysis. The first four minutes (minutes 1–4) were designated the training period and included repeated infusions of US, CS and/or control solutions. During training, all infusions were 10 μ l in volume; fetuses in treatment groups involving the presentation of two stimuli thus received a combined volume of 20 μ l of fluid. The next five minutes (minutes 5–9) were designated the delay period, and comprised no infusions. The final minutes of the observation session (minute 10) was designated the test period, and commenced with a single 20 μ l infusion of the CS (sucrose).

A separate set of analyses was performed to address the issue of whether fetuses in different groups became activated upon reexposure to the CS. The occurrence of behavioral activation was assessed by comparing fetal activity exhibited during the test period to activity during the delay period. For each group, a 99% confidence interval was constructed around mean fetal activity during the delay period. Fetal activity that exceeded the upper bound of this confidence interval during the test period provided evidence for significant behavioral activation.

RESULTS

Experiment 1

Method. The feasibility of producing conditioned activation of behavior during the prenatal period was assessed in the first experiment. Fetuses were prepared for observation and testing on day 20 of gestation. Ten pregnant rats each provided two fetuses as subjects. One fetus from each mother received four conditioning trials consisting of a 10 μ l infusion of the sucrose CS followed one to two seconds later with a 10 μ l infusion of the lemon US (experimental group). A second fetus from each mother also received stimulus pairings during the training period that consisted of the sucrose CS followed by an infusion of isotonic saline (control group), which does not affect fetal activity. After the 6-min delay period, all fetuses received a single 20 μ l infusion of the sucrose CS.

Results and discussion. During the training period, fetal activity was compared across groups in a 5 (Groups) \times 4 (Trials) repeated-measures ANOVA. Although fetuses in the experimental group appeared to be more active than fetuses in the control group, this trend failed to reach statistical significance; the overall analysis revealed no main or interaction effects. No differences between groups were found during the delay period. In the test period, when all subjects were reexposed to the sucrose CS, a *t*-test revealed significantly greater activity among fetuses

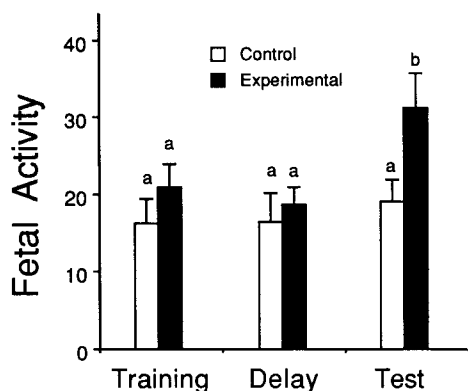


FIG. 1. Number of fetal movements per min during the training, delay and test periods of Experiment 1. Bars represent mean activity; vertical lines depict SEM. Within each period, bars overscored by the same letter did not significantly differ ($p>0.05$).

in the experimental group than in the control group, $t(18)=2.3$, $p<0.05$ (Fig. 1). Comparison of fetal behavior during the delay and test periods revealed that only fetuses in the experimental group exhibited a significant increase in behavior following the last infusion of the sucrose CS.

The results of the first experiment provide initial evidence that conditioning trials in which sucrose was paired with lemon altered the subsequent responsiveness of fetuses to the sucrose stimulus alone. Moreover, this change in response was manifested as an increase in fetal activity, similar to the behavioral activation observed following infusion of lemon alone (18,19). This finding suggests that the behavioral influence of sucrose is altered by a series of trials in which it is paired with lemon. Originally neutral in its effects on behavior, sucrose comes to evoke a conditioned activation of fetal behavior.

Experiment 2

Method. Although the results of the first experiment are suggestive that the association of sucrose and lemon results in conditioning, they do not exclude alternative explanations, such as sensitization to the CS. The results also do not demonstrate that an ordered temporal association of the CS and US are necessary to produce heightened responsiveness to the CS. To evaluate these possibilities, a second experiment with additional controls was conducted. A total of 50 subjects from 25 mothers were assigned to one of five groups ($N=10$ per group). In the first group, fetuses received four conditioning trials during the training period, consisting of a 10 μ l infusion of the sucrose CS followed immediately by a 10 μ l infusion of the lemon US (forward-pairing group). Fetuses in a second group also received four paired infusions during the training period, which consisted of the sucrose CS followed immediately by saline (saline control). In both of these groups, which replicate the two groups of Experiment 1, the training period commenced with the appropriate pair of infusions and included three additional pairings delivered at 1-min intervals. A third group received four 10 μ l infusions of sucrose (CS-alone control), while a fourth group received four 10 μ l infusions of lemon (US-alone control). These groups were included to assess CS or US sensitization. Finally, in a fifth group the temporal association of the CS and US during training was altered by presenting the CS infusion 30 s after each US infusion (backward-pairing). That is, a 10 μ l infusion of lemon was delivered at the beginning of minute 1, 2, 3, and 4, and a

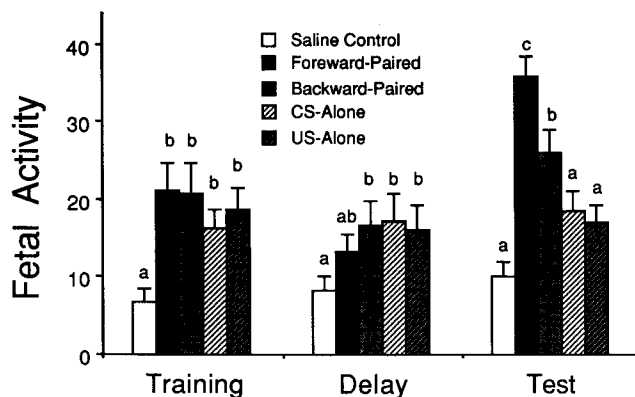


FIG. 2. Number of fetal movements per min during the training, delay and test periods of Experiment 2. Bars represent mean activity; vertical lines depict SEM. Within each period, bars overscored by the same letter did not significantly differ ($p>0.05$).

corresponding 10 μ l infusion of sucrose was delivered at minute 1.5, 2.5, 3.5 and 4.5 of the training period. In all five groups, subjects were reexposed to a 20 μ l infusion of the sucrose CS at the beginning of the test period.

Results and discussion. During the training period, fetal activity was compared across groups in a 5 (Groups) \times 4 (Minutes) repeated-measures ANOVA, which indicated a significant main effect of Groups, $F(4,45)=6.8$, $p<0.001$. Post hoc comparison of means by the method of Newman-Keuls revealed that fetuses in the saline control group were significantly less active than fetuses in all four other groups. No other differences were apparent among other groups, however.

During the delay period, the 5 (Groups) \times 5 (Minutes) repeated-measures ANOVA indicated a significant main effect of Groups, $F(4,45)=3.2$, $p<0.05$. Post hoc comparisons (Newman-Keuls) revealed that fetuses in the saline control group were significantly less active than fetuses in the US-alone, CS-alone or backward-pairing groups. The activity of subjects in the forward-pairing group did not differ from that of any other group.

During the test period, when all fetuses were reexposed to the sucrose CS, a single-factor ANOVA revealed the significant effect of Groups, $F(4,45)=14.7$, $p<0.001$. Post hoc tests (Newman-Keuls) indicated that subjects in the saline control, US-alone and CS-alone groups exhibited relatively low levels of activity following infusion of sucrose. Fetuses in the backward-pairing group exhibited significantly more activity during the test period than fetuses in the other three control groups ($p<0.01$). Fetuses in the forward pairing group exhibited the greatest behavioral activity to the sucrose CS, which differed significantly from the control groups ($p<0.01$) and from the backward-pairing group ($p<0.05$) (Fig. 2). Fetuses in two groups exhibited a significant increase in activity following infusion of the sucrose CS. Comparison of fetal activity during the delay and test periods revealed that fetuses in the forward-pairing and backward-pairing groups both expressed significant behavioral activation. These results confirm that only fetuses that received infusions of both the sucrose CS and lemon US during the training period exhibited conditioned activation of behavior during the test period.

CONCLUSIONS

The principal finding of this study, that pairing a neutral CS with an activity-evoking US results in conditioned activation of

behavior upon reexposure to the CS, was demonstrated in Experiment 1 and replicated in Experiment 2. Further, fetuses in the forward-pairing group of Experiment 2 were more active than fetuses exposed to US-alone or CS-alone during training, indicating that the behavioral activation following reexposure to the CS is not an effect of either CS or US sensitization (2). These results imply that the response of fetuses to the CS during the test period is a conditioned increase in activity.

Fetuses in the backward pairing group also exhibited an increase in activity during the test period, although not as pronounced as the activity expressed in the forward-pairing group. This may indicate that fetuses are not responsive to the order of presentation of CS and US during training. More likely, conditioned activation may be produced with a delay between presentation of CS and US. The protocol employed in this study involved four paired infusions during the training session, with presentation of CS and US reversed and offset by 30 s in the backward-pairing group. Thus three of the infusions of the CS during training were followed 30 s later by an infusion of the US. Further, it is possible that some of the CS solution remains within the oral cavity and is present at the time of the lemon infusion in the backward pairing condition. Therefore, it is parsimonious to assume that this training schedule is capable of producing conditioned activation of behavior with three trials instead of four and with an effective delay between presentation of CS and US that may be less than 30 s. But the training schedule employed in the backward-pairing group is not as effective as the ordered pairing of CS and US in close temporal association.

The lemon solution employed as a US in this experiment is a complex chemosensory stimulus with strong olfactory properties. The olfactory characteristics of the lemon solution contribute to the behavioral response it evokes in the fetal rat. For instance, fetal rats respond to lemon when presented in liquid or gas phase (19) and responsiveness is markedly reduced following transection of the neuraxis posterior to the olfactory bulbs (20). In contrast, the sucrose solution used as a CS in this study has traditionally been viewed as a gustatory stimulus (4, 5, 9). Pre-

vious developmental studies of rats have identified taste buds on the fetal tongue as early as day 20 of gestation (12), but behavioral responsiveness to sucrose has been documented no earlier than 24–48 h after birth (4,5). Infusions of gustatory stimuli, such as sucrose or quinine hydrochloride, do not evoke pronounced increases in fetal motor behavior [(18); unpublished data], so it is more difficult to rely on unconditioned responses to document the capacity of fetuses to detect and respond to these stimuli. The conditioned activation paradigm reported in the present study thus may serve as a behavioral assay for measuring gustatory sensation in utero. The conditioned increase in fetal activity following infusion of the sucrose CS provides the earliest behavioral evidence for a functional gustatory sense in the rat fetus.

Previous studies of learning during the prenatal period have demonstrated that rat fetuses are capable of associating neutral chemosensory cues with the behavioral effects of an aversive stimulus (such as IP injection of lithium chloride) (13,15). In these experiments, reexposure to the CS (either before or after birth) results in reduced activity and/or avoidance of the CS. The findings of the present study are noteworthy, therefore, in demonstrating that rat fetuses can learn and express conditioned increases in activity as well. At the least, this finding indicates that the neural mechanisms exist to process chemosensory stimuli (lemon and sucrose), to associate the temporal conjunction of these stimuli, to retain this association over a brief delay and to express a conditioned response upon reexposure to the CS. The functional expression of conditioned behavioral activation on day 20 of gestation thus occurs at a time when CNS structures, many of which are involved in sensory processing and learning in older animals, are still undergoing rapid differentiation in the rat fetus.

ACKNOWLEDGEMENTS

W.P.S. is supported by grant HD 16102-08 and Research Career Development Award HD 00719-05 from the National Institute of Child Health and Human Development (NIH). S.R.R. is supported by the Center for Developmental Psychobiology at SUNY-Binghamton.

REFERENCES

- Barker, L. M.; Best, M. R.; Domjan, M. Learning mechanisms in food selection. Waco, TX: Baylor University Press; 1977.
- Bitterman, M. E.; LoLordo, V. M.; Overmier, J. B.; Rashotte, M. E. Animal learning: Survey and analysis. New York: Plenum Press; 1979.
- Braveman, N. S.; Bronstein, P. Experimental assessments and clinical applications of conditioned food aversions. New York: New York Academy of Sciences; 1985. (Ann. NY Acad. Sci., vol. 443.)
- Ganchrow, J. R.; Steiner, J. E.; Canetto, S. Behavioral displays to gustatory stimuli in newborn rat pups. *Dev. Psychobiol.* 19:163–174; 1986.
- Grill, H. J.; Norgren, R. The taste reactivity test. II. Mimetic responses to gustatory stimuli in chronic thalamic and chronic decerebrate rats. *Brain Res.* 143:281–297; 1978.
- Johanson, I. B.; Hall, W. G. Appetitive learning in 1-day-old rat pups. *Science* 205:419–421; 1979.
- Johanson, I. B.; Hall, W. G. Appetitive conditioning in neonatal rats: conditioned orientation to a novel odor. *Dev. Psychobiol.* 15: 379–397; 1982.
- Johanson, I. B.; Hall, W. G.; Polefrone, J. M. Appetitive conditioning in neonatal rats: Conditioned ingestive responding to stimuli paired with oral infusions of milk. *Dev. Psychobiol.* 17:357–381; 1984.
- Johanson, I. B.; Shapiro, E. G. Intake and behavioral responsiveness to taste stimuli in infant rats from 1 to 15 days of age. *Dev. Psychobiol.* 19:593–606; 1986.
- Johanson, I. B.; Terry, L. M. Learning in infancy: a mechanism for behavioral change during development. In: Blass, E. M., ed. *Handbook of behavioral neurobiology*, vol. 9, Developmental psychobiology and behavioral ecology. New York: Plenum Press; 1988: 245–281.
- Kehoe, P.; Blass, E. M. Gustatory determinants of suckling in albino rats 5–20 days of age. *Dev. Psychobiol.* 18:67–82; 1985.
- Mistretta, C. M.; Bradley, R. M. Development of the sense of taste. In Blass, E. M., ed. *Handbook of behavioral neurobiology*, vol. 8, Developmental psychobiology and developmental neurobiology. New York: Plenum Press; 1986:205–236.
- Smotherman, W. P. Odor aversion learning by the rat fetus. *Physiol. Behav.* 29:769–771; 1982.
- Smotherman, W. P.; Richards, L. S.; Robinson, S. R. Techniques for observing fetal behavior in utero: A comparison of chemomyelotomy and spinal transection. *Dev. Psychobiol.* 17:661–674; 1984.
- Smotherman, W. P.; Robinson, S. R. The rat fetus in its environment: Behavioral adjustments to novel, familiar, aversive and conditioned stimuli presented in utero. *Behav. Neurosci.* 99:521–530; 1985.
- Smotherman, W. P.; Robinson, S.R. In: Krasnegor, N. A.; Blass, E. M.; Hofer, M. A.; Smotherman, W. P., eds. *Perinatal development: A psychobiological perspective*. New York: Academic Press; 1987:39–60.
- Smotherman, W. P.; Robinson, S. R. Prenatal expression of species-typical action patterns in the rat fetus (*Rattus norvegicus*). *J. Comp. Psychol.* 101:190–196; 1987.
- Smotherman, W. P.; Robinson, S. R. Behavior of rat fetuses fol-

- lowing chemical or tactile stimulation. *Behav. Neurosci.* 102:24-34; 1988.
19. Smotherman, W. P.; Robinson, S. R. Rat fetuses respond to chemical stimuli in gas phase. *Physiol. Behav.* 47:863-868; 1990.
 20. Smotherman, W. P.; Robinson, S. R. Olfactory bulb transection alters fetal behavior after chemosensory but not tactile stimulation. *Dev. Brain Res.* 57:175-180; 1990.
 21. Smotherman, W. P.; Robinson, S. R. Accessibility of the rat fetus for psychobiological investigation. In: Barr, G.; Shair, H.; Hofer, M., eds. *Developmental psychobiology: current methodology and conceptual issues*. New York: Cambridge University Press; 1991: 256-270.
 22. Spear, N. E.; Miller, J. S.; Jagielo, J. A. Animal memory and learning. *Annu. Rev. Psychol.* 41:169-211; 1990.
 23. Stickrod, G.; Kimble, D. P.; Smotherman, W. P. In utero taste/odor conditioning in the fetal rat. *Physiol. Behav.* 28:5-7; 1982.