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Olfactory bulb transection alters fetal behavior after chemosensory but not tactile stimulation

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Rat fetuses respond to an intraoral infusion of lemon extract with an increase in overall activity and facial-wiping behavior. Other studies have suggested a role for olfaction in mediating fetal responses to chemosensory stimuli. In the present study, a micro-knife was used to surgically isolate the main and accessory olfactory bulbs from more caudal structures in the fetal brain. Fetuses that received this transection procedure or a sham treatment showed normal levels of non-evoked motor activity during the period prior to chemosensory infusion. Surgical isolation of the olfactory bulbs had no effect on fetal response to perioral tactile stimulation. Behavioral responses to infusion were diminished but not eliminated in fetuses with olfactory bulb transections. The olfactory bulb, which is functional in spite of its anatomical immaturity, plays a role in the control of fetal behavior.

INTRODUCTION

Neuroanatomical evidence indicates that the olfactory bulbs are not mature in the fetal rat and undergo rapid differentiation during gestational days 19 and 20 (refs. 3, 17). The observation that certain areas of the olfactory bulbs exhibit enhanced metabolic activity late in gestation has been interpreted as evidence for a functional olfactory sense in utero, which most likely involves the accessory olfactory system[16]. Because amniotic fluid is actively ingested and respired by the fetus, odorants in solution can gain direct access to olfactory receptors. Further, blood-borne odorants may be transported to the capillary beds associated with olfactory epithelia and stimulate odor sensation[13]. Prenatal exposure to chemosensory cues can influence postnatal olfactory preferences[7,15,19], suggesting that the fetus is capable of detecting chemical stimuli in amniotic fluid. These findings suggest that olfaction before birth may play an important role in neural development[8].

Experiments involving experimental application of sensory stimuli also have proven useful in studying prenatal behavior and sensory development. Intraoral infusion of milk elicits a stretch response[23] similar to the behavior expressed by neonatal rats following milk letdown during suckling[11]. Application of repetitive tactile stimulation to the anogenital region elicits a leg-extension response in fetuses[25] that resembles the response of pups during episodes of maternal licking[14]. Experimental compression of the umbilical cord evokes a behavioral response that is characterized by stereotypic movements of the trunk, head and forelimbs[26]. Some of these examples of organized fetal responses appear to anticipate postnatal function (e.g. the stretch and leg-extension responses) while others likely serve as ontogenetic adaptations that promote the survival and development of the fetus during prenatal development[24,29].

The behavioral response of fetal rats to experimental presentation of novel chemosensory cues has provided a particularly useful model system for investigating the basis underlying prenatal behavioral organization. This system takes advantage of surgical techniques for externalizing fetal subjects in a benign fluid environment, experimental techniques for delivering controlled volumes of test solutions through a cannula directly into the mouth of the fetus, and direct measures of fetal motor behavior[30]. The typical response of the rat fetus to intraoral infusion of a lemon solution, for instance, is a brief increase in overall motor activity[27]. A principal component of this behavioral activation is the expression of facial-wiping[23], a species-typical action pattern that appears to be an antecedent of postnatal grooming behavior and behavioral responses to aversive chemosensory stimulation[5,9].

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The present study investigates the behavioral and neural systems involved in the control of organized behavioral responses to chemosensory stimulation. Specifically, the role of olfaction in mediating fetal responses to infusion of a novel chemosensory solution (Experiments 1 and 2b) and perioral tactile stimulation (Experiment 2) is assessed in fetuses prepared to isolate the olfactory bulbs from more caudal central nervous system structures.

MATERIALS AND METHODS

Subjects

Female Sprague–Dawley rats (Charles River Laboratories, Wilmington, MA) were time-mated to Long–Evans males to produce subject fetuses for this study. Breeding females were housed in groups of three in polycarbonate cages (33 × 38 × 10 cm) until day 19 or 20 of gestation (presence of sperm in vaginal smear = day 0). Cages were kept in a temperature- and humidity-controlled colony room; lights were programmed to follow a 12-h light (on at 07.00 h)/12-h dark cycle. Fetal testing occurred between 12.00 and 17.00 h. Rats were provided with ad libitum food and water and maintained in accordance with guidelines for animal care established by the National Institutes of Health (PHS Publication No. 86-23).

Preparation for fetal observation

To prepare fetuses for testing, pregnant rats received a chemomyelotomy on day 19 or 20 of gestation. This procedure was performed with the female under ether anesthesia. An injection of 100 μl of 100% ethanol was delivered into the spinal canal between the first and second lumbar vertebrae, producing an irreversible spinal blockade posterior to the site of injection and thereby eliminating sensation from the lower body[20]. The prepared female then was placed in a plexiglas holding apparatus, the uterus externalized through a midventral incision, and the uterus and lower body immersed in an isotonic saline bath maintained at 37.5 °C. The pregnant rat remained in the bath for 20 min before further manipulation, providing ample time to fully recover from the ether anesthetic.

Preparation of each subject fetus involved (a) delivery from the uterus and membranes, (b) central nervous system manipulation, (c) installation of the intraoral cannula, (d) stimulus delivery and behavioral assessment, and (e) killing and preservation for histological examination. To permit direct observation and surgical access, subject fetuses were delivered through a small incision in the uterus and the enveloping embryonic membranes were removed. However, an intact umbilical connection of the fetus to the placenta and uterus was maintained; only fetuses that remained healthy (judged by pink coloration and condition of the umbilical cord and placenta) were included in this study.

CNS manipulations

Each subject fetus was assigned to one of three groups: olfactory-bulb transection (hereafter referred to as TRANSECT), sham-transected controls (SHAM), and unoperated controls (INTACT). A fine knife fashioned from a length of steel wire (diameter = 0.30 mm) was employed to effect transections. The micro-knife was initially positioned with reference to cranial landmarks (on the midline at the posterior margin of the os frontale) and was inserted perpendicular to the cranium to a depth of 3–4 mm. After insertion, the micro-knife was rotated through the coronal plane to left and right. This procedure affected the rostral margin of the forebrain and completely severed neural connections between the olfactory bulbs and the rest of the fetal brain. Fetuses in the sham control group received nearly identical treatment to transected fetuses. The micro-knife was positioned and inserted into the fetal brain at the same coordinates, but the tip of the knife was not rotated. Thus, the connections between the main and accessory olfactory bulbs and the remainder of the brain were spared. A third group of fetuses was tested as unoperated controls and received no CNS manipulation.

Stimulus delivery and behavioral testing

To permit controlled delivery of a chemosensory stimulus, the fetus was fitted with an intraoral cannula fashioned from a 2–3 cm length of PE-10 polyethylene tubing[23]. The cannula was installed in the lower jaw with a small flange resting on the dorsal midline of the tongue in an anterior position[10]. The free end of the cannula then was connected to PE-50 tubing attached to a micrometer syringe containing the test solution. Delivery of the chemosensory stimulus consisted of a 1–2 s pulsatile infusion of 20 μl of the test solution into the mouth of the subject fetus. The test solution was prepared as a 1:3 dilution of pure lemon extract (Schilling) mixed in isotonic saline. Procedures for performing CNS manipulation (TRANSECT and SHAM) and oral cannulation were facilitated by holding the head of the subject fetus gently between thumb and forefinger. Handling of INTACT fetuses occurred only during cannulation. Through all manipulations, care was taken to avoid placing strain on the umbilical cord and to ensure that fetuses remained submerged within the saline bath. Behavioral testing began 5 min after CNS manipulation and cannulation.

In Experiment 1, all movements of the subject fetus were noted by an observer and keyed into a microcomputer serving as a real-time event recorder. This protocol has been employed in previous studies of fetal behavior and found to be highly reliable[21]. The total number of fetal movements provided a measure of overall fetal activity[20]. In both experiments, every instance of a focal pattern of fetal behavior – facial wiping – also was recorded. Facial wiping is a species-typical action pattern expressed by the rat fetus in response to various forms of sensory stimulation[23]. The wiping response consists of the fetus placing one or both forepaws in contact with the side of the face and sweeping them forward in a rostral direction. Facial wiping is readily distinguished from other forms of fetal forelimb movement by its stereotypic form and tendency to occur in a flurry of activity immediately after sensory stimulation. Because facial wiping rarely occurs during spontaneous activity[22], but is reliably elicited by chemical infusion[28], it provides a useful index of fetal responsiveness to stimulation.

Histological verification of olfactory bulb transection

At the conclusion of the testing session, subject fetuses were killed and preserved in buffered formalin for later histological examination. The brain of each fetus was removed, sectioned in the mid-sagittal plane, and examined under a binocular dissecting microscope to determine the extent and location of the knife cut. Figure 1 describes the median and range of olfactory bulb transections portrayed on a mid-sagittal diagram of the fetal rat brain. Only those transected fetuses that met the following criteria were included in the analysis (Experiments 1 and 2b): (a) the cut projected to the base of the brain; (b) the olfactory bulbs were intact rostral to the cut; (b) less than 20% of the forebrain extended rostral to the cut, and (d) the cut exhibited approximate bilateral symmetry.

EXPERIMENT 1: EFFECT OF CNS MANIPULATIONS ON FETAL CHEMOSENSORY RESPONSIVENESS

Introduction

The objective of Experiment 1 was to ascertain whether intact connections between the olfactory bulbs and more caudal neural structures play a role in the responsiveness of rat fetuses to chemosensory stimulation. Specifically, in this experiment fetal rats underwent

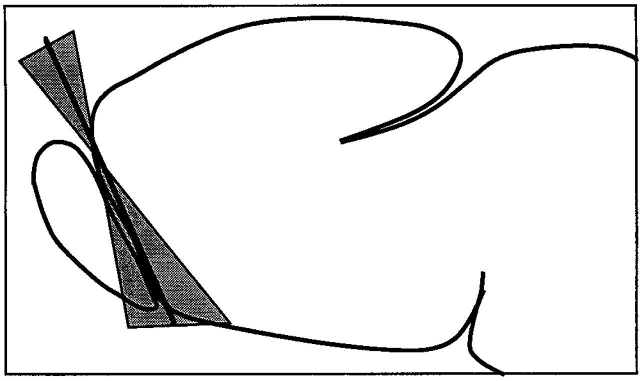

Fig. 1. Location of olfactory bulb transections in the sagittal plane determined by histological examination of fetal subjects after behavioral testing. The heavy line represents the median cut portrayed on a diagram of a medial section of the fetal rat brain. The shaded area represents the rostral-caudal range of cuts among transected fetuses included in Experiments 1 and 2b.

a surgical procedure to isolate the olfactory bulbs prior to chemosensory stimulation and behavioral testing on days 19 and 20 of gestation.

Subjects and methods

A total of 71 pregnant rats provided a single subject fetus on either day 19 or day 20 of gestation in this experiment. All three CNS manipulation groups were represented on both day 19 (12 TRANSECT, 10 SHAM, 12 INTACT) and day 20 (14 TRANSECT, 10 SHAM, 13 INTACT). Each fetus was observed during a 2-min test session, which consisted of a 55-s period preceding stimulus delivery, a 5-s window that included stimulus delivery, and a subsequent 60-s period. Changes in overall fetal activity and the expression of the facial wiping response to infusion were used to measure fetal responsiveness to infusion.

Overall fetal activity was separated into pre-infusion and post-infusion periods for analysis. A three-factor repeated-measures ANOVA compared the activity of fetuses at different ages in different experimental groups across the 12 5-s intervals within each period. Main effects were further analyzed by the method of Newman–Keuls to assess patterns of difference between groups. To measure the effect of the infusion on fetal activity, mean activity exhibited during each 5-s interval following infusion was compared to fetal activity before infusion. This comparison was performed by constructing a 99% confidence interval around the mean level of activity expressed during the last 5-s interval prior to infusion. This confidence interval was extended across the post-infusion period, and activity means that lay above the confidence interval were interpreted as evidence of behavioral activation[31]. The incidence of facial wiping in response to lemon infusion was compared among fetuses exposed to different CNS manipulations by means of a chi-square test of independence.

TABLE I

Incidence of facial wiping by 20-day-old fetuses in control and olfactory bulb transection groups

	Pre-infusion	*Post-infusion*
INTACT	0/13[a]	11/13
SHAM	0/10	7/10
TRANSECT	0/14	6/14

[a] The numerator in each cell is the number of fetuses that exhibited facial wiping; the denominator is the total number of fetuses tested.

Results

Fetuses in the transected and sham groups showed little outward signs of behavioral effect of their respective CNS manipulations prior to stimulation and their behavior appeared similar to the non-evoked activity of intact fetuses[22]. Comparison of spontaneous fetal activity during the 55-s period before infusion confirmed this impression. The three-factor repeated-measures ANOVA (age by CNS manipulation by 5-s intervals) failed to indicate any main or interaction effects ($Ps > 0.05$). On day 19, the mean activity of fetuses during the last 5-s interval before infusion was 1.1 movements, with a 99% confidence interval of 0.3–1.9 movements. On day 20, fetuses exhibited a mean of 0.9 movements during the interval before infusion, with a 99% confidence interval of 0.1–1.7 movements.

The three-factor repeated-measures ANOVA comparing fetal activity during the period after infusion indicated a significant main effect of CNS manipulation $F_{2,63} = 12.8$, $P < 0.01$, as well as the significant interaction of CNS manipulation by 5-s intervals, $F_{22,693} = 1.6$, $P < 0.05$. To simplify presentation, fetal activity before and after infusion was plotted separately for each age of testing (Fig. 2). It should be noted, however, that there was no effect of age or any interactions involving this factor.

In all experimental groups, fetuses exhibited increased activity in response to the lemon infusion. However, the magnitude and duration of behavioral activation elicited by infusion differed between transected fetuses and controls. Among TRANSECT fetuses, comparison of fetal activity relative to pre-infusion confidence intervals indicated that activity remained elevated for only 15–25 s after infusion, compared to the 50–60 s period of behavioral activation exhibited by both INTACT and SHAM fetuses. The degree of overlap between INTACT and SHAM groups during the post-infusion period indicated no systematic difference in patterns of behav-

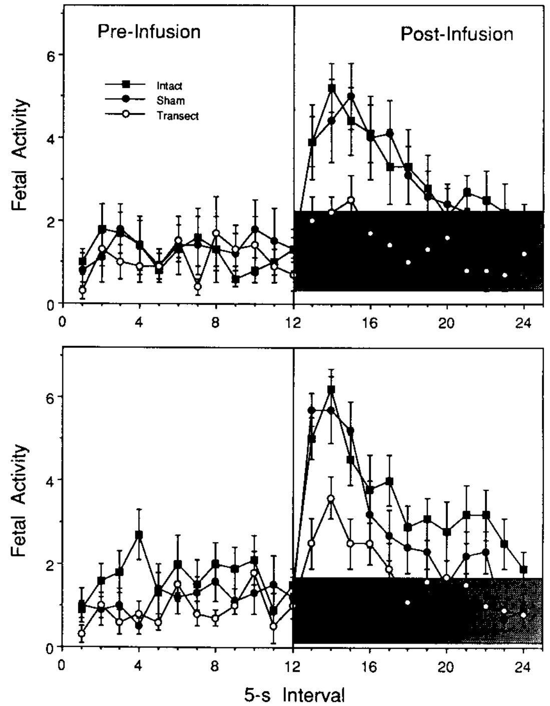

Fig. 2. Overall fetal activity on gestational days 19 (top) and 20 (bottom) expressed as the number of fetal movements per 5-s interval (mean ± S.E.M.) during the 2-min observation session. The 20-μl infusion of lemon was delivered during the last 5-s interval of the first minute of the session. The shaded area depicts the 99% confidence interval around mean fetal activity before infusion.

ioral response of these fetuses. Therefore, the influence of chemosensory infusion on fetal activity was affected by severing neural connections between the olfactory bulbs and the rest of the brain.

There was no evidence that facial wiping was expressed before or after infusion by any fetuses on day 19 of gestation, which is consistent with previous reports on the development of this behavioral pattern[23]. On day 20, facial wiping was not expressed prior to lemon infusion.

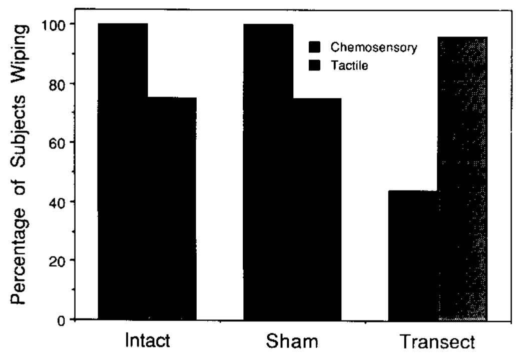

Fig. 3. Percentage of fetuses with various CNS manipulations in Experiment 2b that exhibited facial wiping in response to lemon infusion or perioral tactile stimulation.

However, facial wiping was observed after infusion and the incidence of wiping differed among groups. A chi-square test comparing the incidence of facial wiping in control (SHAM and INTACT) and TRANSECT fetuses was significant, $\chi^2 = 4.0$, $df = 1$, $P < 0.02$ (Table I). Fetuses with olfactory bulb transections were less likely to express the wiping response.

EXPERIMENT 2: RESPONSE OF TRANSECTED FETUSES TO CHEMICAL AND TACTILE STIMULATION

Introduction

The results of the first experiment are consistent with the involvement of the olfactory system in the control of fetal behavior, but do not exclude the possibility that the neural transection procedure produced nonspecific deficits in fetal motor behavior, arousal and/or excitability. Facial wiping, which was affected by the transection procedure in Experiment 1, can be elicited in postnatal animals by a variety of experimental manipulations, including somatosensory stimulation directed to the perioral area. In a classic paper on the development of fetal sensation, Carmichael also reported that guinea pig fetuses exhibit responses resembling facial wiping in response to tactile stimulation[4]. The purpose of Experiment 2 was to document whether facial wiping can be evoked by a perioral tactile stimulus and to determine whether fetal responsiveness to a nonchemical form of stimulation is altered by olfactory bulb transection.

Experiment 2a: response of intact fetuses to perioral tactile stimulation

Subjects and methods

A total of 21 pregnant rats each provided a single fetus on day 20 of gestation to serve as subjects in this experiment. Each fetus was observed in a 2-min observation session. Perioral tactile stimulation was delivered by applying a Von Frey filament (3.7 g compression pressure) to the lateral vibrissal pad adjacent to the corner of the mouth twice in rapid succession. The tactile stimulus was delivered at the beginning of the session and fetuses were observed for 60 s. The occurrence of facial wiping by each subject was recorded.

Results

After application of the perioral tactile stimulus, 15 of 21 (71%) fetuses exhibited the wiping response. Application of punctate tactile stimulation to the perioral area was effective in eliciting this pattern of fetal behavior. Presenting the perioral stimulus by this method results in stimulation to only one side of the face. The forelimb strokes elicited by tactile stimulation typically were unilateral, involving only the forelimb on the stimulated side of the fetus. In contrast, chemosensory stimulation delivered by intraoral infusion typically results in bilateral and synchronized forelimb strokes during a bout of facial wiping[23]. However, the topography of the movement and the pattern of contact between the forelimb and the face accentuate the basic similarity between this response and chemosensory-evoked facial wiping.

Experiment 2b: effect of CNS manipulations on tactile and chemosensory responsiveness

Subjects and methods

A total of 10 pregnant rats each provided six fetuses as subjects on day 20 of gestation. All fetuses were cannulated and prepared by one of the three CNS manipulations described in Experiment 1 (TRANSECT, SHAM or INTACT). Testing occurred in a 6-min observation session, which included delivery of two forms of sensory stimulation to each subject. Chemosensory stimulation was presented in a 20 μl intraoral infusion of lemon extract; perioral tactile stimulation was presented following the methods described in Experiment 2a. Stimulation occurred at the beginning of the second and fourth minutes of the session; half of the subjects received the chemosensory infusion first, half received perioral tactile stimulation first. The combination of three CNS manipulations with 2 orders of presenting stimulation resulted in six treatment groups. Each treatment group was represented by only a single subject fetus within each pregnant rat. The behavior of subjects was continuously monitored during the session for evidence of facial wiping.

Results

Preliminary analysis indicated that the incidence of facial wiping evoked by either chemosensory or tactile stimulation was not affected by the order of testing. Facial wiping was consistently evoked by perioral tactile stimulation across all three CNS manipulations, $\chi^2 = 4.3$, $df = 2$, $P > 0.05$ (Fig. 3). The wiping response also was evoked by chemosensory infusion in all fetuses in the SHAM and INTACT groups, but by only 44% of fetuses in the TRANSECT group, $\chi^2 = 28.1$, $df = 2$, $P < 0.001$. A separate chi-square analysis indicated that TRANSECT fetuses were less responsive to chemosensory infusion than to perioral tactile stimulation, $\chi^2 = 19.9$, $df = 1$, $P < 0.001$.

DISCUSSION

The results of Experiment 1 and the replication provided by Experiment 2b confirm that interruption of neural connections between the olfactory bulbs and the rest of the fetal brain alters the behavior of rat fetuses. Diminished activity and a reduced facial wiping response were evident only in transected fetuses that received a lemon infusion. Olfactory bulb transection had no observable effect on non-evoked fetal behavior (Experiment 1) and 20-day-old transected fetuses remained responsive to perioral tactile stimulation (Experiment 2b). It therefore seems unlikely that the behavioral effects of the transection procedure were due to a nonspecific deficit in fetal motor abilities or to reduced arousal or excitability of fetuses. These results are consistent with the interpretation that information processed rostral to the knife cut, and most probably in the main and/or accessory olfactory bulbs, plays a role in the control of both overall motor activity and the facial wiping response to chemosensory stimuli.

However, olfactory bulb transection did not completely eliminate behavioral responsiveness of rat fetuses. In fact, transected fetuses at both ages continued to exhibit modest behavioral activation after infusion, and some transected 20-day-old fetuses exhibited facial wiping. Because olfactory receptor cell axons travel in a ventral corridor[18], it is possible that some fibers were spared from the transection procedure, permitting olfactory-evoked facial wiping to be expressed by some subjects. An alternative interpretation of these results is that fetal activity in general, and the facial wiping response in particular, is subject to control by multiple sensory modalities. Olfactory bulb transection as performed in this study spares gustatory, trigeminal and tactile sensory pathways from the oral region. The results of Experiment 2 confirm that a stimulus limited to one of these modalities – perioral tactile stimulation – is sufficient to evoke the facial wiping response. Moreover, presentation of a lemon odor stimulus in gas phase can elicit the facial wiping response, but is less effective than presentation of the same stimulus in liquid phase[31]. Taken together, these findings suggest that olfaction contributes to, but is not the sole factor involved in the control of stereotypic fetal responses to chemosensory infusion.

Olfaction is known to be an important sensory modality in the regulation of behavior in the neonatal rat. Odor cues have been demonstrated to guide the newborn pup to the nipple[2], coordinate maternal–infant interactions[1,12], and facilitate recognition of kin[6]. Although the olfactory bulbs are immature[3], the fetus appears to possess a functional olfactory sense in utero[17]. The results of the present study, combined with a growing literature on sensory-regulated behavior during the perinatal period, confirm that the fetus can distinguish chemical cues present in the amniotic environment and that olfactory cues may modify fetal motor behavior before birth.

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