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# Effects of Amniotic Fluid on Opioid Activity and Fetal Responses to Chemosensory Stimuli

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**ABSTRACT:** The present study provides evidence that milk or amniotic fluid (AF) can promote activity in the endogenous opioid system of the E20 rat fetus. Fetal responses to a chemosensory test stimulus (lemon) were reduced after intraoral infusion of milk (Experiment 1). The effect of milk was mimicked by the kappa opioid agonist U50,488 (Experiment 2), and blocked by pretreatment with naloxone (Experiment 3), confirming opioid involvement. E20 fetuses also showed reduced responses after exposure to AF collected on E20 or E21, but not to AF collected on E19 (Experiment 4). The effects of AF on fetal responses were blocked by pretreatment with naloxone (Experiment 5), and by a selective kappa opioid antagonist, but not by a mu antagonist (Experiment 6). These findings suggest that the fetus may experience activation of the kappa opioid system for several days before birth as a consequence of its exposure to AF in utero.  
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**Keywords:** rat fetus; kappa opioid; fetal behavior; facial wiping; amniotic fluid; milk; prenatal development

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Immature organisms face two simultaneous but very different challenges during early development. In order to develop they first must survive and adapt to circumstances in their immediate environment, a process sometimes referred to as ontogenetic adaptation (Smotherman & Robinson, 1988). Organisms also experience new environments in the course of development. But predictable changes in behavior often occur well before the transition from one ontogenetic niche to the next (West & King, 1987), as existing capacities are modified or new capabilities expressed in anticipation or preparation for later functional demands

(Oppenheim, 1982). Because the fetus inhabits a very different environment from the neonate, and birth is relatively abrupt, the distinction between the competing demands of adaptation and preparation may be most sharply defined during the transition from prenatal to postnatal life.

Recent research on prenatal behavioral development has tended to focus on the latter question of developmental continuity: How does fetal behavior reflect and contribute to behavioral capacities of the newborn? This research emphasis has produced a wealth of data on the prenatal antecedents of postnatal behavior, including evidence for precursors of locomotion (Robinson & Smotherman, 1992a), behavioral states (Nijhuis, 1995; Nijhuis, Prechtl, Martin, & Bots, 1982), suckling (Robinson et al., 1992; Smotherman

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& Robinson, 1994), grooming (Robinson & Smotherman, 1991), and maternal–infant interaction (Decasper & Fifer, 1980). The prenatal development of capacities needed by the newborn is well illustrated by research on fetal responses to milk. On the last 2 days of gestation (E20–21), the rat fetus exhibits distinctive behavioral and physiological responses after delivery of a small volume of milk into the mouth. Fetuses can distinguish milk from other chemosensory fluids (Smotherman & Robinson, 1992a), express changes in motor behavior and stereotypical action sequences in response to milk (Robinson & Smotherman, 1994), show specific activation of various neurochemical systems including opioid and dopamine systems that modulate behavioral responsiveness to other forms of stimulation (Robinson, Moody, Spear, & Smotherman, 1993), and exhibit classical conditioning when milk is used as the unconditioned stimulus (Robinson, Arnold, Spear, & Smotherman, 1993). By experimentally exposing the fetus to a fluid that is biologically essential for the newborn, studies such as these have succeeded in identifying behavioral capacities of the fetus that foreshadow functionally important behavior in the infant rat.

Many of the behavioral effects of milk in the fetal rat appear to be mediated by the endogenous opioid system. A momentary exposure to milk (1–2 s) results in a more lasting period of activity (3–5 min) at the kappa subclass of opioid receptors (Robinson & Smotherman, 1994; Smotherman & Robinson, 1992b). Kappa opioid activity, in turn, has been shown to reduce fetal responses to cutaneous stimuli applied to the perioral area of the fetus (Smotherman & Robinson, 1992b), to alter the spatial and temporal organization of motor activity over a period of minutes (Andersen, Robinson, & Smotherman, 1993), and to promote expression of components of neonatal suckling behavior, such as the stretch response of infant rats (Smotherman & Robinson, 1992c). Furthermore, U50,488, a selective agonist of kappa opioid receptors, results in similar changes in fetal motor behavior and sensory responsiveness in the absence of milk (Robinson et al., 1993). These findings imply that both sensory and motor capacities of the newborn, including elements of neonatal suckling behavior, can be expressed by the fetus and are influenced by activity in the kappa opioid system.

Few other stimuli have been shown to produce behavioral effects similar to milk or to evoke opioid responses in the rat fetus. Although the fetal rat can distinguish and respond to a broad range of chemosensory fluids, opioid responses have been demonstrated only to a dilute solution of dimethyl disulfide

(DMDS) (Smotherman & Robinson, 1992a). DMDS is a constituent of pup saliva that appears to facilitate nipple attachment by infant rats (Pedersen & Blass, 1981). Intraoral infusion of DMDS to the rat fetus increases mouthing and rearlimb activity and reduces fetal responsiveness to perioral cutaneous stimuli. These behavioral effects are reversed by administration of nor-binaltorphimine (BNI), a selective antagonist of kappa opioid receptors. These findings suggest that DMDS is like milk in its ability to promote activity in the kappa opioid system of the fetal rat (Smotherman & Robinson, 1992a).

The selective responsiveness of fetal rats to milk and DMDS provides an example of the general principle of anticipatory development or forward reference, which holds that behavioral capacities can develop in advance of functional demands. However, the demonstration that milk can evoke endogenous opioid activity and trigger selective behavioral responses in the fetus does not directly address the more general question of whether features of the prenatal environment may help to shape the development of these behavioral capacities before birth. One possible mechanism by which fetuses come to respond to milk may be exposure to amniotic fluid (AF). The fetus is continuously exposed to AF during prenatal development, although the composition and physical characteristics of AF vary throughout gestation and change dramatically during the last few days prior to birth (Marsh, King, & Becker, 1963; Smotherman & Robinson, 1988). Because AF and milk both are complex biological fluids that are derived, in part, from maternal blood plasma, it is plausible that they may share characteristics or constituents that can produce similar effects on fetal behavior.

Several studies have indicated that AF may provide more than just a physical environment in which fetuses develop. For example, during the first suckling episode, AF appears to play a role similar to the DMDS in pup saliva in guiding newborn rats to locate and attach to the nipple of the lactating mother (Teicher & Blass, 1977). During parturition, pregnant rats lick the newborn pups and their own perineum, thereby distributing AF and saliva over their ventrum (Roth & Rosenblatt, 1967). Newborn pups exhibit a preference to attach to nipples painted with AF over nipples that are washed and painted with distilled water (Teicher & Blass, 1977). Older pups express a similar preference to attach to nipples painted with pup saliva or DMDS (Pedersen & Blass, 1981). Further, when a distinctive odor cue such as citral (an artificial lemon scent) is added to AF before birth, newborn pups can express a preference to attach to nipples painted with

citral (Pedersen & Blass, 1982). These studies indicate that AF has the potential to influence postnatal nipple attachment during the first and possibly subsequent suckling episodes.

A potential relationship between AF and the opioid system also has been suggested by the studies of Kristal and colleagues (Doerr & Kristal, 1989; Kristal, Thompson, & Abbott, 1986). Pregnant rats, like most other mammals, ingest AF and placental tissue during parturition (Kristal, 1991). In turn, ingestion of AF appears to enhance maternal responses to exogenous administration of morphine. Comparable levels of analgesia are produced with lower doses of morphine after ingestion of AF. These findings suggest the existence of a factor or constituent of AF that either promotes activity at opioid receptors or facilitates the action of opioid agonists in the adult rat.

The present study was initiated to explore similarities in the behavioral effects of milk and AF in the rat fetus. Specifically, we sought to determine whether fetal exposure to AF may result in opioid-mediated changes in responsiveness to a complex chemosensory stimulus. A series of six experiments was conducted to (a) measure the effects of milk on fetal behavioral responses to oral infusion of a lemon odor solution, (b) assess fetal responsiveness to the lemon infusion after pharmacological activation of the kappa opioid system, (c) determine whether milk-induced changes in chemosensory responses are mediated by endogenous opioid activity, (d) examine whether intraoral infusion of AF collected at different gestational ages can influence fetal chemosensory responsiveness in a manner similar to milk, (e) assess whether changes in responsiveness promoted by AF are mediated by the opioid system of the fetus, and (f) determine which subclass of opioid receptors, mu or kappa, is involved in the behavioral effects of AF.

## GENERAL METHODS

### Subjects

Fetal subjects were the offspring of Sprague-Dawley rats (Harlan Labs, Indianapolis, IN) bred in our laboratory. A total of 74 pregnant rats provided 350 fetuses used as subjects in this study. Breeding females were housed three per cage, in standard breeding cages (38 × 48 × 20 cm). Room temperature was maintained at 25°C and the light:dark cycle was 12:12 hr. Food and water were available ad libitum. The gestational age of fetal subjects was determined by dating conception through daily examination of vaginal

smears; the presence of sperm in a smear was designated E0 of gestation. Rats were maintained in accordance with NIH guidelines for animal care and use (National Institutes of Health, 1985).

### Prenatal Preparation

Pregnant rats were prepared for fetal testing on E20 of the 22-day gestation. Each pregnant female rat was placed under brief ether anesthesia and prepared by chemomyelotomy, which involves injection of 100  $\mu$ l of 100% ethanol between the first and second lumbar vertebrae. This procedure produces an irreversible spinal blockade which eliminates sensation in the lower portion of the body. The rat then was placed in a holding apparatus and her abdomen immersed in a warm (37.5°C) bath containing a buffered isotonic saline solution (Locke's solution). The pregnant female was monitored throughout the test session to ensure completeness of spinal blockade.

To provide experimental access to individual fetal subjects, both horns of the uterus were externalized through a midline laparotomy into the saline bath, and individual fetuses were delivered from the uterus and amniotic sac for direct manipulation and observation. Care was taken to maintain the umbilical connection of each fetal subject to the placenta, which remained within the uterus. The condition of the fetus was monitored throughout the experimental session. A 20-min period elapsed before the onset of any testing to allow the pregnant female and fetal subjects to acclimate to the testing environment.

### Administration of Opioid Agonists and Antagonists

Opioid receptor agonists, antagonists, or vehicle controls were administered to manipulate the opioid system of fetal subjects. Opioid antagonists included naloxone hydrochloride (NAL; Research Biochemicals Inc., Natick, MA), a nonselective opioid antagonist; the selective kappa opioid antagonist nor-binaltorphimine dihydrochloride (BNI; Research Biochemicals Inc.); and the selective mu antagonist [Cys<sup>2</sup>, Tyr<sup>3</sup>, Orn<sup>5</sup>, Pen<sup>7</sup>]-Amide (CTOP; Peninsula Laboratories Inc., Belmont, CA). NAL was administered in a range of dosages (0.1–10.0 mg/kg). Selective antagonist drugs were administered at dosages previously determined to be effective in completely blocking the behavioral effects of selective mu or kappa opioid agonists: BNI = 9.0 mg/kg, CTOP = 6.0 mg/kg (Smotherman & Robinson, 1992b; Smotherman, Simonik, Andersen, & Robinson, 1993). To stimulate

activity at kappa opioid receptors, the selective kappa agonist U50,488 was used (U50; Research Biochemicals Inc.). U50 was administered in a range of dosages (0.0–1.0 mg/kg). Because individual fetuses could not be weighed before testing, a fixed dosage of a drug was administered to each subject based on the mean body weight on E20 of gestation (4.4 g). Stock solutions of each drug were prepared in an isotonic saline vehicle and refrigerated until use. Drugs were administered directly to individual fetal subjects by intraperitoneal (IP) injection of 50  $\mu$ l of the drug solution or vehicle control with a 30-ga hypodermic needle. In all experiments, the observer was blind to the treatments received by fetal subjects.

### **Cannulation and Infusion**

In order to present subjects with two different chemosensory stimuli in the same test session, fetuses were fitted with dual intraoral cannulae (Hall & Rosenblatt, 1977), with the flanged tips resting in an anterior position on the tongue of the subject (Kehoe & Blass, 1985). The cannulae then were attached to micrometer syringes that allowed delivery of a 20  $\mu$ l ( $\pm 1$   $\mu$ l) infusion of chemosensory fluids. Infusions were presented in a 2-s pulse directly into the mouth of the fetal subject. Stimuli infused to fetal subjects included isotonic saline, milk, amniotic fluid (AF), or lemon. The milk used in this study was a commercially available bovine light cream (half-and-half), which has been used as a standard milk stimulus in studies of suckling behavior in neonatal and fetal rats (Hall & Rosenblatt, 1977; Robinson & Smotherman, 1994). AF samples were collected from donor fetuses at one of three gestational ages: E19, E20, or E21. Donor fetuses were obtained from pregnancies other than pregnancies that provided subject fetuses for behavioral testing. The procedure used to collect AF consisted of (a) removing the fetus and placenta from the pregnant female's uterus, taking care that the amnion and chorion remained intact, (b) rinsing the exterior surface of the chorion with isotonic saline and blotting dry, and (c) extracting AF into a test tube. The aliquot of AF was immediately frozen at  $-70^{\circ}\text{C}$  until later use. The lemon solution used as a test stimulus was prepared as a 1:2 dilution of pure lemon odor extract (Schilling brand) in isotonic saline. This stimulus has been reported in previous research to be effective in consistently evoking facial wiping responses in fetal rats (Robinson & Smotherman, 1991; Smotherman & Robinson, 1989). All chemosensory fluids were delivered at fetal body temperature ( $37.5^{\circ}\text{C}$ ).

### **Behavioral Observations**

The behavior of each fetal subject was observed in sessions of 2- or 3-min duration. Behavioral scoring focused on a simple action pattern—facial wiping—which is reliably evoked by lemon infusion in the E20 rat fetus. Facial wiping involves movement of one or both forelimbs along the side of the face, with the forepaws making contact from the ears to the nose (Robinson & Smotherman, 1991). The occurrence of each facial wiping stroke was entered into a real-time event recording system, which preserved information about the number of wiping strokes and the time of their occurrence ( $\pm 0.5$  s). This method of scoring fetal motor behavior is highly consistent between observation sessions (reliability  $> .90$ ). Moreover, because fetuses can express a variable number of wiping strokes after lemon infusion, this behavior provides a continuous measure of fetal responsiveness to chemosensory stimulation and permits the use of parametric statistical procedures (Simonik, Robinson, & Smotherman, 1993).

### **Experimental Design and Data Analysis**

In the experiments where fetal subjects were pretreated with an opioid agonist or antagonist, the observation session began 5 min after injection of the drug or vehicle control. A delay of this length has been found sufficient for selective opioid agonist and antagonist drugs to exert behavioral effects in rat fetuses (Smotherman & Robinson, 1992b; Smotherman et al., 1993). All observation sessions included a 1-min baseline period before the subject was exposed to any chemosensory stimulus. The fetal subject then was observed for 1 or 2 min after the baseline period. In most experiments, subjects received an Exposure infusion of a chemosensory fluid (saline, milk, or AF) followed 1 min later by a test infusion of lemon. In Experiment 2, subjects received a single test infusion at the end of the baseline period. Behavioral scoring concluded 1-min after the test infusion. In each experiment, multiple fetuses were tested from each pregnancy. In order to avoid confounding treatment effects with litter effects, fetal subjects from the same pregnancy were assigned to different treatment groups, with each treatment group represented only once in each pregnancy (Holson & Pearce, 1992) and the order of testing counterbalanced across pregnancies. In some cases, no facial wiping was expressed after the test infusion of lemon by subjects in one or more treatment groups; in these instances the presence or absence of a wiping response (one or more strokes) was determined for

each fetal subject and summarized in a contingency table. These presence/absence response data were analyzed by non-parametric chi-square test of independence. However, in most experiments subjects showed one or more facial wiping strokes, and the effects of various treatments on fetal behavior were assessed by comparing the number of wiping strokes by analysis of variance (ANOVA), with the alpha level set at  $p < .05$ .

## EXPERIMENT 1. MILK EFFECTS ON LEMON-EVOKED FACIAL WIPING

Intraoral infusion of milk alters fetal responsiveness to sensory stimulation. After delivery of milk into the subject's mouth, fetuses are less responsive to tactile stimulation of the perioral area (Robinson & Smotherman, 1994; Smotherman & Robinson, 1992b). The behavioral bioassay used in most previous experiments with fetal rats involves application of a stiff bristle or von Frey filament to the lateral vibrissal pad adjacent to the mouth. Fetal subjects reliably express a facial wiping response to this stimulus, which typically consists of a single unilateral stroke. Because this bioassay provides essentially a presence or absence measure of response, experiments employing the perioral tactile stimulus must use nonparametric statistical tests to compare the relative frequency of responses in different treatment groups. However, milk appears to alter fetal responsiveness to a broad range of sensory stimuli. In addition to perioral cutaneous stimulation, milk reduces responsiveness to novel chemosensory fluids, such as lemon odor extract, delivered into the mouth of the fetus (Smotherman & Robinson, 1992b). Because chemosensory stimulation typically evokes a series of bilateral facial wiping strokes, it can provide a continuous measure of fetal response that permits employment of more powerful parametric statistical tests. Experiment 1 of this study was designed to assess the influence of intraoral milk infusion on chemosensory-evoked facial wiping in the E20 rat fetus.

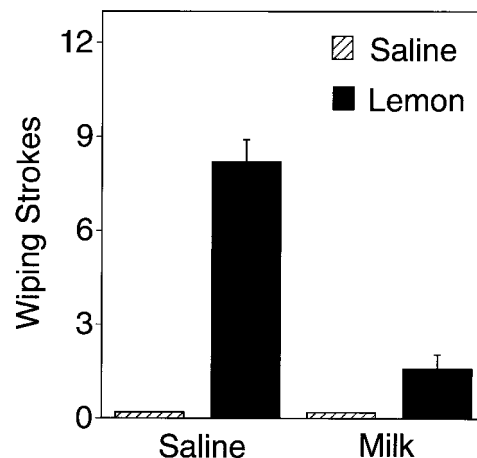
### Methods

A total of 40 fetal subjects from 10 pregnancies was tested in Experiment 1. Subjects were assigned to one of four treatment groups ( $n = 10$  per group) which resulted from the factorial combination of exposure treatment and test stimulus. Following a 1-min baseline period, all subjects received an exposure infusion consisting of  $20 \mu\text{l}$  of either milk or isotonic saline. The exposure treatment was followed 60 s later by a

test infusion of either a lemon solution or isotonic saline. These exposure and test conditions thus yielded four treatment groups: saline/saline, saline/lemon, milk/saline, and milk/lemon. The number of facial wiping strokes evoked by the test infusion was used as a measure of fetal chemosensory response.

## Results and Discussion

None of the subjects in any of the four groups exhibited facial wiping during the 1st baseline min of observation, nor during the 2nd min of observation, after the exposure and before the test infusion. After the test infusion, facial wiping also was not observed after infusion of saline, but was expressed by 17 of 20 fetuses (85%) that received lemon,  $\chi^2(1, N = 40) = 26.2, p < .001$ . Although lemon infusion evoked facial wiping in most subjects, the number of facial wiping strokes expressed appeared to vary as a function of their prior exposure to saline or milk. A  $t$  test was used to compare the number of facial wiping strokes evoked by lemon subjects exposed to saline or milk. This analysis indicated significantly more facial wiping in the saline/lemon group than in the milk/lemon group,  $t(18) = 8.0, p < .001$  (Figure 1). These findings demonstrate that exposure to milk reduced fetal responses to an aversive chemosensory stimulus (lemon). The results of Experiment 1 also confirm that infusion of a lemon odor solution into the mouth of the fetus elicits a facial wiping response that can be



**FIGURE 1** Number of facial wiping strokes (mean  $\pm$  SEM) evoked by intraoral infusion of isotonic saline or a chemosensory lemon solution in Experiment 1. E20 fetal subjects were exposed to  $20 \mu\text{l}$  infusions of either saline or milk (bovine light cream) 1 min before the chemosensory test infusion.

used as an effective and continuous measure of sensory responsiveness in the fetal rat (see also Simonik et al., 1993; Smotherman & Robinson, 1990).

## EXPERIMENT 2. KAPPA OPIOID AGONIST EFFECTS ON FACIAL WIPING

Previous studies have shown that milk exerts its effects on many aspects of fetal behavior by promoting activity at kappa receptors of the endogenous opioid system (Smotherman & Robinson, 1992a, 1992b, 1992c). Pretreatment with nor-binaltorphimine (BNI), a kappa receptor antagonist, is effective in blocking the effects of milk on fetal responsiveness to a cutaneous tactile stimulus (Smotherman & Robinson, 1992b). Conversely, administration of U50,488 or U69,593, which are selective kappa receptor agonists, promotes changes in motor behavior and sensory responsiveness that mimic the behavioral effects of milk (Andersen et al., 1993; Robinson, Moody, Spear, & Smotherman, 1993; Smotherman et al., 1993). However, previous work has not specifically addressed whether activity in the kappa opioid system alters fetal responsiveness to stimuli in other sensory modalities, such as complex chemosensory fluids. The aim of Experiment 2 was to determine whether pharmacological activation of kappa opioid receptors is effective in reducing the facial wiping response elicited by lemon infusion in the E20 rat fetus.

### Methods

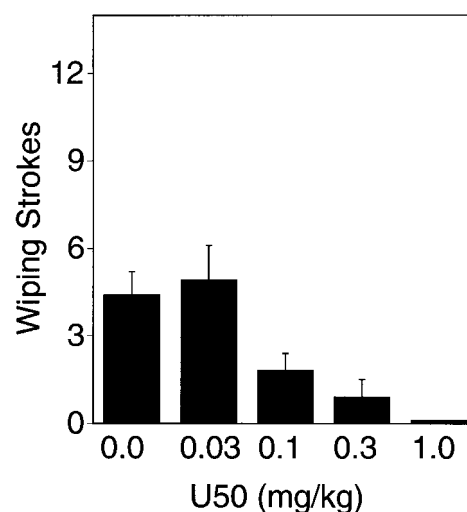
A total of 50 fetal subjects from 13 pregnancies was tested in Experiment 2. Subjects were assigned to one of five treatment groups ( $n = 10/\text{group}$ ) that received different dosages of the kappa receptor agonist U50,488 (U50): 0.0 (saline controls), 0.03, 0.1, 0.3, or 1.0 mg/kg. Five minutes after the treatment injection, all subjects were tested in a 2-min session. After the initial 1-min baseline, subjects received a 20  $\mu\text{l}$  test infusion of the lemon solution, and the number of facial wiping strokes was recorded.

### Results and Discussion

Facial wiping responses to the test infusion of lemon were not expressed by all subjects in Experiment 2. None of the subjects in the highest dosage group (1.0 mg/kg) expressed facial wiping, compared to 2, 7, 8, and 9 subjects in the 0.03, 0.1, 0.03, and 0.0 mg/kg groups, respectively. The incidence of facial wiping among these five groups differed significantly, con-

firmed a dose-dependent effect of kappa opioid activity on facial wiping behavior,  $X^2(4, N = 50) = 25.2, p < .001$ . Among the four groups in which facial wiping was observed, a one-factor ANOVA revealed that the number of facial wiping strokes evoked by lemon also was affected by U50 dosage,  $F(3,36) = 4.8, p < .01$  (Figure 2). Post-hoc comparisons of group means by the method of Fisher PLSD revealed that treatment with the lowest dose of U50 (0.03 mg/kg) did not differ significantly from saline controls (0.0 mg/kg;  $p > .05$ ). However, subjects receiving 0.1 or 0.3 mg/kg U50 expressed significantly fewer facial wiping strokes ( $< 2$  strokes) than subjects in the 0.03 and 0.0 mg/kg groups ( $> 4$  strokes;  $ps < .05$ ).

The results of Experiment 2 indicate that activity at the kappa class of opioid receptors is effective in producing dose-dependent reductions in fetal responses to lemon. The opioid-induced decrement in response is evident not only to perioral cutaneous stimuli, as reported in previous studies (Robinson & Smotherman, 1994; Smotherman & Robinson, 1992b), but also to complex chemosensory stimuli such as lemon (this study). The effect of the kappa opioid agonist on wiping responses thus resembles the effect of oral exposure to milk shown in Experiment 1. This finding adds to a growing list of evidence that the behavioral consequences of milk are mediated through the kappa opioid system of the fetus and neonatal rat.



**FIGURE 2** Number of wiping strokes (mean  $\pm$  SEM) evoked by the test lemon infusion after treatment with an opioid agonist in Experiment 2. Fetuses were pretreated by injection of saline (0.0) or one of four doses of the kappa opioid agonist U50,488 (0.03, 0.1, 0.3, or 1.0 mg/kg) 5 min before testing.

### EXPERIMENT 3. MILK EFFECTS ON OPIOID ACTIVITY

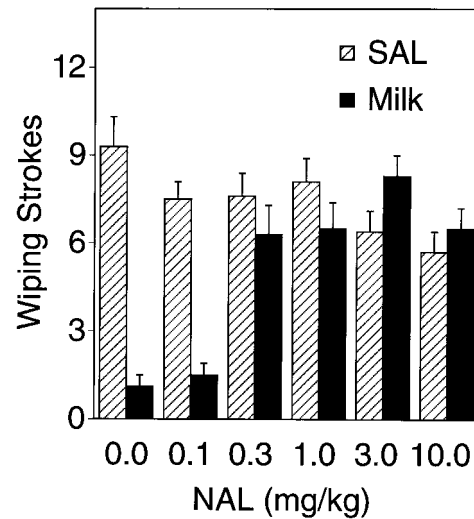
Fetal response to a perioral cutaneous stimulus is reduced after fetal exposure to milk (Robinson & Smotherman, 1994; Smotherman & Robinson, 1992b). The experiments reported in the present study demonstrate that milk also reduces fetal responses to a chemosensory stimulus (Experiment 1), and that similar effects can be produced by pharmacologically activating the kappa class of opioid receptors in the fetus (Experiment 2). These findings imply that the effects of milk on fetal responsiveness to the lemon stimulus are mediated by activity in the endogenous opioid system. The aim of Experiment 3 was to verify involvement of the opioid system by pretreating fetal subjects with naloxone, a nonselective opioid antagonist.

#### Method

A total of 120 fetal subjects from 20 pregnancies was tested in Experiment 3. Subjects were assigned to 1 of 12 treatment groups ( $n = 10/\text{group}$ ) that resulted from the factorial combination of six dosages of naloxone (0.0, 0.1, 0.3, 1.0, 3.0, or 10.0 mg/kg) by two exposure infusions (saline or milk). Only 6 subjects were tested from each pregnancy, each representing different dosage groups in either the saline or milk exposure condition. The observation session began 5-min after the subject was pretreated by IP injection of the appropriate dosage. After a 1-min baseline period, subjects received the exposure infusion of either saline or milk, and 1 min later received the test infusion of lemon.

#### Results and Discussion

None of the subjects in any of the 12 groups exhibited facial wiping during the baseline minute of observation, nor during the 2nd min of observation, after the exposure infusion. However, at least one facial wiping stroke was expressed after the test infusion of lemon by nearly all subjects across the 12 groups. The number of wiping strokes evoked by lemon was compared in a 2-factor  $6 \times 2$  (Dosages  $\times$  Exposures) ANOVA, which indicated a significant Dosage  $\times$  Exposure Interaction,  $F(5,108) = 13.6$ ,  $p < 0.001$ , interaction (Figure 3). To interpret the interaction, a series of  $t$  tests were conducted to assess the simple main effect of exposure within each dosage condition. These comparisons revealed significant differences between saline and milk exposure when fetuses were pretreated with 0.0 mg/kg,  $t(18) = 7.5$ ,  $p < .001$ , or 0.1 mg/kg



**FIGURE 3** Number of wiping strokes (mean  $\pm$  SEM) evoked by the test lemon infusion after opioid receptor blockade and exposure to milk in Experiment 3. Fetal subjects were pretreated by IP injection of saline (0.0) or one of five doses of the opioid antagonist naloxone (0.1, 0.3, 1.0, 3.0, or 10.0 mg/kg) 5 min before testing, and received a 20  $\mu\text{l}$  exposure infusion of either saline (SAL) or milk 1 min before the test infusion.

naloxone,  $t(18) = 8.5$ ,  $p < .001$ . However, facial wiping did not differ between saline- and milk-exposed subjects that were pretreated with higher dosages of naloxone,  $p_s > .05$ .

The pattern of results in Experiment 3 indicated that intraoral infusion of milk results in activity in the endogenous opioid system of the rat fetus. Milk-induced opioid activity reduced fetal responses to the test infusion of lemon. The involvement of the endogenous opioid system was confirmed by the effectiveness of the opioid antagonist naloxone, administered at a dosage of 0.3 mg/kg or greater, to completely block the behavioral effects of milk and reinstate high levels of facial wiping to the lemon stimulus.

Inspection of the behavioral records from Experiments 1 and 3 suggested that fetuses that were exposed to milk may have shown fewer wiping strokes to lemon because the wiping response was delayed. To evaluate this observation, data on the latency between lemon infusion and the initial wiping stroke were combined from Experiments 1 and 3. Only those subjects that were exposed to saline or milk, without opioid drug pretreatment, and which exhibited at least one wiping stroke were included in this analysis. This yielded a total sample of 20 fetuses that received an exposure infusion of saline (10 in Experiment 1, 10 in Experiment 3), and 13 fetuses that received an expo-

sure infusion of milk (7 in Experiment 1, 6 in Experiment 3). Latency data were compared among these three conditions in a *t* test. This analysis indicated a significantly longer latency to wipe in subjects exposed to milk (mean  $\pm$  SEM =  $9.4 \pm 0.6$  s) than in subjects exposed to saline ( $6.9 \pm 0.4$  s),  $t(31) = 3.4$ ,  $p < .005$ . The increased latency to the initial wiping stroke may account in part for the reduced total number of wiping strokes expressed by fetuses exposed to milk.

#### EXPERIMENT 4. AMNIOTIC FLUID EFFECTS ON FACIAL WIPING

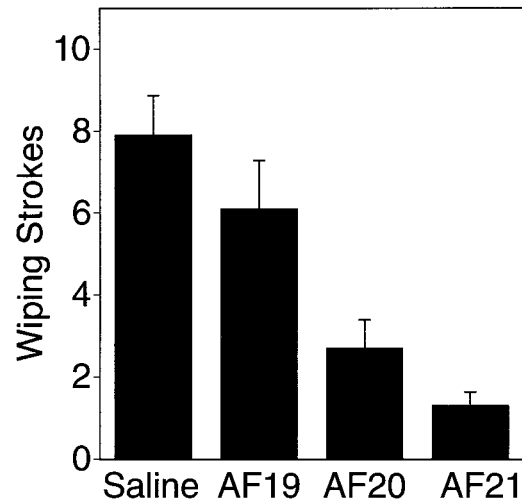
Delivery of milk to the E20 rat fetus or exogenous stimulation of the kappa opioid system results in changes in fetal response to chemosensory stimulation. These findings support published reports that prenatal exposure to milk can reduce fetal responses to cutaneous tactile stimuli (Robinson & Smotherman, 1994; Smotherman & Robinson, 1992b). The developmental question raised by these results is how prenatal exposure to milk—which is never experienced by the fetus under natural circumstances—can result in changes in endogenous opioid activity. One hypothesis to explain the prenatal effectiveness of milk is that the fetus is naturally exposed in utero to other biological fluids, such as amniotic fluid (AF), that produce the same behavioral effects as milk. The aim of Experiment 4 was to determine whether AF, collected at different gestational ages, can alter responses of the E20 rat fetus in a manner equivalent to milk.

#### Method

A total of 40 fetal subjects derived from 10 pregnancies on E20 of gestation was assigned to four exposure groups ( $n = 10$ /group): Saline, or AF collected from fetal rats on gestational Day 19, 20, or 21 (AF19, AF20, or AF21, respectively). Subjects were externalized from the uterus and amniotic sac into the saline bath prior to testing, which ensured that they were not exposed to their own AF during the observation session. After the initial 1-min baseline period, each subject received an oral infusion ( $20 \mu\text{l}$ ) of saline or AF collected at one of three ages. Fetal wiping responses to a test infusion of lemon were assessed 1 min after exposure.

#### Results and Discussion

None of the subjects in any of the four groups exhibited facial wiping during the 1st baseline min of ob-



**FIGURE 4** Number of wiping strokes (mean  $\pm$  SEM) evoked by the test lemon infusion after exposure to amniotic fluid in Experiment 4. One min before the test infusion, each E20 fetal subject received a single  $20 \mu\text{l}$  infusion of either saline or amniotic fluid (AF) collected from other pregnancies on Day 19, 20, or 21 of gestation.

servation, nor during the 2nd min of observation, after exposure to saline or AF. Nearly all subjects expressed one or more wiping strokes after infusion of the test lemon solution. The number of facial wiping strokes evoked by lemon was compared across the four exposure groups in a one-factor ANOVA. The analysis indicated that expression of facial wiping varied significantly among the four groups,  $F(3,36) = 12.4$ ,  $p < 0.001$  (Figure 4). Post-hoc comparisons of group means (Fisher PLSD) revealed that AF19 did not differ from saline,  $p > 0.05$ . AF20 or AF21 reduced facial wiping to lemon relative to saline and AF19,  $p < 0.05$ , but did not differ from each other,  $p > 0.05$ . These results indicate that delivery of AF into the mouth of a rat fetus can produce behavioral effects similar to milk. However, the effectiveness of AF is age-dependent: AF collected on E20 (AF20) or E21 (AF21) reduced facial wiping to the lemon stimulus, but AF19 had no significant effect on fetal response. Because fetal subjects in this experiment were tested at the same gestational age (E20), variation in the effects of AF must reflect differences in the characteristics or composition of AF at E19, E20, and E21 of gestation.

#### EXPERIMENT 5. AMNIOTIC FLUID EFFECTS ON OPIOID ACTIVITY

Experiment 4 demonstrated that experimental presentation of AF collected at E20 or E21 of gestation is

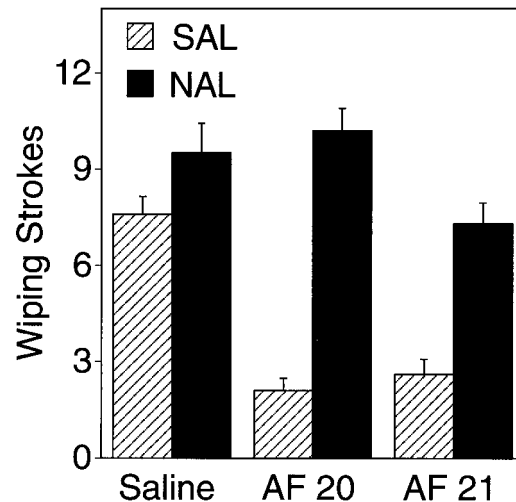
effective in reducing responses to lemon in a manner similar to milk. Milk exerts its effects on fetal responsiveness by promoting activity in the endogenous opioid system (Smotherman & Robinson, 1992a, 1992b, 1992c; Experiment 3). The objective of Experiment 5 was to assess whether AF influences fetal sensory responses through the same opioid mechanism. Specifically, the opioid antagonist naloxone was used to block activity at opioid receptors, preventing a subsequent infusion of AF from engaging the opioid system of the fetus. If behavioral effects of AF are mediated by opioid activity, then naloxone should prevent an opioid-mediated reduction in responsiveness and thereby permit high levels of facial wiping to the lemon solution.

### Methods

A total of 60 fetal subjects derived from 11 pregnancies was prepared for testing on E20 of gestation. Subjects were assigned to one of six treatment groups that resulted from the factorial combination of two pretreatment injections (SAL or NAL) by three exposure infusions (saline, AF20, or AF21). The pretreatment injection was administered 5 min before the observation session; subjects in the SAL control group were pretreated with the saline vehicle and subjects in the NAL group were pretreated with the opioid antagonist naloxone (1.0 mg/kg). After the 1-min baseline period, subjects received an exposure infusion of saline, AF20, or AF21, and facial wiping responses to the lemon test stimulus were assessed 1 min later.

### Results and Discussion

None of the subjects in any of the six groups exhibited facial wiping during the baseline minute of observation, nor during the 2nd min, after exposure to saline or AF, but nearly all subjects expressed at least one wiping stroke after the test infusion of lemon. The number of facial wiping strokes evoked by lemon was compared in a two-factor  $2 \times 3$  (Pretreatment Injections  $\times$  Exposure Infusions) ANOVA. The analysis indicated the significant interaction of Pretreatment  $\times$  Exposure,  $F(2,54) = 11.7, p < 0.001$  (Figure 5). Two one-factor ANOVAs then were conducted to test for the simple main effect of exposure infusion. Among SAL-pretreated subjects, facial wiping varied with exposure infusion,  $F(2,27) = 41.8, p < 0.001$ ; post-hoc comparisons (Fisher PLSD) indicated fewer facial wiping strokes were performed after exposure to AF20 or AF21 than after exposure to saline,  $p < 0.05$ . Among NAL-pretreated subjects, facial wiping to lemon was expressed at control levels. Although the



**FIGURE 5** Number of wiping strokes (mean  $\pm$  SEM) evoked by the test lemon infusion after opioid receptor blockade and exposure to amniotic fluid in Experiment 5. E20 fetal subjects were pretreated by IP injection of saline (SAL) or naloxone (NAL; 1.0 mg/kg) 5 min before testing, and received an exposure infusion of saline or amniotic fluid (AF) collected on Day 20 or 21 of gestation.

test for the simple main effect of exposure was significant,  $F(2,27) = 3.8, p < 0.05$ , pairwise comparisons indicated that subjects exposed to AF20 or AF21 did not differ from saline controls,  $p > 0.05$ . But the lemon test stimulus evoked more facial wiping after exposure to AF20 than after AF21,  $p < 0.05$ . This pattern of results provides clear evidence for opioid involvement in the behavioral effects produced by AF exposure: fetuses that were pretreated with the opioid antagonist naloxone did not exhibit reduced responsiveness to the lemon Test infusion. The slight difference in facial wiping expressed by naloxone-pretreated, AF-exposed subjects suggests that AF collected on gestational Days 20 and 21 may differ in their effects on fetal behavior, perhaps through a non-opioid mechanism.

### EXPERIMENT 6. AMNIOTIC FLUID EFFECTS ON MU OR KAPPA OPIOID SYSTEMS

In Experiment 5, naloxone was effective in reinstating facial wiping responses to the chemosensory lemon solution. Past research with rat fetuses has indicated that two main subclasses of opioid receptors—kappa and mu—can influence fetal behavior and sensory responsiveness. Infusion of milk into the mouth of the rat fetus promotes activity at kappa receptors (Smother-

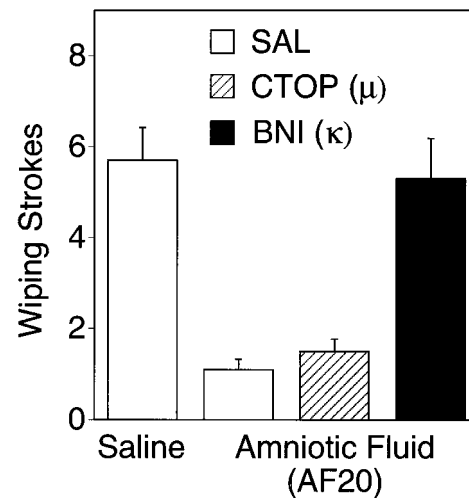
erman & Robinson, 1992b), whereas classical conditioning of opioid activity can result in activity at mu receptors (Robinson et al., 1993). Therefore, it is possible that the opioid responses resulting from prenatal exposure to AF could be mediated by either kappa or mu receptors. The aim of Experiment 6 was to identify the subclass of opioid receptors responsible for the opioid response to AF by pretreating fetal subjects with a selective antagonist of kappa opioid receptors (nor-binaltorphimine diHCl or BNI) or mu receptors (the somatostatin analog CTOP).

## Methods

Forty fetal subjects from 10 pregnancies were prepared for testing on E20 of gestation and assigned to one of four groups ( $n = 10$  subjects/group) that received different combinations of drug pretreatment and AF exposure. In three groups, fetal subjects received a 50  $\mu$ l IP pretreatment injection of the selective kappa antagonist BNI (9.0 mg/kg), the mu antagonist CTOP (6.0 mg/kg), or the isotonic saline vehicle (SAL). Subjects in these three groups received an exposure infusion of AF during the test session; the AF was collected from donor fetuses on E20 of gestation. The fourth group received a pretreatment injection of SAL, and later received an exposure infusion of saline. Pretreatment injections were administered 5 min before the onset of the 3-min test session. Behavioral testing consisted of a 1-min baseline period, an exposure infusion of AF or saline, and measurement of facial wiping responses to the test infusion of lemon 1 min later.

## Results and Discussion

None of the subjects in any of the four groups exhibited facial wiping during the 1st baseline min of observation, nor during the 2nd min of observation, after the exposure infusion of AF or saline. However, at least one facial wiping stroke was expressed by nearly all subjects after the test infusion of lemon. A one-factor ANOVA that compared the number of facial wiping strokes evoked by lemon indicated significant differences among the four groups,  $F(3,36) = 16.7$ ,  $p < 0.001$  (Figure 6). Post-hoc comparison (Fisher PLSD) of the two groups pretreated with SAL confirmed that subjects exposed to AF showed significantly fewer wiping strokes than subjects exposed to saline,  $p < .05$ . Fetuses pretreated with the mu antagonist CTOP showed low levels of facial wiping that did not differ from SAL-pretreated subjects exposed to AF,  $p > .05$ . However, facial wiping was significantly elevated in BNI-pretreated subjects. Among fe-



**FIGURE 6** Number of wiping strokes (mean  $\pm$  SEM) evoked by the test lemon infusion after selective blockade of opioid receptors and exposure to amniotic fluid in Experiment 6. Fetal subjects were pretreated by injection of saline (SAL), the mu opioid antagonist CTOP (6.0 mg/kg), or the kappa opioid antagonist nor-binaltorphimine (BNI, 9.0 mg/kg) 5 min before testing, and received an exposure infusion of saline or amniotic fluid collected on Day 20 of gestation (AF20).

tuses that were exposed to AF, BNI-pretreated subjects showed significantly more facial wiping to the lemon test infusion than SAL- or CTOP-pretreated subjects,  $p < .05$ . Moreover, facial wiping in the BNI group did not differ from the responses of control subjects that were pretreated with SAL and exposed to saline.

The results of Experiment 6 clearly demonstrated that the selective kappa receptor antagonist BNI was effective in blocking the opioid-inducing effects of AF and reinstating facial wiping responses to control levels in fetuses exposed to AF. This finding provides strong evidence that fetal exposure to AF results in activity at the kappa subclass of opioid receptors, which in turn reduces facial wiping responses to chemosensory stimulation in the rat fetus.

As reported in Experiment 3, fetal exposure to milk resulted not only in a reduction in the number of facial wiping strokes evoked by lemon, but an increase in the mean latency between infusion and the initial wiping stroke. To assess whether exposure to AF produced a similar increase in latency to wipe, latency data were combined from Experiments 4, 5, and 6; only those subjects that were exposed to saline or AF (collected on E20 or E21), without opioid drug pretreatment, and which exhibited at least one wiping stroke were included in this analysis. This yielded a total sample of 29 fetuses that received an exposure infusion of saline (10 in Experiment 4, 10 in Experi-

ment 5, 9 in Experiment 6), 26 fetuses that received an exposure infusion of AF collected on E20 (9 in Experiment 4, 9 in Experiment 5, 8 in Experiment 6), and 15 fetuses that received an exposure infusion of AF collected on E21 (7 in Experiment 4, 8 in Experiment 5). Latency data were compared among these three conditions in a one-way ANOVA, which revealed the significant effect of exposure,  $F(2,67) = 6.6, p < .005$ . Post-hoc comparisons (Fisher PLSD) indicated that mean latency to wipe was longer in fetuses exposed to AF ( $8.7 \pm 0.5$  s for E20 AF;  $8.6 \pm 0.6$  s for E21 AF) than in fetuses exposed to saline ( $6.9 \pm 0.3$  s). The increased latency to wipe was comparable in magnitude to that observed after fetal exposure to milk (see Experiment 3).

## CONCLUSIONS

A growing literature concerned with behavioral development of the rat fetus and neonate has reported that a brief exposure to certain chemosensory fluids can result in activation of the endogenous opioid system. In the neonatal rat, milk and a variety of other chemosensory stimuli (sucrose, corn oil) are effective in triggering opioid responses, although the specific class of opioid receptors that mediate these neonatal responses has not been clearly identified (Blass & Fitzgerald, 1988; Shide & Blass, 1989). In the rat fetus, bovine light cream, which is similar in fat and water composition to postpartum rat milk (Hall & Rosenblatt, 1977) consistently evokes activity at the kappa subclass of opioid receptors (Smotherman & Robinson, 1992a, 1992b, 1992c). Unlike postnatal rats, fetuses do not exhibit opioid responses to sugars, fats, or novel chemosensory fluids; apart from milk, the only chemosensory stimulus previously reported to evoke opioid activity in the fetus is dimethyl disulfide (Smotherman & Robinson, 1992a). The consequence of milk or DMDS-induced opioid activity in both the fetus and neonate is reduced responsiveness to cutaneous stimulation. Neonatal rats exhibit longer latencies to withdraw a paw from a thermal stimulus after intraoral infusion of milk or sucrose (Blass, Jackson, & Smotherman, 1991), whereas fetal rats show reduced facial wiping responses to a stiff bristle applied to the perioral area (Smotherman & Robinson, 1992b). The effectiveness of milk to reduce these measures of fetal responsiveness is blocked when subjects are pretreated with the opioid antagonist naloxone (fetus and neonate), or with the selective antagonist nor-binaltorphimine (fetus), which confirms the involvement of the kappa opioid system.

The results of Experiments 1 and 3 extend previous

work by demonstrating that milk also is effective in reducing fetal responses to stimuli in a chemosensory modality. Intraoral infusion of a lemon odor solution consistently evokes a series of bilateral facial wiping strokes, which are similar in form to the single unilateral wiping stroke that is evoked by a perioral cutaneous stimulus (Smotherman & Robinson, 1992b). However, because the lemon infusion typically evokes 5–10 wiping strokes, it provides a continuous measure of fetal responsiveness in lieu of the all-or-none measure of responsiveness that is afforded by perioral cutaneous stimulation. In the present study, this continuous measure of fetal response revealed that milk reduces but does not eliminate the facial wiping response to lemon (e.g., Figure 1). Administration of the kappa opioid agonist U50,488 also reduced lemon-evoked facial wiping in a dose-dependent fashion, and naloxone was effective in blocking the effect of milk, thereby reinstating high levels of fetal response to the lemon stimulus (Figures 2 and 3). Taken together, these findings provide confirmation that fetal behavioral responses to a range of sensory stimuli can be altered by activation of the kappa opioid system.

Other methods of measuring the behavioral effects of opioid activity in neonatal and adult rats have tended to emphasize the antinociceptive or analgesic properties of opioid activity. For example, exogenous opioid agonists, such as morphine, increase the latency to withdraw a paw from a thermal stimulus, suggesting a decrease in pain sensitivity (Blass, Cramer, & Fanselow, 1993; Blass et al., 1991; Giordano & Barr, 1987). The perioral stimulus used to evoke fetal responses in previous studies of milk-induced opioid activity—namely, a stiff bristle applied to the lateral vibrissal pad—also is a cutaneous stimulus, although not necessarily nociceptive. However, the demonstration that milk and amniotic fluid reduce fetal responses to a complex chemosensory fluid such as lemon extract suggests that nociception may not be the only sensory modality that is affected by opioid activity. Rather, endogenous opioid activity appears to exert a more general influence on sensory, motor, and associative behavioral processes. This interpretation agrees with a spectrum of other behavioral effects associated with opioid activity in the fetus, including reorganization of spontaneous motor activity (Smotherman & Robinson, 1992c), expression of species-typical suckling behavior (Anderson et al., 1993), and facilitation of associative learning in both the fetus and neonatal rat (see reviews by Johanson & Terry, 1988; Kehoe, 1988; Robinson & Smotherman, 1995). Thus, apart from the specific effects of opioids on nociception, evidence suggests that the fetal opioid system may play a more general role in regulating the organization

of behavior and responses and in many sensory modalities.

The fetus, of course, is not exposed to milk during normal development in utero. Experiments 4–6 of the present study demonstrate that comparable effects on fetal chemosensory responses occur when the fetus is externalized from its own amniotic sac and reexposed to amniotic fluid. AF collected on Days 20 or 21 of gestation was effective in reducing the facial wiping response of the E20 rat fetus, but AF collected on Day 19 exerted no appreciable effect on fetal behavior. Pretreatment of subjects with the opioid antagonist naloxone prevented AF-induced changes in fetal response, implying that exposure to AF results in opioid activity. Further, the selective kappa antagonist BNI also blocked AF effects on fetal responding, but the mu antagonist CTOP was not effective. These results indicate that, like milk, AF can evoke activity at kappa receptors of the endogenous opioid system of the rat fetus (Smotherman & Robinson, 1992b). However, the ability of AF to evoke activity in the opioid system is dependent on the gestational age of the fluid, suggesting that some change in the composition or physical characteristics of AF late in gestation may be responsible for triggering an opioid response in the fetus. Opioid compounds, notably B-endorphin (Mauri & Volpe, 1994; Moss, Conner, Yee, Iorio, & Scarpelli, 1982), have been identified in AF. However, one would expect endorphins to exert effects principally at mu opioid receptors, not kappa, as was found in this study. Intraoral infusion of milk also has been shown to evoke kappa opioid responses in fetal subjects with an esophageal ligature, thus preventing milk from being ingested (Robinson & Smotherman, 1994). This finding suggests that orosensory characteristics of milk (and possibly AF) may be sufficient to elicit kappa opioid responses. It is possible that an opioid-compound or opioid-inducing factor is absent in AF on Day 19 of gestation in rats, or alternatively, that the factor exists at a concentration too low to effectively engage fetal behavior.

In part, the effect of milk-induced opioid activity to reduce the number of wiping strokes appears to be due to an increased latency to initiate a facial wiping response. Data from Experiments 1 and 3 indicated that fetal wiping responses were delayed by several seconds after exposure to milk. A comparable increase in latency to first wiping stroke was evident after fetal exposure to AF collected on E20 or E21 of gestation (Experiments 4–6). The finding of longer latencies to first wipe is consistent with a body of evidence on the opioid mediation of antinociception in infant and adult animals. For example, exposure to milk results in activity at opioid receptors that increases the latency for

an infant rat to withdraw a paw from a heated surface (Blass et al., 1991; Blass & Fitzgerald, 1988). However, increased latency to the initial wiping stroke also has been reported in previous studies of facial wiping behavior in rodent fetuses. In a developmental study of facial wiping in fetal rats, detailed examination of videotape records revealed that E19 rat fetuses perform forelimb strokes that are poorly coordinated and require more time to establish paw–face contact than E20 fetuses (Robinson & Smotherman, 1991). Precocial rodent species, such as cotton rats (*Sigmodon hispidus*) also show delayed onset of facial wiping strokes when the coordination of this response is disrupted by competing motor behavior (Robinson & Smotherman, 1992b). These contrasting examples illustrate that the reduction in wiping strokes and increased latency to the first stroke may be attributed either to reduced sensory responsiveness or diminished ability to coordinate limb strokes elicited by the lemon test stimulus. The data reported in the present study do not enable us to distinguish between these two competing explanations for the mechanism of kappa opioid action in modulating fetal wiping responses to lemon.

Although it may be a remarkable coincidence, the ability of AF and milk to evoke opioid activity and exert similar effects on fetal responses suggests a developmental link between these two biologically essential fluids. Immediately after birth, newborn mammals must recognize and ingest milk without rejecting milk, the nipple, or other features of the suckling context as novel stimuli. But the newborn has no opportunity for direct experience with milk before birth. This developmental paradox may be resolved by fetal exposure to AF. Fetuses are continually surrounded by and exposed to AF in utero. If prenatal exposure to AF can promote endogenous opioid activity, it may result in a steady state of activation in the kappa opioid system during the 24–48 hr prior to birth. In turn, AF-induced opioid activity could contribute to the transition of the fetus to postnatal life by facilitating the development of components of suckling behavior in advance of birth. Whether the opioid response evoked by AF serves to promote continuity between prenatal and postnatal behavior, or plays a more immediate role in facilitating other aspects of prenatal development, remains to be determined.

## NOTES

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