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Conditioned Opioid Activity in the Rat Fetus

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Classical conditioning in the rat fetus (Embryonic Day 20) was investigated in 4 experiments. Reexposure to a conditioned stimulus (CS; sucrose), after 3 pairings with an unconditioned stimulus (US; milk), reduced fetal facial wiping in a bioassay of perioral cutaneous responsiveness. Reduced responsiveness was evident only in subjects that received paired presentations of the CS and US and cannot be attributed to habituation, sensitization to the CS, or protracted effects of US exposure during conditioning trials. Fetuses attended to the chemosensory, not the tactile, qualities of the sucrose infusion during CS reexposure. Changes in fetal responsiveness resulted from conditioned activity in the endogenous opioid system, specifically at mu opioid receptors. These data confirm that the rat fetus is capable of exhibiting a conditioned opioid response in utero.

One of the most important behavioral events initiated by the infant is locating a nipple, attaching to it, and withdrawing milk, collectively known as suckling (Brake, Shair, & Hofer, 1988). Many of the behavioral components involved in this complex task, such as approaching the nipple and withdrawing milk, can be conditioned in the infant rat pup (Pedersen, Williams, & Blass, 1982). Rat pups also can learn to approach stimuli that have become a signal for the presence of the mother (Brake, 1981; Sullivan, Brake, Hofer, & Williams, 1986). Suckling in the absence of milk has been demonstrated to be an effective reinforcer for the neonate. Pups will learn to traverse a Y maze or will persist in running down an alley for access to a nonlactating nipple by Postnatal Day 7 (Amsel, Burdette, & Letz, 1976; Kenny & Blass, 1977). However, the 7-day-old pup has had literally hundreds of pairings of the nipple with a biologically important stimulus: milk. In fact, pups are able to learn a preference for a previously aversive odor paired with suckling, and this conditioned preference is enhanced by pairing the odor with suckling and milk (Brake, 1981; Brake et al., 1988).

Infusion of milk into the mouth also can serve as an effective reinforcer in infant rats. For example, rat pups can learn to lift their head into a paddle for an infusion of milk within 12 hr after birth (Johanson & Hall, 1979). Milk can support the conditioning of consummatory behavior such as mouthing to a novel odor paired with milk (Johanson, Hall, & Polefrone, 1984). Milk also elicits a number of behavioral changes in the neonatal and fetal rat, including behavioral activation, reorganization of motor activity, expression of stereotypic action patterns, and reduction of sensory responsiveness to a perioral probe (Hall, 1979; Smotherman & Robinson, 1992d). The neonate expresses adaptive behavior at birth for obtaining and responding to milk, implying the development of these behavioral capacities during the prenatal period.

Milk has been shown to activate the endogenous opioid system in perinatal rats. Intraoral infusion of milk results in reduced cutaneous responsiveness to thermal or tactile stimuli in both neonatal and fetal rats (Blass, Jackson, & Smotherman, 1991; Smotherman & Robinson, 1992b). Milk-induced changes in cutaneous responsiveness are reversible with naloxone, confirming opioid involvement. In the fetal rat, which lacks suckling experience, milk infusion promotes activity in the kappa opioid system (Smotherman & Robinson, 1992b). The rewarding properties of milk that support associative learning early in development may be related to milk's ability to engage the endogenous opioid system of the fetus and neonate (Blass, 1990).

Morphine and other agonists that act on the mu opioid system can support conditioned taste preferences in adult rats when given in a relatively low dose; higher doses, however, result in conditioned taste aversion (Lett & Grant, 1989; Mucha & Herz, 1985). A similar finding has been reported in 5-day-old rat pups, which develop a preference for taste or odor cues that have been paired with a low dose of morphine (Blass & Kehoe, 1987). The expression of these conditioned preferences may be associated with opioid activity that occurs when the pup is reexposed to the conditioned stimulus (CS). Rat pups that were treated with morphine to establish an odor preference exhibited longer latencies to withdraw a forepaw from a heated surface upon reexposure to the odor. The effect of the odor on paw withdrawal was blocked with naloxone, indicating that CS reexposure resulted in conditioned opioid activity (Kehoe & Blass, 1989).

Our knowledge about milk's ability to support associative learning early in development derives from experiments using subjects at least 1 day after birth. However, the neonate gains considerable experience with milk and contingencies involving milk in the first 24–48 hr after birth (Brake et al., 1988). We conducted the present study with rat fetuses, which lack a history of reinforcement with milk and stimuli associated with

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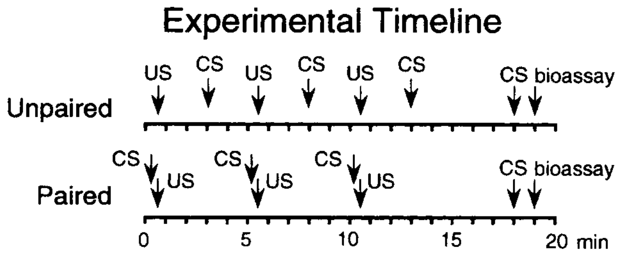

Figure 1. Example of experimental timelines for subjects receiving Unpaired and Paired presentations of conditioned stimulus (CS) and unconditioned stimulus (US) during conditioning trials. (Experimental stimuli consisted of intraoral infusion of sucrose [CS] and milk [US]. Fetal subjects in the Unpaired group received the US followed 2.5 min later by the CS in each of three conditioning trials; subjects in the Paired group received the CS followed 15 s later by the US in each of three trials. In Experiment 1a, fetuses in the CS–Sal group received infusions of sucrose and saline on the same schedule as the Paired group; fetuses in the US-alone group received milk infusions on the same schedule as US presentations in the Unpaired and Paired groups. After a delay of 7.5 min after the last US [Experiments 1a and 1b] or 9.5 min [Experiments 2a and 2b], subjects were reexposed to the CS and 1 min later were exposed to a perioral tactile probe to assess cutaneous responsiveness. In Experiments 2a and 2b, injection of opioid antagonists or saline occurred 5 min before CS reexposure.)

milk, to determine whether milk can support associative learning and conditioned changes in opioid activity. These experiments were conducted with a protocol that permits training and testing of fetal subjects and manipulation of opioid activity within a single session on Embryonic Day 20 (E20; Smotherman & Robinson, 1991b).

General Method

Subjects

Subjects were fetuses of Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA), which were bred in groups of three in plastic breeding cages (36 × 47 × 20 cm). Room temperature remained constant (22 °C) during a 12-hr light–dark cycle with light onset at 8:30 a.m. Food and water were available ad libitum. Vaginal smears were collected daily to determine conception, with the detection of sperm designated as E0. All animals were maintained according to guidelines for animal care established by the National Institutes of Health (1986). To avoid potential confounding of litter and treatment effects, all experimental groups were represented by a single subject within each pregnancy when feasible, and no more than 2 subjects per pregnancy were assigned to a given experimental group. The order of testing each group was counterbalanced within each experiment. A total of 239 fetuses that derived from 82 pregnancies were used as subjects.

Preparation of Fetal Subjects

All fetuses were conditioned and tested during a single session on E20. Direct observation of fetal behavior was made possible by surgical preparation of the pregnant rat and the uterine environment (Smotherman & Robinson, 1991a). Pregnant rats were placed under brief ether anesthesia and received a 100-μl injection of 100% ethanol between the first and second lumbar vertebrae. This method of spinal preparation results in an irreversible blockade of neural transmission at the low thoracic level and eliminates sensation in the lower portion of the rat's body. The pregnant rat was placed in a Plexiglas holding apparatus and immersed to chest depth in a buffered isotonic saline thermoneutral bath (37.5 °C). We externalized the uterus through a low midline incision and delivered individual fetuses from the uterus and amniotic sac into the bath, taking care to preserve the blood circulation in the umbilical cord and the placental attachment to the uterus. Only fetuses that remained fully oxygenated were tested during the course of these experiments. A 20-min period elapsed before the onset of the first observation session to provide time for the pregnant rat and subject fetuses to accommodate to the bath environment.

Stimulus Presentation

Each fetal subject was fitted with a dual cannula constructed from PE-10 polyethylene tubing (Smotherman & Robinson, 1991b), with a flanged tip installed on the midline of the tongue in a midanterior position similar to that described by Hall and Rosenblatt (1977). The two channels of the dual cannula permit independent delivery of chemosensory solutions that serve as the conditioned stimulus (CS) and unconditioned stimulus (US). Each channel of the cannula was connected to a Gilmont micrometer syringe (Barrington, IL) containing one of three solutions: isotonic saline, sucrose (10% weight/weight in isotonic saline; the CS), or milk (commercially available bovine light cream; the US). Each solution was delivered as a 20-μl infusion in a 1–2-s pulse directly into the mouth of the fetus. This protocol permitted precise (±1 μl) delivery of stimulus solutions to the fetus without otherwise interrupting fetal activity.

Conditioning Protocol and Drug Manipulations

Conditioning trials and behavioral testing occurred in a single 20-min (Experiment 1a) or 22-min (Experiments 1b, 2a, and 2b) session. The first 13 min of the observation session comprised a series of three conditioning trials. During these trials, fetal subjects received three presentations of the sucrose (CS) and/or the milk (US). Subjects in the experimental group (Paired condition) received an infusion of the CS followed 15 s later by infusion of the US in each of the three trials. Subjects in control conditions received an infusion of sucrose followed by an infusion of saline (CS–Sal condition), an infusion of milk without other infusion (US-alone condition), or explicitly unpaired presentations in which infusion of the US was followed 2.5 min later by infusion of the CS (Unpaired condition). The parameters of stimulus presentation were based on previous studies of fetal learning that demonstrated that the fetal rat is capable of learning an aversion to sucrose after pairing with a chemosensory stimulus (a lemon infusion) that increases motor activity (Smotherman & Robinson, 1991b). Conditioning trials were scheduled at 5-min intervals; timelines for the Paired and Unpaired groups are depicted in Figure 1. After the third US presentation, a 7.5-min (Experiment 1a) or 9.5-min (Experiments 1b, 2a, and 2b) delay was introduced before reexposure to the CS.

Drug manipulations (Experiments 2a and 2b) involved a single 50-μl ip injection administered after the last conditioning trial, 5 min before reexposure to the CS. Drugs used to manipulate opioid activity included naloxone hydrochloride (NAL, 1.0 mg/kg; Sigma Chemical Co., St. Louis, MO), the kappa opioid antagonist nor-binaltorphimine dihydrochloride (BNI, 9.2 mg/kg; Research Biochemicals, Natick, MA), and the mu opioid antagonists β-funaltrexamine hydrochloride (FNA, 1.0 mg/kg; Research Biochemicals) and the somatostatin analog [Cys,^{2}Tyr,^{3}Orn,^{5}Pen^7]-amide (CTOP; 6.0 mg/kg; Peninsula Labs, Belmont, CA). Administration of BNI and CTOP to fetal subjects in this dosage has been reported to reverse the behavioral effects of agonists selective for kappa and mu opioid receptors, respectively (Smotherman, Simonik, Andersen, & Robinson, 1993). The effectiveness of FNA to block activity at mu opioid activity has been suggested by previous experiments (Smotherman & Robinson, 1992c) and was confirmed in a preliminary experiment. In a dosage of 1.0 mg/kg, FNA antagonized the effects of the mu-selective agonist [D-Ala,^{2}N-Me-Phe,^{4}Gly^5-ol]-enkephalin (DAMGO). DAMGO typically eliminates fetal facial wiping in a bioassay of perioral cutaneous responsiveness (see *Assessment of Conditioning and Data Analysis* section); subjects pretreated with FNA and DAMGO (1.0 mg/kg) 8 min before the bioassay exhibited high responsiveness (10 of 15 subjects), whereas subjects receiving a control injection of isotonic saline with DAMGO continued to exhibit low responsiveness (1 of 15 subjects), $\chi^2(1, N = 30) = 9.2, p < .005$. These data indicate that FNA acts as a mu antagonist in the fetal rat within minutes of ip administration.

Assessment of Conditioning and Data Analysis

Testing took place during the final 2 min of the observation session and was conducted by two persons, an observer and a recorder. The recorder programmed experimental conditions, monitored the schedule of CS and US presentations, and served to keep the observer unaware of drug treatments. Except as required by specific testing procedures in Experiment 1b, each fetus was reexposed to the CS (sucrose) followed 1 min later by assessment of fetal behavior in a bioassay of perioral cutaneous responsiveness. The bioassay consisted of application of a stiff bristle (3.7-g force Von Frey probe) to the lateral vibrissal pad near the corner of the mouth twice in rapid succession. The probe reliably elicits a facial wiping response in unmanipulated fetal subjects on E20 of gestation. This bioassay is sensitive to endogenous opioid activity and exogenous opioid manipulations; facial wiping to the probe is virtually eliminated after intraoral infusion of milk, which promotes kappa opioid activity (Smotherman & Robinson, 1992b), or is reduced in a dose-dependent fashion after administration of various opioid agonists (Smotherman et al., 1993). The presence or absence of facial wiping was assessed during the 1-min interval after application of the perioral probe. Facial wiping was defined as placement of one or both forepaws in contact with the head and movement of the paws, in a rostral direction, in contact with the face (Robinson & Smotherman, 1991). The incidence of facial wiping (number of subjects in each group that exhibited a wiping response in the bioassay) was compared across the various experimental conditions by nonparametric chi-square tests. An experimentwide analysis was conducted to assess changes in the likelihood of fetal subjects to express the wiping response. Where the overall analysis was significant, post hoc comparisons of individual groups were conducted. The level of significance in all tests was set at $p < .05$ (two-tailed).

Experiment 1a: Conditioned Changes in Fetal Response

The purpose of Experiment 1a was to determine whether milk can function as a reinforcer and support conditioned changes in sensory responsiveness in the E20 rat fetus. Associative learning in the fetal rat was demonstrated previously in a single-session paradigm using an artificial US (lemon infusion; Smotherman & Robinson, 1991b). In the present experiment, a sucrose infusion was paired with an appetitive reinforcer (milk) in a series of three trials. Changes in fetal responsiveness in the bioassay were assessed after reexposure to the CS. If contingent presentations of the CS and US result in conditioning, then subjects in the Paired group should express a reduced incidence of facial wiping relative to control subjects in the CS–Sal, US-alone, and Unpaired conditions.

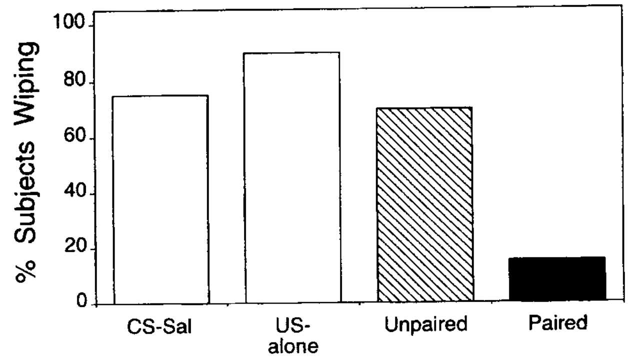

Figure 2. Percentage of fetal subjects that expressed a facial wiping response in the bioassay of cutaneous responsiveness after reexposure to sucrose (the conditioned stimulus; CS) in Experiment 1a. (CS-Sal = group receiving infusions of sucrose and saline. US-alone = group receiving milk infusions. Subjects were divided into paired and unpaired groups according to CS and US presentation.)

Method

Eighty fetal subjects from 20 pregnant rats were assigned to one of four conditioning groups ($n = 20$ per group)—CS–Sal, US-alone, Unpaired, or Paired—which differed in the infusions delivered over the three conditioning trials. After a delay of 5 min, all subjects were reexposed to the CS. Fetal responsiveness in the bioassay of perioral cutaneous sensitivity was assessed 1 min after CS reexposure.

Results and Discussion

The overall chi-square analysis indicated a significant effect of conditioning group, $\chi^2(3, N = 80) = 27.5, p < .001$. Post hoc comparisons revealed that facial wiping was expressed by fewer subjects in the Paired group than in the CS–Sal group, $\chi^2(1, N = 40) = 14.5, p < .001$, the US-alone group, $\chi^2(1, N = 40) = 22.6, p < .001$, or the Unpaired group, $\chi^2(1, N = 40) = 12.4, p < .001$. The incidence of facial wiping in the bioassay, expressed as a percentage of the subjects tested, is shown in Figure 2.

This result confirms that contingent presentations of sucrose with milk result in changes in fetal behavior after reexposure to sucrose. Subjects in the CS–Sal and US-alone groups exhibited facial wiping in response to the perioral tactile probe, indicating that cutaneous sensitivity was not affected by sensitization or habituation to the CS or US. Fetuses that received unpaired presentations of the US and CS also exhibited the wiping response. Because control subjects in the US-alone and Unpaired groups were exposed to milk but continued to express facial wiping in the bioassay, changes in fetal responsiveness during testing could not have been due to protracted effects of milk infusion. Furthermore, the results are consistent with a previous report that sucrose itself has no effect on this measure of fetal responsiveness (Smotherman & Robinson, 1992a). Therefore, the contingency between the CS and US during conditioning trials was responsible for the change in responsiveness to the probe observed in the Paired group.

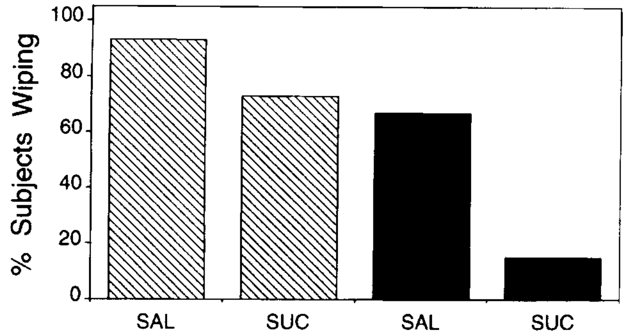

Figure 3. Percentage of fetal subjects that expressed a facial wiping response in the bioassay after reexposure to saline (SAL) or sucrose (SUC) in Experiment 1b. (Hatched bars = fetuses in groups that received Unpaired presentations of the unconditioned and conditioned stimuli during conditioning trials. Solid bars = fetuses in Paired groups.)

Conditioned changes in fetal cutaneous responsiveness in Paired subjects resemble the reduction in responsiveness produced by a single infusion of milk in the E20 rat fetus (Smotherman & Robinson, 1992b).

Experiment 1b: Specificity of the CS

Intraoral infusion of a chemosensory solution presents stimulation in multiple modalities to the fetus (Smotherman & Robinson, 1990). It is possible that the critical component of the CS that evokes the conditioned change in perioral sensitivity is the tactile experience of the infusion rather than chemosensory aspects of the sucrose. The ability of a tactile stimulus to serve as a CS has been observed in other experiments using an artificial nipple as the CS (Robinson, Arnold, Spear, & Smotherman, 1993). The aim of Experiment 1b was to determine whether changes in cutaneous responsiveness after representation of the CS are evoked by the tactile or chemosensory properties of the CS (sucrose).

Method

Sixty fetal subjects from 15 pregnancies were assigned to four experimental groups (n = 15 per group), which derived from the combination of treatment during conditioning trials (Paired or Unpaired) with stimulus presented during testing (sucrose or saline). Paired and Unpaired presentations of sucrose (CS) and milk (US) occurred in a series of three conditioning trials, as in Experiment 1a. A 7-min delay was introduced after the last trial before testing. Subjects received an infusion of sucrose or saline at the beginning of testing, and perioral cutaneous sensitivity was measured in the bioassay 1 min later.

Results and Discussion

The overall analysis indicated significant differences in the expression of facial wiping during testing, $\chi^2(3, N = 60) = 22.2, p < .001$. The incidence of facial wiping in the bioassay is shown in Figure 3. The wiping response was expressed by most fetuses in the Unpaired groups after infusion of sucrose or saline. Post hoc comparisons revealed that facial wiping was expressed by fewer subjects in the Paired sucrose group than in the Unpaired sucrose group, $\chi^2(1, N = 30) = 11.0, p < .001$. The incidence of wiping in the Paired saline group did not differ from that in the Unpaired saline group, $\chi^2(1, N = 30) = 3.33, p > .05$. Comparison of fetuses in the two Paired groups indicated that reexposure to sucrose resulted in a significant reduction in facial wiping relative to infusion of saline, $\chi^2(1, N = 30) = 8.89, p < .005$. These findings indicate that the chemosensory properties of the CS, rather than the tactile sensation provided by intraoral infusion, evoked the conditioned changes in perioral cutaneous responsiveness during testing.

Experiment 2a: Role of Opioids in Conditioning

In Experiment 1a, the contingent presentations of the CS and US resulted in a decrease in facial wiping after reexposure to the CS. Experiment 1b showed that this altered responsiveness was specific to the chemosensory aspects of the CS. Similar changes in sensory responsiveness after intraoral infusion of milk have been found to be mediated by the endogenous opioid system of the fetus (Smotherman & Robinson, 1992b). It is possible that reexposure to a CS that previously was associated with milk results in conditioned activation of the endogenous opioid system. The purpose of Experiment 2a was to assess the role of endogenous opioids in mediating conditioned changes in perioral cutaneous sensitivity in the E20 rat fetus. If reexposure to the CS results in conditioned opioid activity, then naloxone administered before testing should block the expression of a conditioned response and promote facial wiping in the Paired group.

Method

Fifty-four fetal subjects from 15 pregnancies were assigned to four experimental groups derived from the combination of treatment during conditioning trials with drug treatment during the delay before testing: Paired naloxone (n = 15), Paired saline (n = 15), Unpaired naloxone (n = 12), and Unpaired saline (n = 12). Paired and Unpaired presentations of sucrose (CS) and milk (US) occurred in a series of three conditioning trials, as in Experiment 1a. A 7-min delay was introduced after the last trial, and one of two drug treatments (naloxone or saline vehicle) was administered in a 50-μl ip injection 5 min before reexposure to the CS. Naloxone was administered after training to avoid interference with transient episodes of opioid activity evoked by milk infusions during conditioning trials (Smotherman & Robinson, 1992b). The purpose of naloxone administration was to block conditioned changes in opioid activity after reexposure to the CS. All fetal subjects were reexposed to sucrose, and perioral cutaneous responsiveness was measured in the bioassay 1 min later.

Results and Discussion

The overall analysis indicated that the expression of facial wiping in the bioassay differed among the four experimental groups, $\chi^2(3, N = 54) = 25.9, p < .001$ (Figure 4). Fetuses in the Paired saline group seldom exhibited the wiping response, whereas subjects in the Unpaired saline group consistently expressed facial wiping, $\chi^2(1, N = 27) = 16.2, p < .001$. The vehicle control groups confirmed that paired presentations of the CS and US during conditioning trials produced a conditioned change in fetal responsiveness to the perioral probe. Naloxone administration resulted in an increase in the number of fetuses that exhibited wiping in the bioassay, as revealed in a comparison of the Paired saline and Paired naloxone groups, $\chi^2(1, N = 30) = 16.4, p < .001$. The incidence of facial wiping by fetuses in the Unpaired naloxone and Unpaired saline groups was identical, suggesting that this opioid antagonist had no effect on the expression of facial wiping behavior in the absence of conditioning. The results of Experiment 2a suggest that reexposure to the CS resulted in conditioned activation of the endogenous opioid system, which reduced fetal responsiveness in the bioassay.

Experiment 2b: Conditioned Changes in Mu or Kappa Opioid System?

The ability to block conditioned changes in perioral responsiveness with naloxone indicates that reexposure to the CS resulted in an increase in opioid activity. The first presentation of milk to the fetus has been demonstrated to promote activity at the kappa, but not mu, class of opioid receptors (Smotherman & Robinson, 1992b). One might expect that sucrose would promote conditioned changes in activity at the same opioid receptors that are engaged by milk. In this way, sucrose may simply serve as a substitute for milk after conditioning. However, identical patterns of response to the US and CS are not a necessary feature of classical conditioning (Eikelboom & Stewart, 1982; Rescorla, 1988). The purpose of Experiment 2b was to characterize the conditioned opioid response by determining the classes of opioid receptors that are activated after reexposure to the CS. Opioid activity at mu or kappa receptors was manipulated by administration of selective receptor antagonists before CS reexposure. If reexposure to the CS results in conditioned opioid activity at one class of opioid receptors, administration of either selective kappa or mu opioid antagonists before testing should block the expression of a conditioned response and promote facial wiping.

Method

Forty-five fetal subjects from 32 pregnancies were assigned to three drug treatment groups ($n = 15$ per group). All subjects received a series of three Paired presentations of the CS and US during conditioning trials. Five minutes before CS reexposure, each subject received an ip injection of BNI to block kappa opioid activity or FNA or CTOP to block mu opioid activity. The responsiveness of all subjects to the perioral probe was assessed 1 min after reexposure to the CS.

Results and Discussion

The overall analysis indicated that the expression of facial wiping in the bioassay differed among the three groups, $\chi^2(2, N = 45) = 20.6, p < .001$ (Figure 4). After reexposure to the CS, few fetuses treated with BNI exhibited facial wiping to the perioral tactile probe. The wiping response frequently occurred after injection of FNA and CTOP, however. The incidence of facial wiping differed between the BNI and FNA groups, $\chi^2(1, N = 30) = 16.1, p < .001$, and between the BNI and CTOP groups, $\chi^2(1, N = 30) = 13.4, p < .001$. Administration of selective mu antagonists therefore eliminated the

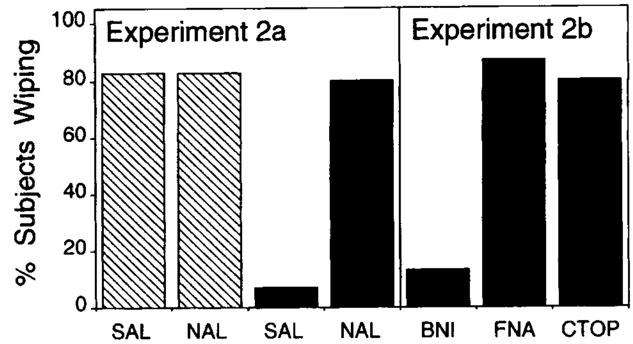

Figure 4. Percentage of fetal subjects that expressed a facial wiping response in the bioassay after opioid treatment and reexposure to sucrose (conditioned stimulus; CS) in Experiments 2a and 2b. (Hatched bars = fetuses in Unpaired presentations of the unconditioned stimuli [US] and CS during trials. Solid bars = fetuses in Paired groups. Fetal subjects in different groups received an injection of SAL [saline vehicle control], NAL [nonselective opioid antagonist], BNI [kappa antagonist], FNA [mu antagonist], or CTOP [mu antagonist] before reexposure to sucrose. NAL = naloxone hydrochloride. BNI = nor-binaltorphimine dihydrochloride, FNA = β-funalhexamine hydrochloride. CTOP = [Cys,^{2}Tyr,^{3}Orn,^{5}Pen^7]-amide.)

conditioned response, whereas the kappa antagonist had no effect on the conditioned reduction in facial wiping. Thus reexposure to sucrose after a series of CS–US pairings promoted a conditioned increase in activity at mu opioid receptors, which resulted in changes in facial wiping to a perioral cutaneous stimulus.

Conclusion

The present four experiments provide a clear demonstration that contingent presentations of sucrose and milk can support classical conditioning in the E20 rat fetus. Fetal conditioning was inferred from a reduction in the facial wiping response to a perioral cutaneous stimulus after reexposure to sucrose. The reduction in responsiveness, which was evident only in subjects that received paired presentations of the CS and US, could not be attributed to habituation, sensitization to the CS, or protracted effects of US exposure during conditioning trials. Fetuses attended to the chemosensory, not the tactile, qualities of the sucrose infusion during CS reexposure. This demonstration of classical conditioning with a chemosensory CS complements other reports that fetal conditioning is effected by pairing an artificial nipple with infusion of milk (Robinson et al., 1993) and implies that the association of a CS with milk can be supported in both chemosensory and tactile modalities. The most noteworthy finding of the present experiments, however, is that pairing a neutral CS with another sensory stimulus that engaged the endogenous opioid system resulted in conditioned opioid activity in the rat fetus. Reexposure to sucrose promoted activity at the mu class of opioid receptors, but only if sucrose had been paired previously with milk. These data provide general support to the conclusion that the rat fetus is capable of acquiring and expressing associative learning in utero (Robinson & Smotherman, in press; Smotherman & Robinson, 1987).

A number of recent studies have documented that the endogenous opioid system of the fetal rat is functional late in gestation and can be accessed by pharmacological treatments (Smotherman, et al., 1993) or sensory manipulations (Smotherman & Robinson, 1992d). Specifically, infusion of milk into the mouth of the fetus results in a brief period of activity in the kappa opioid system, with no evidence of mu involvement. The ability of milk to promote kappa opioid activity is evident upon the fetus's first experience with this biologically important fluid (Smotherman & Robinson, 1992b). Sucrose has no effects on either the mu or kappa opioid system (Smotherman & Robinson, 1992a), but after pairing with milk the sucrose CS promotes activity at mu receptors, with no evidence of kappa involvement. Other learning studies conducted with adult subjects, particularly those experiments involving drug manipulations as the CS or US, have documented that the conditioned response need not resemble the unconditioned response in all details (Eikelboom & Stewart, 1982; Gallagar & Holland, 1992; Rescorla, 1988). However, it should be noted that the US in the present experiment was a sensory stimulus—milk—to which the newborn rat is exposed repeatedly under natural conditions. It remains unclear how the CS can come to promote activity at a different class of opioid receptors than the US.

Several alternative hypotheses may be offered to explain differences in the class of opioid receptors engaged by the CS and US. It is possible that milk infusion results in kappa opioid activity alone only during the fetus's first exposure to milk. Over a series of infusions, milk may come to promote activity in the mu system instead of or in addition to the kappa system. By this view, reexposure to the CS evokes opioid activity at the same class of receptor (mu) as is engaged by the US during the last conditioning trial. A second possibility is that milk continues to engage the kappa opioid system alone, but, through the associative process, the CS comes to potentiate activity in the mu system. After a series of conditioning trials, the mu opioid system may do more than modulate sensory responsiveness; it may play a more fundamental role in facilitating the expression of associative learning in the fetus. Speculation about the underlying neurobiology of learning in the fetus is beyond the scope of this article; however, either of the alternative explanations suggests that a limited number of experiences during the prenatal period may be sufficient to produce lasting changes in the organization of the nervous system and in neurochemical systems that mediate fetal responsiveness to sensory stimulation, fetal ability to acquire and express associative learning, or both. The study of classical conditioning of opioid responses in the rat fetus therefore may provide a useful model system for investigating the neurobiological substrates of associative learning.

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