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Central Administration of the Endopeptidase 24.15 Inhibitor cFP-AAF-pAB Suggests Dynorphin as the Endogenous Ligand Underlying Behavioral Effects of Milk in the Fetal Rat

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cFP-AAF-pAB Endopeptidase 24.15 Kappa Mu Delta Opioid Rat fetus Nor-binaltorphimine CTOP Naltrindole

OVER the past decade, the endogenous opioid system has been found to encompass an assortment of receptor classes and subtypes and their associated ligands. The mu, kappa, and delta systems, which preferentially bind endorphins, dynorphins, and enkephalins, respectively, are three important classes of opioid receptors for which effects on adult behavior have been demonstrated (10). Unlike classical neurotransmitters, such as acetylcholine or catecholamines, which influence neuronal activity on a time scale of milliseconds or seconds, the action of opioid compounds often is longer lasting, on the order of minutes or hours. Following their release at a synapse, the effects of endogenous opioid ligands is terminated by endopeptidases that degrade and inactivate the peptide (8,9). Synthetic compounds are now available that inhibit the

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activity of specific endopeptidases, with the functional effect of prolonging the action of the endogenous ligand at the receptor (8,9).

In vitro binding studies have indicated that receptors of these three opioid systems develop before birth (mu and kappa) or soon after birth (delta) in rats (2,3). Pharmacological manipulation of opioid receptors in fetal rats has confirmed that the mu and kappa systems exert an influence on behavior before birth (21,26). On the last 2 days of gestation (E20–E21), administration of agonists that selectively bind to mu (morphine or DAMGO) or kappa receptors (U50,488 or U69,593) markedly suppress responsiveness of rat fetuses in a behavioral bioassay involving perioral cutaneous stimulation (26). These agonist effects are receptor mediated, as demonstrated by the ability of specific antagonists (CTOP for mu receptors; nor-binaltorphimine for kappa receptors) to reinstate fetal responsiveness in the bioassay after agonist administration (15,24,26). These experimental findings indicate that opioid activity during the prenatal period can alter sensory or motor systems to bring about changes in fetal behavior (1, 21–23).

Sensory manipulations also can bring about changes in cutaneous responsiveness in the fetal rat. Intraoral infusion of a small volume of milk into the mouth of the fetus abolishes the facial wiping response typically elicited by a perioral cutaneous stimulus (21). The facial wiping response, which involves unilateral contact of the forepaw with the stimulated side of the face (19), is suppressed 60 s after delivery of milk, remains reduced 3 min after infusion, and recovers to preinfusion levels by 5 min (19). Milk appears to exert its effects on fetal behavior through activation of the endogenous opioid system: the facial wiping response is expressed if subjects are treated with an opioid antagonist prior to infusion of milk. Specifically, antagonism of the kappa opioid system, but not the mu system, blocks the behavioral effects of milk and permits expression of facial wiping in the bioassay of perioral cutaneous responsiveness (21). These findings, together with data from agonist experiments, suggest that milk engages the kappa opioid system of the fetus to bring about changes in sensory and motor behavior (16,18,21,22).

The objective of the present study was to employ an alternative strategy to confirm the nature of opioid involvement in the prenatal behavioral effects produced by milk. Fetal subjects were treated with a synthetic compound, *N*-(1-(RS)-carboxy-3-phenylpropyl]-Ala-Ala-Phe-p-aminobenzoate (cFP-AAF-pAB), that inhibits the action of endopeptidase 24.15 (EC3.4.24.15) to convert longer chain opioid compounds, including dynorphin A(1–8) and Met-enkephalin-Arg^6-Gly^7-Leu^8 (MERGL), to shorter chain enkephalins (8,9). Through inhibition of endopeptidase 24.15 activity, cFP-AAF-pAB has the effect of prolonging the action of dynorphin and MERGL at kappa and delta opioid receptors, respectively. If milk alters fetal responsiveness in the bioassay by promoting release of longer chain opioid compounds, then fetuses treated with cFP-AAF-pAB should continue to exhibit reduced responsiveness in the bioassay for an extended period of time after infusion. Prolongation of the period of reduced responsiveness after cFP-AAF-pAB administration specifically should implicate the involvement of dynorphin or MERGL in the effects produced by milk. Further, administration of selective opioid antagonists can provide information about which endogenous opioid compounds mediate the effects of milk. These predictions were evaluated in this experiment by using techniques that are well established in our laboratory, which permit administration of drugs and sensory stimuli to individual rat fetuses and direct observation of behavior on E20 of gestation.

METHOD

Subjects

Subjects were the fetuses of pregnant Sprague-Dawley rats (Charles River Laboratories, Wilmington, MA) time mated in our laboratory. A total of 23 pregnant rats provided 106 fetuses as subjects in this experiment. Adult female rats were maintained in groups of three in plastic breeding cages (36 × 47 × 20 cm) under conditions of constant room temperature (22°C), on a 12 L : 12 D cycle (lights on at 0700). Food and water were freely available. Daily vaginal smears during the 4-day breeding period were examined to date conception. The first day in which sperm were detected in a smear was designated embryonic day 0 (E0). Rats were maintained in accordance with guidelines for animal care established by the National Institutes of Health (PHS publication 86-23) and the International Society for Developmental Psychobiology.

Prenatal Preparation

Pregnant females were prepared for testing of fetuses on E20 (birth occurs on E21.5) (18). Under brief ether anesthesia, pregnant rats received a 100 μl injection of 100% ethanol between the first and second lumbar vertebrae. This procedure results in irreversible blockade of the spinal cord at the low thoracic level and eliminates sensation in the lower body of the rat. The pregnant rat was then placed in a holding apparatus and immersed to chest depth in a warm bath (37.5°C) containing buffered isotonic saline. Throughout the observation session, the activity of the pregnant rat was visually monitored to insure the completeness of the spinal preparation. To permit direct access to fetal subjects, the uterus was externalized by midline laparotomy and individual fetuses delivered from the uterus and amniotic sac into the bath, taking care to preserve the umbilical attachment of the fetus to the placenta and uterus. The condition of the fetus was monitored visually during the experiment; only fetuses that remained pink and apparently well oxygenated were used as subjects. A 20-min period elapsed before the onset of experiments to provide time for the pregnant rat and subject fetuses to accommodate to the bath environment.

Cannulation and Infusion

Controlled presentation of milk or isotonic saline into the mouth of individual rat fetuses was accomplished with an intraoral cannula (6). The cannula was fashioned from PE-10 polyethylene tubing (outer diameter = 0.61 mm) inserted through the midline of the lower jaw. The flanged tip of the cannula rested on the dorsal surface of the tongue in an anterior position. The free end of the cannula was connected to a micrometer syringe driven by a rotary syringe pump (7). This system enabled precise infusion (± 1 μl) of a chemosensory solution into the mouth without otherwise interrupting ongoing fetal activity. Infusions consisted of 20 μl of the isotonic saline or milk, which consisted of commercially available bovine light cream, delivered in a 2-s pulse. Test solutions were delivered at fetal body temperature.

Central Administration of Opioid Antagonists and Endopeptidase Inhibitor

Opioid receptor antagonists and the endopeptidase inhibitor cFP-AAF-pAB were administered directly into the central

nervous system of individual fetal subjects via intracisternal injection (IC). A 30 ga hypodermic needle attached to a length of transparent polyethylene tubing (PE-10) was inserted under visual guidance into the foramen magnum between the occipital bone and the first cervical vertebra, with the tip placed in the cisterna magna. Successful placement of the needle into the cisterna magna was immediately confirmed by the appearance of a small volume of cerebrospinal fluid in the tubing. Drug and vehicle solutions were injected slowly (8–10 s pulse) in a standard volume of 1.0 μl with the aid of a 0.2 ml micrometer syringe (Gilmont).

The following drugs were used in this experiment. *N*-(1-(RS)-carboxy-3-phenyl-propyl]-Ala-Ala-Phe-p-aminobenzoate (abbreviated EPI in this report, 15.6 μg per fetus) is an inhibitor of endopeptidase 24.15 (8,12,13). Norbinaltorphimine diHCl (BNI, 20.0 μg) is a kappa opioid antagonist (27). The somatostatin analog (Cys^2, Tyr^3, Orn^5, Pen^7]-Amide (CTOP, 12.5 μg) is an antagonist of mu receptors (Peninsula Labs, Belmont CA) (5). Naltrindole HCl (NTI, 6.3 μg) is an antagonist of delta receptors (Research Biochemicals Inc., Natick, MA) (14). Isotonic saline (SAL) was used as the vehicle in preparing these drug solutions. Dosages of BNI, CTOP, and NTI were based on effects of these compounds reported in previous studies of fetal behavior (15,16,18,21,23,24,28). Some experimental groups received a single IC injection of EPI, one of the three opioid antagonists, or saline. Other groups received an IC injection of EPI followed 2 min later by a second IC injection of one of the three opioid antagonists or saline.

Experimental Design and Behavioral Bioassay

Drug administration and behavioral assessment occurred in a 15-min (single IC injection) or 17-min (double IC injection) test session. Fetuses treated with a single IC injection were treated with saline, one of the three opioid antagonists, or EPI. Fetuses receiving two IC injections were treated with isotonic saline or an opioid antagonist followed 2 min later by injection of EPI. Subjects in all groups received a 20 μl intraoral infusion of milk 5 min after the last IC injection, and their behavior was scored in a behavioral bioassay 5 min and 10 min after infusion. These injection and infusion procedures resulted in the following experimental groups: SAL, EPI, BNI, CTOP, NTI, SAL + EPI, BNI + EPI, CTOP + EPI, and NTI + EPI. One additional control group received a single IC injection of EPI followed 5 min later by intraoral infusion of isotonic saline (EPI-saline).

The behavioral bioassay consisted of application of a cutaneous stimulus to the perioral region of the fetus and assessment of fetal behavioral response. The stimulus was presented by applying a von Frey filament (3.7 g pressure) to the lateral vibrissal pad near the corner of the mouth twice in rapid succession (19). Fetal subjects that are otherwise unmanipulated consistently express a facial wiping response to this cutaneous stimulus, with most subjects exhibiting a single paw–face stroke. Facial wiping was defined as the placement of one forepaw in contact with the side of the face and movement of the forelimb in a rostral direction. This simple action pattern is stereotypic in form, is readily distinguished from general forelimb movements, and occurs rarely during nonevoked fetal activity (17). The presence or absence of a facial wiping response in this behavioral bioassay was scored 5 min and 10 min after intraoral infusion. Statistical comparisons of the number of subjects exhibiting a wiping response were conducted with nonparametric chi-square tests of independence. An alpha level of $p < 0.05$ was used in all tests to judge significant differences among treatment groups.

RESULTS

The expression of facial wiping by fetal subjects in the bioassay of perioral cutaneous responsiveness is summarized in Fig. 1. An overall chi-square analysis comparing the incidence of facial wiping in the bioassay administered 5 min after intraoral infusion indicated significant differences among the 10 experimental groups, $\chi^2(9) = 77.0$, $p < 0.001$. A series of simplified chi-square tests were conducted to address specific experimental questions and patterns of difference among groups. Comparison of responsiveness in the SAL and EPI groups indicated that milk infusion had little effect on the wiping response 5 min after infusion, but treatment with EPI before milk infusion significantly reduced facial wiping behavior, $\chi^2(1) = 14.1$, $p < 0.001$. The effect of EPI on wiping in the bioassay was dependent upon subjects receiving milk;

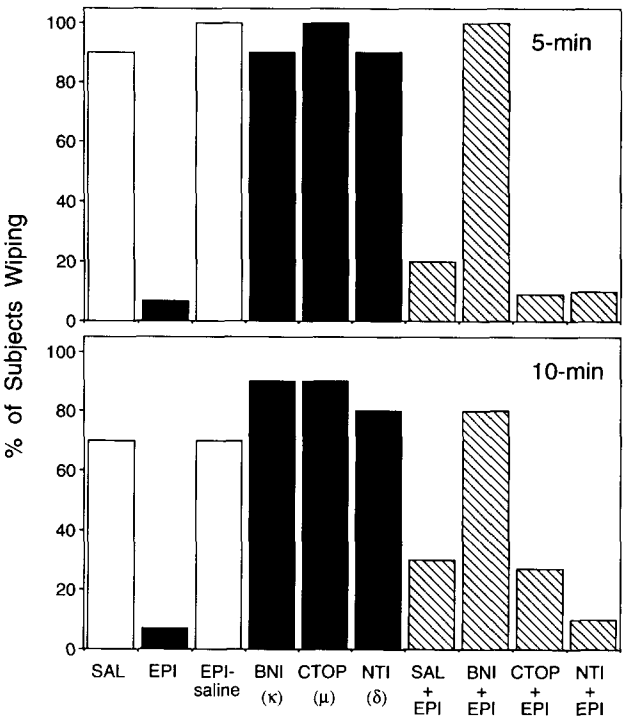

FIG. 1. Percentage of fetal subjects exhibiting facial wiping in the bioassay of perioral cutaneous responsiveness. Subjects were treated by intracisternal injection of saline (SAL), the endopeptidase inhibitor cFP-AAF-pAB (EPI), one of three receptor specific opioid antagonists (BNI: κ, CTOP: μ, NTI: δ), or combined administration of EPI with an opioid antagonist. Perioral responsiveness was measured in the bioassay 5 min (top panel) and 10 min (bottom panel) after a single intraoral infusion of milk. One additional control group (EPI-saline) was treated with EPI and tested 5 or 10 min after infusion of saline. At both time points, EPI resulted in reduced perioral responsiveness relative to SAL controls, suggesting that cFP-AAF-pAB prolonged the effect of milk infusion beyond the normal period of 3 min. The behavior of fetuses in the EPI-saline group indicated that EPI itself had little influence on responsiveness in the bioassay. Similarly, the three opioid antagonists administered alone did not alter fetal responsiveness in the bioassay. The effect of EPI was reversed by administration of the kappa antagonist BNI, but not by the mu antagonist CTOP or the delta antagonist NTI.

fetuses treated with EPI that received an infusion of saline (EPI-saline) all exhibited facial wiping, $\chi^2(1) = 17.6$, $p < 0.001$. Comparison of the three groups treated with each of the opioid antagonists indicated that BNI, CTOP, and NTI did not differ from the SAL group in the expression of facial wiping, $\chi^2(3) = 1.1$, $p > 0.75$. However, comparison of the EPI group with the four groups treated with an opioid antagonist (or saline) before EPI revealed a significant difference in responsiveness in the bioassay, $\chi^2(4) = 33.9$, $p < 0.001$. While the expression of facial wiping was low in the EPI, SAL + EPI, CTOP + EPI, and NTI + EPI groups, a wiping response was expressed by most subjects in the BNI + EPI groups. Specific comparison of the responses in the EPI and BNI + EPI groups confirmed this difference, $\chi^2(1) = 17.6$, $p < 0.001$.

A similar pattern of results was evident in the bioassay administered 10 min after intraoral infusion (Fig. 1). The overall chi-square analysis indicated significant variation in the expression of facial wiping among the 10 groups, $\chi^2(9) = 44.5$, $p < 0.001$. Comparison of SAL and EPI groups indicated significantly reduced facial wiping after treatment with EPI, $\chi^2(1) = 8.3$, $p < 0.005$. The effect of EPI was evident only if fetuses received an infusion of milk, as confirmed by behavioral differences between the EPI and EPI-saline groups, $\chi^2(1) = 8.3$, $p < 0.005$. Administration of the three opioid antagonists did not affect fetal responsiveness in the bioassay, as compared to SAL controls, $\chi^2(3) = 1.9$, $p > 0.50$. Facial wiping was significantly reduced in the EPI, SAL + EPI, CTOP + EPI, and NTI + EPI groups, but was reinstated in the BNI + EPI group, $\chi^2(4) = 18.2$, $p < 0.005$. Specifically, facial wiping by fetuses in the BNI + EPI group was elevated relative to subjects treated with EPI alone 10 min after milk infusion, $\chi^2(1) = 11.0$, $p < 0.001$.

DISCUSSION

A series of published reports have documented that presentation of milk to the near-term rat fetus results in reduced responsiveness to perioral cutaneous stimulation (22,25). Specifically, intraoral infusion of milk virtually abolishes facial wiping in the behavioral bioassay within 60 s of infusion. The effect of milk is still evident 3 min after infusion. However, the milk effect is transient, with facial wiping returning to preinfusion baseline levels when the bioassay is administered 5 min after infusion (21). The effect of milk on facial wiping is evident in the present study in the EPI and EPI + saline groups: subjects that received an infusion of isotonic saline continued to exhibit high levels of facial wiping at both 5 and 10 min after infusion, whereas fetuses that received milk showed reduced levels of wiping behavior.

The finding that EPI was effective in suppressing facial wiping responses up to 10 min after infusion, but only in subjects that received milk, is consistent with the interpretation that milk promotes a transient period of opioid activity that reduces cutaneous responsiveness, and that EPI extends the duration of milk-induced opioid activity. Moreover, the evidence provided by subjects treated with selective opioid antagonists before administration of EPI confirms that milk-induced opioid activity involves kappa receptors. The kappa antagonist BNI blocked the effects of EPI, whereas facial wiping was reduced in fetal subjects treated with the mu antagonist CTOP or the delta antagonist naltrindole before administration of EPI.

The compound cFP-AAF-pAB inhibits the degradation of longer chain endogenous opioids, including dynorphin A(1-8), alpha and beta neoendorphin, and MERGL, by endopeptidase 24.15 (8,9). Dynorphin A preferentially binds to kappa opioid receptors, whereas other endogenous opioids that are degraded by endopeptidase 24.15 either bind nonselectively or show higher affinity for mu or delta receptors (3). In conjunction with previous reports, the present experiment provides three points of evidence that the fetus's initial exposure to milk promotes activity in the kappa opioid system. a) Fetal rats exhibit changes in behavior following infusion of milk that are blocked by treatment with the kappa opioid antagonist nor-binaltorphimine (21). b) The duration of milk effects on fetal behavior are extended beyond their normal recovery by treatment with the endopeptidase inhibitor cFP-AAF-pAB. c) The action of cFP-AAF-pAB to prolong the effect of milk on fetal behavior is blocked by nor-binaltorphimine, but not by mu or delta antagonists. These behavioral effects appear to be mediated by longer chain compounds that bind selectively to the kappa receptor, such as Dynorphin A, or other opioids that bind to kappa, mu, and delta receptors, including alpha and beta neoendorphin. Further, because cFP-AAF-pAB was administered to fetal rats via intracisternal injection, the site(s) of action of the endogenous opioid peptides released after infusion of milk is in the central nervous system of the fetus, most likely in caudal brain structures or the spinal cord. These experimental results imply that the fetus's first exposure to milk, and by implication the newborn rat's first suckling experience, may increase levels of dynorphin in the brain and spinal cord.

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