

# The Analgesia-Enhancing Component of Ingested Amniotic Fluid Does Not Affect Nicotine-Induced Antinociception in Naltrexone-Treated Rats

T. M. ROBINSON-VANDERWERF,\* J. M. DI PIRRO,\* A. R. CAGGIULA† AND M. B. KRISTAL\*

\*Behavioral Neuroscience Program, Department of Psychology, University at Buffalo, Buffalo, NY 14260

†Biopsychology Program, Department of Psychology, University of Pittsburgh, Pittsburgh, PA 15260

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ROBINSON-VANDERWERF, T. M., J. M. DI PIRRO, A. R. CAGGIULA AND M. B. KRISTAL. *The analgesia-enhancing component of ingested amniotic fluid does not affect nicotine-induced antinociception in naltrexone-treated rats.* PHARMACOL BIOCHEM BEHAV 58(1) 147–151, 1997.—Ingestion of amniotic fluid and placenta by rats has been shown to enhance opioid-mediated antinociception but not affect the nonopioid-mediated antinociception produced by aspirin, suggesting specificity for opioid-mediated processes. However, enhancement by the active substance(s) in amniotic fluid and placenta (POEF, for placental opioid-enhancing factor) of antinociception produced by other nonopioid mechanisms has yet to be examined. The present experiments tested whether ingestion of amniotic fluid enhances the antinociception produced by nicotine injection. In Experiment 1A, enhancement of morphine-mediated antinociception by ingestion of amniotic fluid was demonstrated in a hot-plate assay. In Experiment 1B, rats pretreated with naltrexone were given an orogastric infusion of amniotic fluid or control (0.25 ml), then injected with nicotine (0, 0.075, 0.125, or 0.225 mg/kg subcutaneously), then tested for antinociception in a hot-plate assay. Amniotic fluid ingestion did not enhance the antinociception produced by various doses of nicotine. In Experiment 2, rats pretreated with naltrexone were given an orogastric infusion of amniotic fluid (0, 0.125, 0.25, or 0.50 ml) and then injected with 0.125 mg/kg nicotine. None of the doses of amniotic fluid enhanced the nicotine-induced antinociception. The findings of these experiments lend support to our contention that the enhancement by POEF of antinociception is specific to opioid-mediated processes. © 1997 Elsevier Science Inc.

Pain Analgesia Antinociception Amniotic fluid Placenta POEF Nicotine Hot plate Rat

THE INGESTION of amniotic fluid and placenta has been shown to enhance opioid-mediated antinociception such as that produced by morphine injection (1,13–18), vaginal/cervical stimulation (19,23), late pregnancy (17), and foot shock (18). The active opioid-enhancing substance(s) in amniotic fluid and placenta, referred to as POEF (placental opioid-enhancing factor), does not produce antinociception by itself. Evidence suggests that POEF may exclusively enhance opioid-mediated antinociception; the ingestion of amniotic fluid does not enhance the nonopioid-mediated antinociception produced by aspirin (15). The enhancement by POEF of antinociception produced by other nonopioid mechanisms has yet to be fully examined.

Nicotine injection has been shown to produce antinociception in a variety of species (12,20), including mice and rats (2–7, 9–11,20–22,24–26). Evidence suggests a primarily central site of action, because nicotine administered subcutaneously (SC) or intracerebroventricularly (ICV) produced antinociception that was blocked by pretreatment with SC, intraperitoneal, or ICV administration of mecamylamine, a tertiary nicotine receptor antagonist that readily crosses the blood–brain barrier (5,11,22,24–26). Peripherally injected chlorisondamine, a bisquaternary peripheral antagonist specific for nicotine, completely failed to block nicotine-induced antinociception in the hot-plate assay (5), the assay used in the present set of experi-

Requests for reprints should be addressed to Mark B. Kristal, Department of Psychology, University at Buffalo, Buffalo, NY 14260. E-mail: kristal@acsu.buffalo.edu

ments. It should be noted, however, that peripherally injected chlorisondamine did successfully block nicotine antinociception when tested in a hot-water tail-dip assay, suggesting the possible presence of a peripheral component to nicotine's effects on the tail withdrawal response (5). A central site of action is further indicated by studies showing that nicotine-induced antinociception is greatest after injection into the pedunculopontine tegmental nucleus of the mesopontine tegmentum, areas of the ventral and posterior medulla, the central gray, or the subarachnoid space (2,9–11,24).

Nicotine-induced antinociception in the rat may be mediated by several neurotransmitter systems, including adrenergic, serotonergic, and cholinergic muscarinic systems (10,24,26). Although endogenous opioid systems may be involved in nicotine-induced antinociception in mice (3,26) and dogs (12), there is little evidence to suggest opioid involvement in rats. Rats pretreated with the opiate antagonist naloxone SC still exhibited antinociception after either SC or subcortical nicotine injection (11,24–26). However, Davenport, Houdi, and Van Loon (7) found that ICV nicotine pretreatment significantly increased morphine-induced antinociception in rats treated with  $\beta$ -funaltrexamine, an irreversible  $\mu$ -opioid receptor antagonist. They contended that nicotine caused the release of endogenous opioids, which then occupied  $\mu$ -opioid receptor sites and prevented  $\beta$ -funaltrexamine from inactivating those receptor sites. In the present study, all rats were pretreated with the opiate antagonist naltrexone to ensure that any possible endogenous opioid release would not affect antinociception. The presence of nicotine-induced antinociception in these rats would lend support to the findings of others that endogenous opioids are not involved in nicotine-induced antinociception in the rat. The present experiments were conducted to determine whether the ingestion of amniotic fluid by rats enhances the antinociception produced by nicotine injection.

#### EXPERIMENT 1A

Previous testing of POEF enhancement of opioid-mediated antinociception has primarily been conducted using the tail-flick latency test. We decided to use a hot-plate assay in the present experiment because it is frequently used to assess antinociception produced by nicotine (5,9–11,20,22,24). Therefore, it was important to demonstrate the presence of POEF enhancement of opiate-mediated antinociception using the hot-plate assay before testing for POEF enhancement of nicotine-induced antinociception.

#### Methods

**Subjects.** All live animal experimental procedures were approved by IACUC, SUNY at Buffalo, Buffalo, New York. Female Long-Evans rats ( $n = 40$ ) weighing  $266.45 \pm 2.91$  g were used as subjects. All rats were housed in hanging wire-mesh cages ( $24.5 \times 18 \times 18$  cm) in an environmentally controlled room maintained on a 14 L:10 D cycle. Rats had ad lib access to water and chow (Agway Prolab Rat/Mouse/Hamster Formula 3000), except as noted. Prior to testing, daily vaginal smears were obtained to check for normal estrous cyclicity in each rat. Vaginal smears were also taken on the day of testing to assign rats to groups in such a way that would ensure that the groups contained approximately equal numbers of rats in each stage of the estrous cycle. Testing was conducted between 5 and 7 h after lights-on.

**Apparatus.** Pain threshold was assessed by means of a hot-plate algometer (IITC Inc./Life Science Instruments model 39D) maintained at  $52^\circ\text{C}$  (5,10,11). Antinociception was mea-

sured by placing each rat on the center of the hot plate so that all four paws touched the plate simultaneously. As all four paws contacted the surface, a stopwatch was started to measure the latency from paw contact until the rat either licked its hindpaw or jumped. If the rat failed to perform the required behavioral end point within 55 s, testing was terminated.

**Procedure.** A  $2 \times 2$  design was used: drug (morphine sulfate 4.0 mg/kg, vehicle 1 ml/kg)  $\times$  enhancer [0.25 ml amniotic fluid (AF), 0.25 ml saline (SAL)],  $n = 10/\text{group}$ . Prior to testing, subjects were habituated to all testing procedures. Subjects were exposed to a room-temperature plate for 5 min on each of the two days before the day of testing. Each rat was also given one blank orogastric infusion (no fluid was administered) each day for five consecutive days.

On the day of testing, subject rats were food- and water-deprived for a 3-h period. Each rat was then injected with 0.9% saline (1 ml/kg SC) to control for the naltrexone injection given to subjects in experiments 1B and 2. After 25 min, each rat was injected (SC) with morphine sulfate (4.0 mg/kg) or vehicle (1.0 ml/kg saline). Thirty minutes after morphine injection, subjects were given an orogastric infusion of enhancer (0.25 ml AF) or control (0.25 ml SAL). Antinociception was measured on the hot plate 15 min later.

#### Results

A two-way ANOVA showed a significant main effect of drug [morphine > vehicle:  $F(1, 36) = 20.54$ ,  $p < 0.001$ ] and a drug  $\times$  enhancer interaction [ $F(1, 36) = 4.73$ ,  $p < 0.05$ ]. Further probing of the interaction using the Student–Newman–Keuls multiple-range test ( $\alpha$  level =  $p < 0.05$ ) showed that rats given morphine + AF showed a significantly higher level of antinociception than did rats given morphine + SAL, vehicle + AF, or vehicle + SAL (see Fig. 1). Rats that received morphine + AF showed a mean latency to lick their hindpaw or jump of  $27.20 \pm 4.13$  s, whereas rats given morphine + SAL showed a mean latency of  $17.56 \pm 2.11$  s. Rats given vehicle + AF showed a mean latency of  $10.45 \pm 1.27$  s, and rats receiving vehicle + SAL showed a mean latency of  $11.67 \pm 1.33$  s. Therefore, AF (and presumably POEF) enhanced the antinociception produced by a low dose of morphine (4.0 mg/kg).

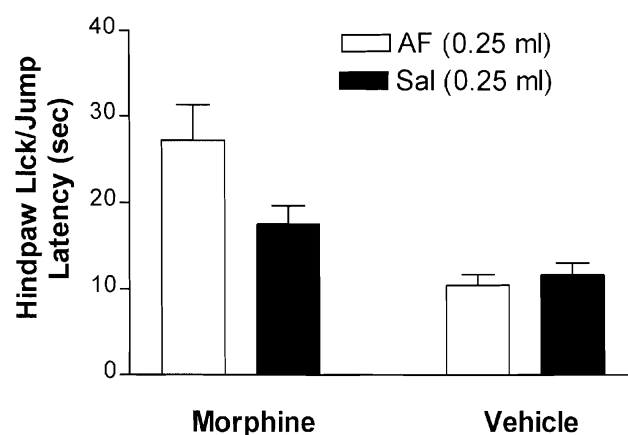


FIG. 1. Mean ( $\pm$ SEM) hindpaw lick/jump latency of rats treated with 4.0 mg/kg morphine (SC) or 1 mg/kg vehicle followed by orogastric infusion of 0.25 ml amniotic fluid or 0.25 ml saline;  $n = 10/\text{group}$ . Latency was significantly greater for the morphine/AF combination than for the others (morphine/Sal = vehicle/AF = vehicle/Sal),  $p < 0.05$ .

## EXPERIMENT 1B

## Methods

**Subjects.** Female Long-Evans rats ( $n = 104$ ) weighing  $261 \pm 1.82$  g were housed and maintained as described for experiment 1A.

**Drugs.** Nicotine bitartrate (Sigma Chemical Co., St. Louis, MO, USA) was diluted to final concentrations of 0.075 mg/ml, 0.125 mg/ml, and 0.225 mg/ml (free base) in a 0.9% saline solution. Drugs were administered SC at a volume of 1 ml/kg. Doses of nicotine higher than 0.225 mg/kg were not used because rats given doses higher than that in pilot tests failed to perform the required behavioral end point within 55 s (i.e., the higher doses produced a ceiling effect). Naltrexone HCl (Sigma Chemical Co.) was dissolved in 0.9% saline solution to a concentration of 1 mg/ml.

**Procedure.** A  $2 \times 3 \times 2$  design was used: drug [nicotine, vehicle cohort (1 ml/kg)]  $\times$  dose (0.075 mg/kg, 0.125 mg/kg, 0.225 mg/kg)  $\times$  enhancer (0.25 ml AF, 0.25 ml SAL), with no repeated measures. The doses of nicotine were tested sequentially; therefore, each dose was run with a corresponding vehicle-injection cohort of 1 ml/kg. For each cell,  $n = 8$ , except for the four groups treated with 0.125 mg/kg nicotine or vehicle, for which  $n = 10$ . Prior to testing, all subjects were habituated to all testing procedures as described in experiment 1A.

On the day of testing, rats were deprived of food and water for 3 h. Each rat was then pretreated with the opiate blocker naltrexone (1 mg/kg SC) (15). Sixty minutes later, each received an orogastric infusion of enhancer (0.25 ml AF) or control (0.25 ml SAL). Ten minutes after the infusion, each rat was injected SC with nicotine (0.075 mg/kg, 0.125 mg/kg, or 0.225 mg/kg) or vehicle (1 ml/kg). Antinociception was measured on the hot plate 10 min after the nicotine injection; pilot data from our laboratory indicated that nicotine-induced antinociception is at its peak at this time.

## Results

There were no significant differences among the three cohort groups of rats receiving vehicle injection + SAL infusion, therefore the groups were combined for purposes of analysis ( $\alpha$  level =  $p < 0.05$ ). There were also no significant differences among the three cohort groups receiving vehicle injection + AF infusion; these three groups also were combined. This reduced the design of the experiment to  $4 \times 2$ : dose (0.0 mg/kg, 0.075 mg/kg, 0.125 mg/kg, 0.225 mg/kg)  $\times$  enhancer (0.25 ml AF, 0.25 ml SAL). A two-way ANOVA revealed a significant main effect of dose [ $F(3, 96) = 13.62$ ,  $p < 0.0001$ ]. There was no significant main effect of enhancer nor was there a dose  $\times$  enhancer interaction. Therefore, amniotic fluid did not enhance the antinociception produced by various doses of nicotine (see Fig. 2).

Pairwise comparisons of the doses of nicotine by the Student-Newman-Keuls multiple-range test ( $p < 0.05$ ) showed that the highest dose of nicotine (0.225 mg/kg) produced significantly greater antinociception than did either vehicle injection ( $10.76 \pm 0.44$  s) or the lowest dose of nicotine (0.075 mg/kg;  $14.66 \pm 1.62$  s). The medium dose of nicotine (0.125 mg/kg) also produced significantly more antinociception ( $24.46 \pm 3.47$  s) than did either the lowest dose or the vehicle. There were no significant differences between the highest and the medium doses or between the lowest dose and the vehicle injection. Although there was a significant dose effect, linear increases in the dose of nicotine did not produce a corresponding linear increase in antinociception in our rats. The dose range was

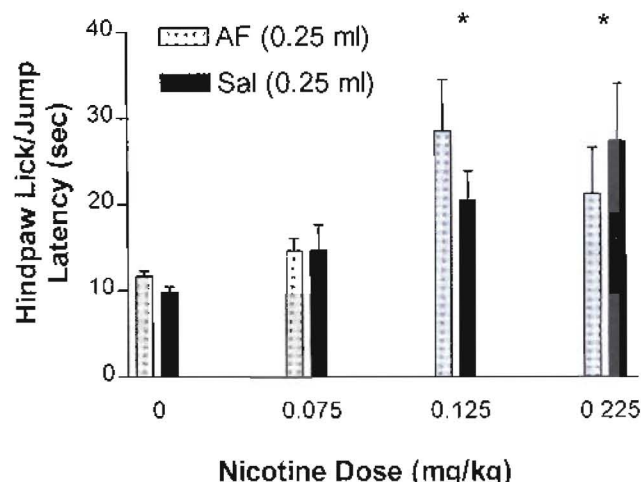


FIG. 2. Mean ( $\pm$ SEM) hindpaw lick/jump latency of rats treated with 0.075 mg/kg ( $n = 8$ /group), 0.125 mg/kg ( $n = 10$ /group), or 0.225 mg/kg ( $n = 8$ /group) nicotine (SC) or 1 ml/kg vehicle ( $n = 26$ /group), after orogastric infusion of 0.25 ml amniotic fluid or saline. All rats were pretreated with 1 mg/kg naltrexone HCl. \*Latency significantly different from that produced by 0.075 mg/kg nicotine,  $p < 0.05$ .

truncated because, as mentioned above, doses of nicotine higher than 0.225 mg/kg could not be administered to our rats without producing a ceiling effect in the hot-plate assay.

It is possible that enhancement of nicotine-induced antinociception by amniotic fluid was not apparent because the amount of POEF contained in the volume of the amniotic fluid infusion used was not appropriate to enhance antinociception in this situation. A dose of 0.25 ml amniotic fluid was chosen for experiment 1B because previous data from our laboratory have shown that 0.25 ml of amniotic fluid significantly enhances the antinociception produced by a threshold dose (3.0 mg/kg) of morphine (14,16). We attempted to address the issue of amniotic fluid volume in experiment 2 by injecting rats with a single, effective dose of nicotine (or vehicle) and infusing them with various volumes of amniotic fluid or saline. A dose of 0.125 mg/kg nicotine was chosen for this experiment because it produces a significant level of antinociception without producing a ceiling effect (55 s maximum) in our testing paradigm (see Fig. 2). Therefore, the dose is appropriate for manipulations likely to result in enhancement. That dose of nicotine also produces a level of antinociception roughly equivalent to that produced by 4.0 mg/kg morphine, the dose that produced enhanceable antinociception in experiment 1A.

## EXPERIMENT 2

## Methods

**Subjects.** Female Long-Evans rats ( $n = 132$ ) weighing  $270 \pm 2.14$  g were housed and maintained as described in experiment 1A.

**Procedure.** A  $2 \times 2 \times 3$  design was used: drug (nicotine 0.125 mg/kg, vehicle 1 ml/kg)  $\times$  enhancer (AF, SAL)  $\times$  enhancer volume (0.125 ml, 0.25 ml, 0.5 ml), with no repeated measures. Each cell contained  $n = 10$ , except the four groups receiving 0.5 ml amniotic fluid, for which  $n = 13$ . Prior to testing, subjects were habituated to all testing procedures as described in experiment 1A.

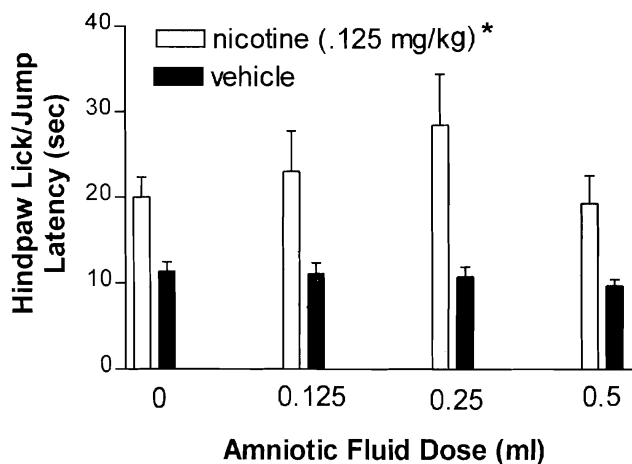


FIG. 3. Mean ( $\pm$ SEM) hindpaw lick/jump latency of rats treated with 0.125 mg/kg nicotine (SC) or 1 ml/kg vehicle after orogastric infusion of 0.125 ml ( $n = 10$ /group), 0.25 ml ( $n = 10$ /group), or 0.50 ml ( $n = 13$ /group) amniotic fluid or saline ( $n = 33$ /group). All rats were pretreated with 1 mg/kg naltrexone HCl. \*Latency produced by nicotine significantly greater than that produced by vehicle,  $p < 0.0001$ .

On the day of testing, each subject rat was deprived of food and water for 3 h. Each rat was pretreated with naltrexone HCl (1 ml/kg SC). Sixty minutes later, each received an orogastric infusion of AF or SAL (0.125 ml, 0.25 ml, or 0.5 ml). Ten minutes after the infusion, each rat was given an injection of either nicotine (0.125 mg/kg SC) or vehicle (1 ml/kg SC). Antinociception was measured on the hot plate 10 min after the nicotine injection.

### Results

There were no significant differences ( $\alpha$  level =  $p < 0.05$ ) among control groups of rats receiving nicotine-vehicle injection and the three volumes of saline infused as a control for amniotic fluid (0.125, 0.25, or 0.5 ml saline); therefore, these groups were combined for purposes of analysis. Likewise, there were no significant differences among rats receiving nicotine injection and any of the three infused volumes of saline; therefore, these groups were also combined for purposes of analysis. A two-way ANOVA revealed only a significant effect of drug [nicotine > vehicle;  $F(1, 120) = 31.31$ ,  $p < 0.0001$ ] (see Fig. 3). Rats receiving nicotine showed a mean latency to lick or jump of  $21.60 \pm 1.78$  s; those receiving nicotine vehicle showed a mean latency of  $10.89 \pm 0.62$  s. There was no significant main effect of enhancer volume, nor was there a drug  $\times$  enhancer volume interaction. Although it may appear in Fig. 3 that rats receiving nicotine + 0.25 ml AF ( $28.46 \pm 5.97$  s) showed a somewhat greater level of antinociception than did rats receiving nicotine + 0.0 ml AF ( $20.00 \pm 2.39$  s), which would indicate enhancement by amniotic fluid, this was not the case. There was no statistically significant difference be-

tween nicotine + 0.25 ml AF and nicotine + 0.0 ml AF, even after analyzing the pairwise comparison with a liberal statistical technique, a one-way ANOVA [ $F(1, 41) = 2.43$ ,  $p = 0.1269$ ]. (A highly conservative  $\alpha$  level of 0.0018 would be required for each pairwise comparison, within the two-way ANOVA, to obtain significance of  $p < 0.05$  with no compounding  $\alpha$  problem.) Varying the volume of amniotic fluid infusion had no significant effect on nicotine-mediated antinociception.

### GENERAL DISCUSSION

The presence of nicotine-induced antinociception in rats pretreated with naltrexone in this study is consistent with the findings of others that antinociception produced by nicotine is not mediated by opioid mechanisms in the rat (11,24–26). Nicotine may indeed cause a release of endogenous opioid, as suggested by Davenport et al. (7), but that release, in rats, apparently does not mediate the antinociception produced by nicotine. In the present study, pretreatment with naltrexone, a general opiate antagonist, should have prevented any endogenous opioid release from producing antinociception.

Nicotine administered SC produced antinociception that was not enhanced by amniotic fluid ingestion. Varying the dose of nicotine or the volume of amniotic fluid did not produce enhancement of nicotine-induced antinociception. It should be noted that although optimum levels of various parameters (hot-plate temperature, dose of naltrexone, interval between injection and test, etc.) were used (as determined by pilot studies, previous research in our laboratory, or references in the literature) and provided support for our hypothesis, these results might not apply to all other combinations of parameters. Nevertheless, amniotic fluid ingestion *did* enhance the antinociception produced by a low dose of morphine. POEF enhancement of morphine-induced antinociception in the present hot-plate study agrees with recent work, using a hot plate, that showed that selective activation of the  $\delta$  opioid receptor with ICV-administered DPDPE produced antinociception that was enhanced by ingestion of placenta (8). Although there is the possibility that the absence of enhancement by amniotic fluid of nicotine-induced antinociception resulted from the assay chosen, this possibility is largely eliminated by the demonstration that the hot-plate assay is sensitive to POEF enhancement of opiate-mediated antinociception. Our results are consistent with earlier work showing that amniotic fluid ingestion did not enhance the nonopioid-mediated antinociception produced by aspirin (15), and further support our contention that the enhancement of antinociception by POEF is specific to opioid-mediated antinociception. These findings also support growing evidence that the benefit of ingestion of amniotic fluid and placenta by parturient mammals is the enhancement of opioid-mediated antinociception that occurs from late pregnancy through parturition (13).

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