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Research Report

Ingestion of amniotic fluid enhances the facilitative effect of VTA morphine on the onset of maternal behavior in virgin rats

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ARTICLE INFO

Article history:
Accepted 13 January 2009
Available online 21 January 2009

Keywords:
Amniotic fluid
Interpeduncular nucleus
Maternal behavior
Opioid
Placentophagia
POEF
Ventral tegmental area
Morphine
Rat

ABSTRACT

Previous research has shown that injection of morphine into the ventral tegmental area (VTA) facilitates the onset of maternal behavior in virgin female rats, and injection of the opioid antagonist naltrexone into the VTA disrupts the onset of maternal behavior in parturient rats. Placentophagia – ingestion of placenta and amniotic fluid, usually at parturition – modifies central opioid processes. Ingestion of the active substance in placenta and amniotic fluid, Placental Opioid-Enhancing Factor (POEF), enhances the hypoalgesic effect of centrally administered morphine, and more specifically, enhances δ- and κ-opioid-receptor-mediated hypoalgesia and attenuates μ-opioid-receptor-mediated hypoalgesia. POEF (in placenta or amniotic fluid) ingestion does not, by itself, produce hypoalgesia. In the present study, we tested the hypothesis that ingestion of amniotic fluid enhances the facilitative effect of opioid activity (unilateral morphine injection) in the VTA on the rate of onset of maternal behavior. Virgin female Long-Evans rats were given one intra-VTA injection of morphine sulfate (0.0, 0.01, or 0.03 μg, in saline) and an orogastric infusion of 0.25 ml amniotic fluid or saline once each day of the first three days of the 10-day testing period. Subjects were continuously exposed to foster pups that were replaced every 12 h; replacement of pups was followed by a 15-min observation period. Maternal behavior latency was determined by the first of two consecutive tests wherein the subject displayed pup retrieval, pup licking in the nest, and crouching over all foster pups, during the 15-min observation. We confirmed the previous finding that the VTA injection, alone, of 0.03 μg morphine shortened the latency to show maternal behavior and that 0.0 μg and 0.01 μg morphine did not. Ingestion of amniotic fluid (and therefore POEF) facilitated the onset of maternal behavior in rats receiving an intra-VTA microinjection of an otherwise subthreshold dose of morphine (0.01 μg).

1. Introduction

Rats at parturition show an almost immediate onset of appropriate maternal behavior. In contrast, the onset of maternal behavior in virgin rats that are continuously exposed to relatively constant-age foster pups (a procedure referred to as "concaveation", i.e., "with pups") is slow to appear, and is usually measured as the latency, in days, to the appearance of

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doi:10.1016/j.brainres.2009.01.015

pup licking, pup retrieval and crouching over pups (Rosenblatt and Lehrman, 1963; Wiesner and Sheard, 1933). Depending on the strain of the virgin, this can take from 4 to 10 days. Maternal behavior is induced by stimuli emanating from the pups, and can be enhanced by the sensitization of the maternal neural substrate by the mother's hormones, neurohormones, and neural stimulation (for review, see González-Mariscal and Poindron, 2002; Kristal, 2009).

Plasma and brain levels of endogenous opioids increase over the course of pregnancy, peak during parturition, and then decline to pre-pregnancy levels during lactation (Petraglia et al., 1985; Wardlaw and Frantz, 1983). These changes in endogenous opioids are consistent with "pregnancy-mediated analgesia", the elevation in pain threshold (hypoalgesia) during labor and delivery (Gintzler, 1980), which is greatly enhanced in the periparturitional period by the opioid-modifying effect of afterbirth ingestion (Kristal, 1998; Kristal et al., 1990a, 1990b).

Endogenous opioids have been shown to affect the rapid onset of maternal behavior at parturition in both a positive and negative fashion (Mann and Bridges, 1992; Mann et al., 1991; Rubin and Bridges, 1984; Thompson and Kristal, 1996). Central opioids can affect the onset and maintenance of maternal behavior. The nature of this modulation depends on the site of action. Increased opioids in the medial preoptic area (MPOA) inhibit maternal responsiveness; the MPOA is an area widely accepted to be crucial for the mediation of maternal behavior (Mann and Bridges, 1992; Mann et al., 1991; Numan 2006; Rubin and Bridges, 1984). In contrast, morphine injected into the ventral tegmental area (VTA), which modulates motivation and reward, facilitates the onset of maternal behavior in virgin female rats and injection of the opioid antagonist naltrexone into the VTA disrupts the onset of maternal behavior in parturient female rats (Thompson and Kristal, 1996).

Placentophagia (ingestion of afterbirth material) occurs in most nonhuman and non-aquatic mammalian species at parturition (Kristal, 1980, 1991, 1998). Ingestion of placenta or amniotic fluid modulates opioid-mediated events: (a) it enhances opioid-induced hypoalgesia (Kristal et al., 1985, 1986a, 1986b) whether the pain relief is produced by endogenous (Kristal et al., 1990a, 1990b, 1986a, 1986b) or exogenous opioids (Kristal et al., 1985, 1986a, 1986b), (b) it does not affect nonopioid-induced hypoalgesia (Kristal et al., 1990a, 1990b; Robinson-Vanderwerf et al., 1997), and (c) it does not produce hypoalgesia by itself (without the existence of an underlying opioid hypoalgesia) (Kristal, 1991, 1998). Furthermore, ingestion of placenta enhances δ- and κ-opioid-receptor-mediated hypoalgesia and attenuates μ-opioid-receptor-mediated hypoalgesia (DiPirro and Kristal, 2004), which is consistent with Gintzler's research (Gintzler, 1980; Gintzler and Liu, 2001) showing that the spinal mechanisms of periparturitional hypoalgesia (pregnancy-mediated analgesia) are mediated by δ- and κ-opioid receptors, but not by μ-opioid receptors (Gintzler and Liu, 2001). The putative component of ingested afterbirth material that affects central opioid phenomena has been termed Placental Opioid-Enhancing Factor (POEF) (Kristal et al., 1988). POEF itself does not reach the brain, but apparently works centrally by activating gastric vagal afferents (Abbott et al., 1991; Aicher et al., 1991; DiPirro et al., 1991; Robinson et al., 1995; Tarapacki et al., 1992). Although the modification of central opioid activity by POEF ingestion is not specific to the periparturitional period, to rats, or even to females (Abbott et al., 1991), a principal adaptive advantage of the POEF mechanism apparently is that it provides greater opioid-mediated hypoalgesia at parturition while requiring a lower level of opioid; a higher level of opioid release would interfere with the efficient execution of maternal behavior at and immediately after parturition (Tarapacki et al., 1995; for review, see Kristal, 1991, 1998).

Because POEF enhances opioid-mediated hypoalgesia during labor and delivery, we hypothesized in the present study that placentophagia, and therefore ingested POEF, orchestrates the opioid role in the onset of maternal behavior. We predicted that ingested amniotic fluid would enhance the facilitative effect of VTA morphine on the onset of maternal behavior in virgin female rats. Specifically, we hypothesized that (a) the ingestion of amniotic fluid alone (in the absence of opioids) would not facilitate the onset of maternal behavior, (b) the injection of morphine into the VTA would facilitate the onset of maternal behavior (a confirmation of the findings of Thompson and Kristal [1996]), and (c) the ingestion of amniotic fluid would hasten the onset of maternal behavior above and beyond the facilitation produced by increased opioid activity in the VTA, i.e., that virgin rats treated with VTA morphine and that had ingested amniotic fluid would become maternal in fewer days than would those receiving VTA morphine alone, and that those receiving VTA morphine alone would become maternal in fewer days than would those not receiving VTA morphine.

2. Results

Of the 116 rats tested, 58 were found to have properly placed VTA cannulae after histological examination. The low success rate resulted from the use of a 4° tilt for the electrode carrier. Of those 58, 7 were excluded from statistical analysis because they engaged in cannibalism of pups, and 2 because of loss of cannulae during the testing period. Data from the remaining 49 rats were used for the statistical analysis (see Fig. 1). The subjects were distributed as follows: saline infusion — 0.0 μg morphine injection, $n=9$; AF infusion — 0.0 morphine injection, $n=7$; saline infusion — 0.01 μg morphine injection, $n=10$; AF infusion — 0.01 μg morphine injection, $n=6$; saline infusion — 0.03 μg morphine injection, $n=11$; AF infusion — 0.03 μg morphine injection, $n=6$.

The results showed no relationship to stage of the estrous cycle at the time the subjects became maternal. Furthermore, the procedures did not produce any apparent change in estrous cyclicity.

Microinjection of morphine into the VTA significantly facilitated the onset of maternal behavior in a dose-dependent fashion (see Figs. 2 and 3). To determine the effect of VTA morphine injection, the doses were compared across infusion conditions. The 0.03-μg dose group showed significantly shorter latencies than did the 0.00-μg dose (vehicle) group ($\chi^2=6.79$, $df=1.6$, $p<0.05$, Median test). The 0.01-μg dose, when pooled over infusion conditions, produced a Median latency that was not significantly different

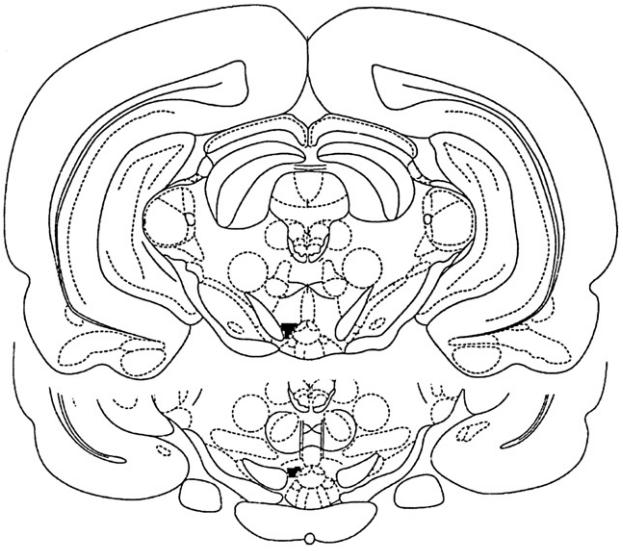

Fig. 1 – Composite diagrams of accurate cannulae placements in the ventral tegmental area. The anterior–posterior coordinates of the diagrams, relative to bregma, were –6.04 mm for the top section and –6.30 mm for the bottom section.

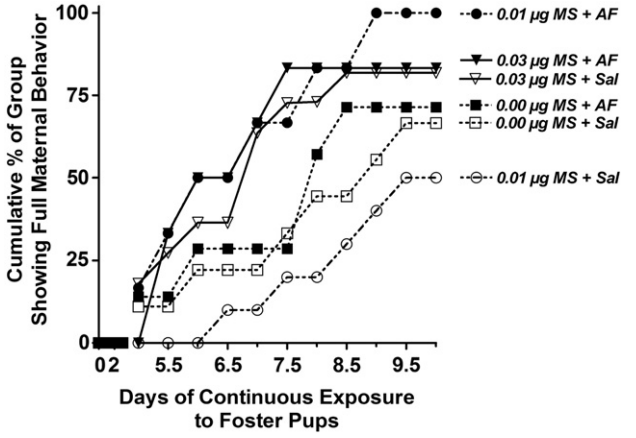

Fig. 3 – Cumulative percent of groups of rats showing full maternal behavior. Ventral tegmental area (VTA) injections of morphine occurred on Days 1, 2 and 3. MS = morphine sulfate; AF = orogastric infusion of 0.25 ml amniotic fluid. Intra-VTA injection of 0.03 µg morphine facilitated the onset of maternal behavior over 0.0 µg morphine (both infusion conditions). AF ingestion turned the otherwise ineffective 0.01-µg dose of morphine into one that strongly facilitated the onset of maternal behavior.

from that of either the 0.00-µg dose or the 0.03-µg dose because of the discrepancy between the latencies of the different infusion-treatment groups for that dose. In the saline-infusion condition, the 0.01-µg dose produced a Median latency (9.75) that was not significantly different from that of the 0.00-µg dose (9.0), but was significantly

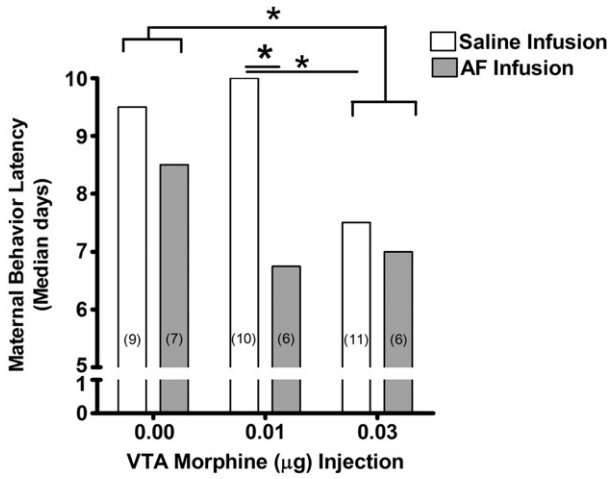

Fig. 2 – Effect of orogastric infusion of amniotic fluid (AF) on the facilitation by intra-VTA injection of morphine on the onset of maternal behavior in rats. *Represents a significant difference between the 0.01 and 0.03 µg morphine doses in the saline orogastric-infusion condition ($p<0.05$, Kruskal–Wallis Test), a significant difference between 0.0 and 0.03 µg morphine doses across the infusion conditions ($p<0.05$, Median Test), and a significant difference between amniotic fluid and saline infusions for the 0.01-µg dose of morphine ($p<0.05$, Kruskal–Wallis Test). The number of rats in each group is presented in parentheses.

higher than that of the 0.03-µg dose (7.0, $\chi^2=5{,}79$, $df=1$, $p<0.05$, Kruskal–Wallis test) and of that of the 0.03-µg dose pooled across infusion conditions ($\chi^2=7.45$, $df=1$, $p<0.01$, Kruskal–Wallis test): latencies for 0.0 µg morphine = 0.01 µg morphine > 0.03 µg morphine (see Figs. 2 and 3). Overall, these findings replicated those of Thompson and Kristal (1996): a dose of 0.03 µg morphine, injected into the VTA facilitates the onset of maternal behavior, but a dose of 0.01 µg of morphine does not.

The individual infusion groups were then compared across morphine doses. The saline-infusion and AF-infusion conditions did not differ in either the 0.0 µg or the 0.03 µg morphine-treatment conditions, and were therefore pooled for each dose of morphine; the insensitivity of nonparametric tests, particularly with small *n*s, made individual group comparisons and the analysis of interactions difficult. The latencies of the group receiving the 0.0 µg dose of morphine (vehicle), pooled across infusions (Mdn = 8.25 days), did not differ significantly from those of the saline-infusion group receiving the 0.01 µg morphine dose (Mdn = 9.75 days).

The analysis of the effect of AF infusion across doses of morphine showed that AF infusion, alone, had no main effect on maternal behavior ($\chi^2=1.76$, $df=2$, $p>0.05$, Kruskal–Wallis test). The maternal-behavior latencies differed significantly depending on the dose of VTA morphine injected into rats that received orogastric AF infusions: latencies for 0.0 µg morphine > 0.01 µg morphine = 0.03 µg morphine. In the amniotic-fluid-infusion condition, the 0.0 µg morphine (vehicle) injection, as expected, did not facilitate the onset of maternal behavior. The latency (Mdn = 8 days) was not significantly different from that of the 0.0 µg morphine dose of the saline-infused group (Mdn = 9 days). Therefore, amniotic fluid (and presumably POEF) ingestion alone had no effect on the onset latency of maternal behavior.

Infusion of amniotic fluid significantly shortened the Median latency to onset (facilitated the onset) of maternal behavior in rats receiving 0.01 μg intra-VTA morphine from 9 days to 6.5 days (χ^2=6.70, df=1, p<0.05, Kruskal–Wallis test), and increased the proportion of the group showing maternal behavior at all in the 10-day test from 50% to 100% (Fig. 3). As stated above, 0.01 μg is a subthreshold dose of morphine that did not facilitate the onset of maternal behavior on its own. The latencies of the groups receiving 0.01-μg (Mdn=6.5 days) and 0.03-μg doses (Mdn=6.5 days) of intra-VTA morphine were not significantly different from each other in the amniotic fluid condition. Infusion of amniotic fluid did not shorten the latency to onset of maternal behavior beyond that facilitation produced by 0.03 μg intra-VTA morphine (Fig. 2); the 0.03-μg dose of morphine may have produced a maximum facilitation (ceiling effect). The facilitation by amniotic-fluid ingestion of the 0.01-μg dose of morphine confirmed our hypothesis that ingestion of amniotic fluid facilitates the onset of maternal behavior beyond that produced by VTA-opioid activity alone.

In summary, with a saline orogastric infusion, a 0.03 μg VTA morphine injection facilitated the onset of maternal behavior whereas a 0.01 μg VTA morphine injection did not. AF orogastric infusion had no effect on maternal behavior in rats receiving either 0.00 μg or 0.03 μg VTA morphine. Finally, AF infusion rendered an otherwise ineffective dose of VTA morphine (0.01 μg) very effective in facilitating the onset of maternal behavior (see Fig. 3).

An interesting side observation was made during the experiment. Of the missed placements, 37 appeared to impinge on the interpeduncular nucleus (IPN) (see Fig. 4).

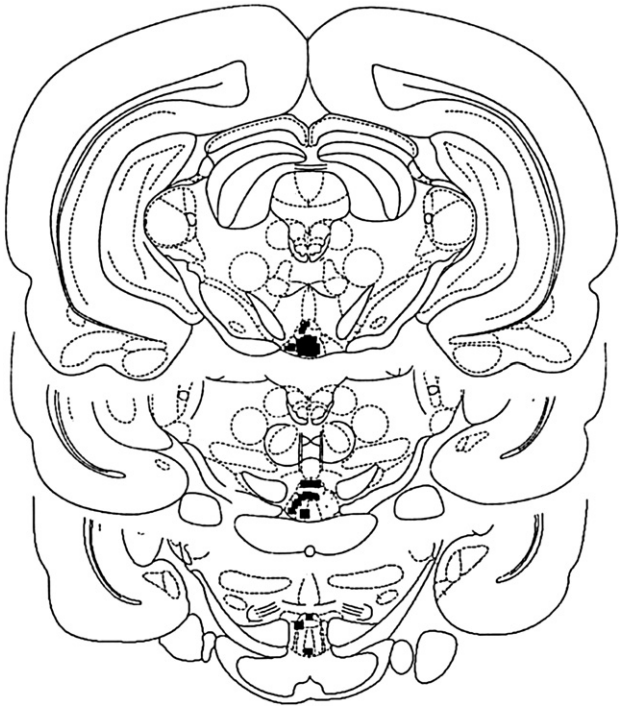

Fig. 4 – Composite diagrams of incorrect cannulae placements that impinged on the interpeduncular nucleus. The anterior–posterior coordinates of the diagrams, relative to bregma, were –6.04 mm for the top section, –6.30 for the middle section, and –6.72 for the bottom section.

The microinjection of morphine in these IPN placements appeared to facilitate the onset of maternal behavior. In rats that received an orogastric saline infusion, the maternal-behavior latencies differed significantly by dose (χ^2=6.99, df=2, p<0.05, Kruskal–Wallis Test) in that 0.01 μg morphine produced significantly shorter maternal behavior latencies (Mdn=4.5 days) than did 0.0 μg morphine (Mdn=9 days; χ^2=5.06, df=1, p<0.05, Kruskal–Wallis Test) or 0.03 μg morphine (Mdn=8.5 days; χ^2=5.64, df=1, p<0.05, Kruskal–Wallis Test). However, there were too few subjects to analyze the effect of the interaction of AF infusion and IPN morphine injection on the onset latency for maternal behavior.

3. Discussion

Thompson and Kristal (1996) showed that VTA microinjection of 0.03 μg and 0.1 μg, but not of 0.01 μg morphine facilitates the onset of maternal behavior. The present study confirmed that 0.03 μg morphine, but not 0.01 μg morphine, injected into the VTA, shortens the latency to onset of maternal behavior in naïve virgins.

We also showed that the ingestion of amniotic fluid alone (in the absence of the opioid activation) does not facilitate the onset of maternal behavior (see Fig. 2), but that the ingestion of amniotic fluid in combination with increased VTA-opioid activity facilitates the onset of maternal behavior beyond that produced by VTA-opioid activity alone. Orogastric infusion of amniotic fluid (and presumably POEF) turned the ineffective (subthreshold) dose of 0.01 μg VTA morphine into a facilitative dose.

The facilitation of the onset of maternal behavior by morphine injected into the interpeduncular nucleus has not been reported previously. However, in one study conducted on sheep (Lévy et al., 1999), a possible connection between the IPN and maternal behavior was suggested, because it is apparently one of the areas receiving olfactory input that is involved in the olfactory memory component of maternal behavior. Furthermore, the IPN has found to be rich in opioid, particularly μ, receptors (Ding et al., 1996) and so it is plausible that this neuroanatomical structure is involved in opioid mediated effects on maternal behavior. However, more work evaluating the structural specificity of this finding is necessary; the earlier VTA-maternal behavior study (Thompson and Kristal, 1996) did not find morphine-induced facilitation of maternal behavior in the missed placements landing in the IPN. In that study, the cannulae were inserted into the VTA vertically from the top of the brain rather than from an angle. The angled approach to the VTA used here would mean that a missed placement that impinges on the IPN still delivers drug into the VTA; the VTA is immediately upstream from the tip of the cannula when the tip falls in the IPN; this does not occur in a vertical approach.

Overall, these results suggest that amniotic fluid (and presumably POEF) ingestion facilitates the onset of maternal behavior by enhancing the action of opioids in the VTA. The effect of the interaction of POEF ingestion and intra-VTA morphine injection on maternal behavior likely results from the modification of mesolimbic dopamine activity. The rewarding effects of intra-VTA morphine (e.g., Bozarth, 1987)

result, primarily, from morphine-induced inhibition of GABAergic neurotransmission in the VTA, which disinhibits mesolimbic dopamine activity (Klitenick et al., 1992). Increases in mesolimbic dopamine activity seem to be involved in the neural mechanisms mediating multiple motivational phenomena, including maternal behavior (Byrnes et al., 2002; Hansen et al., 1991a, 1991b; Stern and Keer, 1999; Stolzenberg et al., 2007). Based on our previous work evaluating the effect of POEF on opioid-mediated hypoalgesia we speculate that POEF enhances the action of VTA morphine on maternal behavior by activating vagal afferent input to neural circuits that feed into mesolimbic neural circuitry (Ter Horst and Streefland, 1994, for review of vagal afferent distribution to the CNS), and specifically potentiating the effects of intra-VTA morphine by modulating δ- or κ-opioid-receptor activity, or both (DiPirro and Kristal, 2004).

Based on these VTA results and our previous work, we propose that ingestion of placenta at delivery and of amniotic fluid during labor and delivery facilitates the onset of maternal behavior by two mechanisms: (a) by increasing approach to pups (because of the attractiveness of afterbirth materials [Kristal et al., 1981; Steuer et al., 1987]); and (b) because of the POEF activity of ingested afterbirth, by modifying CNS opioid circuits influencing maternal behavior, motivation and reward, and hypoalgesia. In this way, placentophagia contributes to the multiple redundant pathways producing rapid onset of maternal behavior at parturition by attracting the mother to the amniotic-fluid-covered neonates, and concurrently enhancing the effect of elevated opioids in the VTA on the facilitation of the onset of maternal behavior that occurs as the young are delivered. It is also possible that POEF helps to facilitate the onset of maternal behavior by attenuating the negative effect of hypothalamic μ-opioid activity on maternal behavior.

4. Experimental procedures

4.1. *Subjects*

Subjects were 116 experimentally naïve, virgin female Long-Evans (hooded) rats, 2–5 months old, weighing 155–300 g. All rats were born and raised in our colony at the University at Buffalo and were the first- or second-generation random-bred offspring of rats purchased from Harlan Blue Spruce stock (Harlan Sprague Dawley, Indianapolis, IN). All procedures were approved by the University at Buffalo Institutional Animal Care and Use Committee.

Rats were maintained in a controlled environment with an ambient temperature of 22°±1 °C, a relative humidity of 40–60%, and a 14-h-on/10-h-off light/dark cycle (lights on at 0500 h, EST). Rats were housed individually in 32×20×20-cm, standing, clear plastic cages, and were allowed ad lib access to food (Harlan Teklad 22/5 Rodent Diet [W] 8640 and Harlan Teklad 2018) and water, except where otherwise stated.

As is commonplace in our laboratory, all rats were monitored daily for stage of the estrous cycle by microscopic examination of the cells in vaginal fluid.

4.2. *Stereotaxic surgery*

All rats were implanted with a single, permanent, guide cannula through which morphine could be injected directly into the left VTA. Surgery was performed while rats were anesthetized with ketamine (75 mg/kg, IP) augmented with xylazine (10 mg/kg, IP). All rats received the topical analgesic Marcaine® (bupivacaine hydrochloride and epinephrine, 50% dilution of 2.5% solution) applied to the eardrum and the incision site.

A 22-ga stainless-steel cannula (Plastics One, Inc., Roanoke VA) was inserted 1 mm dorsal to the left VTA (AP=−6.0 mm from bregma; L=−0.6 mm from the mid-sagittal suture; DV=−8.4 mm from dura), with the incisor bar positioned at the interaural line and the stereotaxic arm at an angle of 4° to the left of vertical. The coordinates were modified from the stereotaxic atlas of Paxinos and Watson (1986). The internal cannula used to deliver the drug to the VTA was cut 1 mm longer than the tip of the guide cannula. The guide cannula was anchored to the skull with dental polymer affixed to three 0–80 stainless-steel screws. At the end of surgery, a stainless-steel obturator, cut flush with the guide cannula, was inserted into the guide cannula.

4.3. *Drug and drug injections*

The nonselective opioid-receptor agonist morphine sulfate (Sigma-Aldrich Inc., St. Louis MO) was used. The doses, 0.0, 0.01, and 0.03 μg, in saline, were chosen in accordance with the study by Thompson and Kristal (1996).

Morphine sulfate solution was injected with a Harvard microinfusion pump (Models 944 and 935) at a volume of 0.56 μl and a rate of infusion of approximately 1 μl/min. During the injection, the rat was free to move about its home cage. After the injection, the internal cannula was left in position in the VTA for a period of 30 s to allow for drug dispersal from the cannula tip. The internal cannula was then replaced with the obturator in order to minimize the backflow of drug into the guide cannula.

4.4. *Habituation*

Each subject was habituated to all testing procedures for 5 days before the stereotaxic surgery and then for 2 days before the start of testing. Rats were habituated to (a) the experimenter (handheld for 4–5 min/day); (b) removal of obturator (cannula was unscrewed once/day); (c) the microinjection procedure (5 min/day); (d) and the orogastric intubation procedure. All rats were moved to the testing room and placed into testing cages 24 h before the start of the experiment to habituate them to the testing room.

4.5. *Stimulus pups*

Stimulus pups were foster rat pups obtained from the litters of time-bred donors maintained in a separate colony room. Exposing maternally naïve adults to foster

pups for a prolonged period necessitates replacing the pups at regular intervals to insure continued stimulus quality and survival of the pups because the subjects neither lactate nor necessarily behave maternally. Pups were exchanged every 12 h with pups that had been fed and cared for by their natural mother (donor) over the preceding 12 h (at minimum) (Kristal et al., 1981; Rosenblatt and Lehrman, 1963; Steuer et al., 1987; Wiesner and Sheard, 1933). Pup replacement occurred at the start of each observation period.

4.6. *Amniotic fluid collection and administration*

Amniotic fluid was harvested on Day 21 of pregnancy (presence of sperm = Day 1) from donors euthanized with CO_2. Amniotic fluid was immediately frozen (−40 °C) and stored for later use during experiments. For administration, frozen amniotic fluid was warmed for 15 min to 37 °C in a heating block, drawn up in a 1 ml, 0.5 ml, or 0.25 ml plastic or glass syringe to the appropriate volume and immediately administered to the subjects via an orogastric infusion (Kristal et al., 1988). Subjects were infused with 0.25 ml heated amniotic fluid or saline through an 11.4-cm length of PE 140 tubing that was inserted into the stomach. Rats were handheld during the intubation procedure. We do not find it necessary to administer AF in doses indexed by body weight. The dose of amniotic fluid is based on the previous finding that 0.25 is the optimum dose for the enhancement of a low, suprathreshold dose of morphine, and corresponds to the amount delivered with each neonate (Kristal et al., 1988). Furthermore, our research has also shown that POEF activates receptors in the gut (Robinson et al., 1995; Tarapacki et al., 1992) rather than being absorbed into the system; indexing doses by body weight is a procedure reserved usually for substances that are absorbed when administered systemically.

4.7. *Apparatus*

Observation cages, in which experimental rats were housed during the testing period, were 10-gal glass aquaria with a mesh partition, reducing the size to 34 × 29 × 38 cm. Rats were prevented from seeing one another by cardboard partitions placed between the cages. No more than four rats were observed at a time.

4.8. *Maternal behavior tests*

During the behavior observations, the presence or absence of full maternal behavior was noted. At the start of each 15-min observation period, four freshly fed and cared-for age-matched foster pups (3–8 days old) were scattered about the observation cage and the presence of pup retrieval to the nest, pup licking, and crouching over the pups was scored. When a rat showed all three of these pup-directed behaviors consistently, in two consecutive 15-min observation periods, she was considered fully maternal. The dependent measure derived from the maternal-behavior test was latency in days to show full maternal behavior (the frequencies, durations, and latencies of various components of maternal behavior do not vary significantly in concaveated rats, so latency to onset of full maternal behavior is usually the dependent variable of choice in such maternal-behavior studies). Each rat was assigned the latency, to the nearest 0.5 days, that corresponded to the first observation period of the two consecutive observation periods in which she showed full maternal behavior. Any rat that had not expressed maternal behavior by the end of the 10th day was assigned a latency of 10 days. The protocol used to score full maternal behavior corresponds to the standard maternal-behavior test protocol used in our laboratory during concaveation (Steuer et al., 1987), and is consistent with the procedures used in other laboratories.

In addition to latency to show full maternal behavior (sensitization latency), general activity, spontaneous retrieval, and cannibalism were noted. There were no spontaneous retrievers in our study and seven rats that cannibalized. The seven rats that cannibalized were distributed in a nonsystematic fashion among the groups. In Long-Evans (hooded) virgin female rats, we have observed incidence of spontaneous retrieval to be usually quite low (<1%).

4.9. *Testing timeline and design*

Each subject was habituated for 5 days before the stereotaxic surgery. After the surgery each subject received a recovery period of 5–7 days followed by another 2 days of habituation. Testing began on the next day (8–10 days after surgery).

Two 15-min behavioral observations were made on each of the 10 consecutive days: one between 0900 h and 1000 h (during lights on) and one between 2100 h and 2200 h (during lights off). On the first 3 morning tests, each subject received a microinjection of morphine sulfate solution, or vehicle, into the VTA. Fifteen minutes after the microinjection the subject received an orogastric infusion of either 0.25 ml amniotic fluid or saline. Therefore, every rat received both an orogastric infusion and a VTA injection. Twenty minutes after the orogastric infusion each subject was presented with pups and observed for 15 min for the occurrence of maternal behavior. Food was removed 1 h before the observation period and restored immediately after the observation period. Although there is a specific onset latency and duration of the effect of POEF (Doerr and Kristal, 1989), the order of injection and infusion was found in our previous studies not to be relevant, and the precise time line and number of treatments used here was based on an attempt to replicate those used by Thompson and Kristal (1996).

The design of this experiment was a 2 × 3 factorial design: Enhancer (0.25 ml amniotic fluid, 0.25 ml saline, infused orogastrically) × Drug Dose (0, 0.01, 0.03 μg morphine in 0.56 μl saline, injected into the VTA).

4.10. *Histological examination*

At the conclusion of the study, each rat was euthanized with CO_2 and injected through the guide cannula with 0.1–0.5 μl

methyl blue dye. The brain was then removed, frozen (−20 °C), and cut into 40-μ sections. Every 4th or 5th section that showed the cannula track was mounted and saved; a subgroup of those was stained with cresyl violet. Placements were considered to be accurate if dye was found in the VTA (Fig. 1). Data from any rat with an inaccurate cannula placement were excluded from statistical analysis of the VTA results.

4.11. *Statistical analysis*

Nonparametric analyses were used to analyze the differences in Median response latencies between amniotic fluid-infused and saline-infused groups at each dose of morphine. The data contained many latency scores that were "at ceiling", (i.e., 10 days), which truncated the distribution of scores and led to a violation of the assumption of interval measurement necessary for parametric analysis. Therefore, nonparametric tests (Kruskal–Wallis test and Median test) were applied.

Acknowledgments

This research was supported by NSF Grant IOB-0445679 awarded to M.B.K. The authors thank Reema T. Cheema, Eric T. Hillery, Seth W. Hurley, Ksenia Kastanenka, and Thom P. Smith for their assistance.

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