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**Research Report**

# Effect of amniotic-fluid ingestion on vaginal–cervical-stimulation-induced Fos expression in female rats during estrus

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**ABSTRACT**

Placental Opioid-Enhancing Factor (POEF) is a substance found in amniotic fluid (AF) that, when ingested, potentiates opioid-mediated, but not non-opioid-mediated, hypoalgesia. Vaginal–cervical stimulation (VCS) produces a stimulus-bound, partially opioid-mediated hypoalgesia that previous research has shown to be potentiated by AF ingestion. To understand the mechanism of opioid enhancement by POEF we investigated the pattern of neural activation after a bout of VCS that produced hypoalgesia, with and without co-administration of AF. Specifically, virgin Long–Evans rats showing vaginal estrus were handled briefly (control) or received VCS (75 g pressure, 1 min), in a pattern that approximated early parturition rather than copulation, using a spring-loaded glass-rod probe. Rats were given an orogastric infusion (0.25 ml) of either AF or 0.9% saline resulting in four groups (VCS or handling; AF or saline). Rats were perfused 90 min after treatment and tissue was processed by immunohistochemistry for Fos. The number of Fos-immunoreactive cells was counted in structures previously shown to express Fos in response to VCS (the medial preoptic area, MPOA; the ventrolateral portion of the ventromedial hypothalamic nucleus, vlVMH; the arcuate nucleus, ARC). We found that this pattern of VCS did not produce a significant increase in Fos expression in the MPOA and vlVMH unless it was paired with AF. VCS produced a significant increase in Fos in the ARC. The interaction of AF and VCS on Fos expression in the MPOA suggests that POEF may enhance vaginal–cervical sensory input at parturition to facilitate sensitization of the MPOA, and presumably facilitate maternal-behavior onset.

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**1. Introduction**

At delivery there are numerous redundant factors that lead to the immediate and appropriate expression of maternal behavior in the rat. Among these factors are vaginal–cervical stimulation from pup expulsion and uterine stretch, ingestion of afterbirth materials (amniotic fluid, placenta and fetal

membranes) and opioid-mediated events including increased pain threshold and facilitation of maternal behavior (for review, see Kristal, 2009).

The pelvic, pudendal, and hypogastric nerves have been shown to carry sensory information from the genitalia to the CNS (Peters et al., 1987). More recently, the vagus nerve has also been shown to transmit sensory information from

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vaginal-cervical stimulation (VCS): (a) complete spinal transection attenuated, but did not block, reflexive pupil dilation to VCS. Blocking of the pupil reflex was not accomplished until a spinal transection was paired with bilateral vagotomy (Komisaruk et al., 1996). (b) After section of the pelvic, pudendal and hypogastric nerves, analgesia from VCS was not blocked until subsequent bilateral vagotomy occurred (Cueva-Rolón et al., 1996). And (c) women with complete spinal transections show a response in the nucleus of the solitary tract (NTS) to VCS in PET and fMRI imaging studies (Komisaruk et al., 2004; Komisaruk and Whipple, 2005) as well as report a subjective sensation of orgasm. These data indicate that the vagus nerve plays a role in VCS in general, but also in the analgesia component more specifically.

This interpretation is consistent with experiments from other research that show that vagal stimulation has effects on pain transmission that include: inhibition of background activity of spinothalamic tract neurons (Ren et al., 1991); attenuation of morphine-induced hypoalgesia from bilateral vagotomy at several different levels (Randich et al., 1991); and mediation by spinal opioids and serotonin and norepinephrine (Liu and Gintzler, 1999; Ren et al., 1989). These data, taken together, indicate that vagal stimulation is not only sufficient to produce an opioid-mediated hypoalgesia on its own but that the vagus participates in the response to morphine injections through modulation of spinal neurochemicals.

VCS has been shown to facilitate the onset of maternal behavior in pseudopregnant rats (Graber and Kristal, 1977) and lamb acceptance in ewes (Keverne et al., 1983). VCS also produces a partly opioid-mediated hypoalgesia (Hill and Ayliffe, 1981; Gintzler and Komisaruk, 1991) that is modulated by steroid hormones and stimulus intensity (Crowley et al., 1976) as well as potentiated by amniotic fluid ingestion (Kristal et al., 1986b; Thompson et al., 1991).

Ingestion of afterbirth materials (placentophagia) occurs in most nonhuman mammalian species during parturition (Kristal, 1980). In nonpregnant rats, ingestion of either placenta or amniotic fluid (AF) has been shown to potentiate opioid-mediated increases in pain threshold from systemic morphine injection (Abbott, et al., 1991; Kristal et al., 1986a), central morphine microinjection (DiPirro et al., 1991), central injection of  $\delta$ - and  $\kappa$ -opioid agonists (DiPirro and Kristal, 2004), and footshock (Kristal et al., 1985). The potentiating effect of ingested afterbirth material can be blocked by a selective gastric vagotomy (Tarapacki et al., 1992).

Ingestion of AF seems to act only by potentiating opioid-mediated events because it has no effect on its own (Kristal et al., 1985, 1986a), is naloxone reversible, and does not enhance nicotine-mediated (Robinson-Vanderwerf et al., 1997) or aspirin-mediated hypoalgesia (Kristal et al., 1990). The active substance in afterbirth material has been named Placental Opioid-Enhancing Factor (POEF) (Kristal et al., 1988).

Numerous experiments have used VCS as a stimulus for Fos expression (Pfaus et al., 1996; Tetel et al., 1993) but these experiments have used a pattern of VCS that mimics copulation by using a stimulus male or repeated short duration pressure delivered by the experimenter. This VCS is likely quite different from that experienced during parturition and may present a ceiling effect for studies investigating facilitating influences on the response. Moreover, to the best

of our knowledge, there have been no studies using a parturitional model for application of VCS. Accordingly, the present experiments used a modified application of VCS that was selected to more closely resemble the longer duration, less oscillating stimulus that would occur during expulsion of a fetus. Moreover, the experiments were designed to provide the first evaluation of Fos expression associated with ingestion of AF.

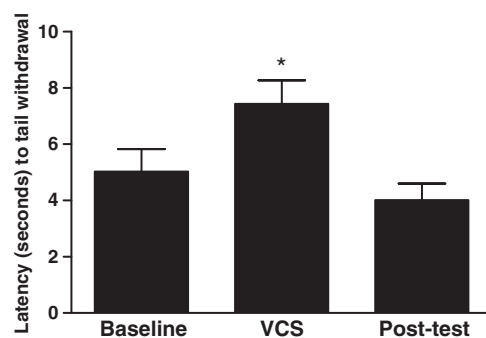
## 2. Results

An initial experiment was performed to test whether the VCS parameters used by us produced hypoalgesia that was at all similar to that reported using more copulation-like parameters (Hill and Ayliffe, 1981). To this end, rats ( $n=5$ ) were tested in the hot-water tail-dip assay for VCS-induced hypoalgesia. Rats were tested 20 min before, during and 20 min after VCS application. As illustrated in Fig. 1, VCS produced by 75 g of pressure increased tail-flick latency over baseline, but the effect of VCS did not persist into the final test (one-way repeated measures ANOVA,  $F_{2,8}=37.17$ ,  $p<0.01$ ).

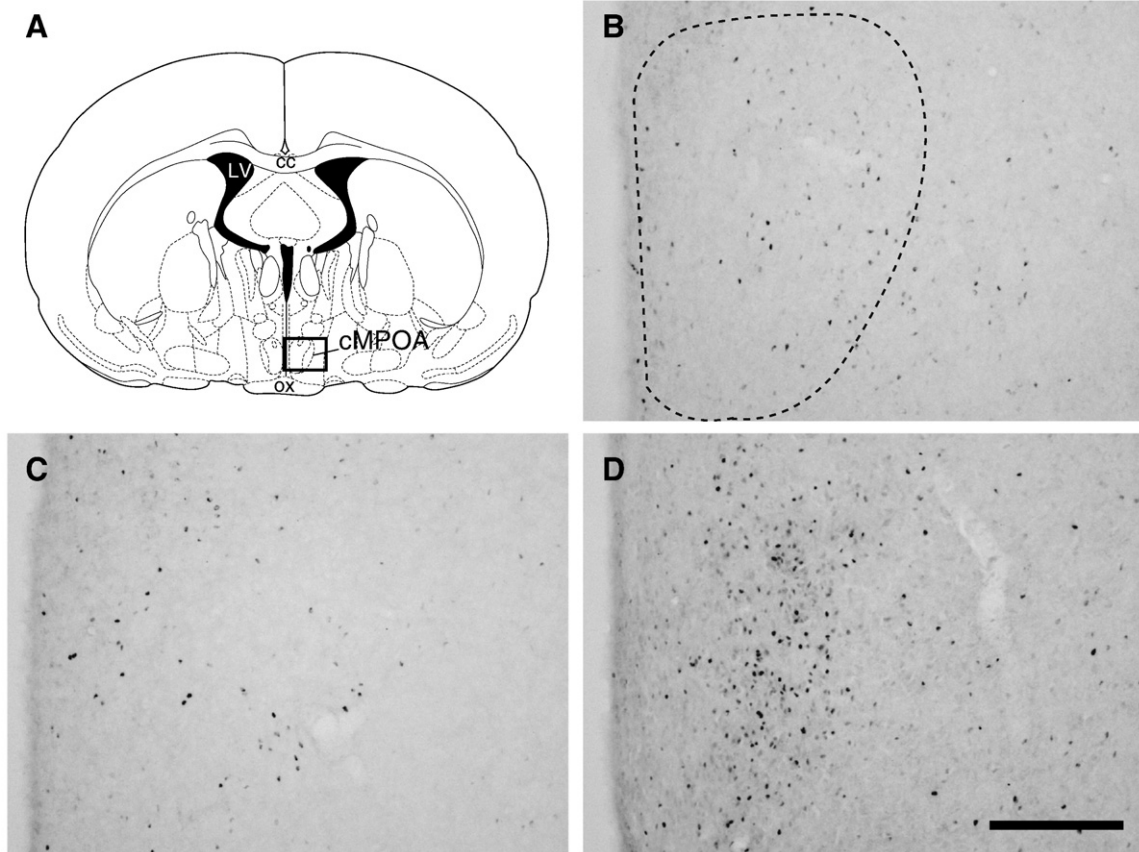
Once the hypoalgesic efficacy of 75 g of VCS was confirmed, we determined the pattern of Fos expression in rats ( $n=4-5$  per group) after orogastric infusions of amniotic fluid or saline, with or without VCS. Our analysis included brain areas associated with maternal behavior as well as those implicated in the response to VCS or, more generally, pain responsiveness.

The medial preoptic area (MPOA) has been shown to be crucial for maternal behavior expression in rats (for review, see Kristal, 2009; Numan, 2006). In the caudal MPOA (cMPOA) there were significant elevations of Fos expression due to AF ( $F_{1,14}=10.037$ ,  $p\leq 0.006$ ) and VCS ( $F_{1,14}=22.5$ ,  $p\leq 0.001$ ) and a significant interaction between AF and VCS ( $F_{1,14}=11.124$ ,  $p\leq 0.005$ ) (Fig. 2). A post-hoc test indicated that the main effect of VCS was driven by the VCS+AF group mean (Fig. 3) because VCS in the absence of AF failed to produce detectable increases in Fos.

The ventromedial hypothalamus (VMH), particularly the ventrolateral subregion (vlVMH) has been identified as a

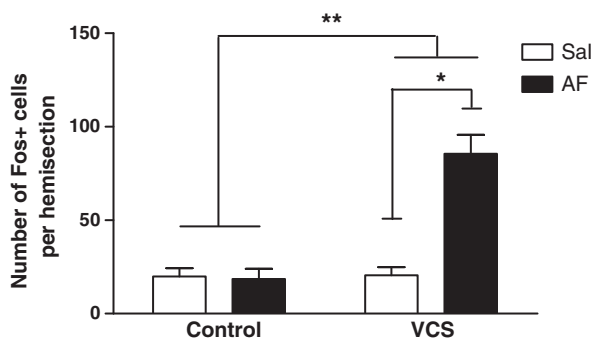


**Fig. 1 – Hypoalgesic effect of 75 g pressure VCS in the hot-water tail dip-assay. Values are the latency (Mean ± S.E. M. sec) to withdraw the tail from 52 °C water. Data are shown from rats ( $n=5$ ) 20 min before, during, and 20 min after VCS. An asterisk is used to indicate differences ( $p<0.05$ ) from all other groups.**



**Fig. 2** – Fos expression in the cMPOA. Panel A shows an atlas diagram (modified from Paxinos and Watson, 2005) of the approximate location of the sections in the analysis. A box shows the approximate area of the micrographs. Representative micrographs from rats in the Handling + AF (B), VCS + Sal (C), and VCS + AF (D) groups are shown. The dashed line in panel B shows the region of interest included in the analysis. Abbreviations: cc, corpus callosum; LV, lateral ventricle; cMPOA, caudal medial preoptic area. Scale bar is 200  $\mu\text{m}$ .

component of the circuit mediating the lordosis response (Pfaff and Sakuma, 1979a,b). Results obtained in this region were similar to the cMPOA (Fig. 4) in that there was a significant elevation of Fos expression due to VCS ( $F_{1,14}=18.63$ ,  $p\leq 0.001$ ) and a significant interaction between AF and VCS ( $F_{1,14}=10.05$ ,  $p\leq 0.007$ ), but we did not find a significant main effect of AF ( $F_{1,14}=3.448$ ,  $p\leq 0.08$ ) (Fig. 5). These



**Fig. 3** – Mean ( $\pm$  S.E.M.) number of Fos-immunoreactive cells in the cMPOA. Data are shown from rats ( $n=4-5$  per group) that were handled briefly (control) or given 75 g pressure VCS after orogastric infusion of saline or AF. \*  $p<0.01$ , \*\*  $p<0.001$ .

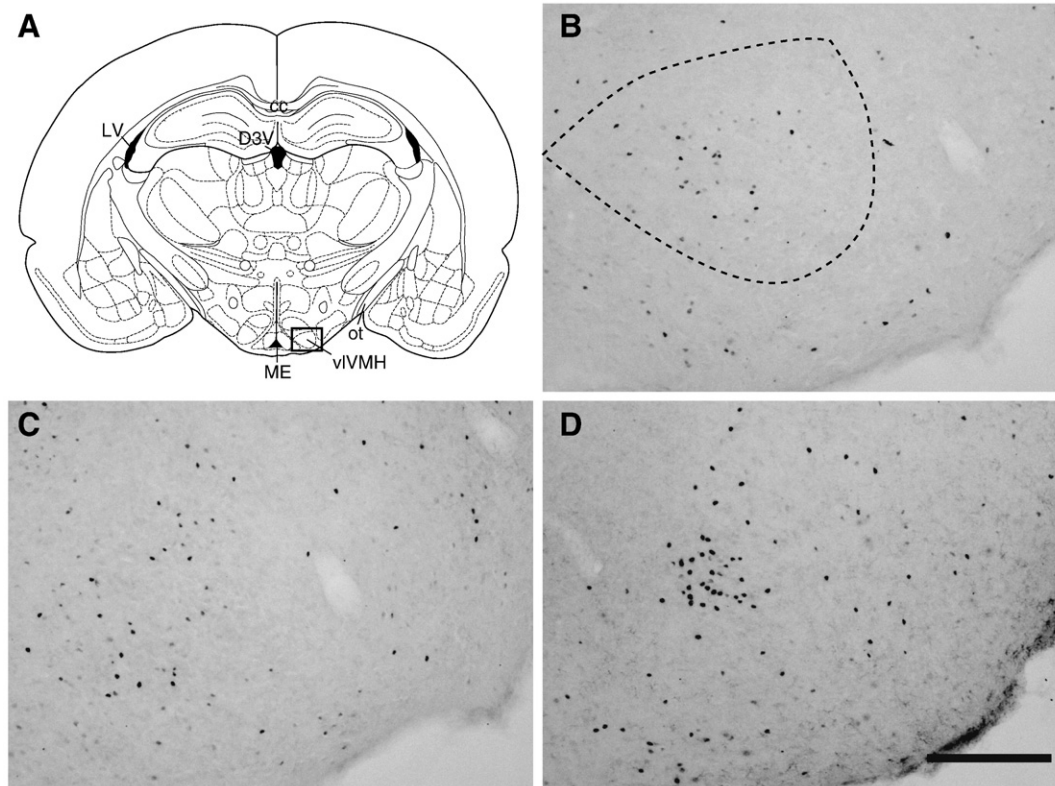
data show that POEF ingestion modulates VCS-induced Fos expression in the vVMH.

In the arcuate nucleus there was a significant elevation of Fos expression due to VCS ( $F_{1,14}=5.072$ ,  $p\leq 0.005$ ) but no main effect of AF and no significant interaction between AF and VCS (Fig. 6). These data indicate that, unlike the other brain areas examined, AF did not modulate VCS-induced Fos in the arcuate nucleus (Fig. 7).

The nucleus of the solitary tract (NTS) receives sensory information from the vagus nerve. Although parturition-induced Fos has been shown in the NTS (Lin et al., 1998) and many effects of VCS and AF are mediated by the vagus nerve, we did not find reliable increases in Fos at either of the two levels of the NTS we examined. Several nuclei related to hypoalgesia were examined, either qualitatively (ventral tegmental area rostromedial medulla), or quantitatively (periaqueductal gray), but there were no significant results from these areas.

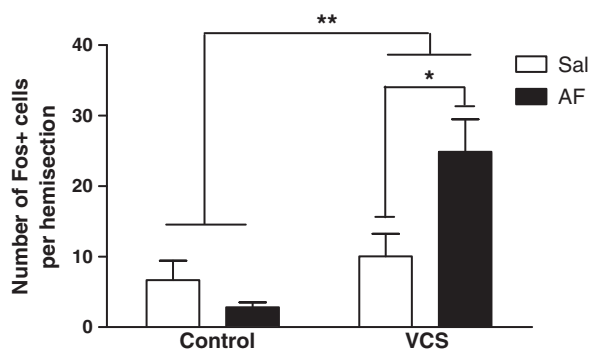
### 3. Discussion

The present studies are the first to investigate the effect of AF ingestion on Fos expression in the rat CNS. These experiments



**Fig. 4** – Fos expression in the vVMH. Panel A shows an atlas diagram (modified from Paxinos and Watson, 2005) of the approximate location of the sections in the analysis. A box shows the approximate area of the micrographs. Representative micrographs from rats in the Handling+AF (B), VCS+Sal (C), and VCS+AF (D) groups are shown. The dashed line in panel B shows the region of interest included in the analysis. Abbreviations: D3V, dorsal third ventricle; LV, lateral ventricle; ME, median eminence; ot, optic tract. Scale bar is 200  $\mu\text{m}$ .

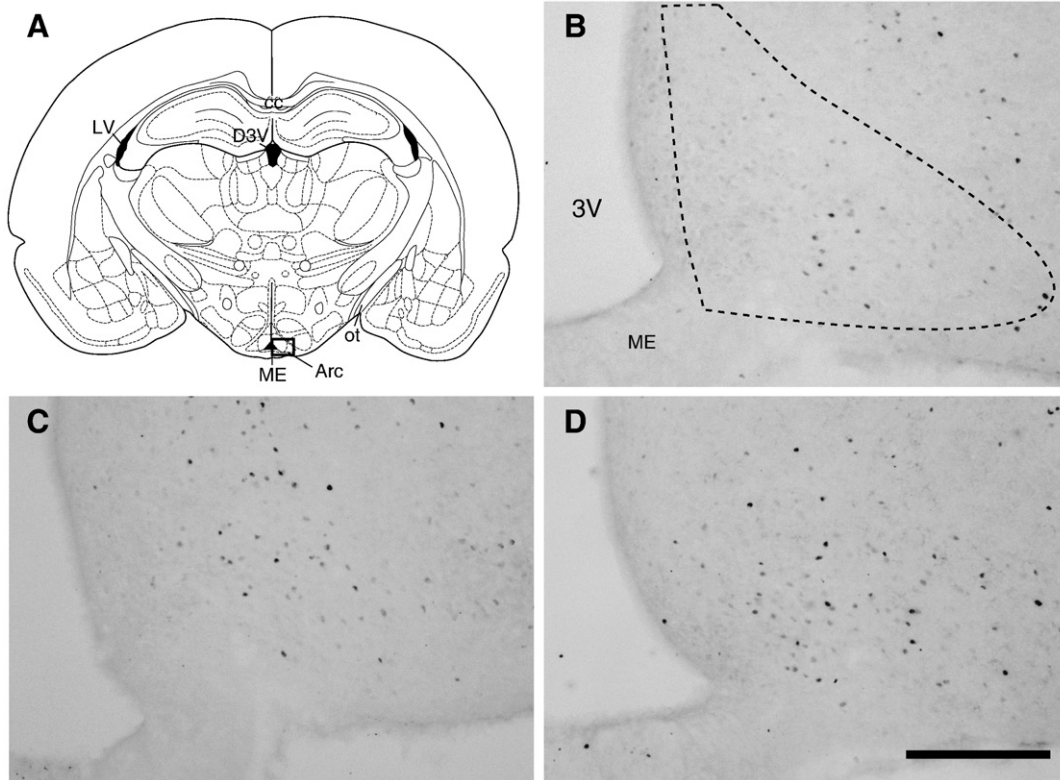
demonstrated that AF, given alone, had no detectable effect on Fos expression, but that it produced a marked increase in Fos in several structures when paired with VCS. The interaction between VCS and POEF (the active component in AF and placenta) ingestion in the MPOA is especially noteworthy because it suggests that these factors facilitate maternal-behavior onset at parturition by increasing activity in the MPOA, a structure that has been shown to be crucial for the



**Fig. 5** – Mean ( $\pm$  S.E.M.) number of Fos-immunoreactive cells in the vVMH. Data are shown from rats ( $n=4-5$  per group) that were handled briefly (control) or given 75 g pressure VCS after orogastric infusion of saline or AF. \*  $p < 0.01$ , \*\*  $p < 0.001$ .

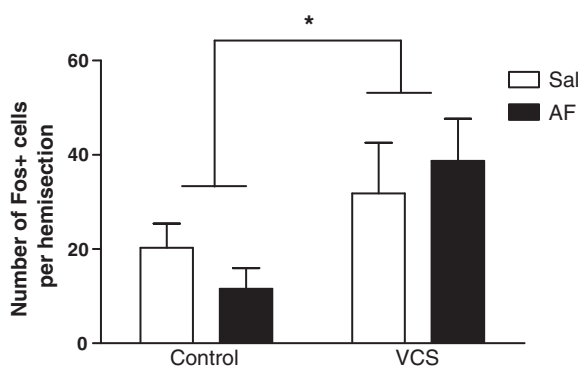
expression of maternal behavior (Numan, 1974, 2006; Numan et al., 1977).

The present data are the first to explore a VCS pattern that is arguably more like that occurring during parturition than during copulation, and the first to demonstrate a dissociation between VCS-induced hypoalgesia and changes in forebrain Fos expression. Our stimulation paradigm that used lower VCS pressure (75 g) and longer duration (1 min of continuous pressure) is not typical in Fos literature on VCS. Previous studies have used higher pressure (150–250 g) and trains of short duration (2-sec applications) stimulation to mimic the type of stimulation that is received during copulation. Rather than replicate this pattern we wanted to approximate the stimulation that occurs during the early stages of fetal expulsion by increasing the duration of application. Initial testing of the VCS-induced hypoalgesia showed that 75 g of pressure was sufficient to produce hypoalgesia in the hot-water tail-dip assay. Nevertheless, this VCS, when given in the absence of AF, was not sufficient to produce observable differences in Fos, even in brain areas shown to express Fos after copulation-like VCS. This lack of Fos, however, allowed us to observe an interaction between VCS and AF that might not have been observable otherwise. Indeed, many effects of POEF involve enhancement of an effect (e.g., pain threshold) and a less intense stimulus is required to avoid a ceiling that could otherwise mask the effect of POEF.



**Fig. 6 – Fos expression in the arcuate nucleus of the hypothalamus.** Panel A shows an atlas diagram (modified from Paxinos and Watson, 2005) of the approximate location of the sections in the analysis. A box shows the approximate area of the micrographs. Representative micrographs from rats in the Handling + AF (B), VCS + Sal (C), and VCS + AF (D) groups are shown. The dashed line in panel B shows the region of interest included in the analysis. Abbreviations: 3 V, third ventricle; arc, arcuate; D3V, dorsal third ventricle; LV, lateral ventricle; ME, median eminence; ot, optic tract. Scale bar is 200  $\mu\text{m}$ .

Afterbirth materials are typically only available for consumption at parturition, which is also the time that maternal behavior begins in primiparous rats. AF (and presumably POEF) ingestion has been found to potentiate the facilitative effect on pup-exposure-induced maternal behavior of a unilateral morphine microinjection into the ventral tegmental area (Neumann et al.,



**Fig. 7 – Mean ( $\pm$  S.E.M.) number of Fos-immunoreactive cells in the arcuate nucleus of the hypothalamus.** Data are shown from rats ( $n=4-5$  per group) that were handled briefly (control) or given 75 g pressure VCS after orogastric infusion of saline or AF. \*  $p < 0.01$ .

2009; Thompson and Kristal, 1996). These results indicate that ingestion of POEF plays a role in the expression of maternal behavior at parturition through modulating opioid-mediated effects on maternal behavior as well as hypoalgesia. Our Fos data now suggest that one site in the brain at which this phenomenon occurs is the cMPOA.

Examination of the vVMH revealed a similar increase in Fos-immunoreactive cells after the combination of AF and VCS. Although the VCS used here was intended to resemble more closely the stimulation that occurs during parturition, the effects observed in the vVMH are most likely related to the role of the VMH in mating behavior. Previous studies demonstrated VCS- or mating-induced Fos in the VMH (Calizo and Flanigan-Cato, 2003; Pfau et al., 1996; Quysner and Blaustein, 2001) and this structure has a well-documented role in the control of the lordosis reflex (Pfaff and Sakuma, 1979a,b; Daniels et al., 1999). Whether our VMH data reflect lordosis involvement or involvement of the VMH in the regulation of maternal behavior (Bridges and Mann, 1994; Bridges et al., 1999; Mann and Babb, 2004), still remains to be determined.

The NTS, especially the caudal portion of the structure, is the primary site of vagal input to the CNS (Loewy, 1990). Given previous suggestions of the importance of the vagus nerve in the response to VCS (Hubscher and Berkeley, 1995; Cueva-Rolón et al., 1996; Komisaruk et al., 1996; Komisaruk et al., 2004; Komisaruk and Whipple, 2005) and to the POEF effect (Tarapacki

et al., 1992), it was surprising that we did not observe VCS- or AF-induced Fos in the NTS, even when the treatments were combined. The lack of differences in Fos expression may relate to the VCS paradigm used. Even though our procedure produced hypoalgesia it may not have been sufficient to produce changes in Fos in this area. Specifically, the procedure may not have sufficiently mimicked all the parameters of VCS during early stages of delivery, thereby activating only a subset of the structures involved. It is also possible that a different staining procedure would have revealed changes in Fos in the NTS, but our ability to detect the interaction between AF and VCS in other regions suggests that a similar interaction is not occurring in the NTS. However, we feel that our results neither support nor disconfirm the suggestion, based on indirect data, that the afferent signals from VCS are mediated by the NTS.

Examination of the arcuate nucleus of the hypothalamus revealed an increased number of Fos-positive cells after VCS, but we found no effect of AF ingestion and no interaction. The VCS-induced increase is consistent with previous studies of the arcuate (Yang et al., 1999). It is important to note, however, that our examination of the effect of AF ingestion does not rule out an effect within the arcuate nucleus that is more selective to specific populations of cells. The arcuate is not a homogeneous population, but can be subdivided anatomically or neurochemically (for review, see Chronwall, 1985). Examination of a subset of these neurons may reveal differences that were not apparent in the present analysis. Accordingly, we cannot completely rule out a role of the arcuate nucleus in the affect of AF ingestion.

These data show that VCS-induced Fos in the cMPOA and vVMH can be potentiated by afterbirth ingestion. When these results are applied to the facilitative effect of POEF on maternal-behavior onset it is clear that VCS and afterbirth ingestion at parturition interact to not only produce a potentiated hypoalgesia during delivery, but also to increase the activity of the MPOA to facilitate the expression of maternal behavior. Furthermore these data are the first to show an anatomical substrate for POEF activity.

## 4. Experimental procedures

### 4.1. Subjects

Subjects were experimentally naïve, virgin female Long–Evans (hooded) rats, 2–3 months old at the start of the experiment, weighing 150–250 g, and had been purchased at 60 days of age (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 ambient temperature of  $22 \pm 1$  °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  $32 \times 20 \times 20$  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 tap 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. Estrus was defined as a smear with

predominantly cornified cells, possibly some nucleated cells, but no leucocytes.

### 4.2. Habituation

All rats were allowed to acclimate to our laboratory for 7 days after arrival. Each subject was habituated to the following testing procedures for 5 days prior to testing: (a) the experimenter (handheld for 5 min/day); (b) restraint for VCS (handling was done using the black cotton sock that was used for restraint during VCS); (c) and the orogastric intubation procedure (11.4 cm length of PE 140 tubing was inserted into the stomach 1/day).

### 4.3. Amniotic fluid collection and administration

Amniotic fluid was harvested on Day 21 of pregnancy (presence of sperm=Day 1) from donors euthanized with CO<sub>2</sub>. AF was immediately frozen ( $-20$  °C) and stored for later use during experiments. For administration, frozen AF was warmed for 15 min to 37 °C in a heating block, drawn up into a 0.25 ml glass syringe to the appropriate volume and immediately administered to the rats via an orogastric infusion (Kristal et al., 1988). Orogastric infusion was accomplished by infusing 0.25 ml heated AF 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. The dose of AF was based on previous studies showing that 0.25 ml is the optimum volume for enhancement of a low, suprathreshold dose of morphine, and roughly corresponds to the amount delivered with each neonate (Kristal et al., 1988).

### 4.4. Hot water tail-dip assay

The hot-water tail-dip assay is a quick, easy, and innocuous algometric test (Hahn, 1985) that we have used numerous times in our laboratory (e.g., Martin et al., 2001; Thompson et al., 2004). For testing, the home cage of each rat was moved into a separate testing room, and returned to the colony room afterward. To verify hypoalgesia with our stimulus parameters (75 g pressure, 1-min bout) we tested naïve female rats in estrus ( $n=5$ ) for tail withdrawal latency from  $52 \pm 1$  °C water (water bath model 181, Precision Scientific Inc.) 20 min before VCS, during VCS and 20 min after VCS. Each test consisted of 4 trials with 30 s between trials. A trial was terminated after 1 min to prevent tissue damage and any residual water was lightly dried with a soft lab tissue (Kimwipes). A score was calculated for each test by excluding the first and averaging the last 3 measurements.

### 4.5. Vaginal-cervical stimulation

For VCS and handling application, the home cage was moved to a separate testing room. All rats were restrained by being put into a black cotton sock head first with the tail and anogenital region outside of the sock. VCS was applied continuously for 1 min by inserting a glass-rod probe fitted into a spring-loaded glass tuberculin syringe (calibrated to deliver 75 g of pressure) into the vagina. Handled (control) groups were restrained in the sock and handheld for 1 min. After treatments were applied, the home cage was moved back to the subject room until perfusion.

#### 4.6. Perfusion and sectioning

Ninety minutes after treatment, rats were anaesthetized with ketamine (75 mg/kg, IP) augmented with xylazine (10 mg/kg, IP) and transcardially perfused with heparinized saline (0.9% NaCl) followed by 2% paraformaldehyde. After perfusion, brain tissue was collected and stored in 2% paraformaldehyde overnight. Brains then were transferred into 20% sucrose solution for 1–2 days before sectioning. Brains were frozen, cut on a rotary cryostat into 3 serial sets of 40- $\mu$ m sections, and stored in cryoprotectant (pH 7.2, phosphate buffered solution containing 30% ethylene glycol, 300 g sucrose and 10 g polyvinylpyrrolidone per liter) at  $-20^{\circ}\text{C}$  for 18–20 days before being processed by immunohistochemistry as described below.

#### 4.7. Fos immunocytochemistry

Brain sections were removed from cryoprotectant and washed for 1 h in tris-buffered saline (TBS) changing the solution every 10 min. After initial washes, sections were incubated in hydrogen peroxide (3% in TBS) for 15 min. Sections then were washed briefly in TBS before being incubated with rabbit anti-Fos [EMD Chemicals, San Diego, CA; 1:10,000 in TBS with 0.2% Triton X-100, 3% normal donkey serum (NDS)] for 1 h at room temperature then overnight at  $4^{\circ}\text{C}$ . Sections were removed from the primary antibody and washed in TBS for 1 h, changing TBS every 10 min before being incubated for 2 h with biotinylated donkey anti-rabbit (Jackson ImmunoResearch, West Grove, PA; 1:1000 in TBS with 0.2% Triton X-100 and 3% NDS). After another set of three, 10-min washes in TBS, the sections were incubated with an avidin-biotin complex (Vector Laboratories, Burlingame, CA) for 1 h at room temperature. Sections then were washed twice in TBS before a third 10-min wash in 50 mM Tris. Immunoreactivity was visualized by reacting the sections with 3,3'-diaminobenzidine (0.2 mg/mL) and 0.025%  $\text{H}_2\text{O}_2$  in 50 mM Tris for 20 min. The reaction was stopped with repeated, brief TBS washes before the sections were floated onto Superfrost Plus slides (VWR, West Chester, PA) and coverslipped with Permount (Electron Microscopy Sciences, Hartfield, PA).

#### 4.8. Testing timeline and design

On the testing day, estrous stage was assessed by vaginal smear; only those females in estrus were tested. Those rats being tested had their food removed 2 h before treatments were applied to prevent stomach contents from interfering with AF administration; water was available ad lib throughout the experiment.

When rats were identified as being in estrus they were randomly assigned to one of four groups in a  $2 \times 2$  factorial design: VCS+AF, VCS+Sal, Hand+AF, Hand+Sal.

At the beginning of the test, AF or saline was heated and administered as described above. Ten minutes after orogastric infusion the subject was moved, in the home cage, to the testing room and VCS or handling was applied.

Ninety minutes after VCS rats were perfused and tissue collected and stored as described above.

#### 4.9. Data analysis

Tissue sections were examined using a Nikon Eclipse 80i microscope (Nikon Inc, Melville, NY) at  $10\times$  magnification. Images for analysis were captured using a Nikon DS-Fi1 camera (Nikon Inc) or RT KE Spot camera (Diagnostic Instruments, Inc, Sterling Heights, MI). After slides were coded so that the experimenter was unaware of experimental condition, pictures were collected of sections that were subjectively assessed as having the most Fos expression within each animal for each area of interest. Using observable landmarks and guidance from a rat brain atlas (Paxinos and Watson, 2005), ImageJ software (Abramoff et al., 2004) was used to set a threshold of detection and count the number of Fos-immunoreactive cells in 4–6 hemisections (cMPOA, v1VMH, arcuate) or sections (NTS) per area from each rat. Manual corrections were made to account for overlapping cells that would have been counted as one cell or not counted at all. All counting was done on images that were randomized and coded such that the experimenter was not aware of treatment group or rat of origin. An average number of Fos-immunoreactive cells in each area was calculated for each rat and used in the statistical analysis described below. One rat was excluded from the analysis because of levels of staining that were as much as 2.5 standard deviations from the Mean (with the subject included). Data from this rat were excluded from all analyses, even those unaffected by its inclusion.

Statistical analyses were performed by GraphPad Prism Pro 5.00 (GraphPad Software, Inc). One-way repeated measures ANOVA was used to evaluate effects of VCS on latency in the tail-dip assay. Two-way ANOVAs (Handling or VCS  $\times$  saline or AF) were used to test for differences in the number of Fos-immunoreactive cells per section or hemisection. Statistically significant differences ( $p < 0.05$ ) were subsequently analyzed using Bonferroni posthoc tests.

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