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Highlights

- Males of some biparental species eat placenta when their young are born.
- We studied the effects of placentophagia in sexually naive male California mice.
- Placenta treatment had no effect on paternal behaviors.
- Placenta treatment decreased latencies to approach pups and novel objects.
- Placenta treatment decreased fos expression in bed nucleus of the stria terminalis.

Consequences of Placentophagia by Adult Virgin Male California Mice (*Peromyscus californicus*)

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1. Introduction

Placentophagia, or ingestion of the afterbirth, is commonly performed by parturient females of most eutherian species, with some exceptions (e.g., pinnipeds, cetaceans, humans: Kristal, 1980; Young & Benyshek, 2010). The functional significance of placentophagia is unclear, but proposed explanations include avoiding predators or pathogens and meeting general or specific nutritional demands (reviewed by Kristal, 1980; Kristal et al., 2012). Studies on the effects of maternal placentophagia in several mammalian species have revealed that this behavior can modulate pain sensitivity and maternal motivation (reviewed by Kristal, 1991). For example, in rats (*Rattus norvegicus*) and cows (*Bos spp.*), placentophagia enhances opioid-mediated analgesia through an opioid-enhancing factor (POEF) produced by and found in the placenta (Hoey et al., 2011; Kristal, 1991; Kristal et al.,, 2012; Pinheiro-Machado et al., 1997). This hypoalgesic effect is mediated by the vagus nerve, may occur as soon as 5 minutes after ingestion, and can last for approximately one hour (Doer & Kristal, 1989; Tarapacki et al., 1992). Placentophagia-induced hypoalgesia was recently identified as being potentially mediated by δ-opioid receptor activation (Thompson et al., 2018). Decreased pain sensitivity during parturition may facilitate labor, as neonates are expelled more quickly (Kristal, 1991). Interestingly, POEF is found in placental tissues even of species that typically do not ingest placenta (i.e., dolphins, humans), suggesting that this substance is highly conserved among placental mammals (Abbott et al., 1991).

The placenta is an endocrine organ that produces many of the protein and steroid hormones involved in the onset and maintenance of maternal and paternal care in mammals (e.g., progestogens, estrogens, lactogens: Malassine et al., 2003). Although adult, sexually inexperienced female rats do not express high levels of spontaneous maternal-like behavior (i.e.,

47 alloparental behavior), this can be modified with exposure to pups and placenta, or with oral
48 administration of placenta. For example, exposure of adult female virgin rats to pups smeared
49 with placenta and amniotic fluid shortens the latency for the expression of alloparental care (i.e.,
50 maternal sensitization) (Kristal et al., 1981). Additionally, ingestion of placenta and amniotic
51 fluid by adult virgin female rats enhances the stimulatory effect of intracerebroventricular
52 morphine treatment on pup-induced maternal behavior (Neumann et al., 2009). Thus,
53 placentophagia by some female mammals may induce physiological and behavioral changes that
54 promote maternal care and, as a result, offspring survival.
55 Males of some mammal species, too, ingest placenta at the birth of their young. In the
56 uniparental (i.e., only one parent, the mother, provides offspring care) Siberian hamster
57 (*Phodopus sungorus*), males ingest experimentally presented placenta only if they are present at
58 the birth of their pups (Gregg & Wynne-Edwards, 2006). Similarly, male rats, which are
59 commonly averse towards the afterbirth, will begin to eat placenta after continuous exposure to it
60 (Abbott et al., 1991). In several biparental (i.e., both males and females care for their young)
61 mammals, males, in addition to females, sometimes ingest placenta during the birth of their
62 offspring. Among primates, placentophagia by males has been observed in the common
63 marmoset (*Callithrix jacchus*: T. E. Ziegler, pers. comm.), cotton-top tamarin (*Saguinus oedipus*:
64 T. E. Ziegler, pers. comm.), and silvery marmoset (*C. argentata*: J. A. French, pers. comm.), as
65 well as in some human populations (Coyle et al, 2015; Marraccini & Gorman, 2015). In
66 biparental rodents, placentophagia by males has been reported in dwarf hamsters (*Phodopus*
67 *campbelli*: Gregg & Wynne-Edwards, 2005; Jones & Wynne-Edwards, 2000), California mice
68 (*Peromyscus californicus:* Lee & Brown, 2002; Perea-Rodriguez & Saltzman, 2014), and prairie
69 voles (*Microtus ochrogaster*: K.L. Bales, pers. comm.) (but see McGuire et al., 2003).

Studies in dwarf hamsters and California mice indicate that adult males, similar to adult females, respond differently to placenta depending on their reproductive condition. In these two species, males are more likely to ingest placenta when housed with their pair-bonded, gestating mates and when they become fathers than when they are sexually inexperienced (Gregg & Wynne-Edwards, 2005; Perea-Rodriguez & Saltzman, 2014). These findings suggest that in at least some biparental mammals, males naturally become attracted to placenta during their mates' pregnancy and may commonly ingest placenta during the birth of their offspring. Still unknown, however, are the potential behavioral and/or physiological changes that males undergo as a consequence of ingesting placenta, and whether these changes influence the males' responses to their young.

In this study, we sought to characterize the behavioral and neural responses to an unfamiliar pup after oral administration of conspecific placenta to adult, virgin male California mice. We analyzed the presence of the protein Fos, the product of the c-Fos immediate-early gene that is commonly used as a marker of neuronal activity (Hoffman & Lyo, 2002), in key brain areas involved in paternal care in rodents. Adult virgin males were used because they are highly variable in their behavioral responses to pups, whereas virtually all California mouse fathers show pronounced, rapid-onset paternal care (de Jong et al., 2009; de Jong et al., 2012; Gubernick & Nelson, 1989; Horrell et al., 2017). We speculated that behavioral and neural effects of placentophagia were likely to be mediated by steroid hormones, and steroids can exert both rapid, transient effects via non-genomic mechanisms and delayed, more sustained effects via changes in gene expression (McEwen, 1991). Therefore, we analyzed responses to pups at three time points: 1, 7, and 24 h after placenta administration.

We hypothesized that the physiological changes resulting from ingestion of placenta lead to changes in both neural and behavioral responses to pup-related stimuli. We predicted that mice treated with placenta would approach pups more rapidly, would spend more time engaging in caretaking behaviors, and would express more Fos-immunoreactivity (Fos-ir) in brain areas positively linked to paternal care (ventral bed nucleus of the stria terminalis, medial preoptic area), as well as reduced Fos-ir in brain areas commonly activated by aversive stimuli (paraventricular nucleus of the hypothalamus, amygdala), compared to controls treated with oil vehicle only. Finally, we predicted that placenta ingestion would exert these behavioral and neural effects specifically in response to a pup as opposed to a neutral novel object.

2. Methods

2a. Animals

We used male California mice born and reared in our breeding colony at the University of California, Riverside that were descended from mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC). Mice were housed in standard, shoebox-style, polycarbonate cages (44 x 24 x 20 cm) containing aspen shavings for bedding and cotton wool for nesting material, with *ad libitum* access to food (Purina Rodent Chow 5001) and water. Lighting was on a 14:10 light:dark cycle, with lights on from 05:00 until 19:00 h. Ambient temperature and humidity were kept at approximately 23°C and 70%, respectively. Mice were checked twice daily and weighed twice weekly, and cages were changed weekly.

Mice were weaned at 27-31 days of age and housed in same-sex groups of three or four age-matched individuals; these groups contained no more than two siblings from any one litter. As mice reached the age of sexual maturity (~90 days: Gubernick, 1988), male groups were

115 divided into pairs of unrelated males. We chose adult males specifically because we wanted to
116 test animals at a stage when they would naturally search for mates, reproduce, and ingest
117 placenta.
118
119 2b. Experimental Design
120 Virgin male California mice were treated with either placenta homogenized in sesame oil
121 or oil alone via oral gavage (see below). Beginning 1, 7, or 24 h later, each mouse underwent a
122 1-h behavior test with either a 1- to 4-day-old pup or a control novel object - a pup-sized, oblong
123 glass marble. Immediately following the behavior test (i.e., 2, 8 or 25 h after placenta or oil
124 treatment), mice were euthanized and their brains were harvested for immunohistochemical
125 analyses (see below). Each virgin male mouse was tested under a single treatment condition
126 (placenta or oil), at a single time point (1, 7, or 24 h after gavage), and with a single test stimulus
127 (pup or marble). At the time of testing, mice had never been exposed to pups (other than their
128 own littermates) or marbles. The resulting sample sizes for each treatment, time point, and
129 stimulus type are shown in Table 1.
130 Mice assigned to the *placenta* group were administered a single near-term placenta (from
131 a gestating female no more closely related to the male than second cousin) homogenized in
132 sesame oil. Mice in the *control* group were administered sesame oil alone. We administered
133 placenta (or oil) via oral gavage because virgin male California mice are not likely to voluntarily
134 ingest placenta (Perea-Rodriguez & Saltzman, 2014; Perea-Rodriguez & Saltzman, unpub. data).
135 Mice from the two treatments did not differ in age at the time of testing (placenta: 158.9 ± 4.3
136 days, mean ± SEM; oil: 162.9 ± 5.2 days; $p=0.63$, $T=0.46$, $df=1$; unpaired T-Test).
137

2c. Placenta Collection

As previously described (Perea-Rodriguez & Saltzman, 2014; Perea-Rodriguez et al., 2018), placentas were collected from multiparous (2-7 previous litters) females 1-3 days prior to their estimated parturition date, determined by the date of their previous parturition and assessment of changes in female body mass based on measurements taken every 3-4 days. Fetuses were inspected visually to confirm that they were near-term and immediately euthanized with an intraperitoneal injection (0.1 mL) of pentobarbital sodium (Fatal-Plus: Vortech Pharmaceuticals, Dearborn, Michigan, USA). Placenta donors were euthanized using CO_2 inhalation, and placentas were removed and immediately stored at -70° C.

2d. Oral Gavage

Oral gavage was performed as previously described (Perea-Rodriguez et al., 2018) using a 5 cm length of Silastic® laboratory tubing (1.57 mm inside diameter x 2.41 mm outside diameter; Dow Corning, Copley, Ohio, USA) fitted onto an 18-gauge sterile needle; the needle's tip (~ 0.5 cm) had been filed off to avoid puncturing the tubing and injuring the animal. The needle was attached to a sterile 1 mL syringe containing either a single placenta (~0.4 g, and 0.1-0.2 mL in volume) homogenized in sesame oil (total volume: 0.5 mL) or 0.5 mL sesame oil alone. This volume was selected based on the size of the stomach and to minimize any discomfort to the mice. We used oil as a vehicle because we anticipated that hormonally mediated effects of placentophagia would likely be related to steroid hormones (Cornil & Charlier, 2010), as these hormones readily cross the blood-brain barrier and are biologically active follow ingestion; steroid hormones are hydrophobic and therefore oil-soluble.

Additionally, the sesame oil facilitated the passage of the placental tissues through the gavage apparatus.

Mice underwent oral gavage between 08:30 and 09:30 h. We treated animals in the morning because this is the time of day when California mice are most likely to give birth (within a few hours after lights-on: Lee & Brown 2002; Perea-Rodriguez & Saltzman, unpub. data) and therefore to ingest placenta. Each male mouse was first housed alone for 30 min in a clean isolation cage containing fresh bedding, food, and water. Placentas were thawed on ice, homogenized in 0.1-0.2 mL of sesame oil using a mortar and pestle, and collected using the sterile syringe, which was then attached to the 18-gauge needle fitted with the Silastic tubing; air bubbles were avoided as much as possible. Mice were lightly anesthetized using isoflurane (Minrad, Orchard Park, NY, USA) and held vertically as the tubing was carefully inserted into the esophagus and the contents of the syringe delivered over approximately 5-10 s. The recovery time from anesthesia was between 60 and 180 s, at which point animals were observed in their isolation cages for 10 min before being returned to the colony room.

2e. Behavior Testing

Each animal underwent a behavior test in the colony room during the lights-on phase of the light:dark cycle, beginning at 09:30-10:30 h (1 h after oral gavage), 16:30-17:30 h (7 h after gavage), or 09:30-10:30 h the next day (24 h after gavage). At the outset of each test, a 1- to 4-day-old pup (no more closely related to the male than second cousin) or a clean, pup-sized, oblong, glass marble was placed at the opposite end of the male's isolation cage from the focal animal. Each mouse was exposed to its respective stimulus for 60 min before being euthanized for tissue collection (see below). Behavior tests were videotaped, and the initial 20 minutes were

later scored using JWatcher software (Blumstein & Daniel, 2007). Behaviors scored were latency to approach the pup or marble, duration of investigating (i.e., sniffing) the pup, and duration of huddling + licking the pup (i.e., paternal behavior). All videos were scored by a single observer, who was blind to the animals' treatment.

2f. Brain Collection, Immunohistochemistry, and Fos-ir Quantification

Immediately after each hour-long behavior test, the focal mouse was deeply anesthetized with 10% pentobarbital (Vortech, Dearborn, Michigan, USA; 0.5 mL, i.p.) and perfused transcardially, first with 0.1M phosphate-buffered saline (PBS) and subsequently with 4% paraformaldehyde (PFA) (de Jong et al., 2009). Brains were placed in 4% PFA for 1 h immediately after perfusion to further increase tissue robustness. After the additional fixation period, brains were removed from PFA and stored in 0.1M PBS at 4°C until further processing. Brains were later cryoprotected in 30% phosphate-buffered sucrose for 2-4 days, embedded in optimal cutting temperature compound, frozen, and sliced into 30 μm sections on a cryostat set at -19°C. Five series of brain sections were collected sequentially and stored in 0.1M PBS with 0.01% sodium azide until staining occurred.

Fos immunohistochemistry was performed as previously described (de Jong et al., 2009). After pre-incubation with PBS containing 0.1% bovine serum albumin and 0.3% Triton-X-100 (i.e., PBS-BT), slices were incubated in a 1:10,000 dilution of rabbit-anti-c-Fos antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) in PBS-BT overnight. The next day, after removal of excess antibody through a series of PBS washes, the slices were incubated with donkey-anti-rabbit antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) in a 1:1,500 dilution with PBS-BT for 90 min. Signaling was enhanced using ABC-vector (1:800

206 dilution in PBS-BT, Vectastain Elite Kit, Vector Laboratories, Burlingame, CA, USA) before
207 being stained with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich, St. Louis, MO,
208 USA) in 0.6% Tris-buffer.

209 Using fine brushes, stained slices were mounted onto glass slides coated with gelatin and
210 chrome alum. Mounted slices were air-dried overnight, cleared using a range of alcohols, and
211 embedded in Entellan New (EMS, Hatfield, PA, USA) before being coverslipped. Micrographs
212 of stained and mounted brain slices were taken using a digital camera (Canon EOS 40D)
213 attached to a microscope (Leica Leitz DMRB). Micrographs of the medial preoptic area
214 (MPOA), the dorsal (dBST) and ventral (vBST) regions of the bed nucleus of the stria terminalis,
215 the paraventricular nucleus of the hypothalamus (PVN), and the central (CeA) and basolateral
216 (BLA) nuclei of the amygdala were taken for each brain (Figure 1). Because no brain atlas was
217 available for *Peromyscus* when the study was performed, brain regions/nuclei of interest were
218 located based on a standard atlas of the mouse brain (Paxinos & Franklin, 2004), as in previous
219 studies (de Jong et al., 2009; de Jong et al., 2012).

220 ImageJ software (1.46r; National Institutes of Health, USA) was used to count the
221 number of Fos-ir neurons in a 200 x 200 μm square in a representative area of neurons in each
222 region. The person counting was unaware of the treatment and stimulus condition of each
223 animal. Some of the brain sections were not usable due to problems during the sectioning or
224 staining process, so these were excluded from the analyses. The final sample sizes are presented
225 in the results.

226

227 2g. Statistical Analyses

All statistical analyses were performed using R statistical software (R Core Team, 2014). Behavioral and immunohistochemical data were tested for normality using Shapiro-Wilk tests. Bartlett's tests were used to determine homogeneity of variance. Because data collection and immunohistochemical staining for the three time points were performed separately, data from each time point were analyzed independently. Normally distributed data (latency to approach stimuli, all Fos-ir data) were analyzed by 2-way ANOVAs, with treatment (placenta, oil) and stimulus (pup, marble) as factors. If a significant ($p \leq 0.05$) treatment x stimulus interaction was found, we performed post-hoc pairwise comparisons using Tukey's HSD tests. Tukey's HSD tests performs all pairwise comparisons while controlling the probability of making Type I errors. Non-normal data (duration of huddling + licking pup, duration of investigating pup) were analyzed using Mann-Whitney U tests to compare behavioral responses in placenta- vs. oil-treated mice within each stimulus condition.

3. Results

3a. Behavioral Responses to Stimuli

Among the mice tested with a pup at each time point, the proportion that showed paternal behavior (i.e., licking and/or huddling pup) did not differ between placenta- and oil-treated males (all p-values>0.50, Fisher's Exact test for each time point; Table 1). Additionally, placenta treatment did not affect the total duration of caretaking behavior (huddling + licking) that mice engaged in during the pup test at any time point (all p-values>0.40; Mann-Whitney U test for each time point; Figure 2). At 7 h post-gavage, placenta-treated mice approached their assigned stimuli more quickly than oil-treated mice (main effect of treatment: $F_{1, 25}=4.22$, $p=0.05$; 2-way ANOVA); however, this effect did not differ between males tested with pups and those tested

with marbles (main effect of stimulus: p=0.15; treatment x stimulus interaction: p=0.43). Latencies to approach pups or marbles did not differ significantly between placenta- and oil-treated mice at either of the other time points (1 h: main effect of treatment: p=0.54; main effect of stimulus: p=0.50; treatment x stimulus interaction: p=0.66; 24 h: main effect of treatment: p=0.63; main effect of stimulus: p=0.71; treatment x stimulus interaction: p=0.56; 2-way ANOVA for each time point; Figure 2). Finally, placenta treatment had no effect on the total duration of time mice spent sniffing pups at any of the time points (all p-values≥0.3; Mann-Whitney U test for each time point).

3b. Neural Responses to Stimuli

In general, total Fos-ir in the brain areas investigated was lower in mice treated with placenta than in those treated with oil; however, most of our planned analyses did not reach statistical significance (Table 2). Treatment with placenta significantly altered neural responses to stimuli in the dBST at both the 1 h and 7 h time points. Placenta-treated mice tested 1 h after oral gavage had significantly lower Fos-ir in the dBST than oil-treated controls (main effect of treatment: $F_{1, 20}$=4.51, p=0.04; 2-way ANOVA; Table 2, Figure 3). At this time point, Fos-ir in the dBST was not influenced by stimulus type (main effect of stimulus: p=0.54), nor by an interaction between treatment and stimulus (p=0.87). At the 7 h time point, placenta-treated mice still showed a reduction in Fos-ir in the dBST compared to oil-treated controls (main effect of treatment: $F_{1, 18}$=4.13, p=0.05), and this effect differed between males exposed to a pup and those exposed to a marble (treatment x stimulus interaction: $F_{1, 18}$=7.33, p=0.01; 2-way ANOVA). Among placenta-treated mice, those exposed to a pup 7 h after gavage tended to show a reduction in dBST Fos-ir compared to males exposed to a marble, but this reduction was not statistically significant (p=0.06, Tukey's HSD test); no such effect was seen in oil-treated

animals (p=0.69) nor did any additional pairwise comparison reach statistical significance. At 24 h post-treatment, Fos-ir in the dBST was not significantly influenced by a main effect of treatment or stimulus, or by an interaction between these two factors (all p-values >0.33).

Fos-ir in the MPOA, vBST, PVN, BLA, and CeA was not significantly affected by treatment (all p-values >0.07; Table 2, Figure 3). However, 1 h after gavage, Fos-ir in both the BLA and CeA was significantly higher in mice exposed to a pup than in those exposed to a marble (BLA: main effect of stimulus: $F_{1, 20}$=4.60, p=0.04; CeA: main effect of stimulus: $F_{1, 20}$=5.71, p=0.02; 2-way ANOVAs), compared to mice exposed to a marble (novel object). Neither of these effects differed between placenta- and oil-treated mice (p-values >0.12).

4. Discussion

In this study, we aimed to identify possible neural and behavioral consequences of placenta ingestion (i.e., placentophagia) by adult virgin males of a monogamous, biparental rodent species. Specifically, we sought to investigate the possible role placentophagia might play in facilitating pup-directed care in the California mouse, as males of this species ingest placenta during the birth of their offspring (Lee & Brown, 2002; Perea-Rodriguez & Saltzman, 2014) and engage in extensive paternal behavior (Gubernick & Alberts, 1987). We hypothesized that the physiological changes resulting from ingestion of placenta lead to changes in both neural and behavioral responses to pup-related stimuli.

The majority of our analyses found that placenta treatment had no effect on paternal behavior. The small number of statistically significant results indicate that 7 hours after treatment, placenta-treated virgin male mice showed reduced latencies to approach both pups and novel objects (marbles) compared to oil-treated mice. In addition, placenta treatment reduced

298 pup- and marble-induced activation (Fos-immunoreactivity) of the dorsal region of the bed
299 nucleus of the stria terminalis (dBST) both 1 and 7 h after treatment. At the 1-h time point,
300 placenta-treated mice had reduced Fos-ir in response to both pup and marble stimuli, compared
301 to oil-treated mice. Taken together, these findings indicate that ingesting placenta does not
302 produce any major effects on paternal care but may reduce responsiveness of the dBST as
303 rapidly as within 1 h and for as long as at least 7 h. Ingestion of placenta did not alter pup-
304 directed care or neural activity in other brain regions, including the PVN, BLA, CeA, vBST, and,
305 most strikingly, the MPOA, which has been implicated in paternal behavior in California mice
306 and other biparental mammals (Bales & Saltzman, 2016; Horrell et al., 2018; Saltzman &
307 Ziegler, 2014).

308 In two biparental species, prairie voles and California mice, fatherhood modulates stress
309 reactivity and anxiety-like behaviors, suggesting that males modify how they perceive potentially
310 aversive or novel stimuli with changes in reproductive state or reproductive experience (Bardi et
311 al., 2011; Chauke et al., 2012; Lieberwirth et al., 2013). In the same two species, paternally
312 responsive males have increased Fos-ir in the medial posteromedial and medial BST after
313 exposure to pups, compared to parentally unresponsive males (de Jong et al., 2009; Kirkpatrick
314 et al., 1994). The BST is a limbic forebrain structure that has been linked to paternal care, stress,
315 anxiety, and aggression in California mice and other species (Bester-Meredith & Marler, 2003;
316 Davis & Marler, 2004; Davis et al., 2010; de Jong et al., 2009; Gungor & Paré, 2016; Trainor et
317 al., 2010). Neurochemical changes in the BST can alter an animal's behavioral response to
318 unpredictable, threatening, and aversive stimuli (i.e., unconditioned fear) (Walker & Davis,
319 1997). In rodents, the BST contains dorsal and ventral regions that differ in their
320 electrophysiological properties (Egli & Winder, 2003; Frazier et al., 2006). The dorsal and

ventral BST also respond differentially to stressors, possibly due to their dissimilar inputs from other brain nuclei and to their sensitivity to certain neurotransmitters and neuropeptides (Daniel & Rannie, 2016); however, both regions show increased Fos-ir under stressful conditions (Di Bonaventura et al., 2014). Thus, the reduced activity in the dBST seen in placenta-treated mice, as well as the shorter latencies of these mice to approach pups and marbles, may be associated overall with increased motivation to interact with environmental stimuli, regardless of whether the stimuli are pup-related.

Studies on the consequences of placenta ingestion suggest that placentophagia by mothers may trigger behavioral and physiological changes that positively affect their offspring (e.g., Abbott et al., 1991; González-Mariscal et al., 1998). In the case of males, a study on rats, which are uniparental, showed that virgin males experience hypoalgesia after ingesting placenta (Abbott et al., 1991). Recently, we showed that oral administration of placenta to male California mice, irrespective of reproductive experience, increased exploration of a novel space (an open-field arena) but had no effect on paternal behaviors (Perea-Rodriguez et al., 2018). Similarly, in the present study, placentophagia did not enhance pup-directed care, but it decreased latencies to approach novel stimuli (pups and marbles) and led to changes in neural activity in a brain nucleus heavily involved in regulating responses to a variety of environmental stimuli, including pup-related and other social stimuli.

Some important caveats should be kept in mind when interpreting the results of this study. First, we evaluated effects of placentophagia only in virgin males, rather than in fathers, because fathers typically show maximum paternal care. In our recent study, however, behavioral effects of oral treatment with placenta did not differ among California mouse fathers, first-time expectant fathers, and virgin males (Perea-Rodriguez et al., 2018). Second, although Fos

expression has been linked to changes in neuronal activity, this is not always the case; Fos may or may not be expressed when neurons undergo changes in electrical activity or gene expression (Hoffman & Lyo, 2002). Third, the sample sizes in this study were relatively small. Fourth, the oral gavage procedure by which we administered placenta eliminated possible effects that placenta and amniotic fluid may have via olfactory or accessory olfactory pathways, and the oil preparation used may have limited absorption of some of the chemicals found in placenta and amniotic fluid, such as peptide hormones. Fifth, placentophagia may have affected how mice responded to pups and marbles through neural changes in brain nuclei that were not investigated in this study (e.g., subregions of the amygdala and BST). Finally, although the oral gavage procedure does not produce any significant changes in corticosterone secretion in California mice (unpub. data), the procedure itself could have produced or inhibited any effects of placenta ingestion, an issue that our experimental design was unable to address.

5. Conclusions

In conclusion, we found that placentophagia by adult, virgin male California mice did not lead to significant changes in paternal care. Placenta administration did, however, transiently reduce males' latencies to approach an unfamiliar pup or a novel object, and reduced Fos-immunoreactivity in the dorsal region of the bed nucleus of the stria terminalis after exposure to each of these stimuli. Thus, our results are consistent with findings from a previous study (Perea-Rodriguez et al., 2018) suggesting that ingestion of placenta may reduce neophobia and anxiety-related behavior in males, but not paternal behavior *per se*.

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Figure 1: Brain nuclei in which Fos-immunoreactivity was quantified. dBST: dorsal bed nucleus of the stria terminalis; vBST: ventral bed nucleus of the stria terminalis; MPOA: medial preoptic area of the hypothalamus; PVN: paraventricular nucleus of the hypothalamus; BLA: basolateral amygdala; CeA: central nucleus of the amygdala.

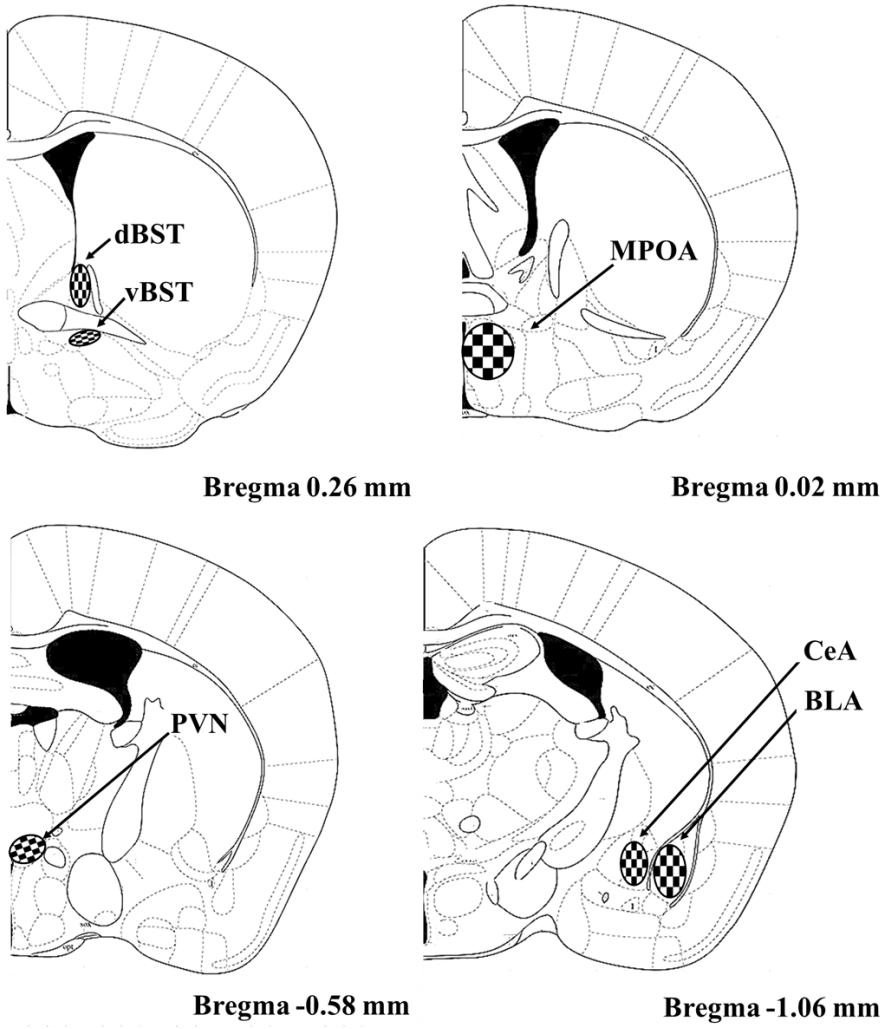

Figure 2: Behavioral responses to a 1- to 4-day-old pup (top) and an oblong, pup-sized glass marble (bottom) by virgin male California mice 1, 7, or 24 after treatment with oil or placenta. Bars represent 1st quartiles, medians, and 3rd quartiles. The asterisk indicates a significant difference between treatments ($p \leq 0.05$).

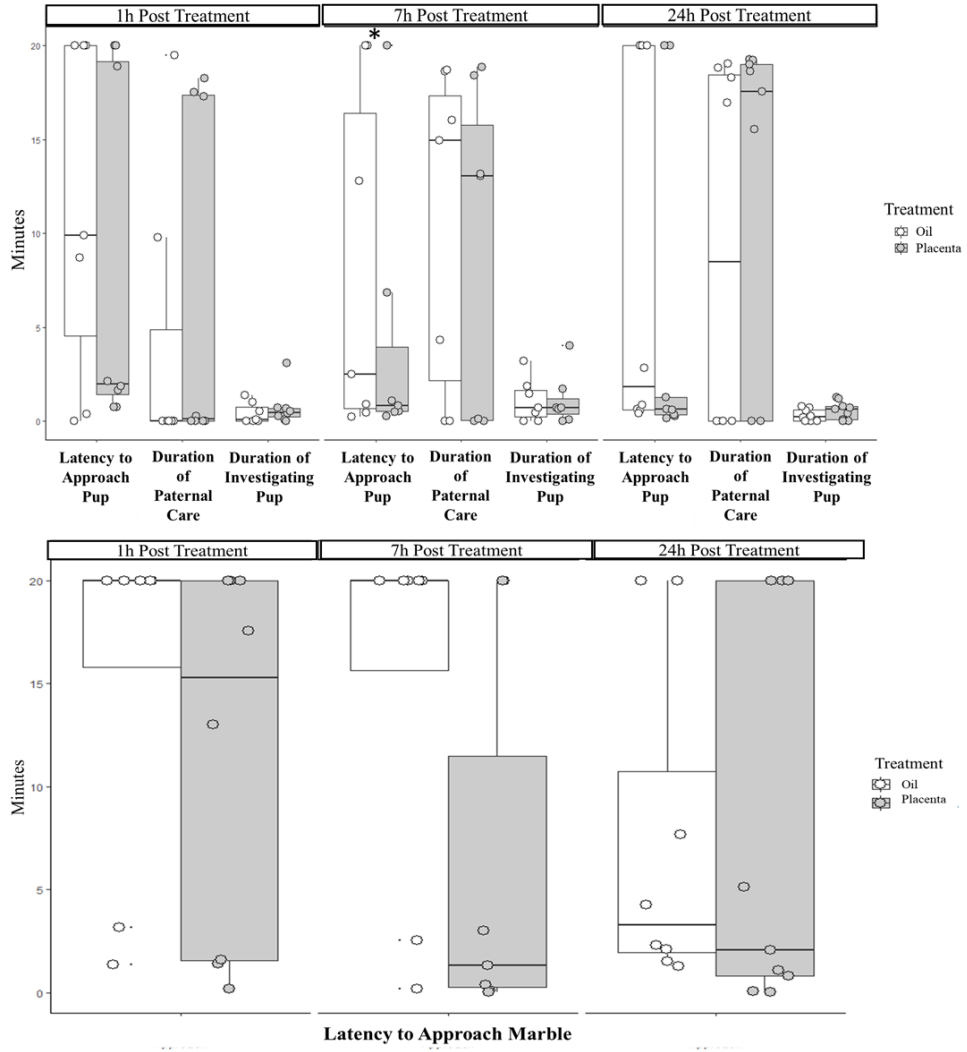

Figure 3: Representative photomicrographs of Fos labeling in the dorsal bed nucleus of the stria terminalis of virgin male California mice exposed to a pup or a marble for 1 h, beginning 1 h following oral treatment with placenta or oil. Each image in the right-hand column shows a higher magnification (100x) of the adjacent image (25x), with arrows indicating the black nuclear staining of Fos-positive neurons. AC - anterior commissure; LV - lateral ventricle. Ovals indicate the area sampled.

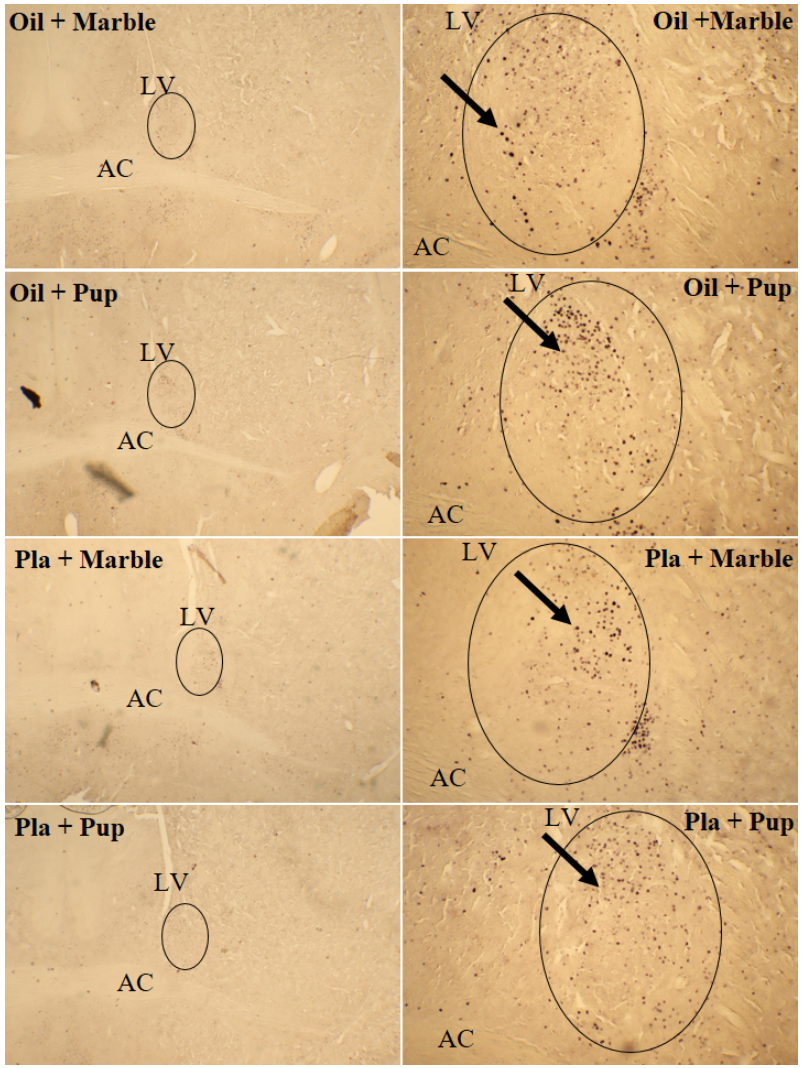

Table 1: Sample sizes of placenta-treated and oil-treated virgin male California mice per time point and stimulus. Bold numbers represent the number of mice tested with a pup that showed paternal behavior (huddling and licking pup).

1 h Post-treatment		
	Stimulus	
Treatment	Pup	Marble
Placenta	8; **4**	6
Oil	7; **2**	6

7 h Post-treatment		
	Stimulus	
Treatment	Pup	Marble
Placenta	7; **5**	6
Oil	7; **5**	5

24 h Post-treatment		
	Stimulus	
Treatment	Pup	Marble
Placenta	9; **6**	7
Oil	8; **4**	7

Table 2: Numbers of Fos-positive neurons following exposure to a pup or control object (marble) at each of three time points after treatment with placenta or oil. Data were analyzed using 2-way ANOVAs. Means, standard errors, and sample sizes are shown, as well as p-values for main effect of treatment, main effect of stimulus, and treatment x stimulus interaction. P-values ≤ 0.05 are shown in bold. MPOA – medial preoptic area of the hypothalamus, dBST – dorsal bed nucleus of the stria terminalis, vBST – ventral bed nucleus of the stria terminalis, PVN – paraventricular nucleus of the hypothalamus, BLA – basolateral amygdala, CeA – central nucleus of the amygdala.

Brain Area	Time Post-treatment	Marble		Pup		P-Value		
		Oil	Placenta	Oil	Placenta	Treatment	Stimulus	Treatment * Stimulus
MPOA	1h	14.75 ± 4.73 n=6	22.75 ± 6.10 n=6	27.83 ± 5.02 n=6	19.00 ± 3.25 n=6	0.93	0.35	0.10
	7h	10.10 ± 2.35 n=5	13.08 ± 3.21 n=6	7.33 ± 1.20 n=6	7.91 ± 2.85 n=6	0.46	0.13	0.64
	24h	15.60 ± 5.71 n=5	13.14 ± 2.81 n=7	17.62 ± 4.92 n=8	13.00 ± 2.49 n=8	0.65	0.85	0.78
dBST	1h	36.00 ± 5.48 n=6	27.58 ± 2.95 n=6	34.08 ± 4.40 n=6	24.25 ± 3.93 n=6	**0.04**	0.54	0.87
	7h	24.80 ± 5.42 n=6	26.91 ± 3.80 n=6	30.50 ± 2.86 n=6	12.80 ± 1.49 n=5	**0.05**	0.26	**0.01**
	24h	18.00 ± 5.83 n=5	27.57 ± 6.58 n=7	25.87 ± 5.56 n=8	24.5 ± 6.12 n=8	0.75	0.85	0.33
vBST	1h	12.33 ± 1.97 n=6	10.91 ± 1.43 n=6	11.33 ± 1.92 n=6	14.25 ± 1.27 n=6	0.78	0.40	0.27
	7h	8.60 ± 1.81 n=5	11.66 ± 2.11 n=6	10.08 ± 1.47 n=5	7.10 ± 0.92 n=5	0.49	0.80	0.93
	24h	9.87 ± 1.57 n=4	11.25 ± 1.25 n=2	10.33 ± 1.45 n=3	12.2± 3.10 n=5	0.49	0.80	0.93
PVN	1h	37.60 ± 4.61 n=5	27.7 ± 2.22 n=6	53.83 ± 11.03 n=6	34.54 ± 6.65 n=6	0.07	0.07	0.37
	7h	22.90 ± 2.14 n=5	27.36 ± 4.01 n=6	20.58 ± 6.56 n=6	24.16 ± 5.06 n=6	0.41	0.59	0.93

	24h	24.85 ± 3.54 n=7	23.50 ± 5.50 n=6	26.57 ± 5.69 n=8	35.42 ± 5.38 n=7	0.72	0.23	0.31
BLA	1h	24.83 ± 2.52 n=6	24.00 ± 4.52 n=6	39.91 ± 5.43 n=6	30.41 ± 6.63 n=6	0.31	0.04	0.39
	7h	21.90 ± 5.08 n=5	20.41 ± 3.23 n=6	21.08 ± 5.85 n=6	20.90 ± 1.17 n=5	0.85	0.99	0.88
	24h	21.33 ± 3.17 n=6	29.00 ± 5.95 n=7	33.60 ± 3.60 n=5	36.66 ± 11.02 n=6	0.60	0.21	0.69
CEA	1h	28.41 ± 2.33 n=6	27.50 ± 2.50 n=6	41.41 ± 3.46 n=6	31.16 ± 4.98 n=6	0.12	0.02	0.19
	7h	40.40 ± 6.23 n=5	43.83 ± 7.65 n=6	30.83 ± 4.20 n=6	28.00 ± 5.16 n=5	0.30	0.33	0.65
	24h	28.66 ± 4.40 n=6	30.28 ± 2.54 n=7	28.00 ± 5.16 n=5	33.16 ± 9.32 n=6	0.81	0.88	0.80