TITLE

Neural responses to pup calls and pup odors in California mouse fathers and virgin males

ABSTRACT

The onset of mammalian maternal care is associated with plasticity in neural processing of infant-related sensory stimuli; however, little is known about sensory plasticity associated with fatherhood. We quantified behavioral and neural responses of virgin males and new fathers to olfactory and auditory stimuli from young, unfamiliar pups in the biparental California mouse (*Peromyscus californicus*). Each male was exposed for 10 minutes to one of four combinations of a chemosensory stimulus (pup-scented or unscented cotton [control]) and an auditory stimulus (pup vocalizations or white noise [control]). Behavior did not differ between fathers and virgins during exposure to sensory stimuli or during the following hour; however, males in both groups were more active both during and after exposure to pup-related stimuli compared to control stimuli. Fathers had lower expression of Fos in the main olfactory bulbs (MOB) but higher expression in the medial preoptic area (MPOA) and bed nucleus of the stria terminalis medial division, ventral part (STMV) compared to virgins. Lastly, males had higher Fos expression in MPOA when exposed to pup odor compared to control stimuli, and when exposed to pup odor and pup calls compared to pup calls only or control stimuli. These findings suggest that the onset of fatherhood alters activity of MOB, MPOA and STMV and that pup odors and vocalizations have additive or synergistic effects on males' behavior and MPOA activation.

KEY WORDS

Paternal behavior, olfaction, vocalizations, Fos, main olfactory bulb, medial preoptic area, bed nucleus of stria terminalis *Peromyscus californicus*

INTRODUCTION

In female mammals, the onset of parenthood can be associated with pronounced changes in the mother's behavioral responses to young, which may shift from avoidance or aggression to attraction and nurturance (Numan et al. 2006; Duarte-Guterman et al. 2019). The onset of motherhood is also accompanied by plasticity in neural circuits subserving sensory processing, cognition, affect, motivation and reward (Kinsley et al. 2008; Leuner et al. 2010; Lambert 2012; Numan 2012; Glasper et al. 2019; Rogers and Bales 2019; Tasaka et al. 2020). This plasticity is mediated largely by the neuroendocrine changes that occur during pregnancy, parturition and lactation (Levy et al. 2004; Marlin et al. 2015; Dunlap and Liu 2018) and facilitates the expression of appropriate behavioral responses to offspring (Numan and Insel 2003; Kinsley et al. 2008).

In the 5-10% of mammalian species in which both mothers and fathers provide care for their young (i.e., biparental species; Kleiman and Malcom 1981), males, like females, may exhibit pronounced changes in their behavioral responses to infants as they become parents. For example, in several biparental rodents, pup-naïve virgin males may behave affiliatively, indifferently or aggressively when tested with infants, whereas fathers are consistently attracted to and nurturant toward both related and unrelated infants (California mouse, *Peromyscus californicus*: de Jong et al. 2009; Chauke et al. 2012; Jasarevic et al. 2013; Rosenfeld et al. 2013; Horrell et al. 2017; Mongolian gerbil, *Meriones unguiculatus*: Elwood and Ostermeyer 1984; dwarf hamster, *Phodopus campbelli:* Wynne-Edwards 1995; Mandarin vole, *Microtus*

47 *mandarinus*: Yuan et al. 2019). The mechanisms underlying this shift in behavioral responses to
48 infants are not well understood. However, fatherhood-associated neural plasticity has been
49 described in several biparental rodents and is likely mediated in part by the neuroendocrine
50 changes associated with copulation, cohabitation with a pregnant or parturient female, and/or
51 exposure to pups (reviewed in Dulac et al. 2014; Elwood and Stolzenberg 2020; Horrell et al.
52 2021).

53 An important determinant of behavior toward infants is detection and processing of
54 infant-related sensory stimuli, especially odors and vocalizations (Hofer et al. 2001; Levy et al.
55 2004; Levy and Keller 2009; Shair 2018). Chemosensory stimuli can act through both the
56 accessory and main olfactory systems to influence the activation of maternal care (Levy and
57 Keller 2009), but the exact nature of this influence differs among species. For example,
58 hyposmia, induced by various experimental procedures, reduces latency to maternal behaviors in
59 virgin female rats (*Rattus norvegicus*) and rabbits (*Oryctolagus cuniculus*) but increases the
60 likelihood that virgin female house mice (*Mus*) will behave aggressively towards pups (reviewed
61 in Levy and Keller 2009). In addition to odors, rodent pups emit ultrasonic vocalizations that
62 elicit parental care (Blumberg and Sokoloff 2001; Ehret 2005).

63 Few studies have sought to compare the salience of olfactory versus acoustic stimuli for
64 parental care, but those that do have shown that pup chemosensory and acoustic cues can have
65 synergistic effects on maternal behavior. In C57BL/6 mice, for example, the presence of both
66 stimuli, but not either stimulus alone, elicits approach by mothers (Okabe et al. 2013), and
67 female Australian sea lions (*Neophoca cinerea*) are more likely to approach a pup model
68 accompanied by pup odors and pup vocalizations compared to a model accompanied by pup
69 vocalizations alone (Wierucka et al. 2018). The relative importance of chemosensory and

auditory stimuli for parental behavior has not been investigated in California mice, the species used in the present study; however, both sensory modalities are known to play important roles in other forms of social behavior in this species (Pultorak et al. 2017; Kalcounis-Rueppell et al. 2018; Rieger et al. 2021; Bester-Meredith et al. 2022). Moreover, in rodents, both olfactory and acoustic information are processed in brain regions important for the expression of parental behavior (Cohen et al. 2011; Choi et al. 2018).

The onset of maternal care in female rodents is associated with plasticity in the circuitry involved in processing sensory stimuli from infants and assigning hedonic value to them (Kinsley and Bridges 1990; Olazábal et al. 2013; Dulac et al. 2014; Marlin et al. 2015; Schiavo et al. 2020). For example, house mouse mothers are better able to detect pup calls, and are more attracted to pup calls, compared to virgins (Ehret et al. 1987; Tasaka et al. 2020). Additionally, house mouse mothers show extensive plasticity in the olfactory bulbs, which is thought to enhance mothers' ability to discriminate pup odors, and they are more attracted to pup odors compared to virgin females (Levy et al. 2004; Liu et al. 2006; Belnoue et al. 2016; Vinograd et al 2017).

The role of infant odors and vocalizations in parental care by fathers has received less attention than in mothers, but these stimuli appear to be important for paternal care. In the biparental prairie vole (*Microtus ochrogaster*), MOB lesions in virgin males increase aggression towards pups, which is usually a rare behavior even in virgin males of this species (Kirkpatrick et al. 1994a). Furthermore, in the uniparental house mouse, fathers housed with their mate and pups prefer sounds similar to pup ultrasonic vocalizations over control sounds (Ehret and Koch 1989; Ehret 2005). Several brain regions that process sensory information also play key roles in the activation of paternal behavior. Through different pathways, responses to chemosensory and

93 acoustic stimuli are conveyed to the amygdala, which projects to the bed nucleus of the stria
94 terminalis (BNST) and medial preoptic area of the hypothalamus (MPOA) (Dulac et al. 2014;
95 Horrell et al. 2019). These latter two regions are activated in response to pups in male rodents
96 (Numan 1988, 1990; Rosenblatt and Ceus 1998; Sheehan and Numan 2002; Numan and Insel
97 2003; de Jong et al. 2009; Dulac et al. 2014; Horrell et al. 2017, 2018) and, along with the
98 amygdala, have been shown by lesion studies to play a causal role in parental behavior (reviewed
99 in Bales and Saltzman 2016). The MPOA is also connected to areas of the reward circuitry, (e.g.,
100 nucleus accumbens [NAcc], Li and Fleming 2003; Horrell et al. 2019; Numan 2012, 2020),
101 suggesting that the MPOA acts to promote parental behavior through increased motivation. Very
102 little is known, however, about plasticity in males' behavioral and neural responses to pup-
103 related stimuli in males of biparental species, or about potential interactions among pup stimuli
104 in different sensory modalities.

105 In this study we assessed effects of fatherhood on behavioral and neural responses to
106 olfactory and acoustic cues from pups in the California mouse, a monogamous, biparental rodent.
107 Fathers provide extensive care for their offspring, engaging in all the same types of parental
108 behavior as mothers except for lactation (Dudley 1974; Gubernick and Alberts 1987), and care
109 from both parents can have important consequences for offspring survival and development
110 (Dudley 1974; Gubernick et al. 1993; Cantoni and Brown 1997; Gubernick and Teferi 2000;
111 Wright and Brown 2002; Frasier et al. 2006). However, whereas fathers exhibit strong attraction
112 and nurturant behavior toward both familiar and unfamiliar pups, virgin males show highly
113 variable responses to experimentally introduced pups, ranging from nurturance to avoidance to
114 aggression (de Jong et al. 2009, Chauke et al. 2012; Jasarevic et al. 2013; Rosenfeld et al. 2013;
115 Horrell et al. 2017). Correspondingly, after exposure to unfamiliar pups, fathers show greater

activation of the MPOA and BNST compared to virgin males, as indicated by higher expression of Fos, the protein product of the immediate-early gene c-fos, (de Jong et al. 2009; but see Horrell et al. 2017). The role of sensory plasticity in mediating these effects of fatherhood is unknown. Therefore, we tested the hypotheses that 1) fathers are attracted to acoustic and olfactory stimuli from pups compared to control stimuli, whereas this attraction is less pronounced or absent in virgin males, 2) differential behavioral responses to pup stimuli in fathers and virgin males are associated with differences in neural responses to these cues in brain regions associated with paternal care, reward, and/or fear and anxiety, and 3) pup calls and pup odors act additively or synergistically on behavioral and neural responsiveness.

METHODS

Animals

We used California mice that were bred at the University of California, Riverside (UCR) and were descended from mice purchased from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, USA). Mice were housed in 44 x 24 x 20 cm polycarbonate cages with aspen shavings for bedding and cotton for nesting material and had *ad libitum* access to food (Purina 5001 Rodent Chow) and water. The ambient temperature was maintained at approximately 23 °C, humidity was at approximately 65%, and lights were on a 14:10 h cycle (lights on at 2300 h). All procedures were approved by UCR's Institutional Animal Care and Use Committee and were conducted in accordance with the recommendations of the *Guide for the Care and Use of Laboratory Animals*. UCR is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

At weaning age (27-31 days), prior to the birth of younger siblings, mice were removed from their parents' cage and housed in single-sex groups of 2 to 4 age-matched mice until they were used in this study. We used 64 males and 64 females that originated from 27 families.

Surgeries and Pairing

At 85-135 days of age, males were paired with unrelated females that had undergone ovariectomies (virgin males) or sham ovariectomies (fathers) 10 days earlier. As previously described (Zhao et al. 2018), females were anesthetized with 2.5% isoflurane vapor, and an approximately 1cm midline incision was made. The right and left ovaries were located and, for ovariectomies, removed using microscissors. The abdominal muscle was closed using absorbable sutures, and the skin was sealed using tissue glue. Females were given 5 mg/kg carprofen (Carprieve [Norbrook Laboratories; Overland Park, KS, USA]) S.C. every 12 h for 48 h for analgesia and housed in isolation for 7 days to allow for recovery. Females were then housed with their original same-sex cage mates for 3 days before being paired with a male.

Fathers and virgins were paired when they were 104.03 ± 2.19 and 99.67 ± 2.22 days old (mean ± SE), respectively (t-test: $t = -1.40$, $df = 59$, $P = 0.17$). Each male was randomly assigned to either the virgin group (i.e., housed with an ovariectomized female) or the father group (housed with a sham ovariectomized female) except that if two males from the same litter were used, they were assigned to different groups. Opposite–sexed pair mates were no more closely related than first cousins. All pair cages were checked daily for the presence of pups.

Stimulus Exposure

Each male underwent a single stimulus-exposure test, after which it was perfused and its brain collected for immunohistochemistry (see below). Fathers were tested when their first litter of pups was between 4 and 6 days old, and virgin males were tested at a matched time point to ensure that age and time since pairing did not differ between the groups. In each test, the mouse was exposed to one olfactory stimulus (pup-scented cotton or clean cotton; see below) and one acoustic stimulus (pre-recorded pup vocalizations or white noise), which produced 4 stimulus combinations: Control (clean cotton and white noise), Call (clean cotton and pup vocalizations), Odor (pup-scented cotton and white noise), and Call + Odor (pup-scented cotton and pup vocalizations). Fathers and virgins had been pair-housed with females for 50.19 ± 13.56 days and 49.93 ± 10.13 days (mean ± SE), respectively, at the time of testing (t-test: $t = -0.08$, $df = 61$, $P = 0.93$). Mice were assigned to stimulus treatments randomly.

Between 0800 and 0900 h (during the light phase of the cycle) on the day of testing, the male mouse was placed individually in a 12 x 7.5 x 5.25 cm polycarbonate cage with shavings, food and water. The cage was placed in a corner of a black acrylic open-field arena (1 x 1 x .5 m) in a sound-attenuated and anechoic room. In each corner of the arena, the floor contained a circle (Ø: 6.5 cm) of 5-mm holes. The arena was raised 10 cm off the floor, and a speaker (UltraSoundGate BL Pro, Avisoft Bioacoustics, Glienecke, Germany) was positioned beneath the holes in the corner of the arena that contained the test cage. Stimulus exposure began 110 minutes after the mouse was placed in the cage, to allow for dissipation of any peaks in Fos expression in the brain related to home-cage events or handling (de Jong et al. 2009).

We prepared olfactory stimuli within 5 minutes before use. To avoid introducing spurious odors, the experimenter wore fresh gloves and selected a cotton ball from a sealed storage area. If the male was to be exposed to a control olfactory stimulus (clean cotton), the cotton ball was

placed in a stainless-steel wire-mesh tea ball (Ø: 6 cm), which was stored in a clean, sealed container until the test began. If the male was to be exposed to a pup-odor stimulus, the cotton ball was wiped 30 times across the ventrum and anogenital region of an unrelated, 3- to 7-day-old pup (mean ± SE = 4.38 ± 1.03 days). The cotton ball was rotated after each wipe. The cotton ball was then placed in a tea ball, identical to the one used for clean cotton, which was stored in a clean, sealed container until the test began.

Acoustic stimuli were prepared in advance of the study. The control stimulus consisted of a 25-s sound loop of 6 pulses of white noise followed by a 1-s pause, which is consistent with the calling pattern of young California mouse pups (Johnson et al. 2017; Wilson et al. 2022). The pup acoustic stimulus consisted of a 25-s sound loop of an isolated 4-day-old pup, unrelated to the male subjects, recorded using a BAT miniMIC (Binary Acoustic Technology, Tuscon, AZ; USA) and Spectr III software (Spectral Analysis, Digital Tuning, and Recording Software; Binary Acoustic Technology, Tuscon, AZ, USA). Acoustic stimuli were played through the speaker located under the test cage at 68 dB SPL measured 1 inch from the speaker, which is consistent with the normal volume of California mouse pup calls at the same distance (pers. obs.).

To begin the stimulus exposure, the cotton-containing tea ball (i.e., the “odor ball”) was placed in a standardized position in the front left corner of the cage above the speaker, and the acoustic playback was immediately started. Both the olfactory and the acoustic stimuli were presented to the male for 10 minutes. The acoustic playback was then turned off, and the odor ball was quickly removed from the cage. Sixty minutes after the end of the stimulus exposure, the mouse was deeply anesthetized with pentobarbital (Fatal-Plus solution, Vortech Pharmaceuticals, Dearborn, MI, USA) and perfused transcardially with 0.1 M phosphate

buffered saline (PBS) followed by 4% paraformaldehyde. The brain was removed rapidly and fixed in 4% paraformaldehyde for 2 days at 4 °C. The brain was then cryoprotected in 30% sucrose and frozen in cryoprotectant (30% sucrose, 30% ethylene glycol) at -20 °C. Because production of fecal boli is often used as a metric of anxiety (Archer 1973; Gentsch et al. 1981), the shavings from each male's test cage were saved, and fecal boli were collected and counted.

Immunohistochemistry

Immunohistochemistry protocols were adapted from methods previously established in our lab for this species (de Jong et al. 2009; Horrell et al. 2017). Immunohistochemistry was performed in batches containing one brain from each of the 8 reproductive status x stimulus treatment groups. Three to 5 days prior to slicing, brains were thawed and transferred into 30% sucrose at 4 °C. Brains were sectioned (40 μm) using a Leica CM1950 cryostat (Leica Biosystems, Deer Park, IL, USA) set at -20 °C. Brain sections were incubated overnight with polyclonal rabbit anti-cFos (1:2,500; Synaptic Systems, Göttingen, Germany) followed by incubation with goat anti-rabbit IgG, Alexa Fluor 555 (1:500; Thermo Fisher Scientific, Waltham, MA, USA) for 90 minutes. Procedures using Alexa Fluor and all subsequent procedures were conducted with minimal ambient light. Sections were mounted on slides with EMS Shield Mount with DABCO (Electron Microscopy Sciences, Hatfield, PA, USA) and stored covered at 4 °C. Images of the brain regions of interest were taken between 16 and 22 h after tissue was mounted, using a Zeiss LSM 880 inverted microscope (Carl Zeiss Microscopy, LLC, White Plains, NY, USA).

Fos immunoreactivity was quantified in regions associated with sensory relay (main olfactory bulb [MOB] granule cell layer, basolateral amygdaloid nucleus [BLA] and basomedial amygdaloid nucleus [BMA]), parental behavior (bed nucleus of the stria terminalis medial

division, ventral part [STMV], medial preoptic area [MPOA]), reward (nucleus accumbens [NAcc] shell), and fear/anxiety (anterior hypothalamic nucleus [AHN]) (reviewed in Horrell et al. 2019). Brain regions were located by cross-referencing *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin 2013) for *Mus musculus* and images of Nissl-stained California mouse sections (Mikula et al. 2007 [http://brainmaps.org]). QuPath 3.0 (Bankhead et al. 2017) was used to quantify the number of Fos-positive cells by outlining in each brain region of interest a 200 x 200 μm square in the area with the highest density of Fos-positive cells. Scorers were blind to parenthood status and stimulus treatment during quantification of Fos immunoreactivity. Data for each region for each male were averaged from two sections from each hemisphere. Technical problems resulted in a small number of unusable images (see Fig. 1 for final sample sizes).

Behavior Measurements

Mice were video-recorded throughout the 10-minute stimulus-exposure period as well as the subsequent 60 minutes. Video recordings were scored using Behavioral Observation Research Interactive Software (BORIS53) (Friard and Gamba 2016). All behaviors scored were mutually exclusive of one another. For the 10 minutes of stimulus exposure, we scored behavior continuously to quantify latencies to listen (ears perked in the direction of the acoustic stimulus), sniff the odor ball (nose < 4 cm from the ball, with whiskers moving up and down), and handle the odor ball (front paw(s) on ball), and the total durations of time spent listening, sniffing the ball, and handling the ball. Additionally, we measured the total time spent in active behaviors (i.e., locomoting, autogrooming and nest building) and resting (lying down with little or no head movement). Durations of active and resting behaviors did not include time spent interacting with

stimuli as specified above. For the hour following stimulus exposure we performed instantaneous scans every 5 minutes and scored the subject's behavior as either active or resting. Because the exact amount of time that stimuli were presented varied slightly across tests, the time spent in each activity was normalized across all recordings by dividing the total time of the activity by the duration of stimulus exposure and multiplying by 600 seconds ([Σ behavior (s) / stimulus presentation (s)] * 600 s).

Statistical Analyses

Analyses were performed in STATA 15 (StataCorp LP, College Station, TX, USA). Assumptions for linear mixed-effects models (LMMs) and ANOVAs were checked by evaluating quantile-quantile plots and through Shapiro-Wilk analyses. Fos expression in MOB, NAcc, MPOA, AHN, BLA, and BMA, and fecal bolus counts, were square root transformed, and Fos expression in STMV was log transformed to meet assumptions for parametric tests. Significance was assessed based on $\alpha = 0.05$ (two-tailed).

LMMs were used to assess the effects of male reproductive status (father vs virgin), stimulus treatment (Control, Call, Odor, Call + Odor), and their interaction on Fos expression in the brain regions of interest (see above). Immunohistochemistry batch (the group with which each brain underwent immunohistochemistry) was included as a random variable for analyses of Fos expression. Two-way ANOVAs were used to assess the effects of the same independent variables on latency to interact with the pup stimuli (listen, sniff the odor ball, handle the odor ball) and on number of fecal boli. Non-significant ($P > 0.05$) interactions were removed from the final models for both Fos expression and fecal bolus counts.

Durations of each behavior during stimulus exposure and counts of behaviors in the 60 minutes following stimulus exposure were analyzed using non-parametric tests because measures did not meet parametric assumptions and were resistant to transformation. Mann-Whitney U tests were used to compare behavior between fathers and virgin males, and Kruskal-Wallis tests were used to compare behavior among stimulus treatments. When results were significant, Dunn's pairwise comparisons were performed. Lastly, Pearson's correlations were used to assess associations between Fos expression and behavior for fathers and virgin males separately.

RESULTS

Behavior

All males spent time in close proximity (less than 4 cm) to the odor ball, and all males exposed to pup odors sniffed the ball, although latency to approach the ball and time in proximity to the ball were highly variable. Neither reproductive status nor stimulus treatment influenced latency to sniff the odor ball (two-way ANOVA: $F_{31,4} = 1.15$, $P = 0.35$; reproductive status: $z = 1.04$, $P = 0.32$; stimulus treatment: $z = 1.20$, $P = 0.33$), latency to handle the odor ball ($F_{22,4} = 0.55$, $P = 0.93$; reproductive status: $z = 0.01$, $P = 0.93$; stimulus treatment: $z = 0.73$, $P = 0.55$) or latency to listen ($F_{22,4} = 0.26$, $P = 0.90$; reproductive status: $z = 0.31$, $P = 0.58$; stimulus treatment: $z = 0.22$, $P = 0.88$; Table 1). The interaction between reproductive status and stimulus treatment was not significant for any behavioral latencies. Fathers and virgin males did not differ in the amount of time they spent sniffing the odor ball (Mann-Whitney, $z = 0.61$, $P = 0.54$), handling the odor ball ($z = 1.05$, $P = 0.29$) or listening ($z = 0.48$, $P = 0.63$). Similarly, stimulus treatment did not affect the amount of time mice spent sniffing (Kruskal-Wallis, $\chi2 = 5.66$, $P = 0.13$) or handling ($\chi2 = 3.30$, $P = 0.35$) the odor ball or listening ($\chi2 = 4.04$, $P = 0.34$; Table 1).

297 Active behaviors observed during stimulus exposure included behaviors that would be
298 expected had a live pup been emitting cues: males often appeared to be searching the cage
299 (perhaps for a pup), and would sometimes shred or pile shavings, as is observed when captive
300 California mice build nests (Gubernick and Alberts 1987). Fathers and virgin males did not differ
301 in the amount of time they were active (Mann-Whitney, $z = -0.27$, $P = 0.79$) or resting ($z = -0.48$,
302 $P = 0.63$) during the stimulus exposure or the number of 5-minute scan samples in which they
303 were active ($z = -1.14$, $P = 0.26$) during the 60 minutes following stimulus exposure. Stimulus
304 treatment, in contrast, influenced the amount of time males rested during stimulus exposure
305 (Kruskal-Wallis, $\chi2 = 8.44$, $P = 0.038$) and tended to influence the amount of time males were
306 active during stimulus exposure ($\chi2 = 6.94$, $P = 0.074$). During exposure, males in the Control
307 treatment rested more than males in the Call, Odor and Call + Odor treatments (Dunn's post-hoc
308 P's ≤ 0.02) and tended to be less active than males in the Odor and Call + Odor treatments
309 (Dunn's post-hoc P's ≤ 0.03). Stimulus treatment also influenced the number of 5-minute scan
310 samples in which males were active after stimulus exposure ($\chi2 = 11.63$, $P = 0.009$): males in the
311 Call + Odor treatment were more active than males in the other three treatments ($P \leq 0.003$).
312
313 **Fos Expression**
314 Reproductive status influenced Fos expression in the MOB (LMM, model: $\chi2 = 14.76$, $P =$
315 0.005), STMV (model: $\chi2 = 36.72$, $P < 0.0001$), MPOA (model: $\chi2 = 118.74$, $P < 0.0001$), and
316 BLA (model: $\chi2 = 9.88$, $P = 0.043$). Virgin males had higher Fos expression than fathers in the
317 MOB (effect of reproductive status: $\chi2 = 13.44$, $P = 0.0002$), whereas fathers had higher Fos
318 expression than virgins in the STMV (effect of reproductive status: $\chi2 = 3.63$, $P < 0.0001$) and

MPOA (effect of reproductive status: $\chi 2 = 108.70$, $P < 0.0001$) and tended to have higher expression in the BLA (effect of reproductive status: $\chi 2 = 3.52$, $P = 0.061$) (Fig. 2).

Stimulus treatment influenced Fos expression in the MPOA (effect of treatment: $\chi 2 = 11.32$, $P = 0.01$): males in the Odor and Call + Odor treatments had higher MPOA Fos expression than males in the Control treatment, and males in the Call + Odor treatment had higher MPOA Fos expression than males in the Call treatment (Fig. 3). Stimulus treatment did not affect Fos expression in the MOB, STMV, or BLA, and neither stimulus treatment nor reproductive status influenced Fos expression in the NAcc (LMM, model: $\chi 2 = 1.81$, $P = 0.77$), AHN ($\chi 2 = 9.26$, $P = 0.10$) or BMA ($\chi 2 = 4.48$, $P = 0.34$). There were no significant interactions between reproductive status and stimulus treatment for any brain region.

Fecal Boli

The model for number of fecal boli produced across the entire 2-h test was not significant (two-way ANOVA df = 58, n = 63, z = 1.60, P = 0.19). Although the main effect of reproductive status was significant (z = 5.39, P = 0.024, square-root-transformed X ± SE: fathers = 4.35 ± 0.19; virgins = 3.70 ± 0.20), stimulus treatment was not (z = 0.33, P = 0.80, square-root-transformed X ± SE: Control = 4.15 ± 0.29; Call = 3.81 ± 0.29; Odor = 4.04 ± 0.28; Call + Odor = 4.15 ± 0.27).

Correlations Between Behavior and Fos Expression

Correlations between individual animals' behavioral and neural responses to stimulus exposure differed between fathers and virgin males (Table 2). Numerous significant correlations were found in fathers. Across all four stimulus treatments, fathers that spent more time sniffing the

odor ball had greater Fos expression in the NAcc, STMV and MPOA, and tended to have greater Fos expression in the MOB. Fathers that spent more time handling the odor ball had greater Fos expression in the BLA and BMA. Additionally, fathers that were more active during the stimulus exposure tended to have higher Fos in the AHN, and fathers that rested more during the stimulus exposure had lower Fos in the STMV and MPOA and tended to have lower expression in the BMA (Table 2A). In virgin males, only one significant correlation was found: virgins that were more active during the period of stimulus exposure had higher Fos expression in the MOB (Table 2B).

DISCUSSION

Olfactory and acoustic plasticity in males of biparental species are likely to be similar to females, in view of previous work demonstrating similarities in the neural basis of maternal and paternal care (Leuner et al. 2010; Tachikawa et al. 2013; Rymer 2020). Studies of maternal behavior in rodents indicate that neural plasticity in the medial preoptic area (MPOA) during the transition to parenthood leads to changes in the valence of pup sensory cues such that these stimuli switch from eliciting avoidance or aggression to eliciting an approach response (Lambert 2012; Rogers and Bales 2019; Elwood and Stolzenberg 2020; Numan 2020). In our study, each male was placed in a small cage to limit its ability to avoid the stimuli and presented with a combination of a single acoustic cue and a single olfactory cue. We show that pup calls and pup odors elicit comparable behavioral responses in this test paradigm but somewhat different neural responses in male California mice, and that simultaneous exposure to pup calls and odors has more pronounced effects on neural activation in the MPOA, a brain region associated with paternal care, than exposure to pup calls alone.

Effects of Reproductive Status

Although California mouse fathers consistently engage in paternal behavior towards unrelated pups under experimental conditions, pup-naïve virgin males behave less predictably, either avoiding or attacking experimentally presented pups or engaging in paternal behavior (Gubernick and Nelson 1989; de Jong et al. 2009; Horrell et al. 2017). Similarly, we recently found that California mouse fathers and virgin males respond differently to sensory cues from unrelated pups: when adult males were exposed to odors and/or vocalizations from unrelated pups, in a large arena that afforded them greater behavioral flexibility than the test cages in the current study, fathers, but not virgins, spent more time in proximity to and in contact with the pup stimuli than with simultaneously presented control stimuli (Arquilla, A.M., Wilson, K.M., Razak K.A., Saltzman W., submitted). Despite these previous observations, we found that reproductive status did not influence males' behavioral responses to sensory stimuli from unrelated pups when the males were presented with a single stimulus combination in a small cage. In contrast, reproductive status did affect neural activation as well as associations between neural and behavioral responses to stimuli. Although several differences in Fos expression were found between fathers and virgins, these differences were not specific to pup-stimulus exposure. This opens the possibility that changes in neural processing that occur at the onset of fatherhood lead to high activation of regions associated with parental behavior, even in the absence of exposure to pups or pup-related stimuli.

Our finding that fathers had higher Fos expression in the bed nucleus of the stria terminalis medial division, ventral part (STMV) and medial preoptic area (MPOA) across treatment groups, compared to virgin males, is consistent with previous reports of the effects of

388 reproductive status on these regions. Although the entire bed nucleus of the stria terminalis
389 (BNST) has been implicated in parental care behaviors, there is strong evidence that the ventral
390 part in particular is important for the maintenance of parental behavior in mothers (reviewed in
391 Numan 2020). The importance of the BNST and MPOA in mediating the onset of pup attraction
392 and inhibition of pup avoidance in fathers has been established through studies of biparental
393 male rodents (Kirkpatrick et al. 1994b; Lambert et al. 2013), including previous studies in
394 California mice, which have shown that lesions to the MPOA reduce paternal behavior (Lee and
395 Brown 2002, 2007) and that fathers have higher STMV and MPOA Fos expression compared to
396 virgins when exposed to a live pup secured in a mesh ball (de Jong et al. 2009).
397 The MPOA and BNST are important for the expression of parental behaviors through
398 their role in suppressing avoidance of pups and activating attraction to pups (Bales and Saltzman
399 2016; Horrell et al. 2019; Rogers and Bales 2019). The MPOA and BNST receive information
400 about olfactory and auditory cues and project to the ventral tegmental area, which activates the
401 reward circuitry through mesolimbic dopaminergic projections to the nucleus accumbens (NAcc)
402 (reviewed in Horrell et al. 2017; Rogers and Bales 2019; Elwood and Stolzenberg 2020).
403 Additionally, the MPOA and BNST inhibit aversion to pups through GABAergic signaling
404 between these two regions as well as between the MPOA and the medial amygdala (Rogers and
405 Bales 2019, Numan 2020).
406 The mechanisms underlying neural plasticity in the MPOA and BNST in response to
407 fatherhood have been the focus of several studies in rodents, which have implicated both steroid
408 hormones and neuropeptides (see Horrell et al. 2021 for review). For example, in California
409 mice, fatherhood is associated with a reduction in progesterone, oxytocin and vasopressin
410 receptor mRNA in the BNST (Perea-Rodriguez et al. 2015) and increased activity of aromatase,

411 which converts testosterone to estrogen, in the MPOA (Trainor et al. 2003). Neural plasticity in
412 these regions can also be influenced by previous experience. For example, high-stress early-life
413 environments can alter estradiol-regulated RNA transcripts in the MPOA of male rats (Eck et al.
414 2022). In addition, neural plasticity can be influenced by experiences associated with the onset of
415 fatherhood, such as mating, cohabitation with a (gestating or lactating) female, and exposure to
416 pups (reviewed in Horrell et al. 2021). At the cellular level, plasticity can involve changes in
417 production, survival, morphology and gene expression of neurons (Horrell et al. 2021).These
418 general mechanisms of neural plasticity may contribute to plasticity in sensory systems. For
419 example, Seelke et al. (2018) explored differences in gene expression in the MPOA of prairie
420 vole fathers, virgin males and paired males and found differences in gene ontology annotations
421 for olfactory behavior, as well as for many other behaviors and neural functions.
422 Fathers in our study tended to have higher Fos expression than virgin males in the
423 basolateral amygdala (BLA), which is also important for mediating parental responsiveness to
424 offspring (Numan 2012). Neurons in the BLA are activated in response to environmental stimuli
425 that may or may not relate directly to stimuli from pups, such as novel environments and
426 approach/avoidance conflict (Hale et al. 2006; Reznikov et al. 2008). Mechanistically, the BLA
427 processes information received from the olfactory and auditory pathway (Numan 2012; Grimsley
428 et al. 2013; Gadziola et al. 2016; Rogers and Bales 2019). It plays an important role in assigning
429 valence to sensory information and relaying information to several regions, including the NAcc -
430 ventral pallidum reward circuit (Numan 2012; Dulac et al. 2014; Rogers and Bales 2019). The
431 trend toward greater activation of the BLA in fathers than in virgin males may reflect differences
432 in the valence of pup-related cues between fathers and virgins. This line of reasoning is further

supported by the finding that BLA activation and time spent handling the ball were positively associated for fathers but not virgins.

Interestingly, we did not find a difference between fathers and virgin males in Fos expression in the NAcc, which suggests that reproductive status might not influence the extent to which pup calls and odors *per se* are rewarding. This result is in line with our previous finding that NAcc Fos expression did not differ between California mouse fathers and virgin males that had been exposed to a live pup in a mesh tea ball, which permitted exposure to auditory and olfactory cues but prevented direct contact (de Jong et al. 2009). The NAcc is part of the mesolimbic reward circuitry and may respond to pup sensory cues via projections from the MPOA and STMV (Numan and Numan 1997; Kaufling et al. 2009). Although it is frequently included in neural models of maternal care (e.g., Rogers and Bales 2019; Numan 2020), its role across sexes and species has proven to be ambiguous (Horrell et al. 2019). For example, in female Sprague-Dawley rats NAcc lesions cause significant disruptions to normal maternal behavior (Li and Fleming 2003), while NAcc lesions in male California mice cause only minor reductions in paternal behavior (Lee and Brown 2007). A possible explanation is that reward may be derived from the behavior of caring for pups, which is initiated in response to sensory cues, rather than from perception of the cues themselves. This possibility requires further investigation.

Our decision to focus on the granule cell layer of the main olfactory bulbs (MOB) was based on previous work showing high activity in this region in new house mouse fathers (Mak and Weiss 2010). We also found, when blind to male reproductive status and treatment, that Fos expression was much higher in this layer compared to others. The granule cell layer plays a role in lateral inhibition, with GABA as the dominant neurotransmitter (Nagayama et al. 2014), and

plasticity in this region aids in odor discrimination (Gheusi et al. 2000; Mandairon and Linster 2009). Fathers had lower Fos expression in the MOB compared to virgins, even though the two groups of males displayed comparable durations of sniffing and handling the odor ball. While this pattern has not been reported previously for the MOB, others have reported lower activation of the accessory olfactory system in house mouse fathers compared to virgins (Nakahara et al. 2016; Isogai et al. 2018). This finding, along with significant effects of reproductive status on Fos expression in other brain regions (MPOA and STMV) in our study, strengthens the case that the detection and/or perception of pup stimuli changes with fatherhood and does not necessarily depend on the acute amount of stimulus exposure.

Effects of Stimulus Modality

As predicted, we found that some aspects of fathers' and virgins' behavioral and neural responses to stimuli differed among treatment groups, with males exposed to the combination of pup calls and pup odors exhibiting the highest number of significant differences compared to mice exposed to neither pup calls nor pup odors. Mice rested less during the exposure period when they were exposed to any pup stimuli (Call, Odor, Call + Odor) compared to no pup stimuli, and mice exposed to Call + Odor remained significantly more active after the stimulus exposure period compared to all other treatment groups. While studies evaluating possible causal relationships are still needed, the effects of stimulus treatment on neural activity in the MPOA complement these behavioral findings. Although Fos expression in the MPOA did not differ between males exposed to the Call and Odor treatments, males in the Odor treatment, but not those in the Call treatment, had higher MPOA Fos than those exposed only to control stimuli. Moreover, the neural response to pup calls was potentiated by simultaneous exposure to pup

odor, as mice in the Call + Odor treatment had significantly higher Fos in the MPOA than those in the Call treatment. In contrast, simultaneous exposure to calls did not affect the response to odors, as MPOA Fos expression did not differ between males in the Call + Odor and Odor treatments. These differences suggest that pup odor is a more potent stimulus than pup calls and, together with the behavioral data, suggest that pup odor and pup calls can have an additive or synergistic effect, rather than redundant effects, on behavior and neural activation of the MPOA in males.

In house mice, isolated pup vocalizations elicit retrieval from mothers and fathers Ehret and Koch1989), and mothers have better neural entrainment in the auditory cortex to acoustic stimuli within the natural frequency range of pup vocalizations, compared to virgin females (Liu et al. 2006). Given these previous results, we were somewhat surprised to find that pup calls did not activate brain regions associated with paternal care unless paired with pup odor, although pup calls alone elicited more active behavior in males during stimulus exposure compared to the control. These findings could relate to differences in the functional role of pup calls versus pup odors. Rodent pup calls reliably elicit parental approach, the appetitive component of reward (Ehret 2005), but rodent pups stop vocalizing or reduce their vocalization rate with the introduction of a parent or family odor (Shair 2018; Wilson et al. 2022). Thus, calls are not associated with the consummatory component of reward (Ehret 2005), which may be more relevant for the activation of brain regions important for parental care.

The main olfactory system has received little attention in relation to parental care in males; however, studies of the accessory olfactory system have begun to elucidate potential mechanisms through which perception of chemosensory cues differs with the onset of fatherhood. Because the chemosensory stimulus was presented in an enclosed ball in this study,

the mice were not able to have direct contact with the stimulus and therefore might not have been able to detect it with the vomeronasal organ (VNO) or, consequently, to process the stimulus in the accessory olfactory system. However, the relative wealth of studies of the accessory olfactory system provides good context for discussing the effects of pup olfactory stimuli observed in our study. Genetic or surgical ablation of the VNO increases paternal care in virgin male house mice (Tachikawa et al. 2013; Wu et al. 2014; Isogai et al. 2018), but olfactory bulb lesions, which impact both chemosensory systems, increase aggression towards pups in virgin male prairie voles (Kirkpatrick et al. 1994a, b).

The transition to fatherhood in house mice is accompanied by a dampening of the VNO response to pups, which is likely mediated by the down-regulation of specific odorant receptors within the VNO (Nakahara et al. 2016; Isogai et al. 2018). Specifically, when exposed to unrelated pups, virgin male house mice have higher Fos expression in the VNO and lower Fos expression in the MPOA than fathers (Tachikawa et al. 2013), which aligns with our findings of higher Fos expression in the MOB and lower Fos expression in the MPOA in virgin males compared to fathers. We also found that Fos expression in the STMV, MPOA and NAcc was positively correlated with time spent sniffing the odor ball in fathers, while Fos expression in the MOB was positively correlated with activity levels in virgins. These findings suggest that, like the accessory olfactory system in male house mice, activity of the main olfactory system is dampened during the transition to fatherhood in male California mice, an effect that might facilitate the inhibition of aggression and activation of affiliation toward pups.

Based on their findings in house mice, Tachikawa et al. (2013) suggested that in males, the accessory olfactory pathway processes aversive chemosensory stimuli from pups while the MOB may process attractive chemosensory stimuli from pups. We find mixed support for this

hypothesis, since fathers that spent more time sniffing the odor ball had greater Fos expression in the reward circuit (NAcc) and tended to have greater Fos expression in the MOB. However, fathers also had lower Fos expression in the MOB compared to virgins, which seems to contradict Tachikawa et al.'s (2013) hypothesis. The effect of male reproductive status on the accessory olfactory pathway has not been explored in biparental rodents, and this is the first study, to our knowledge, to explore neural responses to isolated pup stimuli in the MOB. Thus, the relationship between the main and accessory olfactory pathways in mediating parental behavior remains unclear but presents a promising avenue of research.

Our results in the MOB also contrast with findings by Mak and Weiss (2010), who reported that house mouse fathers had more neurogenesis in the olfactory bulbs when they were housed with their mate and first litter for the first two days after birth compared to fathers that were removed shortly after parturition. However, there are key differences between Mak and Weiss's (2010) study and ours. Most notably, we compared fathers to virgins, while Mak and Weiss (2010) compared fathers housed with or without their mate and pups. The potential effects of housing with a pregnant or parturient mate and/or initial interactions with pups could affect results, since copulation alone can be enough to alter behavioral responses to pups (Elwood and Stolzenberg 2020). Including a virgin male group in a similarly designed study would allow us to test this possibility.

A more complete understanding of the effects of fatherhood on males' responses to isolated sensory cues from pups will come from exploring additional mechanisms of neural plasticity. This could be done, for example, by quantifying receptors for neuropeptides and hormones thought to play a role in the onset of paternal care (e.g., testosterone, estrogen, oxytocin, vasopressin) in regions that receive and integrate sensory information (reviewed in

548 Bales and Saltzman 2016; Horrell et al. 2019, 2021). Although we were unable to analyze
549 auditory structures in the brain for technical reasons, we would expect to see the transition to
550 fatherhood to affect, for example, the auditory cortex and inferior colliculus, based on their
551 neural connections to the MPOA, BNST, BLA, BMA and medial amygdala (Numan 2020) and
552 given the high degree of connectivity among primary cortices (Campi et al. 2019). Additional
553 work to further explore auditory brain regions, using techniques such as auditory brainstem
554 recordings and electrophysiology, is forthcoming. Such studies will shed additional light on the
555 present findings, in particular, the surprising finding that fathers had lower expression of Fos in
556 the MOB than virgins, despite positive correlations between sniffing behavior and activation of
557 paternal behavior and reward circuits. It would also be informative to examine effects of
558 additional environmental and experiential correlates of the transition to fatherhood, such as
559 formation of a pair bond, copulation, or cohabitation with a pregnant female.
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939 TABLES

940 Table 1. Total durations of behaviors during 10-min stimulus exposures and number of scan
941 samples in which behavior was observed during the 60 min after stimulus exposure (median, 1st
942 and 3rd quartiles). Bolded values are significantly different from all other stimulus treatments (P
943 < 0.05).
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	Parenthood status		Stimulus treatment			
Variable	Father (N=30)	Virgin (N=29)	Control (N=14)	Call (N=14)	Odor (N=14)	Call + Odor (N=17)
<u>During stimulus exposure</u>						
Sniff ball (s)	7.5 (0.0, 40.7)	14.7 (0.0, 67.7)	0.0 (0.0, 16.9)	10.3 (0.0, 77.7)	10.8 (1.7, 63.0)	25.7 (8.0, 45.7)
Handle ball (s)	0.0 (0.0, 31.4)	0.0 (0.0, 117.1)	0.0 (0.0, 0.0)	0.0 (0.0, 117.1)	0.0 (0.0, 27.0)	9.4 (0.0, 41.2)
Listen (s)	0.0 (0.0, 40.8)	0.0 (0.0, 48.4)	0.0 (0.0, 0.0)	6.5 (0.0, 48.4)	7.8 (0.0, 55.1)	8.5 (0.0, 49.1)
Activity (s)	0.0 (0.0, 52.1)	0.0 (0.0, 52.3)	0.0 (0.0, 0.0)	0.0 (0.0, 46.1)	31.4 (0.0, 82.9)	3.3 (0.0, 82.3)
Rest (s)	558.3 (170.5, 600.0)	429.8 (213.1, 599.0)	**600.0 (583.1, 600.0)**	427.5 (181.1, 600.0)	415.0 (77.9, 594.8)	523.9 (162.2, 592.0)
<u>After stimuli removed</u>						
Activity (count)	5.5 (1.0, 9.0)	9.0 (2.0, 12)	7.0 (2.0, 12.0)	8.5 (5.0, 12.0)	8.5 (1.0, 12.0)	**2.0 (1.0, 7.0)**

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Table 2. Correlations between Fos expression (number of Fos-positive neurons per 200 x 200 µm square) and behavior duration (s) for (A) fathers and (B) virgin males. Significant correlations ($P < 0.05$) are indicated in bold, and non-significant trends ($0.05 < P < 0.07$) are indicated in italics. For each correlation, Pearson's *r* (top line) and *P*-value (bottom line) are shown.

Fos-IR/ Behavior (s)	MOB	NAcc	STMV	MPOA	AHN	BLA	BMA
A. Fathers	N = 30	N = 29	N = 30	N = 30	N = 29	N = 27	N = 27
Sniff odor ball	*0.346*	**0.420**	**0.804**	**0.569**	0.070	0.100	0.196
	0.061	**0.024**	**< 0.001**	**0.001**	0.717	0.619	0.327
Handle odor ball	0.015	-0.045	0.153	0.172	0.090	**0.404**	**0.602**
	0.939	0.818	0.419	0.363	0.644	**0.037**	**0.001**
Listen	-0.074	-0.092	0.299	0.250	0.142	-0.192	-0.080
	0.697	0.635	0.109	0.183	0.466	0.341	0.692
Activity	0.061	-0.036	0.182	0.158	*0.366*	0.037	0.036
	0.751	0.853	0.337	0.403	*0.051*	0.855	0.857
Rest	-0.246	-0.200	**-0.507**	**-0.380**	-0.293	-0.212	*-0.379*
	0.190	0.299	**0.004**	**0.038**	0.123	0.289	*0.051*
B. Virgin males	N = 29	N = 29	N = 29	N = 29	N = 29	N = 25	N = 26
Sniff odor ball	-0.076	0.100	-0.134	-0.256	0.007	-0.272	0.089
	0.697	0.604	0.489	0.180	0.971	0.189	0.666
Handle odor ball	0.004	-0.062	-0.105	-0.282	-0.217	-0.228	-0.047
	0.985	0.749	0.587	0.139	0.259	0.273	0.820
Listen	0.052	-0.231	-0.020	0.077	0.128	-0.040	0.228
	0.790	0.228	0.917	0.6902	0.507	0.851	0.263
Activity	**0.481**	-0.036	-0.086	0.162	0.004	-0.111	-0.855
	0.008	0.853	0.658	0.401	0.985	0.599	0.678
Rest	-0.177	0.149	0.097	0.072	-0.005	0.265	-0.011
	0.358	0.442	0.616	0.711	0.980	0.201	0.957

FIGURES

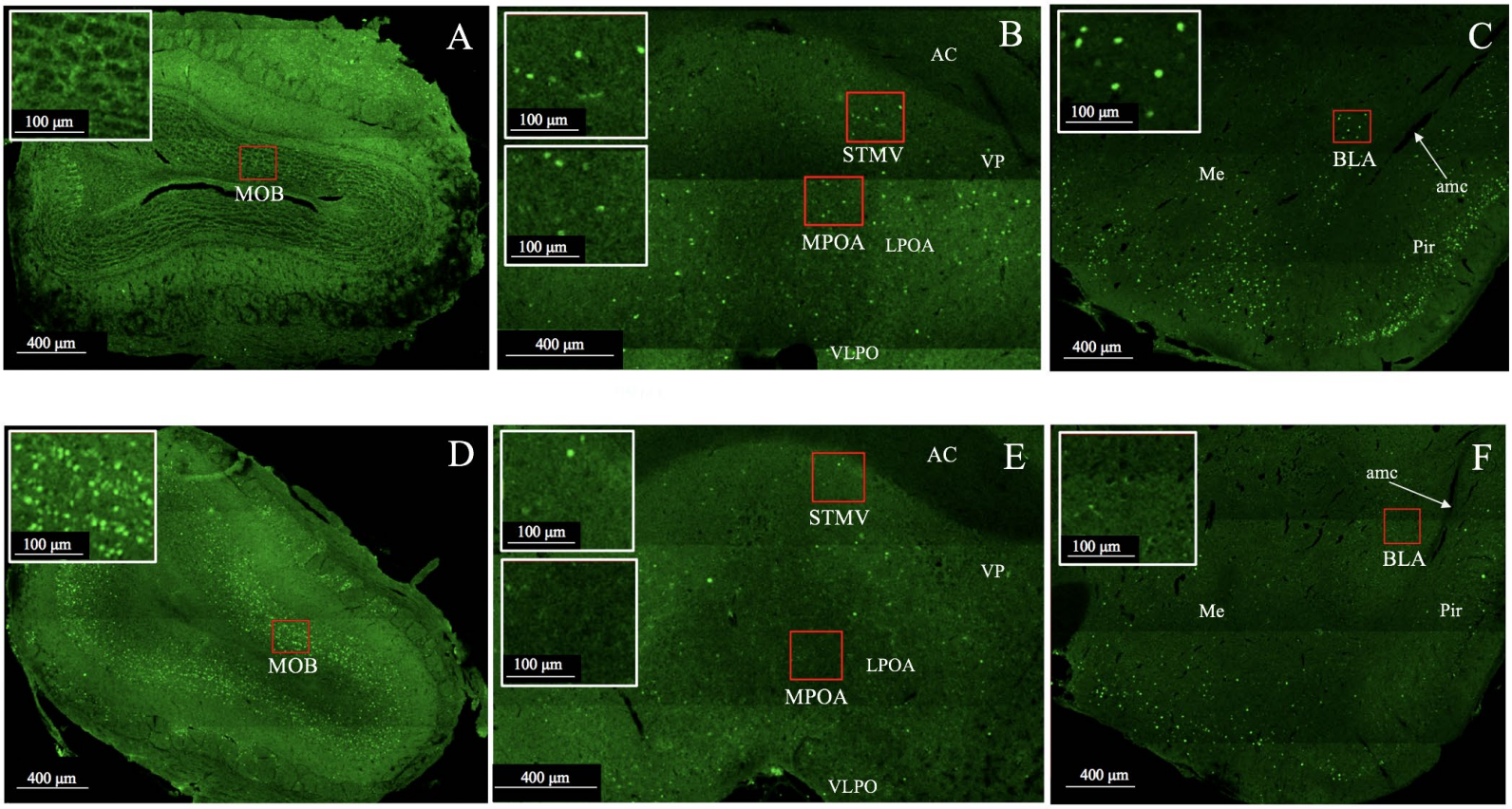

Figure 1. Representative photomicrographs of coronal brain sections (40 µm thick) showing Fos staining in the main olfactory bulbs (MOB; A and D), bed nucleus of the stria terminalis medial division, ventral part (STMV) and medial preoptic area (MPOA; B and E), and basolateral amygdala (BLA; C and F) of California mouse fathers (A-C) and virgin males (D-F). Magnified images are of the area outlined by the red box (200 x 200 µm) in each photomicrograph. In images B and E, the top inset in each photomicrograph corresponds to the STMV and the bottom inset corresponds to the MPOA. AC = anterior commissure, amc = amygdalar capsule, LPOA = lateral preoptic area, Me = medial amygdaloid, Pir = piriform cortex, VLPO = ventrolateral preoptic nucleus, VP = ventral pallidum.

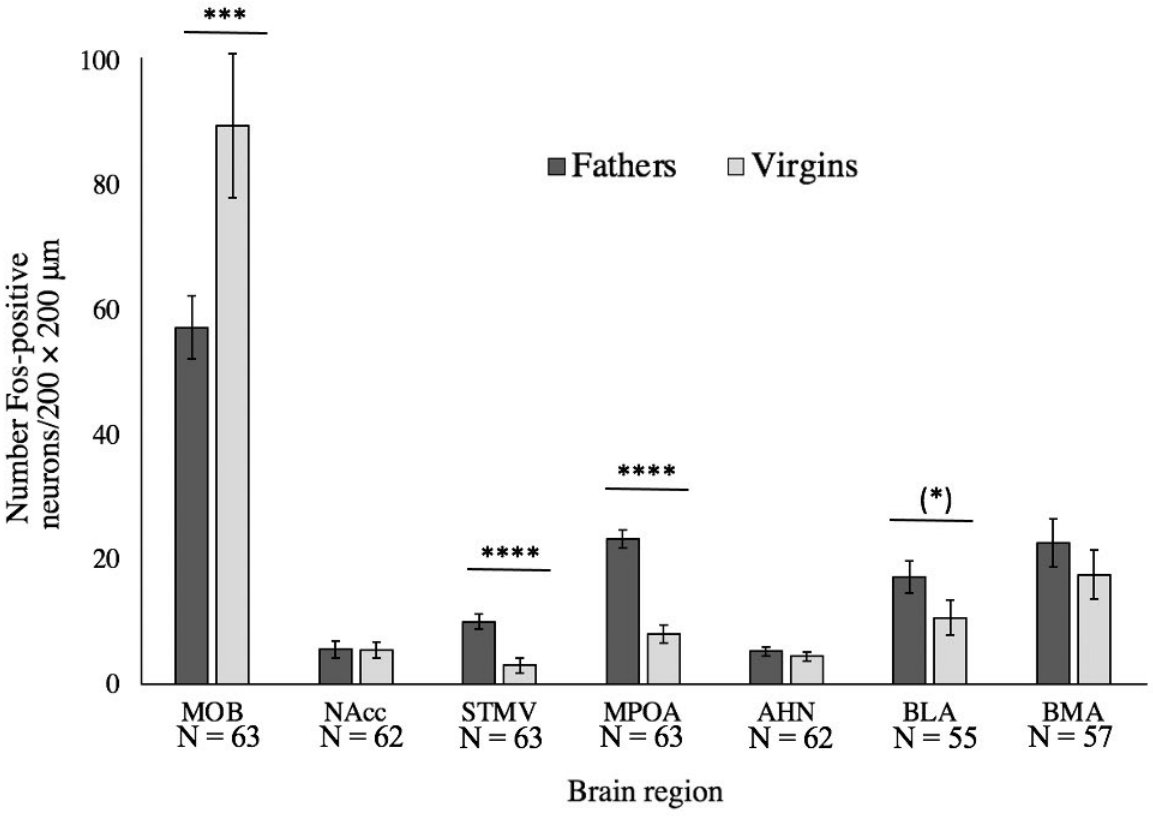

Figure 2. Fos expression (mean ± SE) in fathers and virgin males collapsed across all four stimulus treatments. MOB[a] = main olfactory bulbs, NAcc[a] = nucleus accumbens, STMV = bed nucleus of the stria terminalis medial division, ventral part, MPOA[a] = medial preoptic area, AHN[a] = anterior hypothalamic nuclei, BLA = basolateral amygdala, BMA[a] = basomedial amygdala. Data shown are not transformed. [a]Immunohistochemistry batch contributed significantly to the model. LMMs; (*)P< 0.065, *** P < 0.001 ****P < 0.0001.

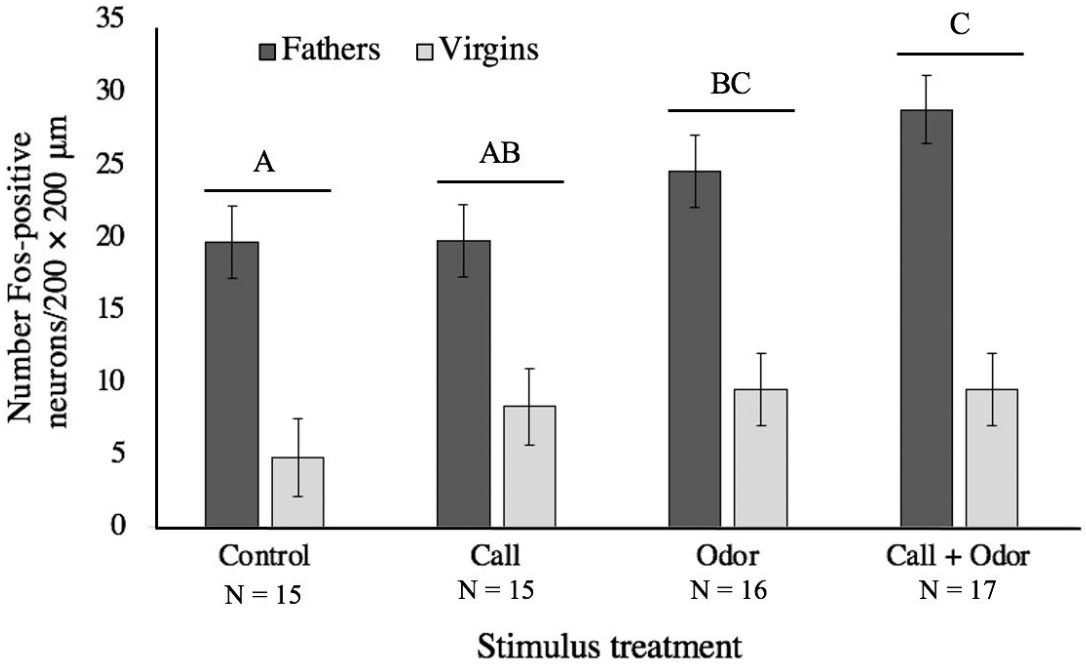

Figure 3. Fos expression (mean ± SE) in the medial preoptic area by stimulus treatment for fathers and virgin males. Analysis of stimulus treatment (LMMs) included data from both fathers and virgins, but data are shown separately because fathers had significantly higher Fos expression in the MPOA than virgins across all stimulus treatments ($\chi 2 = 108.70$, $P < 0.0001$). Letters denote significant differences among treatments based on post-hoc pairwise comparisons: bars with the same letter do not differ significantly ($P < 0.05$). Data shown are not transformed.