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Mapping a Pup-responsive Pathway from the Medial Preoptic Area to the Ventral Tegmental Area.

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Mapping a pup-responsive pathway from the medial preoptic area to the ventral tegmental area.

A Thesis Presented
by
MATIAS LEANDRO ANDINA

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for the degree of

Master of Science

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Neuroscience and Behavior
Mapping a pup-responsive pathway from the medial preoptic area to the ventral tegmental area.

A Thesis Presented
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The research in this thesis would not be possible without the help of many people. I want to thank Dr. Pereira for the opportunity, nothing would be possible without her. I want to thank Dr. Bergan for continuous support, sharing reagents and the hydraulic injector. I want to extend thanks to the members of the Bergan lab for help with surgeries. Dr. Andrew Farrar also helped me with my first surgeries. Many thanks to Dr. Moorman for endless access to the cryostat. I want to thank Matheus Macedo-Lima for help with immunohistochemistry and continuous support. Dr. James Chambers greatly helped me with the microscope routine and training.
Maternal behavior is the complex array of caregiving behaviors females display towards offspring. In rats, the transition to motherhood depends on the action of various hormones, especially estradiol near parturition, which primes the maternal circuitry to respond to pups upon first encounter at parturition with appropriate maternal behavior. Although virgin rats avoid pups, new mothers are highly motivated to interact with pups, and their maternal behavior depends on the functional interaction between the medial preoptic area (mPOA) and the ventral tegmental area (VTA). However, a precise mapping of the VTA-projecting mPOA neurons remains to be elucidated. To determine whether pup-responsive neurons in the mPOA...
project to the VTA, we injected the retrograde tracer Fluorogold (FG) into
the VTA of new mother and virgin female rats. Six days later, females
were exposed to 3 pups for 5 minutes, and their brains processed to visu-
alize FG and c-Fos immunostaining. In addition, we further characterized
the molecular phenotype of these neurons by performing immunohistochem-
istry against estrogen receptor alpha (Esr1). As expected, the behavior of
postpartum and virgin females toward pups was different. Mothers readily
approached pups and displayed maternal behavior, whereas virgins avoided
interaction with pups. Despite these disparate responses to pups, no dif-
fferences were found in the number and distribution of mPOA→VTA
neurons. In addition, in both postpartum and virgin females, a signifi-
cant proportion of these pup-responsive mPOA→VTA projecting neurons
also express Esr1. Further functional interrogation of these c-Fos+/Esr1+
mPOA→VTA neurons in virgins and mothers might elucidate distinct cir-
cuit dynamics potentially underlying their behavioral differences towards
pups.
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CHAPTER 1
INTRODUCTION

Maternal behavior is the complex array of caregiving behaviors mothers display towards offspring, essential for the survival of the young. In rats, the onset of maternal behavior is facilitated by the hormonal changes occurring during late pregnancy and parturition (Bridges, 1984). Although virgin rats normally avoid pups, new mothers readily approach pups and contingently perform high levels of caregiving behaviors, including retrieval of pups to the nest site, nest building, and licking and nursing of pups (Kuroda, Tachikawa, Yoshida, Tsuneoka, & Numan, 2011; Numan & Insel, 2003). Estrogen and progesterone priming, with downstream consequences for prolactin and oxytocin systems, prepare the maternal circuitry to respond to infant stimuli upon first exposure at parturition with appropriate maternal behavior (Rosenblatt & Siegel, 1975; Siegel & Rosenblatt, 1978). Once established, maternal behavior is subsequently regulated by the continuous sensory experience of interaction with the developing young (Bridges, 1978).

Extensive research has shown that the medial preoptic area (mPOA) plays a central role in orchestrating maternal behavior (Kuroda et al., 2011; Numan & Insel, 2003; Pereira & Ferreira, 2016). Interference with mPOA function or with mPOA lateral efferent connections disrupts both the onset
and early expression of maternal behavior (Arrati, Carmona, Dominguez, Beyer, & Rosenblatt, 2006; Fleming, Miceli, & Moretto, 1983; Miceli & Malsbury, 1982; Numan, 1974, 1996; Numan & Callahan, 1980; Numan, Corodimas, Numan, Factor, & Piers, 1988; Numan, Rosenblatt, & Komisaruk, 1977; Pereira & Morrell, 2009). Interference with mPOA function also affects responding on behavioral tasks that assess pup-mediated reinforcement, further characterizing a role of the mPOA in motivational aspects of maternal behavior (Lee, Clancy, & Fleming, 1999; Pereira & Morrell, 2010). Immediate early gene studies revealed increased activity of c-Fos, a marker of neuronal activity, in the mPOA of postpartum females after mothers physically interact with pups and display maternal behaviors (Lonstein & De Vries, 2000; Tsuneoka et al., 2013). Substantial evidence further indicates that the mPOA regulates reinforcing properties of pups and active components of maternal behavior (i.e., retrieving, and licking and grooming) through projections to components of the mesolimbic dopamine (DA) system, the ventral tegmental area (VTA) and the nucleus accumbens (Fang, Yamaguchi, Song, Tritsch, & Lin, 2018; Kohl et al., 2018; Numan & Stolzenberg, 2009). Accordingly, the motivational impairments in rat maternal behavior after pharmacological interference with accumbens DA are remarkably similar to the effects of mPOA functional inactivation (Keer & Stern, 1999; Numan & Insel, 2003; Numan & Stolzenberg, 2009; Pereira & Morrell, 2011). Furthermore, DA is released in the nucleus accumbens, as
measured by microdialysis and fast scan cyclic voltammetry, of postpartum rats and primed virgins during interaction with pups (Afonso, King, Chatterjee, & Fleming, 2009; Afonso, Shams, Jin, & Fleming, 2013; Champagne, 2004).

The mPOA is a heterogeneous structure containing multiple anatomically and functionally diverse subregions that express a variety of neurotransmitters and neuropeptides, such as neurotensin, galanin, preproenkephalin, oxytocin, calbindin, and cocaine- and amphetamine-related transcript (Tsunekawa et al., 2013, 2017). The mPOA contains receptors for all the hormones involved in maternal behavior, including receptors for estradiol, such as estrogen receptor alpha (Esr1) (Fang et al., 2018; Shughrue, Lane, & Merchenthaler, 1997; Simerly, Swanson, Chang, & Muramatsu, 1990; Tsuneoka et al., 2017). Extensive research has demonstrated that estrogen surge during late pregnancy and parturition acts on mPOA neurons to facilitate the onset of rat maternal behavior (Numan et al., 1977; Rosenblatt & Siegel, 1975). The mPOAEsr1 population has been shown to be mostly GABAergic (80%) and to co-expresses galanin (~50%) (Han, Li, Wang, Wei, & Xu, 2017; Tsuneoka et al., 2017; Wei et al., 2018).

Recent studies in mice have revealed a role of mPOA\(^{Esr1} \to \) VTA projections in promoting social and maternal behaviors, showing that estrogen sensitive, estrogen receptor alpha (Esr1) expressing mPOA neurons project to
VTA and influence motivated social behavior via dopaminergic modulation in the nucleus accumbens (Fang et al., 2018; Kohl et al., 2018; McHenry et al., 2017; Wei et al., 2018). However, a detailed characterization of the mPOA→VTA circuitry relevant to maternal behavior in the rat remains to be fully elucidated. Here, we used a combination of anatomical tract-tracing, double-label c-Fos and Esr1 immunohistochemistry, and behavioral analyses to determine if the mPOA → VTA neurons that are recruited with pup presentation are sensitive to estrogens.
CHAPTER 2
MATERIALS AND METHODS

2.1 Animals

Adult virgin and primiparous Sprague-Dawley female rats (250-400 g body weight, Charles River Laboratories) were used in this study. All females were kept on a 12-h light/dark cycle (lights on at 0400 AM) at 22±1 °C, with ad libitum access to water, rat chow and sunflower seeds. Late pregnant females were housed in individual clear Plexiglas cages (47 x 25.5 x 20.3 cm), lined with fresh Sani-Chips® bedding (P.J. Murphy Forest Products Corp, Montville, NJ) and containing Eco-Bedding nest-building material (Fibercore LLC, Cleveland, Ohio). Postpartum females and litter remained undisturbed for the first 24 h after parturition (birth = postpartum day 0, PPD0) to ensure maternal behavior establishment. On PPD1, litters were culled to 12 pups (5-7 males, 5-7 females) per mother rat, and all mothers remained and were tested with their own pups. All procedures were approved by the University of Massachusetts Amhersts Institutional Animal Care and Use Committee in compliance with the NIH Guide for the Care and Use of Laboratory Animals.
2.2 Stereotaxic Injections of Fluorogold

Virgin and PPD1 females were anesthetized with Isoflurane (4% induction, 2% maintenance), and positioned in a stereotaxic apparatus (David KOPF Instruments, USA). Animals were given bilateral injections of the retrograde tracer FluoroGold (FG, 2% solution, Fluorochrome Inc., Englewood CO, USA) into the VTA (AP: -5.6 mm (from Bregma), ML: ±0.8 mm (from midline), DV: -8.3 mm (from the skull); Paxinos & Watson, 2013) through a 30-40 µm thick pulled glass capillary (DMN3000203GX, Drummond) using a hydraulic micromanipulator (MO-10, Narishige, Japan). Each side received 100-125 nl total volume of FG over 1 min. After the injection, the capillary was left in place for 20 min to allow time for diffusion of the tracer, and then slowly retracted. Incisions were cleaned and sutured with absorbable stitches. FG injections in virgin females were time-matched (within 24 hours) with those of the maternal animals. Animals were behaviorally tested 6-7 days later to allow satisfactory axonal transport of FG to cell bodies.

2.3 Behavioral Test and Analysis

One day before testing, virgin or postpartum females with their pups were housed in individual clear Plexiglas cages (38.5 cm W x 48.5 cm L x 20.5 cm H). The pups were marked on their back using a non-toxic marker to
facilitate tracking of their position during the test. On PPD7-8, pups were separated from their mother for 2 h and kept together and warm. After a 2 h mother-litter separation, three pups from the litter were returned to the maternal cage and placed individually into each corner of the test cage away from the mother. The behavior of the mother and pups was recorded for 5 minutes. After the test, the pups were removed, and the mother remained in the cage for 1 h before being sacrificed for histological analysis. Virgin animals were tested with age-matched pups from a donor mother.

All behavioral interactions were recorded from the top of the cage using a Logitech C270 USB Webcam (Logitech International S.A., Lausanne, Switzerland) connected to a computer running the open source software Bonsai (Lopes et al., 2015). The lid of the cage was replaced by a height extension (17.8 cm) during testing that prevented escape. Video acquisition, behavioral annotation, and analysis were performed using a semi-automated custom pipeline written in Bonsai, MATLAB, and R (https://github.com/matiasandina/MLA2_Tracking). Briefly, each video frame was analyzed through different HSV thresholding filters, and the largest binary polygonal region for each binary layer (i.e., each animal) was kept. The coordinates of each regions centroid were considered to be the raw x, y values of each animals position. Additionally, the major axis of the polygonal region was kept. Raw positions were then inspected for missing values,
and manually corrected during data processing. The following distances were derived for every recorded frame: the Euclidean distances between the adult and each pup, and between pups. In addition, the arena was divided into four virtual quadrants, and the animal position was cataloged for each frame to one specific quadrant. The proportion of test time in which 2 or more pups were in the same quadrant (pup-pup proximity fraction), the proportion of test time in which the adult-pup distance was less than half of the adults median major axis (adult-pup proximity fraction), and the difference between the sum of pup-pup distances at the first and last frame of the test (total change in pups position) were calculated. The number of pup-directed (retrieving, sniffing, licking, hover-over, nest-building) and other behaviors not directed to the pups (rearing and self-grooming) were manually labelled by observation of the video recordings. From this annotation, the duration and latency of each scored behavior was calculated.

2.4 Immunohistochemistry and Image Analysis

One hour after behavioral testing, animals were deeply anesthetized with sodium pentobarbital (100 mg/kg), and transcardially perfused with 0.01 M PBS and 4% paraformaldehyde in 0.1 M PB. After perfusions, brains were postfixed overnight in 4% paraformaldehyde, and then cryoprotected in 30% sucrose in 0.1 M PB at 4 °C until they sank. Brains were sec-
tioned into 40-µm thick cross sections using a cryostat (Leica 3050 S, Germany). One series containing one every other two sections throughout the mPOA and VTA was processed for immunohistochemistry. Sections from the mPOA were immunostained with both c-Fos and Esr1 antibodies. Injection sites were corroborated by FG fluorescence and TH immunostaining in VTA sections. Alternate brain sections throughout the mPOA were immunostained for the 67,000 mol. wt. isoform of glutamate decarboxylase (GAD67). According to the Paxinos and Watson rat brain atlas (Paxinos & Watson, 2013), 6 sections were selected from the mPOA at the following coordinates: 0.24, 0.00, -0.24, -0.48, -0.72, and -1.08 mm from Bregma. The free-floating sections were stored 0.1 M PB until IHC processing. Controls for immunocytochemical specificity included sections incubated without primary antibodies.

2.4.1 c-Fos, Esr1, TH IHC

Free floating sections were washed with 0.1 M PB (3 x 15 min), blocked in 10% normal donkey serum (D9663, Millipore-Sigma) for 2 h at room temperature, and then incubated with primary antibodies in 0.3% PBT for 1 h at room temperature, followed by 36 h in agitation at 4°C. The primary antibodies used were: guinea pig anti-c-Fos (1:500, 226 004, Synaptic Systems), rabbit anti-Esr1 (1:5000, 06-935, Millipore-Sigma), and mouse anti-TH (1:5000, 22941, Immunostar). Sections were then washed with 0.1%
PBT (3 x 15 min), incubated with secondary antibodies for 1 h at room temperature. The secondary antibodies used were: donkey anti-rabbit Alexa Fluor 594 (1:500, A-21207, ThermoFisher), donkey anti-guinea pig Alexa Fluor 647 (1:800, 706-605-148, Jackson ImmunoResearch), and donkey anti-mouse Alexa Fluor 647 (1:500, A-31571, ThermoFisher). Sections were finally washed with 0.1% PBT (3 x 10 min), mounted on gelatin-coated slides and coverslipped with glycerol.

2.4.2 GAD67 IHC

Free floating sections were subjected to a heat-mediated antigen retrieval step (50 mM citrate buffer at 90 °C, 20 min), followed by the IHC protocol described above with the following minor modifications. Specifically, none of the solutions contained Triton, and the sections were first incubated for 1 h in mouse anti-GAD67 (1:1000, G5419, Millipore) at room temperature, followed by 18 h in agitation at 4 °C.

2.4.3 Quantification of VTA-Projecting mPOA neurons expressing c-Fos, Esr1, and/or GAD67

Mounted sections were imaged in a confocal microscope (A1R, Nikon, Tokyo, Japan) with NIS-Elements imaging software (RRID: SCR_002776). Co-expression of FG and molecular markers within representative mPOA
sections were unambiguously confirmed by 20X z-sectioning analysis of a squared region of 0.41 mm$^2$, collected in 11 increments of 2.12 µm throughout the z axis. Cell-counting for c-Fos and Esr1 labelling and co-localization analysis was performed using a semi-automated pipeline in NIS-elements software (Nikon, Tokyo, Japan) and custom R scripts. Briefly, mean intensity in z was subtracted from all z planes within each z-stack, followed by median filtering and manual thresholding to obtain 3D binary masks (putative positive cells in each channel). These binary masks were exported as individual channels (i.e, FG, Esr1, and c-Fos) and their intersections (e.g, FG and c-Fos intersection). Percentages of marker expression were calculated based on the total number of c-Fos+ cells, unless otherwise indicated. Only one side per mPOA section was counted per animal. Cell-counting for GAD67 was performed by manual inspection of the z-stacks.

2.4.4 DAPI Staining

To quantify the number of cells throughout the mPOA, separate virgin females (n=2) were perfused, and their tissue processed for 4’,6-Diamidino-2-phenylindol (DAPI, a cell nuclear marker) staining. Briefly, free-floating mPOA sections were briefly dipped in 0.1M PBS, and then incubated in DAPI diluted in 0.1 M PBS (0.1%, D1306, Invitrogen, USA) for 20 min at room temperature. After rinsing with 0.1M PBS, sections were mounted, coverslipped with glycerol, and imaged in the confocal microscope. Large
tiled images were taken with 10X magnification, and four square-shaped areas (306 x 306 µm) per mPOA section were randomly selected for analysis. Counting was performed by manual inspection following automatic denoising and intensity thresholding methods by the NIS software.

2.5 Statistical Analysis

Behavioral comparisons between virgin and postpartum females were performed by t-test or Mann-Whitney U-test, when the assumptions for parametric testing were not met. Behavioral data was subjected to principal components analysis (PCA), with a first component (PC1) explaining 42% of the variance of the data. Correlations between behavioral and IHC data were conducted with Pearson $r$ tests comparing PC1 with the mean number of cells. The average number of mPOA cells single, double- and triple-labelled with FG, c-Fos, and/or Esr1 per section was compared between virgin and postpartum females using Pearsons chi-squared tests. As sections at similar levels throughout the mPOA were obtained from all animals, the number of cells single-, double- or triple-labelled with FG, c-Fos and/or Esr1 was compared between virgin and postpartum females with repeated measures mixed ANOVAs, with 6 mPOA AP positions as the within-subject factor. All statistical analysis was performed in R, code is available upon request. Statistical significance was set at $p<0.05$. 

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CHAPTER 3

RESULTS

Histological analysis of sections stained for TH confirmed FG infusion sites within one unilateral VTA in 7 postpartum females and 5 virgin females. Included injections had a dense core of FG contained within the VTA. The size of injections did not differ systematically between groups. Females with misplaced injections were excluded from histological data analyses (n=9).

3.1 Behavioral assay

Consistent with previous studies (Fleming & Rosenblatt, 1974; Pedersen, Ascher, Monroe, & Prange, 1982), we observed differences in the display of caregiving behaviors between virgin and postpartum females during a 5 min test, in which three pups were individually placed into each corner of the female’s cage except the one closest to the female (Figure 1A,B,C). As shown in Figure 1D,E, mothers readily retrieved and grouped the pups in the nest (Mann-Whitney U test, U=100, p<0.001), expressed more nest building (U= 90, p=0.004 ), and spent significantly more time in close proximity to their pups compared to the virgin group (U = 103, p<0.001). In contrast, virgins spent significantly more time sniffing the pups than mothers (Mann-Whitney test, U = 3, p<0.001, Fig. 1D). Thereafter, virgin females mostly avoided interactions with pups (Figure 1B,C,D). No virgin
females retrieved or attacked pups during the test. No significant differences were found between groups in any other behaviors measured, including rearing and self-grooming (Mann-Whitney U test: all p>0.05; Fig. 1D). Although pups did move during the test, the distance between them was smaller when mothers retrieved them versus their self-grouping behavior (Welch’s t-test, t = -2.93, p=0.009, Fig. 1F-H). Similarly, pups exposed to mothers spent a higher fraction of the test in the same quadrant (Mann-Whitney test, U = 96, p = 0.004, Fig. 1F).

3.2 DAPI Results

Analysis of DAPI staining indicated a small but significant increase in the number of cells in the AP axis, with caudal regions having greater number of cells ($\beta = 33.893$, $t(91) = 27.288$, p<0.001).

3.3 Analysis of mPOA→VTA projecting neurons

To map the anteroposterior distribution of mPOA→VTA neurons activated during maternal behavior, virgin and maternal female rats were first injected with the retrograde tracer Fluorogold (FG) in the VTA and exposed 6 days later to 3 pups for 5 minutes.
3.3.1 Distribution of mPOA → VTA neurons

Consistent with previously reported data, the number of neurons in the mPOA projecting to VTA was abundant (Mahler & Aston-Jones, 2012), with the greatest number found within the middle portion of the mPOA (at ∼ 0.48 mm caudal to Bregma), especially in the central nucleus of the medial preoptic nucleus and neighboring regions (Figure 2A,D). Although there was no difference in the number of FG+ cells between virgin and postpartum females (mothers: 1940 ± 273; virgins: 1808 ± 374), a significant main effect was found in the distribution of mPOA→VTA projections along the anterior-posterior axis of the mPOA ($\chi^2(5, N = 86) = 35.434, p<0.001$), with sections at ∼ 0.48 mm caudal to Bregma containing the highest numbers of VTA projecting neurons. No signs of gliosis in the mPOA due to FG toxicity were found.

3.3.2 c-Fos activation in mPOA→VTA neurons

We found no significant differences in the number of mPOA-c-Fos neurons (2209 ± 262 in mothers versus 2736 ± 393 in virgins) or their anteroposterior distribution between mothers and virgin females ($\chi^2(1, N = 86) = 1.667, p=0.197$).

In both groups, a significant number of mPOA neurons projecting to VTA was activated in the presence of pups (c-Fos+/FG+, 231/783, 29.5% in
postpartum females and 265/693, 38.2% virgin). However, there was no significant difference in the number of FG+ mPOA cells that also expressed c-Fos between virgin and postpartum females ($\chi^2(1, N = 86) = 0.344, p=0.557$). Correlation analysis indicated that c-Fos activation of mPOA→VTA was unrelated to behavior ($r(10) = 0.151, p=0.64$).

3.4 Esr1 expression in mPOA-c-Fos→VTA

Recent studies have shown a critical role of mPOA$^{Esr1} \rightarrow$VTA neurons in mice retrieval behavior (Fang et al., 2018; Wei et al., 2018). To assess whether maternally relevant mPOA→VTA neurons in the rat (i.e., FG+/c-Fos+ cells) are also responsive to estrogens, we also stained for Esr1 in the same tissue sections (Figure 2B,C).

The distribution of mPOA neurons expressing Esr1 varied along the anterior-posterior axis ($\chi^2(5, N = 86) = 146.879, p<0.001$), with the highest number of mPOA$^{Esr1}$ neurons located between Bregma level -0.48 to -0.72 mm (Figure 2D, Tukey paired multiple comparisons, -0.48 and -0.72 individually against all other sections, p <0.05). No group differences were found in the total number of mPOA$^{Esr1}$ neurons (mothers: 6417 ± 482; virgins: 6458 ± 453) or when analyzing by section ($\chi^2(1, N = 86) =0.157, p=0.692$). Examination of the number of cells double-labeled with c-Fos revealed that Esr1 is expressed in approximately half of mPOA$^{c-Fos}$ neurons. The dis-
tribution of these estrogen sensitive maternally relevant neurons was also highest between Bregma level -0.48 to -0.72 mm, with up to 75% of c-Fos+ cells co-expressing Esr1 (Figure 2E, main anteroposterior effect: $\chi^2(5, N = 86) = 126.903, p<0.001$; Tukey paired multiple comparisons: -0.48 and -0.72 against all other sections, $p<0.05$). In addition, the majority of c-Fos and Esr1 expressing mPOAVTA projecting cells was also found at 0.48 mm caudal from Bregma ($\chi^2(5, N = 86) = 12.818, p=0.025$; Tukey paired multiple comparisons: -0.48 vs all $p<0.05$, Figure 2E). No significant differences were found between virgin and postpartum groups in the number of double-, or triple-labeled cells for Esr1 ($\chi^2(1, N = 86) = 0.532, p=0.466$; Triple: $\chi^2(1, N = 86) = 0.128, p=0.720$).

3.5 GAD67 expression in mPOA $\rightarrow$ VTA neurons

Given that a significant proportion of neurons in the mPOA synthesize the inhibitory neurotransmitter GABA, it is likely that many mPOA$\rightarrow$ VTA neurons are GABAergic. We examined this possibility by performing antibody staining against GAD67 in alternate sections of the mPOA. GAD67 expression was detected in $\sim 50.3 \pm 4.7\%$ of the mPOA$\rightarrow$VTA cells.
Figure 1: Behavioral differences between mothers and virgins. A) Schematic of the test arena and derived variables. Three pups (p1, p2, p3) were individually positioned in three corners of the arena away from the female. B) Representative 2D density maps showing the cumulative position of a mother and a virgin interacting with pups during the behavioral test (warmer colors represent greater time). C) Temporal distribution of the behaviors displayed by mothers and virgin females during the test. Each row represents one animal. Maternal behaviors (i.e., retrieving, hover-over, and nesting) were pooled together (red). D) Time spent by mothers (white) and virgins (black) on pup-directed (retrieving, hover-over, and nesting) and other behaviors. E) Fraction of the test in which the female is in close proximity to pups (within pink region in A, see methods). F) Fraction of the test in which at least two pups were in the same quadrant. Insets represent examples of pups located in the same quadrant (top) or scattered on the arena (bottom). G) Perimeter of the triangle formed by the pups as a function of time during the test, in the presence of a mother (white) or a virgin female (black). Individual points represent the group mean and shaded area the group mean±SEM interval. H) Difference between the perimeters of the triangles formed by the pups in the last and first frame of the test. Individual points in scatter plots represent each animal, and horizontal lines indicate group medians, unless otherwise noted. *Significant difference at p<0.05.
Figure 2: Maternally relevant mPOA→VTA projecting neurons. A) Experimental design with FG injection in the VTA and qualitative scale of the FG signal distribution in mPOA. Numbers represent mm from Bregma. B) Euler diagram showing the mean distribution of cells in the mPOA of mothers (white) and virgins (gray). Cell numbers were normalized to the triple colocalization value for each group. The formulas show the calculation for the colocalization with FG and/or Esr1s of maternally relevant cells as shown in E. C) Representative immunostaining of mPOA cells that are FG-labelled (retrograde labeling from VTA) shown in cyan, Esr1 immunostaining signal shown in red, and c-Fos immunostaining signal (i.e., cells activated by the experience of interacting with pups) shown in white.
Maternally relevant mPOA→VTA projecting neurons, continued. The hollow arrow with solid border shows double labeling of mPOA Esr1+ and FG+ cells, the hollow arrow with dashed border indicates colocalization of mPOA c-Fos+ and FG+ cells, and the white filled arrow shows triple colocalization of mPOA FG labeled, Esr1+, and c-Fos+ cells. Rightmost panels are digitally augmented regions around each arrow. **D)** Number of cells for each channel along the mPOA anteroposterior axis. **E)** Double and triple labeling of maternally relevant neurons as calculated in **B** along the mPOA anteroposterior axis. In both **D)** and **E)**, individual points represent each animal, lines represent the predicted values, and the shaded areas outline the 95% confidence intervals of each locally weighted scatterplot smoothing (loess) fit. Different letters indicate significant differences between groups detected by post hoc Tukey’s HSD comparisons. All microscopy images shown are maximal intensity projections of a 20X z-stack.

Figure 3: Representative immunostaining of GAD67. mPOA cells that are FG-labelled (retrograde labeling from VTA) shown in cyan, and GAD67 immunostaining signal shown in magenta. White arrow shows double-labelled neurons. Maximal instensity projection of 60X z-stack. Scale bar represents 50 µm.
Maternal behaviors can be separated into those behaviors in which the mother is actively performing highly motivated, pup-directed, caregiving behaviors (e.g., retrieving, licking, and nest building) and those in which the mother remains quiescent (e.g., nursing). Because previous evidence has shown involvement of the mPOA→VTA in active, but not passive maternal behaviors (Fang et al., 2018; Kohl et al., 2018; Numan & Insel, 2003; Seip & Morrell, 2009; Wei et al., 2018), we employed a 5 min behavioral task to capture initial female-pup interaction, which in mothers would likely include retrieving and grouping the pups in the nest, licking them and nest building. Indeed, during the test, mothers readily performed active caregiving behaviors, whereas virgins mostly avoided pups (Fig. 1). Albeit short, our behavioral task proved to be sufficient to induce c-Fos expression in the mPOA following pup exposure.

We successfully implemented a semi-automatic method using the open-source software Bonsai to track interactions between an adult female and three pups. This method reliably detected the position of freely moving animals in real time, and could be easily scaled up to track a greater number of animals, using commercially available hardware and open-source software (Lopes et al., 2015).
Although virgin and postpartum groups displayed profound behavioral differences during the test, the number and molecular identity of the mPOA neurons that expressed c-Fos following exposure to pups was not different between groups. This finding may appear to contrast previously reported data showing that pup stimuli activates higher c-Fos expression in the mPOA of rats who display maternal behavior than in nonmaternal virgin rats who avoid the pups (Kalinichev, Rosenblatt, Nakabeppu, & Morrell, 2000; Numan & Numan, 1995; Sheehan, Cirrito, Numan, & Numan, 2000), and that c-Fos expression in the mPOA is closely tied to the performance of active components of maternal behavior (Lonstein, Simmons, Swann, & Stern, 1998; Numan & Numan, 1995). However, there are stark differences with the experimental protocols utilized by these prior studies, including longer mother-litter separation periods and later reunion with pups for at least 2 h, allowing expression of the full repertoire of maternal behaviors, that may explain the lack of difference in c-Fos expression between our groups (Kalinichev et al., 2000; Numan, Numan, Marzella, & Palumbo, 1998; Sheehan et al., 2000). In addition, a detailed anteroposterior and mPOA subregion analysis in mice suggests that the dorsomedial mPOA is the only subregion that presents pup-induced c-Fos differences between virgins and mothers following a 30 min social interaction with pups (Tsuneoka et al., 2013). Our anteroposterior analysis was restricted to the median preoptic nucleus and central regions of the mPOA, and consistent
with Tsuneoka et al. (2013)’s findings did not reveal pup-induced c-Fos differences between virgin and postpartum rats. A more detailed subregion analysis of our mPOA tissue samples may reveal differences between groups.

We noticed high variability in the number of mPOA c-Fos cells in the virgin group, which could be due to the fact that females in this group might have been at different stages of their estrous cycle. In this study, we did not monitor the virgins estrous cycle, and thus, it is possible that the lack of difference between groups was confounded by this. In this sense, the gene expression, protein levels, and distribution pattern of Esr1 in the hypothalamus and mPOA has been shown to change with fluctuations in circulating steroid hormone levels (Liu & Shi, 2015; Vastagh & Liposits, 2017).

Using the retrograde tracer FG, we were able to map the distribution of mPOA neurons projecting to the VTA (Figure 2A). We noticed a high-density region around the central nucleus of the mPOA, with highly ramified, intensely labeled neurons. The number and anteroposterior distribution of FG+ neurons within the mPOA was similar between groups. This suggests that mPOA→VTA projections are already present in virgins, likely playing a role in motivated social behaviors (McHenry et al., 2017). Consistent with previous reports, we found that Esr1 is expressed in
∼60% of mPOA cells that are retrogradely labeled from VTA (Fang et al., 2018). Our analysis revealed that only 11% of these mPOA$^{\text{Esr1}}$→VTA also express c-Fos. This mPOA population likely plays a role in orchestrating motivated responses to pups. Future studies employing molecular strategies will evaluate whether these mPOA cells belong to the same neuronal population/cluster in both groups, but mediate distinct forms of motivated responses to pups, or belong to different neuronal populations in mothers and virgins.

Close to one third of neurons in the mPOA express Esr1 (Fang et al., 2018), and mPOA subregions are highly diverse in terms of the molecular markers that define clusters of neurons (Tsuneoka et al., 2013, 2017). Thus, one possibility could be that pup-induced c-Fos expression in the mPOA did not occur in the same population of Esr1+ neurons in mothers and virgin females. A recent neuropeptide characterization of mPOA neurons revealed that Esr1 partially overlaps with several neuropeptides, including neurotensin and galanin (Tsuneoka et al. 2017). Moreover, a recent study from the Dulac group provides molecular evidence indicating that different subpopulations of mPOA$^{\text{gal}}$ neurons express c-Fos in virgin and postpartum females after interaction with pups. Alternatively, pup-induced c-Fos expression occurred in the same neuronal population within the mPOA in both groups. In support for this possibility, mPOA$^{\text{Esr1}}$ neurons have been shown to increase their activity during approaching, sniffing and re-
trieving of pups in spontaneously retrieving virgin female mice (Fang et al., 2018). Moreover, a recent experiment using a catFISH strategy showed that mPOA$^{\text{Esr1}}$ neurons activated in response to a female or pups are largely distinct (i.e., stimuli-specific) (Wei et al., 2018). Also, maternally-relevant mPOA$^{\text{gal}}$ neurons appear to contribute to behavior in a segregated fashion, with each pool largely projecting to a specific area and directing a specific module of maternal behavior (Kohl et al., 2018). Thus, if pup-induced c-Fos expression occurred in the same neuronal population in our study, the observed behavioral differences could have arisen due to different circuit properties of these neurons. In this sense, estradiol primed mPOA→VTA neurons have enhanced calcium activity and change their firing pattern, increasing the number of evoked action potentials (McHenry et al., 2017). Increased sensitivity to appetitive cues (e.g., pups) could be due to an A-type K+ channel conductance that allows rapid membrane repolarization and increased firing rate following estrogen action on the mPOA (McHenry et al., 2017). Interestingly, motherhood induced regulation of potassium channels has been reported (Driessen et al., 2014).

Finally, the use of c-Fos as a marker of neuronal activation in our experimental design may have limited the detection of other maternally-relevant neuronal populations by excluding other critical signaling pathways involved in maternal behavior, such as PKA, FosB, Egr1, pERK, pSTAT (Brown et al., 2017; Numan & Insel, 2003; Sheehan et al., 2000; Stack & Numan,
Moreover, the use of immediate early genes to detect subsets of neurons engaged during maternal interactions is sensitive to the duration of mother-pup interactions. Specifically, 2 h continuous interaction with pups produces a 3-fold increase in the number of c-Fos and Egr1 expressing mPOA neurons, with c-Fos remaining elevated as long as the female rats are with pups and engaging in maternal behavior, and FosB showing at increase after 4-6 h long interactions (Numan et al., 1998; Stack & Numan, 2000). It is possible that the temporal course of c-Fos activation within the mPOA following interaction with pups is different between mothers and virgins. Such temporal dynamics of transcription factor activation may underlie plasticity events that reflect and might affect how the network will respond to future encounters with pups.

Consistent with recent studies that posit a key role for the mPOA$^{\text{GABA}} \rightarrow$ VTA$^{\text{GABA}}$ pathway in maternal behaviors (Fang et al., 2018; McHenry et al., 2017), we found that $\sim 50\%$ of mPOA$\rightarrow$ VTA projections express GAD67, suggesting an inhibitory projection. Activity of these mPOA$^{\text{GABA}}$ neurons may result in the disinhibition of VTA$^{\text{TH}}$ neurons via inhibition of local VTA$^{\text{GABA}}$ neurons, with the subsequent release of DA in the nucleus accumbens (Tan et al., 2012). Our approach using the retrograde tracer FG does not allow us to determine the identity of the VTA neurons receiving projections from the mPOA. It is noteworthy that a significant number of mPOA$\rightarrow$ VTA neurons are not GABAergic, and may be glutamatergic neu-
rons (Geisler, Derst, Veh, & Zahm, 2007). As glutamate has been shown to regulate activation of DAergic neurons in VTA (Sesack & Grace, 2010), mPOA$^{\text{Glu}} \rightarrow \text{VTA}$ might also be important for maternal motivation. Because mPOA neurons project to VTA$^{\text{TH}}$, VTA$^{\text{GABA}}$, and VTA$^{\text{glut}}$ neurons (Beier et al., 2015; Faget et al., 2016; Geisler et al., 2007; Tobiansky et al., 2013; Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012), future studies using rabies-assisted trans-synaptic labeling will help dissect the contributions of discrete pup-responsive mPOA projections to distinct VTA subpopulations in either mother and virgin groups. Future studies using a greater array of candidate genes (see Tsuneoka et al., 2017), ex vivo and awake behaving recordings paired with functional manipulations can elucidate whether differences in network functions between virgin and postpartum female rats underlie their unique responses to pups.
BIBLIOGRAPHY


