THE AFFERENT CONNECTIVITY OF THE MEDIAL PREOPTIC AREA IN THE CALIFORNIA MOUSE

By

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Abstract

The medial preoptic area of the hypothalamus is known to play an important role in the onset and maintenance of maternal and paternal behavior in rodents; however, little is known about the specific neural circuitry of which this area is a component. Here we report the first study of the afferent connectivity of the medial preoptic area in the California mouse, Peromyscus californicus. This species is a valuable model for studying parental behavior because it is monogamous and exhibits paternal care, which is unusual among mammals. Fifty uL of cholera toxin B unit (a retrograde tracer) conjugated to Alexa 647 was injected into the preoptic area. The entire brain was sectioned into 40 um slices, and every other slice was stained with SYTOX Green (a nucleic acid stain) and imaged at 4x using confocal laser scanning microscopy. Our analysis will allow us to identify which brain areas project to the medial preoptic area, enabling us to better understand the function of this brain region. Ultimately, this work will contribute to our understanding of the neural mechanisms underlying paternal care in males.

Acknowledgments

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Introduction

Parental care plays an important role in the survival of offspring in mammalian species. All mammals exhibit maternal care, which is care provided by the mothers, but only a subset of these taxa, 5-6%, also exhibit paternal care, which is care provided by the fathers (Kleiman & Malcolm, 1981). It has been proposed that there is limited paternal care since males generally lose reproductive success when they invest time and energy in rearing their young instead of mating with other females (Kurland & Gaulin, 1984).

The California mouse, Peromyscus californicus, is a valuable rodent model for studying the neural circuitry underlying paternal care. The species is both monogamous and biparental, meaning both parents provide care for their offspring. Paternal behavior may include, but is not limited to, nest building, as well as licking, retrieving and huddling the pups (Gubernick & Alberts, 1987). Paternal behavior in P. californicus improves the survival of the offspring. In one study, for example, some females were able to raise their pups without the father, but paternal care significantly enhanced the offspring's survival (Cantoni & Brown, 1997; Gubernick & Teferi, 2000; Wright & Brown, 2002). These findings demonstrate the importance of paternal care. This type of care has also been shown to influence hormone levels in adolescent California mice by increasing their plasma testosterone levels (Becker et al., 2010). In addition, studies in mandarin voles have shown that father-deprived pups of both sexes showed higher levels of anxiety in an open-field test and engaged in less social interaction compared to pups that were raised by both parents (Jia et al., 2009).

Behavioral changes occur in male P. californicus when they become fathers. When exposed to a pup before fatherhood, a virgin male may attack and even kill the pup. In a study of P. californicus, only 19% of virgin males acted paternally when exposed to 1- to 3-day-old

unfamiliar pups, in comparison to 80% of males who had been fathers for a day (Gubernick & Nelson, 1989). Other studies done in P. californicus showed that 65-75% of males attacked or ignored unfamiliar pups and did not show paternal behavior prior to the birth of their own pups (Gubernick, Schneider & Jeannotte,1993). Males that did not display paternal behavior prior to the birth of their offspring became paternal after the birth, but the study reported that the presence of the mother was necessary to maintain this paternal behavior.

The same stimuli in the environment can have different effects on sexually naive male mice vs males that are fathers. It has been shown in house mice (Mus) that chemical cues from the pups detected by the vomeronasal organ activate the accessory olfactory pathway in virgin male C57BL/6 mice, but not in fathers (Tachikawa, Yoshihara & Kuroda, 2013). This implies that the downregulation of the accessory olfactory pathway might play a role in the transition from attack to paternal behavior, which is mediated by certain brain regions in the hypothalamus.

One of the brain regions that has been strongly implicated in maternal care in rodents is the medial preoptic area of the hypothalamus (MPOA). The preoptic area is located in the rostral part of the hypothalamus and is highly complex, with many nuclei that have different functions. In rats (Rattus norvegicus) and mice, indicators of neuronal activity, such as expression of the immediate-early gene Fos or uptake of radioactive glucose, are elevated in the MPOA after females are exposed to pups and/or engage in maternal care (Komisaruk et al., 2000; Lonstein & De Vries, 2000; Okabe et al., 2013). MPOA lesions reduce components of maternal care in female rats (Fleming et al., 1983; Franz et al., 1986; Lee et al., 1999; Noonan & Kristal, 1979; Numan & Callahan, 1980; Numan et al., 1988; Olazábal et al., 2002; Stack et al., 2002; Terkel et al., 1979), as does infusion of GABA agonists into the MPOA (Arrati et al., 2006). Kindling of the MPOA increases female rats' preference for pup-associated environments in conditioned

place-preference paradigms (Morgan et al., 1997; Morgan et al., 1999), while MPOA lesions reduce the number of bar presses rat dams perform to gain access to pups (Lee et al., 1999). MPOA lesions also disrupt maternal behavior in other rodent species, including house mice (Tsuneoka et al., 2013), Siberian hamsters (Mesocricetus auratus; Miceli & Malsbury, 1982), and California mice (Lee & Brown, 2002, 2007).

As in maternal care, the MPOA has been demonstrated to be critical for paternal care in a number of rodent species. Indicators of neural activity increase in the MPOA following exposure to pups in male house mice (Tsuneoka et al., 2015), prairie voles (Microtus ochrogaster) (Kirkpatrick et al., 1994), North American deermice (Peromyscus maniculatus; Lambert et al., 2013), and California mice (De Jong et al., 2009; Horrell et al., 2017; Lambert et al., 2013). MPOA lesions disrupt paternal care in male California mice, rats, and mice (Lee & Brown, 2002, 2007; Rosenblatt et al., 1996; Sturgis & Bridges, 1997; Tsuneoka et al., 2015). Optogenetic activation of the MPOA decreases infanticide in male mice (Tsuneoka et al., 2015), and specific neurons in the MPOA that express galanin are activated during male and female parenting in mice (Wu, Autry, Bergan, Watabe-Uchida, & Dulac, 2014). Genetic ablation of these neurons in the MPOA impairs parental behavior in males and females.

It is known that the medial preoptic area of the hypothalamus plays an important role in the onset and maintenance of maternal and paternal behavior in rodents, but limited data is available on the afferent circuitry of this region, especially in the context of parental care (Chiba & Murata 1985; Cservenák et al., 2017; Dobolyi et al., 2014; Simerly & Swanson 1986). Studies in rats have illustrated the inputs to a part of the MPOA, the medial preoptic nucleus. One of the observations made was that function of the medial preoptic nucleus might be influenced by the medial nucleus of the amygdala and accessory olfactory bulb through relay neurons in the bed

nucleus of the stria teminalis (Simerly & Swanson, 1986). Another study using a wheat germ agglutinin conjugated with horseradish peroxidase (WGA-HRP), which is used in neuronal transport studies, allowed for the illustration of the afferent and efferent connections of the MPOA. This study was able to confirm previous results from other experiments using the same marker, such as projections to the MPOA from the lateral septal nucleus, medial amygdaloid nucleus and subiculum (Chiba & Murata, 1985).

In the present study, for the first time, the afferent connectivity of the medial preoptic area in P. californicus was investigated. Results will allow us to better understand the function of this brain region and the various inputs it receives from other brain regions. The MPOA indirectly receives various sensory cues that have been shown to affect paternal behavior. Chemosensory cues such as olfaction play an important role in recognizing offspring in many amphibians, fish, birds and insects (Rosenblatt, 1967; Mennella & Moltz, 1988; Lévy, Keller & Poindron, 2004; Schulte et al., 2011). In some mammals the vomeronasal pathway and the olfactory system can inhibit paternal behavior in virgin males, but olfactory cues stimulate parental behavior in parents (Lévy, Keller & Poindron, 2004). In view of these findings, we predicted that some of the inputs the MPOA receives come from the amygdala, hypothalamus, thalamus and septum, since these brain regions all indirectly respond to these cues. Also, previous studies in rats, Syrian hamsters and prairie voles have shown projections to the MPOA from these brain regions (Chiba & Murata,1985; Kirkpatrick, Kim, & Insel, 1994; Wang & Swann, 2006).

Methods

Animal

The subject was an adult male California mouse that was born and raised in our breeding colony in the Spieth Vivarium at the University of California, Riverside (UCR) and descended from California mice purchased as adults from the Peromyscus Genetic Stock Center (University of South Carolina, Columbia, SC, USA). Animals in our colony are housed in 44 × 24 × 20 cm polycarbonate cages containing aspen shavings and cotton wool for nesting material, with food (Purina Rodent Chow 5001) and water available ad libitum (Perea-Rodriguez et al., 2018; Zhao et al., 2018). Colony rooms are kept on a 14:10 light: dark cycle (lights on from 0500 h to 1900 h), with temperature and humidity at 21°C and about 55% respectively. At 27 days of age, prior to the birth of the next litter of siblings, the animal was removed from the parents' cage and housed in a group of four same-sex, age-matched, unrelated juveniles. All procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the UCR Institutional Animal Care and Use Committee. UCR is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Stereotaxic Surgery and Tracer Injection

At 186 days of age, the adult male mouse was anesthetized with isoflurane and placed in a rodent stereotaxic frame with continuous isoflurane delivery. Bregma and Lambda were positioned into the same horizontal plane, and a small craniotomy was performed directly over the MPOA using the following coordinates: Anterior-Posterior = 0.06 mm rostral of bregma, Medial-Lateral = 0.05 mm from the midline, Dorsal-Ventral = 0.71 mm past the skull. A 30-gauge, 1 uL Neuros Hamilton syringe was inserted into the preoptic area, and 50 uL of a 2% solution of cholera toxin B unit (a retrograde tracer) conjugated to Alexa 647 was injected into the preoptic area over 5 min. Ten minutes after the termination of injection, the syringe was removed. The wound was

closed with tissue glue. Carprofen (0.1 mL of a 1:50 dilution) was injected subcutaneously before removal of the mouse from isoflurane, and the subject was returned to a cage with the age-matched conspecific virgin male from its home cage as prolonged periods of isolation are stressful for California mice (Chauke et al., 2012). The group size was reduced from 4 to 2 after surgery.

Retrograde transport typically requires seven days after injection of tracer, the male was heavily anesthetized with a 0.2 mL intraperitoneal injection of sodium pentobarbital and perfused transcardially with 0.1 M phosphate buffered saline (PBS) and 0.4% paraformaldehyde (PFA) (De Jong et al., 2009; Horrell et al., 2017). It was necessary to wait a week for retrograde transport because this seems to be the standard length of time as seen in other studies (Wang & Swann, 2006). The brain was removed, post-fixed in paraformaldehyde overnight, and cryoprotected in 30% sucrose prior to being sectioned into 40 um slides collected in 6 series using a cryostat. Alternate slices were permeabilized with 0.3% Triton X and stained with SYTOX Green (1: 30,000, ThermoFisher Scientific, Chino, CA, USA) before being dehydrated with alcohol, cleared with xylene and permeabilized on gel-alum coated slides. Slices were imaged using laser scanning confocal microscopy at 4x, and a whole-slice composite was created in Microsoft PowerPoint. Because there is no brain atlas for California mice, *The Mouse Brain in Stereotaxic Coordinates* (Paxinos and Franklin, 2013) for *Mus musculus* was used to locate brain regions, as done previously in our lab (De Jong et al., 2009; Horrell et al., 2017). Density of staining in various brain regions was determined by visual inspection. The cerebellum was not included in the analysis, as previous reports indicate no connections between the cerebellum and the MPOA (Chiba & Murata, 1985; Simerly & Swanson, 1986; Wang & Swann, 2006).

Results

Injection Sites

Four brain slices were found to have a vivid illustration of the injection site, with the retrograde tracer clearly seen in the medial preoptic area as a red stain (Fig. 1). Thirty-one brain slice composites were analyzed in addition to the four composites clearly showing the injection site.

Afferent connections to the medical preoptic area in *Peromyscus californicus*

Fig. 2 shows composite brain slices with dense afferent projections to the MPOA from certain regions within the adult male mouse brain. The figure shows only the slices with the highest density of cell bodies labeled with retrograde tracer, which are the septum, thalamus and amygdala (i.e., CTB neurons with four plus signs as depicted in Table 1). Additional projections were observed from various regions in the brain, specifically the hypothalamus, epithalamus, and cerebrum, but these areas had lower densities of retrogradely labeled cells. In total, there were 6 regions that showed projections to the medial preoptic area.

Septum

The septum was one of the three brain regions that had a high density of projections. Three areas within the septum had a high density of retrogradely labeled cells, with the highest in the ventral part of the lateral septal nucleus. Fewer labeled cells were found in the intermediate part of the lateral septal nucleus, and the least were found in the dorsal part of the lateral septal nucleus.

Thalamus

Of all brain areas, the thalamus had the most nuclei with densely packed retrogradely labeled cells. Density was highest in the central and medial parts of the mediodorsal thalamic nucleus, the parafascicular thalamic nucleus, the posteromedian thalamic nucleus, the paraventricular thalamic nucleus and the anterior part of the paraventricular thalamic nucleus. The second-most-labeled areas were the anteromedial thalamic nucleus and the ventral part of the anteromedial thalamic nucleus. The least dense area within the thalamus was the intermediodorsal thalamic nucleus.

Amygdala

The amygdala was one of the three brain regions that had a high density of projections. The anterolateral part of the amygdalohippocampal area and the posterior part of the basolateral amygdaloid nucleus were densely packed with retrogradely labeled cells. Density was somewhat lower in the ventral part of the basolateral amygdaloid nucleus and the posterior part of the basomedial amygdaloid nucleus, followed by the posterolateral cortical amygdaloid area and the posteromedial cortical amygdaloid area. Density of labeling was lowest in the rostral amygdalopiriform area.

Hypothalamus

The hypothalamus, in comparison to the septum, thalamus, and amygdala, had a lower density of projections. Within the hypothalamus, four areas contained retrogradely labeled cells. The most densely labeled region was the posterior hypothalamic nucleus. The dorsal hypothalamic area

and the dorsal posterior hypothalamus area had the next densest region of labeled cells, and the lateral hypothalamic area had the lowest density.

Epithalamus

Overall, the epithalamus had a lower density of projections than did the septum, thalamus and amygdala. The retroflexus was the only area within the epithalamus that consistently had retrogradely labeled cells.

Cerebrum

The cerebrum had the lowest density of projections, of the brain regions that showed any retrograde labeling. The piriform cortex consistently showed retrogradely labeled cells within the cerebrum, while other parts of the cerebrum did not seem to express any labeled cells.

Abbreviations used in the figures

AM anteromedial thalamic nucleus

AMV anteromedial thalamic nucleus, ventral part

AHiAL amygdalohippocampal area, anterolateral part

BLP basolateral amygdaloid nucleus, posterior part

BLV basolateral amygdaloid nucleus, ventral part

BMP basomedial amygdaloid nucleus, posterior part

DA dorsal hypothalamic area

fr fasciculus retroflexus

IMD intermediodorsal thalamic nucleus

LH lateral hypothalamic area

LSD lateral septal nucleus, dorsal part

LSV lateral septal nucleus, ventral part

LSI lateral septal nucleus, intermediate part

MDC mediodorsal thalamic nucleus, central part

MDM mediodorsal thalamic nucleus, medial part

PaF parafascicular thalamic nucleus

PH posterior hypothalamic nucleus

PHD posterior hypothalamic area, dorsal part

Pir piriform cortex

PLCo posterolateral cortical amygdaloid area

PMCo posteromedial cortical amygdaloid area

PoMn posteromedian thalamic nucleus

PV paraventricular thalamic nucleus

PVA paraventricular thalamic nucleus, anterior part

PVP paraventricular thalamic nucleus, posterior part

RAPir rostral amygdalopiriform area

Location	*CTB-Neurons*
Septum	
-LSD	+
-LSV	++++
-LSI	++
Hypothalamus	
-DA	++
-LH	+
-PH	+++
-PHD	++
Amygdala	
-AHiAL	++++
-BLP	++++
-BLV	+++
-BMP	+++
-PLCo	++
-PMCo	++
-RAPir	+
Thalamus	
-AM	+++
-AMV	+++
-IMD	++
-MDC	++++
-MDM	++++
-PaF	++++
-PoMn	++++
-PV	++++
-PVA	++++
Epithalamus	
-fr	+++
Cerebrum	
-Pir	++

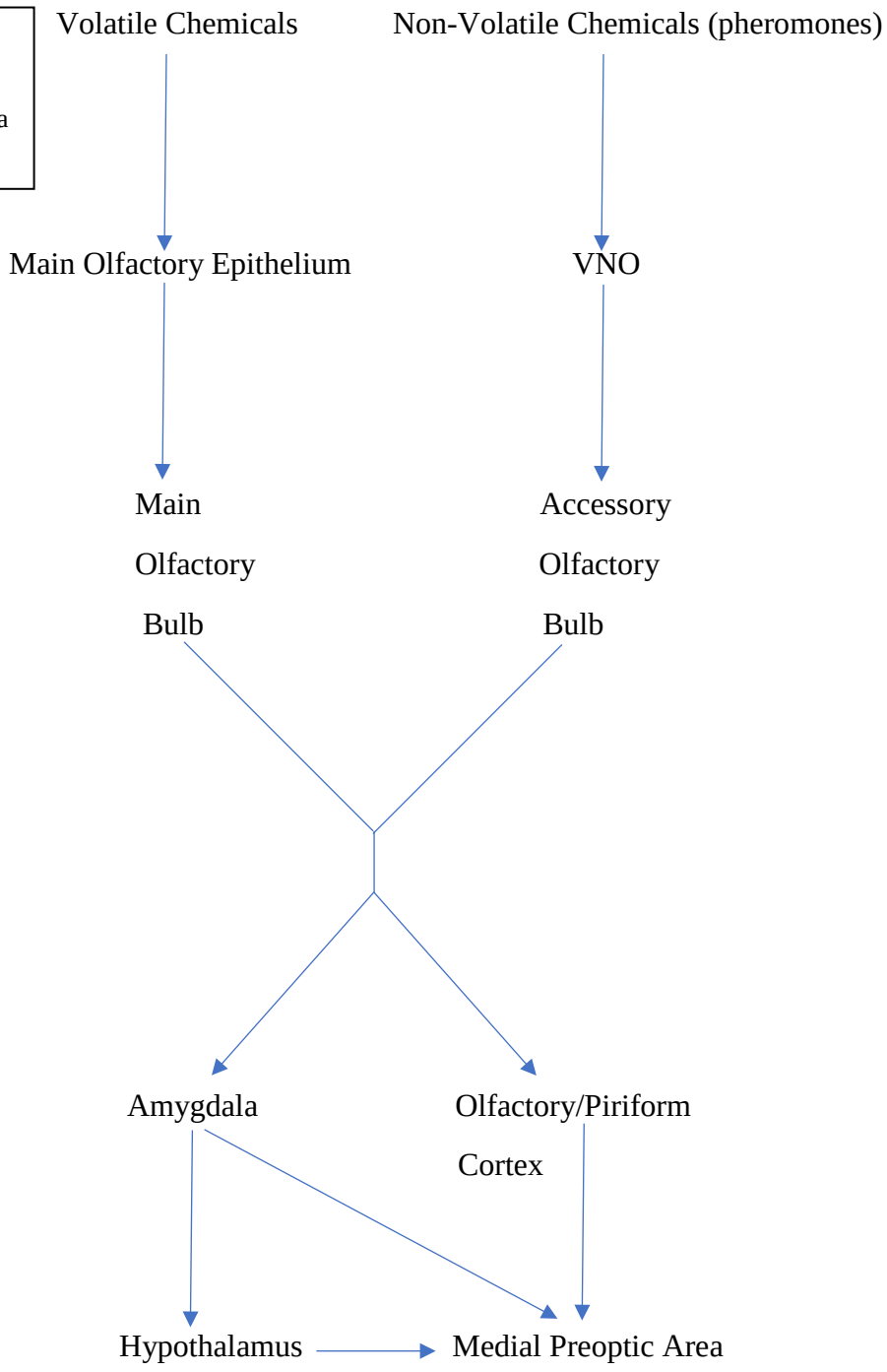
Table 1.

Density and distribution of CTB labeled neurons with retrograde tracer injections in medial preoptic area

-The more "+" signs, the higher the density of labeled cells

Table 2.

Vomeronasal organ (VNO) pathway to the medial preoptic area (Sokolowski & Corbin, 2012).



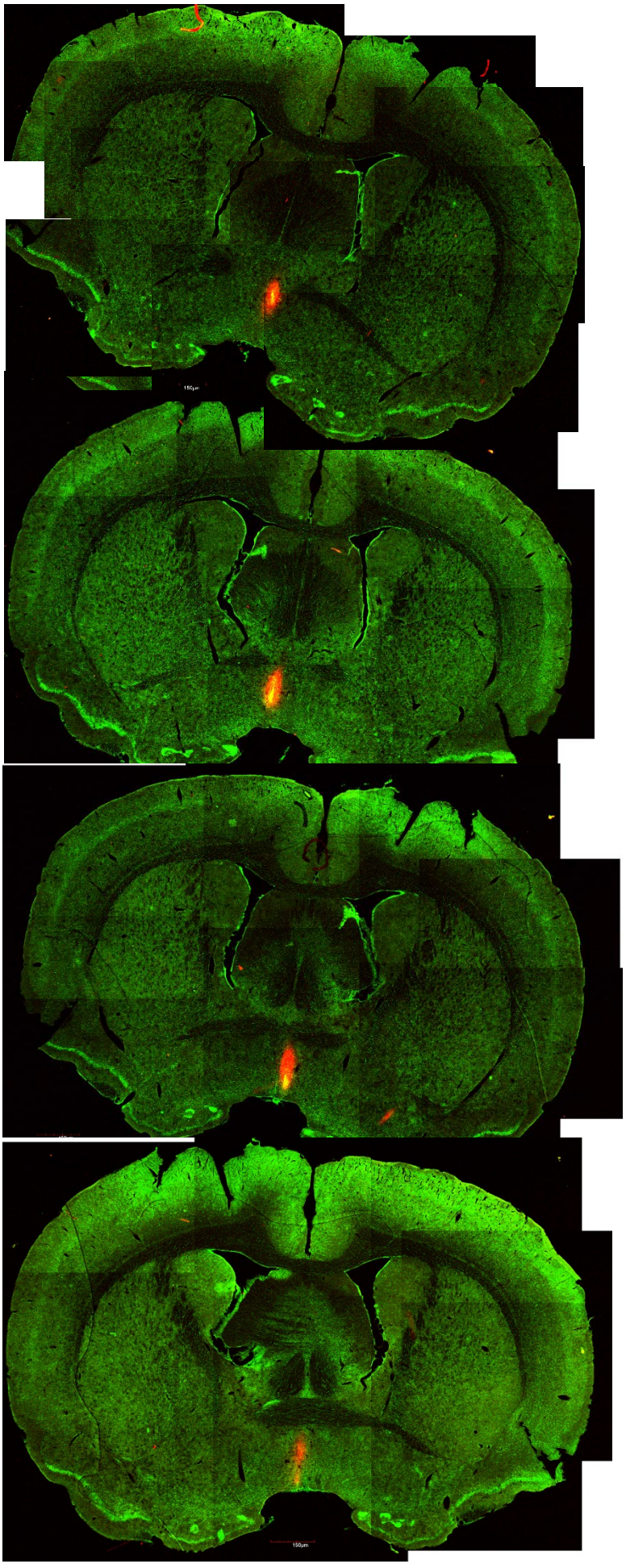
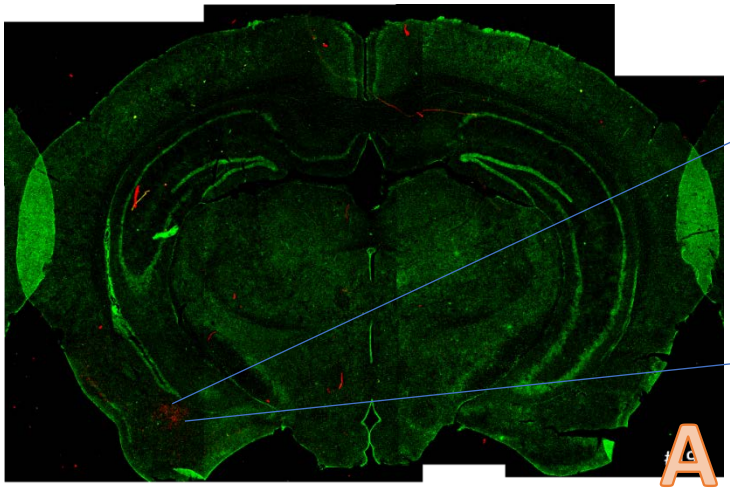


Figure 1.
Whole slice composites of injection site in four brain slices imaged using confocal microscopy 4x.



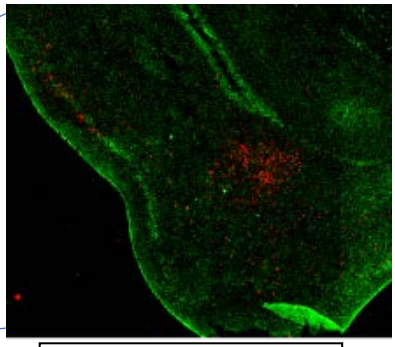
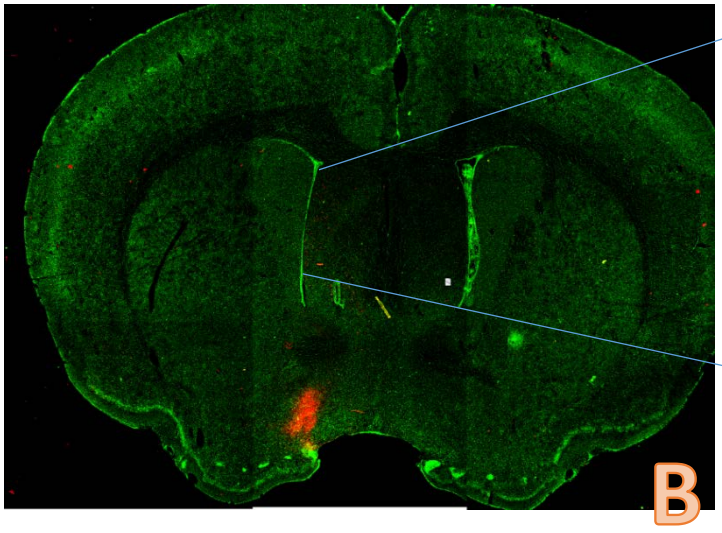
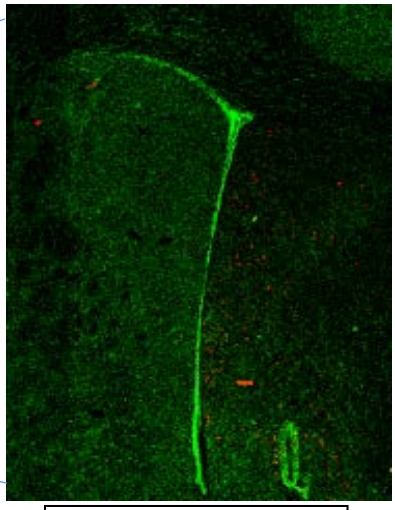


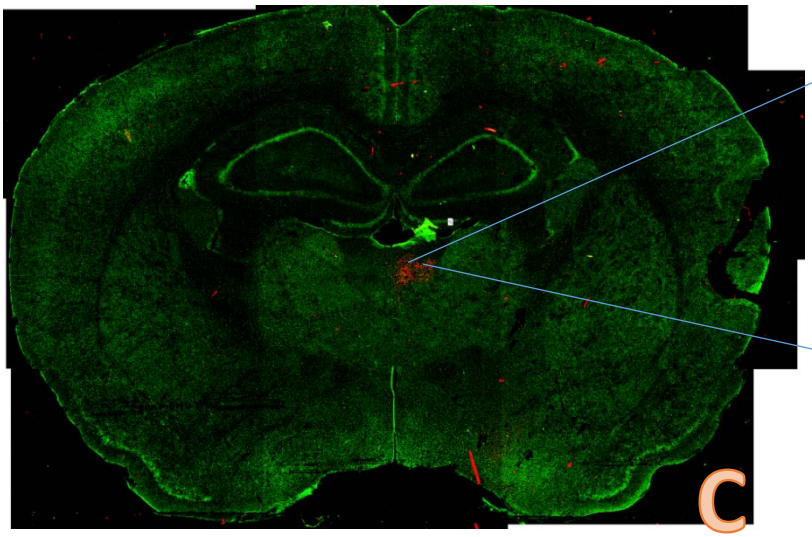
Figure 2.

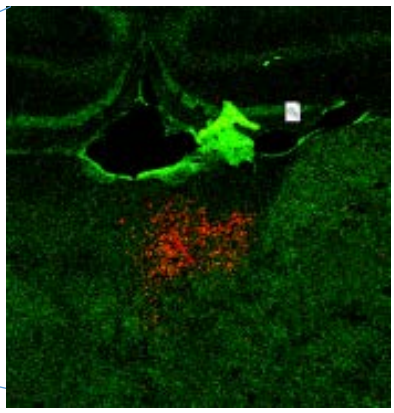
(A) Projections to MPOA from amygdala





(B) Projections to MPOA from septum





(C) Projections to MPOA from thalamus

Discussion

This study showed the afferent projections to the medial preoptic area (MPOA) from various brain regions in an adult male *Peromyscus californicus*, using a retrograde tracer, cholera toxin B unit, that labels only cell bodies and not fibers. The data confirmed findings from other rodent species that the MPOA receives input from many areas in the brain. Although the majority of the projections were found to originate in the septum, amygdala, and thalamus, there were projections from the hypothalamus, epithalamus and cerebrum as well. The results confirm previous findings in male Syrian hamsters that showed afferent projections to the medial preoptic nucleus (a part of the MPOA) from the septum, hypothalamus, amygdala, and thalamus (Wang & Swann, 2006). A study in rats found projections to the MPOA from specific regions within the septum and thalamus such as the lateral septal nucleus and parafascicular thalamic nucleus, which was confirmed by our results (Chiba & Murata, 1985). Another study in rats found similar results as ours, such as major inputs from many limbic regions, such as the amygdala and the ventral lateral septal nucleus (Simerly & Swanson, 1986). Our results did not seem to differ from other studies in any notable ways, but a study in Syrian hamsters claimed they did not see many nuclei in the thalamus projecting to the MPOA (Wang & Swann, 2006). In contrast, out of all the brain regions in our study that were found to project to the MPOA, the thalamus had the most nuclei with retrogradely labeled cells. Other studies in rats also did not find any projections from the piriform cortex and fasciculous retroflexus as we did (Chiba & Murata, 1985; Simerly & Swanson, 1986; Wang & Swann, 2006).

There is an important organ, the vomeronasal organ (VNO), that is located in the base of the nasal septum and is involved in the detection of non-volatile chemicals in rodents. Once the non-volatile chemicals enter the VNO, the inputs are sent to the accessory olfactory bulbs, which in

turn send projections directly or indirectly to brain structures such as olfactory/ piriform cortex and the amygdala (Sokolowski & Corbin, 2012). According to our results, both the amygdala and piriform cortex project to the MPOA. Ablation of the vomeronasal organ in virgin male mice abolished infanticidal responses and caused the mice to behave more parentally (Tachikawa, Yoshihara & Kuroda, 2013). The results suggest that in sexually naïve males, the downregulation of the VNO can eradicate pup directed aggression. A study in female rats infused a GABA antagonist into the accessory olfactory bulb and results showed increased maternal behavior in virgin rats (Carretero, Segovia, Gomez & Del Cerro, 2003). This suggests once again the importance of olfaction in parental behavior which is perhaps why we saw projections from the piriform cortex.

Our study also found the lateral septum projecting to the MPOA. The lateral septum has strong projections to the hypothalamic region of the brain, which is part of the limbic system. The lateral septum is known for modulating sociability (Talishinsky & Rosen, 2012). Perhaps the function of this brain region affects the parental behavior of the mouse since it sends inputs to the MPOA. Studies in other parts of the limbic system, such as the amygdala, have shown that lesions in the lateral amygdala reduced maternal behavior in rats (Lee, Clancy & Fleming, 2000). The results were surprising because the lateral amygdala had not been implicated with maternal behavior before. The findings suggest that communication between the amygdala and MPOA might be crucial for the rodent to express parental behavior. The importance of the amygdala has also been implicated in paternal care. A study in prairie voles showed that axon-sparing lesions of the medial nucleus of the amygdala decreased paternal behavior (Kirkpatrick, Kim, & Insel, 1994).

Since the thalamus is a motor and sensory relay system, perhaps projections from this brain region to the MPOA mediate parental behavior. Previous studies in virgin prairie voles have shown that pup exposure elevated Fos-ir in the nuclei of the thalamus, paraventricular nucleus and the nucleus reuniens (Kirkpatrick et al., 1994). These results suggest that the thalamus is active during pup exposure, helping confirm our findings that the thalamus interacts with the MPOA.

Our study also found projections from the epithalamus, specifically from the fasciculus retroflexus. This area is a primary output from the habenula, which is important in encoding the motivational value of stimuli. This suggests that the area might affect paternal behavior because it might affect the motivation of the mouse to engage with the pup.

One limitation of our study is the small sample size. Typically, at least 40 animals are used for a study like this (Chiba & Murata, 1985). Future research can increase sample size to gather more data. In addition, the findings of our study showed the afferent connectivity of the MPOA by using a retrograde tracer, but we did not look for the specific functional connectivity of the MPOA. To discern which connectivities are involved in which functions of the MPOA, retrograde tracing can be paired with markers of neuronal activity, such as c-fos. It would be interesting to see if the results between males and females and fathers and virgins would be different both with the c-fos and without.

In summary, this is the first description of the afferent projections to the MPOA in the California mouse, a monogamous, biparental species that is a valuable model for studies of parental behavior. Understanding the circuitry associated with parental care can help us better understand the function of this brain region and see what regions it interacts with in the California mouse brain.

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