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**Molecular Dissection of Neural Circuits Underlying
Parental Behavior in Mice**

A dissertation presented

by

Zheng Wu

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Neurobiology

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Molecular dissection of neural circuits underlying parental behavior in mice

Abstract

Mice display robust and stereotyped behaviors towards pups: virgin males typically attack pups, while virgin females and sexually experienced males display parental care. I show here that virgin males that are genetically impaired in vomeronasal sensing do not attack pups and are parental, suggesting a key role of the vomeronasal system in controlling male infanticide. In addition, we have identified putative vomeronasal receptors (or receptor groups) for the detection of pup odors, thus uncovering new tools for the molecular and genetic dissection of male infanticide. Further, we have uncovered galanin-expressing neurons in the medial preoptic area (MPOA) as key regulators of male and female parental behavior. Genetic ablation of MPOA galanin- neurons results in dramatic impairment of parental responses in both virgin females and sexually experienced males. In addition, optogenetic activation of these cells in virgin males suppresses infanticide and induces pup grooming. Thus, MPOA galanin-expressing neurons emerge as an essential node of regulation of innate behavior in the hypothalamus that orchestrates male and female parenting while opposing vomeronasal circuits underlying infanticide. Our results provide an entry point for the genetic and circuit-level dissection of mouse parental behavior and its modulation by social experience.

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Chapter I. Introduction

Social behavior

Understanding the nature of behavioral circuits is a major goal of neuroscience. Many key questions are still elusive and await elucidation: how is sensory information encoded by the brain in order to lead to specific behavioral responses, how does the physiological state of the animal modulate behavior, what defines and characterizes a behavioral neural circuit, and how learning and genetic predispositions interact in the development of behavior circuits and in the execution of behavior. One distinct class of behavior, social behavior, is of special interest and importance to neuroscientists. Social behavior refers to a repertoire of behaviors taking place between members of the same species, ranging from basic interactions between two individuals such as sexual and aggressive behavior, to behaviors in complex societies including social hierarchy, division of labor, and collective action. Different forms of social behavior are expressed in virtually all animals (except extremely rare cases of solitary animals with asexual reproduction, such as parthenogenesis in sharks (Chapman et al., 2007)), ensuring the reproductive success of the individuals and the continuation of the species. As Charles Darwin pointed out, “the struggle almost invariably will be most severe between the individuals of the same species, for they frequent the same districts, require the same food, and are exposed to the same dangers” (Darwin, 1964). In this severe competition for resources and mates, some of the most dramatic and complex forms of behavior have evolved in order to perpetuate the genes of individuals.

Although biologists have had a long interest in animal behavior, the study of animal behavior only emerged as a discrete biological science, namely ethology, since the work of Konrad Lorenz, Karl von Frisch and Nikolaas Tinbergen. Even though ethology involves the study of both individual and social behavior, much of the work of its founding members focused on innate social responses including imprinting, communication and supernormal stimuli. As Tinbergen proposed, animal behavior can be understood on four levels of inquiry: causation, ontogeny, survival value and evolution, which were named after him as “Tinbergen’s four questions” (Tinbergen, 1963). Based on a different perspective, these four questions can be categorized as the proximate causes (causation and ontogeny) and the ultimate causes (survival value and evolution).

Proximate causes include genetic, anatomical and physiological mechanisms that underlie the behavior (causation) and developmental changes in individuals which lead to its current form (ontogeny). For example, a mother house mouse displays maternal care towards its infants to ensure their survival. The adult neural and endocrine circuits through which maternal care is based, the facilitation by sensory cues from the pups and the hormonal changes through gestation, parturition and lactation address the causation of maternal behavior, whereas the ontogeny concerns the development and formation of circuits underlying maternal behavior from embryonic stage through the life span of individual female mouse.

In contrast, the ultimate questions explore the functional utility of the behavior (survival value) and its evolutionary history in a species over generations (evolution, or phylogeny). In the case of maternal behavior, the query of the ultimate causes

investigates the benefits and costs of maternal care, how maternal care for the young promotes the survival of an individual's offspring, how females compete and cooperate with males over parental care and how maternal behavior evolved over generations (Royle et al., 2012).

Research on the proximate causes of social behavior has been trying to address two general lines of key questions: the modulation of behavior by physiological state of the animal and the sensory control of the behavior. The line along the modulation of physiological state studies how hormones and experiences influence the physiological underpinning of animal behavior. In particular, the study of hormones has established the central role of sex hormones in reproductive social behavior in terms of masculinity and femininity (Neill and Knobil, 2006). It is generally accepted that perinatal exposure to high levels of estrogen, which is converted by an enzyme aromatase from testosterone, masculinizes the brain and specifies the neuronal circuit for male-typical behavior, whereas the lack of exposure leads to the development of a circuit responsible for female-typical behavior (Morris et al., 2004).

Recent advances have allowed us to visualize the distribution and projection of hormone receptors- and aromatase-expressing cells by genetically modifying their gene loci. This revealed extensive and novel sexual dimorphism of androgen receptor and aromatase-expressing cells (Cooke et al., 1998; Shah et al., 2004; Wu et al., 2009; Yang et al., 2013). For example, more aromatase-expressing cells are observed in the caudal hypothalamus in females compared with males, while an opposite expression bias towards male was found in the bed nucleus of stria terminalis and medial amygdala (Wu

et al., 2009). Further, androgen receptor knockout out mice were found to still exhibit masculine sexual and territorial displays, but with striking deficits in specific components of these behaviors. These results confirmed that estrogen converted from testosterone masculinizes the brain and behavior, and suggests that androgen controls the levels of male behavioral displays through androgen receptor (Juntti et al., 2010).

Although prenatal hormonal exposure primarily specifies the animal for male or female typical behavior, for a given individual, neural circuits for the behaviors of the opposite sex still exist and can be induced under certain circumstances. Females of some species can also exhibit male-like mounting behavior and conversely, some males are able to display female-typical sexual behavior (Beach, 1947; Vasey, 2002). Moreover, experiments using genetic manipulations showed that female mice with disrupted vomeronasal signaling display unique characteristics of male sexual behavior, demonstrating that neuronal circuits underlying male-specific behaviors still exist and are functional in females (Kimchi et al., 2007).

The study of sensory processing, on the other hand, addresses the primary sensory mechanism and the downstream neural pathways processing social cues. The founders of Ethology, Lorenz and Tinbergen, through the study of supernormal stimuli in food-begging behavior of Herring Gull and egg-rolling behavior in Graylag Goose, discovered that under certain circumstances, specific sensory stimuli termed releasers elicit the stereotypical display of social behavior (Tinbergen, 1951). In particular, a distinct class called pheromones emerged in the survey of chemical cues. Providing information about social and sexual status of conspecifics, these cues were discovered to elicit marked

changes in animal behavior and endocrine status (Dulac and Torello, 2003). These influences were classified in the forms of releaser effect and primer effect (Halpern, 1987), although the influences are often mixed and complex. In rodents, pheromone releasers elicit rapid behavioral responses including inter-male aggression, maternal aggression, male copulatory behavior and ultrasonic vocalization, whereas primers induces relatively slow behavior response with endocrinological changes, such as induction of estrus and estrus synchrony by male pheromones, and acceleration of puberty in females by male pheromones (Halpern, 1987).

Despite their key roles in chemical communication and regulation of social behaviors, the chemical nature of the pheromones is still poorly understood in mammals except for a few examples. Among the identified pheromones are aphrodisin, which is a protein secreted in hamster vaginal discharge and elicits male copulatory behavior, 2MB2, which is emitted by female rabbit and induces oral grasping in pups, MTMT, which enhances male urine attractiveness to females, and the protein components of a class of major urinary protein (MUP) complex, which promotes inter-male aggressive behavior (Chamero et al., 2007; Lin et al., 2005; Schaal et al., 2003; Singer et al., 1987).

The vomeronasal organ (VNO) is thought to be the major sensory organ detecting pheromone information (Dulac and Torello, 2003), although several studies provided direct evidence that the main olfactory epithelium also plays a key role in the detection of social cues (Liberles and Buck, 2006; Lin et al., 2005; Mandiyan et al., 2005; Schaal et al., 2003; Yoon et al., 2005). Since the two major classes of mammalian vomeronasal receptors V1R and V2R families were first cloned (Dulac and Axel, 1995; Herrada and

Dulac, 1997; Matsunami and Buck, 1997), over 250 vomeronasal receptors have been identified in the mouse VNO (Zhang et al., 2010). Genetic manipulations of the vomeronasal signaling have generated fascinating insights regarding the regulation of social behavior in mice. Male mice deficient in TRPC2, an ion channel specifically expressed in VNO receptor neurons and essential for VNO sensory transduction, fail to display male-male aggression. Instead, *Trpc2*^{-/-} males initiate sexual and courtship behaviors toward both males and females introduced in their cage. These results demonstrated that vomeronasal signaling is essential for the sex discrimination of conspecifics and for the control of gender-specific behavior (Leypold et al., 2002; Stowers et al., 2002). Further, female mice with disrupted vomeronasal signaling display unique characteristics of male sexual behavior, indicating that although male-specific circuits are normally repressed by VNO-mediated pheromone inputs, neuronal circuits underlying male-specific behaviors exist in females (Kimchi et al., 2007).

To understand how pheromone signals are encoded and integrated in the central nervous system, electrical responses were recorded from single neurons in the accessory olfactory bulb (AOB) and hypothalamic area that receives vomeronasal inputs. In an AOB recording while the animals are freely engaged in reproductive social behavior, individual AOB neurons were found to respond selectively to stimulus animals of specific combinations of sex and strain, implying a sensory basis for detecting conspecific individuals (Luo et al., 2003). By recording from the AOB of anesthetized animals in response to urine and saliva, a later study confirmed that the AOB reliably distinguishes the sex and the strain of stimulus animal, and further showed that a substantial fraction of AOB neurons respond selectively to predator odors, indicating a surprising role of the

vomeronasal system in interspecific recognition (Ben-Shaul et al., 2010). Recording in the ventrolateral part of the ventromedial hypothalamic nucleus (VMHvl) in awake, behaving mice showed that cells in the VMHvl showed distinct excitatory and inhibitory responses to male or female encounter. Further, cells that are excited by males are actively suppressed during encounters with females but not vice versa, implying a close neuroanatomical relationship but an asymmetric inhibition between aggression and reproductive circuits (Lin et al., 2011).

Along with these experiments, genetic and optogenetic manipulations of hypothalamic areas have begun to uncover defined neuronal populations that are critically involved in social behavior. Lin et al. found that that optogenetic, but not electrical, stimulation of the VMHvl induces violent aggressive display even towards females and inanimate objects. Meanwhile, Pharmacogenetic silencing of VMHvl through the activation of ivermectin-gated chloride channel (GluCl $\alpha\beta$) reversibly inhibits inter-male aggression (Lin et al., 2011). Further, when a genetically defined population in the VMHvl, the progesterone receptor-expressing cells, were ablated genetically by caspase 3-mediated apoptosis, females showed diminished sexual receptivity whereas males exhibited defects in both mating and aggression (Yang et al., 2013), indicating the same brain area is able to controls distinct behavior in either sexes.

Studies with anatomically and increasingly molecularly defined populations provide initial entry points to analysis of circuits that direct reproductive social behaviors. However, many questions remain to be answered: what the exact inputs and outputs of these defined populations as circuit nodes? What is the circuit basis of the competitive

interplay between two or more motivated behaviors, such as the case between aggression and reproductive behavior? How do these hypothalamic circuits interact with the rest of the brain including cortical or striatum areas to drive a coordinated behavior display? These questions will require systematic dissection of the molecularly defined behavior circuits. Approaches including Channelrhodopsin-asssitaed circuit mapping, cell-type specific neuron activation and inhibition, assessing function of specific connection using photostimulation of axonal projection, cell-type specific gene knockouts and genetic ablation, all of which have been recently elegantly applied in the dissection of hunger circuit (Atasoy et al., 2012; Sternson, 2013; Wu et al., 2012), will be particularly valuable for the dissection of neural circuits underlying social behavior.

Social behaviors are long thought to belong to a type of behavior in which genetic factors play a dominant role during its development. This idea has been embraced by many ethologists including Konrad Lorenz and Nikolaas Tinbergen, who described these behaviors as “instinctive” or “innate” (Tinbergen, 1951): It appears that the stereotypical display of social behavior, elicited by specific aspects of the environment through the “innate releasing mechanism”, stem in-born from the animals without prior instructions or experiences.

While the innate aspect of social behavior is likely to be true in some cases, for example birds which develop normal song without auditory feedback (Kroodsma and Konishi, 1991), the social responses of many animal species are critically dependent on the early social or sensory experience of the individual, sometimes in a subtle manner. For example, the sexual behavior of male gelada baboon can develop along one of two

distinct modes. In one mode, males grow rapidly at puberty, become almost twice the size of females, and acquire, defend and breed with a harem of females. But when many such males are present, males adopts a different growth trajectory and behavior, such that they maintains a similar size with females and mate with them when the dominant males are not paying attention (Dunbar, 1984).

An even more striking example comes from Gottlieb's classical study of mallard ducklings. After hatching, ducklings appear to have a species-specific preference for the maternal call of their own species. However, since duck embryos begin to vocalize several days prior to hatching, Gottlieb suspected that this preference might result from these prenatal vocalizations. When duck embryos are devocalized in the late stages of incubation and reared in isolation, mallard ducklings does not develop the selectivity for the maternal call of their own species (Gottlieb, 1971). Moreover, when devocalized mallard embryos are exposed to chicken maternal call, after hatching they could develop a preference for the chicken call over the calls of their own species (Gottlieb, 1991). These results demonstrated that stereotypical and species-specific social behaviors that appear to be innate or instinct do not imply the absence of developmental plasticity and experience-dependent modulation.

Nonetheless, Gottlieb found that when devocalized mallard embryos were exposed to their own vocalization for just 10min/hour, they were prevented from developing a preference for the chicken maternal call. Conversely, even four days of continuous exposure to the chicken maternal call was unable to induce a preference for chicken call in ducklings that were not devocalized (Gottlieb, 1991). These results indicated a strong

tendency towards the preference for their own calling in spite of the plasticity. In another set of classical studies by Peter Marler and colleagues, male swamp sparrow (*Melospiza georgiana*) and song sparrows (*M. melodia*) were reared under identical conditions and in complete isolation from adult conspecific songs. Although abnormal song features resulted from the isolation, such as reduced numbers of notes per song and longer durations of notes and inter-note intervals, many species differences were also retained that matched differences in the natural singing behavior of the two species, including song repertoire size and song duration (Marler and Sherman, 1985).

These experiments, while acknowledging the strong influence of social experiences in the development of behavior, indicated that the genetic predisposition of animals still plays a critical and sometimes dominant role in social behaviors. In placing behaviors on a continuum where some behaviors are more nature-dependent and others are more nurture-dependent, we could potentially appreciate more the relative importance of heredity and experience in the study of various kinds of behaviors, and comprehend better the intricate and complex interactions between genotype and environment.

In a handful of areas, the influence of experience on social behavior was further explored in great detail and some of the molecular mechanisms have been revealed. For example, in vervet monkeys (*Cercopithecus aethiops sabaeus*), it was found that the average amount of contact that mothers made with their infants in the first six months of life could be predicted by the amount of contact the females had experienced as infants (Fairbanks, 1989). This effect was also shown later in rats: individual differences in licking/grooming behavior in mothers are stable and are readily transmitted to the next

generation (Champagne et al., 2003a; Francis et al., 1999). Offspring of mothers that display high levels of licking/grooming and arched-back nursing were found to have altered DNA methylation at a glucocorticoid receptor (GR) gene promoter, as compared to mothers which exhibited lower levels of these maternal interactions. DNA methylation of this locus in turn regulates GR expression and hypothalamic-pituitary-adrenal (HPA) responses to stress (Weaver et al., 2004). As variations in maternal care are strongly associated with the endocrine responses to stress (Francis et al., 1999), this could serve as the molecular basis for the transmission of individual differences in maternal behavior. Together, these findings revealed a fascinating mechanism with which social experience or other forms of environmental effects alter the epigenetic state of genes and enable the stable transmission of behavior phenotypes.

In the following paragraphs, I will focus on a specific social behavior, parental behavior, which constitutes the major topic of this dissertation. While a large body of literature examined parental behavior in the context of selection and evolutionary history, my dissertation will center on its neurobiological mechanisms. Although understanding the phylogeny and evolution of parental behavior provides a framework to interpret observations and manipulations of behavior, it is of critical importance to understand at the level of neural circuits of individual animals how sensory information and physiological state of the animals are integrated to generate appropriate parental interactions.

Overview of parental behavior

Parental behavior is defined as any behavior that an individual exhibit to increase the survival of conspecific young. It can be exhibited by biological parents or foster parents including virgin animals. Parental behavior ranges from egg-laying site selection, nest building, burrowing, egg attending, and brooding in oviparous animals to food provisioning, nursing, defending offspring, and even teaching skills in viviparous animals in which fertilized eggs develop within female reproductive tract. Parental behavior occurs in a variety of vertebrates and invertebrates, including insects, arachinids, mollusks, fishes, amphibians, reptiles, birds and mammals (Royle et al., 2012). However, it is most common and highly developed in birds and mammals, in which one or both parents provide elaborate forms of care.

Young animals after hatching or birth usually vary in their maturity and dependence on the parents, which in turn leads to different levels and forms of parental care. Precocial animals are relatively mature and mobile, and usually able to forage on their own after a short period of time after hatching or birth, whereas altricial animals are usually immobile, and need nourishments and care for a extended period of time. There is no clear dichotomy between these two states, but different species utilize different strategies in their reproduction. For example, like most rodents, newborn house mice are hairless and immobile, with their eyes closed and rely on their mother's milk and warmth to survive. However, newborn guinea pig pups are well-developed with hair, teeth, claws, and partial eyesight; they are immediately mobile and begin eating solid food shortly after birth (Harkness and Wagner, 1995). In precocial animals such as sheep, a selective

bonding is formed between the mother and her lamb at its birth, and the mother will only care for her own lamb and reject others (Carter and Keverne, 2002). In many nidifugous birds that leave the nest shortly after hatching and are usually precocial, another type of interesting bonding, filial imprinting, occurs between the young and the parents, such that the young animals will imprint on the first suitable moving objects within a critical period and follow them around to acquire behavioral characteristics (Bolhuis, 1999).

The contribution of the two parents in the parental care varies greatly across different taxa and species. In arthropods that provide parental care, the care provider is strongly biased toward females. In teleost fish, males more often than females provide care including nest building, egg attending and transporting newly hatched young, if some type of parental care does occur in that species (Reynolds et al., 2002). In extreme cases of paternal care, the eggs are laid in male seahorse and pipefish's enclosed brood pouch, fertilized and nourished for several weeks until their hatching (Stölting and Wilson, 2007; Vincent et al., 1992). Over 90% of the birds are biparental such that both parents share the responsibilities by building a nest, incubating eggs, and defending and feeding the young. Consistent with this biparental nature, males and females appear to be equally capable of providing care (Ketterson and Nolan, 1994).

In mammals, maternal care is the most common form of parenting. This is not surprising since gestation and nursing occurs almost exclusively in females, which renders them more suitable for the role of care provider, and makes it more costly for them to abandon the offspring. However, in many species, males also assist and invest significantly in the care, by carrying, warming, feeding and defending their young. For example, among the

most studied biparental mammals are the prairie voles, the males of which show virtually all of the female-typical parental care except nursing (Lonstein and De Vries, 1999). Interestingly, some other closely related voles in the same genus, such as the mountain and meadow voles, are uniparental (Insel and Young, 2001; Oliveras and Novak, 1986). Cross fostering experiments showed that when male meadow voles are reared by prairie voles, they exhibited significantly more paternal care to their offspring than in-fostered counterparts, even though still less than male prairie voles (McGuire, 1988). This result once again indicated the influence of early social environment on parental behavior in addition to genetic differences between congeneric species.

Parenting is strongly correlated with, if not directly influenced by the animal's mating system, characterized by the number of mates one animal has and the stability of the mating pairs. Promiscuous animals do not form long-term mating pairs and one sex usually mates with more than one animal of the opposite sex. In these animals such as many insects, parental care is strongly female-biased if there is parental care at all. In polygamous animals, a relatively stable bond forms between one individual and multiple partners of the opposite sex, most often in the form of one male and several females.

Polygamy is the most common mating system in mammals of which females almost always provide care. Monogamous animals have a stable one-one pair bonding for at least a breeding season or for life. These animals are usually biparental such as prairie voles (Insel and Young, 2001), Californian mice (Gubernick and Alberts, 1987), and many avian species. Interestingly, the neuropeptide vasopressin, facilitates both pair bonding and paternal behavior in prairie voles (Insel and Young, 2000; Wang et al.,

1994), suggesting the circuits of these two behaviors might be closely associated and mediated by common modulators.

Maternal aggression evolved as a special form of parental behavior against intruders, whether they are predators or conspecifics. For example, female membracid bugs usually take evasive action when approached by a predator. However, after they lay eggs, they usually remain with their eggs in the presence of predator and may even counter-attack the predator (Hinton, 1977). The most elaborate and complex form of maternal aggression occurs in female mammals which undergo drastic changes in social behavior associated with pregnancy and subsequently with parturition and lactation (Svare, 1981). Lactating female rodents such as house mouse, rats, hamsters are found to be more aggressive than nonlactating resident females (Erskine et al., 1980; Siegel et al., 1983; Svare et al., 1981). In contrast to male aggression which is usually directed against males only, both males and females are attacked by lactating females, in a display thought to protect the young from conspecific infanticidal males and females (John and Corning, 1973; Scott, 1966; Svare et al., 1981).

In some cases, other than providing care, the adults might adopt a different strategy by abandoning, killing or cannibalizing their own offspring or alien young. In the case of animal's own offspring, abandonment or infanticide occurs when the offspring is too dense, some offspring appear to be unfit or parents are unable to care for all the offspring, and thus appears to be an adaptive strategy by the parents to optimize their own fitness and future reproduction (Royle et al., 2012). Infanticide also occurs in animals which have not sired offspring and particularly in virgin animals. For example, after male lions

take control of a pride, which is a family group of lions, they usually kill the cubs and impregnate the females (Pusey and Packer, 1994). A high incidence of infanticide in virgin male mice is also observed, compared to high parental care in females and sexually experienced males (Brooks and Schwarzkopf, 1983; Svare and Mann, 1981). In these cases, infanticide is considered a strategy to eliminate competitors and to allow the females to become receptive sooner for the propagation of their own young (Mennella and Moltz, 1988a). Interestingly, it has been reported that virgin males stop committing infanticide and become paternal toward pups in a transient period after mating with a female, starting approximately at the time of birth until the weaning of pups (Labov, 1980; vom Saal, 1985; Saal and Howard, 1982), implying yet another adaptive strategy to prevent the males from harming their own offspring. While the exact mechanism remains elusive, strain, breeding condition and social experience were all suggested to play important roles in determining the likelihood of a male to exhibit paternal or infanticidal behaviors (Labov et al., 1985). Additionally, in some other animals such as rats, virgin males usually display aversive responses towards foreign pups, exhibiting neither paternal nor infanticidal behavior. However, paternal responses are usually induced by a period of cohabitation with pups (Rosenblatt, 1967).

Modulation of parental behavior by the physiological state

To address the neurobiological basis of parental behavior, two lines of inquiry have examined the physiological states and sensory inputs that regulate the parental behavior. From this body of work, tentative neural models have been proposed to characterize the circuit that controls and modulates parental care and infanticide (Numan, 2006; Numan and Insel, 2003; Numan and Stolzenberg, 2009). Most of our mechanistic understanding of parental behavior comes from laboratory and field study of mammals, primarily rodents including rats and mice (Numan and Insel, 2003). With a few exceptions such as guinea pigs, the young of most rodents such as rats and mice are altricial and requires intensive care from the parents to ensure their survival and growth. Typical parental behavior includes building nests, retrieving and carrying the pups to the nest, grooming the pups and crouching over them with nursing posture (Lonstein and De Vries, 2000; Svare and Mann, 1981). In most cases the females lactate and provide the majority of the care, while the contribution of the males range from helping in the delivery and full parenting of the pups, to shared or partial care, to neglect and even aggression (Brown, 1993; Lonstein and De Vries, 2000). It is intriguing that the respective roles of the two parents in the care of offspring exhibit such dramatic sex differences.

The laboratory rat has been the most described species in the literature of parental behavior. Virgin male and female rats usually avoid physical contact with foreign pups even though they start to interact with pups and eventually exhibit parental care after continuous exposure to pups. This effect was termed “sensitization” (Rosenblatt, 1967).

In contrast to virgin female rats, postpartum females exhibit robust maternal care towards pups.

In recent years, the mouse has become the focus of laboratory research as the most genetically accessible mammal, and provided new insights into the molecular and cellular aspects of animal reproductive behavior. Mice exhibit great variability in their parental behavior according to their strain, age and breeding condition (Lonstein and De Vries, 2000; Svare and Mann, 1981; Svare et al., 1984). However, in laboratory mouse strains, sexually inexperienced males typically attack and sometimes cannibalize the pups (Brooks and Schwarzkopf, 1983; Svare and Mann, 1981), whereas sexually inexperienced females, although unable to lactate, exhibit spontaneous and stereotyped displays of maternal care: they build nests, retrieve and carry the pups to the nest, groom the pups and crouch over them with nursing posture (Lonstein and De Vries, 2000; Svare and Mann, 1981). In addition, domestication had a strong influence on mouse behavior such that virgin females of wild derived mice generally commit infanticide, resembling the behavior of virgin wild-derived and lab male mice (McCarthy and vom Saal, 1985).

The prevailing model for reproductive social behavior is that perinatal exposure to sex hormones specifies the neuronal circuit for male- or female- typical behavior (Morris et al., 2004). As described below, maternal behavior in adults is also strongly mediated by hormonal changes associated with pregnancy, lactation and parturition, a regulation usually termed as activational effects. In addition, male parental behavior and infanticide are also influenced by hormonal changes in response to female and pup stimuli (Brown, 1993).

In a classical study by Terkel and Rosenblatt (Terkel and Rosenblatt, 1968), virgin female rats injected with blood plasma from a parturient female showed an earlier onset of maternal behavior than control groups, indicating a humoral factor in the regulation of maternal behavior. Through pregnancy, plasma estrogen level remains low in the beginning stage, but then rises and reaches a plateau till parturition. In contrast, progesterone level is high throughout the first part of pregnancy, then declines abruptly before parturition (Numan and Insel, 2003). Prolactin is released in two daily surges in the first half of pregnancy and then remains low in the second half until parturition. Treating virgin females with a regimen mimicking this pattern indeed facilitates the display of maternal behavior in ovariectomized nulliparous female rats (Moltz et al., 1970).

While male rats are usually less likely to show parental care to pups and more likely to exhibit infanticide (Jakubowski and Terkel, 1985), males castrated at birth or receiving gonadotropin antiserum in infancy exhibit enhanced parental care than males exposed to neonatal androgen normally (McCullough et al., 1974), once again confirming the organizational role of perinatal androgen. Interestingly, however, the masculinizing effect of neonatal androgen on female rats seem to be equivocal and small, and it appears that the androgen-sensitive period in the female rat exists prenatally, if there is any (Bridges et al., 1973; Ichikawa and Fujii, 1982; Quadagno and McCullough, 1973).

Using molecular biology and mouse genetics, more recent studies have confirmed the critical roles of estrogen and prolactin in maternal behavior (Champagne et al., 2003b; Lucas et al., 1998). As lactating rats showed stable variations in their pup

grooming/licking behavior, these natural variations in maternal care were found to be associated with differences estrogen receptor α (ER α) expression in the MPOA, such that females with high levels of grooming/licking exhibited increased levels of ER α expression (Champagne et al., 2003b). While prolactin receptor (*Prlr*) knockout female mice suffer a failure of embryonic implantation and fail to give birth, *Prlr*^{+/−} and *Prlr*^{−/−} virgin females as well as *Prlr*^{+/−} mothers showed maternal defects when presented with foster pups, compared to wildtype controls (Lucas et al., 1998).

Two closely related neuropeptides, oxytocin and vasopressin, were found to be implicated in parental behavior. Classical experiments using intracerebroventricular injection showed that oxytocin induces a rapid onset of maternal behavior in virgin female rats that have been ovariectomized and primed with estrodiol (Pedersen et al., 1982). Accordingly, intracerebroventricular administration of oxytocin antagonist in postpartum females significantly lowered their frequencies of pup licking and grooming (Champagne et al., 2001). Although initial observations of postpartum oxytocin knockout female mice did not find any maternal defects (Nishimori et al., 1996; Young et al., 1996), oxytocin knockout nulliparous females exhibited less pup licking retrieving behavior than wildtype controls (Pedersen et al., 2006). These minor defects in oxytocin knockout females are possibly due to the high binding affinity of vasopressin for oxytocin receptor (Caldwell et al., 2008). Indeed, both postpartum and virgin oxytocin receptor knockout females were found to exhibit deficits in maternal behavior, including significantly longer latency to retrieve the pups or to crouch over the pups and shorter duration of crouching (Takayanagi et al., 2005).

Vasopressin (AVP), on the other hand, has been implicated in the central regulation of paternal behavior in males. Paternal behavior has been investigated primarily in monogamous, biparental rodents such as prairie voles. In this species, males and females display almost identical repertoire of parental behavior, including nest building, pup grooming and huddling over the pups (Lonstein and De Vries, 1999). Because vasopressin plays an essential role in the pair bonding in prairie voles (Winslow JT, Hastings N, Carter CS, Harbaugh CR, 1993), and males become more paternal after mating when vasopressin release is up-regulated in the lateral septum (Bamshad et al., 1994), vasopressin was hypothesized to be involved in paternal care in male prairie voles. Indeed, sexually inexperienced males with AVP injection in the lateral septum were shown to spend more time contacting and crouching over pups, whereas injection of AVP antagonist induced an opposite effect (Wang et al., 1994). Furthermore, AVP injection in polygamous, uniparental meadow voles inhibited pup-directed aggression in previously pup-aggressive males, and induced paternal behavior in previously nonpaternal males (Parker and Lee, 2001). These multi-facet roles of oxytocin and vasopressin in parental care and pair bonding, and their timed release during mating suggest a coordinated and shared mechanism of neuropeptides in the regulation of affiliative social behavior.

As in rats, neonatal administration of testosterone in female mice significantly increases their rate of infanticide in adulthood when primed again with testosterone (Gandelman, 1972). Moreover, treating intact adult female mice also induces a significant but smaller increase in infanticide, suggesting an activational effect of testosterone in infanticide behavior that is facilitated by neonatal testosterone exposure. Interestingly, however, such treatment failed to induce infanticide in intact males and in males gonadectomized

in adulthood, even though testosterone treatment promotes infanticide when given to adult, neonatally gonadectomized males (Gandelman and Vom Saal, 1975). This has led to the hypothesis that infanticide is induced when adult animals are exposed to testosterone for the first time. However, this effect is somewhat attenuated by neonatal testosterone exposure (Gandelman and vom Saal, 1977). This hypothesis was corroborated by examining the influence of intrauterine position on the parental responses later in adult: males which were flanked by two female fetuses in uterus, and were therefore exposed to relatively low testosterone level during fetal development, were significantly more likely to exhibit infanticide than were males which developed between two male fetuses (Perrigo et al., 1989). Strikingly, males that were flanked by two male fetuses were found to be more aggressive towards other males in adults, which is opposite to the effect of pup-directed aggression (Saal and Grant, 1983). In a natural mouse colony, a deme is generally composed of a dominant male, several subordinate males, several females and their litters (DeFries and McClearn, 1970). The underlying adaptive value of this negative correlation between infanticide and inter-male aggression might be that since most of the offspring are likely sired by the more aggressive, dominant male, attenuated infanticide could prevent him from killing his own offspring, whereas the inclination of infanticide in less aggressive, subordinate males could help eliminate the pups of his competitors (Perrigo et al., 1989).

Progesterone, whose decline in the last stage of pregnancy has been shown to induce maternal care in females, was also found to be critical in modulating male infanticide. Progesterone receptor knockout virgin males were shown to exhibit no infanticide

behavior and little aggression towards pups. Instead, these males display elevated parental care towards foster pups (Schneider et al., 2003).

Mating experience strongly modulates the parental behavior of rodents and mediates the on/off switch of parental care and infanticide in males. It was shown that fathers of CFLP strain in mice, when housed with a single lactating female, contribute to the care of the young. Moreover, males and females did not appear to differ in the overall incidence of retrieving, nest building, licking, and huddling over the pups (Priestnall and Young, 1978). This observation was later confirmed in CF-1 mice and the mechanism of the transition was further explored in greater detail. Male CF-1 mice stop committing infanticide and become paternal toward pups in a transient period after mating with a female, starting at the approximate time of birth until the weaning of pups (vom Saal, 1985; Saal and Howard, 1982). The coincidence of the suppression of infanticide in males and the presence of their own pups likely provides a timing mechanism, which prevents males from harming their own offspring. Mechanistic studies confirmed that concurrent exposure to testosterone is required in virgin males to exhibit infanticide. But castrated and hypophysectomized males showed a behavior pattern similar to intact males, suggesting neither testosterone nor pituitary hormones mediates the mating-induced behavior switch (Perrigo et al., 1989). Furthermore, male mice still exhibit behavioral transition even when they are kept in constant darkness, even though the transition is slower than those kept in a regular 12:12 light/dark cycle or under constant light (Perrigo et al., 1991). Interestingly, wild females which are typically infanticidal follow a similar transition pattern in accordance with their parturition and lactation, with

a surprising elevation of infanticide throughout their pregnancy (McCarthy and vom Saal, 1985; Soroker and Terkel, 1988).

Sensory control of parental behavior

The nature of sensory stimuli that trigger or modulate parental behavior represents another key question to understand the neural mechanisms underlying parental behavior. This line of research addresses the identity of sensory receptors and brain areas involved in detecting pup-related social cues, the connectivity between these areas, and the neural encoding and integration of social signals. The sensory control of behavior and modulation of animal's physiological state are closely related to each other. Through primer effects, sensory inputs from pups can lead to modulation of physiological state, whereas the physiological states can also modulate sensory processing. For example, even though ovariectomized, hypophysectomized, and intact females do not show any significant difference in their latencies to the onset of maternal behavior (Rosenblatt, 1967), intact females still show a higher level of maternal behavior than ovariectomized females (LeRoy and Krehbiel, 1978), suggesting hormonal influence in this "sensitization" process directed by sensory exposure.

The majority of the research on the sensory control of parental behavior has been done in rats. In a classical study, the function of different sensory modalities in the maternal behavior of postpartum female rats was assessed by crude surgical removal or nerve transaction, where vision was removed by enucleation of the eyes, olfaction was removed by cauterization of the olfactory bulbs, and tactile inputs for different areas in the anterior head were eliminated by transection of somatosensory nerves (Beach and Jaynes, 1956). It was found that no single modality is essential for the behavior: blind, anosmic or anaptic females, each retrieved the pups in a fashion not different from the controls.

However, as more sensory modalities were affected by deprivation, the maternal behavior showed progressively more defects. For example, The combination of anosmia and tactile deprivation resulted in a more pronounced defect in retrieving than did the loss of either sensory system alone, and the defect is even more severe when all three sensory inputs were eliminated.

This study was largely confirmed by a similar experiment which systematically removed vision, audition or olfaction in virgin female rats exposed to foster pups (Herrenkohl and Rosenberg, 1972). However, it was also found that deafening produced by destruction of the basilar membrane increased the latency of retrieving and sniffing. Experiments in which recordings of ultrasonic calls were played to lactating females showed searching behavior is facilitated by the pup vocalization (Allin and Banks, 1972; Smotherman et al., 1974). Further exploration of the role of tactile inputs found that injection of lidocaine into the mystacial pads, which anesthetize the snout, resulted in defects in retrieving behavior. However, repeated tests with pups override the local anesthesia of the snout and eventually made the anesthetized females maternal, reflecting an experience-dependent training process and possible compensation by other modalities (Kenyon et al., 1981).

The investigation of the role of olfactory inputs in parental behavior led to some controversy. Bulbectomized primiparous females rats showed normal maternal behavior postpartum in a study by Fleming and Rosenblatt (Fleming and Rosenblatt, 1974). However, later studies using similar procedures reported severe deficiencies in maternal behavior and litter growth and survival in postpartum females (Benuck and Rowe, 1975;

Kolunie and Stern, 1995). Olfactory bulbectomy is a crude experiment in which both the main olfactory bulbs and the accessory olfactory bulbs are likely to be damaged, which makes the results difficult to interpret. When the vomeronasal inputs were assessed alone by vomeronasal nerve cuts, a more rapid onset of maternal care for foster pups was found in virgin female rats (Fleming et al., 1979). Strikingly, in Wistar rats in which virgin males usually commit infanticide, surgical removal of the vomeronasal organ resulted in a significant decrease of infanticide behavior, suggesting a critical role of the vomeronasal inputs in infanticide in rats (Mennella and Moltz, 1988b).

Different results emerged when the role of various sensory modalities were examined in mice. In contrast to the multisensory control of maternal behavior in rats, removal of the olfactory bulbs in lactating and virgin female mice leads to complete loss of maternal behavior and cannibalism of the young (Gandelman et al., 1971). In the olfactory bulb, adult-born neurons are continuously generated in the subventricular zone (SVZ) and migrate anteriorly through the rostral migratory stream (RMS) into the olfactory bulb, where most of them become granule cells and are incorporated into the circuit (Ming and Song, 2005). *In vivo* time-lapse imaging of these new-born granule cells showed that their dendritic spines were significantly more stable in lactating mothers compared with virgin females, whereas the spine stability of the resident granule cells remain unchanged, indicating an enhanced integration of adult-born neurons into the olfactory circuit of lactating mothers (Kopel et al., 2012). While lactating female rats are unable to locate a lost pup from its odor alone, lactating female mice were found to be able to locate pups using olfactory cues when they were tested in a two-way alternative choice task in which one choice was associated with pup odors (Smotherman et al., 1974). In addition,

ultrasonic calling was also found to be an effective directional cue in the presence of olfactory cues (Smotherman et al., 1974).

In summary, experiments in rats suggested that maternal behavior is under the control of multiple sensory inputs including vision, olfaction, somatosensation and audition, but no one sensory system is indispensable for the exhibition of maternal care. However, results in mice regarding the role of the olfactory inputs indicate that olfactory inputs are critical for maternal care, and maternal behavior may utilize distinct sensory information in different species.

A model for the neural control of parental behavior

Studies of different sensory inputs and the modulation of parental behavior by physiological state have provided valuable insights into the mechanisms underlying the control of parental behavior. However, for us to acquire a circuit-level knowledge of parental behavior, it is crucial to identify the neural populations involved in parental behavior, to elucidate how they encode pup stimuli and respond according to different physiological states, and to understand how they interact anatomically and functionally with each other.

Most of the brain areas involved in parental behavior were discovered in rats using electrolytic lesion, chemical lesion, electrical stimulation or hormone implants. Virgin female rats with electrolytic lesion in the corticomedial amygdala were found to have a shorter latency in starting to exhibit maternal behavior than controls (Fleming et al., 1980). This result was later replicated by injecting excitotoxic chemical NMDA into the medial amygdala (MeA), a more restricted lesion sparing fiber of passage, resulting in virgin females with significantly shorter latency to the onset of maternal behavior than control (Numan et al., 1993). Conversely, when the long-term excitability of the medial amygdala was induced by a kindling procedure, rats were found to have a longer sensitization period (Morgan et al., 1999). These results demonstrated that the medial amygdala, which receives direct projection from the accessory olfactory bulb, processes sensory cues leading to the inhibition of maternal behavior, and mediates the initial avoidance responses in virgin female rats.

Another structure, the medial preoptic area (MPOA) and the adjoining ventral bed nucleus of stria terminalis (vBNST), was found to be critically involved in the positive regulation of maternal behavior in rats. An early study by Fisher tentatively implicated the MPOA in parental behavior, such that males stimulated with sodium testosterone sulfate in the MPOA induced parental responses including nest building, retrieving and grooming of young (Fisher, 1956). Later studies confirmed this critical role and found that lesion of the MPOA leads to maternal deficits in a variety of conditions including in “sensitized” virgin females, pregnant females at parturition and lactating females (Lee et al., 2000; Numan, 1974; Numan et al., 1977). Small electrolytic lesions in the MPOA further uncovered that animals which failed to show retrieval and nest building behaviors tend to have a greater area of lesion within the more dorsal part of the MPOA, and the size of the lesion seems to be positively correlated with the defects of maternal behavior (Jacobson et al., 1980). Chemical lesion of the MPOA, which spared fibers of passage, confirmed these results (Numan et al., 1988). Since the ventral BNST is adjacent to the dorsal MPOA, it is often affected in these lesion studies (Numan et al., 1988). The role of the vBNST in maternal behavior was then tested by targeted lesion and it was found that vBNST lesion also disrupts maternal behavior although in a less severe manner (Numan, 1996). Moreover, bilateral injections of prolactin in the MPOA facilitates the onset of maternal behavior, whereas the injection of a prolactin receptor antagonist disrupts its onset, although these injections may spread out and affect the neighboring areas (Bridges et al., 2001, 1990). Immediate early genes (IEGs) including *c-fos*, *fosB*, and *Egr1*, which are proximate readouts of neural activity (Sheng and Greenberg, 1990), are also induced in the MPOA and vBNST during maternal behavior in rats (Numan et al., 1998). In

addition, *fosB* knockout female mice was shown to exhibit defects in maternal behavior (Brown et al., 1996).

Further studies using immediate early genes and anterograde/retrograde tracers have mapped a series of candidate regions that may be involved in the facilitation of maternal behavior in rats. Active brain areas in the display of maternal behavior include the MPOA, the vBNST and the intermediate part of the lateral septum (Fleming et al., 1994; Stack and Numan, 2000). Using a double-labeling procedure to detect both c-Fos and a retrograde tracer, wheat germ agglutinin (WGA), injected into the candidate targets of the MPOA, a study found that the active MPOA neurons during maternal behavior project to the anterior hypothalamic nucleus (AHN), the ventral tegmental area (VTA), retrorubral field (RRF) and periaqueductal grey (PAG) (Numan and Numan, 1997). It has been widely accepted that dopamine neurons in the VTA and their projections to nucleus accumbens (NAc), amygdala, prefrontal cortex (PFC), and other forebrain regions are critically involved in reward process and proactive motivational behavior (Kelley and Berridge, 2002). Indeed, extracellular dopamine level in NAc increases significantly when a lactating female grooms and licks its young (Champagne et al., 2004).

Inactivation of the VTA projection neurons by muscimol or baclofen disrupts maternal behavior in postpartum rats (Numan et al., 2009). Depletion of dopamine in the ventral striatum or lesion of dopamine neurons in the VTA causes a persistent deficiency in pup retrieval, which is a component of the active maternal response (Hansen et al., 1991a, 1991b). Using selective D1 or D2 dopamine receptor antagonist, it was found that dopamine action on D1 receptors in NAc is essential for pup retrieval. Although the mesolimbic dopamine pathway regulates reward and motivation in general and thus

unlikely to specifically control maternal behavior, it is hypothesized that the MPOA interacts with the VTA-NAc dopamine pathway to initiate and facilitate maternal behavior in rats (Numan, 2006; Numan and Insel, 2003; Numan and Stolzenberg, 2009).

Conversely, a few brain areas were found to be active in the aversive response of virgin females to foster pups, opposing the effect of the facilitative MPOA-VTA-NAc pathway. In addition to the MeA, these areas include the anterior hypothalamic nucleus (AHN), the dorsal premammillary nucleus (PMd), ventral part of the lateral septum (LSv) and the parvocellular part of the paraventricular hypothalamic nucleus (PVNp) (Sheehan et al., 2000). Many of these areas project to one another: MeA projects to AHN and LSv, and LSv in turn projects to AHN (Canteras et al., 1995; Swanson and Cowan, 1979). Further, most of these areas are shown to be involved in defensive social encounters, stress or anxiety (Canteras et al., 1997; Dielenberg et al., 2001; Duncan et al., 1996; Kollack-Walker et al., 1997; Silveira et al., 1993), suggesting a general circuit for aversive/defensive behavior is likely mediating the initial aversion of pups in virgin female rats. Indeed, bilateral chemical lesioning the dorsal hypothalamus and the anterior hypothalamic area stimulated a rapid onset of maternal behavior in estrogen-treated, nulliparous rats (Bridges et al., 1999). Later experiments confirmed the inhibitory effect of AHN, and also showed that unilateral lesion of MeA and AHN/VMH (VMH: ventromedial hypothalamus) in one hemisphere or unilateral lesion of MeA together with a contralateral lesion of AHN/VMH induces early onset of maternal behavior. Noting that the MeA projection to AHN/VMH is mostly ipsilateral, these results suggest that the MeA-AHN pathway while promoting aversive response, also suppresses the circuits that facilitate maternal behavior (Sheehan et al., 2001).

Thus far, a hypothetical neural model of parental behavior in rats has emerged (Figure 1.1), according to which the MPOA/vBNST-VTA-NAc pathway mediates proactive maternal responses and the VNO-MeA-AHN pathway regulates the aversive behavior towards pups which counteracts the former (Numan, 2006; Numan and Insel, 2003; Numan and Stolzenberg, 2009). In male and most female virgin rats, the aversive circuit, which is primarily activated by vomeronasal inputs, is dominant and drives aversion towards pups. In postpartum and “sensitized” females, hormonal and experience dependent factors modulate the circuits such that the MPOA/vBNST-VTA-NAc pathway becomes active.

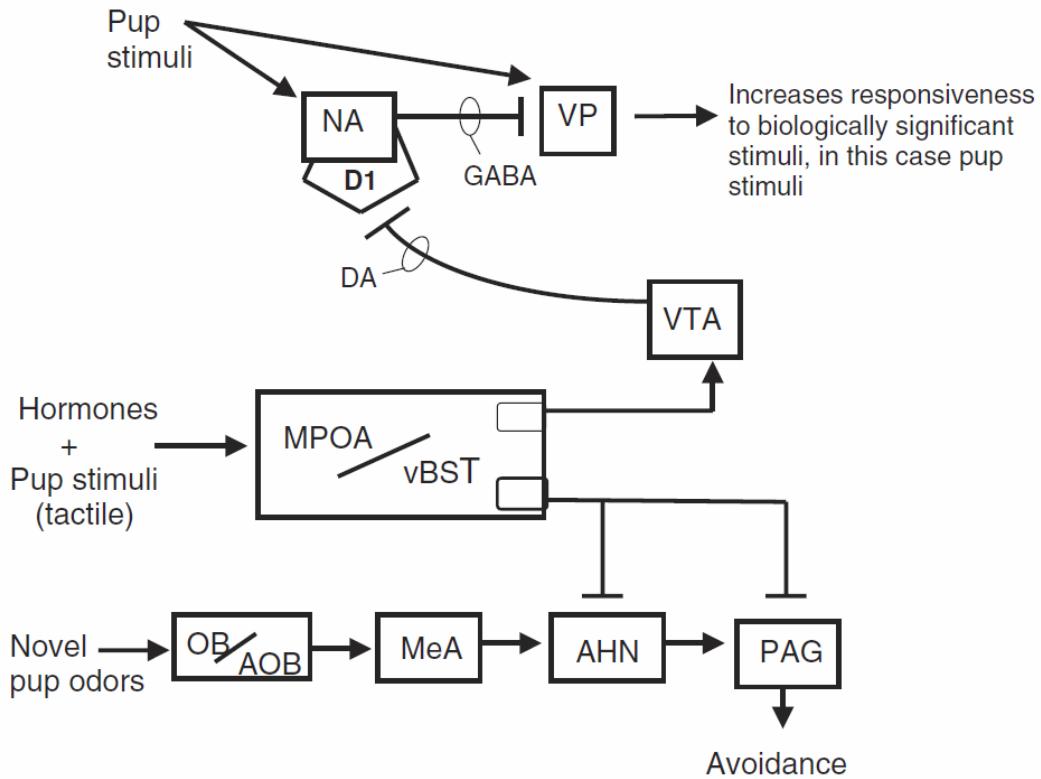


Figure 1.1: Neural model of the regulation of maternal behavior (Numan, 2006)

Two opposing circuits mediate the avoidance and maternal behavior in rats.

While it is tempting to apply this model derived from several decades of study in rats to other rodents or animals, obvious differences in parental behavior are present in many species and must be considered. For example, while virgin female rats typically exhibit avoidance behavior towards foreign pups, virgin female mice are usually spontaneously maternal. In addition, although virgin males of many rat strains avoid contact with pups, virgin male mice usually commit infanticide and male prairie voles are primarily paternal (Lonstein and De Vries, 2000).

Unlike the systematic analysis of maternal behavior in rats, neural circuits in other rodents have not been explored in detail. Recent advance in genetics and molecular biology has favored mice as a more genetically accessible model organism than the rat. Albeit limited, the studies in mice have generated some different results than in rats while confirmed others. For example, a different role of olfactory inputs was identified in the mouse than in the rat, such that olfactory bulbectomy in the mouse eliminates maternal behavior and even induces infanticide in both virgin and postpartum females (Gandelman et al., 1971). In contrast, when the vomeronasal organ is surgically ablated in virgin male mice, pup-directed aggression is abolished and parental behavior occurs simultaneously, confirming that the vomeronasal pathway is critical for pup-directed aggression in mice (Tachikawa et al., 2013). Moreover, a handful of studies also confirmed the critical role of the MPOA in maternal behavior. *c-fos* and *fosB* in the MPOA was found to be induced by maternal interaction with foster pups in nulliparous females (Brown et al., 1996; Calamandrei and Keverne, 1994; Tsuneoka et al., 2013). Bilateral excitotoxic lesion of

the MPOA in females also disrupts maternal behavior and induces infanticide (Tsuneoka et al., 2013).

Even though extensive research in rats has provided a general framework for the study of maternal behavior, and a limited number of studies has shed light on the emerging circuit analysis in mice, the identity of specific neural circuits underlying parental behavior as well as infanticide remains elusive. The MPOA and other hypothalamic areas are very heterogeneous structures (Simerly et al., 1986), raising the question of the molecular identity of the specific neural populations involved in control of parental behavior. Further, how do different components of parental and infanticidal circuits connect with each other and function in accordance to sensory stimuli and the animal's physiological state? How do neural populations encode sensory information and how are they modulated by hormones and social experience? All these questions require detailed examination and a molecular dissection of the neural circuits underlying parental behavior in mice.

This dissertation aims to evaluate the role of the vomeronasal inputs and mating experience in the regulation of infanticide and parental behavior, to identify vomeronasal receptors responsible for the detection of pups cue and to examine their roles in pup-directed behaviors (Chapter II). Further, this dissertation aims to identify and characterize molecularly defined cell populations that control parental behavior, and to confirm their role by cell-type specific ablation and optogenetic activation (Chapter III). Finally, we will discuss the implications of these findings and future directions (Chapter IV).

Chapter II. Sensory and experience-dependent regulations of parental behavior

Introduction

Pheromones are defined as a class of species- and gender-specific chemical cues that provide information about social and sexual status of individuals among the animal group, and play key roles in driving and modulating social behavior (Dulac and Torello, 2003). Pheromones are thought to be used by most animal species, from single-cell organism to higher mammals. In particular, classical studies of insect pheromones have provided a basic framework for our understanding of pheromone communication (Wilson, 1963). Pioneering work by E. O. Wilson and colleagues in ants demonstrated how pheromones can induce highly stereotypical behaviors including territory marking, colony identification, social hierarchy, reproductive status and mating rituals (Hölldobler and Wilson, 1990). Pheromone detection in insects usually involves ultrasensitive receptors. For example, gyplure, the sex pheromones of gypsy moths, elicits male sexual responses in quantities less than 10^{-7} µg (Jacobson et al., 1960).

Mammalian pheromones have been shown to elicit rapid behavioral responses as well as relatively slow behavior responses associated with endocrinological changes (Dulac and Torello, 2003; Halpern, 1987). In rodents, rapid behavioral responses elicited by pheromones include inter-male aggression, maternal aggression, male copulatory behavior and ultrasonic vocalization. The relatively slow and long-lasting effects, termed

“primer effect”, include induction of estrus and estrus synchrony by male pheromones, and acceleration of puberty in females by male pheromones (Halpern, 1987).

Although the nature of many pheromones remain elusive, a few chemicals have been identified so far as potent regulator of social behaviors in mammals. Aphrodisin, which is a protein secreted in hamster vaginal discharge, elicits male copulatory behavior (Singer et al., 1987). 2MB2, emitted by female rabbit, induces oral grasping in rabbit pups (Schaal et al., 2003). MTMT, a sex-specific compound in male urine, enhances male urine attractiveness to females (Lin et al., 2005). The protein components of a class of major urinary protein (MUP) complex were found to promote inter-male aggressive behavior (Chamero et al., 2007).

The vomeronasal system has been shown to play a key role in the regulation of social behavior (Dulac and Torello, 2003; Halpern, 1987). Unlike other sensory modalities, there is no direct cortical projection of the vomeronasal inputs, suggesting their unique role in mediating basic survival and reproductive behaviors. The VNO projects to the accessory olfactory bulb (AOB), which in turn projects to the medial amygdala (MeA), the bed nucleus of the accessory olfactory bulb (BAOT), the bed nucleus of stria terminalis (BNST) and the posteromedial cortical amygdala (PMCo), and then to various hypothalamic and other areas (Segovia and Guillamón, 1993a). Males with impaired VNO signaling exhibit mounting behavior toward both males and females indicating a critical role of the vomeronasal pathway in gender identification (Stowers et al., 2002). Further, VNO-deficient females display striking male-like mounting and courtship

displays, suggesting that the vomeronasal signaling constitutively represses circuits underlying male-specific behaviors in the female brain (Kimchi et al., 2007).

In addition to its role in adult-adult communication, classical studies in rats have implicated the vomeronasal pathway in the control of parental behavior. Male rats in which the VNO has been surgically removed exhibit reduced infanticidal behavior and a faster induction of paternal behavior (Mennella and Moltz, 1988b). Chemical lesion of the MeA induces significantly faster onset of maternal behavior in rats (Numan et al., 1993). Conversely, long-term increase in the excitability of the MeA causes a longer sensitization period (Morgan et al., 1999). In addition, lesion of the BAOT significantly reduces the latency for induction of parental behavior in both virgin male and female rats (Del Cerro et al., 1991; Izquierdo et al., 1992). Recent experiments using VNO surgical removal confirmed its role in pup detection in mice (Tachikawa et al., 2013). Using c-Fos protein expression as a neuronal activity marker, the study showed that areas along the vomeronasal neural pathway were more strongly activated in virgin males than in fathers after exposure to pups. Further, surgical ablation of the vomeronasal organ in virgin males resulted in loss of infanticide and induction of parental behavior.

Parental behavior is also strongly modulated by mating experience in rodents including rats and mice. Under the combined influence of estrogen, progesterone and prolactin, postpartum female rats and mice exhibit robust maternal care towards pups (Lonstein and De Vries, 2000). Virgin rats and mice exhibit different behavioral responses to pups, however. Virgin male and female rats usually avoid physical contact with foreign pups, but after continuous exposure to pups, they start to approach, interact with them and

eventually exhibit parental care (Rosenblatt, 1967). On the contrary, virgin laboratory mice display a drastic sex difference in their interaction with pups: While most adult virgin females are spontaneously maternal, adult virgin males are typically infanticidal (Lonstein and De Vries, 2000).

Mating experience alters the male behavior towards pups in an interesting and dramatic fashion. Approximately two weeks after mating, infanticide behavior is inhibited in males and substituted by paternal care (Labov, 1980; vom Saal, 1985; Saal and Howard, 1982). The inhibition of infanticide subsides for approximately three weeks, which correlates with the weaning time for the pups. The coincidence of the suppression of infanticide in males and the birth of their own pups likely provides an adaptive mechanism that prevents a male mouse from killing its own pups, but successfully eliminates pups sired by others. Two general mechanisms have been hypothesized for this radical behavior shift (Elwood and Ostermeyer, 1984). Firstly, a time-dependent synaptic or transcriptional change may occur that is triggered by mating. The second hypothesis is that the female releases chemical cues during pregnancy that prevent the male mouse from killing her offspring, which can be viewed as a female counter-strategy against infanticide (Mennella and Moltz, 1988a). The results examining these two hypotheses have been controversial: Studies in CF-1 mice showed that ejaculation but not intromission results in the reduction in infanticide (vom Saal, 1985). In contrast, other studies have suggested that it is the post-copulatory cohabitation with the female that suppresses infanticide in mice (Elwood and Ostermeyer, 1984). Yet other studies have suggested that both mechanisms may have an effect (Elwood, 1985; Labov, 1980). Recent experiment in C57BL/6 mice confirmed the presence of both mechanisms, and

further suggested that the reduced activation of vomeronasal system in fathers by pup cues might be involved in the behavioral transition (Tachikawa et al., 2013). However, the neural mechanism of this time-dependent behavior switch requires further examination.

In this chapter, I aim to use genetic methods to re-evaluate the role of the vomeronasal pathway in infanticide and parental behavior of males, and to explore mechanisms underlying mating-induced behavior switch in males.

Results

The vomeronasal pathway is critical for male infanticide

First, we used genetic tools to confirm the role of VNO inputs in the behavior of virgin adult mice towards pups. Previous observations indicated that the interpretation of the behavioral phenotypes resulting from VNO surgical ablations published in the literature may be inaccurate due to bleeding and obstruction of the nasal cavity, resulting in behavior phenotypes similar to that of olfaction-deficient mice such as severe defects in sexual and aggressive behavior (Kimchi et al., 2007). TRPC2 is a cation channel expressed specifically in VNO receptor neurons and its genetic ablation impairs vomeronasal signaling (Liman et al., 1999; Stowers et al., 2002). Individually housed adult *Trpc2*^{-/-} virgin males and females and *Trpc2*^{+/+} virgin littermates were presented with four 1- to 3-day-old C57BL/6J pups placed at the far corner of the cage. Animals that retrieved all four pups to the nest within 30 minutes and crouched over pups were scored as “parental”. Animals that attacked the pups within 30 minutes were considered “infanticidal”. Lack of parental or attack display was scored as “ignore”. In contrast to *Trpc2*^{+/+} littermates, *Trpc2*^{-/-} virgin males showed a dramatic reduction in infanticidal behavior (Figure 2.1). In addition, a large fraction of *Trpc2*^{-/-} virgin males exhibited parental care typically seen in females and sexually experienced males (Figure 2.1). Quantification of the behavior towards pups showed that *Trpc2*^{-/-} males retrieved pups with shorter latency (Figure 2.2), were more active at building nests, and spent more time in the nest, crouching over the pups and grooming them than *Trpc2*^{+/+} males (Figure 2.3).

Trpc2^{-/-} males, while clearly parental for all tested parameters, displayed overall less parental care than *Trpc2*^{-/-} females (Figure 2.3).

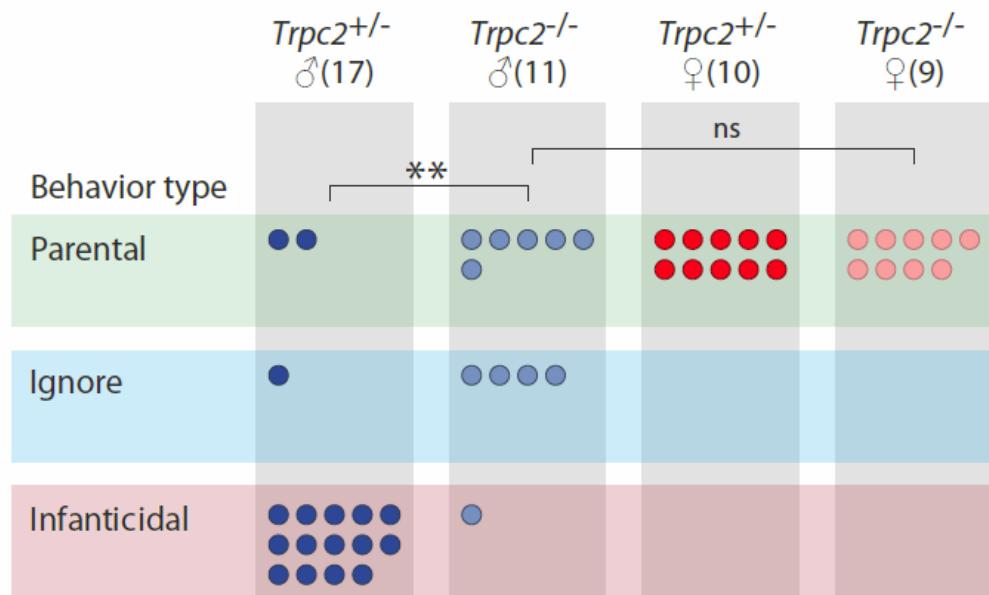


Figure 2.1: Behavior of *Trpc2*^{-/-} and *Trpc2*^{+/-} virgin males and females toward pups

Behavior analysis of *Trpc2*^{-/-} and *Trpc2*^{+/-} virgin males demonstrates significantly different responses to pups in the presence or absence of VNO signaling. Chi-square test with Bonferroni correction, ** $P<0.01$.

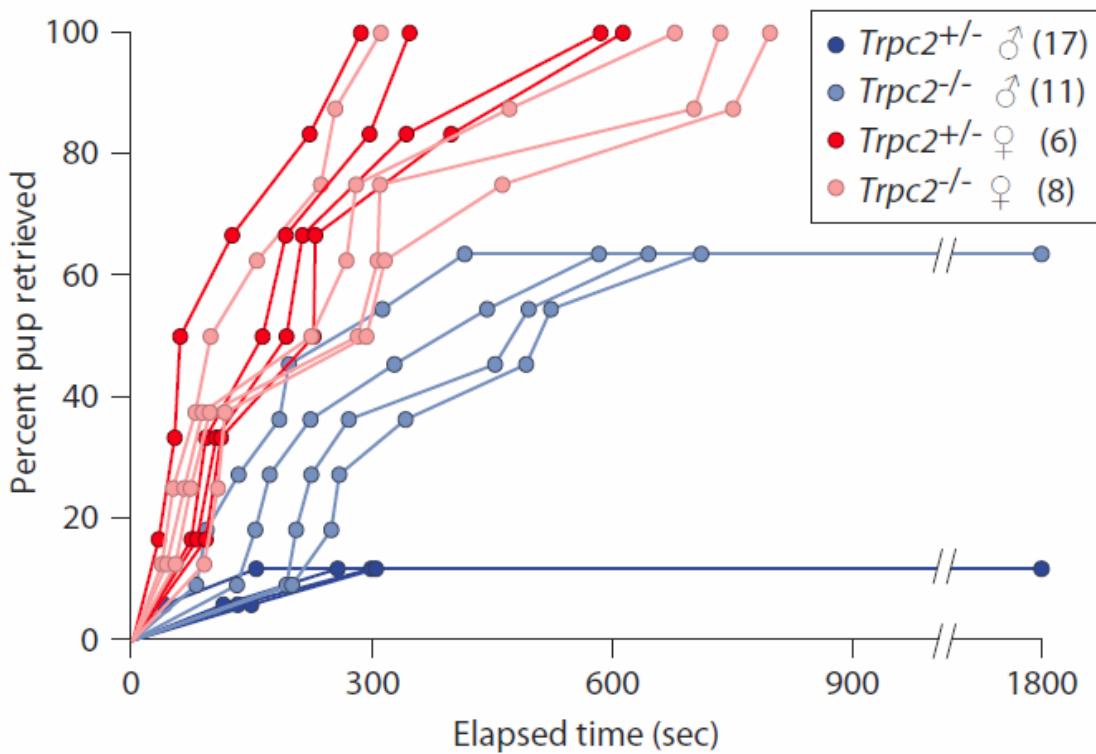


Figure 2.2: Pup retrieving of $Trpc2^{-/-}$ and $Trpc2^{+/-}$ males and females

Each animal was presented with four pups. Each curve plots the retrieving latencies of all the animals in one group for one pup on the x-axis, and the cumulative retrieving percentage of that group for that pup on the y-axis. The retrieving curves of the $Trpc2^{-/-}$ males are different from those of the $Trpc2^{+/-}$ males. Kolmogorov-Smirnov test, $P<0.05$.

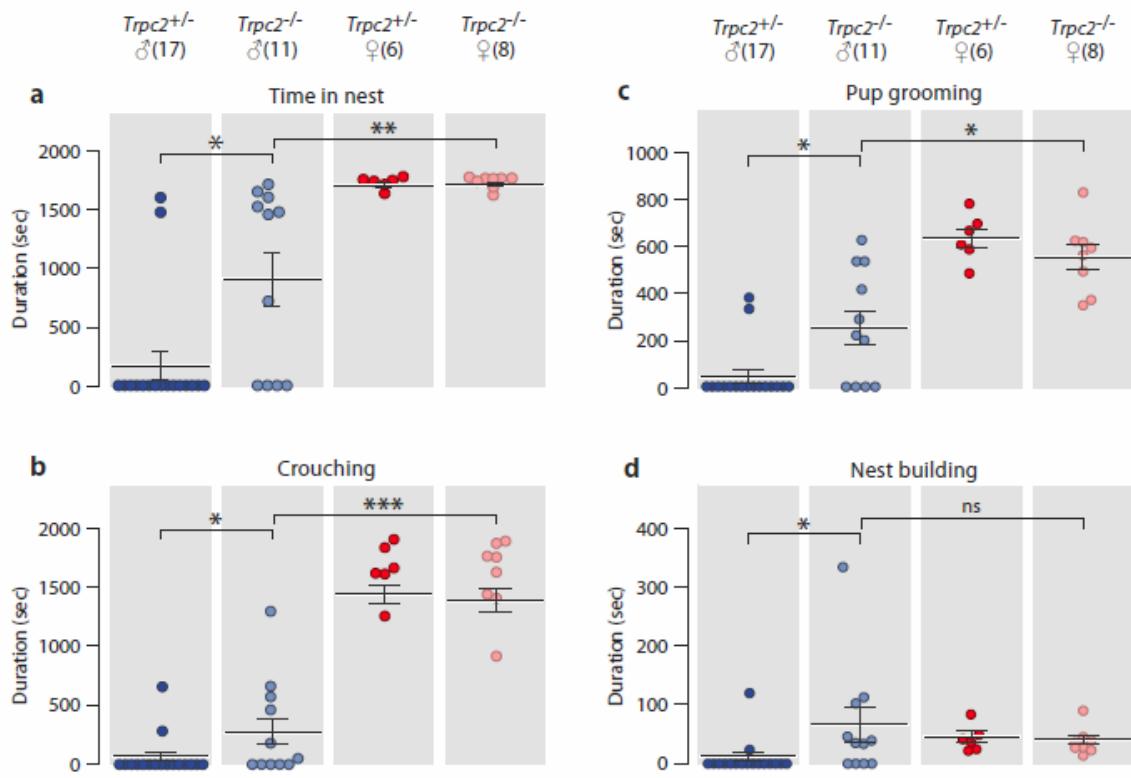


Figure 2.3: Comparison of time in the nest, crouching, pup grooming and nest building of *Trpc2^{−/−}* and *Trpc2^{+/−}* males and females toward pups

The *Trpc2^{−/−}* males spend significantly more time in the nest, crouching, pup grooming and nest building than the *Trpc2^{+/−}* males, but less than *Trpc2^{−/−}* females. Mean±SEM. Mann-Whitney test with Bonferroni correction, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, ns. not significant.

Mechanism of mating-induced behavior switch

In the next set of experiments, we aimed to investigate the post-mating switch from infanticide to paternal care originally reported in the wild house mouse and the CF-1 mouse strain (Labov, 1980; vom Saal, 1985). *Trpc2^{+/−}* males were allowed to mate with females, and housed together with their mates until they were tested at different time points. Control *Trpc2^{+/−}* virgin males were kept in their cages without contact with females after weaning. Control males as well as the majority of experimental males tested 1-2 days, or 10-12 days after mating committed infanticide (Figure 2.4). In contrast, when tested at time periods surrounding the birth of pups, most males tested at Day 17-20 failed to exhibit infanticide with half displaying paternal behavior, and all the males tested at Day 25-27 were paternal (Figure 2.4). Since males tested at Day 17-20 were separated from the females before they gave birth, the switch of the male behavior does not seem to depend on exposure to pups. It is unclear however, whether this change in behavior results from an intrinsic timing mechanism or from the effects of cohabiting with pregnant females.

The next experiment, performed in collaboration with Anita Autry and Brenda Marin-Rodriguez, addressed this question more specifically. Males were mated with females at Day 0 and housed together for different durations, but all tested for their parental behavior at Day 24 post-mating (Figure 2.5). Around 50% of the males showed a complete reversal of their behavior after only one day of housing with their mates, implying an intrinsic time-dependent mechanism triggered by mating. Gradual decrease in infanticide and increase in paternal behavior were also observed as the males increase

their length of cohabitation with the females, suggesting that cohabitation with a pregnant female and/or pups still makes important contribution to the emergence of paternal behavior (Figure 2.5).

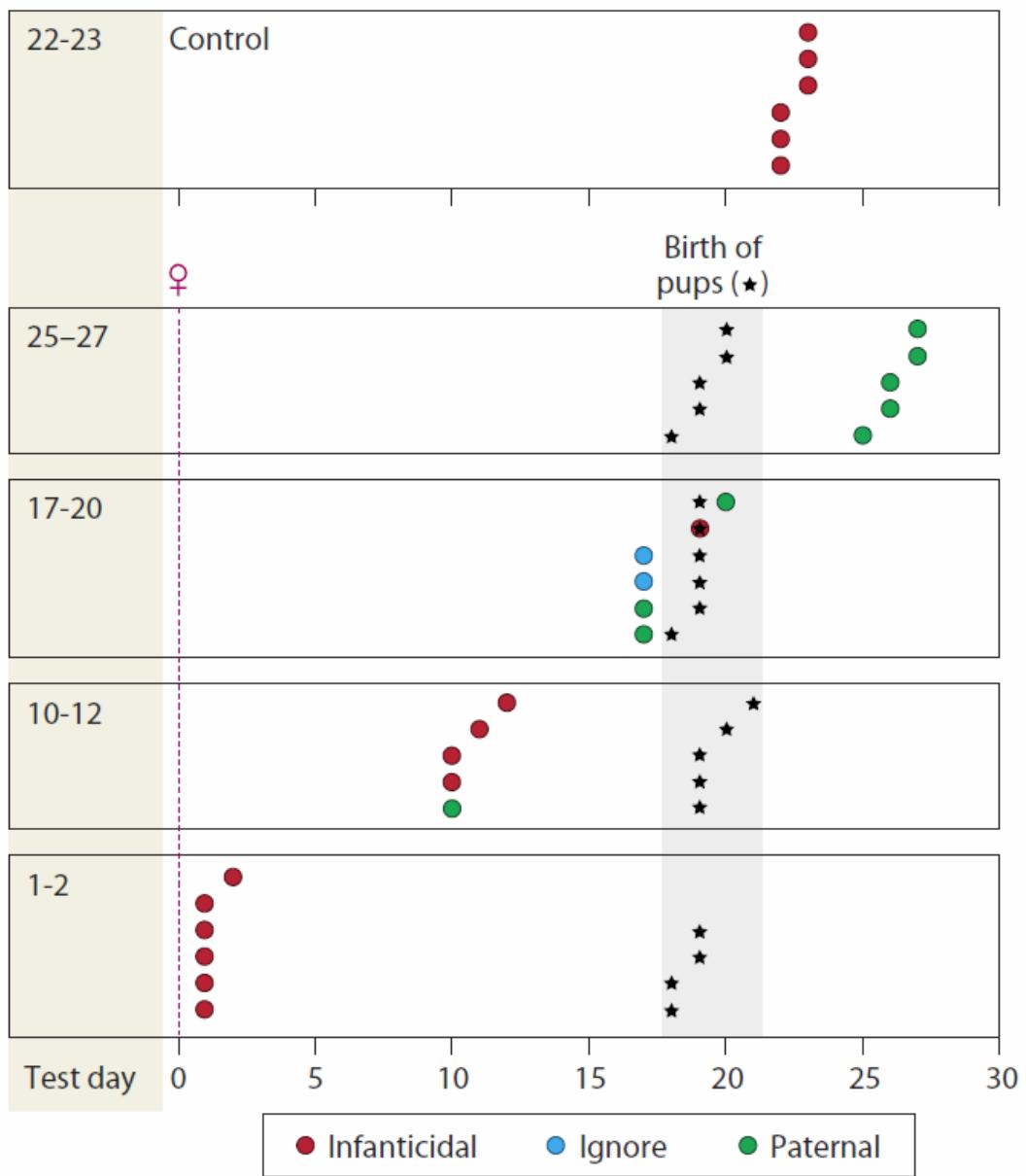


Figure 2.4: Behavior switch of *Trpc2*^{+/-} males after mating

Behavior of *Trpc2*^{+/-} males tested at different days after mating with females. Males mated on Day 0 except virgin controls, which were individually housed from Day 0 throughout the test. Male behavior switches from infanticidal to parental at a time period after mating that corresponds to the birth of pups.

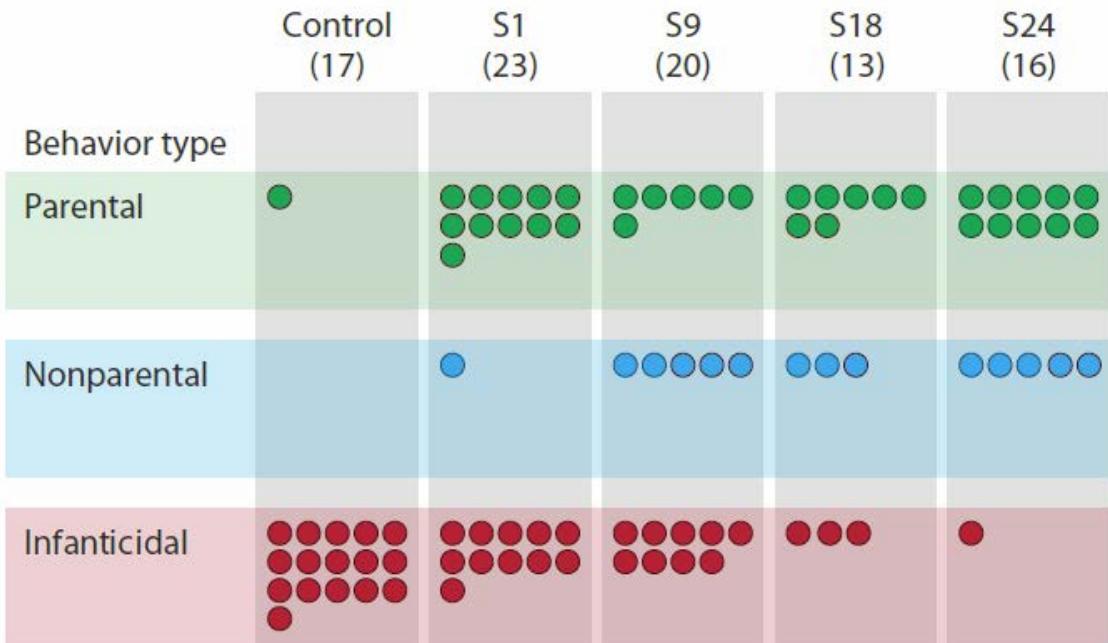


Figure 2.5: Behavior switch of *Trpc2*^{+/−} males after increasing durations of cohabitation with females after mating

The control group is made up of sexually naïve male mice which have never been exposed to females. The S1, S9, S18 and S24 groups had one, nine, eighteen and twenty-four days of cohabitation with females, respectively. All the groups were tested on Day 24.

Discussion

The behavior characterization of *Trpc2*^{-/-} virgin animals showed that the vomeronasal pathway is critical for the exhibition of infanticide behavior in males, and is dispensable for the display of parental behavior in virgin females. In addition, most of the knockout males exhibit paternal care, suggesting a possible role of the vomeronasal signaling in the suppression of paternal care in males. The parental care exhibited by *Trpc2*^{-/-} males, however, is overall still less pronounced than *Trpc2*^{+/+} females, such that the duration of pup grooming and crouching and time spent in the nest are significantly smaller in knockout males than in females. These results indicate that while the vomeronasal system is critical in specifying the behavioral sex dimorphism of virgin males and females towards pups, other factors possibly including sex hormones and other sensory modalities are likely to be involved as well.

Interestingly, the *Trpc2*^{-/-} female behavior is not significantly different from the heterozygous controls in our behavior assay. Previous studies testing *Trpc2*^{-/-} females in seminatural environment for prolonged period of time found that in the first and second days after birth, both *Trpc2*^{-/-} and *Trpc2*^{+/+} mothers spent a similar amount of time with their pups (Kimchi et al., 2007). However, during the following days, *Trpc2*^{-/-} females exhibited a significant decrease in time spent in the breeding nest, compared to *Trpc2*^{+/+} females. Since the females were tested in the presence of many other adults in a complex environment, this decrease in time spent with the offspring may indirectly result from the high level of social interactions of the *Trpc2*^{-/-} females with other adult animals.

Together, these results indicate that basic components of maternal behavior remain largely unaffected in *Trpc2*^{-/-} females, although vomeronasal signaling may play a role in the long-term maintenance of maternal care.

Our investigation of the post-mating switch from infanticide to paternal care after 3 weeks showed that a complete reversal of behavior occurred in about half of the males if they have been housed only one day with their mates, indicating an intrinsic time-dependent mechanism triggered by mating that does not require the continuous presence of females. However, cohabitation with the females also led to further decrease in infanticide and increase in paternal behavior, suggesting that cohabitation with a pregnant female and/or pups makes important additional contributions to the emergence of paternal behavior.

The exact nature of this time-dependent mechanism, however, remains elusive. Previous experiments have implicated the vomeronasal signaling pathway in the behavioral transition from infanticide to parenting, such that neural activity shown by c-Fos is greatly diminished along the vomeronasal pathway including in the VNO epithelium of fathers compared to virgin controls (Tachikawa et al., 2013). However, it is unclear how mating may cause a down-regulation of VNO response to pup cues in fathers. Mating is able to induce a series of hormonal changes in males, including elevated levels of testosterone, luteinizing hormone (LH) and prolactin (Brown, 1993), although these changes tend to be transitory and difficult to account for the transition in behavior over the course of weeks. Interestingly, wild females, which are typically infanticidal, follow a similar transition pattern in accordance with their parturition and lactation, with a

surprising elevation of infanticide throughout their pregnancy (McCarthy and vom Saal, 1985; Soroker and Terkel, 1988).

In summary, these experiments demonstrated that the vomeronasal system plays a key role in the on/off switch of parental behavior that establishes its sex specificity. Further, two opposing behavior circuits appear to co-exist in the male brain, in order to regulate infanticide and parenting behaviors according to the social context. In virgin males, vomeronasal circuits activated by pup cues elicit infanticide while pathways underlying parenting behavior remain silent. In contrast, mated males transiently repress vomeronasal-evoked infanticide and instead activate parenting circuits in response to pup signals.

Materials and Methods

Parental behavior assay

Trpc2 knockout mice of C57BL/6J x129/Sv mixed genetic background were generated previously in our laboratory (Stowers et al., 2002). Animals were maintained on 12h: 12h light/dark cycle (lighted hours: 02:00-14:00) with food and water available *ad libitum*. 2- to 4-month-old, *Trpc2*^{+/−} and *Trpc2*^{−/−} virgin male and female littermates were individually housed for approximately one week before the test.

Behavior assays started at the beginning of the dark phase and were performed under dim red light. 1- to 3-day-old C57BL/6J pups were used as the standard pup intruder in all the behavior assays performed in this study. The pups are of a different strain from the *Trpc2*^{−/−} animals and therefore are not related to the resident animals. The pregnant females were separated from the stud before parturition, so the pups are not exposed to their fathers and do not carry any adult male odor. Four C57BL/6J pups were introduced to the home cage of each animal and placed at the farthest corner from the resident's resting nest. The first investigation marked the beginning of the assay, which then extended until 30 minutes after all the pups were retrieved, or until the resident attacked and wounded the pups, or for 30 minutes in case neither of above happened. When a pup was attacked, the assay was ended immediately and the wounded pup was euthanized. Each test was videotaped (Sony DCR-HC65 camcorder in nightshot mode or Geovision surveillance system) and the following behavior of the adult residents were scored by an experimenter blind to the genotype: latency to retrieve each pup (picking up a pup with its mouth and

carrying it to the nesting area), latency to attack (biting a pup, confirmed by examining actual wounds on the pup), grooming (sniffing and licking a pup), crouching (extending its limbs, assuming a nursing-like posture and huddling over at least 2 pups), nest building (collecting and arranging nesting material and making a nest) and time spent in the nest. Grooming, crouching, time in the nest and nest building were scored as duration during the 30-minute recording after all the pups were retrieved using the Observer 5.0 software (Noldus Information Technology). The latencies to retrieve or attack pups were recorded in seconds.

Parental behavior assay for mated males: *Trpc2^{+/}* virgin males were individually housed for 3-5 days and then paired with females. In the next few days the females were checked daily for vaginal plugs. Once a plug was spotted, the day was marked as Day 0 for the mating pair and that pair was randomly assigned to a group for different length of cohabitation (1-2 days, 10-12 days, 17-20 days or 25-27 days). According to their group, the males were tested one day after the females and their litters (if any) were removed from their home cage. For example, animals tested on Day 1 were separated from their mates on Day 0. The animal tested on Day 20 was separated from its mate on Day 19 and was not exposed to its own litter. The negative controls for this essay were individually housed *Trpc2^{+/}* virgin males.

Chapter III. Vomeronasal receptors for pup detection

Introduction

The vomeronasal organ (VNO) has long been implicated in the detection of pheromones (Halpern, 1987). Experiments based on the differential screening of cDNA libraries prepared from single VNO neurons uncovered a novel gene family encoding G-protein-coupled receptors (GPCRs), the V1Rs (Dulac and Axel, 1995). Remarkably, the V1Rs do not show significant sequence homology with the olfactory receptors (ORs). In addition, each individual receptor gene is only expressed in a small subset of VNO neurons in the apical neuroepithelium, suggesting that the V1Rs may be a family of putative mammalian pheromone receptors. When a genomic region that contains a cluster of 16 intact V1r genes is deleted in mouse, the mutants exhibited deficits in male sexual behavior and maternal aggression (Del Punta et al., 2002). Moreover, the VNO neurons of these mice were unable to detect specific pheromonal ligands shown by electrophysiological recording. In another study, single-cell electrophysiological recording and calcium imaging showed that a V1R receptor, V1rb2, detects a pheromone, 2-heptanone, at physiological levels (Boschat et al., 2002). Together, these results further verified the role of V1Rs as pheromone receptors.

A second major class of mammalian vomeronasal receptor gene family, named V2Rs, was subsequently cloned. The V2Rs were found to be expressed in the basal zone of the VNO (Herrada and Dulac, 1997; Matsunami and Buck, 1997). Recently, a third family of mouse putative VNO receptors, the formyl peptide receptors (FPR), was cloned and

found to be selectively expressed in VNO neurons in patterns similar to those of V1Rs and V2Rs (Liberles et al., 2009; Rivière et al., 2009). Through data mining of the mouse genome, over 250 putative pheromone receptor genes that are expressed in the mouse VNO have been identified so far (Zhang et al., 2010).

Historically, a functional dichotomy was proposed between the two olfactory systems and the chemosignals they detect: the main olfactory system is thought to process mostly volatile cues while the vomeronasal system primarily detects non-volatile pheromones (Halpern, 1987). However, more recent studies have provided some direct and indirect evidence that the main olfactory epithelium can also detect pheromonal ligands and mediate social behavior. For example, 2MB2, a volatile molecular in rabbit milk inducing stereotypical oral grasping, presumably acts through the main olfactory epithelium without body contact (Schaal et al., 2003). MTMT, which enhances urine attractiveness to female mice, induces robust electrophysiological responses in a small subset of mitral cells in the main olfactory bulb (Lin et al., 2005). In addition, the vomeronasal pathway was presented as the major inputs for the luteinizing hormone-releasing hormone (LHRH)-expressing neurons in the rostral hypothalamus, which control and regulate reproductive functions including the onset of puberty (Neill and Knobil, 2006). However, in contrast to this established notion, cell-type specific tracing using conditional pseudorabies virus found that the major inputs for LHRH neurons project from a discrete population of olfactory sensory neurons, with either absence of, or only minor connectivity with the vomeronasal system (Boehm et al., 2005; Yoon et al., 2005). This anatomical connectivity between the main olfactory system and hypothalamic neurons involved in the control of reproduction is also functionally demonstrated by the

behavioral phenotype of mouse mutants impaired in olfactory detection (Mandiyan et al., 2005; Yoon et al., 2005). Finally, unlike odorant receptors, trace amine-associated receptors (TAARs), which are expressed in the main olfactory epithelium were reported to detect social cues in male urine (Liberles and Buck, 2006). These results indicate that the concept of a functional dichotomy between the two olfactory systems is actually inaccurate, and suggest that the neural encoding of social cues in mammals involves the integration of both olfactory and vomeronasal cues.

Although the nature of the chemical ligands and their paired receptors has been elusive, a handful of receptor-ligand pairs have been identified. In addition to the V1rb2 receptor/2-heptanone pair (Boschat et al., 2002), VNO neurons expressing V2r1b (also known as Vmn2r26) were found to detect major histocompatibility complex (MHC) peptides at subpicomolar concentrations (Leinders-Zufall et al., 2009). A specific V2R receptor, V2Rp5, was shown to regulate female reproductive behavior through the detection of exocrine gland-secreting peptide 1 (ESP1) (Haga et al., 2010). In a recent effort by Isogai et al. in our laboratory, the response profiles of 88 individual vomeronasal receptors to a wide range of physiologically relevant stimuli have been characterized, generating the most comprehensive functional map of VRs to date (Isogai et al., 2011). Consistent with previous results using calcium imaging of vomeronasal receptor neurons (He et al., 2008), this study found that the detection of sex-specific cues relies on a small and specific subset of VNO neurons. In addition, this study identified single V1Rs activated by sulphated steroids, which are thought to account for the majority of VNO neuronal activation by female urine through V1Rs (Holekamp et al., 2008). Further, the results suggest that V1Rs and V2Rs may use different strategies in the encoding of

chemosignals: individual V1Rs detect the physiological status of an animal, whereas individual V2Rs encode information about the identity of emitters, such as the sex of a conspecific or the predator/competitor nature of a heterospecific.

The genetic and surgical ablations of the VNO have demonstrated the critical role of vomeronasal signaling in infanticide behavior. But the identity of the vomeronasal receptor(s) to pup stimuli, and the nature of the corresponding ligand(s) have not been characterized. In collaboration with Yoh Isogai, a postdoc in the lab, I set out to identify the vomeronasal receptors for pup cues and to characterize their role in pup-directed behavior. Elucidating the nature of the receptors used for pup detection will help us understand the mechanism with which chemical cues influence social behavior, and provide an entry point to the dissection of its downstream pathway and the central circuits underlying pup-directed behavior.

Results

Previous studies have established *Egr1* activity as a robust and reliable readout of pheromone-evoked VNO activation (Isogai et al., 2011). Putative pup receptors were identified by double mRNA *in situ* hybridization in male and female VNO sections with probes against *Egr1* and vomeronasal receptor genes. As the vomeronasal pathway plays a critical role in establishing the behavioral dimorphism in males and females towards pups, the VNO neurons may exhibit different activity pattern in response to pup cues in two sexes, serving as a sensory basis for the behavioral sexual dimorphism. As previous experiments suggested, the detection of specific cues relies on a small and specific subset of VNO neurons (Isogai et al., 2011). We predict that only a small number of receptor neurons are activated by pup cues. In that case, one or two receptors can be selected to validate their functional role in infanticide and parental behavior by creating receptor knockout animals. This project is done in collaboration with Yoh Isogai: I breed all the experiment animals for *in situ* hybridization; Together we ran the pup exposure essay; Yoh performed the mRNA *in situ* hybridization experiments to identify the putative receptors, while I generated the constructs of the receptor knockout.

Firstly, the overall VNO response to pups was characterized in males and females. Adult virgin animals were exposed to 1- to 3-day-old C57BL/6J pups and allowed to freely behave. Their VNOs were then collected and analyzed for *Egr1* activation. It is estimated that pup cues activate approximately 4.5% of all the VNO neurons in males, and 1.1% in females. Consistent with our previous results that vomeronasal signaling is critical for infanticide behavior but not required for parental behavior in females, pup stimuli

activated approximately 4-fold more VNO neurons in males than in females, suggesting a sex difference in the information encoding on the vomeronasal receptor level (Figures 3.1, 3.2).

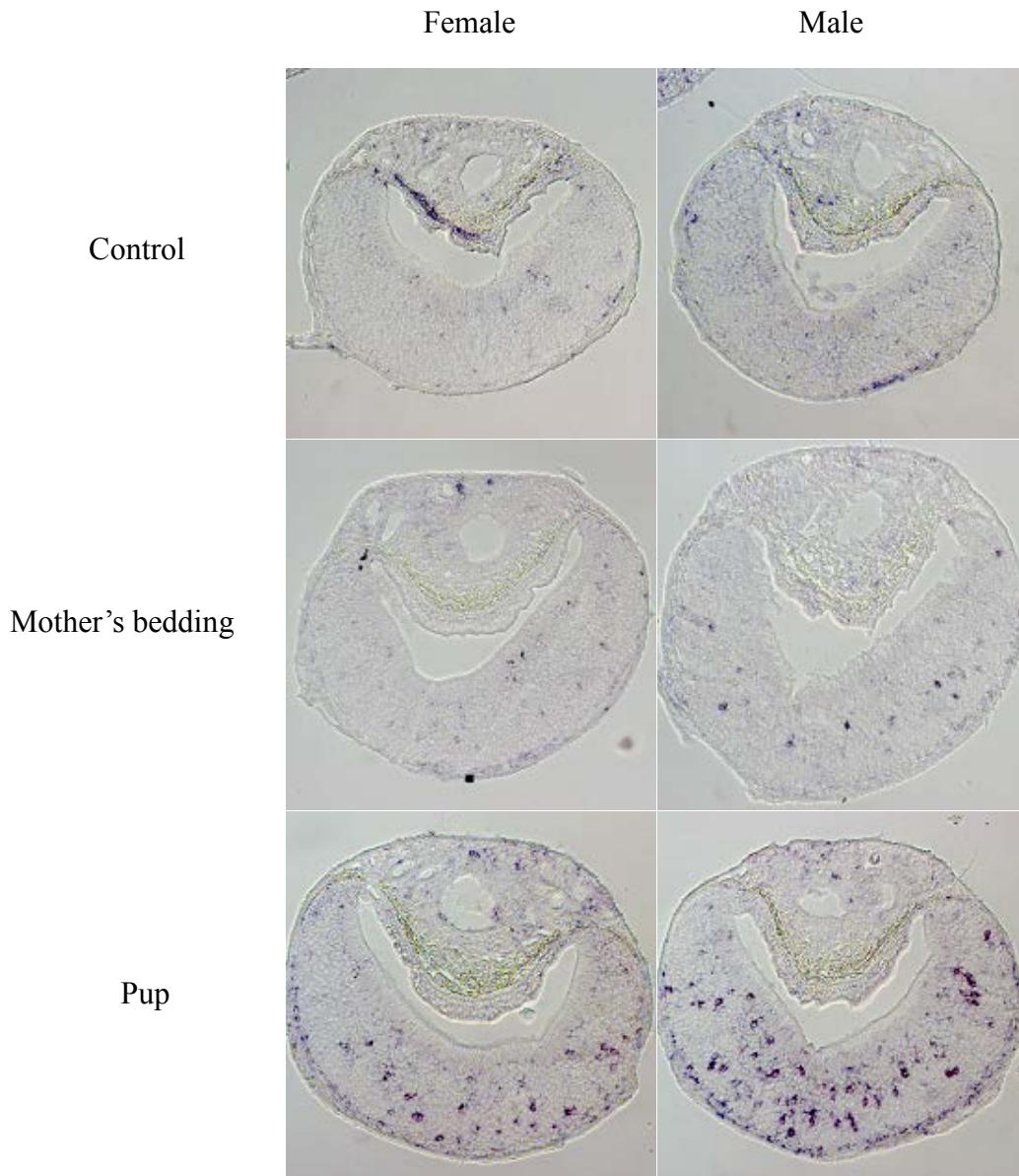


Figure 3.1: *Egr1* activation in VNO sections of virgin males and females exposed to pups, mother's bedding and fresh bedding control

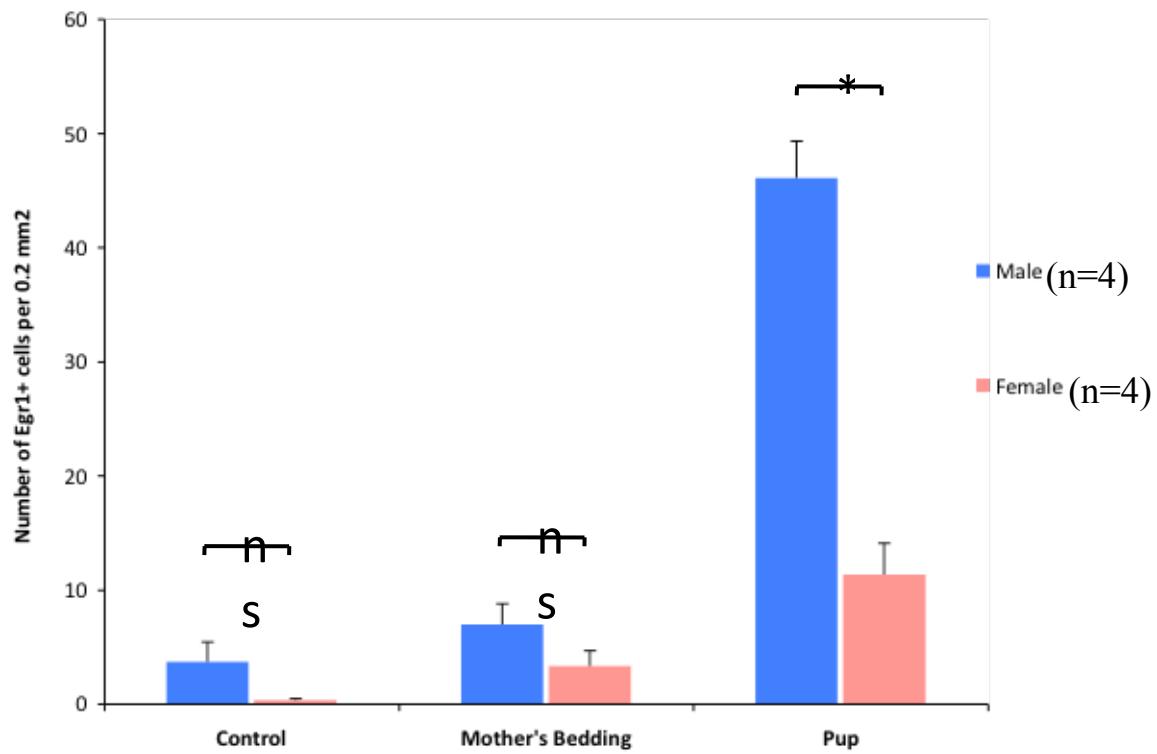


Figure 3.2: Number of *Egr1*+ cells in VNO sections in response to pup stimuli

Virgin males and females are exposed to pups, mother's bedding and fresh bedding control.

Mean+SEM. * $P<0.05$, ns. not significant. 0.2 mm² of VNO area contains approximately 1000

VNO neurons (Materials and Methods).

A hierarchical approach was then used to identify VRs activated by pup cues. V1R neurons with cell bodies in the apical layer of the sensory epithelium express the G-protein α -subunit $G\alpha_{i2}$, whereas V2R neurons in the basal layer express $G\alpha_o$ (Dulac and Torello, 2003). A third receptor family expresses formyl peptide receptors (FPRs) (Liberles et al., 2009; Rivière et al., 2009). The co-expression of *Egr1* with $G\alpha_{i2}$, $G\alpha_o$ or FPRs was analyzed to determine whether the putative pup receptors belong to V1R, V2R or FPR family. Both V1R and V2R but not FPR-expressing cells were activated by pup cues (Figure 3.3). The identity of the activated VRs was then narrowed down with probes against V1R and V2R subfamilies or clades (Figure 3.4). Double labeling was found between *Egr1* and receptors in V1rc and V1ri subfamilies of V1r family, and Clade 3 and Clade 6 of V2r family. These subfamilies and clades recognize a variety of male, female and heterospecific cues (Figure 3.5) (Isogai et al., 2011).

These clades were then characterized by probes specific for single receptors. For all vomeronasal receptor genes whose expression can be confirmed in our mapping, the specificity of their probes was tested by double *in situ* hybridization using DIG and FITC probes. Some of the receptor gene sequences are too similar with each other for us to generate specific probes for single receptors. In these cases the receptors are listed together as a group.

Using this approach, four major vomeronasal receptors (or receptor group) were identified to detect pup cues (Figure 3.6). These receptors include V1rc1/30 and V1ri9 of the V1r family and Vmn2r65 and Vmn2r88 of the V2r family. Interestingly, although the combination of this set of receptors is unique, not a single one of these receptors appears

dedicated to pup stimuli since they can also be activated by other conspecific or heterospecific odors. V1rc1/30 is also activated by mammalian non-predators, V1ri9 is activated by *M. Spicilegus*, Vmn2r65 is activated by adult female cues and Vmn2r88 is activated by adult male cues. The activation of both V1Rs (many ligands are steroid derivatives) and V2Rs (many ligands are peptides) indicates that pups emit a complex mixture of chemical cues. While single VRs are expressed on average in ~0.3% of all the VNO receptor neurons, the two V2Rs that we identified as responsive to pups cues appear more highly represented in the VNO (~1.5% for Vmn2r88 and ~0.5% for Vmn2r65). In addition, V2Rs were found to be more specific than V1Rs, such that individual V1Rs tend to be activated by a variety of cues whereas V2Rs are mostly activated by single stimuli (Isogai et al., 2011). Further, as V1Rs tend to detect cues regarding the physiological state of the animal and V2Rs encode the nature of the stimuli (male or female, predator or competitor), the two V2Rs might be crucial in detecting the characteristic ligands representing the pup cues, hence prompting us to specifically follow up on their functional characterization, rather than on the identified V1Rs.

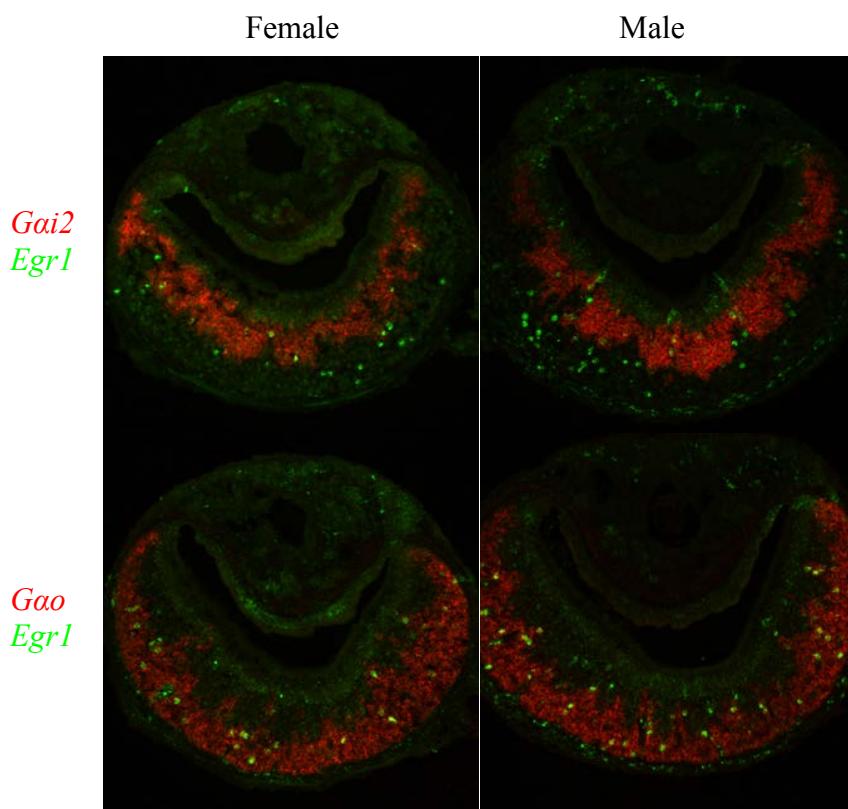


Figure 3.3: VRs in both $G\alpha_{i2}$ and $G\alpha_o$ zones are activated by pup cues.

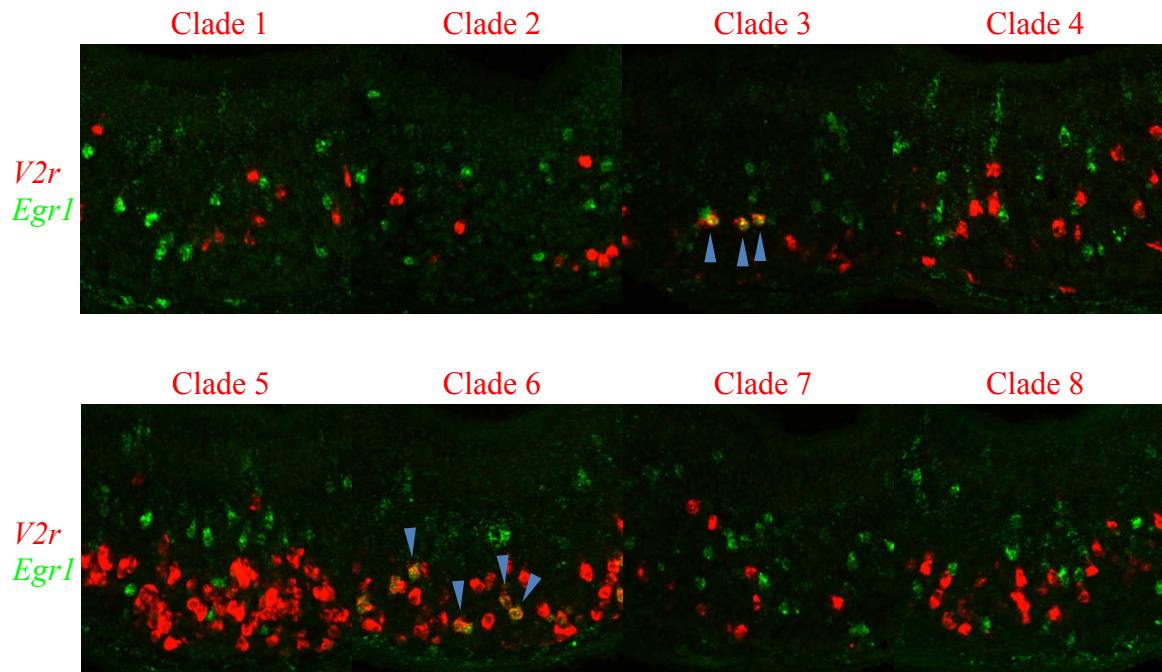


Figure 3.4: Mapping with probes against eight receptor clades in V2R family.

Co-labeling was found in V2r Clade 3 and Clade 6. Arrowheads indicate overlap between *V2r* and *Egr1*.

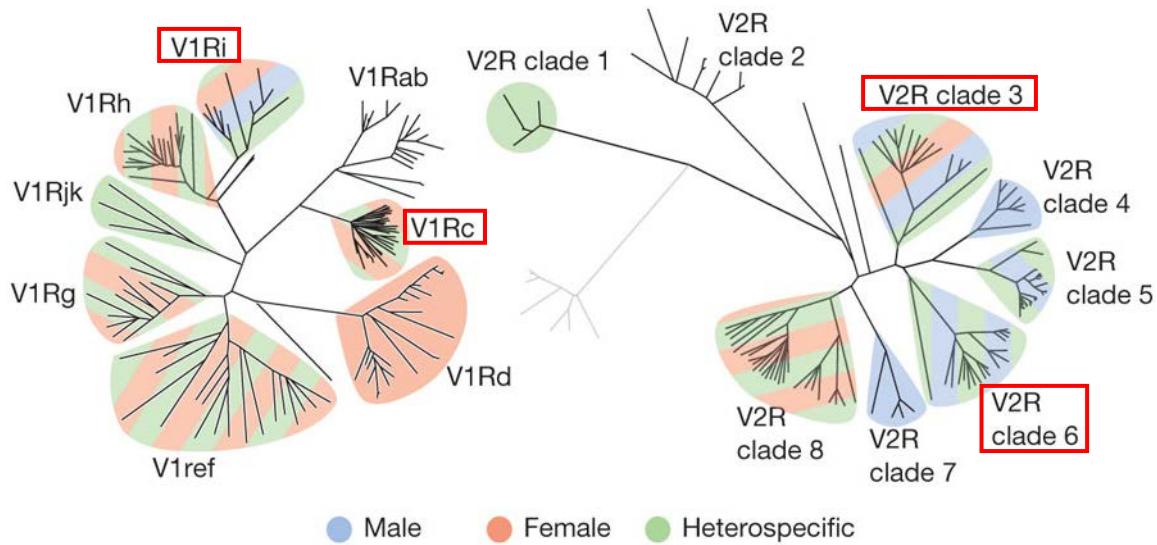


Figure 3.5: Clade-level maps of V1R and V2R activation and the clades that detect pup cues

(Adapted from Isogai et al., 2011)

Clades that detect pups are in red boxes. Detection for male, female or heterospecific cues is color coded. Hatched patterns indicate responses to multiple types of cues.

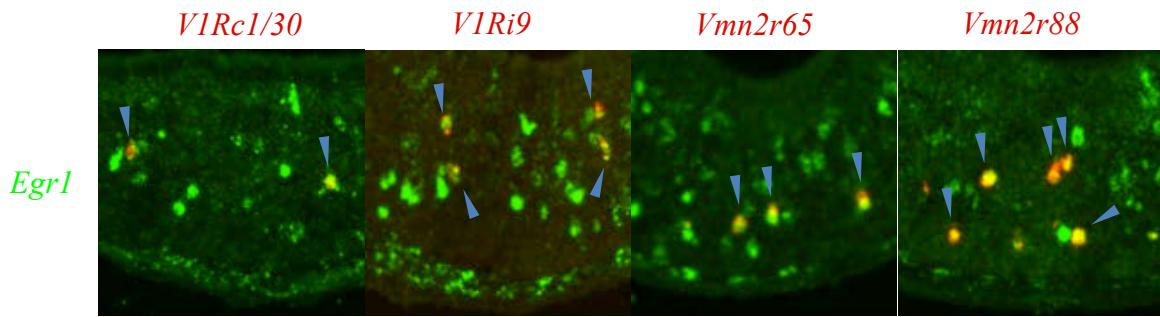


Figure 3.6: VR Mapping with probes against single receptors.

Co-labeling was found in *V1rc1/30* and *V1ri9* of the *V1R* family and *Vmn2r65* and *Vmn2r88* of the *V2R* family. Arrowheads indicate overlap between *Vr* and *Egr1*.

To verify the function of these putative pup receptors and to study how single receptor affects the detection of sensory stimuli and parental behavior, the two V2R receptors, Vmn2r88 and Vmn2r65, were chosen to generate receptor knockouts. Vmn2r88 and Vmn2r65 knockout mice were generated using a λ phage-mediated recombineering approach (Materials and Methods; Figures 3.7, 3.8). Since the seven-transmembrane domain is critical for the membrane expression and the function of vomeronasal receptors (Dulac and Torello, 2003), knockouts were generated by deleting the genomic region containing exons encoding the seven-transmembrane. The germ line transmission for both animals was confirmed by PCR amplification of the knockout loci. Knockout animals are viable and fertile and do not appear to exhibit obvious behavior deficits. The characterization of the behavior of the receptor knockouts is in progress.

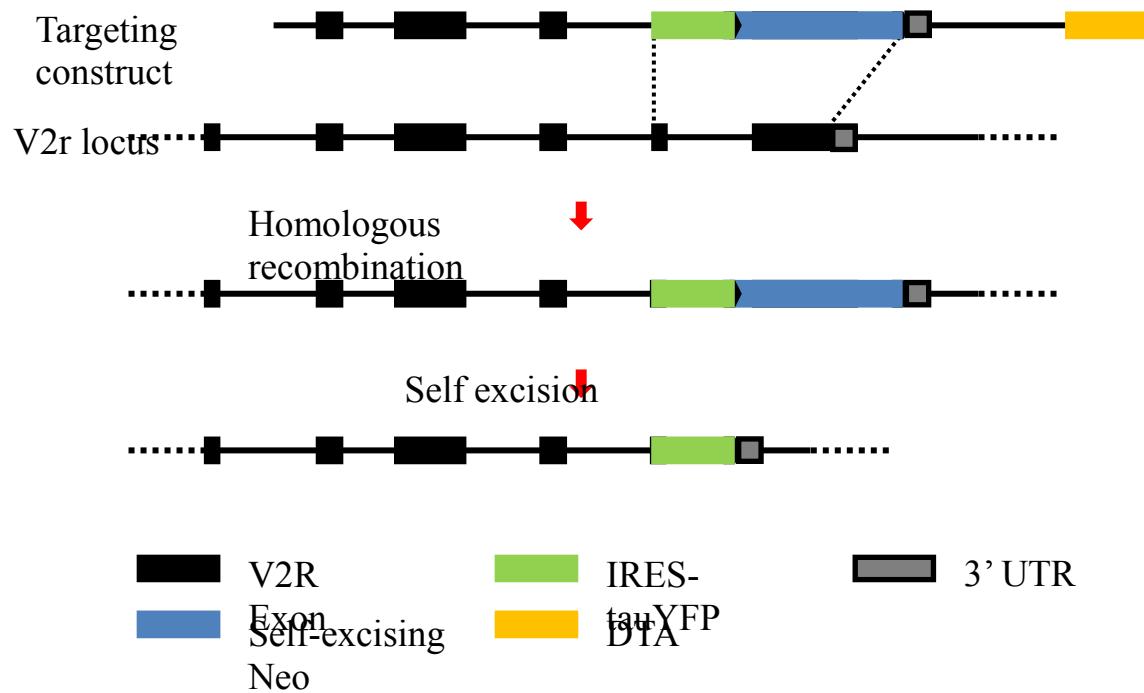


Figure 3.7: Schematic illustration of the knockout strategy for Vmn2r88

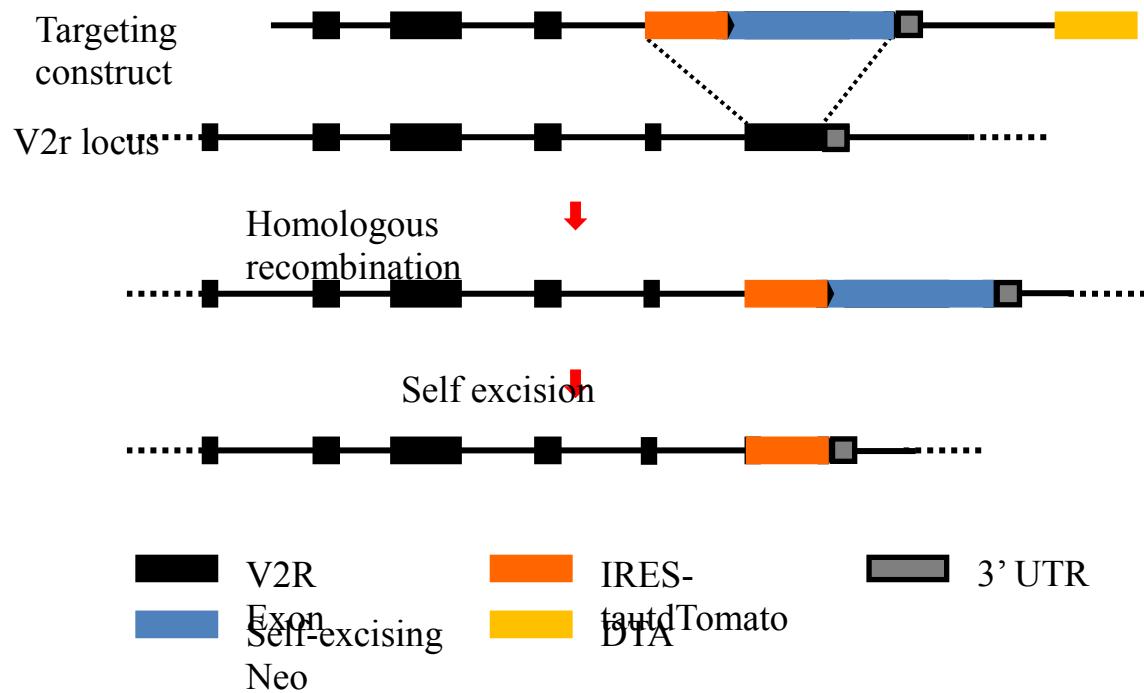


Figure 3.8: Schematic illustration of the knockout strategy for Vmn2r65

Discussion

By characterizing the vomeronasal receptors that detect pup cues, we have shown that pup stimuli preferentially activate vomeronasal receptor neurons in virgin males, consistent with the behavior phenotype of *Trpc2*^{-/-} animals. Further, we have identified three putative pup receptors and one unresolved receptor group, of which two belongs to the V1R family and two belongs to the V2R family. Because individual V1Rs tend to detect the physiological status of an animal and V2Rs encode information about the identity of emitters, activation of both V1Rs and V2Rs by pup stimuli suggests that pup cues are a complex chemical mixture with various components indicating the nature of the stimulus.

In addition, although the combination of these receptors is unique, none of the single receptors appear to be dedicated to pup stimuli: V1rc1/30 is also activated by mammalian non-predators, V1ri9 is activated by *M. Spicilegus*, Vmn2r65 is activated by adult female cues and Vmn2r88 is activated by adult male cues. Since pups are under constant exposure to their mothers, it is possible that they may utilize or carry over some of the adult signals, especially those of adult females which activate Vmn2r65. If this is the case, it presents an interesting phenomenon that individuals use foreign but physiologically relevant odors to represent their own identity.

Because pup cues activate around 4% of the VNO receptor neurons in males, and the eight receptors represent approximate 3% of all the receptor populations (Vmn2r has an usually high representation of 1.5%), we may have identified most of the receptors

activated by pup cues. It is possible that other pup receptors exist besides these receptors that we identified so far, since our probe set may not cover the entire repertoire of the vomeronasal receptor genes.

The necessity of two V2R receptors in pup-directed behaviors is now being tested in both males and female knockout animals. Because the VNO and the downstream pathway are highly activated in infanticidal males, we expect that male infanticide behavior may be preferentially affected by the deletion of these two V2R alleles. It is possible that each pup receptor is necessary for infanticide behavior and therefore a single knockout will cause behavioral defects. However, these receptors could also act in an additive, redundant fashion, in which case we can only observe a phenotype in the double receptor knockout, or when even more pup receptors genes are deleted. Meanwhile, a complimentary approach using VR-driven ChR2 expression and light-mediated activation of the pup receptors, could potentially address the issue of sufficiency of these receptors for parental behavior or infanticide. Once the role of the receptors are confirmed, the specific downstream targets of these receptors can also be traced by ChR2-assisted mapping, which will provide an entry point for the dissection of the central circuits regulating infanticide and parental behavior. The exact role of these receptors in pup detection and the mechanism with which these receptors mediate parental behavior and infanticide still await further investigation.

Materials and Methods

mRNA in situ hybridization and image analysis

Fluorescent mRNA in situ hybridization was performed as described (Isogai et al., 2011). The dissected VNOs were embedded in OCT (Tissue-Tek) and frozen with dry ice. 16 μ m cryosections were used for RNA in situ hybridization at 68°C. The probes were detected using horseradish peroxidase (POD)-conjugated antibodies (anti-FITC-POD, Roche; anti-DIG-POD, Roche). The signals were amplified using TSA Biotin plus kit (PerkinElmer) and subsequently visualized with Alexa Fluor 488-conjugated streptavidin (Invitrogen). Tissues were mounted with Vectashield (Vector labs) containing 8 μ g/ml DAPI. All the microscopy images were acquired using LSM510 or AxioImager Z2 (Zeiss). For single color in situ hybridization images, quantitation was conducted using a minimum of 10 VNO sections per animal and a minimum number of 3 animals. Because 0.2 mm² represents average area of medial cryostat sections of the VNO and contains approximately 1,000 VNO cells, we used the number of *Egr1*+ cells per 0.2 mm² as the unit for cell counting. For dual color in situ hybridization, we quantified the co-localization of *Egr1* and receptor signals over four sections per VNO, for a minimum of three animals.

Generation of the receptor knockout animals

A λ phage-mediated recombineering approach was used to generate the Vmn2r65 and Vmn2r88 receptor knockouts. Vmn2r88 gene fragment was retrieved from BAC clone

RP24-161J9 of a C57BL/6J mouse BAC library. A 2.2kb sequence including Exon 5 and Exon 6 which encodes the transmembrane domain was deleted and replaced by IRES-tau-YFP-ACN. Plasmid PL611 was used to retrieving the targeting construct with a 5.8kb long arm and a 3.2kb short arm. Electroporation of ES cells and drug-selection were performed by the Genome Modification Facility (GMF) at Harvard University: Linearized targeting construct was electroporated into 129 ES cells and positive clones were selected by neomycin resistance. Two positive clones carrying a targeted allele were injected into C57BL/6J blastocysts to produce male germline chimeras. These chimeras were crossed to C57BL/6J females, and germ line transmitted animals were selected by PCR verification of the knockout locus. Using a similar approach, Vmn2r65 gene fragment was retrieved from BAC clone RP23-221021. Exon 6 which encodes the transmembrane domain was deleted and replaced by IRES-tau-tdTomato-ACN. Targeting construct has a 5.6kb long arm and a 2.7kb short arm.

Chapter IV. Characterization of the central circuits underlying parental care and infanticide

Introduction

The characterization of the central circuits underlying social behavior aims to define the relevant neural populations and brain regions, to characterize their cellular property and connectivity, and to understand how they process pertinent sensory stimuli, respond to hormonal and homeostatic regulation and generate appropriate behavioral and endocrinological responses to conspecific stimuli. Historically, the dissection of central circuits controlling social behavior has followed three distinct but inter-related routes: characterization of brain regions under steroid hormone regulation, identification of areas activated by social cues, and direct brain electrical/chemical stimulation to elicit or block social responses, all of which have provided valuable insights regarding the neural mechanism of social behavior.

Steroid hormones have been shown to play critical roles in establishing anatomical and functional sex dimorphism that is required for the sex specificity of social behavior, and the identification of brain regions sensitive to steroid hormones has provided a natural point of entry into central circuits underlying social behavior (Cooke et al., 1998). The sex steroids' organizational of the brain in a way such that perinatal exposure to high levels of estrogen, converted by an enzyme aromatase from testosterone, masculinizes the brain and specifies the neuronal circuit for male-typical anatomy and behavior, whereas the lack of exposure leads to the development of a female-typical circuit. A series of

areas have been identified by examining the anatomical dimorphism and the expression of estrogen receptors, androgen receptor and aromatase. These areas include the medial preoptic area (MPOA), the anteroventral periventricular nucleus (AVPe), the medial amygdala (MeA), the posterodorsal medial amygdala (MePD), the bed nucleus of stria terminalis (BNST) and the lateral septum (LS), all of which are part of the brain limbic system (Morris et al., 2004; Segovia and Guillamón, 1993b, 1996). Indeed, many of these areas are shown to be critically involved in social behaviors. For example, lesion of MPOA drastically disrupts male copulatory behavior (Arendash and Gorski, 1983a). Bilateral lesion of the MeA in rats causes a significant increase in ejaculatory latency, although copulatory behavior is not abolished (Perkins et al., 1980). Lesion of a subnucleus of the MPOA, the sexually dimorphic nucleus of the preoptic area (SDN-POA), induces lordosis in male rats treated with estrogen and progesterone (Hennessey et al., 1986).

By genetically modifying androgen receptor, aromatase and progesterone receptor gene loci, recent advances have allowed mouse geneticists to visualize the distribution and projection of these areas, and revealed novel sites of sexual dimorphism in the brain (Shah et al., 2004; Wu et al., 2009; Yang et al., 2013). For example, several clusters of androgen receptor-expressing neurons in the basal forebrain were found to have an elevated number in males than in females (Shah et al., 2004). In addition, more aromatase-expressing cells are observed in the caudal hypothalamus in females compared with that in males, while an opposite expression bias towards male was found in the BNST and the MeA (Wu et al., 2009).

Genetic manipulation of hormone receptors as well as corresponding brain areas also provides further insights regarding the central circuits of reproductive social behaviors. For example, although androgen receptor knockout out mice exhibited striking deficits in specific components of sexual and territorial displays, these behaviors were found to be overall male-specific. This result confirmed that estrogen converted from testosterone masculinizes the brain and behavior, and showed that androgen controls the levels of male behavioral displays through androgen receptor (Juntti et al., 2010). Further, when a genetically defined population in the VMHvl, the progesterone receptor-expressing cells, are ablated genetically by caspase 3-mediated apoptosis, females showed diminished sexual receptivity whereas males exhibited defects in both mating and aggression (Yang et al., 2013), indicating the same brain area is able to controls distinct behavior in either sexes.

Social cues activate their corresponding sensory areas and a variety of hypothalamic areas, which provide another point of entry for the central circuits of social behavior. Not surprisingly, many of these areas exhibit anatomical sexual dimorphism and are rigorously modulated by sex hormones as well as neuropeptides (Segovia and Guillamón, 1993b, 1996). Immediate early gene mapping has been particularly informative in the survey of active brain areas involved in a certain behavior. Immediate early genes are a class of genes whose expression is transiently induced by a wide range of stimuli, and can be used as an approximate marker for neural activity (Sheng and Greenberg, 1990). For example, c-Fos activity mapping has implicated the MPOA/vBNST in the positive regulation of maternal behavior as well as identified other areas that may inhibit maternal response in rats (Numan et al., 1998; Sheehan et al., 2000). Recently, by using c-Fos

immunostaining, ESP1 is found to induce elevated activity in cells of the MPOA and the VMH, but not in V2rp5-deficient mice which cannot detect ESP1, corroborating the observation that ESP1 is involved female sexual behavior (Haga et al., 2010). In particular, using cellular compartment analysis of temporal activity by fluorescent in situ hybridization (catFISH), Lin et al. compared *c-fos* expression during consecutive mating and fighting displays in the same animal. It was found that sexual and aggressive behaviors recruit overlapping but distinct sets of neurons in brain regions including the VMHvl, ventral premammillary nucleus (PMv), and the posteroverentral and posterodorsal medial amygdala (Lin et al., 2011).

Because the vomeronasal pathway plays a key role in the control of social reproductive behavior, brain areas receiving vomeronasal inputs, including the MeA, the BAOT, the BNST, the PMCo and the MPOA, the VMH, the AHN, the PMd of the hypothalamus were explored in detail. For example, lesion of the medial part of the BNST causes defects in copulatory behavior in male rats, such as increased length of intromission intervals and post-ejaculatory refractory periods (Emery and Sachs, 1976). Lesion of the BAOT significantly reduces the latency for induction of maternal behavior in both naïve male and female rats (Del Cerro et al., 1991; Izquierdo et al., 1992).

From a gain-of-function perspective, direct stimulation of brain areas has provided a unique approach in the dissection of brain circuits and identified discrete brain regions whose activation results in the display of specific behavior. Perhaps one of the most famous example of brain stimulation comes from the classical experiment by Odds and Milner, in which electric stimulation of the septal area was found to induce positive

reinforcement learning in rats (Olds and Milner, 1954). In the study of reproductive social behavior, various brain stimulation experiments have been shown to induce diverse responses such as sexual behavior, aggression, and maternal responses. Electrical stimulation in the anterior dorsolateral hypothalamus was found to produce a significant increase in sexual capacity of male rats (Vaughan and Fisher, 1962). Electric stimulation of the MPOA was also shown to facilitate sexual behavior in male rats, by eliciting a reduction in both the number of mounts and intromissions preceding ejaculation, and in some animals a shorter latency to approach and mounting of the female (Malsbury, 1971). In addition, stimulation of various distinct hypothalamic sites induced a range of behavior response including attack, social grooming and teeth-chattering (Lammers et al., 1988). Further, maternal behavior has been elicited during chemical stimulation with sodium testosterone sulfate in an area roughly identified as the MPOA. Interestingly, chemical stimulation induced or accentuated all components of maternal behavior, including a persistent retrieving and pup grooming (Fisher, 1956).

In recent years, the discovery and modification of light-activated rhodopsins have provided a cell-type specific and temporally precise method for neural activation as well as silencing (Boyden et al., 2005). This approach has been applied in the dissection of a variety of neural circuits including those of feeding, sleep/awake regulation, anxiety and reward (Adamantidis et al., 2007; Atasoy et al., 2012; Jennings et al., 2013; Tye et al., 2011). However, its use has been rather scarce in the study of social behavior, except in one case where localized, though non cell-specific optogenetic stimulation was found to potently induce aggressive behavior (Lin et al., 2011). Interestingly, this study found that

optogenetic, but not electrical, stimulation of the VMHvl induces violent aggressive display even towards females and inanimate objects.

Using some of these approaches, previous studies have led to many insights into the neural circuits of parental behavior. In female rats and mice, the MPOA in the rostral hypothalamus and the dopamine system have been implicated in the control of maternal behavior (Brown et al., 1996; Calamandrei and Keverne, 1994; Numan, 1974; Numan and Stolzenberg, 2009; Tsuneoka et al., 2013). Meanwhile, opposing the effect of the facilitative role of the MPOA and its downstream pathway, a few other brain areas were found to be active in the aversive encounter of virgin female rats with foster pups including the MeA, the AHN and the PAG (Sheehan et al., 2000). However, the exact neural populations and the mechanisms that underlie the distinct behaviors of males and females, and of males with different social experiences, remain largely unknown. The identification of neuronal circuits controlling the ability of males and females to display parental behavior should help elucidate the basic neural mechanisms underlying an essential social behavior and provide novel insights into the regulation of sexually dimorphic brain functions.

In this chapter, I aim to identify and characterize genetically defined neuron population(s) that controls parental behavior. Using genetic methods to specific ablate or to activate these neuron population(s), I wish to confirm their causal role in the behavior control and provide further insights about the neural mechanism of parental behavior and infanticide.

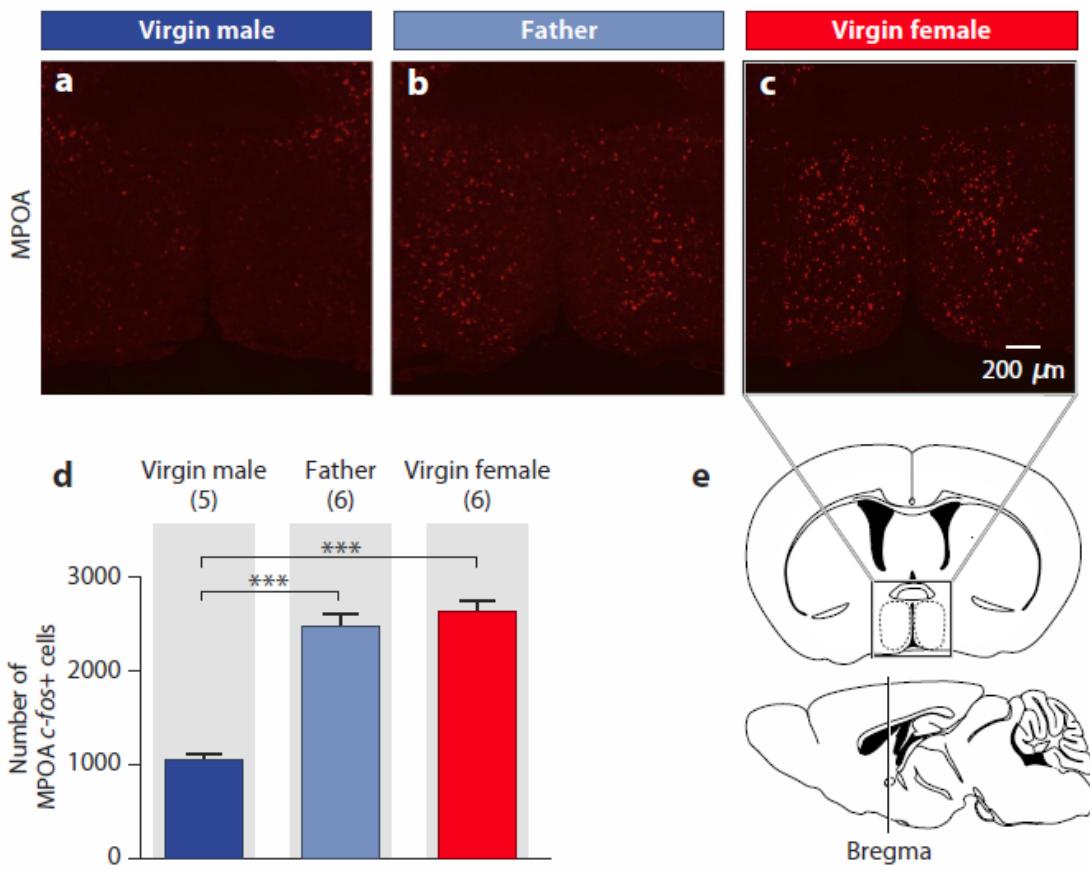
Results

Neuronal activation during parenting

To identify brain regions involved in the regulation of parental care, we compared the brain activity patterns of infanticidal virgin males versus maternal virgin females and paternal fathers using the induction of the immediate early gene *c-fos* as readout of neuronal activation. Male and female mice were exposed to C57BL/6J pups and allowed to freely interact with the pups, sacrificed 35 minutes after the onset of behavior, and brains were analyzed for *c-fos* expression using *in situ* hybridization. We focused our analysis on hypothalamus, amygdala and other regions known to be involved in the control of innate behaviors, including the MPOA, the AVPe, the BNST, the MeA, the PMCo, the NAc, the VMH, the PVN, the lateral septal nucleus, the suprachiasmatic nucleus, anterior basomedial nucleus and dorsomedial hypothalamic nucleus.

Fathers and virgin females activated similar brain areas after parental care, and the activation of these areas remains reduced or diminished in virgin males. In particular, we observed a striking increase in the number of *c-fos*+ cells in the MPOA of maternal virgin females and paternal fathers compared to infanticidal virgin males (Figure 4.1). The ventral BNST/dorsal MPOA is thought to play an important role in maternal behavior in rats and mice (Calamandrei and Keverne, 1994; Numan and Stolzenberg, 2009). In addition, this brain region is involved in several other functions such as the control of male copulatory behavior (Arendash and Gorski, 1983b; Dominguez and Hull, 2005), thermoregulation (McAllen et al., 2010), and GnRH secretion (Jennes and Conn, 1994). Accordingly, the MPOA is a highly heterogeneous structure (Simerly et al., 1986), which

receives and sends inputs from and to a large number of brain regions (Simerly and Swanson, 1986, 1988). The identity of the cell population governing parental behavior is unknown.



The activated MPOA cells were characterized using double fluorescent *in situ* hybridization with *c-fos* and a series of molecular markers (for a list of genes, please see Materials and Methods), including hormone receptors, neuropeptides, genes associated with available Cre lines and with distinct MPOA expression according to the Allen Brain Atlas (Lein et al., 2007). Many genes with sparse and distinct expression patterns rarely overlap with *c-fos*, whereas genes that overlap with *c-fos* tend to be expressed extensively in large percentages of neurons for a given area. From this search, we uncovered the neuropeptide galanin (*Gal*) as a candidate marker for the *c-fos*⁺ cells in the MPOA of both parental males and virgin females: $51.7\% \pm 1.4\%$ of MPOA *c-fos*⁺ cells in females and $39.3\% \pm 1.5\%$ in fathers also express *Gal* (Mean \pm SEM, paired t test, $P < 0.001$; Figure 4.2). Further, $39.1\% \pm 2.3\%$ of MPOA *Gal*⁺ cells in females and $25.1\% \pm 2.8\%$ in fathers also express *c-fos* (Mean \pm SEM, paired t test, $P < 0.001$ for females, $P < 0.05$ for fathers; Figure 4.2a, 2.2b, 2.2d). In contrast, significantly less *c-fos* expression was identified in virgin males compared to females or fathers that were exposed to pups. Only $11.2\% \pm 1.2\%$ of MPOA *Gal*⁺ cells in virgin males express *c-fos*, which is not significantly different from chance level (Mean \pm SEM, paired t test, $P > 0.05$; Figure 4.2d).

Interestingly, we identified a mild but significant sexually dimorphic expression of *Gal* by mRNA *in situ* hybridization (Figure 4.3): *Gal*⁺ cells in the MPOA of virgin females are 18.0% less than those of virgin males and 12.9% less than those of fathers. *Gal*⁺ cell numbers do not differ significantly in virgin males and fathers, suggesting sexual experience or pup exposure does not affect the number of MPOA *Gal*⁺ cells per se.

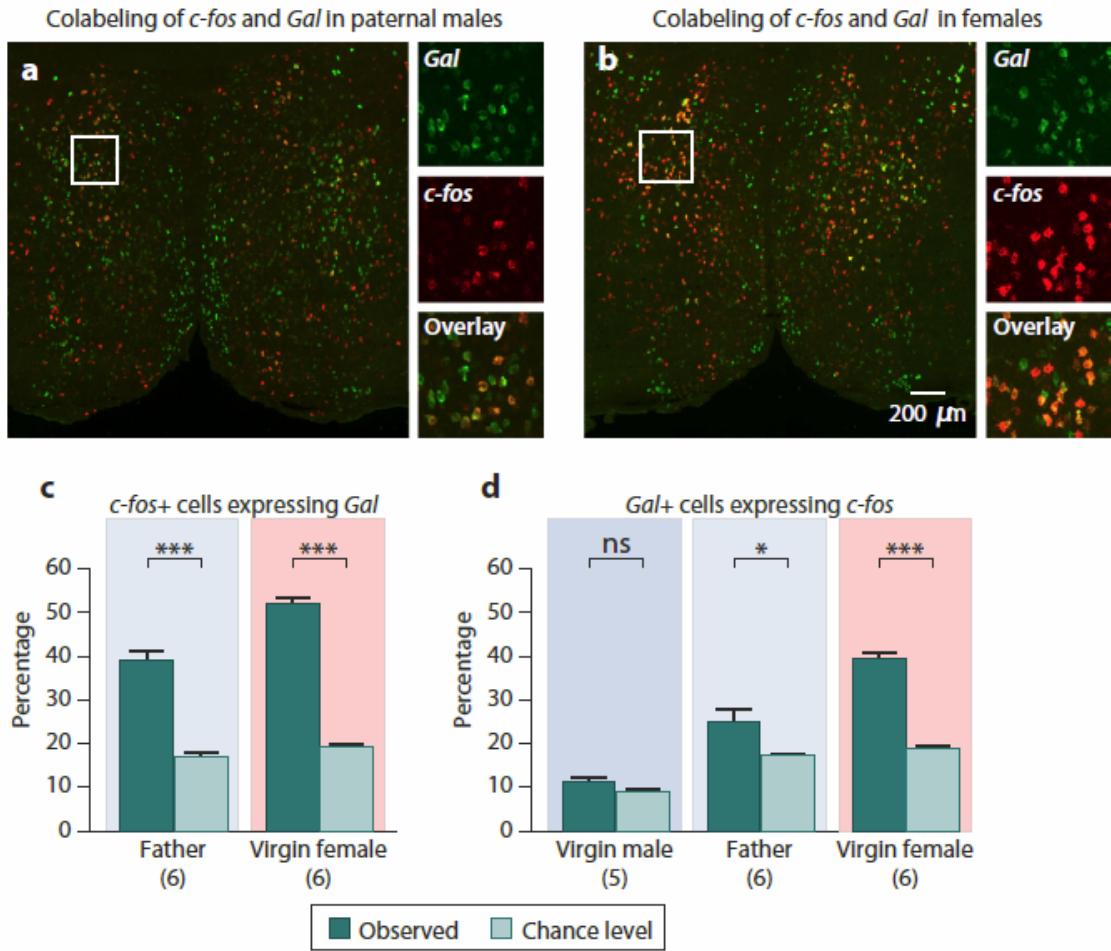


Figure 4.2: galanin (*Gal*) is a candidate marker for the *c-fos*+ cells in the MPOA of both parental males and virgin females

a, b, Colabeling of *c-fos* and *Gal* shown by *in situ* hybridization in the MPOA of fathers and virgin females after interaction with pups.

c, d, Percentage of *c-fos*+ cells expressing *Gal* and percentage of *Gal*+ cells expressing *c-fos* in virgin males, fathers and virgin females compared to calculated chance level. Mean+SEM, paired t test, * $P<0.05$, *** $P<0.001$, ns. not significant. The chance level is calculated as $N_{Gal\ cell} \cdot N_{c-fos\ cell}$ cell / ($N_{Gal\ cell} + N_{c-fos\ cell}$)² for each animal.

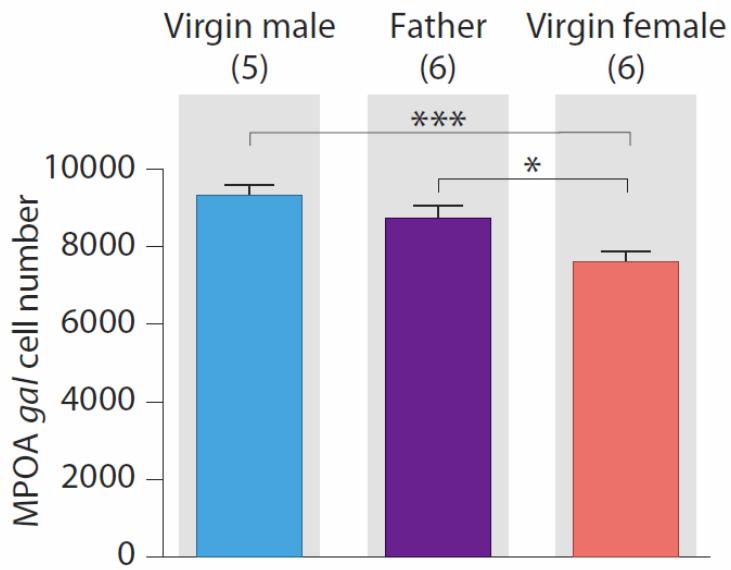


Figure 4.3: sexual dimorphism expression of galanin (*Gal*) in the MPOA of Virgin males, fathers and virgin females.

Mean+SEM, one-way ANOVA followed by Bonferroni's multiple comparison, $*P<0.05$,

$***P<0.001$.

Galanin is expressed in several brain areas, and has been involved in the modulation of multiple physiological functions including nociception, sleep, feeding and energy balance, thermoregulation, osmotic regulation, water intake, and reproduction (Mechenthaler, 2008). Galanin is also co-expressed by prolactin-secreting cells in the pituitary gland and is involved in the control of lactation (Wynick et al., 1998). Most *c-fos*⁺ and *Gal*⁺ cells in the MPOA express *Gad1* (Figure 4.4), indicating that they are likely GABAergic inhibitory neurons.

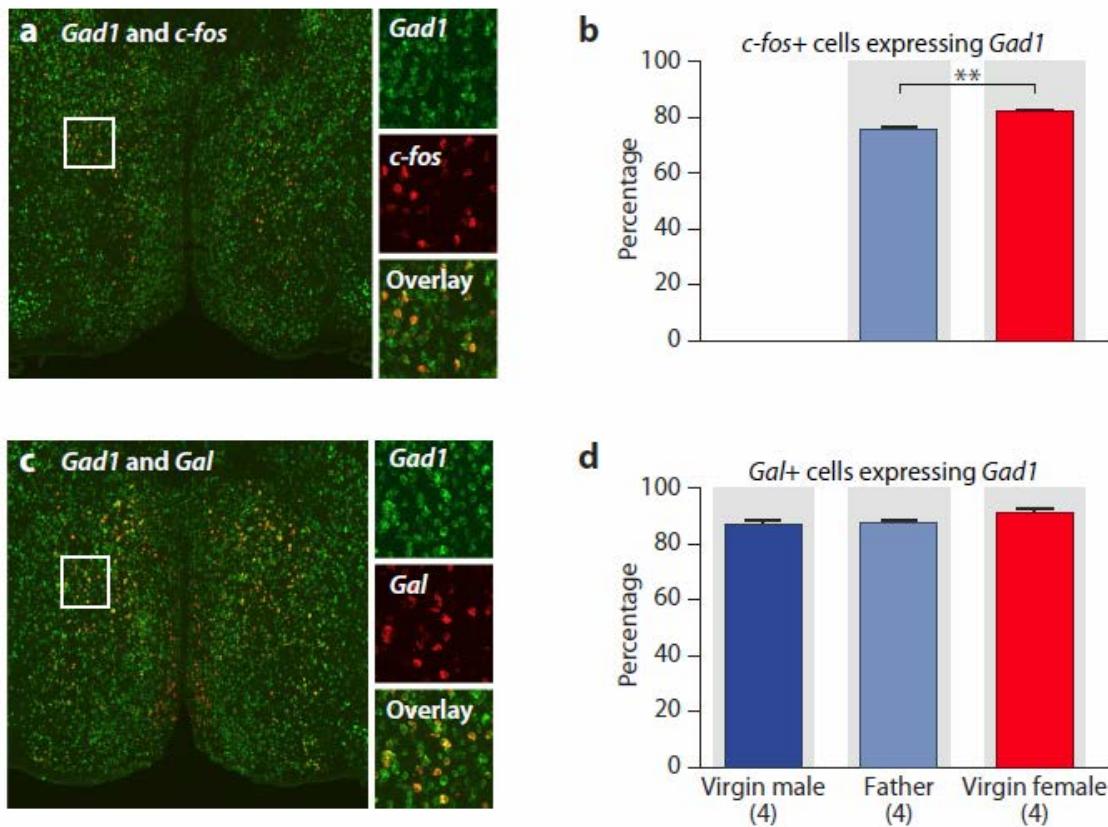


Figure 4.4: Most *c-fos*+ and *Gal*+ cells in the MPOA express *Gad1*

a, *Gad1* and *c-fos* mRNA double *in situ* hybridization in a virgin female after interaction with pups.

b, Percentages of *c-fos*+ cells expressing *Gad1* in fathers and virgin females. Mean+SEM, t test, **P<0.01

c, *Gad1* and *Gal* mRNA double *in situ* hybridization in a virgin female.

d, Percentages of *Gal*+ cells expressing *Gad1* in virgin males, fathers and virgin females.

Mean+SEM, one-way ANOVA, P>0.1.

Ablation of MPOA Gal+ neurons

We next aimed to investigate the requirement of the MPOA *Gal*+ neurons in the control of parental behavior of virgin females, and of sexually experienced males. To specifically ablate MPOA *Gal*+ neurons, we used a BAC transgenic mouse line in which a Cre recombinase cassette followed by a polyadenylation sequence is inserted at the ATG codon of the first coding exon of the *Gal* gene. Gal-Cre mice were injected bilaterally in the MPOA with a recombinant adeno-associated viral vector expressing a Cre-dependent diphtheria toxin A fragment (AAV-DTA) (Figure 4.5). Gal-Cre negative littermates receiving the same treatment were used as controls. On average, AAV-DTA eliminated around 60% of the *Gal*+ cells in the MPOA compared to average controls (Figure 4.6a, 2.6b). We verified that an independent MPOA cell population expressing the gene encoding the thyrotropin-releasing-hormone (*Trh*) was not affected by the targeted ablation (Figure 4.6c).

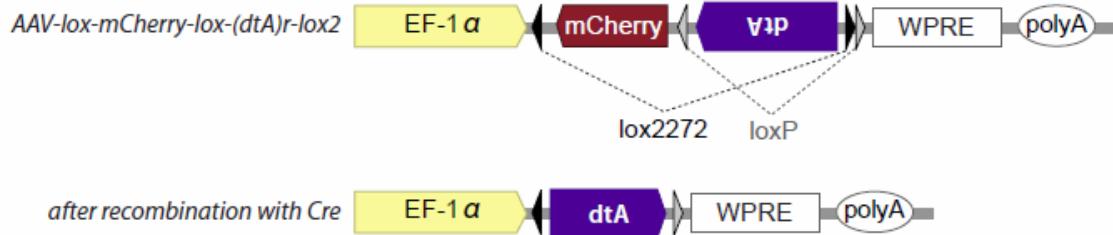


Figure 4.5: Schematic illustration of the Cre-dependent AAV-DTA construct

DTA is doubly flanked by two sets of incompatible lox sites and inverted to enable transcription after Cre-mediated recombination. Note that mCherry is expressed in all viral infected cells in a non-conditional fashion.

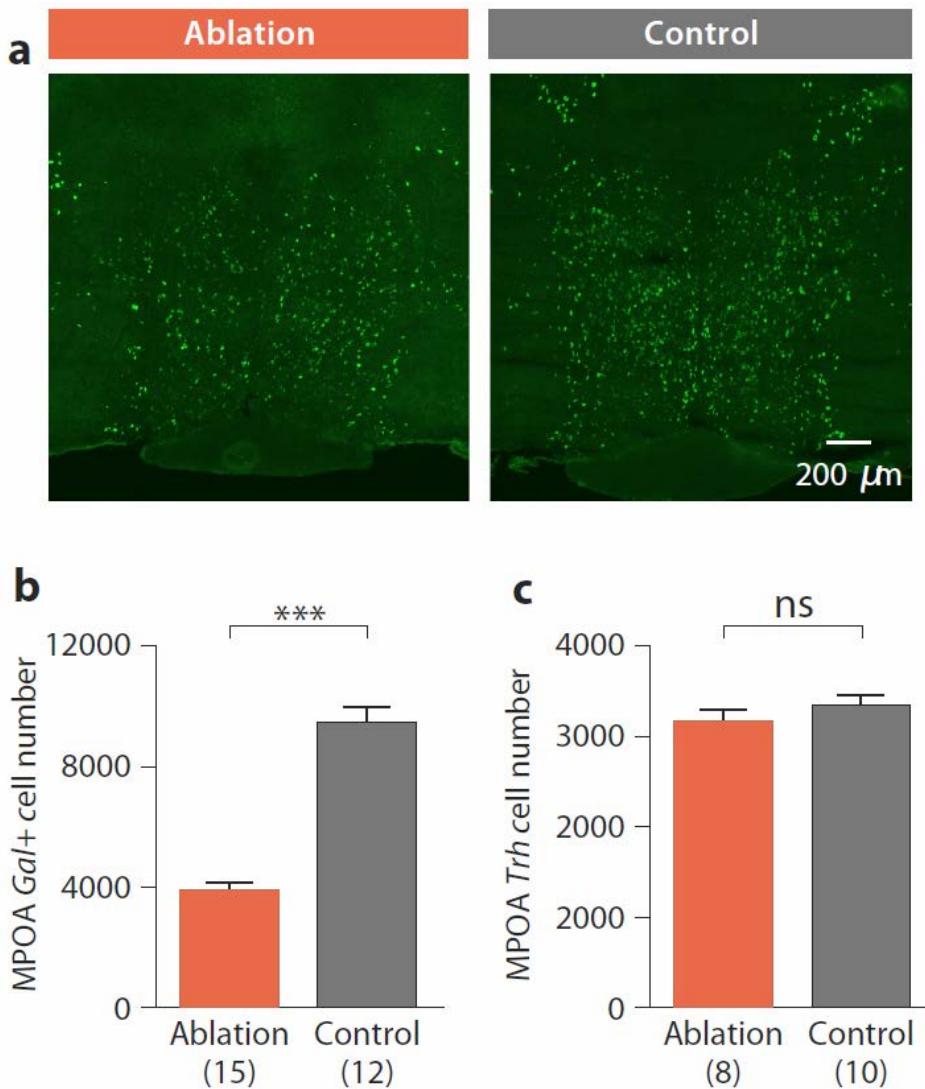


Figure 4.6: AAV-DTA specifically ablates ~60% of the *Gal*+ cells in the MPOA

a, mRNA *in situ* hybridization shows *Gal* expression in the MPOA of ablated and control males

b, Number of MPOA *Gal*+ cells in ablation group compared to controls. Mean+SEM, t test,

$***P < 0.001$

c, Number of MPOA *Trh*+ cells in ablated males and controls. Mean+SEM, t test, $P > 0.2$.

Virgin females showed a striking reduction in maternal behavior and an emergence of infanticidal behavior associated with the loss of MPOA *Gal*⁺ neurons (Figure 4.7). The duration of overall maternal interaction calculated as the cumulative time spent crouching, grooming pups and nest building appeared positively correlated with the number of remaining *Gal*⁺ cells (Figure 4.7a; N=23, P<0.05, R=0.46). Moreover, a global scoring of female behavior with pups showed that virgin females with low ablation efficiencies of MPOA *Gal*⁺ cells were maternal, while ablation efficiencies above 50% were associated with loss of maternal care and emergence of infanticidal behavior (Figure 4.7b). Detailed analysis of maternal behavior in virgin females in which at least 50% *Gal*⁺ neurons had been ablated showed that crouching, nest building, retrieval to nest and overall maternal interaction were significantly reduced compared to controls (Figures 4.8, 4.9). Thus, MPOA *Gal*⁺ cells represent an essential neuronal population for the proper control of maternal behavior in virgin females.

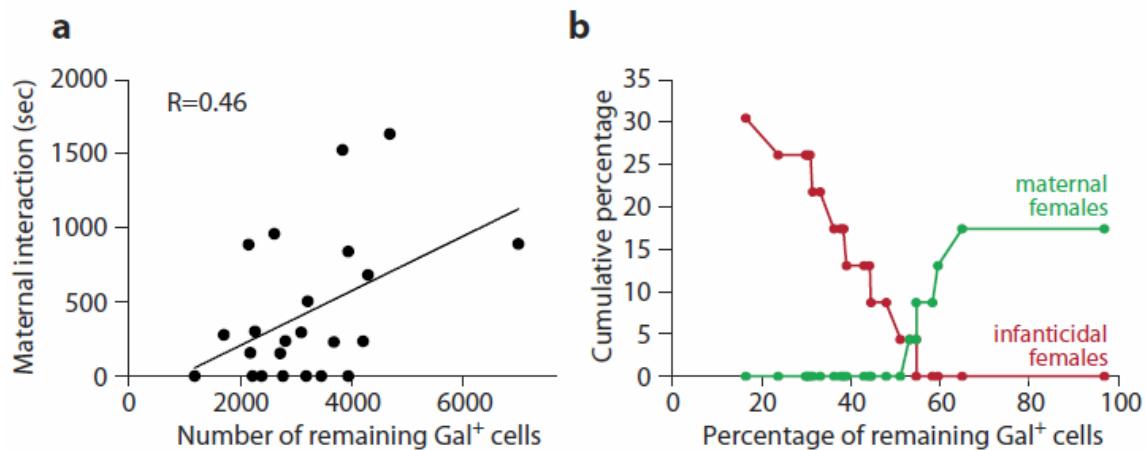


Figure 4.7: Ablation of MPOA *Gal*⁺ neurons impairs maternal behavior in virgin females

a, Linear regression of maternal interaction and the number of remaining MPOA *Gal*⁺ cells in ablated virgin females. Pearson correlation, N=23, $P<0.05$, $R=0.46$.

b, Cumulative percentages of infanticidal and maternal females in relation to percentage of remaining *Gal*⁺ cells, N=23. Reference cell number (100%) is the average MPOA *Gal*⁺ cell number in the control group.

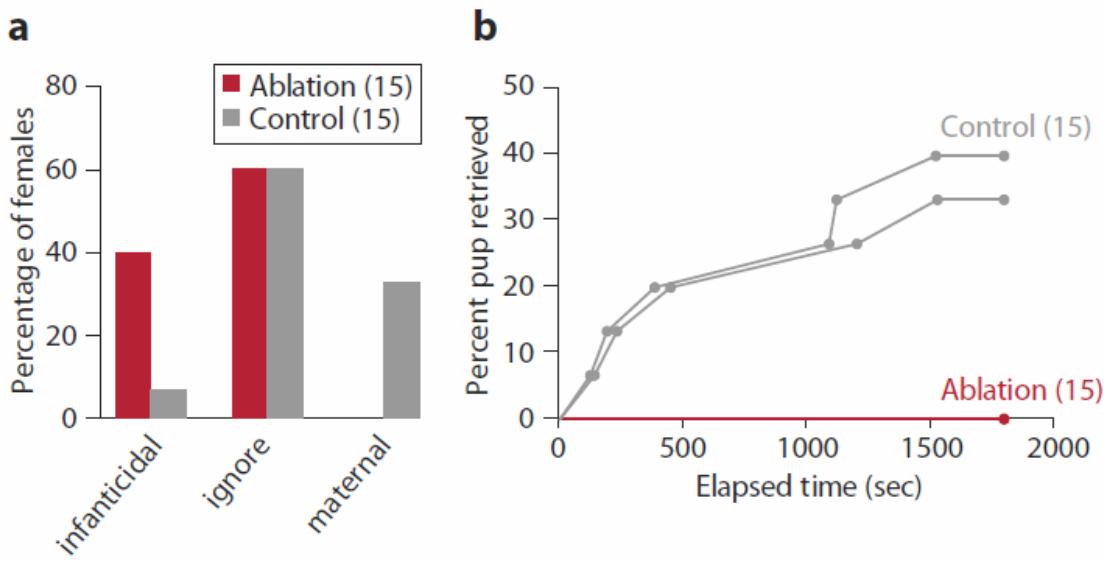


Figure 4.8: Behavior type and pup retrieving of ablated females comparing to controls

a, Behavior type of ablated females with over 50% ablation efficiency (N=15) compared to control (N=15). Chi-square test, $P<0.05$.

b, Every female was presented with two pups. Each curve plots the retrieving latencies of all the animals in one group for one pup on the x-axis, and the cumulative percentage of animals of that group that retrieved the pup on the y-axis. Kolmogorov-Smirnov test, $P=0.14$ for 1st pups, $P=0.31$ for 2nd pups.

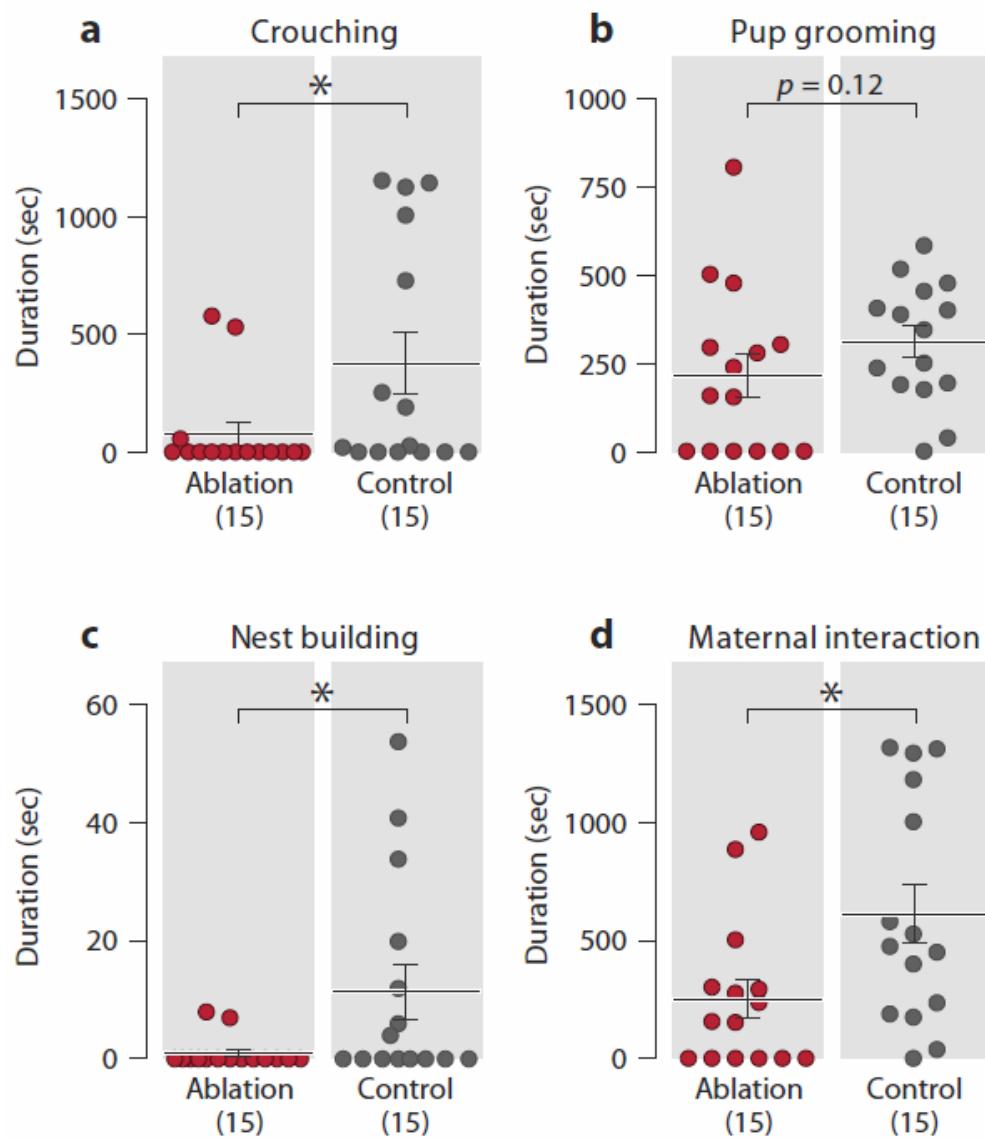


Figure 4.9: Duration of crouching, pup grooming, nest building and maternal interaction in ablated and control groups.

Crouching, nest building and overall maternal interaction were significantly reduced in females with over 50% *Gal*+ cell ablation compared to controls. Mean \pm SEM. Mann-Whitney test,
 $*P<0.05$.

We then tested the requirement of *Gal*⁺ neurons for parental behavior in males. Virgin males were paired with females from one week after injection until pups were born. The males were then individually housed and tested for parental behavior. As with females, the disappearance of parental behavior in males was associated with loss of over 50% of *Gal*⁺ cells (Figure 4.10). Behavior assays in mated males showed that 14.3% of males in which over 50% MPOA *Gal*⁺ neurons were genetically ablated (N=14) displayed paternal behavior 3 weeks after mating, compared to 75% of the littermate controls (N=12; Fisher's exact test, P<0.01; Figure 4.11a). Animals with DTA ablation behaved differently than controls in all measurements of paternal behavior including crouching, pup grooming, nest building, retrieval to nest and overall paternal interaction (Figures 4.11, 4.12).

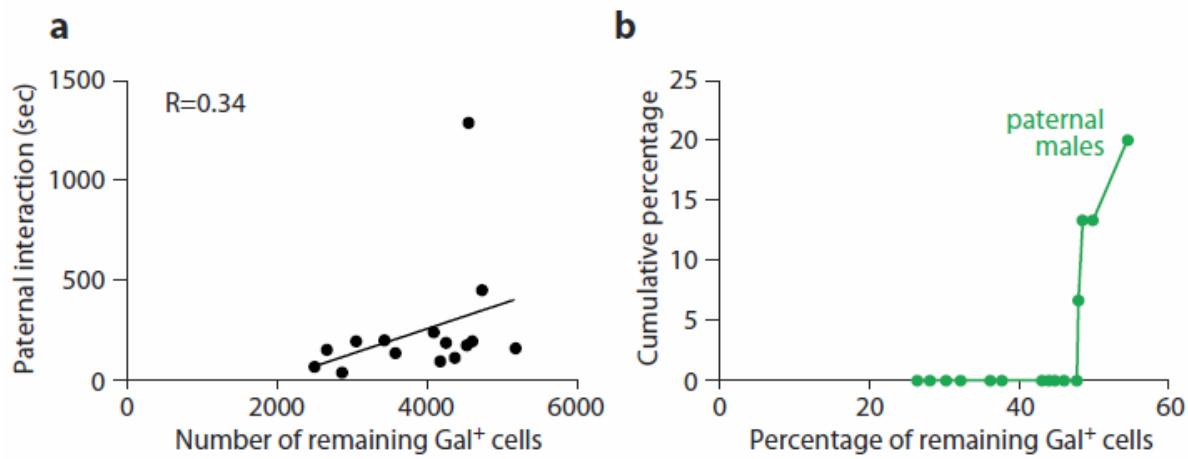


Figure 4.10: Ablation of MPOA Gal⁺ neurons impairs paternal behavior in fathers

a, Linear regression of paternal interaction and the number of remaining Gal⁺ cells in the MPOA in ablated males. Pearson correlation, N=15, $P=0.21$, $R=0.34$.

b, Cumulative percentages of paternal males in relation to the percentages of remaining Gal⁺ cells in ablation group, N=15.

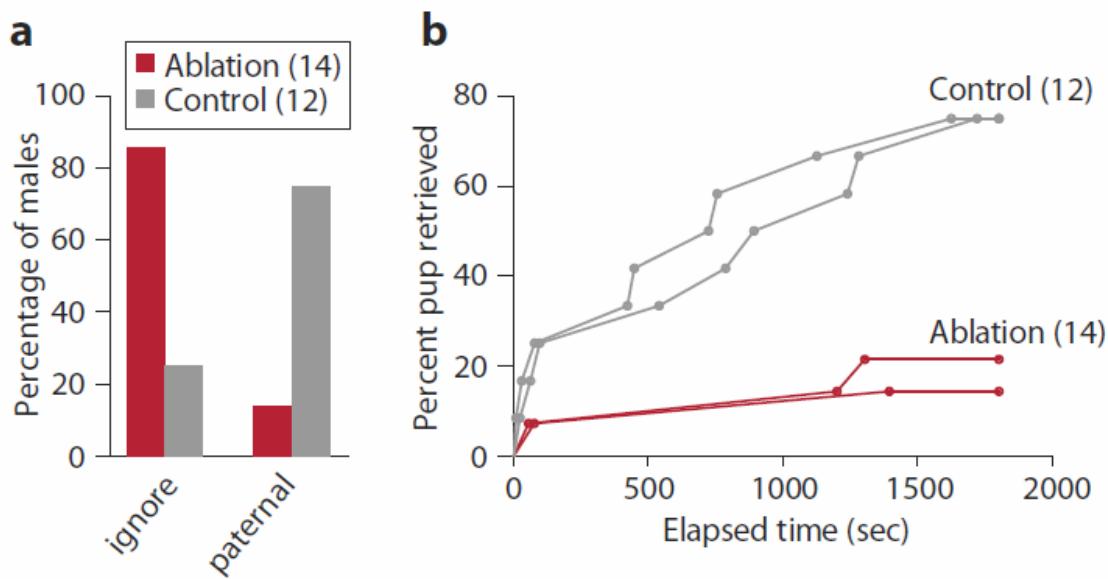


Figure 4.11: Behavior type and pup retrieving of ablated males comparing to controls

a, Behavior type of ablated males with over 50% ablation efficiency (N=14) compared to control (N=12). Fisher's exact test, ** $P<0.01$.

b, Every male was presented with two pups. Each curve plots the retrieving latencies of all the animals in one group for one pup on the x-axis, and the cumulative percentage of animals of that group that retrieved the pup on the y-axis. Kolmogorov-Smirnov test, $P<0.05$ for both pups.

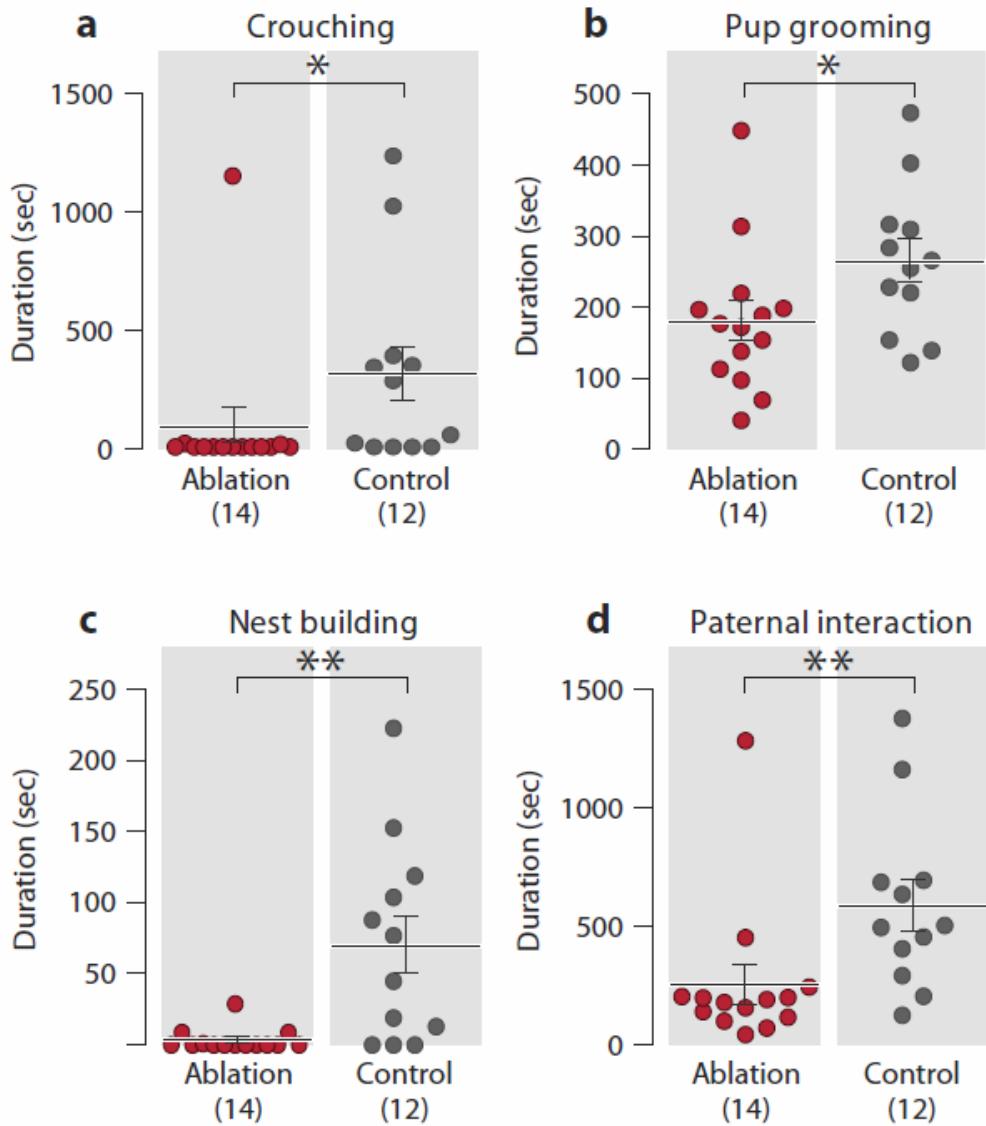


Figure 4.12: Duration of crouching, pup grooming, nest building and paternal interaction in ablated and control male groups.

Crouching, pup grooming, nest building and overall paternal interaction were significantly reduced in males with over 50% *Gal+* cell ablation compared to controls. Mean \pm SEM. Mann-Whitney test, * $P<0.05$, ** $P<0.01$.

These results indicate that MPOA *Gal*⁺ cells are required for the display of parental behavior in both virgin females and sexually experienced males. Remarkably, the specific ablation of *Gal*⁺ cells seems to affect all aspects of parental behavior, including retrieving, crouching, grooming and nest building. However, while a significant fraction of virgin females with strong reduction in *Gal*⁺ neurons displayed infanticide, none of the treated mated males were infanticidal. This result suggests that a reciprocal inhibition of maternal and infanticidal circuits may exist in virgin females, while, in males, mating may unlock *Gal* neuronal activation and independently suppress infanticidal circuits.

Activation of MPOA Gal⁺ neurons

To address whether the activation of MPOA *Gal*⁺ neurons is sufficient for the suppression of infanticide and the display of parental behavior, males were tested for their behavior toward pups while the *Gal*⁺ neurons were activated optogenetically. Gal-Cre virgin males were injected bilaterally with a recombinant AAV expressing Cre-dependent channelrhodopsin-2 fused with enhanced yellow fluorescent protein (AAV-ChR2:EYFP) in the MPOA and implanted with an optic fiber. Negative controls were Gal-Cre negative littermates receiving the same treatment. After recovery, males were tested with C57BL/6J pups. In stimulation trials, blue light was delivered to the MPOA in 30ms pulses at 20Hz for 1-4s each time, with an estimated power output of ~10-20mW at the fiber implant tip, whenever the male touched a pup with its snout. Post-hoc mRNA *in situ* hybridization confirmed specific *ChR2:EYFP* expression in the MPOA *Gal*⁺ cells (Figure 4.13). Strong *c-fos* induction was also observed in MPOA *Gal*⁺ cells of the

Gal::ChR2 males, but not the control males after light stimulation in awake behaving animals (Figure 4.14; $33.5\%\pm3.3\%$ for Gal::ChR2 males, 6 animals; $4.1\%\pm0.2\%$ for controls, 8 animals; Mean \pm SEM, t test, $P<0.001$).

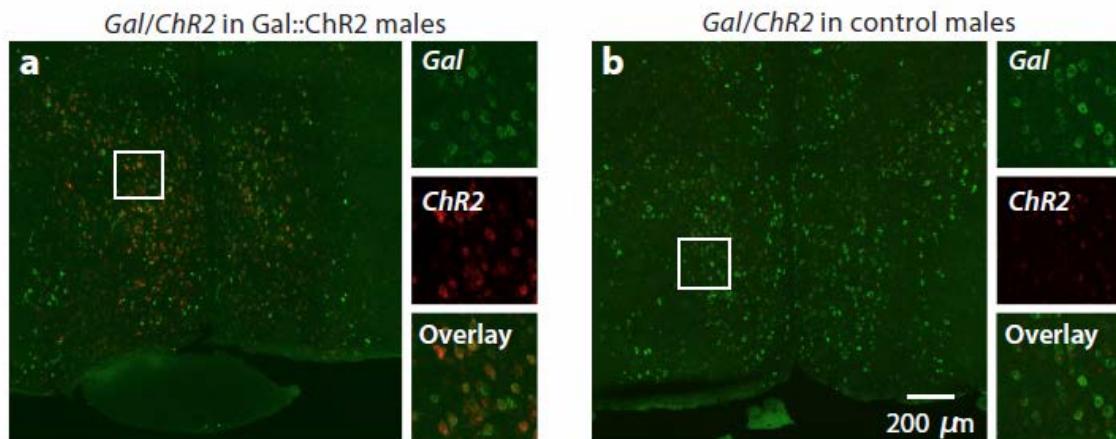


Figure 4.13: Colabeling of *Gal* and *ChR2:EYFP* in the MPOA of the Gal::ChR2 and control males shown by mRNA *in situ* hybridization.

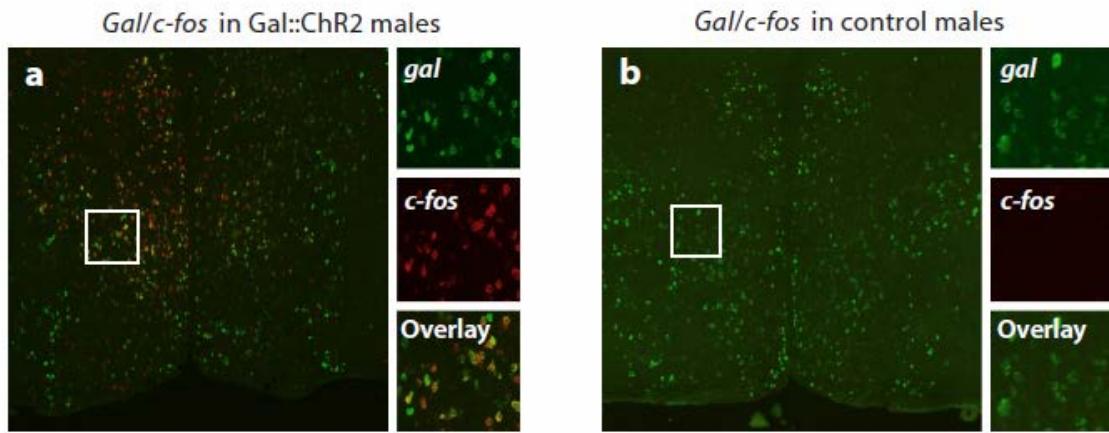


Figure 4.14: Colabeling of *Gal* and *c-fos* in the MPOA of light illuminated Gal::ChR2 and control males shown by mRNA *in situ* hybridization.

We first investigated whether *Gal*⁺ cell activation made virgin males less prone to commit infanticide. Each male was tested multiple times with stimulation (stim) and no-stimulation (no stim) trials randomly assigned in 1:1 ratio (Methods). Light stimulation of MPOA *Gal*⁺ neurons in Gal::ChR2 males inhibited infanticide in 16 of the 18 trials (6 animals, 2-4 trials per animal), whereas the same group of animals committed infanticide in 18 of the 19 trials without stimulation (Figure 4.15). Loss of infanticide was not due to pup avoidance, as light stimulated Gal::ChR2 virgin males displayed frequent and lengthy bouts of pup grooming and interactions with pups that were not observed in control groups (Figures 4.16, 4.17, 4.18). In contrast, light stimulation did not significantly alter the behavior of the control virgin males (Figures 4.16, 4.17, 4.18).

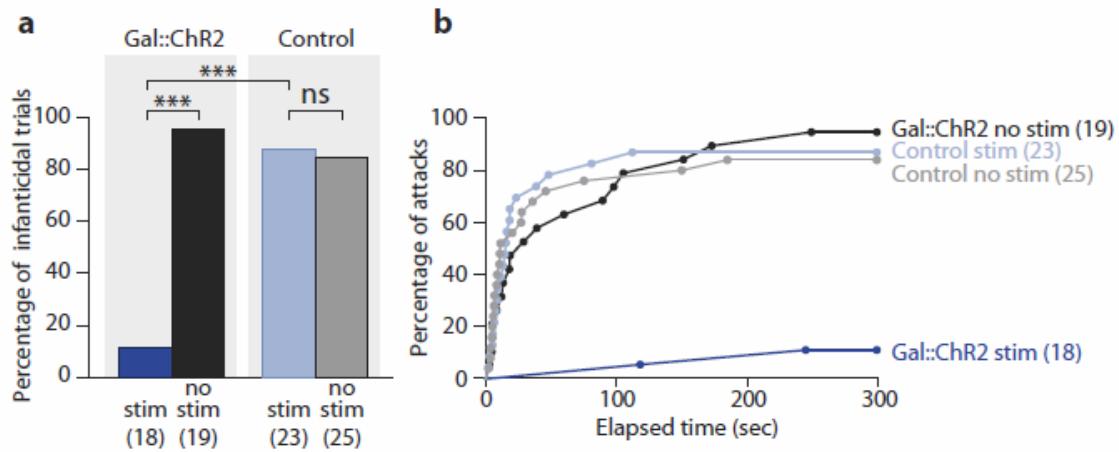


Figure 4.15: Light stimulation in Gal::ChR2 males inhibits infanticide

a, Percentage of infanticidal trials. Fisher's exact test with Bonferroni correction, *** $P < 0.001$, ns. not significant.

b, Each curve plots the latencies to attack on the x-axis for all the animals in one group, and the cumulative percentage of animals that attacked the pup on the y-axis. Gal::ChR2 stim trials are significantly different from Gal::ChR2 no stim and control stim trials. Kolmogorov-Smirnov test with Bonferroni correction, $P < 0.001$.

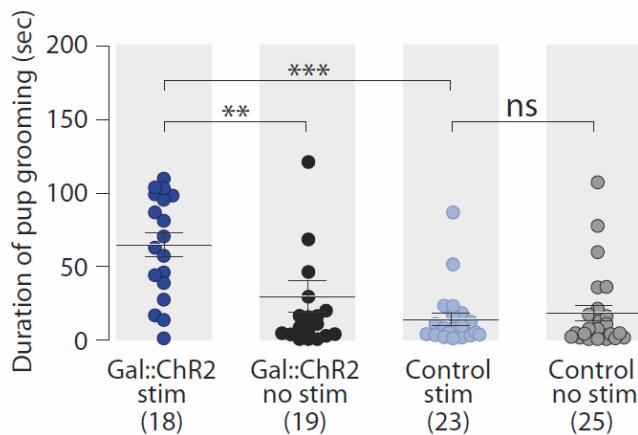


Figure 4.16: Duration of pup grooming in Gal::ChR2 males comparing to control groups.

Gal::ChR2 stim trials are significantly different from Gal::ChR2 no stim and control stim trials.

Mean±SEM; Mann-Whitney test with Bonferroni correction. ** $P<0.01$, *** $P<0.001$, ns. not significant.

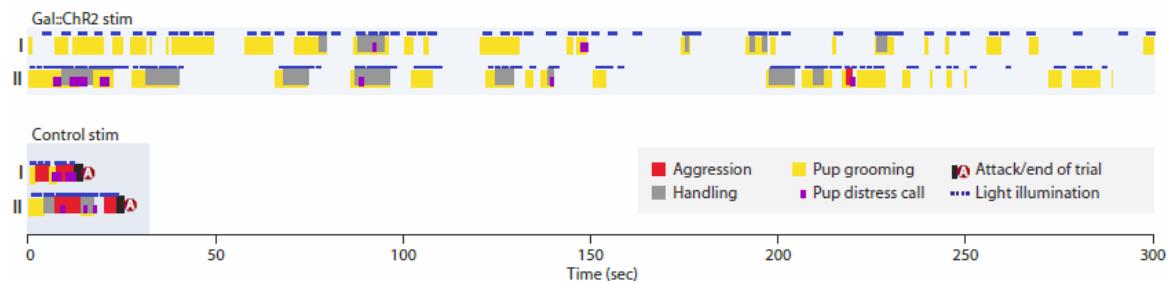


Figure 4.17: Sample behavior raster plot of Gal::ChR2 stim and control stim trials.

Different behavior elements are color coded. Note that two behavior elements (such as pup grooming and handling) can happen at the same time.

Figure 4.18: Behavior raster plots of Gal::ChR2 and control virgin males.

Each row represents a single trial lasting for 5 min or until the male attacked the pup. Trials are grouped by experiment conditions and sorted by trial length. Roman numerals indicate the sample trials shown in Figure 4.17.



Figure 4.18 (continued): Behavior raster plot of Gal::ChR2 and control virgin males

The effect of light stimulation was then tested on the parental behavior of mated, paternal males. Gal::ChR2 and control males were mated with females and tested with two C57BL/6J pups 3 to 4 weeks after mating. Measures of parental care showed that light stimulation elicited strikingly elevated pup grooming in Gal::ChR2 males compared to no stimulation (Figures 4.19a, 4.20, 4.21). The parental behavior of control animals was not altered by light illumination. Interestingly, the strong induction of active pup grooming in ChR2 stimulated males was seen at the expense of crouching, suggesting that crouching fails to emerge if ChR2-stimulated males are directed toward persistent licking and grooming (Figures. 4.19b, 4.20, 4.21). In the absence of pups, experimental light stimulation did not obviously alter baseline behavior, although we observed in some animals an occasional increase in locomotion and rearing activity induced by prolonged light stimulation. These results indicate that optogenetic activation of MPOA Gal+ cells is sufficient to suppress infanticide in males, and to induce pup grooming.

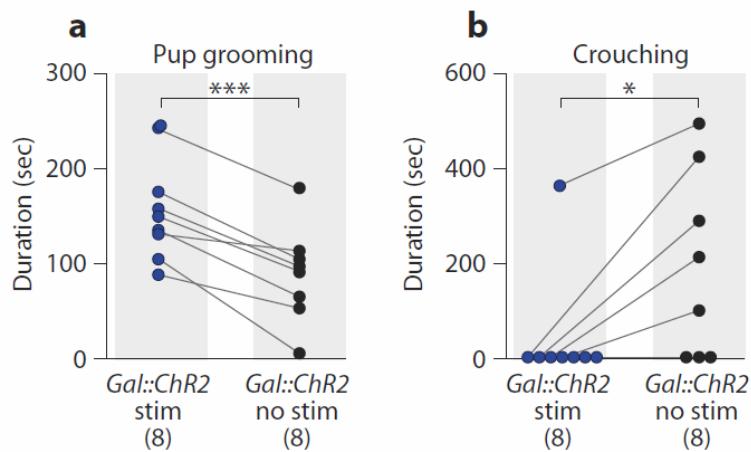


Figure 4.19: Duration of pup grooming and crouching in Gal::ChR2 and control fathers.

a, Duration of pup grooming in the tests of fathers. Paired t test, *** $P<0.001$.

b, Duration of crouching in the tests of fathers. Paired t test, * $P<0.05$.

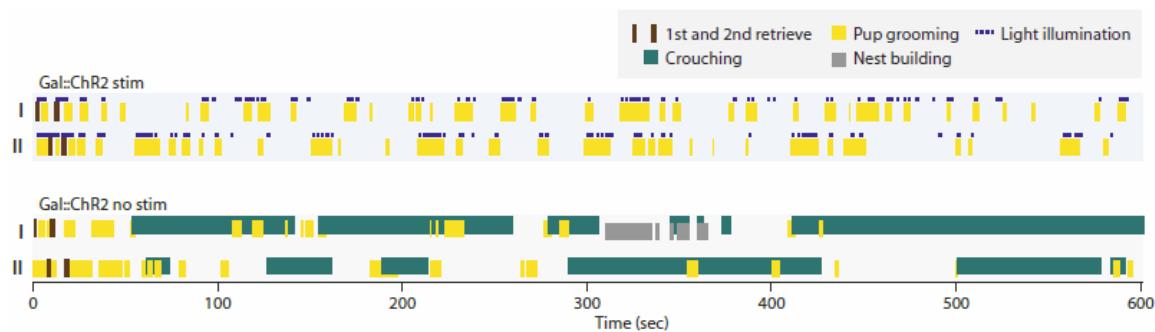


Figure 4.20: Sample behavior raster plot of Gal::ChR2 fathers with and without light illumination.

Figure 4.21: Behavior raster plot of Gal:::ChR2 and control fathers.

Each row represents a 10-min trial. Trials are grouped by experiment conditions. Roman numerals indicate the sample trials shown in Figure 4.20.

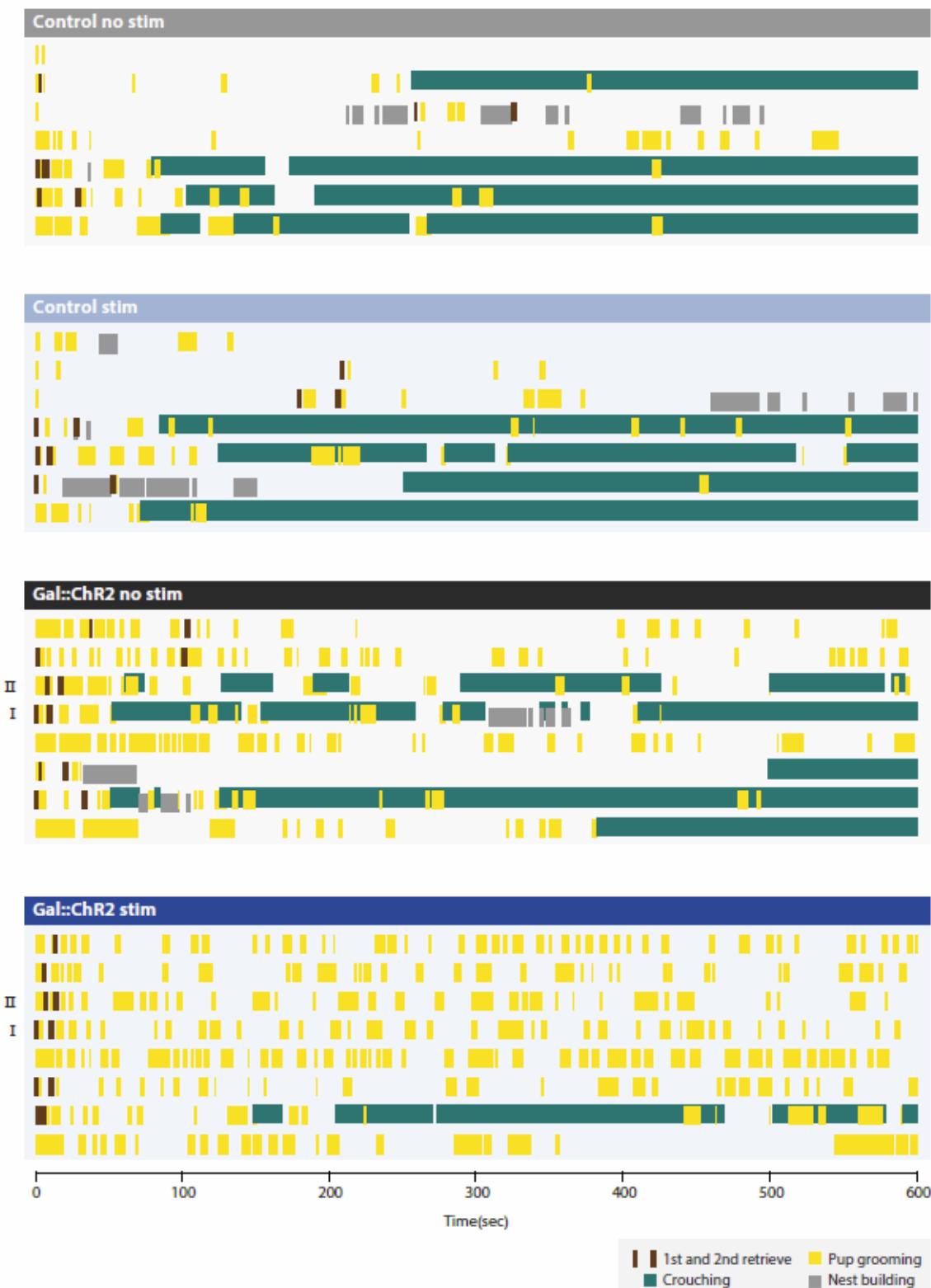


Figure 4.21 (continued): Behavior raster plot of Gal::ChR2 and control fathers.

Discussion

Our data provide significant insights into the control of two antagonistic social behaviors in mice: parenting versus infanticide. Our results suggest that, while vomeronasal circuits in virgin males trigger infanticidal behavior in response to pup cues, this response is silenced in virgin females and mated males, and neuronal pathways underlying parental care are activated instead. We show here that galanin-expressing cells in the MPOA are necessary and sufficient to control parental behavior in mice, and repress infanticide in virgin females, thus acting as a central node of regulation of social interactions with pups. The manipulation of this genetically defined neuronal population switches on or off the behavior of mice towards pups, providing a valuable entry point for further dissection of the neural circuits underlying parental care and their modulation by social experience.

Interestingly, the loss of function of MPOA *Gal*⁺ neurons leads to a reduction in all tested aspects of parental behavior, while activation of MPOA *Gal*⁺ neurons triggers pup grooming but no other parental displays such as nest building or crouching. An understanding of the natural pattern of MPOA *Gal*⁺ neuron activity during parental care, particularly during intense care such as grooming, versus more passive display such as huddling with pups, may help optimize the ChR2-mediated stimulation of MPOA *Gal*⁺ neurons and, in turn, its behavioral outcome. In addition, although MPOA *Gal*⁺ neuronal activity appears essential for all tested aspects of parenting behavior, some behavioral displays may require simultaneous activation of additional neuronal populations.

The concept of command neuron was initially proposed by Wiersma and Ikeda to describe how five interneurons of the crayfish nerve cord caused bilateral rhythmic

swimmeret movements (Wiersma and Ikeda, 1964), such that the stimulation of any of these neurons led to the execution of a specific behavior that is equivalent to its natural pattern. Later, Kupfermann and Weiss proposed a more rigorous definition: The activity of command neurons had to be both necessary and sufficient for the behavior that they command (Kupfermann and Weiss, 1978). In a strict term, no single neuron or single set of neurons is sufficient for a behavior. All neurons function in a network: The activation of any given neuron will be unlikely to result in behavioral outcome in the absence of its downstream targets or upstream inputs. However, the converging feature of these neurons is that they integrate all the pertinent sensory information and trigger appropriate motor pattern, which distinguishes them from other circuit component for a specific behavior. According to this criterion, it remains to be demonstrated whether or not the MPOA *Gal*⁺ neuron population meets the full description of “command neurons” for parental care. Indeed, although MPOA *Gal*⁺ neuron ablation leads to deficits in all tested aspects of parental behavior, stimulation of these cells in the experimental paradigm used to far appears to trigger pup grooming but no other parental displays.

From our results, the relationship between circuits mediating parental care and infanticide, appear complex and modulated by social experience. Activation of MPOA *Gal*⁺ neurons suppresses infanticide in virgin males, indicating that this neuronal population may be able to directly repress centers driving attack towards pups. Indeed, the loss of function of MPOA *Gal*⁺ neurons impairs parental behavior as well as elicits infanticide in virgin females. However, ablation of MPOA *Gal*⁺ neurons, leads to deficits in paternal behavior but does not elicit infanticide in mated males, suggesting that circuits underlying infanticide are silenced in mated males through an independent mechanism.

The observation that mating does not induce an increase in the MPOA *Gal*⁺ cell number in males further supports this hypothesis, although transcriptional or synaptic changes of these cells may occur. The further understanding of the coordinated control of parental care and infanticide will require the identification of neural populations controlling infanticide, corresponding to role of the MPOA *Gal*⁺ neurons in parental care.

In addition, circuit-level analysis of MPOA *Gal*⁺ neurons will help determine the mutual connections between these two antagonistic circuits, and assess the connectivity with other amygdala, hypothalamic and basal ganglia areas participating in the control of parental care (Numan and Stolzenberg, 2009; Numan et al., 2010). The MPOA receives and sends inputs from and to a large number of brain regions (Simerly and Swanson, 1986, 1988). Retrograde tracing with true blue, SITS, or wheat germ agglutinin showed that the MPOA receives inputs from all major areas of the hypothalamus, from limbic regions including the amygdala, ventral subiculum, ventral lateral septal nucleus and the BNST, and from the NAc, the VTA, the PAG and the raphe nuclei. (Simerly and Swanson, 1986). Anterograde tracing using lectin *Phaseolus vulgaris* leucoagglutinin (PHA-L) found that the MPOA projects to many hypothalamic nuclei including the AVPe, the PVN, the VMH, the DMH, the arcuate nucleus (Arc), the ventral premammillary nucleus (PMv), and other areas such as the BNSY, the VTA, and the PAG (Simerly and Swanson, 1988). Interestingly, many of the areas, including the PVN, the BNST, the VTA and the PAG, form reciprocal connections with the MPOA and have been involved in the regulation of parental care or the aversive responses to pups in rats (Numan, 2006; Numan and Stolzenberg, 2009; Sheehan et al., 2000). Since the MPOA is a highly heterogeneous structure (Simerly et al., 1986), it would be important to trace the

specific projection of the *Gal*⁺ neurons for us to understand the connectivity of circuits underlying parental behavior.

Finally, since the MPOA Gal⁺ neurons are primarily GABAergic, the function of these neurons in the control of parental behavior may be executed through the neurotransmitter GABA, the neuropeptide galanin, or both. As neuropeptides act on a slower time scale than neurotransmitters in general, the fast response we observed in the ChR2-mediated activation experiment suggests a direct role of GABA in this behavior. However, the neuropeptide may still play important modulatory roles. A variety of hormones and neuropeptides, including estradiol, testosterone, prolactin, progesterone, and oxytocin, have been shown to modulate parental care according to the physiological state of the animal and its social context (Bridges et al., 1985; Champagne et al., 2001, 2003b; Insel and Young, 2001; Lucas et al., 1998; Pedersen et al., 1982; Schneider et al., 2003; Trainor and Marler, 2001). Galanin is expressed in several brain areas, and has been involved in the modulation of multiple physiological functions including nociception, sleep, feeding and energy balance, thermoregulation, osmotic regulation, water intake, and reproduction (Mechenthaler, 2008). In particular, galanin is co-expressed by prolactin-secreting cells in the pituitary gland and is involved in the control of lactation (Wynick et al., 1998). In *Gal* knockout mice, pituitary prolactin mRNA level and protein content are reduced by 30–40% compared with wild-type controls, resulting in a failure of lactation in mutant females. However, it is unclear whether Gal is directly involved in other behavioral components of maternal responses such as pup retrieving or grooming. It would be interesting to determine whether Gal, together with other peptides potentially

co-expressed in MPOA *Gal*⁺ neurons, are new players in the regulation of parental behavior.

Materials and methods

RNA in situ hybridization

Fresh brain tissues were collected from animals housed in their home cage or 35 minutes after the start of the behavior tests when *c-fos* expression is analyzed. The dissected brains were embedded in OCT (Tissue-Tek) and frozen with dry ice. 20 μ m cryosections were used for RNA *in situ* hybridization. Adjacent sections from each brain were usually collected over a few replicate slides to generate copies for staining with multiple probes.

Fluorescent mRNA *in situ* hybridization was performed largely as described (Isogai et al., 2011). Complementary DNA of *c-fos*, *Gal*, *Trh*, *Gad1*, *Vglut2*, *EYFP*, *ChR2* and other candidate MPOA molecular markers (*Esr1*, *Esr2*, *Cyp19a1*, *Ar*, *Pgr*, *Prlr*, *Hcrt*, *Cartpt*, *Tac1*, *Penk*, *Th*, *Peg10*, *Pvalb*, *Calb1*, *Calb2*, *Vip*, *Nos1*, *Cck*, *Sst*, *Nts*) were cloned in approximately 800-base-pair (bp) segments to pCRII-TOPO vector (Invitrogen). Antisense cRNA probes were synthesized with T7 or Sp6 polymerases (Promega) and labeled with digoxigenin (DIG; Roche), fluorescein (FITC; Roche) or dinitrophenol (DNP; PerkinElmer). Where necessary and possible, a cocktail of 2–4 probes were generated covering different segments of the target mRNA to maximize detection.

mRNA hybridization was performed with 0.5–1.0 ng/ μ l cRNA probes at 68°C. The probes were detected using horseradish peroxidase (POD)-conjugated antibodies (anti-FITC-POD at 1/250 dilution, Roche; anti-DIG-POD at 1/500 dilution, Roche; anti-DNP-POD at 1/100 dilution, PerkinElmer). The signals were amplified using Biotin conjugated tyramide (PerkinElmer) and subsequently visualized with Alexa Fluor 488-conjugated streptavidin or Alexa Fluor 568-conjugated streptavidin (Invitrogen), or directly

visualized with TSA plus cyanine 3 system or TSA plus Fluorescein system (PerkinElmer). Tissues were mounted with Vectashield (Vector labs) containing 8 μ g/ml DAPI.

Image analysis and cell counting

All the microscopy images were acquired with AxioImager Z2 and AxioVision software with a 10X objective (Zeiss). Brain areas were determined based on landmark structures and white matters such as the ventricles, anterior commissure and optic tract, with the occasional assistance of Nissl staining and other area-specific molecular markers on adjacent sections when necessary. Areas of interest in the *c-fos* expression analysis included the MPOA, anteroventral periventricular nucleus, bed nucleus of stria terminalis, medial amygdala, posteromedial cortical amygdala, nucleus accumbens, lateral septal nucleus, suprachiasmatic nucleus, paraventricular nucleus, anterior basomedial nucleus, ventromedial hypothalamic nucleus and dorsomedial hypothalamic nucleus. After manual assignment of brain structures, automated cell counting was performed using ImageJ with custom-written macro scripts. Sample images were manually counted by experimenters blind to the test condition to verify the reliability of automated cell counting. For a given brain area, the absolute cell number was determined by summing up the cell counts of all the sections deemed as part of that area, adjusted by the number of the slicing replicates collected in cryosectioning.

Ablation of MPOA Gal-expressing cells

The Gal-Cre transgenic mouse line (STOCK Tg(Gal-cre)KI87Gsat/Mmucd, identification number 031060-UCD) was imported from the Mutant Mouse Regional Resource Center (http://www.mmrrc.org/catalog/sds.php?mmrc_id=31060). The Cre expression was verified by the GENSAT (http://www.gensat.org>ShowMMRRCStock.jsp?mmrc_id=MMRRC:031060) and by our laboratory. The imported line was in an FVB/N-Crl:CD1(ICR) mixed genetic background and backcrossed to C56BL/6J genetic background in our breeding colony. The animals used in the ablation study came from the F1 generation.

The rAAV8/EF1 α -mCherry-FLEX-dtA (AAV-DTA) construct was generated using the A subunit of the diphtheria toxin gene from a PGKdtabpA plasmid (Addgene plasmid 13440) (Soriano, 1997). The recombinant vectors were then serotyped with AAV8 coat proteins and packaged by the viral vector core at the University of North Carolina. AAV-DTA (4×10^{12} viral particles/ml) was injected bilaterally in the MPOA of Gal-Cre males in the amount of 0.8 μ l on each side (bregma: 0.0mm, midline: +0.5mm; dorsal surface: -5.0mm) with Nanoject II injector (Drummond Scientific). The total volume was delivered in 18nL pulses every 10 seconds over 7 minutes and the glass pipette was left in place for 3 minutes. The negative control group consisted of Gal-cre negative littermates receiving the same treatment.

After surgery, each female was individually housed for a week of recovery, then group housed in 4-5 animals per group for two weeks, and then separated again for one week before behavior tests. The four weeks of recovery enables optimal DTA expression and cell ablation before behavior testing. Each female was tested with two C57BL/6 pups in

their home cage, in a similar manner as described earlier (see Page 55). The first contact made by the female initiates the trial and the females are allowed up to thirty minutes to retrieve both pups. Animals that retrieved both pups to the nest or built a new nest around the pups within 30 minutes and crouched over pups were considered “maternal”. Animals that attacked the pups within 30 minutes were scored as “infanticidal” (attack is aggressive biting of the pups and causes wounding to the pups, usually accompanied by audible pup vocalization). All the other females were categorized as “ignore”. All the trials were video recorded and analyzed by an observer blind to the test condition.

Males were allowed about one week of recovery after surgery and then paired with females until the females gave birth (~3 weeks). 1-2 days after the pups were born, males were separated from their mates and litters, individually housed for 2-3 days and tested in a 30-minute behavior assay with two C57BL/6J pups. All the tests of the males were carried out in a similar manner. The brains were harvested after behavior assays for histological analysis.

ChR2-mediated cell activation

The AAV-EF1 α -DIO-hChR2(H134R):EYFP (AAV-ChR2:EYFP) construct was a gift of Dr. Karl Deisseroth (Gradinaru et al., 2009) and the recombinant AAV vectors were serotyped with AAV5 coat proteins and packaged by the viral vector core at the University of North Carolina. Gal-Cre males were tested with pups and the infanticidal ones were selected for surgery. 0.8 μ l of AAV-ChR2 (4×10^{12} viral particles/ml) was

injected bilaterally into the MPOA of Gal-Cre males (bregma: 0.0mm, midline: +0.5mm; dorsal surface: -5.0mm) using Nanoject II injector (Drummond Scientific). After injection, a small plastic adaptor holding an optical fiber (300 μ m diameter; Polymicro technologies) was implanted above the MPOA and affixed to the skull with dental cement (bregma: 0.0mm, midline: +0.2mm; dorsal surface: -4.2mm). The implant was positioned close to the midline to cover the MPOA in both hemispheres and lowered to a depth of approximately 0.8mm above the center of the AAV injection. A threaded plastic cap (Plastics One) was used to cover the implant during recovery and between experiment sessions. Gal-Cre negative males treated with the same procedure were the negative controls.

The males were individually housed and tested after at least 2 weeks of recovery. Before stimulation, the implant was connected to an optical fiber (300 μ m diameter, Polymicro technologies), which was connected in turn to a blue laser via an optical commutator permitting free movement of the animals. The optic fiber was flexible and long enough (~2 meters) to allow the animal to freely behave and interact with the intruder. A custom written Matlab program was used to control the frequency and the duration of the light stimulation. Both Gal::ChR2 and control animals were tested for 2-4 trials with stimulation (stim) and no stimulation (no stim) trials randomly assigned in 1:1 ratio. In each trial, a C57BL/6J pup was introduced to the male's home cage and blue light (473nm) was delivered in 30ms pulses at 20Hz for 1-4s whenever the male contacted the pup with its snout. The light power exiting the fiber tip was at ~10-20mW, ensuring a light intensity above ~1.0mW/mm² over the entire MPOA (Yizhar et al., 2011). There

was almost no leakage of light from the optic fiber or the adaptor. Each trial was up to 5 minutes but when the male attacked and wounded the pup, the trial was ended and the pup was euthanized immediately. All the trials were videotaped and analyzed by an observer blind to the experiment condition. The following behavior was scored and quantified: pup grooming (male sniffing and/or licking a pup), handling (male holding or moving a pup with its forelimbs, sometimes while grooming the pup), aggression (male grabbing and restraining a pup, grooming it violently and attempts to bite, often causing pup distress call) and pup distress calls (made by pups mostly during male's aggressive interaction, only audible calls were recorded).

For paternal behavior assays, the Gal::ChR2 and the control males were paired with females. After their pups were born, the females and the pups were removed and the males were tested in their home cage by introducing two C57BL/6J pups. Each male was tested in two 10-minute trials with one stim and one no stim trial in randomized order. Blue light is delivered when the males sniff or lick the pups. None of the males committed infanticide or displayed obvious aggression. Retrieving, pup grooming, crouching and nest building behaviors were scored and quantified as described above (see Page 55).

After behavior assays, the brain tissues of these animals were harvested after a standard *c-fos* induction protocol to analyze the efficiency of viral infection and cell activation. A train of light was delivered in 30ms pulses at 20Hz for 2s, repeated every 10s for 15 minutes, at experimental light intensity. Co-labeling between *Gal*, *ChR2:EFYP* and *c-fos* was analyzed by mRNA *in situ* hybridization. Two Gal::ChR2 animals with less than 20%

of MPOA *Gal*⁺ cells expressing *c-fos* were discarded from the group (six animals were kept in the group). The fiber implants from both Gal::ChR2 and control animals were verified for efficient light transmission.

Chapter V. Conclusion and future directions

This dissertation investigated the sensory and experience-dependent control of parental behavior and identified the MPOA *Gal*⁺ cell population as its central regulator. Using *Trpc2*^{-/-} animals, I showed that virgin males that are genetically impaired in vomeronasal signaling no longer commit infanticide and are parental, whereas the parental behavior in females are largely unaffected. We confirmed the mating-induced behavior switch from infanticide to paternal care in males and showed that both intrinsic time-dependent mechanism and cohabitation with females contribute to this behavioral switch.

These experiments demonstrated that the vomeronasal system plays a key role in the on/off switch of parental behavior, and enables the control of its sex specificity. Further, two antagonistic behavior circuits appear to co-exist in the male brain to regulate infanticide and parenting behaviors according to different social context. In virgin males, vomeronasal circuits activated by pup cues elicit infanticide while pathways underlying parenting behavior remain silent. In contrast, mated males transiently repress vomeronasal-evoked infanticide and instead activate parenting circuits in response to pup signals.

For us to understand the differential activation of males and females by pup stimuli and the resulting behavioral sex dimorphism, it will be informative to characterize the neuronal responses by recording from the neurons along the vomeronasal pathway such as the AOB, the MeA and the BNST. In addition, to understand the mechanism of the

mating induced behavior switch in males, it will be particularly useful to examine the changes in gene expression profile or synaptic plasticity in these areas, as the vomeronasal pathway has been implicated in the switch (Tachikawa et al., 2013).

By characterizing the vomeronasal receptors that detect pup stimuli, we have shown that pup stimuli preferentially activate vomeronasal receptor neurons in virgin males than in virgin females. Further, we have identified seven putative pup receptors and one receptor group, of which six belongs to the V1R family and two belongs to the V2R family. The function of the two V2R receptors is now been verified by testing the pup-directed behavior in both male and female knockout animals. Since the vomeronasal pathway is highly activated by pup cues in infanticidal males than in parental females, we expect that male infanticide behavior may be preferentially affected by the deletion of these two V2R alleles.

In addition to the generation of the receptor knockouts, a complementary approach could potentially address the sufficiency of these receptors in inducing parental behavior or infanticide. These receptors can be stimulated in a natural encounter by driving ChR2 expression with vomeronasal receptors and stimulating their activities with light illumination. Once the role of the receptors are confirmed, the specific downstream targets of these receptors can also be traced by ChR2-assisted mapping, which will provide an alternative route for the dissection of the central circuits regulating infanticide and parental behavior.

Finally, we have identified galanin-expressing neurons in the MPOA as key regulators of male and female parental behavior. Genetic ablation of MPOA galanin- neurons results in

dramatic impairment of parental responses in both virgin females and fathers. In addition, optogenetic activation of these cells in virgin males suppresses infanticide and induces pup grooming. These results established the MPOA galanin-expressing neurons as an essential node of regulation of innate behavior that orchestrates male and female parenting while opposing vomeronasal circuits underlying infanticide, and provided an entry point for the genetic and circuit-level dissection of mouse parental behavior and its modulation by social experience (Figure 5.1).

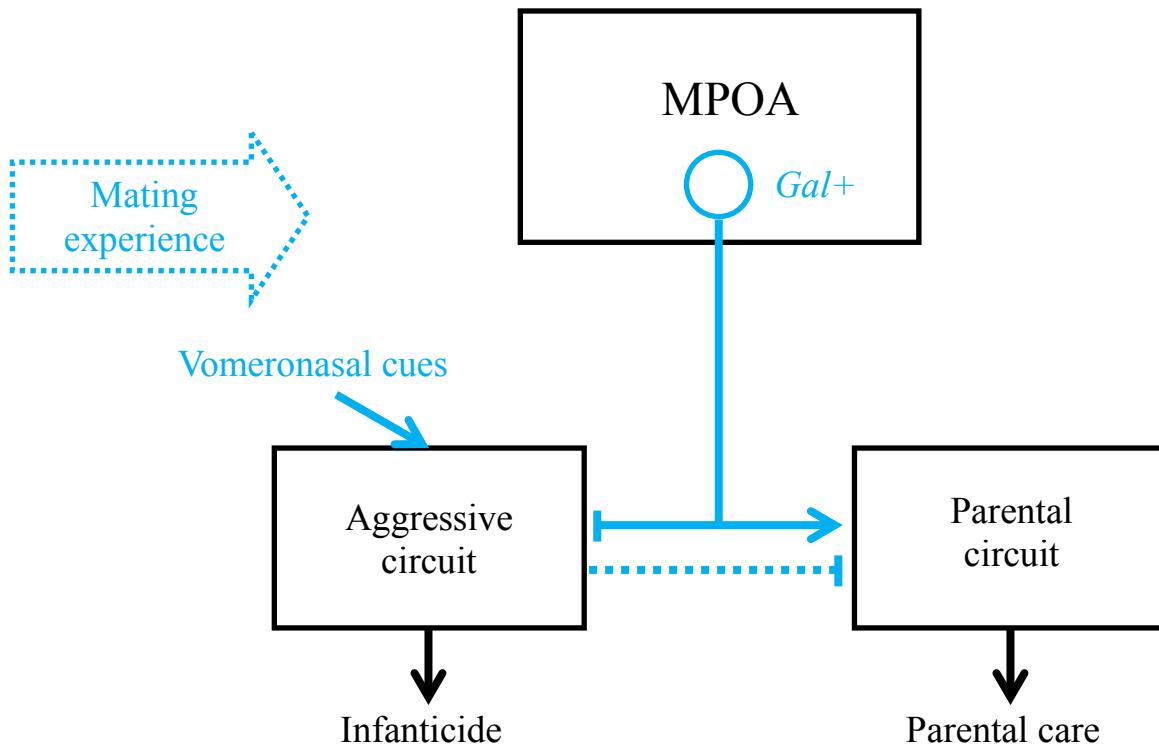


Figure 5.1: Neural model of parental behavior and infanticide in mice.

The vomeronasal inputs activate the aggressive circuit in males and induce infanticide. When vomeronasal inputs are removed, the parental circuit is disinhibited and leads to paternal care. This switch of behavior also occurs naturally under sexual experience, resulting from both an

intrinsic timing mechanism in males and the cohabitation with females. Furthermore, galanin neurons in the MPOA are critical for parental behavior, such that the ablation of them causes defects in parental care, and their activation suppresses infanticide and induces pup grooming.

The relation between the MPOA *Gal*⁺ cells and the vomeronasal pathway remains elusive, however. It would be interesting to characterize the activity of the MPOA *Gal*⁺ cells in *Trpc2*^{-/-} animals in response to pups. Moreover, cell-type specific tracing from the MPOA *Gal*⁺ cells could reveal their connections with other areas that are implicated in the control of parental care and candidate areas involved in the opposing circuit underlying infanticide. In addition, although mating does not induce an increase in the MPOA *Gal*⁺ cell number in males, it could be interesting to examine other possible modulation by mating experience, such as synaptic, transcriptional or epigenetic changes.

For a mechanistic understanding of infanticide and the analysis of its circuit, it will be crucial to identify the area and the neuron populations that control infanticide. Such populations can be identified by a similar approach using immediate early gene mapping with a list of candidate molecular markers, or a recent approach analyzing total transcripts of the areas activated in infanticide by capturing phosphorylated ribosomes (Knight et al., 2012). Once the critical neuron population for infanticide is identified and confirmed, we will be able to examine its possible connection and interaction with the MPOA *Gal*⁺ cells. A detailed analysis of their connectivity, their responses to sensory inputs, and their modulation by social experience, will help reveal the entire brain circuits and the complex control of parental behavior and infanticide.

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