Mechanistic and Circuit-Based Studies of Infant-Directed Behaviors

Mechanistic and Circuit-Based Studies of Infant-directed Behaviors

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Abstract

Male mice display stereotyped behaviors towards pups that vary depending on their mating status: virgin males typically attack pups, while sexually experienced males display parental care. This behavioral phenomenon is referred to as the 'parental switch'. This thesis details the projection patterns of MPOA Gal+ neurons to known target sites, in order to better understand how this cell population regulates the many behaviors needed for pup-directed care. In addition, we present brain areas that send projections unto a population of cells modulating pup-directed neglect/attack. Further, I explore the role of adult neurogenesis in preventing infanticide and promoting pup-directed care in male mice. Since mating is necessary for the parental switch, this was done by quantifying rates of proliferation and integration in mated animals as compared to control animals. Lastly, I show functional studies that prevented adult neurogenesis from occurring during and after mating and subsequently tested the response to pups. Ablation of adult neurogenesis near the time of mating led to an increase in pup-directed attacks, supporting the hypothesis that adult neurogenesis underlies the behavioral changes associated with the parental switch.

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CHAPTER I: INTRODUCTION

"Much of human behavior can be explained by watching the wild beasts around us. They are constantly teaching us things about ourselves and the way of the universe, but most people are too blind to watch and listen."

— Suzy Kassem (Rise Up and Salute the Sun: The Writings of Suzy Kassem)

In biology, behavior has been broadly defined as "the internally coordinated responses of whole living organisms to internal and/or external stimuli" (Levitis, Lidicker, Freund, & Freund, 2009). In almost all environmental contexts, behaviors such as eating are necessary for an individual to survive, but except in very few species (Chapman et al., 2007), a host of other behaviors are important for organisms to interact with one another and form communities that help that given species to continue existing. These set of behaviors are called social behaviors, and they encompass a wide range of complex, stereotyped interactions such as aggression, mating and parenting. Scientists have long been interested in how and what regulates stereotyped-behaviors that are seen not just in humans but across the animal kingdom. Our knowledge of what a behavior is and what underlies its expected expression has changed with time as new methodology gave rise to new ways of thinking, aspects which I will describe in the first part of this introduction. In the second part, I will discuss the circuitry underlying infant-directed care and infant-directed aggression, which represent the specific behaviors mostly relevant to my work. Finally, in a third part, I will discuss how and where adult neurogenesis was discovered as well as some putative functions for this phenomenon, an issue relevant to data presented in the 4th chapter of this thesis.

1- Social Behavior: Overview & New Approaches for Studying Social Behavior Circuits

The field of ethology focuses on the study of animal behavior, usually in natural conditions. One of the founders of the field of ethology, whose observational approach helped shape the modern fields of behavioral ecology, evolutionary biology and behavioral neuroscience was Nikolaas Tinbergen. Tinbergen thought that when studying a behavior, four crucial questions should be asked (Tinbergen, 1963). Firstly, what are the causes and mechanisms underlying this behavior? For example, what stimulates a behavior to occur and how can the behavioral outcome be modified? This question also ponders on the molecular mechanisms, social context and physiological elements that allow the expression of a behavior. The second question probes at the development of a behavior. For example, is the behavior changing with age, and what are the necessary experiences (including but not limited to environmental factors during specific developmental periods) for the behavior to be expressed as expected in the first place? Thirdly, what is the purpose of this behavior in the context of an animal's fitness? Lastly, what evolutionary history and evolutionary benefit does a behavior possess?

Tinbergen used these questions to study the behavior of many species and his findings helped the scientific community better understand some of the general factors that elicit behaviors in individuals and groups. Tinbergen's greatest discovery was that of 'sign stimuli'. Sign stimuli are defined as an essential feature of a stimulus that is needed to elicit a response in an animal. An expansion of this concept was the 'supernormal stimulus' in which an artificial stimulus leads to an exaggerated response when compared to the natural stimulus normally encountered by the animal. An example of this was an experiment in which Tinbergen made eggs out of plaster of

different sizes and color intensities to study what factors motivated a bird to sit on an egg and found that birds preferred sitting on the largest eggs available with the most saturated colors and would choose such an egg over their own smaller, less-saturated hatched egg (Tinbergen, 1951).

In the spirit of understanding behavior in general, it is important to ask: what is a necessary component for a given behavior to occur in an individual? Though, humans have long known that an intact brain is necessary for behavior to occur, the study of brain lesions has helped further uncover the functional role of specific brain regions. An example of a human brain lesion that yielded insight into the function of a particular brain region was the curious case of Phineas Gage. In 1948, Gage was working at a construction site when a steel rod pierced through his cheek into the frontal lobe of his brain and out of his skull. Unexpectedly, Phineas Gage lived and remained fully conscious and in perfect health throughout the ordeal. It was only after his injury healed that anybody realized that his personality had completely changed (Macmillan, 2000). The once compliant, calm-mannered foreman was now erratic and completely consumed by angry, profane-laden outbursts. This case study was important to psychology as it helped us see that even some aspects of a human's personality are tied to the functioning capacity of the frontal lobe and that lesioning this area partially can radically-affect the way a person acts.

Other accidental lesions in humans and lesions of specific brain regions in non-human test subjects have helped us understand that the brain is necessary for the expression of the different senses and motor actions. For example, lesioning the V5 region of the visual cortex

leads to impairments in motion perception but no other effects on vision (Shipp, de Jong, Zihl, Frackowiak, & Zeki, 1994). Physical lesions have therefore been a crude but helpful way to understand the broad-spectrum function of anatomical regions in the brain.

Though the lesioning of some parts of the central nervous system (especially those encoding the senses or directly severing areas with motor neurons) often resulted in similar results, the medical practice of human lobotomies made clear that there was a lot we did not know about how the brain works. Lobotomies and brain lobectomies were once popular procedures (especially after the Gage case-study emerged) that were used to improve the pathological personalities of psychiatric patients and to stop debilitating seizures in others (Miller, 1967). For example, patient H.M. had disabling seizures that seemed to come from a region that we now refer to as the hippocampus. Surgeons removed his hippocampus and now H.M. could not form new memories which led scientists to study this region's role in memory formation (Beecher & Millner, 1957).

Lesioning other parts of the nervous system, such as that of sensory organs, have provided valuable insights into a phenomenon called 'critical periods.' A critical period is a transient state in the life of an animal in which it is particularly sensitive to specific environmental stimuli (reviewed in (Hensch, 2004)). Consequently, lesions of sensory organs can affect whether a behavior occurs at all, especially if an animal is deprived of sensory information at specific developmental timepoints (called critical periods). For example, the effects of monocular deprivation are a fruitful example of this phenomenon. Initially studied in cats, monocular deprivation for 2 months led to aberrant organization of the ocular dominance columns (Wiesel

& Hubel, 1963), In a more recent example performed in mice, suturing one eye shut in four-week old mice lead to permanent visual defects even once the sutures are removed and even though the eye has no abnormalities that would prevent vision (Fu & Gordon, 1997). Lesions and sensory deprivation during critical periods have taught us that the brain controls sensory and motor function, as well as behavior (reviewed in Scott, 1962, Knudsen, 2004 & Hensch, 2004). However, behavior has proven to be a complex problem to understand. Indeed, sensory organs integrate sensory input and send signals that are then processed by different parts of the brain. Those brain areas, in turn, integrate these signals and relay a decision, expressed as a reaction coordinated by motor outputs. Due in part to the vast number of different areas and neurons involved, and to the complex wiring and integration processes, we still do not understand the entire process by which behavior is elicited and orchestrated.

Behavioral observations in their naturally occurring environment and the effects of brain lesions have changed how we approach the study of the brain, but we owe a great deal of our modern understanding of the brain to the work of people who helped elucidate its structural composition at a microscopic level. Almost a century ago, the neuroanatomist physician Ramon y Cajal forever changed how we conceive the underlying structure of the brain. Using a groundbreaking technique called Golgi staining (Golgi, 1873) that sparsely but very clearly labels neurons, Cajal discovered that the brain was composed of neurons that were connected to each other in what we now refer to as circuits (Ramón y Cajal, 1891). Circuits are the structural networks of how brain function is organized in many brain areas, including brain areas connected to sensory organs or containing motor neurons. Cajal made hundreds of detailed drawings of his stained brain slices which led us to better understand how the sensory

areas were organized. Thus, his findings allowed scientists to ask more specific questions about how sensory information was encoded and potentially processed (Ramón y Cajal, 1923). For example, his thorough neuroanatomical comparisons of the olfactory systems of different species led him to conclude that connectivity of the cortex and the olfactory bulb could indicate a reciprocal and bidirectional exchange of information and further proposed, by inferring from his connectivity maps, that olfactory granular interneurons transfer information from the cortex to the mitral/tufted cells, which are the primary recipients of information coming in from olfactory sensory neurons (Ramón y Cajal, 1891). Since the connectivity was at least partially untangled, he and other researchers could now create and test more specific hypothesis alike the one explained above. Cajal's detailed drawings also helped future scientists correlate distinct cellular morphology to specific functions. Cajal's work is continued today with more modern technology, for example by scientists who invented a technique called Brainbow that uses the genetic expression of different fluorescent proteins to distinguish cell morphologies. Or, the inventive alterations and usage of the viruses as tracers (Wickersham et al, 2007; Nassi, Cepko, Born, & Beier, 2015; Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012). Along with many other approaches, these new approaches help us further our understanding of how neurons are connected into complex circuits.

Another paradigm-shifting discovery that changed how we understand the brain's control of social behaviors was the discovery of the effect of hormones, particularly steroid hormones, on brain function and behavior**.** Because of the popular agricultural practice of castration and the human equivalent (eunuchs and operatic castratos), we have long known that some factor in testicles affected behavior and physiology. Castration of adult humans like eunuchs, decreased

sex drive, reduced sexual function and increased longevity. Humans castrated before puberty such as castratos did not undergo voice changes in puberty and had distinct morphological features such as large ribs and long arms that were different from the average non-castrated human male (Barbier, 1996). In agriculture, castration has been performed to decrease aggression in males and prevent intermale aggression in a herd. In 1849, these observations led the physiologist Berthold to start to untangle the underlying endocrinological reasons for these changes by studying capon birds (Berthold & Quiring, 1849). He had castrated capons which led to their red head cone appearing deflated. He then implanted testes from other birds into the abdomens of the castrated birds and found that the birds redeveloped the male-specific characteristics. Post-mortem analysis unveiled the testes had redeveloped their blood supply (Brown-Sequard, 1889). This finding led Brown-Séquard to an experiment in which he injected himself with an aqueous solution of rodent testes and claimed that he subsequently felt more physical strength and rejuvenating stamina (Brown-Sequard, 1889).Though his finding was not replicated, it led to other scientists studying the role of injecting thyroid extracts into thyroidectomized monkeys led to a recovery of the symptoms of thyroidectomy, which led to cures for humans with thyroidectomy, as well as the continued research into what are now called hormones.

Hormones are regulatory substances produced in organisms that travel mostly via the bloodstream to exert a variable set of actions by binding to their target hormone receptors (Tata, 2005). This classical research eventually provided us with the structure of hormones and the ability to make synthetic hormones. Since hormones seem to modulate behaviors and the brain underlies behavior, scientists started to question how hormones affected the brain and

started injecting hormones into the brain and finding behavioral changes when injecting in some brain regions. Those findings and some of the structural findings made by scientists like Cajal eventually led to the discovery of hormone receptor throughout the body and the understanding that hormones exert their actions in cells via these receptors The mechanisms of action of hormonal modulation in the brain are not yet completely understood. However, scientists also coined the concept of 'pheromones' which are a type of hormone called an ectohormone, which is secreted or excreted outside of the body in order to affect another individual's behavior (Doty, 2014). These pheromones are sensed through vomeronasal receptors (C Dulac & Axel, 1995; Herrada & Dulac, 1997; Matsunami & Buck, 1997; Ryba & Tirindelli, 1997) located in the vomeronasal organ (VNO) (Catherine Dulac & Torello, 2003). An example of pheromones affecting behavior via these vomeronasal receptors, mice gather pheromonal cues from conspecifics that can provide information needed for recognition of an individual in order to avoid inbreeding (Sherborne et al., 2007). Since this is an oversimplification of the role and understanding of hormones in neuroscience and general biology gathered through thousands of equally-important experiments, here is further material for more in-depth, independent exploration of the topic: Review by (Mucignat-Caretta, 2014; Pfaff, 2017; Tata, 2005).

The combination of hormonal modulation, lesions, anatomical circuit knowledge of brain structures and recording neuronal activity (as well as artificially eliciting it to test connectivity) from known structures to understand how the cells interacted with one another led scientists to further refine the types of questions they could ask about the brain and behavior.

At this junction, scientists wondered how subpopulations of cells might interact and whether there were specific markers that could be used to label specific target subpopulations of neurons.

The discovery that cells express genes and proteins that exert functional changes was used by some neuroscientists to explore whether radiolabeled product from the enzymatic activity of a neuronal marker, Tyrosine Hydroxylase, could label specific functionally-related and/or region-specific populations of neurons (Coyle & Axelrod, 1972). This led to the development of region-specific genetic markers, cell-type markers and activity markers for brain cells that could be unveiled by *in situ* hybridization and immunohistochemistry (Houben, Vandenbroucke, Verheyden, & Denef, 1993). Some markers were found to be transiently expressed directly after neuronal firing, these markers were called immediate early genes. When IEG activity markers are combined with the use of region-specific markers, scientists can unveil active populations of neurons and with recent methodology are now able to target and manipulate specific cell populations that share a genetic marker (Strader *et al.*, 1991; reviewed by Tsien, 2016). These new methodologies have allowed researchers to think of the brain function as not just contiguous, firing neurons in a circuit, but rather as larger populations of neurons interacting with other populations and the composition of these population dynamics creating diverse, modifiable networks, through which information can be processed and interpreted to ultimately exert diverse functions while also retaining the ability to modulate the network of interactions to be able to respond to changing environmental challenges.

The advent of genetic engineering applications in mammalian systems has allowed researchers to observe and probe neural circuits in a more specific manner. For example, researchers can use transgenic mice, engineered to express fluorescent proteins under the control of specific gene promoters to visualize a particular neuronal populations (van den Pol & Ghosh, 1998). Once populations could be specifically targeted, their necessity could be probed by specifically expressing toxins in these populations. For example, the use of diphtheria toxin (Kohlschütter, Michelfelder, & Trepel, 2010), allowed for the ablation of genetically-defined neuronal populations to ascertain their role in selected social behaviors (Wu, Autry, Bergan, Watabe-Uchida, & Dulac, 2014). To then test for sufficiency of those neuronal population, researchers can artificially activate or temporarily inhibit genetically-defined neural populations using chemogenetic approaches such as DREADDs (Strader et al., 1991; Tsien, 2016). Furthermore, the discovery and genetic introduction of a light dependent membrane channel, Channel Rhodopsin (Boyden, Zhang, Bamberg, Nagel, & Deisseroth, 2005; X. Li et al., 2005; Nagel et al., 2005), expressed in a genetically defined subset of neurons allowed for a better temporal and spatial resolution in functional studies enhancing our understanding of neural circuits. Consequently, this led to a more thorough and complex understanding of neural circuit mechanisms regulating social behavior (Wu et al, 2014).

The advent of visualization, ablation and activation of genetically specific neuronal populations allowed for a better understanding of the functional architecture of the brain. However, another classical approach to understand the intricacy of neuronal connections and circuits is electrophysiology, a method allowing for the direct recording of cellular electric activity. This technique was limited for a long time to the recording of only one cell at a time, a long a

complex process but current technologies now allow scientists to record the activity of hundreds of cells simultaneously with high temporal resolution (Buzsáki, 2004). Moreover, Calcium (Ca2+) imaging, an approach combining biochemical and genetic engineering paired with new imaging technique such as endoscopic microscopy (Bocarsly et al., 2015) now allows for the recording of the activity of specific neuronal population. For example, this has been used to dissect the discreet activity of neural circuits in the Medial Amygdala (MeA) of awake behaving mice performing social interactions across a span of several months (Y. Li et al., 2017). This study is a great example of how these technological advances have allowed researchers to better understand the spatio-temporal relationships between cellular activity of a circuit and behaviors.

To conclude, one of the goals of neuroscience is to understand the neural circuits and mechanisms underlying all behaviors in their full complexity. To this end, new computational methods have been developed to model and simulate neural networks (Schliebs & Kasabov, 2014). However, researchers are confronted to a conundrum, on one side we have not yet reached a complex and data-rich understanding of most neural circuits, while on the other end modern approaches such as whole brain circuit tracing projects generate difficult-to-analyze data in almost intractable quantities (Sejnowski, Churchland, & Movshon, 2014). Future research will surely resolve both and allow for a more complete understanding of the neural mechanisms underlying behaviors.

2- Infant-directed Behavior and Related Circuitry in Rodents

The behavior that I seek to understand in this thesis is how adult animals interact with infants of their own species. Particularly, I will focus on rodents as mice and rats are a canonical model of the mammalian brain that have provided many insights into how other conserved behaviors are orchestrated (Kohl et al., 2018; Lin et al., 2011; Shah et al., 2014; Wu et al., 2014).

Within the large array of social interactions that animals exhibit, parenting is a social behavior composed of a number of particularly interesting characteristics. For example, parenting is composed of nurturing and protection of altricial offspring, along with training and education of these infants. In contrast to other social behaviors, such as aggression or mating, parenting is a behavior of particular interest due to its long-term interactions between parents and infants that can span multiple years. Moreover, it presents a complex phenomenon to study as the nature of parenting varies from mono-parental to bi-parental care or even multi-parental care where multiple individuals participate in the protection of the young (Michael Numan, 2014; Michael Numan & Insel, 2003). In rodents, the North American deer mice (*Peromyscus maniculatus*) is mono-parental (Mihok, 1979), whereas the California mice (*Peromyscus californicus*) are monogamous, mate for life and are bi-parental (Gubernick & Alberts, 1987). Finally, parenting is by nature an unequal behavior where the parents pay a cost to raise the infants; this has led to the hypothesis that the control of parenting might have led to the development of altruism behaviors (Preston, 2013). Similarly, the circuits responsible for parental care are thought to underlie a primordial template from which other intraspecific bonding behavior may have emerged (Michael Numan & Young, 2016).

However, why would such a behavior emerge in the first place? Indeed, from the point-of-view of the individual, parenting is a costly sacrifice in resources, time and energy. The offspring, however, benefits fully from receiving care, and this in turn maximizes its chance of survival (Michael Numan & Insel, 2003). This is exacerbated in mammals where in-utero development has a high cost for the gestating individual. From an evolutionary perspective, one might wonder why parenting would emerge, as the cost of raising infants is high and reduces the fitness of the parent. Therefore, a conflict will arise between maximizing species' fitness and the cost to the parents' fitness. In many species, regulating behaviors such as abandonment or infanticide can offset the parenting costs and can be triggered by environmental factors. In mammals, the cost of gestation seems to have tipped the balance heavily towards parenting behavior in maternal individuals, while males exhibit a range of different infant-directed behaviors ranging from infanticide to parenting (Lukas & Huchard, 2014; Stockley & Hobson, 2016). This delicate balance is probably finely regulated by evolution for different species making the study of the underlying neural circuits even more fascinating. However, our current understanding of pup-directed behavior is incomplete despite many recent advances in the field, which will be discussed below.

For mammals, our current understanding relies mostly on the study of maternal care, and it is unclear whether paternal care relies on the same neural mechanisms and circuits. Moreover, even less studied is the counter strategy to parenting: infanticide (Hausfater, 1984). It was first though that this behavior was abnormal and pathological, but Sarah Hrdy's work in langurs along with many more studies in insect, fish, amphibians, birds, rodent, felines and primates have now provided support to the concept that this is very likely an adaptive behavior

(Hausfater, 1984; Hrdy, 1977; Lukas & Huchard, 2014). The exact selective pressures acting upon the display of this particular trait are unclear. However, when males perform infanticide the loss of pups will induce an early estrus unto the female whose pups were killed and increase that male's chances of reproduction (Lukas & Huchard, 2014; Opie, Atkinson, Dunbar, & Shultz, 2013).

In this thesis, the experiments focus on the parenting behavior of *Mus musculus.* Mice, like all mammals, carry out pup development through in-utero gestation. Moreover, lab mice are of particular interest as they exhibit stereotyped pup-directed behavior instinctively. In adult mice, males and females display different behavioral responses when exposed to pups. Adult females show parental behavior, whereas, adult virgin males exposed to pups display pup-directed aggression known as infanticide (Brooks & Schwarzkopf, 1983; Svare & Mann, 1981). Mated males start displaying parental behavior upon the birth of their young and remain parental until the weaning of their offspring (Mennella & Moltz, 1988; vom Saal, 1985; vom Saal & Howard, 1982). This is known as the parental switch and will be the main focus of this thesis. Since mice have altricial young, mouse parenting is a complex and time-consuming endeavor. Not only does good mouse parenting require feeding, protecting, and taking care of their young, but also crouching, grooming, nest-building and pup retrieval. Moreover, the onset of parental behavior is affected by factors such as the animal's sex, age, social experience and internal state (Mennella & Moltz, 1988).

In females rodents, pregnancy is heavily regulated by a complex system of hormonal changes, which allow the body of the mother to adapt and carry through the development of the soon-

to-be-born infant (Kohl, Autry, & Dulac, 2017; Moltz, Lubin, Leon, & Numan, 1970). Of particular interest are the changes in progesterone which progressively increase during pregnancy and which during the peripartum will decrease drastically (Kohl et al., 2017; Michael Numan & Insel, 2003). This progesterone decrease triggers pulsatile releases of oxytocin (OTX), which coordinate uterine contractions (Higuchi, Honda, Fukuoka, Negoro, & Wakabayashi, 1985; Kohl et al., 2017). Following birth, prolactin (PRL) and OTX will work in concomitance to trigger milk production and milk ejection, respectively (Kohl et al., 2017; Michael Numan & Insel, 2003; Robinson, 1986). These hormonal stimulation patterns are of importance to the behavior of parenting, as it is known that some of these hormonal fluctuations trigger changes that are necessary for mothers of some animal species to express parental behavior (Moltz et al., 1970; M Numan, Rosenblatt, & Komisaruk, 1977; Terkel & Rosenblatt, 1968, 1972).

In female mice, the internal hormonal state of the individual has direct effects on the quality of parenting. For example, stressed or virgin females are less maternal than mated females (Kohl et al., 2017; Weaver et al., 2004). Moreover, studies have found that reproducing artificially the steroidal hormonal changes of pregnancy in virgin female mice increased the quality of parenting behavior by shortening pup retrieval latency (Michael Numan & Insel, 2003). This is probably due to similar effects described in rats, where prolactin and estrogen receptors can modulate maternal responses (Moltz et al., 1970). Moreover, the expression patterns of the receptors, for the hormones mentioned previously, are still poorly documented making it even more difficult to decipher the underlying mechanisms. This and other studies on hormonal and neuromodulator effects indicate a dependency of maternal behavior with internal state of the animal, such as whether the animal is stressed (Kohl et al., 2017). However, while the hormonal

neuromodulation of parenting has been well studied in females, males have received less attention. While males do not undergo the hormonal changes associated with pregnancy, male rats undergo mating-induced neuroendocrine responses such as oxytocin release (Waldherr & Neumann, 2007). This in turn could be creating changes in pup-directed behavior at a later timepoint, however, to my knowledge there is not a similar study in male mice regarding oxytocin. There exists some evidence for a testosterone release, but it has been found to dependent of the genetic background of the male (James, Nyby, & Saviolakis, 2006).

Another key element underlying paternal behavior is the regulation of infanticide by the vomeronasal system. Studies have shown that the surgical or genetic removal of the vomeronasal organ, responsible for sensing non-volatile chemicals, inhibited the stereotyped infanticidal responses of virgin mice (Tachikawa, Yoshihara, & Kuroda, 2013; Wu et al., 2014). While the mechanistic cause of this is still unknown, the length of co-habitation of males with pregnant females seems to positively correlate with the increased likelihood that males will display paternal care (Wu et al., 2014) and this phenomenon might be due to vomeronasally-processed cues sensing the pregnancy; Moreover, ejaculation during mating is a necessary component for the behavioral paradigm seen in male mice where virgin males switch from infanticide to parenting after mating (vom Saal, 1985). These findings indicate that the response of a male to pups is not just state-dependent but also affected by environmental factors.

As mentioned previously, parenting is a complex behavior. Moreover, sensory systems play an important role in pup-directed behavior. A classical study assessed the necessity of different sensory modalities for the display of parental behavior in postpartum female rats (Beach &

Jaynes, 1956). In this experiment, researchers either surgically remove sensory organs or cut nerves that underlie their function. The result of these sensory ablations was that removing just one sensory modality, regardless of which one it was, did not eliminate parental care as measured by pup retrieval latency. However, ablation of two sensory modalities at the same time did produce defects in parental care (Beach & Jaynes, 1956). Auditory cues also seem to play an important role in parenting as pup vocalizations seem to lead to sustained changes in the auditory cortex of new mothers that make mothers more responsive to pup calls (Cohen & Mizrahi, 2015; Cohen, Rothschild, & Mizrahi, 2011; Liu, Linden, & Schreiner, 2006; Liu & Schreiner, 2007; Shepard, Chong, & Liu, 2016; Tasaka et al., 2018), and these cortical changes seem to be modulated by oxytocin (Marlin, Mitre, D'amour, Chao, & Froemke, 2015). Olfaction's role in parenting has not been as complicated to elucidate as audition's. When female nulliparous mice and virgin female mice have their olfactory bulbs removed, they eat their offspring (Gandelman, Zarrow, Denenberg, & Myers, 1971). However, removing the olfactory bulbs, removes both the ability to process pheromones via the Accessory Olfactory Bulb (AOB), as well the ability to process volatile cues through the Main Olfactory Bulb (MOB). In virgin female rats, cutting the vomeronasal nerves led to a faster onset of pup-directed care when compared to controls (A. Fleming, Vaccarino, Tambosso, & Chee, 1979). In female rodents, the ablation of the main olfactory bulb does not eliminate parenting (though some aspects of parenting are negatively affected) (A. S. Fleming & Rosenblatt, 1974), whereas equivalent VNO disruptions in virgin males lead to a reduction in pup-directed attacks. However, the role that the MOB plays in the behavior of male mice towards pups remains to be discovered.

A long-known central component of parenting control is the medial preoptic area (MPOA) in the hypothalamus. The hypothalamus is an area containing multiple nuclei involved in a range of functions. It is involved in the control of homeostatic functions such as sleep or hunger. But also plays a role in the neuroendocrine control of behaviors such as stress. It also plays a role in the modulation of social behaviors such as parenting, mating or aggression (Bridges, Mann, & Coppeta, 1999; Kohl et al., 2017). When this positive regulator of parenting is ablated many aspects of maternal behavior are severely disrupted (Michael Numan, 1974; Wu et al., 2014) among which the motor related parenting behavior are disrupted (Lee, Clancy, & Fleming, 2000). Moreover, cells in this area highly express pregnancy related hormonal receptors, and direct artificial hormonal stimulation increases parenting (Rosenblatt & Ceus, 1998; Rosenblatt, Olufowobi, & Siegel, 1998). However, the MPOA is involved in many other behaviors such as reproduction (Arendash & Gorski, 1983; Dominguez & Hull, 2005; Jennes & Conn, 1994), aggression (Hammond & Rowe, 1976). To understand the role of the MPOA in the control of parenting, Wu et al. identified a subpopulation of MPOA neurons activated during parenting and showed that it expressed the neuropeptide galanin. Using functional manipulation of MPOA Gal+ neurons, Wu et al were able to demonstrate that Gal+ cells are necessary for the induction of parenting behavior (Wu et al., 2014). More specifically, the ablation of this cell population inhibits parenting behavior such as nest building, pup retrieval or grooming in both sexes. Prior to this study, it was believed that the MPOA was influencing parenting through activation through glutamatergic excitatory projections (Michael Numan & Insel, 2003), however the MPOA Gal+ population is mostly composed of inhibitory projections (Kohl et al., 2018). This finding implies that the role of the MPOA in regulating parenting is through the

inhibition, or disinhibition of another circuit, leading the way to further deciphering of the circuit underlying the modulation of parental behavior (Kohl et al., 2018).

As we discussed above, the lack of the VNO also eliminated infanticide in virgin male mice (Tachikawa et al., 2013; Wu et al., 2014). Unlike the MOE that signals to the main olfactory bulb (MOB), and then to the olfactory cortex, the VNO signals to the accessory olfactory bulb which in turns projects towards the medial amygdala (MeA), the bed nucleus of the stria terminalis (BNST) and the hypothalamus. Isogai et al studied the circuits underlying the VNO modulation of infanticide and parenting behaviors (Isogai et al., 2018). They discovered 7 vomeronasal receptors activated by the presence of new born pups and were able to find that the sensed pup cues included hemoglobin and a large salivary protein, neither of which are pup-specific. When they genetically ablated cells expressing two of the seven receptors, they noticed that infanticidal behavior towards pups was reduced. However, the non-specific nature of the sensed the pup cues made them research further the exact set of signals inducing infanticide. By using a clever approach of silicone-molded pups swabbed with salivary extracts, they uncovered that only molds shaped as pups, or containing partial elements of a pup shape induced aggressive behaviors (Isogai et al., 2018).

Trying to understand the role of the hypothalamus in infanticide, studies looked at the differential activation of regions in virgin males versus parental females presented with a pup (Tachikawa et al., 2013; Wu et al., 2014). They showed that the PVN, DM, VMH and posterior hypothalamus were more active in infanticidal males (Tachikawa et al., 2013). Finally, Wu et al showed that the optogenetic activation of the MPOA Galanin+ neuronal population induced a

partial parenting response (pup grooming) and inhibition of infanticide in virgin males. To conclude we have seen that both parenting and infanticide are modulated through the internal state of the mouse, controlled -at least partially- through sensory stimuli, and orchestrated by specific cell populations in the hypothalamus. Though in the lab mouse infanticide is not seen in females, across the animal kingdom this phenomenon is not uncommon (Dias & Crews, 2008; Godwin, Crews, & Warner, 1996). Moreover, because both behaviors are antagonistic they must repress one another. Since we know that ablation of MPOA Galanin+ neurons leads to infanticidal behavior in virgin female mice (Wu et al., 2014), we know that MPOA Gal+ cells are inhibiting infanticidal circuits. Therefore, the evidence discussed above provides support that animals exhibit a bipotential state and that evolution has finely-tuned this bipotential switch to be used in a way that most increases fitness.

As mentioned above, male mice undergo a fascinating phenomenon called the parental switch. Virgin male mice are infanticidal, but a most interesting phenomenon is observed when a virgin male mate with a female. After a period of about 21 days, it's behavior switches from infanticidal to parenting (vom Saal, 1985; vom Saal & Howard, 1982). The mated male will then exhibit traits such as pup retrieval, grooming or nesting. To this day it remains unclear what sensory stimuli and internal state changes control this switch. This time approximately corresponds to the gestation time of the mouse female. Even more fascinating is the reversal of this parenting behavior after 50-60 days when the pups are weaned. Continuing to utilize this behavioral switch paradigm seen in male mice, we can tease apart the circuits underlying both infanticide and parenting as well, as which circuit disruptions affect expected pup-directed behaviors to better understand the complex interactions of these two circuits.

In future chapters of this thesis, I will explore adult neurogenesis as a potential molecular mechanism underlying this behavioral switch.

3- Adult Neurogenesis: History, Location and Function

"Once the development was ended, the founts of growth and regeneration of the axons and dendrites dried up irrevocably. In the adult centers, the nerve paths are something fixed, ended, and immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree."

-Ramon y Cajal (1928)

The quote above was dubbed the 'central dogma of neurobiology' for almost a century. By the time the neuroscientist Ramon y Cajal expressed the words above, several scientists had already found evidence of adult-born cells in the brains of rodents (Allen, 1912; Sugita, 1918). and a decade after, also in humans and teleosts (Kershman, 1938). However, Cajal's prominence in the scientific community and deeply-held dogma, as well as flawed techniques used to study the existence of adult-born cells resulted in the concept of adult neurogenesis being denied and ignored for almost a century, despite the overwhelming evidence supporting the phenomenon.

As early as 1912, researchers had already seen mitosis occurring in the brains of rats 120 days after birth (Allen, 1912), which is well into sexual maturity for rats (Adams & Boice, 1983). By 1938, a group of researchers even claimed to have seen "indifferent" cells distributed throughout the adult brains of teleosts and humans and also claimed that these cells had the ability to turn into neurons or glia (Sugita, 1918). Unfortunately, the belief that the brain of the adult mammals was structurally constant was already universally held by the main scientific figures of the time (Cajal, 1928; Compston & Cajal, 2002; Kölliker, 1854). At the time the birth of new neurons (aka neurogenesis) was thought to happen throughout brain development in the

womb and stop prior to puberty (Cajal, 1928; Jacobson, 1970). This belief was born of and supported by years of observations made by Koelliker, and Cajal, who had studied, using sparse staining, the development of the central nervous system of rodents and humans. Since their personal observations did not uncover mitotic elements or cells having the morphology of neurons in developmental stages in adult brains, they concluded that the brain's architecture remained fixed from soon after birth until death(Cajal, 1928; Compston & Cajal, 2002; Kölliker, 1854).

Lack of support from the scientific community did not prevent scientists in the 1950s and 1960s from continuing to explore the possibility of adult neurogenesis in the mammalian brain. In 1959, Dr W.A. Bryan used colchicine to halt cell division in rat brains and found cells that had been undergoing mitosis in 1+ year old white rats (Bryans, 1959). Bryans noted that the cells were located in the walls that outlined the lateral ventricle; nowadays, this region is called the subventricular zone. This result combined with previous findings led Bryans and other scientists to hypothesize that these adult-born cells, that were generated in the subventricular zone might migrate to the cerebrum to become neurons there (Bryans, 1959; Hamilton, 1901; Sugita, 1918). The premise was promising, but a looming question still remained unanswered: Are these cells neurons or are they glia? The development of tritiated thymidine autoradiography used in combination with electron microscopy would soon unveil the answer.

Tritiated thymidine or [3 H]-thymidine is a type of radiolabeled thymidine that incorporates into the DNA of dividing cells. Its presence can be detected by measuring the ionizing radiation of cells that have incorporated the thymidine. Tritiated thymidine therefore allowed researchers

to determine when and where cells were born, as well as visualize the progeny of recently-divided cells (Sidman, Miale, & Feder, 1959). Though this tool was originally used to study perinatal development in rodents (Sidman et al., 1959), Dr Smart used it to study the proliferation of adult-born cells in the subventricular zone of mouse pups. He was able to confirm that cells born in the subventricular zone indeed migrated to other brain regions in pups but was not able to replicate his findings in adults (Smart, 1961). Shortly after, Joseph Altman made landmark discoveries using thymidine autoradiography in adult rats (J Altman & Das, 1965; Joseph Altman, 1962b, 1962a, 1966, 1969; Joseph Altman & Das, 1967) and adult cats (Joseph Altman, 1963). In rats, he found that new cells were present in the neocortex (Joseph Altman, 1963, 1966), dentate gyrus (J Altman & Das, 1965; Joseph Altman, 1963) and olfactory bulb (Joseph Altman, 1969). He also confirmed that cells from the subventricular zone migrated to the olfactory bulb in adult rats (Joseph Altman, 1969). In cats, he found adult-born cells in the neocortex (Joseph Altman, 1963). Altman hypothesized that these cells were neurons that were crucial for learning and memory but never discovered evidence to support these claims (Joseph Altman & Das, 1967). Though Altman's results were largely dismissed by his contemporaries (Jacobson, 1970; Walton & Weiss, 1971), some of his claims were directly supported by the electron microscopy studies led by Michael Kaplan.

Dr Kaplan and his team found that tritiated thymidine-labelled cells in the olfactory bulb and dentate gyrus of adult rats had the ultrastructural characteristics (such as synapses and dendrites) that were unique to neurons and were incompatible with astrocyte and oligodendrocyte morphology (M. Kaplan, 1984; M. S. Kaplan, 1977). Using the same techniques, Kaplan also showed that mitosis occurred in the subventricular zone of adult macaque monkeys

(M. Kaplan, 1983) and provided key evidence for the foundation of adult neurogenesis in primates.

This evidence should have led the scientific community to further explore adult neurogenesis, but unfortunately, these discoveries were disregarded when a prominent developmental primatologist, Pasko Rakic, published a study in which he used tritiated thymidine to label entire adult rhesus monkey brains and claimed that no heavily-labelled cells with neuronal morphology were found in any major structures and subdivisions of the brain including the visual, motor, and association neocortex, hippocampus or olfactory bulb (Rakic, 1985b, 1985a). Rakic did not try to counter the evidence for adult neurogenesis found in rodents but claimed that this was unique to species that never stopped growing and therefore, whose brains never reached adulthood (Rakic, 1985b). Though he later changed his stance and published papers supporting adult neurogenesis in primates (Kornack & Rakic, 1999), the damage had been done and adult neurogenesis was not widely accepted as a relevant or important phenomenon in the field of neurobiology.

It was not until the 1990s that the development of new cell labelling methods and the discovery that neurogenesis was regulated by psychological states and environmental stimuli led the scientific community to believe that the addition of adult-born neurons to mammalian brains was an interesting and worthwhile phenomenon to study. In particular, Arturo Alvarez-Buylla showed neuronal migration from the SVZ to the OB of rats by using SVZ cells from adult mice carrying a neuronal-specific transgene that was grafted into the brain of the adult recipient (Lois & Alvarez-Buylla, 1994). The results of this experiment were further validated by the

development of the synthetic thymidine analogue BrdU (5-bromo-3'-deoxyuridine). BrdU's development led to a technique that allowed the clear distinction of adult-born neurons and glia without the use of autoradiography or time-intensive, resource-intensive electron microscopy (Nowakowski, Lewin, & Miller, 1989). BrdU incorporates into cells during the S-phase of mitosis and also serves as a marker of proliferating cells and their resulting progeny (Nowakowski et al., 1989). Importantly, BrdU labelling also allows for co-labelling with markers of specific cell types, as well a signal that can be quantified to estimate adult-born cells. The use of BrdU labelling to study adult neurogenesis was first pioneered in Fred Gage's lab (Kuhn, Dickinson-Anson, & Gage, 1996). Their data supported adult neurogenesis in the dentate gyruses of adult rats and also uncovered an age-related decline of adult neurogenesis. This immunohistochemical technique was further advanced by the development of cell-type specific markers that could uncover the nature of adult-born cells. Markers such as NeuN (Kuhn et al., 1996; Mullen, Buck, & Smith, 1992) stain the nuclei of mature neurons, while others such as PSA-NCAM serve as markers for immature neurons (Doetsch, García-Verdugo, & Alvarez-Buylla, 1997; Seki & Arai, 1999). Markers for nonneuronal cell types such as oligodendrocytes (Kornack & Rakic, 1999; Sommer & Schachner, 1981) and astrocytes (Choi, 1986; Woolley, Mcewen, Gould, & Cameron, 1993) were particularly important in the reaffirming and expeditious creation of data supporting life-long, continuous adult neurogenesis in many mammalian species (including humans), because if an adult-born cell expresses neuronal markers but lacks glial markers, the labelling serves as supporting evidence that the cell is a neuron and not a glial cell (Eriksson, Perfilieva, & Björk-Eriksson, 1998).

Next, I would like to summarize the main findings of the last decades dictating the location and potential functions seen in the sites of adult neurogenesis previously mentioned. Though adult neurogenesis has been seen in other regions, most scientists agree that active adult neurogenesis in normal/baseline conditions is spatially-restricted to two "neurogenic" brain regions, the subgranular zone (SGZ) in the dentate gyrus of the hippocampus and the subventricular zone (SVZ) of the lateral ventricles (Gage FH, 2000). Adult neurogenesis in other non-canonical brain regions is thought be limited during regular physiological conditions but can be induced by causing a brain injury (Gould, 2007).

In both rodents and humans, cells generated in the SGZ generally become excitatory granule cells that integrate locally into existing circuits (Gage, Deng, & Aimone, 2010; Gage FH, 2000). Conversely, cells generated in the SVZ travel through the rostral migratory stream (RMS) to reach the olfactory bulb to become mostly inhibitory interneurons (granule cells or periglomerular cells) that can integrate into regions in the accessory olfactory bulb or main olfactory bulb of rodents (Gage et al., 2010; Gage FH, 2000). The process of proliferation in adult neurogenic niches has been found to be regulated by a variety of intrinsic factors such as hormones, cytokines and neurotrophins (Zhao, Deng, & Gage, 2008). Moreover, physiological state and environmental stimuli also seem to affect the proliferation rates of adult-born neurons and long-term survival and integration into already-existing neural circuits (Gage et al., 2010; Zhao et al., 2008). For example, the rate of adult-born cell birth (aka proliferation) is increased after exercise (Praag & Kempermann, 1999) in the hippocampal SGZ niche, whereas an enriched environment promotes the continued survival of adult-born neurons for weeks after cell birth occurs (Kempermann, Kuhn, & Gage, 1997). Androgen exposure also promotes

the survival of adult-born neurons in male rats (Duarte-Guterman, Hamson, Wainwright, BioRxiv, & 2019, 2018). In the SVZ, elevated prolactin increases proliferation (Shingo et al., 2003); androgen exposure seems to promote proliferation in female rodents (Larsen, Kokay, & Grattan, 2008), whereas mating increases integration in males (Unda, Portillo, Corona, & Paredes, 2016). Though we have evidence of what regulates adult neurogenesis, the ultimate purpose and function of adult-born neurons is still largely unknown and contentiously debated.

There is amassing evidence to support the idea that adult olfactory bulb neurogenesis is necessary in providing structural integrity to the olfactory bulb by replenishing interneuron populations (Lazarini & Lledo, 2011). Adult olfactory bulb neurogenesis is also thought to be involved in the maintenance of short-term olfactory memories, long-term associative olfactory memories that required active learning and olfactory fear conditioning (reviewed by (Lazarini & Lledo, 2011)). Moreover, olfactory bulb neurogenesis seems to regulate pheromone-related behaviors, such as mating and social recognition (Feierstein et al., 2010) and recognition of adult offspring by fathers (Glasper, Kozorovitskiy, Pavlic, & Gould, 2011; McCarthy, 2010). Hippocampal adult neurogenesis, on the other hand, is thought to be necessary for long-term spatial memory retention, spatial pattern discrimination, contextual fear conditioning, dissolution of short-term memory traces and potentially to reorganization of associative memories (Gage et al., 2010; Ming & Song, 2011).

Given this wide range of potential functions and regulators, adult neurogenesis could underlie a behavioral paradigm that has not yet been explored with this mechanism in mind: the switch from infanticide/neglect to parental in mated male mice. The potential role of adult

neurogenesis has been explored in studies with pregnant and peripartum females but has led to conflicting results (Leuner & Sabihi, 2016). Only a handful of papers have looked at the correlation of adult neurogenesis and male parenting, and these papers have focused on the recognition of offspring as adults regulated by hippocampal prolactin-induced proliferation (Shingo et al., 2003) and extent of proliferation when exposed to pups(Lieberwirth & Wang, 2012; Ruscio et al., 2008). Since mating is necessary for males to undergo the parental switch (vom Saal, 1985), I set out to explore the possibility that adult neurogenesis from mating is orchestrating a behavioral shift in fathers by making changes in either or both the canonical target sites of adult neurogenesis in rodents: the olfactory bulb and the dentate gyrus.

Thesis Aims

1. This dissertation aims to explore aspects of the circuit elements underlying pup-directed aggression/neglect and parenting by elucidating the inputs of a population of cells active during infanticide. Conversely, a deeper understanding of how MPOA Gal+ neuron projections are organized is meant to yield information as to how different aspects of parental care might be modulated (Chapter II).
2. This dissertation also aims to explore whether adult neurogenesis occurring during mating in male mice could underlie a behavioral paradigm seen in males towards infants. To this end, this dissertation explores the state-dependent survival and brief characterization of mating-born cells in mated animals as compared to controls at the time fathers would interact with their own pups for the first time (Chapter III). Thirdly, this dissertation aims to explore the effect of eliminating adult neurogenesis in the SVZ and DG of mating animals to explore the effects of this ablation on the onset of paternal care as compared to controls (Chapter IV).

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Introduction

Parental behavior is essential for survival of infants in mammals, as well as in many species of birds, amphibians, reptiles, fish, and even some insects. All mammal species require parental care, but the extent of care varies: for example, in species with altricial young such as humans and mice, adults must invest a significant amount of energy in feeding, protecting, and taking care of their young to ensure proper development and survival of the infant. Moreover, the onset of parental behavior is affected by factors such as the animal's sex, age, social experience and internal state (Mennella & Moltz, 1988). In adult mice, males and females display different behavioral responses when exposed to pups. Adult virgin females and mothers show parental behavior such as crouching, grooming, nest-building and pup retrieval. By contrast, adult virgin males exposed to pups display pup-directed aggression known as infanticide (Brooks & Schwarzkopf, 1983; Svare & Mann, 1981). Mated males, however, start displaying parental behavior upon the birth of their young and remain parental until the weaning of their offspring (Mennella & Moltz, 1988; V. Saal, 1985; F. S. vom Saal & Howard, 1982). This behavioral shift is thought to give males a reproductive advantage because they are able to eliminate foreign offspring while protecting their own (Hausfater, 1984; Rees, 2010). This switch provides an interesting experimental paradigm to explore the functions and regulations of neural circuits underlying parental behavior, as well as opposing circuits that promote infanticide and neglect.

The most critical brain area involved in the control of parental care is, first and foremost, the MPOA, which have been shown to be necessary different aspects of parental behavior (Beach & Jaynes, 1956). Other regions involved in parental care are the nucleus accumbens (NAc), the retrorubral formation (RRF), ventral tegmental area (VTA), the lateral habenula (lHb), the paraventricular nucleus of the hypothalamus (PVN) (Insel & Harbaugh, 1989), and the periaqueductal grey (PAG) (Corodimas, Rosenblatt, Canfield, & Morrell, 1993; Insel & Harbaugh, 1989; Michael Numan & Insel, 2003). The NAc, VTA and RRF are thought to be implicated in parental responsiveness motivation (Michael Numan & Insel, 2003), which is thought to be regulated by inputs from the PVN and lHb (Corodimas et al., 1993). Lastly, the RRF and PAG are believed to be involved in the motor aspects of parental care (Michael Numan & Insel, 2003).

Similarly, the brain areas involved in the regulation of pup-directed aggression and neglect have been mostly studied via the use of lesions and expression of immediate early genes (IEG) such as c-fos, which are commonly used as molecular markers for neuronal activity (A. Lee, Clancy, & Fleming, 2000; Sheng & Greenberg, 1990). In rats, virgin females are often neglectful towards pups (Rosenblatt, 1967). In virgin female rats neglecting pups, c-fos immunoreactivity is found in several hypothalamic areas: ventromedial hypothalamus (VMH), lateral septum (LS), paraventricular nucleus (PVN), dorsal premammillary nucleus (PMd) and anterior hypothalamus (AH), and in areas that are part of the vomeronasal system, such as the medial amygdala (MeA) and the principal nucleus of the bed nucleus of stria terminalis (BNST) (T. P. Sheehan, Cirrito, Numan, & Numan, 2000). The vomeronasal system detects pheromones by the vomeronasal organ (VNO), which sends information to the Accessory Olfactory Bulb (AOB) (Dulac & Torello, 2003). The AOB then projects to the Medial Amygdala (MeA), which has been linked to social

recognition in mice (Ferguson, Aldag, Insel, & Young, 2001). In infanticidal males, the MeA, BNSTpr, AH, and the VMH were differentially labeled by c-fos compared to non-attacking animals (Tachikawa, Yoshihara, & Kuroda, 2013). Moreover, lesioning the AH and VMH in virgin female rats resulted in a quicker onset of parental behavior (T. Sheehan, Paul, Amaral, Numan, & Numan, 2001) and lesioning the MeA made them maternal (Fleming, Vaccarino, & Luebke, 1980; M Numan, Numan, & English, 1993). Male rats (Mennella & Moltz, 1988) and virgin male mice who have undergone VNO genetic (Wu, Autry, Bergan, Watabe-Uchida, & Dulac, 2014) or surgical (Tachikawa et al., 2013) ablation show neglect or parental care towards pups. These results imply that areas of the hypothalamus play a key role in the proper function of the neglect circuit and that pup cues sensed by the VNO are necessary to activate the infanticide/neglect circuit. However, the exact connections that cause pup cues to be interpreted as aversive have remained largely unknown. In 2014, a publication from the lab (Wu et al., 2014) identified Galanin (Gal) neurons in the Medial Preoptic Area (MPOA) as necessary and sufficient for the display of paternal behavior in mice. In this study, mice expressing CRE recombinase under a Gal promoter (Gal::CRE) received MPOA injections of a virus that expressed diphtheria toxin in a CRE-dependent manner. When MPOA Gal+ ablation levels were more than 50%, virgin females displayed attack behavior, and parents of both sexes displayed neglect. Wu et al., then activated MPOA Gal+ neurons by shining blue light unto the MPOA of Gal::CRE mice injected with a CRE-dependent channelrhodopsin (ChR) virus (Wu et al., 2014). The activation caused virgin male mice to abruptly stop attacking pups. Since parental behavior is a complex set of many behavioral steps and these behaviors are all affected by MPOA Gal+ cell ablation to varying degrees, this finding suggests that MPOA Gal+ neurons play

a direct or indirect role in all the different behavioral components of parenting. To better understand how different subsets of parenting were modulated, previous Dulac lab postdoctoral fellow, J. Kohl, traced the projections of MPOA Gal+ neurons and found that they project and form synapses in 18 different sites in the brain (Kohl et al., 2018). Using this finding, our goal was to map the topography of different subsets of Gal+ neurons in order to determine whether all MPOA Gal+ neurons modulate all parental behaviors by projecting to all or several functionally-relevant sites, or if each behavioral component is controlled by a different subset of MPOA Gal+ neurons.

In a parallel, unpublished study, previous Dulac lab postdoctoral fellow, Anita Autry, used a molecular strategy based on fluorescent in situ hybridization (fISH), laser capture microscopy and gene expression analysis to identify a candidate population expressing the IEG c-fos during infanticide. She found that a subset of cells expressing the neuropeptide urocortin3 (Ucn3) in the perifornical area (PeFA) is activated in infanticidal animals and not in parental animals. Importantly, other findings in the study suggest that the PeFA Ucn3+ neurons are a necessary part of the neural circuit that promotes negative infant-directed behaviors in adult mice. Ucn3 is a neuropeptide of the corticotropin releasing factor (CRF) family that binds selectively to the CRF2 receptor. In female rats, PeFA Ucn3+ neurons show activation after acute stress (Venihaki et al., 2004), and Ucn3+ neurons do not seem to become activated when animals are adrenalectomized (Jamieson, Li, Kukura, Vaughan, & Vale, 2006). Functional studies of Ucn3 by different groups provided contradictory results: infusion of Ucn3 peptide into the brain lead to attenuation of anxiety displays (Venihaki et al., 2004), but whole brain overexpression of Ucn3 in mice was reported to cause an increase in stress-related behavior and deficits in spatial

memory (Neufeld-Cohen et al., 2012). Overexpression of Ucn3 in the PeFA lead to increases in anxiety displays in mice (Kuperman et al., 2010). Though most of the focus of the Ucn3+ neuron literature has been on its role on stress, caudal PeFA Ucn3+ neurons were reported to promote eating when stimulated by neuropeptide y (Gillard, Dang, & Stanley, 1993; Stanley, Magdalin, Seirafi, Thomas, & Leibowitz, 1993). The relationship between Ucn3, stress, and parental neglect is fascinating, as stress is known to play an important part in the negative regulation of parental behavior, especially given findings that increased amounts of stress have been correlated with decreased parental behavior and increased displays of pup-directed neglect/aggression (Liu et al., 1997; Parent et al., 2005). Our goal in this project was to create a map of all the inputs of PeFA Ucn3+ cells in order to determine how PeFA Ucn3+ cells are modulated to better understand their role in the circuit organization of pup-directed aggression and/or pup-directed neglect.

In this chapter, I discuss my PhD work directed at better understanding the circuitry underlying the regulation of pup-directed behavior. In collaboration with two postdoctoral fellows in the lab, I participated in work aimed at elucidating connectivity of two neuronal populations (MPOA Gal+ and PeFA Ucn3+) with opposite functions in regulating parental behavior. Together with Dr. Kohl, I helped optimize the methodology to visualize the branching patterns of subpopulations of MPOA Gal+ neurons, which in turn helped identify the logic of neural connectivity of this neuronal population.

Together with Dr. Autry, I quantified and analyzed the monosynaptic inputs of PeFA Unc3 neurons in adult male and female mice and tried to identify circuit differences that may underlie the sexually dimorphic response to pups.

Results

Analysis of MPOA Galanin+ Projections

As mentioned in the introduction, it has been known for a long time that the main area that controls parenting behavior is the MPOA. Within this area, our lab has shown that the subpopulation of cells expressing Galanin (referred to as MPOA Gal+) are necessary for parenting (Wu et al., 2014). However, to understand the mechanism through which this population controls parenting, it is critical to understand the patterns by which MPOA Gal+ cells project to target sites. This is why Dr. Kohl, at the time a postdoctoral fellow in the laboratory, had created a map of the projections of the MPOA Gal+ cells, which uncovered 18 target sites. Moreover, though his experiment allowed Dr. Kohl to visualize all MPOA Gal+ projections at once, it did not provide information about axonal branching of the MPOA Gal+ cells and thus, did not let us visualize whether a population of MPOA Gal+ cells was sending projections to more than one of the target sites. Stated otherwise, MPOA Gal+ projection patterns could follow any of these three scenarios: First, it is possible that each MPOA Gal+ neuron has extensive axonal branching so that each MPOA Gal cell sends projections to all target sites involved in orchestrating a parental behavior. A second possibility is that each MPOA Gal+ cell sends projections to only one target. Third, a mixture of the previous two scenarios is possible. To understand the output logic, we would need to visualize the branching patterns of MPOA Gal+ subpopulations to particular output areas. This is where I helped Dr. Kohl with this experiment; I decided to target four of the 18 areas. The technique we used to tease apart output populations is the injection of retrograde tracers such as CTB (Wang, Chen, & Lin, 2015).

In this experiment, we used two modified CTB that were tagged with fluorescent reporters, Alexa 488 and 647. One of the issues with CTB is that it is readily taken up by most axons that it comes in contact with, therefore, the injections were performed in lsltdtomato x Gal::CRE mice. This way, we could measure the co-labelling between the two output areas in the tdTomato expressing cells, which were the MPOA Gal+ cells (Schematize in Figure 2.1A).

I chose areas that had the most innervation from MPOA Gal+ neurons (the Medial Amygdala (MeA), rostral periacqueductal gray (rPAG) and two subregions in the caudal PAG: ventrolateral PAG (vlPAG) and dorsolateral PAG (dlPAG)). All of these areas have distinct functions according to the literature (Kim et al., 2015; Kollack-Walker & Newman, 1995; Lin et al., 2011; Lonstein, Simmons, & Stern, 1998; Lonstein & Stern, 1998; Unger et al., 2015; Wang et al., 2015). One pairwise combination was the MeA and the rPAG. Indeed, the postereodorsal MeA (MeAPD) is essential for intermale aggression and mating (Kim et al., 2015; Kollack-Walker & Newman, 1995; Lin et al., 2011; Unger et al., 2015) whereas rPAG lesions facilitate maternal behavior in rats (Lonstein et al., 1998; Lonstein & Stern, 1998). The second pairwise combination was the dlPAG and the vlPAG, both being in the cPAG. dlPAG has been shown to be active during defensive behaviors (Wang et al., 2015), while vlPAG has been implicated in maternal behavior (Lonstein et al., 1998; Lonstein & Stern, 1998). In his projection map, Johnny noted that MPOA sent projections to caudal PAG but this bregma coordinate contains several behaviorally-distinct PAG subregions. Both subregions are involved in decision making in response to a threat; vlPAG is activated by stimuli for which a passive coping behavior, such as freezing, is the primary response, and dlPAG is activated by stimuli to which an active defensive reaction, such as an attack or escape, is the primary response (Keay & Bandler, 2015).

In order to control for the uptake efficiency of the different CTB by every area I co-injected both tracers in the same area, and then measured the number of MPOA Gal+ that uptook both CTBs. For all controls this resulted in a neuronal subset in the MPOA Gal+ cells with a very high co-labeling by both CTBs. For the MeA, the result was 89% of co-labeling of the MPOA Gal+ cells by both CTB-647 and CTB-488. The rPAG co-injection resulted in 93% co-labeling of the MPOA Gal+ cells by both CTB-647 and CTB-488 (Figure 2.1D). The dlPAG resulted in 92% co-labeling of the MPOA Gal+ cells by both CTB-647 and CTB-488, and finally, the vlPAG co-injection resulted in 90% co-labeling of the MPOA Gal+ cells by both CTB-647 and CTB-488 (Figure 2.1E). All controls were performed only one time. Furthermore, the vlPAG and dlPAG areas are extremely close to each other, and I needed to ensure that the injection of both tracers would remain within the bounds of the target area. To this end, I performed the following experiment: I injected both areas with different volumes of tracers, and subsequently imaged the resulting spread of the tracers. I concluded that 9nl of CTB lead to a maximal spread in either vlPAG or dlPAG without spreading onto the other target subregion (Figure 2.1B).

Next, I tested the hypothesis that the different areas could receive input from different subpopulation of MPOA Gal+. To this end I injected MeA and rPAG with two different CTB (n=3) and did the same for vlPAG and dlPAG (n=5). Interestingly, both results showed a fairly low overlap of the tracers within the MPOA Gal+ cells. The MeA and rPAG lead to an 11% co-labeling of the MPOA Gal+ cells by both CTB-647 and CTB-488, whereas the vlPAG and dlPAG had a 25% co-labeling of the MPOA Gal+ cells by both CTB-647 and CTB-488. Therefore, these results show that there is little branching of MPOA Gal+ cells to more than one target site, especially given that the vlPAG and dlPAG are part of the same area. Kohl continued these

experiments testing more pairwise interactions and found that this logic of local laterization between target sites was similar throughout all areas he targeted (full results published in (Kohl et al., 2018)). Kohl also examined the following pairwise combinations: PAG & VTA, VTA & MeA and PAG & RRF. For all these combinations, Kohl chose areas that have opposite putative roles in pup-directed care. For example, lesioning the PAG facilitates maternal responses in lactating rats even when exposed to predator odors (Sukikara et al, 2010), whereas lesioning the VTA negatively affects infant care by impairing pup retrieval in female rats (Hansen et al, 1991). For the PAG & VTA pairwise combination, Kohl found 7% co-labeling of the MPOA Gal+ cells by both CTB-647 and CTB-488. Like the PAG lesion, lesioning the MeA accelerates the onset of maternal behavior in virgin female rats (Numan et al, 1993; Sheehan et al, 2001). Lastly, cell projection from the RRF to the MPOA are found to be differentially active during maternal behavior in female rats (Numan & Numan, 1997). For the MeA & VTA pairwise combination, Kohl found 5.5% co-labeling of the MPOA Gal+ cells by both CTB-647 and CTB-488. For the PAG & RRF pairwise combination, Kohl found 11% co-labeling of the MPOA Gal+ cells by both CTB-647 and CTB-488.

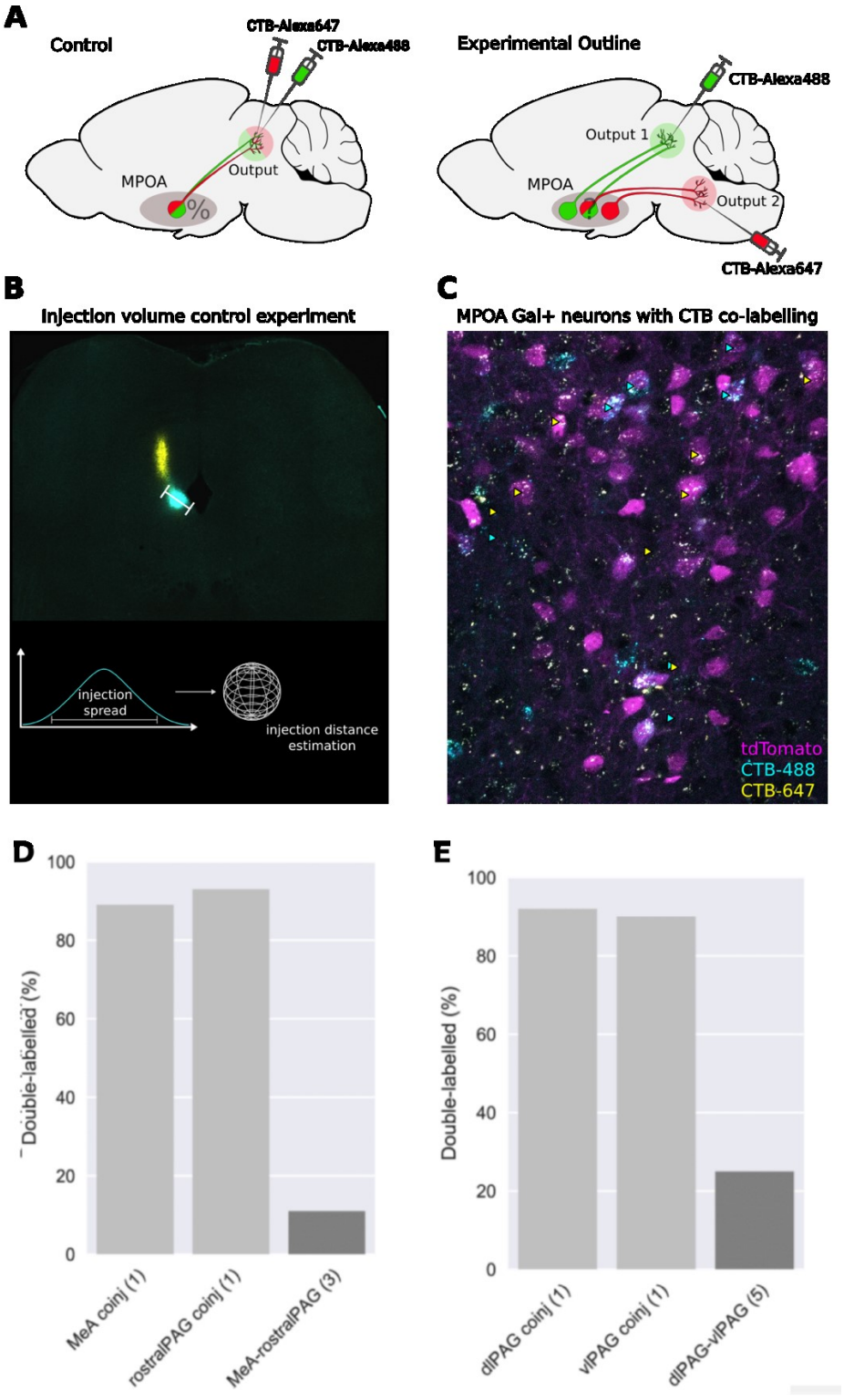

Figure 2.1: Topographical Maps of Select MPOA Gal+ Outputs

Figure 2.1 (Continued) **A.** Experimental diagram of dual CTB injections to determine topography of MPOA Gal+ outputs. Control experiment consist of co-injecting CTB-488 and CTB-647 in the same area, followed by a quantification of the amount of overlap in MPOA Gal+ cells. The experimental design consists of injecting two different areas, one with CTB-488 and the other with CTB-647. Then the amount of MPOA Gal+ cells that show signal for both CTB are counted. **B.** Representative image of CTB injection in the vlPAG and dlPAG. The spread of the two CTBs was quantified using Zen and extrapolated to maximize area coverage while minimizing leakage to other area. **C.** Representative image of the uptake of CTBs by MPOA Gal+ cells. In yellow CTB-488, in cyan CTB-647 and in magenta tdTomato. Arrow colors indicate cells with positive signal from the corresponding CTB. **D.** Quantification of co-labelled cells in co-injection controls and MeA-rPAG (n=3) injections. **E.** Quantification of co-labelled cells in co-injection controls and vlPAG-dlPAG (n=5) injections.

Analysis of Inputs of Ucn3+ PeFA Neurons

As presented in the introduction of this chapter, virgin male mice behave differently towards pups than virgin female mice. Specifically, virgin males display pup-directed aggression, whereas virgin female mice will exhibit pup-directed care. A. Autry, a previous postdoctoral fellow in the laboratory, discovered that a cell population of the PeFA expressing Ucn3 may function as a central node regulating pup-directed aggression/neglect (unpublished data). In this study, we were interested in understanding the input connectivity of this particular cell population. This could shed light on how this node is modulated by sensory or physiological information, a critical question towards understanding the complete circuit of infanticide. Because of the sex dimorphism of infanticide, another question is whether the Ucn3+ cells of the PeFA are differentially connected in male and female brains. Therefore, we decided to perform the following experiment comparing the sex-specific map of inputs of PeFA Ucn3+ cells. To create these input maps, we decided to use an approach that utilizes the capacity of a conditional Rabies Virus (RV) to travel trans-synaptically in a retrograde manner (Watabe-Uchida, Zhu, Ogawa, Vamanrao, & Uchida, 2012; Wickersham et al., 2007). We utilized a RV engineered to lack two key genes (Wickersham et al., 2007). First it was pseudotyped with the avian virus envelope protein A (envA), preventing the infection of mammalian cells by this RV. Therefore, the infection in the mammalian brain can only occur in cells engineered to express the cognate receptor TVA. Second, the gene encoding the RV envelope glycoprotein (RG) was replaced with the fluorescent reporter eGFP. The envelope glycoprotein is necessary for RV transsynaptic spread. Therefore, the virus can only travel trans-synaptically from cells expressing the RG gene. In order for the PeFA Ucn3+ neurons to both express EnvA and RG, we

utilized an approach performing an infection with an AAV virus inducing the expression of TVA and mCherry and another AAV inducing the expression of RG (Watabe-Uchida et al., 2012). In both viruses, the expression of the proteins are only active in the presence of CRE recombinase, through the use of the highly specific FLEX switch (Watabe-Uchida et al., 2012). Therefore, both starter infections by AAVs were performed in a Ucn3::CRE mouse line (summarized in Figure 2.2A). This allowed us to specifically trace the input projections targeting the PeFA Ucn3+ neurons. Autry performed the surgeries and the microscopy, and I performed the quantification and analysis of this experiment. Cell positive for the expression of eGFP were counted manually in across the brain of the animal. In order to normalize for animal and experiment variation, the number of positive cells in an area was normalized by the total number of cells counter in the brain. We discovered that there was direct innervation of PeFA Ucn3+ neurons from several hypothalamic areas including the Paraventricular Nucleus (PVN), the MPOA, anterior hypothalamus (AH), arcuate nucleus (Arc), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), peduncular lateral hypothalamus (PLH), lateral hypothalamus (LH) and posterior hypothalamus (PH). There were also inputs from the lateral septum (LS), zona incerta (ZI), as well as the medial amygdala (MeA) and lateral amygdala (LA). Among those areas, some are of particular interest such as the PVN and LS, involved in stress/aversion (Swanson & Sawchenko, 1980); the VMH and PAG involved in aggression; MeA involved in the vomeronasal system (Figure 2.2B and C).

Utilizing the same technique, we also investigated the sex dimorphism between virgin male and virgin female circuits. First, we found that the main input area is the PVN for both sexes. (Figure 2.2C). Furthermore, the PVN, MPOA, VLPO and LH show statistically significant sex differences

(Figure 2.2C). On average, PVN inputs made up 22.95% ± 4.19% of all monosynaptic inputs of PeFA Ucn3+ cells in males and 44.92% ± 8.09% in females. The MPOA was the 2nd largest PeFA Ucn3+ direct input contributor with 9.06% ± 2.08% of the inputs in males and 2.84% ± 0.03% in females (Figure 2.2C). Therefore, in female mice, Ucn3+ cells had a higher proportion of inputs from the PVN (p value was 0.049) and LH (p value was 0.038) than those seen in male mice. Conversely, in male mice, the MPOA (p value was 0.011) and VLPO (p value was 0.031) participated to a higher degree in the map of inputs (Figure 2.2C). The MPOA input involvement is interesting as these cells are largely GABAergic and as stated previously, necessary for the orchestration of parental behavior (Kohl et al., 2018; Wu et al., 2014); given the data, one could hypothesize that the MPOA inhibits PeFA Ucn3+ cells.

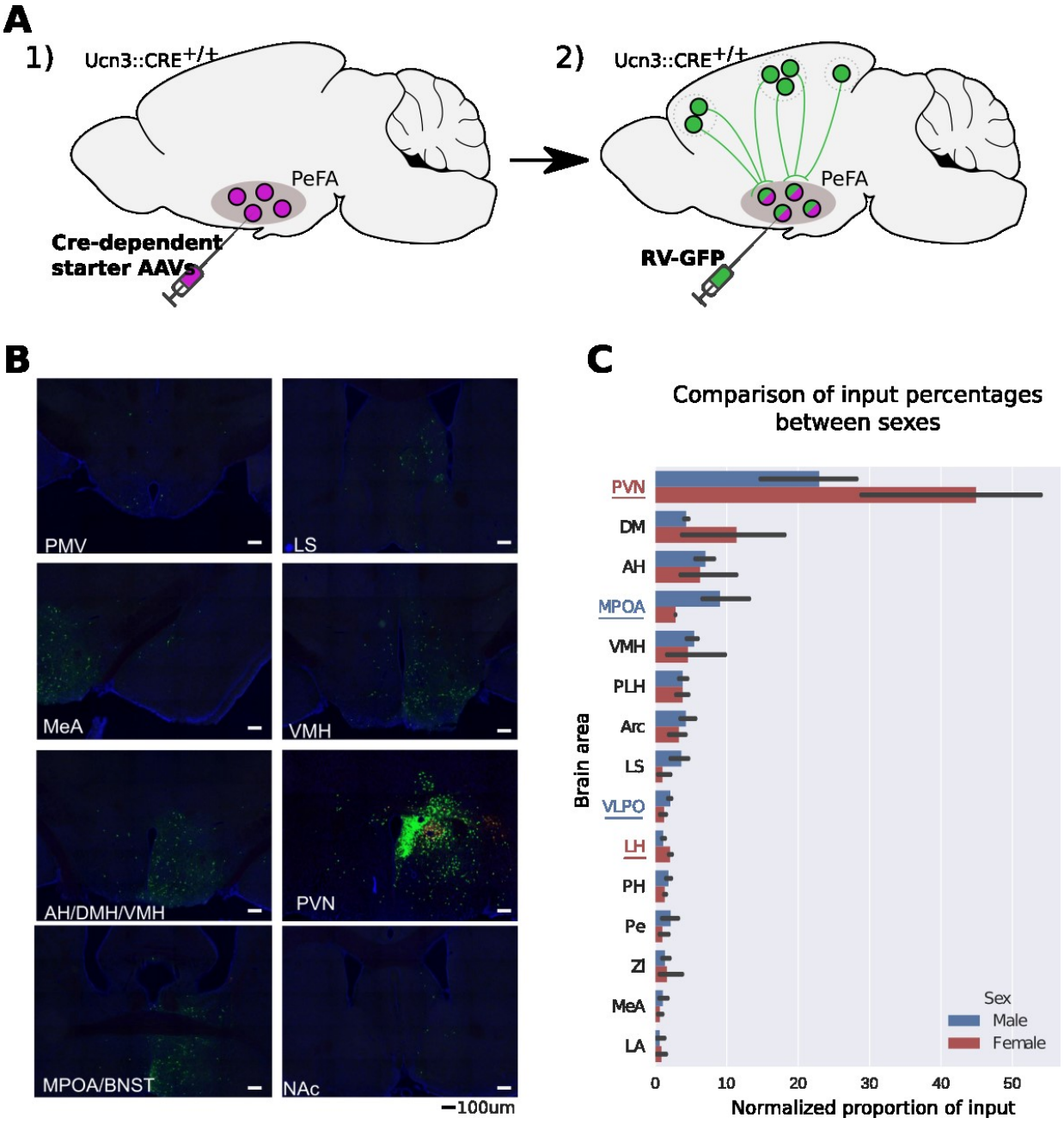

Figure 2.2: PeFA Ucn3+ Input Map

A. Experimental diagram of injection paradigm. 1. PeFA injection of CRE-dependent starter AAVs. 2. PeFA injection of EnvA, G-deleted rabies virus (RV-GFP). **B.** Representative images of labeled cells by the RV carrying GFP. Areas shown: PMV, LS, MeA, VMH, AH/DMH/VMH, PVN,

Figure 2.2 (Continued) MPOA/BNST, NAc. **C.** Comparison PeFA Ucn3+ map of inputs in virgin males (blue) versus virgin females (red). Significant sex dimorphic differences are underlined and colored correspondingly to which sex had an increase in this area. Error bars are the standard error (SEM).

Discussion

Analysis of MPOA Galanin+ Projections

In this study, we looked at the projection patterns of MPOA Gal+ neurons. To this end, we utilized approach similar to the study of Agrp neurons in the Arc for the hunger circuit namely the co-injection of different CTB tracers to uncover branching patterns of the MPOA Gal+ cells (Wang et al., 2015). Interestingly, as in the Arc Agrp study, we found that retrogradely-labelled cell bodies from each of the target site projections generally occupied discreet, non-overlapping subpopulations in the population sending projections, which for us was the MPOA Gal+ cells. As described in the results section of this chapter, Kohl also examined the following pairwise combinations: PAG & VTA, VTA & MeA and PAG & RRF. In these combinations, he also found that non-overlapping MPOA Gal+ cells sent projections to discreet target sites (Kohl et al., 2018). Only a small subset of MPOA Gal+ neurons that took up tracers was found to project to more than one output area. In the case of MeA and PAGr pairwise combination, little bifurcation was seen indicating that independent MPOA Gal+ subpopulations mostly send their projections to non-overlapping target areas. Though the same result is generally true for the pairwise combination of vlPAG and dlPAG, there was more bifurcation seen for this result than any other combination. However, this was the only combination that had two target sites in subregions of a larger anatomical region. The difference could therefore be due to a subregion-specific trend in which two, functionally-distinct subregions in an area share slightly more inputs homology than two, functionally-distinct areas. Overall, these results suggest that MPOA Gal+ neurons are organized in distinct subsets that project to mostly non-overlapping target areas (Kohl et al., 2018). The larger-scale hypothesis that was produced by Kohl from this data

was that some of these non-overlapping populations of MPOA Gal+ neurons control specific subsets of parenting via their target-site projections. In consequent experiments, Kohl found that the MPOA Gal+ projections to the PAG and MeA overlapped with cfos during parental care. Using fiber photometry, he then found that the MPOA Gal+ neurons projecting to the PAG were specifically activated during pup grooming, whereas MPOA Gal+ neurons projecting to the MeA were active during most behaviors encompassed by parental behavior, such as crouching nest building, grooming etc. When he optogenetically-activated MPOA Gal+ PAG projections suppressed pup attacks in virgin males and increased pup sniffing in both males and females. Activation of MPOA Gal+ MeA projections had less drastic effects as it only decreased the amount of time females spent in their nest. These fascinating results support the hypothesis that non-overlapping populations of MPOA Gal+ neurons control specific subsets of parenting via their target-site projections (Kohl et al., 2018).

Analysis of Inputs of Ucn3+ PeFa Neurons

We used a conditional rabies virus to create a map of inputs of PeFA Ucn3+ cells in virgin male and female mice. The inputs of these cells were previously unknown. We uncovered the PVN as the largest input area to the PeFA Ucn3+ neurons. Moreover, we found the existence of direct innervation unto PeFA Ucn3+ neurons from areas with many functions including but not limited to stress/aversion/social behavior (PVN and the Lateral Septum (LS) (T. P. Sheehan et al., 2000; Swanson & Sawchenko, 1980)), parenting/sexual behavior/thermoregulation/(MPOA) (Beach & Jaynes, 1956; Magoun, Harrison, Brobeck, & Ranson, 1938; Wu et al., 2014), aggression (Ventro Medial Hypothalamus (VMH) and PAG), and social recognition (MeA) (T. Sheehan et al., 2001). Those areas are also interesting as many have been directly linked with parenting behavior. OTX secreting PVN neurons have been implicated in maternal behavior and lactation (Marlin, Mitre, D'amour, Chao, & Froemke, 2015; Wu et al., 2014). The MPOA is a long known key node in parental behavior (Beach & Jaynes, 1956). The LS has been implicated with maternal aggression (G. Lee & Gammie, 2009). The VMH has been shown to regulate maternal behavior in rats (Bridges, Mann, & Coppeta, 1999). Finally the MeA has been shown to inhibit maternal behavior in rats (T. Sheehan et al., 2001). Moreover, the MeA is involved in the vomeronasal pathway, which has been shown to be necessary for the display of pup-directed aggression (Tachikawa et al., 2013; Wu et al., 2014). Moreover, Autry showed that PeFA Ucn3+ neurons show c-fos expression during pup-directed aggression and a stressed state can affect parenting (Murgatroyd & Nephew, 2013; Pedersen, Caldwell, McGuire, & Evans, 1991), these inputs coming from areas involved in aggressive behavior and stress/aversion are particularly interesting. Vomeronasal inputs are also interesting because VNO ablation in virgin male mice

leads to decreased levels of pup-directed aggression (Tachikawa et al., 2013; Wu et al., 2014). However, as these areas are also involved in other behaviors such as feeding or mating, functional experiments would be needed to conclude whether the activation of the PeFA Ucn3+ neurons is directly related to stress or aggression activated neurons in those areas, as well as to determine whether the sex-differences in the inputs are behaviorally-relevant to pup-directed neglect or infanticide.

In conclusion, this chapter has discussed some of the circuitry involved in regulating the pup-directed behaviors of parenting and infanticide/neglect. In the parenting circuit, the projection patterns seem to indicate a mostly-independent control by different subpopulation of MPOA Gal+ cells. In the infanticide circuit, we have unraveled a new set of monosynaptic inputs for a potentially central node, the PeFA Ucn3+ cells.

Interestingly, a central area to both behaviors is the MPOA, which projects inhibitory outputs to the PeFA Ucn3+ cells, potentially regulating the display of infanticidal behavior. This will lead to further experiments to decipher how both circuits might regulate each other and lead to a better understanding of the bipotential circuits regulating those two behaviors.

Materials and Methods

Cholera Toxin Subunit-B Tracing in MPOA Galanin+ Cells

In mice expressing tdTomato in Gal+ (Gal::Cre +/−; loxP-Stop-loxP-tdTomato +/−), I performed pairwise injections of 27 – 55 nl of 0.5% (wt/vol) fluorescently labelled cholera toxin B subunit (CTB-488, Thermo Fisher C22841, CTB-647, Thermo Fisher C34778) in known MPOA Galanin+ target sites. Each dye was injected into a different output site. I injected CTBs in the following MPOA Gal+ output area combinations: a) posteriodorsal Medial amygdala (MeApd) and rostral periacquetal grey (rPAG) b) ventrolateral PAG (vlPAG) and dorsolateral PAG(dlPAG).

After one week, tissue was collected, perfused, fixed with PFA and sliced with a vibratome into 60 µm sections. All injection sites and corresponding MPOAs were imaged at 20x resolution using a LSM 880 confocal microscope. After imaging the tissue, I quantified the number of MPOA Gal+ somas that had taken up either one or both tracers to determine the topography of different populations of MPOA Gal+ neurons, as defined by their projection patterns. Moreover, for all injections, I used ZEN Imaging Processing software to measure the spread of different volumes in each of the areas where a tracer was injected to ensure that the volumes had not spread beyond the anatomical area of interest. In control experiments, a 1:1 mixture of CTB-488 and CTB-647 was injected into MeA or PAG. This methodology was inspired by that seen in Wang et al, 2015 but volumes of CTB injected were changed to ensure CTB did not spread beyond the target site (Wang et al., 2015).

Trans-synaptic Retrograde Tracing in Perifornical Area

Ucn3::Cre mice of 8–12 weeks of age were used in input tracing experiments. Dr. Autry injected 100 – 150 nl of a 1:1 mixture of AAV-FLEX-TVA-mCherry and RG glycoprotein (AAV-FLEX-RG) into the PeFA. After waiting two weeks, Dr. Autry injected 250 – 300 nl EnvA-pseudotyped, G-deleted, GFP-expressing rabies virus (EnvA-ΔG rabies) in the PeFA. After the second surgery, mice were singly-housed and given a five-day recovery period. Mice were then euthanized and perfused. Brains were collected and sliced into 60 μm sections using a vibratome. During imaging, Dr. Autry imaged every second section at 20x resolution using a LSM 880 confocal microscope, and I counted the cells manually and performed all statistical analyses.

For quantification, GFP+ cells in both hemispheres were counted across the brain. Cells were assigned to brain areas by using DAPI staining and the Paxinos Mouse Brain Atlas (3rd edition), to identify anatomical landmarks in the corresponding tissue. I quantified the number of PeFA starter cells (mCherry+ & GFP+ cells) and input cells across the brain of three control virgin male mice that do not express CRE recombinase, as well as three Ucn3::CRE virgin female mice and three Ucn3::CRE virgin male mice.

For the analysis, I started by counting the total amount of input cells (GFP+ only cells) in each brain region normalized by the total amount of input cells across all regions per brain to be able to measure the relative fraction of inputs for each area to the PeFA Ucn3+ neurons. The data was then grouped and averaged by sex. Each area of the normalized input fraction for both sexes was then tested for significance using a Student t-test. This experimental paradigm was

modified from that used in Kohl et al. to best ensure comparability of results (Kohl et al., 2018). The only modifications done to that protocol were the amounts of viruses injected were reduced in this experiment and the quantification was done manually in this experiment.

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Introduction

In Chapter I, I discussed how parenting and infanticide seem to exist in a bipotential state. In male mice, a fascinating process occurs, in which mating leads virgin males to change their behavior towards pups from infanticide to parental care. This behavioral shift takes almost three weeks to occur, which coincides with the pregnancy length of mice (vom Saal, 1985). Mated males start displaying parental behavior upon the birth of their young and remain parental until the weaning of their offspring (vom Saal and Howard, 1982; vom Saal, 1985; Mennella and Moltz, 1988) and this event was shown to occur in male mice even if they do not cohabitate with females after mating suggesting the existence of some internal clock regulating this process (vom Saal, 1985). However, an open question remains as to what mechanism causes this switch**.** For example, behavioral changes directly elicited by hormonal spikes typically occur within half an hour to several hours (Numan and Insel, 2003) and chromatin remodeling-based changes such as histone acetylation could elicit behavioral effects within hours (Levenson *et al.*, 2004). Though these processes cannot be excluded as potential mechanisms underlying the parental switch, their effects can last several weeks. The experiments outlined in this chapter and chapter 4 seek to explore adult neurogenesis as a potential mechanism underlying the parental switch seen in male mice.

In the rodent brain, there are two undisputed neurogenic niches in adults, the subventricular zone (SVZ) near the thalamus and the subgranular zone (SGZ) in the dentate gyrus (DG)

(Palmer, Takahashi and Gage, 1997; Feierstein, 2012). While newborn cells in the hippocampus are integrated locally, the cells born in the SVZ travel via the rostral migratory stream (RMS) to the olfactory bulb (OB) to establish connections (Kempermann, 2011). Between three to eight weeks after their birth, neurons born in the DG are integrated into existing circuits and their firing patterns are indistinguishable from mature granule cells (reviewed in Gage, 2000). The cells born in the SVZ migrate to the OB and some of them are already integrated into circuits by three weeks (Alvarez-Buylla and Lim, 2004). Both of those adult neurogenesis phenomena correlate well with the three weeks timing of the parenting switch. Moreover, adult neurogenesis has been shown to be triggered by a host of behaviors, including social behaviors such as mating (Feierstein, 2012). Other phenomena, such as the memory formation involved in conspecific recognition (Raam *et al.*, 2017), also requires adult neurogenesis. Moreover, as I have discussed in Chapter I, hormones play a key role in controlling parental behavior. To this end, adult neurogenesis has been linked to hormonal changes, such as the rise of prolactin during pregnancy in females (Mann and Bridges, 2001; Oboti *et al.*, 2011). However, since males do not experience the hormonal changes produced by pregnancy, it was intriguing to consider what other molecular mechanisms could underlie this drastic behavioral change toward the pup. Also, some labs had already hypothesized that mating-induced adult neurogenesis could give rise to neurons needed in partner recognition and parental behavior (Bonfanti *et al.*, 1997). To understand how adult neurogenesis could make a circuit affecting preexisting circuits involved in pup directed behaviors, we need to look in more detail at how the proliferation and circuit integration of those cells happens.

In the DG, radial glia-like “stem-cells” called type 1 cells divide asymmetrically to give rise to type 2a glia-like cells which transiently and locally proliferate. Type 2a cells then differentiate into Type 2b cells which are determined to be neurons. Finally type 2b cells will become mature into “neuroblast” like cells which will migrate and be integrated into existing circuits. Those cells will exhibit a mature dendritic tree and have characteristics of granule cell chromatin (Kempermann, 2011). As mentioned before, this process comprising proliferation and integration requires between 3 to 8 weeks (Kempermann, 2011). In the SVZ, the process is slightly different. A niche of cells, maintained in the highly vascularized ventricular zone, control the niche’s ability to proliferate. These cells, called ‘B cells’, asymmetrically divide to produce ‘C cells’. Most ‘C cells’ symmetrically divide in large numbers forming a proliferating pool. Subsequently, most of the new proliferating pool of ‘C cells’ differentiate into type ‘A cells’, whereas the remaining ‘C cells’ become oligodendrocytes in the cortex (Kempermann, 2011). After proliferation and differentiation, type ‘A cells’ travel a large distance through the Rostral Migratory Stream (RMS) for several days until they reach the OB. In the OB, they differentiate further into mostly granular and periglomerular interneurons (Kempermann, 2011). Of interest to this chapter is the fact that there exist three main types of OB interneurons: Calbindin- (CB), Calretinin- and Tyrosine Hydroxylase- (TH) expressing cells. The new cells integrated into the circuit can start producing inhibitory outputs as early as two weeks after their genesis (Bardy *et al.*, 2010). Moreover, it is largely believed that cells that are not recruited to join existing circuits do not survive to be integrated (Gould *et al.*, 1999).

The mating-induced parental switch approximately correlates with the amount of time needed for newly-generated neuroblasts to become interneurons integrated into and affecting existing

circuits. To test this idea, Dr. Autry performed an initial set of experiments to assess the role of mating-induced neurogenesis in the establishment of parental behavior in male mice. First, she aimed to validate the idea that mating can increase integration of adult-born cells in the DG and OB. To this end, she labelled newly-born cells, by injecting control virgin males and male mice that were mated, with the synthetic nucleoside analog, 5-Bromo-2-deoxyuridine (BrdU). BrdU is incorporated into the DNA of cells in S-phase and allows for tracking of cells undergoing mitosis in the presence of the chemical (Eriksson *et al.*, 1998). Autry's injection paradigm consisted of injecting the male mice every 2 hours with BrdU for a total of 6 hours. During that time vaginal plugs were verified, and only males that mated during this period remained part of the experiment. Autry then waited 24 days and compared the amount of BrdU+ cells in the DGs of virgin versus mated male mice and found that mated animals had a larger number of BrdU-labelled cells. Furthermore, she observed a high number of BrdU labelled cells in the OB of mated animals, although this was not quantified. While these results are promising, the timing of the injection paradigm was questionable as it could potentially lead to large amounts of cells captured unspecifically before and after mating. Moreover, the cell integration in the OB was not quantified. For these reasons it seemed important to repeat this experiment with a precise injection of BrdU at the time of mating, and a careful quantification of potential differences in the OB integration. I performed these new experiments, which are described in the first part of this chapter. Moreover, to explore the potential source of differences in integration rates, I also quantified differences in proliferation rates between mated and control animals. Finally, I looked at different cell type markers expressed in the OB of mated males three weeks after

mating in order to find markers that were differentially expressed in cells born directly after mating.

Results

Comparison of Integration Rates of the Cells Born after Mating

In order to confirm and pursue the experiments initiated by Dr. Autry, I performed an experiment to compare the amount of cell integration in mated males three weeks after mating compared to control groups. To this end, I devised a methodology to target the cell population born during the mating event. This consisted of injecting BrdU within five minutes of the ejaculation of male mice. In order to acquire the integration differences due to mating, I analyzed the following two controls as well: unmated virgin males who roamed freely in their home cage and virgin males exposed to an estrus female but prevented from mating. All mice were allowed to engage in their assigned behavior for one hour, the average time it takes for a sexually-inexperienced male to complete mating. Since non-copulatory exposure to a female is not sufficient to cause the infanticide-to-parenting behavioral shift in virgin males (Brown, 1986), the female exposure group is a good null control to assess the role of social exposure in cell integration. Twenty-five days after BrdU injection, the mice were sacrificed, and I performed a comparative analysis of integrated BrdU positive cells in the DG (Figure 3.1), AOB (Figure 3.2) and MOB (Figure 3.2). To this end, brain slices of the OB and DG were immunostained for BrdU and imaged using confocal microscopy. I manually counted the BrdU+ cells (mated n=8, female exposure control n=8 and baseline control n=8, cohort size determined by power analysis) with a DAPI counterstain within sub-regions of the MOB, the AOB and the DG. Importantly, the MOB is composed of five main sub-region layers, the Granule Cell Layer (GCL), the Internal Plexiform Layer (IPL), the Mitral Cell Layer (MI), the External

Plexiform Layer (EPL) and the Glomerular Layer (GL). I observed that while cells were found in all of those sub regions in the three groups, only the GCL had a significant increase in mating-born cells when compared to controls (p-value < 0.05; Mann-Whitney U test) (Figure 3.2). In the AOB, cells were also present in all groups and the mated group had the largest amount of BrdU+ cells of any group, with a statistically significant increase compared to both female exposure and virgin male (p-value < 0.05; Mann-Whitney U test) (Figure 3.2). Outliers were excluded from this data set if they were three or more standard deviations from the mean, as recommended by Dr. Zhengzheng Liang and Dr. Catherine Dulac. Finally, in the DG, all groups showed new born cells and the mated animals had significantly more BrdU+ cells than either of the control groups and all groups were significantly different from each other as tested by a Mann-Whitney U test (Figure 3.1). The increases in the GCL of the MOB and AOB are interesting as granule cells are a major class of GABAergic interneurons in the OB. The newborn neurons in this area could signal the rearrangement of inhibitory circuits potentially underlying the behavioral switch induced after mating.

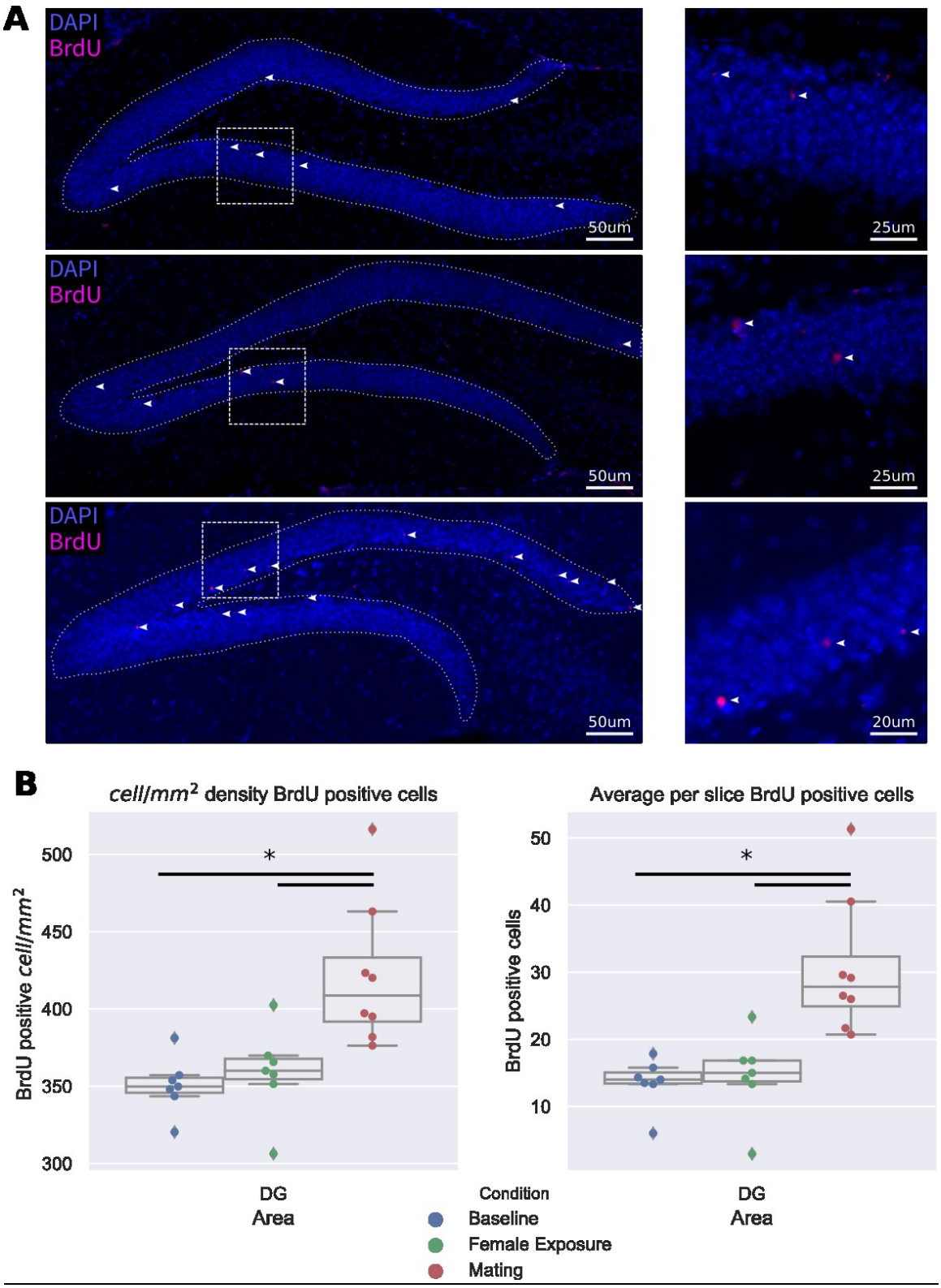

Figure 3.1: Quantification of Newborn Cells at their Hippocampal Target Sites 3.5 weeks Post-behavior

Figure 3.1 (Continued) Baseline: virgin males in experimental setup without intruders. Female Exposure: virgin males exposed to an estrus female. Mating: virgin males mated with an estrus female for the first time. **A.** Confocal images (20x on LSM880) of BrdU+ cells (magenta) with a DAPI counterstain (blue) in DG of males after treatment. **B.** Quantification of BrdU+ cell survival in DG of male mice 3.5 weeks after treatment. Statistical significance performed with a Mann-Whitney U test. Left, cell count was normalized for the area counted. Right, cell count was normalized by the number of slices counted.

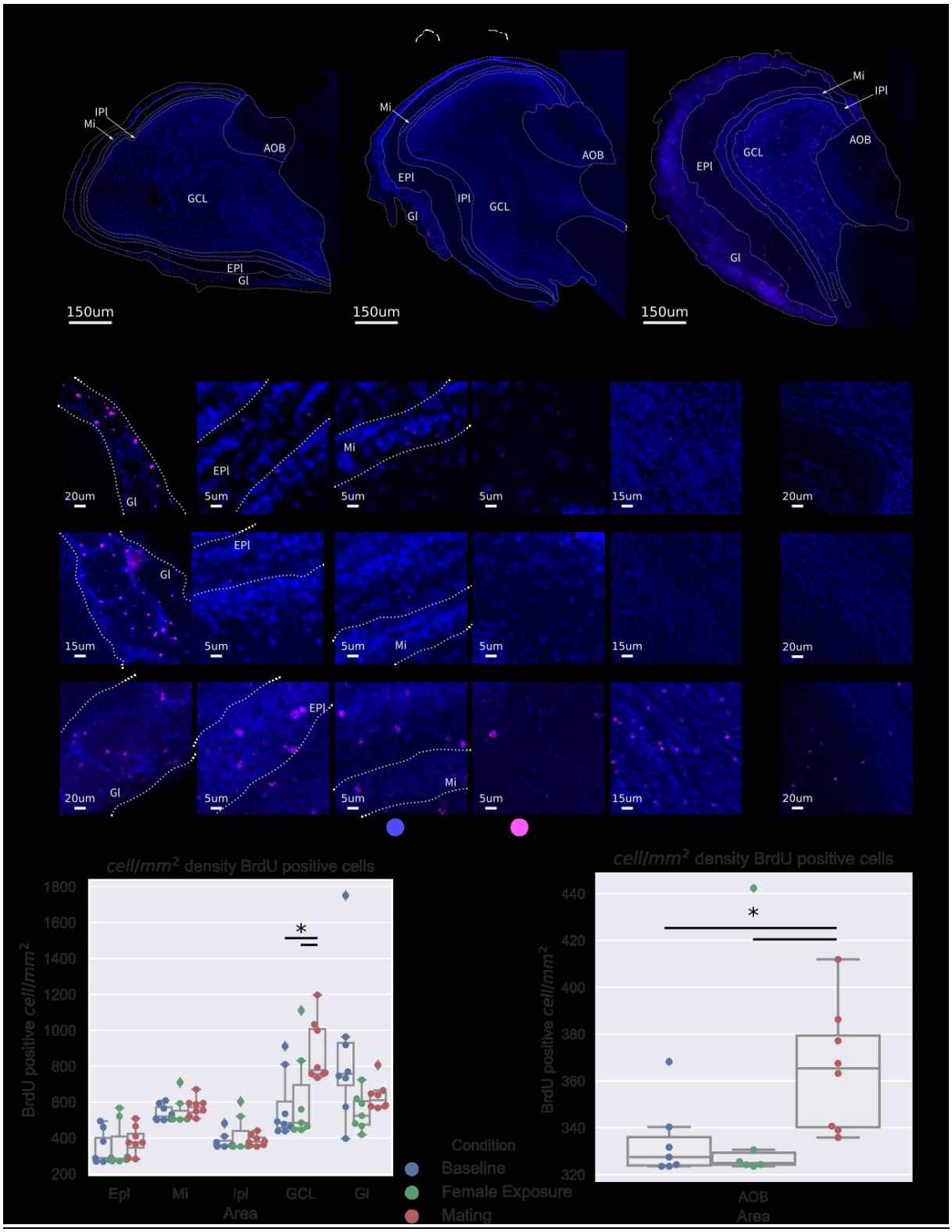

Figure 3.2: Quantification of Newborn Cells at their Olfactory Bulb Target Sites 3.5 weeks Post-behavior

Figure 3.2 (Continued) Baseline: virgin males in experimental setup without intruders. Female Exposure: virgin males exposed to an estrus female. Mating: virgin males mated with an estrus female for the first time. **A.** Representative images of areas in bulbs imaged (10x confocal microscopy on LSM880) 3.5 weeks after males of the 3 behaviors were injected with BrdU. **B.** Magnification of different areas in olfactory bulbs from A. **C.** Quantification of BrdU+ cells survival in different areas in the main olfactory bulb. Statistical significance performed with a Mann-Whitney U test **D.** Quantification of BrdU+ cells survival in the accessory olfactory bulb. Statistical significance performed with a Mann-Whitney U test.

Comparison of Proliferation Rates of the Cells Born after Mating

There might be two potential causes for measured increase in the integration of adult-born cells. First, the same number of cells may be born in every paradigm but mating produces factors that allow the cells to survive differentially. A second possibility is that mating induces an increase in the amount of proliferation in one or both of the neurogenic niches, and this difference is perpetuated and leads to increased integration. To determine which of these scenarios explains the increased integration seen in mated animals, I compared the proliferation rates in males in the following groups from the anterior to posterior SVZ: mating (n=3), female-exposure (n=3) and baseline (n=3) (Figure 3.3). In this experiment, virgin male mice were either mated with a receptive female, exposed to a receptive female across a barrier that prevented mating or placed in the experimental setup in their home cage. All mice were allowed to engage in their assigned behavior for one hour. After an hour, the mice received a BrdU intraperitoneal injection and were subsequently euthanized and perfused twenty-two hours later. The SVZs of the mice were then stained for BrdU. Quantification of the average total number of BrdU cells in each SVZ revealed no significant differences across the three groups. This data was analyzed using student t-tests and the corresponding p values are as follows: Baseline vs Female Exposure: the two tailed p value equals 0.2057 Not significant; Baseline vs Mated: the two tailed p value equals 0.1480 Not significant & Female Exposure vs Mated: the two tailed p value equals 0.4565 Not significant (Figure 3.3).

I also compared the average distributions across the SVZ (rostral to caudal) of BrdU+ cells in mated versus control animals and found that the data was not normally distributed. Indeed, as

we are measuring the number of cells across Bregma coordinates, there is no reason to assume that this would follow a normal distribution. Therefore, this data was analyzed using a multivariate Kolmogorov Smirnov test. Interestingly, the female exposure group had a distribution that was significantly different from the baseline group (p value was 0.03), as well as significantly different from the mated group (p value was 0.0005). However, the baseline and mated group were not significantly different from one another (p value was 0.44) (Figure 3.3).

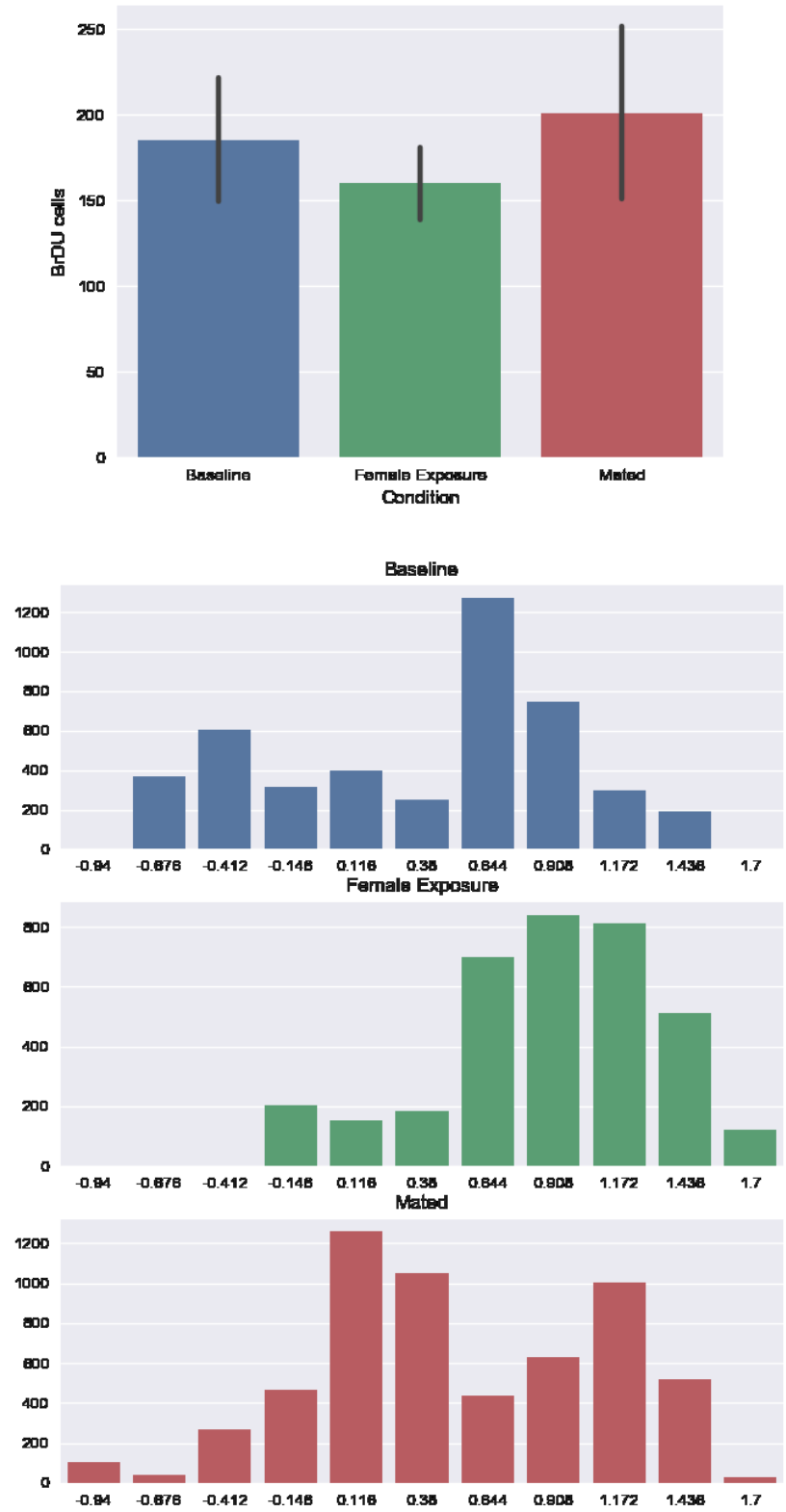

Figure 3.3: Comparison of Proliferation Rates in the SVZ Neurogenic Niche

Figure 3.3 (Continued) Baseline: males allowed to roam freely in their cage. Female Exposure: males exposed to an estrus female. Mating: mated allowed to mate with a receptive female. **A.** Quantification of BrdU+ cells in the entire SVZ across different behavioral conditions (Baseline n=3, Female Exposure n=3, Mated n=3). Statistical significance performed with a Student's t-test. **B.** Distribution of BrdU+ cells in SVZ bregma coordinates across different behavioral conditions (Baseline n=3, Female Exposure n=3, Mated n=3). Statistical significance performed with a KS test.

Comparison of Proliferation Rates in SVZ Sub-regions to Predict Markers in Maturity

As stated above, the mated group had increased integration of newborn neurons in the GCL but no increased proliferation as compared to controls was measured in the SVZ. However, this does not mean that there were no differences in the types of new born cells being produced in the niche. Indeed, the SVZ gives rise to cells from four main subpopulations named as follows: the dorsal wall (DW), lateral wall (LW), medial wall (MW) and ventral tip (VT). Some of these subpopulations have been linked to unique integration patterns as defined by their final location and expression combinations of tyrosine hydroxylase (TH) and calretinin (CR) (Alvarez-Buylla *et al.*, 2008). Calretinin marks neural subsets that contain calcium-binding proteins involved in calcium signaling (Briñón *et al.*, 1997). Tyrosine Hydroxylase is a marker for dopamine, norepinephrine and epinephrine-containing neurons and endocrine cells (Smith *et al.*, 1991). In an effort to predict which markers are expressed by mating-born cells that migrated to the OB (Merkle, Mirzadeh and Alvarez-Buylla, 2007), I performed a comparative analysis of the proliferation of the different neurogenic subpopulations (Figure 3.4). In this experiment, I had three groups of mice (n=3 per group). Each group performed one of the following behaviors for one hour: mating, exposure to estrus female across a divider (female exposure) and baseline, exactly as in previously-described experiments. At the end of the behavior period, mice were injected with BrdU and euthanized twenty-two hours after the injection. The SVZ was collected for each animal and the tissue was then stained for BrdU. I then imaged SVZs from Bregma 0.86mm to Bregma 0.14mm in which the neurogenic niche is known to have morphologically-distinct subpopulations, each of which expresses predictable combinations of cell type markers in their maturity (Merkle, Mirzadeh and Alvarez-Buylla,

2007). I predicted that this experiment would help narrow the search for integration markers in the olfactory bulb for cells born directly after mating. Indeed, differential proliferation in one of the sub regions could have predicted or indicated a unique integration marker in mature integrated adult-born cells from mating. However, upon quantifying the subpopulations for all the mice across all categories, I found no significant differences in any of the subpopulations across any of the experimental groups (Figure 3.4). This result could be due to the fact that I only counted a small, limited region of the SVZ and that a more thorough count of subpopulations across the entire SVZ might may further reveal a difference in the proliferation yield of discrete subpopulations in the SVZ of mated animals relative to controls. However, to my knowledge, no other study had quantified these subpopulations beyond the Bregma coordinates used. Therefore, this analysis did not yield any insights into specific markers of newborn neurons after mating.

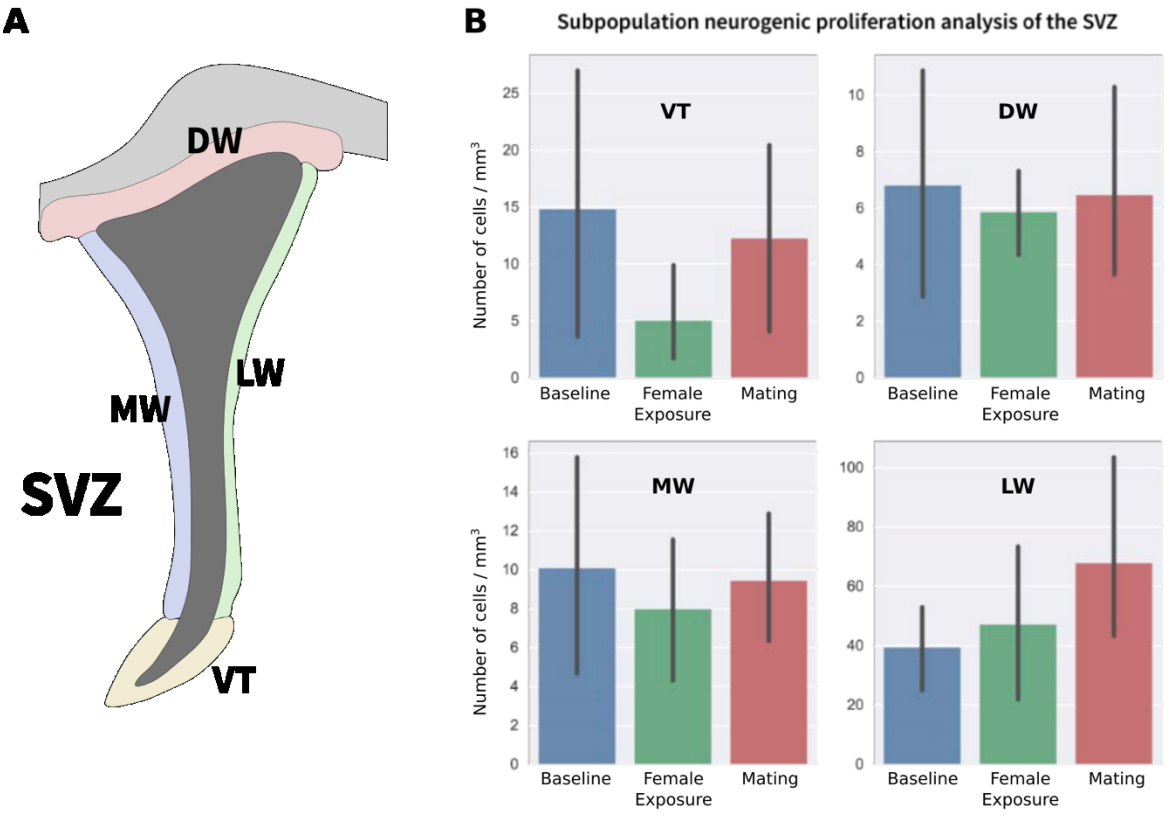

Figure 3.4: Comparison of Differences in Subpopulations of the SVZ Neurogenic Niche

A. Schematic representation of distinct subpopulation within the neurogenic niche of the SVZ.

B. Quantification of BrdU+ cells across different behavioral categories performed 22h prior to tissue collection. BrdU+ signal was counted per each subpopulation of the SVZ (graph not shown) and since no differences were seen across any area, the subpopulation counts were aggregated in each mouse to search for overall proliferation differences (Baseline n=3, Female Exposure n=3, Mated n=3). Statistical significance performed with a Student's t-test.

Characterization of Cells Born Directly after Mating

Since no cell populations were differentially proliferating across the different experimental groups, I could use this phenomenon to predict potential integration markers present. Therefore, I decided to continue trying to uncover which markers could be differentially expressed by the cells born directly after mating with immunohistochemistry of common markers from the literature (Batista-Brito et al, 2008). As mentioned in the introduction, most adult born cells in the SVZ in steady state conditions give rise to inhibitory granular interneurons. Therefore, I wanted to determine if the same was true in animals after mating. Moreover, I also wished to uncover the types of interneurons present (Figure 3.5). First, I sought to determine whether cells born directly after mating were neurons or non-neuronal cells. I used NeuN as a marker for mature neurons and GFAP as a marker for astrocytes. I injected BrdU in males, exactly as explained in the integration experiment above, and waited 25 days prior to collecting the tissue. It was then stained via immunohistochemistry for BrdU along with NeuN and GFAP. One of the potential pitfalls of using GFAP was that GFAP is also a marker for proliferating cells. However, since twenty-five days had elapsed since BrdU injection, none of the GFAP/BrdU double-labeled cells would have still been proliferating and would very likely be astrocytes. My NeuN/BrdU+ quantification (n=3) indicated that most (~93%) of the BrdU+ cells were colabelled with NeuN. This result did not seem to vary across mated, female exposure and baseline individuals. My GFAP/BrdU+ quantification (n=3) indicated that most of the BrdU+ cells were not colabelled with GFAP (less than 1% colabelling) and were therefore not adult-born astrocytes (Figure 3.5).

After demonstrating that the newly-generated cells were largely neurons, I next explored whether these cells expressed inhibitory markers such as GABA or GAD65/67 or whether they expressed excitatory markers such as VGLUT2. I first attempted a GABA/BrdU double immunohistochemical assay that was unsuccessful. I then performed Gad6567/BrdU immunohistochemical assay, as well as a VGLUT2/BrdU immunohistochemical assay and found that the BrdU+ mating-born cells were colabelled with VGLUT2 an average of 1.3% of the time across all areas of the OB (n=3). The Gad65/67 overlapped with BrdU+ cells an average of 72% of the time across all areas of the OB (n=3). This recapitulates previous findings that most of the adult born cells in the OB are inhibitory (Moreno *et al.*, 2009) (Figure 3.5).

Lastly, I also successfully performed double immunohistochemical assays of BrdU and the following markers: tyrosine hydroxylase (TH) and Calbindin (CB). Tyrosine Hydroxylase is a marker for dopamine, norepinephrine and epinephrine-containing neurons and endocrine cells (Smith *et al.*, 1991). Calbindin marks neural subsets that contain calcium-binding proteins involved in calcium signaling (Briñón *et al.*, 1997; Bastianelli, 2003). Calbindin and Tyrosine Hydroxylase overlap with BrdU+ cells varied across the different subregions of the MOB, so I did not average the percentages as for the markers above; the findings were as follows: In the Glomerular layer, Calbindin had a 79% overlap with Brdu+ cells. In the Granule Cell Layer, Calbindin had a 8% overlap with Brdu+ cells. Finally, there was no overlap between Calbindin and BrdU in the Accessory Olfactory Bulb. For TH, the Glomerular layer had an overlap of 6% with Brdu+ cells and there was no overlap in the Granule Cell Layer or in the Accessory Olfactory Bulb (Figure 3.5).

Overall, these results indicate that the vast majority of OB mating born cells are inhibitory neurons.

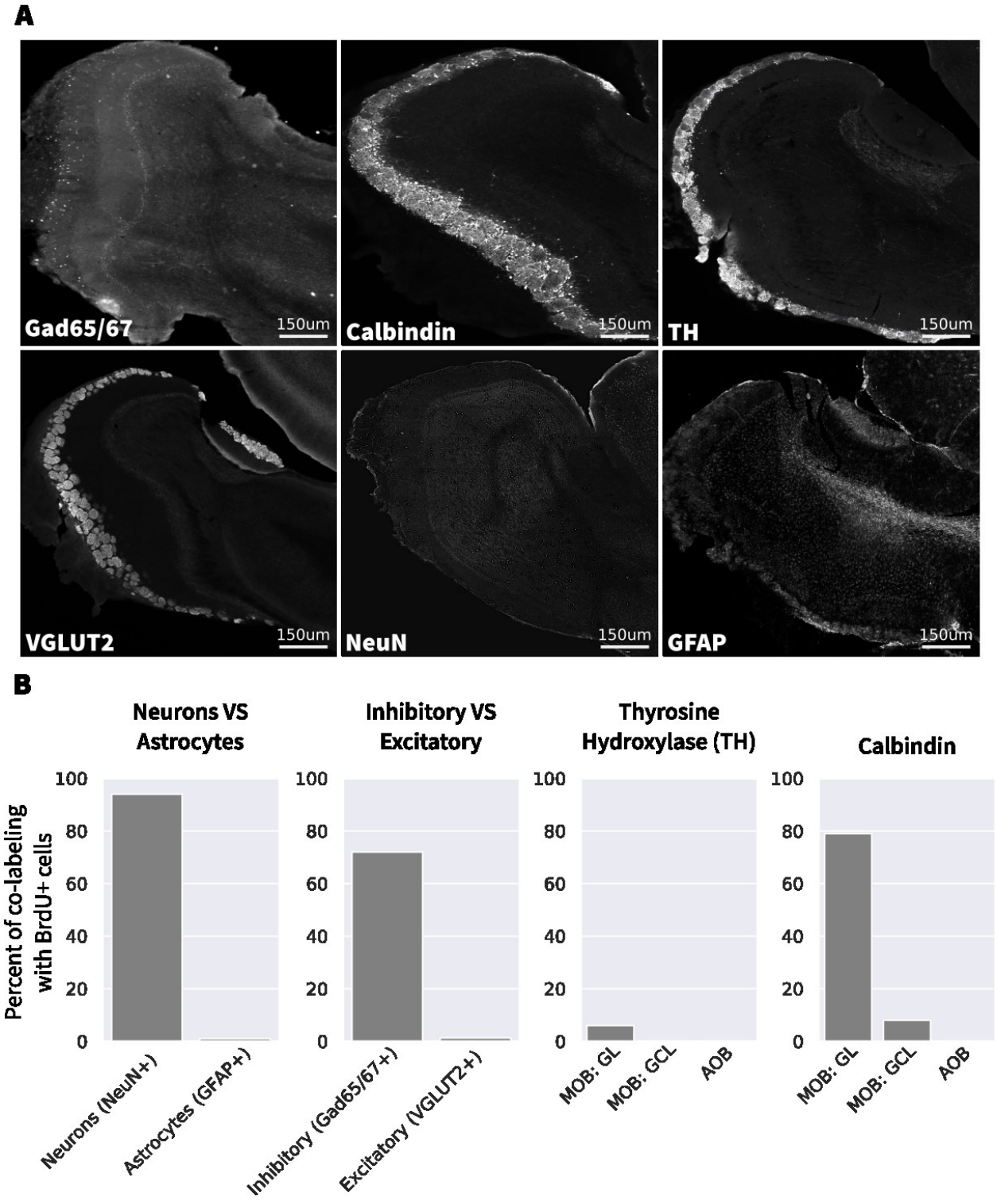

Figure 3.5: Characterization of Cells Born Directly after Mating

Figure 3.5 (Continued) **A.** Representative images of the different markers used in this study. In **B.** from left to right: Quantification of percent of OB BrdU+ cells born after mating colabelling with either NeuN (marker for mature neurons) or GFAP (astrocyte marker) (n=5). Quantification of percent of OB BrdU+ cells born after mating colabelling with either VGLUT2 (excitatory marker) or Gad65/67 (inhibitory marker) (n=3). Quantification of percent of OB BrdU+ cells born after mating colabelling with Tyrosine Hydroxylase (dopamine interneuron marker) in different regions of the bulb (n=3). Quantification of percent of OB BrdU+ cells born after mating colabelling with Calbindin (interneuron marker, periglomerular marker) in different regions of the bulb (n=3).

In summary, I have shown that there are changes in integration rate in the DG and MOB of mated animals that are present at the time of pup exposure. However, a pilot study of proliferation did not uncover differences at the level of the SVZ.

Discussion

In the experiments outlined in this chapter, I sought to determine whether adult neurogenesis occurring during mating led to an increase in cell proliferation in the SVZ and/or an increase in integration of newly born neurons several weeks later in the OB and DG, at a time coinciding with the birth of pups produced from the mating event. These experiments were designed to answer the question of whether cells born directly after mating were present at a timepoint that could provide support for their potential function in creating circuit changes that underlie the parental switch seen in male mice.

As we have seen, the rate of integration of adult-born cells in mated versus control animals showed that mated animals had significantly more cells in the granule cell layer of their main and accessory olfactory bulbs as well as in the DG of the hippocampus. This increase of cell integration in the GCL of the MOB, the AOB and the DG of mated animals is interesting because it indicates that there are inhibitory neurons born around the time of mating in the OB and granule cells in the DG that differentially survive and get incorporated into existing circuits. These modified circuits could be responsible for the behavioral changes seen in mated males from infanticidal to parental behavior. One question was whether these cells were the result of an increased proliferation, or an increased survival in the region they are integrated in. I compared the proliferation rates 22 hours after BrdU injection in mated animals and found that mated animals did not have significantly higher levels of SVZ proliferation as compared to controls. Importantly, proliferation rates were not quantified in the DG in this experiment.

Therefore, the increase in integration probably resulted from an increase in survival of mating born cells in the OB.

Following this result, I hypothesized that while the total number of cells might not be different, the area they were born in the SVZ might help predict the markers these cells express upon maturation. As discussed in the result and introduction of this chapter, different subregions of the SVZ contribute differentially to the adult born cell types integrating in the OB. However, I did not observe any significant differences in the proliferation across the different subregions when the experimental groups where compared to each other. I, therefore, decided to look at the expression of different markers in integrated cells born directly after mating. Characterization of those cells in the olfactory bulb found that these cells were mostly inhibitory neurons as indicated by the high overlap with Gad65/67, NeuN and BrdU. The other used markers showed small overlaps with BrdU+ cells. A recent publication has found new potential markers for this area by using single cell sequencing (Tepe *et al.*, 2018).

While those experiments uncovered a difference in the number of integrated cells in the OB and the DG, in concordance with Dr. Autry's preliminary experiments, they do not provide direct functional evidence to support that they underlie the parental switch. The evidence provided here does, however, support the idea that there are cells born during the period of mating that are mature and have differentially survived until a timepoint in which males would interact with their own pups for the first time. Cells born from mating have been investigated by others at different timepoints and no concrete discoveries have yet uncovered their function. Some studies have shown that cells born during mating help establish stereotyped

sexual function and sexual motivation (Waterston *et al.*, 2002; Unda *et al.*, 2016). No previous study, apart from Autry's preliminary experiments, had assessed the rate of integration of cells born directly after mating at a timepoint that could be functionally relevant to parenting. However, some studies had found tangentially-related evidence such as conflicting reports on the proliferation rate of males exposed to their offspring for the first time. In one study, they showed that exposure to pups for 20 min led to an increase of cell proliferation in the DG (Ruscio *et al.*, 2008). However, a more recent study found that a pup exposure for 20 minute, or a chronic exposure of 20mn/day did not lead to an increase in cell proliferation in the DG (Lieberwirth and Wang, 2012). Importantly, these studies did not control for the males' prior experiences, which could be the root of the discrepancies. Finally, studies showed that fatherhood lead to a decrease in the proliferation of cells in the DG, without affecting the proliferation in the SVZ nor the in the OB (Glasper *et al.*, 2011; Lieberwirth *et al.*, 2013).

Despite the absence of SVZ proliferation differences between the baseline and mated experimental groups, it is interesting to ponder the hypothetical models that could explain how an integration increase in the granular layer of the MOB, the AOB or the DG could possibly exert a functional difference affecting existing circuits involved in pup-directed behavior. First, I will discuss how in the AOB inhibitory granule cells and inhibitory periglomerular cell can prevent pup-directed attacks. As discussed in the introduction, the accessory olfactory system is necessary for pup-directed aggression in male mice (Wu *et al.*, 2014; Isogai *et al.*, 2018), therefore, a change in the circuitry of pup-sensing could affect the behavior displayed.

In the AOB, both adult-born periglomerular cells and adult-born granule cells inhibit mitral cells. In virgin males attacking pups, the IEG c-fos is highly expressed in the AOB (Tachikawa et al, 2013). However, fathers mated several weeks prior show a statistically significant decrease in AOB c-fos (Tachikawa et al, 2013). This difference could be explained by a subset of granule cells born directly after mating inhibiting other granule cells and mitral cells that were previously relaying VNO-sensed cues that led to pup-directed attack. However, this explanation would not explain the results seen in the VNO in previous studies where the VNO of fathers had significantly less activity to pups than that of virgin males (Tachikawa et al, 2013). Mustafa Talay, is hypothesizing that the GABAergic AOB periglomerular cells may inhibit vomeronasal sensory neurons (VSN) via GABA release that reaches GABAergic metabotropic receptors on the axon terminals of the VSNs (personal communication, Dr Talay). Though this hypothesis is still unsubstantiated, it does provide cohesiveness to the findings in this thesis in the context of previous published work (Tachikawa, et al, 2013; Wu et al, 2014; Isogai et al 2018).

Second, I will discuss how an increase in the GCL can potentially create a change to facilitate parenting. When the function of the main olfactory system is disrupted, female mice display impaired maternal behavior (Wang and Storm, 2011). Moreover, lesions to the piriform/entorhinal cortex impair pup-seeking motivation and pup-retrieval in female mice (Koch and Ehret, 1991). I hypothesize that MOB granule cell inhibition of mitral cells that is orchestrated by inputs from the entorhinal and piriform cortex are leading to a facilitation of parental care.

The GCL is largely composed of granular interneurons and these granule cells lack an axon. Mammalian olfactory bulb granule cells are not only fascinating in their morphology, but also in their function as their dendrites can process both synaptic input and synaptic output (Egger et al, 2005). Their morphology consists of mostly short proximal dendrites and a long apical dendrite that goes through the granule cell layer and interacts with mitral/tufted cells in the mitral cell layer. Olfactory bulb granule cells are usually inhibitory (Balu, Pressler and Strowbridge, 2007) and as was seen in the characterization experiment above, that is true of the BrdU+ cells I am seeing in the MOB granule cell layer. Approximately three weeks after their birth, these cell integrate into existing circuits and have formed inhibitory projections (Bardy *et al.*, 2010). These projections will then connect to the lateral dendrite or soma of mitral cells in the MOB (Shepherd, 2004; Balu, Pressler and Strowbridge, 2007). Moreover, the inhibition that these cells can exert is controlled by projections from the piriform/entorhinal cortex, which has been shown to be essential for the appetitive component of parental behavior in female mice (Koch and Ehret, 1991). The mitral cells receive excitatory inputs unto their primary dendrites from olfactory sensory neurons, and send their axons to the olfactory cortical regions, piriform cortex, medial amygdala and the anterior cortical amygdala (Kang, Baum and Cherry, 2011). Of note is the projection back to the piriform cortex, which could be creating a regulation loop. Since the MeA is an input area for MPOA Gal+ cells, I hypothesize that the granule cells born directly after mating inhibit mitral cells signals to MeA that were providing inhibitory inputs to MPOA Gal+ cells and, thus, by disinhibition, promote parental care in the parenting circuit. However, it is important to state that without more information to confirm the sequential

direct connectivity, all the hypotheses above are merely speculative and will require significantly more work to determine their ultimate validity.

To summarize, while these models are very hypothetical, the cells born directly after mating, could disrupt vomeronasal signaling to affect consequent perception of pups to prevent attack, as well as promote parental care by disinhibitions of the parenting circuit stemming from changes in the MOB. While both of these models involve the changes seen in the olfactory bulb, they do not account for the integration changes in the DG. Hippocampal neurogenesis is thought to be necessary for recognition of conspecifics (Raam et al, 2017) and the modulation of social aggression (Leroy et al, 2018). While the exact mechanisms are still unclear, it is involved with formation of new memories (Wei *et al.*, 2011). In Isogai et al. (2018), they demonstrated that many of the pup cues necessary for pup-directed aggression are also present in the mother. We could hypothesize that during mating, some of this odor cues are also present on the female and that a memory or engram of these combinatory odor profiles formed. The response to this memory could be controlled by the excitatory granule cells born directly after mating in the DG which target CA1 and CA2 neurons. CA2 neurons can be directly inhibited by the enthorhinal cortex and excited by the adult-born dentate gyrus granule cells (as reviewed in Dudek et al., 2016) in the presence of specific hormones (Leroy et al 2018), thus keeping tight regulation of whether these neurons fire. A group of CA2 neurons sends inhibitory projections to the lateral septum; chemogenetic inhibition of the CA2 synapses unto the lateral septum (LS) neurons inhibits social aggression tested using resident-intruder assays (Leroy et al, 2018). Though classic experiments support the role of LS activity as inhibiting aggression (Brady & Nauta, 1953; Wong, 2016), the researchers that found these connections between CA2 and

LS, also found that these LS neurons that were inhibited by CA2 were tonically inhibiting VMHvl, and thus the disinhibition drives aggression (Leroy et al, 2018). Of note, the main input to the adult-born granule cells largely GABAergic but changes over time (Tozuka et al, 2005). For example, though the first inputs are GABAergic the GABA exerts an excitatory effect before the new DG cells develop their glutamatergic neurotransmitter feedback (Tozuka et al, 2005). This input comes largely from the enthorhinal cortex (as reviewed in Dudek et al., 2016; Tozuka et al, 2005), which as stated previously plays a role in parental care motivation (Koch & Ehret, 1991). Thus, I hypothesize that in male mice, new DG cells born directly after mating can control the aggression directed at a pup by firing when overstimulated by hormonal modulation such as in the presence of an adult male or when their input from the entorhinal cortex changes. The engram from mating likely provides a protective effect to the pup and inhibits rather than excites the DG cells that can lead to aggression mediated by a DG-CA2-LS-VMHvl pathway. The maintenance of the mating memory by mating or continuous exposure to pups is likely needed to maintain this indirect inhibition of pup-directed aggression, thus providing an alternate mechanistic explanation underlying the parental switch.

To conclude, the exact mechanism is unknown, however, the three models presented above are not mutually exclusive and could work together to elicit a behavioral change.

Materials and Methods

Animals

All male mice used were between 10-14 weeks old. Animals were kept on a 12 h:12 h light/dark cycle with food and water available ad libitum. All behavioral components of experiments were done during the lights of cycle starting at 5pm. All experimental paradigms were approved by the Harvard University Institutional Animal Care and Use Committee (IACUC).

BrdU injection Paradigm for Analysis at Target Sites

The following injection paradigm was modified from the Abcam staining protocol: https://www.abcam.com/protocols/brdu-staining-protocol

To determine if mating leads to a rise in survival of mating born cells in the hippocampus and olfactory bulb, I labelled cells undergoing mitosis during three different one-hour behaviors by injecting male mice with 5-Bromo-2-deoxyuridine (BrdU). There were three groups of males, each of which underwent a different behavior. The categories were baseline, mated and female exposure. Mated animals were placed into a cage for an hour with a female mouse in estrus to facilitate mating. After mating occurred, the males received a 150mg/kg intraperitoneal injection of BrdU and were then individually housed for 25 days. After 25 days males were euthanized via an intracardial perfusion while under anesthesia. Brain tissue was subsequently collected. Brain tissue was then fixed overnight in a 4% solution of paraformaldehyde. Tissue was embedded in a 2% agarose gel and sliced into 50-micron slices using a vibratome. Bulbs were sliced sagittally and all sections that remained attached to the cortex were collected. The

dentate gyrus was sliced coronally from Bregma -1.06mm to Bregma -2.80mm. One fourth of all tissue slices then underwent immunohistochemical staining for BrdU, with the exception of slices with AOB, for which one out of every three slices obtained were stained for BrdU.

BrdU Injection Paradigm for Analysis in Subventricular Zone

The following injection paradigm was modified from the Abcam staining protocol: https://www.abcam.com/protocols/brdu-staining-protocol

In this experimental paradigm, I labelled cells undergoing mitosis during three different one-hour behaviors by injecting male mice with BrdU. There were three groups of males, each of which underwent a different behavior. The categories were baseline, mated and female exposure. Mated animals were placed into a cage for an hour with a female mouse in estrus to facilitate mating. After mating occurred, the males received a 150mg/kg intraperitoneal injection of BrdU and were then euthanized 22 hours after the injection. Brain tissue was subsequently collected. Brain tissue was then fixed overnight in a 4% solution of paraformaldehyde. Tissue was embedded in a 2% agarose gel and sliced coronally into 50-micron slices using a vibratome. Tissue collected was the subventricular zone from Bregma 1.18mm to Bregma -0.20mm. One fourth of all tissue slices then underwent immunohistochemical staining for BrdU.

Tissue Preparation and Immunohistochemistry

The BrdU immunohistochemistry protocol used was made by Jenelle Wallace, member of the Murthy Lab at Harvard University. In the above experiments, all animals were perfused

transcardially with 1x phosphate buffered saline (PBS) for five minutes and then the tissue fixed with 4% paraformaldehyde. Brains tissue was collected and post-fixed in 4% paraformaldehyde overnight. After transferring the tissue to PBS overnight, brains were embedded in 2% agarose. This agarose block was mounted unto a Leica vibratome and sliced into 50micron sections.

For immunostaining, I used 12-well culture plates and permeabilized sections using 0.3% Triton X-100 in PBS for 30 min. For proper BrdU staining, the tissue was treated with 2 N HCl for 30 min at 37°C to denature the cellular DNA. The blocking step was done by keeping tissue overnight in blocking buffer which was made as follows: 0.3% Triton X-100, PBS and 2% normal goat serum in PBS. Primary antibody incubation was done over two overnights at 4°C at an antibody concentration of 1:200 in 0.3% Triton X-100 and 10% goat serum. Secondary antibodies were added for 2 h at room temperature at an antibody concentration of 1:500. Rat monoclonal antibody to BrdU (1:200, Abcam) was used to detect adult generated cells. Other primary antibodies used were as follows: mouse anti-NeuN, rabbit anti-GFAP, rabbit anti-VGLUT2, rabbit anti-Gad65/67, mouse anti-Calbindin and rabbit anti-Tyrosine Hydroxylase. For secondary antibodies, I used the following: goat Alexa-488 anti-mouse 1:500, goat Alexa-555 anti-rat 1:500, and goat Alexa-647 anti-rabbit 1:500. Tissue sections were mounted unto Superfrost Plus slides and let to dry overnight. The slides were then layered with DAPI-containing Vectashield mounting medium (Vector Laboratories, H-1200) and coverslipped.

Blinding Protocol

To avoid any potential bias, I was blinded in all these experiments at the tissue collection stage. After obtaining brain tissue from each mouse and placing it in a vial of paraformaldehyde, I

would label each vial to indicate the behavior group the mouse belonged to, as well as a number I had given each mouse in a group. These vials were then given to Stacey Sullivan, who would keep the label I placed and replace it with a letter code that she assigned to it. When I turned in all quantifications for all animals in an experiment, Stacey would provide the code map that linked her given code letter to the labels I had originally given the mice.

Microscopy

All brains were imaged using the Zeiss Axioscan Scan.Z1 slide scanner fluorescent microscope at 10x resolution. For characterization experiments, all slices were imaged using a Zeiss LSM 880 confocal microscope. For the confocal imaging, Z stacks were gathered with focal planes no greater that 4 microns per plane to ensure colabelling quantification accuracy.

For the proliferation and integration experiments, every fourth section was imaged, with the exception of AOB-containing slices, where every third section was imaged. For characterization experiments, every eight section was imaged, with the exception of AOB-containing slices where every sixth slice was imaged. All quantification was done manually. BrdU signal that colabelled with DAPI was counted as positive signal.

Statistical Analyses

The integration data was analyzed using Mann-U Whitney tests. Proliferation data averages in the SVZ were analyzed using Student's t-tests. Proliferation distributions were analyzed using a multivariate Kolmogorov Smirnov test.

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CHAPTER IV: FUNCTIONAL STUDIES INVESTIGATING ROLE OF ADULT NEUOGENESIS ON ONSET OF PATERNAL CARE

Introduction

As shown in Chapter III, mating correlates with changes in the integration of adult-born cells in the main and accessory olfactory bulb and dentate gyrus in mated males compared to controls. However, the experiments of Chapter III did not assess whether cells born directly after mating were necessary for the change of behavior happening during the parental switch. Using methodology successfully shown to ablate newly-born neurons through irradiation that consequently led to behavioral deficits (Wei *et al.*, 2011), Autry performed a pilot experiment to test whether ablating the neurogenic niches to prevent cells from being born during mating could lead to a change in pup-directed behavior in male mice (Figure 4.1A). More specifically, she irradiated the brains of males prior to mating to eliminate all neurogenic niches, thus, preventing the birth of cells born after mating (Figure 4.1B). She then proceeded to test if these males displayed parental behavior 24 days after mating (Figure 4.1A). To this end, she tested parental behavior by performing a pup assay, which consists of placing 2 pups opposite to the tested male's nest in its cage and recording the infant-directed responses. There are three possible behaviors each male might perform when encountering a pup: attack, ignore or retrieve. Males that had been irradiated mostly ignored the pups, or in other words, showed neglect. This initial result was encouraging and suggested that adult-born neurons may be necessary for the state-dependent, pup-directed behavioral switch from infanticide to parenting that male mice undergo after mating (Figure 4.1C). However, irradiation is a highly

toxic method that induces cellular damage across the entire neural population of the brain (Wojtowicz, 2006). Irradiation potentially had off-target effects such as inflammation and brain damage that could have affected the health and subsequent motivation of animals to perform most tasks. Therefore, these experiments could not lead to a clear conclusion on whether the neglect showed by males was due to the irradiation or to the loss of cells born during or directly after mating.

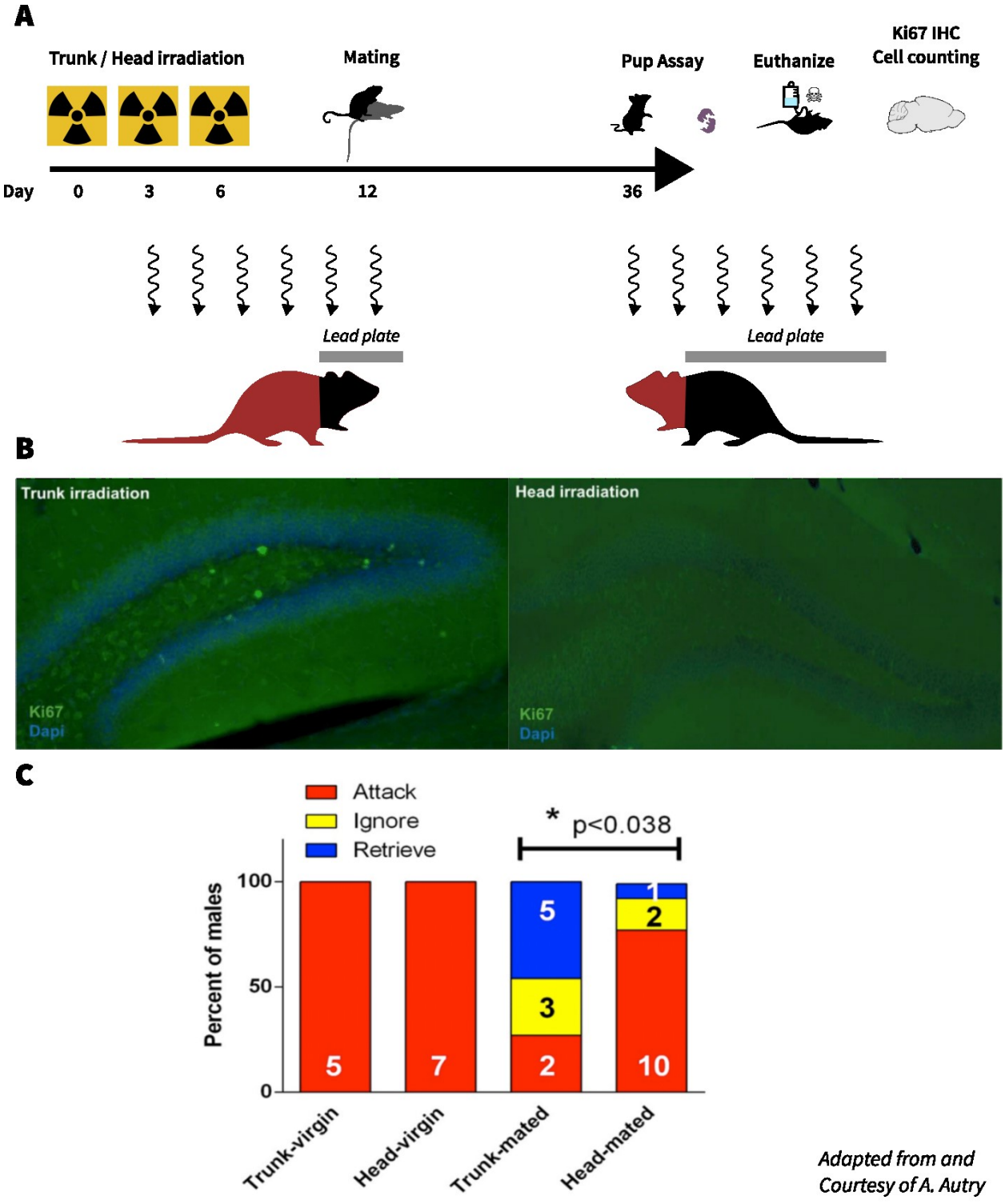

Figure 4.1: Irradiation-Induced Ablation of Neurogenic Niches Prevents the Parenting Switch.

Figure 4.1 (Continued) Experiment performed by and data courtesy from Dr. Autry. **A**. Schematic of the experiment performed by Dr. Autry. Virgin male mice were irradiated 3 times at day 0, 3 and 6 with either their head or their trunk protected by a lead plate. At day 12, males were mated to a female and 24 days later (day 36) parental behavior was tested in the groups via a pup assay. Following this, irradiated mice were euthanized and their brains collected and stained for Ki67. **B.** Representative images of Ki67 in the DG of trunk-irradiated vs head-irradiated mice. **C.** Results of the pup assay performed on the four tested groups, head-virgin, trunk-virgin, head-mated and virgin-mated. Mated trunk-irradiated mice are significantly more parental than mated head-irradiated mice.

Following on this work, I decided to ablate cells born after mating using an alternative approach. First, I decided to use a more targeted chemical ablation of dividing cells through the use of Arabinosyl cytosine (AraC). For future experiments, I also sought to establish a genetic ablation technique that utilizes the transient expression of TK in GFAP+ newborn cells.

AraC is a cytosine analog which is incorporated into the DNA of dividing cells. Carrying a arabinoside sugar instead of a deoxyribose, the incorporation of this chemical slows down DNA and RNA polymerase, prevents the completion of the S phase of mitosis and results in apoptosis (Perry, Doll and Freter, 2012). Here, by infusion of this chemical into the ventricles connecting the neurogenic niches, dividing cells will incorporate this chemical and enter apoptosis. AraC infused into the ventricles is able to efficiently target the neurogenic niches in the SVZ and DG if infusions are continued for 7-14 days, as has been utilized in previous experiments (Doetsch *et al.*, 1999; Doetsch, García-Verdugo and Alvarez-Buylla, 1999; Ghanbari *et al.*, 2015). This approach allows for a time controlled, and fairly specific ablation of the neurogenic niches. However, this approach still requires extensive surgeries that are discussed in the methods section and might lead to altered behaviors. Here, I present the effects of the AraC ablation of the neurogenic niche prior to mating on pup-directed behaviors as well as experiments whose results guided this experimental paradigm.

A different ablation approach is the use of a time-controlled genetic ablation of the neurogenic niche. In the early 1990s, researchers generated a transgenic mouse line that allows for the ablation of adult neurogenesis and is referred to as GFAP::TK (Bush et al, 1998; Garcia *et al.*, 2004; Saxe *et al.*, 2006). This mouse line utilized the enzyme Thymidine Kinase (TK) which

phosphorylates the small molecule Ganciclovir (GCV), causing it to become a thymidine analog. The phosphorylated GCV then incorporates into the DNA of dividing cells causing failures in S phase that lead to apoptosis (Garcia *et al.*, 2004; Saxe *et al.*, 2006). Since GFAP is expressed in progenitor cells of adult-born neurons in the subgranular zone of the DG, and the SVZ, this approach allows a selective, inducible inhibition of adult neurogenesis (Garcia *et al.*, 2004).

While the genetic approach seems to present clear advantages, I could not directly utilize this mouse line for this study. The display of infanticidal behavior by virgin males is variable across different laboratory mouse strains. The virgin males in the mouse line that contained the GFAP-TK cassette did not display infanticide. Therefore, I could not perform experiments regarding the parental switch. However, I shall present my efforts regarding backcrossing of this GFAP-TK mouse line into the TrpC2+/+ mouse strain that has been used across this study and in which most virgin males exhibit pup-directed aggression.

Results

In order to determine whether cells born directly after mating are necessary for the parental switch in male mice, I performed ablation experiments followed by behavioral assays. As discussed in the introduction, there exists several possible ablation paradigms to target adult neurogenesis, ranging from global or targeted irradiation methods (Wojtowicz, 2006) to controlled genetic ablation (Saxe *et al.*, 2006). One commonly used ablation approach is the intracerebroventricular infusion of the drug AraC (Doetsch *et al.*, 1999; Doetsch, García-Verdugo and Alvarez-Buylla, 1999; Ghanbari *et al.*, 2015). However, when deciding how to ablate cells born during or directly after mating, it was unclear what the best infusion paradigm would be. Injecting prior to or after mating could both be hypothetically valid approaches to ablate the cells born during or directly after mating. Importantly, if the ablation paradigm takes place prior to the mating event, the goal is to deplete the niche so that no cells are born during mating or directly after, as the niche will not have yet recovered from the ablation (Kempermann, 2011). Therefore, I devised the following experiment: I fit singly-housed, virgin male mice with unilateral cannulas that allowed for the input of either saline or AraC directly into the ventricles associated with the SVZ. After a weeklong recovery period, mice were separated into two groups. During a different set of days per paradigm, one group received bi-daily infusions of saline via their cannula, whereas the other group received volumetrically-equivalent, bi-daily AraC infusions. All mice were injected intraperitoneally at a specific time discussed below with the thymidine analog of BrdU to label dividing cells. To determine the most effective timing to administer AraC via a cannula, I concurrently tested the ablation efficiency of three different time window. The first, which I refer to as a 7d ablation consisted of

injecting AraC for 7 days prior to BrdU injection. Upon the last injection of AraC mice were injected with BrdU and sacrificed 22h after this last injection. The second, which I refer to as 12d ablation consisted of the same experiment, except that for 5 days following the BrdU injection, the mouse then received an AraC-BrdU (or PBS-BrdU) injection, and where sacrificed 22h after the last injection. Finally, I tested a 21d post-mating ablation paradigm where male mice were allowed to mate. Following ejaculation, males were injected with BrdU and for the next 21days they were then infused with AraC or PBS. For each ablation efficiency paradigm, I immuno-stained against BrdU and counted positive cells in the SVZ, RMS and DG of AraC-treated versus control PBS-treated mice. The 7d ablation lead to a reduction of 50% of BrdU positive cells, while the 12d ablation led to an 80% reduction (Figure 4.2).

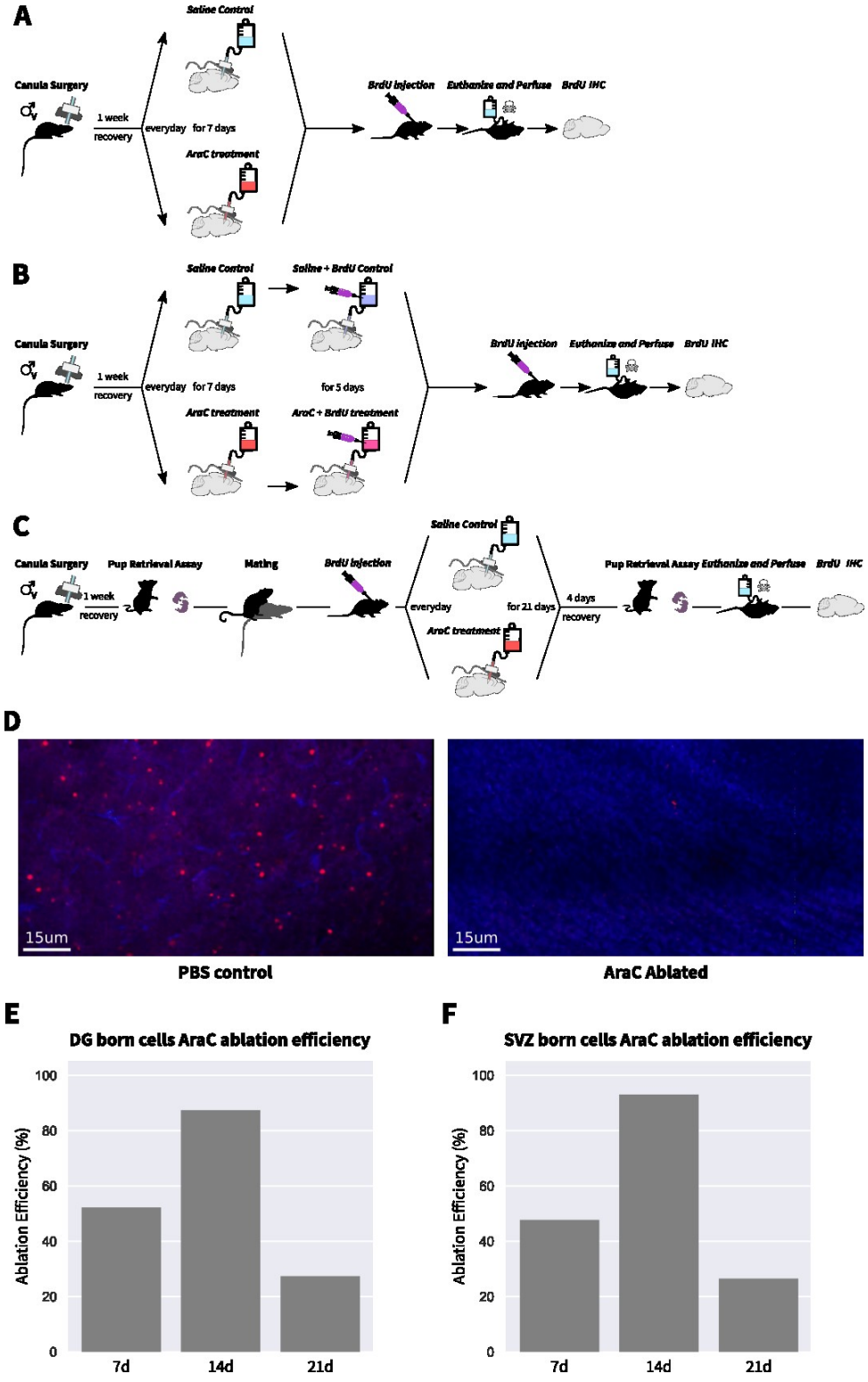

Figure 4.2: Ablation Efficiency Tests to Ablate Adult-born Cells Using AraC Infusions & BrdU

Figure 4.2 (Continued) **A-C.** Schematic representation of the different ablation paradigms tested. **D**. Representative images of the 14d ablation experiment performed on mated males. **E.** Quantification of BrdU+ cells in the DG of mice that underwent 7-day, 14-day and 21-day ablation efficiency experiments in (Saline n=2; AraC n=2) **F**. Quantification of BrdU+ cells in the SVZ of mice that underwent 7-day, 14-day and 21-day ablation efficiency experiments (Saline n=2; AraC n=2)

Dr. Autry, in her irradiation ablation experimental paradigm, utilized another marker of proliferating cells, Ki67. In order to compare the two ablation strategies, I repeated the 12d ablation using Ki67 as the proliferation marker rather than BrdU+. Autry found a 60% ablation efficiency in the DG when using a whole head irradiation, whereas my 12d ablation paradigm resulted in an average 86% reduction of Ki67+ cells in the DG and 93% in the RMS (Figure 4.3) suggesting an even higher efficiency than the X-Ray irradiation. Finally, the 21 days ablation that began after mating led to the worst efficiency of all tested paradigms with only a 28% reduction in cells born directly after mating. Moreover, only 20% of the animals survived the 21day treatment. The 12d ablation prior to mating was thus favored and selected over the 7d and 21d ablation paradigms.

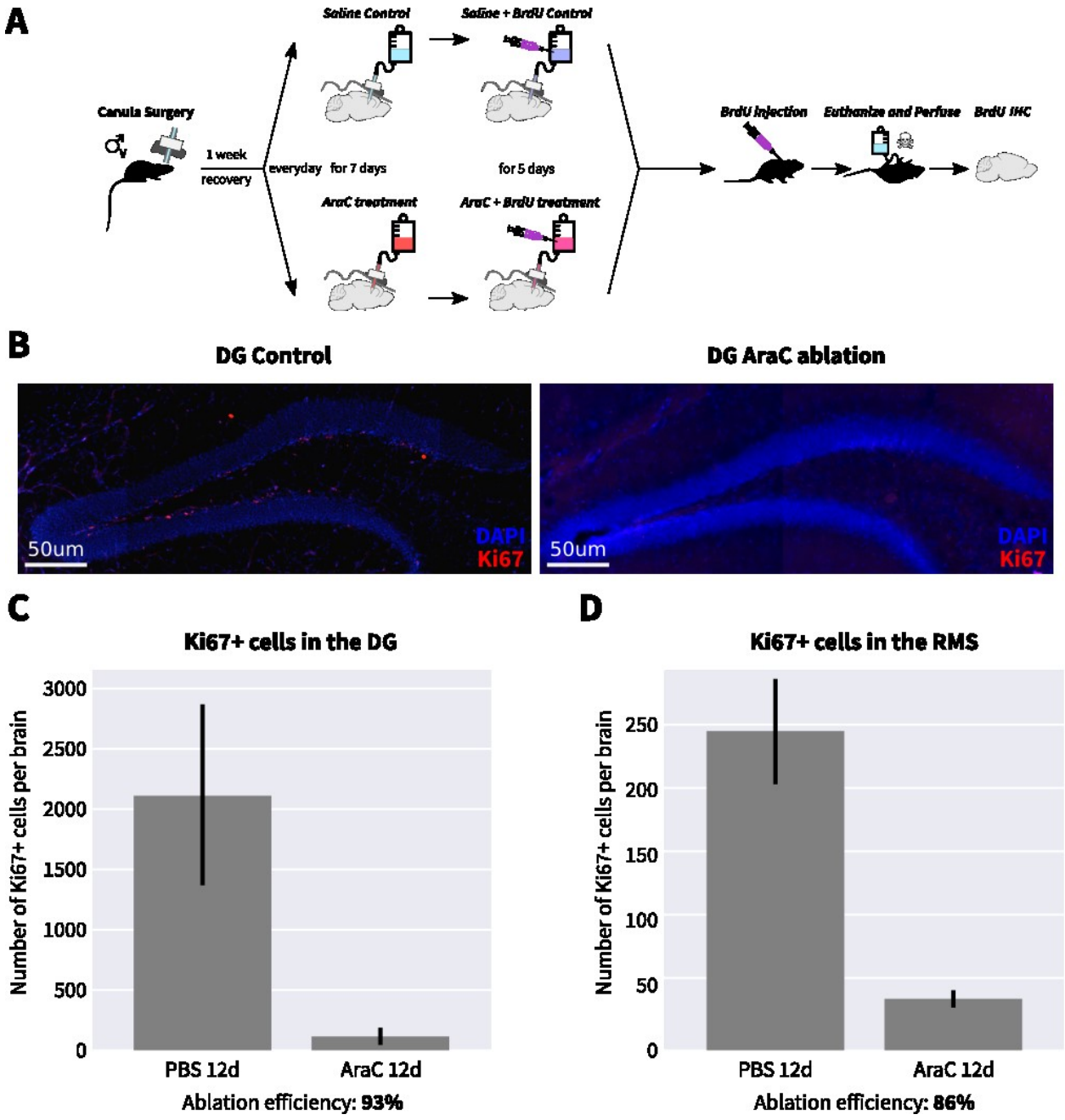

Figure 4.3: Ablation Efficiency Tests to Ablate Adult-born Cells Using AraC Infusions & Ki67.

A. Schematic representation of the ablation experiment performed for the Ki67 stain. **B**. Representative images of Ki67 staining in control and ablated animal. **C**. Quantification of Ki67+ cells in the DG of mice that underwent the 12-day ablation efficiency experiment. **D**.

Figure 4.3 (Continued) Quantification of Ki67+ cells in the RMS of mice that underwent the 12-day ablation efficiency experiment.

Having determined the best timing to ablate the neurogenic niche, I proceeded to perform the ablation of cells born immediately after mating and to subsequently test the impact on pup-directed behavior. Using the same surgical methodology to inject AraC and the 12d paradigm, I devised the following blinded experiment: Virgin male mice were mated with a receptive female and were injected intraperitoneally with the thymidine analog BrdU upon ejaculation to label cells born directly after mating. These groups are referred to as 'Mated-AraC' and 'Mated-PBS', respectively. Moreover, another control was performed where virgin mice were injected with BrdU without mating. This group is referred to as 'Virgin-AraC'. To control for the surgical procedure and effects of AraC infusions, two other groups of mice were added, mated-no-experimental-manipulation and virgin-AraC-treated males. In the 'No experimental manipulation' group, males were mated with a receptive female and were separated directly after ejaculation; no injections or cannulas were used on this group.

All males were then individually housed for 24 days and subsequently tested in a pup assay, as described in the introduction, and their responses to pups were recorded. All males with implanted cannula also underwent a mating assay and resident-intruder assay described in details in the methods of this chapter. Brains from mice with implanted cannulas were then collected, sliced and stained and quantified for BrdU expression in the OB. BrdU+ cells in the OB of males was quantified to determine the average extent of ablation of integrated neurons born during and directly after mating. On average, the AraC-treated groups had 81% less BrdU+ cells in the olfactory bulb than the PBS-treated group. Fischer exact tests were used to analyze all behavioral data.

In the pup assay, all the Mated AraC-treated animals were infanticidal. In the PBS-treated group, five animals displayed parental behavior, four animals ignored pups and twelve were infanticidal. In the Virgin-AraC group, thirteen animals were infanticidal towards pups and three animals ignored pups. In the 'No Experimental Manipulation' mated group, four animals were parental, four animals ignored pups and eight were infanticidal. Virgin-AraC and Mated-AraC are not significantly different (p-value = 0.0936). Virgin-AraC and Mated-PBS were also not significantly different in their response to pups (p-value = 0.1657). Mated-AraC and Mated-PBS males were significantly different in their response to pups (p-value = 0.0016). 'No Experimental Manipulation' and Virgin-AraC are not significantly different (p-value = 0.1351). 'No Experimental Manipulation' and 'Mated AraC' are significant different (p-value = 0.0007). 'No Experimental Manipulation' and 'Mated PBS' are not significantly different (p-value = 0.7463). All the results are summarized in Figure 4.4.

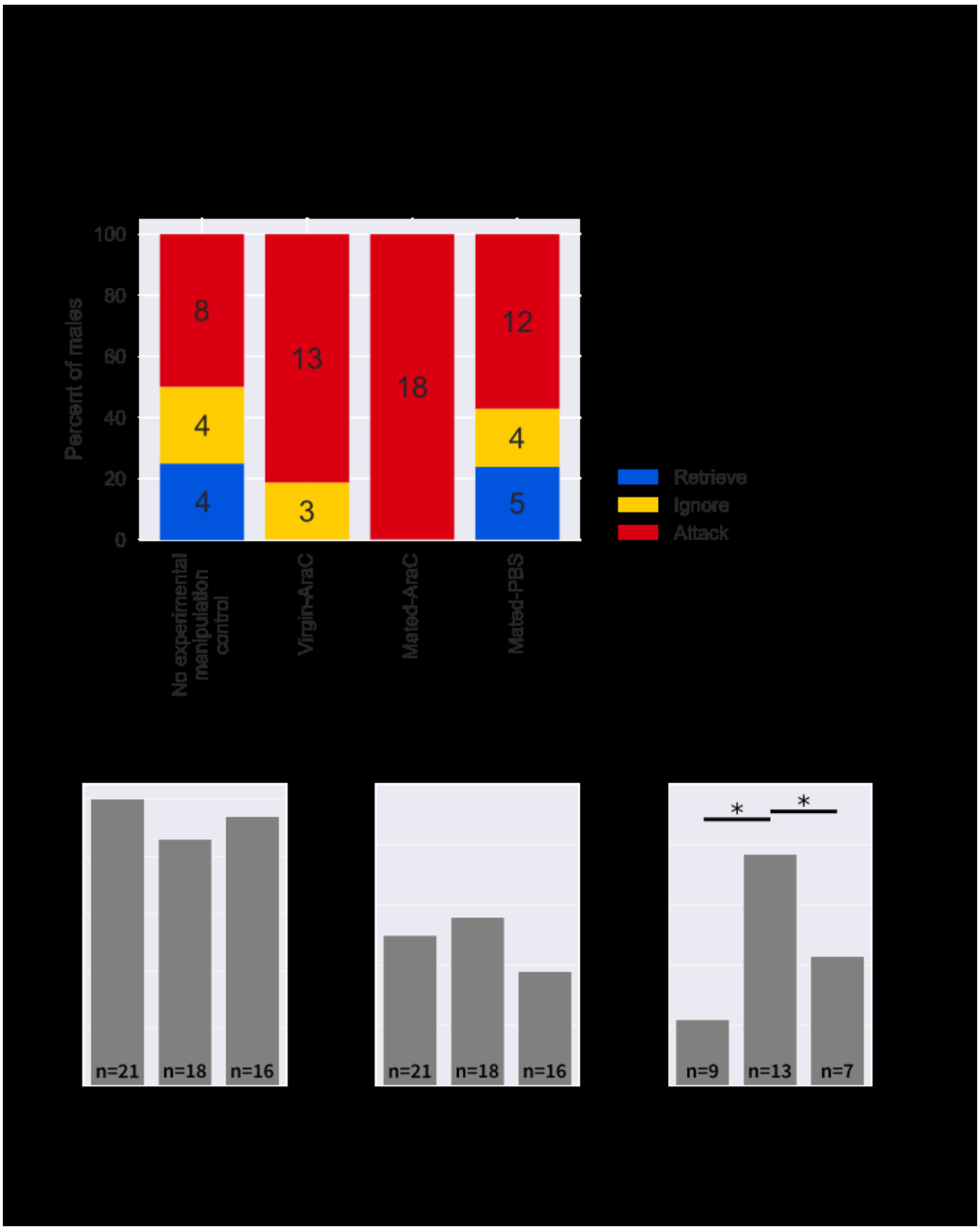

Figure 4.4: General Pup-directed Behaviors in I.c.v Cannula Experiment:

A. Quantification of pup assay results of Mated-AraC treated males (n=18), Virgin-AraC (n=16) treated males, Mated-PBS treated males (n=21) and male mice that were mated but underwent no cannula implantations or injections (n=16). **B**. Quantification of percent of mice who

Figure 4.4 (Continued) attempted to mate in each group of mice with cannulas. The experimental groups are the following: Mated-AraC treated males (n=18), Virgin-AraC (n=16) treated males and Mated-PBS treated males (n=21) **C.** Quantification of percent of resident mice that attempted to mount intruder mice in icv cannula experiment. The experimental groups are the following: Mated-AraC treated males (n=13) and Virgin-AraC (n=7) treated males & Mated-PBS treated males (n=9).

Following those results, a careful behavior analysis of recorded pup assays revealed that the duration of time spent by males with implanted cannulas sniffing and grooming pups was not significantly different across groups. Moreover, the attack latency towards pups of infanticidal males with implanted cannulas was not significantly different across groups. The analysis of recorded mating assays revealed that the number of males that mounted females was not significantly different across groups (Figure 4.4) and the duration of time spent by males with implanted cannulas sniffing and grooming females was not significantly different across groups. The analysis of recorded mating assays revealed that the quantity of mounting attempts towards females was not significantly different across groups. Moreover, the duration of time spent by cannula-bearing males mounting females while engaging in pelvic thrusting was not significantly different across groups. The analysis of the resident-intruder assays revealed that the duration of time spent by cannula-bearing males sniffing and grooming intruder males was not significantly different across groups. Moreover, attack latencies and attack durations of resident males towards intruder males were also not significantly different across groups. However, Mated-AraC resident males attempted to mount intruders at a significantly higher proportion than Mated-PBS and Virgin-AraC males (Figure 4.5). The details of these behavior analyses are presented in Figure 4.5.

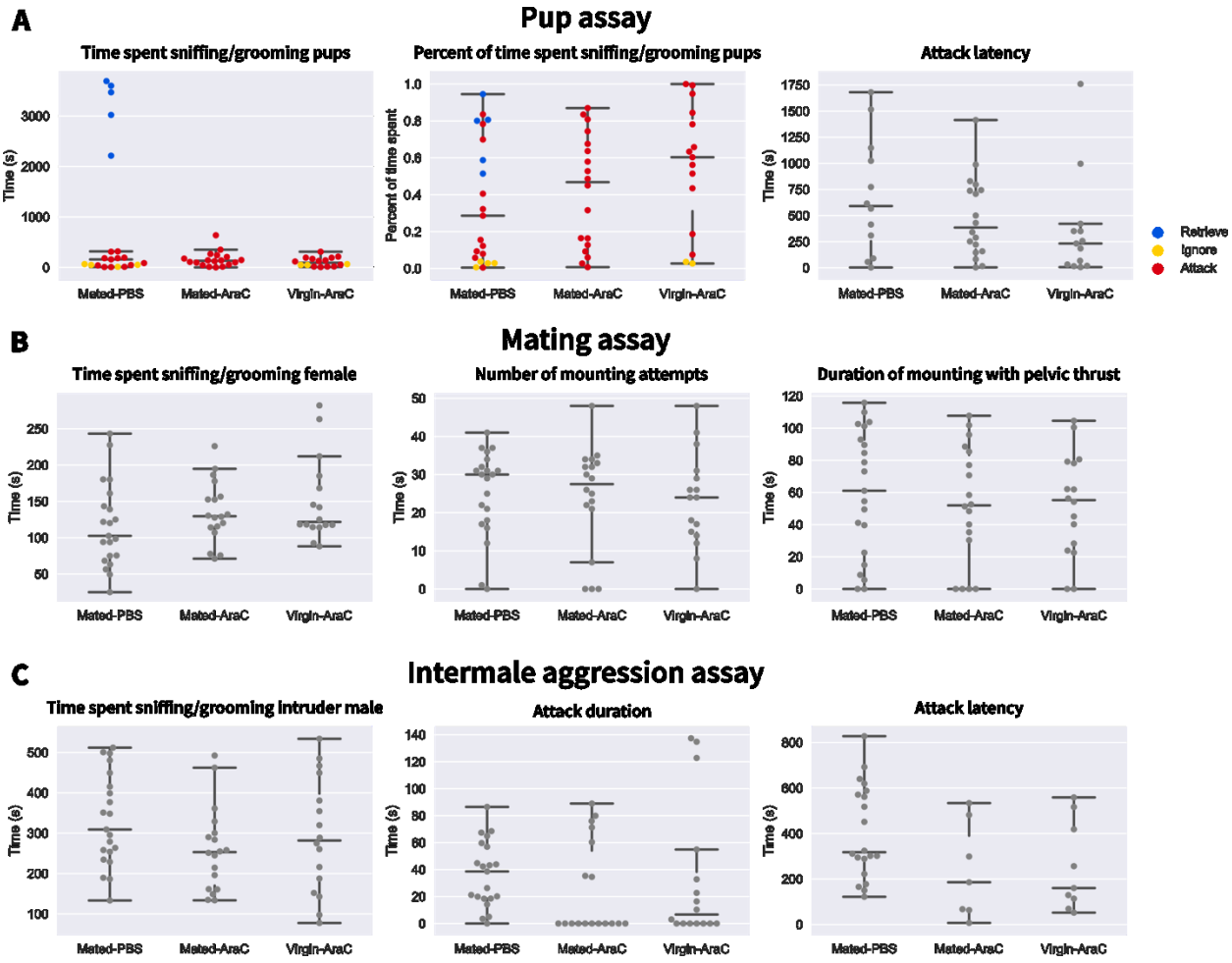

Figure 4.5: In-depth Analyses of Behavioral Assays

In depth behavior analysis of the different behavioral assays. **A.** Analysis of the pup assay. In the first two panels, data points are colored by the overall behavior of the mouse. **B**. Analysis results of the mating assay. **C**. Analysis results of the intermale aggression assay.

These results seem to indicate that the ablation of the neurogenic niche had a significant effect on the capacity for the parenting switch to occur. Indeed, Mated-AraC males were behaving more similarly to virgin males. However, the non-significant difference between Mated-PBS and Virgin-AraC might indicate that this phenomenon may be partially due to the infusion treatment with AraC or could also be due to undue stress caused to first surgery cohorts as I improved my surgical technique. Since stress can affect a response to pups, I have included a timeline of surgeries to compare the cohorts results across linear time to verify behavioral biases that could be explained by older cohorts being significantly impacted by stress as compared to the later cohorts (Figure 4.6). Compared using a Fischer exact test, the oldest cohorts are significantly different than the most recent cohorts supporting the hypothesis that variable handling caused greater stress to the first cohorts (Figure 4.6).

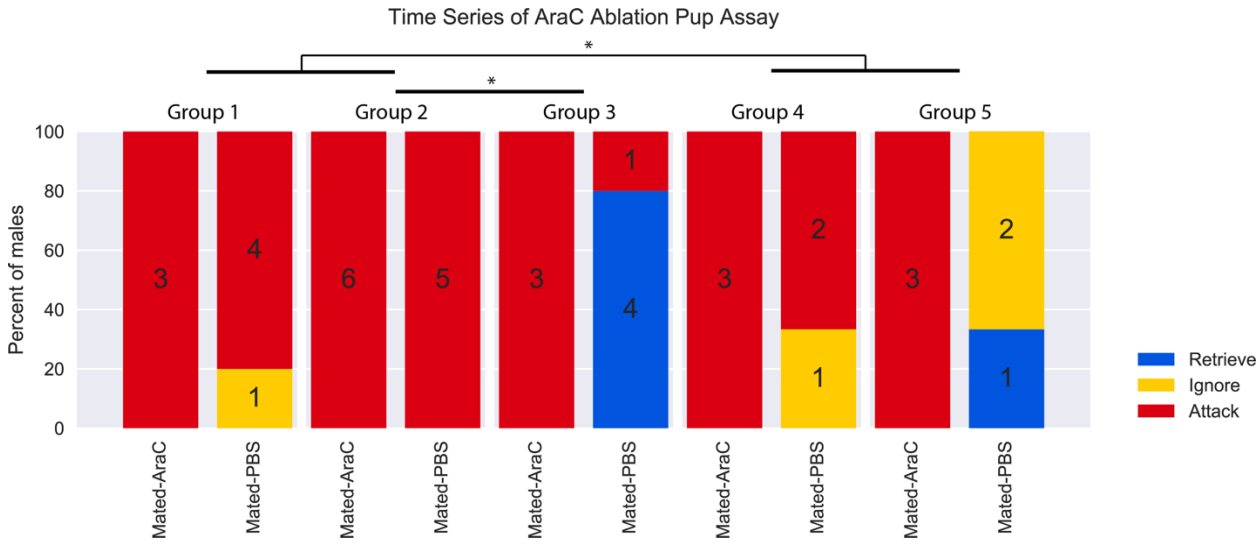

Figure 4.6: Comparison of Different Pup Assay Trials of Cannula Experiment

Results of the pup assay performed on the different groups that underwent cannula surgeries to compare the cohorts results across different groups sorted by time. Fischer exact test * = $p<0.05$.

Establishing GFAP-TK Line

The drawback of the GFAP-TK mouse line is that virgin males of this line did not initially display pup-directed aggression. I made this observation when I crossed GFAP-TK+ females to 129sv males upon arrival. The males in the litters of these crosses were genotyped using PCR for the presence of Thymidine Kinase (TK), and the TK+ adult, virgin males (n=6) were tested in a pup assay to ensure they were infanticidal prior to sexual experience. All the TK+ virgin male mice displayed parental behavior towards pups, and thus could not be used for an ablation experiment investigating the parental switch in their current genetic background.

The lab strain used to study pup-directed aggression is the TrpC2+/+ line as virgin males in this strain are highly infanticidal. In order to transfer the genetic cassette into the TrpC2+/+ genetic background I performed a classic genetic backcrossing experiment where GFAP-TK+ females were crossed with TrpC2+/+ males. The offspring were then tested for the presence of the GFAP-TK cassette by PCR as described above. The resulting TK+ virgin males were then subjected to a pup assay to analyze pup-directed behavior. Since GFAP-TK males are sterile, the TK+ female siblings of the males were selected for further crossing with TrpC2+/+. The goal for this strain to be used in further experiments is to reach a 50% infanticide rate in virgin males. The first and second backcrosses both yielded parental virgin male offspring. However, the third backcross was partially successful, yielding a group of males which were 31% infanticidal (4 out of 13 males), 8% ignoring/neglectful (1 out of 13 males) and 61% parental (8 out 13 males) (Figure 4.7).

In order to utilize this genetic line in an ablation experiment to study the involvement of neurogenesis in the parenting switch I needed to first determine the most effective ablation paradigm in GFAP-TK+ animals. To this end, I performed the following experiment: TK+ males were divided into two groups (n=3 per group). The control group was given peanut butter once a day for 10 days. The ablation group was given peanut butter mixed with 7.5mg of valganciclovir (VGCV) each day; VGCV is a bioavailable form of Ganciclovir which reacts with TK to form a Thymidine analog that will incorporate into dividing cells and cause apoptosis. All mice were injected with BrdU on the 5th day, as well as on the final day of the experiment. This injection paradigm was meant to allow for the visualization and quantification of different pulses of neurogenesis during the experiment. After the 10^{th} day of treatment, all mice were sacrificed within 22h from the last BrdU injection. On average, VGCV-treated animals had 66% fewer BrdU+ cells in the RMS than controls (Figure 4.7). A student t-test was used to analyze this result; the difference was found to be significant with a two-tailed p value of 0.0260. This preliminary experiment is encouraging. Indeed, the treatment using oral delivery of VGCV through peanut butter is not invasive and lead to a significant reduction in neurogenesis after 5 days.

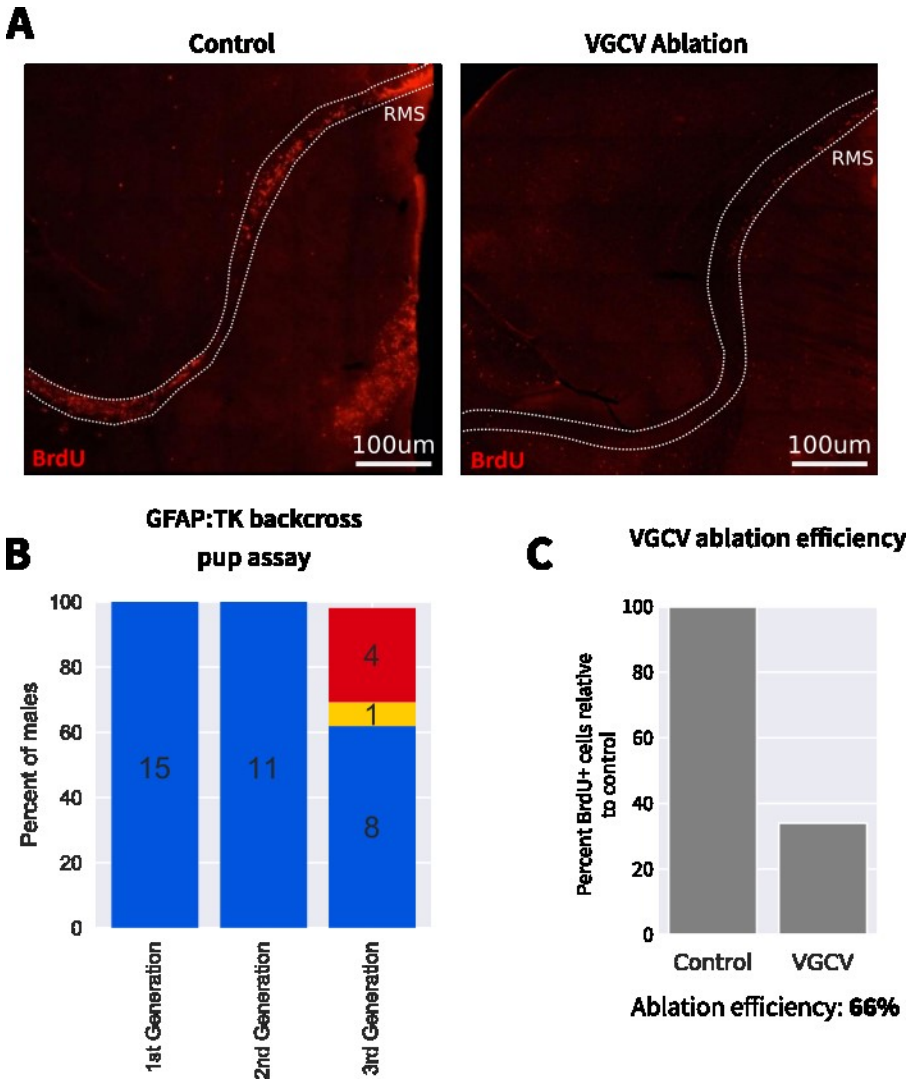

Figure 4.7: Pup Assay Changes Across Backcrossed Generations of GFAP-TK Mice.

A. Representative images of GFAP-TK ablation. **B**. Quantification of pup assay results of the adult virgin male offspring of subsequent generations of backcrosses of GFAP-TK+/- female mice to TrpC2+/+ males. The first backcross had n=15 males, the second had n=11 males and the third had n=13 males. **C**. Quantification of the ablation efficiency of the GFAP-TK system using VGCV in peanut butter. n=3 for both groups.

Discussion

Here, I set out to investigate the necessity of neurons born directly after mating for the parental switch. Inspired by Autry's irradiation experiments that demonstrated an increase in neglect towards pups in irradiation-treated mated male mice and utilizing a previously reported neurogenic niche ablation method (Doetsch *et al.*, 1999; Doetsch, García-Verdugo and Alvarez-Buylla, 1999; Ghanbari *et al.*, 2015), I tested differently-timed ablation paradigms to find the experimental design that maximized the ablation of cells born directly after mating. The best paradigm consisted of exposing the neurogenic niches to AraC for 12 days prior to mating with a continuation of treatment 3 days after mating, leading to an average ablation of 87% of the adult-born cells. Using this paradigm, I then tested whether the cells born directly after mating were necessary for the parenting switch. In this experiment, AraC-treated, mated male mice and virgin AraC-treated mice were compared to PBS-treated mated mice.

Interestingly, I found that the ablation of the neurogenic niche led to a drastic change in AraC-treated mated males. The AraC-treated mated group was significantly different from PBS-treated mated males and exhibited 100% of pup-directed aggression. This stark contrast with the normal parental behavior of mated males suggests that neurogenesis at the time of mating, or near the time of mating, is necessary for the parenting switch. However, the virgin AraC-treated males were not significantly different from either the males injected with PBS or males that did not undergo surgical procedures, which is likely due to a slight increase in males ignoring the pups, a phenotype that is more ambiguous but as stated previously, likely due to stress caused to initial cohorts as I improved my surgical technique with experience.

The AraC infusion experiment -while more targeted than whole-head irradiation- the procedure likely has side effects. Importantly, mice undergo an intense surgical procedure where a cannula is implanted into their brains, which causes some damage in the cortex where the metal infusion cylinder passes through to reach the target location in the ventricles. Moreover, both the cells born in the DG and the SVZ are ablated to a large extent and this does not allow us to understand the individual effect of ablating just one of the two areas and the resulting effects on the parental switch.

In the discussion of Chapter 3, I proposed three potential models that could explain how cells born directly after mating could regulate the parenting switch. The first model hypothesized how a quantitative difference in cells born directly after mating in the AOB could modulate pup perception by blocking vomeronasal signals that elicited attack. Briefly, the model hypothesized that AOB granule cells and periglomerular cells born from mating inhibited previously existing populations of granule cells and mitral cells to block VNO-sensed cues that elicited attack. The second model hypothesized how cells born directly after mating that integrate into the GCL in the MOB could facilitate paternal care by inhibiting mitral cells projecting to MeA cells that were directly inhibiting MPOA Gal+ cells and through this disinhibition promote parental care. Finally, the third model proposes that cells born directly after mating in the DG participate in the formation of an olfactory-based engram that is recalled three weeks later upon pup exposure and this recollection inhibits pup-directed aggression via a DG-CA2-LS-VMHvl pathway, that regulates whether social aggression will occur when in a novel situation.

Here, my experiment ablated the cells born directly after mating that were integrating into the MOB, the AOB as well as the DG. Therefore, the experimental manipulation I used does not provide the information needed to state whether one of the three models is more likely than the others, or if they could work together to modulate pup-directed behavior. Moreover, the experiment presented here only considered ejaculation from mating as a critical event for the parenting switch, but there are other factors such as cohabitation with a pregnant female that also affect pup-directed responses from mated males (Wu et al, 2014), and the potential neurogenesis-dependence of the effect of cohabitation has not yet been explored. Moreover, the survival of new born cells in the MOB and the AOB is correlated with their stimulation (Kempermann, 2011). Therefore, it will be interesting to include further experiments exploring the effect of cohabitation on the fate of neurons born directly after mating. This could help to further understand the potential neurogenesis-based contributions of the MOB or the AOB in this behavior switch.

This experiment also leads to another interesting result, which is that mated males with their neurogenic niche ablated displayed unexpected sexual behavior towards other males three weeks later. Normally, when confronted with intruder males in an intermale aggression assay, most TrpC2+/+ resident males will display aggressive behaviors, while others will largely ignore the intruder male (personal communication, Dr Bambah-Mukku). Here, AraC treated mated males that would normally ignore the other male, displayed in a large majority (77%) sexual behaviors towards other males as measured by mounting attempts with a pelvic thrust, as well lower-than-expected fighting rates among all resident males (Figure 4.5) towards intruder males. While this is not related to the parental switch, these results might be indicative of a

critical period during the first sexual experience of the male that is dependent on neurogenesis for future stereotyped behavior towards males. Moreover, though it is possible that general olfactory processing may have been affected by the AraC treatment, the mating assay result shows that all groups of males mate normally with females. Since mating requires VNO function, this result tells us that the mice still possess at least a partial ability to process olfactory cues. Moreover, time spent sniffing male intruders and female intruders did not differ among the AraC treated or control groups, further supporting the hypothesis that AraC treatment did not eliminate olfactory processing (Figure 4.5).

While those results are encouraging, the AraC approach has several drawbacks presented above. Thus, I set out to utilize a genetic model to ablate the neurogenic niche in a timely manner. This approach would allow to ablate the niche with minimal intervention and in a highly accurate cell type and time dependent manner. Utilizing the GFAP-TK mouse line, I tested one ablation paradigm which was successful in ablating both the DG and SVZ niches with a 66% ablation efficiency. However, when GFAP-TK virgin males mice were subjected to a pup assay, they displayed parental behaviors. Therefore, the genetic cassette needed to be backcrossed into the model strain used to study pup directed aggression, TrpC2+/+. After the 3rd backcross, virgin male mice possessing the GFAP-TK cassette started to display pup-directed aggression, a highly encouraging result. This crossing strategy will need to be continued until at least 50% of the virgin males display pup-directed aggression in a stable manner. Finally, while this mouse line will provide researchers with a more accurate and less invasive paradigm to study the involvement of neurogenesis on the parenting switch, it will not by itself be appropriate to tease apart the role of SVZ versus DG born neurons. Therefore, further

experiments using SVZ and DG specific markers should be devised to ablate one or the other, followed by careful behavioral observation.

To conclude, neurons born directly after mating appear to be necessary for the parental switch to occur as expected, but outstanding questions remain to be answered along with new paradigms to be tested, in order to reach a clear understanding of the exact mechanisms underlying the parental switch and the role of adult neurogenesis in this behavioral phenomenon.

Materials and Methods

Animals

All male mice used were between 10-14 weeks old. Animals were kept on a 12 h:12 h light/dark cycle with food and water available ad libitum. All behavioral components of experiments were done during the lights off cycle starting at 5pm. All experimental paradigms were approved by the Harvard University Institutional Animal Care and Use Committee (IACUC).

Intracerebroventricular Cannulation and Infusions

Mice were anesthetized using isoflurane were then implanted with steel guide cannulas (Plastics One) that targeted to the right ventricle (Coordinates: -0.4 mm anteroposterior, 1.0 mm laterally to bregma, and -2.3 mm below the skull). Mice were then given 1 week of recovery in a clean, new cage. AraC infusions were then given every 12hours at a rate of 50ng of AraC per day. Timeline for delivery and concentrations have been modified from (Singer *et al.*, 2009)

Blinding Protocol

To avoid any potential bias, I was blinded in all these experiments after the cannula implantation. The substances that were to be infused were provided to Stacey Sullivan, and Stacey substituted the substance identification level with a code for each vial and placed all substances in identical vials labelled only with its assigned code. When I turned in all quantifications for all animals in an experiment, Stacey would provide the code map that linked her given code to the substance name.

BrdU injection paradigm

At the end of the infusion treatments, one group given AraC infusions and another group given PBS infusions were mated with estrus females, and a third group which underwent AraC infusions was placed in the same experimental setup but did not receive any access to female mice. Males in this strain faint upon ejaculation. Ejaculation was confirmed by a visual inspection of the female genitalia. Afterwards, all males received a 150mg/kg intraperitoneal injection of 5-Bromo-2-deoxyuridine and were individually housed for 25 days. Mice then underwent behavioral assays and were subsequently euthanized.

Behavioral assays observations

All behavioral assays were started three hours after the beginning of the dark phase and were performed under dim red light. In all mice who underwent cannula implantation, the assays were videotaped (Sony DCR-HC65 camcorder in nightshot mode, Microsoft LifeCam HD-5000). The behaviors were then scored using the Observer 5.0 or XT 11 software (Noldus Information Technology).

Parental Behavior Assay

Ten-week-old to fourteen-week-old Trpc2+/+ virgin males were individually housed for 25 days before the tests after completing infusion treatments. One-day to three-day-old C57BL pups were used as pup intruders in this behavior assay. The pups are not related to the resident animals. Two pups were placed at the farthest corner from each adult male's nest. Olfactory investigation marked the beginning of the parental behavior assay. The assay length was 30

minutes if pups were neither wounded nor retrieved. If both pups were retrieved, the assay extended for another 60 minutes. If the pups were attacked or blood was seen, the assay was ceased immediately, and the wounded pup was euthanized. Animals that retrieved both pups to the nest within 30 minutes were categorized as “Retrieve”. Animals that attacked a pup within 30 minutes were categorized as “Attack”. Animals that neither retrieved both pups to the nest or attacked them within 30 minutes were categorized as “Ignore”. In parental animals, the following behaviors were collected: amount of time adult spent in the nest, amount of time adult spent outside the nest, pup retrieval latency, time spent building a nest, time spent grooming and/or licking pups. Sniffing and grooming was collected for all animals regardless of their reaction to pups. In infanticidal animals, latency to attack was collected for each animal, as well as behaviors that foreshadow aggression such as ‘tail rattling’. This behavioral assay approach and metrics were identical to the ones described in Wu *et al.* (2014)

Mating Behavior Assay

Ten-week-old to fourteen-week-old Trpc2+/+ virgin males were individually housed for 25 days before the tests after completing infusion treatments. 14-20 weeks old, receptive Trpc2+/+ females were used. For each male, a female was placed at the farthest corner from each adult male’s nest. Interactions were recorded for 15 minutes. The following behaviors were quantified and analyzed: sniffing/grooming duration, number of mounting attempts and duration of mounting with pelvic thrust. This behavioral assay approach was identical to the one described in Wu *et al.* (2014)

Resident-Intruder Assay

Ten-week-old to fourteen-week-old Trpc2+/+ virgin males were individually housed for 25 days before the tests after completing infusion treatments. Three-month-old to nine-month-old castrated males of C57BL/6 background were used. For each cannulated male, an intruder male was placed at the farthest corner from each cannulated male's nest. Interactions were recorded for 15 minutes. The following behaviors were quantified and analyzed: sniffing/grooming duration, number of mounting attempts, attack duration and latency to first attack. This behavioral assay approach was identical to the one described in Wu *et al.* (2014)

Tissue Preparation and Immunohistochemistry

In the above experiments, all animals were perfused transcardially with 1x phosphate buffered saline (PBS) for five minutes and then the tissue fixed with 4% paraformaldehyde. Brains tissue was collected and post-fixed in 4% paraformaldehyde overnight. After transferring the tissue to PBS overnight, brains were embedded in 2% agarose. This agarose block was mounted unto a Leica vibratome and sliced into 50micron sections. For immunostaining, I used 12-well culture plates and permeabilized sections using 0.3% Triton X-100 in PBS for 30 min. For proper BrdU staining, the tissue was treated with 2 N HCl for 30 min at 37°C to denature the cellular DNA. The blocking step was done by keeping tissue overnight in blocking buffer which was made as follows: 0.3% Triton X-100, PBS and 2% normal goat serum in PBS. Primary antibody incubation was done over two overnights at 4°C at an antibody concentration of 1:200 in 0.3% Triton X-100 and 10% goat serum. Secondary antibodies were added for 2 h at room temperature at an antibody concentration of 1:500. Rat monoclonal antibody to BrdU (1:200, Abcam) and anti-

Ki67 (1:200, Abcam) were used to detect adult generated cells. Tissue sections were mounted unto Superfrost Plus slides and let to dry overnight. The slides were then layered with DAPI-containing Vectashield mounting medium (Vector Laboratories, H-1200) and coverslipped. BrdU staining protocol used was established by Jenelle Wallace from the Murthy lab in Harvard University.

Microscopy

All brains were imaged using the Zeiss Axioscan Scan.Z1 slide scanner fluorescent microscope at 20x resolution. Every eight section of the olfactory bulb was imaged to quantify ablation of BrdU in the granule cell layer of the MOB in AraC treated animals as compared to PBS-infused controls. All quantification was done manually. BrdU signal that colabelled with DAPI was counted as positive signal.

Statistical Analyses

All behavioral assay data was analyzed using a Fischer Exact tests. Ablation efficiency averages were analyzed using Student's t-tests.

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CHAPTER V: CONCLUSIONS AND FUTURE DIRECTIONS

This dissertation investigated different aspects of infant-directed behavior in adult mice. I have presented work that helped elucidate the projection logic of MPOA Gal+ cells to better comprehend how they might regulate different aspects of parenting behavior. Moreover, I have also presented data exploring the map of direct inputs of PeFa Ucn3+ neurons, which are likely a central node in the display of pup-directed aggression and neglect. These circuit-based experiments were followed by experiments aimed at elucidating the molecular mechanisms underlying the parental switch in male mice from infanticidal to parental behavior. Due to its correlative timeline, adult neurogenesis was explored as a potential mechanism underlying the parental switch. Though my studies of adult neurogenesis have not yielded irrefutable evidence of the involvement of adult neurogenesis in the parental switch, the data presented are strongly suggestive of its involvement in the behavioral paradigm. I have discovered that adult born cells during mating integrated at a higher rate in the OB of mice at a timepoint relevant to parental care. Moreover, I confirmed Dr. Autry's results that more mating-born cells were integrated in the DG approximately three weeks after mating. Finally, I explored whether mating-born cells are a necessary component of the parental switch phenomenon by ablating this population in the SVZ and DG of virgin males prior to mating.

Working alongside Kohl, I performed tracing experiments necessary to understand the branching pattern of MPOA Gal+ neurons. The results I obtained, along with others that Kohl gathered, demonstrated that MPOA Gal+ neurons may modulate the display of different aspects of parental care via distinct inhibitory subpopulations that each send outputs to

different brain areas involved in parental care. Working with Autry, I analyzed the map of inputs that projected onto the PeFa Ucn3+ neurons, a previously unknown input population. We discovered which regions project unto PeFa Ucn3+ neurons. Notably, PVN neurons provided the largest input contribution to PeFa Ucn3+ neurons, followed by the MPOA as the second largest input contributor. Moreover, we explored the sexual dimorphism of the inputs of PeFA Ucn3+ cells by comparing the quantity of inputs from each region in males and females. We discovered that the PVN contributed twice the proportion of PeFa Ucn3+ inputs in females as compared to males, whereas the MPOA projections composed three times more of the projections in male compared to female brains. Moreover, Autry used *in situ* hybridizations and immunostainings against candidate markers and found that the PVN inputs were largely comprised of AVP-expressing cells, whereas MPOA inputs were largely comprised of Galanin-expressing cells (Autry et al., unpublished manuscript).

Branching off from the work I performed with Dr. Kohl and Dr. Autry, I explored adult neurogenesis as a potential mechanism that could underlie the parental switch. A common phenomenon in evolution is known as co-option (linked to neuroscience in Hoke et al, 2019). It is the process by which an already existing protein, function or circuit is co-opted to perform a new function, for example, through new wiring of protein regulation or neuronal circuits. For example, the gene *oskar* was passed down from bacteria living near eukaryotic germlines to insects via horizontal gene transfer, and it was co-opted in insects for germ cell formation (Blondel et al, 2018). The experimental results of this thesis support the hypothesis that evolution co-opted this mechanism to generate a behavior switch in the male. In my experiments, I have shown that adult-born cells, created during or near an ejaculatory

experience induced by mating, incorporated in larger numbers than control groups in the MOB and the DG three weeks after mating. In my pilot proliferation experiment, I found this increase in integration was not correlated with an increase in proliferation in the SVZ following a mating event. Therefore, the increase in integration must be due to other external factors that increased the survival of those cells. Given the role of androgen surges in the survival of adult-born cells (Hamson et al., 2013), one potential hypothesis is that the rise of testosterone seen in recently-mated males (Batty, 1978) is responsible for the differential survival of mating-born cells several weeks after mating. Alternatively, hypothalamic inputs could be releasing factors after mating to promote survival; a similar example of this has been seen in hypothalamic POMC+ neurons projecting to the SVZ to promote proliferation in adult neurogenesis that correlates with satiety (Paul, Chaker & Doetsch, 2017). In another relevant discovery, oxytocin expressing neurons in the PVN send projections to the hippocampus that have been found to mediate adult neurogenesis proliferation and survival (Yu-Ting et al, 2017).

In my experiments, the comparative increase in integration was not uniform throughout the OB, as only the AOB and the granule cell layer of the MOB showed increases in integrated mating-born cells. Finally, I characterized, using immunohistochemistry, the mating-born cells in the OB and confirmed that most of the cells were inhibitory neurons.

When future experiments perform niche specific ablations to ascertain the role of each neurogenic niche in the parental switch, it will become of interest to find ways to manipulate the mating-born cells of behaviorally-relevant regions to test their sufficiency and role in the parental switch and beyond. In order to do this, researchers will need to find specific markers

that label cells in a temporally specific manner and allow for their subsequent harvest. One potential experiment that can be done to collect these mating-born cells and find specific markers for them is the following: Utilizing a dox-dependent Nestin::CRE mouse line and injecting doxycycline during a time window that captures mating in order to tag cells expressing nestin near mating at a later timepoint. Three weeks later, MOB, AOB and DG tissue can be collected separately and an RNA sequencing experiment such as DroNc-Seq, a nucleus RNA sequencing approach (Habib et al., 2017) can be performed. This could lead to the discovery of differentially-expressed markers in mating-born neurons and subsequently allow the probing needed to ascertain the exact functional role of these cells.

I presented a set of experiments that aimed at probing the necessity of mating-born cells in the stereotyped execution of the parental switch. Using an established neurogenesis ablation paradigm of cannula-delivered AraC, I tested different ablation paradigms aiming at ablating the mating-born cells. I discovered that the best ablation method consisted of injecting AraC in the ventricles for 12 days prior to mating, followed by 2 more days of treatment. Using this paradigm, I inhibited adult neurogenesis by depleting the neurogenic niches in male mice that were subsequently mated and their interactions with infants observed and quantified three weeks post-mating. I demonstrated that mated males treated with AraC displayed significantly more pup-directed aggression than was expected given their mated state, whereas mated mice treated with PBS were not significantly different than mated male mice that did not undergo cannulation. These results indicate a potential necessary role of neurogenesis for the establishment of the parenting switch in male mice. However, this ablation technique depleted cells in both the SVZ and DG neurogenic niches. Due to the increase in integration in both the

DG and MOB, further experiments ablating only one of the two niches will need to be performed to tease apart further the contribution of the mating-born cells in the parental switch.

Lastly, I obtained a mouse line with a genetic construct containing an inducible cytotoxin under the control of a neural "stem cell" promoter, GFAP. The GFAP-TK mouse line has been previously utilized in multiple studies to ablate the neurogenic niches successfully (Garcia, Doan, Imura, Bush, & Sofroniew, 2004; Saxe et al., 2006). However, when I tested the virgin males of this line for the display of pup-directed aggression, all TK+ males instead showed pup-directed care. In order to establish a GFAP-TK mouse line that displayed the parental switch, I have performed backcrosses with the TrpC2+/+ strain. After the third backcross, a TK+ population of virgin males displayed some pup-directed aggression. These backcrosses will need to be continued until at least 50% of the virgin males display pup directed aggression. Moreover, I performed pilot ablation efficiency experiments which validated the use of the GFAP-TK system in ablating adult-born cells. After backcrossing ends, this mouse line will likely be useful for testing the effect on the parental switch of neurogenesis ablation when paired with cohabitation with a pregnant female. Using this paradigm, no injections, cannulas or surgeries will be needed and cohabitation can occur as cage fellows will not remove cannulas or other headgear from experimental subjects.

For a mechanistic understanding of the role of neurogenesis, it will be crucial that further research assess the exact functional role of cells integrated in the OB and the DG. Through careful characterization, spatially-limited ablations, tracing experiments and genetically defined

perturbations of integrated mating-born populations, it will be possible to understand the role of mating-born neurons as well as the circuitry changes they may create to exert change. The results of those experiments will in turn allow for further hypotheses concerning the role of these cells in the parental switch to be formulated, as well leading to a better understanding of this fascinating phenomenon.

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