ANALYSIS OF FUNCTIONAL CIRCUITRY AND THE EFFECT OF ACTIVITY ACROSS DEVELOPMENT IN THE MOUSE ACCESSORY OLFACTORY SYSTEM

by

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ABSTRACT: ON THE ANALYSIS OF FUNCTIONAL CIRCUITRY AND THE EFFECT OF ACTIVITY ACROSS DEVELOPMENT IN THE MOUSE ACCESSORY OLFACTORY SYSTEM

The vomeronasal system (VNS) is a chemosensory system designed for the detection of chemical messengers dissolved in bodily secretions which help to identify kin, conspecifics, and predators in the animal’s environment. Although a growing body of evidence over the last two decades has revealed a very important role for the VNS in many stereotypical behaviors which are essential to the animal’s survival, our understanding of the detailed organization of the circuitry of this system, and its development, remains limited. Consequently, the goal of this dissertation is to examine the development of connectivity from the vomeronasal organ to the accessory olfactory bulb to determine the fundamental principles of adult connectivity in this circuit, and to manipulate sensory activity to understand the role that activity might play in anatomical development of vomeronasal projections to their targets in the accessory olfactory bulb.

There are several anatomical challenges we faced when trying to visualize connectivity in a select population of neurons in the VNS. We utilized two tools to overcome some of these barriers. First, we took advantage of a transgenic mouse line whose vomeronasal sensory neurons (VSNs) expressing the V2r1b-receptor also expressed tau-GFP to visualize a specific population of sensory neurons in the vomeronasal organ (VNO) and their projections in to the accessory olfactory bulb (AOB). Second we developed a local electroporation technique enabling us to label specific populations of mitral cells in the AOB receiving common input. This method is described in detail in Chapter 2 where we show the versatility of this method, not only for use in the AOB, but in many other brain systems and with a diverse set of dyes and calcium indicators.

We exploited these two tools to examine both the gross anatomical development of the VNO and the AOB, as well as the development of connectivity between them in Chapter 3. Our results show that the first few post-natal weeks represent a dramatic period of growth in both the
VNO and the AOB. In addition, we show that axons of VSNs expressing the V2r1b-receptor undergo a striking and rapid period of refinement and coalescence during the first four post-natal days of the animal’s life. Subsequently, mitral cell dendrites, which initially promiscuously send out multiple dendritic branches, begin to ramify and form tufts in specific glomeruli following this period of axonal refinement into well-defined glomeruli. Finally, our results support the hypothesis that mitral cells precisely project their dendrites to target glomeruli receiving input from sensory neurons expressing the same receptor type.

Finally in Chapter 4, we explored the role of activity in modulating the development described in Chapter 3. We demonstrate that the duct connecting the VNO to the external world is indeed open and thus that external ligands can access the VNO and bind to VSNs, as early as postnatal day 0. Further, we demonstrate that VSNs are capable or releasing neurotransmitter onto mitral cells at these early postnatal ages. These results suggested that early activity in this system might help regulate development. To test this hypothesis we needed a ligand that would activate a known subset of VSNs. We determined the concentration of a major histocompatibility complex peptide known to activate V2r1b-receptors and found it to be more than 1,000 fold above what would likely activate these sensory neurons. We show that these peptides are capable of inducing immediate early genes in downstream mitral cells and then employed the use of two of these peptides to selectively manipulate the activity of sensory neurons expressing the V2r1b-receptor. Although strong sensory activity did not affect the number of VSNs expressing the V2r1b-receptor, it did significantly alter axonal refinement and coalescence in AOB, resulting in delayed pruning and formation of well-defined glomeruli.

Taken together, the results from the chapters in this dissertation suggest that connectivity between mitral cells and sensory neurons in the vomeronasal organ is specifically targeted and that early activity may influence the development of this circuit to ensure proper connectivity providing a substrate for information processing in this system.
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The work presented herein is based on several manuscripts that have been published or have been submitted. These are:

Chapter 2 is a modified version of:


Chapter 3 and 4 each contain work from:


The appendix is a modified version of:

# List of Abbreviations

AOB – Accessory Olfactory Bulb  
BNST – Bed Nucleus of the Stria-terminalis  
CNS – Central Nervous System  
DAG – Diacylglycerol  
ESP – Exocrine Gland-Secreting Peptides  
FSH – Follicle-Stimulating Hormone  
GL – Glomerular Layer  
GC – Granule Cell  
GCL – Granule Cell Layer  
H – Hypothalamus  
IP$_3$ – Inositol Trisphosphate  
LH – Luteinizing Hormone  
LHRH- Luteinizing-Hormone-Releasing Hormone  
MC – Mitral Cell  
MCL – Mitral Cell Layer  
MHC – Major Histocompatibility Complex  
MOB – Main Olfactory Bulb  
MUP – Major Urinary Protein  
NAOT – Nucleus of the Accessory Olfactory Tract  
NCAM – Neural Cell Adhesion Molecule  
NMJ – Neuromuscular Junction  
OR – Olfactory Receptor  
ORN – Olfactory Receptor Neuron  
OSN – Olfactory Sensory Neuron  
PNS – Peripheral Nervous System
PG – Periglomerular
PLC – Phospholipase C
TRPC – Transient Receptor Potential Channels
VA – Vomeronasal Amygdala
VN – Vomeronasal Nerve
VNO – Vomeronasal Organ
VR – Vomeronasal Receptor
VNS – Vomeronasal System
VSN – Vomeronasal Sensory Neuron
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1.0 Introduction

One of the fundamental goals of neurobiology is to understand how sensory systems integrate information to affect behavior. As is the case for any sensory system, our ability to elucidate the principles which govern its function is dependent, at least at some level, on our understanding of the neuronal architecture of the system. In other words, the characterization of neuronal structure is paramount in understanding its function. This important interplay between structure and function has been recognized since the time of Cajal’s early drawings, yet there are still gaps in our understanding of the basic organization principles of several sensory systems. This is especially true of the vomeronasal system (VNS). Despite our somewhat limited understanding of the neuronal architecture of the VNS and its development, it is clear that it plays an essential role in directing the overall behavior of the organism. In fact, the ability of the VNS to detect chemical cues in the environment is essential to the survival of the animal.

Throughout this introduction, I will discuss many of the seminal papers published over the past decades that have given us a glimpse into the mysteries of this sensory system and its role in regulating animal behavior. We will focus primarily on the vomeronasal system in rodents and compare and contrast it with the better-studied main olfactory system to see how discoveries and breakthroughs in that system paved the way, in many respects, for unlocking the principles of the organization and function of the vomeronasal system. I will discuss the general anatomical organization of the vomeronasal organ as well as the mechanisms of pheromonal signal transduction through vomeronasal sensory neurons and their receptors. I will also highlight several of the chemicals found to act as ligands for sensory neuron receptors as well as their effect on behavior through downstream projections to the accessory olfactory bulb and further central brain areas.
1.1 The Organization of the Accessory Olfactory System

1.1.1 The Vomeronasal Organ

The vomeronasal organ (VNO), which is also known as Jacobson’s organ, named after the Danish anatomist Ludvig Jacobson, was first described in 1813 after Jacobson extensively studied a large number of animals, both domesticated and wild (Trotier and Doving, 1998). His drawings and thorough anatomical observations revealed a richly innervated organ that was hidden in the nasal septum, escaping the attention of countless anatomists over the years. Enclosed in a bony capsule, he found a cigar-shaped tubular structure (Figure 1.1A), one on each side of the nasal septum, which were opened to the nasal cavity through a small duct. Coronal sectioning of the organ revealed a crescent-shaped lumen (Figure 1.1B) which was highly innervated, suggesting to Jacobson that it may have a sensory function (Jacobson et al., 1813; Broman, 1920)

Despite Jacobson’s rigorous and thorough description of the structure of the VNO across species, the function of the organ only began to be revealed 100 years after his death. Beginning in the 1950s and 1960s, behavioral endocrinologists were attempting to explain behavioral effects controlled by chemical stimuli found in the urine of conspecifics, which disappeared upon lesions of the olfactory bulbs. By the 1970s, many of these effects that previously had been attributed to the main olfactory system were hypothesized to rely on the function of the vomeronasal organ and projections to the accessory olfactory bulb (Winans and Scalia, 1970; Raisman, 1972; Scalia and Winans, 1975). This paved the way for many studies which tested this hypothesis, thereby elucidating the many and important anatomical and physiological differences between the accessory and main olfactory systems in mammals.
In contrast to the main olfactory epithelium (MOE), which has passive access to odorants in the dorsal caudal area of the nasal cavity, the entry of pheromones to the VNO is believed to be controlled actively through a “suction mechanism” causing fluids to be sucked into the lumen of the VNO. This is due to the fact that the VNO contains highly vascularized tissue and large blood vessels just lateral to the lumen (Figure 1.1B) which are innervated and regulated by the autonomic nervous system (Eccles, 1982). As a result, independent control of vasoconstriction (which causes inflow of fluid at the duct), and vasodilatation (which causes fluid outflow at the duct), results in the production of a pump-like mechanism to actively deliver chemical stimuli to sensory neurons residing in the sensory epithelium and therefore suggests regulation of VNO function based on the behavioral state of the animal (Meredith and O’Connell, 1979).

1.1.2 Vomeronasal Sensory Neurons and Signal Transduction

Although the basic sensory codes have been known for decades in sensory systems such as the auditory or visual system, it has only been in the past two decades or so that the principles of chemosensory coding has been elucidated for the olfactory system. The early 1990s marked the arrival of the molecular area of research into the chemical senses with the discovery of odorant receptors genes in 1991 by Linda Buck and Richard Axel (Buck and Axel, 1991). For the first time, a new molecular basis for odor recognition based on a multi-gene family of olfactory G-protein-coupled receptors revolutionized our understanding of olfactory perception. For example, whereas the recognition of sound frequency is determined by the location of the displacement of sterocilia along the basilar membrane in the cochlea (Ehret, 1978), olfactory recognition is based on the existence of a family of approximately 1,000 genes in mice coding for olfactory receptors.
Figure 1.1 - Anatomical Structure of the Vomeronasal Organ. A) Schematic showing the relative locations of the vomeronasal organ in the dorsal portion of the nasal septum and the accessory olfactory bulbs located in the caudal dorsal portion of the olfactory bulbs (Red). B) Coronal section of the snout of a mouse to visualize the VNO and nasal septum. C) Zoom of B displaying vomeronasal sensory neurons in the sensory epithelium projecting their dendrites towards the lumen. Scale bar = 100 μm.
The characterization of this gene family, along with the analysis of the expression patterns of these genes, began to illuminate the new and complex system of olfactory coding. Evidence even as early as the mid-1980’s implicated G-proteins in olfactory signal transduction (Pace et al., 1985; Sklar et al., 1986) suggesting they share the same seven-transmembrane-domain sequence motif (Kobilka, 1992). Later, it was suggested that each olfactory sensory neuron is likely to express only one or very few of the 1,000 receptor genes (Chess et al., 1994; Kishimoto et al., 1994) with rare simultaneous expression of different receptor genes in a given sensory neuron (Serizawa et al., 2000; Rawson et al., 2000).

These studies in the main olfactory system motivated many of the efforts which ultimately led to the identification and characterization of vomeronasal receptors. One might expect vomeronasal sensory neuron receptors to share some homology with other chemosensory receptors found in olfactory sensory neurons. Although in many respects, the vomeronasal system shares many characteristics with the main olfactory system since both are chemosensory systems, the detailed organization of these two systems is quite different. Early anatomical differences were seen with the discovery of the dichotomous nature of the chemoarchitecture of the VNO. In depth structural examination of the vomeronasal epithelium revealed sensory neurons with an apical pole, dendritic processes extending out towards the lumen (Figure 1.1C), and simple columnar tissue very similar to olfactory sensory neurons (Read, 1908; Moulton and Beidler, 1967; Graziadei, 1977) with a few notable differences. First, unlike the olfactory sensory neurons which display a terminal knob containing cilia protruding into the olfactory mucosa, VSN extensions have a bundle of long microvilli instead of cilia. Second was the subtle difference that there appeared to be two types of sensory cells in the vomeronasal epithelium. Histological studies by Ciges and colleagues in 1977 revealed the presence of highly segregated lamination between the apical and basal portion of the epithelium.
(Ciges et al., 1977). However, it wasn’t until nearly 20 years later that genetic studies began to unveil the details of the subtle structural difference in the sensory layer of the VNO.

As the paradigmatic genetic discovery of olfactory genes began to be harnessed to elucidate the sequence of VN genes, many early attempts to identify them based on olfactory receptor homology proved unsuccessful because there appears to be little homology between odorant receptors expressed by olfactory sensory neurons and vomeronasal receptors expressed by vomeronasal sensory neurons (Dulac and Axel, 1995). Using an ingenious approach, they differentially screened cDNA libraries constructed from single sensory neurons in the VNO, and compared them to one another. They detected only one difference, a novel family of 30-100 genes encoding seven-transmembrane putative VN receptors (VN1, later called V1R) likely coupled to G-proteins (Dulac and Axel, 1995; Del Punta et al., 2000) which is now thought to be about 240 genes which encode full length reading frames. So began the discovery of the genes that encoded putative vomeronasal receptors.

Within two years, three groups had simultaneously discovered and cloned a second family of about 120 putative VN receptor genes (excluding pseudogenes, see (Shi and Zhang, 2007) that came to be called V2Rs (Matsunami and Buck, 1997; Herrada and Dulac, 1997; Ryba and Tirindelli, 1997; Berghard and Buck, 1996). Interestingly, in situ hybridization experiments using V1R and V2R probes against mRNA expression in VSNs revealed expression in a sub-population of VSN restricted to the apical portion (Figure 1.2) of the sensory layer of the VNO for V1Rs (Buck, 1995; Dulac and Axel, 1995; Dulac, 1997), and V2Rs expressed in the basal portion (Herrada and Dulac, 1997; Ryba and Tirindelli, 1997). These findings finally explained the early anatomical differences seen in the chemoarchitecture and suggested that there may be two independent divergent signal transduction pathways accomplished by spatially segregated neuronal circuits (Herrada and Dulac, 1997).
Figure 1.2 - Vomeronasal Sensory Neurons and their Projections. Vomeronasal sensory neurons (VSNs) reside in the vomeronasal organ (VNO), which contains a two-layered sensory epithelium. The apical layer of this epithelium contains VSNs expressing receptors from the V1R family (red) which utilize the Gi2α variant of the G-protein and send their axonal projections to the rostral region of the accessory olfactory bulb where they terminate in discrete regions of neuropil called glomeruli (dashed circles). The basal layer expresses receptors from the V2R family (blue) utilizing the Goα variant of the G-protein and project to the caudal aspect of the AOB.
Further evidence for this came when examining the axonal projections of sensory neurons from the vomeronasal organ to the accessory olfactory bulb. Sensory neurons expressing the V1R family projected their axons centrally to the anterior/rostral aspect of the AOB (Figure 1.2) whereas V2R expressing neurons project to the posterior/caudal aspect of the AOB (Jia et al., 1997). Additionally, comparisons these two families revealed no significant homology to either olfactory receptors or with each other (Matsunami and Buck, 1997). Although both families were members of the G-protein coupled receptor superfamily, V1Rs predicted structure consisted of a seven trans-membrane domain with a relatively short N-terminal extracellular domain (Figure 1.2) and corresponded to G_{i2α} expressing sensory neurons (Saito et al., 1998), whereas members of the V2R family had an extremely long N-terminal domain (Figure 1.2) and corresponded to G_{oα}-protein expressing neurons (Herrada and Dulac, 1997). These results supported a dual perspective concerning VSN processing with apical and basal populations of VSNs expressing two different families of putative receptors and two different G-proteins projecting to distinct sub-regions of the accessory olfactory bulb (Halpern and Martinez-Marcos, 2003).

However, recent work has revealed a more complicated story concerning receptor expression in the VNO. Recent work by Linda Buck and colleagues has reported the existence of a third family of candidate chemosensory vomeronasal receptors containing five members of the formyl peptide receptor (FPRs) family (Liberles et al., 2009). The expression patterns of these receptors in sensory neurons appears in both the apical and basal layers of the VNO and they are not co-expressed with other chemoreceptors of the V1R or V2R family, making them distinct with regard to both receptor expression and expression pattern. In addition, the most recent work examining response profiles of VSNs expressing a given receptor revealed heterogenous peptide specificities suggesting that some V2R expressing cells may express different receptors or receptor combinations violating the one neuron one receptor hypothesis.
that has dominated vertebrate olfaction (Leinders-Zufall et al., 2009). These results taken together suggest that the simple dual perspective of VNO processing may not entirely accurate at least with regard to certain receptor families or receptor sub-families (see Boschat et al., 2002).

Studies investigating the transduction mechanism of OSNs revealed a dependence on expression of the G-protein named $G_{olf}$ which uses adenyl cyclase III to generate the second messenger cAMP which in turn activates cyclic nucleotide gated channels (Reed, 1992; Ronnett and Snyder, 1992; Ronnett et al., 1993). However, the search for similar machinery in the transduction mechanism of VSNs was not successful. Instead, it was found that VSN transduction relies on a different G-protein than $G_{olf}$, namely $G_\alpha$ (Berghard and Buck, 1996; Berghard et al., 1996) to activate the phospholipase C (PLC) pathway and inositol triphosphate (IP3)/diacyl-glycerol (DAG) production (Krieger et al., 1999; Runnenburger et al., 2002) as second messengers to activate a non-specific cation conductance via transient receptor potential channels or TRP2C channels (Lucas et al., 2003). Inactivation of these receptor channels in TRPC2-deficient mice resulted in either absent or strongly diminished pheromone-induced VNO field potentials (Leypold et al., 2002) and the inability to respond to cues present in dilute urine (Stowers et al., 2002). These studies uncovered a separate and distinct transduction mechanism of VSN activation compared to the main olfactory system and continued to add to the list of important differences in the organization and function of these two chemosensory systems.

1.1.3 The Nature of Pheromonal Stimuli

Since the early 1970’s the dual olfactory hypothesis, first described by Raisman and colleagues in 1972 (Raisman, 1972), suggested that because there existed two distinct parallel pathways from the VNO and the main olfactory epithelium into the telencephalon and
diencephalon, that each system was in turn responsible for a completely distinct behavioral function. In this way, it was postulated that the main olfactory system was responsible for the detection and identification of volatile air-borne odorants while the accessory olfactory system detected and identified non-volatile secreted pheromones.

Classically, the term pheromone was used to describe “biological compounds that are secreted and have defined physiological or behavioral effect on an individual of the same species” (Karlson and Luscher, 1959). However, this classical distinction between general odorants and pheromones can, in some cases, be blurred. For example, some pheromones can be volatile compounds while some odorants can act as pheromones by inducing behavioral or physiological changes in much the same way as a pheromone (Touhara and Vosshall, 2009). As a result, there has been some desire in the field to extend the strict classical definition mentioned above to include the diverse functional roles of pheromones than are included in the classical definition (Herness, 2006).

To add to the complexity, as further studies began to elucidate the function of putative pheromones in the VNS, it became clear that the VNS did not have a complete monopoly on pheromone detection and that the VNO might detect some non-pheromone odorants. For example, the VNO has been shown to be capable of detecting common odorants such as ethyl acetate, ethyl propionate, ethyl vanillin, and butanone (Trinh and Storm, 2003) that are not typically thought of as pheromones, with certain volatile odorants and odorant mixtures eliciting calcium responses (Sam et al., 2001) and electro-olfactogram responses from VSNs (Trinh and Storm, 2003). Similarly, the main olfactory system has been shown to mediate pheromone responses, including the “nipple-search pheromone” in rabbits (Hudson and Distel, 1986) as well as to the pheromone androstenone in pigs (Dorries et al., 1997). Therefore, it seems more accurate to view the rodent VNO as a detector of chemicals dissolved in the bodily fluids which
are sucked into its lumen and produce specific behavioral and endocrine effects (Mombaerts, 2004)

What is the nature of these chemicals? Early work revealed that urine and urine derived compounds elicited activation of vomeronasal sensory neurons in culture (Hagino-Yamagishi et al., 2001) and in slice (Krieger et al., 1999). Further investigation revealed that one of the components of urine which can cause an extension of the estrous cycle, was found to specifically activate the V1Rb2 receptor (Boschat et al., 2002). In addition, VNO slice work confirmed that the apical layer of the sensory epithelium responded via calcium influx to six putative pheromones found in urine (Leinders-Zufall et al., 2000). Taken together, these results strongly suggested that components in urine serve to activate V1Rs expressing VSNs.

Continued work began to reveal the ligands of receptors in the V2R family. Early biochemical studies demonstrated that α2u-globulin, a major urinary protein (MUP) which is part of a diverse family of non-volatile extracellular proteins found in urine, specifically activated cells in the basal layer of the VNO epithelium (Krieger et al., 1999). MUPs are characterized by their ability to bind small primarily hydrophobic molecules (Flower, 1996) and may be responsible for the binding, transport, and delivery of small molecules and peptides in urine (Bocskei et al., 1992) and even may themselves act as pheromones (Mucignat-Caretta et al., 1995). Stowers and colleagues went on to show that purified MUP proteins found in urine activate Vmn2r receptors in the V2R family (Chamero et al., 2007).

Early methods to identify putative pheromones in urine were limited to the examination of the electrophysiological, behavioral, and endocrine effects of purified fractions of urine only, thus limiting the number of compounds present in any given fraction. However, behavior can sometimes depend on blends of pheromones (Wyatt, 2003) which are difficult to obtain from intermediate fractions of purification. As a result, Holy and colleagues undertook a de novo
screen by collecting chromatographic fractions of urine resulting in the identification of many new “subfractions” that collectively contained many distinct compounds (Nodari et al., 2008). As a result, they could selectively test combinations of compounds to identify responses dependent on pheromonal blends. This resulted in the identification of a large family of sulfated steroid compounds found in urine which caused significant increases in the firing rates of VSNs and corroborated previous results in goldfish (Sorensen et al., 1995) and lampreys (Sorensen et al., 2005). Non-sulfated variants of these same molecules failed to activate these neurons. Given that some of these sulfated glucocorticoids are elevated several fold in animals following acute stress, these results suggest these chemical messengers might provide information about the physiological status among conspecifics (Nodari et al., 2008).

Another class of molecules found in urine which was hypothesized to play a role as sensory stimuli for VSNs, was the peptide ligands of the major histocompatibility complex (MHC) class I molecules. Their established role in the immune system to convey information about the genetic identity of an individual made them an excellent candidate to provide information about conspecifics through the VNS. Two of these short 9 amino-acid long peptides, SYFEITHI and AAPDNRETF, were shown to activate VSNs expressing the V2R family of receptors (Leinders-Zufall et al., 2004) and were later shown to specifically activate the V2r1b receptors at sub-picomolar concentrations (Leinders-Zufall et al., 2009). These experiments motivated many of our experiments in which we used these two peptides to drive sensory activity in our V2r1b-GFP transgenic mouse line (See Chapter 4).

Urine is not the only secreted bodily fluid that has been found to contain small molecules capable of activating sensory neurons of the VNS. Recordings from single neurons in the accessory olfactory bulb of mice engaged in natural behaviors revealed that investigations of the face and mouth areas evoked some of the most robust increases in firing rates (Luo et al., 2003a). Later, it was shown that male tear fluids contain chemical signals, one of which came to
be called the exocrine gland-secreting peptide 1 (ESP1) which is a 7-kDa peptide. Exposure to this peptide induced c-Fos expression in the basal layer of the VNO (Kimoto et al., 2005) and was shown to selectively activate the V2Rp5 receptor in female mice (Haga et al., 2010). It is thought that ESP1 is secreted from the eyes of male mice and is transferred to the female vomeronasal organ during facial contact to elicit a behavioral response (See Section 1.2).

As described earlier, the discovery of a third, small family of vomeronasal receptors, named formyl peptide receptors (FPRs), which likely bind to formyl-peptides, suggests they might provide a selective advantage to the animal through the ability to recognize sensory ligands not detected by the V1R or V2R family. Given the function of FPRs in host defense against pathogens in the immune system (Migeotte et al., 2006) and the presence of formylated peptides in bacteria and mitochondria, it raises the possibility that FPRs, and this small family of chemoreceptors, might play a role in the identification of decay in a food sources or even diseased states in conspecifics, therefore imparting a novel chemosensory function to rodents (Liberles et al., 2009). However, their exact function has not been established to date.

Possibly the most important functional difference between ORs and VRs, besides the ligands that bind to them, are the differences in their response profiles to their respective ligands. Unlike OSNs which often respond to a broad array of related odorants (Zhao et al., 1998; Duchamp-Viret et al., 1999; Krautwurst et al., 1998), VSNs peptide responses are much more specific and much more highly sensitivity, with EC_{50} values as low as 10^{-14} M, making these chemosensors some of the most sensitive detectors known to exist in mammals (Leinders-Zufall et al., 2000; Leinders-Zufall et al., 2009). In addition, the specificity of the response appears to be invariant of a vast range of ligand concentrations. This differs from the apparent OSN coding scheme which appears to use combinatorial coding in which OSN tuning curves result in the recruitment of additional OSNs at higher ligand concentrations (Malnic et al., 1999; Firestein, 2001). In contrast, the apparent goal of VSN response constancy in the face of
varying concentrations may be to provide stable representations in the ensembles encoding a given ligand over time (Leinders-Zufall et al., 2009).

In summary, the last two decades have revealed an incredibly diverse set of ligands that vomeronasal sensory neurons are capable of detecting and responding to. From large complex proteins such as MUPs and ESPs, to sulfated steroids, to small, <1 KDa peptides like MHC-I and formyl peptides, the VNS is capable of detecting a broad array of chemical signals derived from multiple bodily secretions. Although there is some overlap in the functions of odorants and pheromones, generally these two groups of chemical messengers are derived from different sources and bind to structurally different receptors in different gene families in mammals. Similarly, the coding schemes of the main olfactory system versus the accessory olfactory system appear to be different even at the level of the sensory neurons themselves to accomplish different goals with the information provided by chemical messengers.

1.1.4 Projections to the Accessory Olfactory Bulb

The axons of vomeronasal sensory neurons fasciculate giving rise to the vomeronasal nerve (VN), which courses along the nasal septum and passes through an opening in the inner edge of the cribriform plate (lamina cribosa) and then along the dorsal aspect of the main olfactory bulb and enters the AOB dorsally (Figure 1.3A & B). Once their axons enter the bulb, they defasciculate into separate bundles of fibers which distribute to more than one glomerulus (Cajal, 1902). Glomeruli in the AOB appear as small, roughly spherical regions of neuropil which are diffusely organized compared to glomeruli in the MOB which are much more distinct and consistent in size and shape (Meisami and Bhatnagar, 1998).

Early studies using Nissl staining or cytochrome oxidase methods to stain and examine glomeruli in the AOB were difficult to interpret because glomeruli were not well distinguished due to faint staining and the sparseness of the periglomerular cells that, in the
main olfactory bulb, completely encircle the glomeruli. However, these studies concluded that
the rodent AOB contains several hundred glomeruli with sizes ranged from 25-50 microns. This
makes these glomeruli fewer in number and smaller in size than the approximate 2,500 MOB
glomeruli at an average diameter of approximately 140 microns in adult rats (Meisami and
Sendera, 1993). Using the Golgi method, Takami and colleagues examined the terminations of
axonal arbors in individual glomeruli in rodents and found a high degree of variability in both the
volume and fashion in which they occupied a given glomerulus, some branching in all the
glomerular volume, others instead occupying discrete portions of it. The function of these
differences in sensory termination remains unresolved (Takami and Graziadei, 1990; Takami

In the MOB, olfactory sensory neurons expressing a particular odorant receptor send
projections to the bulb where they converge to only one or two glomeruli such that each
glomerulus represents a single type of odorant receptor. In the AOB, early work using
transgenic mice in which axons of a single receptor class were modified to express LacZ,
showed axons expressing a given receptor converged onto approximately 10-30 glomeruli in the
AOB residing within spatially restricted domains (Belluscio et al., 1999; Rodriguez et al., 1999).
Further work by Wagner and colleagues examined the projections of VSN axons expressing
different V1Rs differentially labeled with fluorescent proteins. They reported a glomerular map
divided into multiple non-overlapping domains, with each V1R subfamily randomly clustering
within a given domain (Wagner et al., 2006). Termination of V2R-expressing VSN axons has not
been similarly examined.

Del Punta and colleagues observed small punctuate areas within a LacZ-positive
glomerulus that were immuno-positive for presynaptic markers NCAM or synaptotagmin but
LacZ-negative, suggesting to them that glomeruli receive input from more than one sensory
neuron type. More recent work has suggested that all afferents to a given glomerulus are from
vomeronasal sensory neurons expressing the same receptor type (Del Punta et al., 2002). If true, this would make connectivity in the AOB functionally similar to the connectivity in the MOB. However, it remains unclear the degree to which VSN axonal input to the AOB resembles that of the MOB and it may be that the wiring scheme of the VSN inputs relies on receptor subfamilies rather than individual receptors as in the main olfactory bulb. Recent work suggesting that VSNs may express multiple vomeronasal receptors further complicates this analysis, as all axons entering a given glomerulus may express one receptor, but be heterogeneous in their expression of a second receptor.

**The Circuitry of the Accessory Olfactory Bulb**

The principal neurons in the AOB receive direct glutamatergic synaptic input from the axons of vomeronasal sensory neurons within a given glomerulus. These neurons, whose somata are between 10 and 20 microns, are called mitral cells. As early as Cajal’s early work, it became clear that rodent mitral cell dendritic morphology differed significantly from mitral cells in the MOB. MOB mitral cells project a single primary dendrite to one or a few glomeruli with approximately 50 mitral cells receiving convergent input from approximately 5,000 axons of olfactory receptor neurons all expressing the same odorant receptor (Shepherd, 2004; Schoppa and Urban, 2003). Mitral cells of the accessory olfactory bulb however, exhibit a more complex primary dendritic structure with as many as ten first order dendrites innervating and forming synapses with a similar number of glomeruli (Ramon y Cajal S, 1911; Takami and Graziaidei, 1990; Takami and Graziaidei, 1991) and as many as 20 mitral cells connected to each glomerulus (Meisami, 1989).

It remains unclear to what degree the approximately 5,000 mitral cells of the AOB innervate glomeruli receiving homogenous input from sensory neurons expressing the same receptor. Early work (Rodriguez et al., 1999) as well as more recent work from the Dulac lab
Figure 1.3 - Anatomical Structure of the Accessory Olfactory Bulb. A) Schematic showing the relative locations of the vomeronasal organ and the accessory olfactory bulbs located in the caudal dorsal portion of the olfactory bulbs (Red). B) Sagittal section of the brain illustrating the location of the AOB with regard to the main olfactory bulb and cortex. C) Zoom of B to visualize the layers of the AOB, namely the glomerular layer (which receives VSN input), the mitral cell layer (in which the principle neurons reside), and the granule cell layer. Scale bar = 200 µm.
(Wagner et al., 2006) suggest that in the rostral AOB mitral cell dendrites target glomeruli innervated by sensory neurons expressing different receptor types. Although this work suggests that the dendrites of a given mitral cell may always send its dendrites to glomeruli innervated by VSNs expressing VRs in the same family. Mitral cells targeting glomeruli receiving input from the V2R family of receptors have also been examined by Del Punta and colleagues and they claim the principal dendrites of a given mitral cell all project to glomeruli of the same molecular identity (Del Punta et al., 2002). Although more work is need to answer this question definitively, it appears clear that mitral cell dendrites are not randomly targeting glomeruli and that dendritic targeting likely represents an important feature of AOB function. We examine this question further with our own data on mitral cell dendritic morphology and targeting in Chapter 3, and discuss in more detail in Chapter 5.

Surrounding individual glomeruli are another cell type of the glomerular layer (Figure 1.3C) of the AOB called periglomerular (PG) cells. These neurons are highly variable in size, yet relatively small (5-8 microns) and form semi-lunar shells around the glomeruli projecting short dendrites which extend into the periglomerular region (Pinching and Powell, 1971). Functional studies have shown PG cells to be inhibitory neurons through mainly GABAergic synapses in the AOB (Takami et al., 1992; McLean and Shipley, 1992). Although their physiology has not been well described in the AOB, in the MOB it has been shown that some PG cells receive direct sensory neuron input as well as input from mitral cell dendrites (Kasowski et al., 1999; Toida et al., 2000) enabling them to participate in interactions both at the level of sensory nerve terminals as well inhibition onto mitral cell tufts. In this way, they may play a particularly important role in processing VSN inputs to the bulb through tuft-specific inhibition.

In a thick layer deep to the layer of mitral cell bodies (Figure 1.3C) is another inhibitory cell type in the AOB which appears to play a critical role in certain pheromone-mediated behaviors, namely granule cells (GCs) (Kaba et al., 1994a). AOB granule cells appear to have the same general morphology as those found in the MOB except that they are smaller with less
extensive arborization (which can extend laterally 50-200 µm) and are more sparse compared to the MOB (Takami et al., 1992). Their cell bodies are also very small (6-8 µm) and appeared as grains to early microscopists, hence the name granule cells.

One of the most notable features of granule cells which was described by the earliest anatomists is that they lacked an axon and thus stood as an exception to the classical "law of dynamic polarization" (Ramon y Cajal S, 1911). However, as further studies began to examine their physiology and ultrastructure, it became clear that olfactory bulb granule cells released neurotransmitter from their dendrites. Early intracellular and extracellular recordings from MOB mitral cells recorded long-lasting inhibition following stimulation of the lateral olfactory tract (Phillips et al., 1963) and recordings from GCs themselves suggested that this inhibition could be mediated by their output (Shepherd, 1963). Later, electron microscopy using serial reconstructions confirmed that indeed mitral cell secondary dendrites and granule cell dendritic spines form reciprocal synapses with each other (Rall et al., 1966) with approximately ~100 GCs for each mitral cell (Shepherd, 2004).

In this way, granule cells mediate both lateral inhibition between mitral cells (Yokoi et al., 1995) and self (recurrent) inhibition of mitral cells. In the AOB, there is no direct evidence that lateral inhibition between mitral cells exists, however, Kaba and colleagues have shown that recurrent inhibition is critical for the induction of pheromonal memories, such that suppression of GABA-mediated inhibition of mitral cells facilitates the induction of specific olfactory memories (Kaba et al., 1994a). Although our understanding of granule cell mediated effect on mitral cell processing in the AOB remains limited, it appears that as in the main olfactory bulb, it plays an important role in the processing of pheromonal information in the VNS.

1.1.5 Downstream Projections from the Accessory Olfactory Bulb

In mammals, mitral cell axons leave the AOB forming the accessory olfactory tracts and project to the bed nucleus of the accessory olfactory tract (NAOT), the bed nucleus of the stria
terminalis (BNST), and the medial nucleus and the posteromedial nucleus of the amygdala otherwise known as the “vomeronasal amygdala” (Figure 1.4) (Broadwell, 1975; Davis et al., 1978; Scalia and Winans, 1975; Winans and Scalia, 1970). Recently, these connections have been investigated using wheat-germ-agglutinin conjugated to horseradish peroxidase which was exposed to VSNs or injected into the AOB to observe second-order terminals in each pathway and confirmed previous results of connectivity (Itaya, 1987). From these limbic regions, projections are directed towards the ventromedial and medial preoptic areas of the hypothalamus (Meredith, 1998; Brennan and Kendrick, 2006; von Campenhausen and Mori, 2000) which appear to be involved in reproductive and social behaviors (Kevetter and Winans, 1981a; Kevetter and Winans, 1981b; Petrovich et al., 2001).

Interestingly, some of the specific regions of the limbic system which receive AOB input appear to control different behavioral responses. For example, the NAOT has been suggested to regulate maternal behaviors (Del Cerro et al., 1991), whereas the BNST may function to regulate copulatory behaviors (Emery and Sachs, 1976). Neurons in the medial preoptic area of the hypothalamus, when stimulated, release luteinizing hormone releasing hormone (LHRH) which in turn stimulates the anterior pituitary gland to release luteinizing hormone (LH) and the follicle-stimulating hormone (FSH) (Wu et al., 1997). Evidence strongly suggests that LHRH regulates mating behavior in both males and females (Fernandez-Fewell and Meredith, 1995), although recent work suggests this may be mediated by the main olfactory system as well (Boehm et al., 2005; Yoon et al., 2005).

As a result, some have postulated that these functional differences regulated by different central brain areas receiving VNS input may be the result of differential axonal projection patterns at the level of mitral cells in the AOB. One might expect the system to maintain a complete segregation of V1R and V2R families through the AOB to downstream regions in order to accomplish this. Although it appears this segregation is not completely maintained in that
Figure 1.4 - Outputs of the AOB. The axonal projections of the primary output neurons of the AOB, mitral cells, project to several central brain regions such as nucleus of the accessory olfactory tract (NAOT) and the bed nucleus of the stria-terminalis (BNST). They also project to the medial and posteromedial cortical nuclei of the amygdala, also known as the “vomeronasal amygdala” (VA) which relays information to the hypothalamus (H) which directly elicits endocrine and ultimately, behavioral responses.
clusters of neurons in the amygdala may combine information from both families (von Campenhausen and Mori, 2000; Salazar and Brennan, 2001; Martinez-Marcos and Halpern, 1999; Mohedano Moriano et al., 2007; Mohedano-Moriano et al., 2008), these studies provide evidence that the two families do project to specific areas in the amygdala and hypothalamus.

In this way, there may be a partial functional segregation with regard to these two pathways that may, in part, mediate these functional differences seen in behavior (Mohedano Moriano et al., 2007; Mohedano-Moriano et al., 2008; Tirindelli et al., 2009). Thus, the VNS pathway bypasses typical higher centers common to the visual or the auditory system and consequently drives innate and stereotyped behavioral and neuroendocrine responses that will be discussed further in the next section.

1.2 The Vomeronasal System and Behavior

Over the course of the last fifty years, the pheromonal effects on mouse ecology mediated by the vomeronasal system have been thoroughly investigated producing an enormous collection of data on the role of this system in mammalian behavior. We will discuss much of this data, primarily examining the role of the VNS and the complex array of behaviors, social interactions, and endocrine changes it is thought to mediate. Although this review is not exhaustive, it will give a broad overview in order to demonstrate the vast body of evidence implicating the VNS in complex intra-species and inter-species communication, which are essential to the animal’s survival.

Some of the earliest studies demonstrating the role of pheromones in communication between conspecifics were a series of studies in the 1950s and 1960s exploring the importance of hormonal changes on animal behavior. These studies resulted in the identification of a number of hormonal effects mediated by the VNS. For example, early studies revealed that
female rodents housed in close quarters would modify or suppress their estrous cycle in response to these close quarters (Van der Lee and Boot, 1955) and was later named the Lee-Boot effect. However, further studies showed that exposure of male urine to grouped females resulted in restoration and synchronization of these cycles in females (Whitten effect) (WHITTEN, 1958) or even that exposure to male urine could accelerate puberty onset in adolescent females (Vandenbergh effect) (Vandenbergh, 1969).

Probably the most studied of these effects is the Bruce effect in which exposure of a recently mated female mouse to a male other than the stud male that impregnated her, prevents the implantation of fertilized egg in the uterus and ultimately, the abortion of the pregnancy (Bruce, 1959). These pheromonal effects were thought to be initiated by stimuli present in urine and postulated to be mediated via the vomeronasal system (Tirindelli et al., 2009). As a result, these early studies argued for a role of the vomeronasal system in responding to cues present in urine and eliciting endocrine responses and innate behaviors in the receiver. In this way, they paved the way for future work examining the specific function of vomeronasal organ projections in behavior.

1.2.1 VNO Ablation Studies

The earliest work elucidating the functional role of the VNS in the behavior of the animal was 35 years ago in 1975 by Winans and colleagues in which vomeronasal nerve cuts resulted in severe mating deficits in sexually experienced male hamsters (Powers and Winans, 1975). In 1982, a procedure was perfected for the removal of the VNO through the mouth (Wysocki et al., 1982). The advantage of this approach was that the procedure spared the olfactory epithelium, allowing neuroscientists to examine the dependence of many stereotypical behaviors on the function of the vomeronasal organ. These included behaviors such as reproductive and copulatory behaviors, aggression and territorial marking behaviors, as well as social interactions.
and neuroendocrine effects (Wysocki and Lepri, 1991). What followed was an explosion of studies examining the effect of vomeronasal organ removal on behavior in several species which I will discuss in the following sections.

1.2.1.1 Hormonal Responses in Males and Females

Both male and female endocrine states are sensitive to stimulation provided by the opposite sex and these intricate changes play an important role in the reproductive state of the animal. This includes the modulation of testosterone levels in males as well the orchestration of many types of hormonal and behavioral responses effecting female reproductive patterns (Wysocki and Lepri, 1991). Indeed, VNO ablation studies have revealed profound reductions in the normal occurrence of pregnancy failures upon exposure to a non-stud male, suggesting a necessary role for the vomeronasal system in the Bruce effect (Kelliher et al., 2006).

Similar studies demonstrated the role of the VNS in mediating hormonal changes in females. For example, females housed in cages containing a large number of females produce a chemical messenger in their urine which has been shown to inhibit sexual maturation in other females (Drickamer, 1977). Yet studies examining this effect in females whose VNO had been removed found that the detection of a crowded condition and the subsequent release of this chemical cue was eliminated, such that even the urine from these animals did not delay the onset of puberty in control females (Lepri et al., 1985).

In male mice, other work has shown that exposure of a novel female or her urine resulted in an increase in LH followed by an increase in plasma testosterone levels in males. (Macrides et al., 1975). Several studies examined this effect in male mice whose VNO has been removed and found that they fail to report these hormonal changes after exposure to an anesthetized novel female or her urine, although exposure to awake females apparently presented enough cues to evoke these responses (Coquelin et al., 1984; Wysocki et al., 1983). Given the strong presence of AOB projections found in the hypothalamus, these studies
continued to support a role for the vomeronasal system in mediating the hormonal state of the animal.

1.2.1.2 Sexual and Reproductive Behaviors

One of the most thorough studies was performed by Meredith and colleagues in which they demonstrated that VNO removal prior to sexual experience had severe effects on male hamster mating behavior compared to previously experienced males which displayed minimal deficits (Meredith, 1986). Similarly, early work examining several aspects of male mouse sexual behavior suggested that they depended on the function of the VNS. For example, removal of the VNO in male mice resulted in severe impairments in copulation with females compared to controls (Clancy et al., 1984). Others have also seen significant negative and enduring effects on nearly every measure of male sexual behavior in adult males whose VNO had been removed at an early age, including mounting, intromissions, and successful copulations (Bean and Wysocki, 1985).

Another important component of sexual behavior in male mice is the emissions of ultrasonic 70-kHz vocalizations in the presence of female mice or their urine (Nyby, 1979; Holy and Guo, 2005). This behavior was shown to be affected by the removal of the VNO, especially in sexually naïve males compared to males which had interactions with females prior to VNO removal, indicating associative learning as well as other olfactory cues may also be important in this behavior (Wysocki et al., 1986). Finally, female sexual receptivity marked by lordosis (defined as the hind end elevated and back slightly arched to allow for successful copulation) was impaired in female hamsters lacking a functional VNO (Mackay-Sim and Rose, 1986).

Taken together, these ablation studies provide strong evidence for a primary role for the VNS in sexual and reproductive behaviors in both males and females.
1.2.1.3 Aggression and Territorial Marking Behaviors

Other stereotypical male mouse behaviors such as territorial urine marking and aggressive behaviors towards other males have been investigated following VNO ablation. For example, all these behaviors were shown to be significantly diminished in males who had had their VNO removed suggesting these normal male behaviors were dependent on the vomeronasal system (Maruniak et al., 1986). VNO removal resulted in significant deficits in territorial markings, however, as with many of the effects described previously, territorial marking seems to be affected most in sexually naïve males although experienced males do show depressed marking behaviors (Labov and Wysocki, 1989).

Inter-male aggression, which is also a well-established stereotypical male behavior, has been examined by a number of groups upon the removal of the VNO. For males, this is usually assayed by introducing a male intruder into a cage containing a singly housed male resident. After a period of olfactory investigation, the resident male initiates a series of attacks whose latency and duration represent a standard measure for male aggressive behavior (Connor and Lynds, 1977; Maruniak et al., 1986). In all cases, VNO removal caused either a significant reduction or complete elimination of aggression towards other males depending on the animal’s prior experience with invading males. These mice display the most severe deficits compared to males who had not experienced inter-male aggression prior to VNO removal (Clancy et al., 1984; Maruniak et al., 1986; Bean and Wysocki, 1989).

However, male mice are not alone in their display of aggressive behaviors towards other mice. Early experiments showed that female mice also display aggressive behaviors, particularly lactating females towards intruder males placed in the cage with their nursing pups (Svare and Gandelman, 1973) and it was suggested that chemical cues may play some role in mediating these behaviors. Later, studies provided evidence to support the hypothesis that these maternal behaviors were highly dependent on a functional VNS. Although VNO removal
prior to mating did not affect nest building, pup weights, or pup discrimination (Wysocki et al., 1986; Lepri et al., 1985), it did eliminate the display of maternal aggression behaviors (Bean and Wysocki, 1989).

This rich body of evidence on the behavioral effects of lesioning the vomeronasal system make clear that this system plays a very important role in many stereotypical behaviors that are essential to the survival of the animal, including numerous behavioral and physiological effects. However, these studies suffer from several disadvantages arising from the technique used for VSN removal. For example, trauma associated with the procedure including the removal of the VNO can cause excessive tissue damage due to ischemia or scarring. In addition, partial or incomplete lesions can result confounding the conclusions one may draw from the results observed. The introduction of selective genetic manipulations such as knock-outs of specific genes along with the discovery of the mechanisms underlying vomeronasal receptors neuron responses has opened the door for more rigorous investigation of the specific roles of the main and accessory olfactory system in mediating animal behavior.

1.2.2 Genetic Knock-Out Studies

As discussed in Section 1.1.2, the discovery of a distinct and separate signal transduction mechanism in VSNs utilizing G\(_0\) and G\(_i\)-proteins to activate the phospholipase C (PLC) pathway and IP3/DAG production (Krieger et al., 1999; Runnenburger et al., 2002) to ultimately open TRPC2 channels for sensory signaling (Liman et al., 1999), opened the door for their exploitation. This included genetic manipulation by ablation of genes encoding specific signal transduction molecules involved in the vomeronasal pathway only. By far, the most genetically targeted protein in this pathway has been the TRPC2 channel, which is expressed exclusively in sensory neurons of the vomeronasal organ (Zufall et al., 2005). Genetic ablation of TRPC2 channels does not result in a complete attenuation of the electrophysiological responses of VSNs to urine or its components but the vast majority of VSNs no longer are
capable of responding to sensory stimuli (Kelliher et al., 2006; Stowers et al., 2002; Leypold et al., 2002), making this manipulation an excellent model for selectively studying VNO function.

1.2.2.1 Sexual and Reproductive Behaviors

As described previously, the Bruce effect, or selective pregnancy block, is one of the best known examples of pheromone-mediated effects on female sexual reproduction. After early studies by Hilda Bruce, further work by others confirmed that the formation and maintenance of this pheromonal recognition memory is dependent on the vomeronasal system (Brennan et al., 1990; Hudson, 1993; Kaba et al., 1994a). Furthermore, others have shown that MHC peptide ligands from the strange male can mediate the Bruce effect in female mice (Leinders-Zufall et al., 2004; Boehm and Zufall, 2006). Surprisingly, transgenic mutants lacking functional TRPC2 channels did not display significant deficits in pregnancy blocks and showed a normal Bruce effect compared to profound deficits in the occurrence of pregnancy failures in VNO lesion studies as described above (Kelliher et al., 2006). These studies suggest a TRPC2 independent signal transduction mechanism that may be important for the formation of pheromonal recognition memories that affect female sexual reproduction.

Likewise, the genetic ablation of TRPC2 channels appears to greatly affect some, but not all features of sexual behaviors in males. For example, as mentioned above, VNO removal in sexually naïve hamsters was found to abolish male sexual behavior (Meredith, 1986). However, TRP2C knock-out male mice displayed innate sexual behavior that was indistinguishable from control males (Stowers et al., 2002). However, knock-out males even displayed sexual behavior including mounting and thrusting towards male intruders instead the typical inter-male aggression behaviors (Stowers et al., 2002; Leypold et al., 2002), similar to what was seen in VNO ablated males (Kimchi et al., 2007).

Female sexual receptivity to males also was not altered in TRPC2 knock-outs, however these mice did show a reduction in female-specific sexual behavior. Surprisingly, their sexual
behaviors began to resemble that of males (Kimchi et al., 2007). That is, female knock-out mice displayed several male sexual and courtship behaviors such as mounting, pelvic thrusts, solicitation, anogenital investigation, and emission of ultrasonic vocalizations towards both male and female mice. This striking behavioral phenotype was also seen in adult females whose VNO had been removed and did not appear to result from hormonal changes (Kimchi et al., 2007). These findings suggest a whole new way of understanding sexual dimorphism and the role of the VNS in gender specific behaviors. Indeed, they support a hypothesis in which gender-specific circuits mediating male and female behaviors exist in the brains of both sexes, which are then turned on or off by gender specific modulators. These data also suggest that the VNS is one of these sensory modulators, which in females, may act to detect specific chemical cues which act to repress male-like sexual and courtship behaviors in females.

In addition, other genetic knock-out studies ablating genes encoding putative pheromone receptors have revealed the role of specific receptors and their ligands in rodent sexual and reproductive behavior. For example, the simultaneous deletion of a group of approximately 16 V1R genes encoding their respective pheromone receptors resulted in a significant decrease in male copulatory behavior. Taken together with the studies described earlier from TRP2C knock-out animals, these results suggest an important role for the VNS in male reproductive activity likely through V1R expressing VSNs but that signaling through the VNO is not required for initiating sexual behavior.

Some of the most recent work has examined the role of a specific putative pheromone and its receptor in regulating sexual-behaviors in female mice. As described previously, ESP1 is a 7-kDa peptide that is released into male tear fluids and stimulates vomeronasal sensory neurons expressing the V2Rp5 receptor in female mice (Kimoto et al., 2005; Kimoto et al., 2007). Kimoto and colleagues further examined the role of this peptide in female behavior by utilizing a transgenic mouse line deficient for the V2Rp5-receptor. Whereas wild-type females showed increased lordosis behavior and successful intromissions by males as a result of ESP1-
stimulation, V2Rp5 knock-out female mice did not display increased lordosis behaviors in the presence of ESP1 nor did TRP2C-deficient mice (Haga et al., 2010). In addition, they showed that ESP1 signals are relayed through a highly localized population of second–order neurons in the AOB and finally through the ventromedial hypothalamic nucleus. These results suggest a specific sexually dimorphic circuit from the VNO to the hypothalamus for peptides released in male tears that during investigation and sexual courtships, which ultimately trigger female receptivity and lordosis allowing for successful copulation.

Indeed, strains with low expression of this “sex-pheromone” showed a smaller number of both lordosis responses in females as well as successful intromissions by males. These results are striking and indicate that these strains are sexually less successful than strains with males secreting higher levels of ESP1. In addition, this is the first report of a select vomeronasal receptor detecting and mediating the release of a specific peptide, which particularly effects female sexual behavior through a sexually dimorphic AOB to hypothalamic circuit. In turn, these results provide insight into the mechanisms and circuitry which underlies pheromone-induced behavior in mammals.

1.2.2.2 Aggression and Fear Behaviors

As discussed previously in Section 1.2.1.3, both males and females exhibit stereotypical aggressive behaviors towards intruders. As described earlier, VNO removal nearly abolishes male-male aggression behaviors and several genetic knock-out studies have corroborated these results. For example, male TRPC2 channel mutants also fail to display aggression toward male intruders (Stowers et al., 2002; Leypold et al., 2002). In females, several genetic studies have recapitulated decreased aggression towards intruders in lactating females. The genetic ablation of TRP2 channels (Kimchi et al., 2007; Leypold et al., 2002) also displayed low maternal aggression.
Mutant mice carrying a targeted disruption for genes other than the TRP2C channel have further substantiated these results. For example, female mutant mice, in which the G_{\alpha_{i2}} gene is mutated (Rudolph et al., 1995) resulting in attenuated responses in apical VSNs, showed significantly less aggression towards male intruders despite unaltered sexual-partner preferences (Norlin et al., 2003). These results taken together, suggest a possible role for apical VSNs expressing the V1R family of receptors in mediating maternal aggression in lactating females.

Male mutants that fail to express receptors capable of binding MHC Class Iib molecules also exhibit specific defects in male-male aggression behavior (Loconto et al., 2003). Interestingly, synthetic MUP proteins alone, which have been shown to activate V2R family receptors, were sufficient to induce male-male aggression which was mediated exclusively through the VNS (Chamero et al., 2007). This group found at least two MUPs that independently promoted aggressive behavior in males through VNO and not MOE circuitry, although others have shown the main olfactory system is also necessary for male aggressiveness in both ablated (Keller et al., 2006) and mutant mice (Mandiyan and Coats, 2005). As a whole, these data suggest there may be multiple pathways which promote aggressive behaviors in male mice mediated by several different pheromones by both the vomeronasal and main olfactory systems.

The body of evidence discussed thus far primarily examined the role of the VNS in mediating various behaviors towards conspecifics. However, potential predators also emit chemical messengers that warn receiving species of danger. These “fear-evoking odors” have been called kairomones, which differ from pheromones which mediate intra-species communication, in that they mediate inter-species communication. Recently, these kairomones (which are released from multiple species such as cats, rats, and snakes), were examined in
mice and found to require the function of sensory neurons in the VNO (Papes et al., 2010). In addition, isolation and purification of these ligands from cats and rats revealed that these ligands belong to the MUP family and are homologues of the aggression-promoting mouse pheromones described previously. These MUPs were shown to be sufficient to initiate defense behaviors such as freezing, fleeing, or fighting depending on the context (Eilam, 2005).

In summary, we are only beginning to unravel the complexity of the vomeronasal system and the multifaceted assortment of behaviors it is involved in orchestrating. Evidence from many studies examining the vomeronasal system across many different species using ablation and genetic approaches has revealed a chemosensory system capable of detecting a broad array of chemosignals found in several different bodily secretions and involved in regulating many important stereotypical social and reproductive behaviors. Indeed, the VNS is capable of mediating such mutually exclusive behaviors as pheromone-mediated intra-species behaviors as well as kairomone-promoted inter-species fear-evoking behaviors. The fundamental mechanisms of how this neural circuit differentiates various conspecific and species-specific cues to elicit particular behaviors in response to its environment continue to be an area of intense interest.

One of the most important principles gleaned from these behavioral studies and the harnessing genetic tools to manipulate the function of different rectors and signal transduction molecules, is that specific stereotypical behaviors appear to depend on a select subset of sensory neurons and even in some cases, solely on a single receptor type and its respective ligand. This suggests that sensory information detected in the periphery by the VNO directly influencing behavior is relayed through particular VSNs expressing select receptor types. Taken together with work suggesting that VSN axons converge and target specific glomeruli in the AOB and that mitral cell dendrites target glomeruli receiving input from particular VSNs, these results support a model in which well conserved specific neural circuits in the vomeronasal system are used to induce innate behaviors in rodents.
If this is indeed the case, the accurate relay of specific pheromonal information to downstream effector brain areas would rely heavily on the formation of proper connectivity between VSN axons and their respective target glomeruli as well as the precise targeting of mitral cell dendrites to glomeruli receiving specific input. Yet our understanding of the principles of this connectivity as well as the mechanisms by which the developing vomeronasal system ensures this specificity is very limited, which brings us to the goal of the body of work presented here in this dissertation.

1.3 The Goals of this Dissertation and Summary of Findings

Thus far we have discussed the general organization of the vomeronasal system within distinctive brain structures and the large chemical variability this system is capable of detecting, leading to marked changes in animal behavior and endocrine status. Anatomical, lesion, molecular, and electrophysiological studies have uncovered an unexpected diversity and complexity underlying the function of this system (Dulac and Torello, 2003), yet it is also surprising how poor our understanding of the basic circuitry of this system remains. Although the fundamental circuitry from sensory activation in the periphery, to inputs in the brain have been well-established in other sensory systems, including the main olfactory system, there is relatively little data to this end in the vomeronasal system.

For example, as described in Section 1.1.4 and Section 1.1.5, there is still some debate on the molecular homogeneity of VRNs that innervate a given glomerulus as well as the degree to which mitral cell dendrites target glomeruli receiving homogenous input in the bulb. Although it is clear from several studies that connectivity between VSNs and AOB mitral cells is not random, the detailed organizational principles of sensory input to the brain in this circuit are not well understood. In addition, our understanding of the development of this circuitry in young
animals to ultimately give rise to proper connectivity in this system remains poor. As with other sensory modalities in the brain, the characterization of these principles in the VNS, across development and in the adult, will be essential to ultimately understanding the underpinnings of pheromonal processing through this system, all the way from detection to behavior.

Our goal was to explore the growth and development of this circuit from birth into adulthood, to not only identify some of the fundamental principles for the wiring of this system, but to elucidate the mechanisms by which this connectivity is established. Therefore, the goals of the dissertation were the following: 1) to develop a method that would enable us to examine and investigate both cellular morphology and neuronal connectivity in specific brain circuits. 2) to examine the early development of connectivity between the vomeronasal organ and the accessory olfactory bulb. 3) to observe the role that sensory activity plays in the early development of connectivity.

1.3.1 Chapter 2: A Method for Studying Neuronal Circuit Architecture

The method described in Chapter 2 is a technique capable of overcoming several of the logistical barriers to visualizing and recording from specific cell populations in a given neuronal circuit in vitro. This method requires only basic electrophysiological equipment and we have demonstrated that that this technique is highly versatile in that it can be used with a diverse set of fluorescent tracers and calcium imaging dyes. We report its use in visualizing neuronal circuits for targeted whole-cell patch clamp recordings as well as for labeling specific populations with calcium imaging dyes for population level physiological analysis. Finally, we illustrate its use in several brain areas including the cortex, the hippocampus, the main olfactory bulb, and the accessory olfactory bulb.

The method we developed is a useful technique for any brain region as we illustrate and elaborate on in Chapter 2; however its primary intended use for us was to ultimately provide us with a tool to examine the connectivity in specific circuits of the vomeronasal system which we
described in Chapter 3. In addition, this method also provides other advantages for labeling and recording from specific neurons based on connectivity, especially within the main and accessory olfactory systems, and has since been utilized by other members of the Urban lab for other studies, which are not discussed in this dissertation.

1.3.2 Chapter 3: Analysis of the Development of the Vomeronal System

The research described in Chapter 3 characterizes the overall development of the connectivity between the vomeronasal organ and the accessory olfactory bulb in part using the method described in Chapter 2. In addition, we use a transgenic mouse line created by Mombaerts and colleagues (Del Punta et al., 2002) in which GFP is inserted into the gene coding for the V2r1b receptor. As a result, the fluorescent GFP label is present in the cell bodies, axons, and dendrites of all V2r1b-expressing VSNs. This enabled us to visualize and count these cells in the VNO and to measure properties of their axonal terminations in the AOB.

In this chapter, we first describe the gross anatomical development of both the vomeronasal organ and the accessory bulb illustrating the dramatic period of growth and development that occur in this system during the first few post-natal weeks of the animal’s life. These results set the stage for subsequent experiments by establishing the critical time window to examine the pre-synaptic and post-synaptic development in this system.

Consequently, we examined the refinement of axonal projections from V2r1b-expressing vomeronasal sensory neurons in the vomeronasal organ to the AOB during this critical time period and describe an early period of axonal coalescence during the first four post-natal days. Further examination of the post-synaptic targeting of dendrites also revealed a substantial period of pruning and refinement of dendritic clusters in respective glomeruli which peaks just after the time period of axonal development. Finally, we employ our electroporation method to examine mitral cell connectivity to glomeruli receiving input from V2r1b-expressing sensory neurons and report that mitral cells preferentially target glomeruli receiving homogenous input.
1.3.3 Chapter 4: The Role of Sensory Activity in the Development of the VNS

The results described in Chapter 3 reveal an important period of growth and refinement in this system both in terms of axonal coalescence and dendritic pruning to ultimately establish precise connectivity between peripheral sensory neurons and the output neurons of the accessory olfactory bulb. One interesting question is what role, if any, activity might play in this development to help establish this specificity in connectivity. For example, one might hypothesize that activity would accelerate synaptic refinement through increasing the rate by which hebbian-like mechanisms establish proper connections.

We first provide evidence that the vomeronasal organ is indeed functional at birth, both with regard to access to external sensory stimuli and physiologically with sensory neurons capable of releasing neurotransmitter onto post-synaptic mitral cells. This then prompted us to investigate the endogenous ligands for the V2r1b-receptor, namely two MHC class I peptides. We provide the first evidence that at least one of these peptides is indeed present in urine at sufficient concentrations to activate sensory neurons and that these ligands are capable of inducing immediate early gene activity in downstream neurons of the accessory olfactory bulb.

Finally, the work of Chapter 4 culminates in the demonstration of the effect of select sensory stimulation using these peptides on the number of sensory neurons expressing the V2r1b receptor as well as their axonal development and coalescence in the accessory olfactory bulb. We report that excess sensory activity does not significantly affect receptor expression in VSNs but does significantly alter axonal coalescence into well-defined glomeruli early in development. Surprisingly, instead of accelerating this refinement, we report that activity delays this refinement resulting in over-exhuberant projections in the AOB. Taken as a whole, these results point to a critical time period of development during the first few post-natal weeks in which vomeronasal sensory neurons establish precise connections with mitral cells in the accessory olfactory bulb.
2.0 A Simple Method Of In Vitro Electroporation Allows Visualization, Recording, And Calcium Imaging Of Local Neural Circuits

2.1 Abstract

Since Cajal’s early drawings, the characterization of neuronal architecture has been paramount in understanding neuronal function. With the development of electrophysiological techniques that provide unprecedented access to the physiology of these cells, experimental questions of neuronal function have also become more tractable. Fluorescent tracers that can label the anatomy of individual or populations of neurons have opened the door to linking anatomy with physiology. Experimentally however, current techniques for bulk labeling of cells in vitro often affect neuronal function creating a barrier for exploring structure-function questions. Here we describe a new technique for highly localized electroporation within a cell or cell population that enables the introduction of membrane impermeable charged dyes including dextran-conjugated fluorophores, hydrazide tracers, and calcium indicator dyes in vitro. We demonstrate that this technique is highly versatile, allowing for labeling of large or small areas of tissue, allowing for the investigation of both cellular morphology and physiological activity in identified neuronal circuits in acute brain slices. Furthermore, this approach allows subsequent targeted whole-cell patch recording based on well-defined connectivity as well as assessment of physiological activity in targeted circuits on a fast time scale.
2.2 Introduction

Understanding neural circuit function is greatly facilitated by the simultaneous analysis of anatomical and physiological properties of the elements in these circuits. Previous approaches to this problem have focused mostly on simultaneous recordings of random pairs or triples of neurons (Wang et al., 2006; Le Be and Markram, 2006). Although powerful, this approach is limited in that the number of cells being recorded from is low, particularly when the specific neurons one wants to analyze are sparse and not easily identified by somatic position and shape. Alternative techniques enabling the examination of neural circuits and pathway tracing in vivo have several limitations, especially for slice electrophysiology.

In vivo bolus or electroporation loading techniques prior to brain removal (Stosiek et al., 2003; Bonnot et al., 2005; Nagayama et al., 2007), are inefficient for targeted labeling of specific neuronal populations. Additionally, some individual neurons in particular circuits may be impossible to load, especially in deep brain areas. Alternative approaches in vitro carry with them their own limitations. Lipophilic carbocyanine dye traces can readily be taken up by nearby neurons in vitro, and have been widely used to label and trace individual neuronal morphology (Honig and Hume, 1986; Honig and Hume, 1989; Vonbartheld et al., 1990; Del Punta et al., 2002). However, these dyes have relatively slow diffusion rates and can often take hours or even days to label an entire neuron, which limits their ability to be used in targeted patch clamp recording experiments and also excluding the possibility of calcium dye loading and imaging of physiological activity.

The development of electroporation techniques that allow for the delivery of macromolecules through the creation of transient pores in the plasma membrane, have been used in several brain preparations such as the whole cerebellum (Yang et al., 2004), cerebellar organotypic slice cultures (Murphy and Messer, 2001), the spinal cord (Bonnot et al., 2005), the
brainstem (Barker et al., 2009), and the thalamic reticular nucleus (Pinault, 1996). However, these techniques often require the use of complex electroporation/pressure ejection machinery (Barker et al., 2009) or the placing of the slice between electrode plates (Bonnot et al., 2005), platinum wires (Murphy and Messer, 2001), or in an electroporation chamber (Bright et al., 1996), all of which can affect neuronal health, and accessibility for subsequent recording.

Here we describe a technique, adapted from previous in vivo work (Nagayama, Zeng et al. 2007) for labeling neurons that allows for subsequent targeting of the labeled cell for physiological analysis without the use of complex electroporation chambers or apparatuses. Specifically, we show that a local electroporation technique using a simple glass electrode filled with a charged dye can be used in in vitro brain slices to target and load a wide range of tissue areas to label either large or small populations of neurons that have overlapping axons and/or dendrites. This technique can be used to deliver virtually any charged dye into populations of neurons for visualization of specific neuronal subtypes and circuits, targeted whole-cell patch clamp recordings, and calcium imaging within a given circuit. Dye uptake and diffusion is rapid over long distances allowing for visualization and calcium imaging of processes hundreds of microns away from the electroporation site within the duration of a typical acute slice experiment. This approach will facilitate recording from pairs and even groups of neurons that are connected anatomically, allowing experimenters to sidestep labor intensive recording techniques to find connected pairs in slice, and rapidly identify microcircuits of interest.
2.3 Materials And Methods

2.3.1 Dye Solution Preparation

Several charged dyes were successfully used with our protocol (Table 1) such as dextran-conjugated dyes (Invitrogen) including Alexa Fluor 488, 594, and 647, Rhodamine Ruby Red and Oregon Green Bapta. We also used several hydrazide tracers (Invitrogen) such as Alexa Fluor 350, 488, 594 and 647. All dextran-conjugated dyes were generally soluble in aqueous buffers in 0.1M phosphate buffer (pH = 7.2). Given that their solubility decreases as their molecular weight increases, solutions were made for maximum solubility according to their molecular weight (50mg/ml for 10,000 MW dextrans, 25mg/ml for 70,000 MW dextrans, etc.) Solutions were then vortexed and sonicated and in some cases heated to increase solubility. If insoluble particles were seen at the tip of the electrode, the solution was centrifuged at 12,000 x g for 5 minutes before filling the electrode. Hydrazide dyes were made at 1mM in internal buffer solutions containing the following (in mM): 150 D-gluconic acid, 10 HEPES, 2 KCl – pH adjusted to 8.05 with KOH.

2.3.2 Electroporation protocol

Glass electrodes were pulled to a tip size of 1-5 µm and filled with 8-10 µl of dye solution and held with an MXP-Probe Holder (Siskiyou Design Instruments, Grant Pass, OR). A silver wire was inserted into the electrode so that the end of the wire was close (< 1 mm) to the tip of the electrode and immersed within the dye solution. The silver wire was connected to an SIU (stimulus isolation unit) (AMPI, Israel) controlled by TTL (transistor-transistor logic) pulses from an ITC-18 data acquisition board (Instrutech, Port Washington, NY) and the ground wire was connected to the bath ground. The electrode was then placed in the brain slice (~ 10 – 20 µm deep) in an area of interest. Different stimulus protocols were developed depending on experimental requirements. A high intensity stimulus protocol optimal for large scale loading of
Table 1
Comparison of Dyes Used

<table>
<thead>
<tr>
<th>Part #</th>
<th>Dextran Dyes</th>
<th>Concentration</th>
<th>Vortexed</th>
<th>Sonicated</th>
<th>Centrifuged</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>D1817</td>
<td>Rhodamine Ruby Red</td>
<td>12% in PBS</td>
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<td>✔️</td>
<td></td>
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<tr>
<td>O6798</td>
<td>Oregon Green Batpta</td>
<td>5% in PBS</td>
<td>✔️</td>
<td>✔️</td>
<td></td>
<td>10,000</td>
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<tr>
<td>D22913</td>
<td>Alexa Fluor 594</td>
<td>7% in PBS</td>
<td>✔️</td>
<td>✔️</td>
<td>✔️</td>
<td>10,000</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Part #</th>
<th>Hydrazide Dyes</th>
<th>Concentration</th>
<th>Vortexed</th>
<th>Sonicated</th>
<th>Centrifuged</th>
<th>MW</th>
</tr>
</thead>
<tbody>
<tr>
<td>A10439</td>
<td>Alexa Fluor 350</td>
<td>1mM in IBS</td>
<td>✔️</td>
<td></td>
<td></td>
<td>350</td>
</tr>
<tr>
<td>A10436</td>
<td>Alexa Fluor 488</td>
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<td>✔️</td>
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<td>570</td>
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<tr>
<td>A10438</td>
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<td>760</td>
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<td>1mM in IBS</td>
<td>✔️</td>
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<td>1,300</td>
</tr>
</tbody>
</table>

Table 2.1 – Dyes Used for Electroporation. Both dextran and hydrazide dyes were used successfully for both electroporation protocols. Dextran dyes were dissolved in phosphate buffer solution (see methods) and generally required at least sonicating and vortexing prior to being loaded in the electrode to reduce dye aggregates that would clog the electrode. Hydrazide dyes were dissolved in internal buffer solution (see methods) and were far less likely to aggregate, requiring only sonication. They provided the best results for repeated electroporation and multi-dye electroporation experiments.
many neurons in a given area consisted of 1200 current pulses, given at 2 Hz, each 25 msec in duration at 30 to 40 µA each. Low intensity protocols, ideal for preserving the health of individual cells for electrophysiological recordings consisted of 100 current pulses, given at 2 Hz, each 25 msec in duration at 1-2 µA each.

2.3.3 Electrophysiology

Slices sectioned from young mice (post-natal day 12 to 30) were superfused with oxygenated Ringer’s solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 1MgCl$_2$, 25 glucose, 2 CaCl$_2$, warmed to 34-36º C. Whole-cell voltage recordings were obtained from the somata of identified filled cells and recordings were established using pipettes (resistances of 2-8 MΩ) filled with a solution containing the following (in mM): 120 potassium gluconate, 2 KCl, 10 HEPES, 10 sodium phosphocreatine, 4 Mg-ATP, and 0.3 Na$_3$GTP, adjusted to pH 7.3 with KOH. Voltage and current clamp recordings were performed using a MultiClamp 700A amplifier (Molecular Devices, Union City, CA). Data were filtered (4 kHz low pass) and digitized at 10 kHz using an ITC-18 (Instrutech, Mineola, NY) controlled by custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR). Noisy stimulus pulses (DC = 400 pA, variance = 40 pA) were generated by convolving frozen white noise with an alpha function (Mainen and Sejnowski, 1995; Galan et al., 2008; Galan, 2008).

2.3.4 Immunohistochemistry

After electroporation, each 300 µm section was fixed for 24 hours in 4% PFA and transferred to a 30% (in 0.1 M phosphate buffer) sucrose solution. Slices were then sectioned at 50 µm using a Leica cryostat SM2000R (Germany). Sections were transferred to 1 ml of 0.1 M phosphate buffer (PB). The solution was then replaced with 300 µl of PB with 2% normal donkey serum and 0.1% Triton X-100 at room temperature for 1hr. The sections were then washed 3X with PB incubating for 5 minutes each wash. The solution was then aspirated and
replaced with the primary antibody (300 µl of 1:1000 Kv. 1.2 mouse antibody, NeuroMAB) in PB with 2% normal donkey serum and 0.05% Tween 20. After washing 3X again in PB, the solution was aspirated and replaced with the secondary antibody (300 µl of 1:600 donkey antibody mouse conjugated 488, Invitrogen) with 2% normal donkey serum and 0.05% Tween 20 and incubated for 1 hr in the dark and finally washed 3X in PB. The sections were then mounted in gelvatol for confocal imaging.

2.3.5 Calcium and Confocal Imaging

After electroporation and uptake of calcium dye, extracellular stimulation of a nearby region was elicited using a theta glass electrode connected to an SIU box controlled by TTL pulses. Increases in fluorescence in loaded cell were visualized by a back-illuminated, cooled CCD camera (Princeton Instruments, Cascade 512B) in 3-s-long movies acquired using custom software written in Igor Pro. Movies were analyzed by calculating the \( \Delta F/F \) versus time and analyzed to identify individually activated cells. Slices were then fixed in 4% paraformaldehyde for 24 hours and mounted for later confocal imaging. Confocal image stacks were acquired using a Zeiss LSM 610 Meta (1024 x 1024, 12-bit images) using 488, 561, and 633 lasers and BP 500-550IR, BP 575-630 IR, and 672-704 filters. Images were then processed and stacked using the Zen 2007 Software (Zeiss).
2.4 Results

2.4.1 Electroporation Enables Uptake of Various Dyes in Multiple Different Circuits

Slices containing either the cortex, the hippocampus, the main olfactory bulb, or the accessory olfactory bulb from mice were used to test the local electroporation method. Dextran-conjugated dyes were initially used to test the protocol in each brain area. Immediately after completion of the protocol, dye was present throughout the tissue in a larger area surrounding the electrode location, which eventually dissipated leaving only the area of tissue which had taken up the dye. The number of loaded cells varied greatly with the number of pulses given and with stimulation intensities. We eventually established two protocols, one with a greater number of pulses with higher amplitudes for loading large areas and numerous cells and another with much fewer pulses with smaller amplitudes for more focal targeting and labeling of a single or a few cells (see methods).

As a result, we were able to electroporate both large areas of the cortex, hippocampus and main olfactory bulb (Figure 1A-D), and also target loading to a fairly small and well-defined area using the low intensity protocol. As an example, we found that we could electroporate a single glomerulus (25 µm) as seen via a GFP-transgenic mouse line (Del Punta et al., 2002) in which sensory neurons expressing the V2r1b receptor type also express tau-GFP allowing us to observe axonal terminations in individual glomeruli in the accessory olfactory bulb (Figure 1E). In some instances, many cells deep to the stimulation electrode were labeled following electroporation. For example, in the CA3 region of the hippocampus, a confocal stack acquired starting just below the site of electroporation showed dense labeling of many cells (Figure 1B). Anterograde and retrograde projections were clearly visible in all circuits. Indeed we were able to visualize processes from loaded cells up to several hundred microns away from the site of
Figure 2.1 – Electroporation of Various Circuits. A) Coronal slice containing barrel cortex was electroporated in layer 2/3 labeling the processes of several neurons in other cortical layers. B) Coronal slice containing hippocampus was electroporated in area CA1 labeling multiple pyramidal cells and their projections. C) Sagital slice containing the main olfactory bulb. A single glomerulus was electroporated labeling periglomerular, external tufted, and mitral cells all innervating the electroporated glomerulus. D) Coronal slice containing somatosensory cortex was electroporated in layer 5 labeling pyramidal cells and their projections towards layer 1. E) Sagittal slice containing the accessory olfactory bulb in a GFP-transgenic mouse line. A GFP-positive glomerulus was targeted and electroporated. F) A mitral cell labeled by electroporating the glomerulus in E shown beside a second GFP-positive glomerulus.
electroporation (Figure 1D) as well as visualize the distinct morphology of individually loaded cells and their processes (Figure 1F). In all these examples, dye loading was complete within 25–30 minutes following the beginning of the electroporation.

### 2.4.2 Electroporation Can Provide for Discrete Labeling of Specific Neuronal Circuits

We used acute coronal brain slices to test whether this technique was capable of targeted electroporation of specific processing units, for instance the mitral cell to glomerular layer connections within the olfactory bulb. Three consecutive glomeruli were identified under DIC and targeted for electroporation using three separable hydrazide dyes (Alexa Fluor 488, 594, and 647). Each glomerulus was electroporated separately with a different electrode using the high intensity stimulation protocol (see methods). High intensity protocols typically labeled 3-10 mitral cells per glomerulus whereas low intensity protocols typically labeled only 1–2 (see Figure 5 and 6).

We found that each glomerulus was individually labeled with surrounding periglomerular and external tufted cells (Figure 2A) as well as connected mitral cells for each glomerulus (Figure 2B). We found little to no overlap between electroporated glomeruli at the level of the glomerular layer for each labeled glomerulus (Figure 2A) consistent with the known anatomy of this system (Schoppa and Urban, 2003). The resultant mitral cell loading labeled unique populations with each population connecting exclusively to the corresponding electroporated glomerulus (Figure 2C). As a result, this technique allowed us to fluorescently identify individual processing units in the olfactory bulb.

Following electroporation, we also performed immunohistochemistry on sections containing labeled cells. After staining for the potassium channel Kv. 1.2, we found that we could examine the expression of local potassium channels in specific populations of neurons by observing the overlap between labeled neurons via electroporation (Figure 3A) with
Figure 2.2 – Specificity of Electroporated Circuits. A) A coronal slice showing three consecutive glomeruli electroporated with Alexa 448, 594, and 647 hydrazides respectively. B) Confocal image of the mitral cell layer with each representative population of mitral cells innervating each glomerulus from A labeled separately. C) Confocal image stitched from A and B displaying both the glomerular and mitral cell layers of the section. High intensity stimulation protocols were used for each glomerulus (1200 current pulses, 25 msec in duration at 2 Hz, 30 to 40 µA).
immunohistochemistry (Figure 3B). Indeed, we observed overlap between labeled cells and Kv. 1.2 staining as seen in Figure 3C, where two electroporated external tufted cells also stained positive for Kv. 1.2 (arrows). This illustrates that this technique provides a useful tool for examining the overlap of the expression of individual proteins within local neuronal circuits of interest.

2.4.3 Rapid Uptake and Diffusion of Dye After Electroporation

One of the major limitations of previous dye loading techniques such as the use of lipophilic dyes, including DiI, is the relatively slow uptake of dye and subsequent loading of somata and processes. For imaging approaches to be useful in slice electrophysiology experiments, both rapid uptake and diffusion of excess dye are essential for overall slice quality, the quality of whole-cell recordings, and calcium imaging of cellular activity. To assess the time required for dye uptake we acquired images at a series of time points following electroporation.

Immediately after the electroporation protocol we observed bright fluorescence through the tissue in the region in which the electrode had been placed. In a few minutes following electroporation, the fluorescence became less uniform presumably as the dye in the extracellular space diffused away, leaving behind labeled processes. Detectable levels of dye were seen in the somata of cells ≈200 µm away immediately after the completion of the electroporation protocol (data not shown). Sufficient levels for targeted whole-cell patch recordings were seen at 5 minutes post-hoc (Figure 4B & C).

We quantified this observation by calculating the ratio of pixel intensities across the cell body over the pixel intensities at a fixed point in the surrounding tissue. This ratio increased dramatically at each time point (Figure 4B) as the dye diffused towards the cell body, and the dye in the surround tissue was cleared. We found nearly complete diffusion of dye by
Figure 2.3 – Electroporation-Coupled Immunohistochemistry. A) Confocal images stack of a coronal slice containing the main olfactory bulb in which one glomerulus was electroporated labeling several periglomerular and external tufted cells. B) Confocal image stack of the same slice in A which was also stained for Kv. 1.2 potassium channels. Arrows indicated two nearby Kv. 1.2-positive cells. C) Overlay of A & B illustrating the expression of Kv. 1.2 potassium channels by these two external tufted cells which were also labeled via electroporation.
Figure 2.4 – Rapid Uptake and Diffusion of Dye. A) Schematic illustrating the experimental design to observe and evaluate loading at various time intervals in the main olfactory bulb. The mitral cell layer was imaged after electroporating a single glomerulus. B) Graph of the ratio of pixel intensities measured at the soma divided by the background plotted against time after completion of the electroporation protocol. C-D) Fluorescent images of the mitral cell layer taken at 5, 10, and 20 minutes after electroporation of a glomerulus. Inset) The difference in pixel intensities between the cell body (black bar), and the surrounding tissue (yellow bar) was calculated for each time point.
20 minutes after electroporation (Figure 4C-D). All experiments including calcium imaging and targeted whole-cell patch clamp recording were performed 15-25 minutes post electroporation.

For usefulness of this technique purely for anatomical purposes, we also wondered what fraction of the cells from the population projecting to a target region were labeled after a single electroporation protocol. As before, the glomerular system provided a perfect setting to answer this question by electroporating the same glomerulus twice with two differently colored dyes. To do this, we used a theta glass electrode, filling one chamber with Alexa 594 hydrazide, and the other chamber with Alexa 488 hydrazide (Figure 5A). We then electroporated a random glomerulus using the high intensity protocol with the red chamber only and observed the number of labeled periglomerular, external tufted, and mitral cells (Figure 5B).

Without moving the electrode, we then electroporated the same glomerulus with the same protocol using the green chamber to then observe the labeled cells from the second electroporation epoch (Figure 5C). If each electroporation protocol was 100% effective in labeling all cells projecting to a given glomerulus, we would expect all yellow cells. In fact, when we counted the number of labeled cells in each channel, we saw that, on average, 93% percent of labeled cells (29 Red, 32 Green in Glomerulus 1, 39 Red, 41 Green in Glomerulus 2), were labeled during the first electroporating epoch while the second electroporation epoch only labeled a combined 5 additional cells in both glomeruli (Figure 5D). This suggests that, at least for the high intensity protocol, each electroporation epoch is effective in labeling the vast majority of cells projecting into a target region.

**2.4.4 Electroporation Enables Targeted Whole-Cell Patch Clamp While Preserving Cell Viability**

Many slice electrophysiology experiments require the ability to target and record from cells based on their connectivity. This includes single cells or pairs of cells that innervate a
Figure 2.5 – Completeness of Electroporation Protocol. A) Schematic illustrating the experimental design using a theta glass electrode, each chamber filled with a differently colored dye. A given glomerulus was electroporated first with the red chamber and second with the green chamber. B) Confocal image stack of the red channel from two adjacent glomeruli which had been electroporated labeling many periglomerular, external tufted, and mitral cells. C) Confocal image stack of the green channel from the same two glomeruli in panel B. D) Overlap of both channels to observe the number of cells labeled only after the second electroporation protocol was given (green cells) and those labeled by both (yellow).
particular layer or which send their dendrites to a particular layer. One example of such a system is the mouse olfactory system where the primary output neurons of the olfactory bulb, mitral cells, target a single primary dendrite typically to one glomerulus. Individual glomeruli are innervated by olfactory sensory neurons expressing a single receptor gene (Ressler et al., 1994; Mombaerts et al., 1996) such that all mitral cells innervating a given glomerulus receive homotypic input. Thus targeting cells based on the glomerular termination of their dendrites allow analysis of neurons that receive the same inputs and should have comparable receptive fields.

To determine if this technique would enable us to not only label multiple mitral cells innervating a given glomerulus, but to also use this fluorescence to target and record from those cells, we first electroporated random glomeruli in the main olfactory bulb using the low intensity stimulation protocol. We observed loading of (in most cases) surrounding periglomerular cells, external tufted cells, and typically 1-2 mitral cells (Figure 6A1 & A2, Figure 7A & B). We used fluorescence to target labeled mitral cells/tufted cells for whole-cell patch clamp to record and fill these cells with a second fluorescent dye (Figure 6C1 & C2, Figure 7A & B). For patched mitral cells, we injected both square current pulses and filtered white noise current pulses (generated by convolving white noise with a 3 msec alpha function), to probe the responses of electroporated cells to various inputs (Figure 7C & D).

To assess the effect of electroporation on the intrinsic properties of these mitral cells, we recorded the changes in membrane potentials in current clamp to 25 pA hyperpolarizing currents for 500 msec (Figure 7E). Input resistances were calculated when the membrane potential reached steady state (Figure 7F). The membrane time constant (\( \tau \)) was calculated by fitting the membrane potential trace between the initial potential and the steady state potential using a mean-square error function (Figure 7G). We found no significant differences in the input resistance (control \( R=19\pm7 \) M\( \Omega \), electroporated \( R=37\pm34 \) M\( \Omega \), \( P=0.29 \) ANOVA) or membrane
Figure 2.6 - Targeted Whole-Cell Patch Clamp via Electroporation. A1 & A2) Fluorescent images of two separate slices in which a single glomerulus was electroporated with Rhodamine Ruby Red (A1) and Oregon Green Bapta 488 (A2) labeling external tufted and mitral cells. B1 & B2) Fluorescence enabled targeted whole-cell patch clamping and filling with a second fluorescent dye, Alexa 350 (B1) and Alexa 594 (B2). C1 & C2) Overlay of A1 vs B1 and A2 vs B2 confirm cell identity.
Figure 2.7 – Electrophysiological Recordings from Electroporated Cells. A & B) Two neurons which were electroporated and filled using a low intensity stimulus protocol (100 current pulses, 25 msec in duration at 2 Hz, 1-2 μA). Fluorescence was used to target individually labeled cells for whole-cell recordings. C & D) Cells were injected with noisy (C) or plain (D) current pulses (see methods) to evaluate the health of electroporated cells. E – G) The intrinsic properties of electroporated mitral cells were compared to control mitral cells. 25 pA hyperpolarizing currents were given and compared to control cells (E) and used to calculate and compare input resistances (F - control R=19±7 MΩ, electroporated R=37±34 MΩ, P=0.29 ANOVA) and the membrane time constants (G - control τ=14±4.5 ms, electroporated τ=17±9.8 ms, P=0.56 ANOVA).
time constants (control $\tau = 14 \pm 4.5 \text{ msec}$, electroporated $\tau = 17 \pm 9.8 \text{ msec}$, $P=0.56$ ANOVA) between mitral cells recorded under control conditions ($N=5$) and those recorded after they had been electroporated ($N=4$). In addition, these values are consistent with previously reported values \textit{in vitro} (Margrie et al., 2001a).

2.4.5 Electroporation of Calcium Indicators Enables Calcium Imaging of Local Neuronal Circuits

We found that both high and low intensity protocols worked well with multiple dextran conjugated dyes and hydrazide dyes (see Table 1) and allowed for multi-dye loading within a given circuit. However, these fluorescent dyes only allow for morphological reconstruction and targeted whole-cell patch clamp recording of labeled neurons. To determine if our technique would also allow for the examination of physiological activity of multiple neurons within a circuit, we proceeded to label populations of neurons with a calcium indicator dye that serves as a proxy for neuronal activity via calcium imaging (Tank et al., 1988; Kerr et al., 2005; Ohki et al., 2005).

We choose dextran-conjugated Oregon Green Bapta as a calcium indicator and electroporated within the mitral cell layer in the accessory olfactory bulb. We found intense labeling of both mitral cells and granule cells within the bulb as a result (Figure 8A). In order to evoke activity within these neurons and to test whether the electroporated calcium indicator retained its calcium sensitivity, we placed a theta glass stimulation electrode nearby labeled mitral and granule cells (Figure 8B). Stimulation evoked calcium transients in nearby cells that had been loaded with Dextran-OGB (Figure 8C & D) indicating that electroporation of calcium-sensitive dyes could be used to study the physiological activity of targeted populations of neurons.
Figure 2.8 – Calcium Activity in Local Neuronal Circuit. A) Sagittal slices containing the accessory olfactory bulb were electroporated using Oregon Green Bapta in two separate locations in the mitral cells layer labeling both mitral cells and granule cells. B) Fluorescent image of a stimulating electrode (blue star) nearby several labeled mitral cells. C) Image stack of the average delta F/F at frame 11 during stimulation showing the increase in fluorescence of nearby mitral cells. D) The average delta F/F as a function of time calculated by subtracting the delta F/F of the background (yellow line) from the cell body (black line).
2.5 Discussion

2.5.1 Summary of Findings

In summary, we provide a powerful technique for linking anatomical study of microcircuits with physiological exploration of the properties of the cells within those circuits. The paucity of tools available to slice electrophysiologists for the simple and efficient introduction of fluorescent dyes into specific groups of neurons within a circuit without compromising the cellular function of the neurons has rendered some questions in slice electrophysiology out of reach. We have shown that using simple glass electrodes and small current pulses, we could effectively expel a given dye while simultaneously electroporating local cell membranes to label and identify neurons in any given neural circuit.

Furthermore, we demonstrate that the described method is highly versatile with regard to the brain areas that can be electroporated and also with regard to the diverse sets of fluorescent dyes and indicators that can be used. In addition, this technique provides a high degree of specificity of neurons targeted in a wide variety of circuits enabling the rapid identification of neurons while preserving cell viability for whole-cell recording of individual neurons. Taken together, this method provides the ability to examine neuronal morphology, target specific neurons for whole-cell patch clamp recording, and assess the physiological activity of groups of neurons using calcium sensitive dyes.

2.5.2 Pitfalls and Limitations

As with any technique or method used in conjunction with acute slice preparations, the probability of success is directly proportional to the quality of slice health. Although we have found our technique to be highly reproducible and consistent across brain areas and trials, we
found that our ability to use this technique in combination with whole-cell patch clamp recording or calcium imaging was dependent on initial slice quality.

Furthermore, because electroporation functions by creating transient pores in the membranes of cell bodies, dendrites, and axons, cell function is affected, at least transiently. We have shown here that after electroporation, several measures of cell viability and health, such as input resistance and the membrane time constant remain relatively indistinguishable from control cells. However, we cannot exclude the possibility that some measure of cell function is not transiently or permanently affected as a result of these short current pulses, although it seems reasonable to assume that this effect is no different than multiple trials of current injection in a typical whole-cell attached recording common to electrophysiology.

2.5.3 Significance for Studying the Vomeronasal System

Although we describe in detail the variety of brain areas within which this technique can be used successfully, particular brain areas lend themselves to taking advantages of the strengths of this technique based on their anatomy. This is especially the case with the main and accessory olfactory systems. As described earlier, both of these systems share many anatomical similarities. For example, in both systems sensory neuron axons terminate into well-defined spherical collections of neuropil called glomeruli where they synapse with the dendrites of the main output neurons of the bulbs, mitral cells. These glomeruli represent anatomical features within these circuits which can be exploited using the described electroporation technique to label subsets of neurons receiving identical input as seen in Figure 2.2.

Combined with the anatomical advantages presented in the accessory olfactory system, the technique described here represents several methodological possibilities for studying various aspects of AOB function. For example, in the main olfactory bulb, recent work has revealed that mitral cells have intrinsic biophysical diversity and that this diversity as a result of
differential expression of voltage-gated ion channels (Padmanabhan and Urban, 2010) which results in increases in information carrying capabilities by these diverse populations. In addition, further work has shown that mitral cells which innervate the same glomerulus and therefore receive common input (sister mitral cells) have differential phases responses that could possibly be a result of differences in intrinsic properties of sister mitral cells (Dhawale et al., 2010).

However, questions about biophysical diversity among mitral cells in the accessory olfactory bulb, or the coding schemes and firing rate properties of sister mitral cells, have not been examined in the accessory olfactory bulb. The technique described here would enable several approaches to examine questions about mitral cell diversity. For example, electroporation of a given AOB glomerulus labeling several sister mitral cells would allow for the identification of these cells post-hoc as well as coupling with immunohistochemistry to examine differential expression of various channels and receptors in sister mitral cells or other cell types innervating a given glomerulus as illustrated in Figure 2.3.

Likewise, we have illustrated the usefulness of this technique for targeted whole-cell patch clamp recording of specific neurons in a given circuit. In the MOB, whole-cell in vitro recordings have been used to evaluate mitral cell output spike patterns to examine correlations and differences in mitral cell intrinsic properties (Padmanabhan and Urban, 2010). Our electroporation technique would facilitate whole-cell recordings from both sister and non-sister mitral cells to examine the output properties of various mitral cells or peri-glomerular or tufted cells. Similarly, electroporation of calcium-dye indicators in a given glomerulus or multiple glomeruli facilitates simultaneous examination of activity in populations of sister or non-sister mitral cells. These experiments provide the advantage of observing the responses of many neurons simultaneously as opposed to one or a few using patch-clamp recording techniques. Experiments such as these may continue to divulge the properties of the processing units in the accessory olfactory bulb and the coding schemes of pheromonal processing in the AOB.
Finally, we have shown the usefulness of this method to examine the anatomical connectivity in various circuits. This proves especially useful to examine ongoing questions of mitral cell connectivity in the AOB. As described in Section 1.1.5, it remains unclear the degree to which mitral cells targeting glomeruli receiving homogenous input, especially with regard to different receptor types. In the next chapter, we will describe the use of this technique to examine questions of anatomical connectivity in the accessory olfactory system, specifically with regard to mitral cell dendritic targeting of glomeruli receiving input from sensory neurons expressing the V2r1b receptor type.
3.0 Characterization of Vomeronasal Organ to Accessory Olfactory Bulb Circuitry Across Development in Mice

3.1 Abstract

The mammalian accessory olfactory system is specialized for the detection of chemicals that identify kin and conspecifics. The principle neurons of the accessory olfactory bulb (AOB), mitral cells, process incoming sensory information about chemicals and it is thought that the anatomical organization of this circuit is essential for this information transfer. However, the development of this anatomy, as well as the mechanism by which this anatomy provides a substrate to filter sensory information, is poorly understood. We show here that connectivity between mitral cells and axons from sensory neurons in the vomeronasal organ is specifically targeted. We further investigated the development of this specification and show that initially, mitral cells promiscuously send out individual dendritic branches which only after the formation of well-defined glomeruli, elaborate tufts in specific glomeruli that contain axons from sensory neurons expressing the same receptor gene. Finally, synapse maturation occurs at or just prior to this period of refinement. These results suggest that neuronal connectivity within the AOB is highly specified in order to provide a substrate for proper informational processing in this system.
3.2 Introduction

The anatomical organization of the brain is a necessary prerequisite for understanding its function. The mammalian accessory olfactory bulb (AOB), a CNS structure involved in the identification of chemicals that signal the social and reproductive status of conspecifics (Dulac and Torello, 2003), is a model system well-suited to study the relationship between circuitry and function. Individual AOB principle neurons, mitral cells, are extraordinarily sensitive and selective to socially relevant ligands and mixtures (Luo et al., 2003a), yet this system presents several anatomical challenges towards providing highly specified input onto these cells.

Unlike their counterparts in the main olfactory epithelium, where axons expressing a given odorant receptor converge to innervate just one or two glomeruli, axons of vomeronasal sensory neurons (VSNs) expressing a single receptor type or perhaps the same set of receptors (Leinders-Zufall et al., 2009), diverge and innervate between 6-30 glomeruli in the AOB. Axons of VSNs that express the same receptor project precisely such that single glomeruli are believed to contain a single type of sensory neuron axon (Rodriguez et al., 1999; Belluscio et al., 1999). Moreover, unlike their counterparts in the MOB, AOB mitral cells have multi-tufted morphology, and receive input from 3-6 glomeruli (Ramon y Cajal S, 1911; Takami and Graziadei, 1990).

Despite this anatomical complexity, neuronal connectivity within the AOB appears to be remarkably precise with dendrites of individual mitral cells receiving input from highly specific sets of glomeruli. This dendritic targeting results in a single mitral cell receiving input from glomeruli that are either homogeneous with respect to the receptor type expressed by the sensory neurons innervating them (Del Punta et al., 2002), or which receive input from VSNs expressing receptors in the same family (Wagner et al., 2006).
In the present study, we examined the development of anatomical and functional connectivity of VSNs to AOB mitral cells in young mice. We found that, in accordance with previous studies, mitral cell dendrites did not form tufts in random glomeruli but instead targeted specific glomeruli containing axons of sensory neurons expressing the same receptor gene. We further investigated how this specification occurs over the first few post-natal weeks. We found that both the VNO and AOB both underwent dramatic growth during this period. VSN axons expressing the V2r1b receptor gene rapidly coalesced into well-defined glomeruli during the first four post-natal days. Interestingly, the dendritic branches of mitral cells only begin to form well-defined dendritic tufts in glomeruli after post-natal day four. Furthermore the miniature synaptic amplitudes and kinetics of excitatory inputs onto mitral cells did not change during development. Taken together, these data point to precision and specificity in the development of the AOB, which could provide a stable, specific, anatomical structure to facilitate AOB computations.

3.3 Materials and Methods

3.3.1 VNO and AOB Tissue Collection and Immunohistochemistry

Animals at all ages were perfused first with buffer containing 1% NaCl (2 minutes) then with 4% PFA (2 minutes), and their brains were dissected and fixed overnight before being transferred to a 30% sucrose solution. Each brain was sectioned at 75 µM using a Leica cryostat SM2000R (Germany) and the sections placed in 1 ml of PB solution. They were then washed 3X with PB incubating for 5 minutes each wash. The solution was then aspirated and replaced with the primary antibody (300 µl of 1:1000 GFP rabbit antibody) in PB with 2% normal donkey serum and 0.05% Tween 20 and incubated for 1 hour at room temperature. After washing 3X again in PB, the solution was aspirated and replaced with the secondary antibody
(300 µl of 1:600 donkey anti-rabbit conjugated 488, Invitrogen) with 2% normal donkey serum and 0.05% Tween 20 and incubated for 1 hr in the dark and finally washed 3X in PB. The sections were then mounted in gelvatol for confocal imaging and analysis.

The heads were also fixed overnight in 4% PFA before being transferred to 0.1M phosphate buffer (pH = 7.2). The heads were mounted against a 4% agarose block and sectioned at 200 µM using a vibratome and the sections were also transferred to 24 well plates containing net wells with 1 ml of PB solution. The sections were washed 3X with PB incubating for 5 minutes each wash. The solution was then aspirated and replaced with the primary antibody (300 µl of 1:10,000 goat anti-GFP, Abcam) in PB with 2% normal donkey serum and 0.05% Tween 20 and incubated at room temperature for 3 hours. After washing 3X again in PB, the solution was aspirated and replaced with the secondary antibody (300 µl of 1:600 donkey antibody goat conjugated 488, Invitrogen) with 2% normal donkey serum and 0.05% Tween 20 and incubated for 2 hr in the dark at room temperature and finally washed 3X in PB. The sections were then mounted in gelvatol for confocal imaging and analysis. For both AOB and VNO sections, the final secondary contained 1:40,000 Hoechst (Invitrogen).

3.3.2 Imaging and Analysis of Axonal Coalescence

Consecutive sections containing AOB and GFP-positive glomeruli were imaged using a Zeiss LSM 610 Meta and processed using Zen 2007 software. Image stacks were median filtered using ImageJ software with a Gaussian width of 2.0 pixels. Maximum intensity z-projections of each stack were imported into custom written software in Igor Pro. Individual glomeruli were analyzed by placing the coordinates of a semi-circle such that the arc of the semi-circle was oriented ventral to the section with the straight-edge cutting off the axons just above the glomerulus. This ensured that each concentric semi-circle expanded throughout the axonal terminations in the glomerulus while excluding the axons superficial to the glomerulus.
Sholl analysis was performed such that the final concentric semi-circle encompassed the entire glomerulus. Histogram plots of the number of pixels crossing threshold as a function of distance were calculated and the width of the peak of each histogram plot was measured at half of the maximum amplitude. In cases where it was difficult to determine a single glomerulus vs. multiple glomeruli, consecutive sections were examined for evidence of a second glomerulus.

3.3.3 Slice Preparation

Sagittal olfactory bulb slices (280-350 μm thick) were prepared from young transgenic V2r1b mice (Del Punta et al., 2002) post-natal day 0 to 33 as described previously (Urban and Castro, 2005). Mice were anesthetized (0.1% ketamine/0.1% xylaxine; ~ 3mg/kg, i.p.) and decapitated. Olfactory bulbs were removed and sectioned on a vibratome while submerged in ice-cold oxygenated Ringer’s solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 1MgCl$_2$, 25 glucose, 2 CaCl$_2$. All procedures were in accordance with the guidelines of Institutional Animal Care and Use Committee of Carnegie Mellon University.

3.3.4 Electrophysiology

Whole-cell voltage recordings were obtained from the somata of identified AOB mitral cells. Slices were superfused with oxygenated Ringer’s solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO$_3$, 1.25 NaH$_2$PO$_4$, 1MgCl$_2$, 25 glucose, 2 CaCl$_2$ warmed to 34-36º C. Whole-cell recordings were established using pipettes (resistances of 2-8 MΩ) filled with a solution containing the following (in mM): 120 potassium gluconate, 2 KCl, 10 HEPES, 10 sodium phosphocreatine, 4 MgATP, and 0.3 Na$_3$GTP, adjusted to pH 7.3 with KOH along with neurobiotin and Alexa Fluor 594 for visualization purposes. Each cell was held for approximately 30 minutes to allow for dye diffusion. Voltage and current clamp recordings were performed using a MultiClamp 700A amplifier (Molecular Devices, Union City, CA). Data were low pass
filtered (4 kHz) and digitized at 10 kHz using an ITC-18 (Instrutech, Mineola, NY) controlled by custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR).

3.3.5 Mitral Cell Reconstruction and Tuft Analysis

After fixation, slices that contained neurons filled with neurobiotin were stained using the avidin-biotin peroxidase reaction to produce an insoluble brown 3,3’-diaminobenzidine precipitate. The sections were then mounted in gelvatol and examined under DIC. Their dendrites were reconstructed using Neurolucida 8.11 (MicroBrightField) and analyzed using Neurolucida Explorer. Density calculations were performed in Matlab using XYZ coordinates of traced mitral cells in Neurolucida. Sliding windows of 5, 25, and 50 µm were used and a density histogram was calculated for each of the window sizes. The mean of the histogram at two standard deviations above the mean was calculated in order to compare across age groups.

3.3.6 Calcium Imaging

Calcium signals in AOB slices were imaged using methods previously described in (Kapoor and Urban, 2006). Briefly, slices were loaded in a chamber containing 500 µl of Ringer’s solution, 1.5 µl of pluronic (Invitrogen) and 5 µl of a 5mM solution (in DMSO) of fura-2AM (Invitrogen). Slices were incubated in this solution for 60-90 minutes and oxygenated with humidified air. After loading, extracellular stimulation of a GFP-positive glomerulus using a theta-glass stimulating electrode connected to an SIU box, elicited decreases in fluorescence in several mitral cells. This was visualized in 3-sec long movies acquired using a back-illuminated,
cooled CCD camera (Princeton Instruments, Cascade 512B) and custom software written in Igor Pro. For each movie the $\Delta F/F$ versus time was calculated to identify individually activated mitral cells. These cells were then targeted for whole-cell patch recording and filled with Alexa 594 hydrazide (Molecular Probes) and Neurobiotin (Vector). After recording, the slices were then fixed in 4% paraformaldehyde and the mitral cells were imaged using confocal microscopy (LSM 610 Meta).

### 3.3.7 Electroporation

Slices containing AOB were examined under fluorescence (488nm) to identify GFP-positive glomeruli innervated by V2r1b-GFP expressing sensory neurons. Glass electrodes were pulled to a tip size of 3-7µm and filled with 5-10% solutions of Dextran conjugated Alexa 594 (Molecular Probes). The tip of the electrode was placed in the center of a GFP-positive glomerulus. The electroporation protocol consisted of 1200 pulses, (25msec duration, 1-2µA), at 2 Hz were given by an SIU box controlled by TTL pulses from the ITC-18 data acquisition board (Hovis et al., 2010). This typically resulted in fluorescent dye loading of 6-10 mitral cell bodies and their dendrites per slice. This tissue was fixed in 4% paraformaldehyde for approximately 24 hours and examined post-hoc using confocal imaging (LSM 610 Meta Axioscope 2). Single images, image stacks, and 3D reconstructions of slices were taken and compiled using Zen 2007 software.
Figure 3.1 – The AOB Grows Dramatically During the First Four Post-Natal Weeks. A) The accessory olfactory bulb (blue lines) the glomerular layer (red lines) and individual GFP-positive glomeruli (green lines) were traced using Neurolucida for each section of the brain containing AOB across multiple animals to quantify the change in volume across development. B) Actual fluorescent image stained with VGlut2 to visualize glomerular layer and AOB boundary. C) Change in the volume of the AOB across development (black bars) and the change in volume of the glomerular layer across development (red bars). D) The percentage of the AOB that is comprised of the glomerular layer for each age group across development. Scale bar = 100 μm.
3.4 Results

3.4.1 Gross Anatomical Development of the VNO and AOB

We desired to investigate the development of connectivity from the vomeronasal organ (VNO) to the accessory olfactory bulb (AOB) in mice over the course of developmental change early in the animal's life. In order to identify the critical time window of growth and development in this system, we first examined the gross anatomical changes in both the VNO and the AOB during the first four post-natal weeks of the animal's life. We utilized a transgenic mouse line whose sensory neurons express a specific sensory receptor, namely the V2r1b receptor, also express tau-GFP (Del Punta et al., 2002). This provided us with the ability to visualize sensory neurons expressing the V2r1b receptor in the VNO, as well as their axonal projections innervating the AOB. Animals from post-natal day zero to post-natal day thirty (P0 – P30) were perfused, dissected, and examined to evaluate the various stages of development for the vomeronasal system.

For each age group, the VNOs and the AOBs (n = 3-5 from each group) were assessed. For each animal, the AOB was removed and sectioned on a sliding Leica sliding microtome SM2000 (Germany) at 75 µm and then mounted for imaging. For each section, the layers of both the accessory olfactory bulb were traced using Neurolucida (Figure 3.1A,B). We then calculated the total volume of each section as well as the entire AOB in cubic microns and compared these across development (Figure 3.1C). We found that during the first twelve to fourteen post-natal days, both the glomerular layer and the AOB undergo a dramatic period of growth, which afterwards, remains relatively constant (Figure 3.1C), consistent with previous results seen in the rat (Roos et al., 1988). However, when we compared the relative percentage of bulb occupied by the glomerular layer, we found that it remained relatively constant across development (Figure
Figure 3.2 – The VNO Grows Dramatically During the First Four Post-Natal Weeks. A) Confocal image stack of a 200 μm section of VNO from a post-natal day zero animal and V2r1b-GFP expressing sensory neurons. B) Confocal image stack of a section from a post-natal day fourteen VNO. C) Confocal image stack of a section from a post-natal day 492 VNO. D) The number of V2r1b-GFP expressing sensory neurons per VNO across development. E) The change in the average volumes of VNOs reconstructed across development. Scale bar = 50 μm.
2D). Thus, both the glomerular layer and the AOB undergo a dramatic period of growth during the first two weeks of the animal’s life, but this growth is proportional relative to each other.

For each animal, the vomeronasal organ was also removed, fixed, and sectioned (200 µm) using a Leica vibratome VT1000S (Germany). The entire VNO of each animal was also traced using Neurolucida and the total number of V2r1b expressing neurons were counted for each. This then allowed us to reconstruct the entire volume of each VNO (Figure 3.2A) and we calculated the volume in cubic microns of each VNO as well counted the total number of V2r1b expressing sensory neurons and compared them across development (Figure 3.2B). We found that, between P0 and P14, the VNO grew dramatically both in terms of overall volume, similar to previous reports in the rat (Weiler et al., 1999), and in terms of V2r1b expressing VSNs. These changes plateaued by post-natal day fourteen (Figure 3C,D). These results, taken together, suggest that the first few post-natal weeks are a critical time window for growth and development in the vomeronasal system.

3.4.2 The Development of VSN and Mitral Cell Connectivity

The results from the previous experiments suggest that the first 12-14 post-natal days of the animal’s life may be a critical time period for the development of connectivity within this system. To examine how the pre-synaptic axons of sensory neurons were developing during this time period, we again utilized the V2r1b transgenic mouse line in order to visualize the development of GFP-positive axons during this time period. We performed confocal imaging on the glomerular layer of animals during the first few post-natal days of the animals life to examine the terminations of vomeronasal sensory neuron axons in glomeruli. We found that during the first four post-natal days, VSN axons expressing the V2r1b receptor gene rapidly coalesced into well-defined glomeruli (Figure 3.3) which after P5, remained relatively constant in terms of both shape and size.
Figure 3.3 – V2r1b-Expressing Sensory Neuron Axons Coalesce During the First Four Post-Natal Days. Single projections of confocal image stacks taken of V2r1b-expressing sensory neuron axons and their terminations in the accessory olfactory bulb across the first four post-natal days of life (P0-P3). Scale bar = 20 μm.
Figure 3.4 – Quantification of Axonal Coalescence Across Development. A1) Confocal image stack of V2r1b-expressing sensory neuron’s axonal terminations in a post-natal day zero mouse. A2) Histogram distribution using Sholl analysis for the glomerulus shown in A1. B1) Confocal image stack of axonal terminations in a post-natal day three mouse. B2) Histogram distribution using Sholl analysis for the axonal arborization shown in B1. C1) Confocal image stack of axonal terminations in a post-natal day thirty-six mouse. C2) Histogram distribution using Sholl analysis for C1. D) Quantification of the half peak width from each histogram across animals at three different ages, P0-1 = 25.75 μm +/- 2.32 (n=4), P2-3 = 17.58 μm +/- 0.94 (n=6), Adult = 16.9 μm +/- 0.123 (n=5), p<0.01.
In order to quantify this effect, we performed Sholl analysis on confocal image stacks in young and adult animals (Figure 3.4A1-C1) and calculated the histogram of the number of intersections as a function of distance with increasing semi-circle size. We then measured the full width of the histogram at half the maximum amplitude representing a given glomerulus (Figure 3.4A2-C2) and plotted these versus age (Figure 3.4D). As one might expect, the half-width was significantly more dispersed at post-natal day zero to one (25.75 µm ± 2.32, n=4) than in animals at post-natal day two to three (17.58 µm ± 0.94, n=6, p<0.01) or animals older than post-natal day thirty (16.9 µm ± 1.23, n=5). These results suggest that the first few post-natal days of the animal’s life are critical for the coalescence and refinement of vomeronasal sensory neuron axons into glomeruli in the accessory olfactory bulb.

Given that sensory neuron axons do not terminate in well-defined glomeruli at birth, we postulated that the dendritic morphology of mitral cells might also be unrefined compared to the highly precise targeting of tufts in adolescent mice. Slices containing AOB were obtained from mice during the first two post-natal weeks and mitral cells were targeted for whole-cell patch recording and filled with neurobiotin and Alexa Fluor 594. Cells were allowed to fill for ~30 minutes and the slices were then fixed, stained using the avidin-biotin peroxidase reaction, and mounted to be traced using Neurolucida (Figure 5). Interestingly, we found that early in development (P0-P4) mitral cell dendrites lacked elaborate tufts seen in their mature counterparts (P9-33). Instead, mitral cells projected multiple dendrites with simple point endings. After P4, these endings began to ramify in well-defined tufts in their respective glomeruli.

To quantify this, we assessed the changes in clustering of dendritic endings from Neurolucida tracings of mitral cells across development. First, we compared the average distributions of distances between end points within the glomerular layer across development.
Figure 3.5 – Mitral Cell Dendritic Morphology Undergoes Dramatic Refinement During the First Four Post-Natal Weeks. A) Neurolucida reconstructions of mitral cells across development (P2-P33). Mitral cells were targeted, patched, and filled with neurobiotin. Slices were fixed post-hoc and stained to be traced and reconstructed using Neurolucida. B-D) Point ending distributions across development. Neurolucida tracings were used and each point-endings within the glomerular layer was identified. The histogram of all the distances between each point ending was calculated and averaged for P2 (B, n=3), P14 (C, n=3), and P30 and older (D, n=3) mitral cells. Scale bars = 50 μm.
Figure 3.6 – Clustering and Elaboration of Mitral Cell Dendrites Into Tufts Occurs During the First Four Post-Natal Weeks. A1-A3) Neurolucida tracing of a P33 mitral cell’s dendrites within the glomerular layer only. Sliding windows (green box) of 5, 25, and 50 μm per side (A1-A3 respectively) were used to calculate the number of points contained within the box as it moved through glomerular layer tracings of mitral cells across development. B) 3D plot of the distribution in space of point endings within the glomerular layer of two mitral cell tracings, P2 (Red) and P33 (Black). C) Density histograms for each of the three sliding windows were calculated for each trace. The average density value at two standard deviations above the mean was calculated for each trace and averaged for each age group (P2-4, P9-14, P30 and older). These averaged values were plotted versus the size of the sliding window for each age group. D) Bar graph illustrating the average density values using a 25 μm sliding window versus age group with significant differences between P2-4 (n=4) and both P9-14 (n=4) and P30 (n=3) and older (two-tailed t-test).
The point endings in young mitral cells (P0-P4) were distributed uniformly in space (Figure 5B). The spatial distribution of point endings became increasingly bimodal with age (P5-P30, Figure 5C, D) indicative of tuft ramification within glomeruli and the first peak of these distributions coincide with the average size of glomeruli in the AOB.

Using sliding windows of 5, 25, and 50 µm (Figure 6A1-A3 respectively), we counted the number of points within each window and compiled histograms of point densities as a function of distance as the window moved across a given mitral cell tracing from three different age groups (P2-4, P9-14, P30 and older). We reasoned that increasing the size of the sliding window would result in a longer tail of the distributions in older animals due to the clustering of their tufts. As expected, the average values across age groups became more distinct as the size of the sliding window was increased (Figure 6C) and was significant for young mitral cells (P2-4) compared to both P9-14 and P30 and older mitral cells using a sliding window of 25 µm (Figure 6D) indicative of the clustering of tufts within glomeruli during development.

### 3.4.3 Adult Connectivity in the AOB is Non-Random

We utilized two separate techniques to examine connectivity in adolescent mice (P12-P20) using the transgenic mouse line in which sensory neurons expressing the V2r1b receptor, also express tau-GFP. This mouse line provides a valuable tool to examine the internal organization principles of the accessory olfactory bulb by enabling us to identify individual glomeruli, which are much less well-defined than those of the MOB (Belluscio et al., 1999), on the basis of labeled axons terminating in round, glomerular like structures (Figure 1). In addition, we could identify glomeruli receiving input from sensory neurons expressing a known receptor gene.

In an initial set of experiments, we utilized a calcium imaging technique to identify and target mitral cells connected to a specific glomerulus for patch-clamp recording to observe and
Figure 3.7 – Targeted Whole-Cell Patch Clamp Recording via Calcium Imaging Reveals Precise Adult Connectivity. A) Schematic illustrating experimental design. Theta-glass stimulating electrode is placed in identified GFP-positive glomerulus in slice loaded with Fura-2AM. Stimulation results in activation as observed via calcium imaging in mitral cell layer allowing for targeted whole-cell patch clamp recording and filling for morphological reconstruction. B1) Fluorescent image of fura-2AM loading in the mitral cell layer of the AOB. B2) Overlay of B1 (green), stimulation induced activity of several mitral cells (blue) and targeted patch-clamp recording and filling (red). B3) Fluorescent image of stimulation electrode in a GFP-positive glomerulus (top), and a targeted patched mitral cell (bottom). B4) Response of mitral cell to stimulation of the GFP-positive glomerulus via the stimulation electrode. C1) Reconstruction of mitral cell post-hoc and its morphology. C2) Confocal image stack of the stimulated GFP-positive glomerulus and the mitral cell innervating it. C3) Confocal image deeper in the slice of a second GFP-positive glomerulus also innervated by the filled mitral cell. Scale bar = 20 μm.
evaluate their morphology (Figure 3.7A). In slices loaded with the calcium indicator Fura-2AM (see methods), electrical stimulation of GFP-positive glomeruli resulted in calcium transients in mitral cells (Figure 3.7B1 & B2), which were then targeted for whole-cell patch clamp (Figure 3.7B3). To confirm cell identity and connectivity with the stimulated glomerulus, we stimulated and recorded the response in patched mitral-cells (Figure 3.7B4). These mitral cells were filled with neurobiotin and Alexa 594 and allowed to fill for ~30 minutes. Slices were then fixed, mounted, and imaged post-hoc to examine dendritic morphology.

To confirm that mitral cells which were targeted for whole-cell patch clamp innervated the stimulated glomerulus, we examined confocal image stacks of fixed slices and reconstructed their morphology in 3-dimensions (Figure 3.7C1). Surprisingly, we found that when we examined the morphology of mitral cells post-hoc via confocal imaging, the dendrites of mitral cells which innervated the stimulated glomerulus (Figure 3.7C2), also appeared to innervated other GFP-positive glomeruli in the slice (Figure 3.7C3). This suggested that mitral cell dendrites were targeting glomeruli receiving input from sensory neurons expressing a specific receptor type.

In order further confirm that mitral cell dendrites were targeting specific glomeruli, we utilized an electroporation technique (Hovis et al., 2010) that enabled us to target and fill mitral cells whose dendrites terminated in a given GFP-positive glomerulus. Slices containing AOB were examined under fluorescence to identify GFP-positive glomeruli. Electrodes filled with a charged Alexa Fluor 594 (AF594) dye were placed in the center of an identified GFP-positive glomerulus. Current pulses effectively drove dye into the electroporated membranes of axons and dendrites within the glomerulus. This typically labeled of 6-10 mitral cell somata and dendrites, the vast majority of which had a tuft in the electroporated GFP-positive glomerulus.
Figure 3.8 – Electroporation Confirms Highly Precise Adult Connectivity in the AOB. A) Slice electroporation of an identified glomerulus innervated by V2r1b-GFP expressing sensory neuron axons labels several downstream mitral cells and their subsequent dendrites with a red fluorescent dye. B) Higher magnification view of A showing the targeting of a single GFP-positive glomerulus revealing the morphology of several labeled mitral cells. C) Higher magnification view of C showing multiple dendrites of labeled mitral cells targeting other GFP-positive glomeruli. D & E) Higher magnification view of C showing two individual GFP-positive glomeruli along with multiple red fluorescent dendrites innervating them. Note size of scale bars.
(Figure 3.8A,B). After electroporation, the neurons were allowed to fill for ~30 minutes and then fixed and mounted for confocal imaging.

To evaluate the targeting of connectivity within the glomerular layer of mitral cell dendrites, the dendritic tufts of electroporated mitral cells were examined. As expected, nearly all electroporated mitral cells innervated the electroporated, GFP-positive glomerulus. Since AOB mitral cells innervate multiple glomeruli, we also examined the tufts that were not in the electroporated glomerulus. We examined more than 20 tufts of mitral cells from multiple slices and found that 21 out of 22 of those tufts terminated in other, GFP-positive glomeruli (Figure 3.8C, D & E). It could not be confirmed whether the remaining tuft terminated in a GFP-positive glomerulus. (Data not shown).

Using two different techniques to examine the morphology of mitral cells innervating GFP-positive glomeruli, we found that all of the cells which clearly innervated either the stimulated or electroporated glomerulus also innervated other GFP-positive glomerulus. Although the possibility that some mitral cells may innervate glomeruli receiving heterogeneous input cannot be rule out, these results taken together, strongly suggest that the majority of mitral cells which target and innervate a glomerulus receiving input from V2r1b-expressing sensory neurons, also project their other dendrites to other glomeruli receiving homogenous input.

3.5 Discussion

3.5.1 Summary of Findings

We have described a dramatic period of growth and organization of the accessory olfactory system during the first two post-natal weeks of life that correspond with previous
results examining the growth of the VNO and ABO (Jia et al., 1997; Roos et al., 1988; Weiler et al., 1999). Early in this development (P0-P4), we show that the axons of sensory neurons undergo a rapid maturation from irregular, poorly-defined structures to well-defined glomerular-like structures. Although we showed that mitral cell dendrites during this period contact these axons and form functional synapses with them, it is only after the formation of well-defined glomeruli that mitral cell dendrites target specific glomeruli containing axons of sensory neurons expressing the same receptor gene to ramify and form elaborate tufts in.

This study suggests that the first few post-natal weeks are a critical time period of development for both the vomeronasal organ and the accessory olfactory bulb. During this epoch, VSN axons and mitral cell dendrites achieve a high degree of specificity in the synaptic connections between them, despite the complex anatomical architecture of the AOB. This anatomical precision reflects an important prerequisite within this system during development and is essential to understanding of how this system is processing chemical information in the adult.

3.5.2 Pitfalls and Limitations

In this chapter, we took advantage of a transgenic mouse line as well as a technique we developed and described in Chapter 2, to examine the development of V2r1b-expressing sensory neurons and the connectivity of mitral cells who received input from them. These tools afforded us the ability to examine the specifics of the development of the vomeronasal system at the level of individual receptor neurons and their respective connections. However, these two advantages also represent limitations for our study.

On one hand, we have described here the first detailed examination of the development of a specific subset of sensory neurons expressing a given receptor both with regard to the expression of this receptor across development, as well as their axonal refinement in the
accessory olfactory bulb. However, we cannot rule out the possibility that the timeline of
development for other sensory neurons expressing different receptor type may be different.
Indeed, previous studies in the main olfactory bulb have shown that glomerular maturation
proceeds along different time courses, even for receptors type which are very similar (Zou et al.,
2004). It certainly may be the case that this is true of other receptors types of the V1R family or
even similar receptors subclasses of the V2R family.

Similarly, our electroporation method enabled the first detailed morphological
examination of multiple mitral cells all receiving input from the same glomerulus. We have
demonstrated in this chapter that mitral cells receiving input from glomeruli innervated by V2r1b-
expressing sensory neurons project subsequent dendrites to other glomeruli receiving
homogenous input. However, the degree to which the wiring logic of mitral cells receiving input
from other receptor types resembles this logic remains unclear. Previous work suggest the logic
may be slightly different with regard to the V1R family of receptors (Wagner et al., 2006). Here
they suggest that mitral cells do not targeted completely homogenous glomeruli with respect to
receptor type, but rather to glomeruli receiving input from sensory neurons expressing different,
but closely related receptor types. Further experiments utilizing our electroporating method with
other transgenic mice for the V1R family of receptors would be required to determine the
answer to this question.

Furthermore, we were also limited in our ability to examine the development of specific
subsets of mitral cell dendritic morphology. Ideally, we would like to examine the refinement of
mitral cell dendritic tufts terminating in GFP-positive glomeruli across development using our
electroporation method. However, there were several anatomical and logistical barriers that
made these experiments intractable. First, as we demonstrate in Figure 3.5, very young mitral
cells lack elaborate tufts innervating a given glomerulus, making it very difficult to determine if
the mitral cell in question is in fact receiving input from the electroporated glomerulus. Second,
as we show in Figure 3.3, glomeruli from very young animals are difficult to identify in order to accurately electroporate due to their dispersed morphology, especially at very young ages (P0-P2). Third, we found that identification of individual glomeruli via fluorescence under the microscope during experiments nearly impossible until much later ages (P11-P12), making these experiments intractable until the very latter stages of mitral cell development. This might be due to the expression levels of receptors at early ages or due to increased axonal arborization and therefore GFP signal at later stages of development.

Finally, although our results strongly suggest that mitral cell connectivity with regard to the V2r1b-receptor type is homotypic, this is a difficult hypothesis to prove, and much easier to disprove. We did not observe any examples of mitral cells whose dendrite clearly innervated a GFP-positive glomerulus also innervate a GFP-negative glomerulus. Yet we cannot say with 100% certainty that this in fact, never happens. We can only claim that for the vast majority of mitral cells innervating glomeruli receiving input from V2r1b-expressing sensory neurons, they desire to innervate other like glomeruli with respect to receptor type.
4.0 Activity Dependent Modulation of Development

4.1 Abstract

The mammalian accessory olfactory system is specialized for the detection of chemicals that identify kin and conspecifics. Vomeronasal sensory neurons (VSNs), residing in the vomeronasal organ, send axonal projections and synapse with principle neurons of the accessory olfactory bulb (AOB), mitral cells, which process incoming sensory information about chemicals in the environment. It is thought that the anatomical organization of this circuit is essential for this information transfer. However, the development of this anatomy, as well as the mechanisms by which this anatomical development is established, remains poorly understood. We show here that the vomeronasal duct is open at birth, exposing sensory neurons to external chemical stimuli, and that these sensory neurons are capable of releasing neurotransmitter to downstream neurons as early as the first post-natal day. Using major histocompatibility complex class I (MHC-1) peptides to activate a selective subset of VSNs during the first few post-natal days of development, we show that increased activity results in exuberant VSN axonal projections and delayed axonal coalescence and refinement into well-defined glomeruli in the AOB. Finally, we show that mitral cell dendritic development occurs just after the refinement of their pre-synaptic partners in order to establish precise connectivity with glomeruli receiving input from sensory neurons expressing the same receptor type. These results suggest that neuronal connectivity within the AOB is highly specified in order to provide a substrate for proper informational processing in this system.
4.2 Introduction

The mammalian accessory olfactory system, comprised mainly of the vomeronasal organ (VNO) and the accessory olfactory bulb (AOB), is a sensory system involved in the identification of chemicals that signal the social and reproductive status of conspecifics (Dulac and Torello, 2003). Individual vomeronasal sensory neurons (VSNs) residing in the VNO, bind chemical stimulants from the external environment and send axonal projections to the AOB which provide direct excitatory input onto principle neurons called mitral cells. These cells are extraordinarily sensitive and selective to socially relevant ligands and mixtures (Luo et al., 2003a), yet in the AOB there are several anatomical challenges towards providing highly specified input onto these cells.

Unlike their counterparts in the main olfactory epithelium where axons expressing a given odorant receptor converge to innervate just one or two glomeruli, axons of vomeronasal sensory neurons expressing a single receptor type or perhaps the same set of receptors, diverge and innervate between 6-30 glomeruli in the AOB. Axons of VSNs that express a given receptor project precisely such that single glomeruli are believed to contain a single type of sensory neuron axon (Rodriguez et al., 1999; Belluscio et al., 1999). Moreover, unlike their counterparts in the MOB, AOB mitral cells have multi-tufted morphology, and receive input from 3-6 glomeruli (Ramon y Cajal S, 1911; Takami and Graziadei, 1990). This unique anatomy is thought to play a critical role in the computations performed by the AOB (Dulac and Torello, 2003), but the mechanisms governing the establishment of this circuit are not well understood.

In the present study, we examined the development of the vomeronasal system and studied the effects of activity on the early development of anatomical and functional connectivity of VSNs to AOB mitral cells. We found that, contrary to previous reports, the
vomeronasal duct connecting the VNO to the external environment is open at birth allowing chemical stimuli to reach the VSNs in very young animals. Moreover, as early as post-natal day zero, VSNs are capable of releasing glutamate to activate downstream mitral cells in the accessory olfactory bulb. These observations indicate that activity may have effects on development; we therefore stimulated the system to explore the role of activity using two major histocompatibility complex (MHC-I) peptides known to activate a subset of vomeronasal sensory neurons (Leinders-Zufall et al., 2009). We found that at least one of these peptides is present in urine from males and females at sufficient concentrations to activate sensory neurons and is capable of inducing immediate early gene expression in downstream neurons in the AOB.

We exposed newborn pups to higher concentrations of these peptides across the first few post-natal days to examine the effect that excess sensory activation had on the development of VSNs. We expected sensory activation to accelerate refinement and coalescence of axonal terminations of VSNs in the AOB. Surprisingly, we found that sensory activation resulted in the opposite, namely, over-exuberant axonal projections and delayed axonal coalescence into well-defined glomeruli during the first four post-natal days. Finally, we report that mitral cell dendritic endings undergo a dramatic period of refinement and clustering just after pre-synaptic coalescence of sensory neuron axons. We show that VSN to mitral cell connectivity is precise, with mitral cell dendrites targeting glomeruli receiving input from sensory neurons expressing the same vomeronasal receptor type. Taken together, these data point to precision and specificity in the development of the AOB, which could provide a stable, specific anatomical structure to facilitate AOB computations.
4.3 Materials and Methods

4.3.1 Slice preparation

Sagittal olfactory bulb slices (280-350 μm thick) were prepared from young transgenic V2r1b mice (Del Punta et al., 2002) post-natal day 0 to 33 as described previously (Urban and Castro, 2005). Mice were anesthetized (0.1% ketamine/0.1% xylazine; ~ 3mg/kg, i.p.) and decapitated. Olfactory bulbs were removed and sectioned on a Leica vibratome (Germany) while submerged in ice-cold oxygenated Ringer’s solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1MgCl₂, 25 glucose, 2 CaCl₂. All procedures were in accordance with the guidelines of Institutional Animal Care and Use Committee of Carnegie Mellon University.

4.3.2 Electrophysiology

Whole-cell voltage recordings were obtained from the somata of identified AOB mitral cells. Slices were superfused with oxygenated Ringer’s solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃, 1.25 NaH₂PO₄, 1MgCl₂, 25 glucose, 2 CaCl₂, warmed to 34-36º C. Whole-cell recordings were established using pipettes (resistances of 2-8 MΩ) filled with a solution containing the following (in mM): 120 potassium gluconate, 2 KCl, 10 HEPES, 10 sodium phosphocreatine, 4 MgATP, and 0.3 Na₃GTP, adjusted to pH 7.3 with KOH. Voltage and current clamp recordings were performed using a MultiClamp 700A amplifier (Molecular Devices, Union City, CA). Data were low pass filtered (4 kHz) and digitized at 10 kHz using an ITC-18 (Instrutech, Mineola, NY) controlled by custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR). In some experiments in which membrane current was recorded, mitral cells were held at -90 mV to facilitate the recording of glutamate-mediated EPSCs. Otherwise, the neurons were maintained at resting membrane potential (-50-60mV).

4.3.3 Urine Collection and Purification
Urine was collected from male and females separately that were 3-9 months old housed in metabolic cages and was stored at -20ºC until use. Urine was then thawed and centrifuged at 500-g for 5 minutes and the supernatant was filtered through 0.2 µm filter paper. Female and Male urine were mixed in a 1:1 ratio for all experiments. Urine was then purified in 12-mL Sep-Pak Discovery DSC-18 Cartridges (Supelco, Bellefonte, PA). Cartridges were primed with 3 ml of methanol followed by washing with 5 ml of di-water with 0.1% TFA before addition of the urine samples onto the column. Urine samples were then washed with 1 ml of 2% NaCl in TFA. Finally, the peptide was eluted using consecutive additions of 1 ml of 75% Acetonitrile/ 25% Water with 0.1% TFA, (5X) with each fraction collected in conical tubes. In order to determine which fractions contained the peptide of interest, pure urine samples were spiked with derivitized pure peptide to observe, via fluorescence, which fractions contained the peptide.

4.3.4 Peptide Stock Concentration Determination and Derivitization

Stock concentrations of pure peptide (Genscripts) were made by dissolving ~5mg of peptide in H₂O. Protein concentration was determined spectrophotometrically using a Thermo Scientific NanoDrop ND-1000 spectrophotometer (Wilmington, DE) assuming a molar extinction coefficient of 1280 cm⁻¹ M⁻¹ (λ = 280 nm) using the Beer-Lambert Law (A = εlc). The peptide was derivitized by adding 10 µl of dansylchloride solution (2.5 mg/ml in acetonitrile) followed by incubation at 37º for 30 minutes.

4.3.5 Lyophilization and Reversed Phase Liquid Chromatography

Fractions containing the peptide of interest were frozen at -80ºC and lyophilized using a Labconco Freezone 4.5 freeze dry system (Kansas City, MO), and the resulting solid was reconstituted in 100 µl of water with 0.1% acetic acid. Each sample was then run through High Performance Liquid Chromatography (HPLC) on an Alliance Waters 2695 separation module (Milford, MA) using a C18 Symmetry 300 Column (3.9 mm x 150 mm, 5 µM beads). Gradient
elution was carried out at a flowrate of 1.0 ml/min with a mobile phase from 0 to 50% of 0.1% TFA in Acetonitrile vs. 0.1% TFA in H₂O in 20 minutes. Fluorescence of the eluate was monitored with a Waters 996 Photodiode Array Detector using an emission wavelength of 220 nm. Fractions were collected about a minute and a half before and after the peak. The resulting fractions were lyophilized again and reconstituted in 50 µl of 0.1% acetic acid in H₂O for LC-MS.

4.3.6 LC-ESI-MS

Peptide quantification was performed by running the samples on a Michrom BioResources, Magic 2002 HPLC through a Magic MS C18 column (1.0 mm x 150 mm, 5 µM beads) using a gradient elution with a flow rate of 75 µl/min from 0 to 40% acetic acid in acetonitrile vs. 0.1% acetic acid in H₂O in 40 minutes. For ESI-MS spectra, MS/MS was performed using a Thermo-Fisher LCQ ESI/APCI Ion Trap mass spectrometer for the parent mass of 553.7 m/z, with an isolation width of 3.0 and collision energy of 31%. Chromatograms were collected for each sample as it came off the column. Chromatogram peaks filtered to peptide fragment mass ranges and were averaged and smoothed using a 7-point Gaussian filter, then integrated using Thermo XCalibur Qual Browser software and plotted vs. concentration to form a calibration curve. A linear regression of this curve was fitted and extrapolated to determine peptide concentration in pure urine samples. Both spiked urine samples and pure urine samples were prepared, purified, and quantified the exact same way.

4.3.7 VNO and AOB Tissue Collection and Immunohistochemistry

Animals at all ages were perfused first with buffer (2 minutes) then with 4% PFA (2 minutes), and their brains were dissected and fixed overnight before being transferred to a 30% sucrose solution. The heads were also fixed overnight in 4% PFA before being transferred to 0.1M phosphate buffer (pH = 7.2). Each brain was sectioned at 75 µm using a Leica cryostat SM2000R (Germany) and the sections placed in 1 ml of PB solution. The heads were mounted
against a 4% agarose block and sectioned at 200 µm using a vibratome and the sections were also transferred to 24 well plates containing 1 ml of PB solution. For both VNO and brain sections, they were then washed 3X with PB incubating for 5 minutes each wash. The solution was then aspirated and replaces with the primary antibody (300 µl of 1:1000 GFP Mouse antibody, Invitrogen) in PB with 2% normal donkey serum and 0.05% Tween 20. After washing 3X again in PB, the solution was aspirated and replaced with the secondary antibody (300 µl of 1:1000 donkey antibody mouse conjugated 488, Invitrogen) with 2% normal donkey serum and 0.05% Tween 20 and incubated for 1 hr in the dark and finally washed 3X in PB. The sections were then mounted in gelvatol for confocal imaging and analysis.

4.3.8 VSN Axonal Coalescence Imaging and Analysis

Confocal image stacks were acquired using a Zeiss LSM 610 microscope using a 40X oil objective. Image stacks were median filtered using ImageJ software with a Gaussian width of 2.0 pixels. For analysis of axonal arborization, maximum intensity z-projections of each stack were imported into custom written software in Igor Pro. Individual glomeruli were analyzed by placing the coordinates of a semi-circle such that the arc of the semi-circle was oriented ventral to the section with the straight-edge cutting off the axons just above the glomerulus. This ensured that each concentric semi-circle expanded throughout the axonal terminations in the glomerulus while excluding the axons superficial to the glomerulus. Sholl analysis was performed such that the final concentric semi-circle encompassed the entire glomerulus. Histogram plots of the number of pixels crossing threshold as a function of distance were calculated and the width of the peak of each histogram plot was measured at half of the maximum amplitude. In cases where it was difficult to determine a single glomerulus vs. multiple glomeruli, consecutive sections were examined for evidence of a second glomerulus.
4.4 Results

4.4.1 The Vomeronasal System is Functional at Birth

There has been some debate over whether or not the mouse vomeronasal system is functional at birth and therefore plays some role in the behavior of nursing pups. Previous work has shown that in E18 and E19 mouse fetuses, the duct that connects the vomeronasal organ to external sources of stimuli is not patent (Coppola and O’Connell, 1989). Further work showed that even on the first day after birth (P0), the duct primordium remains immature, suggesting that it could not transmit odorants from the nasal cavity (Coppola et al., 1993). However, to our knowledge, a direct test of whether or not the VNO lumen has access to external stimuli just after birth has not been performed.

To determine whether or not the vomeronasal duct is open at birth, thereby allowing for access of external sensory stimuli, we exposed very young pups (P0) to a solution containing Alexa Fluor 594 hydrazide dye. We utilized a transgenic mouse line whose sensory neurons which express the V2r1b receptor, also express tau-GFP, enabling us to visualize these sensory neurons via fluorescence (Del Punta et al., 2002). Using a paintbrush, we painted only one nostril with a solution containing the Alexa dye (Figure 4.1A) three times over the course of a half an hour and then sacrificed the animal an hour later. We then fixed and sectioned the face, and imaged these sections to examine for evidence of dye in the VNO. As expected, we observed bright red fluorescence throughout the main olfactory epithelium that lines the nasal cavity, but only on the side corresponding to nostril exposed to the Alexa (Figure 4.1B). However, we also observed fluorescence in the VNO lumen indicating that the dye had gained access to the VNO. We also observed a small number of VSNs that had taken up the dye and thus were fluorescent. These cells were morphologically similar to and located in the same regions of the VNO as the GFP-expressing sensory neurons (Figure 4.1C & E). We did not observe this
Figure 4.1 - The Vomeronasal Duct Is Open At Birth. A) Schematic of the experimental design. Alexa Fluor 594 Hydrazide dye was painted onto one of the nostrils of a P0 mouse exposing the sensory neurons of one-half of the VNO to the dye for 1 hour. B) Confocal image of a coronal section of the face of an exposed animal illustrating the presence of dye contacting not only the epithelium of the nasal cavity (upper arrow), but also the lumen of the VNO (lower arrow). C) Higher magnification view of B. D) Higher magnification view of the control VNO where only native GFP-expression can be seen in the VSNs. E) Higher magnification view from C showing the exposed VNO which not only contains native GFP-expressing sensory neurons (upper arrow) but some sensory neurons which have been exposed to and taken up some dye (lower arrow). Scale bar = 100 μm.
fluorescence on the side of the animal in which the nostril had not been directly painted and exposed to the dye (Figure 4.1D, n=3 animals).

This result suggested that the duct leading to the VNO is indeed open enough at birth allowing for access to external stimuli as early as post-natal day zero. However, this does not necessarily mean that these sensory neurons are capable of signaling to downstream neurons in the accessory olfactory bulb. In order to determine whether VSN to mitral cell synapses were functional at the early post-natal stage, we performed whole-cell voltage clamp recordings from mitral cells in the accessory olfactory bulb. Axons of VSNs terminate and synapse onto mitral cell dendritic tufts providing the only known source of excitatory input onto these output neurons (Brennan and Keverne, 1997a). We recorded miniature synaptic currents in the presence of gabazine (10 µM) while holding at -90 mV to increase our ability to detect small excitatory events.

We observed mEPSCs in mitral cells from animals as young as post-natal day zero, indicating that the synaptic circuits between the vomeronasal sensory neurons and their post-synaptic mitral cells were indeed functional at birth (Figure 4.2A). In addition, we found that the properties of these synaptic events at P0 were nearly identical to inputs seen at post-natal day twelve (Figure 4.2A & B). We calculated the average amplitude of these events as well as their decay time across cells at three different age groups and found no significant difference between either the amplitude or decay time of events across age groups (Figure 4.2C & D). Taken together, our results suggest that not only is the vomeronasal system open to external stimuli at birth, but that this system is also online at birth and capable of sending signals about the periphery to the brain via synaptic connections with mitral cells in the accessory olfactory bulb. We next undertook experiments to determine whether a peptide ligand for an identified class of VSNs may act as an environmental cue to regulate development of VSN projections to the AOB.
Figure 4.2 - Immature Mitral Cells Form Functional Synapses at or Prior to Birth. A) Example recording of mEPSCs from a P0 mitral cell. B) Example recording from a P12 mitral cell. All cells were held at -90mV to amplify events and events were recorded in the presence of 10 μM gabazine. C) Distribution of the average amplitudes of events at three different age groups (P0-1, n=5, 13.7 pA +/− 1.52; P5-6, n=5, 15.8 pA +/− 3.23; P11-12, n=6, 14.2 pA +/− 2.20). Each value was the average amplitude for a given cell from a minimum of one and a half minutes of data. D) The average decay time for all the events of each cell at each of the three different age groups (P0-1, n=5, 6.4 msec +/− 0.92; P5-6, n=4, 8.1 msec +/− 0.75; P11-12, n=6, 6.9 msec +/− 0.43).
4.4.2 MHC-I Peptides Are Present in Urine and Induce c-Fos Activation in Downstream Neurons

The first two post-natal weeks of a rodent’s life represent a dramatic period of growth and development in both the VNO and the AOB (Roos et al., 1988; Weiler et al., 1999; Jia et al., 1997). Given that the vomeronasal system is able to receive chemical stimuli and that VSNs are capable of activating AOB mitral cells at very young ages, we wondered what role, if any, activity might play in the development of this system. We wanted to manipulate activity in a specific way, namely by activating GFP-labeled sensory neurons expressing the V2r1b receptor. This would allow us to visualize the effect of activity on these sensory neurons and their axonal projections to the accessory olfactory bulb via the transgenic mouse line.

Previous work has shown that peptide ligands of major histocompatibility complex class I (MHC-I) molecules function as olfactory cues for subsets of vomeronasal sensory neurons (Leinders-Zufall et al., 2004; He et al., 2008). Specifically, two MHC peptides in particular have been shown to evoke calcium influx in vomeronasal sensory neurons which express the V2r1b receptor, namely the peptides SYFPEITHI and AAPDNRETF (Leinders-Zufall et al., 2009). V2r1b-expressing sensory neurons have been shown to be extremely sensitive to both of these MHC-I peptides, with EC\textsubscript{50} values of \(\sim3\times10^{-14}\) M and \(\sim8\times10^{-13}\) M respectively. For these peptides to play a role in regulating development, they would need to be present in the animals environment. Thus, we tested if either of these peptides is present in mouse urine at sufficient levels to activate these sensory neurons under normal conditions.

Prior to running the urine samples, we ran samples containing known concentration of stock pure peptide (Figure 4.3A) to determine the retention time of the peptide of interest. Combining urine from both males and females, we first purified 500 µl samples by running them through DSC-18 cartridges (Figure 4.3B) prior to running it on a column using reversed-phase.
Figure 4.3 - Identification and Concentration Determination of MHC-I Peptide in Urine. A) Peptide stock concentrations were determined spectrophotometrically using the Beer-Lambert Law. B) Urine samples and pure peptides samples were each purified in 12-ml Sep-Pak DSC-18 cartridges. C) Fractions containing the peptide of interest were combined and lyophilized and the resulting solid was reconstituted. D) Afterwards, each samples was then run through HPLC collecting fractions surrounding the peak retention time. E) These samples were lyophilized again and reconstituted before being run through mass spec. F) Samples were run through LC-ESI-MS and chromatograms were collected for each sample, filtered and integrated to determine peptide quantities.
liquid chromatography. We then lyophilized the samples (Figure 4.3C) and ran the samples through HPLC and collected the fractions surrounding the retention time observed from previous runs of spiked samples (Figure 4.3D). After purifying and collecting the peptide of interest from HPLC, the samples were then lyophilized and reconstituted (Figure 4.3E) for analysis using electrospray ionization mass spectrometry in order to isolate and observe the peptide of interest (Figure 4.3F & Figure 4.4A). We then quantified the amount of the peptide present in each sample by integrating the chromatogram peak filtered to the mass ranges of the fragments of the peptide (Figure 4.4B). We compared this value to a calculated calibration curve of 500 µl samples of urine spiked with a known concentration of pure stock peptide, running them in triplicate and all through the same purification and fractionation procedures to calculate the average native peptide concentration in pure urine samples (Figure 4.4C). We found the native concentration to be ~5 nM, far greater than the picomolar sensitivity of these receptors, suggesting that upon exposure to urine, animals are presented with sufficient concentrations of this peptide to activate V2r1b-expressing sensory neurons.

Although previous work in acute slice has shown the activation of V2r1b-expressing sensory neurons by these two MHC-I peptides (Leinders-Zufall et al., 2009), it remained unclear whether these peptides would be able to induce activity downstream of these sensory neurons themselves in vivo. In an initial set of experiments, we applied these peptides to determine whether they would activate AOB mitral cells. Following previous work that has demonstrated that activation of AOB mitral cells results in expression of the immediate early gene c-Fos (Guo et al., 1997; Inamura et al., 1999; Yamaguchi et al., 2000), we tested whether application of a solution containing a mixture of the two MHC-I peptides at 250 µM to the naris of P15-18 old animals (3 applications using a paint brush over the course of 30 minutes, 1 hour before sacrificing), increased expression of c-Fos (Figure 4.5A).
Figure 4.4 - MHC-I Peptide is Present in Urine at Sufficient Concentrations to Activate Sensory Neurons. A) Examples MS spectrum of using electrospray injection MS/MS at the 553.7 m/z peak. Collision energy was used to fragment the peptide for identification (see arrows). B) Example chromatogram from A (250 nM), which was filtered to peptide fragment mass ranges and then integrated. C) Calibration curve created from the integration of chromatogram peaks across a range of spiked urine concentrations (50 nM, n=3, 5,318,503 counts +/- 770,200; 125 nM, n=3, 14,155,185 counts +/- 750,388; 250 nM, n=3, 24,637,306 counts +/- 1,716,317). After performing a linear regression, the line equation was then used to calculate the average native concentration of peptide in pure urine samples (red dot).
We observed c-Fos expression in the expected (caudal) region of the AOB in both water exposed control animals as well as peptide exposed animals and in all layers of the bulb, including the glomerular layer, the mitral cell layer, and the granule cell layer (Figure 4.5B & C). However, we found that on average, there was a two-fold increase in the number of mitral cells expressing the immediate early gene c-Fos after exposure to the MHC-I peptides compared to those painted with water (Figure 4.5D). These results confirmed that exposure to the mixture of peptides elicited an increase in the activity of vomeronasal sensory neurons and, in turn, the activity of downstream mitral cells. Given that we and other observe vomeronasal receptor expression in very young animals (P0-4) (Jia et al., 1997), it seems likely that their sensitivity is similar in young animals making these peptides an ideal substrate for examining the effect sensory activity plays in the development of this system.

### 4.4.3 Sensory Activity Affects Axonal Targeting but not Receptor Expression

In order to examine the effect that prolonged activity had on the development of this system, we took newborn litters of pups and separated the litter into two halves, one half with the birth mother, the second half with a surrogate mother who had had a litter within the past two days. In the experimental group, pups were exposed three times a day to the mixture of the two peptides (250 µm) by painting the noses of each pup as well as the mothers face and underbelly with the solution containing the two peptides. The control group was exposed in the same way, only using water as the solution. After each 24 hour period, a pup from each group was perfused, and the brain was dissected out and sectioned and stained with antibodies against GFP, with the average experiment lasting 5 days (litter of 10 pups). The head (containing the vomeronasal organ) was also preserved, sectioned, and stained with antibodies against GFP.
Figure 4.5 - MHC-I Peptides Induce c-Fos Activation in Downstream Neurons in the AOB. A) Schematic illustrating the exposure of the VNO to either MHC-I peptides or water control to examine the activation of c-Fos in downstream neurons in the AOB. B) Confocal image of a sagittal section containing AOB from a control, water exposed animal (Green=GFP, Red=c-Fos, GL=Glomerular Layer, MCL=Mitral Cell Layer, GCL=Granule Cell Layer). C) Confocal image of a section containing AOB from a peptide exposed animal. D) Normalized c-Fos expression in the mitral cell layer across animals of water exposed control animals (n=5, 191.6 cells +/- 21.6) compared to peptide exposed animals (n=6, 387.8 cells +/- 29.8, p=0.0006). Scale bar = 50 μm.
We manually traced each section in order to reconstruct and calculate the entire volume of the VNO at different ages (Figure 4.6A), as well as to visualize and count the number of V2r1b-expressing sensory neurons in each section (Figure 4.6B). Although we did see a greater number of V2r1b-expressing sensory neurons in animals which had been exposed to the peptides, specifically at P1 and P2 (Figure 4.6C), this effect was not significant (p = 0.55, n=3 and 0.25, n=4 respectively). In addition, when we calculated the total volume of each VNO and compared them between conditions at each age, we saw no change in the volumes of the VNOs (Figure 4.6D). These results suggest that activity does not change the number of VSNs that express V2r1b or result in an increase in vomeronasal organ size.

Previous work in our lab has shown that the first four post-natal days represent a dramatic period of refinement and coalescence of axonal arborizations terminating in the accessory olfactory bulb (See Chapter 3). We wondered if increased activity would accelerate this refinement across development as seen through the V2r1b-GFP axons terminating in glomeruli in the AOB. In order to examine axonal terminations in the accessory olfactory bulb, we examined sections which had been sectioned and stained for GFP. Confocal image stacks of glomeruli from across animals under each condition were taken at different ages to observe axonal coalescence during the first few post-natal days of the animal’s life.

For each animal, three consecutive sections were imaged, beginning with the first section containing substantial GFP-positive axons terminating in the AOB. Image stacks were then imported to ImageJ and median filtered before creating z-projections of each section and then compared across the first four days of development (Figure 4.7A-D). Histograms displaying two separable peaks were analyzed as two individual glomeruli or if a second glomerulus was identified in a consecutive section. Surprisingly, activity did not appear to accelerate axonal refinement but instead seemed to result in increased axonal exuberance, and the retardation of glomerular coalescence.
Figure 4.6 - Activation of V2r1b-expressing Sensory Neurons Does Not Significantly Increase Number of V2r1b-expressing VSNs. A) Point by point reconstruction of a complete VNO traced using Neurou lucida and reconstructed using Igor Pro Software. B) Confocal image stack of a 200 μm section of VNO from a transgenic animal whose V2r1b-expressing sensory neurons also express tau-GFP. Scale bar = 20 μM. C) The number of V2r1b-expressing sensory neurons across development in water exposed control animals (black bars; P1, n=3, 86 cells +/- 4.2; P2, n=4, 116.8 cells +/- 8.3; P3, n=3, 134.6 cells +/- 1.6) and peptide exposed animals (red bars; P1, n=3, 94.3 cells +/- 12.2, p=0.55; P2, n=4, 131 cells +/- 7.5, p=0.25; P3, n=5, 131 cells +/- 3.65; P4-5, n=3, 134.5 cells +/- 8.8). D) Comparison of the volume of the VNO’s across ages in control (P1, n=3, 49,375,000 μm³ +/- 1,380,063; P2, n=4, 65,050,000 μm³ +/- 9,058,528; P3, n=4, 59,870,700 μm³ +/- 2,946,470) and peptide exposed animals (P1, n=3, 56,979,833 μm³ +/- 4,453,998; P2, n=4, 60,502,225 μm³ +/- 4,029,919; P3, n=5, 60,960,420 μm³ +/- 2,491,808).
Figure 4.7 - The Effect of Sensory Activity on Axonal Arbors in the AOB Across Development. A) Confocal image stacks of glomeruli at post-natal day one from water exposed (top row) and peptide exposed (bottom row) animals. 
B) Image stack examples from post-natal day two control and exposed animals. C) Image stack examples from post-natal day three animals. D) Image stack examples from post-natal day four animals. Scale bar = 50 μm.
In order to quantify this effect, we performed a modified Sholl analysis on each glomerulus excluding the incoming axons (see methods). We calculated the histograms of the number of pixels over threshold for increasingly expanding rings across conditions and ages (Figure 4.8A&B). We found that on average, the distributions from exposed animals were broader and more diffuse compared to their water exposed counterparts (Figure 4.8C). We measured the width of these distributions at half of their maximum count and found a significant difference in these distributions throughout the first three post-natal days (Figure 6D, p = 0.0029, p = 0.0076, p = 0.001 respectively). These results suggest that strong activation of these sensory neurons does affect the normal developmental targeting of VSN axons to their glomerular targets in the AOB. Specifically, axonal arbors are over-exhuberant and less precise in their targeting, and their refinement is delayed.

4.1 Discussion

4.5.1 Summary of Findings

We have described the neonatal vomeronasal system in mice in which vomeronasal sensory neurons at birth are exposed to external stimuli and capable of neurotransmitter release on post-synaptic targets in the brain. In addition, we demonstrated that at least one MHC-I peptide was present in urine at concentrations more than 1,000-fold above what is likely to activate specific vomeronasal receptors. Moreover, application of these peptides to the nose was able to activate immediate early gene expression in AOB mitral cells. Utilizing these peptides as a means of increasing sensory activity in a subset of vomeronasal sensory neurons, we showed that activity did not significantly affect V2r1b-expressing sensory neuron number or volume of the VNO, but did significantly alter the targeting and refinement of axons from these neurons in the AOB.
Figure 4.8 - Activity Significantly Affects Axonal Exuberation and Coalescence in the AOB. A) Example confocal image stacks of glomeruli across three ages in water exposed control animals. B) Example confocal image stacks of glomeruli from peptide exposed animals. C) Histogram distributions using Sholl analysis for the two example glomeruli above each histogram (black=control animal, red=exposed animal). D) Quantification of the half peak width from each histogram across animals at three different ages comparing water controls (Black squares; P1, n=5, 15.2 μm +/- 1.2; P2, n=9, 13.9 μm +/- 1.4; P3, n=9, 12.8 μm +/- 0.7) with exposed animals (Red triangles; P1, n=6, 23.6 μm +/- 1.7, p=0.0029; P2, n=8, 21.7 μm +/- 2.1, p=0.0076; P3, n=9, 23.6 μm +/- 1.1, p=0.001). Scale bar = 20 μm.
This study suggests that the first two post-natal weeks are a critical time period of development and growth consistent with previous reports for both the growth of the vomeronasal organ (Weiler et al., 1999) and the accessory olfactory bulb (Roos et al., 1988). During this epoch, VSN axons and mitral cell dendrites achieve a high degree of specificity in the synaptic connections between them, despite the complex anatomical architecture of the AOB. This anatomical precision reflects an important prerequisite within this system and is essential to understanding of how this system is processing chemical information.

4.5.2 Pitfalls and Limitations

In this chapter, we provide the first direct evidence to suggest that the VNO, contrary to previous reports, is open enough at birth to allow it access to external stimuli. In addition, we show that very young VSNs are capable of releasing neurotransmitter that is seen even in the presence of a GABA\textsubscript{A} antagonist. Taken together, this data suggests that the vomeronasal system is indeed functional at birth. However, we do not directly test this hypothesis. Although, we show that all of prerequisites for a functional circuit are in place at birth, this does not necessary mean that the system is in fact binding to pheromones and responding by activity in downstream areas. Nevertheless, we cannot rule out the possibility that young VNS is not signaling pheromonal information to downstream brain areas. In order to test this hypothesis, it would require an in vivo or ex vivo prep enabling whole-cell or extracellular recordings from downstream areas responding to application pheromones to the VNO just after birth.

Likewise, we show in Figure 4.5 that two MHC-peptides are capable of eliciting expression of the immediate early gene c-Fos in downstream neurons in the accessory olfactory bulb at post-natal day 15-18. However, we do not demonstrate this effect in very young animals. The assumption that this circuit is functional and responding to these two MHC-peptides at birth seems reasonable given that we see V2r1b-receptor expression at birth (See Chapter 3) along
with our results revealing an effect of two MHC-peptides on axonal coalescence in the AOB. However, we do not directly test this hypothesis by exploring c-Fos activation in response to sensory activity in very young animals. Although there have been no reports examining c-Fos expression in very young animals in the accessory olfactory bulb, in the main olfactory bulb mitral cells at birth have been shown to express c-Fos in response to exposure of a novel odor (Allingham et al., 1999) or decrease expression as a result of naris occlusion (Klintsova et al., 1995). We are currently performing experiments examining c-Fos expression in very young animals (P2/P3) but have not collected this data as to include it in this manuscript.

One of the most intriguing results present in this Chapter is the striking effect that excess sensory activity had on axonal refinement in the AOB, resulting in delayed pruning and coalescence into well-defined glomeruli compared to control animals. One potential pitfall in our experimental design was the concentration of peptide used in our exposure experiments. We used a solution containing a mixture of each peptide at a concentration of 250 µM. This is more than 1,000-fold higher than the concentration we measured in urine samples and 10,000-fold greater than 100,000-fold higher than what these neurons are capable of detecting. As a result, this raises the possibility that the effect we describe here is an artifact an overly strong sensory stimulus.

There are several reasons we do not think this is the case. First of all, exposures only consisted of a few seconds of exposure per animals three times a day. This means that each individual animal only received a few minutes a day of direct exposure to this concentration. Subsequent exposure, due to residual peptide on other pups faces or the mother’s belly would be markedly diluted. Second, although our data suggests that the vomeronasal duct is open enough at birth to allow access to stimuli (Figure 4.1), we also observed a much larger number of sensory neurons labeled in older animals (P3-P5) compared to P0 (data not shown). These results suggest to us that the duct, though open at birth, is not as patent as older animals (P5).
This hypothesis is further supported by previous work showing that the VNO duct at birth is more constricted and lined with cellular debris compared to P5 animals (Coppola et al., 1993). These results suggest that sensory neurons may not have complete and unrestricted access to sensory stimuli at birth, requiring that the sensory stimulus be strong in order to activate the vast majority of sensory neurons expressing the V2r1b-receptor.

Likewise, our study does not examine the length of the observed effect on VSN axonal refinement in the AOB or the possibility that certain distinct sensitive periods may exist. In the main olfactory bulb, sensory deprivation via naris occlusion has been shown influence glomerular maturation during sensitive periods of different lengths, even for closely related receptor types (Zou et al., 2004). This would require an incremental experimental design such that sensory exposure would not start until later times point for some animals and would be an interesting question to examine for this effect.

Finally, our results do not prove that activity is necessary for glomerular development in the vomeronasal system. Our results only suggest that excess sensory activity is sufficient to effect glomerular formation on the AOB. In order to test this hypothesis, ideally one would need a means of selectively depriving a subset of neurons expressing a given receptor type. This would require a functional mutation of V2r1b-expressing sensory neurons in our case, which at this point is not available. Alternatively, one could completely remove all sensory activity through naris occlusion to observe the effect this has on axonal maturation on the AOB. Although these experiments would certain lends insights into the role of sensory activity in this developmental process, they suffer from alternative interpretations due to the extent of the manipulation.
5.0 General Summary and Discussion

5.1 Relevance and Summary of Findings

Early investigations of the functional circuitry and development of the vomeronasal system were limited by the inability to visualize individual sensory neurons expressing a specific receptor or the morphology of mitral cells that synapsed specifically with them. With the discovery of the receptor genes expressed by VSNs (Dulac and Axel, 1995), the door was opened for the use of genetic manipulations to visualize sensory neurons expressing a given receptor type via the expression of fluorescent marker proteins. We have taken advantage of these advances in the body of work presented here by utilizing a transgenic mouse-line in which sensory neurons expressing the V2r1b-receptor also expressed the fluorescent protein GFP (Del Punta et al., 2002). This provided us with ability to visualize the cell bodies, dendrites, and axons of a specific subset of sensory neurons.

Combining the use of these transgenic animals with the development of a method for selectively labeling local neurons and their processes by localized electroporation as described in Chapter 2, we selectively labeled mitral cells which received homogenous inputs as described in Chapter 3. Previous methods seeking to examine mitral cell connectivity to specific glomeruli were limited to imprecise lipophilic tracers (Del Punta et al., 2002) or the very inefficient
approach of randomly patching and filling neurons and then tracing their connections (Wagner et al., 2006). Our method overcomes the problems associated with these techniques barriers by locally electroporating dendrites contained within a given glomerulus resulting in the complete labeling of multiple live mitral cells innervating a specific glomerulus. In this way, our results describe the first morphological examination of multiple mitral cells connected to a given glomerulus across different animals (Figure 3.8). Furthermore, our results lend strong support for the hypothesis that, at least for mitral cells receiving input from V2r1b-expressing sensory neurons, mitral cells target their dendritic tufts throughout development in such a way as to receive homogenous input. These results suggest that vomeronasal circuitry, at some level, resembles that of MOB circuitry through the convergence of receptor-specific inputs onto a small number of output neurons in the brain. The difference is that this specificity appears to be achieved at the level of individual glomeruli in the MOB, rather than at the level of second order neurons (mitral cells) in the AOB.

Another advantage this mouse line afforded us was the ability to visualize and count sensory neuron cell bodies as well as observe their axonal projections to glomeruli in the AOB. This provided us with a tool to examine the development of sensory neurons in the VNO across development, as well as the process of glomerular formation in the accessory olfactory bulb. We found that both the volume of the vomeronasal organ, as well as the number of V2r1b-expressing sensory neurons, grew substantially during the first month of life, especially during the first two post-natal weeks (Figure 3.2). Previous studies in the rat and opossum have found similar results revealing that receptor expression begins early in development (P0-P3) (Jia et al., 1997) and that the largest change in VNO volume and sensory neuron number occurs during the first three post-natal weeks (Weiler et al., 1999).

We observed a similar timeline for development in the accessory olfactory bulb, with the first four post-natal weeks displaying continual and rapid growth of both the glomerular layer and
the overall volume of the AOB. We found that the first post-natal week represented the largest overall change (Figure 3.1), similar to previous reports in the rat (Roos et al., 1988). However, very little data exists examining the formation of glomeruli across development in the AOB. Our data indicate that the first four post-natal days correspond to the critical period of axonal coalescence into well-defined glomeruli (Figure 3.3) which is very similar to what is seen reports describing the structure and emergence of specific glomeruli in the main olfactory system (Potter et al., 2001).

By what mechanism can the development of this circuitry be altered? A number of factors govern the development of neuronal circuits during early development including activity and molecular mechanisms. Thus, one of the most intriguing hypotheses would be an activity dependent mechanism by which early sensory activity regulates proper connectivity between the sensory periphery in the VNO and the brain. One of the best ways to test this hypothesis is by selectively activating a sub-set of sensory neurons while having a way to visualize the effect on axonal coalescence and pruning in the AOB. However, we needed a ligand which was known to activate V2r1b-receptors and ideally one which is present in the animal's environment at concentrations capable of activating these sensory neurons.

Recently, Zufall and colleagues identified a set of MHC-peptides ligands which were detected at subpicomolar concentrations by vomeronasal sensory neurons expressing the V2r1b-receptor (Leinders-Zufall et al., 2009). In addition, they determine that V2r1b-expressing sensory neurons responded to either the peptides AAPDNRET (35%) or SYFPEITHI (55%) or to both peptides (8%). These results taken together provided a quantitative foundation for understanding the coding and recognition of MHC-peptides by V2r1b-expressing sensory neurons. However, it remained unclear at what concentrations these peptides were present at in mouse urine, if they were even present at all.
Here we have described the first report of the native concentration of a mammalian pheromone secreted in the animal’s environment. In other sensory systems, our understandings of the natural stimuli that serve to induce peripheral transduction are essential to understanding perception and sensory neural activity. For example, in the rat somatosensory system, rats use their whiskers to determine and compare the roughness or texture of surfaces (Guic-Robles et al., 1989; Brecht et al., 1997). Different surface textures mechanically induce different whisker vibrations in turn producing a “kinetic signature” encoded in neuronal activity and ultimately leading to texture discrimination (Arabzadeh et al., 2004). Consequently, efforts have been made to understand the nature of these external stimuli using high-speed videography to determine how different textures affect the biomechanics of vibrissa (such as vibrissa resonance) to drive sensory representations (Ritt et al., 2008).

In the vomeronasal system, it is known that small molecules ligands act as sensory signals to induce peripheral transduction at the levels of individual receptors and that these detectors are extremely sensitive and highly specific (See Chapter 1). Although several of these ligands and their respective receptors have been identified (Boschat et al., 2002; Liberles et al., 2009; Haga et al., 2010; Leinders-Zufall et al., 2009), very little is known about the native concentrations of these sensory signals that animals must detect and are exposed to under natural conditions. The lack of this fundamental information represents a void in our understanding of the nature of the native environmental cues detected by this sensory system.

By analyzing samples of urine combined from both male and female mice, we determined the concentration of the SYFPEITHI peptide to be approximately 5 nM (Figure 4.4). Although this concentration may seem extremely low, considering the subpicomolar sensitivity of these neurons to this peptides (Leinders-Zufall et al., 2009), this value is more than 1,000-fold higher than what is required to activate these neurons. It is interesting to speculate that these peptides may act as a conspecific marker to confer identity; however further work would be
needed to determine the concentrations of this peptide in other strains/species. Similarly, our experimental design provides no way of examining gender differences in the secretion of these peptides, and it certainly could be the case that this peptide is secreted differentially between males and females, or across development, which is masked by our experimental design.

The peptide concentration we report suggests that very young mice are exposed to concentrations of these MHC-peptides capable of activating sensory neurons expressing the V2r1b-receptor. In addition, this dissertation provides the first evidence to support the hypothesis that the duct connecting the vomeronasal organ to outside stimuli is open and exposed to external sensory stimuli at birth (Figure 4.1). This data, taken together with our results that show that very young sensory neurons are capable of releasing neurotransmitter to downstream mitral cells (Figure 4.2), raises the possibility that sensory-driven activity could help to regulate the development described in Chapter 3. In the end, the advantage of our study was that we were enabled to examine the development of specific neurons in the VNO as well as the effect that selective activation of these neurons had on targeting and pruning during development.

We found that excess sensory activity, despite having no effect on VNO volume or the number of sensory neurons expressing the V2r1b-receptor (Figure 4.6), did have a significant effect on the degree to which of VSN axonal arbors coalesced into well-define glomeruli during the first few post-natal days (Figure 4.7 & 4.8). Although excess sensory activity did not appear to alter gross targeting of axons to glomeruli, it did result in over-exuberant axonal branching in the AOB as well as delayed refinement compared to control animals. This is the first report providing evidence of any role of activity in glomerular development in the vomeronasal system. Specifically we show first evidence for the existence of a critical period for VSN axonal refinement.
5.2 In Vitro Electroporation Technique

5.2.1 Current Electroporation Techniques

In Chapter 2, we described a novel method for the labeling of specific microcircuits *in vitro* with various dyes and demonstrated its usefulness in a variety of brain areas. Despite being designed to overcome technical barriers we faced in labeling specific neurons in the accessory olfactory bulb circuit, we believe that this technique is broadly useful in visualizing neurons in any brain area by labeling them with many different fluorescent molecules, including calcium indicators. In addition, this technique provides several advantages to previous electroporation and electrophysiological methods.

Electroporation is a technique that is widely used both *in vitro* and *in vivo* for rapid delivery of large macromolecules such as DNA and siRNA (Chu et al., 1987; Haas et al., 2001; Tabata and Nakajima, 2001; Potter et al., 1984; Boudes et al., 2008), fluorescent dyes (Pinault, 1996; Lodovichi et al., 2003), and calcium indicators (Nagayama et al., 2007; Bonnot et al., 2005; Fujiwara et al., 2009) into cell populations. However, as described in Chapter 2, other approaches are limited in their ability to target specific populations and in their requirements for complex apparatuses. Others have used single-cell electroporation techniques for the loading of calcium indicators in individual neurons and also found they could preserve cell viability as seen via whole-cell recordings and evoked calcium transients (Nevian and Helmchen, 2007). Although this technique is useful for individual or small subsets of cells, it prevents the experimenter from labeling large populations of cells based on connectivity.

We have developed a technique that utilizes relatively low current injections from a simple glass electrode which not only eliminates the need for chambers or metal plates, but also enables targeted electroporation of small or large areas and structures in brain slices. This technique did not require building a complex electroporation apparatus and instead used
equipment already used by electrophysiologists, namely glass electrodes a stimulus isolation unit and a bath ground used for slice electrophysiology. We have described two primary uses for this technique in slice electrophysiology; targeted labeling with hydrazide and dextran-conjugated fluorescent fluorophores for slice physiology and anatomical examination only, and targeted labeling of calcium dye indicators for evaluation of physiological activity of multiple neurons within a given circuit. There are several advantages to each of these methods over previously described recording, neuronal tracing, and calcium imaging techniques.

5.2.2 Comparison with Previous Electrophysiological Techniques

Throughout the nervous system, investigations into cell-specific changes in facilitation, depression, synchronization, activity-dependent gating of information, and many others, have gleaned information gained by utilizing techniques involving pairs and triple recordings of neurons in areas such as prefrontal cortex, hippocampus, somatosensory cortex and the olfactory bulb (Hu et al., 2009; Arevian et al., 2008; Pelkey and Mcbain, 2007; Wang et al., 2009). This often requires recording from connected pairs of neurons or neurons projecting or receiving input from common areas.

The technique described in Chapter 2 provides several advantages to blind paired, triple, and even quadruple recordings from random neurons within a circuit. Electroporation of a given area of tissue provides the ability to identify neurons with overlapping axons and dendrites increasing the probability of identifying connected pairs of cells. Similarly, it provides the ability to identify even groups of neurons innervating a particular area, for example a particular layer of cortex or neurons which project to a particular region of CA1. Finally, it also provides the ability to target and record from neurons receiving the same input, for example from a particular glomerulus or alternatively to record from a nearby cell which has not been labeled, thus allowing for recordings from cells receiving input from two different glomeruli.
This technique also provides several advantages to current cellular tracing techniques. Whereas biocytin tracers fill the entire cell allowing for the reconstruction and analysis of morphology post-hoc, they prevent the ability to target recording based on fluorescence. Dil and related lipophilic tracers have been widely used to study the morphology of neurons. Neurons readily take up these dyes into their membranes following injected into a given region of tissue. Thus this approach can be used to label neurons that have axons and/or dendrites in a given location. However, this technique also prevents the targeted recording of labeled neurons via fluorescence, as complete loading of individual cells is often extremely slow, compromising cell viability in acute slice experiments and because delivery of lipophilic dyes is often imprecise.

Electroporation provides the best of each of these techniques, ensuring rapid uptake and dye diffusion for targeted recording from labeled cells while also providing for a variety of tracers for the reconstruction and analysis of morphology of electroporated cells.

In addition to characterizing neurons based on their structure, certain indicators can link neuronal activity to changes in fluorescence through intracellular reporter molecules (Helmchen et al., 1996). Calcium indicators are one such family of reporters, acting as a versatile tool in both in vivo and in vitro preparations to examine physiological activity that results in elevation of intracellular calcium. Most calcium indicators are available in several forms including membrane impermeable carboxylic acids and also membrane permeable forms in which the carboxyl groups are reacted to form ester groups have been widely used (Tsien, 1981; Grynkiewicz et al., 1985). Incubation of tissue with these forms allows these compounds to enter cells. Once internalized, these ester derivatives are cleaved by intracellular esterases, liberating the calcium-sensitive fluorescent indicator, providing widespread, surface loading of many cells within a given slice. However, these techniques often require long incubation times in detergents (Thomas et al., 2000; Kapoor and Urban, 2006), which can compromise slice viability and prevents these techniques from being used on older animals. In addition, this
technique only provides for non-specific loading of superficial cells. In contrast, the technique described here eliminates the need for long incubation times, enables loading of circuits even in older animals, and provides the possibility of penetration and loading of many deep cells in a specific population of interest.

5.3 The Development of the Vomeronasal System

5.3.1 Axonal Targeting During Development

In Chapter 3, we described a striking period of growth and development in the vomeronasal system, particularly for the vomeronasal organ and sensory neuron projections to the accessory olfactory bulb. In the first post-natal week the number of sensory neurons expressing the V2r1b-receptor more than doubled. In addition, we show that the first few post-natal days are a critical time period for axonal coalescence into well-defined glomeruli in the AOB.

The gross anatomical development of the vomeronasal system we describe in Chapter 3 has several parallels with development in the main olfactory system. In the rat, the overall volume of the main olfactory bulb has been shown to increase over 7-fold in the first thirty post-natal days, after which it remains constant (Rosselli-Austin and Altman, 1979), very similar to the data we report here. In addition, the mean areas of olfactory glomeruli increased 7-fold as well, indicating that similar to the growth we report here, even during this period of growth the ratio of glomerular layer volume to bulb volume remains relatively constant.

Likewise, the timeline of the development of sensory neuron axons have also been reported in the main olfactory system in opossums, rats, and mice. For example, mature and seemingly functional olfactory sensory neurons have been reported in the olfactory epithelium
as early as embryonic day 17 (Graziadei et al., 1980). In addition, coalescence of OSN axons into glomeruli across development in the main olfactory bulb has been shown to occur during the first few post-natal days (Potter et al., 2001). Evidence also suggests that these receptor neuron axons play a pivotal role in glomerular formation in that glomeruli have been shown to contain axons that are extremely homogenous in terms of OR expression despite some axons following tortuous paths before terminating in the target glomerulus (Treloar et al., 1999).

Given these similarities in the developmental time course of MOB and AOB, it is possible that, as in the main olfactory bulb, pre-synaptic axonal sorting in the AOB into specific glomeruli may be determined by the identity imparted by the receptor itself to guide axons and mediate homotypic interactions (Feinstein and Mombaerts, 2004). Similarly, other studies have suggested that the relative levels of guidance molecules expressed by OSN axons determine pre-target axonal sorting and ultimately, the projection sites for their targeting in the bulb (Imai et al., 2009).

In addition, activity dependent mechanisms mediated by particular ion channels such as HCN channels, which have been suggested to mediate axon outgrowth and coalescence in olfactory sensory neurons (Mobley et al., 2010), may regulate VSN axon outgrowth and refinement in the AOB. Likewise, the time course of glomerular maturation of V2r1b expressing receptor neuron axons, may proceed along different time courses compared to the maturation of other receptor types (Zou et al., 2004) and could possibly be guided by receptor axon-glial interactions (Bailey et al., 1999).

5.3.2 The Role of Activity in ORN Axon Refinement

In Chapter 4, we investigated the role that activity might play in the development of VNO to AOB connectivity, particularly with regard to axonal refinement and coalescence into well-defined glomeruli in the glomerular layer of the AOB. By selectively stimulating a sub-set of
vomeronasal sensory neurons using two MHC-I peptides, we observed the effect that high levels of activity had on refinement of V2r1b-expressing axons. Although we found that excess activity did affect axonal refinement in the AOB, it did not accelerate coalescence but rather resulted in over-exuberant projections whose pruning and refinement was delayed compared to control animals. In this way, we have shown that excess activity can affect the refinement of precise connectivity which exists between VSNs and mitral cells as described in Chapter 3.

The generation of precise adult connectivity is a prerequisite for a properly functioning adult nervous system. While building the nervous system during development, axon branches that are formed in excess are fated for removal in a process called axon pruning (Low and Cheng, 2005). The developmental phenomena of axon pruning of exuberant axonal projections in order to provide connectional specificity, is a normal hallmark of the developing nervous system (Luo and O’Leary, 2005). Many neural systems in both the central and peripheral nervous system display this common feature of axon pruning and synapse elimination due to an increased number of axonal arbors in a target region during development. For example, in the visual system (Katz and Shatz, 1996), the hippocampus (Bagri et al., 2003), at the neuromuscular junction (Nguyen and Lichtman, 1996) and in the cerebellum (Crepel, 1982; Hashimoto and Kano, 2005). In addition, these mechanisms provide a basis for plasticity, learning, and growth in the adult, or in response to damage or injury.

Much of the early work done contributing to our understanding of axon refinement has been through work done at the neuromuscular junction (NMJ) in the peripheral nervous system. Due to its accessibility and relative simplicity, it is a model system ideally suited to examine the local pruning of axons and synapse elimination. Even though a typical NMJ is innervated by a single motor neuron axon in maturity (Lichtman and Purves, 1980), early in development a given junction may be innervated by two or more motor neurons, each occupying similar synaptic areas (Balice-Gordon and Lichtman, 1993). However, as development continues, a
relative shift in area occurs through an activity-dependent mechanism in which the strong synapses are typically preserved and this axon extends to occupy the sites remaining from the withdrawal of the other, now receding axon (Gan and Lichtman, 1998; Walsh and Lichtman, 2003). These studies in the peripheral nervous system (PNS) suggest a direct role for activity in selecting a presynaptic axon by a winner-take-all mechanism followed by elimination of weaker partner(s).

Similarly, in the central nervous system (CNS), activity appears to be central in the regulation of axon refinement. For example, in the visual system, the segregation of eye-specific inputs from the two eyes into eye-specific domains in the LGN, and subsequently in primary visual cortex, were postulated several decades ago to be controlled by an activity-dependent Hebbian-like mechanism (Hebb, 1949). Since this prediction, many seminal papers have illustrated the role of neural activity in driving this eye-specific segregation during the critical period (Katz and Shatz, 1996; Penn et al., 1998; Stellwagen and Shatz, 2002).

For example, early work by Hubel and Wiesel in monkeys and kittens demonstrated that monocular deprivation during an early period in post-natal development results in a profound loss of retinal ganglion cell axonal terminals in the geniculate nucleus, whereas geniculate terminals with input from the non-deprived eye take over much of the space that would have normally been occupied by terminals from the deprived eye (Wiesel and Hubel, 1963; Hubel and Wiesel, 1970; Le Vay et al., 1980). These experiments strongly supported the hypothesis that competitive mechanisms via early visual experience during a critical period were necessary for the maintenance and full development of highly specific connectivity between the retina and downstream areas.

Later, further work showed that even prior to eye opening and the onset of native visual input, that network generated spontaneous activity was essential for the early segregation of
retinal ganglion inputs (Wong, 1999; Tian and Copenhagen, 2003). Interestingly, these spontaneous retinal waves have been shown to lead to strong correlations between the activity of neighboring retinal ganglion cells even before eye opening, leading to an activity dependent mechanism of axon elimination and eye specific segregation of retinogeniculate axonal arborizations (Meister and Schatz, 1991; Wong et al., 1993). Indeed, in the absence of retinal activity, retinal ganglion axonal arbors are not properly pruned and segregated in the lateral geniculate nucleus (Penn et al., 1998; Rossi et al., 2001). These results suggest that both the amount of neural activity as well as the correlation in activity between axons that innervate the same target region may be integral in driving these elimination events (Luo and O'Leary, 2005).

However, there is also considerable evidence suggesting that the establishment of early axonal targeting is dependent on mechanisms other than sensory activity and may depend rather on molecular guidance mechanisms. For example, in the visual system, the initial formation of ocular dominance columns via segregation of geniculocortical axons in visual cortex has been shown to be established even in of absence of spontaneous correlated activity (Crowley and Katz, 1999). Likewise, in the main olfactory system, it is clear that molecular guidance mechanisms are essential to guide and establish olfactory sensory neurons axon convergence into glomeruli in the olfactory bulb (Mombaerts, 2006; Cho et al., 2009).

For example, receptor swap experiments where the coding sequence of one receptor is replaced with the coding sequence of another, resulted in sensory neurons projecting to glomeruli distinct from either of the wild-type glomerular locations (Mombaerts et al., 1996). Similarly, genetically modified mice in which the rI7 receptor was substituted into the M71 receptor locus, determined both the glomerular convergence of OSN axons into anatomical locations consistent with the rI7 receptor as well as the post-synaptic innervation by mitral cell dendrites which now responded to rI7 ligands (Belluscio et al., 2002).
These results suggest that the olfactory receptor plays an instructive role in axon targeting, however, it seems likely that they are not the only determinant of initial axon targeting (Feinstein and Mombaerts, 2004). Indeed, several studies have revealed a graded expression of various axon guidance cues along differential axis of the olfactory bulb, such as Slit and its receptor Robo-2 (Cho et al., 2007; Cloutier et al., 2004) and the chemorepellant Sema3A (Schwarting et al., 2004; Schwarting et al., 2000; Taniguchi et al., 2003) which presumably play an instructional role in the targeting of OSN axons. In addition, targeted disruption of cAMP-dependent protein kinase and cAMP response element-binding protein shifted the targeting of OSN axons in the bulb, suggesting that it is OR-derived cAMP signals that determine the target destinations of OSNs (Feinstein et al., 2004; Imai et al., 2006).

Although molecular guidance mechanisms play an integral role in the establishment of proper axonal targeting in several brain areas, this does not rule out the possibility that the refinement process after the initial establishment of axonal targeting is not activity-dependent. Indeed, in the main olfactory bulb, work has shown that the maturation of specific glomeruli in the bulb is influenced by the loss of sensory activity. Naris occlusion, performed at post-natal day zero, appears to preserve the immature condition of the M71 and M72 glomerulus into adulthood (Zou et al., 2004). Likewise, OR-tagged transgenic mice that had a gene-targeted deletion of Kv 1.3 potassium channels displayed altered synaptic refinement of axonal projections from OSNs causing the formation of supernumerary glomeruli that failed to undergo neural pruning over development (Biju et al., 2008).

In addition, it appears early sensory activity is required for the initial establishment of precise afferent innervation of glomeruli by OSN axons in the bulb, at least with respect to certain receptor types. Mice lacking the olfactory CNG channel, which fail to exhibit odor-evoked response, maintain convergence to appropriate target glomeruli (Lin et al., 2000). Similarly, mice deficient in G_{olf}, which are anosmic, display unaltered olfactory sensory neuron projections.
in the bulb (Belluscio et al., 1998) however, OCNC1 mutant mice, which display unresponsive olfactory sensory neurons, show altered patterns of axonal convergence for M72-expressing OSNs but not for OSNs expressing the P2-receptor (Zheng et al., 2000).

Furthermore, random inactivation the OCNC1-gene in random OSNs results in unusual patterns of axonal projections and the slow and specific deletion of these OCNC1-deficient neurons that is dependent on odorant exposure and can be reversed by odorant deprivation (Zhao and Reed, 2001). This suggests that odor-evoked activity is critical for neuronal survival and axonal targeting in the MOB at least for some receptor types. Similarly, inhibition of synaptic release via the conditional expression of tetanus toxin in a subset of OSNs alters glomerular targeting of OSN axons (Yu et al., 2004). In addition, this group also demonstrated in the same study that the conditional hyperpolarization of OSNs by overexpression of the inward rectifying potassium channel Kir 2.1, inhibiting both odor-evoked and spontaneous activity, resulted in delayed entry of sensory axons into the bulb as well as a developmental defect in the organization of glomeruli. Therefore, there is considerable evidence to suggest an important role for activity in establishing and maintaining precise connectivity in the main olfactory system.

In the results described in Chapter 4, we see several similarities and differences compared to these results observed in the main olfactory bulb. On one hand, our results suggest that the initial targeting of axonal terminations to target glomeruli in the AOB remains relatively intact during periods of high activity. We observed no significant deficits in gross axon targeting, such as ectopic V2r1b-expressing axons appearing in the rostral portion of the AOB. We found relative glomerular locations to be the same in both control and sensory exposed mice, similar to the results described above in the MOB.

However, unlike previous reports in the MOB, in which increasing sensory activity resulted in the formation of small, heterogeneous and supernumerary glomeruli compared to
wild-type (Biju et al., 2008), we did not find a significant difference in the total number of glomeruli formed by V2r1b-expressing sensory neurons in exposed versus control animals (See Figure 4.7 & 4.8). Instead, we observed what appeared to be reduced axonal pruning and slower coalescence during development in exposed animals compared to controls.

These results suggest that initial axonal targeting of VSN axons to target glomeruli in the AOB may also be guided by molecular guidance cues as has been suggested by other studies (Knoll et al., 2001; Knoll et al., 2003; Walz et al., 2002). However, similar to other circuits described earlier, the refinement and coalescence of VSN axons into well-defined glomeruli may rely, at least partially, on an activity-dependent mechanism. Although our data does not define the length of time sensory activity can alter axonal refinement, our work is the first to suggest that a critical period may exist during the first few post-natal days in vomeronasal system development.

### 5.3.3 Dendritic Targeting and Refinement

Similar to the axonal development of sensory neurons, AOB mitral cell dendritic development, as we describe in Chapter 3, resembles that of the MOB. The coalescence of dendritic processes into glomeruli appears secondary to the arrival and development of olfactory sensory neuron axons (Treloar et al., 1999). After the coalescence of olfactory sensory neuron axons into glomeruli early in development, immature mitral cells exhibit multiple primary dendrites which innervate several adjacent glomeruli that, with maturation, retract to leave a single primary dendrite bearing a glomerular tuft (Malun and Brunjes, 1996). Immature mitral cells have been shown to extend multiple dendrites into the glomerular layer as late as P5 and show mature morphology by P10-15 (Blanchart et al., 2006; Matsutani and Yamamoto, 2000).

These dendritic endings also appear to cross the olfactory nerve layer/external plexiform layer border and unite with OSN axons by E16 (Blanchart et al., 2006) with electron microscopy...
identifying the appearance of functional synapses just prior to birth (Blanchart et al., 2008; Treloar et al., 1999; Hinds and Hinds, 1976). Electrophysiological studies have demonstrated monosynaptic olfactory receptor neuron input onto external tufted cells from the earliest time point studied (P1) consistent with the idea that these early circuits are functional (Grubb et al., 2008). These results are strikingly similar to the results presented in Chapter 4, suggesting similar mechanisms may exist.

Although little is known about the role of olfactory sensory neurons in regulating mitral cell development, in vitro studies have suggested a role for trophic activity in the main olfactory epithelia which promote embryonic mitral and tufted cell dendritic elaboration (Tran et al., 2008). Given the presence of synaptic contacts at or before birth in the present study, it seems likely that contact-mediated interactions between sensory neurons and mitral cells may play a role in mitral cell dendritic targeting and morphology. Activity-dependent regulation of connectivity also has been implicated in the differentiation and pruning of secondary dendrites in mitral cells of the main olfactory bulb with OCNC1KN animals displaying slightly delayed dendritic pruning (Lin et al., 2000). Furthermore, unilateral naris closure at P1 results in an increase in the percentage of mature mitral cells having multiple primary dendrites (Matsutani and Yamamoto, 2000). Therefore, it may be likely that each of these mechanisms play a role in regulating the dendritic development of mitral cells in the accessory olfactory bulb.

5.4 The Role of the Young VNS in Behavior

Our results suggest that despite previous reports that the vomeronasal system duct is immature and not patent at birth (Coppola et al., 1993; Coppola and Millar, 1994), it is open enough that small molecules from the environment can access sensory neuron receptors in the
vomeronasal organ. Previously, Coppola and colleagues tested the functionality of vomeronasal system by injecting fluorescent beads into the amniotic fluid surrounding the mouse fetus. After examination of sections in all regions of the nasal cavity, fluorescence was found in all regions of the nasal cavity except the lumen of the VNO (Coppola and O'Connell, 1989). Coppola and colleagues later examined serial sections of the VNO duct from E19 through P25. Although the duct was clearly obstructed at E19, its morphology had significantly changed at P1 although they claimed was still immature and did not become patent until sometime after the first day of life.

However, we directly tested whether the VNO was indeed open enough to allow for access to external stimuli. It has been demonstrated by other groups that fluorescent dyes could be used as a means for evaluating access to both the VNO lumen and the olfactory epithelium in adults. For example, adult guinea pigs were allowed to investigate urine containing rhodamine and were shown to exhibit fluorescence in their vomeronasal organs but not the olfactory epithelium (Wysocki et al., 1980). Our results here, suggest that although the VNO duct may be at an immature stage just after birth, the duct is still capable of allowing hydrophilic molecules as large as 1,000 Da access to the lumen. This suggests that molecules having properties similar to those of the MHC peptides used in this study also will be able to access the bulb. Alternatively, the possibility that these stimuli may reach the VNO receptors by some other route other than the VNO duct cannot be ruled out.

The results here raise the question of whether the accessory olfactory system plays a role in regulating behavior in early post-natal life. The main olfactory system is known to be critical in the survival of newborns in several species. For example, olfactory cues have been implicated in species such as the wallaby to direct the neonate to its mother's pouch (Schneider et al., 2008; Schneider et al., 2009), in kittens in the guidance of nipple attachment and suckling (Raihani et al., 2009), and in rabbits the role of the “nipple-search pheromone” in nipple
attachment of pups (Hudson et al., 2002). Although it is clear that pheromones are essential in triggering this behavior (Hudson and Distel, 1983), there is no direct evidence that the accessory olfactory system is essential in mediating it.

Instead, studies have suggested that these behaviors do not depend on the vomeronasal to accessory olfactory bulb system (Hudson and Distel, 1986) and that maternal odors induce immediate early genes in the main but not accessory olfactory bulbs in neonatal ferrets (Chang et al., 2001). Based on the results presented here, the circuitry of the vomeronasal system is functional, yet highly imprecise at early time points of post-natal life. Given the time course of the morphological refinement that we see, both with regards to VSN axonal coalesce and mitral cell dendritic clustering, as well as the effect of activity we show here, it may be that exposure to stimuli in the post-natal animal is critical for directing the specificity of this connectivity. We propose that if the accessory olfactory system functions in processing maternal olfactory information in newborn pups it certainly is quite different than the function of this circuit in adult animals.

In summary, the mouse vomeronasal system appears to undergo a remarkable period of growth and development early in the animal’s life. We have demonstrated that during this early period of development, precise connectivity is achieved between VSNs in the VNO and mitral cells in the AOB through VSN axonal coalescence into well-defined glomeruli and mitral cell dendritic refinement and targeting to specific glomeruli. In addition, we have provided some of the first evidence suggesting the existence of an early critical period in the vomeronasal system in which early sensory activity may help drive the formation of this precise connectivity which is likely to underpin proper pheromonal processing in this system.
I. Appendix I: Recurrent Dendrodendritic Inhibition Of Accessory Olfactory Bulb Mitral Cells Requires Activation Of Group I Metabotropic Glutamate Receptors

I.1 Abstract

Metabotropic glutamate receptors (mGluRs) modulate neural excitability and network tone in many brain regions. Expression of mGluRs is particularly high in the accessory olfactory bulb (AOB) – a central nervous system structure critical for detecting chemicals that identify kin and conspecifics. Because of its relative simplicity and its direct projection to the hypothalamus, the AOB provides a model system for studying how mGluRs affect the flow of encoded sensory information to downstream areas. We investigated the role of group I mGluRs in synaptic processing in AOB slices, and found that under control conditions, recurrent inhibition (RI) of principal neurons (mitral cells) was completely eliminated by the mGluR1 antagonist LY367385 (100 µM). In addition, the group I mGluR agonist DHPG (20 µM) induced a dramatic increase in the rate of spontaneous IPSCs. This increase was dependent on voltage gated calcium channels, but persisted even following blockade of ionotropic glutamatergic transmission and sodium channels. Taken together, these results indicate that mGluR1 plays a critical role in controlling information flow through the AOB, and suggest that mGluR1 may be an important locus for experience-dependent changes in synaptic function.
I.2 Introduction

The mammalian accessory olfactory bulb (AOB) is a CNS structure involved in identifying chemicals that signal the social and reproductive status of conspecifics (Stowers et al., 2002; Dulac and Torello, 2003). Individual AOB principal neurons (mitral cells) receive excitatory inputs from vomeronasal axons in a small number of glomeruli (Meisami and Bhatnagar, 1998). In contrast to the broadly tuned mitral cells of the main olfactory bulb, AOB mitral cells are exquisitely selective for specific, socially relevant ligands or mixtures (Luo et al., 2003b). Such selectivity likely indicates a sparse representation of pheromonal information – that is, any given mitral cell is activated only rarely, and the responses of a small number of mitral cells are likely to be behaviorally relevant. Consistent with this, AOB mitral cells are immediately presynaptic to brain areas that cause the changes in behavioral, hormonal and metabolic states associated with pheromones, including the hypothalamus and amygdala (Kevetter and Winans, 1981a; Kevetter and Winans, 1981b).

The throughput of information from the AOB to these downstream structures is controlled, in large part, by recurrent inhibition (RI) of mitral cells by granule cells – a population of axon-less, spiny, GABA-ergic interneurons that inhibit mitral cells via reciprocal dendrodendritic synapses. (Isaacson and Strowbridge, 1998; Schoppa et al., 1998; Taniguchi and Kaba, 2001; Urban, 2002). This relatively simple relay-like relationship between the AOB and downstream structures motivates the hypothesis that recurrent inhibition serves a sensory “gating” function (Best et al., 2005; Brennan and Keverne, 1997b; Brennan et al., 1990). Behavioral and physiological experiments support this notion, as changes in the strength of recurrent inhibition are thought to underlie specific forms of olfactory learning (Brennan and Keverne, 1997b; Hayashi et al., 1993a; Kaba et al., 1994a). Moreover, these changes depend
critically on activation of metabotropic glutamate receptors (mGluRs), which are richly expressed in the AOB (Ohishi et al., 1998; Ohishi et al., 1993; Sahara et al., 2001).

In the present study, we examined the role of mGluRs in mediating recurrent inhibition of AOB mitral cells. Given the high expression levels of mGluRs in the main and accessory olfactory bulbs, and the fact that activation of these receptors is proposed to be important in the induction of experience-dependent changes in information processing in the AOB, we reasoned that they may play an important role in regulating recurrent inhibition. Surprisingly, and in contrast to what we observe in the MOB, we found that RI of AOB mitral cells requires activation of the group I metabotropic glutamate receptor mGluR1. This requirement for mGluR1 was dramatically reduced when extracellular magnesium concentration was lowered, suggesting that mGluR1 activation enhances the coupling of NMDA receptors to inhibition. Inhibition was also induced by direct application of the group I mGluR agonist DHPG, even following blockade of ionotropic glutamatergic synaptic transmission and sodium spikes. These results suggest that mGluR activation is critical for regulating granule cell excitability, and that the mechanisms of recurrent inhibition show interesting differences in the MOB and AOB. Finally, these data point to group I mGluRs as a critical locus for controlling information flow through the AOB.

I.3 Materials and Methods

I.3.1 Slice Preparation

Methods are as described previously in Urban and Castro (2005). Briefly, sagital olfactory bulb slices (300-350 μm thick) were prepared from young mice [postnatal day 14 (P14 to P28)]. Mice were anesthetized (0.1% ketamine/0.1% xylaxine; ~3mg/kg, i.p.) and decapitated. Olfactory bulbs were sectioned on a vibratome while submerged in ice-cold oxygenated Ringer’s solution containing the following (in mM): 125 NaCl, 2.5 KCl, 25 NaHCO₃,
1.25 NaH$_2$PO$_4$, 1MgCl$_2$, 25 glucose, 2 CaCl$_2$. In some experiments, 0.5 mM ascorbate, 1mM pyruvate, and 2mM myo-inositol were added to the slicing medium. All animal care was in accordance with the guidelines of Institutional Animal Care and Use Committee of Carnegie Mellon University.

I.3.2 Electrophysiology

Whole cell voltage recordings were obtained from the somata of identified AOB mitral cells (Stuart and Spruston, 1995). Slices were superfused with the oxygenated Ringer’s solution described above, warmed to 34-36º C. Whole-cell recordings were established using pipettes (resistances of 2-8 MΩ) filled with a solution containing the following (in mM): 120 postassium gluconate, 2 KCl, 10 HEPES, 10 sodium phosphocreatine, 4 MgATP, and 0.3 Na$_3$GTP, adjusted to pH 7.3 with KOH. Voltage and current clamp recordings were preformed using a MultiClamp 700B amplifier (Molecular Devices, Union City, CA). Data were filtered (4 kHz low pass) and digitized at 10 kHz using an ITC-18 (Instrutech, Mineola, NY) controlled by custom software written in Igor Pro (Wavemetrics, Lake Oswego, OR). In all experiments in which membrane current was recorded, mitral cells were held at -40 mV to facilitate the recording of GABA$_A$-mediated IPSCs.

I.3.3 Data Analysis

Miniature IPSCs were detected using custom written functions in IGOR pro (Wavemetrics, Lake Oswego, OR), which implemented the algorithm described by (Kudoh and Taguchi, 2002). Event rates for control conditions were calculated in a 1 minute window prior to the addition of drug. For drug conditions, a window three minutes after drug addition was used to calculate event rate. For analysis of the onset kinetics of the enhanced rate of IPSCs, sigmoids were fit to plots of IPSC rate (calculated in 500 ms bins) vs. time. These sigmoids
were normalized by their maxima and aligned to the first point where the derivative of a given fitted sigmoid was non-zero. From these aligned curves, a mean sigmoid was generated according to $y = 1/(\exp(t_{\text{half}}/\text{rate})$, where the parameters $t_{\text{half}}$ and $\text{rate}$ were averages of these same parameters from the individual sigmoids. For more standard measures of time-to-peak of these same IPSC rate data (the values reported in the text) an alternative procedure was used. The standard deviation of IPSC event rate (also calculated in 500 ms bins) was calculated for a one minute period prior to DHPG addition, and onset was defined as first time point when IPSC event rate exceeded the mean of the baseline period by two standard deviations. The mean event rate and standard deviation were then calculated for a period of two minutes following the onset (defined as above), and peak time was defined as the first point following onset when this mean event rate was achieved and maintained within one standard deviation of this value for at least 10 seconds (20 samplings of the rate). Data are reported as mean ± SEM unless otherwise indicated. Statistical significance was assessed using paired or unpaired t-tests as appropriate.

I.3.4 Drugs

APV, CNQX, bicuculline, and Cyclopiazonic acid (CPA) all were obtained from Sigma–Aldrich (St. Louis, MO) and used at concentrations of 50, 20, 10, and 30 µM, respectively. Gabazine, DHPG and LY367385 were obtained from Tocris (Ellisville, MO), and used at final concentrations of 10, 20 and 100 µM, respectively. 3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine (MTEP) (Calbiochem, La Jolla, CA) was used at 2µM and prepared from a 25 mM stock in 100% DMSO. CPA and LY367585 were prepared in stocks of 18 and 100mM respectively, both in 100% DMSO. The DHPG stock was prepared in dH$_2$O at 100 mM. The voltage gated calcium channel blockers TTX, Nickel, and Cadmium were used at concentrations of 1µM, 100µM, and 30µM respectively.
I.4 Results

I.4.1 Recurrent Inhibition of AOB Mitral Cells Requires mGluR1 Activation

In mitral cells of the MOB and AOB, action potentials or large membrane depolarizations evoke long-lasting inhibition mediated by GABA_A receptors (Margrie et al., 2001b; Isaacson and Strowbridge, 1998; Schoppa et al., 1998; Taniguchi and Kaba, 2001). This form of inhibition, in which activity in an excitatory neuron results in self-inhibition dependent on dendrodendritic synapses with inhibitory interneurons, is typically termed recurrent inhibition (RI). We adhere to this nomenclature in the remainder of the text to differentiate RI from inhibition that is evoked pharmacologically and not strictly by spiking in a presynaptic excitatory neuron. In an initial set of experiments, we characterized RI in AOB mitral cells. In voltage clamp, a 100 ms depolarization of mitral cells from -40mV to +20 mV evoked a slow outward current (Fig I.1A) consisting of many discrete outward synaptic events superimposed on the slower current (Fig I.1A). This current was eliminated by addition of the GABA_A specific antagonist gabazine (SR95531, 10μM), indicating that these voltage steps evoke recurrent inhibition (Fig I.1A). In similar experiments, trains of seven action potentials at 40Hz were evoked in mitral cells by current injection, and recurrent inhibition was observed as a slow, gabazine-sensitive hyperpolarization (Fig I.1B). Recurrent inhibition in this case was quantified as the voltage integral following the final spike in the series of action potentials (from 70 ms following the final injected current step to 1130 ms following this step; this latter number corresponded to the end of the sweep). We typically saw asynchronous IPSP-like events lasting most or all of this period (see, for example, both control cases in Fig I.1B). These results demonstrate that both action potentials and membrane depolarization evoke long-lasting recurrent inhibition in AOB mitral cells, consistent with previous results (Taniguchi and Kaba, 2001).
Figure I.1 - The mGluR1 antagonist LY367385 eliminates recurrent inhibition in AOB mitral cells. A) Single sweeps showing currents evoked by depolarization of a mitral cell from -40 mV to 20 mV. Action currents during the depolarization are blanked for better visibility. Black: control sweep showing slow outward component and discrete outward events. Gray: sweep showing blockade of the slow and discrete outward currents with addition of LY (100μM). Red: sweep showing lack of effect of SR95531 (gabazine,10μM) added after LY. B) Single sweeps from current clamp experiments in which recurrent inhibition was elicited by current pulses (3ms x 40 Hz). Top: traces showing the effect of LY on recurrent inhibition. Note the discrete events evident in the control case that are eliminated by LY addition. The black shaded area corresponds to the difference in voltage integrals used to measure changes in recurrent inhibition between the control and LY cases. Middle: occlusion-type experiments in which recurrent inhibition was first completely blocked by SR and LY subsequently added. The gray shaded area corresponds to the difference between voltage integrals for the control and SR cases. The red shaded area corresponds to the difference between voltage integrals for the SR and SR + LY cases. C) Traces (each an average of 10 sweeps) demonstrating the lack of effect of LY367385 on recurrent inhibition in the main olfactory bulb (MOB), (n=5). Stimulation protocol and integral measurement are identical to those shown in B. Traces below are averages of 10 sweeps under control (black) and LY cases D) Summary data for experiments similar to those shown in B. The colors of the group labels correspond to the colors of the shaded regions shown in B (n=8 for LY-control and n=7 for (SR+LY – SR alone) group), except for the (MTEP-control), group (n=7), which has no corresponding trace in B. E) Summary of individual experiments showing the occlusion effect of SR on LY. Red lines and markers indicate mean ± SEM for all experiments. (Control: -536.36 ± 160.52 mV ms, SR: 355.90 ± 306.67 mV ms, SR+LY: 459.14 ± 264.49 mV ms, n=7).
We next tested whether recurrent inhibition was modulated by activation of mGluRs in AOB slices. Adding the mGluR1 antagonist LY367385 (LY, 100µM) to the bathing medium resulted in a decrease in RI that was comparable to the decrease seen with the addition of gabazine (Δ voltage integral = 1338.48 ± 351.74 mV·ms (LY, n=7), 988.63 ± 193.79 mV·ms (gabazine, n=8), p=.40), suggesting that a large fraction of recurrent inhibition required activation of mGluR1 (Figs 1.1B,1C). By contrast, the selective mGluR5 antagonist MTEP (2 µM) had no effect recurrent inhibition (Fig 1.1D).

It is possible, though unlikely, that blockade of mGluR1 upregulates a depolarization-evoked inward current that masks recurrent inhibition. To test whether the mGluR1-dependent component of RI is the same as the GABA-A dependent component, we first blocked inhibition with gabazine, and then added LY (an example is shown in the bottom of figure 1.1B). Under these conditions, addition of gabazine completely occluded the subsequent effect of LY (Fig 1.1A, B, and C). Taken together, these data demonstrate that mGluR1 activation is necessary for recurrent inhibition in AOB mitral cells in control conditions. By contrast, in experiments in MOB mitral cells, LY had no effect on recurrent inhibition (control voltage integral: -1377.19 ± 452.51 mV·ms; LY voltage integral: -1360.51 ± 481.80 mV·ms, p = 0.92 (n=4)) (Fig 1.1C, see also (Heinbockel et al., 2006)), showing that the specific involvement of mGluR1 in recurrent inhibition is unique to AOB mitral cells.

I.4.2 Activation of mGluRs Evokes Spontaneous mIPSCs in Mitral Cells

Given the strong dependence of recurrent inhibition on mGluR1, we next sought to determine whether direct activation of group I mGluRs is sufficient to evoke IPSCs in mitral cells. Mitral cells were voltage clamped at -40 mV during addition of the broad-spectrum group I mGluR agonist DHPG (20 µM). DHPG resulted in a more than a ten-fold increase in the rate of spontaneous outward events, with the event rate increasing from an average of 0.41 ±0.20 Hz
Figure I.2 - DHPG enhances the rate of spontaneous IPSCs in mitral cells. A) Bath application of DHPG (20 μM) greatly increases the occurrence of spontaneous IPSCs in mitral cells. Addition of DHPG in general evoked a slow inward or outward current followed by a rapid transition to a high rate of IPSCs. Traces on the bottom of A show expanded versions of the control and DHPG conditions. B) Cumulative probability plot of inter-event intervals for control and DHPG conditions (n=6). Inset shows mean event rate for the two conditions (control: 0.41±0.2 Hz, DHPG: 5.86 ± 1.26 Hz, n=6, p<0.01). C) Cumulative probability plot of event amplitudes for control and DHPG conditions indicating no significant differences. (Inset shows average IPSC waveforms for control and DHPG conditions.) D) Mean increase in IPSC rate across all mitral cells (n=6). The mean trace was generated from normalized and aligned sigmoid fits to plots of IPSC rate vs. time, as described in the text. X error bars are ± SEM. E) Examples of sigmoid fits to IPSC event rates for three representative mitral cells. F) Raw data of the cell indicated by the gray line in figure E (different cell than in A). Data segment is 20 seconds following the earliest observed increase in IPSC event rate (as defined in Materials and Methods).
to 5.86 ± 1.26 Hz (n = 6, p<.01, t-test) (Fig I.2 A,B). These events were completely blocked by 10 μM bicuculline (Figs I.3B,C) indicating they are mediated by GABA_A receptors. In addition, the kinetics and amplitudes of these events were nearly identical for the baseline and DHPG cases (see cumulative amplitude distribution in figure I.2C and corresponding traces in the inset), suggesting that DHPG is causing release of GABA at reciprocal synapses onto mitral cells. Amplitude, rise time, and decay time histograms of observed events were unimodal (data not shown), consistent with a single presynaptic source. The effect of DHPG persisted in the presence of the glutamate receptor blockers APV (50µM) and CNQX (20µM) (Fig I.3A), indicating that the effect was at least partially independent of glutamate release from mitral cells. However, the rate of DHPG-evoked IPSCs was lower when ionotropic glutamate receptors were blocked than in the control case (control + DHPG: 5.89 ± 1.26; APV+CNQX+DHPG: 2.40 ± 0.72 Hz, n=6, p<0.03).

It is worth noting that the ~15 pA slow inward current observed in figure I.2A was not observed in all mitral cells. Changes in steady state current evoked by DHPG were heterogeneous: in some cells DHPG evoked slow inward currents, and in others slow outward currents. The mean change in mitral cell holding current was -9.4 ± 10.6 pA (n=8), which is a smaller and less reliable effect than observed in MOB mitral cells (Ennis et al., 2004). In addition, we failed to find a strong relationship between the magnitude of DHPG-evoked current and the DHPG evoked increase in IPSC rate within cells (R^2 = 0.16 for event rate vs. holding current). By contrast, the very rapid onset of enhanced IPSCs was seen in all mitral cells tested (Fig I.2 D,E,F). The mean time to peak event rate from onset across all cells was 24.3 ± 6.3 s (n=6) (see Materials and Methods for definitions of onset and peak).
Figure I.3 - The increase in IPSC rate with DHPG addition occurs via a mechanism presynaptic to mitral cells. A) Sample sweep in which APV (50 μM) and CNQX (20 μM) were bath applied for 10 minutes prior to addition of 20 μM DHPG (baseline period is truncated). B) Sample sweep showing complete absence of spontaneous and DHPG-evoked IPSCs in 10 μM bicuculline. As above, bicuculline addition preceded DHPG addition by 10 minutes (baseline is truncated). C) Summary data of effects of synaptic blockers on DHPG-induced IPSCs.
I.4.3 The Effect of the mGluR-Evoked Increase in the Rate of IPSCs is Primarily Mediated by mGluR1

DHPG is a broad spectrum group I mgluR agonist (Ito et al., 1992), and therefore activates both receptors in this class – mGluR1, and mGluR5 (Conn and Pin, 1997). To determine which of these mGluRs contributes to the increase in mitral cell IPSCs, we performed experiments in which DHPG was added in the presence of LY367385 (LY), or 3-((2-Methyl-1,3-thiazol-4-yl) ethynyl) pyridine (MTEP), specific blockers of mGluR1 and mGluR5, respectively. When DHPG (20 μM) was added to the bathing medium in the presence of 100 μM LY, the rate of mitral cell IPSCs did not increase (LY alone: 0.35 ± .23 Hz, LY+DHPG: .19 ± .08 Hz, n=5, p=.53) (Fig I.4A,D). By contrast, the rate of IPSCs was enhanced when DHPG was added in the presence of 2 μM MTEP (MTEP alone: 0.27 ± .10 Hz, MTEP + DHPG: 2.88 ± .46 Hz, n=4, p=.0009) (Fig I.4B,D). These results indicate that DHPG-evoked IPSCs are due to the effect of DHPG on mGluR1.

I.4.4 DHPG Evoked IPSCs Require Calcium Influx, But Not Sodium Spikes

As noted above, and similar to what others have seen in MOB (Heinbockel et al., 2004), we sometimes observed a slow DHPG-evoked inward current in mitral cells that accompanied the increase in IPSCs (see, for example, figure I.2A). Hence, a potential mechanism for the increased barrage of spontaneous IPSCs is direct depolarization of mitral cells, with concomitant elevated mitral cell firing rates. Thus, we next tested whether action potentials are required for the increase in IPSC rate by mGluR agonists by including 1μM TTX in the bath solution prior to addition of DHPG. Under these conditions the rate of mitral cell IPSCs was still increased (baseline: .04 ± .02 Hz, DHPG: 1.99 ± .76 Hz, p<.05, n=4) (Fig I.5), indicating that sodium spikes are not required for mGluR activation to evoke IPSCs. Since the IPSC rate was lower in TTX than control conditions, sodium channels and spontaneous spiking by granule cells
Figure I.4 - The effect of the mGluR evoked increase in the rate of IPSCs is mediated primarily by mGluR1. A) Bath application of DHPG (20 μM) in the presence of mGluR1 antagonist LY (100 μM) eliminated the increase of spontaneous IPSCs in mitral cells. B) Bath application of DHPG in the presence of mGluR5 antagonist MTEP (2 μM) still showed a significant increase in the occurrence of IPSCs in mitral cells. In both cases, slices were bathed in the individual antagonist for 15 minutes before addition of the antagonist plus DHPG. C) Expanded version of traces showing individual events in two conditions: IPSCs in the presence of the individual antagonist alone (left) and with addition of DHPG along with the antagonist (right). D) Cumulative probability plot of inter-events intervals for control DHPG conditions (black line), DHPG in the presence of MTEP (dashed line), and DHPG in the presence of LY (gray line). Inset shows mean event rates for all conditions (LY baseline: 0.35 ± 0.23 Hz, LY + DHPG: 0.19 ± 0.08 Hz (n=5), MTEP baseline: 0.27 ± 0.10 Hz, MTEP + DHPG: 2.88 ± 0.46 Hz (n = 4).
are likely to play a role in setting the rate of IPSCs. By contrast, when the calcium channel blockers cadmium (30 μM) and nickel (100 μM) were included in the bath, DHPG did not cause a significant increase in IPSC rate (baseline: 0.34 ± 0.09Hz, DHPG: 0.33 ± 0.17 Hz, p>0.05, n=5) (Fig I.5).

I.4.5 Release from Internal Stores Is Not Required For DHPG-Evoked Inhibition

In a number of cell types, activation of mGluRs is coupled to release of calcium from internal stores either via second messengers or calcium influx through voltage gated calcium channels (Takechi et al., 1998; Finch and Augustine, 1998; Chavis et al., 1996; del et al., 1999). The experiments above indicate that calcium influx through voltage gated calcium channels is necessary to evoke recurrent IPSCs, but does not address the question of whether GABA release also depends on release of calcium from internal stores. To test this possibility, we bath applied the sarcoplasmic reticulum calcium pump inhibitor CPA (30 μM) to deplete internal calcium stores prior to adding DHPG. Enhanced rates of IPSCs were still observed under these conditions, with a mean DHPG-evoked rate (8.23 ± 2.27 Hz, n = 5) (Fig I.6), comparable to that observed under control conditions (5.86 ± 1.26 Hz, n=6). This indicates that DHPG likely acts by causing a direct, calcium-dependent depolarization in granule cells – possibly activation of a calcium conductance, or closure of a potassium channel (see (Schoppa and Westbrook, 1997) and that GABA release is then triggered by voltage-gated calcium current in granule cells.

I.4.6 Mechanisms of mGluR1 Action in Granule Cells

In the main and accessory olfactory bulbs, recurrent inhibition evoked by mitral cell spiking is mediated predominantly by granule cells, the major interneuron population (Schoppa et al., 1998; Isaacson, 2001; Isaacson and Strowbridge, 1998; Price and Powell, 1970a; Price and Powell, 1970b). Given that we saw complete elimination of recurrent inhibition in AOB mitral
Figure I.5 - The increase in spontaneous IPSCs is dependent on voltage gated calcium channels, but not sodium channels. A) Sample sweep in which DHPG was bath applied following addition of TTX. Bottom shows expanded sections from the baseline period and TTX addition period. B) Sample sweep in which DHPG was bath applied following addition of cadmium and nickel. C) Summary data for the effects of voltage gated channel blockers on DHPG-induced IPSCs.
Figure 1.6 - DHPG evoked IPSCs do not depend on internal calcium stores. A) Application of DHPG in the presence of CPA (100 μM) still induced a large increase in the occurrence of IPSCs in mitral cells. The slice was bathed in CPA for 20 minutes before the application of CPA plus DHPG. B) Cumulative probability plot of inter-event intervals for CPG + DHPG (black line) and control CPA (gray line). Inset shows events rates for both conditions, (CPA only: 0.37 ± 0.17 Hz, CPA + DHPG: 8.23 ± 2.27 Hz, n=5).
cells with blockade of mGluR1 (Fig I.1) and that DHPG-evoked IPSCs in mitral cells persisted when ionotropic glutamateric transmission was blocked (Fig I.3), we reasoned that granule cells are likely to be the major cell type contributing to the large increase in miniature IPSCs observed in our experiments. In addition, the persistence of DHPG-evoked spontaneous IPSCs in the presence of TTX, but their elimination by blockers of VGCCs is consistent with studies in granule cells demonstrating that local or global calcium spikes are potential triggers of transmitter release (Egger et al., 2003; Egger et al., 2005; Pinato and Midtgaard, 2005; Zelles et al., 2006; Pinato and Midtgaard, 2003). Thus, one possible mechanism for the action of DHPG is via direct depolarization of granule cells. In this scenario, glutamate released from mitral cells would bind to metabotropic receptors on granule cells, and cause a local or global calcium elevation sufficient to evoke GABA release from granule cells. To test this possibility, we recorded from granule cell somata in the presence of blockers of fast glutamatergic transmission (50 μM APV, 20 μM CNQX) and added DHPG (20 μM). In 4 granule cells tested, we observed a depolarization of 6.79 ± 0.79 mV by the third minute after DHPG addition – an effect which persisted for the duration of drug addition (Fig I.7A). Granule cells were also depolarized by 5.15 ± 0.42 mV (n=5) by DHPG when MTEP (2μM) was included in the bath solution (Fig I.7B), indicating that activation of mGluR1 is sufficient for the effect. By contrast, no significant depolarization was observed when mGluR1 was blocked by LY367385 prior to DHPG addition (n=5) (Fig I.7B). It is worth noting granule cell spines are electrotonically isolated from the granule cell soma (Woolf et al., 1991; Egger and Urban, 2006; Rall and Shepherd, 1968), and that depolarization observed at the soma may be an underestimate of voltage changes more local to spines and associated release machinery.

Studies in both the main and accessory olfactory bulb have demonstrated that NMDA receptors (NMDARs) – likely those present on granule cell spines - are important for recurrent inhibition of mitral cells (Chen et al., 2000). In control conditions, blockade of NMDARs results in
Figure I.7 - DHPG depolarizes granule cells. A) Left: group data showing granule cell depolarization with addition of DHPG (20 μM) starting at t=0 (n=4, mean depolarization = 6.79 ± 0.79; shaded areas correspond to ± SEM). Right: example experiment. Negative-going deflections are hyperpolarizing pulses used to monitor input resistance. B) Same layout and experiment design as in A, only with 2 μM MTEP (n=5) or 100 μM LY367385 (n=5) included in the bath to block mGluR5 or mGluR1, respectively. Right: example experiment showing persistence of DHPG-evoked granule cell depolarization when mGluR5 is antagonized. C) Effectiveness of LY367385 in blocking recurrent inhibition at normal (1.0 mM, n=7) and low (0.2 mM, n=6) magnesium concentrations. D) Single sample sweeps showing recurrent inhibition following 7 evoked spikes at 40 Hz in 0.2 Mg2+. Gray, control; Red, following LY alone; Black, LY+gabazine.
near-complete elimination of action potential evoked recurrent inhibition; some of these findings also suggest that calcium influx through NMDARs can be directly coupled to transmitter release (Halabisky et al., 2000; Chen et al., 2000). Given that NMDAR activation is require for recurrent inhibition in control conditions, our results (Fig 1.1) suggest that activation of mGluR1 may play a modulatory, but still critical role in evoking transmitter release from granule cells.

One possibility we considered was that due to its depolarizing effect on granule cells, mGluR1 may facilitate voltage dependent calcium influx that is directly coupled to GABA release. Specifically, mGluR1 may depolarize granule cells by an amount sufficient to relieve magnesium blockade of NMDARs and facilitate reciprocal communication via the “classical” ionotropitc pathway. One prediction of this model is that under conditions permissive for NMDA receptor activation, mGluR1 antagonists should be relatively less effective at eliminating spike-evoked recurrent inhibition. To test this, we evoked action potentials (7 spikes at 40 Hz) in mitral cells under low magnesium (0.2 mM) conditions and measured the magnitude of recurrent inhibition before and after addition of LY367385. In agreement with our proposed mechanism, LY did not cause a significant change in recurrent inhibition in 0.2 mM magnesium (Figure 1.7C,D; 76 ± 24% of control post LY) compared to the change observed in 1.0 mM magnesium (Figure 1.7C, Figure 1.1; 28 ± 17% of control post LY, p=0.012, n=6).

I.5 Discussion

I.5.1 Summary of Findings

We report two main findings in this study. First, under physiological conditions, mGluR1 is required for recurrent inhibition of AOB mitral cells. Second, direct activation of group I mGluRs evokes robust GABA-ergic inhibition of mitral cells via a presynaptic mechanism that depends on voltage gated calcium channels.
Metabotropic glutamate receptors are expressed throughout the CNS and couple glutamate binding to a host of changes in the intrinsic and synaptic properties of neurons. These changes include spatially localized increases in intracellular calcium via activation of ryanodine receptors (Takechi et al., 1998; Finch and Augustine, 1998; Chavis et al., 1996; del et al., 1999), up or down-regulation of potassium and calcium conductances (Charpak et al., 1990; Schoppa and Westbrook, 1997; Heinbockel et al., 2006; Chavis et al., 1998; Fagni et al., 2000), and reduction in the strength of electrical synapses (Landisman and Connors, 2005). These cellular responses often serve to regulate network “tone” and modulate the propagation and extent of neural activity. In the main olfactory bulb, activation of mGluR1 is known to increase mitral cell excitability, and contributes a slow, phasic component to the responses of mitral cells to olfactory nerve stimulation (Ennis et al., 2006; Heinbockel et al., 2004; De Saint and Westbrook, 2005).

Much of the interest in metabotropic glutamate receptors in the olfactory bulb stems from their dense expression in this structure, and from reports of their role in regulation AOB-dependent behaviors. Mitral cells express at least four mGluRs including mGluR1, 2, 7, and 8 (Masu et al., 1991; Martin et al., 1992; Shigemoto et al., 1992). mGluR2s have been localized to granule cells and their activation is reported to underlie suppression of granule cell inhibition of mitral cell inhibition that is linked to forms of olfactory memory (Kaba et al., 1994b). Despite the considerable attention that has been given to the role of specific activation of mGluR2 in this form of olfactory memory (Kaba et al., 1994b; Hayashi et al., 1993b) synaptic release of glutamate from mitral cells is likely to activate both class 1 and class 2 mGluRs are thus understanding the role that both these receptor types play in regulation of olfactory bulb neurons is critical. In this regard, our observation that activation of mGluR1 results in effects on granule cell activity and inhibition at reciprocal synapses that oppose the effects reported for mGluR2 activation raises interesting questions about how these receptors influence mitral cell activity.
mGluR1 immunoreactivity in the AOB is concentrated largely in the mitral-tufted cell layer, with less robust staining observed in the granule cell layer (Sahara et al., 2001). Given that we observed granule cell depolarization in response to DHPG (Fig I.7), and that recurrent inhibition was eliminated by an antagonist of mGluR1 but not mGluR5 (Fig I.1), our results are consistent with expression pattern in which mGluR1 is highly localized to granule cell spines contacting mitral cell dendrites.

Our observations that mGluR activation is required for recurrent inhibition is particularly surprising, since experiments in both MOB and AOB have demonstrated that reciprocal inhibition of mitral cells is eliminated by blockade of AMPA and NMDA receptors (NMDARs). Our interpretation of this finding is that activation of mGluR1, by glutamate released from mitral/tufted cells, is necessary to create conditions favorable for dendrodendritic inhibition via the “classical” reciprocal ionotropic pathway — a result supported by the low magnesium experiments of figure I.7. This may result from direct, mGluR dependent depolarization of granule cells, similar to what we observed in figure I.7. With typical magnesium concentrations (our bath solution contained 1 mM Mg$^{2+}$), NMDA receptors in granule cells will be blocked by Mg$^{2+}$, especially at the hyperpolarized resting potentials (~80 mV) typical of these cells. The depolarization of granule cells by mGluR activation may thus be required to relieve this Mg$^{2+}$ block, which is in turn required to elicit GABA release from granule cells.

It is interesting that the direct effects of DHPG (depolarization of granule cells, inconsistent changes in mitral cell holding current) occur on the timescale of minutes, while the synaptic effects of DHPG (increase in mitral cell IPSC rate) occur with considerable delay but rapid onset (~20 seconds). Although we did not test directly how these two effects are interrelated, we offer two speculations on how the timescales can be reconciled. First, assuming that granule cell voltage gates recurrent inhibition by the NMDAR dependent mechanism described in the Results above (Fig I.7C), DHPG evoked IPSCs will not begin until relatively depolarized
potentials are reached. However, given the negative slope conductance of the NMDAR occurring at \(-65\) mV, when these potentials are reached, a large fraction of NMDARs may become available very rapidly – resulting in rapid onset of the increase in spontaneous IPSCs. Another possibility is that the rapid onset of IPSCs may reflect a population-level phenomenon, such as the onset of synchrony among granule cells.

We also observed that direct application of DHPG evoked IPSCs in mitral cells even when excitatory synaptic transmission was blocked (Fig I.3). This observation indicates that activation of mGluR1 by synaptically-released glutamate may result directly in GABA release without requiring activation of AMPA and NMDA receptors. However, bath application of an mGluR agonist is likely to cause stronger mGluR1 activity than glutamate supplied by physiological release from mitral cells. Nevertheless, the results from these agonist experiments are important for understanding the cellular consequences of activating mGluR1. In particular, the results of the experiments shown in figures I.5 and I.6 indicate that activation of group I mGluRs evokes release in a manner that is dependent on voltage gated calcium channels, but independent of sodium spikes and internal calcium stores. These observations suggest that mGluRs are likely to elicit the kind of large depolarization required to open voltage gated calcium channels coupled to release. However, since we observe only \(~7\) mV depolarization in granule cell somatic recordings, it is possible that granule cell dendrites are substantially more depolarized by mGluR activation than are granule cell somata. Alternatively, mGluRs may facilitate activation of calcium channels by voltage independent mechanisms. Several studies have shown that granule cells exhibit low threshold calcium spikes that can be localized to granule cell spines, or can propagate along the granule cell apical dendrite (Egger et al., 2005; Egger et al., 2003). In summary, we propose that mGluR1 activation in granule cells may act in two capacities: first, they may play a facilitating role in recurrent inhibition by partially relieving magnesium block of NMDA receptors, and second, under conditions of high
network activity, they may be capable of evoking release in a manner that is independent of activation of ionotropic glutamate receptors.

A number of studies have examined the extent to which metabotropic glutamate receptors control information throughput in sensory systems – a function sometimes referred to as “gating”. In the thalamus, for example, mGluRs are coupled to presynaptic release from GABA-containing interneurons, and strongly modulate feedforward information flow to higher cortical areas (Cox and Sherman, 2000; Govindaiah and Cox, 2004). Similarly, in the main olfactory system, a presynaptic group II mGluR has been shown to be critical for habituation of the odor-induced heart-rate orienting response (Best et al., 2005). The AOB provides perhaps the most tractable example of a feedforward system in which information flow is controlled by mGluRs. Classically, work has addressed how activation of mGluR2 in granule cell spines results in diminished recurrent inhibition of mitral cells. This reduction in inhibition is believed to be a neural substrate of the Bruce effect – the failure of embryo implantation following exposure of a female rodent to pheromones of non-stud males (Brennan and Keverne, 1997; Hayashi et al., 1993; Kaba et al., 1994). Regardless of the precise mechanisms underlying this behavior, the Bruce effect highlights the importance of controlling the propagation of activity in the AOB, since presumably small subsets of active mitral cells can communicate signals that result in terminated pregnancy. The requirement for mGluR1 in recurrent inhibition observed in the present study suggests that this receptor plays a powerful role in controlling how pheromone detection is coupled to behavioral and endocrine responses.
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