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ROLES OF EMX2 IN ODORANT RECEPTOR GENE EXPRESSION AND OLFACTORY SENSORY NEURON AXON GROWTH

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ABSTRACT OF DISSERTATION

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The Graduate School

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A dissertation submitted in partial fulfillment of the
requirements for the degree of Doctor of Philosophy in the
College of Medicine Department of Physiology
at the University of Kentucky

By
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The sense of smell relies upon the detection of odorants by neurons located in the nasal cavity. These neurons, referred to as olfactory sensory neurons (OSNs), line the olfactory epithelium and extend axons that make synaptic connections with mitral/tufted cells in the olfactory bulb. The mechanisms by which these synaptic connections form remain largely unknown. The development of these synaptic connections relies on the axons of immature OSNs innervating the olfactory bulb. The primary goal of this dissertation was to identify components of the mechanisms used by immature OSN axons to innervate the olfactory bulb. To accomplish this goal, a knockout mouse model was used. OSN axons, of *Emx2* knockout mice fail to innervate the olfactory bulb. As EMX2 is a transcription factor, this model was used investigate the possible causes of the defective OSN axon growth. To gain a better understanding of OSN axon growth, differences in expression of axon growth and guidance genes in immature and mature OSNs was investigated. This analysis revealed that many axon growth and guidance genes are differential expressed, and helped to identify immature OSN specific genes. The data also revealed a previously unrecognized developmental stage, termed nascent OSNs, identified by the expression of *Cxcr4*. Analysis of *Emx2*$^{-/-}$ mice revealed that EMX2 is necessary for OSN survival, odorant receptor expression and expression of the axonogenesis related gene *Ablim1*. EMX2 is necessary for the expression of many odorant receptor genes; however the loss of odorant receptor expression does not explain the axon growth defects. Apoptosis is increased in *Emx2*$^{-/-}$ mice, an outcome that may be due to the failed axon growth. Analysis of axon guidance gene expression identified a large reduction in *Ablim1* expression in *Emx2*$^{-/-}$ mice. *Ablim1* is expressed by immature OSNs, placing it in the proper cell type to regulate OSN axon growth. The loss of *Ablim1* expression in *Emx2*$^{-/-}$ mice indicates defective signaling in the axon growth cone and a possible mechanism regulating OSN axon growth into the olfactory bulb. The data presented in this dissertation provide new insight into the regulation of odorant receptor gene expression and OSN axon growth.

Keywords: Axonogenesis, Growth cone, Odorant receptor, Transcription factor, Axon guidance

Jeremy C. McIntyre

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To my Wife and Children

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Chapter 1

Introduction

Purpose

The primary goal of this dissertation was to identify critical components of the mechanisms by which immature olfactory sensory neuron (OSN) axons grow to the olfactory bulb. The main tool used was the *Emx2* knockout mouse, in which OSN axons fail to innervate their target tissue, the olfactory bulb. The project had three components. The first was to identify differences in expression of axon growth and guidance genes in immature and mature OSNs (Chapter 2). Axon growth requirements differ between these two populations of OSNs so genes expressed specifically in immature OSNs are most likely to be important for the innervation of the olfactory bulb. The second and third components directly investigated potential causes for the defect in OSN axon growth that occurs in the absence of *Emx2*. Chapter 3 addresses the hypothesis that EMX2 is necessary for odorant receptor gene expression. Odorant receptors are critical for OSN axon growth and coalescence into glomeruli. Chapter 4 addresses the development of OSNs in *Emx2*$^{-/-}$ mice and the hypothesis that EMX2 regulates the expression of axon guidance genes, leading to defective axon growth in *Emx2*$^{-/-}$ mice.

Importance of olfaction

The ability to interact with the surrounding world depends on an organism's ability to convert stimuli into neural signals. This is achieved through specialized sensory systems: vision, hearing, smell, taste and touch.

The sense of smell relies on a specialized type of neuron, the olfactory sensory neuron (OSN), to detect environmental chemicals and transmit that information to the olfactory bulb. The sense of smell has some characteristics that set it apart from other senses: 1) it is the only system in which the cell body of the sensory neuron is located in the periphery, has direct contact with the external environment, and also extends an axon into the central nervous system and 2) olfactory sensory neurons are continually replaced. The sense of smell serves to regulate and modulate behavioral responses to

environmental chemicals. Chemical detection is important for recognizing multiple types of hazards, such as spoiled foods, fire, and predators. The sense of smell is also important for individual recognition and social behavior in many animals. Many mating and aggression behaviors rely on the sense of smell. In addition, the sense of smell mediates many feeding behaviors. For example, newborn anosmic mice often starve because the loss of odor input impairs their suckling behavior.

In order to organize sensory information, neurons of the various sensory systems project axons that form topographic maps in the brain. The logic of this process is easy to understand for stimuli that have an inherent spatial dimension. Several sensory systems develop such that the organization of the sensory detector cells in the periphery is directly mapped in the central nervous system. The visual system creates such maps, in which the spatial relationships between neurons in the retina are maintained in their axonal projections to the either the tectum (non-mammalian vertebrates) or the lateral geniculate nucleus and the superior colliculus (mammals) and then relayed to the visual cortex. The axonal connections between the retina and the tectum are well understood. The maintenance of neuronal organization allows retinal images to be recreated directly in the higher areas of the brain and is achieved by specific targeting of retinal axons. In order to explain how the visual map could form, Roger Sperry proposed the chemoaffinity theory (Sperry 1963). In this theory chemical labels mark position across both the retina and the tectum (also known as the superior colliculus in mammals), and axons find their correct position in the tectum based on their position in the retina. The expression of Eph receptors and their binding partners, ephrins, in both the retina and the tectum create such a system (Cheng et al., 1995; Dresher et al., 1995). Eph receptors and ephrins are divided into A and B subfamilies; preferential binding occurs within the families (Klein 2004). In a somewhat simplified explanation, Eph receptors and ephrins are expressed in gradients across the nasal-temporal (A subfamily) and dorsal-ventral (B subfamily) axes of the retina (Braisted et al., 1997; Hindges et al., 2002). Their binding partners are in turn expressed in gradients across the dorsal-ventral and anterior-posterior axes of the tectum. The expression of these molecules in gradients provides specific targeting instructions so that neuronal organization in the retina is maintained in the tectum. Further refinements to this mechanism arise from that fact that both the A and B subfamilies have subtypes

that are also expressed in patterns that contribute to the specificity of axonal targeting. Other non-classical guidance cues, such as Wnt3 and frizzled receptors, also help to refine this map (Schmitt et al., 2005)

The olfactory system, however, does not create a spatially defined topographic map. Physical relationships of neurons located in the olfactory epithelium are not maintained in their projection to the olfactory bulb. Instead, the olfactory map appears to solely represent the quality dimension of the odor stimulus. Axons of OSNs dispersed throughout large portions of the olfactory epithelium coalesce to form the glomeruli of the olfactory bulb (Ressler et al., 1994; Vassar et al., 1994). Axonal coalescence is determined by the identity of the odorant receptor expressed by each OSN. Each OSN expresses only 1 odorant receptor gene (out of the ~1000 odorant receptors contained in the mouse genome), allowing the innervation of each glomerulus to be homogeneous with respect to odorant receptor identity (Mombaerts et al., 1996; Feinstein et al., 2004). This organization has two advantages. First, input signals can be amplified by convergence. Second, the response pattern for each odorant creates a unique "odotopic" map across the population of glomeruli (~1800 in the mouse) (Figure 1.1) (Sharp et al., 1975, 1977; Stewart et al., 1979). That odorants stimulate particular areas of the olfactory bulb reproducibly across individuals has been verified through multiple techniques, including mitral cell recordings (Mori et al., 1992), activation of immediate early genes (Onoda, 1992; Guthrie et al., 1993), optical imaging of either endogenous reporters (Rubin and Katz, 1999; Uchida et al., 2000), or of genetically modified reporters (Bozza et al., 2004; Soucy et al., 2009), and functional magnetic resonance imaging (Yang et al., 1998; Schafer et al., 1996). Limited conservation of the odotopic map has also been observed across species (Johnson et al., 2009; Soucy et al., 2009). However, molecular investigations have revealed that glomerular positions are not fixed; variations are seen in glomerular positioning across individuals (Royal and Key 1999; Schafer et al., 2001; Strotmann et al., 2000). Although glomeruli that respond to certain odorants are located in similar positions in the olfactory bulbs of different animals there appears to be no precise chemotopic organization of the glomeruli in the olfactory bulb. Glomeruli are only roughly organized by the chemical structures of odorants (Mori et al., 2006; Johnson et al., 2009). For example, in rats, glomeruli that respond to aliphatic acids show a dorsal

to ventral progression with respect to increasing numbers of carbons (Johnson et al., 2009). However, glomerular positioning does not necessarily correlate across chemical structures; glomeruli that respond to aldehyde compounds are not segregated from those responding to ketone compounds (Soucy et al., 2009). A caveat to the identification of the "odotopic map" is that most of the underlying experiments were performed in animals that are essentially genetically identical. In a more genetically diverse population the similarity of the odotopic map may not be as robust across individuals. It has been observed that different strains of mice differ in their response patterns to particular odorants (Sicard et al., 1989). Different strains of mice also exhibit differences in the odorant receptors they express (Feinstein and Mombaerts, 2004). Some odorant receptors exhibit amino acid differences between mouse strains, even to the extent that some are not functional in some strains. These natural occurring polymorphisms in odorant receptor identity will give rise to distinct glomeruli, and would therefore cause differences in the glomerular map across genetically diverse animals.

It has been hypothesized that the organization of glomeruli must have some importance; otherwise the similarity of bulbar activity patterns across animals would not be expected if the regional location of glomeruli were not somewhat conserved (Johnson and Leon, 2007). Indeed, the importance of the regional location of glomeruli has been demonstrated for particular behaviors. For example, functional studies have shown that the dorsal domain of the olfactory bulb is responsible for modulating fear responses in mice (Kobayakawa et al., 2007). Genetically modified mice (termed ΔD) were generated in which OSNs in the dorsal region of the olfactory epithelium were ablated, resulting in a loss of glomeruli in the dorsal domains of olfactory bulb (Kobayakawa et al., 2007). When ΔD mice were exposed to the chemical trimethyl-thiazoline, derived from fox anal glands, they did not show the fear responses seen in wild-type mice. Further testing showed that the ΔD mice were able to detect and discriminate trimethyl-thiazoline and were able to learn to avoid it (Kobayakawa et al. 2007). This work shows that the olfactory bulb may have two modalities, one that drives associative/discrimination abilities and one regulating innate behaviors. The activation of glomeruli in particular domains of the olfactory bulb may regulate innate behaviors through genetically programmed neural circuits connecting to higher brain regions.

The development of the olfactory system is of vital importance to the formation of a functional odotopic map. To create a functional map, OSN axons must grow out of the olfactory epithelium, turn and course through a mesenchymal layer, cross the cribriform plate of the skull, travel across the surface of the bulb, and make synaptic connections with dendrites of mitral tufted projection neurons and periglomerular interneurons of the olfactory bulb. Like all projection neurons, OSN axons must find the correct target, foregoing inappropriate locations via recognition of positive and negative cues in the surrounding environment (Tessier-Lavigne and Goodman, 1996). In order to achieve the correct synaptic connections, OSN axons employ a network of signaling molecules acting to regulate the guiding tip of growing axons, termed the growth cone (Forscher and Smith, 1988). Gene expression by OSNs therefore plays a critical role in determining the responses of OSN axons to guidance cues. The molecular mechanisms used by OSNs share common elements with other types of neurons, but also contain elements unique to OSNs. Most neurons, including OSNs, rely on guidance cues, either classical or non-classical, to guide the growing axons to their target tissues. Additionally, neuronal activity is important for maintaining synaptic connections. However, OSNs have a very unique component regulating axon growth, the odorant receptor. The development and maintenance of the glomeruli in the olfactory bulb is dependent on all of these components.

Classical and non-classical axon guidance

Over 100 years ago, Ramon y Cajal described the axonal growth cone and used the terms chemotaxis and chemotropism to describe axon growth. Since then research has confirmed Cajal's descriptions and shown that axon guidance involves the coordination of both short-range and long-range chemical cues that can act as either attractants or repellents (Sperry, 1963, Tessier-Lavigne and Goodman, 1996). Long-range cues are secreted, diffusible cues, while short-range cues are membrane bound, either to other cells or to an extracellular matrix. Repulsive cues lead to destabilization of the actin network and collapse of the growth cone, while attractive cues stabilize and promote actin tread-milling, causing the growth cone membrane to extend the axon (Chisholm and Tessier-Lavigne, 1999). During growth axons respond to multiple types of guidance cues

and by integrating the different cues can grow over long distances to the correct target. For example, a long-range repellent can "push" the axon from behind through a corridor that is marked by a short-range attractant. Local repellents around the permissive corridor serve to keep the axon in the corridor while a long-range attractant at the end "pulls" the axon through (Tessier-Lavigne and Goodman, 1996). The ability to respond to extracellular guidance cues is driven by the types of receptors that each neuron expresses. Research on axon growth has identified four groups of extracellular cues and receptors considered to be the "classical" guidance cues. (1) The semaphorin family consists of several related proteins that typically function as repulsive cues and can either be secreted or membrane bound (Luo et al., 1993; Chedotal et al., 1998; Raper, 2000). Membrane bound semaphorins bind to a family of receptors called plexins, while secreted semaphorins bind to neuropilin receptors in complex with plexin receptors (Chen et al., 1997; Nakamura et al., 1998; Tamagnone et al., 1999). (2) Netrins are secreted signals that can be either attractive or repulsive (Serafini et al., 1994; Mitchell et al., 1996). The attractive effects of netrins are mediated by Dcc receptors, while repulsive effects typically occur through netrin binding to the Unc5 family of receptors (Leonardo et al., 1997; Hong et al., 1999). (3) Slits are secreted repulsive cues that bind to the ROBO receptors (Kidd et al., 1998; Brose et al., 1999; Nguyen Ba-Charvet et al., 1999). (4) Finally, Ephrins and Eph receptors are membrane bound guidance cues that typically mediate growth cone collapse through contact repulsion but can also act as cell adhesion molecules (Holmberg et al., 2000; McLaughlin et al., 2003; Fuller et al., 2003; Klein 2004). While each of these guidance cues typically acts in the fashion described, many also have been shown to mediate the opposite effect under certain conditions. Regulation of targeted axon growth is not limited to these classical guidance cues. While these cues were among the first identified, research on both *in vivo* and *in vitro* axon growth implicates several other types of molecules. Cell adhesion molecules, neurotrophic factors, morphogens, and Wnts have all been shown to function as guidance cues (Charron and Tessier-Lavigne, 2005).

The signaling pathways that link guidance cue receptors with cytoskeletal rearrangement in axon growth cones converge on common mechanisms. The canonical signaling of most guidance cue receptors is through regulation of monomeric G-protein

signaling. In general, ligand binding that stimulates receptor activation of Rac and Cdc42 GTPases or inhibits RhoA GTPases produces axonal outgrowth and attraction (Kozma et al., 1997, Liu and Strittmatter, 2001, Hu et al., 2001). Cues acting in the opposite fashion typically promote repulsive or growth inhibiting affects. For example, semaphorin binding to Plexin B receptors directly inhibits Rac and activates RhoA leading to growth cone collapse (Hu et al., 2001). Slit repulsion of axons occurs through Robo receptors in part by reducing Cdc42 activity (Wong et al., 2001). Receptor activation can also activate adaptor proteins that then interact with GTPases. For example, binding of EphA receptors activates the adaptor protein, ephexin, which in turn activates RhoA (Shamah et al., 2001). Receptor regulation of GTPases controls cytoskeleton dynamics in the growth cone, causing attraction by extension of the membrane, or repulsion through growth cone collapse.

The signaling network necessary to control the actin and microtubule network is quite extensive. The GTPases are important signaling molecules; however, they do not directly alter actin and microtubule dynamics. Instead the GTPases activate or inactivate downstream kinases, such as myosin light chain kinase, LIM kinase and Rho-associated kinase (Edwards et al., 1999; Sanders et al., 1999). These kinases in turn act on proteins that affect myosin and actin dynamics such as actin related protein 2/3, myosin regulatory light chain, cofilin, gelsolin, and collapsin response mediator proteins (Patel and Van Vactor 2002). By regulating actin-binding proteins, the stability of actin in the growth cone can be altered to either promote extension or collapse. Decreases in retrograde actin flow, decreased depolymerization, and increased actin nucleation all lead to growth cone extension. Increased retrograde flow, increased depolymerization and decreased nucleation all lead to growth cone collapse (Patel and Van Vactor, 2002). Axon growth is therefore the result of integrating multiple guidance cue signals into a summation of cytoskeletal extension and retraction that determines the direction and speed of growth.

The actin network is not the only cytoskeletal element that determines axon growth. Changes in microtubule dynamics in the growth cone also regulate axon growth. Microtubules project from the axon shaft to the central domain of the growth cone and into the actin network of the growth cone where they support axon extension (Zhou and Cohan, 2004). Attractive guidance cues that promote axon turning often do so by

stabilizing the microtubule network. This leads to actin stabilization on the side of the growth cone nearest the guidance cue while the far side is still actively growing. The difference in actin dynamics across the growth cone results in turning towards the guidance cue (Buck and Zheng, 2002; Gordon-Weeks, 2004). Repulsive guidance cues work in the opposite fashion, leading to the local destabilization of microtubules and resulting in growth cones turning away from the cue (Challacombe et al., 1997; Williamson et al., 1996).

Olfactory sensory neuron development and axon growth

The olfactory epithelium is a pseudostratified tissue containing neurons, multipotent progenitor cells, and supporting cells. This organization provides for the continuous replacement of OSNs, which have a short life span, presumably due to their exposure to damaging agents that enter the nasal cavity. The OSN is therefore an advantageous model of the transition between immature and mature neurons because both are always present. Additionally, the processes of axonal growth and guidance can be studied in adult animals, as newly born neurons must extend axons that innervate the correct target in order to maintain the odor quality map across the glomeruli of the olfactory bulb. Most of the events that occur during adult OSN neurogenesis likely recapitulate development. The hypothesis that some events may be unique to axon growth in the adult tissue environment is as yet unproven.

The pseudostratification of the olfactory epithelium also allows for identification of the different cells types by their position in the epithelium and expression of cell type specific markers (Figure 1.2). Located against the basal membrane are the horizontal basal cells, which include the most primitive population of progenitor cells. These cells express Keratins 5 and 14 and are characterized by slow turnover rates (Carter et al., 2004; Leung et al., 2007). Above them lie the globose basal cells, a heterogeneous population that contains at least two stages of progenitor cells, the transit amplifying cells and the immediate neuronal precursor cells (Caggiano et al., 1994, Cau et al., 2002). The transit amplifying cells can be identified by the expression of Ascl1 (Mash1) while the immediate neuronal progenitors can be identified by the expression of Neurog1 (Ngn1). Globose basal cells can also be identified by the expression of Ccnd1, a marker for

proliferating cells. The progenitor cells give rise to immature OSNs, identified by their expression of Gap43 (Verhaagen et al., 1989; Huard et al., 1998). It is not known if the immediate neuronal precursor cells undergo cell division before differentiating into OSNs. Immature OSNs are very abundant in both the embryonic and regenerating adult olfactory epithelium (Verhaagen et al., 1990, Schwob et al., 1995). In contrast, mature OSNs, identified by the expression of olfactory marker protein (OMP), predominate in undamaged adult olfactory epithelium.

During development the olfactory placode invaginates to form the olfactory pit (Cuschieri and Bannister, 1975). The olfactory epithelium forms from the olfactory pit. OSNs begin to be produced around embryonic day 9 (E9) and the first axons leave the olfactory pit at E10 (Hinds, 1972). These pioneer axons grow through the mesenchyme between the olfactory pit and the presumptive olfactory bulb. At E11 these pioneer axons reach the rostral telencephalon, the area that will become the olfactory bulb. When OSN axons first reach the rostral telencephalon their growth pauses until E12 when the axons begin to penetrate the basal lamina surrounding the forming olfactory bulb (Hinds, 1972; Gong and Shipley, 1995; Treloar et al., 1996). This pause in axon growth may be analogous to delays seen in other neural tissues, such as the dorsal root entry zone where dorsal root ganglion axons pause before entering the dorsal mantle layer (Pindzola et al., 1993; Watanabe et al., 2006). Within the dorsal spinal cord, the bi-functional axon guidance cue NETRIN 1 inhibits DRG axons early in development and generates the waiting period. As the early OSN axons penetrate this basal lamina they begin to grow around the entire surface of the bulb, forming the outer olfactory nerve layer. When they near the region where they will form a glomerulus, OSN axons grow deeper into the bulb and form the inner olfactory nerve layer. The first synapses become visible at E15, with the emergence of proto-glomeruli seen around E16 (Treloar et al., 1999; Shay et al., 2008). While glomeruli begin to develop embryonically, glomerular structure and homogeneity is not fully mature until several weeks after birth (Royal and Key, 1999).

The formation and maintenance of these precise OSN axon projection patterns is a complex process utilizing several different mechanisms. Several studies have focused on the effects of classical guidance cues by using targeted gene deletions in mice. Thus far, these studies have not identified any single cue solely responsible for innervation of the

olfactory bulb or glomerular formation. However, several of the cues appear to regulate positioning of certain glomeruli or innervation of regions of the bulb. Semaphorins are an example. Targeted deletions of several semaphorin and neuropilin genes result in aberrant growth of some OSN axons into ventral regions of the olfactory bulb. Semaphorins also appear to restrict axon growth to the glomerular layer, as an increased number of axons grow deeper into the olfactory bulb in knockout animals (Schwarting et al., 2000, Walz et al., 2002; Cloutier et al., 2002; Cloutier et al., 2004; Schwarting et al., 2004). Another example is the Eph receptors (Eph) and ephrins (Efn). Targeted deletions of *EfnA5* and *EfnA3* lead to a posterior shift in a subpopulation of glomeruli. Inversely, the overexpression of *EfnA5* leads to an anterior shift in glomerular position (Cutforth et al., 2003). Slit signaling also has a role in OSN axon growth. Deletion of *Slit1* or its receptor, *Robo2*, causes a subset of OSN axons that normally innervate the dorsal olfactory bulb to form glomeruli in the ventral olfactory bulb instead (Cho et al., 2007). Studies with targeted deletions of cell adhesion molecules, including *Ncam, Ocam*, and *Cntn4*, also show minimal changes in glomerulus formation (Treloar et al., 1997; Montag-Sallaz et al., 2002; Walz et al., 2006; Kaneko-Goto et al., 2008). The non-classical guidance cue, insulin-like growth factor (IGF), has a broader role in the innervation of the lateral olfactory bulb. Targeted deletion of the insulin-like growth factor 1 receptor, expressed by OSNs, resulted in the loss of innervation of the lateral olfactory bulb (Scolnick et al., 2008). Double-targeted deletions of both insulin-like growth factor 1 and insulin-like growth factor 2, expressed by the olfactory bulb, resulted in a similar phenotype. Guidance cues regulating innervation of the dorsal or medial olfactory bulb have not yet been identified. Glomerular homogeneity of axonal convergence in mice lacking guidance cue receptors was normal in all cases investigated thus far. Taken together, these experiments suggest that multiple types of guidance cues play a role in forming the odotopic map and may be important for establishing regions to which OSNs axons target. The data do not, however, reveal any roles for guidance cues in the homogeneity of OSN axon coalescence or the ordering of neighbor relationships between glomeruli.

Odorant receptors regulate axon growth

OSN axon behavior is also regulated by components of the odorant signal transduction pathway. The first piece shown to be crucial for axon coalescence and glomerular position was the odorant receptor itself (Mombaerts et al., 1996). The first experiments to show this used a series of gene swaps where the coding sequence of one odorant receptor replaces the coding sequence of a different odorant receptor. In these experiments, OSNs expressing the donor odorant receptor from the host receptor locus did not coalesce with OSNs expressing either the donor odorant receptor or the host odorant receptor from their endogenous loci, but rather they coalesced into a novel glomerulus (Mombaerts et al., 1996, Feinstein and Mombaerts, 2004, Feinstein et al., 2004). These data imply that other factors, such as OSN position, amount of odorant receptor protein and onset of odorant receptor expression, work along with odorant receptor identity to regulate glomerulus formation (Feinstein and Mombaerts 2004, Mombaerts 2006).

Though odorant receptors have an important role in the coalescence of OSN axons into glomeruli, odor-stimulated electrical activity does not. The absence of the guanine nucleotide binding protein GNAL (also known as G_{olf}) or the cyclic nucleotide gated channel subunit CNGA2 prevents odor-stimulated electrical activity in OSNs, but does not prevent glomerulus formation. These studies provided support for the idea that glomerular formation does not depend on odor-evoked electrical activity of OSNs (Belluscio et al., 1998; Lin et al., 2000; Zheng et al., 2000). However, cAMP production in OSN axons does appear to be important for glomerular position and homogeneity. During odorant stimulation, odorant receptors activate GNAL and stimulate cAMP production through adenylate cyclase type 3 (ADCY3). Targeted deletions of *Adcy3* severely disrupted glomerular development, suggesting that the generation of cAMP by ADCY3 is a major component directing OSN axon growth. If deletion of *Gnal* does not disrupt glomerulus formation, how then can odorant receptor-stimulated cAMP production regulate axon growth? A second type of G-protein a subunit is also capable of coupling odorant receptors to adenylate cyclases (Katade et al., 2004). This subunit, *Gnas*, is expressed at high levels in the olfactory epithelium during development, largely

because it is expressed abundantly in immature OSNs. It appears that GNAS signaling downstream of the odorant receptors during axon growth explains why the loss of Gnal has minimal effects on axon guidance (Zou et al., 2009). The mechanism of activation of the odorant receptors during development and in OSN axons is unknown. One hypothesis is that odorant receptors have different levels of constitutive activity, thereby creating different amounts of cAMP in subtypes of OSNs (Imai et al., 2006; Chesler et al., 2007; Col et al., 2007; Zou et al., 2007). One proposed mechanism for the action of cAMP is through transcriptional regulation. Different levels of cAMP within groups of neurons have been linked to specific levels of guidance cue gene expression (Imai et al., 2009). One of these genes linked to cAMP, *Nrp1*, appears to regulate glomerular positioning along the anterior-posterior axis of the olfactory bulb (Imai et al., 2006, Imai et al., 2009). Expression of other axon guidance molecules such as, *Plxna1, Kirrel2, Kirrel3, Cntn4, EphA5*, and *EfnA5* have also been linked to odorant receptor activity and cAMP stimulation (Imai at al., 2006; Col et al., 2007; Imai and Sakano 2008; Serizawa et al., 2006; Kaneko et al, 2008; Imai et al., 2009). Comparisons of mRNA abundance levels between OSNs expressing an odorant receptor that cannot stimulate heterotrimeric G-proteins with OSNs expressing a constitutively active GNAS protein reveal differential expression of axon guidance genes between the those two groups of OSNs (Imai et al., 2009). Some axon guidance genes were preferentially expressed in cells with high cAMP levels, while others were expressed in cells with low cAMP. Mechanistically, this system relies on the odorant receptors displaying different levels of activity, which has not been conclusively shown. However, differential amounts of axon guidance gene expression in response to either high or low cAMP would provide a broad control mechanism for odorant receptor-mediated growth of OSN axons. Supporting the hypothesis that cAMP generated from odorant receptors regulates axon growth, genetically reduced expression of an odorant receptor in a subset of OSNs caused their axons to form novel glomeruli that were homogenous and distinct from glomeruli formed by axons of the same odorant receptor expressed at normal levels (Feinstein et al., 2004). Reducing the amount of an odorant receptor in an OSN presumably reduced the amount of cAMP so this phenomenon could be consistent with the hypothesis that the level of cAMP in OSN axons helps determine their glomerular target.

Changes in gene expression may not be the only method by which odorant receptors and cAMP direct OSN axon growth. In general, cAMP is itself a potent stimulator of axon extension and growth cone turning (Johnson et al., 1988; Song et al., 1997). Signaling events that increase cAMP are also able to modulate responses to guidance cues (Chalasani et al., 2003). For example, increases in cAMP are able to convert the usually repulsive semaphorin signal into an attractive signal. Other possible mechanisms whereby OSN axon behavior is controlled by odorant receptors via mechanisms that do not involve cAMP signaling have not yet been disproved (Feinstein and Mombaerts, 2004). The role of odorant receptors and cAMP in regulating gene expression does not exclude guidance cues from have direct roles in controlling OSN axon behavior. For example, in a combined hypothetical model odorant receptors may exhibit different levels of activity producing different levels of cAMP that regulates differential axon guidance gene expression across the OSN population. Differences in axon guidance gene expression establish gradients of responsiveness to guidance cues, thereby targeting axons to broad regions of the olfactory bulb. Once the axons reach the correct area of the olfactory bulb, odorant receptor signaling (either directly or through cAMP) in the growth cone and axon drives axonal coalescence. Defects in axonal coalescence lead to the formation of heterogeneous glomeruli, i.e. different axon populations coalescing within a glomerulus (Feinstein and Mombaerts, 1994; Col et al., 2007; Zou et al., 2007).

Neuronal activity and glomerular maintenance

Does neural activity play no role in glomerular formation? Earlier studies with targeted deletions that blocked odorant-evoked action potentials found no defects in glomerular formation (Lin et al., 2000). A loss of odorant-evoked action potentials, however, does not necessarily mean that OSNs axons cannot transmit signals across their synapses. To address this issue, genetically modified mice in which tetanus toxin light chain, which blocks synaptic release, was expressed in OSNs, were developed (Yu et al., 2004). The *Omp* promoter was used to drive expression of this toxin in all OSNs. In a second experiment the promoter of the odorant receptor *Olfr17* was used to drive expression of the toxin only in a subset of OSNs. Blocking synaptic release in all OSNs had no effect

on glomerular formation. In contrast, when synaptic release was blocked only in OSNs expressing *Olfr17*, the Olfr17 glomeruli developed normally but disappeared with age (Yu et al., 2004).

In a second mouse model OSNs were silenced by expressing the inward rectifying potassium channel KIR2.1 (Yu et al, 2004). Overexpression of the KIR2.1 channel hyperpolarizes the neurons and prevents the firing of both odor-evoked and spontaneous action potentials (Ehrengruber et al., 1997; Johns et al., 1999; Yu et al., 2004). This technique addressed the importance of a more cell autonomous effect of neuronal activity on axonal growth. Mice overexpressing KIR2.1 in all OSNs exhibited a delay in axon innervation of the olfactory bulb along with decreased innervation of the dorsal bulb of adult animals (Yu et al., 2004). Overexpression of KIR2.1 in a subset of OSNs also affected glomerular formation and maintenance. *Olfr17* neurons expressing KIR2.1 failed to enter the olfactory bulb and form glomeruli during development. Specific overexpression of KIR2.1 in *Olfr17* neurons after development also resulted in the disappearance of the *Olfr17* glomerulus with age (Yu et al., 2004). These data also support a hypothesis that neural activity may be important within OSNs as it may help set the expression levels of axon guidance genes. This effect of neuronal activity is seen in other neural systems as well (West et al., 2001; Hanson and Landmesser, 2004; Jassen et al., 2006).

These data show that synaptic release is not necessary for development of glomeruli in either a non-competitive (all OSNs silenced), or competitive (specific OSNs silenced) environment. However, glomerular maintenance in a competitive environment depends on activity. In other words, there is activity-dependent competition between OSNs for space in the glomerular layer of the olfactory bulb that acts to refine the odotopic map. This mechanism is reminiscent of the activity dependence needed for map refinement and synapse maintenance common to other areas of the brain (Meister et al., 1991; Feller et al., 1996; Ruthazer et al., 2003; Hua et al, 2005; Zhang and Poo, 2001).

A unique type of map

As previously mentioned, neural maps can be classified into two categories. (1) Continuous maps are those in which the physical relationships of sensory cells in the

periphery are maintained in the CNS. The retinotopic map is a classic and well-studied continuous map. (2) Discrete maps are those in which the spatial organization in the target field represents discrete qualities of stimuli and not the spatial organization of the receptive field. Both the olfactory and taste systems generate discrete maps in the brain. The development of the odotopic map has several features that distinguish it from continuous map development. For example, development of the retinotopic map relies on target-derived expression of a gradient of guidance cues. Axons extend from the retina to specific location-dependent regions in the tectum; neurons located in the nasal retina project axons to the posterior tectum, while neurons in the temporal retina project axons to the anterior tectum. The growth of these axons is dependent on the target-derived expression of eph receptors and ephrins. The odotopic map differs in that the target tissue does not generate the glomerular structures. Glomeruli do not exist before innervation and are not specific targets for OSN axons. Rather than converge onto a target (a glomerulus), OSN axons coalesce to form a glomerulus whose location does not appear to stipulated by the target tissue other than it must occur in the glomerular layer. In fact, OSN axons are able to coalesce and form glomeruli in the absence of their synaptic targets, either the mitral-tufted cells or the local interneurons (Bulfone et al., 1998). The ability of OSN axons to regulate coalescence is even more dramatically demonstrated by the finding that OSN axons segregate by general type and even form odorant receptor-specific proto-glomeruli in the complete absence of the olfactory bulb (St John et al., 2003; Imai et al. 2009).

Regulation of odorant receptor gene expression

The odorant receptor gene family is the largest contained in mammalian genomes, with ~1000 and ~350 functional genes in rodents and humans, respectively (Buck and Axel, 1991; Firestein, 2001; Rouquier and Giorgi, 2007). An individual OSN only expresses one allele of one odorant receptor gene (Chess et al., 1994; Strotmann et al., 2000; Ishii et al, 2001). Additionally, odorant receptors are only expressed in restricted regions of the olfactory epithelium along the dorsomedial-ventrolateral axis (Ressler et al., 1993; Vassar et al., 1993; Kubick et al., 1997 Miyamichi et al., 2005). These regions are referred to as odorant receptor expression zones. Once a functional odorant receptor is selected,

expression of other odorant receptors appears to be silenced through a negative feedback signal (Feinstein et al., 2004; Lewcock and Reed, 2004; Serizawa et al., 2003; Shykind et al., 2004).

How odorant receptor gene choice is achieved is unknown. Early mechanistic hypotheses that proposed DNA re-arrangement or the use of a single control element now seem unlikely. The cloning of mice by transfer of mature OSN nuclei produced animals that expressed the full complement of odorant receptors (Eggan et al., 2004; Li et al., 2004). This result argues that singularity of odorant receptor expression is not achieved through DNA re-arrangement. A unique, conserved element, termed the H-region, was found on chromosome 14 that regulated the expression of a cluster of odorant receptor genes located 75 kb away (Serizawa et al., 2003). This element was proposed to regulate expression of all odorant receptors by acting in trans on odorant receptor genes located on other chromosomes (Lomvardas et al., 2006). However, targeted deletion of the mouse H-region only affected the expression of the odorant receptor genes located closest to it on chromosome 14 (Fuss et al., 2007). While a single region now seems unlikely to control expression of all odorant receptors, it is possible that multiple H-like domains that control expression of clusters of odorant receptor genes exist. At least one other cryptic or displaced odorant receptor gene control region has been found in the mouse genome (Bozza et al., 2009).

Putative odorant receptor promoters are located immediately upstream of the transcriptional start site of odorant receptor genes. The majority of these putative promoters contain both homeodomain and Olf-1/Early B-cell factor (O/E)-like transcription factor binding sites. O/E-like sites bind the Ebf family of transcription factors, which have been shown to regulate olfactory specific expression of other genes, including *Omp* and *Adcy3*. Several homeobox transcription factors are able to bind to putative odorant receptor promoters, including one, LHX2, which may regulate expression of some odorant receptors (Hirota 2004, 2007; Hoppe et al., 2006; Kolterud et al., 2007). Mutation or deletion of one or both of these sites in the putative odorant receptor promoter abolished expression of *Olfr151* (M71) from transgenes, while the same mutations in the endogenous promoter region reduced *Olfr151* expression three-fold (Rothman et al., 2005). While other factors are likely involved, the *in silico*

prediction of putative promoters appears to have been successful in identifying sites important for regulating odorant receptor gene expression (Michaloski et al., 2006).

Defective OSN axon growth

The growth of OSN axons through the basal lamina of the olfactory bulb is a critical step in the development of the olfactory system. The molecular mechanisms underlying OSN axon growth into the developing olfactory bulb are unknown. However, several transcriptions factors appear to regulate axon growth into the olfactory bulb. Targeted deletions of *Dlx5, Fezf1, Klf7, Arx* or *Emx2* all cause OSN axons to fail to innervate the olfactory bulb (Yoshida et al., 1997; Levi et al., 2003; Long et al., 2003; Yoshihara et al., 2005; Hirata et al., 2006; Laub et al., 2006). *Dlx5, Fezf1, Klf7* and *Emx2* are expressed in the olfactory epithelium, mainly in immediate neuronal precursor cells and immature OSNs. *Klf7* and *Dlx5* are also expressed in the olfactory bulb, but *Fezf1* is not and *Emx2* is expressed in the bulb only transiently during early development. *Arx*, which is expressed in the olfactory bulb but not in the olfactory epithelium, produces the same phenotype when it is deleted. The evidence that defects in either the OSNs or the bulb yield similar phenotypes gives rise to the hypothesis that these transcription factors regulate expression of a signaling pathway between the olfactory bulb and OSN axons.

The role of *Emx2* in development

EMX2 is a homeobox transcription factor first identified in Drosophila. Homeobox transcription factors are typically important for body segmentation. The *Drosophila* gene, *empty spiracles* (*ems*), was found to regulate development of the head and antennal structures of the embryonic fly (Walldorf and Gehring, 1992). In postembryonic flies ems has been shown to be a critical factor for olfactory projection neuron development. *Drosophila* lacking *ems* fail to develop the normal number of lateral projection neurons, while anteriodorsal projection neurons show dendritic targeting defects such as failing to innervate the correct glomeruli (Lichtneckert et al., 2008). In the mammalian nervous system, *Emx2* expression is largely restricted to the forebrain. Both progenitor cells and post-mitotic neurons express *Emx2*. Targeted deletions of mouse *Emx2* result in widespread defects in development of several organs systems and homozygous knockout

animals die shortly after birth (Pellegrini et al., 1996, Yoshida et al., 1997). In brains of *Emx2* knockout (*Emx2*$^{-/-}$) mice the medial limbic cortex and the hippocampus are reduced, and the dentate gyrus is absent (Pellegrini et al., 1996, Yoshida et al., 1997). In addition, the axonal projections of several types of neurons are altered. Axons projecting from the entorhinal cortex are properly oriented towards the dentate gyrus; however, after crossing the hippocampal fissure they fail to exhibit their normal laminar distribution (Savaskan et al., 2002).

Summary

Investigation of axon growth and guidance cue gene expression in OSNs revealed that most of these genes are differentially expressed in immature and mature OSNs (Chapter 2). In fact, these data revealed a previously unrecognized developmental stage consisting of nascent immature OSNs defined by expression of *Cxcr4*, a chemokine receptor that regulates axon growth (Chapter 2). EMX2 proved to stimulate expression of the majority of odorant receptor genes, but this could not explain the defect in OSN axon growth in *Emx2*$^{-/-}$ mice (Chapter 3). EMX2 proved to be necessary for the survival of mature OSNs, but not proliferation of new OSNs (Chapter 4). The abundance of *Ablim1*, an axonogenesis related mRNA, was greatly reduced in *Emx2*$^{-/-}$ immature OSNs. The loss of *Ablim1* implies defective signaling in the growth cone and therefore provides a probable explanation for the inability of *Emx2*$^{-/-}$ deficient axons to innervate the olfactory bulb (Chapter 4).

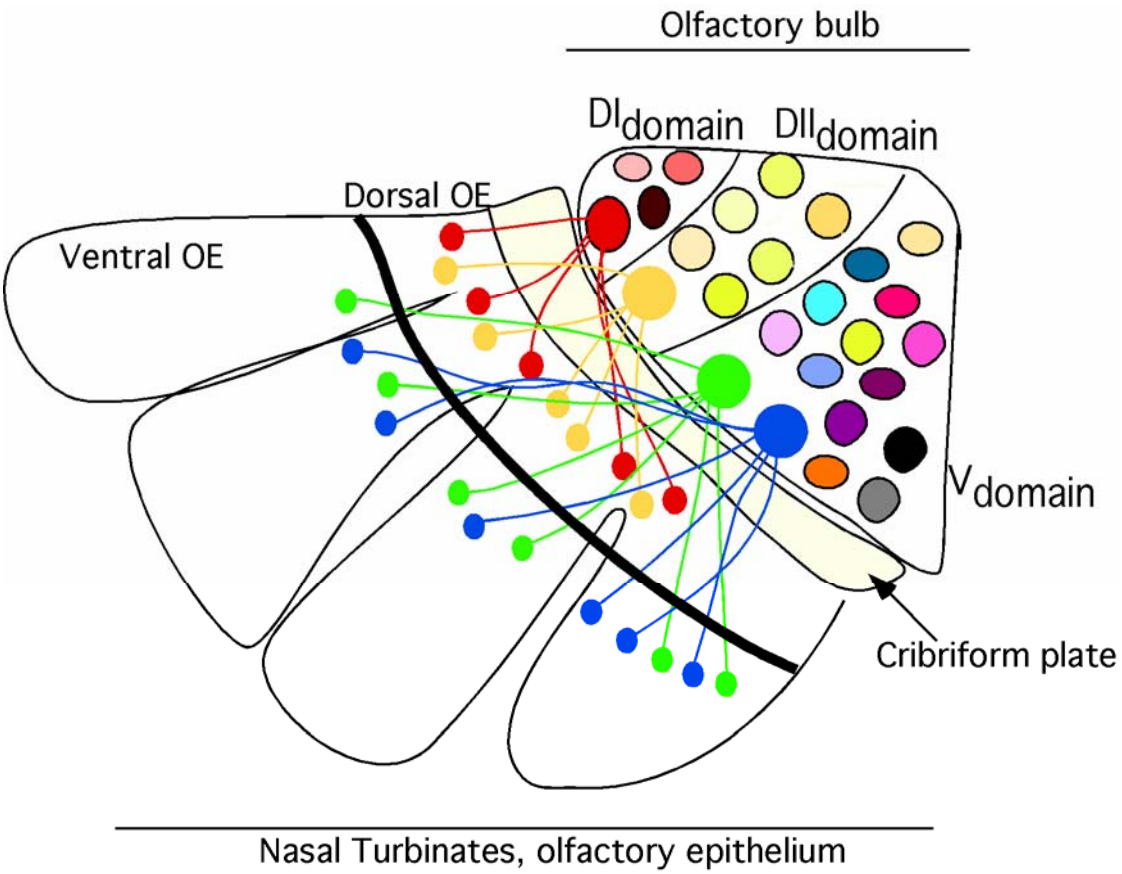

Figure 1.1 Organization of the glomerular map, in sagittal view

OSNs expressing different odorant receptors are distributed throughout broad zones in the olfactory epithelium creating ~1000 subpopulations of OSNs in inbred mice. Four populations, red, yellow, green and blue, represent this organization here. While the neurons expressing a given odorant receptor are scattered throughout the epithelium their axons coalesce into odorant-specific formations, termed glomeruli, where the axons form synapses with both projection neurons and interneurons of the olfactory bulb. OSNs in the dorsal epithelium (red, yellow), project axons to the dorsal olfactory bulb (DI and DII domains), while OSNs in the ventral epithelium (green, blue) project axons to the ventral bulb. Within the dorsal olfactory epithelium OSNs expressing Class I odorant receptors (red) and Class II odorant receptors (yellow) are intermixed even though their glomeruli are not. OSNs expressing Class I odorant receptors (red) project axons to the DI domain and OSNs expressing Class II odorant receptors (yellow) OSNs project axons to the DII domain. OE, olfactory epithelium

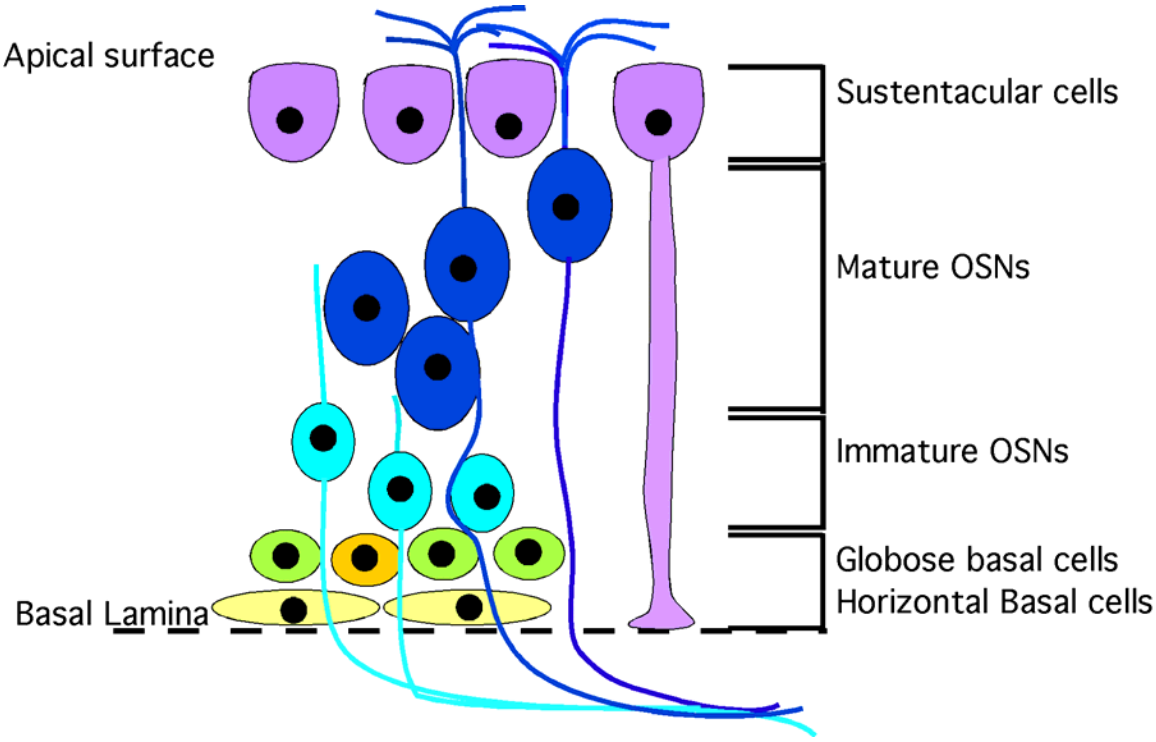

Figure 1.2 Schematic of the olfactory epithelium

The olfactory epithelium is pseudostratified, and cell types can be identified by cell body location and specific markers. Horizontal basal cells express *Keratin5* and *Keratin14*. Globose basal cells (yellow) are a heterogeneous population. Transit amplifying cells (orange) are *Ascl1* positive, while immediate neuronal precursors (green) are *Neurog1* positive. Immature OSNs (light blue) are situated more apically, and are *Gap43* positive. Mature OSNs (dark blue) are the most prevalent cell type in the normal adult epithelium, marked by expression of *Omp*. The most apically located cell bodies are the sustentacular cells (purple), which extend processes to the basal lamina. Sustentacular cells can be identified by expression of cytochrome P450 genes such as *Cyp2g1*.

Chapter 2

Axon growth and guidance genes identify nascent, immature, and mature olfactory sensory neurons

INTRODUCTION

The major task of neural development is to generate the synaptic circuits that provide the basis for the complex functions of the nervous system. Most neurons extend axons that grow to appropriate targets via recognition of positive and negative cues in the surrounding environment (Tessier-Lavigne and Goodman, 1996). As a neuron matures the shift from axon elongation to axon homeostasis is reflected by changes in gene transcription (Skene and Willard, 1981a.b; Li et al., 1995; Smith and Skene, 1997; Blackmore and Letourneau, 2006). Expression of genes associated with axon outgrowth decreases while expression of genes involved in growth inhibition increases. To assess the changes in guidance cue signaling between immature and mature neurons I compared the expression of a large number of axonal growth and guidance genes in olfactory sensory neurons (OSNs).

The synaptic targets of OSNs are the dendrites of projection neurons and interneurons in the glomeruli of the olfactory bulb (Pinching and Powell, 1971; Royet et al., 1988). Glomeruli have specific identities and locations, defined by the innervation of each glomerulus solely by the axons of OSNs expressing the same odorant receptor, but the process is not fully understood (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al 1996; Strotmann et al., 2000; Schaefer et al., 2001; Kobayakawa et al., 2007; Soucy et al., 2009). Studies of mice with targeted deletions of single classical guidance cues or cell adhesion molecules have not revealed major defects in glomerular formation or location (Treloar et al., 1997; Cloutier et al., 2002; Montag-Sallaz et al., 2002; Schwarting et al., 2000; Walz et al., 2002; Cutforth et al., 2003; Cloutier et al., 2004; Schwarting et al., 2004; Walz et al., 2006; Cho et al., 2007; Hasegawa et al., 2008; Kaneko-Goto et al., 2008). These experiments suggest that classical guidance cues may be important for guiding axons to regions of the bulb and restricting axon growth to the glomerular layer,

but do not yet show that these cues determine the fine-scale positioning of glomeruli. Odorant receptor-mediated signaling and neuronal activity are alternative mechanisms for determining glomerular location. Odorant receptor identity itself is a crucial component of axon convergence into glomeruli and the precise location of glomeruli (Mombaerts et al., 1996; Feinstein and Mombaerts; 2004; Feinstein et al., 2004). Glomerular position and homogeneity of glomerular innervation appear to depend on cAMP levels and the activation of GNAS and ADCY3 located in OSN axons (Belluscio et al., 1998; Lin et al., 2000; Zheng et al., 2000; Yu et al., 2004; Imai et al., 2006; Chesler et al., 2007; Col et al., 2007; Zou et al., 2007). Odorant receptor-mediated cAMP signaling regulates the expression of some axon guidance and cell adhesion molecule genes affecting axonal pre-target sorting, glomerulus formation and glomerulus positioning (Imai et al., 2006; 2009; Serizawa et al., 2006; Kaneko-Goto et al., 2008).

The diversity and complexity of potential mechanisms regulating the growth of OSN axons argues for a more complete understanding of axon growth and guidance genes expressed by immature and mature OSNs. Recent evidence indicates that OSNs express several hundred genes related to axon growth and guidance (Sammeta et al., 2007). I hypothesized that many of these genes are differentially expressed between immature and mature OSNs. Distinguishing the axon guidance capabilities of immature and mature OSNs will help identify mechanisms of OSN axon growth and maintenance. Herein I demonstrate differences in the abundance of axon growth and guidance mRNAs between immature and mature OSNs, including the discovery that nascent OSNs can be identified by expression of two axon initiation genes but not by the canonical marker of immature OSNs, *Gap43*.

MATERIALS AND METHODS

In situ hybridization and immunofluorescence

Male C57Bl/6J mice, ages postnatal day 0 (P0) or ages P21-P25, were used for in situ hybridization, which was performed as described previously (Shetty et al., 2005; Yu et al., 2005). A detailed protocol is available from the authors. Briefly, mice were anesthetized via intraperitoneal injection with ketamine hydrochloride (10mg/ml) and xylazine (1mg/ml) in 0.9% saline (0.01mL/g of body weight) and transcardially perfused with 4% paraformaldehyde. The maxillary and anterior cranial region of the head (snout) was dissected free and fixed in 4% paraformaldehyde overnight, followed by decalcification in EDTA overnight, cryoprotected in sucrose, embedded in OCT and stored at -80˚C. Coronal sections 10um thick were cut on a cryostat and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Digoxygenin-labeled riboprobes were prepared from cDNA fragments ranging from 400bp-1000bp in size. Most mRNAs were detected with a single riboprobe, however to increase signal strength two riboprobes were pooled to detect some mRNAs. Sense controls were invariably negative.

For immunofluorescence, 10 μm cryosections were prepared using the same methods as for in situ hybridization, except that fixation was 1.5 hrs in 4% paraformaldehyde. Slides were washed 3 times for 10 min in 1x PBS followed by blocking at room temperature for 30 min with 5% normal donkey serum, 0.4% Triton 100-X, in 1x PBS. The following primary antibodies were used; goat anti-CXCR4 (1:250, Abcam, ab1670, amino acids 14-40 of mouse CXCR4); rabbit anti-GAP43 (1:200; Millipore, AB5220); and mouse anti-NCAM1 (1:1000; Sigma-Aldrich, C9672). Secondary antibodies, all used at a dilution of 1:500, were DyLight 549 donkey anti-goat, DyLight 488 donkey anti-rabbit, and DyLight 488 donkey anti-mouse from Jackson Immunoresearch Laboratories, Inc. The use and specificity of GAP43 and NCAM1 antibodies has previously been demonstrated (Akins and Greer, 2006; Dudanova et al., 2007). The CXCR4 antibody has also previously been used and antibody staining replicates Cxcr4 expression detected by in situ hybridization (Nishiumi et al. 2005).

Digital images were acquired with either a SPOT 2e camera (Diagnostics Instruments, Inc., Sterling Heights, MI) mounted on a Nikon Diaphot 300 inverted

microscope or a Spot 2e camera on a Nikon Eclipse Ti-U inverted microscope. Processing of images to adjust size, brightness, and contrast was done in Adobe Photoshop and organization of figures was done in Deneba Canvas. All procedures described using mice were approved by an Institutional Animal Care and Use Committee and conformed to NIH guidelines.

Olfactory Bulbectomy

Adult male C57BL/6 mice (6 weeks) were anesthetized with ketamine/xylazine as described above. A midline sagittal incision was made in the scalp to expose the cranium and a 2-mm hole over one bulb was drilled into the skull using a diamond-tipped burr. Eight mice were subjected to unilateral bulbectomy by aspiration. Gelfoam soaked in sterile saline was used to fill the cavity and the skin was sutured with 6-O Ethilon suture. Recovery from surgery was aided by warming, subcutaneous injection of 0.5 ml saline, and maintenance on buprenorphine for 48 hrs. Food and water were supplied ad libitum.

RNA Isolation and Quantitative RT-PCR

Eight mice were euthanized seven days after bulbectomy. The septal epithelium and olfactory turbinates were dissected into 700ul of ice-cold TriReagent (Molecular Research Center, Inc, Cincinnati, OH) and homogenized using a polytron. RNA was then extracted using the TriReagent protocol supplied by the manufacturer. The yield and quality of RNA samples was determined with a UV-spectrophotometer and a model 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

Primers with melting temperatures between 58-60˚C were designed using Primer Express software (Applied Biosystems, Foster City, CA) and purchased from Integrated DNA Technologies (Coralville, IA). Complementary DNA was prepared by reverse transcription of 0.5ug of total RNA using Superscript II reverse transcriptase and random hexamers (Invitrogen, Carlsbad, CA) in 50ul reactions. Amplification of samples was performed in triplicate using an ABI 7700 Sequence Detection System. Samples were run using Sybr Green 2x Master mix (Applied Biosystems, Foster City, CA). Thermal cycler conditions were 95˚C for 15min, then 45 cycles of 95˚C for 15s, 60˚C for 1 min. Melt

curve analysis was used to confirm that only a single product was generated in each reaction. The mean of each triplicate set was calculated and these data were normalized using the geometric mean of four control mRNAs in each tissue sample; *Actb* (actin, beta), *Hprt1* (hypoxanthine guanine phosphoribosyl transferase 1), *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase), and *Ubc* (ubiquitin C). Ipsilateral samples from bulbectomized mice were compared against contralateral samples using one-tailed paired t-tests. Correction for multiple testing was done using Holm's step-wise correction method (Holm, 1979; Draghici, 2003).

RESULTS

Most axon guidance genes are developmentally regulated

I hypothesized that immature and mature OSNs differ in expression of axon growth and guidance genes because the needs of their axons differ. Directed by data from expression profiling studies of the olfactory epithelium or of purified samples of OSNs (Shetty et al., 2005; Sammeta et al., 2007), I selected 36 genes that encode proteins involved in axon growth and guidance and tested their expression patterns in the olfactory epithelium. Twenty-two mRNAs were differentially abundant between immature and mature OSNs. Seventeen mRNAs were detected only in immature OSNs, five mRNAs only in mature OSNs, another thirteen mRNAs in both immature and mature OSNs, and one mRNA in the lamina propria (Table 2.1). All but two, *Ncam2* and *Nrp2*, were expressed uniformly across the odorant receptor expression zones of the olfactory epithelium, indicating that few genes correlate with this zonal organization and its effects on axonal connections to the olfactory bulb. The zonality of *Ncam2* and *Nrp2* had previously been established (Yoshihara et al., 1997, Norlin et al., 2002).

Maturation results in the loss of guidance cue local signaling

The mRNAs whose expression was detected primarily in immature OSNs encode guidance cue receptors and intracellular signaling molecules (Figure 2.1). In fact, of the mRNAs that encode intracellular signaling proteins that control the behavior and extension of growth cones, all were detected in immature OSNs and weakly, if at all, in mature OSNs. *Ppp2cb*, the catalytic subunit of protein phosphatase 2A, a protein important for promoting neuritogenesis, was expressed by immature OSNs (Figure 2.1B). Transcripts for *Marcskl1*, encoding a protein similar in function to *GAP43*, were similarly enriched in immature OSNs (Figure 2.1C). *Ablim1*, which mediates axon guidance and specifically the attractive effects of netrin in *C. elegans*, was specific to immature OSNs (Figure 2.1D) (Lundquist et al., 1998; Erkman et al., 2000; Gitai et al., 2003). The related gene, *Ablim2*, was detected at similar intensities in both mature and immature OSNs (Figure 2.1E). While ABLIM2 has been shown to bind F-actin, (Barrientos et al., 2007) whether ABLIM2 is a mediator of signals that control growth cone behavior is as yet

untested. Three members of the dihydropyrimidinase-like family; *Crmp1, Dpysl3* and *Dpysl5*, which encode dihydropyrimidinase-like proteins (also known as collapsin-response mediator proteins) that mediate growth cone collapse and turning in response to semaphorins, were detected only in immature OSNs (Figure 2.1F-H). Another member of this family, *Dpysl2*, was detected strongly in immature OSNs and weakly in mature OSNs (Figure 2.1I). I also tested the expression of four stathmin genes whose encoded proteins interact with the microtubule network to regulate axon extension and turning (Sobel, 1991; Ozon et al., 1997; Grennigloh et al., 2003). *Stmn1* and *Stmn2* were expressed exclusively in immature OSNs, as previously shown (Camoletto et al., 2001; Pellier-Monnin et al., 2001), consistent with their roles in promoting axonal growth for other types of neurons (Morii et al., 2006) (Figure 2.1J-K). *Stmn3* and *Stmn4* were expressed in both immature and mature OSNs (Figure 2.1L-M). STMN3 and STMN4 act to reduce axon branching, a property consistent with expression that spans the differentiation boundary into mature OSNs, which have relatively few branches (Baldassa et al., 2007; Cao et al., 2007; Poulain and Sobel, 2007). Taken together, these findings indicate reduced local signaling by guidance cue receptors in mature OSNs, suggesting a maturational shift in the type of signaling mediated by guidance cue receptors in OSN axons.

Immature OSNs express a unique set of guidance receptors and cell adhesion molecules

Several guidance cue receptors and a cell adhesion molecule were only detected in immature OSNs. The semaphorin receptors *Plxnb1* and *Plxnb2*, and the plexin domain containing receptor, *Plxdc2*, were detected in immature OSNs (Figure 2.2A-C). Another semaphorin receptor, *Nrp1*, gave a mosaic pattern among immature OSNs (Figure 2.2D). This pattern is likely determined by odorant receptor signaling (Imai et al., 2006; 2009). I also detected three cell adhesion molecules, *Chl1*, *Nfasc1*, and *Dscaml1* only in immature OSNs (Figure 2.2E-G). In contrast, *Dscam* was detected in both immature and mature OSNs. In addition to its role as a cell adhesion molecule, DSCAM also acts as a receptor for netrin-1 and can mediate axonal turning responses (Ly et al., 2008). Overall, these findings indicate that immature OSNs detect different guidance cue signals than mature OSNs.

Axon initiation genes identify nascent immature OSNs

Two mRNAs shared a novel expression pattern. *Dbn1* and *Cxcr4* were expressed primarily in a thin band of cells just above the basal cell layer. Alternate sections labeled for these two mRNAs and for *Gap43*, the canonical marker of immature OSNs, appeared to indicate that cells expressing *Dbn1* and *Cxcr4* overlapped with the basal end of the immature OSN layer, though occasional basal cells also expressed *Dbn1* and *Cxcr4* (Figure 2.3A-D). Cells expressing *Cxcr4* and *Dbn1* formed a more continuous layer than *Neurog1* positive basal cells, which occur in clusters in age P21 mice from our colony, suggesting that *Cxcr4* and *Dbn1* positive cells are more numerous (Figure 2.3E-G). Indeed, cells expressing *Cxcr4* were more abundant than *Neurog1* positive cells (8.7 ± 0.8 per 0.1mm, n = 2 mice versus 2.8 ± 0.5 per 0.1mm, n = 3 mice), further indicating that cells expressing *Cxcr4* could not consist solely of the immediate neuronal precursor type of globose basal cell. Neither could more apically located CXCR4 positive cells solely be a subset of *Gap43* positive immature OSNs because cells immunoreactive for both CXCR4 and GAP43 were rare (0.9 ± 0.6 per 0.1mm, n = 2 mice) (Figure 2.3H-L). Therefore, though many CXCR4 immunoreactive cells had short apical and basal processes, few could be identified as immature OSNs (Figure 2.3H-L). CXCR4 immunoreactive processes could be seen exiting the olfactory epithelium and entering olfactory nerve bundles along with NCAM positive axons, confirming that these basal processes were nascent axons (Figure 2.3M-O). I conclude that *Cxcr4* and *Dbn1* are expressed by cells that are transitioning from globose basal cells into OSNs, and that these nascent OSNs are beginning to extend axons and dendrites.

Expression of *Cxcr4* by cells in the olfactory epithelium led us to search for cells expressing the CXCR4 agonist, CXCL12. *Cxcl12* was expressed nearby in a developmentally regulated pattern. At age P21 (Figure 2.4C, D), *Cxcl12* mRNA was detected deep in the bone and cartilage below the lamina propria, but at P0 (Figure 2.4A, B), *Cxcl12* was detected in cells of the lamina propria directly below the basal lamina of the olfactory epithelium. CXCR4/ CXCL12 signaling is therefore properly oriented to promote the extension of nascent OSN axons out of the olfactory epithelium.

Taken together, these data indicate that newly formed “nascent OSNs”

specifically express genes involved in the initiation of axon extension and neuronal migration (Shirao et al., 1992; Ishikawa et al., 1994; Toda et al., 1999; Lieberam et al., 2005; Chalasani et al., 2007; Miyasaka et al., 2007; Geraldo et al., 2008; Zhu et al., 2009) and are consistent with the interpretation that the immature OSN layer has an age gradient, with the youngest OSNs located most basally.

Receptors for inhibitory signals, and cell adhesion molecules, predominate in mature neurons

Mature OSNs expressed several guidance cue receptors that were not detected in immature OSNs. *Plxna3*, a receptor for the secreted semaphorin 3, was expressed only by mature OSNs (Figure 2.5A). Of the ephrins and eph receptors I tested, *Efna3, Epha5*, and *Epha7*, were detected only in mature OSNs (Figure 2.5B-D). Lastly, *Unc5b*, which mediates inhibitory effects of netrin, was expressed by mature OSNs (Figure 2.5E).

Seven receptor mRNAs were detected at approximately equal levels in immature and mature OSNs. The semaphorin receptors *Plxna1* and *Plxna4* were expressed in both cell types, with *Plxna1* exhibiting a punctate staining pattern and *Plxna4* showing more uniform expression (Figure 2.6A, B). The semaphorin receptor *Nrp2* was detected in both immature and mature OSNs (Figure 2.6C), and as shown previously, was limited to the ventral region of the olfactory epithelium (Norlin et al., 2002). *Efna5* was also expressed in both immature and mature OSNs (Figure 2.6D). The cell adhesion molecules *Ncam1, Ncam2, Dscam*, and *Nrxn1* were detected in both cell types (Figure 2.6E-H), and as shown previously, *Ncam2* expression was restricted to the ventral olfactory epithelium (Yoshihara et al., 1997). While clearly detectable in mature OSNs, *Ncam1* and *Nrxn1* gave slightly stronger labeling in the immature OSN layer.

Immature OSN mRNAs increase after bulbectomy

The interpretations of the expression patterns I observed depend upon correct identification of mature and immature OSNs. To confirm the cell type identification I used olfactory bulbectomy, which results in the death of mature OSNs and an increase in the production of immature OSNs in a relatively synchronous wave that appears to peak

at about seven days after bulbectomy (Schwob, 2002; Shetty et al., 2005). The mRNAs I detected anatomically as enriched in immature OSNs should be more abundant in the olfactory epithelium following bulbectomy, and conversely, mature OSN-specific mRNAs should decrease. Unilateral bulbectomies were performed on 6wk old C57Bl/6 mice and changes in mRNA abundance were measured by quantitative RT-PCR for 10 mRNAs. As expected, *Omp* abundance was 5 fold less in olfactory epithelium ipsilateral to the ablated olfactory bulb compared to contralateral olfactory epithelium ($t = -7.73$, $n = 6$ mice, $p < 0.0005$). *Cbr2* was used as a negative control because it is specific to sustentacular cells, which are unaffected by bulbectomy (Monti Graziadei and Graziadei 1979; Costanzo, 1985; Yu et al., 2005). As expected, *Cbr2* mRNA abundance was unaltered by bulbectomy ($t = 1.57$, $n = 6$ mice, $p > 0.1$). In contrast, *Ablim1, Marcksl1, Plxnb1*, and *Dpysl3* gave statistically significant increases (Table 2.2). These data validate the identification of immature OSNs by anatomical position.

DISCUSSION

Based on the different growth requirements of immature and mature axons I hypothesized that differences in gene expression would help define the signaling networks used. Using OSNs as a convenient source of tissue where mature and immature neurons coexist, I found maturational differences in gene expression. I discovered that expression of *Dbn1* and *Cxcr4* define a population of nascent OSNs in transition from globose basal cells to immature OSNs. Immature OSNs express a larger variety of mRNAs for intracellular axon guidance signaling proteins than do mature OSNs. While mature OSNs express few intracellular axon guidance signaling genes, they do express guidance cue receptors and cell adhesion molecules in similar numbers to immature OSNs and many of these are shared between the two developmental stages. The expression patterns I observed indicate that OSN axon growth to the olfactory bulb occurs in several phases, and implicate certain gene products as critical regulators in each phase.

The ability to identify mRNAs enriched in immature OSNs due to the position of immature OSN cell bodies in the pseudostratified olfactory epithelium was confirmed using data from recently bulbectomized mice in which mature OSNs are largely absent and immature OSNs are increased. First, I verified bulbectomy-induced increases for four mRNAs. Second, expression profiling of olfactory epithelia from bulbectomized mice detected increases in other mRNAs I tested, including *Dpysl3, Ablim1, Dbn1, Cxcr4, Gap43, Marcksl1, Ppp2cb*, and *Stmn1* (Table 2.1) (Shetty et al. 2005). In contrast to the increase in immature OSNs after bulbectomy, mature OSNs decrease, so the same expression profiling data also detected decreases in mRNAs detected only in mature OSNs including, *Efna3, Epha7*, and *Plxna3*. The evidence, therefore, argues that I was able to correctly identify by in situ hybridization mRNAs expressed primarily by immature or mature OSNs.

Maturation is marked by changes in the axon guidance signaling network

The majority of mRNAs encoding axon guidance-related intracellular signaling proteins were detected only in immature OSNs. Of 14 tested, only three such mRNAs, *Dpysl2,*

Stmn3 and *Stmn4*, were detected in both immature and mature OSNs, and even these were more abundant in immature OSNs. The maturational reduction in expression of these types of genes coincides with the loss of the growth cone and the need to regulate its cytoskeletal dynamics. Nine mRNAs for proteins that are known to regulate actin and microtubule dynamics in response to guidance cue activation were detected in immature OSNs. The proteins encoded by these mRNAs have both growth promoting and inhibitory effects. Immature OSNs likely have broad signaling networks to allow for the integration a multiple attractive and repulsive cues. In contrast, mature OSNs express fewer mRNAs encoding intracellular signaling proteins.

The receptors detected specifically in mature OSNs typically mediate repulsive or inhibitory effects. Guidance cue receptors in mature OSNs could help to maintain the position of the axon and its terminals, but expression of most of the downstream signaling molecules that link these receptors to the cytoskeletal dynamics of the axonal growth cone were either absent or decreased. It is therefore possible that guidance cue receptors perform as yet undiscovered functions in mature OSNs that differ from their guidance role in immature OSNs. Recent evidence from other types of neurons indicates that some guidance cue receptors can generate signals that target the nucleus and regulate transcription (Bong et al., 2007; Rhee et al., 2007), suggesting that the retention of guidance cue receptors in mature OSNs corresponds with a change from local control of the cytoskeletal dynamics to sending homeostatic signals back to the cell body and nucleus.

Phenotypically distinct stages of OSN axon growth

OSNs are the only type of neuron in which the cell body exists in the periphery and extends an axon to a synaptic target in the brain, the olfactory bulb. To separate the inputs of more than 1,000 different subtypes, OSNs must segregate and coalesce homogeneously according to their odorant receptor identity. My data support the view that OSN axon growth consists of several phenotypically distinct stages. First, newly born immature OSNs must initiate an axon and extend it through the basal lamina into the lamina propria. I found that a set of basally located nascent OSNs specifically express two genes, *Dbn1* and *Cxcr4*, known to be involved in axon initiation and extension

(Shirao et al., 1992; Ishikawa et al., 1994; Toda et al., 1999; Chalasani et al., 2003; Lieberam et al., 2005; Chalasani et al., 2007; Miyasaka et al., 2007; Geraldo et al., 2008). The expression of *Cxcr4* overlapped only partially with expression of *Gap43* and basal cells expressing *Neurog1* were too few to account for the remainder of cells expressing *Cxcr4*. Therefore some cells expressing *Cxcr4* are not identified by the canonical markers for immature OSNs and the immediate neuronal precursor type of globose basal cell. I conclude that these cells represent newly differentiating, nascent OSNs that are just beginning to extend axons out of the olfactory epithelium. I hypothesize that DBN1 contributes to the initiation of the axon and then CXCR4, responding to activation by CXCL12 secreted by cells in the lamina propria, helps attract the nascent axons through the basal lamina and out of the olfactory epithelium. Given that the expression patterns of other axon growth and guidance genes did not extend more basally than *Gap43* or *Ncam1*, which overlap poorly with *Cxcr4* expression, the data suggest that nascent OSNs might not express classical guidance cue receptors until they transition into *Gap43* positive immature OSNs.

Once they have left the olfactory epithelium proper, OSN axons turn caudally towards the olfactory bulb. The cue, or cues, responsible for this turn of the pioneering axons is unknown, though the migratory mass that accompanies these axons may help provide it (Doucette 1989, 1990). Netrin and CXCL12 are possible cues to attract axons towards the bulb as they both are expressed in the mesenchyme surrounding the olfactory epithelium and enriched near the cribriform plate. The lamina propria in which OSN axons grow provides a favorable environment as it contains laminin, fibronectin and collagen-IV (Gong and Shipley, 1996; Whitesides and LaMantia, 1996).

To reach the olfactory bulb, OSN axons must grow through fenestrations in the cribriform plate that separates the olfactory bulb from the nasal cavity. The fenestrations contain laminin surrounded by chondroitin sulfate proteoglycans (CSPG), growth-inhibiting molecules; thereby establishing boundaries around what should be permissive paths for axons to pass through the cribriform plate (Shay et al., 2008).

Once they reach the olfactory bulb immature OSN axons navigate across the surface in the outer olfactory nerve layer until they reach the appropriate domain where they then defasciculate, enter the inner olfactory nerve layer, re-fasciculate and coalesce

into glomeruli (Au et al., 2002). Expression of guidance cue receptors in immature OSNs may be important for growing to the correct domains. The olfactory bulb expresses multiple guidance cues that appear to establish sub-domains, such as *Sema3a*, *Sema3f*, *Slit-1* and *Netrin-4* (Cloutier et al., 2002; Cho et al., 2007; Williams et al., 2007). I detected strong expression of receptors for these molecules in immature OSNs. Immature OSNs detect SEMA3A via NRP1 and several plexin receptors, signaling events that may help keep immature axons in the outer olfactory nerve layer. The mosaic expression of *Nrp1* in the OE may explain why only some types of OSN axons develop ectopic glomeruli in *Sema3a* knockout mice (Schwarting et al., 2002). An example of guidance cue signaling changes that accompany the transition of OSNs from immaturity to maturity is netrin signaling. The netrin receptors *Dcc* and *Dscam* that mediate axon attraction were detected in immature OSNs, along with *Ablim1*, an important downstream signaling molecule linked functionally to *Dcc* (Astic et al., 2002; Gitai et al., 2003; Ly et al., 2008; Andrews et al., 2008). This suggests that netrin is acting to attract immature OSN axons. Mature OSNs, however, express *Unc5b*, a receptor mediating repulsive effects of netrin. By changing receptor expression OSN axons can use the same ligand to attract immature OSN axons and inhibit the growth of mature OSN axons. In the inner olfactory nerve layer of the bulb axons expressing the same odorant receptor coalesce together to form glomeruli. One proposed mechanism aiding this process is contact-mediated repulsion of Ephrins and Eph receptors (Serizawa et al., 2006). Consistent with this hypothesis, I detected enrichment of Ephrin and Eph receptor mRNAs in mature OSNs.

The signals that cause retention of OSN axons in glomeruli are as yet unknown, though synapse formation and the maturation of the OSN presumably solidify the OSN axon at its target (Kim and Greer 2000; Shetty et al., 2005). Semaphorins expressed in deeper layers of the olfactory bulb and the presence of inhibitory extracellular matrix molecules, such as chondroitin sulfate proteoglycans and tenascin C, surrounding the glomeruli (Shay et al., 2008) are likely candidates for stopping OSN axons at glomeruli and maintaining them there. In addition, mature OSN axons have relatively few branches, consistent with the ability of STMN3 and STMN4 to suppress axonal arborization (Klenoff and Greer, 1998; Yilmazer-Hanke et al., 2000; Baldassa et al.,

2007; Cao et al., 2007; Poulain and Sobel, 2007). My data suggests that once mature and connected to their synaptic targets, OSNs express predominantly inhibitory guidance cue receptors that might help inhibit further axon growth, except that the mature OSNs express few of the necessary signaling protein partners to connect to local cytoskeletal dynamics. Instead, I speculate that these receptors shift their functions, perhaps regulating axon branching or transducing homeostatic signals that have effects both locally and in the nucleus.

Table 2.1 Summary of genes tested

Gene Symbol	OMP+/- ratio	OBX Microarray	Cell type by ISH	Gene Name	Entrez Gene ID
Ablim1	0.20	1.6*	iOSN	actin-binding LIM protein 1	226251
Ablim2	1.50	nd	OSN	actin-binding LIM protein 2	231148
Chl1	0.50	3.1*	iOSN	cell adhesion molecule with homology to L1cam	12661
Crmp1	1.10	1	iOSN	collapsin response mediator protein 1	12933
Cxcl12	0.30	1.2	lamina propria (age P0)	chemokine (C-X-C motif) ligand 12	20315
Cxcr4	0.04	2.1*	iOSN, basal	chemokine (C-X-C motif) receptor 4	12767
Dbn1	0.50	2.6*	iOSN, basal	drebrin 1	56320
Dpysl2	0.90	0.8	OSN	dihydropyrimidinase-like 2	12934
Dpysl3	0.30	1.5*	iOSN	dihydropyrimidinase-like 3	22240
Dpysl5	0.80	nd	iOSN	dihydropyrimidinase-like 5	65254
Dscam	2.00	nd	OSN	down syndrome cell adhesion molecule	13508
Dscaml1	0.80	1.7	iOSN	down syndrome cell adhesion molecule-like 1	114873
Efna3	5.60	0.5*	mOSN	ephrin A3	13638
Efna5	1.70	nd	OSN	ephrin A5	13640
Epha5	50.80	0.4	mOSN	eph receptor A5	13839
Epha7	2.50	0.6*	mOSN	eph receptor A7	13841
Gap43	0.60	1.5*	iOSN	growth associated protein 43	14432
Marcksl1	0.30	1.4*	iOSN	MARCKS-like 1	17357
Ncam1	1.70	0.8*	OSN	neural cell adhesion molecule 1	17967
Ncam2	2.90	0.7*	OSN	neural cell adhesion molecule 2	17968
Nfasc	0.90	nd	iOSN	neurofascin	269116
Nrp1	1.10	1	OSN	neuropilin 1	18186

Nrp2	0.70	nd	OSN	neuropilin 2	18187
Nrxn1	1.60	nd	OSN	neurexin I	18189
OMP	44.40	0.3*	mOSN	olfactory marker protein	18378
Plxdc2	0.50	nd	iOSN	plexin domain containing 2	67448
Plxna1	1.80	nd	OSN	plexin A1	18844
Plxna3	7.60	0.4*	mOSN	plexin A3	18846
Plxna4	4.10	nd	OSN	plexin A4	243743
Plxnb1	0.90	nd	iOSN	plexin B1	235611
Plxnb2	0.90	1	iOSN	plexin B2	140570
Ppp2cb	0.50	1.2*	iOSN	ser/thr protein phosphatase 2a, catalytic subunit, Beta isoform	19053
Robo2	1.30	not on array	OSN	roundabout homolog 2 (Drosophila)	
Stmn1	0.70	1.5*	iOSN	stathmin 1	16765
Stmn2	0.7	1.2*	iOSN	stathmin-like 2	20257
Stmn3	1.90	1	OSN	stathmin-like 3	20262
Stmn4	6.30	0.7*	OSN	stathmin-like 4	56471
Unc5b	3.50	nd	mOSN	unc-5 homolog B	107449

$OMP^{+/-}$ ratio column specifies the degree of enrichment in mature OSNs (Sammeta et al., 2007). OBX (olfactory bulbectomy) microarray column shows fold-changes in mRNA abundance for olfactory epithelium samples at 7 days after OBX (Shetty et al., 2005). nd, not detected or not present on the microarray. *, Significant difference between sham and bulbectomized mice, $p < 0.05$.

Table 2.2 Quantitative RT-PCR results

Gene Name	Gene Symbol	Fold Change	t-statistic	p-value
plexin B1	Plxnb1	1.57	9.4943	0.0005*
olfactory maker protein	OMP	0.19	-7.7311	0.0005*
mARCKS-like protein	Marcksl1	2.49	5.5804	0.0025*
actin-Binding LIM protein 1	Ablim1	2.52	4.7242	0.005*
dihydropyrimidinase-like 3	Dpysl3	2.94	4.3564	0.005*
chemokine (C-X-C motif) receptor 4	Cxcr4	2.95	3.8308	0.01
growth associated protein 43	Gap43	2.58	3.2391	0.025
plexin B2	Plxnb2	1.43	2.7576	0.025
drebrin 1	Dbn1	1.77	2.6478	0.025
carbonyl reductase 2	Cbr2	1.29	1.5793	0.1

Summary of quantitative RT-PCR results comparing mRNA abundance from olfactory epithelia ipsilateral and contralateral to unilateral olfactory bulbectomy. Correction for multiple testing adjusted the a-level to < 0.01.

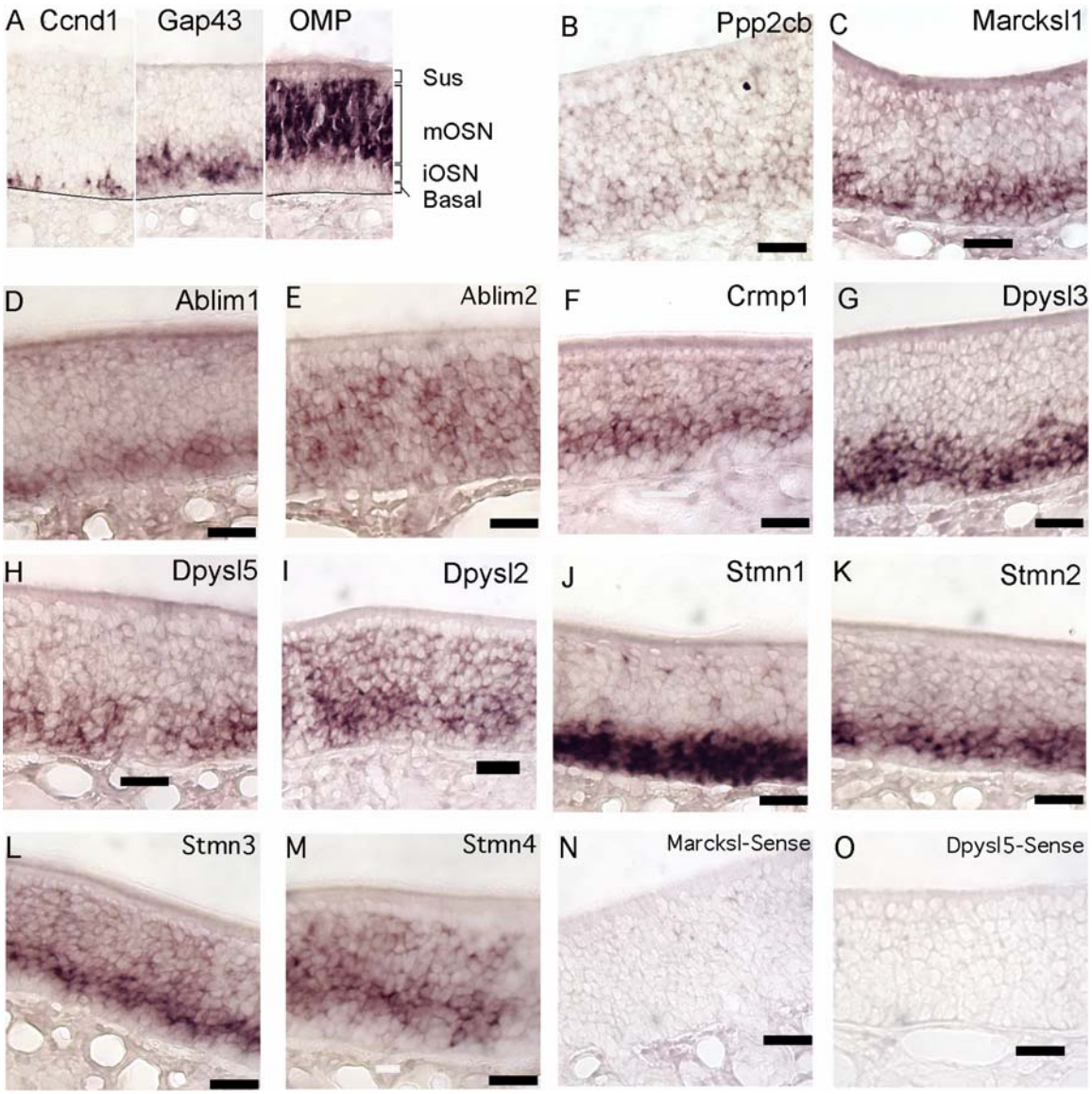

Figure 2.1 Immature OSN enriched mRNAs

Messenger RNAs encoding proteins that regulate the cytoskeleton and growth cone dynamics were primarily expressed in immature OSNs. **A**. Guide to the cell body layers of the olfactory epithelium. *Ccnd1* labels a subset of basal cells; *Gap43* labels immature OSNs; *Omp* labels mature OSNs. Sus, unlabeled sustentacular cell body layer; mOSN, mature OSN cell body layer; iOSN, immature OSN cell body layer; basal, basal cell layer. **B – D**. *Ppp2cb, Marcksl1*, and *Ablim1* were detected in immature OSNs. **E**. *Ablim2* was detected in immature and mature OSNs. **F – H**. *Crmp1, Dpysl3*, and *Dpysl5* were detected in immature OSNs. **I.** *Dpysl2* was detected in immature and mature OSNs. **J – K.** *Stmn1* and *Stmn2* were detected in immature OSNs. **L – M**. *Stmn3* and *Stmn4* were detected in immature and mature OSNs. **N – O.** Examples of the absence of labeling when sense probes were used. Scale bars, 20μm.

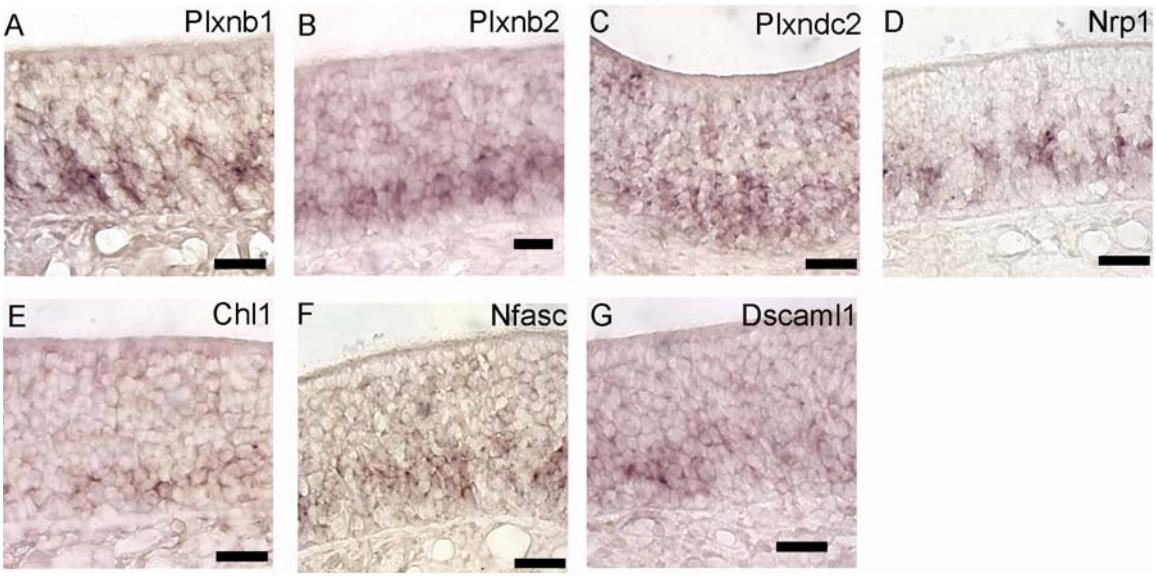

Figure 2.2 Guidance cue receptors enriched in immature OSNs

Guidance cue receptor and cell adhesion molecule mRNAs primarily expressed by immature OSNs. **A – G.** Images of in situ hybridization for *Plxnb1 Plxnb2, Plxdc2, Nrp1, Chl1, Nfasc,* and *Dscaml1*. Scale bars, 20μm.

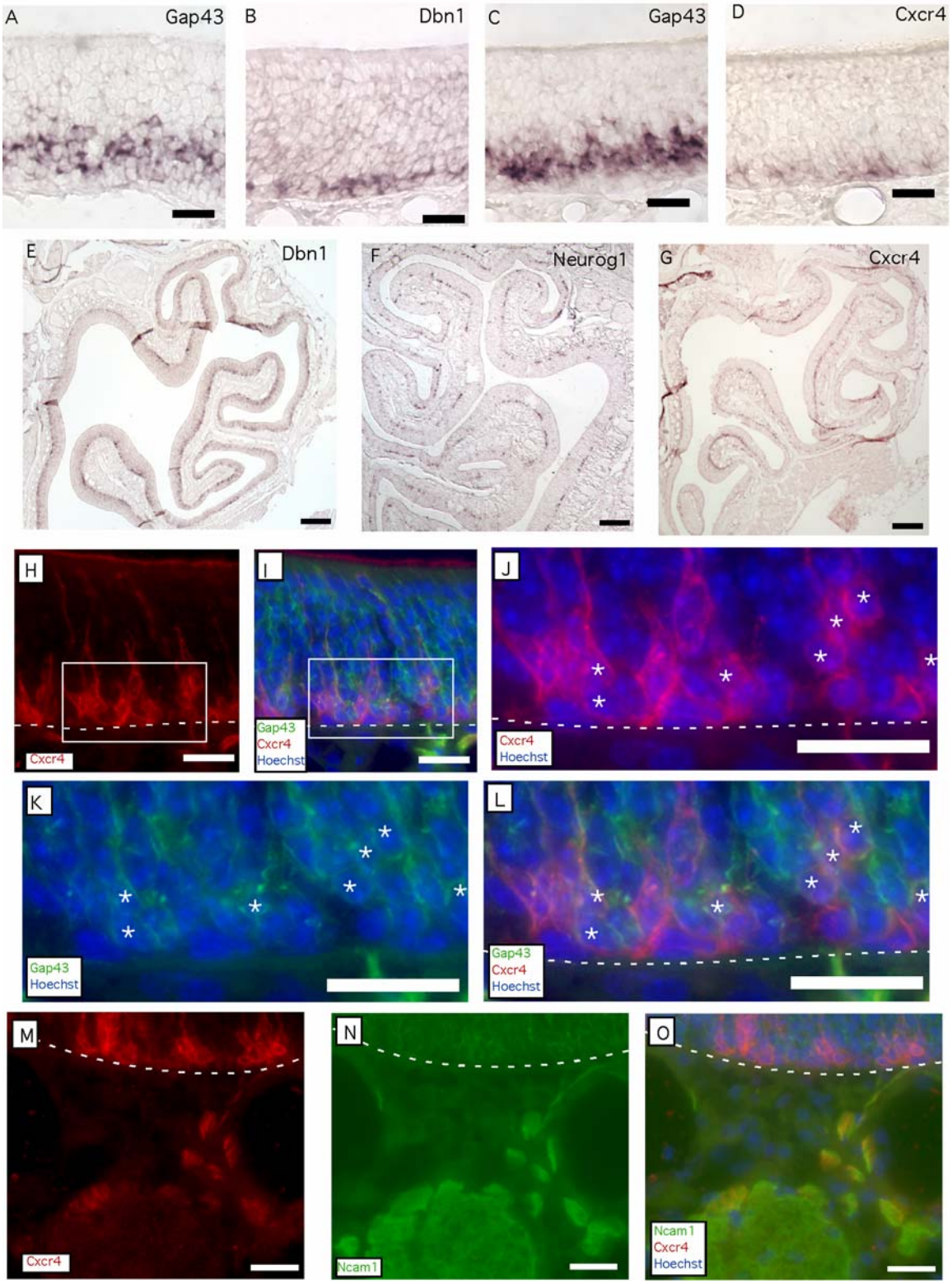

Figure 2.3 Nascent OSNs are identified by Cxcr4 and Dbn1 expression

Figure 2.3 (continued) Nascent OSNs are identified by *Cxcr4* and *Dbn1* expression

Nascent OSNs express axon initiation mRNAs. **A-D**. *Dbn1* (B) and *Cxcr4* (D) mRNAs were expressed in a thin layer of cells that may partially overlap with the basal end of the immature OSN layer marked by adjacent sections hybridized for *Gap43* mRNA (A, C). **E-G**. Cells expressing *Dbn1* (E) and *Cxcr4* (G) formed a nearly continuous layer throughout the olfactory epithelium, compared to the clusters of cells positive for *Neurog1* (F), the canonical marker of immediate neuronal precursors. **H-J**. CXCR4 (red) and GAP43 (green) double labeling in the olfactory epithelium. CXCR4 (H, I) identifies cells located 1 – 3 cell diameters apical to the basal lamina. CXCR4 immunoreactive processes were seen extending to the apical surface of the olfactory epithelium. **J-L.** A region where cells immunoreactive for both CXCR4 and GAP43 were unusually abundant. **M-O**. Fibers immunoreactive for CXCR4 (red) cross the basal lamina and enter olfactory nerve bundles where they are associated with NCAM1 (green) positive axons. Scale bars, **A-D, H-O**: 20μm. E-G: 100μm.

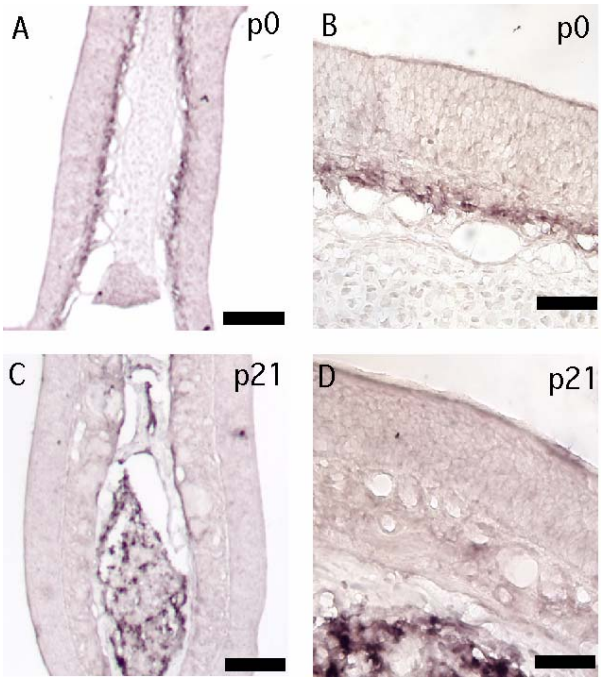

Figure 2.4 Cxcl12 expression in the nasal cavity
Cxcl12 was expressed beneath the olfactory epithelium in an age-dependent pattern. **A, C**. *Cxcl12* was expressed in the lamina propria at age P0. **B, D**. At age P21 *Cxcl12* was instead detected in cells within the bone underlying the lamina propria. Images from the nasal septum are shown. Scale bars: **A - B**, 200μm. **C - D**, 20μm.

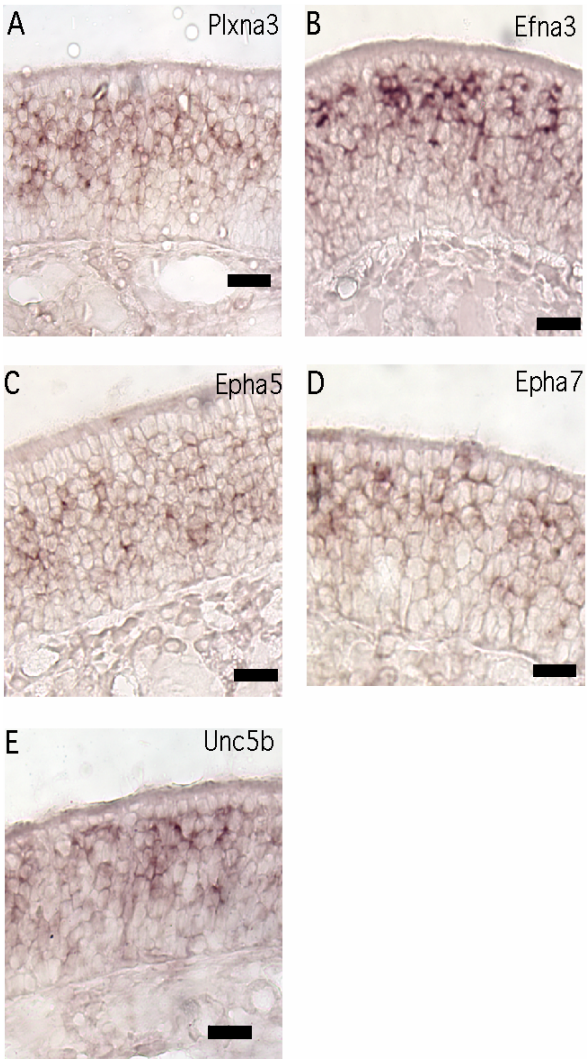

Figure 2.5 Guidance cue receptor mRNAs enriched in mature OSNs
A – E. *Efna3*, *Epha5*, *Epha7*, *Plxna3* and *Unc5b* displayed this pattern of expression. Scale bars, 20μm.

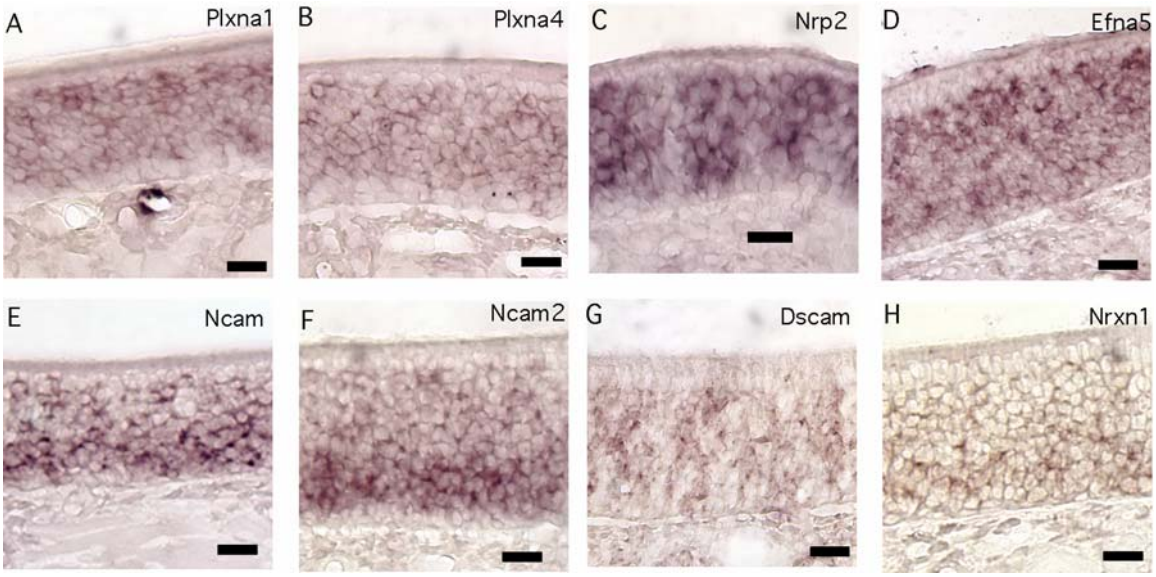

Figure 2.6 mRNAs shared by immature and mature OSNs
Guidance cue receptor and cell adhesion molecule mRNAs detected in both immature and mature OSNs **A – F**. *Plxna1, Plxna4, Efna5, Nrp2, Nrxn1*, and *Ncam1* displayed this pattern. Scale bars, 20μm.

Chapter 3

Emx2 Stimulates Odorant Receptor Gene Expression

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McIntyre JC, Bose SC, Stromberg AJ, McClintock TS. 2008. Emx2 stimulates odorant receptor expression. Chemical Senses 33:825-837.

INTRODUCTION

Odorant receptors (ORs; also known as olfactory receptors) determine the capacity of animals to detect volatile chemical signals. The size of the OR gene family, the largest at more than 1,000 functional genes in several mammalian genomes, correlates with the diversity of the many thousands of volatile chemicals that are potential odorants for mammals (Firestein, 2001; Rouquier and Giorgi, 2007). Although determining which odorants activate each OR is difficult, several studies have now demonstrated that odorants do act as agonists, and even as antagonists, for ORs (Mombaerts, 2004; Krautwurst, 08). In addition to detecting odorant compounds, ORs also play a critical part in the further coding of odor signals via their role in the coalescence of olfactory sensory neurons (OSN) axons into the glomeruli of the olfactory bulb (Mombaerts et al., 1996). All axons terminating in a glomerulus originate from OSNs expressing the same OR protein, allowing the glomerular layer to act as a spatial map of odor quality. This mechanism of encoding odor quality depends on restricting OR expression to a single OR gene in each OSN. In addition, because alleles of an OR gene could encode OR proteins with differing pharmacologies, this logic would work best if OR gene expression was monoallelic, which is indeed the case (Chess et al., 1994; Strotmann et al., 2000; Ishii et al, 2001). This logic is also predicated on an ability of small differences in OR sequence to direct OSN axons to different glomeruli. This also proves to be true (Feinstein and Mombaerts, 2004). Layered on top of these forces dictating the singularity of OR gene choice by OSNs is the phenomenon OR zonality. Every mammalian OR gene investigated thus far is expressed in a circumscribed region of the olfactory epithelium.

For most ORs tested thus far, the expression zone is constrained in the dorso-medial to ventro-lateral dimension, forming a band that stretches the rostro-caudal extent of the tissue (Vassar et al., 1994; Ressler et al., 1994; Kubick et al., 1997; Miyamichi et al., 2005). Whether zonality of OR expression depends on signal gradients that endure throughout life or regional specification laid down during development is not known.

Everything we understand about OR function, from tissue- and spatially-restricted expression patterns to the singularity of expression in OSNs, argues for the evolution of a tightly regulated mechanism for controlling OR gene expression. This mechanism is perhaps the greatest remaining mystery about ORs. It appears to be hierarchical, acting at the zone, OR gene cluster, single OR gene, and allele levels to select a single OR gene, freeing it from the silencing that must otherwise be experienced by OR genes. To what extent the levels in the hierarchy are interdependent is as yet unknown. We do know that at levels below the OR expression zone, the mechanisms have random properties. In addition, the selection of a single OR gene for transcription in OSNs appears to involve several pathways that stimulate transcription and at least one suppressive mechanism whereby the expressed OR protein feeds back negatively upon the expression of other OR genes (Feinstein et al., 2004; Lewcock and Reed, 2004; Serizawa et al., 2003; Shykind et al., 2004). That the overall OR gene selection mechanism is complemented by cell level selection against OSNs that express no OR or multiple ORs may also be possible (Tian and Ma, 2008).

Two novel hypothesized mechanisms for activating transcription of single OR alleles now seem unlikely. A unique and conserved 2 kb sequence on mouse chromosome 14 was discovered to be critical for expression of OR genes in the MOR28 gene cluster, which sits 75 kb away (Serizawa et al., 2003). This sequence, called the H-element, was proposed to act as the factor necessary for the singularity of all OR expression in OSNs, requiring it to act in trans upon ORs on other chromosomes (Lomvardas et al., 2006). This mechanism seems implausible, however, because OR expression is normal in mice lacking the H element, except for reduced expression of the four MOR28 cluster genes nearest the H element (Fuss et al., 2007; Nishizuma et al., 2007). Perhaps instead of selecting individual OR genes, the H-element may be the founding member of a set of enhancer elements that select OR clusters (Rodriguez, 2007). Also out of favor is the

hypothesis that DNA rearrangement might control OR gene expression. Cloning of mice by transfer of mature OSN nuclei resulted in clones with normal OR expression patterns rather than expression of a single OR in all OSNs (Eggan et al., 2004; Li et al., 2004). Unless nuclear reprogramming during early development was able to reverse DNA rearrangements used to select OR genes for expression, this finding argues that OR expression is largely regulated in a more conventional fashion.

Indeed, investigation of putative promoter regions just upstream of predicted transcriptional start sites of OR genes implicate these regions in the control of OR expression. Transgenes carrying as little as a few hundred base pairs of a putative OR promoter are often able to replicate the native expression pattern of the OR gene (Qasba and Reed, 1998; Vassali et al., 2002; Rothman et al., 2005). Two conserved elements within these putative promoters have been identified (Vassali et al., 2002; Hoppe et al., 2006; Michaloski et al., 2006). Most OR genes contain O/E-like sites located upstream of the predicted transcriptional initiation site (Vassali et al., 2002). O/E-like sites are bound by the Ebf family of transcription factors and are present in the putative promoters of many genes whose expression is largely restricted to the olfactory epithelium (Kudrycki et al., 1993; Wang and Reed, 1993; Walters et al., 1996; Dugas and Ngai, 2001). The O/E-like site is therefore likely to contribute to the olfactory specificity of OR expression. Immediately upstream of the O/E-like site(s) typically is a homeodomain-like site that is also implicated in OR gene expression (Vassali et al., 2002 Rothman et al., 2005). This site can bind several homeobox transcription factors and one of them, LHX2, may be necessary for expression of some ORs (Hirota et al., 2004; 2007; Kolterud et al., 2004). Though it is clear that other sites or mechanisms must also help regulate OR gene expression, these two DNA elements and the factors that bind them appear to be important components of the mechanism regulating OR gene expression.

I have investigated a homeobox transcription factor, EMX2, known to bind a putative OR promoter and to be expressed in OSNs (Hirota et al., 2004; Nedelec et al., 2004). EMX2 has important developmental roles in other tissues, most critically in the patterning of cortical areas of the brain and in formation of the urogenital tract (Miyamoto et al., 1997; Polleaux, 2004). I have investigated whether EMX2 is necessary for expression of OR genes in OSNs. It was found that in EMX2 mutant mice the

olfactory epithelium developed normal pseudostratification, except for a reduction in the number of mature OSNs. OR expression, however, was disproportionately affected. The majority of OR genes showed expression in fewer OSNs, while a few OR genes were expressed in more OSNs. These data indicate that EMX2 is necessary for full expression of many OR genes and lend support to the hypothesis that EMX2 does so by acting directly on OR promoters

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MATERIALS AND METHODS

Mice

Mutant mice with targeted disruption of the *Emx2* gene were obtained from the RIKEN Center for Developmental Biology, Japan (Yoshida et al., 1997). *Emx2*$^{-/-}$ mice die soon after birth due to urogenital defects (Pellegrini et al., 1996; Miyamoto et al., 1997). I therefore used mice at embryonic age 18.5 days (E18.5) for my experiments. Embryonic animals were obtained by allowing mating overnight. The morning of vaginal plug detection was considered embryonic day 0.5 (E0.5). Preliminary experiments revealed no differences between *Emx2*$^{+/-}$ mice and *Emx2*$^{+/+}$ mice, so these genotypes were considered phenotypically equivalent in the analyses performed. OMP-GFP mice were obtained from Dr. Peter Mombaerts (Max Planck Institute of Biophysics, Frankfurt, Germany). All mouse procedures were performed in accordance with an approved institutional animal care and use committee protocol.

In situ hybridization

In situ hybridizations were performed as described previously (Yu et al., 2005; Shetty et al., 2005). A detailed protocol is available from the authors. In brief, mouse heads were fixed overnight in paraformaldehyde, cryoprotected, mounted in O.C.T. (Sakura Finetek USA, Inc., Torrance, CA) and stored at –80˚C. Coronal sections of 10 µm thickness were cut on a cryostat and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburg, PA). Digoxygenin-labeled riboprobes were prepared from cDNA fragments that ranged from 500 –1,000 bp in length. In cases where preparing probes that react with more than one OR was unavoidable, the results are described as detection of multiple ORs. Riboprobes were hybridized in 50% formamide in 10 mM Tris-HCl (pH8.0), 10% dextran sulfate, 1X Denhardt's solution, 600 mM NaCl, 0.25% SDS, 1 mM EDTA, and 200 µg/ml yeast tRNA at 65˚C (1 ng/µl per riboprobe). Washes were done in phosphate buffered saline (PBS). Detection was done using an alkaline phosphatase-conjugated antibody to digoxygenin and hydrolysis of nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine. Sense strand probes were used as

controls and were invariably negative. All comparisons between genotypes were done using slides processed together on the same date and under identical conditions. Digital wide-field images were obtained using a Spot 2e camera on a Nikon Diaphot 300 inverted microscope. Images were processed in Adobe Photoshop by adjusting size, brightness and contrast. Images were then combined and labeled using Deneba Canvas.

Cell counts

All cell counts are reported as means with their standard deviations. Counts of OSNs expressing an OR gene were done from in situ hybridization experiments using three *Emx2*$^{-/-}$ and three *Emx2*$^{+/-}$ mice. For each OR tested eight coronal sections were matched for anterior-posterior position. All labeled OSNs, irrespective of location in the olfactory epithelium, were counted and summed across the eight sections. The length of epithelium in each section used was measured to allow calculation of the labeled OSNs per unit distance for each OR tested. To count *Gap43*$^{+}$ immature OSNs, labeled cells in images of in situ hybridization for *Gap43* mRNA were counted in 200 μm long sections of septal epithelia from *Emx2*$^{-/-}$ (n = 2) and *Emx2*$^{+/-}$ (n = 3) mice. To count total cells per linear dimension of the olfactory epithelium, fluorescent images of nuclei stained with Hoechst 33258 were prepared, the location of the basement membrane marked, and nuclei apical to this membrane were counted in 200 μm long sections of the epithelium.

To facilitate the counting of mature OSNs, I bred *Emx2*$^{+/-}$ mice onto an OMP-GFP homozygous background (Potter et al., 2001) to obtain *Emx2*$^{-/-}$*:Omp-GFP*$^{-/-}$*, Emx2*$^{+/-}$*:Omp-GFP*$^{-/-}$, *and Emx2*$^{+/+}$*:Omp-GFP*$^{-/-}$ littermates. These genotypes were used only for accurate counting of GFP fluorescent mature OSNs. Mouse heads were fixed and sectioned as described for ISH. Slides were washed with PBS for 15 min, stained with Hoechst 33258 for 5 min followed by a 5 min PBS wash. Digital dual fluorescent (GFP and Hoechst 33258) images were obtained from the coronal sections matched across genotypes for anterior-posterior position. Cells were counted in 200 μm regions of the dorsal and ventral septum.

Messenger RNA abundance

GeneChip® assessment of mRNA abundance was done using procedures previously established (Shetty et al., 2005; Sammeta et al., 2007). Olfactory epithelium was isolated from mice at age E18.5 using Tri-reagent (Molecular Research Center, Inc). Pooled samples consisting of 2.7 μg of olfactory epithelium RNA from each of three *Emx2*$^{+/+}$ and three *Emx2*$^{-/-}$ mice (n = 3 pools) were prepared. Labeling, hybridization and scanning was performed according to standard Affymetrix protocols by the University of Kentucky Microarray Core Facility using Affymetrix GeneChip® Mouse Exon 1.0 ST Arrays. Affymetrix Expression Console software was used for analysis and generation of gene level RMA values from exon probesets. Gene level data derived from clusters of exons that belong to a single gene are termed transcript clusters. These were analyzed at the Core annotation level (the most conservative level), limiting analysis to exon-level probe sets that map to BLAT alignments of mRNAs with annotated full-length open reading frames (CDS regions). Gene level data were then manipulated in Excel (Microsoft, Redmond, WA). The microarray data have been deposited at Gene Expression Omnibus (accession No. GSE12135). Due to the similarity of some OR genes, a few transcript clusters may detect mRNAs from multiple ORs, a fact that prevents exact identification of every OR affected and, therefore, calculating the exact number of ORs affected.

To eliminate background, any mRNAs that failed to give a signal of at least 9% of the overall mean gene level signal on at least one GeneChip®. This eliminated 1793 transcript clusters. Verification that this eliminated background was done by assessing the correlation between variance and average signal intensity. The size of the variance should become independent of signal intensity at low signals where differences in the biological samples are not the primary source of variation. Testing for differences for each gene was done using Student's t-test at an α level of 0.05, followed by correction for multiple testing using a false discovery rate of 10%. That these criteria were rigorous was indicated by ORs whose p values exceeded 0.05 yet were documented by in situ hybridization to differ between *Emx2*$^{-/-}$ and *Emx2*$^{+/+}$ mice.

Genes

To avoid ambiguity, the official gene symbols provided by the National Center for Biotechnology Information (NCBI) are used for all genes described herein. Table 3.1 lists all genes mentioned in this paper, along with their NCBI Gene IDs and any synonyms with functional significance.

As a comparison for the behavior of OR mRNAs in the microarray data, genes identified by Sammeta et al. (2007) as being expressed primarily in OSNs were used. This population consists of more than 4700 genes that are expressed in both immature and mature OSNs. These mRNAs are sufficiently enriched in purified mature OSNs to indicate that they are more abundant in mature OSNs than in immature OSNs but, like ORs, they are usually present at lower amounts in immature OSNs (Iwema and Schwob, 2003; Sammeta et al., 2007). 600 of these genes were randomly selected to obtain 340 that had signal above background on the exon microarray.

RESULTS

Olfactory epithelia of *Emx2*$^{-/-}$ mice were morphologically normal but had fewer mature OSNs

The nasal cavities of age E18.5 *Emx2*$^{-/-}$ mice contained easily identifiable landmarks and were nearly normal in appearance (Figure 3.1A-B). The most noticeable difference from wild-type littermates was in the shortening of the septum, presumably due to the slightly decreased size of the entire frontal-nasal region of the head. Most importantly for this study, the extent of the olfactory epithelium across the surface of the cavity was normal, and the epithelium contained mature neurons expressing the olfactory marker protein gene (*Omp*) (Figure 3.1). The pseudostratification of the olfactory epithelium was also normal (Figure 3.2A-J). Specific markers for several cell types identified mature neurons (Figure 3.2A-B), immature neurons (Figure 3.2C-D), both immature and mature neurons (Figure 3.2E-F), sustentacular cells (Figure 3.2G-H), and a subtype of globose basal cells (Figure 3.2I-J) in their appropriate positions. However, the thickness of the epithelium was reduced by an average of 15% compared to heterozygous and wild-type littermates (Table 3.2), a statistically significant decrease ($p<0.00001$; Student's $t = 10.266$). A decrease in thickness of the olfactory epithelium indicates that fewer cells are present in the epithelium, often due to a decrease in OSN number. A reduction in mature OSNs was apparent from in situ hybridization for *Omp* in *Emx2*$^{-/-}$ mice compared to wild type littermates (Figure 3.1A, B; Figure 3.2A, B). To more easily quantify this decrease, I bred *Emx2*$^{-/-}$ mutant mice with OMP-GFP mice (Potter et al., 2001). Compared to *Emx2*$^{+/+}$*:Omp-GFP*$^{-/-}$ littermates *Emx2*$^{-/-}$:*Omp-GFP* $^{-/-}$ mice had 42% fewer OMP^{+} mature OSNs (Table 3.2 and Figure 3.1C-D), a significant difference ($p<0.01$; Student's $t = 5.086$). The number of OMP^{+} OSNs in heterozygous *Emx2*$^{+/-}$ *:Omp-GFP*$^{-/-}$ mice did not differ from wild type littermates. The decrease in the number of mature OSNs was shared equally by the dorso-medial and ventro-lateral regions of the epithelium. For example, the average cell counts of OMP+ mature OSNs in dorsal and ventral zones of the septa of *Emx2*$^{-/-}$:*Omp-GFP*$^{-/-}$ mice were 77.5 and 77.0 per mm, respectively.

The loss of mature OSNs appeared to account for nearly all of the decrease in thickness of the epithelium. Total cell counts within the olfactory epithelium were reduced by 17% in *Emx2*$^{-/-}$ mice compared to wild type and heterozygous littermates (Table 3.2), similar to the 15% decrease in thickness. In situ hybridization for markers of immature OSNs, sustentacular cells and globose basal cells labeled cell body layers that were similar in extent to the labeling in littermate controls (Figure 3.2C-J). Counts of immature OSNs by in situ hybridization labeling for *Gap43* mRNA found no difference between *Emx2*$^{+/-}$ and *Emx2*$^{-/-}$ mice, with 390 ± 30 cells and 355 ± 120 cells per mm of epithelium, respectively,

Many ORs were expressed by fewer OSNs in *Emx2*$^{-/-}$ mice

Small upstream regions of OR genes containing the homeodomain-like site that presumably binds EMX2 are often sufficient to support normal expression patterns of OR genes in transgenic mice (Qasba and Reed, 1998; Vassali et al., 2002; Rothman et al., 2005; Hirota et al., 2004). This finding suggests that EMX2 might globally promote OR gene transcription. If so, the absence of EMX2 should reduce OR expression. OR mRNAs are readily detected by in situ hybridization because they are among the most abundant mRNAs in an OSN, so in situ hybridization was used to test whether ORs were expressed in fewer OSNs. I observed little evidence of any decrease in OR mRNA abundance within individual OSNs (insets in Figure 3.3A-B; 3.4A-B), a change that is detected in two ways: as increases in the time necessary for reaction products to become visible and as decreases in signal intensity. Instead, 13 of the 17 ORs tested were detected in many fewer OSNs in *Emx2*$^{-/-}$ mice compared to *Emx2*$^{+/+}$ and *Emx2*$^{+/-}$ littermates (Table 3.3 and Figure 3.3). Conversely, the other four ORs were observed in an increased number of OSNs in *Emx2*$^{-/-}$ mice (Table 3.3 and Figure 3.4), suggesting that not all ORs need EMX2 to help activate their transcription.

ORs from all expression zones and both OR classes were affected

The mammalian OR gene family contains two phylogenetic classes (Glusman et al., 2001; Zhang and Firestein, 2002). Class I ORs appear to be more ancient, having

homology to fish ORs, and nearly all of them are expressed only in the dorso-medial zone of the mammalian olfactory epithelium. Class II receptors evolved more recently, are more numerous, and their expression spans all regions of the olfactory epithelium. I observed a decrease in the frequency of expression for 3 Class I and 10 Class II ORs, while all 4 ORs that increased were from Class II (Table 3.3).

The overall pattern of OR expression in *Emx2*$^{-/-}$ mice appeared normal. Sections from multiple levels of the nasal cavity provided no evidence that the ORs detected in fewer OSNs had merely shifted their expression to different regions or zones in the olfactory epithelium. For the ORs detected with increased frequency, the expression zones were similarly stable, though small expansions may have occurred. For example, the expression of *Olfr15* in the ventro-lateral region in wild type mice spread into the dorso-medial region in *Emx2*$^{-/-}$ mice (Figure 3.4A-B).

Expression of many ORs decreased in *Emx2*$^{-/-}$ mice

To gain a more comprehensive view of whether OR expression depends on EMX2 Affymetrix GeneChip® Mouse Exon 1.0 ST Arrays were used to compare the olfactory epithelia of *Emx2*$^{-/-}$ and *Emx2*$^{+/+}$ mice (n = 3). Unlike other GeneChip microarrays tested, which detect OR mRNAs poorly, this exon microarray detected many OR mRNAs (Shetty et al., 2005; Sammeta et al., 2007). The gene level analysis of these data identified 677 OR transcript clusters, representing 734 OR genes, with mRNA signals above background (Supplemental Table 1). Of these, 336 transcript clusters (representing 365 OR genes) were significantly reduced in the *Emx2*$^{-/-}$ samples. Only 22 transcript clusters were significantly increased. Of the 13 ORs that were decreased in my in situ hybridization data, 9 were significantly decreased and one, *Olfr17*, was not represented on the microarray (Table 3.1). The remaining three that showed decreases by in situ hybridization did not reach significance in the microarray data, an indication that the statistical analysis of the microarray data was conservative. All four ORs that increased in my in situ hybridization data were significantly increased in the microarray data.

The absence of EMX2 disproportionately impacted OR mRNAs compared to

other mRNAs in the olfactory epithelium. The 336 OR transcript clusters that were significantly less abundant in the *Emx2*$^{-/-}$ samples represented 28% of the transcript clusters that had significant decreases. OR mRNAs represent about 10% of the mRNA species expressed in mouse OSNs (Sammeta et al., 2007). OR mRNAs were also the most strongly affected mRNAs. Of the 250 transcript clusters with the greatest fold decreases in this dataset, 217 were ORs. Even more compelling was a comparison of fold changes for all ORs detected on the array against the fold changes detected in an equivalent population of mRNAs - 340 randomly selected OSN-enriched mRNAs (Sammeta et al., 2007). Compared to OR mRNAs, the abundance of these OSN-enriched mRNAs was only slightly decreased by the 42% reduction in mature OSNs (Figure 3.5). To illustrate this fact at the level of individual genes, my cell count data predicted that mRNAs expressed solely in mature OSNs should have decreased by approximately 42%. Indeed, this prediction was borne out as *Omp* mRNA was reduced by 44%, *Adcy3* by 28%, *Cnga2* by 38%, *Ano2* by 56% (Yu et al., 2005), and *Umodl1* by 52% (Yu et al., 2005). These data lead me to conclude that the decrease in mature OSN number could have accounted for only a small fraction of the ORs with decreased expression in *Emx2*$^{-/-}$ mice.

EMX2 regulates OR genes independently of OR gene cluster organization

Most OR genes occur in clusters on the chromosomes. Analysis was performed on four of these clusters: 17-1, 7-3, 11, and 14-1. The absence of EMX2 did not have the same effect on all OR genes within any of these clusters. OR genes whose mRNAs decreased coexisted with OR genes whose mRNAs increased in *Emx2*$^{-/-}$ mice in all four clusters. For example, of the 50 ORs in Cluster 17-1, the microarray detected 3 increases, 16 decreases, 19 that had no significant change, 10 that were not represented on the microarray, and 2 that were not above background. Supplementary Table 2 contains a complete listing of the ORs in these clusters.

DISCUSSION

By comparing expression of *Emx2*$^{-/-}$ mice with wild-type and heterozygous littermates, I detected reduced expression of many ORs and increased expression of a few ORs. Unlike markers of OSN maturity, the reduction in OR expression was disproportionately greater than a 42% reduction in mature OSNs, indicating that the absence of EMX2 is not altering OR expression through some general defect in OSN phenotype. EMX2 therefore appears to contribute to transcriptional activation of many, perhaps most, mouse ORs. I hypothesize that the action of EMX2 on OR expression is direct, consistent with previous evidence that EMX2 can bind an OR promoter and that most of the OR promoter regions predicted thus far have homeodomain-like elements that would be necessary for direct action of EMX2 on OR gene transcription (Vassali et al., 2002; Hirota et al., 2004, Hoppe et al., 2006; Michaloski et al., 2006). A few ORs increased in abundance in *Emx2*$^{-/-}$ mice, arguing that some ORs may be transcribed independently of EMX2. These OR genes appeared to be chosen for expression more often in the absence of EMX2, perhaps compensating for a reduction in the frequency of choice of most other OR genes.

OSN maturity is unaffected in the absence of EMX2

Four lines of evidence argue that a decrement in OSN maturity was not the cause of reduced OR expression. First, the in situ hybridization data indicated that both reductions and increases were due to changes in the number of OSNs expressing an OR rather than in the amounts of OR mRNA per OSN. In other words, the absence of EMX2 altered the frequency with which an OR gene was chosen for expression. Second, the mRNAs of genes expressed specifically in mature OSNs showed reductions in abundance that corresponded closely with the 42% reduction in the number of mature OSNs. In contrast, more than 250 OR mRNAs had reductions of more than 100%, a highly disproportionate effect. Third, the elaboration of cilia is one of the final events in the maturation of OSNs (Cuschieri and Bannister, 1975; Schwarzenbacher et al., 2005), and therefore should be one of the events most susceptible to defective maturation of OSNs, but no evidence of this was observed at the level of expression of cilia-related genes in *Emx2*$^{-/-}$ mice. For example, *Dnali1, Tekt1, Hydin, Ift172, Spag6, Spa17, Ift74, Bbs4,*

Bbs2, and *Nphp1*, which are all documented cilia-related mRNAs expressed by OSNs, were present at normal amounts in the olfactory epithelia of *Emx2*$^{-/-}$ mice (Kulaga et al., 2004; Nishimura et al., 2004; McClintock et al., 2008). Fourth, some ORs showed expression in significantly more OSNs, as would be expected if OR gene choice mechanisms were acting normally and free to favor those ORs least dependent on EMX2. If a general defect in OSN development was affecting OR gene expression, then all ORs should show reduced expression.

Transcription of many OR genes depends on EMX2

Measuring the number of OSNs expressing an OR by in situ hybridization showed decreases for 76% of the ORs tested. The broader experiment using microarray analysis to rapidly test larger numbers of ORs, albeit less sensitive for any given OR mRNA, gave similar results, finding significant decreases in 49% of the OR transcript clusters detected. It is likely that the microarray data underestimated the number of affected ORs. First, both of the ORs that failed to reach significance in the microarray data but were also tested by in situ hybridization were detected in many fewer OSNs in *Emx2*$^{-/-}$ mice. Second, ORs were disproportionately affected in *Emx2*$^{-/-}$ mice compared to other genes expressed primarily by OSNs. Third, homeodomain-like sites are found in the predicted promoter regions of nearly all OR genes analyzed thus far, so if EMX2 is acting directly on OR promoters, the vast majority of OR promoters have potential binding sites for EMX2 (Vassali et al., 2002; Hoppe et al., 2006; Michaloski et al., 2006). These facts argue that EMX2 helps stimulate transcription of at least a majority of OR genes.

Identifying all OR genes affected by the absence of EMX2 was not possible from the data obtained. First, the methods used assessed many, but not all, OR genes. Second, some OR transcript clusters on the exon array detect multiple OR mRNAs due to sequence similarity between certain ORs. For the ORs in this category, therefore, it cannot be certain which of the OR mRNAs represented in a transcript cluster were decreased, forcing us to calculate conservatively. By limiting the calculation to ORs that decreased at least 2-fold in order to avoid counting any ORs that might have decreased due solely to the 42% reduction in mature neurons, the number of ORs for which there is evidence of a decrease was 280. Similarly, microarray data identified at least 19 ORs

whose frequency of expression increased.

The dependence of chemosensory receptor genes on EMX2 may not be limited to OR genes. The microarray data detected significant decreases in abundance in *Emx2*$^{-/-}$ mice for five trace amine-associated receptor (Taar) transcript clusters, representing 7 of the 15 intact mouse Taar genes (Supplementary Table 1). Taar genes are expressed in subsets of OSNs and at least some of them encode proteins that detect amine odors in urine (Liberles and Buck, 2006).

EMX2 appears to be the predominant homeobox protein for OR genes

If EMX2 was not more important for stimulating OR gene transcription than other homeobox proteins, I should not have observed decrements in the expression of most ORs tested. However, the dependence of OR genes on EMX2 was only rarely absolute. Only five of the OR mRNAs tested by in situ hybridization failed to be observed in at least one OSN in *Emx2*$^{-/-}$ mice. Consistent with this observation, some of the OR mRNAs that decreased in the microarray analysis were detected at levels above background in *Emx2*$^{-/-}$ mice. Therefore, it would be expected that other homeobox proteins contribute to OR gene expression. A few dozen other homeobox transcription factor mRNAs are present in OSNs (Sammeta et al., 2007). The most promising candidate is *Lhx2*, a LIM-homeobox transcription factor reported to contribute to OR gene expression (Hirota et al., 2007). Like EMX2, LHX2 binds to an OR promoter that contains a homeodomain-like site (Hirota et al., 2004). In *Lhx2*$^{-/-}$ mice, which die in utero at about age E15.5, differentiation of OSNs appears to be halted at a stage where OR expression has just been initiated and very few mature OSNs form (Kolterud et al., 2004). Only in the dorsal zone of the epithelium do mature OSNs form, and only at 10% of their normal numbers. OR expression can be detected in immature OSNs (Iwema and Schwob, 2003), but if differentiation halts within the immature OSN stage this is a potential explanation for why expression of few ORs can be detected in *Lhx2*$^{-/-}$ mice and correlates exactly with the finding that two Class I ORs normally expressed ventrally cannot be detected in *Lhx2*$^{-/-}$ mice while at least some dorsal zone Class I ORs can be detected, albeit at reduced levels (Hirota et al., 2007). In *Lhx2*$^{-/-}$ mice, therefore, whether decreased expression of ORs could result from the significant reduction in the number of sufficiently

differentiated OSNs, from loss of direct positive action at OR promoters or both is difficult to assess.

For EMX2 the situation is more easily interpreted. Effects on OSN development were limited to a reduction in the number of mature OSNs in *Emx2*$^{-/-}$ mice, so the amount of OR expression measured, which included increased, decreased, and unaffected OR genes, was most likely due to transcriptional events rather than OSN differentiation or survival. Overall, the data are most consistent with the interpretation that the ORs with reduced expression in *Emx2*$^{-/-}$ mice depend on EMX2 to stimulate their transcription. Whether this dependence is direct, as EMX2 binding to the *Olfr151* (M71) promoter would suggest (Hirota et al., 2004), or indirect cannot yet be concluded. However, the effects of EMX2 deletion on OR expression were not due to loss of LHX2. *Lhx2* expression, which is primarily in immature OSNs, was normal in *Emx2*$^{-/-}$ mice (Supplemental Fig. 1). Presuming that EMX2 does act directly on OR promoter elements, then the idea that these other homeobox transcription factors might stimulate the same OR genes as EMX2 at varying efficacies seems reasonable. However, whether these hypothetical mechanisms are normally active or are instead merely compensating mechanisms that are irrelevant in a wild-type mouse is impossible to predict at this time. It should also be noted that the homeodomain-like site of putative OR promoters may not be the only avenue for compensation in *Emx2*$^{-/-}$ mice. At present, I interpret the findings to indicate that EMX2 is the most important homeobox protein for OR genes in general, and that other homeobox proteins can only partially substitute for EMX2 to drive expression of most OR genes.

For OR genes that appeared to be independent of EMX2, their promoters may be more sensitive to other homeobox proteins, such as LHX2, or alternatively, don't depend on homeobox proteins at all (Michaloski et al., 2006). However, the data cannot completely rule out the possibility that these ORs do normally depend on EMX2 and are merely better compensated than other OR genes in the absence of EMX2. This would mean that all ORs normally depend on EMX2 for activation. To clarify these questions, future experiments will need to investigate the ability of EMX2 to act directly on putative promoters of ORs that were sensitive, versus those that were insensitive, to the absence of EMX2.

Implications for OR gene choice

Two of my findings seem relevant to the problem of how an OSN selects an OR gene for expression. First, some ORs showed expression in increased numbers of OSNs in *Emx2*$^{-/-}$ mice. This is consistent with the hypothesis that differentiating OSNs may serially express several ORs before locking in the expression of one OR gene (Shykind et al., 2004). This idea depends on the demonstrated ability of expressed ORs to suppress expression of other OR genes, such that in *Emx2*$^{-/-}$ mice this ratcheting mechanism would have reduced probability of locking on the ORs most dependent on EMX2 (Feinstein et al., 2004; Lewcock and Reed, 2004; Serizawa et al., 2003; Shykind et al., 2004). Alternatives exist, however, such as explanations in which the absence of EMX2 leads to disinhibition or relaxing the competition for some limiting factor, thereby increasing the selection of OR genes for which EMX2 is not the dominant positive factor.

EMX2 has several critical roles in OSNs

The evidence that EMX2 is important for OR gene expression adds to previous evidence that EMX2 is critical for OSN development and function. In addition to altering OR expression, the absence of EMX2 causes OSN axons to terminate at the surface of the olfactory bulb where they form a fibrous cellular mass (Yoshida et al., 1997). OR expression in OSNs that lack contact with their targets is consistent with previous evidence of recovery of OR expression in bulbectomized rodents and with evidence that OR expression precedes contact of OSN axons with the bulb (Strotmann et al., 1995; Sullivan et al., 1995; Konzelman et al., 1998). The lack of axonal contact with the olfactory bulb was therefore unlikely to have caused the changes of OR expression observed in *Emx2*$^{-/-}$ mice.

The data is similarly inconsistent with the interpretation that the axonal targeting defect in *Emx2*$^{-/-}$ mice was caused by the reduced expression of OR genes, largely because I did not find evidence that OSNs lack OR expression or have reduced transcription of the OR gene expressed, but rather the absence of EMX2 changed the frequency with which many OR genes were selected for expression. However, EMX2 has

another putative function in OSNs that may be more relevant. EMX2 is reported to interact with eIF4E and may therefore regulate translation of proteins in OSNs (Nedelec et al., 2004). This interaction was detected in OSN axons, which also contain OR mRNAs (Vassar et al., 1994; Ressler et al., 1994), so it is possible to envision a scenario whereby changes in OR protein translation in OSN axons results in altered OSN axon behavior. ORs are important for the coalescence of OSN axons expressing the same OR, and they might also be involved in the generation of cAMP that is important for OSN axon extension during development (Imai et al., 2006). If translation of OR mRNAs in OSN axons is reduced in the absence of EMX2 then OSN axon behavior could be compromised, leading to defects in both axon extension and fasciculation. However, alternative causes, such as changes in the reception or processing of external guidance signals in *Emx2*$^{-/-}$ mice, are perhaps even more plausible.

The place of EMX2 in the hierarchy of OR gene regulation

EMX2 was not necessary for the zonality of OR gene expression. Neither did it appear to be necessary for the choice of a single OR gene by each OSN, as I would then have expected to observe widespread increases in the frequency of OR expression. The data revealed no evidence implicating EMX2 in regulating clusters of OR genes, in the silencing of OR genes, or in the random inactivation of one parental allele of each OR gene. Instead, I conclude that EMX2 is a transcriptional activator for OR genes. Though it is necessary for producing normal frequencies of expression of many OR genes, it is perhaps best viewed as a permissive factor whose stimulatory action is gated by the contributions of other factors that control the singularity, zonality, and monoallelism of OR gene expression.

Table 3.1 Gene reference table

Gene Symbol	Gene Name	Mouse Gene ID	Chr.	Synonyms
Adcy3	adenylate cyclase 3	104111	12	AC3
Ano2	anoctamin 2	243634	12	Tmem16b, N64J
Bbs2	Bardet-Biedl syndrome 2	67378	8	
Bbs4	Bardet-Biedl syndrome 4	102774	9	
Cnga2	cyclic nucleotide gated channel alpha 2	12789	X	Cnca, Cncg4, OCNC1
Cyp2g1	Cytochrome P450, family 2, subfamily g, polypeptide 1	13108	7	
Dnali1	dynein, axonemal, light intermediate polypeptide 1	75563	4	
Ebf1	early B-cell factor 1	13591	11	O/E-1, Olf-1
Ebf2	early B-cell factor 2	13592	14	Mmot1, O/E-3
Ebf3	early B-cell factor 3	13593	7	O/E-2
Ebf4	early B-cell factor 4	228598	2	Ebf3, O/E-4, Olf-1
Emx2	empty spiracles homolog 2	13797	19	Pdo
Gap43	growth associated protein 43	14432	16	B-50, Basp2, GAP-43
Hydin	hydrocephalus inducing	244653	8	hy-3, hy3
Ift172	Intraflagellar transport 172 homolog	67661	5	Slb, wim
Ift74	Intraflagellar transport 74 homolog	67694	4	Ccdc2, Cmg1
Lhx2	LIM homeobox protein 2	16870	2	LH2A, Lh-2, Lim2, ap, apterous
Ncam1	neural cell adhesion molecule 1	17967	9	CD56, E-NCAM, Ncam
Neurog1	neurogenin 1	18014	13	Ngn1, Math4C, Neurod3

Nphp1	nephronophthisis 1	53885	2	
Olfr121	olfactory receptor 121	258622	17	MOR263-4
Olfr129	olfactory receptor 129	258324	17	MOR263-9
Olfr1440	olfactory receptor 1440	258679	19	MOR215-1
Olfr15	olfactory receptor 15	18312	16	MOR256-17; OR3
Olfr1507	olfactory receptor 1507	57269	14	MOR244-1, Mor28
Olfr1508	olfactory receptor 1508	57270	14	MOR244-2
Olfr151	olfactory receptor 151	406176	9	MOR171-2; M71
Olfr156	olfactory receptor 156	29846	4	MOR262-6; OR37B
Olfr160	olfactory receptor 160	80706	9	MOR171-3; M72; Olfr7b
Olfr17	olfactory receptor 17	18314	7	MOR263-15; P2
Olfr2	olfactory receptor 2	18317	7	MOR103-15; I7; I54
Olfr270	olfactory receptor 270	258600	4	MOR262-9
Olfr272	olfactory receptor 272	258836	4	MOR262-7
Olfr273	olfactory receptor 273	258821	4	MOR222-8
Olfr308	olfactory receptor 308	258614	7	MOR104-1
Olfr544	olfactory receptor 544	257926	7	MOR42-3
Olfr545	olfactory receptor 545	258837	7	MOR42-1
Olfr6	olfactory receptor 6	233670	7	MOR103-16; M50
Olfr615	olfactory receptor 615	259084	7	MOR19-2
Olfr642	olfactory receptor 642	258326	7	MOR13-6
Olfr90	olfactory receptor 90	258469	17	MOR256-21
Omp	olfactory marker protein	18378	7	
Spa17	sperm autoantigenic protein 17	20686	9	Sp17
Spag6	sperm associated antigen 6	50525	16	axoneme protein
Tekt1	tektin 1	21689	11	MT14
Umodl1	uromodulin-like 1	52020	17	Olfactorin, N8

Table 3.1 (continued) Gene reference table. Chr., mouse chromosome.

Table 3.2 Olfactory epithelium cell counts

Genotype	Number of mice	Mean olfactory epithelium thickness (μm)	OMP^+ cell count	Total cell count
+/+	2	98 ± 3	125.5 ± 20.0	1358 ± 61
+/-	5	98 ± 2	137.5 ± 25.5	1360 ± 65
-/-	6	83 ± 3	77.0 ± 16.5	1132 ± 36

Olfactory epithelium thickness and number of mature OSNs (OMP^+) were reduced in *Emx2*$^{-/-}$ mice. Cell counts are means and standard deviations per mm of epithelium.

Table 3.3 ISH results of odorant receptors

Gene Symbol	Class	OSNs/mm (wild-type)	ISH ratio	GeneChip ratio	Region
Olfr2	Class II	2.6	0.03	0.4*	Ventral
Olfr6	Class II	0.8	0.02	0.2*	Ventral
Olfr15	Class II	3.5	5.70	3.1*	Ventral
Olfr17	Class II	0.9	0.10	NP	Ventral
Olfr90	Class II	1.0	2.10	1.5*	Ventral
Olfr129; Olfr121	Class II	2.0	2.10	2.9*	Ventral
Olfr156	Class II	3.1	0.02	0.4	OR37 region
Olfr160; Olfr151	Class II	1.6	0.40	0.3	Dorsal
Olfr270	Class II	0.7	0.07	0.3*	OR37 region
Olfr272	Class II	0.1	0.00	0.5*	OR37 region
Olfr273	Class II	0.5	0.00	0.2*	OR37 region
Olfr308	Class II	0.6	0.00	0.6*	Ventral
Olfr545; Olfr544	Class I	2.6	0.03	0.4*	Dorsal
Olfr615	Class I	1.0	0.00	0.2*	Dorsal
Olfr642	Class I	0.5	0.00	1.0	Dorsal
Olfr1440	Class II	1.2	1.80	1.7*	Ventral
Olfr1508; Olfr1507	Class II	1.9	0.05	0.5*	Ventral

OR mRNAs tested by in situ hybridization. OSNs/mm, the number of OSNs expressing the OR per mm of olfactory epithelium in Emx2+/+ mice. ISH: in situ hybridization. Ratios are *Emx2*$^{-/-}$ divided by *Emx2*$^{+/+}$. *, significant difference between *Emx2*$^{-/-}$ and *Emx2*$^{+/+}$ mice. NP: not present on the microarray. Region: the zone of expression within the olfactory epithelium.

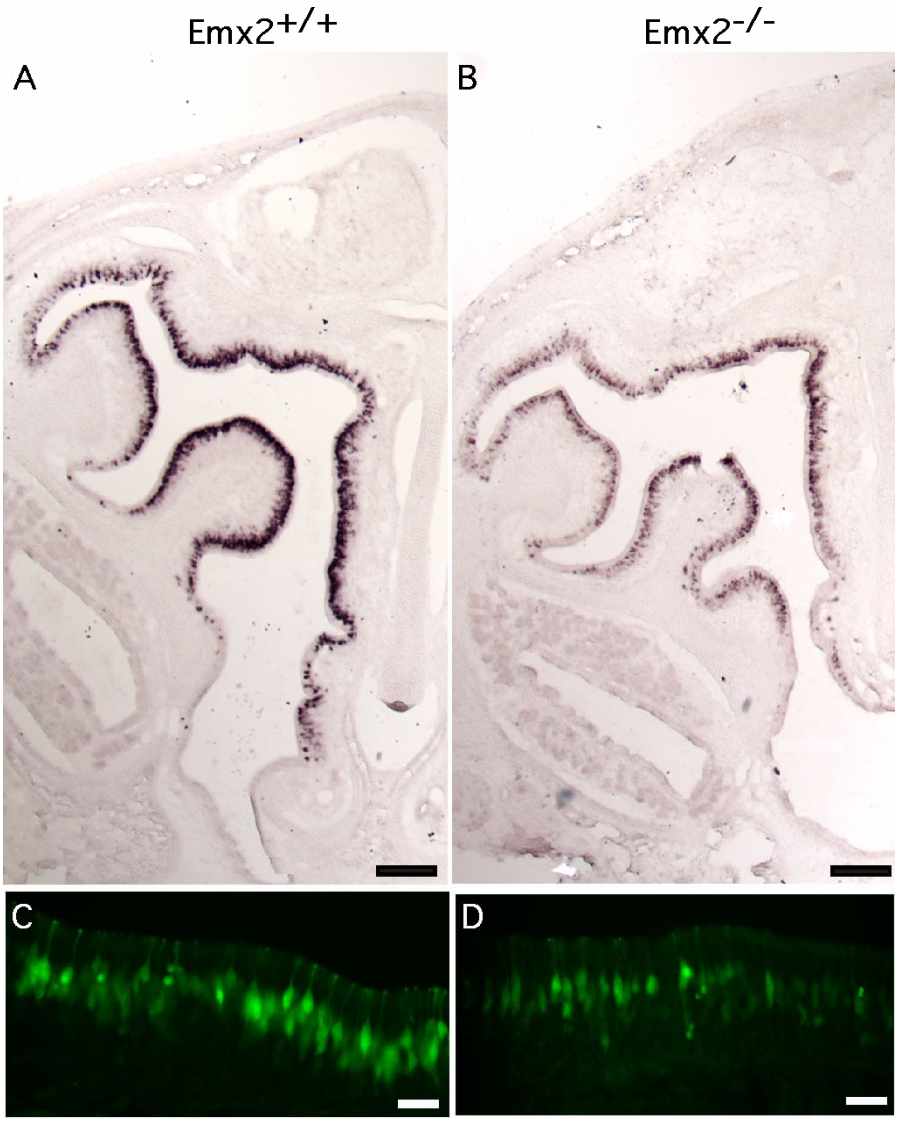

Figure 3.1 *Emx2*$^{-/-}$ olfactory epithelium

Emx2$^{-/-}$mice at age E18.5 had olfactory epithelia containing mature OSNs over the same extent of the nasal cavity as wild type littermates. **A, B**: In situ hybridization for *Omp* mRNA to identify mature OSNs. **C, D:** GFP expression from the Omp locus was used to identify and count mature OSNs. C. *Emx2*$^{+/+}$*:Omp-GFP*$^{-/-}$ genotype. D. *Emx2*$^{-/-}$*:Omp-GFP*$^{-/-}$ genotype. Scale bars, A-B, 200 µm; C-D, 20 µm.

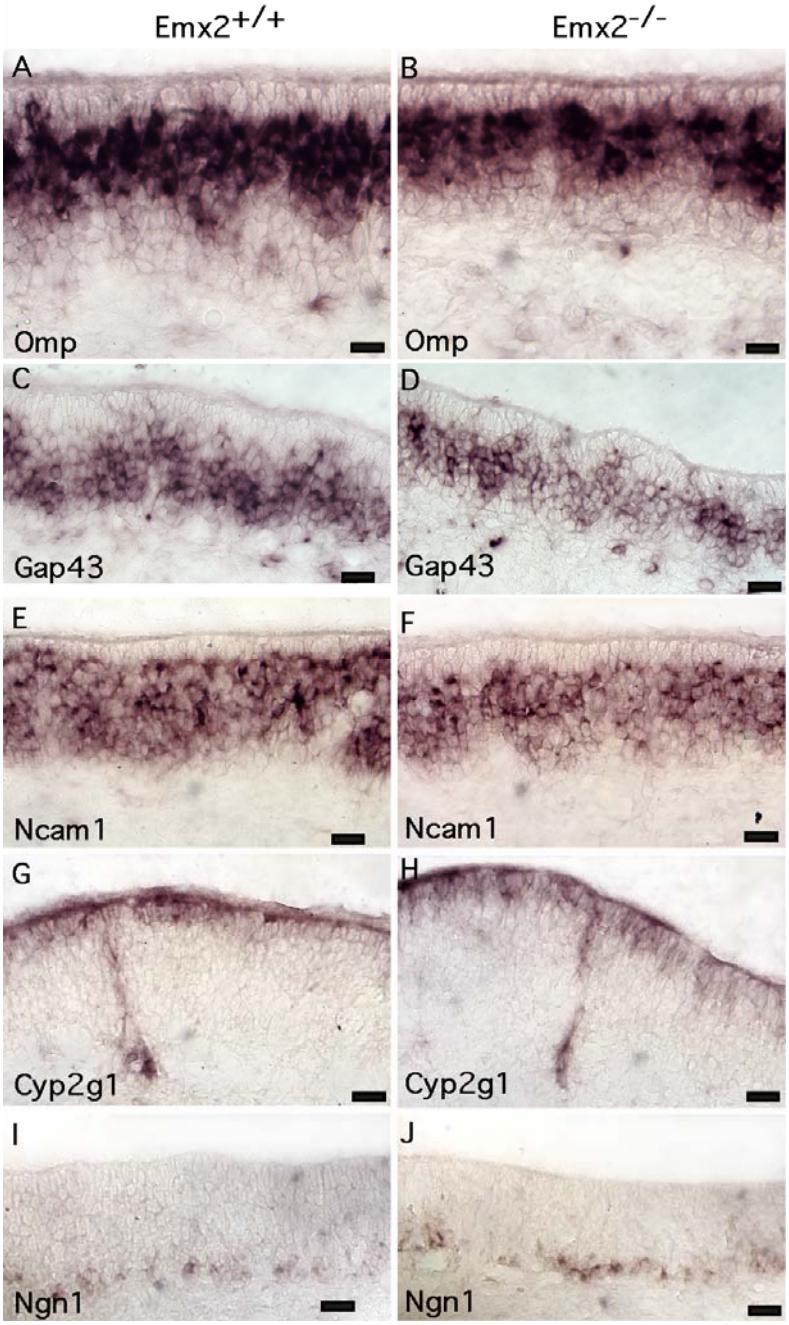

Figure 3.2 Pseudostratification in *Emx2*$^{-/-}$ mice.
Mice lacking EMX2 had normal pseudostratification of the cell body layers in the olfactory epithelium. **A, B:** In situ hybridization for *Omp* mRNA to label mature OSNs. **C, D**: In situ hybridization for *Gap43* to label immature OSNs. **E, F**. In situ hybridization for *Ncam1* to label both developmental stages of OSNs. **G, H**: In situ hybridization for *Cyp2g1* to label sustentacular cells and Bowman's glands (the labeled structure stretching from the lamina propria across the entire depth of the olfactory epithelium. **I, J:** In situ hybridization for *Ngn1* (*Neurog1*) to label a subpopulation of globose basal cells. Scale bars, 20 µm.

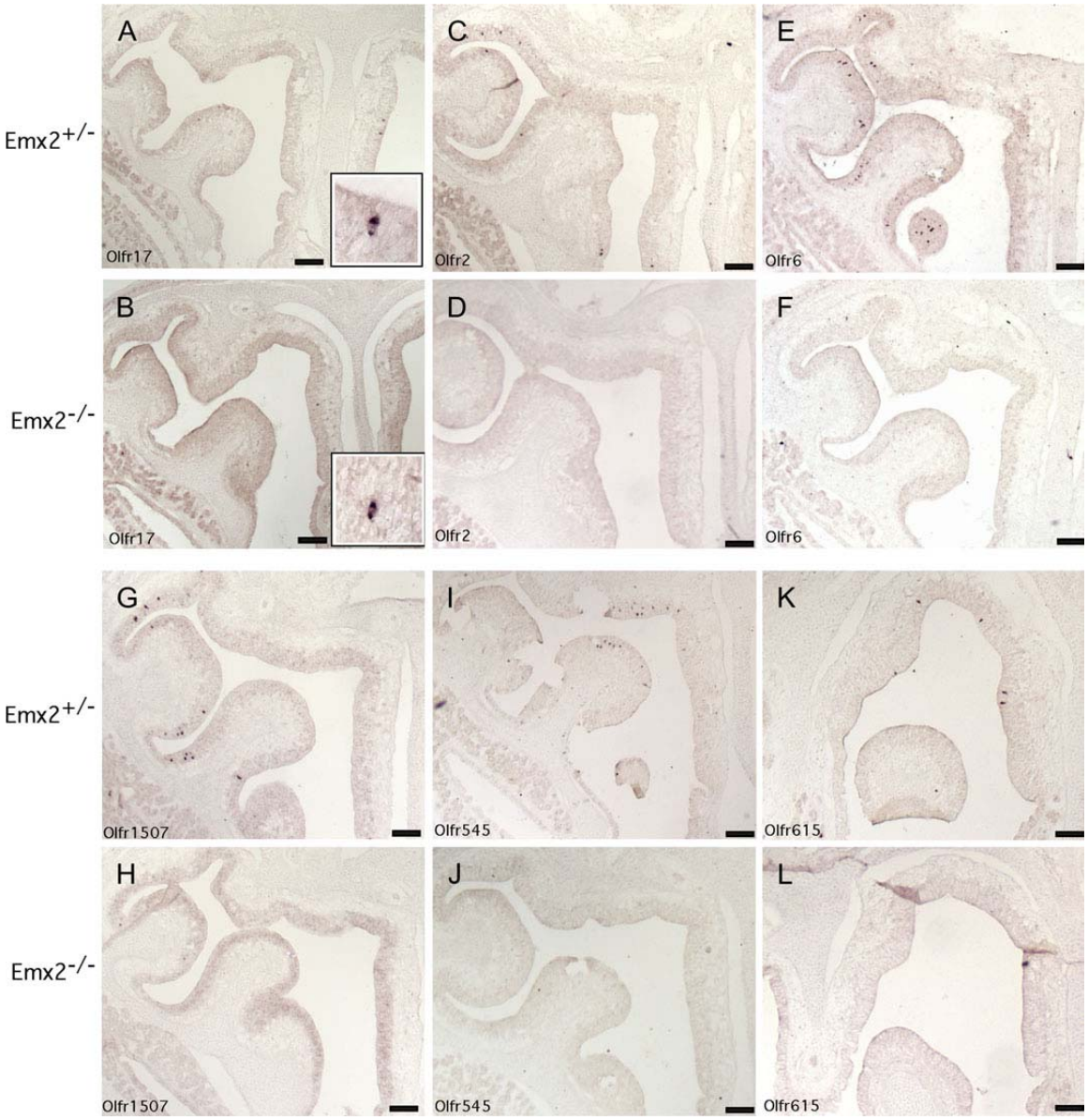

Figure 3.3 ORs with decreased expression
Frequency of expression of many ORs decreased in *Emx2*$^{-/-}$ mice. **A, B:** *Olfr17*, a Class II OR expressed in the ventro-lateral region. Insets, the intensity of signal for an *Olfr17* mRNA within each neuron was not altered by the absence of EMX2. **C, D:** *Olfr2*, a Class II OR expressed in the ventro-lateral region. **E, F**: *Olfr6*, a Class II OR expressed in the ventro-lateral region. **G, H:** *Olfr1507*, a Class II OR expressed in the ventro-lateral region. **I, J:** *Olfr545*, a Class I OR expressed in the dorso-medial region. **K, L:** *Olfr615*, a Class I OR expressed in the dorso-medial region. Half the bilaterally symmetric nasal region is shown in each image, with septum at the right. Scale bars, 200 μm.

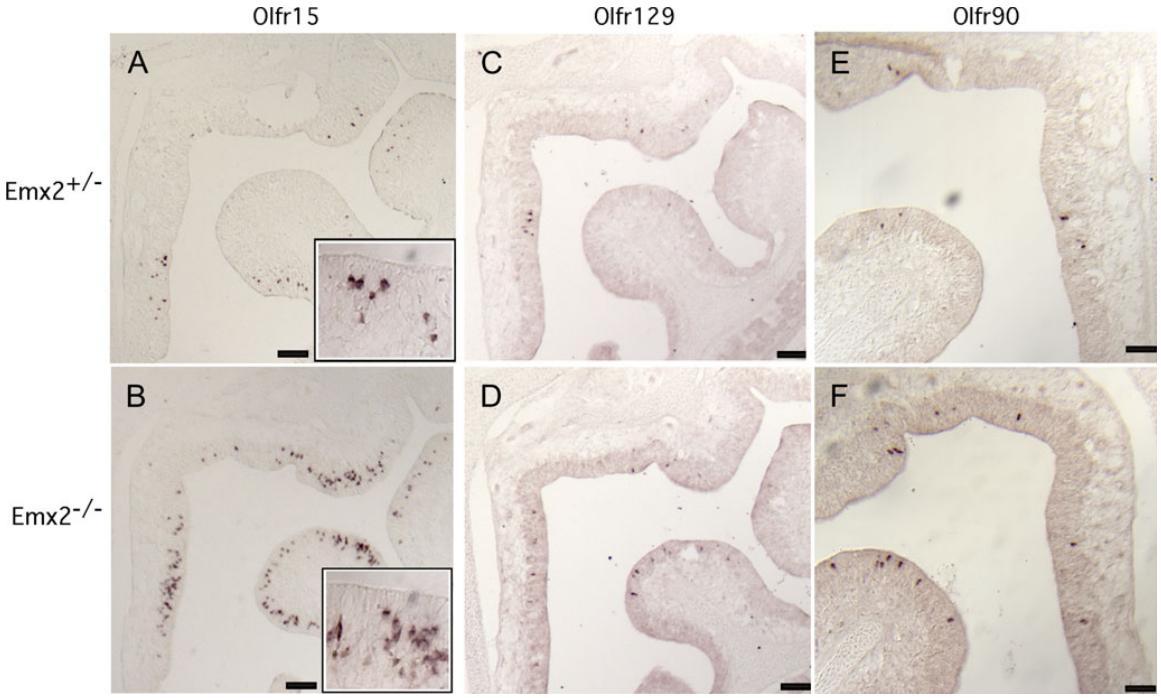

Figure 3.4 ORs with increased expression
Frequency of expression of a few ORs increased in *Emx2*$^{-/-}$ mice. **A, B:** *Olfr15*, a Class II OR expressed in the ventro-lateral region. The region of expression of *Olfr15* appeared to expand in *Emx2*$^{-/-}$ mice. Insets, the intensity of signal for *Olfr15* mRNA within each neuron was not altered by the absence of EMX2. **C, D:** *Olfr129*, a Class II OR expressed in the ventro-lateral region. **E, F:** *Olfr90*, a Class II OR expressed in the ventro-lateral region. Scale bars, **A-D**, 200 μm; **E-F**, 80 μm.

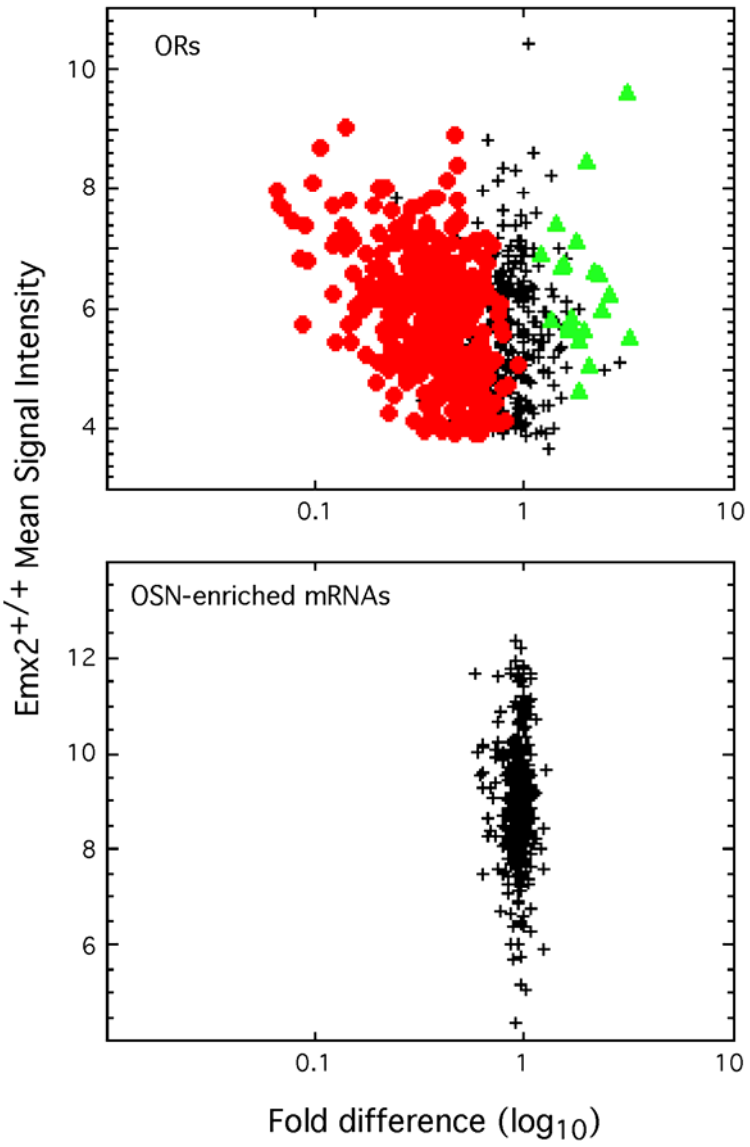

Figure 3.5 ORs are disproportionately affected
Abundances of OR mRNAs were disproportionately altered compared to other OSN-enriched mRNAs in mice lacking EMX2. The mean signals from GeneChip mouse exon arrays for *Emx2*$^{+/+}$ mice (log2) are plotted against the log10 of the fold difference between *Emx2*$^{-/-}$ and *Emx2*$^{+/+}$ mice. Red circles, significantly decreased OR clusters. Green triangles, significantly increased OR clusters.

Chapter 4

EMX2 regulates olfactory sensory neuron survival and expression of *Ablim1*

INTRODUCTION

Empty spiracles homolog 2 (EMX2) is a homeobox transcription factor that is critical for the development of several tissues, including neural tissues (Pellegrini et al., Yoshida et al., 1997; Lopez-Bendito et al., 2002; Ligon et al., 2003; Hamasaki et al., 2004). One of the developmental processes that EMX2 regulates is axon growth and targeting. For example, in *Emx2*$^{-/-}$ mice thalamocortical projections are fewer, are delayed, show fasciculation abnormalities, are often more superficial and often fail to turn medially at the corticostriatal junction (Lopez-Bendito et al., 2002). EMX2 is also required for the entorhinal projections into the dentate gyrus. In the absence of EMX2 entorhinal fibers do not exhibit their normal specificity, a defect that appears to be independent of effects on the migration and differentiation of dentate gyrus granule cells, (Deller et al., 1999; Savaskan et al., 2002). Defects in axon growth in *Emx2*$^{-/-}$ mice are exacerbated by the loss of EMX1 (Shinozaki et al., 2002; Bishop et al., 2003). In *Emx1/Emx2* double knockouts cortical efferent axons fail to enter the internal capsule, while thalamocortical axons fail to enter the cortex (Bishop et al., 2003). The substantial increase in defects in *Emx1/Emx2* double knockouts suggests that the two transcription factors either share a set of target genes or separately drive expression of genes that encode components of a pathway necessary for axon growth. These may be conserved mechanisms, as the *Drosophila* homolog, *empty spiracles* (*ems*), also is necessary for neural development, including proper development of olfactory projection neurons (Walldorf and Gehring et al., 1992; Lichtneckert et al., 2008).

A few axon growth related genes have been identified that may be regulated by EMX2, including *Wnt-1* in the dorsomedial telencephalon and *Crmp1* and *Odz4* in the cortex, however, the mechanisms by which EMX2 regulates axon growth are still largely unknown (Ligon et al., 2003; Li et al., 2006). In *Emx2*$^{-/-}$ mice, olfactory sensory neuron

(OSN) axons failed to innervate the olfactory bulb and instead prematurely terminate in a fibrous cellular mass located between the olfactory bulb and the cribriform plate of the ethmoid bone (Yoshida et al., 1997). As *Emx1* is not expressed in the olfactory epithelium, OSNs provide a cell type in which the effects of EMX2 on axon growth can be studied without influence of EMX1.

Both *Emx2* mRNA and protein are detected in immature and mature OSNs (Nedelec et al., 2004). In the olfactory bulb, *Emx2* expression is low in the proliferative layer, but is detected in subependymal layer and mitral cells in the accessory olfactory bulb early in development, while *Emx1* is expressed in the subventricular zone and mitral cells of the olfactory bulb throughout life (Mallamaci et al., 1998). Expression of *Emx2* decreases after embryonic day 15 and is not detected in olfactory bulb cells of adult mice (Mallamaci et al., 1998; Nedelec 2005). OSN axon growth provides an advantageous model to investigate EMX2 function, in part because the continuous replacement of damaged OSNs means that the role of EMX2 in the development of OSNs is always active. Because *Emx2* is strongly expressed in immature OSNs, the cells responsible for innervating the olfactory bulb, the absence of EMX2 probably causes OSN axon growth defect via cell autonomous causes (Nedelec et al., 2004). This would not be unusual as several aspects of OSN axon growth, such as segregation of axons in the olfactory nerve and the coalescence of axons according to the odorant receptor that each OSN expresses, are independent of bulb-derived cues (St. John et al., 2003; Yoshihara et al., 2005; Imai et al., 2009).

The defective olfactory axon phenotype seen in *Emx2* knockout mice is also seen in targeted deletions of several other transcription factors, including *Dlx5, Fezf1, Klf7* and *Arx* (Levi et al., 2003; Long et al., 2003; Yoshihara et al., 2005; Hirata et al., 2006; Laub et al., 2005; 2006). These transcription factors are expressed in the olfactory epithelium (*Fezf1*), in the olfactory bulb (*Arx*), or in both (*Dlx5* and *Klf7*). That a similar phenotype develops due to changes in either the innervating neurons or the target tissue suggests that the defect could arise from changes in signaling between the incoming axons and their target. For example, defects in Wnt signaling from the olfactory placode to the developing forebrain have been proposed to underlie this phenotype in mice lacking *Dlx5* (Zaghetto et al., 2007). In cases where the defect is due solely to changes in the olfactory

bulb, such as in *Arx* knockouts in which development of multiple cell types in the olfactory bulb is altered, an instructional signal that directs OSN axon growth might have been lost (Yoshihara et al., 2005). In cases where the defect lies solely within the OSN axons, the defect would need to be in the reception or the transduction of the signal. The hypothesis that deletion of *Emx2, Dlx5, Fezf1, Klf7* and *Arx* independently cause defects of critical components of the same signaling mechanism is appealing. Because OSN axons in these knockout mice stall rather than wander into inappropriate locations, I suspect that this putative mechanism controls the robustness of axon growth.

I have previously shown that EMX2 stimulates the expression of a majority of odorant receptor genes (McIntyre et al., 2008). Odorant receptors play several roles in the behavior of OSN axons, being specifically responsible for the coalescence of OSN axons into glomeruli (Mombaerts et al., 1996, Feinstein and Mombaerts, 2004; Feinstein et al., 2004). Odorant receptors also appear to differentially stimulate production of cAMP in OSN axons, thereby directly controlling levels of *Nrp1* expression and the position of glomeruli along the anterior-posterior axis of the bulb (Imai et al., 2006; 2009). However, I hypothesize that the phenotype of OSN axons lacking EMX2 is independent of the effects of EMX2 on odorant receptor expression. I propose that EMX2 also regulates the expression of axon guidance genes important for regulating OSN axon growth.

I found that in *Emx2*$^{-/-}$ mice, fully mature OSNs develop but their survival is reduced. Though the axons of both immature and mature OSNs fail to innervate the olfactory bulb, they do come in contact with the surface of the olfactory bulb. Other aspects of OSN axon behavior, such as segregation by type and expression of axon guidance cue receptors, appeared to be retained in *Emx2*$^{-/-}$ mice. The abundance of nearly all axon growth and guidance gene mRNAs was normal in the OSNs of *Emx2*$^{-/-}$ mice. The exception was the axonogenesis-related gene, *Ablim1*, which could not be detected in immature OSNs of *Emx2*$^{-/-}$ mice. These data suggest a mechanistic explanation whereby the loss of ABLIM1 interrupts the communication of stimulatory guidance cue receptors to the actin cytoskeleton in the growth cone of OSN axons.

MATERIALS AND METHODS

Mice

Genetically modified mice with a targeted disruption of the Emx2 gene were obtained from the RIKEN Center for Developmental Biology, Japan (Yoshida et al. 1997). Animals were maintained as heterozygotes as *Emx2*$^{-/-}$ mice die shortly after birth due to multiple organ defects (Pellegrini et al. 1996; Miyamoto et al. 1997). All studies were performed using animals at embryonic day 18.5 (E18.5). To obtain embryonic mice, heterozygous animals were mated overnight. The morning of detection of a vaginal plug was designated as age E0.5. Previous results showed no differences between *Emx2*$^{+/+}$ and *Emx2*$^{+/-}$ mice (McIntyre et al., 2008), so these genotypes were considered phenotypically identical.

To aid in the identification of mature OSNs and their axons in some experiments, *Emx2*$^{+/-}$ mice were crossed to olfactory marker protein green fluorescent protein (OMP-GFP) mice in which the OMP coding region is replaced by GFP, obtained from Dr. Peter Mombaerts (Max Planck Institute of Biophysics, Frankfurt, Germany). OSNs in OMP-GFP mice exhibit normal axon growth and homogenous coalescence of axons although there is a small increase in the overgrowth of axons past the glomerular layer and deeper into the bulb (Potter et al., 2001; St John and Key, 2005). Consistent with the interpretation that this increase in growth due to the absence of OMP was a small effect, the reduced axon growth phenotype seen in *Emx2*$^{-/-}$ mice was not altered in *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ mice. For example, comparing immunoreactivity for OMP and NCAM1 in *Emx2*$^{-/-}$ mice and GFP fluorescence in *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ mice revealed no difference in the failure of OSN axons to innervate the bulb or the restriction of OSN axons to the fibrous cellular mass that forms anterior and ventral to the olfactory bulb. All experiments with mice were performed in accordance with an approved institutional animal care and use protocol.

In situ hybridization and immunofluorescence

In situ hybridizations were performed as previously described (Shetty et al. 2005, Yu et al. 2005). Briefly, embryonic animals were collected from timed pregnant females,

chilled on ice and decapitated. Embryonic heads were fixed in paraformaldehyde overnight, followed by cryoprotection by washing in 10% for 1 hr, 20% for 1 hr, and 30% sucrose overnight. Following cryoprotection, heads were embedded in OCT (Sakura Finetek USA, Inc., Torrence, CA) and stored at -80˚C. 10 µm were placed onto Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA). Digoxygenin labeled ribo-probes were generated from cDNA fragments of ~400-600bp in length. Hybridization of riboprobes (1 ng/µl) was performed in 50% formamide in 10mM Tris-HCl (pH 8.0), 10% dextran sulfate, 1x Denhardt's solution, 600 mM NaCl, 0.25% sodium dodecyl sulfate, 1 mM EDTA, and 200 µg/ml yeast tRNA at 65˚C. Slides were washed with phosphate-buffered saline (PBS). Following hybridization, detection was performed with an alkaline phosphatase-conjugated antibody to digoxygenin and hydrolysis of nitro-blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphospate p-toluidine. Sense-strand riboprobes were used as controls and were invariably negative. Comparisons between genotypes were made using slides that were processed together under identical conditions on the same date.

For immunofluorescence, 10 µm cryosections were prepared using the same methods as for in situ hybridization, except that fixation was 2 hrs in 4% paraformaldehyde. Slides were washed 3 times for 10 min in 1x PBS followed by blocking at room temperature for 30 min with 2% BSA, 0.4% Triton 100-X, in 1x PBS. For cleaved-caspase 3 and phosphorylated-histone H3 detection, antigen retrieval was performed by incubating slides in sodium citrate buffer at 65˚C for 30 min. The following primary antibodies were used; rabbit anti-ADCY3 (1:200, Santa Cruz; sc-588); guinea Pig anti-mOR-EG (Olfr73) (1:1000; a gift from Dr Yoshihiro Yoshihara,); guinea Pig anti-MOR28 (Olfr1507) (1:1000; a gift from Dr. Yoshihara,), rabbit anti-cleaved caspase 3 (1:200, Cell Signaling, Inc., #96645S); rabbit anti-phosphoHistone H3 (1:200; Millipore, 06-570); rabbit anti-GAP43 (1:200; Millipore, AB5220); rabbit anti-laminin (1:25, Sigma-Aldrich; L9393); mouse anti-NCAM1 (1:1000; Sigma-Aldrich, C9672). The use and specificity of these antibodies has previously been demonstrated (Akins and Greer, 2006; Dudanova et al., 2007; Kaneko-Goto et al., 2008; Rodriguez-Gil and Greer, 2008; Zhao et al., 2008). Secondary antibodies, all used at a dilution of 1:500, were DyLight 549 donkey anti-goat, DyLight 488 donkey anti-rabbit, and DyLight 488 donkey

anti mouse from Jackson Immunoresearch Laboratories, Inc.

For labeling of cell surface carbohydrates with lectin, slides were washed with 3 times for 10 min in 1x PBS then blocked in 2% BSA, 0.3% Triton X-100 in 1xPBS for 30 minutes. The slides were then incubated with 20ug/ml of biotin conjugated *Dolichos bifluros* agglutinin (DBA) (Sigma-Aldrich, L6553-5MG) for 1 hour at room temperature. Slides were then washed 3 times with 0.05% tween-20 in 1x PBS and incubated with either Texas Red-conjugated streptavidin (1:500; Vector laboratories, Inc) for 1 hour. Slides were washed and mounted with Vecta shield.

Digital wide-field images were acquired either with a Spot 2e camera on a Nikon Diaphot 300 inverted microscope or a Spot RT3 camera on a Nikon Eclipse Ti-U inverted microscope. Laser scanning confocal images of dual fluorescence with Adenylyl cyclase 3 was acquired on a Leica TCS confocal system at the University of Kentucky Imaging Facility. Processing of images was done in Adobe Photoshop by adjusting size, brightness and contrast. Images were organized and labeled in Deneba Canvas.

Cell Counts

Counts of specific cell types are reported as means with their standard deviations for three mice per genotype. Cells were counted along the entire length of olfactory epithelium on one side of the septum of 4 sections per animal, and then averaged. The linear lengths of the epithelia counted were recorded and used to normalize the counts. Sections were matched for anterior-posterior position between genotypes.

Microarray Analysis

The generation and transcript level analysis of Affymetrix GeneChip Mouse Exon 1.0 Sense Target Array data used has been described previously (McIntyre et al., 2008). Briefly, equal amounts of RNA were pooled from 3 $Emx2^{+/+}$ and 3 $Emx2^{-/-}$ mice (n = 3 pools). Each pool contained 2.7 μg of olfactory epithelium RNA. Labeling, hybridization, and scanning of arrays were performed according to standard Affymetrix protocols by the University of Kentucky Microarray Core Facility. Additional analysis was performed using Affymetrix Expression Console Software to generate gene-level robust multichip

analysis (RMA) values from exon probe sets. Analysis of these arrays produces gene-level data, termed transcript clusters, which is derived from probe sets within exons. Data was derived from transcript clusters using the most conservative level, Core Annotation, which limits analysis to exon-level probe sets that map to BLAST alignments of mRNAs with annotated full-length open reading frames. Data were organized and analyzed in Excel (Microsoft, Redmond, WA). Raw microarray data have been deposited at Gene Expression Omnibus (Accession No. GSE12135).

As done previously with this dataset, signals from background hybridization were eliminated by deleting the 1793 transcript clusters that failed to produce a signal of at least 9% of the overall mean gene-level signal on at least one GeneChip (McIntyre et al., 2008). Statistical testing for mRNA abundance differences was done using Student's t-test at an α level of 0.05, followed by a correction for multiple testing using a false discovery rate of 10%. Genotype-driven changes in alternative splicing were predicted with Partek® Genomics Suite™ (Partek Incorporated, St Louis, MO). To insure that the predictions of differences in alternative splicing were not contaminated by differences caused by changes in abundance of entire transcripts, only transcript clusters with a p value > 0.4 were considered for exon-level analysis. The exon-level analysis used an α level of 0.05 and a false discovery rate 25%.

RESULTS

Mature OSNs develop in *Emx2*$^{-/-}$ mice

The olfactory epithelia of *Emx2*$^{-/-}$ mice have 40% fewer mature OSNs than wild-type littermates, but the cells in the epithelium still exhibit normal pseudostratification and the mature OSNs continue to express *Omp*, the canonical marker of maturity for these neurons (McIntyre et al., 2008). Consistent with these data, the absence of EMX2 did not prevent expression of other mRNAs enriched in mature OSN, including components of the olfactory transduction pathway. Adenylyl cyclase-3 (ADCY3) immunoreactivity was present in the dendritic knobs of *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ OSNs (Figure 4.1A-H), though the extent of labeling was reduced due to the reduction in mature OSNs (McIntyre et al., 2008). Similarly, immunoreactivity of two odorant receptors was also properly localized to the dendritic knobs of OSNs in *Emx2*$^{-/-}$ mice (Figure 4.1I-P). Both of these odorant receptors, Olfr73 (OR-EG) and Olfr1507 (MOR28), are receptors that are expressed less frequently in *Emx2*$^{-/-}$ mice (McIntyre et al., 2008). The expression at normal locations of two critical components of the olfactory transduction pathway suggests that OSNs of *Emx2*$^{-/-}$ mice should be capable of responding to odorants.

Further evidence of active OSNs in *Emx2*$^{-/-}$ mice was their expression of the activity-dependent genes, *S100a5* and *Kirrel2* (Imai et al., 2007, 2009; Kaneko-Goto et al., 2008). Transcripts from both *S100a5* and *Kirrel2* were detected in OSNs of *Emx2*$^{-/-}$ mice at staining intensities that indicate normal amounts of mRNA within each labeled cell (Figure 4.2B, C). These genes are expressed primarily in mature OSNs (Sammeta et al., 2007: Imai et al., 2007, 2009; Kaneko-Goto et al., 2008), consistent with their expression in fewer cells in *Emx2*$^{-/-}$ mice (Figure 4.2D, E). In addition, I investigated the expression of axon guidance gene *Nrp1*, whose expression is linked to functional odorant receptor signaling, probably in the axons of immature OSNs (Imai et al., 2009). *Nrp1* was expressed in both the mature and immature OSN layers of *Emx2*$^{-/-}$ mice, providing additional evidence of OSN activity (Figure 4.2F, G), albeit activity that is probably independent of odor stimulation. Together, these data suggest that the loss of EMX2 does not prevent the maturation of OSNs or their ability to be stimulated by odorants.

EMX2 controls OSN survival but not basal cell proliferation

The 40% reduction in mature OSNs in *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ mice could be caused by decreased proliferation of basal progenitor cells, increased cell death, or both (McIntyre et al., 2008). Immunoreactivity for phosphorylated histone H3, which increases during the chromatin condensation phase of mitosis, was not altered in basal cells of the olfactory epithelia of *Emx2*$^{-/-}$:*OMP-GFP*$^{-/-}$ mice compared to *Emx2*$^{+/+}$*:OMP-GFP*$^{-/-}$ littermates (n = 3; P = 0.83; Student's t = 0.22) (Table 4.1 and Figure 4.3A, B). These data are consistent with evidence that *Emx2*$^{-/-}$:*OMP-GFP*$^{-/-}$ mice have normal numbers of immature OSNs (McIntyre et al., 2008). In contrast, *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ had a 2.3-fold increase in cleaved caspase-3 immunoreactive cells in the OSN layers of the olfactory epithelium compared to *Emx2*$^{+/+}$*:OMP-GFP*$^{-/-}$ littermates (Table 4.1 and Figure 4.3C, D), a significant increase (n = 3; P < 0.01; Student's t = 5.45). Therefore, an increase in cell death of OSNs was likely responsible for the reduced number of mature OSNs in *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ mice. These data show that while the loss of EMX2 does not prevent the maturation of OSNs, it does affect OSN survival, even at embryonic ages.

OSN axons stop at the surface of the olfactory bulb

The axons of OSNs leave the olfactory epithelium, pass through the cribriform plate of the skull, course across the surface of the olfactory bulb, and eventually coalesce into glomeruli in the outer layer of the bulb. In *Emx2*$^{-/-}$ mice, OSN axons form a fibrous cellular mass just inside the cribriform plate and do not innervate the olfactory bulb (Yoshida et al. 1997). In *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ mice at age E18.5, both immature OSN axons immunoreactive for GAP43 and GFP fluorescent mature OSN axons were found in the fibrous cellular mass (Figure 4.4A-F). The fibrous cellular mass was located anterior and ventral to the olfactory bulb, and OSN axons were not observed traversing across the surface of the olfactory bulb (Figure 4.4G-L). During normal development OSN axons pass through the basal lamina that surrounds the central nervous system and form the olfactory nerve layer just beneath this basal lamina. In both *Emx2*$^{+/+}$*:OMP-GFP*$^{-/-}$ and *Emx2*$^{+/-}$*:OMP-GFP*$^{-/-}$ mice, GFP fluorescent axons formed a normal olfactory nerve layer around the olfactory bulb (Figure 4.4G-I). In *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ mice, GFP fluorescent

axons of mature OSNs contacted the surface of the bulb but failed to penetrate the layer of cells or the basal lamina that surrounds the bulb (Figure 4.4J-L). $GAP43^{+}$ axons of immature OSNs behaved identically.

Emx2$^{-/-}$ OSN axons segregate by type

Even though they failed to innervate the olfactory bulb, OSN axons maintained a segregated organization. I used the lectin DBA, which binds N-Acetylgalactosamine, to preferentially label axons of OSNs in the dorsal olfactory epithelium that project to the dorsal domain of the olfactory bulb, a region that largely overlaps with glomeruli from Class I odorant receptors (Figure 4.5A-C) (Lipscomb et al., 2003; Imai et al., 2009). Even within the fibrous cellular mass of *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ mice, DBA positive axons clustered together rather than being scattered throughout (Figure 5D-F). Although $GAP43^{+}$ immature OSN axons and GFP^{+} mature OSN axons were often differentially abundant in some regions of the fibrous cellular mass, especially posterior regions, the axons of both developmental stages were detected throughout the fibrous cellular mass indicating that the segregation seen with DBA^{+} axons is not between immature and mature OSNs. I found that DBA labeled 79% fewer OSNs in *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ mice compared to wild type littermates (Table 4.1 and Figure 4.5G-L), a significant decrease ($n = 3$; $P < 0.0005$; Student's $t = 11.5$) and nearly twice the reduction in mature OSNs that occurs in *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ mice (McIntyre et al., 2008).

DBA also stains a subpopulation of vomeronasal sensory neurons in the vomeronasal organ (Salazar and Sanchez Quinteiro, 2003). In wild-type littermates, DBA stained vomeronasal sensory neurons located in the basal portion of the vomeronasal organ. Expression of DBA in the basal vomeronasal organ is consistent with strong DBA staining in the posterior accessory olfactory bulb (Lipscomb et al., 2003). In three *Emx2*$^{-/-}$*:OMP-GFP*$^{-/-}$ deficient animals analyzed, the vomeronasal organ was completely devoid of DBA-labeled neurons (Figure 4.6A-D). These results suggest that EMX2 has functional roles in the vomeronasal organ as well as the olfactory epithelium.

Expression of *Ablim1* is greatly reduced in *Emx2*$^{-/-}$ OSNs

Given that each OSN of *Emx2*$^{-/-}$ mice continues to express an odorant receptor (McIntyre et al., 2008), I hypothesized that changes in axon guidance gene expression caused defects in OSN axon growth in *Emx2*$^{-/-}$ mice. I therefore searched my previously published Affymetrix GeneChip Mouse Exon 1.0 ST Array data for differences in the abundance of axon guidance mRNAs and alternatively spliced exons (McIntyre et al., 2008). Predictions of changes in alternative splicing caused by the loss of EMX2 could not be confirmed in the four instances I tested. Gene level analysis, as previously demonstrated, was more successful (McIntyre et al., 2008). Significant decreases in mRNA abundance in *Emx2*$^{-/-}$ mice for 1236 transcript clusters were detected. One of these mRNAs encodes actin-binding Lim protein 1 (ABLIM1), which mediates axon guidance in several organisms (Figure 4.7) (Lundquist et al., 1998; Erkman et al., 2000). In *C. elegans*, UNC-115/ABLIM1 is activated by small monomeric G-proteins, following UNC-6/netrin binding to the receptor UNC-40/DCC (Gitai et al., 2003). Activation of UNC-115/ABLIM1 promotes cytoskeletal changes that form the lamellipodia and filopodia of the growth cone which underlie axon guidance (Yang and Lindquist 2005). In the olfactory epithelia of *Emx2*$^{+/+}$ mice, Ablim1 transcripts were detected exclusively in the immature OSN layer (Figure 4.7A, C). In contrast, *Ablim1* was virtually absent from the olfactory epithelium of *Emx2*$^{-/-}$ mice (Figure 4.7B, D). *Ablim1* may therefore be at least partly responsible for the axon-targeting defect of OSN axons of *Emx2*$^{-/-}$ mice.

DISCUSSION

The reduction in mature OSNs found in *Emx2*$^{-/-}$:*OMP-GFP*$^{-/-}$ mice (McIntyre et al., 2008) proved to be a result of increased apoptosis of mature OSNs rather than a decrease in the production of OSNs from basal cells. Several lines of evidence, including the expression of activity-dependent genes and localization of odorant receptor proteins, indicated that the OSNs of *Emx2*$^{-/-}$ mice become fully mature and are capable of activation by odors. OSN axons, which fail to innervate the olfactory bulb in *Emx2*$^{-/-}$ mice (Yoshida 97), were found to contact but not penetrate into the olfactory bulb. The fibrous cellular mass that consequently forms between the bulb and the cribriform plate contained axons segregated by type, evidenced by concentrations of axons labeled by DBA that label dorsally located OSNs that project axons to the DI domain of the olfactory bulb (Imai et al., 2009). The failure of OSN axon innervation of the bulb was correlated with a loss of expression of *Ablim1*, which encodes an actin-binding protein whose orthologs are important for axon targeting in other organisms. These findings suggest that the loss of any key element linking attractive guidance cues to control of the actin network of axonal growth cones hypothesize, such as ABLIM1, would cause the premature termination of OSN axons.

OSN survival is reduced in the absence of EMX2

The reduction in mature OSNs previously reported (McIntyre et al., 2008) proved to be independent of OSN maturation, at least as evidenced by the expression of known markers of OSN maturity and activity (Serizawa et al., 2006; Imai et al., 2007, 2009), as well as the presence of normal numbers of immature OSNs, numbers of basal progenitor cells, and pseudostratification of the epithelium in *Emx2*$^{-/-}$ mice. Even though many odorant receptors are expressed in fewer OSNs in *Emx2*$^{-/-}$ mice (McIntyre et al., 2008), those odorant receptors selected for expression were properly targeted to the dendrites and cilia of OSNs. I conclude that OSNs in *Emx2*$^{-/-}$ mice are fully mature and have the capacity to respond to odorants.

Instead of altering the production of mature OSNs, the loss of EMX2 significantly reduced mature OSN survival. These results are consistent with the expression pattern of *Emx2*, which is detected abundantly in immature OSNs but not in basal cells, arguing that EMX2 is unlikely to have a direct role in the proliferation of basal progenitor cells. Why

the increase in apoptosis of mature OSNs seen in *Emx2*$^{-/-}$:*OMP-GFP*$^{-/-}$ mice did not stimulate basal cell proliferation indirectly, as happens when large numbers of mature OSNs die after lesion of OSN axons or treatment of the epithelium with an olfactotoxin, is unclear (Costanzo and Graziadei, 1983, Costanzo 1985, Schwob et al., 1995). Perhaps the signaling mechanisms required are not fully functional prior to birth. Nevertheless, the axons in the fibrous cellular mass of *Emx2*$^{-/-}$:*OMP-GFP*$^{-/-}$ mice exhibited intense caspase-3 immunoreactivity, just as severed adult OSN axons do when they trigger OSN apoptosis following olfactory bulbectomy (Cowan et al., 2001; Cowan and Roskams, 2004). During development, OSNs first contact the olfactory bulb at E12 and begin forming synapses and expressing the mature OSN marker OMP at ~E14 (Hinds and Hinds, 1976; Pinchin and Powell, 1971; Farbman and Margolis, 1980; Miragall and Monti-Graziadei; 1982). Therefore, by E18.5 some OSNs in *Emx2*$^{-/-}$ mice have spent as many as six days without making synapses with their target neurons. The olfactory bulb has long been thought to supply trophic support to OSN axons (Schwob et al., 1992; Voyron et al., 1999), an idea that is consistent with my data. Though I cannot yet exclude the alternative that EMX2 has a more direct role in OSN survival, a reasonable hypothesis is that the increase in OSN apoptosis observed in *Emx2*$^{-/-}$ mice is a result of OSN axons failing to innervate and obtain trophic support from the olfactory bulb.

The failure of axons to innervate the bulb does not appear to be a result of altered expression of odorant receptors in *Emx2*$^{-/-}$ mice. Although most odorant receptors are expressed less frequently in *Emx2*$^{-/-}$ mice, some odorant receptors are expressed more frequently, indicating that every OSN still expresses an odorant receptor (McIntyre, 2008). This argues that the role of odorant receptors in controlling OSN axon behavior would not be lost in *Emx2*$^{-/-}$ mice (Mombaerts et al., 1996, Feinstein et al., 2004; Serizawa et al., 2006). Recent work has revealed that signaling by odorant receptors regulates the expression *Nrp1*, and that NRP1 is critical for anterior-posterior positioning of glomeruli (Imai et al., 2006; 2009). OSNs expressing high levels of NRP1 form glomeruli in more posterior positions of the olfactory bulb, while OSNs with low levels of NRP1 form glomeruli in anterior regions. Normal patterns of *Nrp1* mRNA expression were detected in *Emx2*$^{-/-}$ mice. While microarray data did reveal statistically significant changes in the abundance of several axon guidance mRNAs, these differences were small

and in situ hybridization detected these transcripts in the olfactory epithelia of *Emx2*$^{-/-}$ mice. Many of these mRNAs come from genes expressed in mature OSNs, arguing that the decreases in mRNA abundance were due simply to the reduction in mature OSNs. I conclude that the OSN axon-targeting defect caused by the absence of EMX2 either happens in the downstream signaling from axon guidance cue receptors or is entirely independent of these receptors.

Olfactory bulb innervation and *Ablim1*

The defective OSN axon growth observed in *Emx2*$^{-/-}$ mice is also observed after targeted deletions of several other transcription factors, including *Dlx5, Fezf1, Klf7*, and *Arx* (Levi et al., 2003; Long et al., 2003; Yoshihara et al., 2005; Hirata et al., 2006; Laub et al., 2005; 2006). That this defective innervation phenotype is caused by targeted deletions of genes expressed either in the olfactory epithelium (*Fezf1*), in the olfactory bulb (*Arx*) or in both (*Dlx5* and *Klf7*) gives rise to the hypothesis that these transcription factors control expression of genes necessary for signaling between the olfactory bulb and OSN axons. The ability of all of these transcription factors to produce the same phenotype does not result from regulation of one by the others. The abundance of *Dlx5, Klf7* and *Fezf1* mRNAs did not differ between *Emx2*$^{-/-}$ and *Emx2*$^{+/+}$ mice. In fact, the expression of all four of these transcription factors appears to be mutually independent (Kajimura et al., 2007; Merlo et al., 2007; Watanabe et al., 2009). These transcription factors therefore appear to independently regulate the expression of one or more genes that are necessary for OSN axons to innervate the olfactory bulb.

Similar to OSN axons of mice lacking FEZF1, *Emx2*$^{-/-}$ OSN axons contacted the surface of the olfactory bulb but did not penetrate it (Watanabe et al., 2009). OSN axons of both *Fezf1*$^{-/-}$ and *Emx2*$^{-/-}$ mice are able to grow through the basal lamina of the olfactory epithelium, however, suggesting that the presence of a basal lamina around the bulb is not itself limiting. One possible explanation is that these transcription factors regulate the expression of genes need to penetrate the surface of the bulb. Both WNT/β-catenin signaling and secretion of proteases have been implicated in penetration of OSN axons into the bulb (Tsukatani et al., 2003; Zaghetto et al., 2007; Watanabe et al., 2009,). However, no changes in mRNA abundance of the Wingless-related (Wnt), Frizzled-

homolog (Fzd) or matrix metallopeptidase genes known to be expressed in OSNs emerged from the analysis of microarray data comparing gene expression in *Emx2*$^{-/-}$ mice and *Emx2*$^{+/+}$ mice (Tsukantani et al., 2003; Zaghetto et al., 2007; Rodriguez-Gil and Greer, 2008). A more compelling explanation is that axons of OSNs lacking EMX2 are unable to respond to an attractive cue from the olfactory bulb.

I found that *Ablim1* expression was greatly reduced in *Emx2*$^{-/-}$ mice. ABLIM1 regulates growth cone attraction through its interactions with the actin cytoskeleton. Chick retina ganglion cell axons require ABLIM1 for the proper innervation of the contralateral tectum (Erkman et al. 2000). Transfection of chick retinal ganglion cells with a dominant negative ABLIM1 caused incorrect innervation of the ipsilateral optic tract. The *C. elegans* homolog of ABLIM1, UNC-115, is also required for proper axon growth. Mutations in *unc-115* result in the premature termination of axons from the sublateral and phasmid sensory neurons (Lundquist et al., 1998). All neurons exhibited some aspects of normal axon growth in *unc-115* mutants, however those axons that normally make directional changes or substrate changes were unable to do so. Additionally in *C. elegans*, netrin signaling through the UNC-40/DCC receptor has been shown to stimulate UNC-115/ABLIM1 activity and promote growth cone attraction (Gitai et al., 2003). The effects seen in *C. elegans* are similar to the premature termination of OSN axons in *Emx2*$^{-/-}$ mice. DCC expression is detected in the olfactory nerve only during early development, while netrin is expressed in the ventral forebrain during development (Astic et al., 2002; Schwarting et al., 2004). That ABLIM1 could mediate signaling from several guidance cues in addition to netrin is also conceivable. Taken together, these data suggest the hypothesis that the loss of *Ablim1* impedes signaling in the growth cone and prevents OSN axons from innervating the olfactory bulb. That this defect happens primarily in pioneer axons early in development, leading to subsequent innervation failure even of axons less dependent on netrin signaling, is possible.

The effects of EMX2 on innervation of the olfactory bulb appear to be separate from the ability of OSNs axons to fasciculate by type in the olfactory nerve. For example, the ability of DBA positive axons to project together to the dorsal olfactory bulb in wild type animals was recapitulated in the ability of DBA positive axons to locate together in

specific regions of the fibrous cellular mass in *Emx2*$^{-/-}$:*OMP-GFP*$^{-/-}$ mice, rather than being randomly dispersed. This finding argues that OSN axons of *Emx2*$^{-/-}$ mice are still able to sort by subtype even without innervating the olfactory bulb. This is consistent with other data showing that the axons of subtypes of OSNs segregate and even form proto-glomeruli in the absence of the olfactory bulb (St John et al., 2003; Yoshihara et al., 2005; Imai et al., 2009).

Dorsal OSNs are more dependent on EMX2

OSN neurons are not a homogenous population of cells. Expression of several genes differs between OSNs located in the ventral and dorsal regions of the olfactory epithelium. For example, *Ncam2* and *Nrp2* are both expressed in ventrally located OSNs, while *O-macs* and *Nqo1* are expressed by dorsally located OSNs (Yoshihara et al., 1997; Norlin et al., 2001; Oka et al., 2003; Gussing and Bohm, 2004: Yu et al., 2005). Phenotypic differences also exist between OSNs expressing Class I odorant receptors and OSNs expressing Class II odorant receptors in the dorsal olfactory epithelium (Bozza et al., 2009). Dorsal and ventral OSNs also exhibit differences in carbohydrate groups, as demonstrated by DBA staining (Lipscomb et al., 2003). In *Emx2*$^{-/-}$:*OMP-GFP*$^{-/-}$ mice, DBA positive OSNs are disproportionately reduced in the olfactory epithelium compared to the reduction in mature OSNs. This finding correlates with the observation that Class I odorant receptor expression is universally reduced in *Emx2*$^{-/-}$ mice, whereas some Class II receptors increase their frequency of expression (McIntyre et al., 2008). OSNs expressing Class I odorant receptors are found in the dorsal olfactory epithelium, the only exceptions being two Class I odorant receptors that are expressed in OSNs located in the ventral olfactory epithelium (Zhang et al., 2004; Tsuboi et al., 2006; Hirota et al., 2007). DBA positive neurons were also reduced in the vomeronasal organ. Unless one effect of the absence of EMX2 is to suppress the production of proteins glycosylated with N-Acetylgalactosamine, these data argue that dorsal OSNs and basally located vomeronasal sensory neurons are more dependent on EMX2 than ventral OSNs and apical vomeronasal sensory neurons.

Dual roles for Emx2

EMX2 has at least two, and perhaps three, distinct roles in OSNs. EMX2 is necessary for expression of most odorant receptors, for innervation of the olfactory bulb by OSN axons, and as I report here, for OSN survival (McIntyre et al., 2008). EMX2 binds the promoter region of at least one odorant receptor gene that is EMX2-dependent, arguing that its effects on odorant receptor expression are direct (Hirota and Mombaerts 2004). In *Drosophila* the POU gene *pdm3* also exhibits dual roles in regulating odorant receptor expression and axon targeting in olfactory neurons, two processes that are more distinct in flies than in mammals because in flies odorant receptors are not critical to the behavior of OSN axons (Tichy et al., 2008; Dobritsa et al., 2003). Similarly, my data are consistent with the interpretation that EMX2 can regulate odorant receptor expression and axon growth independently. However, the mechanism by which EMX2 contributes to OSN axon innervation of the olfactory bulb remains elusive. The discovery that *Ablim1* expression is greatly reduced in *Emx2*$^{-/-}$ mice provides a testable hypothesis that could explain the axon growth defect of this knockout strain, and perhaps other strains showing the same phenotype. If correct, this idea would indicate that attractive cues from the olfactory bulb are critical for OSN axon innervation of the bulb.

Table 4.1 Apoptotic and proliferating cell counts

Genotype	Number of animals	Caspase3+ cells	Phosphohistone H3+ cells	DBA+ cells
+/-	3	1.74 ± 0.19	2.06 ± 0.23	6.04 ± 0.71
-/-	3	4.1 ± 0.72	2.13 ± 0.45	1.10 ± 0.18

Apoptotic, caspase3 positive cells were significantly increased in *Emx2*$^{-/-}$ mice. Proliferating, phosphohistone H3 positive basal cells were unchanged in *Emx2*$^{-/-}$ mice. DBA positive neurons were significantly reduced in *Emx2*$^{-/-}$ mice. Cell counts are means and standard deviations per 100μm of epithelium.

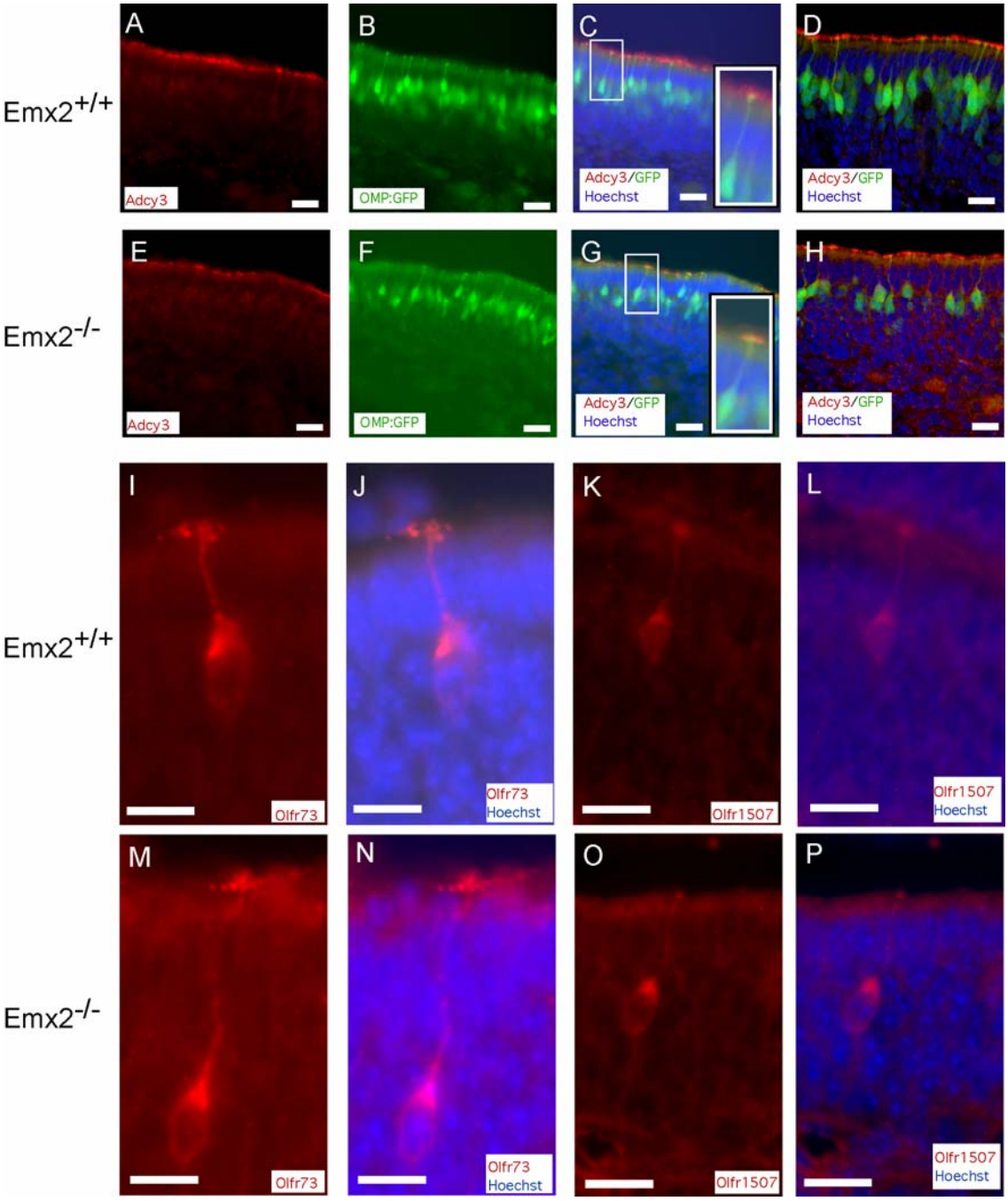

Figure 4.1 ADCY3 and OLFR immunofluorescence
Immunofluorescence for ADCY3 and odorant receptors in *Emx2*$^{+/+}$ and *Emx2*$^{-/-}$ mice. **A-H:** ADCY3 immunoreactivity in dendritic knobs and the overlying cilia layer was apparent in both *Emx2*$^{+/+}$ and *Emx2*$^{-/-}$ OSNs. Insets in C and G show a single GFP positive OSN and ADCY3 staining at the dendritic knob. **D, H:** Confocal image of ADCY3 staining and GFP shows overlap in the cilia layer. **I-P:** Odorant receptor immunoreactivity in *Emx2*$^{+/+}$ and *Emx2*$^{-/-}$ mice. OLFR73 (I, J and M, N) and OLFR1507 (K, L and O, P) immunoreactivity was detected in the dendrites and dendritic knobs of *Emx2*$^{-/-}$ OSNs. Scale bars. **A-C, E-G** and **I-P**, 12.5μm. **D** and **H**, 8μm.

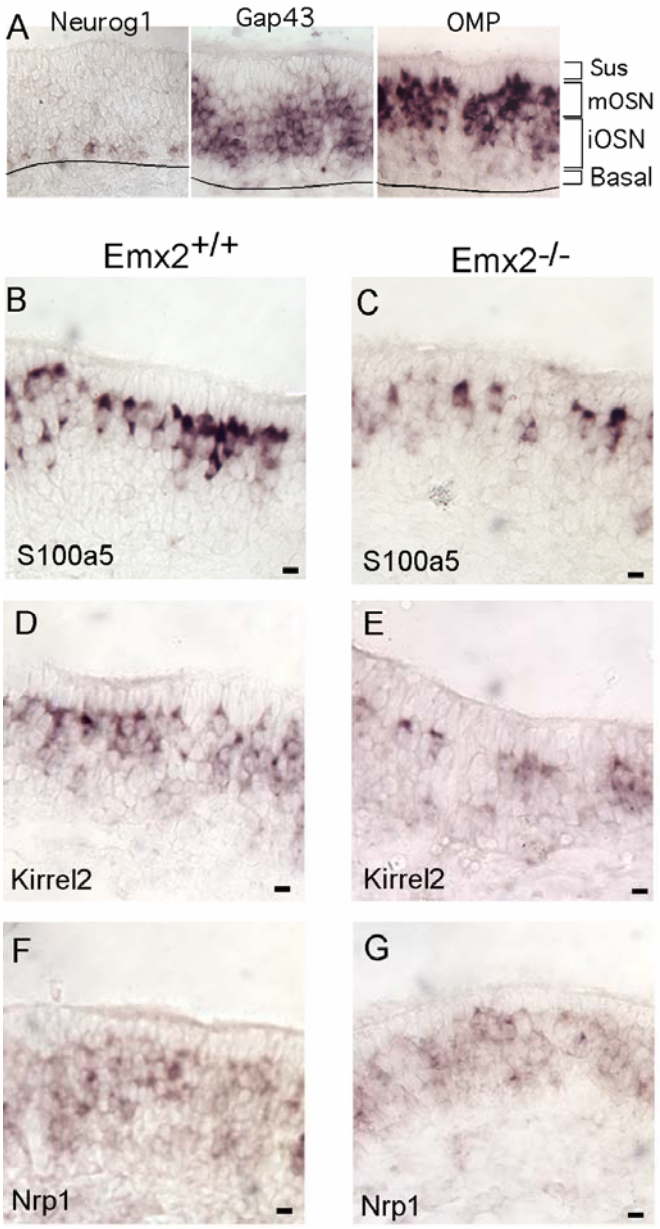

Figure 4.2 Activity-dependent genes expressed in *Emx2*$^{-/-}$ OSNs
A: A guide to the cell layers of the olfactory epithelium in E18.5 mice. *Neurog1* labels a subset of basal cells; *Gap43* labels immature OSNs; *Omp* labels mature OSNs. Sus, unlabeled sustentacular cell body layer; mOSN, mature OSN cell body layer; iOSN, immature OSN cell body layer; basal, basal cell layer. **B, C**: *S100a5* mRNA was detected in mature OSNs of both *Emx2*$^{+/+}$ and *Emx2*$^{-/-}$ mice. **D, E:** *Kirrel2* mRNA was detected in mature OSNs of both *Emx2*$^{+/+}$ and *Emx2*$^{-/-}$mice. **F, G**: *Nrp1* mRNA was detected in its normal mosaic pattern in OSNs of both *Emx2*$^{+/+}$ and *Emx2*$^{-/-}$ mice. Scale bars, 10μm
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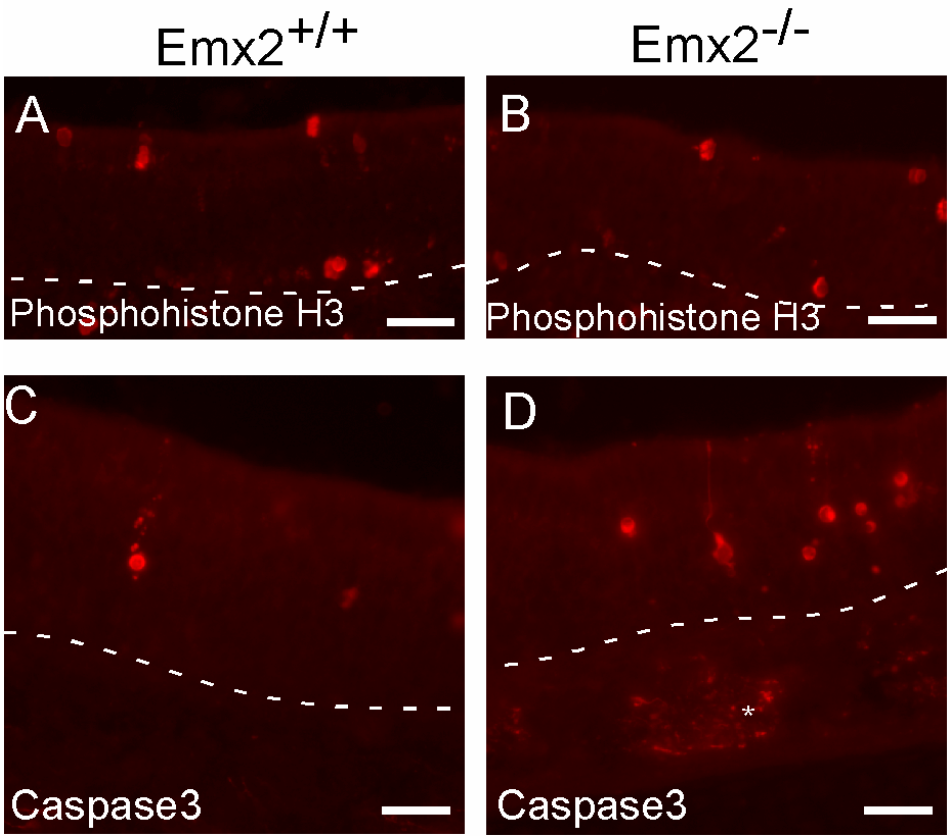

Figure 4.3 OSN survival is reduced in *Emx2*$^{-/-}$ OSNs
A, B: The number of cells immunoreactive for phosphohistone H3 was similar in *Emx2*$^{-/-}$ and *Emx2*$^{+/+}$ mice. Phosphohistone-3 immunoreactivity was located in both the apical sustentacular layer and basal progenitor cell layer. **C, D:** Caspase-3 immunoreactive cells, which were located in the central layers (OSN layers) of the olfactory epithelium, were more abundant in *Emx2*$^{-/-}$ mice compared to wild type littermates. Note the increased immunoreactivity in OSN axon bundles in the lamina propria of *Emx2*$^{-/-}$ mice (asterisk). Dashed lines indicated basal lamina of the olfactory epithelium. Scale bars: **A-B**, 40μm. **C-D**, 20μm.

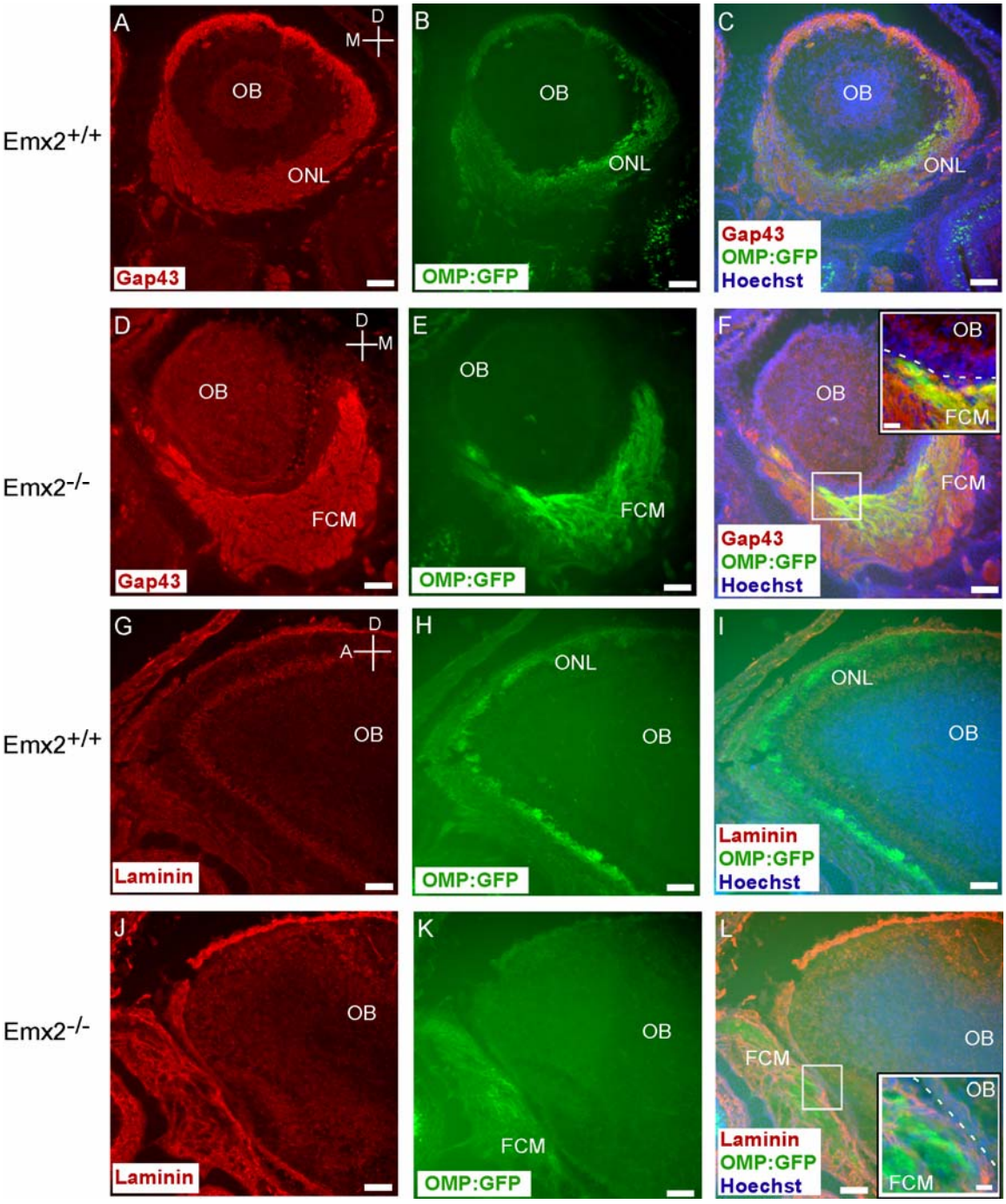

Figure 4.4 *Emx2*$^{-/-}$ OSNs contact but do not innervate the olfactory bulb
A-F: Both GAP43 positive immature OSNs (D) and GFP positive mature OSNs (E) fail to surround and innervate the olfactory bulb in *Emx2*$^{-/-}$ mice as seen in *Emx2*$^{+/+}$ mice (A-C). Inset in F shows that neither GAP43 nor GFP positive axons enter the olfactory bulb but contact the surface of the bulb. **G-L:** Normally, OSNs axons penetrate the basal lamina, immunoreactive for laminin, of the olfactory bulb (G-I). In *Emx2*$^{-/-}$ mice OSN axons do not penetrate the basal lamina and did not grow over the dorsal surface (J-L),

Figure 4.4 (continued)
although they do contact the bulb surface (inset in L). Abbreviations: OB, olfactory bulb. ONL, olfactory nerve layer. FCM, fibrous cellular mass. Orientation: A-C, Dorsal is up, Medial is to the left. D-F, Dorsal is up and Medial is to the right. G-L, Dorsal is up and anterior is to the left. Scale bars, **A-L**, 50μm. Inset in **F** and **L**, 12.5μm

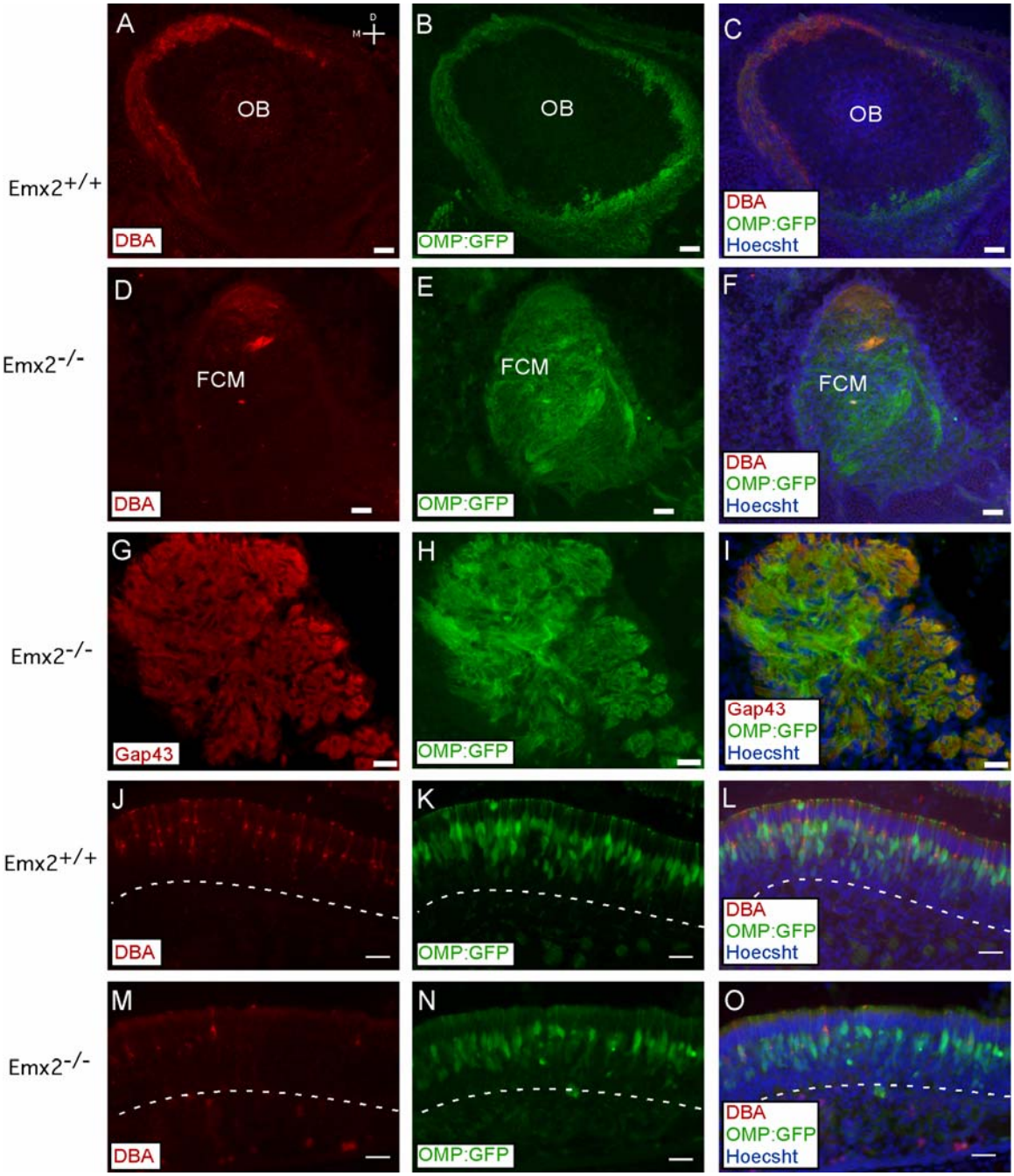

Figure 4.5 DBA positive OSNs are fewer but their axons remain segregated in *Emx2*$^{-/-}$ mice.

A-C: DBA positive axons in wild type mice project to the dorsal bulb. **D-F:** DBA positive axons in *Emx2*$^{-/-}$ were restricted to the dorsal region of the fibrous cellular mass. **G-I:** GAP43^{+} and GFP^{+} axons however overlap throughout the fibrous cellular mass. **J-O:** DBA positive OSN were fewer in *Emx2*$^{-/-}$ mice. Dashed line indicates basal lamina of the olfactory epithelium. Orientation of A-I, Dorsal is up and medial is to the left. Scale bars: **A-F**, 50μm. **G-O**, 25μm.

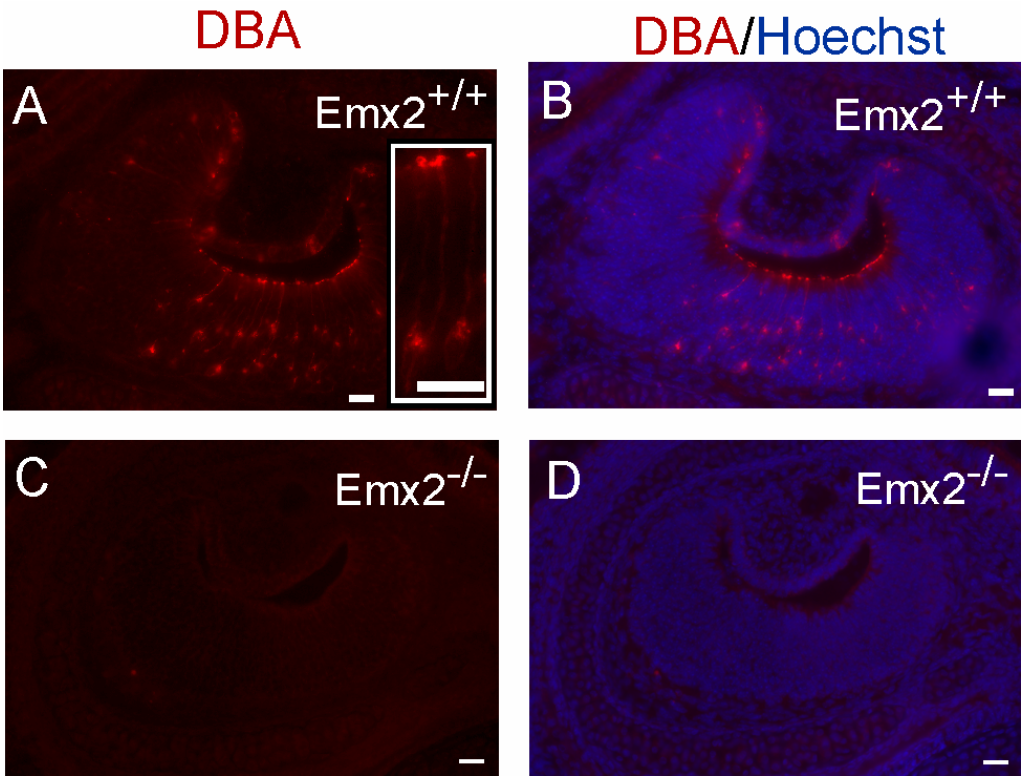

Figure 4.6 DBA positive vomeronasal sensory neurons are absent in *Emx2*$^{-/-}$ mice
A, B: DBA positive neurons are present in the basal regions of the VNO. Inset in A is a higher magnification of DBA positive vomeronasal sensory neurons. **C, D:** No DBA positive neurons were detected in the VNOs of *Emx2*$^{-/-}$ mice (n = 3). Scale bars: 25µm

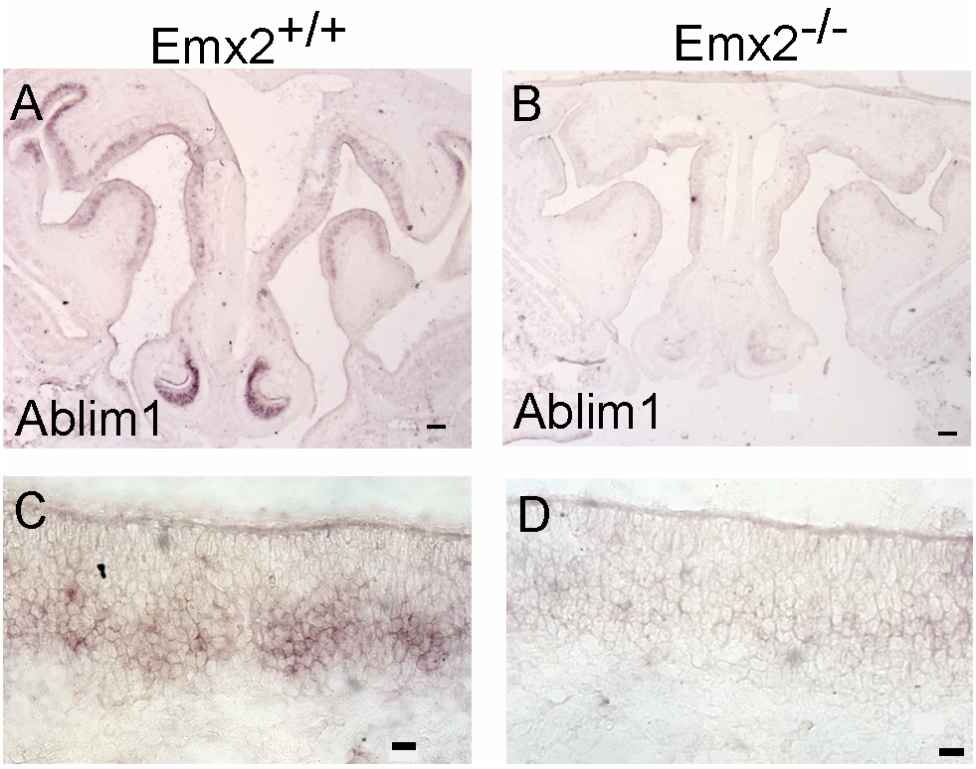

Figure 4.7 Decreased abundance of Ablim1 mRNA in *Emx2*$^{-/-}$ mice
A, B: *Ablim1* mRNA was expressed throughout all regions of the olfactory epithelium and vomeronasal organ at E18.5 but was dramatically reduced in *Emx2*$^{-/-}$ mice. **C, D**. *Ablim1* expression was predominantly located in the immature OSN layer in *Emx2*$^{+/+}$ mice, but not detectable in *Emx2*$^{-/-}$ mice. Scale bars: **A-B**, 100μm. **C-D**, 10μm.

Chapter 5

General Discussion and Conclusions

The previous chapters detail my efforts to aid our understanding of the mechanisms immature OSNs use to innervate the olfactory bulb. In this chapter I will discuss some of the importance of this work and how it will help shape our understanding of various aspects of the olfactory system. Using the *Emx2*$^{-/-}$ mouse, I searched for the gene or genes underlying its defective axon growth. By selecting a mouse that displays a phenotype of interest and then working "backwards", underlying candidate genes were pinpointed for further analysis. Using the advantages of the olfactory epithelium as a model for neurogenesis where immature and mature neurons always coexist, I was able to analyze developmental differences in the expression patterns of axon guidance genes. For *Emx2*$^{-/-}$ mice, axon growth and guidance genes expressed in immature OSNs are better candidates for causing the axon growth defect found in this mouse, as it is the axons of immature OSNs that first innervate the olfactory bulb. This approach proved to be successful as I identified an axonogenesis-related gene, *Ablim1*, whose expression was greatly reduced in *Emx2*$^{-/-}$ mice. *Ablim1* is expressed primarily in immature OSNs and I predict it is therefore important for innervation of the olfactory bulb. Future experiments can now be designed to test this function of ABLIM1 in OSN axon growth. I also discovered that EMX2 is an important regulator of odorant receptor gene expression.

Gene expression correlates with axon behavior

For proper axon function expression of axon guidance gene must be tightly regulated. I have shown that immature and mature OSNs express distinct sets of axon guidance molecules that correlate with the differences in behavior of axons of mature and immature OSNs. In fact, the expression of axon guidance genes enabled me to identify a new population of cells, which I have termed "nascent immature OSNs". This finding alters the traditional view of cellular development in the OSN lineage. In the old view of the OSN cell lineage, immediate neuronal precursor cells, which are *Neurog1* positive, give rise to *Gap43* positive immature OSNs. Here I have shown that an intermediate cell

type exists between *Neurog1* positive cells and *Gap43* positive cells. This cell population is more basally located than *Gap43* positive cells and is more numerous than *Neurog1* positive cells. The nascent immature OSNs express two genes that define this population, *Dbn1* and *Cxcr4*. These genes encode proteins whose known properties predict that they are important for the initiation of OSN axon growth and extension of axons into the mesenchymal tissue of the lamina propria (Toda et al., 1999; Lieberam et al., 2005; Geraldo et al., 2008; Zhu et al., 2009). Immunofluorescence with CXCR4 and GAP43 antibodies identifies a few cells that express both proteins, however, most CXCR4 positive cells are GAP43 negative. That many of these cells have short basal and apical neuritis, presumably the nascent axonal and dendrite, supports the claim these cells are differentiating into neurons and are not progenitor cells. I conclude that immediate neuronal precursors differentiate first into these nascent immature OSNs, and that *Gap43* positive OSNs represent a second stage of immature OSN development. In terms of axon growth, *Cxcr4* positive cells are associated with the first stage of growth, during which the axon exits from the olfactory epithelium proper and extends in the mesenchyme of the lamina propria.

The second stage of axon growth involves growth through the mesenchyme and into the olfactory bulb. Axon growth in immature OSNs shares similarities with other neuronal populations. For immature OSN axons this involves pathfinding to the olfactory bulb. Immature neurons therefore need mechanisms to promote growth and integrate guidance cues. Axon growth of *Gap43* positive immature OSN is marked by expression of a wide variety of axon guidance cue receptor genes, including expression of a variety of receptors for both attractive and repulsive cues. The growth cones of immature OSNs are therefore responsive to both attractive and repulsive cues, such as semaphorins, slit and netrin that are expressed in both the mesenchyme and the olfactory bulb, and also by other OSNs (Williams-Hogarth et al., 2000; Astic et al., 2002; Cloutier et al., 2002; Cho et al., 2007; Williams et al., 2007, Imai et al., 2009). Additionally, the mesenchyme is rich in laminin and other matrix molecules that can either promote or suppress axon growth (Gong and Shipley, 1996; Whitesides and LaMantia, 1996; Kafitz et al., 1997; Shay et al., 2008). Recent research shows that OSN axons begin sorting into distinct populations prior to their glomerular positions, and that some of these cues may be

established by the axons themselves (Imai et al., 2009). Immature OSN axons must therefore also recognize cues necessary for axon fasciculation and defasciculation within the olfactory nerve.

In contrast to immature neurons, mature OSNs have minimal growth requirements. In fact, the expression of guidance cue genes in mature OSNs, even all neurons, is probably highly weighted toward the inhibition of axonal growth. The functions that dominate in mature OSN axons are likely maintaining axon coalescence, position, and synapses. These activities are less dependent on extension mechanisms but may require some relocation of the terminal portion of the axon, and the ability to respond to cues limiting growth out of glomeruli. These tasks are consistent with my observations that the axon guidance cue receptors expressed in mature OSNs typically mediate repulsive or inhibitory behavior, and that expression of intracellular growth cone signaling proteins decreases dramatically. Therefore, the guidance cue receptor genes that are expressed in mature OSNs may be important for the maintenance of axons within glomeruli. Other roles for genes expressed in mature OSNs include the regulation of axon branching, which may be important for synaptic connections between the OSN axons and dendrites of mitral/tufted cells. Perhaps instead of providing axonal growth signals, guidance cues and their receptors serve as axonal/neuronal maintenance molecules in mature neurons.

A persistent idea about OSN axon growth is that expression of axon guidance genes should exhibit zonal distribution. As a whole, my data suggests that zonal expression may not in fact be important for OSN axon guidance. Other sensory maps, such as the retinotopic map, exhibit gradients of axon guidance cues, leading to the notion that the olfactory epithelium would be similar. The in situ hybridization analysis that I performed did not reveal any new zonally distributed genes. Instead of zonal expression patterns, co-expression of axon guidance genes with specific subsets of odorant receptors may be the key to determining the positions of glomeruli (Kaneko-Goto et al., 2008; Imai et al., 2009). *Nrp1*, for example, is expressed throughout the extent of the olfactory epithelium, but is not expressed by all OSNs (Imai et al., 2006; 2009). The same is true to cell adhesion molecule genes *Cntn4*, *Kirrel2* and *Kirrel3* (Serizawa et al., 2006; Kaneko-Goto et al., 2008). Mosaic or differential expression of axon guidance

genes may be more critical for OSN axon growth. It now appears that odorant receptor signaling impacts the expression of multiple axon guidance related genes (Imai et al., 2009). Expression analysis of odorant receptor regulated genes may help to identify whether their encoded proteins are important for growth to the bulb, or for coalescence into glomeruli.

The data from Chapter 2 provide fundamental knowledge of the differential expression of axon guidance genes in OSNs. These data informed my hypothesis that in *Emx2*$^{-/-}$ mice, expression of axon guidance genes in immature OSNs underlies the failure of OSN axons to innervate the olfactory bulb. They led to the identification of reduced expression of *Ablim1* as a probable cause of the axon growth defect in *Emx2*$^{-/-}$ mice (Chapter 4).

Identification of EMX2 as a transcriptional regulator of odorant receptor gene expression

EMX2 is the first transcription factor unequivocally shown to control the expression of odorant receptor genes. Prior claims that another homeobox transcription factor, LHX2, acts similarly are difficult to reconcile against the fact that the absence of LHX2 results in the loss of both *Gap43* positive immature OSNs and Omp positive mature OSNs such that reduced expression of odorant receptors is inevitable in mice lacking LHX2 (Hirota and Mombaerts, 2004; Kolterud et al., 2004; Hirota et al., 2006). Whether the loss of LHX2 prevents odorant receptor expression and therefore inhibits OSN development or LHX2 loss blocks OSN development and subsequent expression of odorant receptors is unknown. Unraveling the role of EMX2 in regulating odorant receptor expression is less complicated. OSN development in *Emx2*$^{-/-}$ mice was largely normal, except for a 40% reduction in mature OSNs. Further analysis showed that OSNs in *Emx2*$^{-/-}$ mice are fully mature and that the decrease in mature OSNs is likely due to increased apoptosis and not defects in development. The loss of EMX2 resulted in reduced expression of the majority of odorant receptors, while the expression of a few increased (Figure 5.1). In demonstrating the dependence of many, but not all odorant receptors on EMX2, my data both support and refine the hypothesized mechanisms by which singularity of odorant receptor expression is achieved and maintained.

Sequence analysis of putative OR promoters found homeodomain binding sites in more than 90% of genes analyzed (Vassali et al., 2002; Hoppe et al., 2006; Michaloski et al., 2006). I used a published list of putative promoters to identify potential differences in the putative OR promoters of genes that were increased and decreased in *Emx2*$^{-/-}$ mice (Michaloski et al., 2006). I discovered that homeodomain sites were present in putative odorant receptor promoters irrespective of whether the receptor's expression frequency increased or decreased in the absence of EMX2. I term these two populations of odorant receptor genes to be EMX2-insensitive and EMX2-sensitive, respectively (Figure 5.2). In the absence of EMX2, expression of EMX2-sensitive odorant receptor genes is reduced. The sensitivity of the ~1,000 mouse odorant receptor genes to the loss of EMX2 varies continuously, from some that are so sensitive that they depend absolutely on EMX2, to some that are only mildly affected by the absence of EMX2, to others that appear to be independent of EMX2 (Emx2-insensitive). Clearly, the odorant receptor genes that are expressed less frequently in the absence of EMX2 have some sort of interaction with EMX2. The ability of EMX2 to bind the putative promoter of one odorant receptor gene supports the conclusion that this interaction is probably direct (Hirota and Mombaerts, 2004). However, the same promoter and a second putative odorant receptor promoter have also proved to be able to bind several other homeobox transcription factors (Hoppe et al., 2003; Hirota and Mombaerts, 2004). These data suggest that the change in expression frequency of each odorant receptor in the absence of EMX2 may represent the ability of each odorant receptor promoter to use these other homeodomain transcription factors in substitution for EMX2. In other words, that all odorant receptors normally depend on EMX2 for their expression is possible. However, what is more likely is that several homeobox transcription factors participate in stimulating the expression of odorant receptor genes, and the discriminating factor is the binding affinity of each odorant receptor promoter for the available homeobox transcription factors.

Interestingly, the increase in the frequency of expression of a small number of odorant receptors in the absence of EMX2 supports a negative feedback mechanism of odorant receptor expression (Serizawa et al., 2003; Shykind et al., 2004 Capello et al., 2009). The expression of a functional odorant receptor provides a negative feedback

signal that prevents the expression of all other odorant receptors. This mechanism also hypothesizes that if a non-functional odorant receptor is selected, the lack of a feedback signal will cause the selection of other odorant receptor genes until a functional receptor is expressed. The increased frequency of expression of a few odorant receptor genes in *Emx2*$^{-/-}$ mice is consistent with these ideas (Figure 5.3). For example, in the absence of EMX2 the transcriptional machinery is much less likely to be recruited to an EMX2-sensitive odorant receptor gene locus even if all other necessary elements are present at this promoter. Without odorant receptor expression, the feedback mechanism would not become activated, other odorant receptor gene loci would not be made inaccessible, and the transcriptional machinery would therefore continue to be recruited to other odorant receptor genes until an EMX2-insensitive odorant receptor is chosen and expressed. Through this switching mechanism EMX2-insensitive odorant receptors would have increased probability of selection and expression.

Widespread gene changes do not underlie the OSN axon growth defect

Analysis of mRNA abundance in *Emx2*$^{-/-}$ olfactory epithelium revealed decreases in approximately 20 axonogenesis-related genes. Of those, expression of 14 genes is predicted in mature OSNs based on additional microarray data (Sammeta et al., 2005). The mRNA abundance changes of these genes were largely proportional to the decrease in mature OSNs, and in situ hybridization studies verified that several were expressed in *Emx2*$^{-/-}$ mice (Table 5.1). Therefore it is likely that the decrease in mature OSNs accounts for the decreased mRNA abundance of these genes. In mice with targeted deletions in *Cntn4, Slit1, Robo2*, or *B3gnt2*, OSN axons continue to innervate the bulb (Henion et al., 2005; Cho et al., 2007; Kaneko-Goto et al, 2008; Nguyen-Ba-Charvet et al., 2008). I conclude that loss of EMX2 likely affects a very specific signaling pathway necessary for innervation of the olfactory bulb. This signaling pathway is likely to act through ABLIM1 (Figure 5.4).

In situ hybridization showed a large decrease in the expression of *Ablim1* in *Emx2*$^{-/-}$ mice. The axon growth defects in *Emx2*$^{-/-}$ mice and *C. elegans unc-115* mutants are strikingly similar (Lundquist et al., 1998). In *unc-115* mutants, neurons showed normal axon growth in most respects. In *Emx2*$^{-/-}$ mice, OSN axons exit the epithelium

and cross through the cribriform plate in normal trajectories. The axons of specific neurons in *unc-115* mutants, however, fail to innervate specific regions or make specific turns. This is the same type of defect seen in OSN axons in *Emx2*$^{-/-}$ mice, which fail to innervate the olfactory bulb even though they come in contact with it. Classical guidance cues often play a role in both attracting axons into new tissue and inducing turning. The secreted guidance cue UNC-6/NETRIN-1 attracts and promotes axon extension and UNC-115/ABLIM1 mediates its effects (Figure 5.4A) (Gitai et al., 2003). Thus, mutations in or loss expression of *Ablim1* may prevent functional guidance cue signaling and alter axon growth (Figure 5.4 B). The reduced expression of *Ablim1* in *Emx2*$^{-/-}$ mice identifies a candidate gene and a probable mechanism for future studies olfactory bulb innervation by OSN axons.

To determine the functionality of ABLIM1 several experiments could be performed. Using a previously published method I attempted to test ABLIM1 function through the creation of a dominant negative protein (Erkman et al., 1998). I obtained an immature OSN specific promoter (Hirata et al., 2006), and placed under it a construct encoding a dominant negative ABLIM1 protein. The dominant negative ABLIM1 would be able to interact with guidance cue receptors but unable to bind to the actin cytoskeleton thus preventing further signaling. Using this construct I had transgenic mice made. Analysis of offspring from three transgenic founders was disappointing, as the transgene was not expressed. This approach still is viable, however, and given the success in affecting axon growth in chick retina cells (Erkman et al., 1998), I continue to predict that a dominant-negative ABLIM1 would interrupt OSN axon growth (Figure 5.4 C). A targeted deletion of *Ablim1* could also achieve similar results. *Ablim1* is alternatively spliced into three variants with unique 5' exons. A knockout mouse lacking the first exon of the longest variant has been produced, but no changes in retina ganglion cell axon growth were observed (Lu et al., 2003). The 3' exons are shared by all three splice variants, and encode the actin-binding domain that is necessary for ABLIM1 function. It is my opinion that the best way to block function of ABLIM1 would be to disrupt the 3' exons encoding the actin binding domains. If mutant *Ablim1* mice produce an axon growth phenotype similar to *Emx2*$^{-/-}$ this would cement the role of ABLIM1 in OSN axon growth. Additionally, these results would provide good evidence for a signaling pathway

between the bulb and OSN axons and hopefully lead to the identification of that pathway. Similarly, restoring *Ablim1* expression to *Emx2*$^{-/-}$ mice could also provide insight into function. Transgenic expression of *Ablim1* with an OSN specific promoter would test the sufficiency of Ablim1 to regulate innervation of the olfactory bulb. If ABLIM1 was capable of restoring OSN innervation a transgenic *Ablim1* mouse on the *Emx2*$^{-/-}$ background could also prove extremely useful for analyzing axon coalescence when odorant receptor expression is perturbed.

Innervation of the olfactory bulb is necessary for OSN survival, even during embryonic development

I have shown that in *Emx2*$^{-/-}$ mice there is increased apoptosis of OSNs. Using an antibody against activated caspase-3 I detected a 2.3-fold increase in dying cells in the olfactory epithelium. Staining in the axon bundle was extremely intense, with many OMP positive fibers co-locating with activated caspase-3 immunoreactivity. During normal development there are peaks of apoptosis at E12 and again at E16 (Voyron et al., 1999). In normal mice apoptosis declines at E18 and stable levels are maintained throughout postnatal development and adult hood. The increase in apoptosis at E16 is likely necessary to remove axons that have not correctly innervated a glomerulus, thereby refining the olfactory map. The use of *Casp3*$^{-/-}$ mice has helped to verify this (Cowan et al., 2001). In *Casp3*$^{-/-}$ mice the number of OSNs is increased, olfactory bulb size is increased but glomerular formation is not as refined compared to wild type littermates. That caspase-3 signaling from the axons leads to apoptosis as also been shown. Olfactory bulbectomy leads to widespread apoptosis of OSNs. In *Casp3*$^{-/-}$ mice, no Tdt-mediated dUTP nick end-labeling (TUNEL, a measure of apoptosis) is seen in OSNs 24 and 48hr after bulbectomy. This demonstrates that although the axons have been severed they are unable to initiate an apoptotic signal to the OSNs. I hypothesize that in *Emx2*$^{-/-}$ mice the lack of innervation induces caspase-3 signaling in the axons leading to increased apoptosis of OSNs.

These data are intriguing for two reasons. First, when viewed in light of other data they support a role for the bulb in supplying a trophic factor necessary for mature OSN survival that is separate from neural activity. Both physical and genetic methods of

neuronal silencing do not affect OSN apoptosis (Lin et al., 2000; Yu et al., 2004). In these models where all OSNs are silenced, OSNs survive long periods. In contrast, regenerated mature OSNs do not survive well following bulbectomy, presumably due to a loss of trophic support (Schwob et al., 1992). My findings appear to support the view that innervation of the bulb is necessary for normal longevity of mature OSNs. Second, the capacity for increased proliferation of OSNs in response to OSN apoptosis may not yet be in place during embryonic development. Counts of phosphohistone H3 positive cells in the basal olfactory epithelium did not show an increase in proliferating cells in *Emx2*$^{-/-}$ mice. In adult mice, apoptosis of OSNs leads to increased proliferation to replace dying cells (Costanzo and Graziadei, 1983; Costanzo, 1985; Schwob et al, 1992; 1995). That *Gap43* positive OSNs are similar between *Emx2*$^{-/-}$ mice and wild type littermates further supports the conclusion that proliferation is not increased. Therefore, I conclude that the lack of innervation leads to increased apoptosis, but the signaling pathway by which apoptosing OSNs stimulate increased OSN production is not yet functional in embryonic development.

Olfactory bulb innervation and axon coalescence are distinct processes in OSNs

To properly form glomeruli OSN axons must innervate the olfactory bulb and then coalesce with other axons expressing the same odorant receptor. In several mouse strains, including *Emx2*$^{-/-}$ mice, where OSN axons fail to innervate the olfactory bulb, the axons do appear to exhibit segregation by type in the fibrous cellular mass in which they terminate (Yoshihara et al., 2005; Imai et al., 2009). In *Emx2*$^{-/-}$ mice I have shown this by demonstrating that DBA positive axons are sequestered rather than being distributed throughout the fibrous cellular mass. This could be further demonstrated in several ways. For example, odorant receptor-tauGFP or tau*LacZ* mice allow for the visualization of all OSN axons expressing a specific odorant receptor. Using these mice, it would be possible to test axonal coalescence in the fibrous cellular mass. Previous studies of other mutant mice with similar phenotypes suggest that odorant receptor-specific proto-glomeruli would form (St John et al, 2003). These experiments are not possible at the moment, as all of the tagged odorant receptors show decreases in expression in *Emx2*$^{-/-}$ mice. An alternative would be the use of odorant receptor antibodies to localize proto-glomeruli.

The identification of genes necessary for innervating the olfactory bulb in *Emx2*$^{-/-}$ mice should make it easier to tease out mechanisms of axon innervation from those of axon coalescence. By replacing the missing axon guidance gene in the *Emx2*$^{-/-}$ background it may be possible to study the effects of altered odorant receptor expression on odotopic map formation. As I propose that the innervation defect is separate from axon coalescence, glomeruli should form in an innervated *Emx2*$^{-/-}$ olfactory bulb. This raises several interesting questions. Would the glomerular map look the same? If Class I odorant receptors are no longer expressed in *Emx2*$^{-/-}$ mice, do glomeruli form in the DI domain of the olfactory bulb (Kobayakawa et al., 2007; Bozza et al., 2009; Imai et al., 2009)? Do large super glomeruli form from odorant receptors with increased expression, or do multiple odorant receptor positive glomeruli form? The answers to these questions would help complete our knowledge of the development of the olfactory map.

Emx2$^{-/-}$ mice may serve as a model for Kallmann Syndrome

Defects in olfactory axon growth and kidney development seen in *Emx2*$^{-/-}$ mice are both symptoms of the human disorder Kallmann Syndrome (MacColl et al., 2002). Migration of OSN axons and GnRH neurons is altered in Kallmann syndrome leading to anosmia and defects in reproductive organ development. Much like the *Emx2*$^{-/-}$ mouse, in Kallmann syndrome OSN axons grow normally to the olfactory bulb but fail to innervate it (Schwanzel-Fukuda et al., 1989). There are currently four Kallmann syndromes in which gene mutations have been identified. The four classified Kallmann syndromes, 1-4, are caused by mutations in *Kal1, Fgfr1, Prokr2*, and *Prok2* respectively. However, mutations in these genes account for only 25-30% of known cases of Kallmann syndrome. *Emx2* has been considered a candidate gene underlying Kallmann syndrome, but no mutations in the exons of *Emx2* were found in 120 patients analyzed (Taylor et al., 1999). It is interesting to note that mutations within the coding region of a gene are not the only mechanism by which a disorder could be caused. DNA changes in either non-coding regions such as the promoter or enhancer element can have significant effects on gene expression. Additionally, a mutation in a transcription factor that controls the expression of *Emx2* could also prevent the expression of genes dependent on EMX2. Thus changes in *Emx2* expression could still be an underlying cause of some types of

Kallmann syndrome and *Emx2*$^{-/-}$ mice could serve as a model for the disease.

Some mechanistic data also exists for one of the causes of Kallmann Syndrome. The *Kal1* gene encodes the ANOSMIN-1 protein, which contains a WAP domain and 4 fibronectin type III domains (common in neural cell adhesion molecules). A mouse or rat homolog to *Kal1* has not yet been identified; however antibodies to the human ANOSMIN-1 do detect a protein of similar size and expression pattern in rodents (Soussi-Yanicostas et al., 2002). ANOSMIN-1 is predicted to be a secreted protein and is able to stimulate neurite extension in multiple cell types and organisms (Soussi-Yanicostas et al., 2002; Gianola et al., 2009; Yanicostas et al., 2009). Analysis of the chick olfactory system has yielded some insights into the mechanism by which ANOSMIN-1 regulates olfactory axon growth (Rugarli et al., 1993). In chick *Kal1* is expressed in the olfactory bulb but expression is not detected in the olfactory epithelium. Cells that express *Kal1* include the mitral cells, which are the synaptic targets of OSN axons. Could ANOSMIN-1 therefore serve as a chemoattractant necessary for innervation of the olfactory bulb? More recently a protein with similar domains to ANOSMIN-1 has been identified in the olfactory epithelium. This protein, UMODL1, is an extracellular membrane bound protein that is expressed in both olfactory and vomeronasal sensory neurons (Di Schiavi et al, 2005). While UMODL1 has a predicted transmembrane domain, no intracellular domains have been identified. This would require UMODL1 to form a complex with another membrane bound protein to form a functional receptor unit capable of generating an intracellular signal. A functional hypothesis is that UMODL1 serves as a co-receptor for ANOSMIN-1 in regulating axon growth (Di Schiavi et al., 2005). However, *Umodl1* is more highly expressed in mature OSNs whose axons have already innervated the olfactory bulb. The DCC receptor contains 6 fibronectin type III domains and 2 immunoglobulin domains. That fibronectin and immunoglobulin domains can interact and activate receptors has been established for other receptor-ligand interactions stimulating axon growth (Kulahin et al., 2007). This suggests the possibility that DCC could serve as a receptor for ANOSMIN-1. It is possible then that ABLIM1 is necessary for signaling downstream of a receptor complex to regulate axon growth into the olfactory epithelium. This could potentially explain the similarities in axon growth defects in OSNs in *Emx2*$^{-/-}$ mice and cases of Kallmann

syndrome. In this case Emx2 may not be directly causal for Kallmann syndrome, but *Emx2*$^{-/-}$ mice may be able to serve as a model for the disorder. Identification of a mutation within OSNs leading to some cases of Kallmann syndrome might reveal a means to treat anosmia associated with the disorder. Given that OSNs continually turnover, a gene therapy that restored the ability of OSN axons to grow into the olfactory bulb could restore some olfactory function to individuals with Kallmann syndrome.

Concluding thoughts

The projects that I have completed add to our understanding of the olfactory system. One of the great mysteries in this field is the regulation of odorant receptor genes. Not only do odorant receptors detect volatile chemicals, their expression forms the very basis of the odotopic map that appears to be critical for odor discrimination. The identification of a transcription factor that regulates odorant receptor expression fills a void in this understanding. EMX2 can best be described as a gatekeeper. It doesn't regulate the singularity or the zonality of expression. These aspects are likely controlled by other factors, probably in part by chromatin remodeling. Without EMX2 some odorant receptors are not expressed and many are expressed much less frequently. Natural variation in EMX2 function or expression could therefore greatly change an organism's olfactory ability. This could account for phenotypic variation in olfactory ability. Putative odorant receptor promoters show a high degree of organizational similarity but their homeodomain binding sites exhibit nucleotide differences. Future studies should investigate whether these differences affect odorant receptor expression. Perhaps polymorphisms in putative odorant receptor promoters account for some of the variation seen in olfactory ability between individuals.

While the sense of smell is often critical for animal survival, in and of itself olfaction is not a vital sensory system for humans. However, olfactory ability is important to the quality to life. The sense of smell is integral to the pleasure of food and drink. It is an informative sense in that it alerts us to spoiled food or an infant that needs a diaper change. The loss of the sense of smell also is a clue to some medical disorders even beyond Kallmann syndrome. Decrements in olfactory ability accompany neural disorders such as Alzheimer and Parkinson disease. Odotopic map formation is the basis of this

sense. The work that I have done adds to our understanding of the integration of olfactory cues into the odotopic map formed in the olfactory bulb. Hopefully, it will lead to the identification of the pathway necessary for OSN axon innervation of the bulb and bring us one step closer to understanding how the entire map develops.

Table 5.1 Axonogenesis transcripts significantly decrease in *Emx2*$^{-/-}$ microarray

Gene Name	Gene Symbol	KO/ WT	GFP+/GFP-	Predicted cell type	Cell type from ISH	ISH in Emx2
slit homolog 1 (Drosophila)	Slit1	0.67	ND	--	ND	ND
actin-binding LIM protein 1	Ablim1	0.73	0.2	iOSN	iOSN	No
RAB3A, member RAS oncogene family	Rab3a	0.85	2	mOSN	ND	ND
SLIT and NTRK-like family, member 3	Slitrk3	0.86	ND	--	ND	ND
UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 2	B3gnt2	0.54	2.2	mOSN	OSN	ND
dopamine receptor 2	Drd2	0.61	25	mOSN	ND	ND
roundabout homolog 2 (Drosophila)	Robo2	0.89	1.3	mOSN	OSN	ND
doublecortin	Dcx	0.82	1.8	mOSN	ND	ND
plexin A3	Plxna3	0.72	7.3	mOSN	mOSN	ND
mitogen-activated protein kinase 8 interacting protein 3	Mapk8ip3	0.9	1.7	mOSN	ND	ND
ets variant gene 4 (E1A enhancer binding protein, E1AF)	Etv4	0.95	2.5	mOSN	ND	ND
contactin 4	Cntn4	0.49	6.5	mOSN	mOSN	Yes
growth associated protein 43	Gap43	0.7	0.6	iOSN	iOSN	Yes
reticulon 4 receptor-like 1	Rtn4rl1	0.76	8.9	mOSN	ND	ND
dihydropyrimidinase-like 5	Dpysl5	0.81	0.8	iOSN	iOSN	Yes
ring finger protein (C3H2C3 type) 6	Rnf6	0.83	1.5	mOSN	ND	ND
syntaxin binding protein 1	Stxbp1	0.81	2.6	mOSN	ND	ND
stathmin-like 3	Stmn3	0.68	1.9	mOSN	OSN	Yes
stathmin-like 2	Stmn2	0.73	0.7	iOSN	iOSN	Yes
neurexin I	Nrxn1	0.73	1.6	mOSN	OSN	ND
drebrin 1	Dbn1	0.81	0.5	iOSN	iOSN	Yes

Table 5.1 (continued)
Axonogenesis transcripts significantly decrease in *Emx2*$^{-/-}$ microarray
This table shows all of the mRNAs related to axon guidance that were significantly decreased in *Emx2*$^{-/-}$ olfactory epithelium. Only a few genes were predicted to be enriched in immature OSNs, and of these Ablim1 was the only mRNA not detected at normal levels by in situ hybridization. The OMP+/- ratio column specifies the degree of enrichment in mature OSNs, thereby predicting the cell type expressing each mRNA (predicted cell type column), data from Sammeta et al. (2007). The last column indicates whether or not mRNA was detected in *Emx2*$^{-/-}$ OSNs. nd, not detected on array or tested.

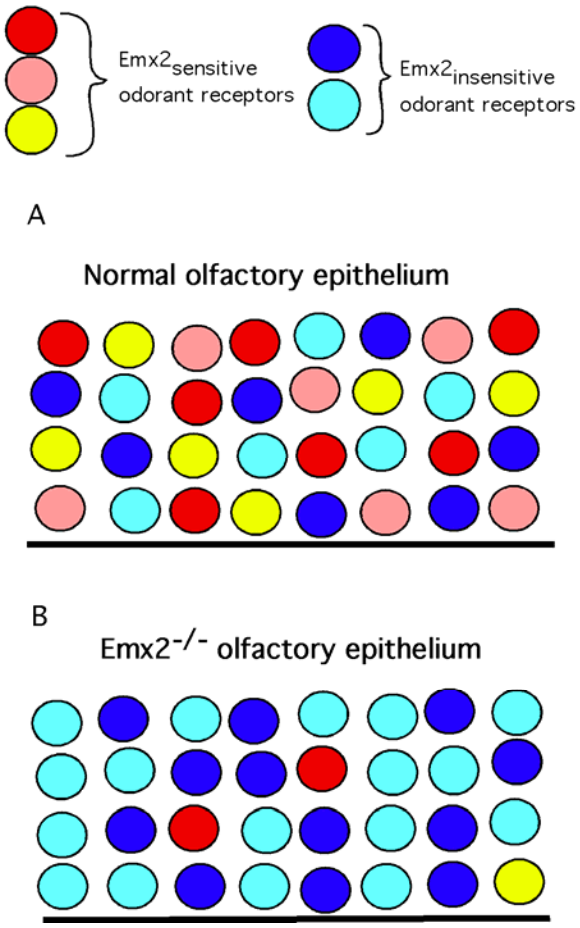

Figure 5.1 Schematic of odorant receptor representation
Odorant receptor gene expression in wild type and *Emx2*$^{-/-}$ olfactory epithelium. **A:** In wild type mice, all odorant receptors are expressed. **B:** In *Emx2*$^{-/-}$ mice, many odorant receptors are expressed less frequently (EMX2-sensitive), while a few are expressed in more cells (EMX2-insensitive).

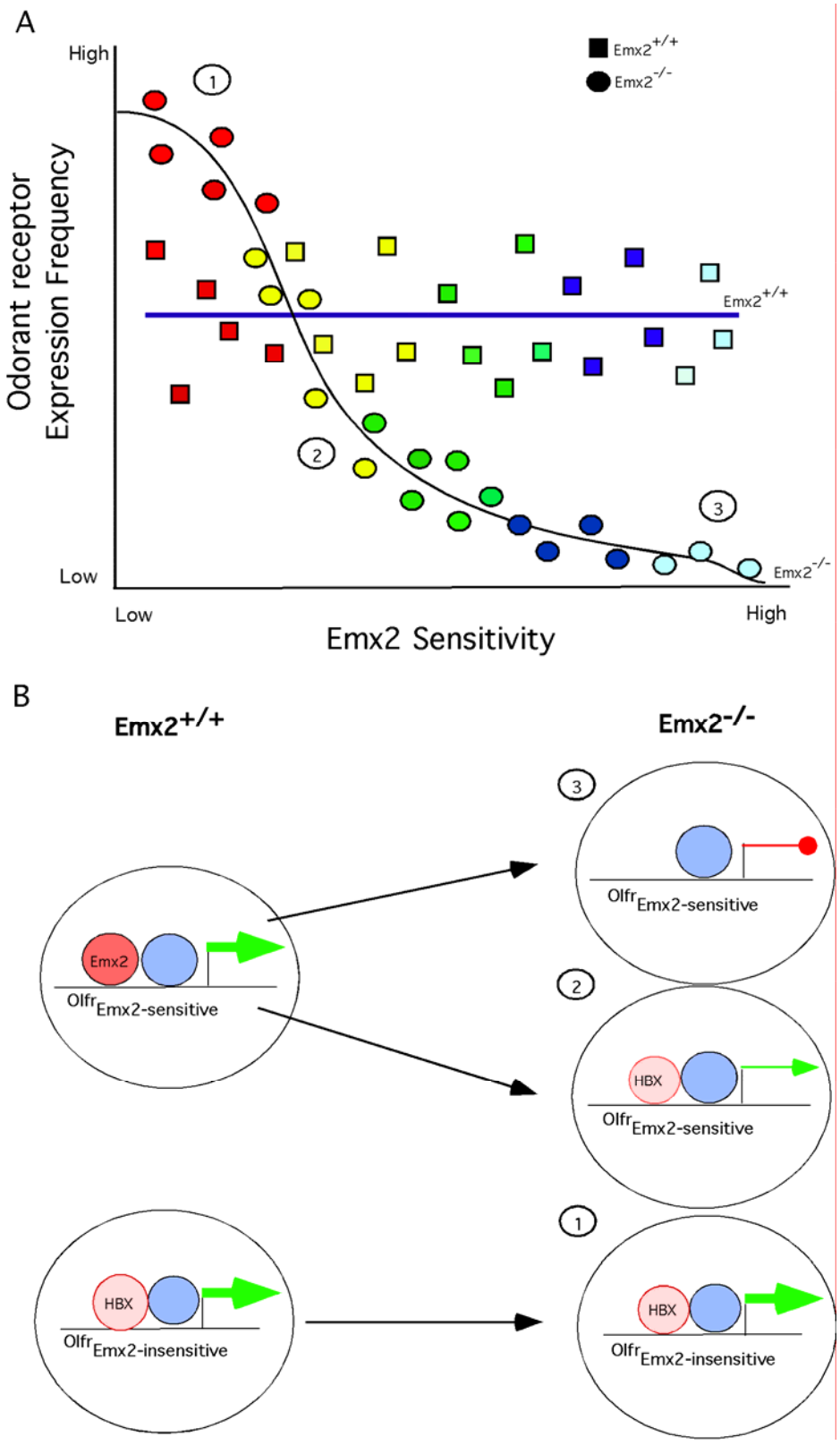

Figure5.2 Model of EMX2 sensitivity

Figure 5.2 (continued)
EMX2 sensitive and insensitive odorant receptors. **A:** Theoretical plot of EMX2-sensitivity against expression frequency. In *Emx2*$^{+/+}$ mice, each of the ~1,000 odorant receptor genes has its own intrinsic level of dependence on EMX2, but expression frequencies are mostly similar. In the absence of EMX2, the expression frequency of odorant receptors least dependent on EMX2 (EMX2-insensitive) increases while the expression frequency of others decreases according to their degree of dependence on EMX2. **B:** In *Emx2*$^{-/-}$ mice, odorant receptors completely dependent on EMX2 are not expressed (3), while those with incomplete dependence are expressed, albeit at lower levels (2). Expression of EMX2-insensitive odorant receptors can be driven fully by other homeobox (HBX) transcription factors.

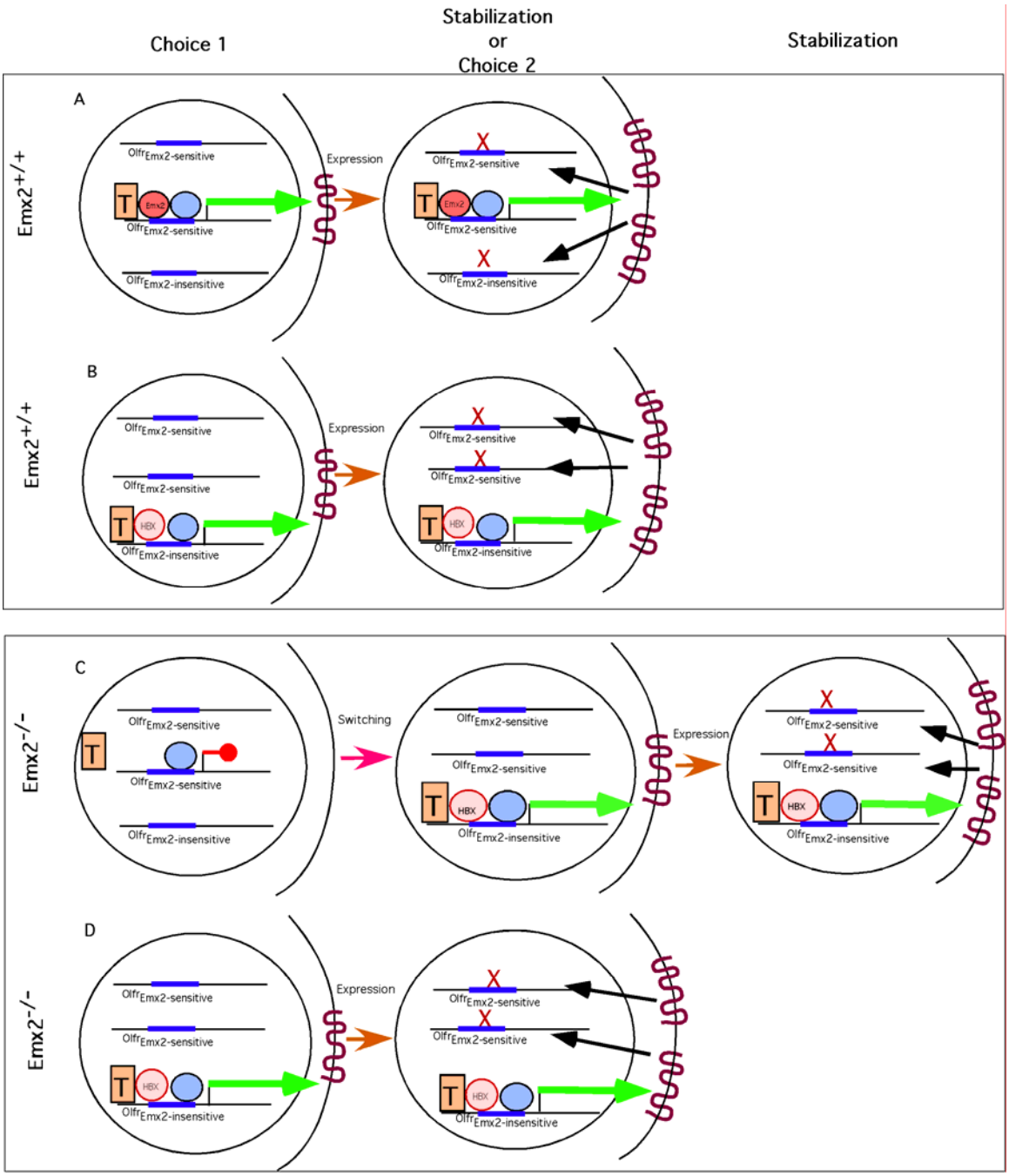

Figure 5.3 Model of Emx2 and odorant receptor gene switching
Increased odorant receptor expression occurs through negative feedback and gene switching. **A, B:** Under normal conditions, a random process in which the mechanism is unknown, selects one odorant receptor gene for expression, the transcriptional machinery (denoted by the Block T) is recruited, and transcription of this gene is strongly stimulated by binding of EMX2 (or some other homeobox transcription factor) to the promoter. A powerful negative feedback signal is produced if the odorant receptor protein is functional (black arrows). Both EMX2-sensitive (A) and EMX2-insensitive (B) odorant

Figure 5.3 (continued)
receptors are expressed through this mechanism. **C, D:** In the absence of EMX2, EMX2-sensitive odorant receptors have a reduced probability of being expressed. If the random process recruits, or attempts to recruit, the transcriptional machinery to an EMX2-sensitive odorant receptor promoter (C), transcription of the selected odorant receptor fails and no negative feedback signal is produced. Without this signal, the random process will select a second odorant receptor (switching). If transcription of this second odorant receptor can be stimulated by another homeobox transcription factor, then this odorant receptor is expressed. **D:** If an EMX2-insensitive odorant receptor is chosen first, gene switching is not necessary. Through feedback and gene switching, EMX2-insensitive odorant receptors are more likely to be expressed in the absence of EMX2 and their expression frequency increases.

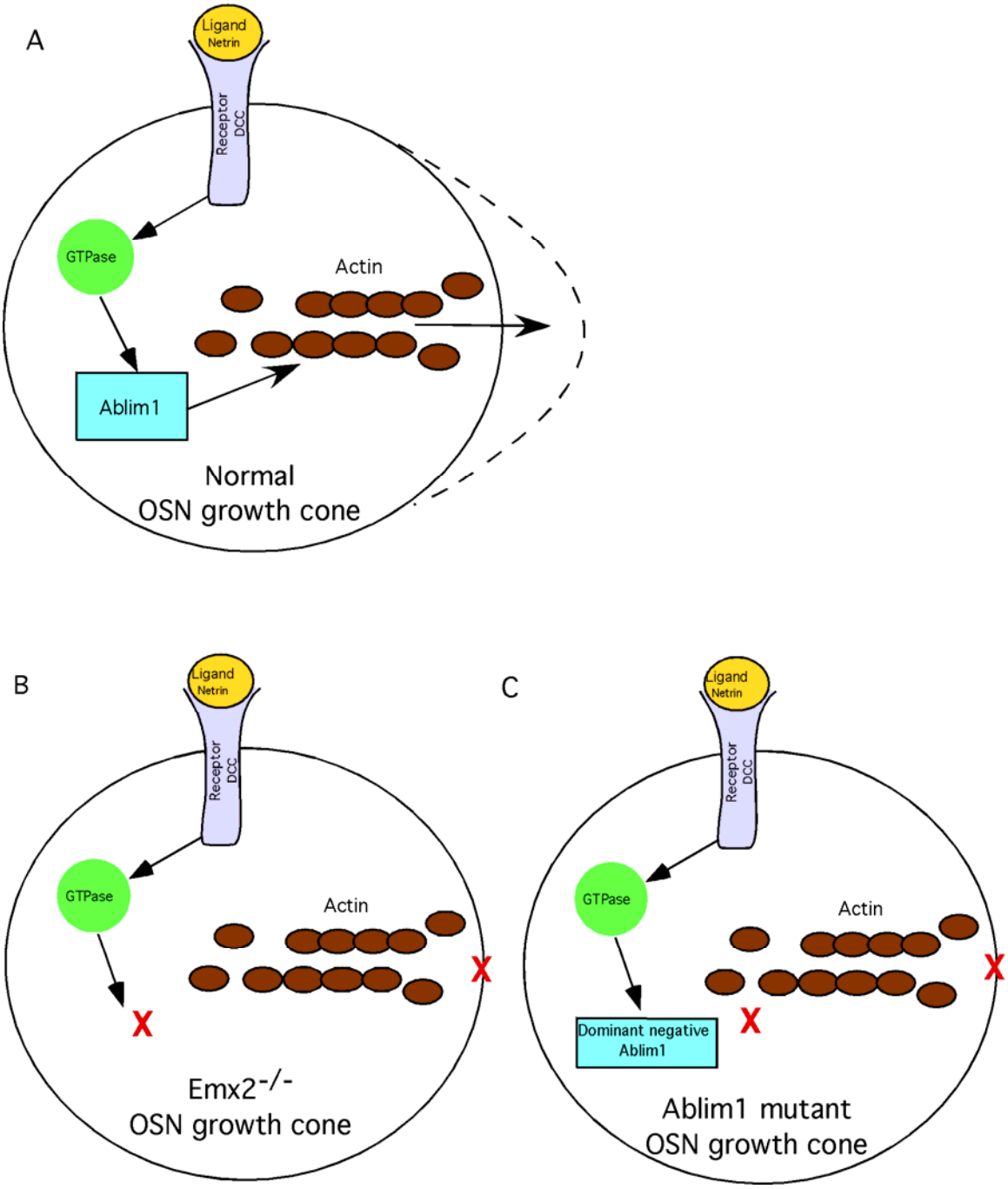

Figure 5.4 Model of ABLIM1 function in axon growth

A: In normal OSNs, ABLIM1 mediates intracellular signaling of axon guidance cues. Ligand binding (netrin-1) to a receptor (DCC) activates a small monomeric GTPase. The GTPase activates ABLIM1, which in turns acts on the actin cytoskeleton. Increases in actin motility push out the cell membrane and extends the growth cone causing it to grow towards its target. **B:** In *Emx2*$^{-/-}$ OSNs, the loss *Ablim1* expression prevents a signal from reaching the actin cytoskeleton. The growth cone is not extended in response to the signal and OSN axons do not innervate the olfactory bulb. **C:** The development of a dominant negative Ablim1 protein will allow this hypothesis to be tested. The dominant negative

Figure 5.4 (continued)
ABLIM1 would still interact with GTPases, but would be unable to interact with the actin network, thus disrupting the signaling pathway. The growth cone would not be extended and OSN axons would not innervate the olfactory bulb.

Appendix

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8/2000-12/22/01	Undergraduate Research Assistant. Bowling Green State University. Advisor: Moira van Staaden Ph.D.

Honors and Awards

4/2009	Student Travel Award. Association for Chemoreception Sciences (AchemS XXVIII April 22-26 2009)
4/2006	Student Travel Award. Association for Chemoreception Sciences (AchemS XXVIII April 26-30 2006)
7/2003-7/2005	Graduate Traineeship, Cellular and Molecular

	Neuroscience of Sensory Systems. NIH-T32-DC-00065 University of Kentucky
4/2004	Student Travel Award. Association for Chemoreception Sciences (AchemS XXVI April 21-25 2004)
8/2002-7/2003	University of Kentucky Graduate School Fellowship.
8/2001-12/2001	James D. Graham Memorial Scholarship. Department of Biology Bowling Green State University
8/2001-12/2001	Suzanne K. Miller Undergraduate Research Assistantship. Department of Biology Bowling Green State University
8/2000-12/2000	Suzanne K. Miller Undergraduate Research Assistantship. Dept of Biology Bowling Green State University
4/1999	Outstanding Poster Award, Sigma Xi Research Competition Bowling Green State University

Society Memberships

1/04-present	Association for Chemoreception Sciences
4/04-6/06	American Association for the Advancement of Science
1/00-12/01	Society for Integrative and Comparative Biology

Publications

Primary Publications

2009 **McIntyre JC**, McClintock TS. EMX2 regulates olfactory sensory neuron survival and expression of *Ablim1*. Neural Dev (In Review)

2009 **McIntyre JC**, McClintock TS. Axon growth and guidance genes identify nascent, immature, and mature olfactory sensory neurons. J Neurosci Res (In Review)

2008. **McIntyre JC**, Bose SC, Stromberg AJ, McClintock TS. Emx2 stimulates odorant receptor gene expression. *Chemical Senses* 33(9): 825-837.

2005. Shetty RS, Bose SC, Nickell MD, **McIntyre JC,** Hardin DH, Harris AM, McClintock TS. Transcriptional changes during neuronal death and replacement in the

olfactory epithelium. *Mol Cell Neurosci* 30(4): 583-600.

2005. Yu T, **McIntyre J,** Bose SC, Hardin D., Owen MC., McClintock TS. Differentially expressed transcripts from phenotypically identified olfactory sensory neurons. *J Comp Neurol* 483(3):251-262.

2003. Bergman DA, Kozlowski CP, **McIntyre JC**, Huber R, Daws AG, Moore PA. Temporal dynamics and communication of winner-effects in the crayfish *Orconectes rusticus. Behaviour* 140:802-825.

Abstracts

2009. **J.C. McIntyre**, S.C. Bose T.S. McClintock. Emx2 stimulates odorant receptor gene expression and controls OSN axon growth. *Chemical Senses*.

2006. **J.C. McIntyre**, T.S. McClintock. Expression of axon growth and guidance genes in immature OSNs. *Chemical Senses*. 31(5): A134-135

2004. **J.C. McIntyre,** T.T. YU, R. Shetty, N. Sammeta, M Smith, T. S. McClintock. Expression profiling of phenotypically identified olfactory sensory neurons. *Chemical Senses*. 30: A82

2004. Yu T., **McIntyre J.C.,** Bose S.C., Hardin D., McClintock T.S. Transcripts enriched in sensory neurons and supporting cells of the olfactory epithelium. *Chemical Senses*. 30: A82

2001. **McIntyre J.C.,** R.W.S Schneider , P.A. Moore. Boundary layer influences of the chemical signal on the antenna of male *A. polyphemus*. *American Zoologist* 40

2001. Kozlowski CP, **McIntyre JC,** Moore PA. The winning effect in agnostic encounters of crayfish is a time dependent phenomenon. *American Zoologist* 40

2000. **McIntyre J.C.,** R.A Zulandt Schneider, RWS Schneider, PA Moore. Orientation mechanisms of juvenile and adult Red Swamp Crayfish, *Procambarus clarkii*, to a distant odor source. *American Zoologist* 39