The role of Kirrel family members during circuit formation of the accessory olfactory system

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Abstract

The ability of animals to detect signals from the environment is regulated by the sensory systems. One of these sensory systems, the accessory olfactory system, controls various social behaviours in mice, such as mating and aggression, which are crucial to their survival. The vomeronasal sensory organ (VNO) contains vomeronasal sensory neurons (VSNs) that detect chemosignals and relay the information to the central nervous system. VSNs project axons that form synapses with dendrites of second order neurons in structures termed glomeruli within the accessory olfactory bulb (AOB). Several axon guidance molecules that control the targeting of VSN axons to specific sub-regions of the AOB have been identified. However, very little is known about the mechanisms regulating the coalescence of axons expressing a specific vomeronasal receptor into glomeruli and subsequent synapse formation between VSN axons and dendrites of second order neurons. Previously, it was shown that two members of the Kirrel family of cell adhesion molecules, Kirrel-2 and Kirrel-3, are differentially expressed in subpopulations of VSNs, thereby defining a molecular code of axonal recognition. Here we show that ablation of Kirrel-3 expression in mice leads to improper coalescence of a specific subset of VSN axons expressing the vomeronasal receptor EC2. Furthermore, germline ablation of Kirrel-2 expression in mice results in the formation of fewer yet larger glomeruli in the posterior region of the AOB. A similar observation is made in mice where Kirrel-2 was specifically ablated in sensory neurons of the olfactory systems, indicating that Kirrel-2 is needed on VSN axons during glomeruli formation. Interestingly, the loss of both Kirrel-2 and Kirrel-3 expression in VSN axons prevents the formation of distinguishable glomeruli throughout the AOB, which is associated with a decrease in the number of excitatory synapses within the AOB. Taken together, our results show that the expression of Kirrels on vomeronasal axons dictates their proper coalescence into glomeruli and is required for neuronal circuit formation within the AOB.

La survie des animaux dépend de leur capacité à s'adapter à l'environnement dans lequel ils fonctionnent à travers la détection de signaux qui contrôlent leurs comportements sociaux. Plusieurs de ces comportements, tels les comportements sexuels et l'agressivité, sont contrôlés par le système olfactif accessoire. La composante sensorielle du système olfactif accessoire, l'organe voméronasal (VNO), contient des neurones qui détectent ces signaux et relaient les données recueillies au bulbe olfactif accessoire (AOB). Pour ce faire, les neurones sensoriels du VNO (VSNs) projettent leurs axones jusqu'à l'AOB, où ils forment des connexions stéréotypées avec les neurones bulbaires au sein de structures appelées glomérules. Plusieurs molécules de guidage axonal pouvant diriger les axones des VSNs vers l'AOB ont été identifiées. Toutefois, les mécanismes moléculaires qui permettent la coalescence d'axones de VSNs exprimant le même récepteur à phéromone, ainsi que la formation de synapses avec les cellules bulbaires de l'AOB, restent inconnus. Deux molécules d'adhésion membres de la famille des Kirrels, Kirrel-2 et Kirrel-3, sont exprimées à différents niveaux sur les axones de sous-populations de VSNs, permettant ainsi la génération d'un code de reconnaissance moléculaire parmi les axones. Dans ce mémoire, nous démontrons que l'ablation de l'expression de la protéine Kirrel-3 chez la souris résulte dans l'incapacité d'une sous-population d'axones provenant des VSNs, exprimant le récepteur EC2, à former des glomérules homogènes. De plus, l'élimination de la protéine Kirrel-2 résulte en un défaut dans la malformation des glomérules de la région postérieure de l'AOB. Nous démontrons aussi que Kirrel-2 est spécifiquement requis dans les cellules sensorielles olfactives pour la formation de glomérules à travers son ablation sélective dans ces cellules. Finalement, l'élimination simultanée de Kirrel-2 et Kirrel-3 chez la souris mène à une perturbation complète de la formation de glomérules et ces défauts sont associés à une diminution dans le nombre de synapses excitatrices formées dans l'AOB. L'ensemble de nos résultats démontre que l'expression de Kirrel-2 et Kirrel-3 sur les axones de VSNs est essentielle pour la formation des circuits neuronaux dans le système olfactif accessoire de la souris.

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For my parents who always support me

even if they do not understand everything in this thesis.

For my brother Kelly and Julie

For Martin who never stops thinking

about science…ever.

Preface

This thesis is original, unpublished work by the author, Alexandra Brignall. Supervision for this thesis was provided by Dr. Jean-François Cloutier and all resources for the research performed in this thesis were supplied by the Cloutier Lab at McGill University. The electron microscopy experiments carried out in this thesis were done in collaboration with the Facility for Electron Microscopy Research (FEMR) and Dr. Louis Hermo at McGill University. FEMR performed the tissue sectioning and provided reagents, and Dr. Hermo assisted with the animal perfusions. Figure 2.1 of the Results is modified from Prince, Brignall et al., 2013. Figure 2.8 of the Results is work performed in collaboration with two undergraduate students, Maddee Nash and James Lee.

Introduction

The ability of mammals to interact with their environment relies on the interpretation of a wide variety of signals detected through various sensory modalities. An accurate representation of these sensory inputs is obtained through the formation of neural maps that allow for the organized relay of information from sensory neurons in the periphery to brain structures that control behavioural responses. Several sensory maps, such as the retinotopic map of the visual system, relay spatial information through the formation of stereotypic connections that preserve spatial order between sensory neurons and their targets in the central nervous system. Maps can also provide information about the discrete qualities of the signals detected, such as in the glomerular map of the olfactory system. The development of neural maps that provide both spatial and qualitative information relies on genetic and activity-dependent mechanisms that ensure the formation of accurate synaptic connections between the peripheral and central nervous system (CNS).

The establishment of the glomerular map in the olfactory systems is crucial for the regulation of a wide variety of innate and social behaviours in animals. In most mammals, the detection of olfactory cues is mainly mediated by two anatomically distinct chemosensory systems, the main and accessory (or vomeronasal) olfactory systems. While these two systems differ by the types of chemosensory receptors they use and the organization of their neuronal circuitry within the central nervous system, there is convincing evidence that they have complementary functions in the regulation of multiple social behaviours in mice, including reproduction and aggression (Boehm et al., 2005, Yoon et al., 2005, Zhang et al., 2005, Keller et al., 2006, Spehr et al., 2006, Keller et al., 2009, Stowers et al., 2013, Matsuo et al., 2015). The olfactory systems are useful models to study the mechanisms that underlie sensory map

formation because of their relatively simple and accessible circuitries. Furthermore, the innate nature of multiple behaviours they control facilitates the examination of the relationship between precise circuit formation and the social behaviours that these circuits regulate.

Although the glomerular maps formed in both the main olfactory system (MOS) and vomeronasal system provide information about the discrete qualities of the chemosensory signals detected, sensory information processing appears to differ between the two systems. In the mouse MOS, olfactory sensory neurons (OSNs) express one of approximately 1200 odorant receptors (OR), and all OSNs expressing the same OR innervate on average two glomeruli per olfactory bulb (OB, or main olfactory bulb, MOB) (Buck and Axel, 1991, Chess et al., 1994, Mombaerts et al., 1996). Dendrites of second order neurons in these glomeruli therefore receive input from a single OR. ORs are not specifically tuned to an odorant but instead bind odorants with varying affinities based on their molecular features (Malnic et al., 1999). This feature enables ORs to bind to multiple ligands and allows a given ligand to activate multiple ORs on different OSNs, depending on the molecular features of the ligand (Malnic et al., 1999). As a result, the MOS must rely on a combinatorial code of OR activation to differentiate between particular odorant molecules (Malnic et al., 1999). Because OSNs cannot discriminate specific odours, a glomerular map of OR activation is relayed to the brain where further processing is needed to obtain the desired response to the stimulus. In contrast, in the vomeronasal system, a large subset of vomeronasal sensory neurons (VSNs) appear to be tuned to specific cognate ligands, suggesting that the integration of stimuli information may happen at the level of the accessory olfactory bulb (AOB) (Leinders-Zufall et al., 2000, Nodari et al., 2008, Haga et al., 2010). In this thesis introduction, we will discuss the mechanisms underlying the formation of

the glomerular map in the vomeronasal system and the importance of this map in regulating social interactions in rodents.

Organization of the vomeronasal system

The accessory olfactory system (AOS) is comprised of the vomeronasal organ (VNO), a chemoreceptive structure located in the base of the nasal septum, and of the AOB, located in the dorso-caudal region of the MOB (Halpern, 1987, Dulac, 2000). The VNO neuroepithelium houses vomeronasal sensory neurons (VSNs) that express receptors capable of detecting chemosignals, including proteins and small organic molecules (Figure 1.1A). While OSNs express one of over 1000 OR genes, each VSNs expresses one or a restricted few VRs from a repertoire of close to 400 functional genes (Dulac and Axel, 1995, Martini et al., 2001, Yang et al., 2005, Silvotti et al., 2007, Young and Trask, 2007, Young et al., 2010). The sensory epithelium can be subdivided into two non-overlapping regions based on the type of vomeronasal receptors expressed by the VSNs. VSNs located in the apical and basal regions of the VNO selectively express members of the vomeronasal receptor 1 (V1R) and V2R families of G-protein coupled receptors (GPCRs) that signal through the Gαi and Gαo proteins, respectively (Dulac and Axel, 1995, Berghard and Buck, 1996, Berghard et al., 1996, Herrada and Dulac, 1997, Matsunami and Buck, 1997, Ryba and Tirindelli, 1997, Pantages and Dulac, 2000, Walz et al., 2002). While most VSNs express V1R or V2R genes, a subset of VSNs exclusively expresses another family of chemosensory receptors known as formyl peptide receptors (FPR) (Liberles et al., 2009, Rivière et al., 2009). The segregated localization of V1R- and V2R-expressing VSN cell bodies in the VNO is maintained at the level of their axonal projections to the AOB. V1R-expressing VSNs that have their cell bodies in the apical layer of the VNO project axons to the anterior portion of the AOB whereas basally located V2R-expressing VSNs

innervate the posterior region of the AOB (Figure 1.1B). In the AOB, VSN axons synapse onto dendrites of mitral cells in neuropil structures termed glomeruli. While the implications of segregating V1R and V2R VSN axonal populations into different regions of the AOB remain unknown, this wiring pattern is not maintained at the level of mitral cell projections, which directly innervate multiple nuclei of the limbic system, bypassing cortical structures (Meisami and Bhatnagar, 1998, Von Campenhausen and Mori, 2000). Stereotaxic injections of anterograde tracers into the glomerular and mitral cell layers of the AOB have revealed significant overlap in the innervation of the amygdala by mitral cells located in the anterior and posterior regions of the AOB (Meisami and Bhatnagar, 1998, Von Campenhausen and Mori, 2000). Neurons located in nuclei innervated by mitral cells project to multiple areas of the hypothalamus that are linked to aggression, parental behaviour and reproduction, including the ventromedial hypothalamus (VMH) (Kevetter and Winans, 1981a, b, Petrovich et al., 2001). Thus, the AOB can be understood as a structure that consolidates chemosensory information from the environment and in turn relays this integrated information to higher brain centres that can direct behavioural outputs.

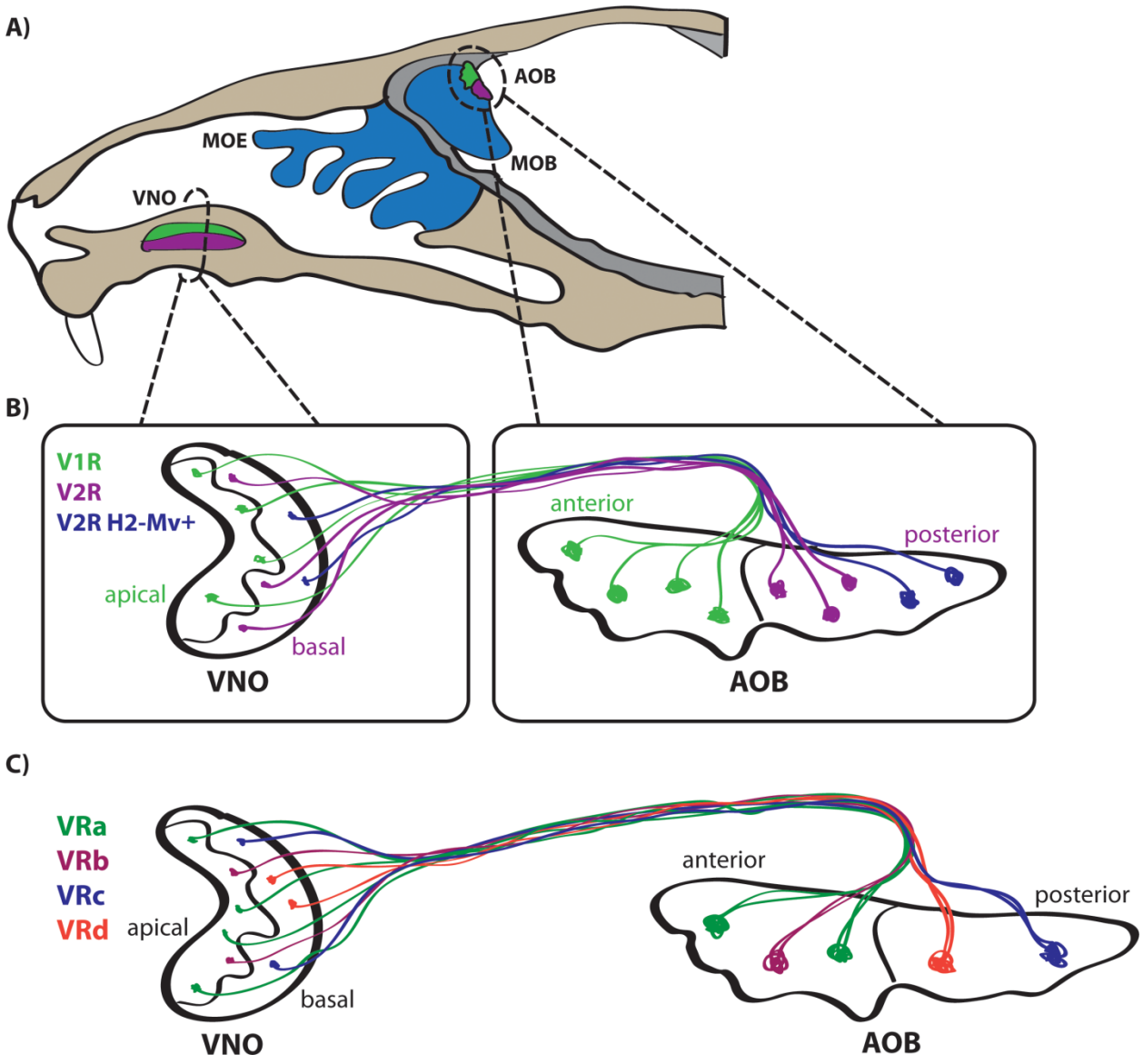

Figure 1.1. General overview of the mouse vomeronasal system.

A) A sagittal view of the mouse olfactory systems. The VNO is located in the base of the nasal septum and the AOB is located in the posterior dorsal aspect of the MOB in the mouse brain. The neuroepithelium of the VNO is divided into apical (green) and basal (purple) regions. This segregation is maintained at the level of the AOB, which is divided into an anterior (green) and posterior (purple) portion.

B) VSNs that have their cell bodies in the apical region of the VNO project their axons to the anterior portion of the AOB (green) and VSNs that have their cell bodies in the basal region project their axons to the posterior portion of the AOB (purple and blue). Apical VSNs express V1Rs and basal VSNs express V2Rs. A third population of VSNs located in the lower sublayer of the basal region of the VNO express V2Rs and H2-Mvs. This population projects its axons to

the posterior subdomain of the posterior region of the AOB (blue). Thus, there is a tripartite organization of the VNO and AOB.

C) The homotypic targeting of VRs. VSNs expressing the same VR project their axons to the same glomeruli within the AOB. Therefore, each glomerulus is a homogeneous structure consisting of one population of VSNs that express the same VR. One population of VSNs can project its axons to multiple glomeruli within the AOB, usually between 10 and 30 glomeruli.

VNO: vomeronasal organ; MOE: main olfactory epithelium; MOB: main olfactory bulb; AOB: accessory olfactory bulb.

Molecular logic of AOB wiring

In the MOS, the main olfactory bulb (MOB) provides a spatial map of olfactory receptor (OR) activation. Here, olfactory sensory neurons (OSNs) expressing the same OR converge onto two main glomeruli at fixed locations within the glomerular layer of the MOB where they innervate mitral cells (Ressler et al., 1994, Vassar et al., 1994, Mombaerts et al., 1996). Each mitral cell projects one apical dendrite to a single glomerulus innervated by axons expressing a given OR. In turn, mitral cells project axons to higher cortical areas where sensory information can be further processed. This one OSN type (one OR) to one mitral cell connectivity is described as a "labelled line" of sensory information processing in the MOS (Luo and Katz, 2004) and allows for spatial recognition of a given OR activation to be relayed to higher brain areas.

A combination of genetic, electrophysiological, and imaging approaches has revealed that the glomerular map in the vomeronasal system differs from the MOS. Genetic labelling of VSN axonal projections has shown that VSNs expressing the same VR innervate as many as thirty different glomeruli within broad but spatially conserved regions of the AOB, with each of these glomeruli containing a single population of VR-expressing axons (Figure 1.1C) (Belluscio et al., 1999, Rodriguez et al., 1999). Ablating expression of the VR in VSNs leads to improper targeting of axons in glomeruli of the AOB indicating that VRs are required for axonal coalescence (Rodriguez et al., 1999). The use of a multireporter transgenic mouse line to label multiple VRs that belong to the same or distinct phylogenetic clade of VRs revealed that VSNs expressing closely related VRs innervate nearby and spatially conserved glomeruli within the AOB (Wagner et al., 2006).

Although VSN connectivity to the AOB is fairly well characterized, the principles underlying mitral cell connectivity remain to be fully established. An analysis of mitral cell dendritic morphology using a genetic labelling approach revealed that mitral cells located in either the anterior or posterior half of the external plexiform layer (EPL) of the AOB can project their dendrites to glomeruli located in the opposite half of the AOB (Figure 1.2B) (Yonekura and Yokoi, 2008). This analysis suggests that the spatial segregation of V1R and V2R glomerular inputs along the anterior-posterior axis of the AOB may not be maintained at the level of the mitral cell layer (Yonekura and Yokoi, 2008).

A series of cell tracing experiments have revealed significant heterogeneity among mitral cell dendritic projections to AOB glomeruli. In contrast to the MOS, where a mitral cell projects its apical dendrite to a single glomerulus, mitral cells in the AOB project their dendrites to multiple glomeruli. Interestingly, while some mitral cells project their dendrites to multiple glomeruli that are innervated by axons expressing the same VR, other mitral cells project their dendrites to several glomeruli with different VR identities (Del Punta et al., 2002b). These observations show that mitral cells can exhibit both homotypic and heterotypic connections with VSNs, suggesting that multiple types of mitral cells may exist in the AOB (Figure 1.2). Furthermore, electrophysiological studies examining the response profiles of mitral cells to different stimuli have revealed functional diversity between mitral cells. In a study by Meeks and colleagues, stimulation of VSNs with sulphated steroids and urine mixes combined with *ex vivo* recordings from mitral cells in the AOB led to the understanding that mitral cells are mostly activated by a single stream of information whereas some mitral cells respond to multiple streams of sensory input (Meeks et al., 2010). Since mitral cells can respond to multiple streams

of sensory input, these results provide further evidence that sensory stimuli processing and integration could happen at the level of the AOB.

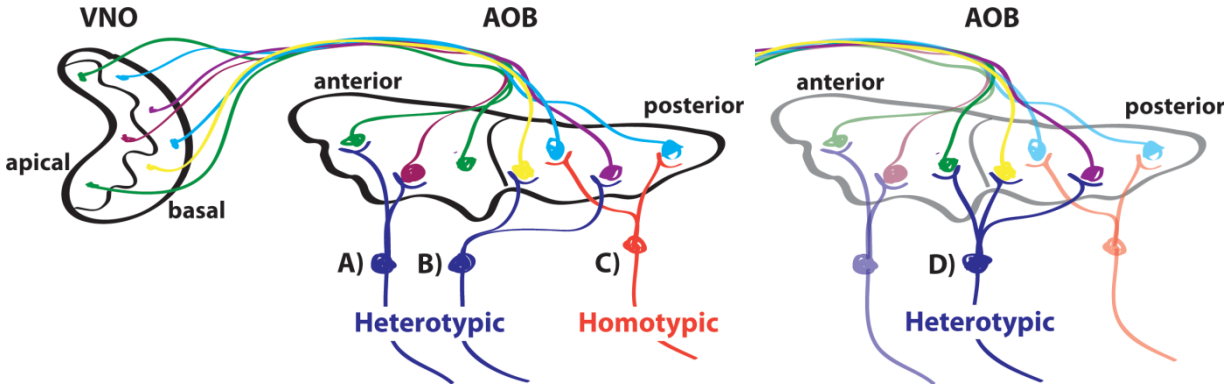

Figure 1.2. Mitral cell connectivity.

Different possibilities of connections between mitral cells and VSNs in the AOB based on morphological, genetic and functional studies (Del Punta et al., 2002b, Wagner et al., 2006, Yonekura and Yokoi, 2008, Meeks et al., 2010). Mitral cells with cell bodies in the external plexiform layer (EPL) of the AOB project multiple dendrites to synapse with VSN axons in glomeruli of the AOB where they are proposed to form heterotypic or homotypic connections with glomeruli.

A) Example of a heterotypic connection where one mitral cell projects its dendrites to multiple glomeruli that are innervated by VSNs that express different VR genes. Heterotypic connections can be made with VSNs that express VRs belonging to the same VR subfamily (ie. the V1Ra subfamily), or with VSNs that express VRs belonging to different VR subfamilies (ie. V1Ra or V1Rb subfamilies). Even though the glomeruli are heterogeneous, the phenotypic quality of the ligands that activate the VSNs which are targeted by the same mitral cell may be similar (ie. glomeruli that are activated by the same donor phenotype are linked together by one mitral cell resulting in a mitral cell that responds to a single stream of sensory information). Another possibility is a mitral cell that connects to heterogeneous glomeruli that are activated by ligands from different phenotypic donors resulting in a mitral cell that responds to and processes multiple streams of information.

B) Mitral cell dendrites can target glomeruli on the opposite region of the AOB to where their cell body is located.

C) Example of a homotypic connection where mitral cells connect to multiple glomeruli that are innervated by VSNs that express the same VR gene.

D) If the segregation between apical and basal VSNs is not present at the level of the mitral cells, a fourth hypothetical possibility of mitral cell connectivity may exist where a mitral cell can project dendrites to multiple heterogeneous glomeruli innervated by VSNs that express different VR families (ie. V1R or V2R).

Mechanisms of glomerular map formation in the AOB

VSNs project their axons in large tightly fasciculated bundles along the medial aspect of the olfactory bulb and turn upon reaching the caudal part of the bulb to innervate the AOB. These axons then segregate into the anterior and posterior regions of the AOB to maintain the spatial separation of apical and basal VSN cell bodies at the level of axonal inputs into the AOB. VSNs expressing the same VR then coalesce to form glomeruli innervated by a single population of VR-expressing axons (Figure 1.3). The segregation of VSN axons within the AOB and their coalescence into glomeruli appear to be regulated through different mechanisms. A combination of attractive and repulsive forces promote the segregation of axons along the anterior-posterior axis while the sorting and coalescence of axons into specific glomeruli is at least in part dependent on cell adhesion molecules expressed at their surface (Figure 1.3B).

In the main olfactory system, there is strong evidence that fasciculation and sorting of OSN axons within the olfactory nerves impinges on the targeting accuracy of these axons in the main olfactory bulb (Imai et al., 2009, Miller et al., 2010). Similarly, the pre-sorting of VSN axons within the vomeronasal tract that project to the AOB may be one mechanism through which segregation of apical and basal VSN inputs occurs at the AOB. However, severe defasciculation of the vomeronasal tract does not significantly affect the anterior-posterior segregation of VSN axons in the AOB, and a clear segregation of basal VSN axons within the vomeronasal tract in the mouse has not been observed (Cloutier et al., 2004, Prince et al., 2013). The tight fasciculation of the vomeronasal tract may instead be required to prevent premature innervation of the MOB by VSN axons projecting to the AOB. Indeed, ectopic innervation of the MOB by VSN axons has been reported in multiple mouse models where defasciculation of the

vomeronasal tract is observed (Cloutier et al., 2002, Cloutier et al., 2004, Takatoh et al., 2008, Degano et al., 2009).

Rather than being pre-sorted within the vomeronasal tract, apical and basal VSN axons appear to differentially respond to a combination of repulsive and attractive cues in the AOB that dictate their segregation within the two regions. One ligand/receptor complex that appears to contribute to this process is the Semaphorin-neuropilin signaling system. Semaphorins are a large family of several different classes of secreted and membrane-associated proteins that are important in mediating many key biological aspects of development (Jongbloets and Pasterkamp, 2014). In some contexts, binding of semaphorins to their receptors located on growing axons, including the neuropilin family, results in growth cone collapse and axon repulsion during nervous system development (Nakamura et al., 2000, Raper, 2000, Jongbloets and Pasterkamp, 2014). The class 3 semaphorin receptor Neuropilin-2 (Npn-2) is selectively expressed in apical VSNs axons, which are repelled *in vitro* by explants of the posterior half of the AOB, suggesting that this region secretes a chemorepellent capable of preventing entry of apical axons into the posterior AOB (Figure 1.3B) (Cloutier et al., 2002). Ablation of Npn-2 leads to mistargeting of apical VSN axons to the posterior region of the AOB, but does not affect the targeting of basal VSN axons (Cloutier et al., 2002, Walz et al., 2002). *In situ* hybridization experiments have shown that multiple members of the class 3 semaphorin family of secreted chemorepellents are expressed by mitral cells of the AOB but no gradient of expression across the anterior-posterior axis was detected (Cloutier et al., 2002). Removal of either Sema3C or Sema3B expression did not affect the targeting of VSN axons (Walz et al., 2007). Sema3F is so far the only member of this family that has been associated with the targeting of VSN axons. Indeed, mistargeting of apical VSN axons in the posterior AOB has been reported in a small subset of Sema3F mutant

mice analyzed (Cloutier et al., 2004). The mild defects observed in Sema3F mutant mice, combined with the lack of a gradient of expression for secreted semaphorins in the AOB, suggest that additional ligands may also contribute to the Npn-2-dependent segregation of apical VSN axons to the anterior AOB.

In addition to apical VSN axons being repelled from the posterior AOB due to Npn-2 activity, apical VSN axons also respond to an attractive signal in the anterior region of the AOB. Apical axons express high levels of the glycosylphosphatidylinositol (GPI)-anchored ephrin-A5 at their surface, while the EphA6 receptor is more highly expressed in the anterior region of the AOB (Figure 1.3B) (Knoll et al., 2001). A subset of EphrinA5 knockout mice exhibit apical VSN axon mistargeting to the posterior AOB defining a requirement for EphrinA5 in VSN axon targeting (Knoll et al., 2001). Furthermore, evidence from *in vitro* stripe assays suggests that Ephrin-A5-expressing VSN axons prefer to grow on cells expressing EphA6, suggesting that ephrinA5-EphA6 interactions promote attraction (Knoll et al., 2001). The interaction of the Ephrin ligand (ephrin-A5) on axons with the receptor (EphA6) in the target region represents a classic example of ephrin reverse signaling whereby the intracellular signal transduction is occurring through the ligand instead of the receptor. Considering that ephrin-A5 is a GPI-anchored protein, reverse signaling in VSN axons is likely to require a co-receptor for ephrin-A5. Potential co-receptor candidates include the receptor tyrosine kinase RET, and the neurotrophin receptors p75 and TrkB, which have been shown to act as ephrin-A co-receptors in the motor and visual systems, respectively (Lim et al., 2008, Marler et al., 2008, Bonanomi et al., 2012).

In contrast to apical VSN axons, which express Npn-2, basal VSN axons express Robo-2, a receptor for the Slit family of repulsive axon guidance cues (Figure 1.3B) (Marillat et al., 2002, Knöll et al., 2003, Cloutier et al., 2004, Prince et al., 2009). Slit-1 is highly expressed in cells

located at the anterior tip of the accessory olfactory bulb, suggesting that a high anterior to low posterior gradient of slit-1 protein is generated in the AOB (Marillat et al., 2002, Knöll et al., 2003, Cloutier et al., 2004, Prince et al., 2009). Furthermore, Slits can repel VSN axons *in vitro*, suggesting that high levels of Slit-1 present in the anterior AOB prevent Robo-2-expressing basal VSN axons from entering this region (Knöll et al., 2003). Indeed, ablating Robo-2 expression in VSNs resulted in the improper targeting of basal VSN axons to the anterior region of the AOB, without affecting the segregation of apical VSN axons (Prince et al., 2009). In addition, Slit-1, but not Slit-2 or Slit-3, is required for the projection of VSN axons into the posterior region of the AOBs (Cloutier et al., 2004, Prince et al., 2009).

An additional layer of axonal organization has been proposed to take place within the posterior region of the AOB. A subset of basal VSNs express members of a family of non-classical class I major histocompatibility *Mhc* genes, known as *H2-Mv* genes, which have been proposed to regulate VR cell surface expression (Ishii et al., 2003, Loconto et al., 2003, Ishii and Mombaerts, 2008). One member of this family, M10.2, is highly expressed in the basal region of the VNO, and M10.2-positive axons project to the most posterior edge of the posterior half of the AOB. This pattern of expression has been suggested to establish a so-called "tripartite organization" of the AOB with V1R-expressing axons in the anterior AOB, V2R-expressing axons in the posterior AOB, and V2R/H2-Mv-positive axons restricted to the most posterior part of the AOB (Figure 1.1B & 1.3A) (Ishii and Mombaerts, 2008). The molecular cues that are involved in specifically targeting axons of V2R/H2-Mv-positive VSNs to the posterior edge of the AOB remain to be identified.

While classical axon guidance molecules play a critical role in the segregation of apical and basal VSN axons within the anterior and posterior regions of the AOB, they are not required

for the coalescence of VSN axons within glomeruli, suggesting that other mechanisms regulate this process. VR expression is required for the formation of discrete glomeruli, and deletion of a V1R led to the broad dispersion of VSN axons in the anterior AOB (Belluscio et al., 1999, Rodriguez et al., 1999). Early after birth, sensory activity can regulate the coalescence and refinement of glomeruli in the AOB. An abnormal increase in VSN activity during this time period results in a delay in coalescence of axons into defined glomerular structures and in exuberant VSN axon projections (Hovis et al., 2012). Interestingly, in the main olfactory system, the expression of several cell adhesion molecules that affect axonal coalescence is regulated by neuronal activity (Serizawa et al., 2006, Kaneko-Goto et al., 2008). Some members of one of these cell adhesion families, the Kirrels, are expressed in VSNs, and their expression is altered in VSNs that lack the TRP2 calcium channel (Prince et al., 2013). A detailed analysis of the expression of Kirrel family members in VSNs revealed that subsets of VSNs express varying levels of Kirrel-2 and Kirrel-3. The differential pattern of expression creates a molecular code by which axons can identify one another and facilitate like axons to coalesce (Figure 1.3B). Ablation of Kirrel-3 led to the formation of larger glomeruli receiving inputs from multiple types of VR in the posterior AOB, demonstrating a role for this family of molecules in VSN axonal coalescence (Prince et al., 2013). Considering the complexity of the glomerular map formed in the AOB, it is very likely that additional families of cell adhesion molecules contribute to the formation of a diverse molecular code among VSN axons coalescing into the AOB.

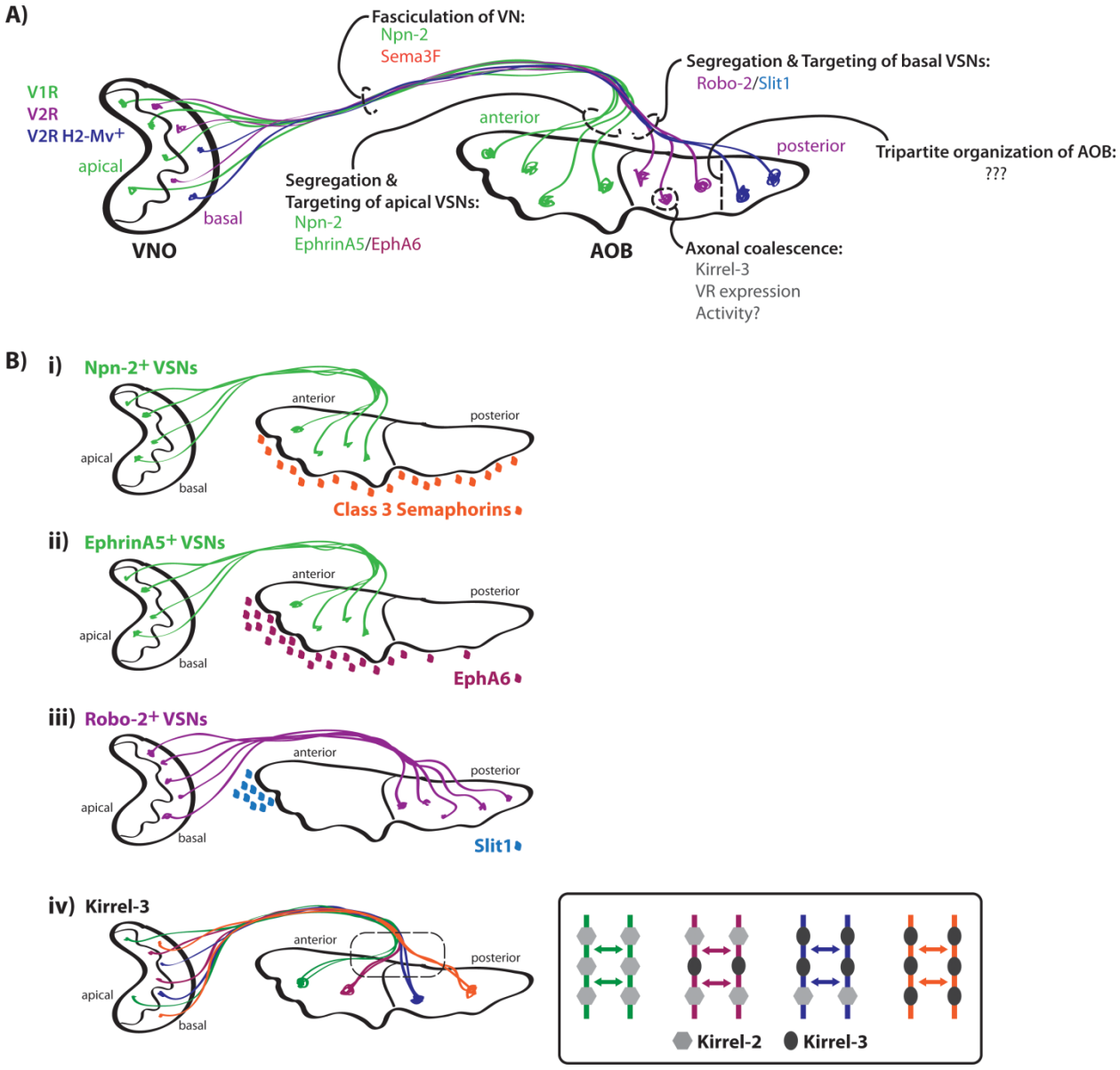

Figure 1.3. Mechanisms and factors involved in vomeronasal circuit formation.

A) General overview of the mechanisms and key factors that control the wiring of VSNs to the AOB and targeting of VSNs within the AOB.

B) Axon guidance molecules which are required for the proper targeting and coalescence of VSNs. i) Npn-2 expression in apical VSNs is required for the proper targeting of apical VSN axons to the anterior AOB. The ligand which mediates Npn-2 dependent axon segregation in the AOB is not known. However, there is expression of class 3 Semaphorins in mitral cells within the AOB. Npn-2^{+} axons could be responding to a gradient of secreted Semaphorins. ii) EphrinA5 expression in apical VSNs promotes the targeting of these axons to the anterior portion of the AOB in response to high expression of the EphA6 receptor in mitral cells of this region. EphrinA5^{+} axons respond to the high EphA6 gradient in the anterior AOB via an attractive

interaction to help segregate apical VSN axons to the anterior AOB. iii) Robo-2 expression in basally located VSNs causes these VSN axons to be repelled to the posterior portion of the AOB in response to a high anterior to low posterior Slit-1 gradient created by Slit-1 expressing cells in the anterior tip of the AOB. iv) Kirrel-3 is required for the coalescence of VSN axons into glomeruli. The differential expression of Kirrel molecules on VSN axons creates a molecular code which dictates their proper coalescence into glomeruli. It is suggested that homophilic interaction between Kirrels drives the convergence of like axons together. Therefore, axons with similar levels of Kirrels will come together forming the target glomerulus.

VNO: vomeronasal organ; AOB: accessory olfactory bulb.

Sensory coding in the vomeronasal system

While genetic and cell labelling studies have provided significant insight into the wiring of the vomeronasal system, an understanding of VSN response to specific ligands to activate stereotypic glomerular maps in the AOB is critical in revealing how sensory information is encoded in this system. The identification of chemosignals that activate VSNs combined with the ability to monitor electrophysiological responses or changes in calcium levels in either VSNs or mitral cells has provided insight into sensory processing in the AOB. To date several ligands capable of activating VSNs have been identified including urine-derived small organic molecules (Leinders-Zufall et al., 2000), sulphated steroids (Nodari et al., 2008, Meeks et al., 2010, Turaga and Holy, 2012), major urinary proteins (MUPs) (Chamero et al., 2007, Papes et al., 2010, Chamero et al., 2011, Kaur et al., 2014), MHC class 1 peptides (Leinders-Zufall et al., 2004), exocrine gland-secreting peptide 1 (ESP1) (Kimoto et al., 2005), and N-formylated peptides (Liberles et al., 2009, Rivière et al., 2009, Bufe et al., 2012, Leinders-Zufall et al., 2014, Bufe et al., 2015).

Several observations suggest that VSNs show a high degree of selectivity in their response to chemosignals. Firstly, V1R VSNs are activated differently than V2R VSNs. For example, three dimensional imaging of VSNs in the VNO in response to urine stimulation using objective-coupled planar illumination microscopy revealed that urine selectively activates V1R VSNs (Holekamp et al., 2008). Interestingly, while whole urine samples specifically activate V1R VSNs, a study by Isogai and colleagues exposed differential roles for apical and basal VSNs. They investigated the response profiles of V1R and V2R VSNs to a wide range of animal cues using egr1 as a marker of VSN activation (Isogai et al., 2011). Results from this study emphasized the difference in activation between V1R VSNs and V2R VSNs and concluded that

V2Rs encode information about the identity of the donor animal, such as a conspecific or predator, whereas V1Rs serve to detect its physiological status (Isogai et al., 2011). Secondly, and more particularly, individual VSNs have been shown to have response profiles that are highly tuned to specific ligands. Simultaneous electrophysiological recordings of a wide range of VSNs in response to mouse urine revealed that different subsets of VSNs respond discriminatorily to either male or female urine (Holy et al., 2000). Furthermore, calcium imaging in organotypic slices of the VNO revealed that low concentrations of small organic molecules from urine activate non-overlapping subsets of VSNs, suggesting that VSNs show a high degree of sensitivity and selectivity to a specific ligand (Leinders-Zufall et al., 2000). Hence, in contrast to the main olfactory system, where OSNs are broadly tuned to respond to many odorant molecules, VSNs are highly tuned to specific ligands.

For the most part, with highly-tuned response profiles, individual ligands tend to activate specific VSN subtypes, and individual VSN populations respond to only specific ligands. Thus, the chemosignal can generally be encoded by the VSN subtype that is activated. This notion is unlike the main olfactory system, where the odor is encoded by the set of OSN types that are activated by a single odorant molecule. Nonetheless, VSNs have also been reported to display combinatorial responses in certain contexts. The detection of cues that encode individual and strain specific information relies on the combinatorial activation of VSNs (He et al., 2008). Interestingly, the detection of MUP ligands, that can both elicit male aggression and allow discrimination of self from non-self, employs different sensory coding strategies to promote these two behaviours. MUPs are a family of proteins abundantly excreted in mouse urine that exclusively activate V2R-expressing VSNs and that induce male-male aggressive behaviour in mice (Chamero et al., 2007). While male-male aggression is induced through a tuned activation

of VSNs by specific MUPs, discrimination of self from non-self relies on the detection of a blend of MUP ligands through combinatorial sensory coding (Kaur et al., 2014).

While these studies have shed light on the highly tuned nature of VSNs to specific ligands, much remains to be learned about the integration of these signals at the level of the AOB. Electrophysiological studies examining mitral cell responses in awake behaving male mice demonstrated that mitral cells are selectively activated when these mice are in contact with a mouse of a given sex or strain (Luo et al., 2003). Furthermore, urine and saliva from donor animals with different sexual and genetic statuses activate different subsets of mitral cells in the AOB (Ben-Shaul et al., 2010). Taken together, these results indicate that identification of an animal's sex and genetic status could be encoded at the level of the AOB. More recently, an elegant study by Hammen et al. using calcium imaging to visualize glomeruli activation in the AOB in response to a wide range of stimuli has provided valuable insight into the organization of the glomerular map in the AOB. Genetic labelling experiments have shown that VSNs expressing closely related VRs innervate nearby and spatially conserved glomeruli within the AOB, suggesting that the glomerular map may be organized to juxtapose inputs of VSNs that recognize ligands with similar structures (Wagner et al., 2006). However, examination of the pattern of activation of glomeruli in response to specific stimuli revealed that the organization of the AOB glomerular map appears to be based on phenotypic rather than molecular similarity between chemosignals (Hammen et al., 2014). Indeed, while glomeruli selectively activated by urine of sexually mature male mice are preferentially located near the posterior border of the anterior AOB, glomeruli selective for urine from sexually mature female mice are preferentially clustered at the anterior edge of the anterior AOB. In contrast, exposure of VSNs to a variety of sulphated steroids showing a range of molecular similarity revealed that juxtaposed glomeruli do

not systematically have similar receptive fields. Furthermore, glomeruli activated by molecularly similar steroid structures do not juxtapose one another (Hammen et al., 2014). These results suggest that glomeruli in the AOB are not organized in a manner that correlates with the similar receptive fields of ligands, but instead by similar phenotypic qualities of the signal donor, such as sex and sexual maturity. Although VRs with similar amino acid sequence homology target closely positioned glomeruli within the AOB, amino acid sequence of a receptor may be more important for axonal targeting than for receptor-ligand tuning. Assessing global activation of VSNs and mitral cells in response to a wide range of natural stimuli has helped reveal the logic of sensory coding in the vomeronasal system and suggests that, in contrast to the MOS where sensory input processing takes place at the level of the olfactory cortex, integration of sensory information takes place at the level of the AOB and is then relayed to the limbic system.

Regulation of aggression by the vomeronasal system

The VNO detects chemosignals that regulate a variety of social behaviours, including mating, social dominance, and maternal care (Wysocki and Lepri, 1991). In addition, the vomeronasal system detects conspecific cues which inform the sex and genetic strain of mice, which are important stimuli initiating instinctive behaviours, such as aggression (Luo et al., 2003, He et al., 2008, Hendrickson et al., 2008, Ben-Shaul et al., 2010). Several studies employing gene inactivation approaches to disrupt VNO function have revealed a critical role for this organ in regulating both male and female aggression. Ablation of the TRP2 ion channel blocks sensory activation of VSNs in response to urine and leads to decreased male-to-male and maternal aggression, along with an inability to discriminate male from female mice (Leypold et al., 2002, Stowers et al., 2002). Several studies support a role for both V1R and V2R-expressing VSNs in the control of aggression by the VNO. Deletion of a cluster of V1R genes leads to

selective defects in male reproductive behaviours and maternal aggression, suggesting that subsets of VRs can mediate specific behaviours (Del Punta et al., 2002a). Blocking V1R signalling through ablation of the trimeric G protein subunit Gαi leads to a reduction in male-to-male aggression in a resident intruder assay (Norlin et al., 2003). Interestingly, ablation of the V2R-specific Gαo subunit, also disrupts male territorial behaviour and maternal aggression (Chamero et al., 2007). Furthermore, ablation of the G-protein subunit γ8, which is preferentially expressed in V2R VSNs display defects in both male and female aggressive behaviours (Montani et al., 2013). Although the effects of V2R gene ablation on aggression remain to be assessed, the absence of V2R localization to the dendritic tip of VSNs in β2m mutants is associated with reduced male-male aggression in these mice (Loconto et al., 2003). Aggressive behaviours in mice have therefore been associated with both V1R and V2R functions.

Although the vomeronasal system is thought to mediate pheromone detection and the regulation of aggressive behaviours in mice, there is evidence that the MOS can also contribute to these processes. Loss of the OR signal transduction molecule type 3 adenylyl cyclase expression in mice led to an inability to detect pheromonal cues and to a lack of male-male aggression and male sexual behaviours (Wang et al., 2006). Furthermore, *cnga2* mutant mice defective in odour-evoked OSN signaling display defects in aggression (Mandiyan et al., 2005). Interestingly, Matsuo et al. demonstrated using conditional knockout strategies that male social behaviours in mice, including male-male aggression, was significantly decreased in mice where OSNs that project to the dorsal zone of the MOB are specifically ablated or have *cnga2* expression ablated. Lesions to the anterior olfactory nucleus (AON) also resulted in similar behavioural phenotypes and loss of male-male aggression which led this group to hypothesize that the MOS to AON pathway is another means of mediating pheromone behaviours

independent of the AOS (Matsuo et al., 2015). It is therefore likely that the control of such a vital innate behaviour implicates the detection of a large array of chemosignals that require both the vomeronasal and MOS.

Our understanding of the principles underlying the control of aggression by the vomeronasal system has been greatly improved through the discovery of chemosignals that can induce aggressive behaviours. The detection of urine represents an important means by which mice communicate with one another and can trigger innate behaviours, including aggression. Urine contains organic volatile compounds, such as 2-*s*-butyl-4,5-dihydrothiazole (SBT), that can promote inter-male aggression (Novotny et al., 1985). As mentioned above, MUPs are a family of proteins excreted in urine that exclusively activate V2R-expressing VSNs and that induce male-male aggression in mice (Chamero et al., 2007). The effect of MUPs is dependent on V2R signalling as ablation of Gαo expression leads to a loss of MUP-induced aggression (Chamero et al., 2011). One member of this family of proteins, MUP20 (also known as Darcin), is sufficient to promote inter-male aggression (Kaur et al., 2014).

Although these studies revealed that isolated MUPs can activate V2R-expressing VSNs, previous observations have shown that low concentrations of urine mainly activates glomeruli located in the anterior region of the AOB, innervated by V1R-expressing VSNs (Holekamp et al., 2008, Hammen et al., 2014). It remains possible that varying concentrations of MUPs can differentially activate V1R and V2R expressing VSNs. Alternatively, the presence of additional compounds in urine that can bind to MUPs could be responsible for the activation of V1R-expressing neurons by urine. In keeping with this possibility, the MUP binding molecule SBT activates VSNs located in the apical region of the VNO that express V1R family receptors (Leinders-Zufall et al., 2000). In addition to acting as a pheromone for intra-species

communication, MUPs can act as kairomones to elicit defensive behaviours to predators in inter-species communication (Papes et al., 2010). A recent study has also demonstrated that MUPs are sufficient for inducing territorial urine countermarking in mice, which suggests a role for these proteins in discriminating between self and non-self (Kaur et al., 2014).

Although the identification of ligands that modulate aggression through the VNO has provided important insight into the regulation of this behaviour, it remains to be determined whether the activation of specific glomerular maps by these ligands is necessary for a behavioural response to take place. In the main olfactory system, disrupting the glomerular map results in a loss of innate avoidance of specific aversive odorants (Cho et al., 2011). Interestingly, the defects observed in the formation of glomeruli in the posterior region of the AOB in *kirrel-3* mutant mice are associated with a loss of male-male aggression in a resident intruder male assay (Prince et al., 2013). Furthermore, *ephrin-A5* mutant mice, which display improper segregation of apical VSN axons in the AOB, also exhibit a severe reduction in conspecific aggression (Sheleg et al., 2015). While these results support an important role for the formation of the AOB glomerular map in the regulation of aggression, further studies combining VNO-specific ablation of these axon guidance proteins and imaging of glomerular activation maps will be needed to conclusively demonstrate that the spatial arrangement of glomeruli in the AOB is necessary to regulate VNO-specific behaviours.

The Kirrel family of cell adhesion molecules

Sensory map formation requires short range signals for refinement of axon targeting. Contact attraction and contact repulsion between axons mediates the fine tune positioning of axons within their target. In the case of the AOS, VSN axons coming from the VNO to their target glomeruli in the AOB must converge with like axons expressing the same vomeronasal

receptor. Therefore, VSN axons must express surface molecules to allow them to interact with other specific axons to align their position within the larger field of axons coming from the VNO. Cell adhesion molecules expressed on the surface of axons that interact either homophilically or heterophilically can mediate contact attraction between like axons. Expression of different molecules creates a molecular code for axonal recognition and allows axons to distinguish between other axons for refinement of position. As shown in the MOS, expression of different axon guidance and sorting molecules can be due to different levels of activity; therefore, axons that have the same activity pattern, or express the same OR (therefore activated by the same ligands), will have similar expression patterns and target the same glomerulus (Sakano, 2010). Furthermore, OSN axons are organized in bundles prior to reaching the MOB and their respective target glomeruli illustrating the importance of local axon sorting in proper circuit assembly (Imai et al., 2009, Imai and Sakano, 2011). Although axon-target interactions mediated by secretion of guidance molecules are key for forming neural circuits, short range axon-axon interactions are also required to refine axon position when building an intricate circuit.

Previously published work in the AOS shows the importance of the cell adhesion molecule, Kirrel-3, in regulating axonal coalescence of VSN axons into glomeruli (Prince et al., 2013). Furthermore, Kirrel expression in the AOS is modulated with the loss of the TRP2 ion channel suggesting activity may regulate Kirrel expression (Prince et al., 2013). Another Kirrel family member, Kirrel-2, is also expressed in the AOS (Prince et al., 2013) and is a perfect candidate molecule to add to the molecular diversity between axons and regulate VSN axon-axon interactions resulting in proper axonal positioning and glomerular formation in the AOS.

The Kirrel family of cell adhesion molecules were first identified in mammals as transmembrane proteins with extracellular immunoglobulin (Ig) domains which are important for forming and maintaining the slit diaphragm and filtration barrier in the kidney (Donoviel et al., 2001). Kirrel-1 (Neph1) is expressed in podocytes where it interacts with Nephrin to form components of the slit diaphragm and mediate filtration barrier function of the kidneys, and therefore, loss of Kirrel-1 expression is associated with proteinuria and perinatal lethality in mice (Donoviel et al., 2001). Three Kirrel family members of the immunoglobulin superfamily of CAMs were identified in mammals, which include Kirrel-1 (Neph1), Kirrel-2 (Neph3) and Kirrel-3 (Neph2) (Sellin et al., 2003). Each family member has five Ig domains in its extracellular region, a transmembrane region and intracellular Growth factor receptor bound protein 2 (Grb2) and Src homology 2 (SH2), Grb2-SH2, binding site and postsynaptic density-95(PSD-95)/Discs large (Dlg)/zona occludens-1 (ZO-1) (PDZ) binding motif (Sellin et al., 2003). Since their initial discovery in the mammalian kidney, comprehensive analysis of Kirrel expression in the developing mouse embryo suggests additional roles to their function in the kidneys due to their wide pattern of expression in other areas, including the developing nervous system (Tamura et al., 2005, Völker et al., 2012). Moreover, Kirrels are expressed in developing sensory systems, such as the olfactory system, during embryonic and early postnatal stages (Morikawa et al., 2007). Altogether, Kirrels are proposed to regulate patterning and morphogenesis of various tissues by acting as cell-cell interactors and cell recognition molecules (Völker et al., 2012).

Concurrent with their wide range of expression, Kirrels are proposed to be involved with synaptogenesis during development of the nervous system (Gerke et al., 2006, Komori et al., 2008, Brusés, 2010), mammalian muscle development (Durcan et al., 2013, Durcan et al., 2014),

β cell function and insulin release in the pancreas (Sun et al., 2003, Fornoni et al., 2010), and haematopoietic stem cell maintenance (Ueno et al., 2003). Furthermore, Kirrels are considered to drive the convergence of axons in the MOS through homophilic interaction as overexpression of Kirrel-2 and Kirrel-3 leads to targeting defects of OSN axons in the MOB (Serizawa et al., 2006). Increasing evidence supports a role for Kirrels in mediating important processes for nervous system development.

Homologues of Kirrels in invertebrate systems also demonstrate their wide range of function in tissue development, especially during nervous system development. The Irre Recognition Module (IRM) proteins, homologues of Kirrels and Nephrin in *Drosophila melanogaster*, orchestrate cell recognition, intercellular adhesion and cell signaling events necessary for tissue morphogenesis and numerous developmental processes (Fischbach et al., 2009, Helmstädter et al., 2014). Heterophilic interactions between IRM proteins and downstream signalling events mediate the patterning of tissue and morphogenesis in the *Drosophila* eye (Bao and Cagan, 2005). Specifically, the IRM proteins are essential for axon pathfinding, cell sorting, and target selection during nervous system and eye development (Fischbach et al., 2009). And as in mammals, the IRM proteins in *Drosophila* also regulate nephrocyte diaphragm formation and myoblast fusion in muscle development (Helmstädter et al., 2014). In *Caenorhabditis elegans,* Kirrel homologue, SYG-1, directs synapse formation and synapse specificity (Shen and Bargmann, 2003, Shen et al., 2004). SYG-1 in the hermaphrodite specific motor neuron L (HSNL) interacts with Nephrin homologue, SYG-2, expressed on vulval epithelial cells to mediate synapse location and initiate synapse formation between the HSNL motor neuron and vulval muscle cells and adjacent target neurons (Shen and Bargmann, 2003, Shen et al., 2004). Kirrel homologues in invertebrates provide valuable insight about Kirrel function and

characterization of these homologues support the notion that Kirrels are important for circuit development in the AOS.

Similar to invertebrate models, the characterization of Kirrel protein interactions and downstream signalling mechanisms during kidney slit diaphragm development have also provided us with insight of how Kirrels could function in other systems. Kirrels interact and signal with multiple different protein families to regulate multiple functions, including actin cytoskeleton dynamics, endocytosis, cell polarity and cell survival at the kidney slit diaphragm (Grahammer et al., 2013). Interacting proteins of mammalian Kirrels include Nephrin (Donoviel et al., 2001), metalloproteinases (Gerke et al., 2005), synaptic scaffold protein calmodulin-associated serine/threonin kinase (CASK) (Gerke et al., 2006), podocin (Sellin et al., 2003), ZO-1 (Huber et al., 2003, Liu et al., 2003), and the cell polarity complex Par3/Par6/aPKC/CDC42 (Hartleben et al., 2008, Huber et al., 2009, Hartleben et al., 2013). The main transmembrane binding partner of the Kirrels in the kidney, Nephrin, is shown to interact with nWASp, Arp2/3 and Nck which are essential mediators of actin dynamics (Huber and Benzing, 2005, Verma et al., 2006). Moreover, interaction of Nephrin with Kirrel-1 leads to Kirrel-1 phosphorylation via Src kinase Fyn and subsequent actin polymerization demonstrating the ability of Kirrels to transduce an outside-in signal (Garg et al., 2007). This provides strong evidence that ligand binding to the Kirrel family and subsequent downstream signalling mediates processes that are important for axon-axon interactions and axon sorting, such as actin polymerization and cytoskeletal remodelling. It was also demonstrated that Kirrels can bind homophilically in trans using *in vitro* cell binding and co-immunoprecipitation assays (Gerke et al., 2003, Minaki et al., 2005, Serizawa et al., 2006, Nishida et al., 2010), and therefore, homophilic cell adhesion between like Kirrels may control their function. Additionally, Kirrel-3 interaction with CASK

(Gerke et al., 2006), a scaffolding protein that is localized to synapses, suggests Kirrels may also regulate synapse development in the nervous system. Taken together, homophilic interaction between Kirrels or heterophilic binding of other ligands to Kirrels may drive key processes for circuit assembly in the AOS.

Other important insight into Kirrel function comes from regulators of gene transcription. The transcription factor Ptf1a directly binds the *kirrel-2* gene and controls its transcription in the CNS during embryonic development (Nishida et al., 2010). Due to Ptf1a's role in cell fate specialization of subsets of neurons in the developing CNS, it is proposed by Nishida et al. that Kirrel-2 expression is a key component for proper NS development. Also, Kirrel-3 is associated with intellectual disabilities (Bhalla et al., 2008) suggesting a critical role for Kirrels during nervous system development and human cognition.

Rationale and Objectives

Overall this thesis highlights the significance of proper circuit formation to create a precise sensory map in order to regulate innate behaviours that are crucial for survival. Despite the fact that the AOS and MOS both detect chemical sensory cues from the environment, the general principles of sensory coding in these two olfactory systems are strikingly different. Development of the AOS circuitry requires multiple events and mechanisms resulting in a fully functional system. Multiple studies have demonstrated the requirement of neuronal activity, VR signal transduction and VR gene expression for the wiring of the vomeronasal system. Classical axon guidance molecules are required for fasciculation, segregation, and targeting of VSN axons to the AOB (Figure 1.3). Once VSNs have reached the AOB and segregated into the anterior and posterior regions, axon sorting molecules are important to drive the coalescence of VSNs into glomeruli. The positioning of axons within the AOB is crucial in order to form the intricate

circuit that is necessary for sensory map formation; however, the mechanisms by which this occurs have not been fully elucidated.

Considering the complexity of glomeruli positioning in the AOB, many factors must exist to regulate axon sorting to form a complete sensory map in the AOB. Recently, the CAM Kirrel-3 was implicated in sorting axons in the AOB (Prince et al., 2013). Prince et al. showed that Kirrel family members, Kirrel-2 and Kirrel-3, are differentially expressed in VSNs and that expression of *kirrel-3* is required for the coalescence of VSN axons into glomeruli. These results led to the hypothesis that the differential expression of Kirrels in VSNs creates a molecular code necessary for the proper coalesce of VSN axons into glomeruli (Figure 1.3B) (Prince et al., 2013). Hence, this thesis looks to identify if the CAM Kirrel-2 is required for wiring the vomeronasal system and further elucidate the mechanism by which the Kirrel family functions in the AOS. In order to address this, in this thesis I will examine 1) is Kirrel-3 required for the early coalescence of VSN axons into glomeruli in the AOB, 2) whether Kirrel-2 expression in VSNs contributes to the segregation of axons and the formation of glomeruli in the AOB, 3) if removal of both Kirrel-2 and Kirrel-3 protein expression results in even greater deleterious effects on glomeruli architecture, and 4) determine if the disruption in glomeruli morphology due to loss of *kirrel* expression also leads to a disruption in the formation of synapses in the glomerular layer of the AOB.

These events which define the connectivity of primary to secondary neurons in the AOS result in principles of sensory map formation for pheromone and chemical stimuli detection. Moreover, the importance of correct wiring in the AOS is underlined by the behavioural defects that arise when the circuitry is disrupted. Processing sensory stimuli to understand and enable us to interact with our environment is required in all animals for survival. How the animal codes

sensory information to mediate instinctive behaviours is a fundamental question which is beginning to be unravelled by studies using the mouse AOS as a model system. The studies discussed and performed in this thesis have provided remarkable insight into how the nervous system codes information to regulate behaviours necessary for animal survival.

Materials & Methods

Animals

All animal procedures used were approved by the Animal Care Facility of the Montreal Neurological Institute and McGill University. *EC2-tau-lacZ* mice (Cloutier et al., 2004), *kirrel-3* mice (Prince et al., 2013), *M72-tau-lacZ* mice (Zheng et al., 2000), *OMP-Cre* mice (Eggan et al., 2004), *S50-tau-lacZ* mice (Bozza et al., 2009), *MOR1-3-IRES-tau-lacZ*, *SP1-tau-GFP* mice (*MOR28-tau-GFP*) (Serizawa et al., 2000, Barnea et al., 2004) and *MOR174-9-IRES-GFP* mice (Cho et al., 2011) have been previously described.

Generation of *kirrel-2*$^{-/-}$ and *kirrel-2*$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice

Kirrel-2$^{-/-}$ mice were generated by blastocyst insertion of embryonic stem (ES) cells that contain the modified *kirrel-2* allele, Kirrel2$^{tm2a(KOMP)Wtsi}$ (Figure 2.2A), creating chimeric mice. Mice which express the modified *kirrel-2* allele are crossed with mice that express *Cre recombinase* under the control of the CMV promoter to excise the coding regions exon 3 and 4 from the *kirrel-2* allele. The ES cells were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). NIH grants to Velocigene at Regeneron Inc (U01HG004085) and the CSD Consortium (U01HG004080) funded the generation of gene-targeted ES cells for 8500 genes in the KOMP Program and archived and distributed by the KOMP Repository at UC Davis and CHORI (U42RR024244).

Kirrel-2$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice were created by crossing mice which express a floxed *kirrel-2* allele (*kirrel-2* lox allele, Figure 2.4A) with mice that express *Cre recombinase* under the control of the OMP promoter (*OMP-Cre* mice, (Eggan et al., 2004)). Excisions at loxP sites lead to the removal of the *kirrel-2* coding region, exons 3 and 4, from OMP positive cells only. The *kirrel-2* floxed allele was generated by crossing mice which express the modified *kirrel-2*

allele (Figure 2.2A and 2.4A) with mice that express flippase to excise at flippase recognition target (FRT) sites leading to a *kirrel-2* allele that has loxP sites flanking the coding region exons 3 and 4.

Perfusion

Prior to perfusion, mice were anaesthetized using Avertin (2,2,2-tribromoethanol and tertamyl alcohol). Mice were perfused transcardially using 0.1M phosphate buffered saline (PBS) to rinse the vasculature and ice cold 4% paraformaldehyde (PFA) in 0.1M PBS solution to fix the tissue. Brains were dissected and put in the fixative solution for 10 minutes post fixation on ice. Following fixation, brains were cryoprotected in 0.1M PBS containing 30% sucrose at 4˚C overnight. Brain tissue was covered in the cryoprotectant, OCT (Optimal Cutting Temperature), and flash-froze using 2-methylbutane on dry ice. Fixed tissue was stored at -80˚C until used.

Immunohistochemistry

Fixed tissue was cryosectioned (20μm) and mounted on Superfrost plus microscope slides (Fischer Scientific). Sections were air dried for 25 minutes, rehydrated with 0.1M PBS and incubated with 0.1M PBS containing 0.5% Triton-100x and 10% fetal bovine serum to prevent any unspecific binding of primary antibodies. Primary antibodies were applied for overnight incubation at 4˚C and used at different dilutions: anti-Kirrel-2 (1:500; R&D Systems), anti-$G_{\alpha i2}$ (1:500; WAKO Chemicals, USA), anti-$G_{\alpha o}$ (1:500; Santa Cruz Biotechnology), anti-olfactory marker protein (OMP) (1:1000; WAKO Chemicals, USA), anti-vesicular transporter 2 (VGLUT2) (1:500; Synaptic Systems, Goettingen, Germany), anti-GFP (1:500; Life Technologies), anti-β-galactosidase (β-gal) (1:500; MP Biomedicals). After primary antibody incubation, sections were washed with PBS and primary antibodies were detected with Alexa-

488 or Alexa-546-conjugated secondary antibodies (1:500; Life Technologies). *Erythrina crystagalli* (EC) lectin (1:1000; Vector Laboratories, Burlingame, USA) and *Bandeiraea simplicifolia* (BS) lectin (1:1500; Vector Laboratories, Burlingame, USA) were applied with the secondary antibodies. All sections were counterstained with Hoechst 33342 (Life Technologies) and mounted with Fluoromount G (Southern Biotech). Images were captured on a Zeiss AxioImager epifluorescence microscope.

Whole mount imaging

Mice were perfused transcardially as described above, and following perfusion, brains were dissected out and imaged. Pictures of GFP positive glomeruli were taken from both olfactory bulbs in the brain. Images were captured on a Zeiss SteREO microscope and AxioCam MRc camera (Stifani Lab).

***In situ* hybridization**

Fresh frozen nasal cavities from post natal day (P) 5 mice were cryosectioned (20µm), fixed with 4% PFA in 0.1M PBS pH 7.4 and rinsed with 0.1M PBS pH 7.4. Sections were then subjected to a 10 minute acetylation with 0.25% acetic anhydride in 1% triethanolamine followed by washes with PBS and 2x standard saline citrate (SSC). Afterwards sections were prehybridized with hybridization solution (5x Denhardt's solution, 100mg/mL baker yeast tRNA, 5x SSC and 50% formamide) at 60˚C followed by hybridization of sections with appropriate cRNA probes diluted in hybridization solution at 60˚C overnight. All solutions used up to and including the hybridization step were made using diethylpyrocarbonate (DEPC) treated water. Following hybridization, sections were subjected to a series of stringency washes with 5x SSC, 2x SSC, 50% formamide in 0.2x SSC all at 60˚C and 0.2x SSC at room temperature. Next, sections were washed in a Tris buffered saline solution (100mM Tris and 150mM NaCl, pH 7.5,

Tris buffer), blocked for 1 hour at room temperature in a 1% solution of blocking reagent (Roche) in Tris buffer, and hybridized Digoxigenin (DIG) labelled RNA probes were detected with an anti-DIG Fab fragment antibody conjugated to alkaline phosphatase (anti-DIG-AP; 1:3000; Roche) that was diluted in Tris buffer and applied to sections for 3 hours at room temperature. Washes in Tris buffer and a second Tris buffered saline solution (100mM Tris, 100mM NaCl with 5mM $MgCl_2$, pH 9.5) followed immunological detection. Finally sections were subjected to a colour reaction by incubation with a colour solution made of NBT/BCIP stock solution (Roche) diluted in Tris buffered saline (100mM Tris, 100Mm NaCl and 5mM $MgCl_2$, pH 9.5) overnight at room temperature. The next day sections were rinsed with PBS and mounted with Mowiol. (Protocol modified from (Giger et al., 1996))

Nonradioactive digoxigenin labelled cRNA probes were produced by *in vitro* transcription using DIG labelling mix (Roche). cDNA clones encoding *kirrel-1, kirrel-2* and *kirrel-3* (Prince et al., 2013) were used to synthesize the probes. Dilutions of cRNA probes used: *kirrel-1*; 1:2500, *kirrel-2*; 1:2500, *kirrel-3*; 1:2500.

Electron Microscopy (performed in collaboration with FEMR at McGill University)

Perfusion: Mice were perfused transcardially using Lactated Ringers solution for the vascular rinse followed by 2.5% glutaraldehyde (Electron Microscopy Sciences) in 0.1M Na cacodylate buffer (Electron Microscopy Sciences) for the fixative. The Lactated Ringer's solution and glutaraldehyde fixative were prepared the day before perfusions and kept at 4˚C.

Tissue processing: Olfactory bulbs were dissected from perfused mice and put in the fixative at 4˚C for overnight fixation. The following day, olfactory bulbs were further dissected to remove excess tissue around the accessory olfactory bulbs and put in 0.1M Na cacodylate buffer and 4% sucrose washing buffer for three days at 4˚C. After tissue samples were washed

repeatedly with washing buffer, tissue samples were post fixed with 1% aqueous osmium tetroxide (Mecalab) and 1.5% aqueous potassium ferrocyanide for 2 hours at 4˚C. Samples were dehydrated by washing with acetone (Fisher Scientific) in increasing concentrations starting with 30%, 50%, 70%, 80%, 90%, and 3 times with 100% acetone. Next, tissue samples were infiltrated and embedded with Epon resin (Mecalab), and polymerized at 60˚C for 48 hours.

*Sectionin*g: Ultrathin sections (90-100nm) were cut from the resin blocks with UltraCutE ultramicrotome (Reichert-Jung). Sections were placed on a 200 mesh copper grid (Electron Microscopy Sciences) and then stained with uranyl acetate (Electron Microscopy Sciences) followed by Reynold's lead (Electron Microscopy Sciences).

Electron micrographs were acquired using the FEI Tecnai 12 120 kV transmission electron microscope equipped with an AMT XR80C CCD camera system. Micrographs were taken at 9,300x and 11,000x magnification.

Analysis of glomeruli in the adult accessory olfactory bulb

Analysis of number and size of glomeruli in the AOB were obtained using ImageJ software. Sagittal sections through the adult AOB were collected on microscope slides and stained with VGLUT2 to delineate all glomeruli in the AOB and BS lectin to delineate the anterior/posterior boundary of the glomerular layer of the AOB. Glomeruli are defined as dense neuropil structures that are surrounded by area that is non-innervated neuropil and are sparsely surrounded by periglomerular cells (Prince et al., 2013). We therefore characterized glomeruli as completely VGLUT2-positive structures that were surrounded by area that was not VGLUT-2 positive. All sections through the lateral to medial axis of the AOB from both bulbs were collected from each mouse examined and used for analysis. The number of glomeruli were counted from each region (anterior or posterior) and taken as an average of all glomeruli counted

per section per mouse. For size analysis, glomeruli were hand traced based on the above definition and the area of each glomerulus was calculated using ImageJ software. Area of glomeruli was taken as an average from all glomeruli measured per section per mouse.

Analysis of synapse number and terminal size in the glomerular layer of the adult accessory olfactory bulb

Analysis of the number of synapses and size of presynaptic axon terminals were acquired using ImageJ software. At least 10 micrographs from the glomerular layer of the AOB in both the posterior and anterior portion were taken at a magnification of 9,300x and analyzed from each mouse examined. The number of asymmetric and symmetric synapses were counted on each micrograph and taken as an average of synapses counted per 25μm^2 per mouse. Synapses were identified as structures that had an identifiable presynaptic area and an identifiable postsynaptic target cell, a clustering of synaptic vesicles in the presynaptic terminal near the presynaptic specialization, and a cleft of space between the pre and post synaptic cell. Asymmetric synapses were defined using the following criteria: the thickness of the post synaptic density is significantly greater than the thickness of the presynaptic density. Symmetric synapses were defined using the following criteria: the post synaptic density is equal thickness to the presynaptic density and both synaptic specializations are thin. The asymmetric synapses were characterized as excitatory glutamatergic synapses between the vomeronasal sensory neuron axons and mitral cell dendrites, and the symmetric synapses were characterized as inhibitory synapses in the glomerular layer of the AOB. The size of the presynaptic terminals was analyzed by tracing the outline of each presynaptic terminal and measuring the area using ImageJ software. The presynaptic terminals were established based on the presence of synaptic vesicles clustered near the presynaptic density, and were delineated by tracing the plasma membrane of

the whole synaptic bouton visible on the micrograph. The area of presynaptic terminals was taken as an average of all areas measured per 25μm^2 per mouse.

Statistical Analysis

Microsoft Excel and GraphPad Prism 6 software were used to analyze data. Two-tailed Student's *t*-test assuming equal variances was performed on data from two different conditions. A p-value less than 0.05 was considered statistically significant. Error bars indicate plus and minus the standard error mean (SEM).

Results

Kirrel expression is required during the initial development of AOB glomeruli

We have previously shown that ablating *kirrel-3* expression leads to improper formation of glomeruli in adult mice (Prince et al., 2013). Since *kirrel-3* is expressed during the initial stage of glomeruli formation (postnatal day (P) 0 to P5, Figure 2.2J & M), we proposed that improper axonal coalescence during early formation of the glomeruli underlies these defects. Alternatively, Kirrel-3 could be necessary for VSNs to maintain the proper structure of glomeruli during adulthood. Thus, to determine the time point at which Kirrel expression is crucial for glomeruli formation, the coalescence of a specific population of VSNs was traced at different stages of AOS circuit development. To track one population of VSNs a tau-lacZ reporter gene construct was expressed under the control of the V2R EC2 promoter (Cloutier et al., 2004). Therefore all VSNs that express the V2R, EC2, will also express β-galactosidase in their axons. Sections from *kirrel-3*$^{-/-}$*;EC2-tau-lacZ* mice were stained for anti-β-galactosidase and anti-OMP to visualize axon targeting and coalescence of the EC2 positive VSNs (Figure 2.1). As early as embryonic day 16 (E16), VSNs have reached the AOB, however, the refinement of their positions within the AOB to form glomeruli occurs postnatally and the glomerular layer does not become distinct until roughly P5 (Salazar et al., 2006). We therefore started examining EC2 glomeruli formation at P5 and continued throughout stages of glomerular maturation to the fully mature glomeruli of the adult at P90 (Figure 2.1A-P). EC2 positive axons were diffuse and glomeruli were not well defined as early as P5 in the *kirrel-3*$^{-/-}$*;EC2-tau-lacZ* mice compared to the compact glomerular structures of control *EC2-tau-lacZ* mice (Figure 2.1A-D). The lack of compact glomeruli in *kirrel-3*$^{-/-}$ mice persisted throughout all stages of glomeruli development (Figure 2.1E-P). Furthermore, at P90, glomeruli were not strictly β-gal positive (Figure 2.1P,

arrowhead) suggesting glomeruli are heterogeneous structures innervated by multiple populations of VSNs in these mice; whereas in controls, glomeruli are strictly homogeneous (Figure 2.1N, arrowhead). Altogether, these results demonstrate that Kirrel expression is required during the early stages of AOS circuit formation when glomeruli are initially being formed, and may contribute to glomeruli maintenance throughout the lifetime of the animal.

Characterization of *kirrel-2*$^{-/-}$ mice

Previous studies demonstrated that Kirrel-2 is expressed in both the main olfactory system (Serizawa et al., 2006) and accessory olfactory system (Prince et al., 2013). However, whether Kirrel-2 has a function in the olfactory systems remains to be determined. To investigate the role of Kirrel-2 in the MOS and AOS mice carrying a *kirrel-2* gene allele where exon 3 and exon 4 can be removed from the coding region was used (Figure 2.2A). These mice were crossed to mice expressing Cre recombinase under a CMV promoter to promote allele excision in germline cells, thereby ablating *kirrel-2* expression throughout the resulting offspring. As described previously, Kirrel-2 is expressed in the glomeruli of the AOB (Prince et al., 2013) and MOB (Serizawa et al., 2006) suggesting Kirrel-2 protein is expressed in the VSN axons and OSN axons that are targeting the AOB and MOB, respectively. Co-immunolabelling of AOB sections from adult wildtype animals with antibodies against Kirrel-2 and the excitatory presynaptic marker VGLUT2 validated these previous findings and confirmed Kirrel-2 expression in VSN axons targeting glomeruli in the AOB (Figure 2.2B & C). While Kirrel-2 is widely expressed in glomeruli of the anterior AOB, it is also detected in a large proportion of glomeruli in the posterior AOB, demonstrating that subsets of both V1R and V2R-expressing VSN axons are Kirrel-2 positive (Figure 2.2B & C). Additionally, Kirrel-2 staining in OSN axon tracts and glomeruli in the MOB of adult wildtype mice confirms Kirrel-2 expression in subsets

of OSN axons targeting the MOB (Figure 2.2F) (Serizawa et al., 2006). In both the AOB and MOB of *kirrel-2*$^{-/-}$ mice, Kirrel-2 protein expression is completely abolished (Figure 2.2D, E & G), confirming the null nature of this *kirrel-2* allele.

Furthermore, *in situ* hybridization verified the absence of *kirrel-2* mRNA expression in VSNs and OSNs (Figure 2.2I, L, N & O). Coronal sections through the vomeronasal organ (VNO) taken from P5 wildtype control littermates and *kirrel-2*$^{-/-}$ mice were probed for *kirrel-1*, *kirrel-2* and *kirrel-3* mRNA expression (Figure 2.2H-M). *Kirrel-1* expression, which is not detected in the vomeronasal organ of wildtype controls, was unchanged in the *kirrel-2*$^{-/-}$ animals (Figure 2.2H & K). However, *kirrel-2* mRNA expression was completely ablated in *kirrel-2*$^{-/-}$ mice while it was expressed in the cell bodies of VSNs in the VNO of controls (Figure 2.2I & L). The pattern of expression of *kirrel-3* was not altered in *kirrel-2*$^{-/-}$ mice (Figure 2.2M). Likewise coronal sections through the MOE from P5 control and *kirrel-2*$^{-/-}$ mice were probed for *kirrel-2* mRNA expression (Figure 2.2N & O). In wildtype mice, *kirrel-2* mRNA is expressed in the OSN layer of the MOE (Figure 2.2N), while it is absent in *kirrel-2*$^{-/-}$ mice confirming the loss of *kirrel-2* mRNA from OSNs in knockout animals (Figure 2.2O). Taken together, both Kirrel-2 protein and mRNA expression is completely removed from the AOS and MOS in *kirrel-2*$^{-/-}$ mice.

Kirrel-2 ablation disrupts formation of glomeruli in the AOB

Since Kirrel-2 is expressed in the axons of VSNs targeting the AOB, it is proposed Kirrel-2 may be required for the proper targeting of axons and formation of glomeruli in the AOB. Moreover, differential expression of Kirrel-2 and Kirrel-3 in VSNs is thought to create a molecular code for axonal recognition, which is important for the proper coalescence of VSNs into glomeruli in the AOB (Prince et al., 2013). To assess Kirrel-2's function in the AOS, we investigated whether Kirrel-2 is required for the proper formation of glomeruli in the AOB. In

order to address this question, sagittal sections through the AOB of wildtype and *kirrel-2*$^{-/-}$ mice were collected and immunolabelled with a VGLUT2 antibody to mark all excitatory presynaptic terminals in the AOB, and therefore, delineate all the glomeruli within the bulb. VGLUT2 colocalizes with the expression of Kirrel-2 within the AOB demonstrating VGLUT2 is marking Kirrel-2 positive glomeruli (Figure 2.2C). The anterior posterior boundary of the AOB is determined by co-staining with BS lectin that labels VSN axons targeting the posterior region (Figure 2.3A & B). Interestingly, glomeruli in the posterior region of the AOB of *kirrel-2*$^{-/-}$ mice appeared larger while the size of glomeruli in the anterior region appeared unchanged compared to controls (Figure 2.3C-F). To examine this change in morphology more closely, we measured the surface area of the glomeruli and counted the total number of glomeruli present in these two regions of the AOB (Figure 2.2G-J). A significant increase in the area of posterior glomeruli was detected (Figure 2.3F & J) which is associated with a decrease in the number of glomeruli within this same region in *kirrel-2*$^{-/-}$ mice (Figure 2.3F & H). These results demonstrate that Kirrel-2 expression is required for the proper formation of glomeruli in the AOB.

To test whether the malformation of glomeruli observed in *kirrel-2*$^{-/-}$ mice results from a loss of Kirrel-2 function in VSN axons, as opposed to being secondary defects in the development of other cell types or structures of the AOB, we ablated *kirrel-2* expression specifically in VSNs and OSNs. Using the *Cre*-lox system, mice that express *Cre recombinase* under the control of the OMP promoter were crossed to mice that have a floxed *kirrel-2* allele resulting in excision at loxP sites flanking exons 3 and 4 of the coding region of the *kirrel-2* allele in OMP positive cells (Figure 2.4A). OMP is a specific marker for the primary sensory neurons of the main and accessory olfactory systems (OSNs and VSNs, respectively). Morphology of glomeruli was examined in *kirrel-2*$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice compared to controls

to see if conditional ablation of *kirrel-2* from VSNs phenocopies what is observed in the *kirrel-2*$^{-/-}$ mice. VGLUT2 and BS lectin staining in the AOB from *kirrel-2*$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice demonstrates that glomeruli in the posterior region of the AOB are misshapen compared to controls (Figure 2.4B-G). Glomeruli were defined as dense neuropil structures completely labelled with VGLUT2 and were traced by following the VGLUT2 positive structures compared to the non-VGLUT2 area in the AOB (Figure 2.4D & E). The number of posterior glomeruli is significantly decreased in *kirrel-2*$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice compared to controls (Figure 2.4F, G & J) and the area of individual posterior glomeruli is significantly increased (Figure 2.4F, G & K). These results recapitulate what is observed in the *kirrel-2*$^{-/-}$ mice confirming the requirement of Kirrel-2 expression in the VSNs for the proper formation of glomeruli in the AOB.

The segregation of apical and basal VSN axons in the AOB is not affected in *kirrel-2*$^{-/-}$ and *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice

During development of the vomeronasal circuitry, many processes occur to allow for the proper targeting of the VSN axons from the VNO to the AOB (Figure 1.2A). Prior to the fine tune positioning of axons within the AOB to form their respective glomeruli, VSN axons must first reach the AOB from the VNO and segregate to either the anterior or posterior region of the AOB. VSNs that express vomeronasal receptors (VRs) that signal through the G protein, Gαi, and have their cell bodies in the apical portion of the vomeronasal epithelium (VNE) target the anterior region of the AOB; whereas basally located VSNs in the VNE project their axons to the posterior region of the AOB and express VRs that signal through Gαo (Dulac and Axel, 1995, Halpern et al., 1995, Berghard and Buck, 1996, Jia and Halpern, 1996, Ryba and Tirindelli, 1997). To investigate if Kirrel-2 is required for early targeting events of VSN axons to the AOB, parasagittal sections of the AOB from *kirrel-2*$^{-/-}$ mice were examined for anterior-posterior

markers. Immunostaining for $G_{\alpha i2}$ to label all apical VSNs targeting the anterior region of the AOB revealed that there is no change in the targeting or segregation of these neurons with the loss of *kirrel-2* expression (Figure 2.5A & C). Similarly no change was observed when basal VSNs positive for $G_{\alpha o}$ which target to the posterior AOB were labelled (Figure 2.5B & D). Strikingly, loss of both Kirrel-2 and Kirrel-3 also had no effect on the segregation of apical and basal VSNs in the AOB (Figure 2.5E-H). Taken together, these results show that Kirrel expression although imperative for glomerular formation is not required for the general targeting and segregation of apical and basal VSN axons to the AOB.

Kirrel-2$^{-/-}$ mice display normal fasciculation of the vomeronasal nerve

The fasciculation of VSN axons to form vomeronasal nerve bundles during their projection to the AOB is a key step during AOS circuit formation. Because interactions between CAMs are known to promote the fasciculation of axons in other systems, such as retinal ganglion cell axons of the optic nerve in the visual system (Missaire and Hindges, 2015), it is possible that the interaction of Kirrel molecules on VSN axons may be required for fasciculation of the vomeronasal nerve. The vomeronasal nerve projects along the medial side of the main olfactory bulb to reach the AOB, and therefore, coronal sections through the olfactory bulbs will provide cross sections of the vomeronasal nerve. Moreover, *Erythrina cristagalli* lectin is a known marker for labelling the vomeronasal nerve (Cloutier et al., 2002), and therefore, can be applied to tissue sections to visualize the fasciculation of the vomeronasal nerve bundles. No mistargeting or straying axons were observed outside of the vomeronasal nerve of *kirrel-2*$^{-/-}$ mice compared to controls (Figure 2.5I & J). Vomeronasal nerve bundles were compact although multiple segregated bundles of the nerve do occur in both *kirrel-2*$^{-/-}$ mice and in controls (Figure

2.5I & J, arrowheads). Thus, Kirrel-2 expression in VSNs is dispensable for the fasciculation of VSN axons to form the compact nerve bundles of the vomeronasal nerve which target the AOB.

Loss of distinct glomeruli in the AOB of *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice

Our previous studies, which identified Kirrel-3 as an important regulator of axon coalescence in the AOB, proposed that the differential expression of Kirrels on VSNs is necessary to create a molecular code for axonal recognition during glomeruli formation (Prince et al., 2013). We have shown that ablating either *kirrel-2* (Figure 2.3) or *kirrel-3* (Prince et al., 2013) affects the formation of glomeruli in the AOB, possibly due to a decrease in the molecular diversity of VSN axons that coalesce together. If expression of both Kirrels is required to create a molecular code of axonal recognition for targeting VSNs to the AOB, ablation of both proteins from VSNs should result in a greater deleterious effect on glomerular formation than the loss of each individual Kirrel protein. *Kirrel-2*$^{-/-}$ and *kirrel-3*$^{-/-}$ mice were crossed to generate *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice. Examination of sagittal sections of the AOB by immunohistochemistry with a VGLUT2 antibody revealed that the glomerular layer of the AOB is severely disrupted in *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice when compared with control mice (Figure 2.6A-F). Contrary to what we observed in *kirrel-2*$^{-/-}$ or *kirrel-3*$^{-/-}$ mice, the boundaries between glomeruli are indistinct in both the anterior region and posterior region of the AOB in *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice which makes it impossible to quantify the size or number of glomeruli in these animals (Figure 2.6E & F). These results demonstrate that Kirrel function in the AOS is additive since loss of both Kirrels has a greater effect on glomeruli formation than the loss of either single protein. Additionally, the results also support the above mentioned model and suggest there is a severe decrease in the molecular diversity between VSN axons with the loss of both proteins leading to imprecise axon sorting.

Synapse formation is altered in *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice

While the general targeting of VSN axons is unaffected in *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice, the gross morphology of glomeruli is severely affected in the AOB of these mice. Since glomeruli represent neuropil structures where VSN axons synapse onto mitral cell dendrites, we examined whether synaptic structures are affected in the AOB of *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice. Electron micrographs taken of the glomerular layer of the AOB (where the dense neuropil of synapses between VSN axons and mitral cells reside) from *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ and control mice were analyzed for the number of asymmetric and symmetric synapses (Figure 2.7A-D). The micrographs were taken from both the anterior and posterior region of the AOB. EM analysis was only performed in *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice and not *kirrel-2*$^{-/-}$ or *kirrel-3*$^{-/-}$ mice due to the greater deleterious effect in glomeruli formation observed in the AOB of *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice. Asymmetric synapses denote the excitatory glutamatergic synapses of VSN axons onto mitral cell dendrites since this is the only excitatory synapse that occurs within the glomerular layer of the AOB (Figure 2.7A & C). Whereas the symmetric synapses examined represent the inhibitory input of periglomerular cells within the glomerular layer of the AOB (Figure 2.7B & D). Interestingly, in glomeruli from *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice there was a significant decrease in the number of asymmetric synapses compared to controls (Figure 2.7E). Furthermore, the presynaptic terminal of asymmetric synapses formed in the AOB of *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice showed an increased average area although not significant (Figure 2.7I). In contrast the number of symmetric synapses was not different between controls and *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice (Figure 2.7F) suggesting the inhibitory synapses that formed were stable. The length of contact between pre and post synaptic cells at the synapse (defined as synapse length) was unchanged between controls and *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice when looking at both asymmetric and symmetric synapses

(Figure 2.7G & H). Strikingly, the area of symmetric presynaptic terminals was significantly larger in the *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice compared to controls (Figure 2.7J & K). These changes in the number and morphology of asymmetric and symmetric synapses demonstrate that ablation of Kirrels affect the formation or maintenance of synapses in the AOB.

Axon targeting in the MOS in *kirrel-2*$^{-/-}$ mice

In addition to being expressed in VSNs, Kirrel-2 is also expressed in OSNs of the main olfactory system and previous reports using gain of function approaches suggest Kirrel-2 also mediates axon convergence of OSNs to target their respected glomeruli (Serizawa et al., 2006). The *kirrel-2*$^{-/-}$ mice therefore could be used to validate these previous findings and address if Kirrel-2 is required for axon targeting events of OSNs using loss of function approaches. To observe if Kirrel-2 plays a role in the targeting of OSNs, we first wanted to identify a population of OSN axons that express high levels of Kirrel-2 in order to track their targeting. The expression of Kirrel-2 in glomeruli innervated by specific populations of OSNs expressing either a tau-GFP or tau-lacZ gene was assessed by double immunohistochemistry. Sections taken from the MOB of *MOR174-9-IRES-GFP*, *MOR1-3-tau-lacZ*, *M72-tau-lacZ*, *S50-tau-lacZ* and *Sp1-tau-GFP* mice were stained with Kirrel-2 and GFP/β-galactosidase antibodies to examine the levels of Kirrel-2 expression in glomeruli innervated by these OSN axon populations (Figure 2.8A-J). Three different populations expressed Kirrel-2 out of the five different OR OSN populations examined. These include the OSNs which express the MOR1-3 OR, the MOR174 OR, and the S50 OR (Figure 2.8B, C, E, G, H, & J). Due to the high intensity staining of Kirrel-2 in the MOR174-GFP population, this reporter mouse line was crossed to the *kirrel-2*$^{-/-}$ mice to address the requirement of Kirrel-2 for the targeting of this OSN population. The two methods used to examine the targeting of the MOR174 positive OSNs to their glomeruli included wholemount

imaging of GFP positive axons in the olfactory bulbs (Figure 2.8K-M) and immunolabelling sections of the olfactory bulbs from *kirrel-2*$^{-/-}$*;MOR174-GFP* mice (Figure 2.8N-P). No gross defects were observed in the formation of glomeruli in these mice (Figure 2.8K-M). The position and size of MOR174 glomeruli in *kirrel-2*$^{-/-}$*;MOR174-GFP* and control mice were similar (Figure 2.8K & M). Furthermore, coronal sections taken from the MOB of *kirrel-2*$^{-/-}$*;MOR174-GFP* mice revealed that the GFP positive axons all targeted to the same glomeruli and the MOR174 glomeruli were positioned in the expected stereotyped locations as observed in control mice (Figure 2.8N-P). These results suggest Kirrel-2 may not be required for the targeting of OSN axons to their glomeruli. However, results are preliminary and further experiments are needed to elucidate the role of Kirrel-2 in the MOS.

Results Figures

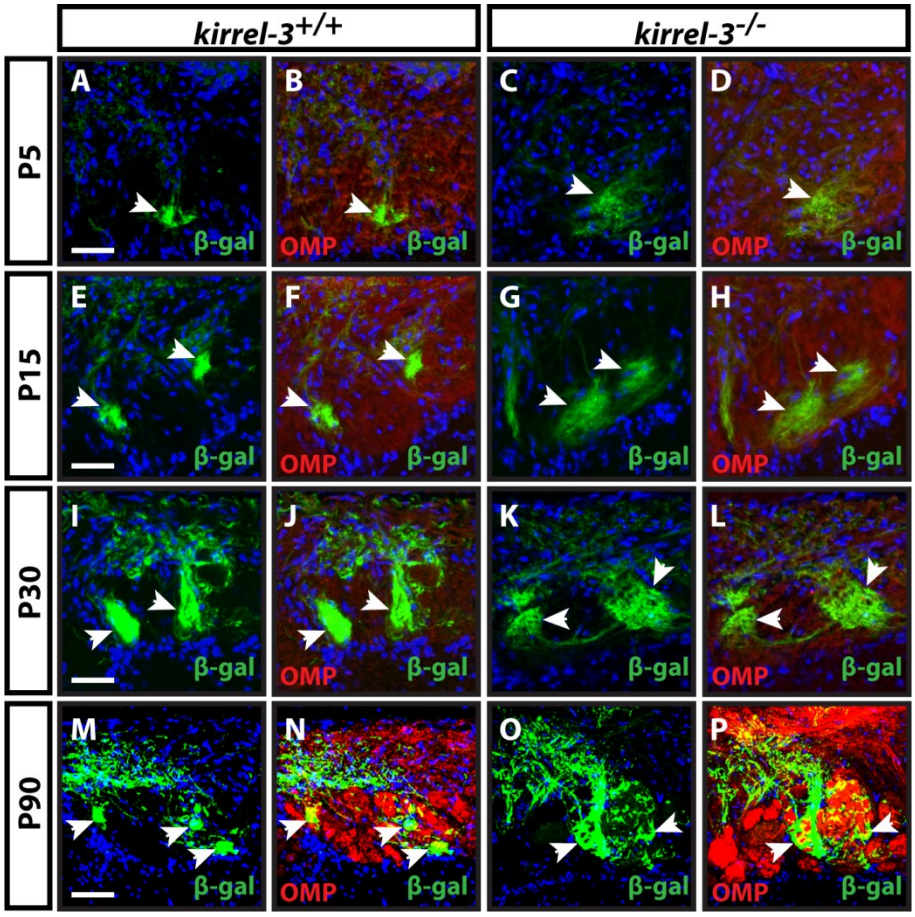

Figure 2.1. Kirrel-3 mediates the coalescence of VSN axons at different stages of development (modified from Prince, Brignall, et al., 2013).

A-P) Parasagittal sections of the AOB from *kirrel-3*$^{+/+}$ (A, B, E, F, I, J, M, N) and *kirrel-3*$^{-/-}$ (C, D, G, H, K, L, O, P) mice at different stages in development, including post natal day 5 (P5) (A-D), P15 (E-H), P30 (I-L) and P90 (M-P). Sections were stained with antibodies against β-galactosidase and Hoechst (A-P) and OMP (B, D, F, H, J, L, N, P). EC2+ axons are labelled with β-galactosidase and the β-gal staining is more diffuse and the EC2+ glomeruli are not well defined in *kirrel-3*$^{-/-}$ mice compared to controls (white arrowheads). The dispersed glomeruli are detected as early as P5 and continue into adulthood in the *kirrel-3*$^{-/-}$ mice whereas EC2+ glomeruli always appear as compact structures in control animals. At P90, glomeruli are not strictly β-gal positive suggesting heterogeneous glomeruli in the *kirrel-3*$^{-/-}$ mice, arrowhead (P). N=3 mice for each condition and for all the different developmental stages. Scale bars: 25μm in A-L, 50μm in M-P. Link to published paper: http://dev.biologists.org/content/140/11/2398.full

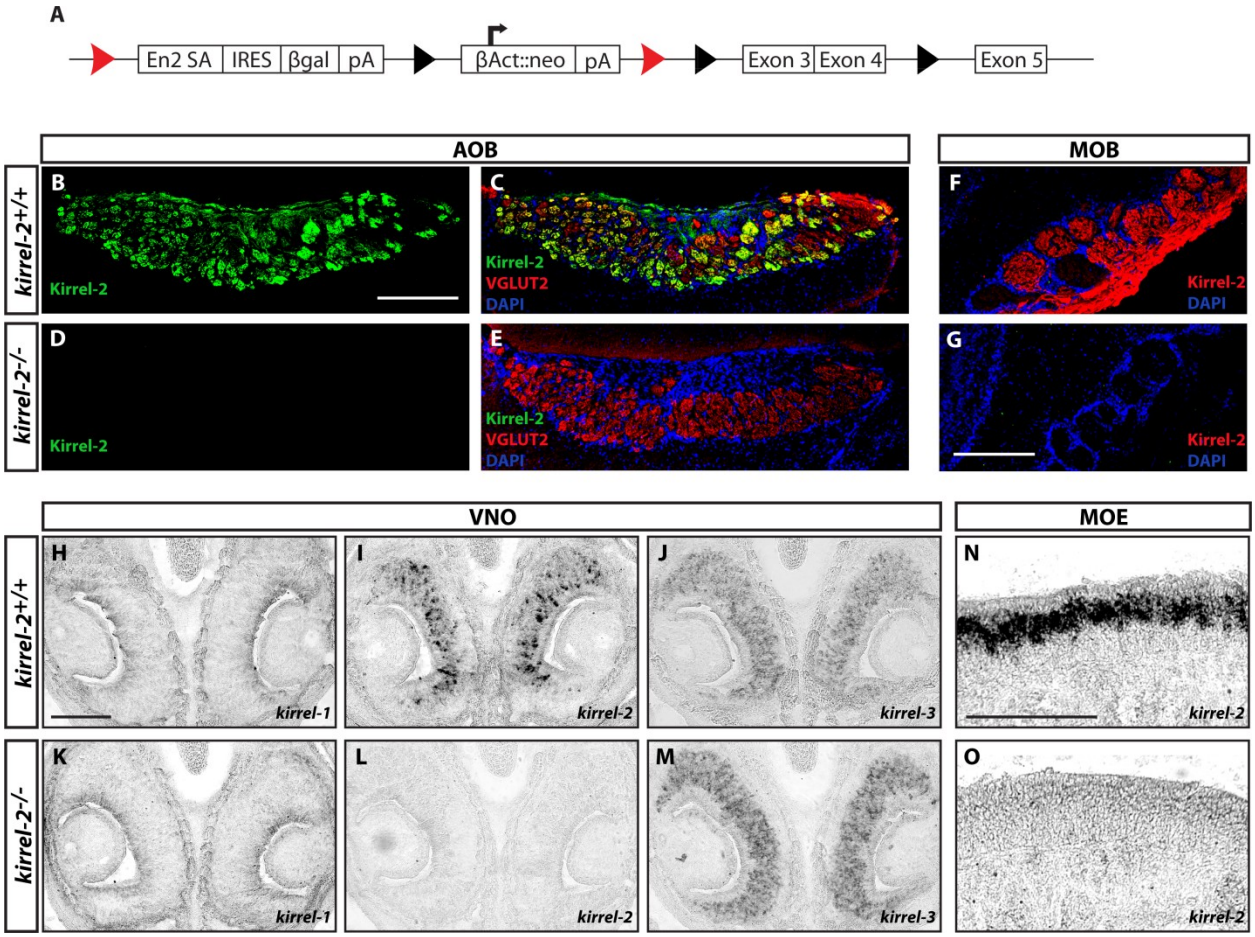

Figure 2.2. *Kirrel-2*$^{-/-}$ mouse characterization.

A) Knock out strategy of the *kirrel-2* allele. Mice that express the modified *kirrel-2* allele are crossed with mice that express *Cre* recombinase under the control of the CMV promoter resulting in excision of the coding region exons 3 and 4 and removal of the β-Actin neomycin resistant (βAct::neo) selection cassette on the *kirrel-2* allele. Red arrows: flippase recognition target (FRT) sites; black arrows: loxP sites.

B-E) Kirrel-2 expression in the AOB is absent in *kirrel-2*$^{-/-}$ mice. Immunohistochemistry on sagittal sections of the AOB taken from adult mice labelled with an antibody against Kirrel-2 in control mice (B). Kirrel-2 is expressed in the VSN axons resulting in labelling of the glomeruli in the AOB and colocalization with excitatory presynaptic marker VGLUT2 in control mice (C). Kirrel-2 is expressed in glomeruli in both the anterior and posterior region of the AOB (B,C). No protein detection of Kirrel-2 in the AOB of *kirrel-2*$^{-/-}$ mice (D,E).

F,G) Kirrel-2 expression is ablated in the MOS of *kirrel-2*$^{-/-}$ mice. A coronal section of the MOB taken from an adult mouse stained with an antibody against Kirrel-2 shows Kirrel-2 is expressed in OSN axons targeting the glomeruli and labels glomeruli in control mice (F). Kirrel-2 expression in the MOB is abolished in *kirrel-2*$^{-/-}$ mice (G).

H-M) *In situ* hybridization on coronal sections of the VNO from P5 mice to detect mRNA expression of *kirrel-1* (H,K), *kirrel-2* (I,L) and *kirrel-3* (J,M) in control and *kirrel-2*$^{-/-}$ mice.

Kirrel-1 is not expressed in VSNs (H,K). Kirrel-2 labels cell bodies of the VSNs in the VNO of control mice (I) but expression is lost in *kirrel-2*$^{-/-}$ mice (L). Kirrel-3 is expressed in VSNs in both wildtype (J) and *kirrel-2*$^{-/-}$ mice (M).

N,O) Coronal sections of the MOE from P5 wildtype and *kirrel-2*$^{-/-}$ mice probed for *kirrel-2* mRNA expression. *Kirrel-2* mRNA labels the OSN layer of the MOE in control mice (N); however, expression is completely ablated in the MOE from *kirrel-2*$^{-/-}$ mice (O). All scale bars are 200μm.

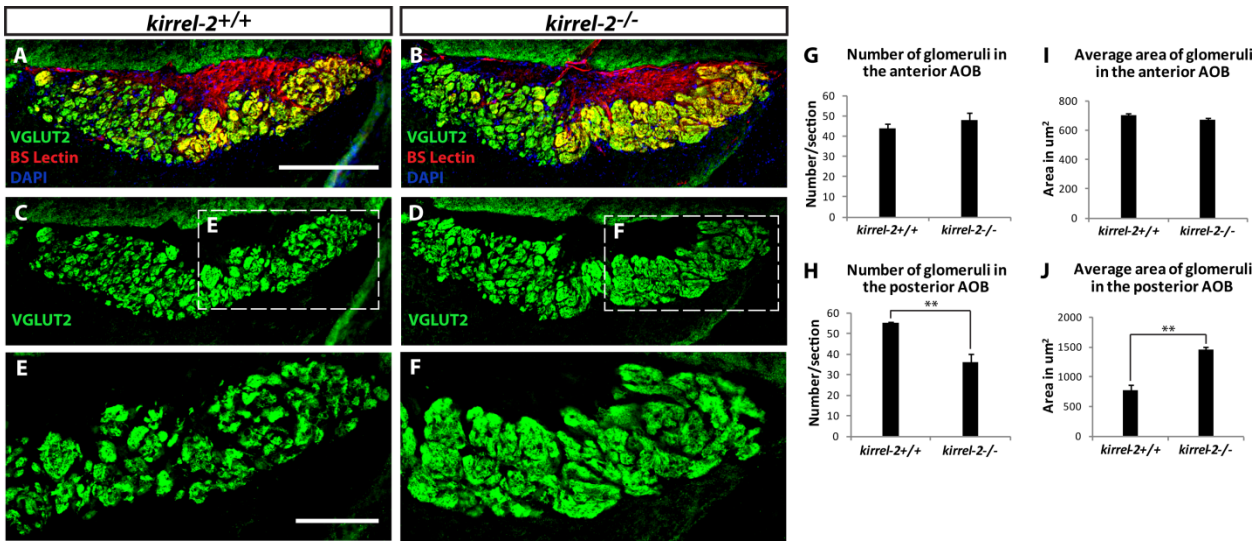

Figure 2.3. Loss of *kirrel-2* expression disrupts formation of glomeruli in the AOB.

A-F) Parasagittal sections of the AOB taken from adult mice. Sections were immunolabelled with antibodies against VGLUT2, BS lectin and Hoechst (A,B) and VGLUT2 only (C-F). Boxes in C and D are magnified in E and F, respectively. Glomeruli in the posterior portion of the AOB of *kirrel-2*$^{-/-}$ mice are significantly larger and less in number when compared to wildtype controls (E,F).

G-J) Quantification of the number and area of glomeruli in adult *kirrel-2*$^{+/+}$ and *kirrel-2*$^{-/-}$ mice. The number of glomeruli in the anterior (G) and posterior region (H) of the AOB in control and *kirrel-2*$^{-/-}$ mice. There is a significant decrease in the number of posterior glomeruli in *kirrel-2*$^{-/-}$ mice compared to *kirrel-2*$^{+/+}$ mice (H) but the number of glomeruli in the anterior region is similar (G). Quantification of the average glomerular area in the anterior (I) and posterior (J) region of the AOB from control and *kirrel-2*$^{-/-}$ mice shows a significant increase in the average area of glomeruli in the posterior region compared to control animals (J). No difference was detected in the average area of glomeruli in the anterior region between control and *kirrel-2*$^{-/-}$ animals (I). Data were analyzed using a two-tailed Student's *t*-test, n=3 mice for each condition, **p-value < 0.008, errors bars: ± SEM. Scale bars: 250μm in A and 100μm in E.

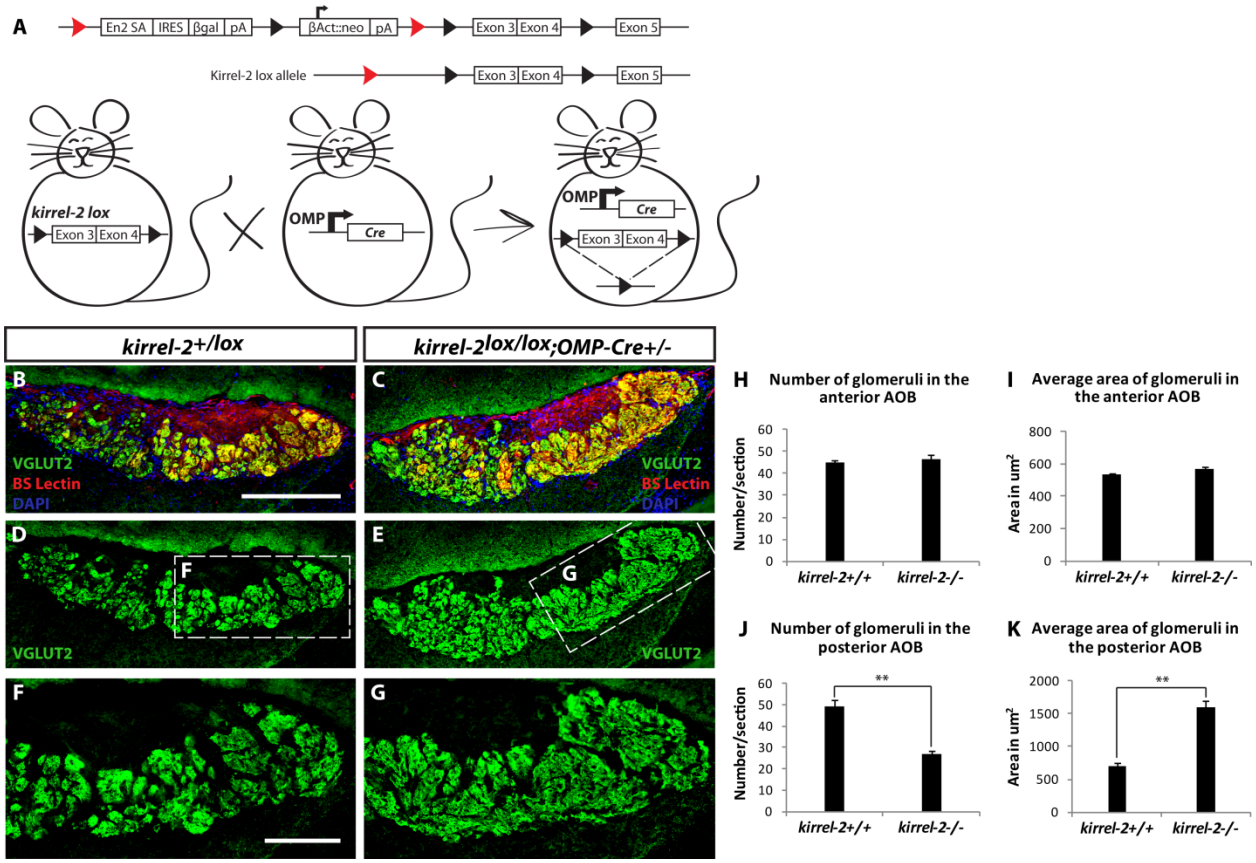

Figure 2.4. Kirrel-2 function is required in VSN axons for the formation of glomeruli.

A) Schematic of the strategy used to generate the *kirrel-2* conditional knockout allele. A *kirrel-2* lox allele is generated by crossing mice with the modified *kirrel-2* wildtype allele (A) with mice that express flippase to excise at the FRT sites (red arrows). The resulting allele is the *kirrel-2* lox allele which has two loxP sites flanking the coding region exons 3 and 4. Mice that express the *kirrel-2* lox allele are crossed with mice that express *Cre recombinase* under the control of the OMP promoter (*OMP-Cre* mice (Eggan et al., 2004)) for specific ablation of *kirrel-2* from OMP positive cells. OMP is a specific marker for the primary sensory neurons of the main and accessory olfactory systems (OSNs and VSNs).

B-G) Parasagittal sections of adult AOB from *kirrel-2*$^{+/lox}$ and *kirrel-2*$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice. Sections are stained with anti-VGLUT2, BS lectin and Hoechst (B,C) and anti-VGLUT2 only (D-G). Boxes in D and E of the posterior region of the AOB are magnified in F and G, respectively. Glomeruli in the posterior region of the AOB taken from *kirrel-2*$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice are significantly larger and contain fewer glomeruli compared to control animals (F,G).

H-K) Analysis of the number of glomeruli (H,J) and area of glomeruli (I,K) in the AOB from control and *kirrel-2*$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice (*kirrel-2*$^{-/-}$ on graphs). Quantifications of the number of glomeruli in the anterior (H) and posterior (J) regions of the AOB show a significant decrease in the posterior region in *kirrel-2*$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice compared to controls (J) but the number of glomeruli is similar in both conditions in the anterior region of the AOB (H). The average area of glomeruli taken from the anterior AOB is similar between wildtype controls and *kirrel-*

2$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice (I). However, there is a significant increase in the average area of glomeruli in the posterior region of the AOB from *kirrel-2*$^{lox/lox}$*;OMP-Cre*$^{+/-}$ mice compared to controls (K). Data were analyzed using a two-tailed Student's *t*-test, n=3 mice for each condition, **p-value < 0.003, errors bars: ± SEM. Scale bars: 250μm in B and 100μm in F.

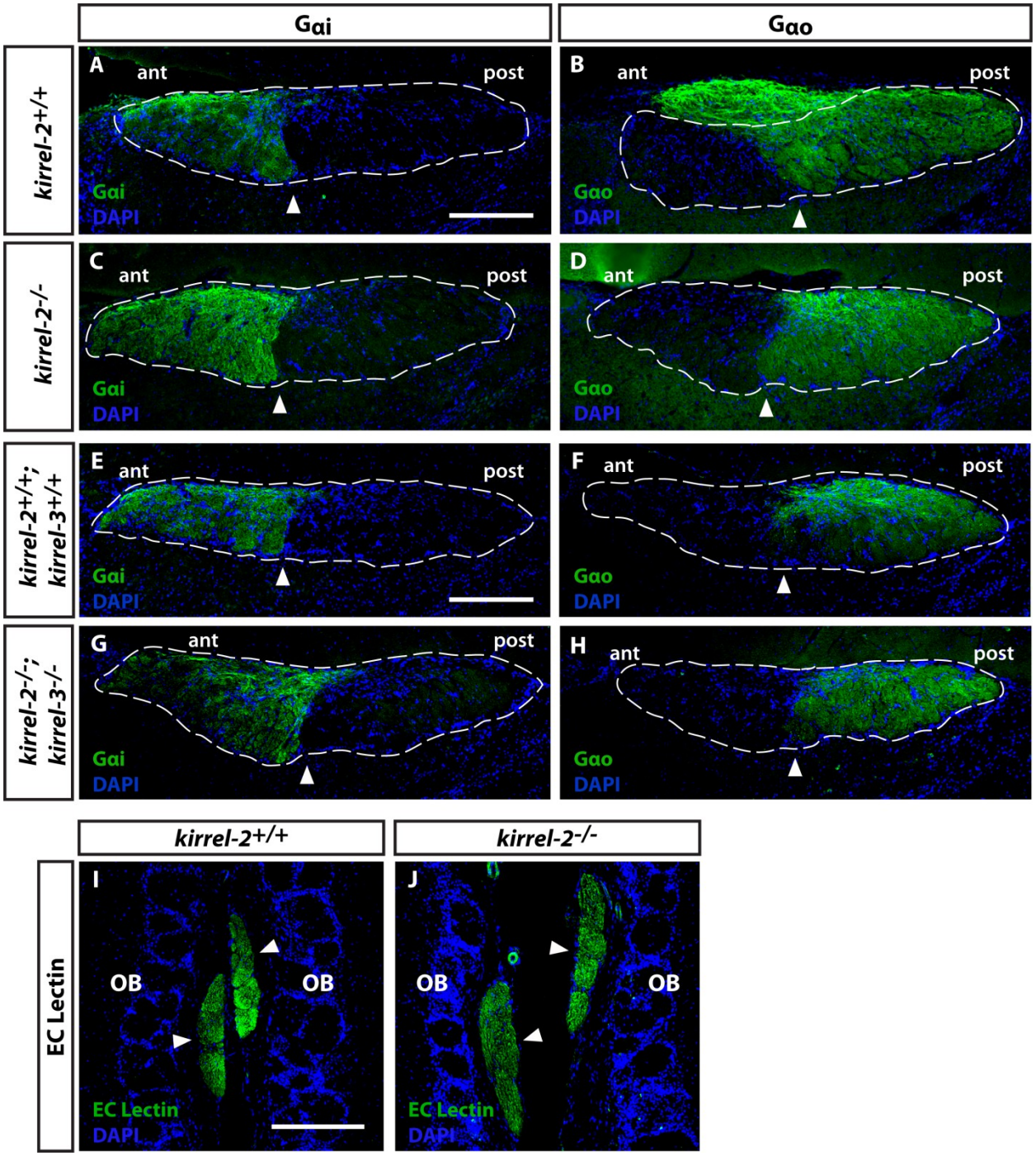

Figure 2.5. Kirrels are not required for the segregation of apical and basal VSNs in the AOB or for the fasciculation of the vomeronasal nerve.

A-H) Parasagittal sections of the AOB from adult *kirrel-2*$^{+/+}$ (A,B), *kirrel-2*$^{-/-}$ (C,D), *kirrel-2*$^{+/+}$*;kirrel-3*$^{+/+}$ (E,F) and *kirrel-3*$^{-/-}$*;kirrel-2*$^{-/-}$ (G,H) mice. Sections are immunolabelled with an antibody for an apical VSN axon marker $G_{\alpha i2}$ (A, C, E, G) and basal VSN axon marker, $G_{\alpha o}$ (B, D, F, H). All sections are labelled with Hoechst (A-H). There are no defects in the targeting or segregation of apical or basal VSN axons to the AOB in *kirrel-2*$^{-/-}$ or *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice

compared to controls. Apical VSN axons target the anterior region of the AOB in both wildtype controls (A,E) and *kirrel-2*$^{-/-}$ and *kirrel-3*$^{-/-}$*;kirrel-2*$^{-/-}$ mice (C,G). Basal VSN axons segregate to the posterior region of the AOB in both wildtype (B,F) and *kirrel-2*$^{-/-}$ and *kirrel-3*$^{-/-}$*;kirrel-2*$^{-/-}$ mice (D,H). Arrowheads denote the boundary between anterior and posterior regions in the AOB. The AOB is outlined in each picture by a white dotted line. N=3 mice for each condition. Scale bars: 200μm in both A and E. Ant: anterior; post: posterior.

I,J) Coronal sections through the olfactory bulbs from adult *kirrel-2*$^{+/+}$ and *kirrel-2*$^{-/-}$ mice. The vomeronasal nerve projects from the VNO to the AOB passing on the medial surface of the main olfactory bulb. Sections are stained with EC lectin to label all nerve fibres of the vomeronasal nerve and Hoechst (I,J). No stray VSN axons are detected coming away from the nerve bundles in *kirrel-2*$^{-/-}$ mice as shown in control mice. Fasciculation of the nerve bundles is similar in *kirrel-2*$^{-/-}$ mice and wildtype controls. Arrowheads point to the vomeronasal nerve bundles. OB: main olfactory bulb. N=3 mice for each condition. Scale bar: 200μm.

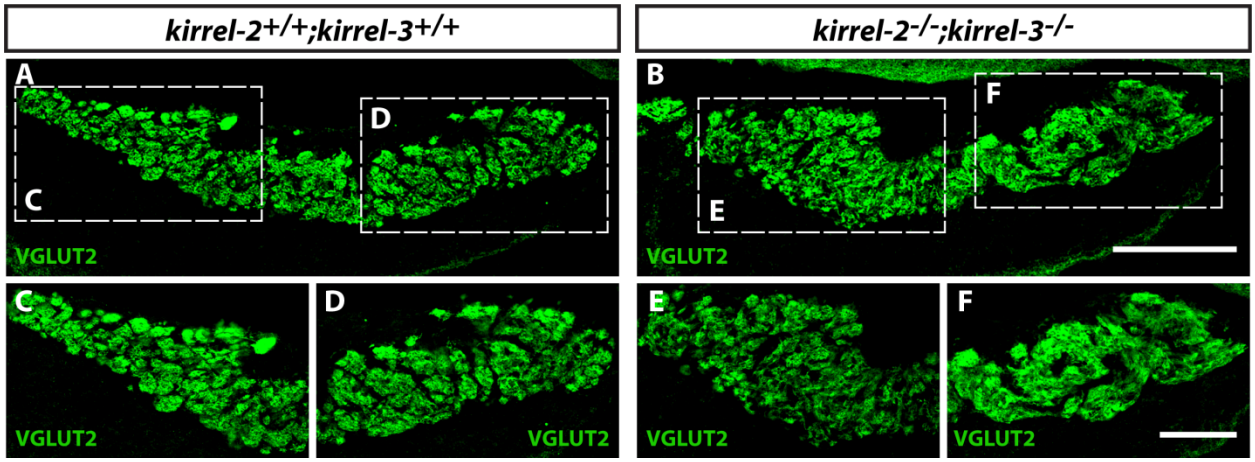

Figure 2.6. Loss of distinct glomeruli in *kirrel-2$^{-/-}$;kirrel-3$^{-/-}$* mice.

A-F) Parasagittal sections of the AOB from adult *kirrel-2$^{+/+}$;kirrel-3$^{+/+}$* (A, C, D) and *kirrel-2$^{-/-}$;kirrel-3$^{-/-}$* (B, E, F) mice. All sections are stained with anti-VGLUT2 (A-F). Glomeruli in both the anterior (C,E) and posterior (D,F) are severely disrupted and difficult to distinguish in the AOB from *kirrel-2$^{-/-}$;kirrel-3$^{-/-}$* mice (E,F) compared to wildtype controls (C,D). Boxes in A and B are magnified in C, D, E and F. Boundaries between glomeruli are blurred and there is a loss of distinct glomeruli in *kirrel-2$^{-/-}$;kirrel-3$^{-/-}$* mice (E,F). Scale bars: 250μm in B and 100μm in F. N=4 mice for controls and n=5 mice for the *kirrel-2$^{-/-}$;kirrel-3$^{-/-}$* condition.

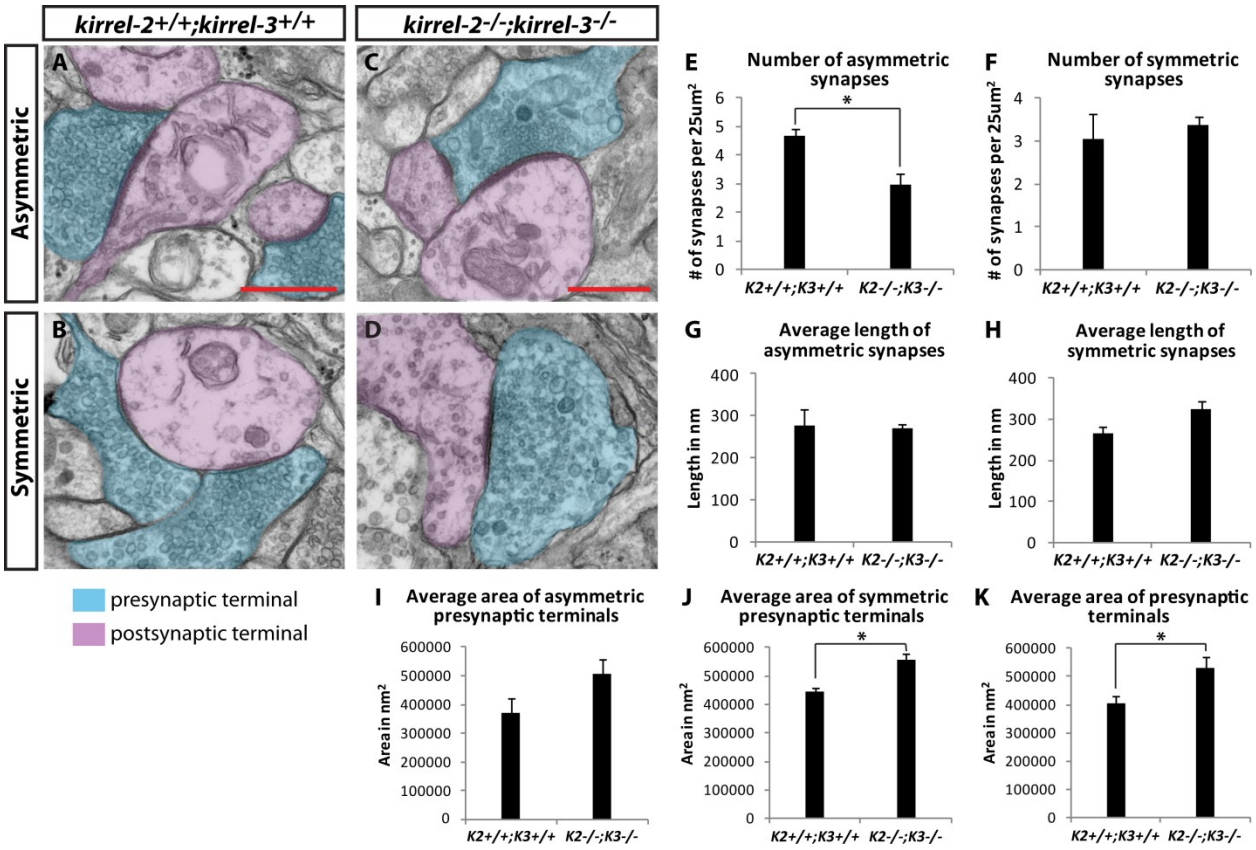

Figure 2.7. Synapse number and size are disrupted with the loss of *kirrel* expression.

A-D) Electron micrographs from the glomerular layer of the AOB from *kirrel-2*$^{+/+}$*;kirrel-3*$^{+/+}$ (A,B) and *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ (C,D) adult mice. Both asymmetric (A,C) and symmetric (B,D) synapses were analyzed. Asymmetric synapses were classified as the excitatory glutamatergic synapses of the VSNs onto mitral cells within glomeruli while the symmetric synapses were classified as inhibitory synapses in the glomeruli of the AOB (see Materials & Methods for criteria). Presynaptic terminals are highlighted in blue and postsynaptic terminals are highlighted in purple. Scale bars: 500nm.

E-K) Quantification of the number of synapses (E,F), length of synapses (G,H) and presynaptic area of terminals (I-K) in the glomerular layer of the AOB. The number of asymmetric synapses is significantly reduced in *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice compared to controls while the number of symmetric synapses is unchanged (F). There is no change between wildtype controls and *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice in the average length of asymmetric (G) or symmetric (H) synapses. Complementarily to the decrease in the number of synapses, there is a significant increase in the area of presynaptic terminals in *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice compared to controls (J,K). Axon presynaptic terminal size at symmetric synapses is significantly higher in *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice compared to the control mice (J) and when asymmetric and symmetric synapses are pooled together, there is a significant increase in the area of presynaptic terminals (K). However, the average area of asymmetric presynaptic terminals is not significantly higher than controls (I). Data were analyzed using a two-tailed Student's *t*-test, n=3 mice for each condition, *p-value < 0.05, error bars: ± SEM.

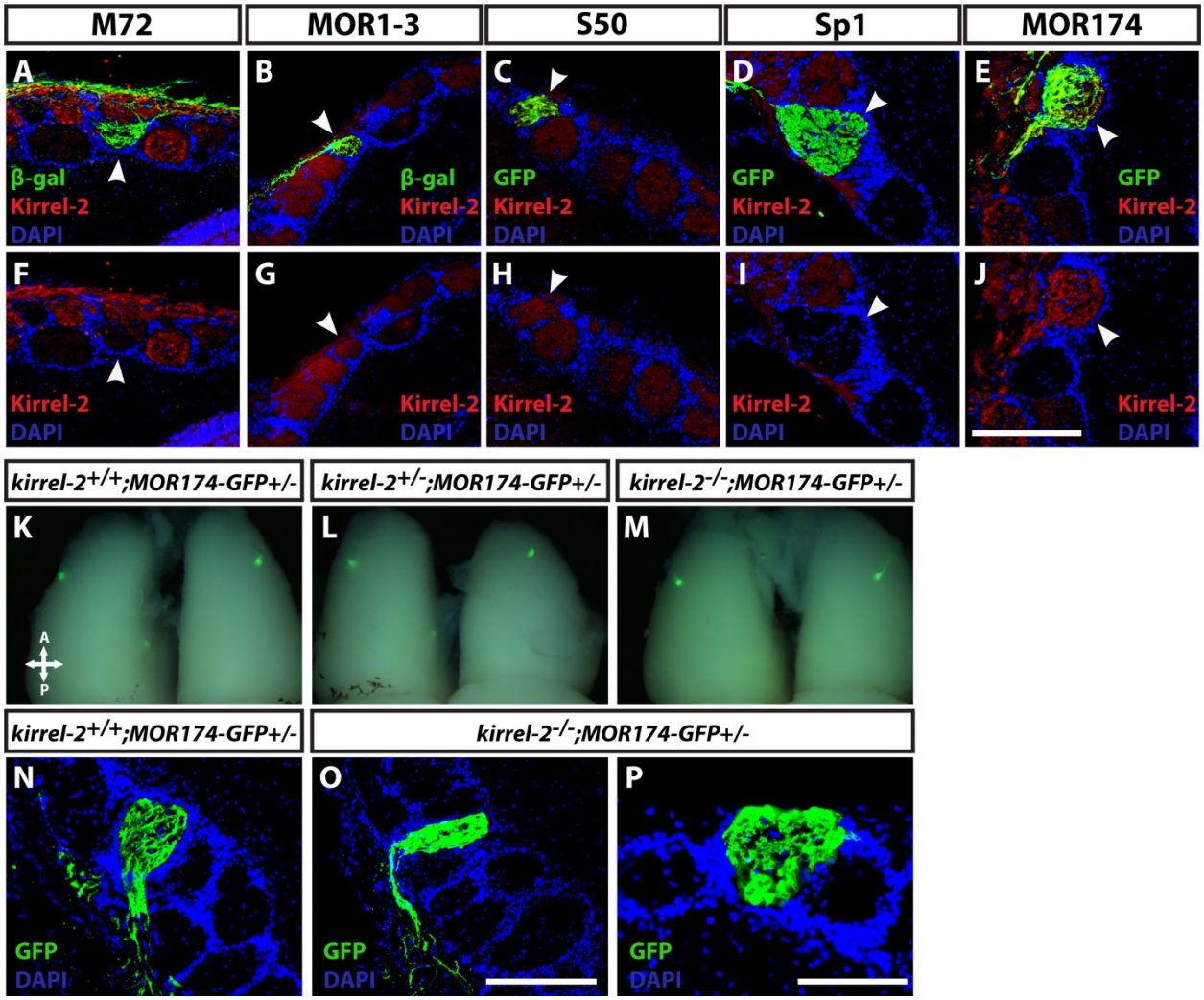

Figure 2.8. Kirrel-2 expression in the MOS is dispensable for axon targeting.

A-J) Kirrel-2 expression in specific populations of OSNs. Coronal sections of the main olfactory bulb were taken from adult mice that express GFP or β-galactosidase under the control of a specific OR promoter. Sections were immunolabelled with anti-β-gal (A,B), anti-GFP (C, D, E) and anti-Kirrel-2 and Hoechst (A-J). Kirrel-2 is not expressed in the M72 population of OSNs (A,F) or the Sp1 population (D,I). Kirrel-2 is highly expressed in the MOR174 population (E,J) and more moderately expressed in the MOR1-3 (B,G) and S50 OSN populations (C,H). White arrowheads denote the glomeruli in the olfactory bulb that are positive for the labelled OSN population. Scale bar: 200μm in J.

K-M) Wholemount images of olfactory bulbs taken from adult *kirrel-2*$^{+/+}$ (K), *kirrel-2*$^{+/-}$ (L) and *kirrel-2*$^{-/-}$ (M) mice that were crossed with the MOR174-GFP mice to trace the size and the positioning of MOR174 OSN glomeruli (K-M). No gross changes between wildtype and *kirrel-2*$^{-/-}$ mice in the positioning or size of GFP+ glomeruli were detected (K,M). N=3 mice for the *kirrel-2*$^{+/+}$*;MOR174-GFP*$^{+/-}$ and *kirrel-2*$^{+/-}$*;MOR174-GFP*$^{+/-}$ condition and n=5 mice for the *kirrel-2*$^{-/-}$*;MOR174-GFP*$^{+/-}$ condition.

N-P) Coronal sections through the olfactory bulb of adult *kirrel-2*$^{+/+}$*;MOR174-GFP*$^{+/-}$ (N) and *kirrel-2*$^{-/-}$*;MOR174-GFP*$^{+/-}$ (O,P) mice. Sections were stained with anti-GFP and Hoechst to label MOR174+ glomeruli and trace the targeting of the MOR174 OSN axons to the glomeruli. All MOR174+ axons target to their respective glomeruli in *kirrel-2*$^{-/-}$ mice (O,P) and no stray axons or mistargeting MOR174 axons are detected similar to controls (N-P). N=2 mice for each condition. Scale bars: 200μm in O and 100μm in P.

Discussion

Kirrels are essential for proper nervous system development

Factors that are fundamental for nervous system circuit development are imperative for the proper functioning of this vital system. Mutations in genes that are responsible for the targeting of axons, or synapse formation and maintenance are detected in many intellectual disabilities and autism spectrum disorders (Hu et al., 2006, Anitha et al., 2008, Südhof, 2008, Suda et al., 2011). Moreover, the *kirrel-3* gene has been associated with intellectual disabilities in humans (Bhalla et al., 2008) indicating the significance of Kirrel function in proper nervous system development. The results demonstrated in this thesis further corroborate the importance of Kirrels in nervous system wiring and facilitating the assembly of a precise sensory circuit necessary for animal survival.

Kirrels mediate VSN axon sorting during AOS circuit formation

The organization of sensory stimuli in the central nervous system is fundamental for interpreting the sensory information our brains receive and process. Therefore, the formation of the sensory map in animals in the AOB is crucial for the processing of stimuli which mediate vital social behaviours. Different mechanisms and various factors are required to form such a precise circuit which connects the VSNs to mitral cells at distinct glomeruli in the AOB. As reviewed in the introduction these include, and most likely are not limited to, classical axon guidance molecules, expression of CAMs on VSN axons, VR expression, and neuronal activity. Furthermore, there are many steps that VSN axons must take to reach their target glomeruli within the AOB, which include exit from the VNO, fasciculation of axons in the VN, and general targeting and segregation of apical and basal VSN populations in the AOB. These processes have

been readily characterized. However, how VSN axons fine tune their position to discrete locations at distinct glomeruli to form a sensory map still remains unclear. Examination of knockout animals for the classical guidance molecules, such as Robo-2 and Slit-1, which are important for the general targeting of VSN axons to the AOB, do not affect the formation of glomeruli which suggests that a different subset of genes are responsible for controlling coalescence of axons (Cloutier et al., 2002, Cloutier et al., 2004, Prince et al., 2009). CAMs are well characterized molecules which are known to mediate axon positioning in various species during nervous system development (Grueber and Sagasti, 2010, Imai et al., 2010, Imai and Sakano, 2011, Missaire and Hindges, 2015). In this thesis we demonstrate that the CAMs Kirrel-2 and 3 are required for axon coalescence and synapse formation or maintenance within the AOB (Figure 3.1).

Genetic knockout strategies enabled the dissection of Kirrel function during circuit formation of the AOS. Ablating *kirrel-2* expression led to the formation of larger and fewer glomeruli. While the absence of both *kirrel-2* and *kirrel-3* expression led to a drastically greater defect in glomeruli formation and a disruption in the number and size of synapses within the glomerular layer of the AOB (Figure 3.1C). Conversely, there were no defects in the fasciculation of the VN or general targeting and segregation of VSN axons to the AOB in these mice (Figure 3.1C). These results suggest a novel role for Kirrel-2, and further elucidate the function of Kirrels in regulating the sorting of VSN axons once they reach the AOB and subsequent synapse formation.

The notion of axon-axon interactions to regulate axonal position within the target field, or axon sorting, has been well studied in various sensory systems. The organization of axonal projections in different sensory systems is necessary to maintain topographic order of sensory

stimuli, and therefore, molecules which mediate this are of particular importance. For example, in the visual system pretarget axon sorting occurs to maintain topographic order of neurons projecting from the retina to second order targets in the brain in many species including mice, frogs, and fish (Scholes, 1979, Lee et al., 2004). Short range contact repulsion between retinal ganglion cell (RGC) axons is carried out by the Ephs and Ephrins family of molecules that are expressed on the surface of RGC axons (Tessier-Lavigne, 1995, Suetterlin and Drescher, 2014). Our results demonstrate that CAMs of the Kirrel family also play a crucial role in the formation of glomeruli within the AOB. The absence of distinct glomeruli in the AOB of *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice further support the concept that short range axon-axon interactions are critical in this sensory system to permit proper sorting of like axons within a target field (Figure 3.2). Additionally, the MOS has been a valuable and well studied tool of axon sorting events during neuronal circuit assembly (Imai and Sakano, 2011). Here, the expression of various CAMs in OSN axons have been shown to be important for the convergence of OSN axons into glomeruli in the olfactory bulb, including Kirrels (Serizawa et al., 2006), BIG-2 (Kaneko-Goto et al., 2008) and protocadherin-α (Hasegawa et al., 2008). In this system, it is proposed that the interaction between CAMs expressed on the surface of axons mediate the convergence or repulsion of different populations of OSNs resulting in the correct targeting of axons to their respected glomeruli (Serizawa et al., 2006, Sakano, 2010, Imai and Sakano, 2011).

Although it is most likely the short range homophilic interaction between Kirrels expressed on VSN axons mediating glomeruli formation in the AOB, there is the possibility that Kirrels are interacting with molecules expressed on the surface of mitral cells to regulate this process. Although we have yet to detect Kirrel-2 or Kirrel-3 expression in mitral cells of the AOB (unpublished observations and (Prince et al., 2013)) it remains possible that low levels of

expression of these two molecules in mitral cells could contribute to glomeruli formation. Another possibility is that Kirrels expressed on VSN axons are interacting with mitral cells to facilitate the formation of glomeruli in the AOB via interactions with other Kirrel binding partners such as Nephrin.

Activity dependent regulation of AOS circuit formation

Activity is an important determinant of circuit formation during nervous system development. The proper connectivity of VSNs in the AOS is dependent on activity (Hovis et al., 2012), although the mechanism of how activity instructs VSN wiring is unclear. On the contrary, methods of how activity dependent circuit formation occurs in the MOS are well studied. In the MOS, the targeting of OSN axons in part depends on activity (Yu et al., 2004, Serizawa et al., 2006, Chesler et al., 2007, Zou et al., 2007). It is well established that neuronal activity in OSNs controls the expression of different axon guidance molecules and CAMs that contribute to the proper targeting of OSN axons to the olfactory bulb (Imai et al., 2006, Serizawa et al., 2006, Dal Col et al., 2007, Imai et al., 2009, Nakashima et al., 2013). For example, spontaneous activity causing activation of ORs and downstream signaling through adenyl cyclase 3 in OSNs results in cyclic adenosine monophosphate (cAMP) production which in turn regulates the expression of Neuropilin-1 and PlexinA1, two axon guidance receptors that are required for OSN axon targeting (Imai et al., 2006, Dal Col et al., 2007, Nakashima et al., 2013). Whereas odour-evoked activity of OSNs regulates the expression of CAMs, such as Kirrels and BIG-2, and Ephrins which are required for the refinement of axon positions within the bulb (Serizawa et al., 2006, Kaneko-Goto et al., 2008). In this case, depending on the stage of OSN targeting, different forms of activity will regulate OSNs to express the necessary molecules to regulate the given stage the OSN is at currently. This suggests that spontaneous activity (when the nose is closed) is

important for early target events such as general targeting of axons to the bulb, and therefore, regulates expression of classical axon guidance receptors. On the other hand, after birth odour-evoked activity is necessary to refine the connectivity between OSNs and mitral cells which is suggested by the changes observed in gene expression of various CAMs.

Similar to the MOS, it is probable that in the AOS odour-evoked activity of VSNs affects the gene expression of CAMs and other molecules that are important for axon positioning and glomerular refinement. During MOS and AOS development, the formation of mature discrete glomeruli does not occur until after birth, usually between P0 to P5 (Potter et al., 2001, Sengoku et al., 2001, Salazar et al., 2006). Furthermore, mitral cell dendritic tufts are being refined to specific glomeruli between P5 to P7 in both olfactory systems (Lin et al., 2000, Hovis et al., 2012). Compellingly, the VNO is open and VSNs can be activated by stimuli at birth, and altered evoked activity in VSNs leads to mistargeting of VSN axons and undefined glomeruli (Hovis et al., 2012). Considering this study from Hovis et al. and the observation that VSN and OSN connectivity to mitral cells matures and is finalized during post natal development, it is likely that evoked activity influences AOS circuit refinement. Interestingly, the expression of Kirrels in the AOS is modulated in the absence of the TRP2 ion channel (Prince et al., 2013), demonstrating that the mechanism by which activity functions to mediate circuit refinement is similar to what is observed in the MOS. Lastly, VR expression is required for the proper targeting of VSN axons (Belluscio et al., 1999, Rodriguez et al., 1999, Del Punta et al., 2002a), and therefore, VR expression could be essential for conveying activity dependent instruction. Taken together, it is likely that odour-evoked activity in VSNs during post natal development is necessary to control gene expression of molecules, such as Kirrels, that are required for fine-tuning vomeronasal circuitry.

Kirrels define a molecular code necessary for the identity of VSN axons

In a vast array of numerous different axon populations targeting the AOB, how do VSNs distinguish themselves from each other to specifically coalesce with axons from the same population and form their target glomeruli? It is well established that the expression of cell surface or transmembrane molecules on axons provides targeting axons with a specific identity, and therefore, allows them to sort accordingly. One clear example of this is exhibited in the development of the *Drosophila melanogaster* nervous system. During development of the mechanosensory system, alternative splicing of the *Down syndrome cell adhesion molecule* (DSCAM) gene results in thousands of different isoforms that are differentially expressed in axons to generate molecular diversity between cells (Chen et al., 2006). The DSCAM splice variants interact homophilically to drive self avoidance of axons from the same cell and repulsion of axons from different cells in order to generate a functional circuit (Chen et al., 2006, Schmucker and Chen, 2009). The idea of axon identity determined by the differential expression of molecules on the surface of axons also is evident in the MOS. Here, OR expression in OSNs is not only necessary for binding odorants, but is also proposed to provide an identity to OSN axons during their targeting to the olfactory bulb since the ORs are expressed in the axons (Bozza et al., 2002). Furthermore, complementary expression of Kirrel-2 and 3, or Ephrin molecules in different populations of OSN axons is suggested to drive the convergence of like axons, and the repulsion of axons from different populations, respectively, during targeting (Serizawa et al., 2006). Similarly, in VSNs Kirrels are expressed in a complementary manner and at various levels in different VSN populations (Prince et al., 2013) suggesting that the differential expression creates a molecular diversity for axons (Figure 3.2).

The differential expression of Kirrels in VSNs allows axons to distinguish themselves from others, but also enables axons expressing the same Kirrel code to interact via homophilic adhesion of Kirrel proteins (Figure 3.2). Homophilic adhesion between Kirrel molecules has been reported in several systems (Gerke et al., 2003, Minaki et al., 2005, Serizawa et al., 2006, Nishida et al., 2010) and downstream signaling of Kirrels is shown to regulate actin polymerization and cytoskeleton dynamics (Grahammer et al., 2013). Therefore, it is likely that Kirrel-Kirrel homophilic interaction between VSN axons leads to changes in cytoskeletal dynamics resulting in the coalescence of axons from the same VSN population. This idea is supported by the observation that diffuse axonal innervation of target glomeruli is observed in *kirrel-3*$^{-/-}$*;EC2-tau-lacZ* throughout different stages of AOS development (Figure 2.1). Additionally, defects in glomeruli formation is only observed in the posterior region of the AOB in *kirrel-2*$^{-/-}$ mice (Figure 2.3), however, in the *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice disruption in glomeruli formation is observed throughout the whole AOB (Figure 2.6). These results suggest that with the loss of one Kirrel only a subset of VSN populations are affected while with the loss of both Kirrels the molecular diversity generated by Kirrel molecules is completely ablated leading to a greater defect in axonal coalescence. It is important to note that the Kirrels are only one family of molecules and cannot account for the wide diversity that must be required to identify the hundreds of VSN populations, thus it is probable that there are multiple other CAMs expressed on VSN axons that could contribute to axonal sorting. As in the *Drosophila* mechanosensory system, thousands of splice variants of DSCAM are required to generate the diversity needed for the recognition of axons in the developing brain (Hattori et al., 2009). One possible candidate molecule that may contribute to sorting and identifying VSN axons is protocadherin-α, which is

expressed in the AOS and is involved with axon convergence in the MOS (Hasegawa et al., 2008).

Synapse formation between VSNs and mitral cells is disrupted with loss of Kirrel expression

CAMs are not only implicated in axon guidance events, but are also actively involved in synapse formation. Numerous families of CAMs have been demonstrated to play a role in regulating the initial formation and maintenance of synapses (Siddiqui and Craig, 2011). Interestingly, ablation of both *kirrel-2* and *kirrel-3* results in a decrease in the number of excitatory synapses and an increase in the area of presynaptic terminals within the AOB (Figure 2.7, Figure 3.1C). Thus, it is possible that the Kirrel proteins are mediating synapse formation in the AOS. However, it is not clear whether Kirrels play a direct effect in synapse formation or maintenance, or whether these defects are the result of an indirect effect of Kirrel function in the VSNs. One explanation of the observed change in synapses is that Kirrel-2 and Kirrel-3 are directly involved in promoting synapse formation and size between VSN axons and dendrites of mitral cells within glomeruli of the AOB. The larger synapse size observed could lead to the formation of larger glomeruli. A second possibility is that VSN axons that are improperly targeted in glomeruli due to loss of Kirrel function form unstable synapses that are pruned away during development, resulting in less synapses. The presence of less synapses could result in more space in the neuropil for existing synapses allowing presynaptic terminals to expand in size. Although we cannot conclude from our results that Kirrels directly regulate synapse formation and maintenance, there is evidence in the literature supporting a role for Kirrels in synaptogenesis.

In the animal model *C. elegans* Kirrel and Nephrin homologues SYG-1 and SYG-2 are required for forming the synapse between HSNL motor neurons and vulval epithelial cells (Shen et al., 2004). Additionally, Kirrels are expressed at the synapse in pyramidal neurons of the hippocampus and colocalize with the synaptic scaffolding protein CASK (Gerke et al., 2006). These studies show a direct role for Kirrel homologues in directing synaptogenesis and provide strong evidence Kirrels may be required in mammalian systems for mediating synapse formation or maintenance. Kirrels could be functioning as trans synaptic binding partners with the mammalian SYG-2 homolog, Nephrin, to mediate synapse formation and maintenance (Shen et al., 2004).

Alternatively, the inability of VSN axons to form stable synapses with mitral cells may be the indirect cause of a decrease in the number of synapses and an increase in presynaptic area observed in *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice. Mitral cell tuft refinement occurs just after the coalescence of VSN axons into glomeruli (Hovis et al., 2012), and therefore, glomerular refinement may be a prerequisite for the formation of stable synapses between VSNs and mitral cells. Consequently, a disruption in VSN axon coalescence could affect mitral cell connectivity and may result in the formation of immature, unstable synapses. Thus, *kirrel-2* and *kirrel-3* ablation would indirectly lead to a greater amount of unstable synapses being pruned away and more space in the neuropil for synapse terminals to spread out.

Kirrel-2 and male-male aggression in mice

The vomeronasal system is a key regulator of aggression which is an important aspect for various social behaviours in mice (as described in the introduction). It is well established that a disruption in the circuitry of the vomeronasal system leads to defects in male-male aggression (as cited in the introduction). Hence, with the phenotype observed in the AOB of *kirrel-2*$^{-/-}$ mice one

would expect to see a disruption in the behaviours mediated by the vomeronasal system. Future experiments could address if the alteration in glomeruli observed with the loss of *kirrel-2* expression is required for detecting the chemosignals which mediate aggressive behaviour in mice. Strikingly, in *kirrel-3*$^{-/-}$ mice which display a similar phenotype as the *kirrel-2*$^{-/-}$ mice, there is a decrease in male-male aggression compared to control animals (Prince et al., 2013). However, Kirrel-3 is expressed in other brain areas that are important mediators of aggression, which includes the medial amygdala and hypothalamus (Tamura et al., 2005, Nelson and Trainor, 2007, Lin et al., 2011). Consequently, it may be that loss of *kirrel-3* from these structures leads to the change in aggression observed in *kirrel-3*$^{-/-}$ mice. Thus, our *kirrel-2*$^{lox/lox}$*;OMP-Cre* mouse model, where *kirrel-2* is specifically ablated in VSNs and where glomerular formation is affected, will be useful to confirm that disruption in the formation of glomeruli in AOS as the cause of the defect in aggressive behaviour.

Kirrel-2 is expressed in specific populations of OSNs in the MOS

Similar to previous reports by Serizawa et al., Kirrel-2 expression is observed on axons of OSNs in the MOS (Figure 2.2F & 2.8A-J). It is possible that Kirrel-2 may function as an axon sorting molecule and provide a surface identity for OSN axons during axonal convergence into glomeruli since Kirrel-2 is expressed at different levels in different populations of OSN axons (Figure 2.8A-J). Although, unlike in the AOS, no gross defect in the targeting of OSNs has been observed thus far using loss of function approaches. The axons of the specific populations of OSNs which were shown to have high levels of Kirrel-2 expression do not display defects in the general size and positioning of glomeruli in the main olfactory bulb in *kirrel-2*$^{-/-}$ mice. However, the fine-tune target selection of distinct populations of axons could be disrupted. It is likely that Kirrel-2 plays a similar role observed in the AOS and is involved with axon sorting during

glomeruli formation, as described previously using gain of function approaches (Serizawa et al., 2006). Further detailed analysis following the targeting of specific populations of OSNs in which Kirrel-2 is expressed is required to address this possibility. Alternatively, Kirrel-2 could be indispensable for axon targeting and may instead drive synaptogenesis in the MOS. Future experiments looking at synapse ultrastructure in the MOS could address this possibility. Further evidence will be required using loss of function approaches to elucidate the role of Kirrels in the MOS and determine if they are important axon sorting molecules.

Conclusion

The ability of mammals to interact with their environment relies on the highly organized wiring of sensory systems in order to encode a clear representation of the outside world in the nervous system. As highlighted above, precise circuit assembly is crucial to facilitate the function of the nervous system. In this thesis, the role of Kirrels during circuit formation of the AOS is examined. Loss of *kirrel-2* or *kirrel-3* expression disrupts the coalescence of VSN axons within the posterior AOB while removal of both Kirrels prevents the formation of distinguishable glomeruli throughout the AOB. Furthermore, the *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice have fewer excitatory synapses and display an increase in the area of presynaptic terminals within the glomerular layer of the AOB. Taken together, these results demonstrate that Kirrel expression on VSN axons dictates their proper coalescence into glomeruli and is required for synapse development within the AOB.

Discussion Figures

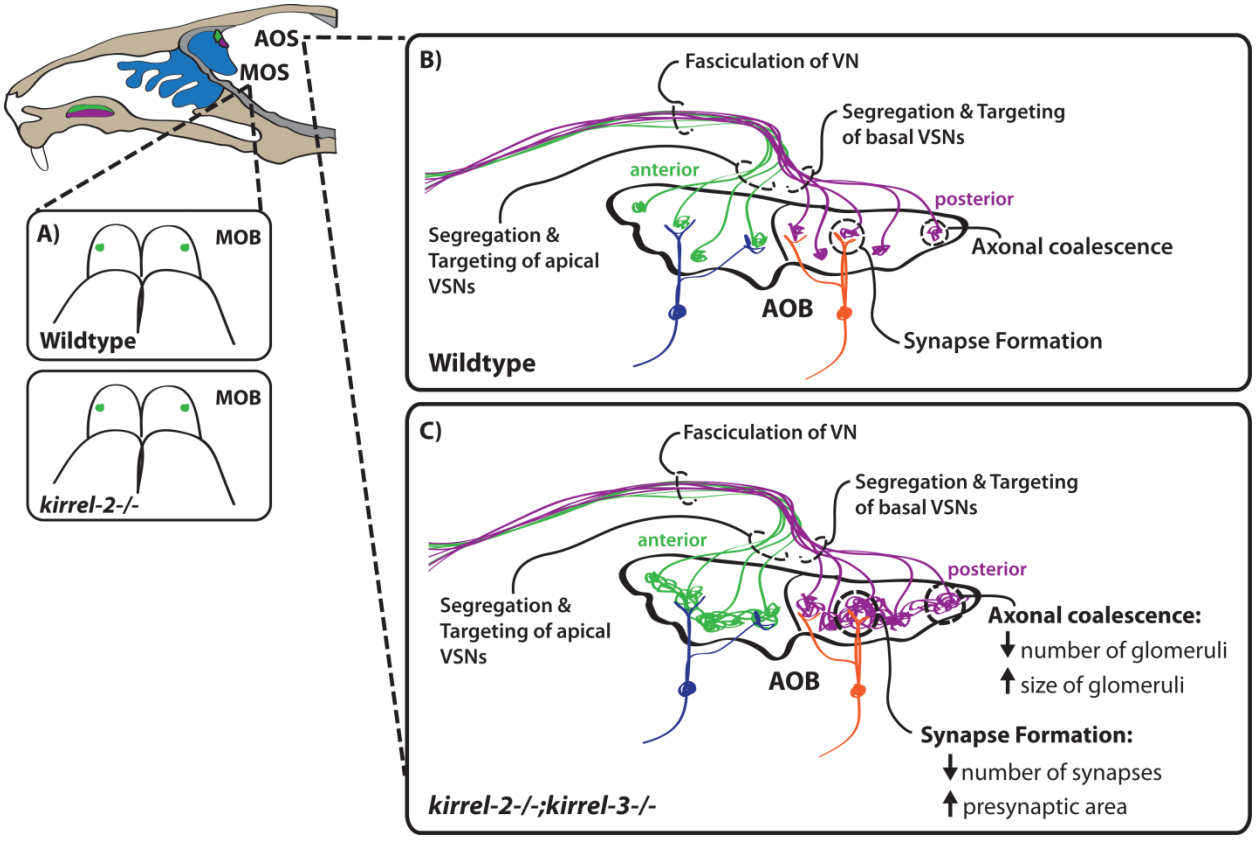

Figure 3.1. Summary schematic of phenotypes observed in the MOS and AOS with the loss of *kirrel* expression.

A) No gross defects are observed in the MOS from *kirrel-2*$^{-/-}$ mice. The positioning and size of MOR174 glomeruli in the MOB are similar in *kirrel-2*$^{-/-}$ and wildtype mice examined. No mistargeting of MOR174 positive axons is observed thus far in the MOB from *kirrel-2*$^{-/-}$ mice.

B) Wildtype phenotype of circuit assembly in the vomeronasal system. The mechanisms regulating axon targeting and glomeruli formation in the AOS are highlighted.

C) Phenotype observed in the AOB with loss of *kirrel* expression. There is a decrease in the number of glomeruli and an increase in size of glomeruli with the loss of *kirrel-2* or *kirrel-3* expression. With the loss of both *kirrel-2* and *kirrel-3*, distinct glomeruli do not form in the AOB. Additionally, there is a decrease in the number of synapses and an increase in the area of presynaptic terminals observed in the glomerular layer of the AOB from *kirrel-2*$^{-/-}$*;kirrel-3*$^{-/-}$ mice. Fasciculation of the vomeronasal nerve and segregation and targeting of VSNs is not disrupted when *kirrel* expression is ablated.

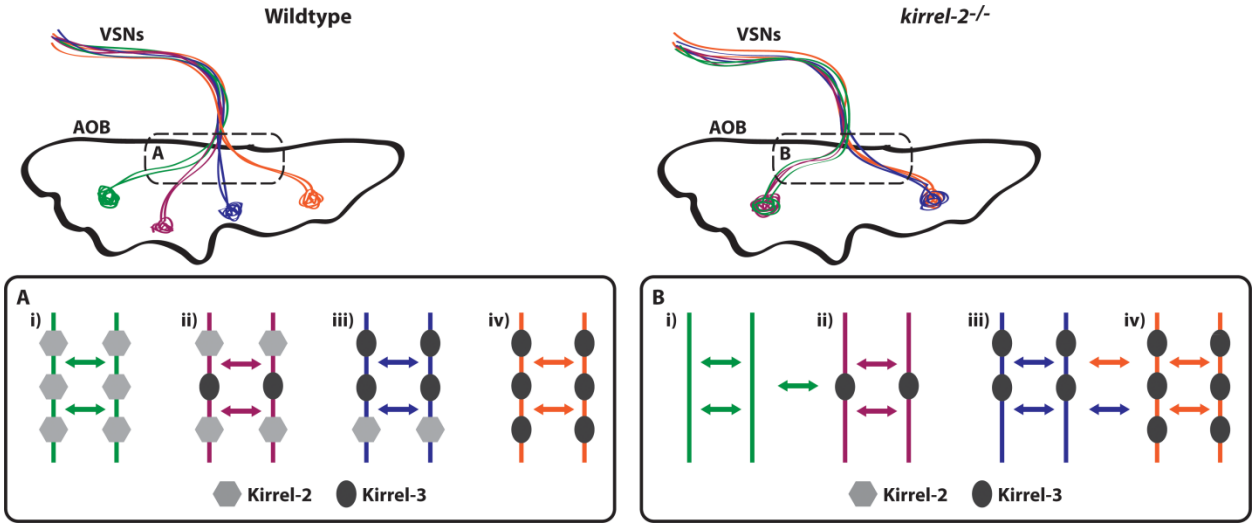

Figure 3.2. Local axon sorting controls the coalescence of VSN axons into glomeruli.

A) The differential expression of Kirrels in VSN axons creates a molecular code for targeting axons to dictate their coalescence into glomeruli. The sorting of axons via interactions between CAMs expressed on the surface of axons is one mechanism which allows axons to fine tune their position within their general target region. Four populations of VSNs have been reported based on the complementary expression of Kirrels which include: i) one population with high Kirrel-2 expression and no Kirrel-3, ii) a second population with high Kirrel-2 and low Kirrel-3, iii) a third with high Kirrel-3 and low Kirrel-2, and iv) a fourth population with only high Kirrel-3 expression (Prince et al., 2013). Kirrels have been shown to interact in trans by homophilic binding of like Kirrel molecules (Gerke et al., 2003, Minaki et al., 2005, Serizawa et al., 2006, Nishida et al., 2010). Therefore, it is proposed that the homophilic interaction of Kirrels drives the convergence of axons together, and allows axons with the same Kirrel identity code to come together and form their target glomeruli. Arrows denote the homophilic interaction and subsequent convergence of axons from the same VSN population.

B) The loss of *kirrel-2* expression decreases the molecular diversity between VSN axons and may result in the coalescence of different populations of VSNs based on the levels of molecules which remain on the surface of axons. This results in the formation of fewer and larger glomeruli which is observed in *kirrel-2*$^{-/-}$ mice. Kirrel-2 ablation leads to only three distinguishable populations of VSNs based on Kirrel expression: i) one population with no expression of either Kirrel-2 or 3, and ii) a second with low Kirrel-3 expression, and iii and iv) a third population with high expression of Kirrel-3. Hypothetically axons that express high levels of Kirrel-3 (orange and blue axons) will homophilically interact and axons with low Kirrel-3 expression (magenta axons) may weakly interact with axons that do not express Kirrels (green axons).

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