Mechanisms underlying pre- and postnatal development of the mouse vomeronasal organ

Raghu Ram Katreddi
University at Albany, State University of New York, rrkatreddi@gmail.com

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Mechanisms underlying pre- and postnatal development of the mouse vomeronasal organ

by

Raghu Ram Katreddi

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Abstract

The Vomeronasal organ (VNO) is a specialized olfactory sensory organ located in the ventral region of the nasal cavity in rodents. The vomeronasal epithelium (VNE) of rodents is composed of 2 major types of vomeronasal sensory neurons (VSNs): 1) VSNs distributed in the apical VNE regions that express vomeronasal type-1 receptors (V1Rs) and the G protein subunit Gαi2, and 2) VSNs in the basal territories of the VNE that express vomeronasal type-2 receptors (V2Rs) and the G subunit Gαo. Besides these two neuronal types, VNE also accommodate a third non-neuronal cell type called Sustentacular cells that lie anatomically above apical and basal VSNs. Close to the lumen. Both V1R+ apical VSNs and V2R+ basal VSNs send their axons to distinct regions of the accessory olfactory bulb (AOB). Together, VNO and AOB form the accessory olfactory system (AOS), an olfactory subsystem that coordinates the social and sexual behaviors of many vertebrate species. In mice, both V1R and V2R+ VSNs form from a common pool of progenitors but have distinct differentiation programs. Interestingly VNO is one of the very few neuronal systems that can show adult neurogenesis throughout the life that is responsible for homeostasis in normal physiological conditions and regeneration in post injury conditions. During development, VNO is formed from the olfactory placode during the secondary invagination of olfactory pit. The vomeronasal epithelium (VNE) is believed to share multiple features with the epithelium of the main olfactory epithelium (MOE), however it has been less characterized compared to MOE.

My doctoral research focused on two different aspects of the VNO development in mice - 1) identifying the mechanisms underlying the cell-fate determination of V1R+ apical and V2R+ basal VSNs, and 2) identifying horizontal basal cells, a second multipotent
stem cell population in the VNO (vHBCs) and study their role in the development and adult neurogenesis in the VNO. We used single cell RNA sequencing of the adult VNO, Cre recombinase induced constitutive and conditional lineage tracing, gain of function and loss of function methods to study these developmental questions of the VNO.

In the first part of my dissertation, we identified the differential expression of Notch1 receptor and Dll4 ligand among the neuronal precursors at the VSN dichotomy. We further demonstrated using loss of function studies that Notch signaling is required for the effective differentiation of V2R+ basal VSNs. Interestingly, gain of function studies gave two different phenotypes depending upon the cell stage where we artificially induced Notch signaling. Ectopic Notch signaling at the immediate neuronal precursors stage diverted them towards an expected basal VSN cell fate, however ectopic Notch expression at much earlier neuronal progenitor’s stage redirected them to a non-neuronal sustentacular cell fate. Together, these results demonstrate that Dll4-Notch1 signaling plays a crucial role in triggering the binary dichotomy between the two main types of VSNs in mice. Nonetheless, further research is needed to understand the formation of non-neuronal sustentacular cells.

The second part of my dissertation showed the presence of vHBCs in the adult VNO similar to the MOE but show much delayed development. Constitutive and conditional lineage tracing techniques in normal physiological conditions reported that vHBCs are multipotent stem cells giving to rise to both neuronal and non-neuronal cells during both early postnatal development and in adult stages of the VNO. Overall, both the studies explored two long standing developmental questions in the VNO that further opened new areas of research.
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Finally, to all of the mice I have sacrificed in the name of science: Thank you.
Preface


2. **CHAPTER 2** was published as Raghu Ram Katreddi, Ed Zandro M. Taroc, Sawyer M. Hicks, Jennifer M. Lin, Shuting Liu, Mengqing Xiang, Paolo E. Forni; Notch signaling determines cell-fate specification of the two main types of vomeronasal neurons of rodents. *Development* 1 July 2022; 149 (13): dev200448. doi: [https://doi.org/10.1242/dev.200448](https://doi.org/10.1242/dev.200448)

3. **CHAPTER 3** is currently unpublished and is ongoing, although the data presented here is of relevance to this doctoral thesis project.

4. **CHAPTER 4**: Open questions to study and concluding remarks on this dissertation work.

5. **CHAPTER 5**: The detailed materials and methods used for all the experiments presented.

6. These studies or articles are being included because they were part of the programmatic line of research that comprised the dissertation and that including them provides a coherent and appropriately sequenced investigation.

7. I was the primary researcher for the work reported in this dissertation.
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CHAPTER 1
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Introduction
The vomeronasal organ (VNO) is a specialized olfactory organ responsible for detecting pheromones and kairomones; stimuli that can trigger a wide range of behaviors [12-16] and hormonal responses [17-19]. Many vertebrates have a vomeronasal organ composed of a sensory and a non-sensory epithelium [20]; however, several adaptive/developmental differences can occur across species [21, 22]. Karl Von Baer’s principles state that the general anatomical features in animal species appear earlier in development than the more specialized features. In line with this, the vomeronasal anlage has been reported as a transient embryonic structure present in many vertebrate species including birds [23, 24], where the VNO does not form, and humans, where a VNO-like structure has been documented only in rare cases [25-27]. This suggests that the embryonic vomeronasal anlage is a general structure that, depending on the animal species, may or may not develop into a functional VNO.

During the early development of the vomeronasal anlage of mice (E10.5-E11.5) some endocrine neurons, called gonadotropin-releasing hormone-1 (GnRH-1) neurons, form proximal to the prospective VNO and then migrate into brain. Once in the brain, these neurons become an integral part of the hypothalamic-pituitary-gonadal axis. GnRH-1 neurons release GnRH to trigger the release of gonadotropins, luteinizing hormone (LH)
and follicle stimulating hormone (FSH), from the pituitary gland. These hormones are critical for proper gonadal function [28-31]. Recent 3D analysis of human embryos by Casoni and coworkers also found that GnRH-1 neurons form proximal to the embryonic vomeronasal structure and migrate to the brain in a process consistent with that in mice [32]. Based on these data, we posit that the embryonic vomeronasal anlage is an evolutionarily conserved structure.

During the late embryonic and adult stages, the mouse VNO is mostly composed of two main classes of vomeronasal sensory neurons (VSNs) that each selectively expresses G protein coupled receptors (GPCRs) encoded by one of the two vomeronasal receptor (VR) gene families: V1R and V2R [6, 8, 33-36]. Hundreds of VSN sub-populations expressing V1Rs or V2Rs bind different ligands, trigger distinct innate behaviors, localize in different regions in the VNO and project to different areas in the accessory olfactory bulb (AOB) [15, 37-41]. V1R positive neurons lie mostly in the apical zone of the vomeronasal epithelium and express the G-protein subunit Gαi2 and neuropilin-2 (Nrp-2) and project to the anterior portion of the AOB [42, 43]. V2R positive neurons lie in the basal zone of the epithelium and contain the G-protein subunit Gαo, the Slit receptor Robo2, and project to the posterior portion of the AOB [44]. A small subset of V2R and Gαo+ VSNs co-expresses another multigene family of nine nonclassical class I major histocompatibility genes, H2-Mv genes. Gαo+ VSNs that are positive for H2-Mv reside in the lower sub layers of the basal zone in the vomeronasal sensory epithelium [45-48].

Approximately a decade ago two independent research groups identified an additional subclass of Gαi2 or Gαo positive VSNs, which express formyl peptide receptors (FPRs)
but do not overlap with V1R or V2Rs in rodents [49, 50]. 7 FPR genes exist in the mouse genome, though five were identified in the VNO. *Fpr-rs1* is co-expressed with the G\(\alpha_o\) subunit in the basal zone, whereas *Fpr-rs3*, *Fpr-rs4*, *Fpr-rs6* and *Fpr-rs7* are co-expressed with G\(\alpha_i2\) in the apical zone [49]. Later studies also revealed that Fpr-rs3 VSNs showed similar physiological properties to the remaining VSN population [51], and Fpr-rs1 VSNs have stereo-selective preference towards D-amino acid containing peptides, which specifically occur in pathogenic bacteria, viruses and fungi [52].

Several studies refer to G\(\alpha_i2^+\) VSNs as apical VSNs, while G\(\alpha_o\) expressing VSNs are often named basal VSNs [53-56]. Even though the spatial distinction is not absolute, in this review we will sometimes refer G\(\alpha_i2^+\) and G\(\alpha_o^+\) VSN populations using the historically accepted apical and basal VSNs nomenclature, respectively.

In this review article, we will discuss some key aspects of embryonic and postnatal VNO development of murine vomeronasal organ, as excellent and extensive reviews exist on postnatal vomeronasal connectivity, adult neurogenesis and function [55, 57-63]. Our goal is to highlight underexplored areas of developmental biology from our perspective.

### 1.1 Ontogeny of the VNO

During embryonic development, both the olfactory and lens placodes share a common origin from the preplacodal ectoderm [64]. Over time, the spatial competence of the olfactory placode becomes restricted and specified to the most antero-lateral side of the head ectoderm [65]. In 1975, Cuschieri and Bannister first described the ontogeny of the mouse VNO [66]. The VNO arises from the olfactory placode, which is visible as a transient thickening in the antero-lateral region of the embryonic head at embryonic day
Previous studies revealed two different phases of olfactory neurogenesis – early/primary neurogenesis and established neurogenesis (Fig 1A, B) [67-69]. By E10-10.5, the olfactory placode invaginates to form the olfactory pit, which begins the early neurogenesis phase [68]. The olfactory pit is divided into anterior-medial sensory epithelium and posterior-lateral respiratory epithelium [70, 71]. By E11.5, the prospective vomeronasal organ thickens at the medial walls of the olfactory pit, which further invaginates toward the mesenchyme to form the vomeronasal groove [66].

In parallel with this stage, Tucker and colleagues identified two distinct populations of neural precursors that give rise to the major neuronal classes (e.g. olfactory sensory neurons, VSNs and GnRH-1 neurons) in the nascent olfactory epithelium. These precursor cells are either 1) slowly proliferating, self-renewing precursors that express Meis1/2 at high levels that are localized primarily in the lateral olfactory epithelium or 2) rapidly proliferating, transit amplifying precursors that express high levels of Sox2 and Ascl1 and are localized primarily in the medial olfactory epithelium [72]. By E12.5, the vomeronasal groove on both sides of the embryo further extends as a tubular structure to form the primitive VNO that separates from the olfactory pit. The VNO, at this developmental stage, is distinguishable into sensory epithelia towards the medial side, and non-sensory epithelia toward the lateral side [66, 73]. As the developing VNO transitions into established neurogenesis, the VNO features three main cells types in the epithelium: 1) Hes-1+ apical proliferative progenitor cells that lie towards the lumen, 2) Ascl1+ basal neurogenic progenitors that arise from apical progenitors and settle towards the basement membrane, and 3) post-mitotic VSNs in the intermediate zone [5, 6, 66, 74, 75] (Fig 1B).
From E13.5, the VNO increases in size and length, such that a cross-section resembles a kidney shape with a crescent shaped lumen separating the sensory and non-sensory epithelia. Unlike the main olfactory epithelium (MOE), blood vessels and perivascular connective tissue in the vomeronasal sensory epithelium are detectable from embryonic to postnatal life [11, 66, 76]. The VNO opens anteriorly and connects to the nasal cavity through a narrow vomeronasal duct. The VNO is also composed of highly vascularized cavernous tissue with large blood vessel lateral to the non-sensory epithelium. The autonomic innervation to the blood vessels can cause vasoconstriction, which then can function as a vomeronasal pump to enable influx of external stimuli [77, 78]. Whether the murine VNO is functional at birth still remains unclear. While Coppola and colleagues showed that the vomeronasal duct is not open at birth [79, 80], others reported that the vomeronasal duct can open to external chemical stimuli at perinatal stages [81]. The development of the VNO is delayed compared to the MOE, as VNO morphogenesis continues extensively in postnatal stages until sexual maturity [82]. After development is complete, the VNO continually generates new neurons throughout life [83]. BrdU based birth dating studies at adult stages highlighted the presence of vomeronasal neurogenic progenitors at the marginal zone between sensory and non-
sensory epithelia and at the central zone close to the basement membrane, which may mediate tissue homeostasis (Fig 1C) [7, 84, 85].
1.2 **Early morphogenesis: Early neurogenesis, GnRH-1 neuron formation, migratory mass and terminal nerve**

Molecularly heterogenous populations of neuronal and non-neuronal cells arise from the olfactory placode as early as E10.5 in mice. These include non-neuronal cells of the putative respiratory epithelium, neuronal stem cells, olfactory sensory neurons, and various migratory neuronal cell types [86]. Migratory neurons formed during the early neurogenesis phase include cells immunoreactive for GnRH-1, Neuropeptide Y, TAG-1, olfactory marker protein (Omp), gamma amino butyric acid (GABA), tyrosine hydroxylase and Islet-1 (Isl1) [29, 87-93] (Fig 1A). Interestingly, most migratory cell populations are still poorly characterized both in terms of molecular markers and function. Some cell
populations include neurons that are part of a complex ganglionic nerve called the terminal nerve [93] and “guidepost” neuronal cells that can aid in the initial formation of olfactory and vomeronasal nerve [86].

In mouse, the GnRH-1 neurons form in the olfactory placode between E10 and E11.5 proximal to the vomeronasal neurogenic area and start migrating by E11.5 from the nasal area to the forebrain [28, 29]. GnRH-1 neurons begin invading the brain around E12.5 and complete their migration to the basal forebrain around E16.5 [29]. Disturbances in GnRH-1 neuronal formation, migration or signaling negatively affect sexual development and fertility of mammals [6, 28-32, 94]. The phenotypic association between anosmia and defective GnRH-1 migration in Kallmann syndrome led to a prevailing model suggesting that migration of GnRH-1 neurons to the brain strictly depends on correct formation of the olfactory/vomeronasal system [30, 95-99]. However, other lines of evidence suggest that GnRH-1 neurons migrate to the brain along the axons of the nervus terminalis or terminal nerve (also cranial nerve-0) [28, 93]. The terminal nerve is still an ambiguous and controversial ganglionic nerve [100-103]. Some reports indicate that this nerve is a transient caudal branch of the vomeronasal nerve [104], while others suggest that the terminal nerve and vomeronasal nerve differ in neuronal composition, genetic expression, and guidance cue response [93]. In fact, Arx-1 null mice, a mouse model that lacks both OB formation and vomeronasal connections to the brain, still have terminal nerve projections to the hypothalamic area and successful GnRH-1 migration to the brain [93]. These data provided strong evidence that formation of the olfactory bulb and connectivity of vomeronasal and/or olfactory neurons to the olfactory bulb are not required for GnRH-1 neuronal migration into the brain. These data suggest that the broadly
accepted causal relationship between defective development of the olfactory/vomeronasal system and aberrant GnRH-1 migration warrants revisiting [105]. Interestingly, a recent publication suggested that GnRH-1 neurons express some vomeronasal receptors [106]. These data suggest that the terminal nerve and GnRH-1 neurons, which are likely present in all vertebrates, could represent an ancestral form of the vomeronasal system. For more extensive reviews on GnRH-1 and terminal nerve please see [30, 101, 107-109].

The mechanisms underlying the formation of various early neuronal and non-neuronal sub populations in the developing olfactory pit remain unresolved. Whether the neurons formed during early and late neurogenesis in the developing olfactory pit have a common genetic lineage also remains a matter of debate. Studies in fish and chick identified the Isl1 transcription factor as a molecular marker for specific subpopulations of cells of the migratory mass (MM). In zebrafish, Isl1 expression colocalize with Gnrh3 neurons, whereas Isl1+cells in chick were found as early migratory neuronal cells, distinct from Lhx2+ and GnRH-1+ neurons of the terminal nerve ganglion [110, 111]. Performing Isl1Cre genetic lineage tracing in mice, we found Isl1Cre recombination/lineage in almost all GnRH-1 neurons, cells of the migratory mass, cells of the respiratory epithelium, in sparse olfactory, vomeronasal neurons and supporting cells [91]. However, Isl1 conditional knockout from GnRH-1 neurons in mice did not cause any disturbance in their formation or migration, leaving the role of this transcription factor in GnRH-1 neurons and other cells formed during the early placodal neurogenesis unresolved [91].

1.3 **Intrinsic and extrinsic factors regulate VNO development**
1.3.1) Transcriptional regulation during early development

As the VNO is formed after a secondary invagination from olfactory pit (Fig 1A, B), all the transcription factors and signaling molecules that affect the formation of the olfactory placode/pit may also control VNO development (Table 1). In this section, we will highlight several studies that showed disruption of olfactory placode/pit and VNO development due to mutation of different transcription factors during the primary neurogenesis phase.

Pax6, a member of the Pax transcription family, is expressed in both olfactory and lens placodes during early development. Grindley and coworkers, (1995) used Pax6 homozygous mutant (Sey) mice to study its role in placodal formation [112]. In these mutants, they found no lens, olfactory placode thickening, nasal cavity, and olfactory bulb formation. Subsequent studies using chimeric embryos composed of both wildtype and Sey (Pax6<sup>-/-</sup>) mutant cells [113, 114] showed that only wild type cells, not Pax6 mutant cells, participate in olfactory placode invagination. These data confirmed a cell autonomous role for Pax6 in olfactory placode formation.

Six1 and Six4 transcription factors, members of Six gene family, are expressed in the pre-placodal region even before the specification of the olfactory placode. Two studies by Ikeda and coworkers reported a shallow olfactory pit invagination in Six1<sup>-/-</sup> mice compared to the wild type mice at E10.5 [67, 68] and showed that Six1 is critical for the differentiation of stem cell progenitors into neuronal precursors. In fact, in Six1<sup>-/-</sup> mice the VNO does not form by E12.5, and the olfactory epithelium degenerates by E14.5. Using a Six1;Six4 double knockout mouse model, Chen et al., (2009) showed synergistic requirements of both Six1 and Six4 for olfactory placode and VNO development [115]. In these double knockout mice, the olfactory placode does not invaginate to form an
olfactory pit. Notably, no defects were observed in the olfactory or VNO development in the Six4<sup>−/−</sup> mice alone, highlighting the functional redundancy of the Six family transcription factors [116].

Sox2, a SoxB1 family member, is also expressed from the early olfactory placode stage and may serve as a stem cell marker of both the olfactory and vomeronasal epithelium [73]. Panaliappan and coworkers reported that conditional ablation of Sox2 disrupts the olfactory epithelium by E10.5 due to increased apoptosis, reduced proliferation and diminished neurogenesis [70]. Sox2 is essential in promoting neurogenic lineage by restricting Bmp4 expression and regulating Hes5 expression in the nasal epithelium [70, 71]. In addition, another study showed the ability of Sox2 to bind to the enhancer elements and induce the expression of Pax6, one of the initial olfactory placodal markers [117].

Foxg1, a Forkhead family transcription factor, is another transcription factor expressed throughout the developing olfactory placode. Duggan and coworkers placed Foxg1 expression upstream to stem cell progenitor marker Ascl1, in the proneural genes cascade those controls both olfactory and vomeronasal neurogenesis [118]. Moreover, studies on Foxg1 constitutive knockout mice showed reduced proliferation and increased apoptosis at the early placodal development [119] and lack of VNO formation.

Dlx5 is a homeobox transcription factor that contributes to the development of the olfactory placode and olfactory bulb in mice [120, 121]. Dlx5 mutant mice display a right-left asymmetry in the nasal cavity formation together with complete absence or rudimentary development of the VNO. Dlx5<sup>−/−</sup> mutants fail to produce olfactory or vomeronasal projections to the main and accessory olfactory bulbs.
All these transcription factors are expressed in the olfactory placode stage, so their mutations also affect VNO formation. However, spatially distinct inductive signals and transcriptional factor networks may be quite critical for the vomeronasal thickening or invagination between E10.5 - E11.5. Nevertheless, Fezf2 remained the only known transcription factor, to our knowledge, that mechanistically contributes to VNO morphogenesis. Both Fezf1 and Fezf2 are closely related zinc finger transcription factors with 97% percent similarity in their zinc finger moiety [122]. Hirata and colleagues showed that Fezf2/Fezfl, is specifically expressed in the VNO at E12.5 [123], while Fezf1 is highly expressed in the MOE and weakly expressed in the VNO [124]. Then, Eckler and colleagues reported that Fezf2 expression is present as early as E10.5 at the vomeronasal thickening of the olfactory pit, which makes it one of the earliest markers for the vomeronasal area [122]. As age increases postnatally, Fezf2 expression becomes restricted to the sustentacular cells. Even though Fezf2-/- mice show normal separation of the VNO from the olfactory pit by E13.5, the VNO degenerates by the date of birth in these mutants. Fezf1-/-;Fezf2-/- double mutants did not reveal obvious synergistic effects in the VNO, but did show increased expression of vomeronasal specific genes in the MOE [122]. This study proposed that both Fezf1 and Fezf2 repress the expression of VSN-related genes in the main olfactory and vomeronasal epithelium, respectively. Nevertheless, the role of Fezf2 in the progenitor cells during early development of the VNO and the mechanisms of neuronal cell death in these knockout mice still remain unknown.

1.3.2) Transcriptional regulation of VNO development during established neurogenesis.
After the invagination of the vomeronasal pouch occurs, migratory neuronal populations leave the VNO toward the brain, and the neurogenesis of VSNs begins (Fig 1A) [2, 6, 91]. During the established neurogenesis phase, the VNO differentiates into medial sensory and lateral non-sensory epithelium with the lumen separating them [66, 73]. Even though only a few neurons form on the non-sensory side, they tend to undergo cell death as age increases [125, 126]. As the stem cells proliferate and differentiate towards VSNs, transcription factors express dynamically at specific temporal stages and play a key role in the VSN development (Table 1).

Ascl1, Ngn1 and Neurod1 are a neurogenic basic-helix-loop-helix (bHLH) family of transcriptional factors that are expressed during VSN development in the same temporal sequence as the MOE [9, 127]. These factors are essential for the specification and differentiation of the neurogenic progenitors. In the VNO, Ascl1 is predominantly expressed by basal progenitors, which further divide and give rise to immediate neuronal precursors that consequently express Ngn1 and Neurod1 [6, 9]. These Neurod1+ precursors mature into the two major types of vomeronasal neurons- \( \text{G}_{\alpha i2}^+ \) apical and \( \text{G}_{\alpha o}^+ \) basal VSNs in the VNO [8]. Ascl1 knockout mice showed drastic reductions in both apical and basal VSN populations highlighting the role of Ascl1 in VSN development [6, 9]. Notably, though very few in number, VSNs can form in Ascl1/Ngn1 double KO mice, suggesting the existence of redundant or compensatory neurogenic factors in the VNO [75].

During embryonic development, the transcriptional regulator Gli3 is expressed along with Hes1 in the apical progenitors in the VNO [6]. Gli3 acts as transcriptional repressor in the absence of Shh signaling, while Hes1 is a bHLH transcriptional repressor
that is essential for repressing neurogenic factors (e.g., Ascl1, Ngn1, Neurod1) [5]. In the VNO, Gli3 showed a similar role to the one described in the cortex in controlling the transitions of stem cells from proliferative to neurogenic program [6, 128]. In fact, characterization of Gli3 null mutants showed a drastic reduction of vomeronasal Ascl1+ neurogenic progenitors and VSNs [6].

The expression of the bHLH transcription factors Hes5 and Hes6 has also been documented in the vomeronasal sensory epithelium; however, loss of function studies are not conducted till now to study their role in VNO development [5, 69, 129].

N-myc is one of the myc proto-oncogene family members that is expressed as early as E9.5 in the olfactory placode at the onset of neurogenesis. Wittmann and colleagues studied the role of N-myc in olfactory and vomeronasal development using N-myc\textsuperscript{Foxg1Cre} conditional knockout mice [69]. N-myc deletion caused a reduction in the proliferation and neurogenesis, with a complete loss of Hes5 positive progenitors in both olfactory and vomeronasal epithelia specifically during established neurogenesis. Furthermore, mutant mice also showed reduced neuronal cell size, ultimately leading to severe atrophy of VNO and olfactory epithelium.

**Dichotomy and maturation of apical and basal VSNs.** Both the MOE and VNE originate from the common olfactory primordium and utilize similar transcriptional cascades to control neuronal specification and differentiation [75]. However, neuronal cell type diversity is one of many prominent features that distinguish VNO from MOE. The VNO contains G\textalpha i2+ apical and G\textalpha o+ basal VSNs as the 2 major neuronal types that are spatially segregated with respect to the basement membrane (Fig 1C). Yet, the
mechanisms that underlie cell fate determination is not fully understood. Bcl11b remains one of the earliest identified transcriptional factors that contributes to Gαi2 vs Gαo+ VSN cell fate determination [8]. Bcl11b expression begins at the vomeronasal grove stage by E11.5 [8]. Newborn Bcl11b−/− mice have a dramatic decrease in mature VSNs due to increased apoptosis. These mutant mice also display a reduction in the number of Gαo+ basal VSNs that selectively express the Tfap2e/AP-2e transcription factor and an increased proportion of Gαi2+ apical VSNs that selectively express the transcription factor Meis2.

These data highlighted the contributions of Bcl11b in the fate choice between Gαi2+ apical vs Gαo+ basal VSNs [130]. Later genetic lineage tracing confirmed that the transcription factor tfap2e/AP-2e is expressed specifically in Gαo+ basal VSN cell lineage [10]. Moreover, characterization of AP-2ε null mice showed that the loss of AP-2ε negatively affects the basal neuronal differentiation/maturation program [10]. In particular, loss of AP-2ε function induced a progressive loss of basal VSNs and aberrant gene regulation. In AP-2ε null mice, many cells that entered the basal program gradually expressed apical genes, such as Gαi2, V1Rs and Meis2. Based on these results, we proposed that AP-2ε is not needed to initiate the apical-basal VSN differentiation dichotomy, but instead is essential to maintain the basal VSN program and prevent the expression of apical VSN genes [10]. What role AP-2ε plays in controlling the chromatin landscape and expression of basal VSN specific genes requires further investigation.

Activating transcription factor 5 (ATF5) is another transcription factor that participates in the maturation of basal specific VSNs [131]. ATF5 is a member of the ATF/cAMP response element-binding (CREB) family of transcription factors and has well-
established prosurvival activity in different organs, including the MOE [132]. ATF5-/− mice showed a dramatic reduction in OMP positive mature VSNs specifically in the basal neuronal lineage due to increased apoptosis [131]. A follow-up study suggested that ATF5 may form a heterodimer with CCAT/enhancer binding protein gamma transcription factor, which then enhances the transcription of vomeronasal receptors [133]. However, the reasons for the specific reduction of $\text{G}_\text{a}^\omega+$ basal VSNs, not $\text{G}_\text{a}^\text{i}^2+$ apical VSNs, in ATF5 knockout mice are still unknown.

A recent study by Chang and Parrilla (2016) further characterized the expression of 28 homeodomain transcription factors in different postnatal VNO cell populations including neuronal progenitors, precursors, neurons and non-neuronal cells [134]. However, specific roles of these proteins in VNO development and maturation require further investigation. In addition to these transcription factors, proteins that comprise a vomeronasal signaling cascade are also vital for the maturation, survival and functionality of the vomeronasal neuronal network in the VNO [15, 37-40, 135-137].

1.3.3) miRNAs in vomeronasal neurogenesis.

Transcription factors are only a part of a complex regulatory system that aid neurogenesis. For example, microRNAs are a major class of non-coding RNAs that regulate the expression of transcription factors. In the MOE, conditional knockout of Dicer complex, an enzyme required for miRNA production, at the neuronal progenitor stage (Foxg1Cre) showed a degeneration of olfactory neuroepithelium [138]. In these mutants, vomeronasal thickening and invagination did not appear to be perturbed at E11.5; however, this study did not report VNO development in the later stages. Notably, Omp
Cre mediated Dicer ablation at a mature neuronal stage did not affect the number of olfactory/vomeronasal neurons, axonal guidance, glomeruli formation or animal behavior. This further underscore the importance of miRNAs during the early stages of neuronal proliferation and differentiation in the olfactory system. A separate study specifically analyzed the role of Dlx5 on miR-9 and miR-200 class miRNAs expression and how they affect the differentiation of olfactory/vomeronasal neurons [139]. Dlx5/- mutant mice showed a downregulation of miR-9 and miR-200 class microRNAs and an upregulation of Foxg1 protein in the olfactory epithelium. Moreover, this study confirmed that these microRNAs can target the 3'UTR of the Foxg1 mRNA to inhibit its translation. This paper not only connects the Dlx5 and Foxg1 regulation in the OE and VNO development, but also highlights how non-coding RNAs can participate in a complex transcriptional regulatory network controlling neuronal development.

1.3.4) Inductive signals in VNO development.

Extracellular cell signaling pathways also play an important role in olfactory placode determination and subsequent differentiation into respiratory, olfactory and vomeronasal epithelia. Inductive signals within the ectodermal cells or between the ectoderm and underlying mesenchyme are essential to induce the spatial and temporal changes in the ectodermal gene expression that are ultimately required for olfactory placodal patterning [64, 140, 141]. For example, culturing olfactory placodal ectoderm in vitro, after separating it from the underlying mesenchyme prior to morphogenesis, prevented the specification of olfactory epithelium [142]. However, recombining and culturing the ectoderm and mesenchyme led to distinct ectodermal thickening, invagination resembling in vivo olfactory pit formation and subsequent sensory neuron differentiation. This experiment
highlighted the importance of epithelial and mesenchymal interactions to induce specific cell fate decisions during the development of olfactory and vomeronasal epithelia. Indeed, studies reported the presence of both juxtacrine and paracrine inductive signals that interact to trigger the expression of specific transcription factors in the epithelia [143]. In this section, we will briefly discuss a few studies that highlighted the role of different signaling pathways underlying VNO development during both early and established phases of neurogenesis (Figs 2, 3).

Fibroblast growth factors (FGFs) are polypeptide growth factors that participate in cell proliferation, differentiation, and organogenesis. Multiple factors like Fgf3, -8, -9, -10, -15, -17 and -18 are expressed near the nasal pit region [144]. Of the various Fgfs, the role of Fgf8 has been studied in more detail. Initial studies showed Fgf8 expression at the rim of the invaginating olfactory pit between E10 - E11 and proposed the presence of primordial neural stem cells of the olfactory epithelium positive for both Sox2 and Fgf8 [2, 145]. Fgf8 loss of function induced the failure of both MOE and VNO development due to increased apoptosis [145]. In a subsequent study in chick and mice, Maier and coworkers (2010) showed that Bmp and Fgf signals specify respiratory and sensory epithelial cell fates, respectively [71]. This study showed that Fgf8 activity restricts Bmp signals to respiratory epithelium, aiding the expression of neuronal genes in the sensory epithelia. However, Forni and coworkers (2013) demonstrated a complex relationship between Fgf8 and Bmp4 signaling pathways with contributions from the underlying mesenchyme as an additional factor in respiratory vs neuronal specification (Fig 2A) [2]. The authors did not find Fgf8 expression in Sox2 or Pax6 positive neural stem cells. Using Fgf8Cre lineage tracing, they suggested that cells positive for the Fgf8 lineage were restricted to the
They also reported Bmp4 expression both in Fgf8 positive respiratory ectoderm along with the underlying mesenchyme. In response to BMP4, strong expression of noggin, a Bmp4 antagonist, was found in a distinct group of mesenchymal proximal to the developing VNO. This Noggin source may facilitate neurogenesis in the sensory epithelium, thus delineating respiratory vs neurogenic domains. The authors also proposed that the effect of Fgf8 inactivation on olfactory and VNO disruption is indirect and due to a broader Bmp4 expression, which expanded into the VNO region preventing neurogenesis (Fig 2B). A separate study probed the role of Fgf3 and Fgf10 in VNO development using Fgf3-/- and Fgf10-/- single mutants and double Fgf3-/-;Fgf10-/- mutation. However, no morphological or functional differences were identified in these mutants [146].

Figure 2: Cartoon summarizing some changes observed in FGF8 null mutants. Fgf8 (blue) is expressed by the cells forming the presumptive respiratory epithelium. Bmp4 (black dotted pattern) is also expressed in respiratory epithelium and by underlying mesenchyme, which further regulate the expression of Bmp4 antagonist Noggin (magenta) close to the GnRH-1 and vomeronasal neurogenic area. The expression of mesenchymal Noggin correlates with the formation of GnRH-1 and vomeronasal neurons. In Fgf8 null mutants, Bmp4 expression expanded into the vomeronasal and mesenchymal area disrupting the noggin source. Fgf8 mutants do not form GnRH-1 neurons and have no vomeronasal neurogenesis. Figure 2 is based on [1, 2].
Do inductive signals aide in defining neuronal identity in postnatal life? The mammalian main olfactory and vomeronasal epithelia display continuous neurogenesis throughout adulthood. However, our knowledge is limited regarding the role of inductive signals that control cell patterning, neuronal differentiation, and neuronal homeostasis in postnatal life. Basal/Gαo+ VSNs that express different V2R receptors appear distributed in specific sublayers of the basal territory of the VNO [47]. Moreover, another study on the MOE showed that the spatial position of stem cells in the MOE can determine the identity of olfactory neuronal subtype [147]. These data suggest that local inductive signals in the main and accessory olfactory systems could play a role in defining the identity of neurons based on their relative positions in the epithelia.

Functionality of the accessory olfactory system relies on the correct development of VSNs, the ability to detect and transduce signals, and the establishment of the correct glomerular map in the AOB [15, 37, 55, 148-152]. Our lab recently reported that active BMP intracellular signaling (phospho Smad1,5,8) is mostly restricted to VSNs in the basal regions of the VNO. Notably, the basal lamina of the vomeronasal epithelium, and the vasculature penetrating the VNE, is a rich source of Collagen-IV (Col-IV), a molecule with high affinity for BMP4 (Fig 3) [153]. By using AP-2εCre mice to conditionally knock out Smad4 in maturing basal vomeronasal neurons, we found that Smad4 mediated TGF/Bmp signaling is important for proper dendritic knob formation, pheromone-induced VSN activation, survival, and correct glomerular formation of Gαo+ basal VSNs in the posterior AOB. However, when we used OmpCre driver to knock out Smad4 from both apical and basal VSNs at a mature stage, we only found glomerular connectivity defects in the posterior AOB that reflected basal VSNs projections. These data suggest that
morphogenic inductive signals in postnatal stages are also critical for proper basal VSNs development and connectivity to the posterior AOB [11].

<table>
<thead>
<tr>
<th>BMP signaling is active proximal to the Collagen-IV rich basal lamina</th>
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<tr>
<td>Vasculature</td>
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<tr>
<td>Col-IV</td>
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<tr>
<td>VSNs</td>
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<tr>
<td>BMP signaling</td>
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<tr>
<td>VSE</td>
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<td>NSE</td>
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**Figure 3:** Schematic illustrating the relation between Collagen IV (Col-IV) positive basement membrane (magenta) and active BMP signaling (green) in the basal VSNs in the adult coronal VNO section. Collagen IV invades the basal regions of the VNO surrounding the vasculature (purple). The basal lamina can aid in sequestering the Bmp that generates Bmp signaling gradients in the basal territories of the VNO with stronger Smad1,5,8, activation (green) close to the basal lamina. VSNs- Vomeronasal sensory neurons; VSE- Vomeronasal sensory epithelium; NSE- Non-sensory epithelium Figure 3 is based on [11].

### 1.4 Conclusion and open questions

Due to its vestigial nature or lack of function in adult humans, investigation of VNO development and function is limited to a small number of labs (grantome.com). Regardless of the formation of a functional postnatal VNO, the presence of embryonic vomeronasal anlage that gives rise to migratory cell populations, such as the GnRH-1 neurons, cells of the migratory mass and the terminal nerve (Fig 1A), does occur in many
animal species [28, 29, 32]. Perturbations affecting the formation or migration of early migratory cells can lead to reproductive disorders like isolated forms of hypogonadotropic hypogonadism with a normal sense of smell and those associated with a lack of sense of smell (Kallmann syndrome) [6, 154-157]. Understanding the mechanisms that lead to the formation and migration of multiple neuronal and non-neuronal populations originating from olfactory placode will exponentially advance our knowledge to discover novel therapeutic strategies for these disorders. In addition, the VNO of rodents is an excellent model system to study neurogenesis, cell fate determination, axon guidance, and behavior [8, 43, 44, 148, 149, 158]. In this review, we highlighted some transcriptional factors, miRNAs and inductive signals that are important for the VNO development.

Until recently, only a few studies explored the molecular mechanisms underlying vomeronasal invagination and segregation from a common olfactory primordium. Fezf2 is shown to have VNO specific expression at the vomeronasal thickening stage during early development [122]. However, in Fezf2-/- knockout mice, initial VNO formation is observable until E13.5, which further suggests that it is not an essential factor in defining and segregating the VNO during the primary neurogenesis stage. In addition, Fgf8 can indirectly influence vomeronasal establishment via Bmp-induced noggin expression in the mesenchyme [1, 2]. We suggest that future investigations into the role of additional intrinsic and extrinsic factors both in the ectoderm and underlying mesenchyme may reveal additional mechanisms involved in the VNO formation. During the established phase of neurogenesis, Bcl11b is critical for the Gαi2+ apical vs Gαo+ basal VSN cell fate determination [8]. However, this view of neuronal cell fate determination seems rather
simplistic. Further studies should identify the mechanisms that induce Bcl11b expression and subsequent apical vs basal VSNs’ fate determination.

In the MOE, studies identified signaling pathways that provide key feedback for regulating olfactory neurogenesis [159]; however, such mechanisms remain unknown in the VNO. One interesting question is to study whether there is one common or distinct negative feedback mechanisms to control neurogenesis of G\(\alpha_i^2\)+ apical and G\(\alpha_o^+\) basal VSNs. Further outstanding questions remain - what signaling mechanisms are involved in the separation of the VNO into sensory and non-sensory epithelium during VNO development and why adult neurogenesis still occurs at the marginal zones of the VNO. Studying the role of cell autonomous factors vs those surrounding the stem cell niche in the adult neurogenesis at marginal zone of the postnatal VNO is a worthwhile endeavor.

Moreover, the role of Sustentacular cells has primarily been studied in the MOE. Few studies highlighted differential expression of metabolizing enzymes and genes in the sustentacular cells of the MOE compared to the VNO [9, 160]. Do sustentacular cells in the MOE and VNO have similar functions? Furthermore, factors that induce specification of neuronal vs sustentacular cells from a common multipotent stem cells are still unknown in the VNO. Interestingly, recent studies from the MOE using post-injury regeneration model found a role of Wnt and Notch signaling pathways in differentiating stem cells towards neuronal and non-neuronal supporting cells respectively [161, 162].

In conclusion, many open questions still remain about the formation of early migratory neuronal populations and the specification of the embryonic vomeronasal structure. Moreover, the key molecular mechanisms controlling neurogenesis and cell differentiation in the postnatal vomeronasal organ are still unresolved. The embryonic
VNO anlage likely gives rise to migratory cells that control sexual development, while the postnatal VNO plays a crucial role in social interactions of animals. Further understanding of the molecular mechanisms underlying the development of the pre and postnatal vomeronasal organ will impact the fields of neural development, evolutionary biology, ethology, and the medical field.

1.5 Rationale and objectives

Multipotent stem cells in the mouse vomeronasal sensory epithelium can differentiate into three different cell types - V1R/Gαi2+ apical, V2R/Gαo+ basal VSNs being the two major neuronal types besides the sustentacular cells being the third supporting non-neuronal cell type. Complex interactions between extrinsic and intrinsic signaling factors control the differentiation of multipotent stem cells to non-neuronal vs neuronal cells and in turn within neuronal cells, apical and basal VSNs. Identifying the mechanisms controlling the stem cell regulation in the VNO can also be extended to understand the normal and abnormal embryonic development in other regions of the central and peripheral nervous systems.

In my doctoral dissertation, I worked on two long lasting questions in the VNO development field. Firstly, in chapter 2, I explored the mechanisms underlying apical vs basal cell fate determination. We performed single cell RNA sequencing (scRNA-seq) using 10X genomics at both early-postnatal and adult ages and identified the expression of Notch signaling related genes, specifically Notch1 receptor and Dll4 ligand at the VSN dichotomy. This suggests that Notch signaling is conserved in apical vs basal VSN cell fate at both embryonic and adult stages. We further used Cre-loxp mouse models to
validate and probe the role of Notch signaling in the VSN neurogenesis specifically at early postnatal age to take advantage of higher rate of neurogenesis. Supporting the transcriptomic data, lineage tracing of neuronal progenitors (Ascl1+ cells) showed traced Notch1+ and Dll4+ cells lying close to each other. If Notch signaling is involved in VSN cell fate determination, then knocking out the receptor should cause a shift in the apical vs basal VSNs formation. To test this, we opted for Notch1 receptor loss of function studies at the neuronal progenitor’s stage (Ascl1+ cells). Similarly, in a parallel study, we also knocked out Rbpj which is a member of transcriptional activation complex in canonical Notch signaling. Both Notch1 and Rbpj knockouts showed a shift in VSN neurogenesis towards apical cell fate. In the contrary, gain of function studies by conditional induction of Notch signaling at neuronal precursors stage (Neurog1+ cells) led the VSN neurogenesis shift towards the basal VSN cell fate. Interestingly, to our surprise, similar Notch gain of function studies at much earlier Ascl1+ progenitor stage, led them to differentiate towards non-neuronal sustentacular cells. Overall, this study suggests that Notch signaling via Notch1-Dll4 at neuronal precursors stage is important for the apical vs basal VSN cell fate determination. Nonetheless, further research is needed to understand if other Notch mechanisms are involved in the formation of non-neuronal sustentacular cells.

In chapter 3 of my dissertation, I worked on identifying horizontal basal stem cells (HBCs) in the VNO and exploring their role in the development. In the main olfactory epithelium, from decades of research, HBCs are known to be second population of multipotent stem cells that are mostly quiescent in normal conditions. However, when there is an injury to the epithelium, HBCs can proliferate and regenerate the main olfactory
epithelium within weeks of time. Surprisingly, except their presence in the VNO from a
1995 study [163], nothing much is known about their role in the VNO. Initially,
immunofluorescence against different HBC markers like p63, Krt5, Krt14, Sox2 identified
these cells in two specific regions of the VNO - 1) on the basement membrane of the non-
sensory epithelium (NSE); 2) on the basement membrane of the marginal zone and
sensory epithelium. We also showed that p63+ HBCs are first seen in the NSE of the
VNO at E15.5 embryonic stage and slowly expand towards the sensory epithelium. This
suggests that HBCs in the VNO are formed at comparatively late stages with respect to
vomeronasal embryonic stem cells and VSNs. Moreover, constitutive P63 Cre lineage
tracing showed that HBCs can give rise to both neuronal and sustentacular cells in the
sensory and only non-neuronal cells in the NSE of the VNO suggesting their role in the
early development of the VNO. As the VNO has the ability to undergo adult neurogenesis
throughout life at the marginal zones (MZ) of the epithelium, we performed conditional
lineage tracing of these cells at 4-5 weeks of age. This study showed the presence of
neuronal and non-neuronal cells in the MZ suggesting that HBCs also have role in the
adult neurogenesis. Overall, our study confirmed the presence of HBCs in the VNO and
revealed their multipotency in both early VNO development and adult stages opening a
new area of research in the VNO.
Table 1: Transcription factors playing a role in olfactory/VNO development.

<table>
<thead>
<tr>
<th>Transcription factor</th>
<th>Phenotype</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Pax6</td>
<td>No olfactory placode</td>
<td>Grindley, Davidson, and Hill 1995</td>
</tr>
<tr>
<td>Sox2</td>
<td>MOE disrupted by E10.5. VNO phenotype not described</td>
<td>Panaliappan et al. 2018</td>
</tr>
<tr>
<td>Six1</td>
<td>No VNO or MOE formation</td>
<td>Ikeda et al. 2010</td>
</tr>
<tr>
<td>Six1; Six4</td>
<td>No olfactory pit</td>
<td>Chen, Kim, and Xu 2009</td>
</tr>
<tr>
<td>Foxg1</td>
<td>No VNO formation</td>
<td>Duggan et al. 2008</td>
</tr>
<tr>
<td>Dlx5</td>
<td>VNO rudimentary or lost</td>
<td>Long et al. 2003</td>
</tr>
<tr>
<td>Fezf2</td>
<td>No VSNs by P0</td>
<td>Eckler et al. 2011</td>
</tr>
<tr>
<td>Ascl1</td>
<td>Drastic reduction in VSNs</td>
<td>Murray et al. 2003</td>
</tr>
<tr>
<td>Ascl1; Ngn1</td>
<td>Reduction in VSNs</td>
<td>Cau, Casarosa, and Guillemot 2002</td>
</tr>
<tr>
<td>N-myc</td>
<td>Reduction in proliferation and VSN neurogenesis</td>
<td>Wittmann et al., 2014</td>
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<tr>
<td>Gli3</td>
<td>Reduction in Ascl1+ cells and VSNs</td>
<td>Taroc et al. 2020</td>
</tr>
<tr>
<td>Bcl11b</td>
<td>Increased Gα2+ VSNs and decreased Gαo VSNs</td>
<td>Enomoto et al. 2011</td>
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<tr>
<td>Tfap2e/AP-2ε</td>
<td>Reduction in basal VSNs and change in basal VSN identity</td>
<td>Lin et al. 2018</td>
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<tr>
<td>Atf5</td>
<td>Reduction in basal VSNs</td>
<td>Nakano et al. 2016</td>
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CHAPTER 2

Notch signaling determines cell-fate specification of the two main types of vomeronasal neurons of rodents.

This chapter was published as Katreddi et al., Notch signaling determines cell-fate specification of the two main types of vomeronasal neurons of rodents. Development 1 July 2022; 149 (13): dev200448. doi: https://doi.org/10.1242/dev.200448

2.1 Introduction: Neural stem/progenitor cells can give rise to multiple neuronal cell types that differ in gene expression, function, and neuronal connectivity. Investigating the molecular mechanisms that establish different neuronal cell fates is crucial to understand how neuronal systems evolve and form and to identify mechanisms underlying neurodevelopmental disorders [164-166]. The vomeronasal organ (VNO) is a specialized chemosensory organ that, in many vertebrate species, is located at the base of the nasal cavity [167]. The VNO is responsible for the detection of semiochemicals, molecules that can trigger stereotypical mating/sex behaviors, parental behaviors, and predator avoidance [12-15, 168]. Most of the vertebrates with a functional VNO have a uniform vomeronasal (VN) system with vomeronasal sensory neurons (VSNs) expressing receptors of the V1R family [169]. However, rodents and some marsupials have developed a more complex binary VN system including a second type of VSNs expressing receptors of the V2R family [170]. In mice, the V1R neurons express Gai2 G protein subunit, Meis2 transcription factor and mostly distributed in the apical territories of VSE, often referred to as apical VSNs. [8]. Conversely, the V2R neurons express Goo G protein subunit, tfap2e (AP-2ε) transcription factor, for the most part, located in
basal regions of the VSE and around the vasculature and are called basal VSNs [8, 10, 171-173]. Apical and basal VSNs detect distinct types of ligands, connect to different areas of the accessory olfactory bulb (AOB) and control distinct behaviors [37-40, 56].

In mice, VSN neurogenesis starts during embryonic development around E11.5 and continues, throughout life, starting from a limited number of progenitors in the marginal zones of the VNO [6, 7, 75, 84, 174]. How the cell fate of apical and basal VSN types is established is not fully understood. A pivotal study by Enomoto and coworkers previously showed that the transcription factor Bcl11b plays a key role in controlling the correct establishment of apical and basal VSNs in the developing VNO [8]. However, which extrinsic signaling pathways control the expression of Bcl11b in the VNO has not been investigated. Moreover, the same study identified, the transcription factor AP-2ε as a potential target of Bcl11b and suggested a role for AP-2ε in controlling basal neuron differentiation. In a follow-up study, we proposed that the AP-2ε is not responsible for initiating the apical vs basal identity bifurcation but is instead crucial for the expression of several genes defining, and maintaining, basal VSN molecular identity [10]. In fact, we observed that after AP-2ε loss of function, basal VSNs lose their expression of basal genes (Gαo and V2Rs) and switch to expressing apical genes such as Gαi2 and V1Rs [10].

In this study, we adopted a single cell sequencing strategy to investigate the mechanisms involved in directing cell fate specification and differentiation of apical and basal VSNs. By following the transcriptomic profile of postnatal stem cells/progenitors and immature VSNs, we identified differential expression of Notch1 receptor and Dll4 ligand across Neurog1/Neurod1 positive VSN precursors. We then adopted both loss of function
(LOF) and gain of function (GOF) in vivo strategies to test the role of Notch signaling pathway in establishing VSN binary differentiation fates. Genetic experiments confirmed differential effects of loss and gain of Notch signaling on the switch between apical and basal VSN cell fate, with Notch LOF directing cells towards apical fate and GOF towards basal fate. Our data characterizing the signaling responsible for more complex VNO development offer a new context in which Notch-mediated cell fate choice is a conserved strategy for establishing binary cellular diversification and increasing the neuronal repertoire in developing neuroepithelia.

2.2 RESULTS

2.2.1 Single cell profiling of the whole adult VNO identifies VSN dichotomy.

In mice, adult neurogenesis occurs throughout life at the marginal zones of the VSE [7, 83]. To identify multiple cell types of the VNO including stem cells, VSNs and non-neuronal cell types, we performed single cell RNA sequencing (scRNA-seq) from whole VNO. We dissociated single cells from a total of 5 male mice at P60 age for the sequencing. For the analysis, we filtered out low quality cells and performed clustering and analysis of the scRNA-seq data using Seurat [175]. A total of 10,582 single-cell transcriptomes passed quality control measures. Based on the expression of the top 2000 highly variable features across the population, cells were clustered in Seurat object 1 and visualized using uniform manifold approximation projection (UMAP) [176] (Fig. 4A). We identified neuronal and non-neuronal cell types in the VNO and annotated clusters based on the following gene expression: Basal cells (Trp63, Krt5), stem cell progenitors (Sox2, Ascl1), neural precursors (Neurog1, Neurod1), immature neurons (Gap43), mature VSNs
(Omp), sustentacular cells (Fezf2, Cyp2a5), olfactory ensheathing cells (S100b, Plp1, Mpz, Sox10), pericytes (Pecam1, Eng, Sox17), vascular smooth muscle cells (Tagln, Acta2), Vegfa+ cells (Vegfa), T cells (Cd3d, Cd3e), B cells (Cd19, Cd79a), macrophages (C1qa, C1qb), monocytes (Chil3, Clec10a, Ccr2) (Fig. 4A, Fig. S1). We focused our further clustering and analysis on stem cell progenitors, neural precursors, and immature neurons with the goal of characterizing the mechanisms underlying VSN cell fate specification. We subset these clusters into Seurat object 2 (Fig. 4B) and re-clustered based on highly variable features among the selected cells. The expression pattern of known apical and basal specific genes (Fig. 4C) further corroborated the scRNA-seq sub clustering [6, 8, 10]. Interestingly, Sox2 expression was found to extend from the Ascl1+ progenitor stage cells to Neurog1+ and some Neurod1+ neuronal precursor stage cells (Fig. 4D, Fig. 5B), similar to the findings in the main olfactory epithelium [127, 177]. In addition, we found that Neurod1 expression, which coincides with Neurog1 in the initial expression, is retained beyond the dichotomy splitting apical and basal VSN lineages (Fig. 4D, Fig. 5B). We labelled Ki67+/Neurog1+/Neurod1+ cells before the dichotomy as immediate neuronal precursors, whereas Ki67-/Neurod1+ cells in apical and basal lineages are considered as post-mitotic apical and basal VSN precursors respectively (Fig. 4B, C).
Figure 4: scRNA-seq identified adult neurogenesis and VSN apical-basal dichotomy in the mouse VNO. 

A) UMAP dimensional reduction plot of Seurat object1 shows neuronal and non-neuronal cell clusters of the VNO. Each colored cluster of cells corresponds to an identified cell type that has similar transcriptomic profile. 

B) Seurat object2 generated from stem cell progenitors, neural precursors and immature neurons of Seurat object1 identifies the VSN apical-basal dichotomy. 

C) Heatmap of known gene expression that is specific to each stage of the VSNs formation. 

D) Feature plots of Sox2, Ascl1, Neurog1 and Neurod1 in Seurat object2. 

E) Summary cartoon depicting dynamic expression of stage-specific transcription factors throughout apical and basal VSN differentiation.
A pseudotime reconstruction was generated by implementing Monocle single cell trajectory analysis on Seurat object 2 [178, 179]. In this analysis, we chose Ascl1-positive neuronal progenitors as a root node (less differentiated stage), and the rest of the single cells in Seurat object 2 were ordered in an unbiased way along the pseudotime. This revealed the formation of a differentiation branched trajectory. This analysis confirmed the validity of the VSN dichotomy obtained from initial Seurat analysis (Fig. S1).

2.2.2 Differential gene expression identifies the expression of Notch signaling related genes at the VSN dichotomy.
To further focus on the origin of the VSN dichotomy, we reclustering Ascl1+ progenitors and VSN neuronal precursors to form Seurat object 3 (Fig. 5A). Feature plots of Ascl1, Sox2, Neurog1 and Neurod1 support the temporal transcriptional cascade as neuronal progenitors proliferate and differentiate into immediate neuronal precursors (Fig. 5B). Further analysis showed, as expected [8, 10], specific expression of the transcription factor Meis2 in post-mitotic (Ki67-negative) cells acquiring apical VSN identity and specific AP-2ε expression in maturing (Gap43-positive) basal VSNs (Fig. 5C). Confirming prior results [10], the expression of the basal-specific gene AP-2ε appears at later stages of maturation compared to apical-specific Meis2. Given a previous study [8] suggesting that Bcl11b is a key gene controlling cell fate choice of apical and basal VSNs, we assessed the spatial expression pattern of Bcl11b in this cell population (Fig. 5D). Initial Bcl11b expression was seen at Neurog1/Neurod1 differentiation stages post Ascl1 expression. On the basal VSN trajectory, Bcl11b expression occurred as a continuum from the dichotomy throughout the immature VSN stage, whereas on the apical VSN trajectory, Bcl11b expression was not detected until early stages of maturation (Fig. 5D,
arrows). Based on this observation, we manually clustered Bcl11b positive and negative cells specifically at the point of separation (dashed boxed area in Fig. 5D and 5Di). Performing differential gene expression analysis between Bcl11b positive and negative cells specifically at this point (Fig. 5Di), we found Notch1 receptor as enriched in the Bcl11b positive cluster, whereas Dll4, which is a Notch ligand, is enriched in the Bcl11b negative cluster (Fig. 5Dii).

Moreover, we also identified Hes5, Hey1, Nrarp, and Ccnd1 genes enriched in the Bcl11b-positive cluster (Fig. 5Dii), which along with Bcl11b are known to be downstream Notch signaling targets [5, 180-183]. Spatial colocalization plots of Notch1 and Dll4 reinforced their mutually exclusive cellular expression even before the apical-basal dichotomy was established (Fig. 5E). Even though Notch1 mRNA could be detected in both apical and basal maturing VSNs after the split (Fig. 5E), Notch downstream targets including Hey1, Hes5, Nrarp and Ccnd1 appeared to be expressed only in Notch1+ but not Dll4+ cells before the VSN bifurcation and in the early stage basal VSN trajectory (Fig. 5F). In addition, Dll4 ligand expression co-occurred along with Meis2, the apical-specific VSN marker, whereas downstream Notch signaling target Hey1 was instead expressed along the basal VSN trajectory before AP-2ε (Fig. 5G, H). Notably, the expression of other known Notch receptors and ligands within the neuronal precursors was found to be minimal (Fig. S2). In addition, many cells that are negative for Notch downstream target genes at the split point are positive for expression of the negative regulator of Notch signaling, Numb, suggestive of post-translational Notch degradation after the split (Fig. S3) [184, 185]. These gene expression patterns suggest that Notch signaling is
selectively activated in the cells acquiring basal identity during the establishment of the apical vs basal dichotomy but not after.
To further validate the occurrence of differential Notch-Delta expression in developing mouse VSNs, we performed immunofluorescence staining for Dll4, Notch1, and Neurod1 on vomeronasal tissue at postnatal day 1 (P1). Imaging confirmed the presence of immunodetectable Notch1 and Delta4 limited to Neurod1 positive precursors in the VNO (Fig. 6A, B, C). This analysis also highlighted the proximity of Dll4+ and Notch1+ cells. These data suggest that differential Delta-Notch expression and activation of Notch signaling, in newly formed VSN precursors, could play a role in establishing a binary outcome in the differentiation of VSNs. Although we obtained scRNA-seq data from adult mice, we chose to further validate and probe Notch1-Dll4 signaling at early postnatal
age to take advantage of higher rate of neurogenesis [186]. Notably, scRNA-seq analysis at P10 showed conserved developmental trajectories of apical and basal VSNs as we

Figure 6: Notch1 and Dll4 immunoreactivity in Neurod1+ cells. A-Aii) Immunofluorescence anti Dll4 and Notch1 shows expression of Notch ligand and receptor in marginal zones (MZ) and basal regions of the VNO at postnatal day1. Inset shows Dll4+ (arrow) and Notch1+ (arrowhead) cells in close proximity. B) Immunofluorescence anti Notch1 (green) and Neurod1 (magenta). Notch1 expression co-occurs in the Neurod1+ stage (see arrow in the magnification). C) Immunofluorescence anti Dll4 (green) and Neurod1 (magenta); Dll4 ligand expression occurs in the Neurod1+ stage (see arrow in the magnification). D-dorsal, V-ventral, L-lateral, M-medial.
described at P60 (Fig. S4). An in depth bioinformatic analysis of p10 VNO scRNA seq data is also available elsewhere [187].

2.2.3 Conditional lineage tracing confirms the formation of Notch1+ and Dll4+ cells from Ascl1+ progenitors.

Temporally controlled genetic lineage tracing of stem cells/progenitors can be used to follow proliferation and differentiation dynamics [188]. We performed conditional lineage tracing of Ascl1CreERT2/R26tdTom P1 pups and collected VNOs at 1, 3, 7, and 14 days post injection (dpi) (Fig. 7A). We performed immunostaining against Ki67, Neurod1 and tdTom to determine: 1) How long does it take for Ascl1 progenitors to differentiate and become post mitotic? and 2) Do Ascl1+ progenitors give rise to both Dll4+ and Notch1+ VSN precursors?

After a single tamoxifen injection, we observed that the number of Ascl1CreERT2 traced cells increased steadily from 30.3±2.5 cells at 1 dpi to 142.1±14.3 cells at 3dpi, 225.1±33.7 cells at 7 dpi and 199.8±10.8 cells at 14dpi (mean±SEM). Notably proliferation decreased with age, as ~88% of the traced cells at 7dpi and a near totality at 14dpi were post-mitotic. (Fig. 7B). These data suggest that Ascl1+ transit amplifying cells [189] of the VNO undergo proliferation before becoming postmitotic neurons.

Analyzing the expression of Neurod1, which is a marker of cells undergoing differentiation (Fig. 4D, 5B), we observed that Neurod1+Ascl1- traced cells transiently increased from 16±1.5% at 1dpi to 39±0.3% at 3 dpi before decreasing to 17±2.2% at 7dpi and 0.3±0.1% at 14dpi (mean±SEM) as the cells start to mature (Fig. 7B). These results together indicate that by 1-2 weeks after the initiation of Ascl1 lineage tracing, most of the cells have committed to neuronal differentiation.
Figure 7: Ascl1 lineage tracing reveals that both Dll4+ and Notch1+ cells are progeny of Ascl1+ cells. A) Ascl1Cre<sup>ERT2</sup>/R26tdTom pups were injected with tamoxifen at P1 and perfused at 1, 3, 7 and 14 days post injection (dpi). B) Plot showing differentiation time course of Ascl1+ traced neuronal progenitors. Data points are % tdTom+ traced cells that are Ki67 positive and Neurod1 positive quantified at different stages. C) Top panel reflects the increase in tdTom+ traced cells from 1dpi to 14dpi. Bottom panel shows immunofluorescence anti Ki67 and tdTom. D) Immunofluorescence anti Dll4, Notch1 and tdTom in Ascl1Cre<sup>ERT2</sup> lineage traced pups at 3dpi. Inset shows both Dll4+ (arrow) and Notch1+ (arrowhead) cells in the marginal zone (MZ) that colocalized with tdTom tracing. E) Immunofluorescence anti Notch Intracellular domain (NICD) and tdTom in Ascl1Cre<sup>ERT2</sup> lineage traced pups at 3dpi. Inset shows tdTom+ cells colocalized with NICD staining in the MZ (arrow). n=3 biological replicates. Data shown as mean±SEM.
We further performed Dll4/Notch1/tdTom immunofluorescence staining after Ascl1Cre\textsuperscript{ERT2}/R26tdTom tracing at 3dpi (Fig. 7D). As expected, we saw Dll4+/tdTom+ and Notch1+/tdTom+ double positive cells mostly in the marginal and basal zones of the vomeronasal sensory epithelium, where most of the postnatal neurogenesis takes place. Moreover, staining for cleaved Notch Intracellular Domain (NICD) confirmed the presence of Ascl1+ traced progeny undergoing active Notch signaling at 3 dpi (Fig. 7E). In summary, these results suggest that Ascl1+ progenitors divide to give rise to Neurod1+ precursors that transiently express either Dll4 ligand or Notch1 receptor and are competent for active Notch signaling.

2.2.4 Loss of active Notch signaling leads maturing VSNs to default to the apical cell fate.

To directly test the role of Notch signaling in controlling the differentiation of VSNs, we conditionally knocked out Notch1 receptor from the Ascl1+ progenitor stage onwards. We used the Ascl1Cre\textsuperscript{ERT2} driver, as both Notch1 and Dll4 expression start immediately after the Ascl1+ stage (see Fig. 5B, 5E). We induced Cre recombination at P1 in both Ascl1Cre\textsuperscript{ERT2}/R26tdTom\textsuperscript{+/−} controls and Ascl1Cre\textsuperscript{ERT2}/Notch1\textsuperscript{fl/fl}/R26tdTom\textsuperscript{+/−} KO pups and analyzed cell phenotypes at 7 days post injection (Fig. 8A). In control pups, 1 week after tamoxifen injection, 50.7±0.4% of the traced cells resulted to be positive for the apical VSN marker Meis2, 33.2±0.8% cells were positive for basal VSN marker AP-2ε and 15.4±0.4% of traced cells didn’t express either Meis2 or AP-2ε+ yet (mean±SEM) (Fig. 8D). In the Ascl1Cre\textsuperscript{ERT2}/Notch1 conditional KOs (cKO), 1 week after recombination, the Meis2+ apical population significantly increased to 66.9±1.7%, whereas the AP-2ε+
basal population decreased to 15.17±1.1% and the rest of the 17.1±0.6% traced cells didn’t express both markers (mean±SEM) (Fig. 8D). In order to follow the cell fate decision of the Meis2/AP-2ε double negative population, we also analyzed the Ascl1CreERT2/Notch1 KO pups at 14dpi (Fig. 8 B, C). Two weeks after recombination, we still detected a higher number of Meis2+/apical cells in the cKOs, however, the population of Meis2/AP-2ε double negative cells was reduced to ~1% of the total traced cells. These data suggest that most of the double negative cells found at 7dpi matured to either apical or basal neurons (Fig. 8Di).

In line with previous observations [8, 10], our scRNA-seq data show that newly formed apical and basal neurons first co-express Gαo and Gαi2 and then, over time, establish either Gαi2/apical or Gαo/basal identity (Fig. 8E). By staining sections of Ascl1CreERT2/Notch1 KO and Ascl1CreERT2/R26tdTom+/− controls at 14dpi against the Gαo/Gnao1 we observed a significantly smaller percentage of Gαo/Gnao1 traced cells in cKOs (Fig. 8 F-H). Similarly, after immunostaining against Gαi2/Gnai2 we observed an increase in the percentage of cells with apical features. (Fig. 8I). These results overall suggest that active Notch signaling, via the Notch1 receptor, is necessary for the activation of the basal differentiation program.
Figure 8: Notch1 LOF biases VSN differentiation to the apical fate.

A) Ascl1Cre<sup>ERT2</sup>/Notch1<sup>fl/fl</sup>/R26tdTom and Ascl1Cre<sup>ERT2</sup>/R26tdTom pups were injected with tamoxifen at the P1 stage and perfused at 7dpi. Ai) Expected results with Notch1 receptor knockout driving the progenitors towards the apical VSN fate.

B-C) Immunofluorescence anti Meis2, AP-2ε and tdTom in Ascl1Cre<sup>ERT2</sup> induced control and Notch1 cKO pups at 14dpi. Arrows highlight traced Meis2+ and arrowheads highlight AP-2ε+ VSNs.

D, Di) Quantification of the percentage of traced VSNs that are Meis2+, AP-2ε+ VSNs and Meis2/AP-2ε double negative cells in control and Notch1 KO mice at 7dpi and 14dpi stages.

E) Feature plots of Gna2 and Gnao1 genes in the Seurat object2 show that both G proteins are transcriptionally active in both VSN branches at the early stages of the dichotomy before getting restricted to apical vs basal VSN branches respectively (see dotted circle).
We then analyzed the effects of induction of conditional Notch1 ablation at a later developmental stage by using Neurog1Cre\textsuperscript{ERT2}/Notch1\textsuperscript{fl/fl}/R26tdTom\textsuperscript{+/-}. We did this to understand whether Notch1 LOF at Neurog1 stage could still deviate the cell fate towards the apical VSNs. We injected tamoxifen at P1 in both Neurog1Cre\textsuperscript{ERT2}/R26tdTom\textsuperscript{+/-} and Neurog1Cre\textsuperscript{ERT2}/Notch1\textsuperscript{fl/fl}/R26tdTom\textsuperscript{+/-} pups and perfused after 1 week to determine the VSN cell fate switch (Fig. S5). At 7dpi, Meis2/\textsuperscript{AP-2ε/tdTom immunofluorescence analysis showed no significant changes in the proportion of Meis2+ vs P-2ε+ VSNs in the cKOs when compared to controls (Fig. S5 B, C, D). These data suggest that activation of Notch1 signaling has the ability to define the VSNs’ fate in a restricted developmental window, whereas activation at later stages has no effect on the cell fate.

The role of Notch signaling in cell fate specification is conserved in many neuronal and non-neuronal systems [190-193]. Studies in retina and spinal cord determined that the forkhead transcription factor Foxn4 has a role in inducing Notch1- Dll4 mediated signaling [193-195]. In both systems, it has been shown that Foxn4 controls the expression of Dll4 and that Foxn4 mutants have aberrant cell fate phenotypes [193, 194]. In line with this, co-expression of Foxn4 and Dll4 in scRNA-seq analysis and
immunofluorescence staining at E14 VNO, suggested a potential role for Foxn4 in VSN differentiation (Fig. S6). However, we found out that Foxn4 null mutants maintained detectable Dll4 expression at E14 stage and we saw no obvious changes in the apical-basal VSN ratio. These data suggest that in the VNO, Foxn4 is dispensable for the induction of Dll4 expression.

2.2.5 The basal VSN differentiation program is established via canonical Notch1 signaling.

Notch signaling can happen either via canonical or non-canonical pathways [196, 197]. In the canonical pathway, once NICD is cleaved after the interaction with ligand+ signal sending cell, it translocates to nucleus to form transcriptional activation complex with Rbpj, MAML and other co-activator factors [197, 198]. To test whether basal VSN cell fate determination is occurring via canonical Notch pathway, we conditionally knocked out Rbpj protein using Ascl1CreERT2 driver at P1 and collected pups 1 week after recombination (Fig. 9A). At 7dpi stage, Meis2/AP-2ε/tdTom immunofluorescence analysis revealed a ~13% increase in Meis2+/apical VSNs in the Ascl1CreERT2/Rbpjfl/fl/R26tdTom1/−cKOc compared to Ascl1CreERT2/R26tdTom1/− control pups. In line with this, AP-2ε+/basal population resulted to be smaller in the conditional Rbpj KOc. Similar to what we observed after Notch1 ablation, we also found a proportion of Meis2/AP-2ε double negative cells in Rbpj KOc (Fig. 9 B, C, D). The highly overlapping results obtained after Notch1 and Rbpj ablation suggest a prominent role of canonical Notch signaling in establishing the basal VSN program.
2.2.6 Ascl1Cre<sup>ERT2</sup> induced Notch1 deletion redirects the cell fate of precursors without changing proliferation or cell death rate.

Notch signaling has well established role in proliferation and differentiation of neuronal progenitors [199]. To test if the increase in apical VSNs’ percentage in the Ascl1Cre<sup>ERT2</sup>
driven Notch1 cKOs is sculpted by changes in the proliferation or cell death, we analyzed Ascl1CreER\textsuperscript{ERT2}/R26tdTom\textsuperscript{+/−} controls and Ascl1CreER\textsuperscript{ERT2}/Notch1\textsuperscript{flo/flo}/R26tdTom\textsuperscript{+/−} KO pups at 1day and 3days post tamoxifen injection (Fig. 10A). Both Ki67/tdTom and cleaved caspase3 (cc3)/tdTom immunofluorescence staining didn’t show any significant changes in rate of proliferation or cell death among traced cells (Fig. 10B).

However, we decided to investigate if we could detect changes in the differentiation trajectory of the proliferative precursors in Notch-1cKOs. For this, we analyzed Bcl11b expression as this is an early marker for basal differentiation and a target of Notch transcription activation complex [8, 183] (Fig. 5B, C, D; Fig 10C). Interestingly at 3dpi, we found a significant reduction in proliferative Bcl11b cells in Notch1 cKO pups compared to controls (Fig. 10D, E, F). These data suggest that Ascl1Cre\textsuperscript{ERT2} driven Notch1 ablation doesn’t affect the overall proliferation or cell death, but prevents Bcl11b expression, which, as previously observed in Bcl11b KO [8] redirects the cell fates towards the Meis2+/apical fate.
No changes in proliferation or cell death in Notch1 cKOs

1 dpi

3 dpi

Proliferative Bcl11b+ cells are putative basal VSNs precursors

Notch1 cKO has reduced number of Bcl11b proliferative cells

Ascl1cko/+/R26tdTom+ (Control) at 3 dpi stage

Ascl1cko/+/Notch1cko/+/R26tdTom+ (KO) at 3 dpi stage
2.2.7 Conditional induction of NICD at the Ascl1 progenitor stage leads to sustentacular cell formation.

Our results upon ablation of Notch1 suggest that without Notch signaling, VSN progenitors’ default towards the apical VSN cell fate. To further test whether active Notch signaling is sufficient to initiate the basal VSN genetic program, we conditionally overexpressed NICD at the Ascl1+ stage, using Ascl1CreE<sup>ERT2</sup>/R26NICD<sup>+/−</sup> pups. We aimed to test if activation of Notch signaling would redirect all the recombined NICD+ cells towards the basal VSN phenotype. In the inducible experiment, we counted GFP positive cells to follow NICD recombined cells as these mice have Cre<sup>ERT2</sup> activated NICD along with nuclear localized EGFP reporter [200]. There were fewer total EGFP+ NICD
recombined cells compared to control Ascl1Cre\textsuperscript{ERT2}/R26tdTom tracing, which may be due to low efficiency in R26NICD recombination as previously reported [201].

We induced Cre recombination at P1 in both control Ascl1Cre\textsuperscript{ERT2}/R26tdTom\textsuperscript{+/-} and Notch inducible Ascl1Cre\textsuperscript{ERT2}/R26NICD\textsuperscript{+/-} pups and assessed cell fate at 7 days post injection (Fig. 11A). We performed immunofluorescence against tdTom/HuC/D and GFP/HuC/D to distinguish between sustentacular cells (HuC/D-) and VSNs (HuC/D+) in control and mutant mice. At 7 dpi in control mice, 99.3±0.1% (mean±SEM) of Ascl1-traced cells became VSNs, and fewer than 1% of the traced cells becoming non-neuronal sustentacular cells (Fig. 11 B, F).

Surprisingly, in Ascl1Cre\textsuperscript{ERT2}/R26NICD\textsuperscript{+/-} pups, we found conditional NICD overexpression in Ascl1+ progenitors primarily induced the formation of non-neuronal sustentacular cells (Fig. 11 C, F). This experiment showed that, in the NICD-inducible pups, only 34.1±1.2% (mean±SEM) of the recombined cells were classified as VSNs (Fig. 11 C, F). At 7dpi, we further analyzed Meis2 expression as it is expressed by sustentacular cells and apical VSNs [134] (Fig. 11 D, E). These data showed that the non-neuronal cells formed after ectopic NICD expression became sustentacular cells. All together, these data suggest that sustained activation of NICD in Ascl1+ progenitors lead them to differentiate into sustentacular cells rather than into neurons.
Figure 11: Ectopic expression of NICD at the Ascl1+ stage diverts the progenitors towards sustentacular cell fate. A) Ascl1Cre$^{ERT2}$/R26NICD and Ascl1Cre$^{ERT2}$/R26tdTom pups were injected with tamoxifen at the P1 stage and perfused at 7dpi. Ai) Expected result with NICD overexpression at the Ascl1+ stage driving progenitors towards the basal VSN fate. B, C) Immunofluorescence of tdTom/HUCD in control and GFP/HUCD in NICD inducible mice at 7dpi. Arrows highlight NICD+/HUCD- sustentacular cells, whereas arrowheads highlight NICD+/HUCD+ VSNs. D, E) Immunofluorescence of tdTom/Meis2 in control and GFP/Meis2 in NICD-inducible mice at 7dpi. Arrows highlight traced Meis2+ apical VSNs in control mice and Meis2+ sustentacular cells in inducible mice. Arrowheads highlight Meis2- basal VSNs in both control and inducible mice. F) Quantification of % traced tdTom+ cells or GFP+ cells that are sustentacular cells or VSNs in control and NICD mice respectively. G) Summary showing NICD overexpression at Ascl1+ stage leads primarily to differentiation of progenitors into non-neuronal sustentacular cells.
2.2.8 Conditional induction of NICD at the Neurog1 precursors stage promote cells towards basal VSN fate.

Based on the intriguing results obtained from the Ascl1+ stage, we decided to test if later NICD activation, in committed (Neurog1+) neuronal precursors, would show a clear role for NICD and Notch signaling in controlling the apical-basal dichotomy. Of note, the Neurog1/Neurod1 stage is coincident to the stage when Notch signaling is more active and when the dichotomy appears to be established (Fig 5 B, D, E, G).

We induced Cre recombination at P1 in both control Neurog1Cre\textsuperscript{ERT2}/R26tdTom\textsuperscript{+/−} and Notch inducible Neurog1Cre\textsuperscript{ERT2}/R26NICD\textsuperscript{+/−} pups and analyzed cell fate 7 days post injection (Fig. 12A). We counted tdTomato+ cells and NICD: EGFP+ cells to follow the recombined cells in control and inducible R26NICD mice respectively. In control Neurog1 traced pups, Meis2/tdTom scoring at 7dpi showed that around 52±0.4% of the cells expressed the apical VSN marker (Meis2+), 46.3±0.3% of the cells are AP-2\varepsilon+ basal VSNs, while the rest of the 1.6±0.4% population didn’t express Meis2/AP-2\varepsilon yet (mean±SEM) (Fig. 12F).

In contrast, in the Neurog1Cre\textsuperscript{ERT2}/R26NICD inducible mice, one week after constitutive NICD overexpression, we found that the Meis2+ apical VSN population significantly decreased to 31.1±3.8%, whereas Meis2/AP-2\varepsilon double negative cells significantly increased to 22.4±4.3% with the rest of the population being AP-2\varepsilon+ (mean±SEM) (Fig. 12 F).
Figure 12: Ectopic expression of NICD at the Neurog1+ stage diverts neuronal precursors towards basal VSN fate. A) Neurog1CreERT2/R26tdTom and Neurog1CreERT2/R26NICD pups were injected with tamoxifen at the P1 stage and perfused at 7 and 14dpi stages. Ai) Expected results shows that NICD overexpression at Neurog1+ stage may drive progenitors towards the basal VSN fate. B-E) Immunofluorescence anti tdTom/Meis2/AP-2ε in Neurog1CreERT2 traced control pups and Neurog1CreERT2/R26NICD mutant pups at 14dpi stage. Image B, C highlights tdTom/Meis2 staining and Image D, E highlights tdTom/AP-2ε staining. Arrows show traced AP-2ε+ basal VSNs and arrowheads show Meis2+ apical VSNs in both control and inducible mice. F, G) Quantification of %traced tdTom+ cells or GFP+ cells at 7dpi and 14dpi that are Meis2+, AP-2ε + VSNs or Meis2/AP-2ε double negative cells in control and NICD mice. Statistical analysis based on arcsine transformed values of the percentage data; unpaired two-tailed t-test; n=3 biological replicates. Data shown as mean±SEM; At 7dpi, the average number of recombined cells in control group: 368.1±23.8; mutant group: 128.3±35.2. At 14dpi, the average number of recombined cells in control group: 509±46.6; mutant group: 148.5±20.7.
To further understand if the NICD induced Meis2/AP-2ε double negative cells can arrive to express the basal maturation marker AP-2ε [10] (Fig. 2C), we also analyzed control and NICD inducible mutants 14 days after tamoxifen injection (Fig. 12 A). Corroborating the 7dpi results, Neurog1CreERT2/R26NICD mutants at 14 days also showed significant decrease in the Meis2+/GFP+ population (Fig. 12 B, C, D, E, G). However, at this stage, there is a significant increase in the AP-2ε+/basal VSNs from 45±0.08% in control pups to 55.7±0.8% in the mutants with the rest of the traced cells being Meis2/AP-2ε double negative (mean±SEM) (Fig. 12 G). These data suggest that induction of Notch signaling at the Neurog1 stage is sufficient to promote the VSNs towards the basal differentiation program.

2.3 Discussion:

The interaction of extrinsic and intrinsic regulators is important for neural stem cells and progenitors to make cell fate decisions and give rise to specific neuronal cell types. The VNE of mice is composed of V1R+/Gαi2+ apical and V2R+/Gαo+ basal VSN sub types. Correct development and function of these two main types of VSNs is important for social, sexual, maternal and predator avoidance behaviors of rodents [37-40]. However, Bcl11b is the only known intrinsic regulator previously shown to affect the initial cell fate decision in the VNO [8]. In the current study, we identified the role of active Notch1 signaling in the cell fate specification of apical and basal VSNs. In the context of T cell development, Bcl11b was previously reported to be a direct downstream Notch signaling target gene [183]. Our results confirm a similar Notch-Bcl11b activation axis also in the VNO.
In this study, scRNA-seq analysis of adult mouse VNO at the neuronal differentiation dichotomy identified specific expression of Dll4 ligand and Notch1 receptor to the apical and basal committed neuronal precursors, respectively. Notably other studies have previously highlighted the expression of Notch1 and Dll4 in the VNO at different developmental stages. In particular, broad Notch1, Dll4 expression have been reported throughout the developing VNO at embryonic stages [202, 203]. However, in postnatal stages, similar to our observations, restricted expression of Notch1 has been previously reported in the marginal zones, the neurogenic areas of the VNO [202, 203]. These data suggested to us to investigate potential roles for Notch-Delta signaling in establishing VSN dichotomy.

Notch is an evolutionarily conserved juxtracrine signaling pathway associated with inhibitory interactions that can determine the cell fates adopted by juxtaposed differentiating cells [199, 204]. Notch inhibitory ligand-receptor interactions rely on non-symmetric expression of a Notch transmembrane receptor and a Notch ligand on neighboring cells. Notch activation in one cell prevents it from assuming the same fate as the neighboring cell expressing the ligand. Notch paralogs (Notch1, Notch2, Notch3, Notch4), three Delta-like ligands (Dll1, Dll3, Dll4) and two Jagged-like ligands (Jagged1, Jagged2) have been identified in mammals [205]. In this study, we performed LOF and GOF experiments at the Ascl1+ neuronal progenitor stage and Neurog1+ neuronal precursor stage to probe the necessity and sufficiency of Notch signaling in establishing neuronal diversity in the VNO. Notch1 receptor knockout from the Ascl1+ neuronal progenitor stage onwards (Ascl1Cre<sup>ERT2</sup> driver) caused most of the developing neurons to enter the apical/Meis2+ differentiation program. This suggests that neuronal
progenitors will default towards apical VSN fate if not instructed otherwise. Vomeronasal precursors with active Notch signaling go on to express Bcl11b [8] and then AP-2ε at a later maturation stage [10]. Notably, Notch signaling can occur in both canonical and non-canonical fashions [196]. By performing Rbpj conditional ablation, a key transcription factor in canonical pathway [198], we showed that the activation of the basal VSN program relies on canonical Notch signaling (Fig. 8, 9).

Activation of signaling pathways at different stages of development can lead to different cellular outcomes. By using Neurog1CreERT2 driver, rather than Ascl1CreERT2, we were able to ablate Notch1 expression at later stages of VSNs neuronal differentiation. Interestingly, Notch1 ablation after Cre recombination induction at Neurog1 stage was no longer effective at altering the differentiation trajectories of the VSNs (Fig. S5). Based on this, we propose that in the developing VSNs, Notch activation has to occur within a critical developmental time window at which cells still retain sufficient cellular plasticity.

To understand, if the differences in the VSN cell types observed after Notch signaling ablation is due to aberrant proliferation or cell death, we quantified these two variables (Fig. 10). Our data showed no differences in cell division or apoptosis. However, we found that 3 days after recombination, most of the proliferative cells resulted to be negative for Bcl11b, which is a known Notch signaling target, with a role in the directing basal VSN differentiation [8, 183]. These data confirm an early role of canonical Notch signaling in directing the expression of alternative differentiation programs starting from proliferative precursors in the VNO.

Ectopic activation of Notch signaling has been previously shown to be able to alter cell fate [206]. Consistently, our conditional ectopic NICD GOF experiments in the VNO
led to distinct phenotypes depending on the timing of NICD induction. NICD activation in Ascl1+ neural committed progenitors, which normally give rise to VSNs (see Fig. 11B), appears to be sufficient to redirect their differentiation towards sustentacular cell fate. However, due to the undetectability of GFP in the early stages like 1 or 2dpi, we could not assess if early changes in the proliferation or cell death compromised the formation of neurons from the recombined cells. This non-neuronal fate may result from NICD-mediated negative regulation of neurogenic transcription factors [207, 208] and by the activation of the alternative sustentacular differentiation program. Notably, it has been shown that NICD over expression in both multipotent horizontal basal cells (HBCs) and globose basal cells of the main olfactory epithelium is sufficient to induce sustentacular cell differentiation [161]. Moreover, our scRNA-seq data also identified the expression of downstream Notch targets in the VNO sustentacular cell population, suggesting a role for Notch signaling in their formation (Fig. S7). Although, Ascl1+ progenitors have more restricted potency than HBCs [162], the phenotypic change of vomeronasal Ascl1+ progenitors in response to NICD implies that, even at this stage, the chromatin landscape is still plastic enough to be rearranged by active Notch signaling [209, 210].

On the other hand, NICD activation at the Neurog1+ stage, did not convert neuronal cells to sustentacular cells, but rather increased the proportion of basal VSNs. This suggests that at the Neurog1+ stage, VSN precursors have reached a level of commitment sufficient to prevent them from being reprogrammed to sustentacular cells [210]. However, at Neurog1 stage, we observed that active Notch signaling is sufficient to divert VSN’s precursors towards the basal VSN fate. After Neurog1 Cre mediated NICD induction we also observed that several VSNs retained the apical identity (Fig. 12 F, G).
Notably, Neurog1 expression starts right after Ascl1, and it is maintained until the apical-basal VSN dichotomy is established (see Fig. 5B). Therefore, Neurog1Cre recombination, and NICD induction, can occur at different stages before or after the differentiation dichotomy occurs. It is therefore possible that the plasticity and the ability of notch signaling to reprogram VSNs to basal fate is limited to a short developmental window [210].

Evolution of new sensory neuronal types in animals can have an important role in determining their social and environmental fitness by expanding their ability to detect, compute, and respond to new stimuli. Vomeronasal receptor types V1R and V2R are functionally and evolutionarily unrelated super families of receptors, and previous studies have highlighted the diversity of V1R- and V2R-positive VSN populations across many vertebrate species [211, 212]. In most mammals (e.g., horse, goat, musk shrew, common marmoset, dog, and cow), few to no V2R genes appear to be expressed. Notably, rodents and opossum have a strikingly expanded repertoire of functional V2R genes, and in these animals, specialized VSNs segregate into V1R+/Gai2+ apical and V2R+/Goa+ basal population [170, 213]. In conclusion, our study has revealed a crucial role for Notch signaling in determining the formation of the two main neuronal cell types of the VNO of mice. Our data suggest an important evolutionary role for Notch signaling in driving the cellular diversity of neuro-epithelia of vertebrates.
Supplementary figures:

Fig. S1: scRNA-seq from p60 mice identifies different cell types in the adult mouse VNO.  
A) UMAP dimensional reduction plot of Seurat object 1 shows neuronal and non-neuronal cell clusters of the VNO. Each color corresponds to a cluster of cells that have similar transcriptomic profile. B) Dot plot visualization of all the genes used to identify cell types in the scRNA-seq data set. Unidentified clusters are not shown here C) Feature plots of various genes related to stem cell progenitors, precursors, immature and mature VSNs and sustentacular cells in Seurat object 1. D) The single cell pseudotime trajectory of Seurat object 2 predicted by Monocle Seurat wrapper and visualized by UMAP. Cells are ordered in pseudotime by choosing Ascl1+ cells as the root node and colored in a gradient from purple to yellow. Di) Dynamic expression of small set of genes as a function of pseudotime.
Fig. S2: Notch1 and Dll4 is the highly expressed receptor and ligand combination at the VSN dichotomy. 

A) UMAP dimension plot of Seurat object 3 from p60 scRNA-seq specifically focusing on the VSN apical-basal split. 

B) Dot plot visualization of all Notch receptors and ligands gene expression in the Seurat object3 highlighting Notch1 and Dll4 expression in Bcl11b+ vs Bcl11b- precursors.
Fig. S3: Expression of Numb and downstream Notch targets are mutually exclusive at the VSN dichotomy. Blended feature plots of Notch target genes Hey1 and, Hes5 vs Numb, which is a negative Notch regulator, from p60 scRNA-seq shows that Notch target genes are not expressed in the same populations as Numb at the VSN dichotomy.
Fig. S4: Integrated scRNA-seq data from P60 and P10 mice shows conserved developmental trajectory across ages. A) UMAP projection of integrated scRNA-seq data from P60 and P10 ages. Each color corresponds to specific cell type B) Integrated UMAP projections of P10 (teal) and P60 (orange) data grouped by age shows overlapping trajectories. C) Feature plots of key developmental markers including Notch signaling pathway genes (Bcl11b, Hey1, Notch1, Dll4) at P60 and P10 shows similar expression pattern across the developmental trajectories.
Fig. S5: Notch signaling knockout induced at Neurog1 stage didn’t affect apical-basal VSN differentiation. **A)** Cartoon summarizing experimental design of Notch1 loss of function study at Neurog1 stage. Neurog1Cre<sup>ERT2</sup>/Notch1<sup>fl/fl</sup>/R26tdTom and Neurog1Cre<sup>ERT2</sup>/R26tdTom pups were injected with tamoxifen at the P1 stage and perfused at 7dpi. **Ai)** The cartoon depicts expected results with Notch1 knockout at Neurog1 stage driving the progenitors towards the apical VSN fate. Red circle highlights Neurog1 stage where recombination is induced. **B)** Triple immunofluorescence of Meis2, AP-2ε and tdTom in Neurog1Cre<sup>ERT2</sup> induced control pups at 7dpi. **Bi, Bii)** Magnification of the box in image B showing Meis2/tdTom and AP-2ε/tdTom double immunofluorescence images respectively. Arrows highlight traced neurons that are Meis2+ apical VSNs and arrowheads highlight traced neurons that are AP-2ε+ basal VSNs. **C)** Triple immunofluorescence of Meis2, AP-2ε and tdTom in Neurog1Cre<sup>ERT2</sup> induced Notch1 conditional KO pups at 7dpi. **Ci, Cii)** Magnification of the box in image C showing Meis2/tdTom and AP-2ε/tdTom double immunofluorescence images respectively. Arrows highlight traced neurons that are Meis2+ apical VSNs and arrowheads highlight traced neurons that are AP-2ε+ basal VSNs. **D)** Quantification of the percentage traced VSNs that are Meis2+ apical, AP-2ε+ basal VSNs and Meis2/AP-2ε double negative cells in Neurog1Cre<sup>ERT2</sup> induced control (gray) and Notch1 KO mice (magenta) at 7dpi stages. Values of traced cells in distinct genetic backgrounds were compared as %.
Percentage values were transformed into Arcsine values. P values were calculated using unpaired two-tailed Student's t-test using the arcsine transformed values; n=3 biological replicates. Both males and females were included in the analysis. Data shown as mean±SEM; *P<0.05, **P<0.01, ***P<0.001. ns- not significant. At 7dpi, the average number of tdTom+ cells in control group: 360.9±12.3; in cKO: 414.45±26.5.
**Fig. S6: Foxn4 is redundant in inducing Dll4 expression**  

**A)** Blended feature plots of Foxn4 and Dll4 from p60 scRNA-seq data show that Foxn4 (arrowhead) is expressed at the apical-basal split along with Dll4 (arrow).  

**B)** Double immunofluorescence of Dll4 and Foxn4 in wild-type VNO at embryonic day 14.5 (E14.5) shows their colocalization (arrows).  

**C, C1)** Immunofluorescence of Dll4 in wild-type and Foxn4 mutant VNOs show detectable Dll4 expression (arrows).  

**D)** Quantification of % Meis2-positive and AP-2ε-positive cells in wild-type and Foxn4 mutant VNOs at E14.5. Percentage values were transformed into Arcsine values.  

P values were calculated using unpaired two-tailed Student's *t*-test using the arcsine transformed values; n=3 biological replicates. Data shown as mean±SEM; *P<0.05, **P<0.01, ***P<0.001. ns- not significant. The average number of Meis2+ and AP-2ε+ cells in control group are 89.2±4.6 and 110.3±4.4 respectively; in mutant Meis2+ and AP-2ε+ cells are 91.2±8.6 and 96.7±9.6 respectively.
Fig. S7: scRNA-seq analysis from p60 mice reveals the expression of downstream Notch targets in sustentacular cells. A) Feature plot of Fezf2 identifies Sustentacular cells in the Seurat object1. B, C) Feature plots of downstream Notch signaling target genes Hey1 and Hes1 show their expression in the Sustentacular cell cluster. D, E, F) Feature plots of Notch signaling receptors Notch1, Notch2 and Notch3 show their expression in the Sustentacular cell cluster. Arrow highlights sustentacular cell cluster in all feature plots.
CHAPTER 3

Characterization of multipotent vomeronasal horizontal basal stem cells

3.1 Introduction:

Stem cells are undifferentiated cells that have the ability to self-renew or differentiate into different cell types of the tissue [214]. Adult tissues can contain stem cells that can either stay mitotically active to maintain tissue homeostasis or in quiescent state [215]. Interestingly, quiescent stem cell population can become active due to extrinsic cell signaling from the surrounding niche or stress signals post injury leading to tissue recovery [216-218]. What molecular mechanisms enable adult stem cells to be in quiescent or active state and how stem cells self-renew or differentiate are important questions to study in stem cell field.

Vertebrate olfactory system is one of the well characterized neuronal system in its ability to turnover new chemosensory neurons throughout the life and to regenerate the sensory epithelium during post injury conditions [219]. In mice, main olfactory system (MOS) and accessory olfactory system (AOS) are the two major sub systems of the olfactory system [73]. MOS consists of the main olfactory epithelium that can detect odorants and send information to main olfactory bulbs, their target region. AOS consists of the vomeronasal organ (VNO) that is responsible for the social and sexual behaviors as it can detect pheromones, kairomones and transmit the information to accessory olfactory bulbs [12, 14]. However, VNO is comparatively less studied in terms of heterogenous stem cell populations, and signaling mechanisms underlying the regeneration of the sensory epithelium [173].
Many studies in the main olfactory epithelium (MOE) highlighted the presence of two distinct types of basal stem cell populations in the adult stages based on their morphology and position in the epithelium - Globose Basal Cells (GBCs) and Horizontal Basal Cells (HBCs) [219-221]. GBCs are mitotically active neural stem cells in normal conditions that lie at the base of the epithelium above HBCs [219]. GBCs are further heterogenous population that can be distinguished based on the transcription factor (TF) expression, potency and ability to self-renew. From upstream to downstream, GBCs comprise of multipotent Sox2/Pax6+ stem cells, Ascl1+ transit amplifying neuronal progenitors and unipotent Neurog1/Neurod1+ immediate neuronal precursors that further divide and give rise to olfactory sensory neurons [74, 75, 177, 222, 223]. HBCs are second population of basal stem cells that lie on the basal lamina of the MOE. These cells are mitotically quiescent, reservoir stem cells that stay dormant in the intact adult olfactory epithelium and express p63 TF specifically ∆Np63α isoform [217, 224]. However, when there is an injury to the sensory epithelium, HBCs can activate, proliferate, and differentiate to regenerate multiple neuronal and non-neuronal cell types of the MOE within weeks of time [161, 162]. Interestingly, during the embryonic development of the olfactory epithelium, HBCs form during late stage of neurogenesis compared to Sox2 positive stem cells that are seen as early as E10 [73, 224].

VNO in mice is bilaterally symmetrical tubular structure present at the base of the nasal cavity that is responsible for the detection of pheromones. It comprises of thick medial vomeronasal sensory epithelium (VSE) and thin lateral non-sensory epithelium (NSE) with lumen separating both the epithelia [173]. Within the VSE, there are 2 major sub types of VSNs – Meis2 TF+ neurons expressing Gai2 subunit and receptors of V1R
family often called as apical vomeronasal sensory neurons (VSNs) and Tfap2e/AP2ε TF+ neurons expressing Gαo subunit and receptors of V2R family called as basal VSNs with respect to the spatial location they occupy in the VSE [33-36]. Multiple BrdU studies in the VNO identified adult neurogenesis in the basal region of the VSE and in the marginal zones between VSE and NSE in physiologically intact and regeneration post-injury conditions [7, 84, 85, 225].

In terms of stem cell population in the adult VNO, even though specific GBCs term is not used like in MOE, the presence of Sox2, Ascl1, Neurog1 and Neurod1+ cells and their role in the VSNs turnover are well characterized [70, 75, 226]. In addition, a recent study from our lab identified the expression of Notch1 receptor and Dll4 ligand within the Neurog1/Neurod1 immediate neuronal precursors at early postnatal stages and showed that Notch1-Dll4 signaling is important for the establishment of Gαo+/V2R+ basal VSNs [226]. In contrary, p63+ HBC population is completely under-explored in the VNO. Till now, we found only one study briefly talking about the presence of Krt14 positive basal cells in the non-sensory epithelium (NSE) and marginal zone of VNO [163]. However, whether there is any role of HBCs in the embryonic/postnatal development of the VNO, adult neurogenesis or regeneration postinjury is completely unknown.

In the current study, we confirmed the presence of vomeronasal HBCs (vHBCs) based on the expression of various olfactory HBC markers and characterized their development from embryonic to adult stages of the VNO. Single cell RNA sequencing (scRNA-seq) of the adult VNO also identified clusters expressing HBC related genes. In addition, using different lineage tracing studies we showed that vHBCs can give rise to non-neuronal and neuronal cell types during both early postnatal development and adult
stages in the VNO. Overall, our study reported the existence of second population of multipotent stem cells in the VNO and opened a new research window to study in terms of regeneration, signaling factors guiding stem cell proliferation vs self-renewal.

3.2 Results:

3.2.1 Olfactory HBC markers identify vHBCs in the VNO.

P63 TF specifically ΔNp63α isoform is one of the initial markers expressed by HBC precursor cells in the MOE [224]. We performed immunohistochemistry against ΔNp63 isoform of p63 TF to identify HBC like cells in the 3-week-old VNO and confirmed p63+ basal cells on the basement membrane of the NSE, marginal zone and parts of the VNO sensory epithelium (Fig. 1A). In the MOE and other p63 related tissues, all basal cells express keratin intermediate filaments Krt5 and Krt14 [224]. Double immunofluorescence against ΔNp63/Krt5 and ΔNp63/Krt14 showed that all p63 cells in the VNO expressed keratin intermediate filaments (Fig. 1B, C). In addition, Sox2/ΔNp63 double immunofluorescence revealed two different types Sox2 population, besides the Sox2+ sustentacular cells in the sensory epithelium - Sox2/ΔNp63 double positive cells which are HBC like cells in the MOE and Sox2+/ΔNp63- cells which can be neuronal progenitors or precursors of the VNO [226] (Fig. 1D).

Interestingly, a closer look at the morphology of the VNO p63+ cells revealed a difference in the cell shape of the basal cells in the NSE versus VSE. In the marginal zone and the basement membrane of the SE, p63+ cells have horizontal shape similar to the HBCs in the MOE (arrow in Fig. 1B), whereas p63+ basal cells in the NSE do not have horizontal shape and look more like the basal cells in the respiratory epithelium.
Henceforth, we call horizontal shape p63 basal cells in the MZ and VSE as putative vomeronasal horizontal basal cells (vHBCs) and p63 basal cells in Figure 13: p63+ cells are expressed in the 3-week-old VNO. A) Nickel DAB immunostaining of ΔNp63 in the 3-week-old VNO. Inset highlights (arrow) the expression of ΔNp63 specifically in the marginal zones (MZ). B) Immunofluorescence anti ΔNp63 (magenta) and Krt5 (green) shows the co-expression of both markers (arrow). C) Immunofluorescence anti ΔNp63 (magenta) and Krt14 (green) shows the co-expression of both markers (arrow). D) Immunofluorescence anti ΔNp63 (green) and Sox2 (magenta) shows three different populations – arrow highlights Sox2 and p63 double positive cells; arrowhead highlights Sox2 only positive neuronal progenitors; and notched arrowhead highlights Sox2 only positive sustentacular cells.

(arrowhead in Fig. 13 B). Henceforth, we call horizontal shape p63 basal cells in the MZ and VSE as putative vomeronasal horizontal basal cells (vHBCs) and p63 basal cells in
the NSE as non-sensory basal cells. In summary, we categorized p63+ cells in the adult VNO into vHBCs and non-sensory basal cells based on their shape and location, and they express all canonical markers of the olfactory HBCs.

### 3.2.2 Characterization of the spatiotemporal developmental pattern of vHBCs.

Once we identified vHBCs and non-sensory basal cells in the adult VNO, we want to investigate their presence at different spatiotemporal stages from embryonic days to postnatal stages in the VNO. We explored p63 expression by considering two different spatial patterns in the VNO - 1) rostral to caudal sections of the VNO and 2) from NSE towards the marginal zone and sensory epithelium of the VNO.

**Figure 14: Dynamic expression of ΔNp63 from rostral to caudal VNO in early embryonic stages.** Top panel shows the expression of ΔNp63 from rostral to caudal sections of the VNO at E14 stage. Arrow shows the ΔNp63 expression only in the rostral sections. Bottom panel shows the expression of ΔNp63 from rostral to caudal sections of the VNO at E15.5 stage. Arrow shows the ΔNp63 expression in all sections. Rostral to caudal differences in ΔNp63 expression can be seen in both stages.
The initial expression of p63 TF in VNO is dynamic and seen as early as E14. Immunohistochemistry of ΔNp63 at E14, showed its expression only in the rostral most sections but not in the caudal sections (Fig. 14). Intriguingly, within 2 days by E15.5, p63 expression is seen in NSE of all sections from rostral to caudal part of the VNO. However, rostral to caudal differences in the expression pattern is still evident at E15.5. At the rostral end of the VNO, p63 expression is seen throughout the epithelium whereas, from medial towards the caudal sections, p63 expression is confined only to the NSE (Fig. 14).

As the p63 expression is expanded throughout the NSE from rostral to caudal ends by E15.5, we further focused specifically on medial sections of the VNO for the later developmental stages. We performed double immunofluorescence against ΔNp63 and
Krt5 at different perinatal ages like E15.5, E18.5, P5, P8, and P23. As the age increases from embryonic to postnatal days, top panel shows the expansion of p63 positive cells (arrow) from non-sensory epithelium (NSE) towards the marginal zone (MZ) and vomeronasal sensory epithelium (VSE). Notably, during the early postnatal stages like P5, we found a sparse population of p63 cells at the MZ and sensory epithelium which are not seen at later stages, suggesting their ability to differentiate. As the VNO ages from P8 to 3-week-old VNO, p63 cells are slowly seen occupying throughout the basement membrane of the sensory epithelium (Fig. 15A). Interestingly, compared to the MOE, p63 development in the VNO is further delayed. In the MOE, p63 cells are seen throughout the basement membrane at 3-week-old stage, whereas in the VNO only part of the basement membrane is occupied by p63+cells at this stage [224].
Besides the p63 expression, following Krt5 immunofluorescence across the perinatal ages also showed a delayed expression pattern (Fig. 15B). At E15.5, the expression of Krt5 in p63 cells is negligible, however, as age increases from E18.5 to P8 stage, Krt5 expression started slowly in all p63 cells. By 3-week-old, most of the p63+ cells expressed Krt5 expression (Fig. 15B). Notably, ΔNp63/Ki67 double

**Figure 16:** ΔNp63 cells are less proliferative at different stages of the VNO development. A,B,C,D,E) Immunofluorescence anti ΔNp63 (magenta) and proliferation marker Ki67 (green) at different ages starting from E15.5, P0, P5, P8 and 3 week old stages. Inset shows proliferative p63 cells (arrows) and non-proliferative p63 cells (notch arrowhead) at different stages of VNO development.
immunofluorescence also showed very few p63+ cells that are proliferative at all perinatal stages suggesting that these cells are less proliferative (Fig. 16).

In summary, the expression of p63 TF from embryonic to postnatal stages of the VNO is dynamic and follows rostral to caudal and NSE to SE spatial patterns. In addition, the Krt5 expression is further delayed compared to p63 cells, suggesting that Krt5 can be an ideal Cre driver to lineage trace only during adult stages but not during the VNO development.

### 3.2.3 Constitutive lineage tracing of vHBCs reveal their role in the early VNO development.

By following the developmental pattern of p63 cells, we observed one interesting aspect during the early postnatal stages of the VNO. At P3-P5 stage, a sparse population of p63+/Krt5- cells are seen in the MZ and VSE (Fig. 15 A, B). However, interestingly, at later postnatal or adult stages, we don’t see any accumulation of p63 cells in the sensory epithelium. This intrigued us to check whether the p63 cells that are seen in the sensory epithelium during the early ages might have differentiated to other cell types in the VNO.

We chose ΔNp63Cre mouse line that has Cre knocked in specifically under ΔNp63Cre promoter to lineage trace and study whether p63+ vHBCs can give rise to any cell types during the early VNO development.
We perfused ΔNp63Cre/R26tdTom mice at 3-week-old stage and looked for endogenous tdTom fluorescence to determine lineage tracing (Fig. 17A). To our surprise, tdTom fluorescence revealed traced neuronal and sustentacular cell types in the sensory epithelium and also traced non-neuronal cell types in the NSE (Fig. 17B). In addition, triple immunofluorescence against Meis2/Tfap2e/tdTom further revealed traced apical and basal traced VSNs in the VSE (Fig. 17B). Notably, ΔNp63/DsRed

Figure 17: Constitutive ΔNp63 lineage tracing till 3-week-old stage showed multipotency role of ΔNp63 cells during VNO development. A) Schematic showing the experimental design - ΔNp63Cre/R26tdTom mice were perfused at 3-week-old stage. B) Immunofluorescence anti ΔNp63 (green) and DsRd (magenta) shows recombined p63 positive traced cells (arrow) and lineage traced neurons and sustentacular cells. C) Immunofluorescence anti Meis2 (green), Tfap2e (cyan), and DsRd (magenta) shows lineage traced apical VSNs (arrows) and basal VSNs (notched arrowhead).
immunofluorescence showed only few p63 cells (Fig. 17A) that are traced suggesting less recombination, similar to other studies that used this mouse line [228]. Overall, constitutive lineage tracing of p63+ cells using ΔNp63Cre line revealed their multipotency during early VNO development for the first time.

3.2.4 Conditional lineage tracing at adult stages identifies neuronal and non-neuronal cell types at the marginal zones.

Adult neurogenesis in the mouse VNO is well established, however the underlying molecular mechanisms are not completely explored. As we see vHBCs lining the
basement membrane including marginal zones, we wanted to next investigate whether p63+ vHBCs have a role in the adult neurogenesis.

To determine whether these p63 cells can give rise to any neuronal or non-neuronal cells in the VNE, we did temporally conditional lineage tracing on K5CreERT2/tdTom mice at adult stages. We induced CreERT2-loxp by injecting tamoxiften at 4 weeks age and perfused the mice 8 weeks post injection (Fig. 18A). Endogenous tdTom fluorescence showed tracing in all p63 cells in the VNO as expected along with two distinct regions in the epithelium (Fig 18B). Firstly, we detected traced non-neuronal cells located above non-sensory basal cells in the NSE. In addition, we found tdTomato+ traced cells in the marginal zone of the VNE (Fig. 18B). Double immunofluorescence against OMP/tdTom confirmed the presence of few neuronal clones at mature stage in the marginal zone (Fig. 18 C,C’,C’’). Triple immunofluorescence against Meis2, Tfap2e and tdTom further revealed the presence of at least Meis2+ apical VSNs (Fig. 18 D,D’,D’’). Besides this, tracing is also seen in many non-neuronal cells in the MZ which can be putative sustentacular cells. In summary, this data shows that vHBCs in the VNE, at a slow pace, can give rise to both neuronal and non-neuronal cells in the epithelium even in the adult stages.
3.2.5 Single cell profiling of the adult VNO identifies p63+ population.

As we characterized the presence and multipotency of p63+ vHBCs in the VNO, we want to explore recently published scRNA-seq data from adult VNO. From the initial analysis, we were able to identify p63 positive cell cluster in the single cell RNA seq data (Fig. 19A, B). Moreover, we also identified few p63/Krt5 positive cells that are proliferative (Ki67+) and lie upstream and close to Ascl1+ stem cell progenitors (Fig. 19B follow notched arrowheads). This may suggest the stem cell potential of p63/Krt5 cells shown in both constitutive and conditional lineage tracing studies earlier (Fig 17, 18).
3.3 Discussion:

P63 is one of the two mammalian paralogues of p53 family of transcription factors that can exist in 6 different isoforms- two different N terminal and 3 different C terminal isoforms [229, 230]. In specific, the N-terminal truncated (ΔN) p63 isoform is mostly expressed in the basal cells of different epithelial tissues and shown to have role both during the development, and homeostasis of the epithelium during the adult stages [224, 228, 231]. In the MOE, which is a pseudo stratified sensory epithelium, many studies characterized ΔN p63+ cells (also called as HBCs) as quiescent stem cell population that have role in the regeneration post injury [217, 224]. Even though VNO shares multiple molecular and morphological features with the MOE, it has been less characterized in terms of stem cell population. In this study for the first time, we identified the stem cell role of p63+ cells in the VNO during both the development and adult stages.

Using scRNA-seq data set of the adult VNO and immunofluorescence validations, we confirmed the existence of p63 cells in the VNO. Interestingly, the shape of these cells varied depending on their presence in the sensory or non-sensory epithelia of the VNO. p63 cells on the basement membrane of the sensory epithelia has horizontal shape like HBCs of the MOE, hence called as vHBCs, whereas p63 in the NSE are called as non-sensory basal cells due to lack of horizontal shape and appear like basal cells of respiratory epithelium (Fig. 13 B). In addition, we opted for constitutive lineage tracing to see if p63 cells can give rise to any cell types in the VNO during development. We used ΔNp63αCre/R26tdTom to lineage trace during development as Krt5 expression is delayed compared to P63. Interestingly, we found endogenous tdTom traced neuronal and non-neuronal cells in both sensory and NSE of the VNO (Fig. 17). Notably, endogenous tdTom
fluorescence is seen in only few VNO sections. Whether this is due to low recombination with this specific mouse line or less role of vHBCs in the early VNO development is not completely known. In addition, we used conditional K5CreERT2/R26tdTom strategy to lineage trace vHBCs specifically during adult stages. Interestingly, after inducing tracing at 4 weeks and a long wait time of 8 weeks, we identified small population of neuronal and non-neuronal cells specifically at the MZ regions (Fig. 18). This is the first time showing the ability of the VHBCs in homeostasis of the VNO. These investigations showed that vHBCs can give rise to both non-neuronal and neuronal cell types during both early postnatal development and adult stages in the VNO.

Overall, our work reported a new second population of p63+ stem cells in the VNO and identified previously unknown role of these cells in both the VNO development and homeostasis during the adult stages. Interestingly, this potential of HBCs in the development or homeostasis in the MOE is unknown. Moreover, our work also encourages to explore new research questions in the VNO field. Firstly, during the embryonic development, we showed that expression of p63 TF is dynamic and followed both rostral to caudal and medial to lateral spatial patterns in the VNO, however, underlying molecular mechanisms are not known. It would be interesting to explore the extrinsic cell signaling pathways and intrinsic transcriptional network that induce the expression of ∆Np63 TF. Notably, during the conditional lineage tracing experiments using K5CreERT2 mice with long wait times, we identified p63 lineage traced neurons in the adult stages, only at the MZ regions of the epithelium. Even though, it is known that MZ regions can undergo adult neurogenesis, it would be interesting to investigate the presence of any signaling factors that are unique to the MZ regions. Lastly, in the current
study, we did not investigate the regenerative potential of the p63 cells, which is a known feature of HBCs in the MOE. It would be interesting to see if vHBCs have the capacity to regenerate the epithelium post injury in the VNO.
Open questions to study

4.1 Discovery of p63+ vomeronasal HBCs role in the development of the VNO may open up new areas of research.

In chapter 3, we showed the presence of p63+ HBCs in the VNO in both non-sensory and sensory epithelium and characterized their expression patterns from embryonic to adult stages. In addition, we also investigated their role in the early development and tissue homeostasis in the VNO. We are the first ones showing that p63+ HBCs are multipotent and have the ability to give rise to neuronal and sustentacular cells in the sensory epithelium and also non-neuronal cells in the non-sensory epithelium. Interestingly, in the main olfactory epithelium, even though many research groups studies the role of HBCs in the regeneration aspects post injury, no one paid attention towards development and tissue homeostasis.

Identification of HBCs in the VNO can promote new areas to research in near future. Immediate question is to investigate whether HBCs have any role in regeneration in the VNO post injuries. It is well established that VNO can regenerate its epithelium following olfactory bulbectomy in both adult and old ages [186, 232, 233], however, the source of its regeneration is not completely known. As we do see the presence of HBCs as a single layer on the basement membrane covering marginal zones and most of the sensory epithelium, it could be interesting to see if p63+ HBCs in the VNO can play any role in the regeneration of the epithelium. Multiple studies have shown successful lesions in the main olfactory epithelium using chemicals like Triton x, methimazole, methyl bromide, ZnSo4 and also surgery models like Olfactory bulbectomy, sensory nerve
transection [161, 234-236]. However, in the VNO, studies showed only surgical models like olfactory bulbectomy and vomeronasal nerve transection to cause epithelial damage and regeneration [186, 232]. Unfortunately, vomeronasal nerve transection may not be consistent due to intricacies in the surgery and gliosis at the injury site. Majority of the chemical lesions to the main olfactory epithelium are due to the toxicity of intermediate compounds during xenobiotic metabolism by specific Cytochrome P450 enzymes in the Sustentacular cells [237]. However, due to low amount and a different spatial localization of these metabolizing enzymes in the VNO, these chemical lesion models may not be ideal to trigger injury in the VNO [160]. In fact, we used methimazole injection to induce injury to both main olfactory and vomeronasal epithelium, using similar protocol used earlier. However, we didn’t see any cell death in the VNO (data not shown), even though main olfactory epithelium showed apoptotic cell death followed by activation of HBCs as previously published [161]. Due to these reasons, we think olfactory bulbectomy surgical model can be ideal to induce damage to the vomeronasal epithelium and check whether HBCs have any role in regeneration.

**Experiment 1**: Inject tamoxifen to lineage trace K5CreERT2/tdTom mice at 4 weeks stage and perform olfactory bulbectomy surgery at 3 days post injection. Perfuse the mice 3 weeks after surgery to do immunofluorescence against tdTom and neuronal and non-neuronal related markers.

**Expected results**: The major goal of doing this experiment is to investigate whether P63+ HBCs in the VNO can regenerate the VNO following olfactory bulb ablation. By injecting
tamoxifen all HBCs express tdTomato fluorescent protein. If these HBCs can regenerate the vomeronasal epithelium after olfactory bulbectomy, we should be able to see red fluorescent neuronal and non-neuronal cells in the VNO. If there are fluorescent cells, then performing immunofluorescence against tdTom and HBC markers (p63, and Sox2), neuronal progenitors (Ascl1), precursors (Neurod1), VSNs (Omp, Meis2, Tfap2e), should reveal whether much detailed information about the potential of vomeronasal HBCs.

Experiment 2: Inject tamoxifen to K5CreERT2/p63^{fl/fl}/tdTom mice at 4 weeks of age to lineage trace activated HBCs and perfuse 2 weeks after injection. Perform immunofluorescence against tdTom and neuronal and non-neuronal related markers.

Expected results: Besides these chemical and surgical models used in the main olfactory epithelium, few studies employed tamoxifen inducible K5CreERT2 system to conditionally knockout p63 transcription factor from HBCs. Notably, this approach can mimic regeneration process in HBCs giving rise to neuronal and non-neuronal cells in the MOE [162, 238]. Taking these observations into consideration, we want to use this genetic model to mimic injury like conditions in the VNO to probe the regeneration ability of vomeronasal HBCs. This way we can not only show regenerative ability, but also explore the mechanisms underlying activation of vomeronasal HBCs. If there are fluorescent cells in the vomeronasal epithelium after 2 weeks, then we can perform immunofluorescence against tdTom and neuronal and non-neuronal markers as suggested earlier.

Experiment 3: Identification of cell signaling pathways underlying the activation of p63 basal cells via single cell transcriptomic analysis.
Studying mechanisms underlying the activation of p63 cells in the VNO during regeneration phase will also address the factors important for quiescence vs proliferation and differentiation. This can be vital information regarding the stem cell’s ability to regenerate even in other tissues. Both intrinsic factors like transcriptional network and extrinsic factors like morphogenic signaling from the surrounding niche plays major role in determining the behavior of the stem cells. Moreover, in the VNO, what signaling pathways guide the stem cells to give rise to only non-neuronal cells towards the NSE Vs apical and basal neurons towards the sensory epithelium is also an interesting question. We want to take the advantage of genetic lesion model we described earlier and do single cell RNA sequencing and ATAC seq to answer these questions.

We will inject 180mg/Kg body weight tamoxifen dose to K5CreERT2/R26tdTom mice (Control group) and K5CreERT2/p63\textsuperscript{fl/fl}/R26tdTom mice (Knockout group) of either sex at 6-8 weeks age. We will euthanize control group mice 3 days after tamoxifen injection to dissociate single cell RNA sequencing and ATAC seq.

**Figure 20: Schematic of single cell RNA sequencing experiments.** After tamoxifen injection, single cells will be dissociated from both control mice and at different time points from conditional knockout mice. Lineage traced cells will be FACS sorted and then send to RNA sequencing and ATAC seq.
cells from the VNO. On the other hand, we will euthanize knockout group mice at 1 day, 3 days, 7 days, and 14 days post injection to check at what stage we can get most of the traced cell types like proliferating HBCs, non-neuronal supporting cells, neuronal progenitors, precursors, and VSNs. After activation, vHBCs undergo dynamic changes in the transcriptional network and go through different cell stages to eventually give rise to mature neuronal and non-neuronal cell types. For example, to give rise to mature VSNs, stem cells have to change into transit amplifying cells, immediate neuronal precursors, immature neurons and eventually mature apical and basal VSNs. Based on different immunofluorescence stainings, we want to select one stage and dissociate their VNO’s to collect single cells (Fig 20). We will use 3 mice in both control and knockout groups. We will FACS sort only tdTom positive cells from the VNO in both control and knockout groups and send cells for single cell RNA seq and ATAC seq. Fastq files will be processed using cell ranger. The output files from both the control and knockout group will be integrated together into one file and will be used for further analysis using Seurat R package [239].

**Expected results and alternatives:** From the integrated single cell RNA sequencing data analysis using Seurat, we expect to identify well separated clusters corresponding to p63 stem cells, non-neuronal cells, neuronal progenitor, immediate neuronal precursors, immature and mature apical and basal VSNs. Within the wildtype single cell data, we also expect to find 2 different clusters one corresponding to the non-sensory basal cells in the NSE and the other one to the vHBCs on the basement membrane of the VSE. Moreover, from the integrated data, using ‘Findmarkers’ function in Seurat, we can determine both global and local differential gene expression for each cluster. Using significant gene
expression data and GO enrichment analysis, we can identify the cell signaling pathways that are unique to each cell type. In addition, dynamic changes in the expression of various transcriptional factors at branching point of the trajectories can be determined. From ATAC seq analysis, we expect to identify chromatin accessibility and gene regulatory elements at single cell level that determine the gene expression and differentiation of neuronal and non-neuronal cell types from multipotent stem cells. Moreover, using ATAC seq analysis, we can also predict the expression of various long and small non-coding RNAs in different cell types during differentiation.

4.2 Future studies related to Notch signaling in the VNO:
Notch signaling is an evolutionarily conserved juxtacrine signaling pathway that is known to have role in stem cell maintenance, proliferation, cell fate choice and differentiation in organogenesis [240]. Specifically, Notch signaling contribution to the cell type diversity in an organ is context dependent. Firstly, it may depend upon the type of the Notch receptor and ligand that two adjacent cells express, as the affinity of the interaction may vary. For example, one study showed that Dll1 ligand activates Notch1+ signal receiving cell in frequency modulated pulses, whereas Dll4 ligand can activate the same Notch1 cell in a sustained, amplitude modulated manner. This differences in the signaling dynamics can further affect the downstream targets that Notch activating complex can activate and the phenotype of the cell [241]. Adding another layer to this, the potency of the cell stage where signaling is occurring may also have affect the output phenotype of the cell due to differences in the chromatin accessibility [242]. Interestingly, our VNO Notch signaling
data can be an excellent example to show how these parameters can alter the final phenotype of the cell.

In the VNO, our initial aim was to identify the mechanisms involved in the apical vs basal VSNs, where our scRNA-seq data identified Notch1-Dll4 signaling. We further demonstrated using loss of function studies that active Notch signaling via Notch1 receptor is required for basal VSN formation. However, when we used gain of function studies to probe whether active Notch signaling is sufficient to drive neuronal progenitors / precursors towards basal VSNs, we were surprised to see two different phenotypes based on the temporal stage of the cell. Induction of NICD at Ascl1+ neuronal progenitors lead them towards non-neuronal Sustentacular cell fate, whereas NICD induction at Neurog1+ neuronal precursors stage (later stage of Ascl1+ cells), lead them towards the basal VSNs. Interestingly, from our Ascl1+ lineage tracing data, it is evident that more than 99% of the traced cells from Ascl1+ stage are neurons. These data shows that Ascl1+ neuronal progenitors are mostly unipotent, however the ability to transform to non-neuronal cells is still intact. Moreover, from the main olfactory epithelium, it is evident that Notch signaling is involved in the formation of sustentacular cells at the multipotent stem...
cell stage (HBCs), even though specific Notch receptor and ligand combination is still unknown [161]. This study along with the VNO scRNA seq data showing the presence of Notch receptors and ligands in the p63+ vHBCs and Sustentacular cells suggests that Notch signaling pathway via different receptor and ligand (not Notch1-Dll4 combination) might be happening at multipotent stage in the VNO (Fig. 21).

**Experiment 4:** Inject tamoxifen to P63CreERT2/RNICODEGFP pups at P3 and perfuse after 7-14 days to quantify total number of GFP+ sustentacular cells and VSNs.

*Expected results:* In control P63CreERT2/tdTom pups, we expect to see both traced VSNs and sustentacular cells, whereas in the P63CreERT2/RNICODE, we would see a shift towards sustentacular cells.

We tried to do this experiment using constitutive P63Cre/RNICODE mice we already have. However, this resulted in lethality by P1-P2 stage due to recombination in all basal cells in the body (data not shown). As most of the vomeronasal HBCs in the sensory epithelium are seen only from P3 onwards, we were unable to see any phenotype.

**Experiment 5:** Inject tamoxifen to P63CreERT2/Rbpjfl/fl/tdTom pups at P3 and perfuse after 7-14 days to quantify total number of traced sustentacular cells and VSNs.

*Expected results:* In control P63CreERT2/tdTom pups, we expect to see both traced VSNs and sustentacular cells, whereas in the P63CreERT2/Rbpjfl/fl/tdTom, we would see a shift towards more neurons.

If P63CreERT2/RNICODE pups show a shift of recombined cells towards sustentacular cells, and P63CreERT2/Rbpjfl/fl/tdTom pups show shift towards more neuronal cells, we can
confirm that a different combination of Notch receptor and ligand may have a role in the cell fate choice of multipotent stem cell into neuronal progenitor or non-neuronal cell.

**Fine tuning of Notch signaling:**

In mammals due to the presence of 4 different Notch receptors and ligands, most of the times, Notch signaling can involve multiple receptors and ligands. For example, during sprouting of new blood vessels during angiogenesis or formation of ventral interneurons of the spinal cord, combination of multiple Notch receptors and ligands are involved [243, 244]. In such situations, fine tuning of the Notch signaling by guiding more affinity towards specific combination of receptor and ligand may play a major role in driving the essential cell fate. For example, the epidermal growth factor-like (EGF) repeats, which are the part of the extracellular domains of the Notch receptors have sequences for O-linked carbohydrate modifications like O-linked fucose, O-linked glucose and O-linked N-acetyl-glucosamine (GlcNAc) [245]. Specifically, Fringe proteins are N-acetylglucosaminytransferase enzymes that can add GlcNAc residues to specific EGF repeats that can modulate the affinity of Notch receptor-ligand combinations [246]. In mammals, three fringe proteins have been identified: Lunatic fringe (Lfng), Manic fringe (Mfng) and Radical fringe (Rfng). Interestingly, studies on fringe proteins highlighted that both Lfng and Mfng can preserve and strengthen interactions between Notch and delta and weaken interactions between Notch and Jag combination [247]. However, Rfng preserves interactions with both Jag and delta ligands. Expression of specific combination of these fringe proteins in the signal receiving cells can enhance only one combination of receptor-ligand interactions while weakening others [247].
granular zone of hippocampus that accommodates adult born Neural stem cells (NSCs), Notch1 receptor and Lfng are expressed in the NSCs, whereas Jag1 ligand is expressed by the newly born progenitors from the NSCs. In addition, granular cells that lie close by to the NSCs also express Dll1 ligand. Interestingly in this study, Lfng is shown to enable NSCs to selectively activate Notch signaling via Dll1, causing stem cell maintenance/quiescence [248]. On the other hand, Lfng can suppress Jag1 induced signaling from the daughter cells thus suppressing their over-proliferation and depletion. Moreover, Lfng knockout showed less NSCs than wild type as they cannot maintain their quiescent state.

![Figure 22: Fringe proteins that modulate Notch signaling are expressed in the VNO. A) Feature plots of Lfng and Mfng fringe proteins at the VSN dichotomy. B) In situ hybridization of Lfng and Mfng fringe proteins in the VNO at embryonic stages. Lfng in E13.5 VNO taken from Allen brain atlas; Mfng in E14 VNO taken from Allen brain atlas.](image)

In the VNO, when the gain of function results at Ascl1+ stage suggested the presence of another Notch1 receptor and ligand combination at multipotent stem cell stage, the immediate question that triggered my interests were how different signal sending and
receiving cells can always interact only specific ways to give rise to basal VSNs and Sustentacular cells. In fact, strong transcriptomic expression of Fringes Lfng, and Mfng in the scRNA-seq data specifically at the VSN dichotomy triggered my interests to study their role in the VNO (Fig. 22A). Moreover, online in-situ data bases like allen brain atlas and genepaint also showed strong expression of Lfng and Mfng in the embryonic VNO at E13.5-E14 stages (Fig. 22B). With these data in hand, I am speculating that these fringes may ensure the interaction between Neurog1+ Notch1 and Dll4+ cells leading to basal VSN formation on one side and Sustentacular formation from the other Notch receptor and ligand combination.

**Experiment 6: Immunofluorescence against GFP, Notch1, Dll4, Neurod1, and other multipotent stem cell markers in Lfng-eGFP mice.**

**Expected results:** This Lfng-eGFP mice [248] can yield important results to further characterize Notch signaling in the VNO. Immunofluorescence against already known Notch1, Dll4, Neurog1 shows whether Lfng is expressed only at Neurog1/Neurod1+ neuronal precursors stage or also seen at any multipotent stem cell stage like p63. As I hypothesized that fringe proteins are only specifically expressed at the VSN dichotomy, I don’t expect Lfng to express in the p63+ HBCs.

**Experiment 7: Characterization of apical, basal VSNs and sustentacular cells in LfnglacZ mutant mice.**

**Expected results:** Immunofluorescence against apical, basal VSNs and non-neuronal sustentacular cell related markers in LfnglacZ mutant mice [248] and wildtype control mice
may reveal any differences between the number of neuronal and non-neuronal cells. I expect to see changes in two different aspects. Lfng knockout may cause reduction in the basal VSNs at the expense of apical VSNs as shown in chapter 2 which is recently published [226]. However, the second interesting aspect that I also expect to see is the upregulation of sustentacular cells at the expense of VSNs overall.

However, as we see strong expression of both Lfng and Mfng as shown in the in-situ hybridization, we may also see compensation from the second fringe Mfng. In this case, it would be better to go for Lfng/Mfng double knockout mice or conditional double knockouts at Ascl1 stage using Ascl1Cre<sup>ERT2</sup>/Lfng<sup>fl/fl</sup>/Mfng<sup>fl/fl</sup>/R26tdTom mice.

Overall, investigating the role of fringe proteins in the VNO may help to reveal the fine-tuning aspects of Notch signaling and how different Notch receptor-ligand interactions at different cell stages can help in maintaining cell diversity in the VNO.

4.3 Concluding remarks:

Genes provide road map for the formation of neuronal circuits during development. How mutations to one or more genes can affect the circuit formation and respective behavior is an active area of research in developmental biology and to understand congenital disorders. Vomeronasal organ is sub-olfactory system that arises from olfactory placode. During the embryonic development of the VNO, vomeronasal neurogenesis is seen as early as E12.5 stage and continues until early postnatal stages. Interestingly, VNO can also undergo neurogenesis throughout the life of the animal specifically at the marginal zones. Studying the development of the VNO can be an excellent model to look at the relation of genes to neural circuits and behavior. Unfortunately, VNO is less studied organ
compared to its placodal counterpart main olfactory epithelium. My doctoral research aims to bridge the gaps in the VNO literature by specifically answering two long standing developmental questions.

To summarize, my research focused on vomeronasal neurogenesis at two different temporal points. In the first project, using scRNA-seq and conditional lineage tracing, we identified the presence of Dll4-Notch1 signaling during the formation of V1R/apical and V2R/basal VSNs in the VNO. We also used loss of function and gain of function studies to further demonstrate that active Notch signaling is required for the cell fate determination of basal VSNs. In addition to this, specifically in the gain of function studies, we also showed that depending upon the potency or cell stage at which we induce active Notch signaling, the phenotype of the cell changed between non-neuronal sustentacular cell and basal VSN.

In the second project, we identified horizontal basal cells, which is a second population of multipotent stem cells in the VNO. Even though lot of literature is available about these cells in the main olfactory epithelium, no one till now studied their role in the VNO. We characterized the development of HBCs from early embryonic to adult stages and showed their ability to give rise to both neuronal and non-neuronal cells during early postnatal development and adult neurogenesis in the VNO. My research also suggested that different combination of Notch receptor and ligand can be involved in the formation of sustentacular cells similar to Dll4-Notch1 at the VSN dichotomy. Overall, my research reveals the potential of Notch signaling in generating cell diversity in the VNO.
4.4 Significance of basic research in the VNO:

During the development of the nervous system, different extrinsic factors guide the formation of neurons from the local stem cell populations. Minor genetic variations like single nucleotide polymorphisms or major mutations that can affect the expression of key genes during this process can significantly affect different aspects of neurogenesis that can ultimately cause neurodevelopmental disorders.

The mouse vomeronasal organ (VNO) is an attractive model system to study the relationship between the genes to the neuronal circuits to the behavior during the development. Interestingly, most of the signaling pathways and the transcription factors that are responsible for the neural stem cell renewal vs differentiation in the central nervous system are also expressed during the development of the mouse VNO. Hence, the findings discovered during vomeronasal neurogenesis can also be translatable to the central and peripheral nervous systems. This way from using VNO as a basic research model, we can identify new factors that may have role in neurodevelopmental disorders.

Interestingly, besides the neurodevelopmental research, it is well established from the neurodegenerative disorders research that both Alzheimer’s and Parkinson’s patients show significant olfactory decline 5-10 years before their diagnosis. However, pathological mechanisms underlying the olfactory deficits in these disorders is not well studied. As VNO is a sub-olfactory system that arises from olfactory placode similar to the main olfactory epithelium (MOE), most of the molecular principles that control neurogenesis vs cell death have common role in both these neuroepithelia. Both the VNO and MOE shows adult neurogenesis throughout their life in mice and have similar stem cell populations that control tissue homeostasis during their adult and old stages. Interestingly, a recently published study also highlighted differences in the VNO directed
social behaviors in the 1-year-old Alzheimer’s mouse model. These factors further highlight that VNO can also be used as a model system to investigate the pathological mechanisms underlying the olfactory deficits that initiate way before the neurodegeneration in these disorders.

Specifically in this dissertation, my doctoral research led to discover how Notch signaling pathway at different stages of the cell development can control the formation of different cell types in the VNO. Even though, Notch signaling pathway is very well known and established pathway elsewhere in the nervous system, showing how same signaling pathway at different cell stages can give rise to different cell types is a novel aspect of my dissertation. Moreover, my study indicate that further experiments should be performed to understand the mechanisms underlying the contextualization and fine tuning of Notch signaling across different cell stages. Thus, studying how Notch signaling can control cell diversity in the VNO is relevant for human health, as the molecular principles discovered in this epithelium can facilitate our understanding of normal and pathological development of multiple neuronal types belonging to the central and peripheral nervous systems.
CHAPTER 5
Detailed materials and methods

Mouse lines: We purchased Ascl1Cre$^{ERT2}$ (Ascl1$^{tm1.1(Cre/ERT2)Jejo}$/J Stock No: 012882), Neurog1Cre$^{ERT2}$ (B6;129P-Tg(Neurog1-cre/ERT2)1Good/J Stock No: 008529), Notch1$^{fl/fl}$ (B6.129X1-Notch1$^{tm2Rko}$/GridJ Stock No: 007181), Rbpj$^{fl/fl}$ (C57BL/6J-Rbpj$^{em2Lutzy}$/J, Stock no: 034200), R26NICD(STOCK Gt(Rosa)26So$^{tm1(Notch1)Dam}$/J, Stock No: 008159), R26tdTom (B6.Cg-Gt(Rosa)26Sortm9(CAG-tdTomato)Hze/J, Stock #007909) Krt5Cre$^{ERT2}$ knock-in (B6N.129S6(Cg)-Krt5$^{tm1.1(cre/ERT2)Blh}$/J Stock No: 029155), Krt5Cre$^{ERT2}$ transgenic (FVB.Cg-Tg(KRT5-cre/ERT2)2Ipc/JeldJ Stock No: 018394), ΔNp63Cre knock-in (B6.129S-Trp63$^{tm1.1(cre)Ssig}$/Stock No: 024564), mouse lines from Jackson Lab. The Foxn4$^{+}/lacZ$ mouse line was generated previously [249]. Genotyping was conducted following the suggested primers and protocols from JAX. Mice of either sex were used for immunohistochemistry and immunofluorescence experiments. All experiments involving mice were approved by the University at Albany Institutional Animal Care and Use Committee (IACUC).

CONTACT FOR REAGENT AND RESOURCE SHARING:
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Paolo Forni (pforni@albany.edu).

METHOD DETAILS
Single-Cell RNA Sequencing
The VNOs of P60 C57BL/6J male mice were isolated and dissociated into single-cell suspensions using neural isolation enzyme/papain (Thermo Fisher, Cat# 88285) in Neurobasal Medium (Thermo Fisher, Cat# 21103049) with 0.5mg/mL Collagenase A, 1.5mM L-cysteine and 100U/mL DNAse I incubated at 37°C. The dissociated cells were then washed with HBSS and reconstituted in cell freezing medium (90% Fetal bovine serum - Thermo Fisher, Cat# 26140079, 10% DMSO - Sigma Aldrich Cat# 472301). Cells were frozen from room temperature to -80°C at a -1°C/min freeze rate. The single cell suspension was sent to SingulOmics for high-throughput single-cell gene expression profiling using the 10x Genomics Chromium Platform.

Single cell library preparation, and sequencing, were conducted by Singulomics Corporation (https://singulomics.com/, Bronx, NY). Cryopreserved, viable single cell suspensions were thawed, washed, resuspended in cell culture media with 0.04% BSA, and counted. Viable cell suspensions were then loaded into the Chromium Controller (10x Genomics, Pleasanton, CA) to generate gel beads-in-emulsion (GEM) with each GEM containing a single cell as well as barcoded oligonucleotides. We made 2 samples targeting 10,000 cells to be captured per sample. Next, the GEMs were placed in the SimpliAmp 96-well Thermal Cycler (Thermo Fisher Scientific, Wilmington, DE) and reverse transcription was performed in each GEM (GEM-RT). After the reaction, the complementary cDNA was amplified and cleaned using Silane DynaBeads (10X Genomics, Pleasanton, CA) and the SPRI select Reagent kit (Beckman Coulter, Indianapolis, IN). Amplified full-length cDNAs from poly-adenylated mRNA were then used to generate 3’ Gene Expression library (Chromium Next GEM 3’ Single Cell Reagent kits v3.1, dual index), following the manufacturer’s instructions (10x Genomics,
Pleasanton, CA). Amplified cDNAs and the libraries were measured by Qubit dsDNA HS assay (Thermo Fisher Scientific, Wilmington, DE) and quality assessed by BioAnalyzer (Agilent Technologies, Santa Clara, CA). Libraries were sequenced on a NovaSeq 6000 instrument (Illumina, San Diego, CA, United States), and fastq files of two samples were generated using Illumina’s bcl2fastq. Fastq files were subsequently processed using 10x Genomics Cell Ranger analytical pipeline (v4.0.0) and mouse mm10 reference. Cellranger aggr was used to aggregate outputs from both the libraries and the aggregated filtered processed files were finally taken as input to do quality control check and further downstream clustering analysis, using Seurat 3.2.3. scRNA-seq data from OmpCreHet P10 animals has been generated as described above.

**Tamoxifen Treatment:** Tamoxifen (Sigma–Aldrich), CAS # 10540–29–1, was dissolved in Corn Oil at 20mg/ml concentration. For all tamoxifen inducible experiments in chapter 2, we injected tamoxifen once intraperitoneally at postnatal day 1 at a dose of 80mg/Kg body weight and perfused at indicated postnatal days. For all tamoxifen inducible experiments in chapter 3, we injected tamoxifen once intraperitoneally at a dose of 180mg/Kg body weight and perfused at indicated postnatal days.

**Tissue Preparation**

For all experiments in chapter 2, collected tissues were perfused with PBS followed by 3.7% formaldehyde in PBS. Noses were immersion fixed in 3.7% formaldehyde in PBS at 4°C for 1-2 hr depending on mouse age. For all experiments in chapter 3, all adult noses were immersion fixed in 3.7% formaldehyde in PBS at 4°C overnight and then transferred to 500mM EDTA solution for 3-5 days to soften the frontal teeth. All samples
were cryoprotected in 30% sucrose in PBS overnight at 4°C then embedded in Tissue-Tek O.C.T. Compound (VWR #Cat 25608-930) using dry ice and stored at -80°C. Tissue was cryosectioned using a CM3050S Leica cryostat at 16µm for VNOs and collected on VWR Superfrost Plus Micro Slides (Radnor, PA) for immunostaining. All slides were stored at -80°C until ready for staining. We included both males and females in our study but didn’t make any sex distinction for the analyses.

**Immunofluorescence**

Citrate buffer (pH 6.0) antigen retrieval was performed [10], for all the antibodies indicated with asterisks (*). Primary antibodies and concentrations used in this study were, Goat anti-AP-2ε (*with antigen retrieval 1:500 and without antigen retrieval 1:200, AF5060, R&D systems), Chicken anti-GFP (1:3000, ab13970, Abcam), Rabbit anti-GFP (1:1000, A-6455, Molecular Probes, Eugene, OR), *Rabbit anti-Ki67 (1:1000, D3B5, Cell signaling Tech), *Mouse anti-Ki67 (1:500, 9449, Cell signaling Tech), *Mouse anti-Meis2 (1:500, sc-515470, Santa Cruz), Rabbit anti-Meis2 (*with antigen retrieval 1:1000 and without antigen retrieval 1:500, ab73164, Abcam), *Mouse anti-NeuroD1 (1:100, sc-46684, Santa Cruz), *Goat anti-NeuroD1 (1:500, AF2746, R&D Systems), *Rabbit anti-Notch1 (1:50, D1E11, Cell Signaling Tech), Goat anti-Dll4 (*with antigen retrieval 1:50 and without antigen retrieval 1:25, AF1389, R&D Systems), *Rabbit anti-NICD activated (1:75, D3B8, Cell Signaling Tech), *Mouse anti-DsRd (1:500, TA180084, Origene), *Rabbit anti-DsRd (1:500, 600-401-379, Rockland), HuC/D 8 µg/ml (Molecular Probes), Rabbit anti-Foxn4 (1:50) [249], *Rabbit anti-Gαo1 (1:1000, PA5-59337, Invitrogen), *Mouse anti-Gαi2 (1:250, clone L5, MAB3077, Millipore), * Rabbit anti-delta N p63 (1:500,
Species-appropriate secondary antibodies conjugated with either Alexa Fluor 488, Alexa Fluor 594, Alexa Fluor 568, Alexa Fluor 680 plus were used for immunofluorescence detection (Molecular Probes and Jackson Immuno Research Laboratories, Inc., Westgrove, PA). Sections were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI) (1:3000; CalBiochem #Cat 268298) and coverslips were mounted with FluoroGel (Electron Microscopy Services, Hatfield, PA #Cat 17985-11). Confocal microscopy pictures were taken on a Zeiss LSM 710 microscope. Epifluorescence pictures were taken on a Leica DM4000 B LED fluorescence microscope equipped with a Leica DFC310 FX camera. Images were further analyzed using FIJI/ImageJ software.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Single-Cell RNA-Seq quality control and cell clustering:** Quality control (QC), and clustering and downstream analysis was performed using Seurat (3.2.3) package in R. Basic filtering was carried out where all genes expressed in ≥3 cells and all cells with at least 200 detected genes were included. QC was based on number of genes and percent mitochondrial genes, where all cells that expressed > 9000 genes and > 5% mitochondrial genes were not included in the analysis. After filtering, 10,582 cells were included for the clustering and analysis. Top 2000 highly variable genes across the population were selected to perform PCA and the first 30 principal components were used for cells clustering, which was then visualized using uniform manifold approximation projection.
(UMAP). Stem cells, neuronal progenitors, precursors and immature neuronal cell types were identified based on the expression of known genes. These cell types were specifically chosen to subset and top 2000 highly variable genes and top 15 principal components were used to cluster and create new Seurat object 2. Similarly, Seurat object3 was created by focusing on clusters only at VSN dichotomy and top 20 principal components were used to cluster and visualize. All downstream analysis that identified Notch1-Dll4 signaling were done on Seurat object 3.

For the integrated P10 and P60 scRNA-seq analysis, we preprocessed and determined highly variable features in P10 and P60 data separately. Seurat integration method was used to identify integration anchors, integrate both data sets and the first 35 principal components were used for downstream cell clustering.

**Pseudotime analysis of cell population.** To further confirm the VSN dichotomy, we used Monocle3 to perform pseudotime analysis, where Ascl1 positive cells were chosen as root node. We used Seurat wrappers package to directly convert Seurat object2 into cell data set format.

**Experimental design, quantification, and statistical analyses of microscopy data.** All data were collected from mice kept under similar housing conditions, in transparent cages on a normal 12 hr. light/dark cycle. Tissue collected from either males or females in the same genotype/treatment group were analyzed together unless otherwise stated; ages analyzed are indicated in text and figures. Measurements of VNE and cell counts were performed on confocal images of coronal serial sections immunostained for the indicated targets. Measurements and cell counts were done using ImageJ. The data are
presented as mean ± SEM unless otherwise specified. Prism 9.0.1 was used for statistical analyses, including calculation of mean values, and SEM. Values of traced cells in distinct genetic backgrounds were compared as % traced. Percentage values were transformed into Arcsine values. P values were calculated using unpaired two-tailed t-test using the arcsine transformed values. Each animal is considered as a biological replicate and both males and females were included in the loss of function and gain of function studies. Data shown as mean±SEM; *P<0.05, **P<0.01, ***P<0.001. ns- not significant. Sample sizes and p-values are indicated as single points in each graph and/or in figure legends.

DATA AND SOFTWARE AVAILABILITY

Accession Numbers

The scRNA-seq data for (P60) mice are available in GEO under accession number GSE190330.

The sRNA-seq data for (P10) mice are available in GEO under accession number GSE192746.


111. Aguillon, R., et al., Cell-type heterogeneity in the early zebrafish olfactory epithelium is generated from progenitors within preplacodal ectoderm. Elife, 2018. 7.


