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Receptor-Mediated Maturation of Olfactory Sensory Neurons: A Time Course Evaluation of Neuronal Maturation in Neomorphic Mutants

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Receptor-Mediated Maturation of Olfactory Sensory Neurons: A Time Course Evaluation of Neuronal Maturation in Neomorphic Mutants

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ABSTRACT

Olfactory receptor (OR) genes comprise the largest gene family in the mammalian genome, with over 1000 coding sequences in mice. Olfactory Sensory Neurons (OSNs) express a single gene from a single allele, ensuring one receptor per neuron. OSN cell bodies reside in spatially segregated domains in the olfactory epithelium that allow for the expression of a restricted subset of ORs. Axons from identical OSNs project to homogenous glomeruli in the olfactory bulb. How OSNs choose a single receptor and then target the bulb to coalesce with like OSNs is poorly understood. Neurons that project axons to a glomerulus, capable of odorant signal transduction, are considered to be "mature." These are distinguished from immature neurons by the expression of Olfactory Marker Protein (OMP), among many other differences. How the process of differentiation to maturity integrates the choice and maintenance of a functional receptor is not known, nor is it known if maturation is a timed or triggered event. These are the topics explored in this study. The impact of a the receptor on maturation is examined in animals that express a class of receptors known as "neomorphic mutants"—they can lock in choice, but are defective in folding, trafficking, and signaling, resulting in poor axon outgrowth and a failure to mature and form a glomerulus. In a time-course experiment using methods of immunohistochemistry and confocal imaging, populations of cells expressing the chimeric receptor M71B2 and the signaling mutant M71(RDY) were compared to cells expressing the wild type receptor M71 at postnatal day (PD) 1 and PD7. By the seventh day in wild type animals, mature OSNs accounted for 10% of total cells, in stark contrast to mutants which contained close to 0% mature cells. In 3-week old animals, wild type OSNs continued to flourish and form a glomerulus, whereas neomorphic mutants decreased in number drastically, most likely dying out and getting cleared. Strikingly, an antibody to the B2AR revealed that the chimeric receptor does not traffic to the plasma membrane but instead gets stuck in ER. These results suggest that a successful receptor plays a critical role in the differentiation pathway of OSNs, and call into question current models that propose receptor switching and OR expression as a post-targeting event.

Acknowledgements

This thesis is dedicated to my family, who has supported me every step of the way, and to Eugene Lempert, who has been by my side as mentor and friend during my time in the Feinstein Lab. I acknowledge Paul Feinstein and all of the members of our lab and thank them for providing me with such a joyous experience that I will always treasure.

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List of Important Abbreviations:

B2AR: Beta 2 adrenergic receptor

GAP43: Growth Associated Protein of 43kD

GFP: Green Fluorescent Protein

H2Bcherry: the histone protein H2B fused to an mCherry fluorescent protein, exciting the red channel to visualize nuclei

M71: a well-characterized murine odorant receptor used as the wild type reference in the current study

M71B2: A chimeric receptor composed of amino acids 1-167 of the M71 odorant receptor fused to amino acids 173-418 of the β2 adrenergic receptor

OE: Olfactory epithelium

OMP: Olfactory Marker Protein

OR: Odorant Receptor

OSN: Olfactory Sensory Neuron

PD: Post natal day

WT: Wild type

INTRODUCTION

The mammalian olfactory system has the power to detect billions of different odors from the environment. Volatile molecules are first received in the nasal cavity, where they bind to G-protein-coupled 7-transmembrane odorant receptors (ORs) on the cilia of olfactory sensory neurons (OSNs) (Buck and Axel, 1991; Menco et al., 1997). Through a complex relay of neuronal circuitry, the brain quickly outputs a behavioral response to the odor that is often critical to an animal's survival. Evading predators, avoiding rancid foods, and attracting a suitable mate are all within the purview of this evolutionarily conserved system. How can an organism provide a unique interface to an astronomical number of novel compounds? Unlike immune receptors which derive their impressive diversity from genetic recombination, odorant receptors are individually encoded in the genome. In fact, odorant receptors comprise the largest gene superfamily in mammals, with mice having a repertoire of over 1000 coding genes distributed in clusters on multiple chromosomes (Buck and Axel, 1991; Sullivan et al., 1996; Zhang and Firestein, 2002).

1.1 Anatomy of the system

The anatomical organization and cell type array of the olfactory system play a critical role in deconstructing the molecular data received from the outside world. Olfactory sensory neurons are born from a population of multipotent stem cells that renew and differentiate throughout adult life. OSNs, their progenitors, and several other supporting cell types are embedded in epithelium that lines an intricate labyrinth of turbinate bones. These provide the surface area to expose millions of receptors to the lumen of the nasal passageway, and vary

greatly in shape from mammal to mammal (Figs. 1 and 2) (Niimura and Nei, 2007). With its soma and single dendrite projecting apically, the bipolar OSNs extend axons to the olfactory bulbs of the forebrain, where they converge on a spherical structure of neuropil called a glomerulus (Klenoff and Greer, 1998). Here, the axon termini of OSNs synapse with second order neurons, which then relay signals to deeper cortical regions of the brain responsible for the recognition and perception of odors. Just as there are hundreds of different odorant receptors encoded in the genome and millions of OSNs expressed in the Main Olfactory Epithelium (MOE), there are close to 1800 glomeruli per olfactory bulb in an adult mouse (Greer et al., 1994; Mombaerts, 2006). The stereotypic organization of these glomeruli provides a topographical map that the brain deciphers as an "odor code" (Strotmann et al., 2000; Malnic et al., 1999). Thus, there is a hierarchy of three agents responsible for parsing out odors: the odorant receptor itself, the OSN that expresses it, and the glomerulus on which it converges.

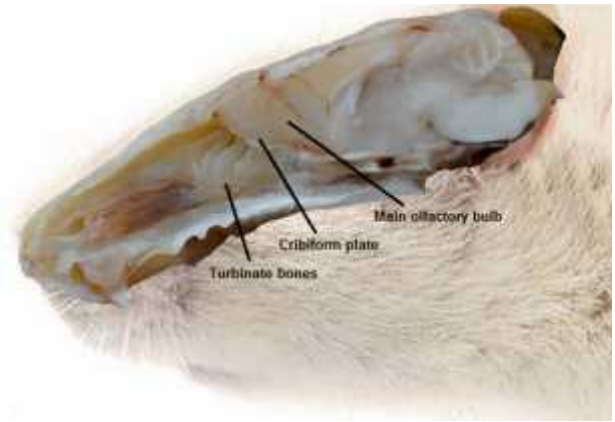

Figure.1. Sagittal view of mouse olfactory components.
Taken from Barrios et al., 2014.

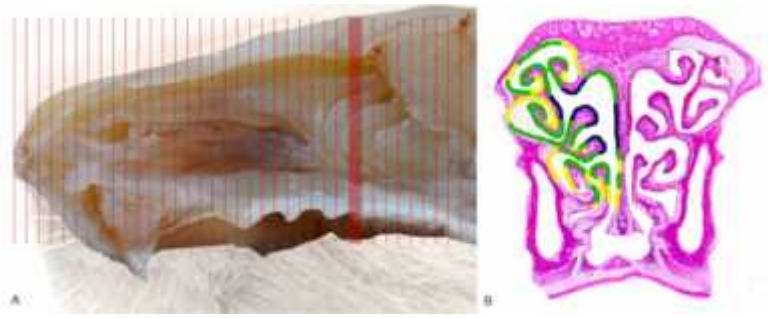

Figure 2. Cross sections of the turbinates (a) Sagittal view of a mouse snout showing turbinate bones with lines of transverse segments. (b) Cross-section of the highlighted segment from (a) visualized with Haematoxylin-eosin-staining. Taken from atlas at http://www.usc.es/anatembriol, Barrios et al., 2014.

Several unusual hallmarks rest at the heart of the functional unison between the molecular mechanisms and gross anatomical organization of this complex system. These defining properties are also the conundrums that continue to baffle the field after twenty years.

1.2 Singular expression

The first of these is an OSN's expression of a singular odorant receptor. It is known that OSNs generally express one gene amongst its available repertoire of thousands—this is referred to as "monogenic expression" (Malnic et al., 1999). Likewise, an OSN only expresses one allele of this gene in what is known as "monoallelic expression" (Chess et al., 1994; Strotmann et al., 2000). These phenomena are collectively referred to as "singular expression" (Vassalli et al., 2002). Many different models attempt to explain how this task is possible.

Not only must an OSN choose a receptor, but it must ensure that this receptor is maintained throughout the cell's life. Once a receptor has been "chosen", there is a mechanism in place that prevents choosing another receptor. By activating this mechanism—which may be linked to or separate from the choice mechanism itself— a receptor is said to have "locked in choice" (Feinstein et al., 2004; Feinstein and Mombaerts, 2004). Odorant receptors and many GPCRs, when flanked by appropriate OR-specific promoters, will lock in choice (Feinstein et al.,

2004a). Even if they fail at making a viable OSN, the deal is done and they do not go back to pick another receptor. However, some argue that switching receptors is possible (Dalton et al, 2013; Shykind et al., 2004). Evidence for this is inconclusive. Other sequences that lack OR character, when placed into an OR locus or flanked by OR promoters, will *not* lock in choice. A classic example is GFP. In such an animal, one can see residual GFP in axons that project to random glomeruli (Feinstein et al., 2004; Feinstein and Mombaerts, 2004). These sequences must bypass or fail to activate necessary components of the system. The qualifications for a sequence to lock in choice are not fully understood.

1.3 Spatial segregation

The second defining property unique to the mammalian olfactory system is the spatially restricted organization of OSNs expressing a given receptor within the epithelium lining the turbinates. There are four distinct but continuous and overlapping "zones" along the dorsal-ventral axis of the epithelium, each home to a subset of OSNs that expresses only certain receptors (Ressler et al., 1993; Vassar et al., 1993; Mayimichi et al., 2005). They are denoted as "Zones 1-4" and are positioned on a roughly dorsal to ventral axis (Fig.3a). Zones are not macroscopically distinguishable structures but domains of cell expression, as the epithelium otherwise seems axially uniform throughout. Up to thousands of cells expressing the same receptor—i.e. identical OSNs—are dispersed in a punctate pattern throughout their respective zones.

Progenitor cells within a zone seem to stochastically choose within a restricted repertoire of receptors; however, the allowable repertoire differs between neighboring zones (Strotmann et al., 1994). That subsets of OSNs are spatially restricted and competent to choose from an invariant zone-specific selection of receptors makes the idea of different cell "types" attractive

(Feinstein, 2004a). A diminished repertoire of fates thus lends a “reduction in complexity” to the feat of choosing one receptor gene among thousands (Rodriguez, 2013).

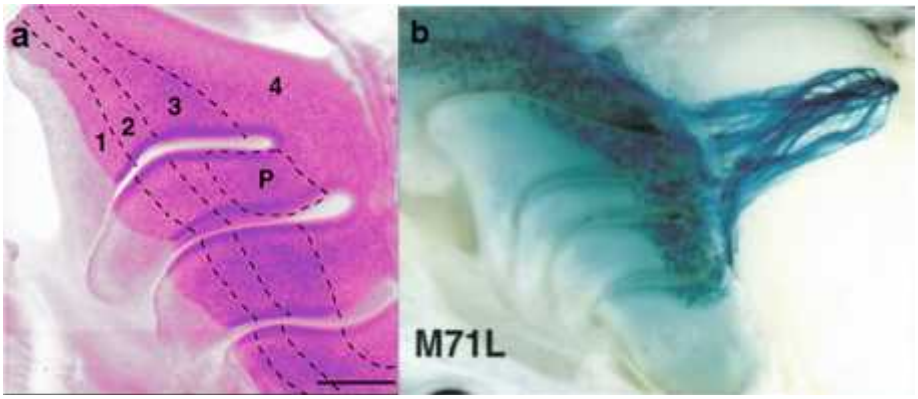

Figure 3. Zonal expression of OSNs. (a) Approximate demarcation of Zones 1-4 on the turbinates**.** Adapted from Vassalli et al., 2002. (b) X-gal stained Wholemount showing expression of odorant receptor M71 cells restricted to “zone 4.” Scale bar 500μm. Taken from Feinstein and Mombaerts, 2004.

1.4 Axon convergence

The last remarkable characteristic of the system’s functional organization is the fact that OSNs expressing the same receptor—from their scattered zonal locations on the turbinates—converge into two homogenous glomeruli on the medial and lateral halves of each olfactory bulb (fig. 4). Here, axon termini of OSNs synapse with dendrites of second-order neurons known as mitral and tufted cells. The anteroposterior and circumferential location of these glomeruli are roughly consistent in species counterparts, although there is local variability, even within both bulbs of a single animal (Conzelmann et al., 2001; Strotmann et al., 2000.) It is known that OSNs from particular “zones” tend to project to regions of the bulb in a dorso-ventral manner; namely, cells from zone 4 tend to project to glomeruli that are more dorsal (Ressler et al., 1993; Vassar et al., 1993) (Fig. 3b). But the mechanisms that govern an OSN’s trajectory to general regions on the bulb from the epithelium are not well-understood, and there is no accepted model.

How axons expressing the same odorant receptor find each other and sort themselves into an exclusive family home on the bulb also remains one of the largest enigmas of the field. Axonal identity is dependent on different components, but the OR itself seems to be of paramount importance. We know that changing just one amino acid in an OR's sequence can project the axons to ectopic glomeruli (Feinstein et al., 2004b; Feinstein and Mombaerts, 2004a). Several schools of thought have emerged over the years offering different models to explain axon targeting. On one side, differing amounts of cAMP signaling provide discreet amounts of downstream effectors such as guidance cues (Imai et al., 2006). On the opposite side, homotypic interactions between adhesion molecules demarcated by receptor shape solely govern the union of like OSNs (Feinstein and Mombaerts, 2004). Though very different, both of these approaches aim at a "unified theory," so to speak. They seek to find a single factor that is used differentially to account for the vast number of converging OSN types. Encoding an additional unique link, since the OR is already unique to the OSN—say, a guidance molecule for every OSN type—would be redundant and would not make biological sense.

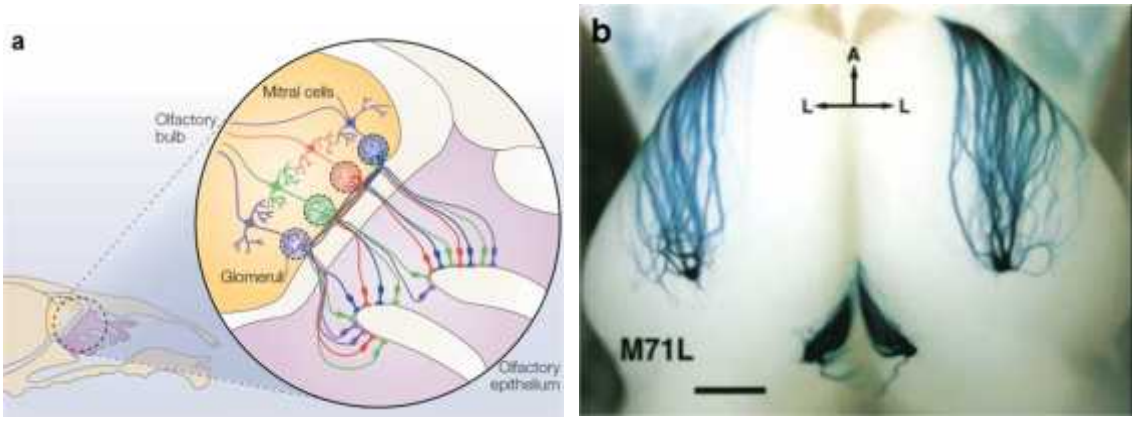

Figure 4. Axons project to two homogenous glomeruli on each bulb. (a) Schematic of different OSNs (denoted by color) projecting to their respective glomeruli on the olfactory bulb. Here, the axon terminals synapse with the dendrites of mitral cells. Taken from Farley, 2004. (b) Dorsal view of olfactory bulbs displaying labeled axons of odorant receptor M71 converging on medial and lateral glomeruli. Wholemount X-gal stain. The OR CDS is followed by gene-targeted IRES-tau-LacZ as a histological marker. Scale bar 500μm. Taken from Feinstein and Mombaerts, 2004.

1.5 Just regular neurons?

Olfactory sensory neurons, although they are epithelial cells thought to be derived from both neural crest and placodal origins, are indeed neurons proper (Suzuki et al., 2015). Like other typical peripheral neurons, they have a designated job to do which is receive chemical ligands on their dendrites, fire an axon potential, and form synapses with interneurons (Brann and Firestein, 2014). OSNs differentiate according to a lineage pathway like any other somatic cell, the only difference being that OSNs form from an adult stem cell niche in specialized epithelium. Like other differentiating cells, they follow a program that is more or less timed and replicable, relying on internal cascades and external signals.

1.6 Maturation, concluding questions, and my project

A terminally differentiated OSN, i.e. a neuron that is fully mature, has achieved several properties of functionality, molecular expression, and physiology that distinguish it from previous stages. Its dendrite has an arboration of cilia that protrude into the nasal cavity, dense in receptors. Its axon has made the complete journey to a home in a glomerulus; specifically in the glomerular nerve layer of the olfactory bulb (Kim and Greer, 2000). It synapses with mitral cells. It has down-regulated Growth-Associated Protein-43 (GAP43), a pan-neuronal molecule expressed in the growth cone of growing neurons and a marker for immature OSNs. In turn, it has upregulated Olfactory Marker Protein (OMP)—notably the "marker" of mature cells. The profile of signaling machinery has also changed. Gs, the G_{α} component of the receptor-coupled heterotrimeric G protein—is expressed in basal cells but then replaced by its homolog, G_{olf}, in mature cells. The mature neuron has upregulated adenylate cyclase, the downstream effector of G_{olf}. It responds to its cognate ligand by firing an axon, thus dispatching a relay that will result in

odor perception. A pressing question then emerges: How do differentiation and maturation integrate the aforementioned unusual hallmarks of olfaction—namely, singular gene choice, zonal restriction, finding like OSNs, and ending up in the "correct" glomerulus on the bulb? Will an OSN mature if it locks in a receptor that misfolds? Must the receptor couple with signaling machinery to mature? Are there checkpoints in the cell's maturation that are contingent on the success of a receptor?

My current study aims to contribute insights to these topics by assessing the maturation status of receptor mutants in a time-course experiment. The mutants I examine, known as "Neomorphs," lock in choice, but are either nonfunctional in folding, trafficking, signaling, or a combination thereof. I show that these receptors don't allow cells to mature, therefore impeding axon growth and ultimate survival. The results suggest that receptors play an integral part in allowing the cell to properly progress, most likely through checkpoints and feedback.

2. BACKGROUND

Olfactory maturation is a topic that is complex and broad in scope. Therefore, considerable background is needed to tie many concepts together in my *Discussion* section.

I will give a brief background of some "basics" about the olfactory system such as its anatomy, cellular makeup, and functions. Then I will discuss anomalies about the system and current models about axon guidance. This section will conclude with a description of Neomorphic mutations and their use in this experiment.

I. Basics of the Olfactory System

2.1 Cell types, cells lineages, and molecular turnovers

The mammalian olfactory epithelium is composed of four cell types that form a pseudostratified epithelium which is interspersed with mucus secreting Bowman's glands (Fig. 5). These cell types are OSNs, basal cells, sustentacular cells, and microvillous, the latter two referred to as "supporting cells." Sustentacular cells are thought to have a similar function to glial cells in the brain. The function of microvillous cells is not well understood. The epithelium lies beneath the lamina propria, a vascularized connective tissue through which axons travel. Cells that enter the neuronal lineage, ultimately to become mature OSNs, move progressively toward the apical side of the epithelium as they differentiate (Yu and Wu, 2015).

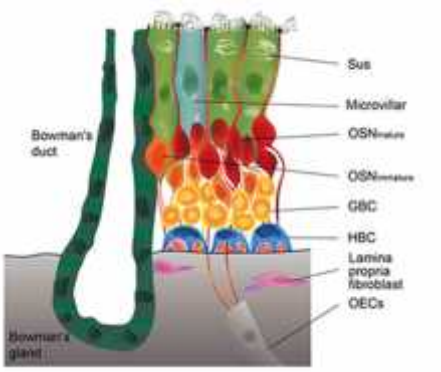

Figure 5. Anatomy of the olfactory epithelium.
Taken from lab website of James Schwob.

As mentioned in the *Introduction*, OSNs come from a population of stem cells that regenerate throughout adult life. Their consistent replacement is needed, as they are subjected to environmental damage from chemicals, infection, and mechanical insult on a constant basis. Therefore, their lifespan is shorter than that of other neurons, ranging from 30-90 days in a clean environment (Yu and Wu, 2015; Brann and Firestein, 2014.)

There are two populations of multipotent stem cells that reside at the basalmost layer of the epithelium: Globose Basal Cells (GBCs) and Horizontal Basal Cells (HBCs). While GBC's are mitotically active, HBCs were originally thought to be quiescent under normal circumstances, as a reserve for regenerating OSNs after severe injury (Yu and Wu, 2015). It has

been shown however that both can give rise to all cell types in the olfactory epithelium (Leung et al., 2007), and that HBC's can give rise to sustentacular cells through "direct fate conversion" without dividing (Fletcher et al., 2017) but I will focus my discussion on GBCs and the neuronal lineage program.

GBCs that are Sox2+/Pax6+ have the totipotent potential to generate multiple lineages, and are the cells that cycle through regeneration (Schwob et al., 2017). These differentiate into transit amplifying cells (TACs) that are $Ascl1^{+}$ (Mash-1) and have committed to the neuronal fate. TACs then become immediate neuronal precursor cells (INP) that express Neurog1, and NeuroD1 and NeuroD2. These are the direct precursors of immature OSNs (Schwob et al., 2017; Yu and Wu, 2015).GAP43

Immature OSNs, which are the subject of this study, express markers Gγ8 and GAP43. The former is a G-protein gamma subunit, and the latter is Growth-Associated Protein-43. Gap43 is a mostly cytoplasmic protein expressed at high levels in the neuronal growth cone and presynaptic terminals during axonal growth (Rosskothen-Kuhl and Illing, 2014). This is no wonder, since immature neurons are the cell stage during which axons grow. GAP43 has many different binding partners, is a substrate for Protein Kinase C, and associates with actin. (Benowitz and Routtenberg, 1997). It is not known how GAP43 and receptor choice and maintenance are related.

The signature of mature OSNs is olfactory marker protein, or OMP, a cytoplasmic protein whose exact role still eludes us. It has been suggested that it plays a role in glomerular refinement (Albeanu et al., 2018) and controlling basal and odor-evoked cAMP signaling (Dibattista and Reisert, 2016). In a developed olfactory system, OMP+ cells cover a broad domain in the epithelium and account for over 90% OSNs (Iwema and Schwob, 2003). During

the process of neuronal maturation, transcripts for intracellular guidance cues are shown to be more abundant in immature cells, while receptors involved in growth inhibition and repulsion are upregulated in mature OSNs, in line with mature OSNs having ceased growth and settled in a glomerulus (McIntyre et al., 2016.). During the maturation process, GAP43 is downregulated and OMP is upregulated. Cellular events that lead to this turnover have not been well-characterized.

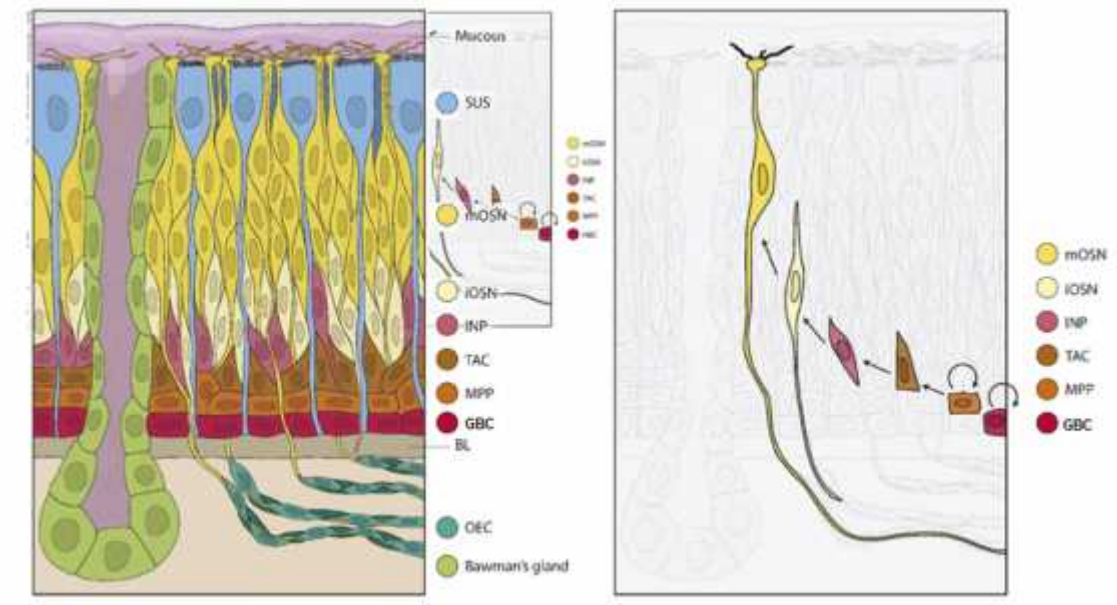

Figure 6. Cell types and lineages in the olfactory epithelium. (a) Pseudostratified epithelium where cells at different developmental stages reside in different layers. (b) Differentiation of a Globose Basal Cell (GBC) into a mature neuron. Adapted from Yu and Wu, 2017.

2.2 *Receptors and Odor signal transduction*

Odorant receptors are Class A Rhodopsin-like GPCRs with a typical 7 transmembrane configuration. Although thousands of sequences of ORs are known from genomic data, no experimental structure has been modeled to date (de March et al., 2015a). ORs share the 7-transmembrane structure as G proteins in general but differ in their amino acid sequence; areas prone to divergent amino acid sequences—namely transmembrane domains III,IV, and V—are likely responsible for odor discrimination (Pifferi et al., 2010).

The signaling cascade that ensues from the binding of an odorous ligand results in a depolarization of the membrane leading to an action potential (Fig.8). Being that odorant receptors are GPCRs, it is not surprising that mammalian transduction machinery is familiar. The cilia extend from the dendritic knob into the external environment, and it is here where odor detection and electrical excitation take place (Fig.7).

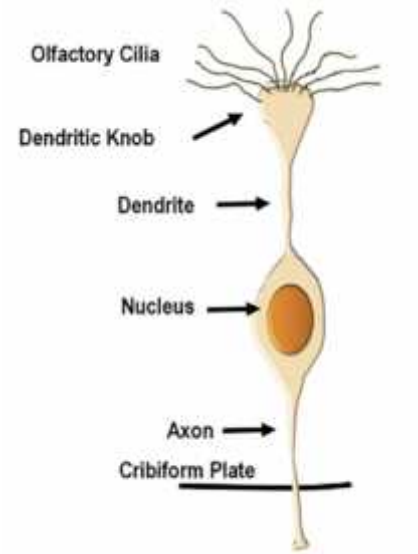

Figure 7. Schematic of an olfactory sensory neuron showing dendritic knob and cilia. Adapted from McIntyre et al., 2016.

When a ligand is bound, an olfaction-specific excitatory G-protein, G_{olf}, becomes activated (Jones and Reed, 1989). Its alpha subunit is a guanosine exchange factor, and dissociates from the other two subunits of the heterotrimeric G protein, β and γ, when in the GTP-bound conformation. G_{olf} alpha activates Adenylate Cyclase type III (ACIII/ADCY3) which in turn catalyzes ATP to cAMP. Increase in cAMP concentration causes the cyclic nucleotide gated channel (CNGA2) to open, generating an influx of Na^+ and Ca^+ that depolarizes the membrane. The calcium ions that enter this channel have excitatory and inhibitory roles (Matthews and Reisert, 2003). Intracellular Ca^{2+} opens Ca^{2+}-activated chloride (CAC) channels which causes further depolarization by an efflux of chloride out of the cell (Pifferi et al., 2010;

Antunes and Souza, 2016). Through two mechanisms, Ca^{2+} mediates adaptation of the signal. One is a Na^{+}/Ca^{+} exchanger, and the other is through calmodulin that activates the plasma membrane Ca^{2+} ATPase (PMCA) pump (Matthews and Reisert, 2003; Pifferi et al., 2010).

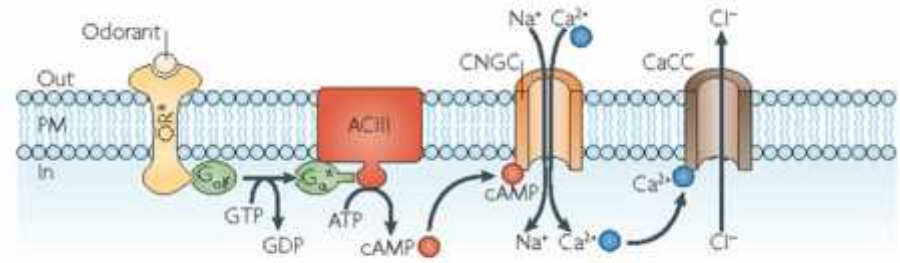

Figure 8. Signal transduction cascade of mammalian odorant receptor. Ligand binds OR; Golf alpha dissociates and activates Adenylate Cyclase III which catalyzes ATP to cAMP; cAMP opens gated nucleotide channel allowing influx of calcium; calcium binds Ca^{2+}-activated chloride which lets out Cl^{-}. Taken from Kaupp, 2010.

Odorous ligands function as agonists that stabilize the receptor in an "active" conformation state. However, it is important to note that in the absence of ligand, ORs are known to spontaneously flip back and forth between the active and active conformations, leading to a baseline level of cAMP signaling (Bond and Ijzerman, 2006; Nakashima et al., 2013) and spontaneous spiking (Movahedi et al., 2016). Moreover, it was shown that different ORs can produce a different level of constitutive signaling that drives fluctuations in membrane current (Reisert, 2010).

Signaling machinery is developmentally regulated, and the ligand-dependent signal transduction described is functional in mature OSNs. G_{olf} is also a marker for maturity, expressed late in development (Tan et al., 2015). A curious transition happens early on wherein OSN progenitor cells express Gnas, the gene for the ubiquitous Gs heterotrimeric G-protein subunit. Later in development, cells "switch" to express Gnal, the gene for G_{olf}. This can be seen in an in situ experiment by Movahedi et al. where an ISH probe for Gs co-localizes to the basal-most layer of the epithelium. There is barely any overlap of Gs transcripts in immature OSNs. In the

same experiment, Gnal transcripts co-localize exclusively with the OMP layer. No G alpha component transcripts are found to co-localize with the GAP43+ layer in this study, indicating that some sort of turnover event is taking place during the immature phase (Movahedi et al., 2016). We do not know the mechanism or cause by which one subunit is downregulated and another is upregulated. This turnover may be impacted by the choice of a successful receptor.

II. Anomalies, Advanced Concepts, and Current Literature

2.3 Some anomalies

OR coding sequences and their promoters have some curious properties. As mentioned in the *Introduction*, OR selection is monoallelic. There is some sort of suppression going on preventing the same receptor to be expressed from both maternal and paternal loci. The same is found to be true of endogenous receptors and transgenes with the same coding sequence (Serizowa et al., 2000).

ORs remarkably cannot be expressed from most non-OR promoters. It was shown that when placed under promoters heavily expressed in ORs like those for the genes OMP and Gγ8, unrelated GPCRs were able to express in every cell while ORs were not, suggesting that the repression is specific to ORs. However, ORs can be made to express under the Tta/TetO system. When the sequence is under the TetO promoter, it is activated by the tetracycline transactivator which is itself driven by an OR tissue-specific promoter (Nguyen et al., 2007; Malnic et al., 2010). This system is now used extensively in olfaction research.

2.4 Components of the signal transduction pathway used non-canonically in receptor choice and axon guidance? A look at some models

The signal transduction pathway of odorant detection is relevant to maturation because we have seen that the onset of expression of the components is phased across differentiation. Several models of receptor choice/maintenance and axon targeting have emerged that involve constituents of the signaling pathway.

The Sakano lab has put forth a model that implicates agonist-independent cAMP signaling as the determinant of axon targeting to the anteroposterior (A-P) axis of the olfactory bulb. In this model, each OR generates a unique level of agonist independent-activity which is translated to cAMP signals by Gs and ADCIII. Through a "noncanonical" signaling pathway involving protein kinase A (PKA) and cAMP response element binding protein (CREB), guidance molecules nrp1 and plxnA1 are differentially expressed, leading to different settlements along the A-P axis. They showed these target "shifts" by generating activity mutants of surrogate B2ARs that couple with Gs in abnormal ways ("high activity" and "low activity"). However, Assens et. Al showed that knockout of nrp1 has no effect on A-P patterning of glomeruli (Assens et al., 2016).

The Sakano model also proposes that agonist-dependent cAMP signaling, namely the canonical pathway, is responsible for regulating the transcription of "glomerular sorting molecules" like kirrel2 and kirrel3. (Nakashimi et al., 2013; Imai et al, 2006).

When the same lab created constitutively active and dominant negative mutants of Gs and downstream effectors like PKA and CREB, they saw aberrant targeting on the bulb (Imai et al., 2006). These mutants were under an OR-specific promoter, which, in any olfaction experiment is confounded by the fact that events prior to OR expression have already happened or leave residual effects. In other words, consequences of reviving a protein that may have taken effect at an earlier state cannot be correlated with manipulations of the protein's natural function

later on. For instance, if proteins in the aforementioned pathway had already had their influence in OSN progenitors, then mutating these proteins at the immature state would not be an accurate measure of their effects. The same goes for when a protein is normally expressed after OR choice is made. This is one of the largest barriers to olfaction experiments: in order to see effects in an OR-expressing type, one must use the OR promoter of that cell type.

Other labs (including the Feinstein Lab) reject the model that differential cAMP levels are responsible for axonal targeting, in favor of an OR-instructed identity. Several experiments were performed to show this. One such study used two distinct receptors, M71 and MOR23, which were crippled by replacing the conserved DRY motif with RDY. The DRY motif is thought to be crucial for coupling with heterotrimeric G proteins. The signaling mutants were co-translated with a constitutively active Gs (caGs) subunit using an internal ribosome entry site (IRES). In this way, the caGs mutant in both cell types would be the sole determinant of cAMP production, as none would result from the receptor signaling. In this experiment, glomerular formation was often hindered by low number of rescued cells, but in heterozygotes for the mutant alleles, axons projected to two fundamentally different areas on the A-P axis, showing that receptor identity plays a role in axon-targeting irrespective of differential cAMP signaling. Another interesting way they "leveled the playing field" in cAMP signaling was by expressing M71 through the early and pan-olfactory promoter O/E2, which made every cell in the entire epithelium express M71 on its surface. OSNs expressing the signaling mutants were rescued by O/E2-M71, which provided the same level of cAMP signaling for both receptors. They each projected to different regions on the bulb (Movahedi et al., 2016). That OSNs incapable of producing cAMP through their "chosen" receptor still projected to different A-P regions lends

credence to the idea that receptor identity is critical to axon targeting and that differential cAMP signaling can be dispensed with.

In the "contextual sorting model" of axon targeting, OR-mediated homophilic and heterophilic interactions provide the means by which axons sort themselves out and coalesce into glomeruli; axonal identity is dependent on the OR molecule and whatever other axons are present (Feinstein and Mombaerts, 2005). Here, axons sample one another at their growth cones, likely through protein complexes that contain odorant receptors and demarcate their three-dimensional structure (Feinstein and Mombaerts, 2005; Feinstein and Mombaerts, 2004a). Candidates for proteins that could "outline" the receptor are cellular adhesion molecules. Data supporting the contextual sorting model comes in a series of OR "swap" and modification experiments. When the coding regions of M71 and M72—sequences that share close homology but form distinct glomeruli—were transposed, it was shown that axons rerouted to donor glomeruli. When various amino acid changes were made, including single substitutions, axons projected to ectopic glomeruli over a range of phenotypes. Some were in different positions on the bulb altogether, whereas some formed "compartmentalized" glomeruli (Feinstein and Mombaerts, 2004a). These results are highly suggestive that the receptor is integral to axonal identity and glomerular targeting.

2.5 Neomorphic Mutants

Six definitive properties that characterize OSNs are that they: (a) lock in gene choice; (b) promote axon outgrowth; (c) develop into mature neurons; (d) have an axonal identity; (e) form glomeruli; and (f) are capable of odorant perception (P. Feinstein, personal communication; Feinstein and Mombaerts, 2005). A special set of receptors, when substituted into an OR locus, produce similar and novel phenotypes that vary by degree in observance and violation of the

aforementioned properties. These have since been termed “neomorphic mutations,” and their behaviors can teach us a great deal about how the olfactory system works (Feinstein et al., 2004b).

Certain GPCRs, when under OR promoters, can function as “surrogate” receptors. Criteria for this substitution are showing strong immunoreactivity in cilia and axons, responding to cognate agonists of the surrogate receptor and not the receptor of the endogenous locus or promoter, and coalescing into glomeruli or at least coalescing to a restricted region on the bulb (Katidou et al., 2018). To date, the best surrogate receptor is the beta-2 adrenergic receptor (B2AR). Not only is it well-characterized, but it is amenable to experiments in which ORs do not work. For instance, it can traffic to the membrane of heterologous cells and be used successfully in vitro. Other surrogates that work as substitutes but less successfully are the Mc4r and Drd1a receptors (Katidou et al., 2018). Notably, these receptors couple to Gnas and Gnal, the G-protein subunits that couple with ORs.

Neomorph Type I mutants are 7-transmembrane receptors that behave poorly when expressed from an odorant receptor promoter and fail in most cases at observing the criteria for OR surrogacy. Examples of this type are the vomeronasal receptor V1rb2 and the RDY mutants like M71(RDY). It is believed that these receptors do not function properly because they do not couple to signaling machinery. They have been shown to project axons to the bulb in very limited capacity---V1RB2 extends axons to the bulb in about 3 out of hundreds of samples (Feinstein et al., 2004b), and I7(RDY) projects axons in a diffuse cloudlike pattern (Imai et al., 2006).

Neomorph Type II mutations are even less effective than Type I. Examples of such receptors are chimeric receptors such as the fusion of M71 and B2AR, or ORs that are crippled

by an epitope inserted into a critical region. One such neomorph of the latter variety is the M72(FLAG) receptor, which has a FLAG-like epitope inside of the C-terminus (Feinstein et al. 2004b). It is likely that these proteins are misfolding in the endoplasmic reticulum (P. Feinstein, personal communication).

The phenotypes of these neomorphic mutants seem to be poor axon outgrowth and decrease in cell number as the animal ages. Studying the consequence of growth and maturation in cells that lock in choice but fail at making a functional receptor can give us insight about where and when certain checkpoints and limiting events may occur.

3. RESULTS

In this cell-counting experiment, one of the main aims was to investigate what percentage of cells is mature as a function of time. It is known that ORs are first detected in mice as early as Embryonic Day (E)11.5-12.5 and that protoglomeruli emerge at E17 as axons are migrating through the neuropil regions of the bulb (Sullivan et al., 1995; Conzelmann et al., 2001; Treloar et al., 2010). By PD1, glomeruli can be seen in small numbers, but they increase in size and definition in the postnatal period (Treloar et al, 2010). In this study, I examine early postnatal mice that may show a combination of embryonic OSNs, as well as OSNs born after birth. Whether these are the same or different is contested and remains an active area of research (Brann and Firestein, 2014; Treloar et al., 2010; Yu and Wu, 2017). Several groups have reported that the time between basal cell division and maturation is about 6-7 days (Iwema and Schwob, 2003; Rodrigues-Gil et al., 2015).

The image data collected in this study displays several trends in epithelial positioning, but it must be noted that my observations are of subtle differences in most cases. While the numbers of cells counted are appreciable, the animal sample size is small. Statements about cell position are qualitative and based on observation, as it was not possible to quantitate the position of every cell and tabulate it into an array.

3.1 Considerably more cells expressing a wild type receptor mature by PD7 compared to cells expressing Neomorph Type I and II receptors

Strains used in this study were mice expressing a wild type receptor with a downstream IRES-tauGFP (which henceforward will be referred to as "wild type"), as well as two neomorphic receptors: M71(IRES)-tauLacZ, and M71B2-IRES-tauLacZ (Fig. 9). The olfactory epithelium of Postnatal Day 1 (PD1) and PD7 mice was sliced into 25μm sections. Cells were visualized by intrinsic GFP or immunohistochemistry to β-galactosidase, yielding green fluorescence, as described in the *Materials and Methods* section. In some instances, cells on every slice were counted; in other instances, cells were counted on every third slice. Immature cells were distinguished as being "green only" by the appearance of fluorescent tau. Mature cells were visualized by a red nucleus resulting from expression of a transgene containing H2BmCherry under the OMP promoter. The total cell counts from two animals of each strain and age were summed, and the total number of mature cells in each group was calculated as a percent (see Table 1 for cell counts).

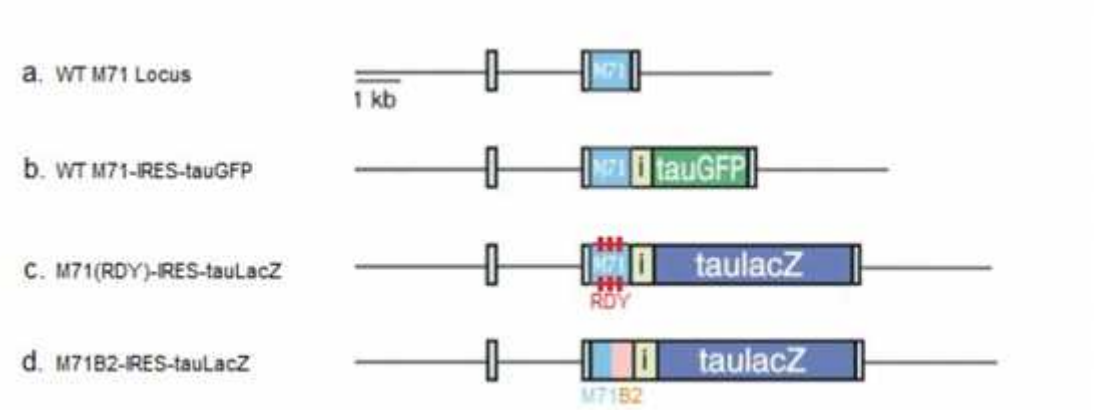

Figure. 9. Schematic of constructs used. (a) The native M71 locus (b) a WT receptor followed by IRES tauGFP .(c) M71(RDY-IRES-tauLacZ. (d) M71B2-IRES-tauLacZ. All of the mutants and histological marker cassettes are gene-targeted into the M71 locus by homologous recombination. Adapted from Feinstein et al., 2004b.

Fig. 10 is a graphical representation of the percentage results. Mature cells comprised 3.16% of PD1 wild-type animals. In this strain at PD7, the percent of mature cells increased considerably to 10.82%. These results are in sharp contrast to what was observed in neomorphic mutants. 1.25% of M71(RDY) PD1 cells were mature—that is, 3 cells out of 240 counted. 0% of mature M71(RDY) cells were observed at PD7. Cells from this second group were counted on every 3rd section so it is possible that some were missed, but they would likely have amounted to a small % of the total. 0% of M71B2 chimeras were mature at both time points.

	WT M71 PD1 (n=2)	WTM7 PD7 (n=2)	M71(RDY) PD1 (n=2)	M71(RDY) PD7 (n=2)	M71B2 PD1 (n=2)	M71B2 PD7 (n=2)
Immature	92	206*	237*	274*	100*	349*
Mature	3	25*	3*	0	0	0
Total	95	231	240	274	100	349

Table 1. Total Cell Counts. For each strain and age, the number of immature and mature cells counted are tabulated and summed. These numbers were used to derive percentages. These numbers represent total counts from the 2 animals used in each group. * represents counts incorporating samples that were counted every 3rd section.

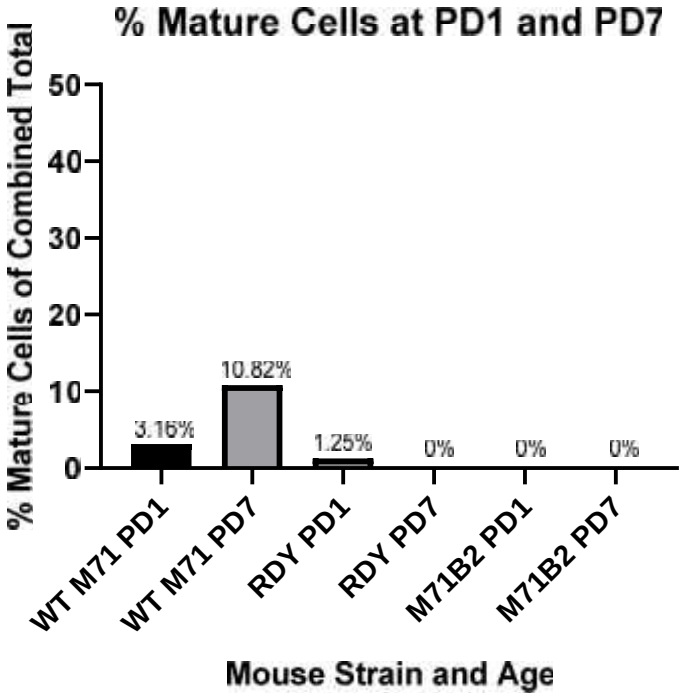

Figure 10. Graphical representation of percentage data showing percent of mature cells in different groups

3.2 *Wild Type M71-expressing animals display a greater increase in total cells after 7 days than do neomorphic mutants*

The way the data were collected in this experiment, namely counting every section versus every 3rd section in different samples, precludes the counts from being representative of total cells. Extrapolation by multiplication to normalize counts gave numbers that seemed too skewed to apply to two samples, so this tactic was abandoned. A better measure of cell number is to look at cells counted in an animal divided by number of sections collected, to generate an average number of cells per section. A "section" in this case is one 25μm slice adhered to a slide. So I generated a percent of cell increase over time by averaging the number of cells per section collected from both animals at PD1 and PD7, respectively (Table 2). The resultant numbers

suggest that wild type OSNs have a greater increase in cell numbers after 7 days than do RDY mutants and M71B2 mutants, with percent increases of 265.63%, 94.59%, and 45.85%, respectively (Table 3; Fig.11). It should be taken into account that alleles containing the LacZ sequence have a preponderance of expression by virtue of chromatin being "more open," or so it is conjectured (P. Feinstein, personal communication). Because of this, I saw more cells on average in the neomorph samples in neonate mice. Comparison of animals within the same strain using cells per slice as a measure is a way to generate ratios that are not confounded by the effect of LacZ.

Animal groups	Total cells counted *	Sections counted	Cells/section	Ave. # cells/section	% Increase from PD1 to PD7
WT PD1 a	56	80	0.7	0.48	
WT PD1 b	29	120	0.26		
WT PD7 a	139	119	1.16	1.755	265.63%
WT PD7 b	85	39	2.35		
M71(RDY) PD1 a	168	84	2	2.125	
M71(RDY) PD1 b	61	27	2.25		
M71(RDY) PD7 a	141	38	3.71	4.135	94.59%
M71(RDY) PD7 b	114	25	4.56		
M71B2 PD1 a	46	15	3	2.4	
M71B2 PD1 b	54	30	1.8		
M71B2 PD 7 a	227	93	2.44	3.5	45.83%
M7B2 PD7 b	119	26	4.57		

Table 2. Total cell counts from all animals and ratios between age. Raw counts are given here and calculated as #cells/slice for each animal. This is then averaged, and a percent increase from PD1 and PD7 for each strain is determined. *=including immature and mature

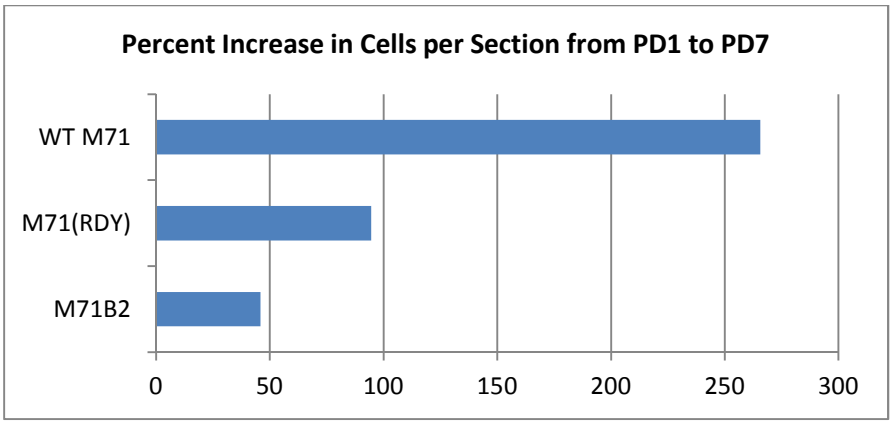

Figure 11. Percent Increase of cells per slide over 7 days. Bar length represents the percent increase in average cells per section after 7 postnatal days

3.3 At PD1, more cells expressing the wild type receptor appear to have migrated toward the OMP+ band compared to cells expressing neomorphic receptors. Of the three strains at PD1, WT expresses the highest number of mature cells

Even at PD1, cells expressing the wild type receptor are generally closer to the OMP+ band than cells expressing the mutant receptors (Fig. 12). Perhaps these cells are growing faster and more effectively because they have a functional receptor that is capable of signaling. This is plausible, since we see in the literature that neomorphic mutants become stunted and eventually fail to thrive (Feinstein et al., 2004b).

Of the three receptor types, OSNs expressing WT receptors represented the highest number of mature cells at this age, albeit still small. As previously stated, incipient glomeruli can be seen at PD1 (Kim and Greer, 2000). No glomeruli were observed, in line with observations that cells expressing the M71 receptor begin to coalesce between PD2 and PD3 (Feinstein et al., 2004b). M71 tends to be a low expresser compared to other receptors like I7 (P. Feinstein, personal communication). It is of note that the histological marker for the WT M71 receptor is intrinsic GFP in this experiment. While bright, it does not have the amplifying effects of

fluorescent secondary antibodies. Low expression and low light taken together render M71 fibers difficult to see at a very young age without amplification. I mention later and show in a figure that axon tracts in the lamina propria can be seen in neomorphic mutants. The reason is because these were visualized with immunohistochemistry. Therefore, the sighting of axon tracts between mutants and wild types cannot be adequately compared. A method for future consideration is to antibody stain against GFP in the wild type strains to better visualize axon fibers.

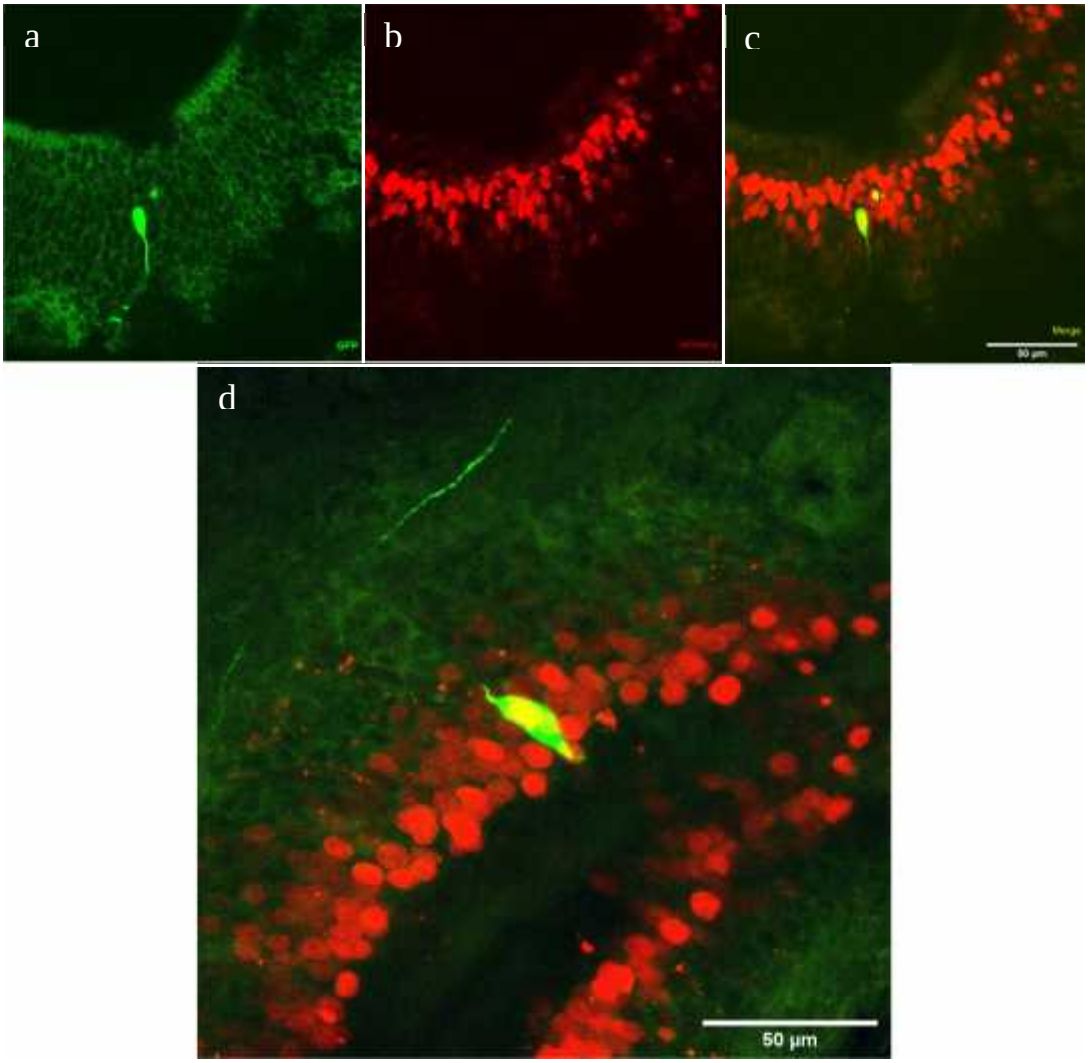

Figure 12. Wild Type PD1 animal displaying mature cells that have a red nucleus. (a-c) split cannels. (d) a soma of a mature cell visible with an axon tract in the lamina propria.

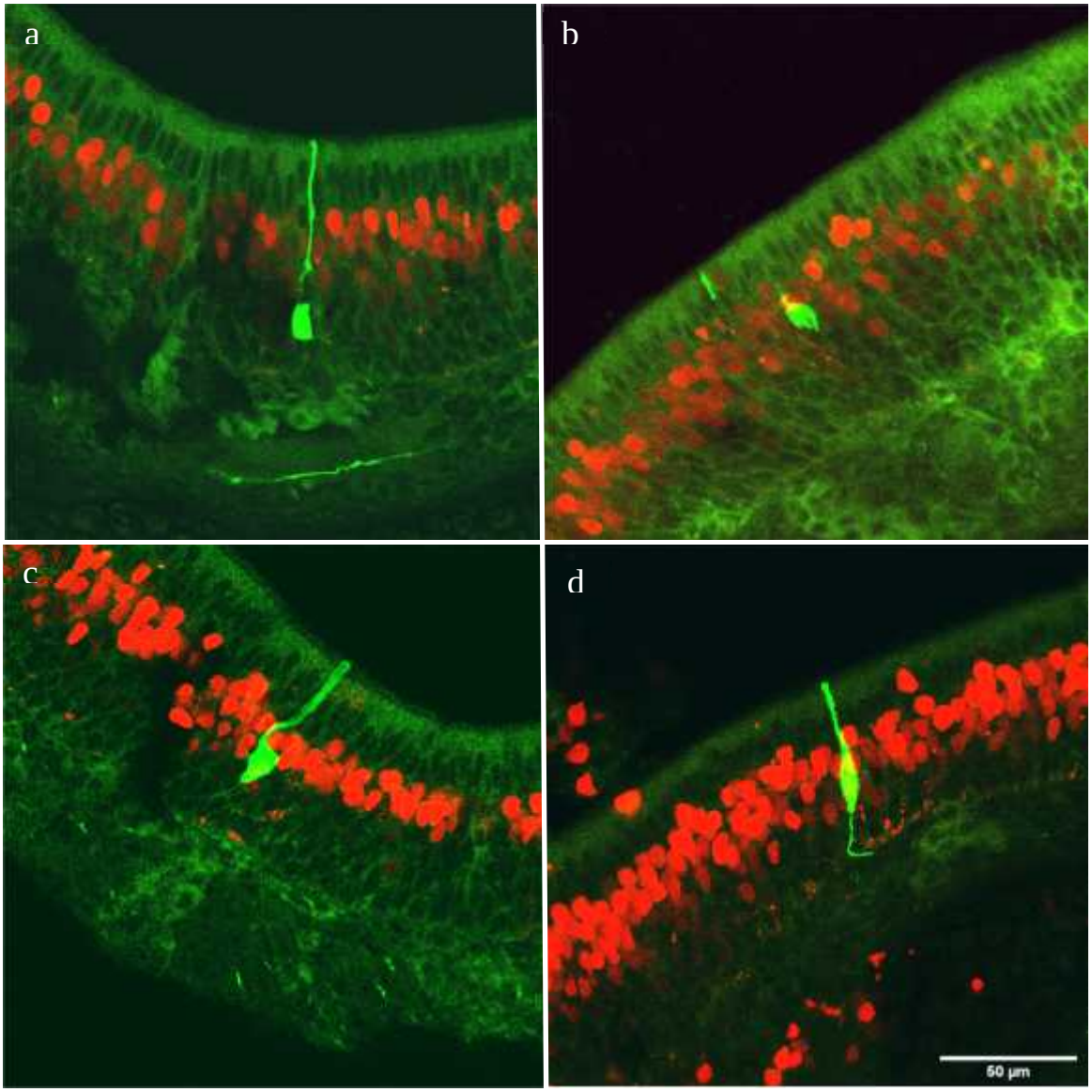

Figure 13. WT M71 PD1 cells typically rest just outside of the OMP+ band and can mature. (a-d) typical and varying location of this strain in the epithelium

3.4 At PD7, cells expressing a wild type M71 receptor are appropriately found in the locale of the OMP+ band. An appreciable number are mature.

There is a marked difference in the placement of WT M71 cells in the epithelium at PD7 compared to PD1 cells, as well as compared to neomorph cells a PD7 (Fig.14). These cells follow a normal developmental pattern and migrate apically as they mature. Many of the OSNs

that are apical and immature are probably in a later stage of immaturity, possibly having begun to transcribe OMP. Mature somas were frequently seen in this group, typically having the appearance of Fig.14c and 14d cells.

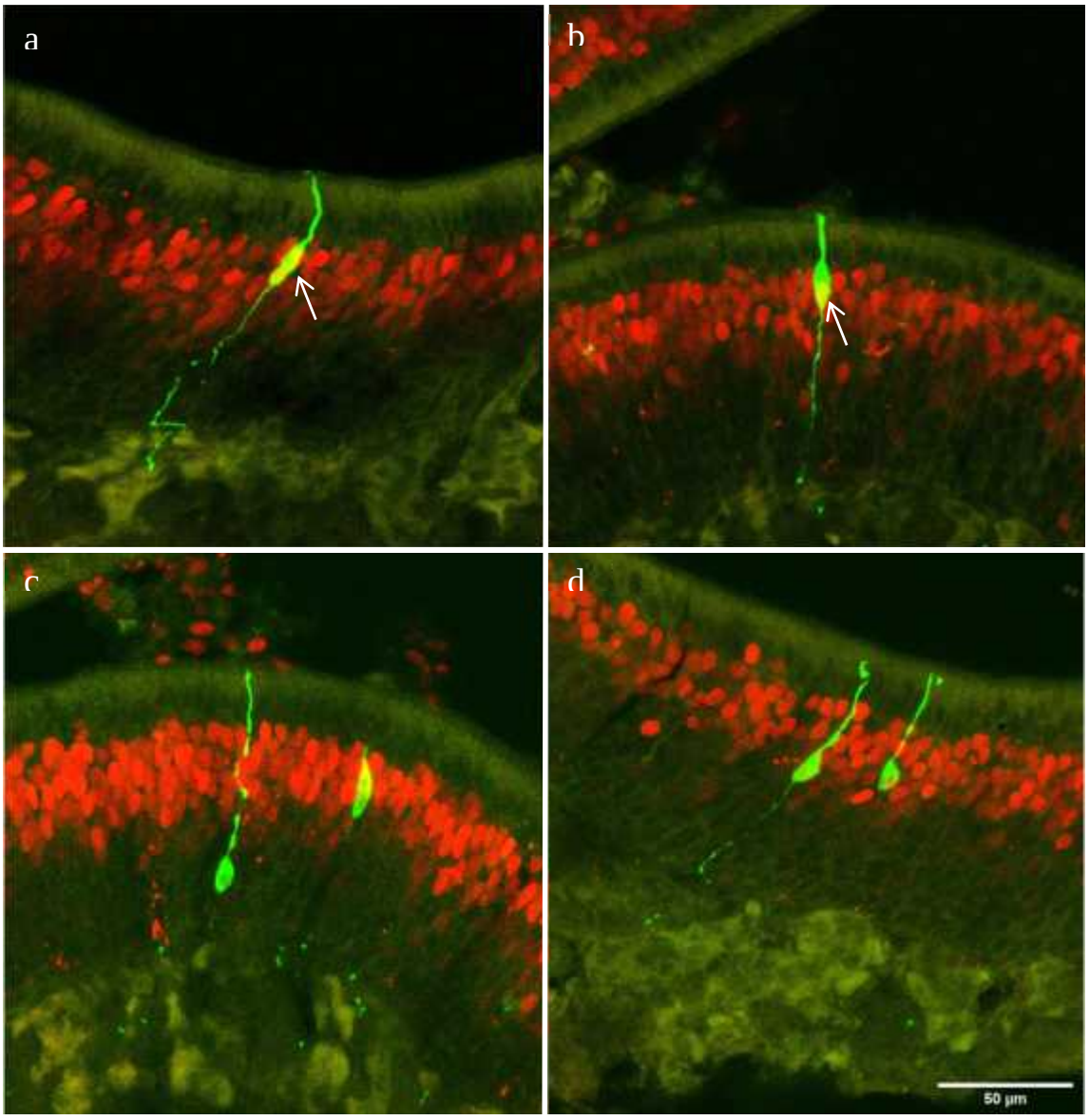

Figure 14. PD7 WT M71 OSNs are typically localized more apically, near or on the OMP+ band. Many are mature

3.5 At PD1, M71(RDY) mutant cells are typically basal to the OMP+ band. They are capable of maturation, albeit extremely infrequently

Similar to WT PD1 cells, M71(RDY) mutants tend to lay basal to the OMP+ band, with variation in position. They display a very subtle tendency to be more basal than WT cells at this age (Fig 15 c-d). It is possible that their signaling mutation leaves these cells slowed or stunted in development. I explore the relationship of signaling and maturation extensively in the *Discussion* section. The fact that three cells were found to be mature is also analyzed later on.

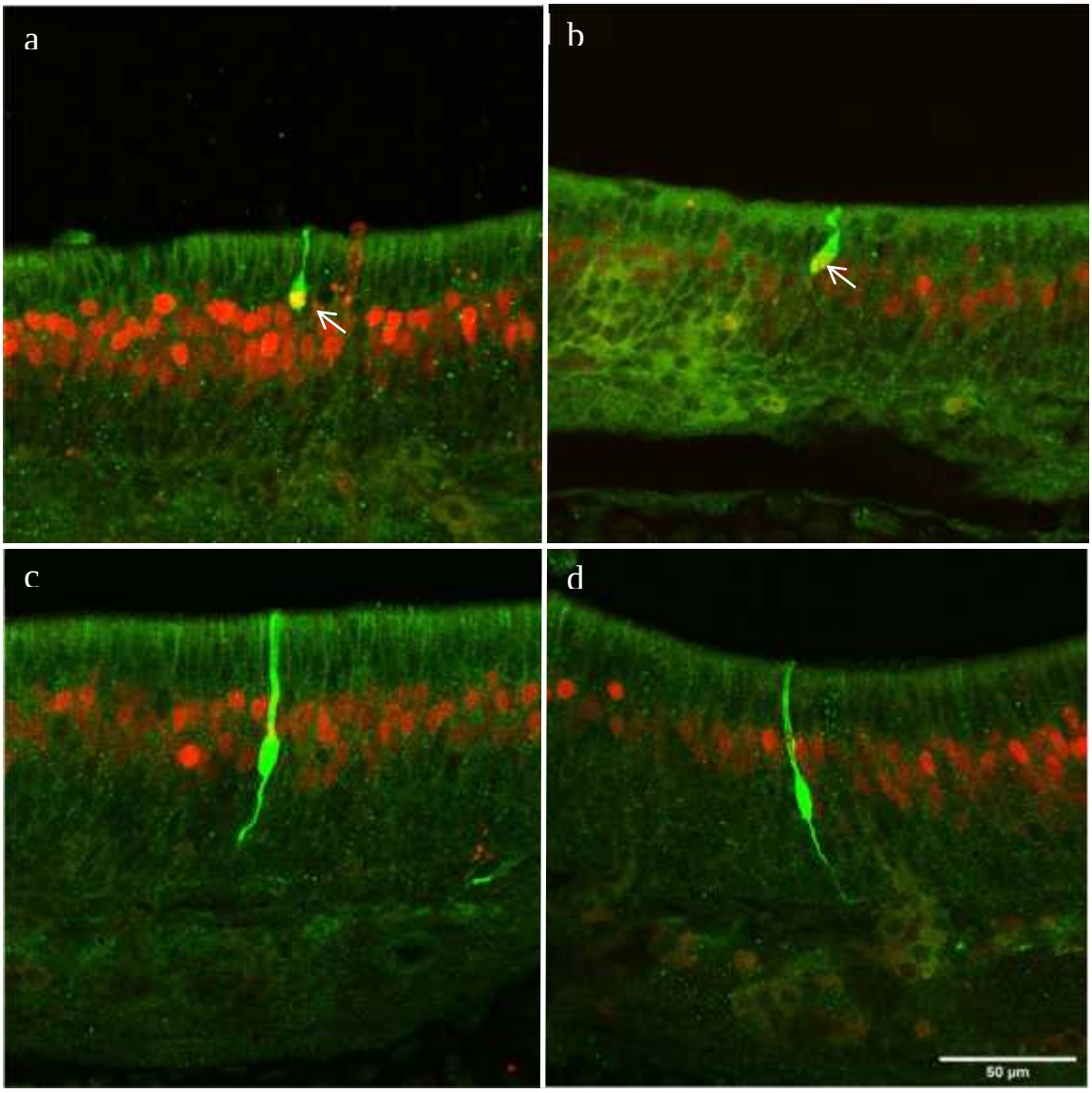

Figure 15. M71RDY PD1 cells displaying typical placement in the epithelium and capability of maturation (a) A mature cell expressing OMP (b) a cell expressing OMP, albeit less than in (a). (c-d) Typical placement of cells in RDY mutants at PD1. They are generally outside of or on the cusp of the OMP+ band.

3.6 At PD7, OSNs expressing the M71RDY receptor are typically more basal than their wild type counterparts, and do not display any considerable differences from M71RDY OSNs at PD1

OSNs expressing the M71(RDY) mutation begin to show a subtle divergence from their wild type receptor counterparts 7 days after birth. Although there is natural variation in cell position on the epithelium, these neomorphic mutants tend to reside more basal to the OMP+ band than WT M71 cells (Fig. 16). Notably, they appear very similar to M71(RDY) cells at PD1, unlike WT PD7 cells which show migration compared to PD1 cells. This result is in line with stunted growth due to a mutated receptor.

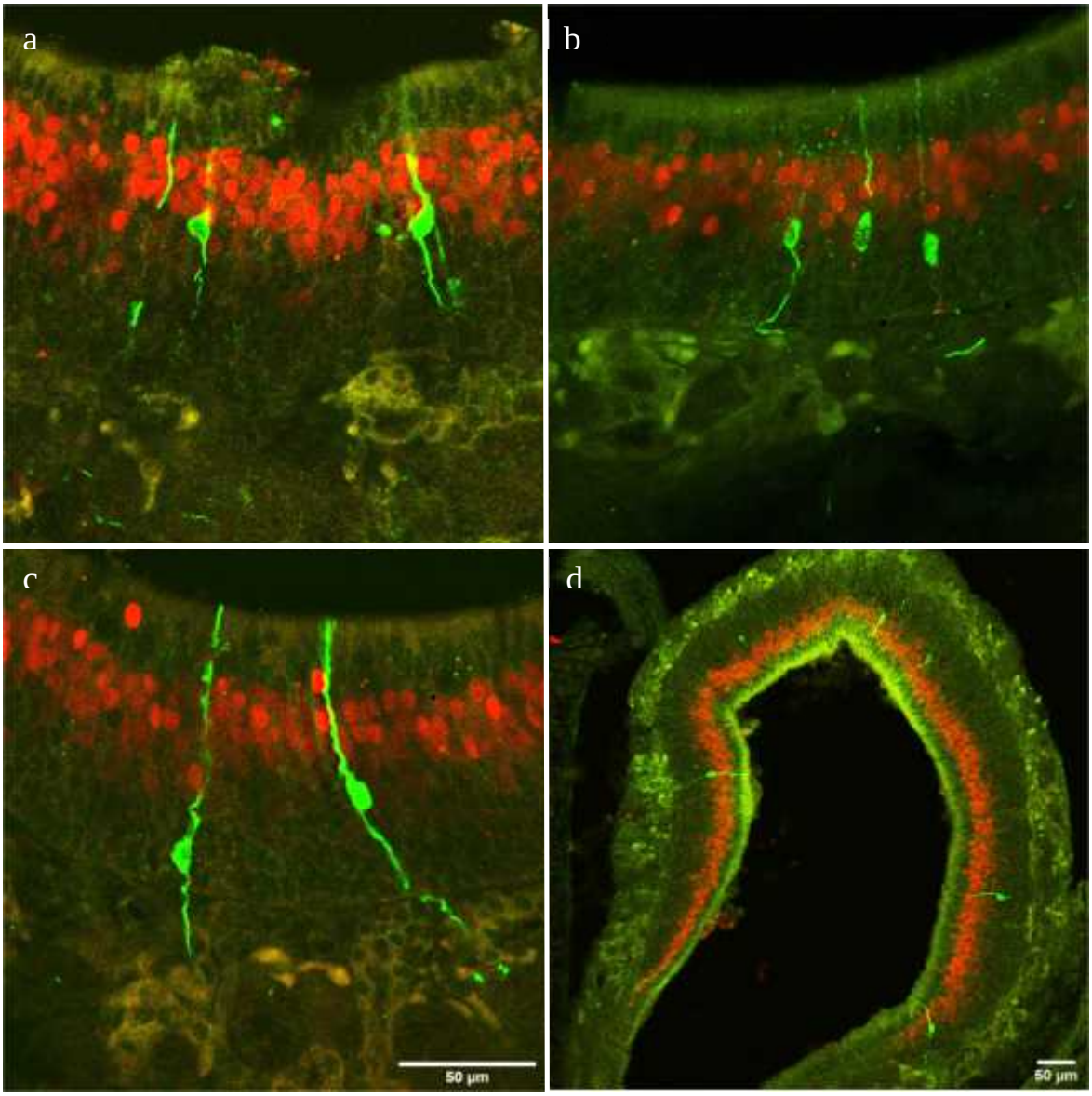

Figure 16. M71RDY PD7 cells lay more basal to the OMP+ band compared to their wild type counterparts, and don't show considerable difference compared to cells at PD1.

3.7 At PD1, OSNs expressing the M71B2 receptor are noticeably more basal to the OMP+ band than their wild type and RDY counterparts. None were found to be mature

M71B2-expressing cells are comparatively basal at PD1 compared to WT and RDY cells at this age, but very subtly. We can see axon tracts in the lamina propria in Fig. 17. This shows that cells are at least attempting to extend axons in the direction of the bulb. As we will later see,

some axons appear to actually touch the bulb from this strain (Fig.21). However, it is likely that these cells were located very posterior, such that the soma was already close to the bulb. Predictably, no mature cells are seen in this group.

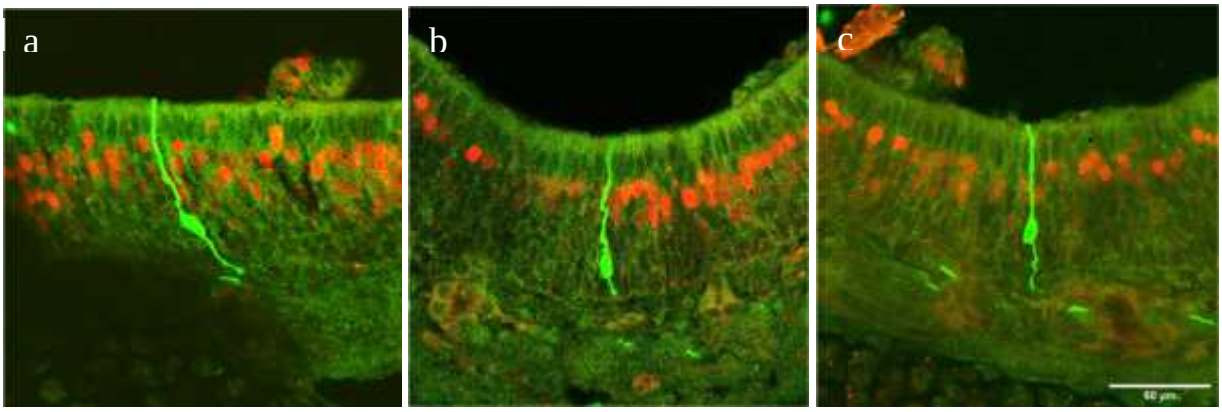

Figure 17. M71B2 PD1 cells reside basal to the OMP+ band in the epithelium and display axon tracts in the lamina propria.

3.8 At PD7, cells expressing the chimeric receptor are still considerably more basal to the OMP+ layer compared to age-matched cells expressing the wild type and RDY receptors. Even at this age, none have matured.

Cells expressing the chimera are noticeably more basal to the OMP+ band than WT OSNs, and even more so than M71(RDY) OSNs. They do not seem to migrate. This is very likely a reflection of their stunted differentiation. Many still reside right just above the basal lamina (Fig.18b). It is unlikely that cells expressing the chimeric receptor were ever successful prenatally. What this means is that there there are no pre-established tracts to follow, if this is in fact how the system works. These cells are likely impeded by cell-autonomous mechanisms, which renders coalescence with neighbors irrelevant.

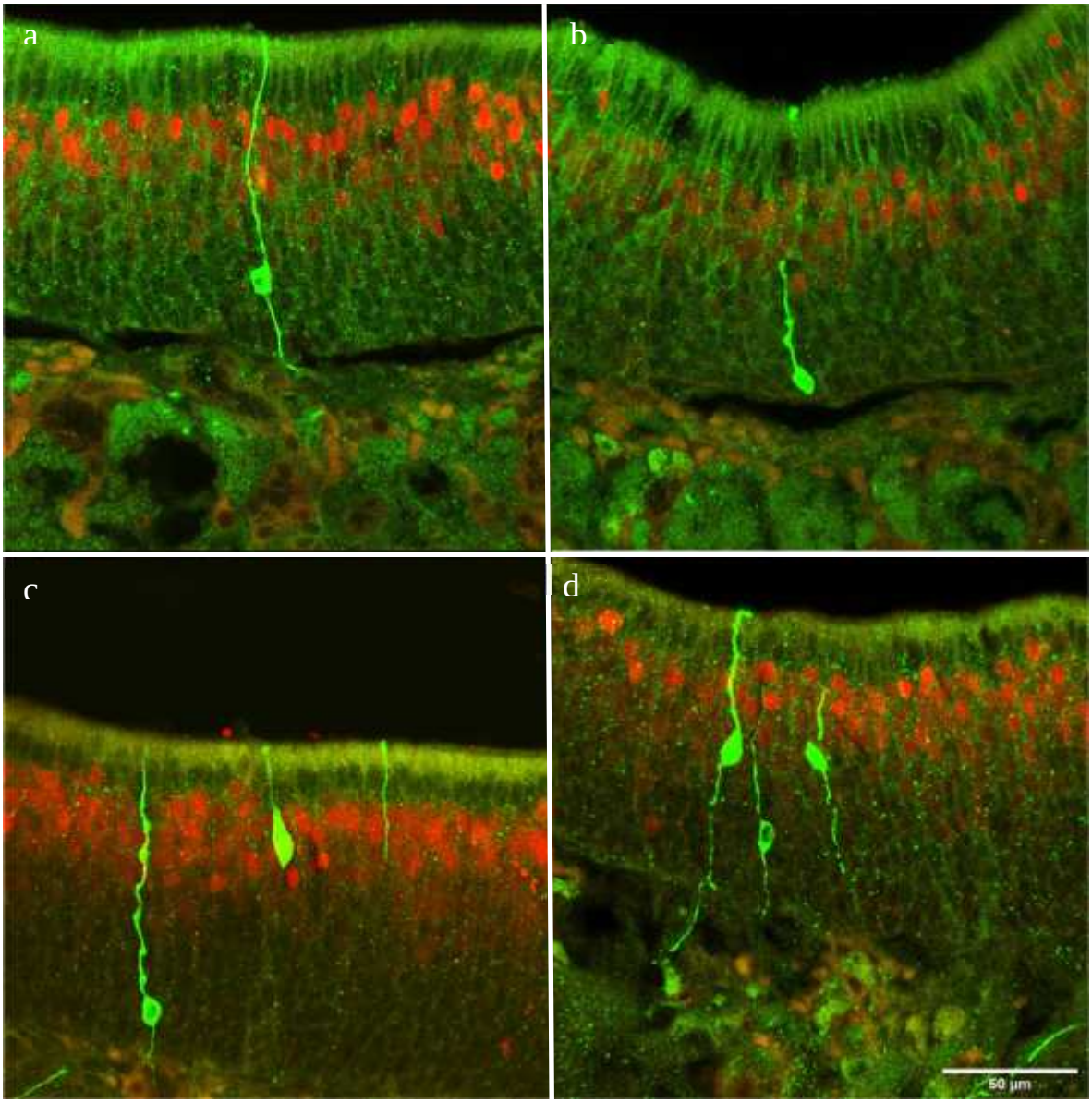

Figure 18. M71B2 cells are typically basal in the epithelium at PD7 and don't display the same migration as WT M71 OSNs
(a-b) PD7 M71B2-expressing cells typically occupy a basal position relative to the OMP+ band. (c-d) There is variation in their position, however, but the top two cells are most representative of cells observed in this group. Green, antibody against LacZ. Red, H2BmCherry.

3.9 In adult animals, the OMP+ band comprises almost the whole epithelium. Cells expressing the M71 receptor tend to occupy the periphery, regardless of maturation status

As an animal develops, its epithelium thickens, and the majority of OSNs become mature (Iwema and Schwob, 2003). Glomeruli innervated by mature OSNs are agents of the brain's odor code, so it is logical that mature OSNs predominate in a fully functional olfactory system. In

developed animals, precursors of mature OSNs typically reside in a thin layer above the basal lamina. Since the OMP+ band occupies almost the entire width of the epithelium (Fig.19), an immature cell's position in relation to the band is of less consequence. A mature cell has cilia protruding from the dendritic knob, which can be observed in Fig. 19b.

Once an animal has its complement of mature OSNs at steady-state level, stem cells are less proliferative than during development, and cells are probably subject to less movement. Unpublished observation from our lab suggests that particular OSN types occupy a typical region on the epithelium. M71 resides basally (Fig. 19a).

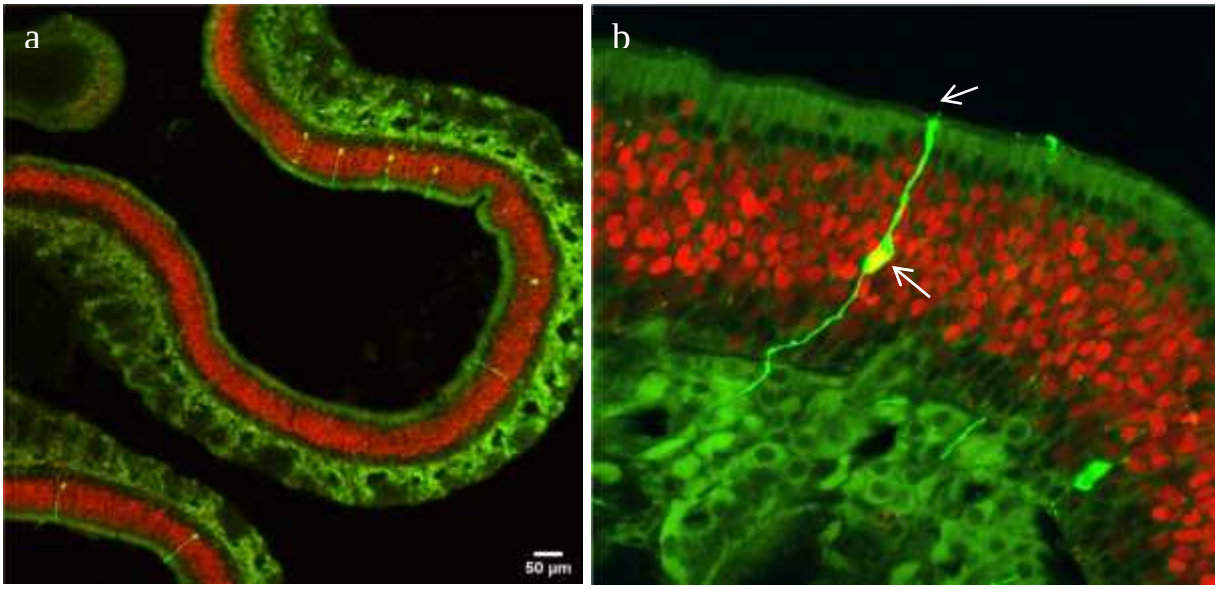

Figure 19. Adult epithelium is mostly composed of OMP+ cells. (a) a zoom-out of epithelium. (b) A mature cell. Taken from a PD29 animal.

3.10 In 3-week-old animals, there are drastically more cells expressing the wild type receptor compared to age-matched neomorphic mutants. Only wild type cells form a glomerulus; axons of neomorphs are absent from the bulb altogether

Total cell counts from WT animals and neomorphic mutants at PD1 and PD7, although displaying different ratios with age, were not radically different from each other, reflecting the shared probability of choosing the M71 locus from the available repertoire at an early age.

At PD1 and PD7, the playing field is still even, so to speak, because numbers of cells expressing a given receptor are still quite low. As the animal ages, however, cells that can mature and form a glomerulus beget other cells to converge, and a steady state is obtained and maintained. We see that this is the case for wild type cells in a 3-week-old animal. They densely populate Zone 4 on the turbinates and form a prominent glomerulus on the bulb (Fig.20c). A startlingly different fate is seen for neomorphic mutants. By 3 weeks, their numbers have dwindled and they scarcely pepper the epithelium. They do not form glomeruli or extend tracts on the bulb (Fig. 20 a-b). Interestingly, the RDY mutants unexpectedly had way fewer cells than the M71B2 mutants. It is possible that the chimeric animal was homozygous and that the animals in the RDY litter were heterozygous. In normal animals, zygosity makes little difference in the appearance of cells projecting to a glomerulus, but it could have had an impact in this case. The sample in Fig.20c was freshly stained and not stored in PFA hence its pink hue compared to the other samples. However, in M71(RDY) P10 animals, many more cells have been documented, comparable to about a third of what we see in the wild type animal (Movahedi, 2016), so it is also conceivable that many cells in this strain simply died off. Compared to the M71B2 strain, it would appear that RDY mutants died off in higher numbers.

Several possibilities may be at play here. Likely, since these cells do not mature or extend axons very far, they either die in a cell-autonomous fashion, or die because they have no home and are out-competed by successful neighbors. When they die, they are probably cleared by ensheathing cells. Since choice is stochastic, roughly the same number of OSNs are choosing the

M71RDy and M71B2 receptors as the wild type. Rather than accumulating, a large majority of OSNs expressing the mutant disappear.

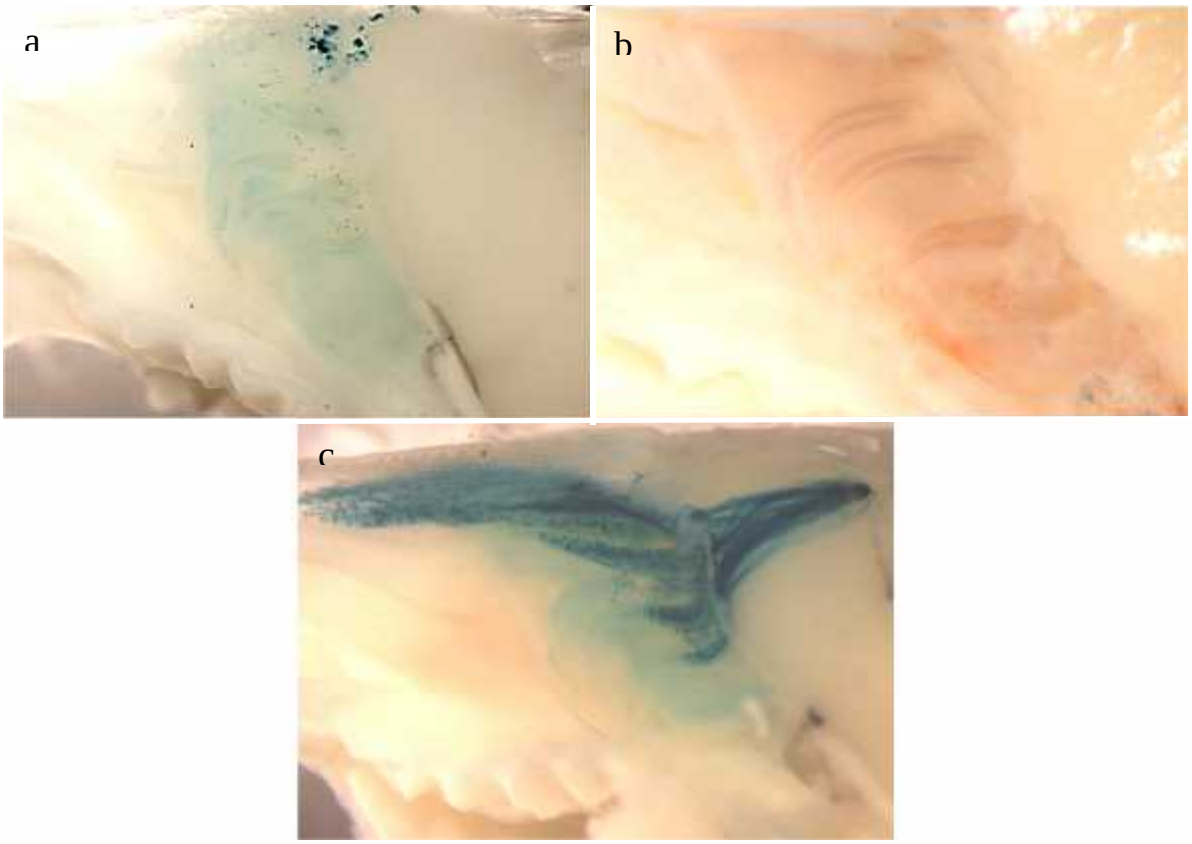

Figure 20. Chimeric and signaling mutants display drastically fewer cells at 3 weeks of age than wild type animals. (a) M71B2 mutant showing a small scattering of cells in Zone 4 (b) Barely any cells are visible. (c) A wild type animal shows plentiful expression and a prominent glomerulus. Wholemount X-gal stains.

3.11 In adult animals, the neomorph cells display poor axon outgrowth. PD1 and PD7 neomorph animals exhibit axon tracts in the lamina propria and occasionally on the bulb. These cells may be dying with age and getting cleared

In young adult mice, there is a striking difference in the morphology of OSNs that express a wild type M71 receptor compared to those expressing the signaling mutant and the chimeric receptor. While the wild type cells extend long axons that project to the olfactory bulb (Fig. 22b, arrow), the mutant OSNs display poor axon outgrowth. In X-gal stained wholemounts where the view is collinear with the axon, we see a stub extending from the cell body of the

neomorphic mutants (Fig. 22a). This is in contrast to what we see in transverse confocal images of PD1 and PD7 mutants (Fig.21).

In neonates, OSNs expressing a neomorphic receptor exhibit axon tracts in the lamina propria (Fig. 21c, 21d), some perhaps reaching the bulb (Fig. 21a, 21b). That the X-gal stained stubs we see at 3-weeks have extended orthogonally and out of view is an unlikely possibility. Therefore, there must be an explanation as to "what happened" to these tracts as the animal aged. As aforementioned, these cells are likely dying and getting cleared.

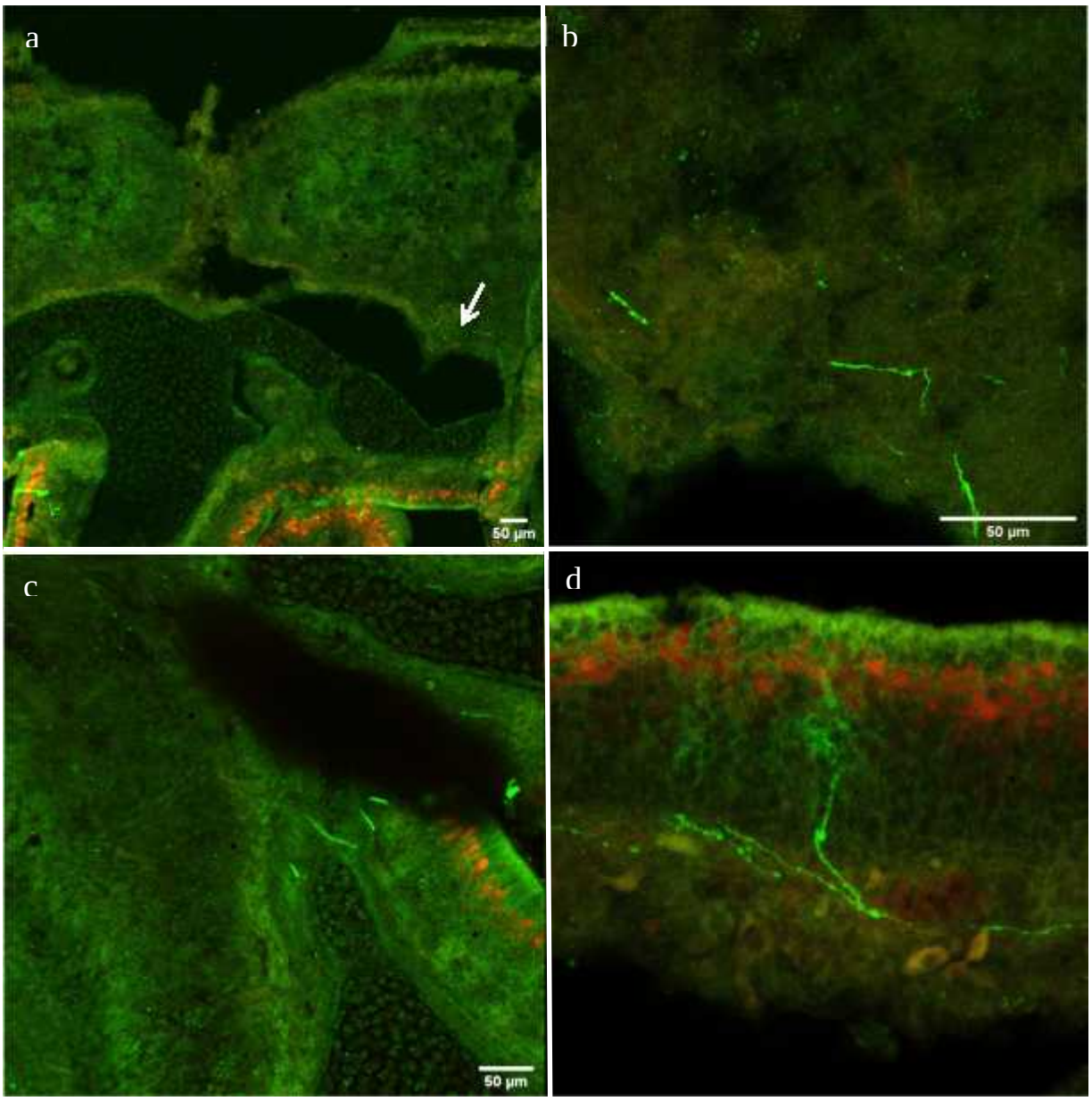

Figure 21. Neonate mutant receptor OSNs display axon pathfinding in the lamina propria and bulb (a-b) axons visible on the bulb of an M71B2 PD1 animal (5X, 25X). (c) axons pathfinding in the lamina propria (LP) of an M71B2 PD7 animal. They appear to be approaching the bulb. (d) axons of OSNs traveling in the LP of an M71(RDY) PD1 animal.

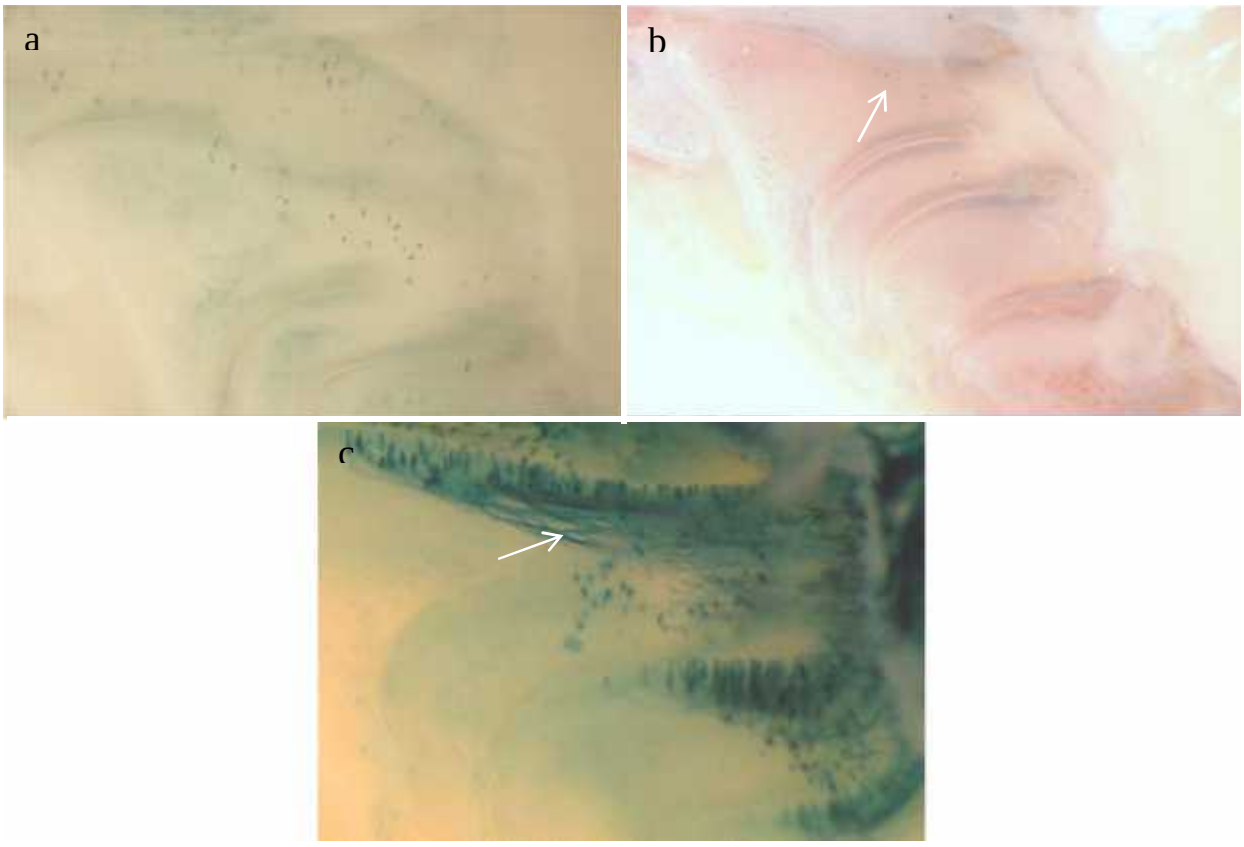

Figure 22. Chimeric and signaling mutants display poor axon outgrowth at 3 weeks (a) Close-up of axons in Fig.20a. In the M71B2 OSNs, axons appear as stubs. (b) The OSNs expressing wild type receptors display long **a**xons that converge on the bulb (see arrow). Wholemount X-gal stain**.**

3.12 The M71B2 chimeric receptor appears to be stuck in the endoplasmic reticulum or apparatus in PD7 animals, compared to adult neurons that express the B2AR receptor throughout the plasma membrane

Perhaps the most intriguing and consequential finding of this experiment is the absence of receptor on the plasma membrane of M71B2-expressing OSNs. Instead, the receptor co-localizes to the endoplasmic reticulum, or possibly the Golgi apparatus (Jeff Martens, personal communication). This can be visualized as a large triangular ring around the nucleus (Fig. 23d). Schwarzenbacher et al. show that in a PD1 animal, receptors line the cilia of an OSN and that as early as E15, protein has co-localized to the dendritic knob (Schwarzenbacher et al., 2005). Likewise, it has been shown that after bulbectomy, regenerating OSNs in the immature state express receptor throughout the plasma membrane (Iwema and Schwob, 2003). Both of these experiments were done using antibody to receptor. So even though the controls I used were not age-matched, it's been demonstrated in the literature that the receptor has left the ER in immature cells and is found on the plasma membrane. Therefore, the M71B2 receptor is an anomaly.

My control animal was a special strain from a previous experiment that expresses two slightly different B2AR receptors: a gene-targeted B2AR-IRES-tauLacZ in the M71 locus, and a B2AR:GFP fusion transgene. The antibody bound both of these and highlighted the entire cell (Fig. 25). A receptor:GFP fusion protein is an informative way of determining where on the cell the protein traffics to, and in this case, both intrinsic GFP and far red from the antibody completely co-localized on both the cell body and the axon (Fig. 25) (Feinstein et al., 2004b). Likewise, we can see the blue cell that is only highlighted by the antibody. Its silhouette is in sharp contrast to that of the M71B2 chimeric receptor in Fig. 23b. Lack of a receptor on the

membrane could have functional implications for the cell at many stages and its apparent stagnation in the ER/Golgi could wreak all flavors of havoc on the cell. It is no wonder that cells choosing this locus have a poor prognosis 3 weeks later.

Another striking find in this experiment is the appearance of tiny nubs on the dendrite in certain M71B2 cells (Fig.24). This seems to indicate that the receptor has left the ER, but in an entirely different way than receptors normally exit. When receptors exit the ER, they are modified in the Golgi and then traffic to the membrane. They normally appear evenly distributed throughout the dendrite, and not as puncta. A proposed explanation for this curious find is offered in the *Discussion* section.

Whether or not the M71(RDY) receptor traffics to the membrane has not been determined in this study because of technical issues and time constraints. The antibody against the C-terminus of the M71 receptor is incompatible with the fixation methods used in this experiment, and did not work at the time of this publication, nor were adequate control animals for WT M71 available at this time.

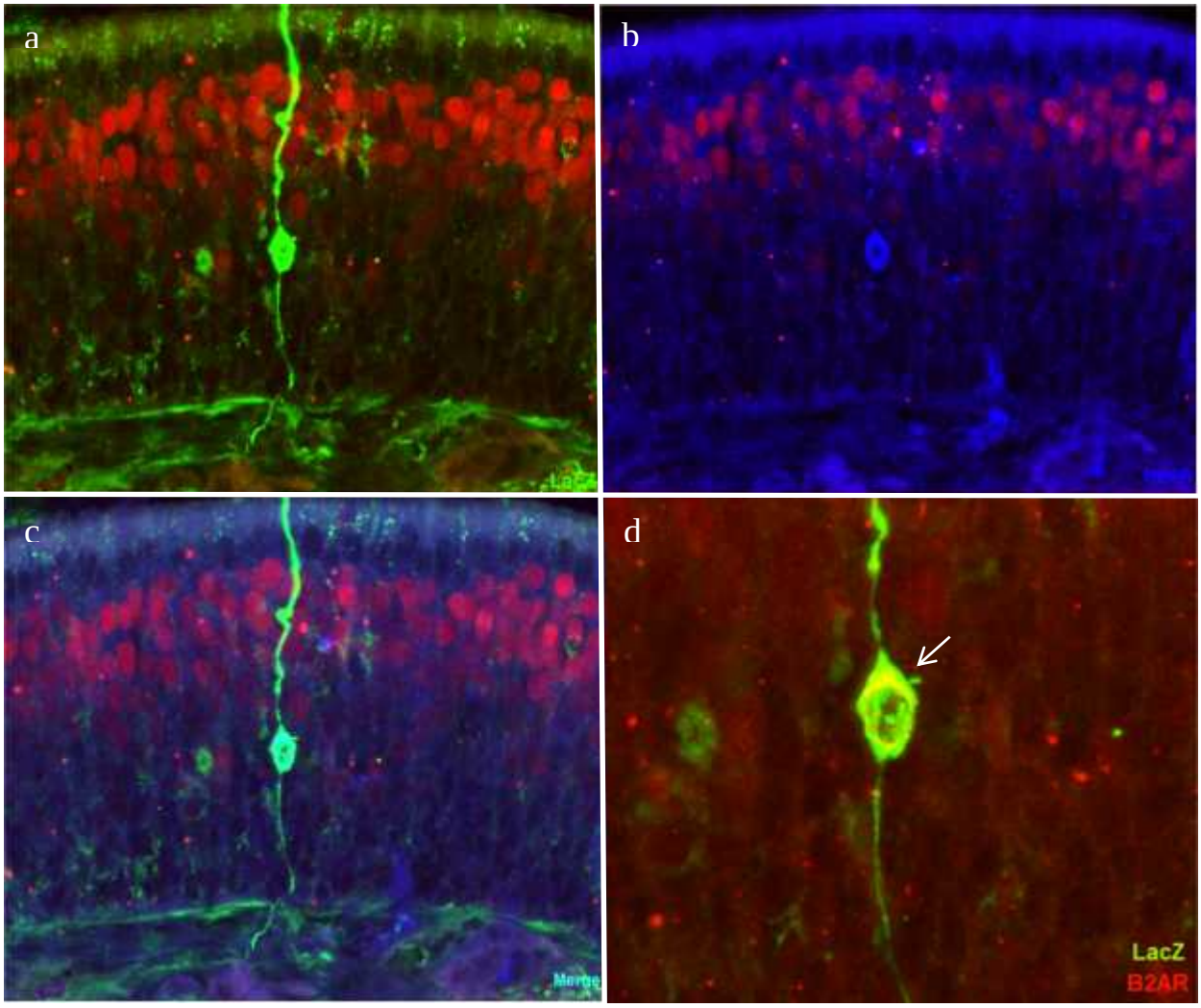

Figure 23. The M71B2 receptor does not reach the plasma membrane but instead localizes in the ER (a) The green channel represents antibody to LacZ; as we see here, fluorescent tau highlights the entire cell. (b) The far red channel highlights antibody to the C-terminus of the B2AR component of the chimeric receptor. (c) Merge of green and far red channels. (d) A close-up of the soma, with the far red channel in red for visualization. Green highlights the entire plasma membrane, whereas the red (appearing as yellow when merged) is inside, surrounding the nucleus only (see arrow). Image in (d) scaled up 2.25X. Cell is from a PD7 animal.

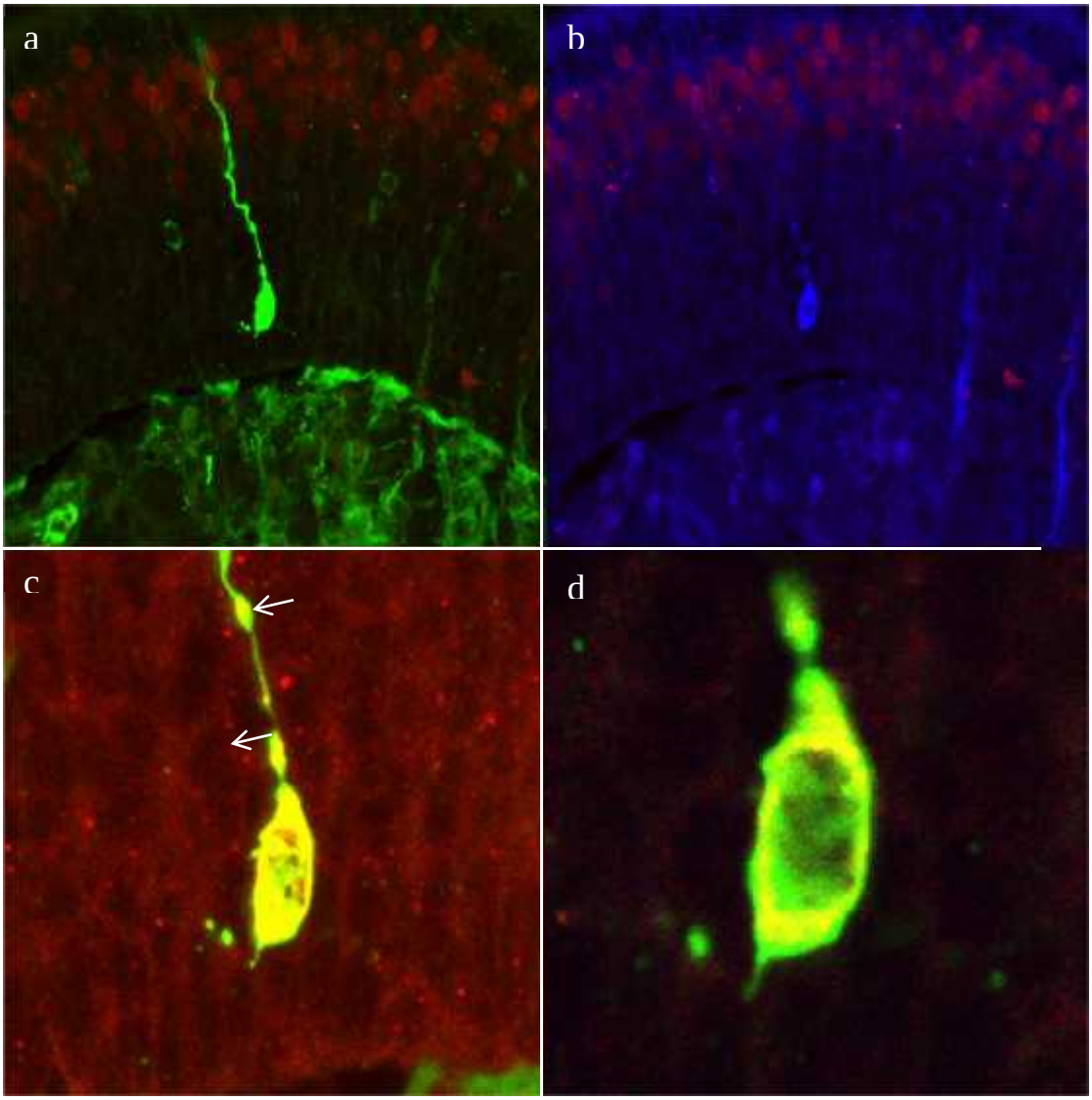

Figure 24. B2AR appears in small "nubs" in the axon. (a) green channel, tauLacZ. (b) far red channel, B2AR (c) Merge and zoom, with the far red in red for visualization. (d) same cell, Z stack, better view of co-localization in the ER.

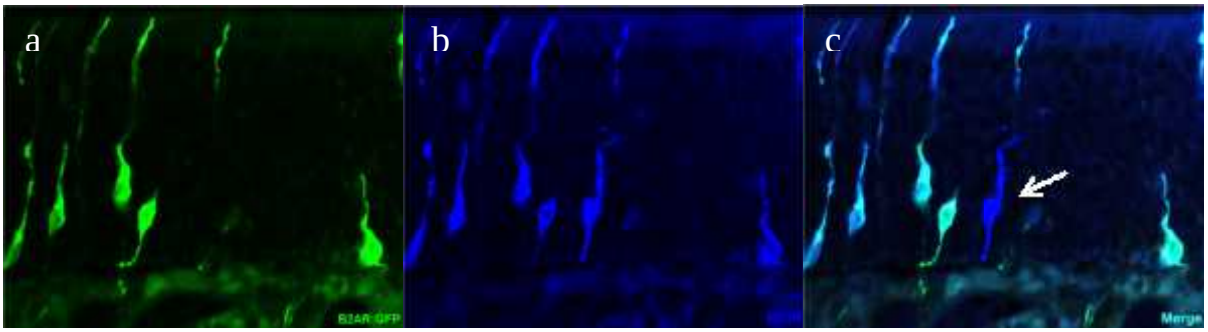

Figure 25. Control animals express the B2AR on the entire plasma membrane (a) Green cells represent the intrinsic GFP of a B2:GFP fusion transgene. The cell puts up a receptor on the entire plasma membrane, as we can see green on both the cell body and axon. (b) The far red channel represents antibody to the B2AR c-terminus. (c) We can see that blue co-localizes completely with green. We can also see a cell that is blue and not green (see arrow). This same animal possesses a gene targeted B2AR-IRES-tauLacZ in the M71 locus,. We can see from this blue cell that B2AR receptor covers the entire plasma membrane. Animals were approximately 3 weeks old.

4. DISCUSSION

4.1 What does "percent of mature cells" mean?

Embryonic development of OSNs may not follow the same program as adult neuronal regeneration, as discussed earlier (Yu et al., 2017). We do not know which of the cells we see are of the first round of "pioneer OSNs" that migrated to the presumptive bulb as embryos, at least in the case of wild type cells. At the ages of PD1 and PD7, many of the OSNs we see may be these primordial cells that have charted the course for subsequent axons, as described in the literature. They may also be cells that have divided after birth.

We must examine what "percentage of mature cells" actually means, since several variables are not constant. If the differentiation timeline is replicable between cells, which it most likely is, then in a hypothetical fixed number of cells that all divided at the same time we would see the same level of development across every cell. In other words, at a given early time point, 100% of a hypothetical population of normal OSNs would be immature, and in several days, 100% would have reached maturity. This is clearly not what is seen. Cells are dividing and adding to the pool continuously and at a nonlinear rate (meaning more cells are added early on than later on), while preexisting cells of that population are already advancing. So in a way, the percent of mature cells is an obscured metric in neonates, because it reflects number of cells added, as well as growth rate. An easier number to define is percentage of *immature* cells in an adult. This number is a reflection of turnover rate, since in adults over 90% of cells of a given receptor population is mature (Iwema and Schwob, 2003). They have reached a "steady state" since the +90% holds for any time point.

If across neonate animals the amount of added cells over time is roughly the same, and the likelihood of choosing a given receptor is the same, then a variable that could account for the difference in percent of mature cells is growth rate. I have provided a schematic to illustrate this concept. In Fig.26, the arrow at the top points to about the seventh day. If the blue lines are cells, then the top graph has about 5 mature cells, and the bottom has zero. In this instance, growth rate is affecting the number of mature cells, when the number of cells added is consistent between samples. As we see in the results of this experiment, mutants have a considerably lower percentage of mature cells than their wild type counterparts. Given that they are all expressed from the same locus and that receptor choice is stochastic, I hypothesize that this difference in neonates is because of differing growth rates. I believe that there is a feedback mechanism that is interrupted which links proper receptor with continued axonal growth.

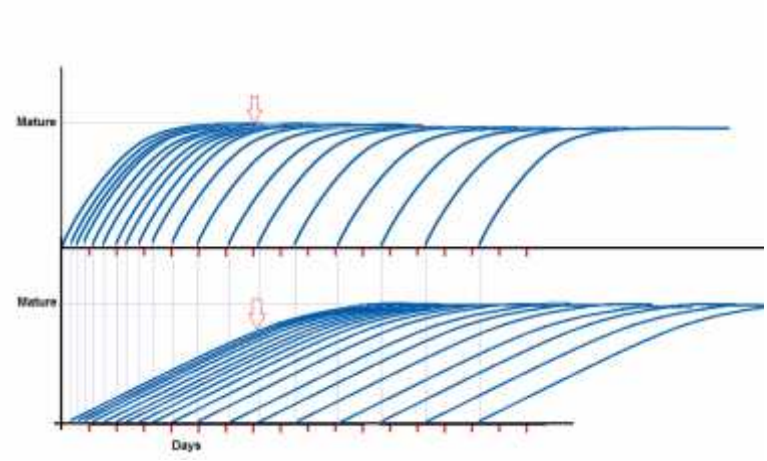

Figure 26. A schematic showing how percent of cells could differ when other variables are not constant. The Y axis represents arbitrary level of development, with "Mature" marked as a line. The X axis is days. Blue lines represent cells added to the pool; their slope is their growth rate. At the arrow, the top graph exhibits a larger percent of mature cells.

4.2 Finding 3 cells versus none in Neomorph Type I and II, respectively

Finding three mature cells in the RDY mutant may or may not be trivial, depending on the explanation offered. It is not a trivial result if it indicates that neomorph Type I–expressing

OSNs are intrinsically *capable* of maturing, and that odds are simply stacked against them in a number of ways under normal circumstances. We've seen in Movahedi et al., 2016, that M71(RDY) mutants can be "rescued" to maturity when supplied with a constitutively active Gs mutant, or when co-expressing M71 under the OE2 promoter. The latter either compensates with its own signaling machinery, or provides a "permissive environment" wherein a "global reduction" of activity in all other cells eliminates competition (Movahedi et al., 2016). Either way, it is speculated that the M71(RDY) receptor provides axonal identity to the cell. Maybe those three cells were mutated in some way that provided a gain of function that allowed for maturation. By a similar token, finding no mature cells in M71B2 mutants could be a comparatively nontrivial result if it indicates that these cells are intrinsically *incapable* of maturing. The protein likely misfolds and does not provide an identity to the receptor. This Neomorphic Type II receptor does not get rescued by OE2/M71 (Paul Feinstein, unpublished data).

If these mutant receptors misfold, mistraffic, or cannot signal, then both of the axon-targeting models described cannot manifest, even if the axon were capable of outgrowth. If receptor doesn't get to the membrane, then there is no identity for the axon and it cannot sort itself through homophilic interactions. Likewise, if there is no receptor, then there can be no baseline signaling to trigger the transcription of guidance molecules. Poor outgrowth precludes either of these models from being confirmed or denied from the present experiment.

4.3 What triggers maturation?

Data suggests that the wild type immature cells at PD1 are maturing by PD7, but some cells may be dying. Counts were taken in every section at PD1, and every 3rd section for one of the PD7 animals and every section for the other. In the PD7 animal examined at every 3rd slice,

12 mature cells were counted. If multiplied by 3, this would amount to about 36 mature cells. The average of immature cells in the PD1animal was about 42, and these numbers are close. However, in the other PD7 animal that was counted every section, only about 12 were counted. This is far off from 42. The latter count is more accurate, since it does not involve extrapolation by multiplication. In this case, it would seem that immature cells die off. Is this because they do not receive survival signals from nearby "like OSNs"? Since no axons were seen on the bulb in wild type animals, it would seem that some homophilic interaction is required *before* reaching the bulb in order to trigger maturation. If having a home in a glomerulus were the trigger for maturation, then I would have seen axons in the bulb of animals having the wild type M71 receptor. It is possible that poor visualization methods precluded the sighting of glomeruli in the bulb, rendering these results inconclusive. I will need to look at more samples and use antibody against GFP to amplify axons. I also plan to X-gal stain sagittally dissected animals. This wholemount method allows the visualization of axons on the bulb from a side view.

Touching the bulb cannot be the trigger for maturation based on this data, since we have seen immature neomorphs touching the bulb, and mature wild type cells *not* reaching the bulb. Both of these results contradict results from the Greer lab which suggest that maturation occurs after axons have reached the olfactory nerve layer of the olfactory bulb, as described shortly (Rodrigues-Gil et al., 2015).

4.4 Nubs in the dendrite?

The "nubs" of the M71B2AR receptor staining in the dendrite differ from what we see elsewhere and what's been reported in the literature about receptor transport and distribution. In the embryonic OR tracing experiment by Scwarzenbacher et al., OR distribution seems to

coincide with ciliary development; antibody staining of receptor shows its accumulation in the knob before becoming coincident with ciliary outgrowth (Schwarzenbacher et al., 2005). Membrane trafficking in cilia is poorly understood and there are different proposed ways it may happen, at least in primary cilia: direct trafficking from the Golgi and docking at the periciliary membrane compartment (PCM) that surrounds the ciliary base, and lateral diffusion from the membrane (Morthorst et al., 2018). Intraflagellar Transport (IFT) is a mechanism by which components of cilia are assembled and by which certain types of cargo are transported. There is also evidence that the IFT plays a role in the transport of certain GPCRs (Morthorst et al., 2018). That ORs were localized in the knob but then co-stained with tubulin in outgrowing cilia made the involvement of these pathways unclear to the Scwarzenbacher team—namely, they couldn't tell if ORs were embedded in compartments in the membrane or trafficking through the IFT. These were also in E12-E19 animals, and it's been mentioned that there is still uncertainty regarding whether postnatal and embryonic OSN development are the same.

How ORs traffic to the membrane is an area of active research. It has been demonstrated that a class of olfactory tissue-specific chaperone proteins called Receptor Transporting Proteins (RTPs) are essential for surface expression of odorant receptors (Saito et al., 2004; Sharma et al., 2017). However, it has been shown that the B2AR traffic to cilia and form glomeruli in the absence of these chaperones (Sharma et al., 2017). The presence of tiny nubs of chimeric receptor in disjointed segments in the dendrite suggests that the cell did not recognize this as an OR and is treating it differently. The nubs appear to be vesicles; since we do not see this elsewhere, I hypothesize that ordinary ORs must be trafficking by lateral diffusion through the membrane. Otherwise we'd see vesicles of receptor as "nubs" with antibody staining in OSNs expressing functional receptors, and not distributed evenly in the dendrite. We see the latter

under normal circumstances. It is also likely that the nubs seen are Golgi outposts (J. Martens, personal communication). Perhaps receptor is stuck in the Golgi and not the ER However, the nubs were seen in only 2 cells of the several cells examined. Reaching the Golgi outposts could have been an outlier situation. I will need to examine more cells to draw any conclusions.

4.5 Timing of OR expression: Refuting current literature?

Since cells expressing a Neomorph Type II do not reach the membrane and do not grow, it seems that there is crosstalk connecting a correct receptor with proper outgrowth. This idea is in conflict with a recent finding that ORs are expressed *after* the axon has reached the bulb. Data from the Greer lab shows through BrDU lineage tracing and ISH that OR mRNAs were detected 4 days after basal cell division, in line with the OSN axon having already reached the cribiform plate and touched the olfactory nerve layer in the bulb (Rodrigues-Gil et al., 2015). If this were how the system worked, then our neomorphic mutants would show proper axonal outgrowth, which they do not. It is likely that the ISH was not sensitive enough to detect transcript level below a certain threshold in their experiment. My data show that the OR expression is intimately connected to axon outgrowth and could not have happened after it.

4.6 Locking in choice, not switching

The results of this experiment refute a commonly held notion that OSNs undergo "switching" when they have chosen an unsuccessful receptor (Shykind et al., 2004; Dalton et al. 2013). First off, we do not see the "null" phenotype which results when a deletion or non-OR coding sequence or the like is expressed with a histological marker. In these cases, the cell does not lock in choice and chooses a second receptor. The marker persists, and axons projecting to random glomeruli in the allowable zone are seen. Clearly the transcripts in null mutant cases are

translated, but an as-yet unknown system in the ER did not “register” them as ORs. The marker proteins are translated from an IRES, as the transcripts are bicistronic. The first sequence in null mutant transcripts is either translated and destroyed, or is bypassed somehow by the ribosome.

If the neomorphs were switching, we would see the same phenotype: The cells would have chosen another receptor and the axons would have grown out to reach the bulb. The tauLacZ would be translated and mark the entire axon. Cells would go on to mature. In this experiment, we see poor axon outgrowth, lack of maturation, and population disappearance likely due to cell death.

One could argue that the M71B2 cells have switched to choose other ORs in the nucleus, since but the ER is flooded, the “new choice” never gets to be translated (P. Feinstein, personal communication). This would be evident from increased mRNA transcripts of a different receptor. To confirm that this isn’t the case, single cell sequencing would have to be performed on these cells.

4.7 Future directions

It will be essential to successfully antibody stain for M71 to see the fate of the M71(RDY) receptor, to determine whether or not this presumed “signaling mutant” is in fact a trafficking mutant. Likewise, it will be important to recapitulate the results of the M71B2 receptor in animals of different ages. As a subsequent experiment, I will co-stain the B2AR with antibodies that stain the ER such as calnexin (Yu et al., 2017) or protein disulfide isomerase (PDI). This would confirm that what we see is in fact co-localization with the ER. I will also co-stain with Golgi markers to see if it is here that receptors are stuck.

It could be interesting to continue this experiment looking at different aged animals as well as embryonic animals. Another future direction is exploring these mutations from a

molecular biology standpoint by using single cell sequencing and in situ hybridization. This could provide information about the choice mechanism and how cells receive feedback from the ER.

5. MATERIALS AND METHODS:

5.1 Mice and Strains Used:

Mice used in this study were of a mixed 129/C57BL/6 background and were bred and maintained in the Laboratory Animal Facility of Hunter College, CUNY. Gene targeted mice were originally derived or procured by Paul Feinstein and made available to me. Procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (NHHHS Publication No. [NIH] 85-23). Briefly, postnatal day 1 (PD1) mice were euthanized by decapitation with a surgical instrument according to the *Guidelines for Euthanasia of Rodent Fetuses and Neonates* (NRC, 2011). PD7 animals were anaesthetized by CO_2 inhalation, and then decapitated by surgical instrument as aforementioned. Animals above 10 days old were euthanized by CO_2 inhalation according to guidelines. Strains analyzed were M71-IRES-tauGFP/OMP H2BmCherry, M71(RDY)-IRES-tauLacZ/ OMP H2BmCherry, and M71B2-IRES-tauLacZ/ OMP H2BmCherry.

5.2 Genotyping:

Animals positive for the gene constructs used in this study were identified by PCR. Presence of the M71-IRES-tauGFP allele was determined by using primers to detect GFP. The M71(RDY)-IRES-tau-LacZ allele was determined by using primers to LacZ, and the presence of the OMP H2Bmcherry construct was detected by primers to the H2B sequence and 5’ end of the

OMP sequence. Mice examined in this study were heterozygous for the respective gene-targeted alleles at the M71 locus and hemizygous for the OMP H2BmCherry transgene.

5.3 Wholemount Staining and Imaging:

X-gal staining was performed on roughly 3-week old mice according to a protocol adapted from Mombaerts et al., 1996. Mice were euthanized and their heads dissected sagittally to expose turbinate bones and olfactory bulbs. Hemi snouts were fixed in 4% paraformaldehyde on ice for 5 minutes, and then rinsed with Buffer A (100 mM phosphate buffer [pH 7.4], 2 mM $MgCl_2$, and 5 mM EGTA), and agitated on a shaker for 10 minutes in new Buffer A at room temperature (RT). Snouts were transferred to Buffer B (100 mM phosphate buffer [pH 7.4], 2 mM $MgCl_2$, 0.01% sodium desoxycholate, and 0..02% Nonidet P40) and agitated for 30 minutes at RT. Samples were transferred to Buffer C (buffer B, with 5 mM potassium-ferrocyanide, 5 mM potassium-ferrocyanide, and 1 mg/ml of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and agitated in the dark at RT overnight to generate a blue precipitate. Samples were imaged under a Zeiss Stemi SV11 dissecting microscope and photographed with a Canon EOS 40D.

5.4 Immunohistochemistry:

1-day and 7-day-old mice were euthanized and dissected by removing nasal bones and frontal bones. Snouts were fixed in 4% paraformaldehyde overnight at -4°C, and then decalcified in 0.5M EDTA [pH 8.0] overnight at 4°C (this step can be skipped for PD1 animals.) Samples were then cryoprotected in 15% and 30% sucrose in DI water at- 4°C for 2 hours then overnight, respectively. Samples were then embedded “nose up” in OTC (Fisher) and stored in -80° until use. 25μm sections were sliced on a Leica CM3050S cryostat and adhered to glass slides (Fisher) and stored at -20° until stained or visualized. For immunohistochemistry: Sections on slides were

blocked for 1 hour at RT in 10% NGS and 0.1% Triton X-100 in 1X PBS. After blocking, slides were incubated with 1:3000 rabbit anti-β-galactosidase (Cappel) in 3% BSA and 0.1% Triton X-100 in 1X PBS overnight at -4°C. Slides were washed three times in 1X PBS and then incubated in Alexa 488-conjugated goat anti-rabbit IgG (Invitrogen) for 2 hours at room temperature, and then washed three times in 1X PBS. To double-stain for tau and the B2AR in the chimeric receptor, the above protocol was used with minor changes to ingredients but with the same temperatures, times, and wash steps. Block buffer contained an additional 2% Donkey serum, and antibody buffer contained an additional 3% NGS. Slides were incubated in rabbit anti-β2AR (1:500, Santa Cruz) and mouse anti-β-galactosidase (1:500, AbCam) primaries. Cy5-goat anti-rabbit IgG (1:500, Invitrogen) and Alexa 488-conjugated goat anti-mouse IgG (1:500, Invitrogen) were used as secondaries. Slides were imaged on a Zeiss LSM 510 confocal microscope using the green, red, and far red channels, when applicable. Images were formatted using ImageJ software.

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