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# Cellular and Synaptic Organization of the Human Olfactory Bulb

Alison Maresh

# CELLULAR AND SYNAPTIC ORGANIZATION OF THE HUMAN OLFACTORY BULB

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Alison Maresh

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**ABSTRACT**

The distribution of cell types and synapses is well characterized in the rodent olfactory bulb (OB), and from that plausible models of odor processing have been constructed. Individual olfactory sensory neurons (OSNs) express only 1 of ~1000 odorant receptors (ORs) and send their axons to specific synaptic targets in the OB glomerular neuropil. Each glomerulus is innervated exclusively by OSN axons expressing the same OR. The distribution of these glomeruli is conserved across animals, as is the numerical relationship between number of expressed ORs and number of glomeruli in the OB. Our objective is to extend such results to the level of the human OB to determine how its cellular and synaptic organization, and more specifically how the number and distribution of its glomeruli, compare to what has been elucidated in mice. As there are ~2,000 glomeruli for ~1,000 ORs in mice, we predicted ~700 glomeruli in humans based on the ~350 intact OR genes identified in the human through genomic studies. Using immunohistochemistry, the organization of cells and synapses in human OBs was evaluated and quantified. While the laminar structure of the OB is broadly conserved between species, in the human OB the laminar organization as well as additional structural features suggest a less rigorously organized OB than in rodents, perhaps suggesting that odor processing in the human OB may be less efficient than in mice. Of particular note, the total number of glomeruli in the human OB differs significantly from predicted and demonstrates a high degree of variability amongst specimens, thus far ranging from approximately 3000 - 9000/OB. These results indicate that the principles of OR-homotypic axon convergence developed from mouse studies may not be readily applicable to the human, and that central processing of odor signals in the human may differ from those characterized in the mouse.

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# INTRODUCTION

## Odors are detected by a complicated chemical sensing system that is conserved across many organisms.

Organisms use their sense of smell to detect their chemical environment, which is a crucial function for survival. At the most fundamental level, even single-celled organisms are able to detect and respond to chemicals in their surroundings. The ability to smell plays a key role in higher organisms that rely on olfaction for processes such as detecting food, staying away from poisonous substances, finding mates, and avoiding predators. While olfaction is the primary modality in many organisms for these survival behaviors, other organisms, such as humans, have grown to rely more heavily on their other senses for these same behaviors.

Despite the large variation in the degree to which different organisms depend on olfaction, the organization of this system appears to be broadly conserved across species. Comparisons have been done between species to evaluate for similarities in several aspects of the olfactory pathway including: receptor genes and receptor gene expression, odorant-binding-induced signal transduction, and even certain components of central organization and neural processing (1-3). The species examined ranged from *Drosophila melanogaster* (fruit fly) in the arthropod phylum to *Rana spp*. (frog), *Carassius aruatus* (goldfish), *Mus musculus clomesticus* (mouse), and *Homo sapiens* (human) in the vertebrate phylum. These studies broadly demonstrated striking similarities at these different levels of odor processing across both species and phyla. This concordance

likely stems from a combination of homology, or inheritance from common ancestors, as well as convergence, or adaptation to the most efficient and functional solution to common environmental demands. There are several species in which there have been extensive studies of the olfactory system in order to gain an understanding of odor processing, including *Drosophila*, rats, and mice. Because they are mammals, most of the assumptions about the organization of the human olfactory system are based on rodent studies.

The unique complexity of the olfactory system is demonstrated superficially by the relatively large number of different receptors that are used to detect odor input as compared to the other senses that organisms use to perceive their environment. Sound is detected by hair cells in the cochlea, which allow for pitch discrimination based on the topography of cells activated by different frequencies of sound waves. The receptor cells for vision are composed of rods and cones which detect light. The topography of the cells on the retina when receptors are activated by light allow organisms to perceive distinct images. Somatosensory detection also occurs through only a few types of receptors; mechanoreceptors detect light pressure, nociceptors detect strong pressure, and thermoreceptors detect heat, all providing the body with information about its physical surroundings. Taste, the other chemical sense, is the only other sense that relies on more than just a few different types of receptors to detect input stimuli, in this case tastants. However, even taste receptors cannot compare in sheer number to odorant receptors; for example mice are thought to have ~36 taste receptors (4), while they have ~1000 odorant receptors (5-7). Because of the large numbers of inputs, mapping and understanding the anatomic structures that integrate molecularly defined inputs at the different levels of

odor processing is of utmost importance for learning how the olfactory system works. Perhaps because of this organizational complexity, gaining an understanding of the olfactory system has lagged behind other sensory systems. Major gains in this area have occurred primarily in the last 10-15 years after the identification of the family of receptors responsible for detecting odorants.

**Odor processing begins at the level of the odorant receptors, which are represented genetically by a large and diverse group of genes.**

Mammalian odorant receptor (OR) genes comprise the largest identified family of genes. They were first described in rats by Dr. Linda Buck and Dr. Richard Axel in 1991 (8). Buck and Axel's original investigations were based on several novel and innovative initial assumptions regarding the genetic, biochemical, and cellular characteristics of the olfactory system. First, that the ORs were likely G-protein-coupled receptors, for which there was preliminary biochemical evidence, and would therefore likely be seven-transmembrane proteins. Second, they predicted that ORs would comprise a large family of genes due to the large number and complexity of chemicals that organisms must be able to detect and discriminate. Finally, they believed that ORs would likely be expressed selectively in OSNs. Their assumptions proved to be correct, and their successful discovery of OR genes earned them the Nobel Prize in Medicine and Physiology in 2004. It also pioneered future studies that have led to an understanding of odor processing and the organization of the olfactory system in mice and rats.

As described by Buck and Axel, ORs are G-protein-coupled receptors with a seven-transmembrane domain. Their coding region is about 1 kb, and contains no

introns. There are several conserved motifs that identify genes as ORs, while the transmembrane domains 3, 4, and 5 are hypervariable, and could likely represent areas that come together to form the odorant binding pocket (9). Signaling occurs via a G-protein/cAMP pathway after binding of an odorant, resulting in depolarization and an increase in spiking frequency. Interestingly, this cAMP pathway has also been shown to play a role in axonal targeting by OSN during the wiring of the olfactory system (10).

After the discovery of the family of OR receptors in rats, work went underway to identify these genes in other vertebrates. Most of this work used the technique of PCR amplification with degenerate oligonucleotide primers for conserved motifs (11), work which was facilitated by the ability to use genomic DNA instead of cDNA, as the coding region of OR genes is intronless. Over a dozen species were selected for study (12, 13), and confirmation of the presence of OR genes was successful in all. However, it was unknown exactly how big this family of genes was, as these studies by no means were exhaustive in identifying OR genes in any given species. Estimates of the complexity of the OR family in rats ranged from 200 (8) to 500-1000 (14).

Comprehensive genomic mining studies to identify the large family of OR genes in mice was first undertaken in 2002 after the release of the Celera mouse genome in 2000 (5, 15). These groups identified ~1300 OR genes, with only ~80% of these, or ~1000 genes, containing intact open reading frames. The remaining ~20% are pseudogenes, containing disruptions in the coding region including insertions, deletion, frame shifts, and premature stop codons. OR genes were found on all 20 pairs of chromosomes in the mouse except for 12 and Y. Most OR genes occur in clusters, defined as groups of five or more genes separated by less than 1 Mb. There are 27

clusters in mice containing 87% of the ORs, with the remaining 13% occurring singly or in smaller groups throughout the genome.

Due to the high degree of similarity amongst genes that are clustered together, Zhang and Firestein (5) hypothesized that local sequence duplication was a likely mechanism contributing to OR family expansion. They also noted that many of the OR gene clusters contain non-OR genes. In 15 out of 27 clusters, these genes encoded retrovirus related Gag proteins, and the density of these genes is twice as high in OR clusters as in the rest of the genome. This data, combined with the fact that OR genes are intronless, has led to the theory that duplication of the OR family may be retrovirus-mediated (16, 17).

This sequencing of genomes from many different organisms within the last several years has allowed for efficient mining of OR genes across multiple species. The first draft of the human genome sequence became available in 2001 by Celera Genomics (18) and the International Human Genome Project Consortium (19). Several groups have used this data to fully identify the large family of human ORs. A first analysis was done by the Weizmann Institute (20, 21), where they found 322 OR genes with intact open reading frames. A second study was performed by the Senomyx group (22) who found 347 candidate human OR genes with intact open reading frames. While, by first analysis, the human genome appears to have ~1000 OR genes, about 60% of these are pseudogenes with fatal errors in their coding region. This is a much higher percentage of pseudogenes than the 20% that has been identified both in mice (5) and rats (23), likely representing humans' decreased dependence on olfaction for survival over evolution compared to other mammals.

The average human OR is 315 amino acids long, with about a dozen conserved sequence motifs defining these sequences as OR genes. These ~350 genes represent 1% if the putative 30,000 genes in the human genome, as well as 1% of the 30 Mb length of genomic DNA. They are found on all chromosomes except for chromosome 22 and the Y chromosome. As in mice, while some genes are located singly throughout the genome, 80% are embedded within 24 larger clusters spread throughout the different chromosomes, and 42% are found in clusters on chromosome 11.

Sequence similarities between OR clusters in humans and mice has allowed for the identification of potentially orthologous OR gene clusters. The cluster at human chromosome 17p13.3 and mouse chromosome 11B3-11B5 (24) shares a high degree of homology and likely represents a common evolutionary derivative. Additional studies have further proven the high degree of homology between human and mouse ORs (25-28), representing further justification for the utilization of rodent olfaction as a model for human olfaction. However, due to genomic changes in both humans and mice since the rodent-primate divergence, it is impossible to identify one-to-one orthologous relationships for most OR genes. In mice, local duplications have dominated the differences seen between species, while in humans, deletions and interchromosomal duplications dominate, resulting in larger OR gene clusters in mice and smaller, more distributed clusters or single genes in humans. Young et al. (29) argues that this represents different evolutionary forces shaping OR gene families in humans and mice, which complicates the often accepted assumptions of common OR-ligand relationships between these two species.

OR proteins are expressed highly in the cilia of OSN dendrites. These cilia project from dendrites into the mucus layer of the olfactory epithelium, located in the posterior nasal cavity of mammals. This mucus layer is where the signal-receptor interaction takes place between odorants dissolved in the mucus and ORs that are found on the surface of cilia. The transcription of OR genes in these OSNs is a highly regulated process, with any single OR gene only being expressed in a subset of OSNs. Furthermore, single-cell RT-PCR data has shown that any given OSN only expresses a single OR (30-32). This idea is the basis for the one receptor-one neuron hypothesis, in which only one out of ~1000 genes in the OR family repertoire is selected for expression in an OSN. In addition, RT-PCR looking at pools of OSNs with polymorphic OR alleles has demonstrated that expression of ORs is monoallelic (33), a conclusion that has been confirmed by several subsequent studies (34, 35). Both maternal and paternal alleles are expressed in separate OSNs in approximately equal numbers.

The genetic regulation of this OR gene selection process is not well understood, though in mouse and rats it has been shown that expression of any single OR only occurs in one of four parallel zones in the olfactory epithelium (36-38). Within each zone, expression of ORs appears to be completely random. While the presence of zones has been described for over a decade, the functional role it plays in odor processing is still unclear.

**The olfactory bulb coordinates sensory input on its way to the olfactory cortex.**

The OSN receives sensory input from odorants via the olfactory receptors, which initiates a G-protein linked signaling cascade. The thin unmyelinated axons of the OSN

then travel through foramen in the cribiform plate and carry the signal to the CNS (Figure 1A). One unique property of OSNs is that they are regenerated throughout the adult life of all studied organisms. Therefore, even beyond the developmental stage, new OSN axons are constantly targeting and integrating into existing central synaptic networks. The rodent olfactory bulb (OB) is the highly organized laminar structure that receives these OSN axonal inputs, making it the first step of odor processing in the central nervous system. The organization of the OB is important to understanding how this level of processing works. Interestingly, the cellular histology has been described long before the understanding of principles of odor coding, starting with the work of Ramon y Cajal in 1911 (39). Both the cellular and synaptic structures in rodent OBs are now well elucidated, and are reviewed in detail by Shepherd and Greer (40).

There are several categories of neurons that play a role in odor processing at the level of the OB. These include input neurons that carry information to the OB, output neurons that carry information out of the OB, and finally short axon intrinsic neurons that participate in the coordination of signals within the OB. Input is received both from OSNs that carry the actual sensory information into the OB from the nose, as well as from regulatory centrifugal fibers consisting of axons from higher centers such as the olfactory cortex, the anterior olfactory nucleus, the basal forebrain, and several parts of the brainstem. Output from the OB is sent through mitral cells and tufted cells, also known as the projection neurons. Axons from these cells leave the OB at the posterolateral surface to form the lateral olfactory tract (LOT), which carries encoded olfactory information to the olfactory cortex. Finally, additional regulation and processing of olfactory signals occurs within the OB through synaptic circuits involving

Figure 1: Overview the Olfactory System and Olfactory Bulb

The OSNs are schematically shown in A. Their cell bodies are located in the olfactory epithelium, where they extend dendrites covered with the OR-expressing cilia into the mucus layer. The OSN axons then travel through foramen in the cribiform plate into the OB, where they reorganize and regroup before finding their specific synaptic targets. The organization within the OB is based on clear laminar layers as shown through DAPI nuclear staining in a coronal section of an adult mouse OB (B). The ONL contains the OSN axons surrounding the OB before entering the GL to find their synaptic targets. Next is the EPL, a cell-sparse area of dendrodendritic synapses, followed by the thin, cell-dense MCL, which contains the cell bodies of the projection neurons, the mitral cells. Deep to this is the IPL, and finally the GCL in the center, which contains the cell bodies of the granule cells, a type of interneuron in the OB. Within the GCL newly generated neurons travel through the RMS to add to and replace existing interneurons in both the GCL and the GL. Image in A adapted from http://www.colorado.edu/epob/epob3730rlynch/image/figure8-18.jpg. OB, olfactory bulb; OSN, olfactory sensory neuron; OE, olfactory epithelium; RMS, rostral migratory stream; GCL, granule cell layer; IPL, internal plexiform layer; MCL, mitral cell layer; EPL, external plexiform layer; GL, glomerular layer; ONL, olfactory nerve layer.

Figure 1:

intrinsic neurons, made up of periglomerular (PG) cells and granule cells.

The layers of the OB are shown in an adult mouse OB in Figure 1B. The most superficial layer is called the outer nerve layer (ONL), and consists of axons reorganizing into functionally related subsets before penetrating into the OB (34, 41). Beneath the ONL lies the glomerular layer (GL). This layer contains glomeruli, described in further detail below, which include the distinct synaptic units between the OSNs and the projection neurons. Below the glomerular layer is the external plexiform layer (EPL) which contains the cell bodies of the tufted cells, as well as the dendrodendritic synapses between projection neurons and granule cells. Next is the mitral cell layer (MCL), containing the cell bodies of mitral cells. The internal plexiform layer (IPL) is a thin layer between the innermost granule cell layer (GCL) and the MCL, containing output axons from the projection neurons on their way to forming the LOT. In the middle of the GCL, which contains the cell bodies of granule cells, is the rostral migratory stream (RMS), through which newly generated neurons travel from the subventricular zone (SVZ) where they are born, to their target destinations in the OB where they differentiate into new PG cells and granule cells.

The complex synaptic circuits formed by these cells hold the key to understanding how odor signals are processed at this level within the OB. A basic scheme of the main synapses is depicted in Figure 2, adapted from Mori et al. (42). Within each glomerulus, the OSN axons synapse with the primary dendrites of mitral and tufted cells. Any given projection neuron only innervates a single glomerulus. However, these projection neurons do send secondary dendrites laterally throughout the EPL where they form

reciprocal dendrodendritic synapses with granule cells; the granule-to-mitral/tufted synapse is inhibitory while the mitral/tufted-to-granule is excitatory.

In addition to the main axodendritic synapses between OSNs and projection neurons within the glomeruli, OSNs also form excitatory axodendritic synapses with PG cells. Also within the glomeruli are reciprocal dendrodendritic synapses between PG cells and the mitral and tufted cells; the mitral/tufted-to-PG synapse is excitatory and the reciprocal is inhibitory.

Within the GL but outside of the glomeruli, PG cell axons form inhibitory synapses both with other PG cell bodies and dendrites, as well as on the primary dendrites of projection neurons as they exit the glomeruli. It can therefore be seen that PG cells are largely inhibitory in nature and are likely involved in negative feedback circuits.

Collaterals from outgoing projection neuron axons on their way to the olfactory cortex form excitatory synapses with the cell bodies of granule cells within the GCL. The axon terminals from incoming centrifugal fibers are found in the GCL, the EPL, and in the extra-glomerular space of the GL, therefore providing regulation of olfactory signals from higher centers at multiple levels within the OB.

**Glomeruli are anatomical structures in the olfactory bulb with topographic specificity that play a functional role in odor processing.**

Glomeruli are the spherical regions within the OB neuropil that represent the primary synapse between OSN axons and projection neuron dendrites. In mammals, input into each glomerulus consists of axons from thousands of OSNs (43, 44). Any one

Figure 2: Synaptic Organization of the Olfactory Bulb

After OSNs enter the OB, they synapse with projection neurons, the mitral cells and tufted cells, within discrete synaptic units called glomeruli. Also participating in the regulation of sensory input at this level are the PG cells, which form feedback circuits within the gomeruli with both the OSNs axons and the projection neuron dendrites. The mitral/tufted cells send only a single primary dendrite into a glomerulus, however they send multiple secondary processes laterally that form synapses with granule cell dendrites. The axons from the mitral and tufted cells then travel to the olfactory cortex where the final level of odor processing takes place. White arrows represent excitatory synapses, black arrows represent inhibitory synapses. OSN, olfactory sensory neuron; GL, glomerular layer; PG, periglomerular cell; M, mitral cell; T, tufted cell; Gr, granule cell. Adapted from Mori et al. (42).

Figure 2

OSN projects to only a single glomerulus (45). When examining the subset of OSNs expressing the same OR in rodents, it can be seen that their axons all project to one, two, or several distinct glomeruli (34, 46-48). The most commonly seen scenario is axons projecting to exactly two glomeruli, one in the medial half of the OB and the other in the lateral half of the OB. This specific targeting of glomeruli by OSN axons is made even more complicated by the fact that the three-dimensional positions of glomeruli are stereotyped from animal to animal within a species. Spatial mapping has been undertaken through a variety of techniques, including 2-deoxyglucose uptake (49-51), optical imaging with voltage sensitive dyes (52, 53), and electrophysiological recordings from projection neurons (54). All studies in all animals have shown that identical odors elicit characteristic spatial patterns of glomerular activity within the OB.

Very little is known about the mechanism of specific glomerular targeting by OSN axons. *In situ* hybridization in rodents has demonstrated that OR proteins are present within the OSN axons and axon terminals (46, 47). Later genetic studies were done in mice in which deleted OR coding sequences were substituted with different receptor sequences resulting in axon convergence to new glomerular targets (55, 56), showing that the OR itself plays a necessary role during axonal targeting. OR guided targeting is presently believed to occur through G protein-coupled signaling via cAMP rather than by the direct action of OR molecules (10). However, the new glomerular targets of these OSN axons were not exactly at the location of the substituted OR glomeruli. This imperfect localization suggests that the ORs are necessary but not

sufficient for exact targeting, and that the process is therefore more complex and likely involves multiple other determinants.

In addition to the targeting specificity of OSNs to glomeruli, there is also evidence of reciprocal specificity, in that all of the axonal inputs to a single glomerulus are specific to OSNs expressing a single OR. Treloar et al. (57) demonstrated this in the mouse by using electron microscopy to show that all axons synapsing within a M72 glomeruli express the M72 OR.

These results further characterize the degree of molecular specificity encoded by the glomeruli, and additionally set up a numerical relationship between ORs and glomeruli. If all OSNs expressing a given OR target a specific number of glomeruli in stereotypical positions in the OB, and all glomeruli are only receiving axonal input from one type of OR, the number of glomeruli should be directly related to the number of expressed ORs in any given species. In mice, there are ~1000 expressed ORs, and the number of glomeruli has been estimated at ~1800 (58). In rats, there are ~1200 expressed ORs (23) and the number of glomeruli in the rat OB has been estimated at ~2400 (59). There is therefore an approximate 2:1 relationship between the number of ORs and the number of glomeruli. This confirms the previously noted observations that OSN axons for any given OR usually project to two glomeruli - one glomerulus medially placed in the OB and one that is laterally placed in the OB (34, 46-48); only rarely do they project to only one or more than two (35).

This specific organization of glomeruli in the rodent OB therefore creates a stereotyped map of ORs at this first level of central processing. It is unclear what role the locations of each OR's glomeruli play in the course of odor processing. However, due to

the ability of PG cells to regulate multiple proximally located glomeruli, as well as the ability of mitral and tufted cells that innervate separate yet adjacent glomeruli to form dendrodentritic synapses with each other via their secondary dendrites, it is indeed likely that the location and organization of the glomerular map has a functional role in regulating and integrating sensory information on its way to the olfactory cortex. Structurally similar odorants have been demonstrated to activate glomeruli in similar OB regions, creating chemotopic maps around the surface of the OB (60-63). This, along with the growing understanding of the synaptic circuits involving closely proximated glomeruli, has popularized the idea that a key function of the glomerulus is to act as a signal-to-noise enhancing device (64). Whatever the function(s) of glomeruli may be, the preservation of their anatomic localization and molecular specificity across all studied species is certainly striking.

## STATEMENT OF PURPOSE AND HYPOTHESIS

### Qualitative evaluation of the primary organization of the human olfactory bulb.

Much work has been done to characterize the synaptic and molecular specificity of the OB glomeruli and their development. Missing from these studies, however, is an analysis of the human olfactory system; a determination of whether the principles of axon convergence and molecular specificity that have been developed in rodent models extend to the human olfactory system. Only a limited number of studies have been reported on the organization of the human OB and these were largely carried out prior to the development of our current molecular reagents or insights into the molecular organization of the OB. Anecdotally, one can argue that the sense of smell in humans has degraded over evolution, but that hypothesis has not been pursued at the level of synapse formation or molecular specificity. Consequently, we are interested in characterizing the primary organization of the human OB to begin to probe the widely held but untested hypothesis that the cellular, synaptic, and molecular organization in the human OB is less precise than that seen in other species.

### Assessing the rodent-based principles of molecular specificity in glomerular maps in the human olfactory bulb.

Rodent studies have shown that the total number of glomeruli in the OB is a direct reflection of the number of intact ORs expressed in the olfactory epithelium; in rodents, there are twice the number of glomeruli as there are OR genes. This leads us to the simple though elegant method of evaluating the human olfactory system to determine just

how similar odor processing is in these two species by counting the number of glomeruli present in human OBs. Genomic mining in the human suggests a total of approximately 350 OR genes with intact reading frames. If the principles of axon convergence occur in the human as they do in the rodent, we predict the human OB will have 700 glomeruli.

# METHODS

## Tissue Procurement and Fixation

Human OBs were obtained through several sources including both during autopsy from post-mortem donors as well as during neurosurgical procedures from live donors. OBs from autopsy were kindly made available by Dr. Jung Kim from the Department of Pathology, Yale University School of Medicine, New Haven, CT, as well as through the National Disease Research Institute, Philadelphia, Pennsylvania. Information regarding age, gender, and relevant medical history was obtained for all donors (Table 1). Exclusions for this part of the study were the presence of symptomatic olfactory dysfunction, neurodegenerative disorders such as Alzheimer's Disease and Parkinson's Disease, and intranasal drug use. Procurement of this tissue and relevant donor information passed HIC approval (#12081), and is exempt from IRB review under federal regulation 45 CFR 46.101(b)(4). The post-mortem interval in these cases was less than 16 hours, and after procurement the OBs were fixed in 10% formalin for 7 to 28 days. After fixation, these tissues were stripped of their meninges and washed two to three times overnight in fresh phosphate buffered saline (PBS).

The live donor OBs were kindly obtained by Dr. Dennis Spencer of the Department of Neurosurgery, Yale University School of Medicine, New Haven, CT, during frontal lobe neurosurgical cases requiring disruption of the lateral olfactory tract (HIC# 12081). OBs were obtained only in those cases in which they would otherwise be sacrificed or discarded during the course of the surgery. These OBs were fixed in 4%

paraformaldehyde for 24 to 48 hours. After fixation, these OBs were stripped of their meninges and washed in PBS overnight.

For qualitative comparisons, mouse OB tissue was also obtained. Adult CD1 mice (Charles River Laboratories) were anesthetized with sodium pentobarbital (80 mg/kg i.p.; Nembutal; Abbott Laboratories, North Chicago, IL), then decapitated. Perfusions were avoided in order to more closely replicate the conditions under which the human OBs were obtained. The mouse brains were removed from their skulls, and their OBs were removed and placed in 4% paraformaldehyde overnight, followed by PBS overnight. All procedures undertaken in this study were approved by Yale University's Animal Use and Care Committee and follow NIH guidelines.

After fixation, all human and mouse OBs were then cryo-preserved in 30% sucrose in PBS for 12 to 24 hours, then sectioned coronally throughout the length of the entire OB on a sliding-freezing microtome (50µm), and stored at -20°C until use. To maintain rostral-caudal order in the human OBs, slices were maintained individually in 48-well plates.

**Immunohistochemistry**

Tissue was removed from -20°C storage and washed in PBS with 0.03% Triton 100-X (PBS-T). For antigen retrieval, OB slices were steamed for 10 minutes in a solution of 0.01M Sodium Citrate, then immediately washed with room-temperature PBS-T. Tissue was blocked with 2% BSA in PBS-T for 45 minutes, then incubated for 48 to 72 hours in primary antibody (see Table 2 for antibodies, concentrations, and sources) diluted in BSA-PBS-T at 4°C. Tissue was then washed in PBS-T, and incubated

in secondary antibody (Table 2) diluted in BSA-PBS-T for 2 hours along with a nuclear marker, DAPI (Sigma) and/or DRAQ5 (Alexis Biochemicals). The sections were then washed in PBS-T, then PBS. In order to eliminate autofluorescence from lipofuscin granules, sections were stained with 1% Sudan Black in 70% Methanol for 5 minutes, then cleared in 70% Ethanol and rinsed in PBS (65). Sections were mounted with GelMount (Bioveda).

**Qualitative Characterization of the Olfactory Bulb**

Antibodies as listed in Table 2 were used to characterize the organization of the OB. MAP2 stains for dendrites, while GAP43 and NCAM are axonal markers. VGlut2 is a synaptic marker specific to OB glomeruli. To look at different classes of neurons, an antibody against calretinin was used to identify neurons with this calcium binding protein, GAD65/67 to identify GABAergic neurons, and TH to identify dopaminergic neurons. Antibodies against specific mouse odorant receptors, mOR50, mOR28, mOR256-17, and mOR267 were used to attempt to look at how axons expressing these individual odorant receptors were organized in the human OB. All staining was done in at least four different human OBs, and presented images are typical unless stated otherwise.

Images were taken with the Leica confocal microscope at different magnifications to assess the immunofluorescent results at different levels of detail. Images were also taken of mouse OB sections stained with the same antibodies for comparison of the distribution of cell bodies, axons, and dendrites.

**Quantifying Glomeruli**

Every sixth section throughout the length of the human OBs was stained with anti-NCAM and anti-VGlut2 primary antibodies. NCAM identifies the OSN axons, while VGlut2 is a synaptic-associated protein that in the OB is only found at the primary synapse between the OSN and the projection neurons, therefore defining the glomerular unit. Glomeruli were identified by co-localization of VGlut2 and NCAM staining.

Overlapping images were taken circumferentially around each section with an Olympus BX51 epifluorescent microscope using the 20X objective. These digitized images were then analyzed using Metamorph software (Molecular Devices, Sunnyvale, CA). Glomeruli were manually circled, while the software calculated total numbers of glomeruli as well as area and length/width diameters of each glomerulus.

In this study, the length of the OB was defined by the distance encompassed by the most rostral and most caudal OB sections that exhibited glomerular staining. The volume was calculated by estimating the shape of the OB to be a cylinder, and the cross sectional area was estimated by averaging the area of 4 slices distributed through the length of the OB. The total counted glomeruli per OB was calculated by first multiplying the total number of counted glomeruli from the sections looked at by the inverse of the fraction of slices counted, usually around 6 as about every $6^{th}$ slice was selected for counting. Finally, to correct for the glomerular overlap between sections, the Abercrombie extrapolation was used: $N = n * (t / ( t + H ))$, where in this case N is the number of glomeruli in the OB, n is the total number of counted glomeruli, t is the width of each section (50μm) and H is the average glomerular diameter.

Statistical analysis was performed using the Prism package (GraphPad Software Inc., San Diego, CA). To look for relationships between the number of glomeruli and the age of the donors, the size of their glomeruli, or the volume of their OB, a linear regression test was performed. To look for significance between the mean number of glomeruli in male donors versus female donors, as well as between "young" donors (<50 years old) and "elderly" donors (>50 years old), an unpaired t-test was performed. There were no significant differences in the viariances in either of these comparisons.

All of the above mentioned procedures, calculations, and analyses were performed by myself. Dr. Diego Rodriguez Gil, a post-doctoral associate in the Greer lab, assisted with the statistical analyses. Instruction and guidance was provided by all members of the Greer lab throughout my work.

**Table 1: Olfactory Bulb Donor Information**

| OB Identification | Source Type | Age of Donor | Gender | Relevant Clinical Information |
|---|---|---|---|---|
| HOB 1 | Surgery | 39 | F | frontal lobe epilepsy |
| HOB 2 | Post Mortem | 89 | M | lung adenocardinoma |
| HOB 6 | Post Mortem | 67 | M | leukemia treated with chemotherapy |
| HOB 7 | Surgery | 66 | M | pituitary tumor |
| HOB 15 | Post Mortem | 70 | M | emphysema |
| HOB 16 | Post Mortem | 85 | F | microscopic polyangiitis |
| HOB 20 | Surgery | 49 | F | frontal lobe glioma |

**Table 2: Antibodies Used for Immunohistochemistry**

| Antibody | Animal | Dilution | Source |
|---|---|---|---|
| MAP2 | chicken | 1:1000 | Sigma |
| GAP43 | rabbit | 1:1000 | Sigma |
| NCAM | mouse | 1:500 | Sigma |
| VGlut2 | rabbit | 1:4000 | Synaptic Systems |
| calretinin | mouse | 1:400 | Chemicon |
| GAD65/67 | mouse | 1:1000 | Stressgen |
| TH | rabbit | 1:1000 | Chemicon |
| mOR50 | guinea pig | 1:3000 | Dr. Richard Axel, Columbia University, New York, NY |
| mOR28 | rabbit | 1:5000 | Dr. Hitoshi Sakano, University of Tokyo, Japan |
| mOR28 | rabbit | 1:5000 | Dr. Richard Axel, Columbia University, New York, NY |
| mOR256-17 | rabbit | 1:800 | Dr. Heinz Breer, University of Hohenheim, Germany |
| mOR262 | rabbit | 1:1500 | Dr. Heinz Breer, University of Hohenheim, Germany |
| chicken-Alexa 555 | goat | 1:1000 | Molecular Probes |
| rabbit-Alexa 555 | donkey | 1:1000 | Molecular Probes |
| mouse-Alexa 488 | donkey | 1:1000 | Molecular Probes |
| guinea pig-Cy3 | donkey | 1:1000 | Jackson Immunoresearch |

# RESULTS

## The laminar organization of the OB is broadly preserved in humans.

The rodent OB is characterized by distinct laminar structures that can be identified using nuclear staining to evaluate the cellular distribution. As can be seen in Figure 3A which depicts the layers of a mouse OB, the glomerular layer is clearly recognizable by the unique distribution of PG cells outlining individual glomeruli. The EPL is an area of sparse nuclear staining as it is the area of dendrodendritic synapses between granule cells and projection neurons. The thin band of dense nuclei represents the concentrated layer of mitral cell nuclei in the MCL. Finally there is another cell-sparse region, the IPL, followed by the cell rich GCL. This is an extremely organized and distinctive pattern, seen consistently in all rodents throughout the rostral-caudal length of the OB.

Figure 3A' demonstrates the same DRAQ5 nuclear staining of a human OB. Clearly the layers are still present, though not as sharply demarcated as in the mouse OB. The GL is separated by the more superficial ONL by the presence of cell-absent spots, the glomeruli. However, unlike in the mouse OB, the PG cells do not form clearly identifiable densities around the glomeruli, making individual glomeruli difficult to visualize in the human GL using nuclear staining alone. Deep to the GL is the EPL, which is seen in this image to be relatively thin compared to the mouse, while the IPL is relatively thick compared to the mouse. However, even within human OBs, there is actually a high degree of variability in the width of the layers.

Figure 3: Laminar Organization of the Human Olfactory Bulb

Nuclear staining with DRAQ5 demonstrates the laminar layers of the mouse olfactory bulb (A) and the human olfactory bulb (A'). While there is preservation of the laminar structure between the two species, the layers are more clearly demarcated in the mouse. In addition, the human OB demonstrates more variability in the width and organization of these layers, which are even sometimes completely absent. Representative coronal OB slices show the structural variations both within single slices as well as between different OBs from different human donors (B-D). In B, there are areas of very clear laminations (red arrow), as well as areas where the layers cannot be distinguished from each other (green arrow). In C, the GL is completely absent around the left side, but in other areas the laminations are very clear (red arrows). D also shows a partially circumferential GL, and in this OB the rest of the layers seem to be completely absent as the GL ends (yellow arrow). GCL, granule cell layer; IPL, internal plexiform layer; MCL, mitral cell layer; EPL, external plexiform layer; GL, glomerular layer; ONL, olfactory nerve layer. Scale bar in A is 50 μm for A, A', scale bar in D is 500 μm for B-D.

Figure 3:

This variability can be seen circumferentially within one OB slice, as well as between slices from OBs from different donors. Figure 3B-D shows examples of slices stained with DRAQ5 from three different donors. In Figure 3B, the GL can be seen around the entire circumference of the OB. The different layers, including the frequently elusive MCL, are clearly demarcated through the entire depth in some areas (red arrow), however seem to blend together deep to the GL in others (green arrow). In Figure 3C, the GL is absent around the left side of the bulb. Despite the absence of glomeruli, there is evidence of lamination suggesting that some level of organization persists. Again, as in the previous image, there are points throughout the rest of the OB's circumference with very clear laminations (red arrow) while in other areas the laminations are less clear.

The final image (Figure 3D) shows another example of an OB with a glomerular layer that is only partly circumferential. However, as opposed to in the previous image, the laminations are completely absent along the top of the slice where there are no glomeruli, and even the density usually seen around the periphery of the central GCL disappears when the glomerular layer ends (yellow arrow). In this OB, the ONL and GL are extremely thick compared to the other OBs pictured.

**Distribution of OSN axons in glomeruli depends on axonal maturity.**

The OB is a dynamic region of the central nervous system; as seen in other species, OSNs are constantly regenerated/replaced and send their newly forming axons through the cribiform plate into the OB, where they seek out their target glomeruli and integrate into the dense synaptic network. To look for evidence of these newly generated

OSN axons in the human OB, sections were co-stained with GAP43, which recognizes immature axons in the process of finding and integrating into their target synaptic networks, as well as NCAM, which stains all axons.

The GAP43 pattern of staining in a mouse OB demonstrates GAP43+ axons clearly co-localizing with the NCAM+ axons in the ONL as well as glomeruli (Figure 4A). This staining pattern is also represented in the human (Figure 4B). While the overall architecture of the OB laminations are not as precise, the distribution of GAP43+ stained axons is similar, with co-localizations seen in the ONL and glomeruli. This image is from HOB 1, taken from a 39 year-old donor, demonstrating clearly that OSNs are generated and replaced in adult humans. The oldest OB examined was HOB 2, taken from an 89 year old donor (Figure 4C). In this OB as well, which qualitatively has even poorer laminations than in the previous OB from a younger donor, GAP43+ axons are still visualized in glomeruli, demonstrating the persistence of OSN proliferation in humans through the entire adult life into old age.

A closer look at a glomerulus as defined by the NCAM stained OSN axons, demonstrates the GAP43 staining around the periphery (Figure 4D). This is consistent with observations in rodents, in which new OSN axons are integrated on the outside of an existing glomerulus, while the more mature axons are more centrally located (66). As additional new axons expressing the same OR arrive at their target, they in turn surround the outside of the glomerulus, and the previous axons that are now maturing and integrating into their synaptic networks become relatively more central.

Figure 4: Presence and Distribution of Newly Generated Olfactory Sensory Neurons Axons from newly generated OSNs are identified by co-localization between GAP43 (red) and NCAM (green). These axons can be seen in abundance in the ONL and glomeruli of the mouse OB (A), showing that newly generated axons are able to integrate into the OB synaptic network. Evidence of OSN regeneration in humans is presented in B-D, where these axons are seen in the ONL and glomeruli similarly as in mice. The OB slice stained in image B is from a 39 year old donor, while the OB in C is from an 89 year old donor, indicating that OSN regeneration continues well into old-age. D is a higher magnification showing that new OSN axons first integrate into the periphery of existing glomeruli, a process that has been previously described in rodents. EPL, external plexiform layer; GL, glomerular layer. Scale bar is 100 μm in A-C, and 25 μm in D.

**Figure 4**

**Glomeruli in the human OB show evidence of compartmentalization.**

Previous work in rodents has demonstrated compartmentalization of glomeruli into axonal and dendritic compartments (67-69); the axonal compartments are where the axodendritic synapses between OSNs and projection neurons take place, while the dendritic compartments are where the dendrodendritic synapses between the PG cells and the mitral and tufted cells take place.

To examine whether there was evidence of compartmentalization in human glomeruli, OB slices were co-stained with NCAM, an axonal marker, and MAP2, a dendritic marker. Figure 5A and B show the distribution of these two markers in the mouse and human OBs respectively. In both species, the MAP2+ dendrites are especially prominent in the EPL, the area of dendrodendritic synapses. Dendrites are also demonstrated extending into glomeruli in the GL, with some degree of co-localization visible even at this low magnification between these MAP2+ dendrites and NCAM+ axons.

By examining higher magnifications of glomeruli (Figure 5C, D), the axonal and dendritic compartmentalizations previously described only in the rodent are demonstrated here in the human OB. The axonal areas are demarcated by the green NCAM+ staining with some evidence of co-localization with the red MAP2+ staining, representing the OSN-projection neuron axodendritic synapses. The dendritic areas are NCAM-, but MAP2+, representing areas of dendrodendritic synapses as previously described. This suggests that at the intraglomerular level, axodendritic primary afferent synapses and local circuit dendrodendritic synapses remain segregated in the human OB and therefore that at this level, processing of odor in the human may be similar to that described in

Figure 5: Evidence of Glomerular Compartmentalization

In rodents, separate axonal and dendritic compartments have been defined in glomeruli, segregating the axodendritic synapses from the local circuit dendrodendritic synapses. The broad distribution of dendrites and OSN axons is demonstrated in a mouse OB (A) using MAP2 (red) and NCAM (green). Nuclear staining with DRAQ5 (blue) is also seen in A, B. As expected, the EPL is rich in MAP2+ dendritic staining, with both MAP2 and NCAM staining seen in the glomeruli. A similar organization is seen the human OB (B) as in the mouse. Higher magnification of glomeruli from the human OB (C,D) shows areas specific to NCAM staining and areas specific to MAP2 staining. These represent axonal and dendritic compartments respectively, suggesting that there is preservation of the synaptic organization within glomeruli between rodents and humans. EPL, external plexiform layer; GL, glomerular layer. Scale bar is 100 μm in A, B, and 10 μm in C, D.

Figure 5:

other species.

**Periglomerular cells in the human OB do not form uniform densities around glomeruli, however they represent a similar heterogeneous population of cells as in rodents.**

As described earlier, glomeruli in the mouse OB can be identified with nuclear stains by their surrounding PG cells, while in the human OB, this method of identifying glomeruli is not as clear. To further evaluate the distribution of PG cells in the human OB, nuclear staining was combined with staining for NCAM, an axonal marker, and VGlut2, a synaptic marker specific to glomeruli; co-localization of these two probes is a more rigorous way of defining glomeruli, and therefore provides a way of visualizing the relationship between PG cells and glomeruli.

The DRAQ5 staining of a mouse OB shows PG cell densities clearly demarcating the borders of glomeruli (Figure 6A). Confirmation of these glomeruli is demonstrated with the added NCAM and VGlut2 staining to this image, clearly showing glomeruli within the PG cell circumferential densities (Figure 6B). However, DRAQ5 nuclear staining from sections of a human OB are far less convincing in their ability to delineate glomeruli through examination of PG cells (Figure 6C,E). There are areas of more sparsely distributed cells, some of which hint at the potential locations of glomeruli, however only rarely do the PG cells identify clear glomerular borders. When NCAM and VGlut2 staining is added to the images (Figure 6D,F), easily identifiable glomeruli are now able to be seen within the PG cells. This PG cell organization appears to be unique in humans compared to mice; while in mice and other rodents the entire GL is defined by

closely approximated glomeruli with evenly distributed PG cells consistently surrounding every individual glomeruli, the human OB GL appears more chaotic in nature. As described in more detail below, glomeruli in the human GL appear to be distributed randomly, often appearing in clusters or as a lone individuals. When in a cluster, there is a relative paucity of directly approximated PG cells relative to the central glomerular bodies. When spaced farther apart, there are large areas of intervening PG cells that have no physical approximation to any glomerulus; it is rare due to the compact nature of the rodent GL to see PG cells that are not directly abutting or within close approximation to a glomerulus. Therefore, while the GL does qualitatively appear to be rich in PG cells in humans, there does not appear to be homogeneity in the distribution of PG cells relative to glomeruli as there does in rodents.

PG cell populations and their chemical heterogeneity have been well described in rodents (70). Their heterogeneity is usually defined by several substances; glutamic acid decarboxylase (GAD), a key enzyme in the synthesis of GABA (71-74), tyrosine hydroxylase (TH), a key enzyme in synthesizing dopamine (75, 76), and several calcium binding proteins, including calretinin (77, 78). As PG cells are primarily inhibitory, it is no surprise that many if not all of them contain GABA and/or dopamine, two of the primary inhibitory neurotransmitters.

By immunohistochemical staining with antibodies for each of these distinct PG cell markers, the relative qualitative distributions of PG cell types can be compared between humans and mice. Figure 7A demonstrates the population of dopaminergic cells in a mouse OB using antibodies for TH, along with NCAM to define the glomeruli. TH+ cells have large cell bodies, and are seen surrounding the glomeruli. More faintly

Figure 6: Defining Glomeruli With NCAM and VGlut2 Co-Localization

Due to the well organized compact nature of glomeruli within the GL in the mouse OB, it is easy to define glomerular borders based on the presence of surrounding PG cell densities using DRAQ5 nuclear staining (A). The locations of these glomeruli are confirmed by the addition of NCAM (green) and VGlut 2 (red) to the DRAQ5 staining (blue) (B) of the same image. However, in the human OB, the glomeruli are not regularly and compactly organized, and the PG cells do not form clear circumferential densities around their borders, making identification with DRAQ5 staining alone difficult (C,E). However, the addition of NCAM and VGlut2 in these same images is able to define the locations of these glomeruli clearly (D,F). In addition to highlighting the degree of variability in the size, shape, and distribution of the glomeruli, this also allows for the better characterization of the relationship between glomeruli and surrounding PG cells, which is also demonstrated to be more variable than compared to mice. EPL, external plexiform layer; GL, glomerular layer. Scale bar in F is 50 μm for A-F.

Figure 6

staining processes can be visualized extending into the glomeruli, representing the PG cell axons that synapse with the dendrites of projection neurons as well as occasionally with the axons of the OSNs. Though not pictured, rare TH+ cells can also be seen in the deeper layers in the mouse OB.

Figure 7B shows TH staining in the human at low magnification. Again, cell bodies are seen in the GL. While many are closely approximating glomeruli, some are more isolated within the GL. This image also depicts several TH+ cell bodies within the GCL. By observation, the percentage of cells in the GL that are TH+ appears to be lower than in the mouse. At higher magnification (Figure 7C) the large-bodied TH+ cells are seen surrounding the glomerulus, with processes extending into the glomerulus; areas of co-localization are present between these TH+ processes and NCAM+ OSN axons.

Calretinin+ PG cells can be seen in the mouse OB surrounding glomeruli defined by VGlut2 staining (Figure 7D). These cells are more abundant than the dopaminergic cells, and have smaller cell bodies. Calretinin+ cells are also found with relatively high frequency in the deeper layers of the OB. Calretinin stains processes very strongly, and even at this low magnification, calretinin+ staining can be seen within the glomeruli.

In the human, the calretinin+ PG cells are also more frequent than dopaminergic PG cells (Figure 7E). These cells seem to be more closely approximated with glomeruli than TH+ cells, only rare calretinin+ cells are seen in the GL that are not directly approximated with a glomerulus. Again, the strongly staining processes are seen in the glomeruli, even at the low magnification the co-localization between VGlut2 and calretinin can be seen. This is better pictured in the higher magnification image (Figure 7F). As in the mouse, these calretinin+ cells have smaller cell bodies and are seen in

Figure 7: Molecular Phenotypes and Distributions of Periglomerular Cells

Large-bodied TH+ (red) cells are seen surrounding glomeruli, as defined by NCAM (green), in the mouse OB (A). A similar distribution is seen in the human OB (B). A closer look at a single glomerulus in the human OB (C) demonstrates the large-bodied TH+ cells surrounding and sending processes into the glomerulus. Calretinin+ (green) cells have smaller cell bodies, and are seen in a mouse OB even more abundantly staining PG cells around glomeruli, in this case identified with VGlut2 (red) (D). A similar distribution is seen in the human OB, with many calretinin+ PG cells surrounding the unevenly distributed glomeruli, into which they are extending many darkly stained processes. This is seen in greater detail in F, which also demonstrates more clearly the smaller cell bodies of calretinin+ cells. The GAD65/67 antibody stains processes very darkly, and the EPL is dense with these GAD65/67+ (green) processes in the mouse OB (G), however abundant GAD65/67 cell bodies can be seen surrounding the VGlut2+ (red) glomeruli. The EPL is similarly dense with processes in the human OB (H), and processes can also be seen around the glomeruli in the GL. At a higher magnification in the GL of a human OB (I), fewer cell bodies are obviously GAD65/67+ as compared to in the mouse, some of the more clearly stained cells are identified with arrows. However, this is likely due to the poor staining quality of the antibody, in fact most PG cells have some degree of co-localization with GAD65/67. The quantities and distributions of these classes of cells are therefore seen to be similar in mice and humans, suggesting similarities between local processing of olfactory signals at this level. DRAQ5 staining of nuclei is blue in all images. EPL, external plexiform layer; GL, glomerular layer. Scale bars are 50 μm in A, D, G, 100 μm in B, E, H, and 25 μm in C, F, I.

Figure 7

fairly high density.

GABAergic cells are characterized by staining with an antibody against GAD65/GAD67. Glomeruli were defined with VGlut2. This antibody has relatively poor staining of cell bodies while staining of the processes is very strong. A large amount of staining of these processes is seen in the EPL of the mouse OB, with cell bodies pictured very densely packed around glomeruli in the GL (Figure 7G). These cells have medium-sized cell bodies, and are the most abundant of the PG cells, which is consistent with the finding is this image.

In the human OB, GAD65/67+ staining is particularly prominent in the EPL as well (Figure 7H). Less densely stained processes can be seen around the glomeruli, however no distinct cell bodies can be identified at this low magnification. At higher magnification, several VGlut2+ glomeruli can be seen surrounded by GAD65/67+ cells (Figure 7I). Due to the poor cell body staining, GABAergic cells are difficult to clearly identify; several of the more prominently stained cells are labeled with an arrow. However, upon close examination, many of the DRAQ5 stained nuclei surrounding these glomeruli demonstrate small but visible co-localization with GAD65/67. This, combined with the clearly identified GAD65/67+ processes surrounding and innervating the glomeruli confirms the localization of GABAergic PG cells in the GL. Unfortunately the staining quality prohibits further speculation about the relative quantities of these cells as compared to rodents.

In summary, all three types of PG cells, including dopaminergic cells, GABAergic cells, and cells expressing calcium binding proteins, are present in the GL of humans. They appear to be present in the same relative quantifications as in mice, with

the exception of the GABAergic cells in which no conclusions could be made due to limitations in the immunohistochemical staining in the human OB. Finally, all three types of cells appear to be distributed in the OB similarly as in mice, and most notably in the GL they are all closely surrounding the glomeruli. While not conclusive, these histological descriptions are consistent with the idea that the same types of synaptic circuits between OSNs, mitral/tufted cells, and PG cells are also present in the human and may play a role in the initial processing of sensory input into the OB. One important observation, however, is that in the GL in the human OB there does not appear to be homogeneity in the distribution of PG cells relative to glomeruli as there does in rodents, and there are large areas within the GL without glomeruli that are still dense with PG cells. Interestingly, while the PG cells directly surrounding glomeruli in the human were stained consistently with the TH, GAD65/67, and calretinin antibodies, only rare PG cells in these intervening areas were stained. It is unclear what the nature of the cells in these areas are or how they may contribute to the synaptic organization in this layer.

**The distribution of glomeruli in the OB is not preserved between specimens.**

Rodents have very specific odor maps, with stereotyped locations of glomeruli for each specific OR that are conserved from animal to animal. For each OR, there are usually two, though sometimes one or a few, glomerular targets, one located on the medial wall of the OB and the second located on the lateral wall. These precise odor maps in mice are composed of regularly-spaced glomeruli evenly distributed almost two-dimensionally in a sheet around the surface of the OB.

To broadly look at the general distribution of glomeruli in the human OB, slices were co-stained with NCAM and VGlut2. By looking at representative low magnification slices arranged rostrally to caudally throughout the length of one of the OB's, HOB1 (Figure 8), it can be seen that the distribution of glomeruli around the OB is very different in humans than in mice. While in mice they are regularly spaced in a thin, circumferential layer, in the human OB glomeruli are often clustered in groups. In the images presented, these clusters are represented by thick NCAM+ axon densities that branch out into large groups of VGlut2+ glomeruli that are often overlapping and that extend the width of the GL deeper into the OB. There are several of these clusters that can be seen throughout the representative slices. They are in different areas of the circumference, though the overall distribution of glomeruli in this OB is lateralized to about half of the overall circumference. This localization remains consistent throughout the entire length of the OB until it tapers off at the most caudal end. Outside of the clusters, the other areas along the circumference seem to have a more uniform thickness, however even here the distribution of glomeruli is not evenly spaced as they are often clumped together or completely absent.

This non-circumferential distribution was seen in all OBs examined, however the extent to which the glomeruli were limited to one area was variable from specimen to specimen, as was the particular area to which they were localized. Representative low magnification slices from several different human OBs, taken from mid-OB except where noted, demonstrate the variety in the distribution around the OB (Figure 9). HOB 20 (Figure 9A) and HOB 6 (Figure 9B) have a similar type of lateralization as was seen in HOB 1 (Figure 8). However HOB 20 has a thick NCAM+ ONL and glomerular layer as

Figure 8: Limited Circumferential Localization of Glomeruli

The localization of glomeruli around the circumference of a single OB, HOB 1, through its rostral-caudal length is shown starting rostrally in A and ending caudally in H. Glomeruli are identified by co-localization of NCAM (green) and VGlut2 (red). Nuclear staining is with DAPI (blue). Clusters of glomeruli can be seen in the GL with intervening areas that are more sparse in glomerular density, unlike the mouse OB in which the glomeruli are regularly spaced, and usually only 1-2 glomeruli thick. The thickness of the GL shown here is variable amongst slices, but the non-circumferential lateralization of glomeruli remains consistent throughout the entire length. Scale bar in H is 500 μm for A-H.

Figure 8:

in HOB 1, while HOB 6 has a very thin ONL and sparse glomeruli. In several of the OBs including HOB 20, the glomeruli could be seen completely circumferentially in the most rostral slices (Figure 9C) until becoming more localized in their distribution. HOB 15 had a particularly large distribution, almost completely circumferential (Figure 9D). HOB 7 and HOB 2 had distributions that each covered about half of the circumference, however, each was distinctive in their localization; in HOB 7 glomeruli were lateralized side-to-side, while in HOB 2 they were lateralized up-to-down (Figure 9E,F). These images highlight the broad spectrum of variations in the glomerular pattern, both in the amount or the circumference surrounded by glomeruli as well as in the localization within the circumference. This variability demonstrates that, as opposed to in rodents, it is unlikely that humans have a stereotypical spatial targeting within the OB for axons expressing specific ORs that is identical amongst all members of the species.

To try to explore this further and examine the localization of specific OR glomeruli, slices throughout the OB were stained immunohistochemically with probes for several ORs (see Table 2). These included antibodies targeting mOR50 (Mori), mOR28 (Sakano), mOR28 (Axel), mOR256-17 (Breer), and mOR262 (Breer). ORs are expressed in the axons of OSNs, so successful staining should demonstrate OR+ axons surrounding the OB in the ONL, as well as OR+ glomeruli representing the glomerular targets for the specific OR. Although these antibodies were developed against mouse ORs, it was hoped that they might recognize human ORs as well, since as previously discussed, there is a high though variable degree of sequence homology between OR genes from the two species. By seeing OR+ glomeruli, this study might be able to confirm molecular specificity of glomerular inputs in the human. In addition, it would describe the

Figure 9: Variability in Glomerular Localizations

The broad range in glomerular distributions is shown in OBs from several different donors. Glomeruli are identified by co-localization between NCAM (green) and VGlut2 (red), nuclear staining is with DAPI (blue). The most rostral tip of several of the OBs, such as HOB 20, exhibited glomeruli around the entire circumference of the OB (C), after which they usually became far more localized. Other than C, all images in this figure are taken from slices that are mid-OB. The most common localization is shown in HOB 20 and HOB 6 (A,B), however the laminar organization is very different between these two OBs, with HOB 20 having a very thick GL and dense glomeruli, and HOB 6 having a very thin GL and sparse glomeruli. In HOB 15 (D), glomeruli are seen surrounding almost the entire OB, with some glomeruli extending out of the GL deeper into the bulb. In HOB 7 and HOB 2 (E,F) glomeruli are seen surrounding about half of the circumference, however in HOB 7 the lateralization is side-to side while in HOB 2 the lateralization is top-to-bottom. While rodents have a very stereotypical odor map that is preserved from animal to animal, because of the broad ranges in the distributions of glomeruli around the OB, it is unlikely that this is the case in humans. Scale bar in H is 500 μm for A-F.

Figure 9:

Figure 10: Single Odorant Receptor Expression in the Olfactory Nerve Layer

In order to examine the distribution of single-OR axons and glomeruli, human OB slices were stained with a mix of antibodies developed against individual mouse ORs, which included mOR50 (Mori), mOR28 (Sakano), mOR28 (Axel), mOR257-17 (Breer), and mOR262 (Breer). Due to the high degree of homology between human and mouse OR sequences, it was hoped that these antibodies would recognize human ORs as well. Evidence of positively stained axons with characteristic knobby densities are present in ONL (A,B), however no glomeruli were visualized. ONL, olfactory nerve layer. Scale bars are 10 μm.

Figure 10:

distribution of specific OR glomeruli in the bulb, and might be able to provide further insight into the numbers of glomeruli targeted by OSN axons for each OR.

Unfortunately, while there was no conclusive evidence of OR+ glomerular staining, there was suggestive OR+ staining in the ONL (Figure 10A,B), which implies that the OR antibodies developed against mouse sequences are able to cross-react with humans. However, due to the lack of identified glomerular staining, no further insight was provided regarding homotypic axon convergence or molecular specificity of the glomeruli.

**Humans have many more glomeruli than models of olfactory processing established in rodent models would predict.**

In rodent models, the total number of glomeruli in the olfactory bulb is a direct reflection of the number of intact ORs expressed in the olfactory epithelium. In both rats and mice, there are twice as many glomeruli as there are OR genes. By evaluating the number of glomeruli in humans, it can be determined whether principles of axon convergence occur in the human as they do in the rodent. We used co-localization of NCAM and VGlut2 to define glomeruli. In addition to counting the glomeruli from representative slices throughout each OB, each glomerulus was circled to measure its two-way diameter and area.

Table 3 shows the final results of this study for the seven OBs that were examined. The average number of glomeruli was 5568 ± 830 (mean ± S.E.M.), many times more than the 700 predicted based on the estimated 350 intact OR genes in humans. In addition, as demonstrated by the large standard error, there was a huge range; the

smallest number counted was 2975 from HOB 6, and the highest 9325 from HOB 20. While based on a small n, these huge counts are many times higher that predicted, and seem to clearly disprove the hypothesis that there would be about 700 glomeruli. This implies that through evolution, in the time since primate-rodent divergence, humans have developed different principles of axon convergence and glomerular targeting, which in turn may reflect a variation in the way odor is processed at this level.

The glomerular diameters were calculated based on two-way measurements across the circled glomeruli, with an average diameter of 59.6 ± 1.4 μm. However, this standard error represents the range of glomerular diameters averaged from each of the OBs. In fact the actual range of diameters was much larger than this, with the smallest measured glomerulus having a diameter of 15.8 μm and the largest measured glomerulus having a diameter of 185 μm. In addition to this huge variation in size, these human OB glomeruli exhibited extreme variations in shape, with often the two axis measurements being extremely disparate for a given glomerulus. However, the two diameters defining the individual glomerular shapes averaged out to within only a few microns for each of the OBs. This indicates that the average two-dimensional shape of glomeruli in the human is circular despite the huge variability between individual glomeruli, and therefore the average structural shape is spherical.

The average length of the OBs, defined by the rostral-caudal distance between the first and last coronal slices that contained glomeruli, was 9.52 ± 0.49 mm. The mean area of the coronal cross-section of the OBs was 5.52 ± 0.32 $mm^2$, and was seen within an individual OB to remain fairly consistent throughout the length except at the most rostral tip, suggesting that the three-dimensional shape of the human OB is more like a cylinder

**Table 3: Olfactory Bulb Analysis Data**

| ID | OB Length (mm) | Average Area of Slice ($mm^2$) | Total OB Volume ($mm^3$) | Average Glomerular Diameter (µm) | Total Glomeruli (corrected) |
|---|---|---|---|---|---|
| HOB 1 | 7.05 | 4.30 | 30.32 | 65.13 | 4595 |
| HOB 2 | 9.60 | 5.99 | 57.50 | 55.15 | 4184 |
| HOB 6 | 9.50 | 5.73 | 54.44 | 56.17 | 2975 |
| HOB 7 | 11.35 | 4.39 | 49.83 | 59.29 | 6530 |
| HOB 15 | 9.40 | 6.28 | 59.03 | 57.44 | 7150 |
| HOB 16 | 9.35 | 5.64 | 52.73 | 62.68 | 4221 |
| HOB 20 | 10.40 | 6.28 | 65.31 | 61.37 | 9325 |
| **Mean ± S.E.M.** | **9.52 ± 0.49** | **5.52 ± 0.32** | **52.74 ± 4.19** | **59.60 ± 1.41** | **5568 ± 830** |

than like an elliptoid. Using this assumption of a cylindrical shape, the mean volume of the OB was calculated to be 52.74 ± 4.19 $mm^3$. Previous MRI imaging has measured the volume of OBs from patients with normal olfaction to be from 59 $mm^3$ to 119 $mm^3$ (79). While this is a bit higher than the volumes seen in our study, this likely reflects the fact that we used a functional definition of volume based on the presence of glomeruli, which is a clear way of identifying the transition between OB and LOT, and which was seen to occur before the anatomic narrowing of OB to LOT was clearly visible.

**There is no relationship between number of glomeruli in human OBs and either age, gender, or OB size.**

There was a huge range in the number of glomeruli counted from these seven human OBs. To look for possible associated factors, the relationship between the number of glomeruli and the age of the donor, the gender of the donor, the average diameter of the glomeruli, and the size of his/her OB was examined. A previous study described an inverse relationship between age and number of glomeruli (80); however, we did not find a correlation between the two variables as measured with a linear regression test ($p=0.39$) (Figure 11A). When placed in two age categories of "young" (age less than 50 years old) and "elderly" (age greater than 50 years old), the average number of glomeruli in OBs from the the "young" group was 6960 ± 2365 ($n=2$), while in the "elderly" group it was 5012 ± 785 ($n=5$) (Figure 11B). While there are fewer glomeruli in OBs from the "elderly" group, using an unpaired t-test this difference was not found to be significant ($p=0.33$). These findings are different from those of Meisami's group, and possible explanations for these discordant conclusions are described in more detail below.

In addition, the data was grouped by gender, with the average number of glomeruli in OBs from female donors calculated as 6047 ± 1643 (n=3), and from male donors, 5210 ± 981 (n=4) (Figure 11C). There is no statistical significance between these groups either (p=0.66). Finally, there was no correlation between glomerular number and average glomerular size (p=0.71) (Figure 11D), or between glomerular number and OB volume (p=0.31) (Figure 11E).

Based on the small n of 7, there is not a large amount of data to support the power of these comparisons. Nevertheless, the only relationships that begin to even support a trend are between number of glomeruli and OB volume, as well as number of glomeruli and age. Only by increasing the number of human OB samples can it be seen whether true relationships develop in these cases. However, it is most likely that the main sources of variation in the number of glomeruli between different individuals lie outside the parameters measured here.

Figure 11: Relationships Between Total Glomeruli and Age, Gender, Glomerular Diameter, and Olfactory Bulb Volume

No significant relationship were found between total glomeruli and donor age ($p=0.39$) (A). There was a trend towards decreasing numbers of glomeruli with increasing age, however even when split into two groups of "young" (age less than 50 years old) and "elderly" (age greater than 50 years old), the difference was not significant ($p=0.33$). The average number of glomeruli in OBs from the "young" group was $6960 \pm 2365$ ($n=2$), while in the "elderly" group it was $5012 \pm 785$ ($n=5$) (B). When grouped by gender, the mean number of glomeruli in OBs from female donors was $6047 \pm 1643$ ($n=3$), and from male donors, $5210 \pm 981$ ($n=4$), which was also not significant ($p=0.66$) (C). Finally, there was no correlation between glomerular number and average glomerular size ($p=0.71$) (D), or between glomerular number and OB volume ($p=0.31$) (E). Linear regression tests were performed to look for significance in A, D, E. Unpaired t-tests were performed for the two-group comparisons in B, C. There were no significant differences in variance for either of these comparisons.

Figure 11:

# DISCUSSION

## The organization of the human OB is preserved, but is less precise than in rodents.

Previous work to describe the human OB histologically was largely undertaken before the availability of our current molecular reagents or insights into the molecular organization of the olfactory bulb. Early immunohistochemical analyses were able to define the presence of synapses and several classes of PG cells, such as those examined here, within the human OB (81-86), but at the time there was very little understanding of the intricate neuronal circuits involved in the OB. In fact, most of these studies were performed before the odorant receptor had even been identified by Buck and Axel in 1991 (8), the discovery that pioneered the understanding of molecular specificity and glomerular odor maps.

Much of the recent work done with the human OB tissue is related to clinical interests in how different diseases, especially neurodegenerative diseases, manifest in the OB. These studies, summarized below, provide little insight into the actual organization of the human OB that is relevant for understanding how it compares to that of the rodent models in which the principles of odor processing were first discovered. Therefore, this current work, while by no means comprehensive, is novel in providing an initial look at the organization of the human OB with regard to these important principles.

The qualitative evaluation of the human OB presented here evaluates several components of OB organization, including the preservation of laminar layers, the presence and distribution of new OSN axons, the compartmentalization of glomeruli, the organization and molecular identity of PG cells in the GL, and finally the distribution of

glomeruli around the OB. Because so much that is now understood about odor processing in rodents has a defined anatomical basis, such anatomical studies of the human OB can provide insight into similarities and differences between odor processing in these two species. Based on these analyses, there are several lines of evidence that support the preservation of OB organization in humans, while several others highlight potential differences between the two.

Definitive evidence is presented here that, as in rodents, OSNs are regenerated throughout the entire adult life of humans, and that these new axons integrate into established glomeruli in a manner typical to what is seen in rodents. This dynamic nature of the sensory input requires the coordination of factors related to axon growth, guidance, and integration within synaptic networks in order to maintain the complex organization within the OB. Any failures in these processes that detract from OB organization may have functional consequences in the efficiency and accuracy of odor processing. While not evaluated here, there is preliminary evidence that in humans, as in rodents, new PG cells and granule cells are also regenerated (87). If true, this would add additional complexity to the dynamic nature of the organization of the human OB.

Olfactory function is known to decline steadily after the age of 40 (88), with a prevalence of olfactory dysfunction in elderly people as high as 70% (89). This occurs despite the ability to regenerate OSNs. Therefore, a potential explanation for this age related decline in olfactory function is in fact an accumulation of organizational errors due to inaccuracies in axon targeting and synaptic integration of these newly generated neurons over time. Due to the complicated integration of sensory information between cells from all layers including PG cells in the GL, mitral and tufted cells, and granule

cells from the GCL, such errors might have organizational consequences throughout the entire OB. In possible support of this hypothesis, there appeared to be qualitative differences between the OBs from younger donors compared to older donors, with older donors demonstrating strikingly less clear laminations in the OB structures. Contributing to this apparent decline in the clear demarcations between cell types and synapses is the presence of glomerular structures that extend beyond the GL into the EPL, observations that were also noted indirectly by several groups of clinician investigators (90, 91). Therefore, while laminar organization is a concept that is preserved in the human OB, because it is such a dynamic structure, perhaps over the long duration of the lifetime of a human inaccuracies accumulate that have anatomic and functional consequences.

At the level of the glomerulus, there were several observations in this study that suggest that the local synaptic regulation of incoming sensory signals is preserved in humans. The PG cells have molecularly defined identities that are similar to that described in rodents with similar distributions surrounding the glomeruli. Additionally, glomeruli have segregated axonal and dendritic compartments that represent, respectively, the axodendritic synapses between OSNs and mitral/tufted cells, and dendrodendritic synapses involved in local PG cell circuits. Despite these important and revealing similarities, there are general organizational differences in the GL between the two species that bear discussion. While the GL in mice is composed entirely of glomeruli with surrounding PG cell densities, as seen in this study, the GL in human OBs contains clusters of glomeruli without the surrounding PG cell densities that are seen in mice, as well as areas of PG cells that have no glomeruli.

The clusters of glomeruli with a relative paucity of surrounding PG cells may be another example of changes in the structural organization of the human OB that may have functional consequences and contribute to the evidence that humans have a weaker sense of smell. PG cells play an important role in local feedback circuits in the GL that may serve to increase the sensory signal as well as potentially inhibit signals from areas of input represent similar but different odors. Therefore, efficiency at this level may be reflected in low odor detection thresholds as well as the ability to discriminate between different odors. Accordingly, having low as well as variable ratios of PG cells to glomeruli, it can be seen that there may be functional consequences in these measures of olfaction.

However, perhaps glomeruli do not necessarily need to be abutting the PG cells with which they form synapses. There are many areas dense in PG cells without proximal glomeruli. PG cells are able to extend long processes, as seen in the TH, calretinin, and GAD65/67 staining presented in this study. Therefore, even without the physical proximity it is possible that these PG cells are involved in local circuits from a greater distance, and the density of PG cells seen surrounding glomeruli in mice is simply to maximize the efficiency of a limited GL space, rather than because it has any functional necessity. However, an argument against this hypothesis is that very few of the many PG cells that were not directly surrounding glomeruli in the human OB were actually labeled with any of the PG marker probes, which are also indications of functionality. In mice, over 80% of PG cells are labeled with any one or more of these probes (70). Perhaps PG cells in the human are participatory but preferentially belong to a category of cells not identified by these markers. Or contrary, these are non-functional

cells that are either remnants of earlier synaptic circuits that no longer exist, or else they could be newer generated PG cells that never found a target synaptic circuit in which to integrate. While the role of these cells is unclear, they are potentially another source of evidence of deviation from the organization of the OB as defined in rodents.

**Large numbers of glomeruli in the human OB suggest a difference in the way that OSN axons find their glomerular targets as compared to rodents.**

One of the most important principles of odor processing in the OB that has been well established in rodents is the idea of molecular specificity; that OSN axons entering the OB form homotypic axon bundles within the ONL with other axons from OSNs expressing identical ORs before penetrating into the OB into their target glomerulus. This is a fundamental principal that identifies glomeruli as functional units and allows for efficient modulation of sensory input at this very first level of odor processing. However, this molecular specificity has not yet been confirmed in the human OB. One simple way to begin to address this is to see whether the numerical relationship between ORs and glomeruli that the principle of molecular specificity establishes is maintained in humans.

Based on rodent models, it was predicted that the human OB would contain ~700 glomeruli, twice the ~350 OR genes that have been predicted based on genomic mining studies. However, counting the glomeruli from seven OBs demonstrated the actual number of glomeruli to range from 2975 to 9325, with an average of 5569. By defining glomeruli by co-localization with NCAM and VGlut2, a synaptic marker that is specific to glomeruli, this study was able to more rigorously delineate glomeruli for counting and measuring than a previous study done by Meisami et al. that attempted to look at numbers

of glomeruli in the human OB (80). This group looked at Nissl staining, and therefore relied on the PG cells to outline glomeruli. As demonstrated here, PG cells are rarely arranged in circumferential densities around regularly sized and shaped glomeruli, which are often clustered together. While the Meisami group found about 8000 glomeruli in young people, which falls within the range identified in this study, they also found a linear decline in glomeruli with age, with only about 2000 glomeruli in the oldest group. While we did not see any clear indication that there was a correlation between age and number of glomeruli, we did see qualitative differences between the OBs from older donors compared to younger donors, with more poorly defined laminar layers and larger variability in the shapes and distributions of glomeruli. Therefore, the fewer number of glomeruli counted by Meisami et al. in this older group is likely a consequence of the decreased organization in the GL, making identification of glomeruli based on PG cells even more difficult.

Using a synaptic marker that is specific to glomeruli, VGlut2, is an unequivocal method of identification. However, there were still many difficulties encountered during the counting process. As can be seen from the images of the human (Figure 8), glomeruli are clustered together, often overlapping within one 50 μm slice. This difficulty was made particularly complicated by the fact that glomeruli in human often have atypical shapes, unlike the almost universal spherical shape seen in rodents. Therefore, with overlapping glomeruli with unusual shapes, it was often hard to define exact borders or to distinguish closely approximated glomeruli from one another. In addition, the compartmentalization within glomeruli established earlier (Figure 5) is also seen with VGlut2 staining, and was sometimes very prominent. This made distinguishing

prominent compartmentalizations within a single glomerulus from multiple smaller clustered glomeruli more difficult. Finally, using the Abercrombie extrapolation might have also introduced some small error into the final calculation. This formula corrects for the idea that three-dimensional objects are often split between slices and would therefore be double-counted. The calculation makes the assumption that the objects counted are spherical, which is demonstrated here to not be the case. However, despite the variability amongst individual glomeruli that was encountered, the two dimensional diameter measurements did average out to very close to a circle in every OB. Lastly, glomeruli were only counted in a total of seven human olfactory bulbs. This is a very small n, and it is likely that the final numbers of glomeruli are not perfectly representative of the population; despite this, the entire range varies significantly from the predicted value, making it extremely unlikely for the principles of axon convergence and OB organization that have been defined in rodents to be identical in humans.

Despite these minor difficulties, this was an extremely rigorous evaluation of the number of glomeruli with truly unexpected results. Why are there so many glomeruli in the human? Does this deviation in organization also imply that there has been a loss of molecular specificity within the human OB? Not necessarily. Perhaps in humans the anatomically based definition of a glomerulus is different, and there has been a fragmentation of the prototypical spherical glomeruli into smaller units. The average glomerular size in humans is ~60 μm, smaller than the 85-100 μm in mice, despite having a larger OB. Perhaps as the size of the glomerulus becomes larger with accumulating axonal inputs, there is a decrease in efficiency of regulation with the surrounding periglomerular cells, resulting in a fragmentation of the glomerulus into smaller units.

The occurrence of sometimes discrete clusters of glomeruli in humans is something that is not seen in rodents, where the glomeruli are almost linearly arranged around the OB. So perhaps what is a discrete sphere in a mouse, is, in humans, a cluster of glomerular units representing a single OR. In this way, not only would there be preservation of homotypic convergence, but there could still be the numerically retained relationship between ORs and glomerular target areas, if there were about 700 of these clusters in the OB. However, even if this were the case, the high variability in circumferential glomerular localization (Figure 9) makes the idea of odor maps that are conserved between individuals extremely unlikely.

Another hypothesis addressing these high glomerular counts in which molecular specificity and organization is maintained is related to the potential emergence of frequent polymorphisms within human olfactory receptor genes. Linardopoulou et al. (92) identified up to 5 amino acid differences in copies of the same OR gene from different individuals. Therefore, because of the unique property of OR gene expression in which only one allele is expressed in any given OSN, polymorphisms in one allele of a certain OR would lead to two distinct cell populations within the group of OSNs that express that OR. It has been additionally discovered that even small changes to the amino acid sequence of an OR can affect the targeting of axons to their glomerulus (93). Taken together, this evidence indicates that there may be distinct glomeruli representing two different versions of a single OR gene. While the presence of polymorphisms alone is unlikely to account for the large numbers of glomeruli seen in human OBs if all other principles of axon targeting are upheld, this presents an interesting point that certainly

adds complexity to the human olfactory system and would indeed account for larger numbers of glomeruli than predicted.

Another potential explanation for the large number of glomeruli is that instead of each OSN axon that expresses any given OR targeting one of two possible glomeruli in the OB, there are many more potential glomerular targets. In this hypothesis, while there is maintenance of molecular specificity in which all axons converging on a single glomerulus express the same OR, there is a loss in the organizational efficiency seen in rodent OBs. For 350 human ORs, with about 5500 glomeruli, that would mean that there would be an average of 16 potential glomerular targets per OR. Part of the elegance of the rodent model is that having only two glomeruli per OR efficiently organizes olfactory input in the central nervous system immediately at the first stage of processing. Therefore, by having so many glomeruli per OB, it can be perceived as a loss in efficiency at this level. As glomeruli are considered functional units in the process of integrating sensory input, such a dispersion of input units would require compensation through more complex local organization in the PG cells and granule cells, or at higher levels in the cortex. If this compensation does not occur in this model, it is possible that such a loss in OB odor map organization may play a role in the human's relatively poor olfactory sense.

The final potential explanation for such a large number of glomeruli in the human OB is that odor processing through evolution has diverged so much from rodents that there is no longer molecular specificity to glomerular targets; axons entering any given glomerulus are no longer from OSNs that express the same OR. With mixed input, all hypotheses about glomeruli acting as functional units, while still likely in rodents, would

no longer be possible in humans. For example it would be impossible for a glomerulus to enhance the signal-to-noise ratio if the input it was receiving was not homogeneous. In this case it would certainly be necessary for sensory integration and odor processing to take place at higher centers, and it certainly implies a loss in efficiency, which would also explain humans' decreased capacity for odor detection and discrimination.

So which of these explanations is correct? Immunohistochemical staining with antibodies to specific ORs would in fact discriminate between many of these hypotheses. As part of this study individual mOR antibodies were used to attempt to answer this question, however the staining was not successful in that it did not identify any glomeruli in the OB. It is likely that the antibodies, which are specific to mouse OBs, were not specific enough to recognize the human OR proteins, or else the staining was localized in glomeruli that weren't represented on any of the slices examined. If a specific OR antibody was developed that successfully stained glomeruli, the results would be extremely informative. Large clusters of fluorescent glomeruli would indicate the fragmentation of glomerular spheres into smaller units. Many glomeruli stained throughout the bulb would indicate that there are more than two potential glomerular targets per OR. Finally, staining of only a part of a glomerulus would indicate a loss of molecularly specific glomerular input.

**High variability in the number of glomeruli indicates that there are many factors that can affect the organization of the OB.**

In addition to the high number of glomeruli counted in each OB, another mystery is the extreme variability in the number of glomeruli between different specimens,

ranging from 2975 to 9325. This is not seen in rodents, in which there is a stereotypical odor map including consistent numbers of glomeruli with specific localizations within the OB. What potential factors could be influencing the number of glomeruli? Glomeruli are composed of synaptic units between OSNs and PG cells, both of which are regenerated throughout life, as well as projection neurons, the mitral and tufted cells, which are stable and not regenerated. The sensory input comes in through the OSNs, without which the glomeruli would not exist. Therefore, any significant loss of OSNs might disrupt the synapses and result in a loss of glomeruli.

OSNs have their cell bodies in the olfactory epithelium (OE) in the back of the nose. This location in the nose is efficient for detecting odors, however for the same reason it is a sensitive area of tissue that is environmentally exposed to many pathogens and toxins that are inhaled with the odorants. While most people experience temporary olfactory dysfunction with minor upper respiratory infections, this is mostly due to the congestion in the nasal passages that is obstructive to sniffing, and normal olfactory function returns as soon as nasal congestion clears. However, people with more significant sinus infections do experience post-infectious parosmia (94), likely related to a disruption OSN integrity and normal OSN replication. In addition, patients who undergo chemotherapy or radiation therapy to the head or neck region also commonly experience parosmia, also likely due to the disruption of OSN replication. It is even possible in these cases that replication of PG cell precursors is affected as well, which might affect central processing of smell, adding another source of disturbance to olfactory processing in these patients.

Based on these lines of clinical evidence, it is appears that disruption of OSNs leads to symptomatic variation in olfaction, however there is limited anatomical evidence of how these changes are manifest in the human OB. There is MRI imaging evidence that patients with post-traumatic and post-infectious olfactory dysfunction have smaller OB volumes (79, 94) than controls. While there was no significant relationship between OB size and number of glomeruli our study, this was one of the relationships in which there was a positive trend, suggesting that with larger power to the study this relationship may become significant. At the molecular and cellular level, a single previous study attempted to look at the affect of cancer treatment, including chemotherapy and radiation therapy of the head and neck, on OSN regeneration and number of glomeruli in the human OB (95) in post-mortem donors. They noticed qualitatively less regeneration based on GAP43 staining compared with controls, but did not note differences in glomerular numbers. However, this evaluation of glomeruli was also qualitative, based on a rating scale, using cellular staining to identify the gloerular structures. As demonstrated earlier, qualitative evaluations of glomeruli, especially when based on PG cell distribution, are unreliable predictors of actual glomerular quantifications.

Interestingly, the donor with the fewest number of glomeruli in our study was a patient who had acute myelogenous leukemia who had undergone chemotherapy. From the limited medical history that was available, it is unclear at what time point relative to death he underwent this regimen. It is also unclear whether he had symptomatic olfactory dysfunction. None of the donors had reported olfactory dysfunction, however several studies documented that olfactory function is rarely addressed in medical practice,

and in fact most patients with olfactory dysfunction are unaware of their condition (89, 96).

So what potential variations could be playing a role in the differences between glomerular numbers for the other OBs evaluated in this study? Based on the small number of OB specimens counted, there was no statistical significance between the numbers of glomeruli and gender of the donor, nor was there a correlation between number of glomeruli and age; however age was one of the comparisons that demonstrated a trend that could potentially be significant with greater power to the study. It does make logical sense that the olfactory system of an elderly person would be more likely to have accumulated a lifetime of infectious and toxic exposures. Age, therefore, can be seen as an indirect measure of these variables, which have been described earlier to likely play a role in OSN integrity and regeneration, and possibly affect the number of glomeruli in the OB. None of the available medical history for the OB donors provided meaningful information regarding potential sources of OSN dysfunction that could lead to changes in the numbers of glomeruli through this predicted mechanism. However, it is likely that potentially relevant aspects of these patients' clinical histories were either unrecognized or unreported, so their absence certainly does not oppose this explanation. The best way to explore this idea further would be to select OBs for evaluation from post-mortem donors who had documented olfactory dysfunction due to trauma, infection, or chemical exposures, as well as the OBs from normal controls with actual documentation of normal olfactory function, and to compare these groups with relation to glomerular number and overall OB organization.

Finally, as an attempt to study "normal" human OB architecture, only OBs that were from donors without neurodegenerative disorders were evaluated to prevent obscuring of the normal and cellular and synaptic organization. There are documented changes in OB architecture from patients with neurodegenerative disease, and in fact most of the current studies of OB organization are currently focused around clinical interests in these diseases, including most prominently Alzheimer's disease (AD) and Parkinson's disease (PD). In both AD and more prominently in PD, olfactory dysfunction is one of the earliest symptoms (97-100), and some have even proposed to use olfactory testing to screen for these diseases in susceptible patient groups before symptomatic onset (101). In AD, neurofibrillary tangles and senile plaques are found in 100% of the OBs of patients during post-mortem analysis (91), while in PD Lewy bodies are also found in 100% of patients OBs (102). An interesting correlation would be to evaluate the OBs from donors with known AD and PD to see how the architectural changes affiliated with these diseases processes affect measures of olfactory processing evaluated in this study.

**So is there histologic evidence to explain a relatively inferior olfactory system?**

There are several measures used to describe the acuity of the olfactory system. One is odor threshold, or detection, another is odor identification, and the third is odor discrimination. Based on genomic mining studies, it is already known that humans have only about one-third the number of OR genes as compared to mice. With fewer ORs, it is likely that humans are not able to recognize as many molecular odorants as mice and

other mammalian species, decreasing our ability to identify odors. In addition, weaker odorant binding to the ORs that are expressed, as well as binding of individual odorants to fewer numbers of ORs, could result both in higher odor thresholds as well as poorer odor discrimination. Therefore, simply looking at the genetics, humans already have a basis for olfactory inferiority in all measures.

Within the central nervous system, odor identification occurs in the olfactory cortex; however in order to correctly identify an odor, the sensory information must be properly integrated within the OB before entering the LOT. In addition, both odor threshold and odor discrimination are dependent upon local circuits within the OB. All of these processes require coordination of input from the central nervous system, sensory input from OSNs, as well as local circuit feedback and regulation through the PG cells and granule cells. Therefore, disruption of the cellular and synaptic organization in the OB would also detract from the human's ability to detect, discriminate, and identify odors. While this study was able to confirm many principles of local odor processing in the OB, it identified several differences between humans and rodents at this level that may in fact support the idea that central processing is in fact less efficient, differences therefore that might begin to explain our poor sense of olfaction. Most importantly, through simple quantification of glomeruli, this study raises interesting and essential questions related to one of these most fundamental concepts of organization in the OB, the idea of homotypic axon convergence and molecular specificity in glomeruli. In conclusion, this study is only a brief and initial step in analyzing the organization of the human OB, however several simple further experiments are proposed that may be able to

answer some of these important questions.

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