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The Role of Frizzled-1 and Frizzled-3 in Olfactory Sensory Neuronal Targeting.

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Mina G. Safain

2009

ABSTRACT

THE ROLE OF FRIZZLED-1 AND FRIZZLED-3 IN OLFACTORY SENSORY NEURONAL TARGETING. Mina G. Safain. Department of Neurosurgery, Yale University School of Medicine, New Haven, CT.

While most areas of the brain do not exhibit profound neurogenesis or continuing synaptogenesis in the adult, there are notable exceptions which include the olfactory and hippocampal regions of the nervous system. In the olfactory system the olfactory sensory neurons continually turn over and extend their axons into the olfactory bulb in a highly precise manner. Some preliminary data exists that suggests the presence of Wnt and Frizzled (Fz) family members in the primary olfactory pathway. Recent evidence has suggested that these proteins could function as guidance cues but these proteins have not been studied in the olfactory system. Therefore this family of proteins, including Fz-1 and Fz-3, could have a role in olfactory sensory neuron targeting. This study proposes to test the hypothesis that the expression pattern and temporal regulation of Fz-1 and Fz-3 is consistent with a role in olfactory sensory neurons axon targeting. Mouse embryos at ages E10, 10.5, 11, and 13 (E0 is the day of conception) were removed, fixed, sectioned on a cryostat, and then thaw mounted onto slides. Immunohistochemistry was performed on the sections and labeled with antibodies for either Fz-1 or Fz-3 and a variety of other antibodies that labeled mature and immature olfactory sensory neurons (GAP 43, NCAM, PSA-NCAM, OCAM, β -Tubulin). Nuclear staining was accomplished by using DRAQ-5. Stained sections were analyzed with a confocal microscope. Fz-1 and Fz-3 were present as early as embryonic day 10-10.5 and expression increased dramatically over the next 3 days of development. These receptors also colocalized with both markers of mature and immature olfactory sensory neurons. These proteins seem to be upregulated and expressed more heavily on olfactory sensory neurons during the onset and throughout the period that is essential for proper glomerular targeting. It is therefore likely that these proteins are involved in some aspect of olfactory sensory neurons intricate navigation from the olfactory epithelium into the glomerular layer of the developing olfactory bulb.

Acknowledgements

I am deeply indebted to my mentors for this project. Dr. Charles Greer and Dr. Diego Rodriguez Gil have been amazing in their mentorship, guidance, enthusiasm, encouragement, and support. There is little chance that this project could have come to fruition without their constant assistance and mobilizing drive. I would also like to thank the other members of the Greer Laboratory for their help, support, and friendship. Furthermore, my family is due an enormous amount of credit for their never ending aid, guidance, open ears, and undying love. Finally, I would like to acknowledge the Office of Student Research at Yale University School of Medicine for their financial support.

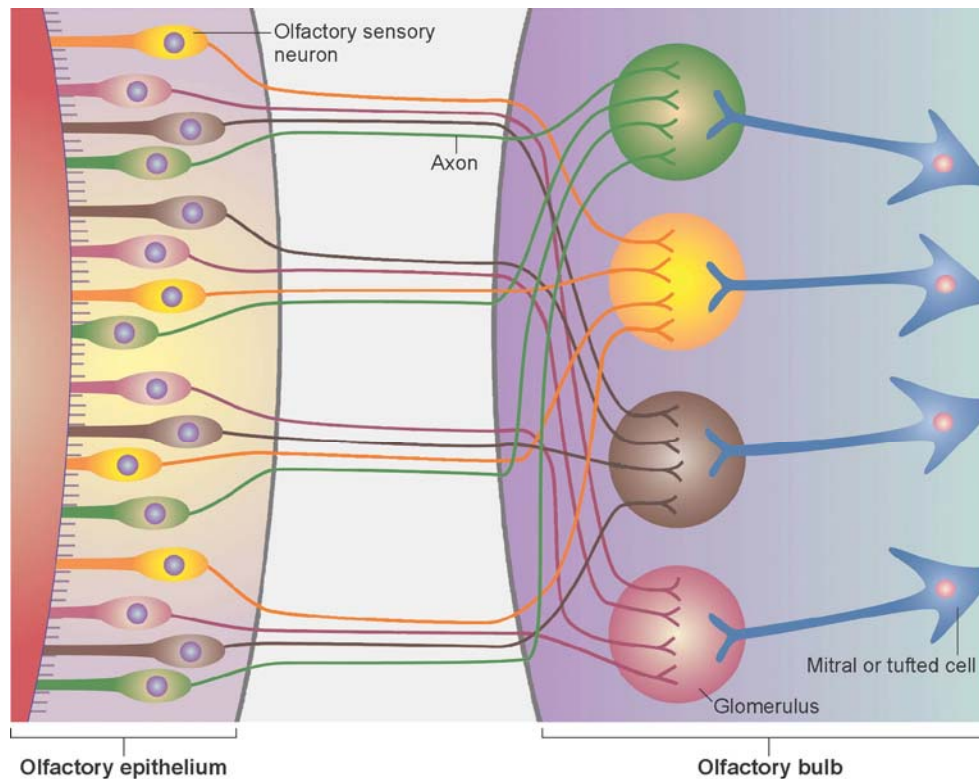
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Introduction

The olfactory system is a specialized system to detect and decode volatile molecules known as odorants. Although the evidence is largely anecdotal, humans are thought to be able to distinguish about 10,000 different odors. Other vertebrates which can have olfactory systems that are more complex than the human by an order of magnitude may be able to detect and distinguish even more. By any measure, the olfactory system is one of the most specific, complex, and arguably one of the most important sensory systems. In rodents, odorant receptors represent the largest known family of genes. This superfamily of genes (over 2.5% of the genome) contains in mice ~1200 genes with open and intact reading frames coding for the 7-transmembrane G-protein coupled odorant receptors found in the olfactory epithelium (1, 2).

The olfactory system can be divided into three major compartments: 1) the olfactory sensory epithelium (OE) where the sensory neurons are located; 2) the olfactory bulb (OB) which receives primary afferent input from the OE and carries out initial coding/processing of odorant information; and 3) the primary olfactory cortex where integration of complex multi-dimensional and combinatorial odorants may occur. The following sections will describe these major compartments of the olfactory system. For aid in a general understanding of the olfactory system a visual schematic is included below that can be followed along with the text that follows. (Image obtained from Momberts. (2006). Axonal wiring in the mouse olfactory system. *Annu Rev Cell Dev Biol.* 22:713-37.)




 Mombaerts P. 2006.
 Annu. Rev. Cell Dev. Biol. 22:713–37

Figure 1. Wiring diagram of the mouse olfactory system. Four different populations of OSNs, each expressing a different OR, are depicted in green, orange, brown, and red. OSNs that express the same OR are scattered in a seemingly random fashion with the epithelium, but their axons coalesce into the same glomeruli in the olfactory bulb. Within a glomerulus, OSN axons synapse with the dendrites of the second-order neurons, mitral or tufted cells, which in turn project their axons to the olfactory cortex. This anatomical organization presents a formidable wiring problem. Figure from Bob Crimi.

Olfactory Epithelium

The olfactory sensory neurons (OSNs) are bipolar neurons characterized by a single short apical dendrite that terminates in an enlarged dendritic knob as it protrudes into the mucus covering the nasal cavity. Extending from the knob are ~10-12 non-motile cilia that increase significantly the surface area of the OSN in the nasal lumen (3). The odorant receptors are inserted into the membranes of the cilia and the knob where they can interact with volatile odorants that partition into the mucous following inhalation. When an appropriate ligand binds, the G-protein coupled receptor initiates a transduction cascade that opens cyclic nucleotide gated channels that allow the influx of Ca^{2+} and depolarization of the OSN. An important principle of OSN organization is that each OSN expresses only 1 of the total 1200 odorant receptors. Therefore, the molecular identity or phenotype of an OSN is determined by which of the 1200 receptors it expresses (4). Extending from the basal pole of the OSN is a single, unbranched, unmyelinated axon coursing from the OE to the OB. The targeting of OSN axons into the OB occurs with startling precision. As mentioned before, there are ~1200 different odor receptors and single OSNs are believed to express only 1 receptor; the axons from all of the OSNs expressing the same odor receptor converge onto only 2 of the 2,000 glomerular targets in each olfactory bulb (5, 6, 7, 8). Therefore, each specific glomerulus is thought to be molecularly homogeneous in that it is innervated only by axons expressing the same odor receptor (9).

It is clear that the odor receptors not only determine the odor response spectra of the OSNs, but that they are also necessary for axon targeting to occur correctly – necessary for axon targeting to occur, but not in and of themselves sufficient to account

for the specificity of targeting (10, 11). In a series of odor receptor swap studies, Feinstein and Mombaerts (2004), and others, have shown that substitution of a different odor receptor can alter the targeting of the axons, but only partially. The redirection of the axons from the OSNs with swapped odor receptors is incomplete leading the authors to suggest that additional mechanisms must also be involved.

Olfactory Bulb

The OB is a derivative of the forebrain and an extremely organized and layered structure. Each OB can be divided into six separate and functionally different layers. These layers include (from superficial to deep): 1) the olfactory nerve layer; 2) glomerular layer 3) external plexiform layer 4) mitral cell layer 5) internal plexiform layer; and, 6) granule cell layer. OSN axons, after penetrating the cribiform plate, approach the OB as the olfactory nerve. Upon contacting the OB they spread across the surface in a complex plexus as the olfactory nerve layer. Within the nerve layer the axons reorganize into molecularly defined fascicles that then innervate single glomeruli, as noted above. Early in development some slight mistargeting of axons and the formation of ectopic glomeruli does occur, but this is resolved within days (12) and the end result is a topography between the OE and the OB that is among the most complex in the nervous system. Mitral cells, whose cell bodies are located in the mitral cell layer, send primary dendrites (that transverse the external plexiform layer) to the glomerular layer where they terminate in a single glomerulus with an intricate network of branches. Tufted cells, a second population of projection neurons, are similarly organized. In the

glomeruli there is a population of interneurons, periglomerular cells, which modulate glomerular activity of the mitral and tufted cells. Finally, granule cells are the largest population of interneurons found in the OB. Their cell bodies are located in the granule cell layer and they extend apical dendrites into the external plexiform layer where they interact with the lateral dendrites of mitral and tufted cells (13).

The synaptic organization of the OB in its most basic form can be summarized as follows: 1.) Primary OSN axons terminate on dendrites of a limited population of mitral and tufted cells in a single glomerulus. 2.) Periglomerular and granule cells act to modulate signals that are presented to the mitral and tufted cells. 3.) Mitral and tufted cells then project their axons to the primary olfactory cortex.

Primary Olfactory Cortex

The axons of the mitral and tufted cells coalesce on the posterolateral surface of the OB and exit via the peduncle as the lateral olfactory tract (LOT). This LOT converges on the primary olfactory cortex, predominately piriform cortex. Collaterals from axons exit the LOT and terminate in Layer Ia of piriform cortex where they synapse on the apical dendrites of Layer II and III pyramidal neurons. The wiring specificity and functional organization of piriform cortex is currently a very topical issue, but remains poorly understood at this time (14).

Adult Neurogenesis

While most areas of the brain do not exhibit profound neurogenesis or continuing synaptogenesis in the adult, there are notable exceptions which include the olfactory and hippocampal regions of the nervous system. This continuing neurogenesis, even into adulthood, is of great interest. In the olfactory system the sensory neurons continually

turnover and extend their axons into the olfactory bulb in a highly precise manner (7, 9, 15, 16). In the olfactory nerve, molecular substrates of growth are expressed in the adult. For example, laminin, PSA-NCAM, tenascin, GAP-43, B50 and others show higher levels of expression in the OB than elsewhere in the adult CNS (16, 17, 18, 19, 20, 21). In addition, lesions of the olfactory nerve initiate a synchronized development of new OSNs (22), and up-regulation of some growth associated molecules (23). In a similar vein, there is an ongoing generation of neurons in the subventricular zone of the lateral ventricles that then migrate along a specialized corridor of PSA-NCAM positive cells into the bulb, termed the Rostral Migratory Stream (RMS) (24, 25, 26, 27, 28, 29, 30, 31). The molecular substrates of the horizontal migratory path appear to be independent of the radial migration that occurs when the migrating cells reach the OB (32, 33). Elsewhere in the CNS, attempts at cellular migration or axonal regeneration encounter inhibitory factors that cause a collapse of growth cones and stop neurite extension (34). Liesi (1985) suggested that a basic difference between regions of the CNS that are permissive of axonal growth or regeneration and those that are not is the molecular phenotype of the glial cell population.

Axon Guidance

Since the early 1990's, a wide range of molecules have been noted to affect axonal growth and guidance (35, 36, 37, 38). These molecules are found in different compartments of the central nervous system and include members that are extracellular matrix proteins, secreted signaling proteins, and membrane bound cell surface receptors. A number of studies carried out in the olfactory system have reported the presence of major axon guidance molecules such as ephrins (39), semaphorins (19, 40, 41), slits (42)

and netrins (43) as well as neurotrophic factors such as NGF (44) and IGF (45) and cell surface carbohydrates (20, 46). Despite recent progress, a comprehensive model of specificity in OSN axon guidance remains elusive.

Throughout the nervous system identifying primary mechanisms of axon guidance and the underlying molecules has been useful in generating developmental models, though they still remain incomplete and not fully understood. The olfactory system, however, remains an enigma; questions such as: which cues guide an OSN axon both out of the epithelium first and then to the OB; or, what mechanism(s) is/are involved in the sorting and targeting of OSN axons remain controversial.

In the past 10 years the olfactory odor receptor (OR) itself has emerged as a favorable candidate for regulating axon guidance in the OSNs. Mombaerts et al. (1996) and Wang et al (1998), deleted odorant receptor coding regions and axons were shown to distribute randomly and failed to converge onto the correct glomeruli. This strengthened the hypothesis that the odor receptor was indeed the sole cue in targeting. However, more recent experiments (10, 11) have shown that although the odor receptor is essential for targeting, it is not the sole determinant for targeting and that there must be some other cue that OSNs receive in order to arrive at the correct glomeruli. Mombaerts et al. used swap experiments, in which the OR coding region of a recipient OR locus is replaced with the coding region of a donor OR by gene targeting, and showed that the OSNs did not target to either the donor or recipient glomeruli but to novel, ectopic glomeruli. With both the following observations, 1.) That OSNs with deleted OR coding regions fail to converge onto the correct glomeruli and 2.) That swapped OR coding regions converge to novel, ectopic glomeruli, it has been concluded that the OR is necessary but not sufficient

for proper targeting. It is this fact that produces an extremely interesting and necessary question, what other cues do OSNs use to target to the correct glomeruli?

Recently, some secreted molecules known by their morphogenic function during development, have also been shown to act as guidance cues. These molecules are grouped into three major families: Wingless-Int (Wnt), Hedgehog, and bone morphogenetic protein (BMP). While preliminary reports suggest the presence of Hedgehog and select BMPs during olfactory system development (47, 48), the Wnt family has not been studied or assessed for a possible role in olfactory system development.

Wnt genes encode a large family of secreted, cysteine rich proteins that act as signaling molecules implicated in patterning of the anterior-posterior axis, cell type specification, cell proliferation and axonal growth (49). Wnt proteins bind to Frizzled (Fz) proteins, which are cell-surface receptors. Fz genes encode seven transmembrane receptor proteins with an amino-terminal extension rich in cysteine residues predicted to be positioned outside of the cell and to interact with Wnt molecules (50). Fz proteins have been suggested to signal through three different pathways termed the Wnt/ β -catenin pathway, Planar Cell Polarity pathway (PCP pathway), or the Wnt/calcium pathway (49). These pathways have been implicated in many different functions including synaptogenesis, cell fate decisions, cell and tissue polarity, dendrite-genesis, and cell movement. In addition to the integral membrane Fz proteins described, several secreted proteins (called FrzB or sFrp), which consist of a cysteine-rich domain very similar to those in Fz molecules have been described. It has been proposed that these molecules would act as inhibitors by binding secreted Wnt proteins through one of three possible

pathways (49).

To date there have been 11 Fz proteins, 19 Wnt proteins, and five sFrp proteins described. This study aims to characterize the expression pattern of two of these Fz (Fz-1 and -3) proteins during olfactory system embryonic development. Specifically, we intend to show first, if these proteins are expressed in OSNs, and second, if their expression pattern on OSNs and their projections suggests a possible role in axon guidance.

These two specific proteins were selected for a few major reasons. Preliminary work in the same laboratory had shown that Fz-1 and Fz-3 were preferentially expressed in the olfactory system when compared to other members within the same family. In addition, it was recognized that these proteins were being expressed at the appropriate time that coincided with a possible role in axon guidance. Furthermore, commercially available antibodies were widely available for these two proteins. It is the hope of this lab to further pursue the characterization of the other members of this family in the future.

STATEMENT OF HYPOTHESIS/PURPOSE

As stated before, some preliminary data exists that suggests the presence of Wnt family members in the primary olfactory pathway. Although the initial data is promising, to begin evaluating whether this family of molecules may be functionally significant, their expression patterns, candidate binding partners and cellular localization needs to be tested in the temporal framework within which OSN axons extended toward the OB, sort into molecularly defined fascicles and subsequently coalesce into molecularly homogeneous glomeruli. We propose to test the hypothesis that the expression pattern and temporal regulation of Fz-1 and 3 is consistent with a role in OSN axon targeting.

Materials and Methods

Animals

Pregnant, time-mated CD-1 mice (Charles River, Wilmington, MA) were anesthetized with sodium pentobarbital (80 mg/kg, i.p.; Nembutal; Abbott Laboratories, North Chicago, IL) prior to cesarean section. Embryos were removed and immersion fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline [PBS; 0.1 M phosphate buffer (PB) and 0.9% NaCl, pH 7.4] at 4°C overnight. Embryos were collected at embryonic day (E) E10, 10.5, 11, and 13, where the day of conception is designated E0. All tissue was rinsed for a minimum of 2 hours in PBS after fixation, before processing. All procedures undertaken in this study were approved by Yale University's Animal Care and Use Committee and conform to NIH guidelines.

Sectioning

Embryos were prepared for sectioning by immersion in 30% sucrose in PBS at 4°C until the entire embryo sank. Embryos were then embedded in OCT compound (Sakura Finetek, Torrance, CA) and frozen in a mixture of 100% ethanol and dry ice. Embryos were then serially sectioned in the coronal plane at 20µm thickness by using a Reichert-Jung 2800 Frigocut E cryostat. Sections were thaw mounted onto SuperFrost plus microscope slides (Fisher Scientific, Pittsburgh, PA), dried on a slide heater, and stored at -20°C until further processing occurred. Multiple embryos at each age (n=7-10 per age) were sectioned both to provide enough tissue for the extensive Immunohistochemistry as well as to reduce the possibility of random binding of antibodies occurring in a specific embryo.

Immunohistochemistry

The 20 μ m cryostat sections were immunostained with antibodies for either Fz-1 or Fz-3 and a selection of additional antibodies that were used to examine colocalization of proteins and provide baseline information about the organization of the developing olfactory system (Table 1). Care was taken as to the species in which the primary and secondary antibodies were raised in order to not allow cross reactivity between them. The tissue was first thawed from -20°C to room temperature and allowed to dry. Sections were then incubated with 2% bovine serum albumin (BSA; Sigma, St. Louis, MO) in PBST [0.1 M PBS buffer, pH 7.4 with 0.3% Triton X-100 (Sigma)] for 30 minutes to block nonspecific binding sites. Incubation in primary antibodies in blocking solution was overnight at room temperature. Sections were washed three times in PBST for 5 minutes each and incubated in secondary antibodies and nuclear markers (diluted 1:1,000 and 1:2,500 respectively) in blocking buffer for 1 hour at room temperature. Sections were washed (as described above), rinsed in PBS, mounted in Gel/Mount mounting medium (Biomedica Corp., Foster City, CA), and coverslipped. Omission of the primary antibody was used to establish the specificity of staining. Antibodies employed as tissue markers exhibited staining patterns consistent with previous reports. Stained sections were analyzed with a confocal microscope. Images were acquired with a Leica confocal microscope, using 40X or 63X oil-immersion objectives. Z-stacks were taken with 0.5 μ m steps between images. Images displayed are either single planes or maximum projections (when indicated), generated with Leica confocal software. Digital images were color balanced using Adobe Photoshop 6.0 (Adobe Systems, San Jose, CA). The composition of the images was not altered in any way.

TABLE 1

Primary Antibody	Dilution	Secondary Antibody	Dilution
Goat anti Frizzled-1	1:100	Donkey anti Goat	1:1000
Goat anti Frizzled-3	1:100	Donkey anti Goat	1:1000
Mouse anti Polysialic Acid acid-Neuronal Cell Adhesion Molecule (PSA-NCAM)	1:200	Donkey anti Mouse	1:1000
Mouse anti β -tubulin	1:200	Donkey anti Mouse	1:1000
Rat anti Neuronal Cell Adhesion Molecule (NCAM)	1:1000	Donkey anti Rat	1:1000
Rabbit anti Growth Associated Protein-43 (GAP-43)	1:1000	Donkey anti Rabbit	1:1000

The two antibodies against the Frizzled receptors were utilized in single and double-label immunohistochemistry studies, together with markers for specific cell types within the olfactory pathway. NCAM, OCAM, as well as OMP are expressed by all mature olfactory sensory neurons. PSA-NCAM, which labels migrating olfactory sensory neurons, and GAP-43 are expressed by maturing but not fully matured olfactory sensory neurons. The specific combinations of primary antibodies reported are given in Table 2.

TABLE 2

1° Antibodies	Ages
Frizzled-1	All ages
Frizzled-3	All ages
Frizzled-1, GAP-43	E11 and E13
Frizzled-1, NCAM	E13
Frizzled-3, GAP-43	E13
Frizzled-3, PSA-NCAM	E13
Frizzled-3, NCAM	E13

Image Preparation

CSLM images were pseudo-colored and merged using Confocal Assistant software. The composition of the images was not altered in any way. Diagrams and figures were constructed using Corel Draw v8.0 Images were analyzed qualitatively; no quantitative analyses were performed. Representative images are presented here.

All procedures in the methods except the initial sacrificing of some animals were completed by the author.

Results

Fz-1 Expression in Early Embryonic Development

Fz-1 expression begins as early as E10 in the OE (**Fig 2**). Expression was restricted to a small number of OSN's located in the dorsal recess of the olfactory pit. Fz-1 is noted to be present throughout the entirety of the cell including the apical dendrite, cell body, and the axons that are exiting the epithelium. Interestingly, in all animals of this age examined, the areas of Fz-1 positive cells were found in the area of the OE closest to the tip of the telencephalic vesicle, which is the place where the OB will be formed. As early as E10.5 some OSN axons positive for Fz-1 can be seen crossing the mesenchyma lying in between the OE and the telencephalic vesicle (**Fig 3**). Furthermore, from this stage on up to E13 mice, there is a dramatic and progressive increase in the number of cells expressing Fz-1 protein (**Fig 3, 4, 5**). With the increase in age of the animals, Fz-1 positive cells spread from being restricted to areas closest to the developing olfactory bulb to being expressed throughout the majority of the OE. Once again, expression in all age groups show that the entire extent of the cell including cilia, apical dendrite, cell body, and axon continue to be Fz-1 positive (**Fig 3, 4, 5**).

At E13, the presumptive olfactory nerve layer (most outer layer of the developing bulb) is stained heavily for Fz-1 (**Fig 6**). Axons are seen extending into the deeper layers of the developing olfactory bulb. The inner most layers of the developing bulb are not stained with Fz-1 (**Fig 6**).

Fz-3 Expression in Early Embryonic Development

Fz-3 is also expressed early in embryonic development in the mouse. Once again, expression was first observed as early as E10 in the Olfactory Epithelium (**Fig 7**). In addition, we again see that areas of the OE that are Fz-3 positive in this early stage are in closest proximity to the developing OB. As observed with Fz-1, there is a similar progressive and dramatic increase in Fz-3 protein expression during E10.5-13 (**Fig 8, 9, 10**). Once again as was seen with Fz-1, Fz-3 expression is restricted to the areas closest to the developing OB in earlier aged animals and becomes more prominent and widespread as the animals age during their embryonic life. By E13 all the cells in the OE express the receptor (**Fig 10**).

Fz-3 positive axons, at age E13, are found heavily staining the presumptive olfactory nerve layer (**Fig 11**). Comparable to Fz-1, axons are seen extending into the deeper layers of the developing olfactory bulb. However, unlike Fz-1, we see that the inner most layers of the developing bulb are in fact stained with Fz-3 (**Fig 11**).

Fz-1 and Fz-3 Colocalize with Markers of both Mature and Immature Olfactory Sensory Neurons

Fz-1 and Fz-3 colocalize with markers of mature, immature and all olfactory sensory neurons including Growth Associated Protein-43 (GAP-43), Neuronal Cell Adhesion Molecule (NCAM), or Polysialic Acid Neuronal Cell Adhesion Molecule (PSA-NCAM) (**Fig 12**). In all stainings, Fz-1 or Fz-3 heavily stains the olfactory

epithelium and axon bundles that are exiting the olfactory epithelium. Fz-1 colocalizes with GAP-43, a marker of immature olfactory sensory neurons, at both E11 (**Fig 12A**) and E13 (**Fig 12B**). In addition, Fz-1 colocalizes with NCAM, a general marker of olfactory sensory neurons, at E13 (**Fig 12C**). Fz-3 colocalizes with two different markers of immature olfactory sensory neurons, PSA-NCAM and GAP-43, at E13 (**Fig 12D and 12E**). In addition, Fz-3 colocalizes with NCAM, a general marker of olfactory sensory neurons at E13 (**Fig 12F**).

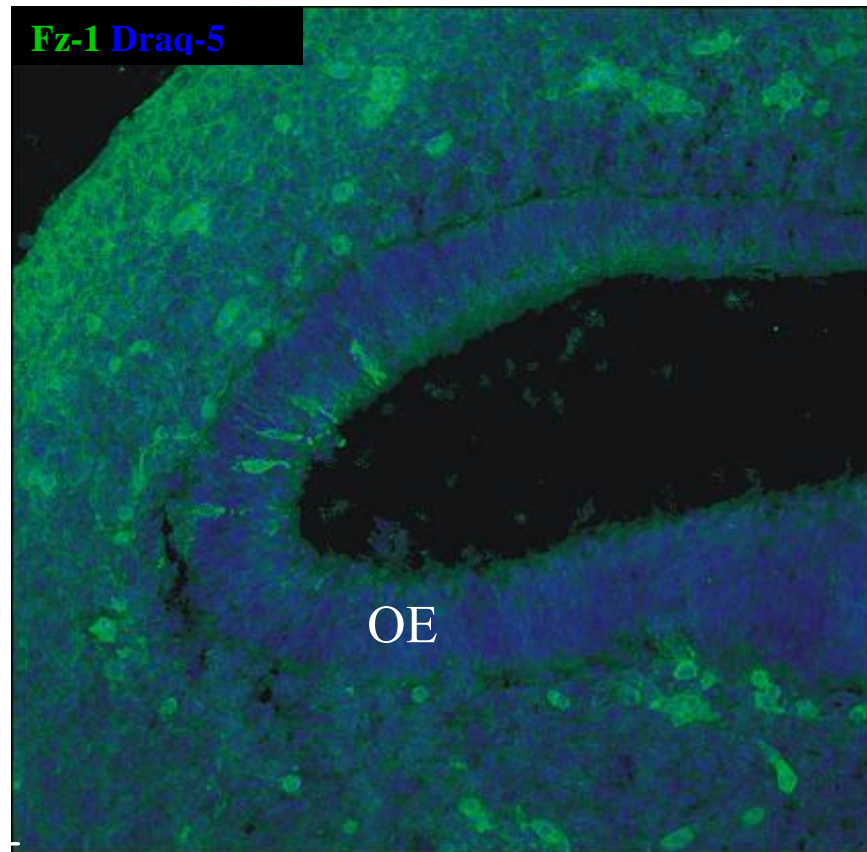


Figure 2: The developing olfactory epithelium of an E10 mouse shown here is stained for Fz-1 (green) and DRAQ5 for nuclear staining (blue). In this projection Fz-1 is clearly expressed in six olfactory sensory neurons. These cells are restricted to a small area in the dorsal recess of the olfactory pit. Nearby is the tip of the telencephalic vesicle, the place where the OB will develop. Fz-1 is noted to be present throughout the entirety of the cell including the apical dendrite, cell body, and the axons that are exiting the epithelium. OE=olfactory epithelium

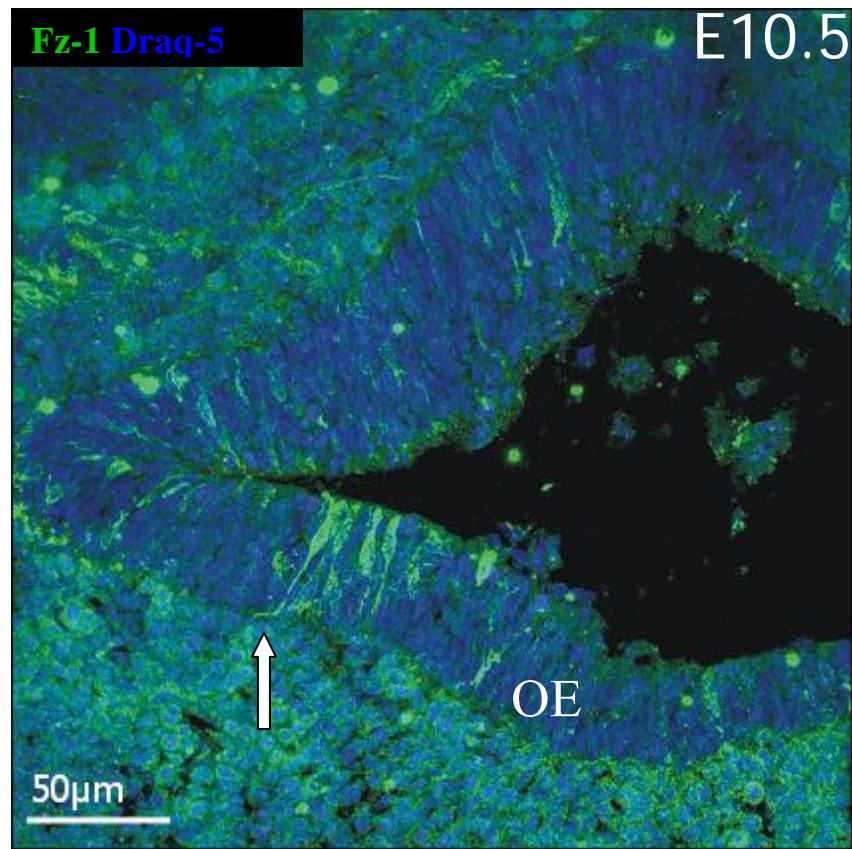


Figure 3: The developing olfactory epithelium of an E10.5 mouse shown here is stained for Fz-1 (green) and DRAQ5 for nuclear staining (blue). In this projection Fz-1 is clearly expressed in a greater number of olfactory sensory neurons than half a day earlier. The cells stained now represent a greater proportion of the olfactory epithelium with the majority of cells still residing closer in distance to the telencephalic vesicle (out of the picture, but located to the left). At this point, some axons exiting the OE and turning dorsally can be observed (arrow). OE=olfactory epithelium

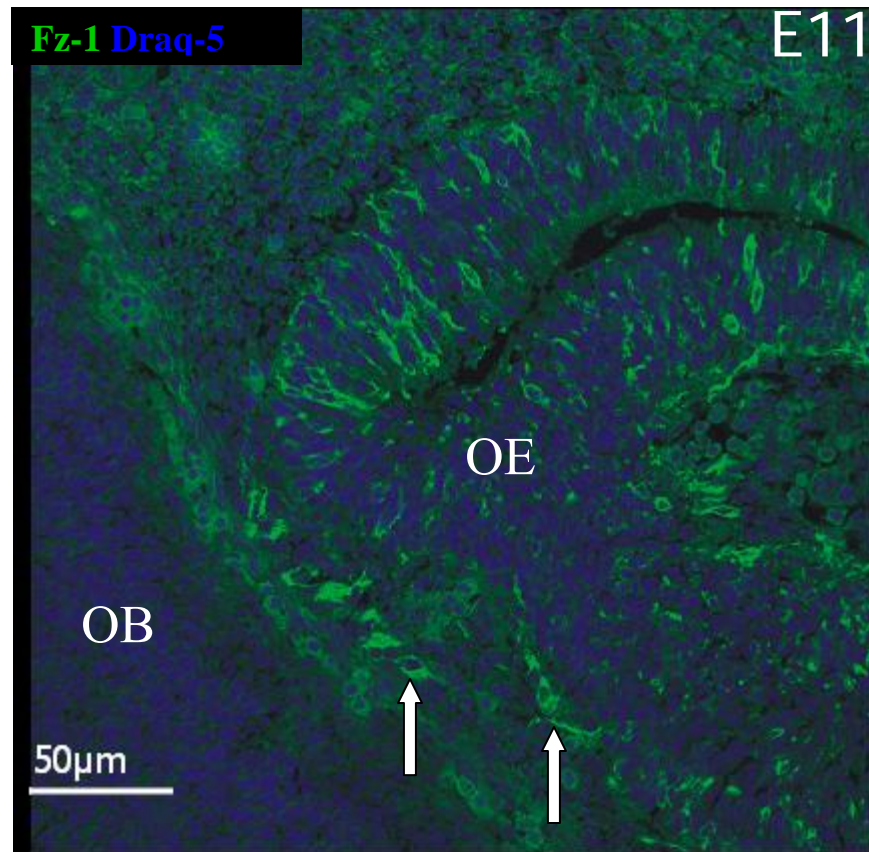


Figure 4: The developing olfactory epithelium of an E11 mouse shown here is stained for Fz-1 (green) and DRAQ5 for nuclear staining (blue). In this projection Fz-1 is expressed in olfactory sensory neurons. Positive Fz-1 cells were observed in all areas of the epithelium which is different than one half to one day earlier where labeled cells were only found a short distance from the telencephalic vesicle (observed on the left). In addition, migrating Fz-1 positive cells are seen migrating out of (arrows) the epithelium. Once again, Fz-1 is being labeled throughout the entirety of the cell including the apical dendrite, cell body, and the axons that are exiting the epithelium. OE=olfactory Epithelium, OB=developing Olfactory Bulb.

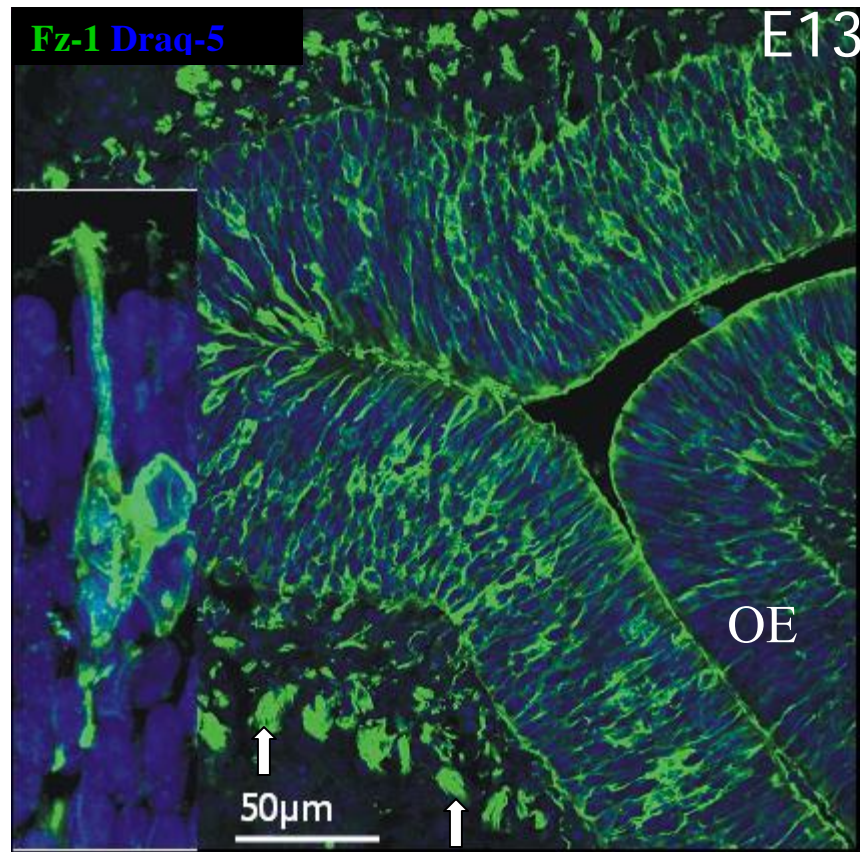


Figure 5: Shown here is the olfactory epithelium of an E13 mouse with clear staining for Fz-1 (green) and DRAQ5 for nuclear staining (blue). In this single confocal section Fz-1 expression has dramatically increased. Labeled OSNs are found in the majority of the epithelium. In addition, axon bundles (arrows) are clearly stained with Fz-1. A magnification of an olfactory sensory neuron is shown with clearly stained axon, cell body, apical dendrite, and a clear dendritic knob with ciliary processes (Magnified picture courtesy of Dr. Diego Rodriguez Gil). OE=olfactory epithelium, AB=axon bundles

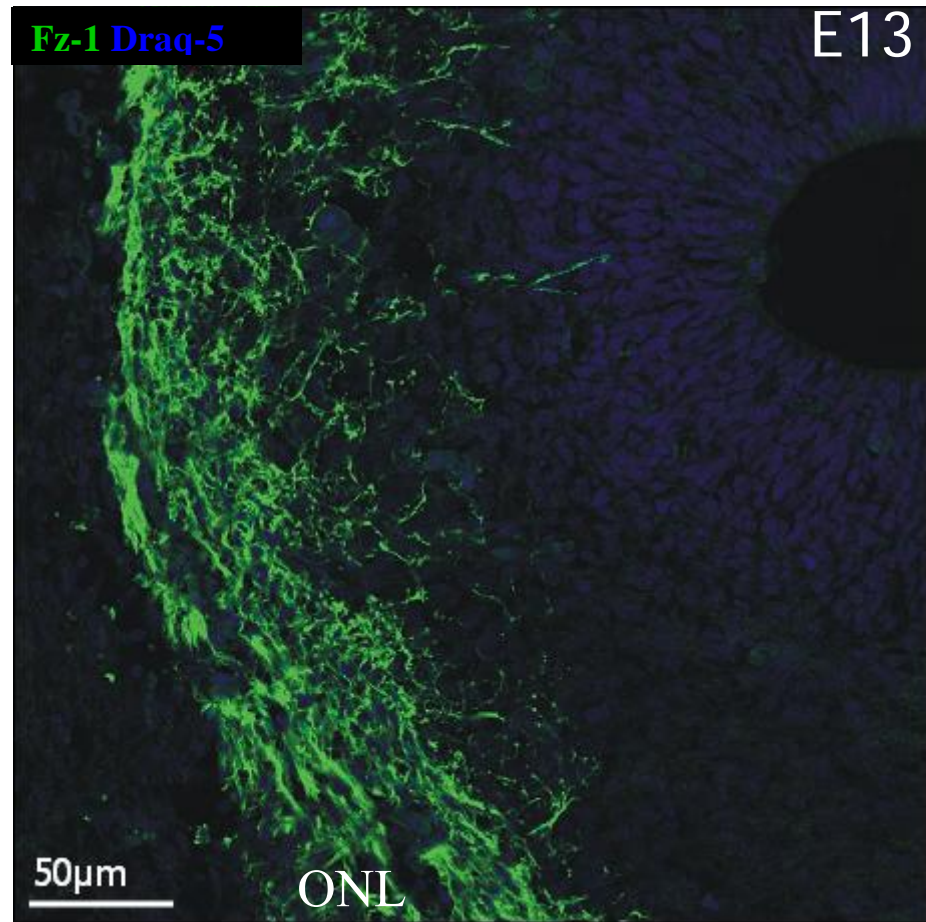


Figure 6: Shown here is the developing olfactory bulb of an E13 mouse stained with Fz-1 (green) and Draq-5 (blue). The presumptive olfactory nerve layer (most outer layer of the developing bulb) is stained heavily for Fz-1. Axons are seen extending into the deeper layers of the developing olfactory bulb. The inner most layers of the developing bulb are not stained with Fz-1. ONL=olfactory nerve layer.

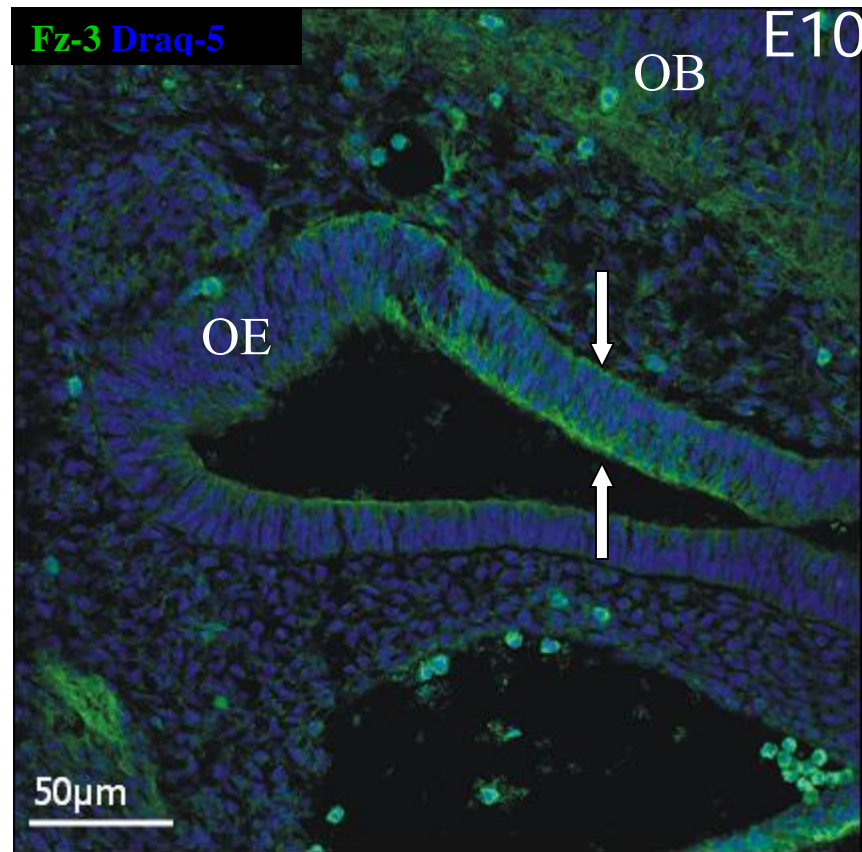


Figure 7: The developing olfactory epithelium of an E10 mouse shown here is stained for Fz-3 (green) and DRAQ5 (blue) for nuclear staining. In this projection Fz-3 is expressed very lightly along the apical and basal borders of the epithelium (arrows). The sections of the epithelium that are stained are constrained to a section of epithelium that is the shortest distance from the telencephalic vesicle. Fz-3 is not staining the mesenchyme nor is Fz-3 labeling cell bodies at this age. OE=olfactory epithelium, OB=developing olfactory bulb

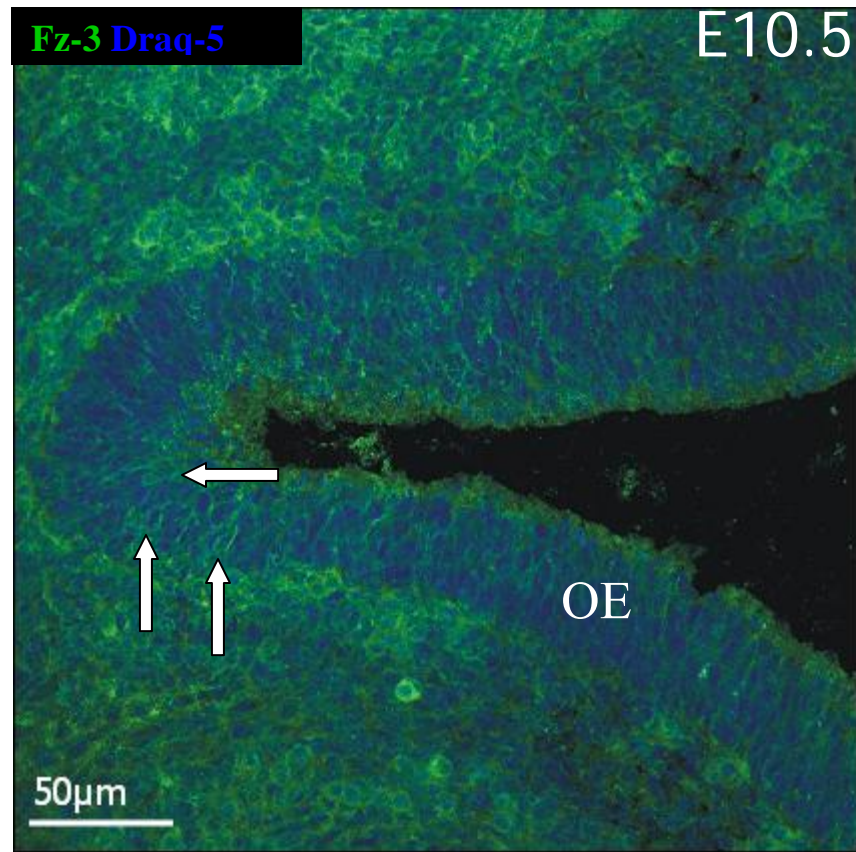


Figure 8: The developing olfactory epithelium of an E10.5 mouse shown here is stained for Fz-3 (green) and DRAQ5 (blue) for nuclear staining. Fz-3 is staining the cell membrane around the cell body lightly (arrows). These cells are restricted to a small area in the dorsal recess of the olfactory pit. This area is close to the tip of the telencephalic vesicle, the place where the OB will develop. OE=olfactory epithelium

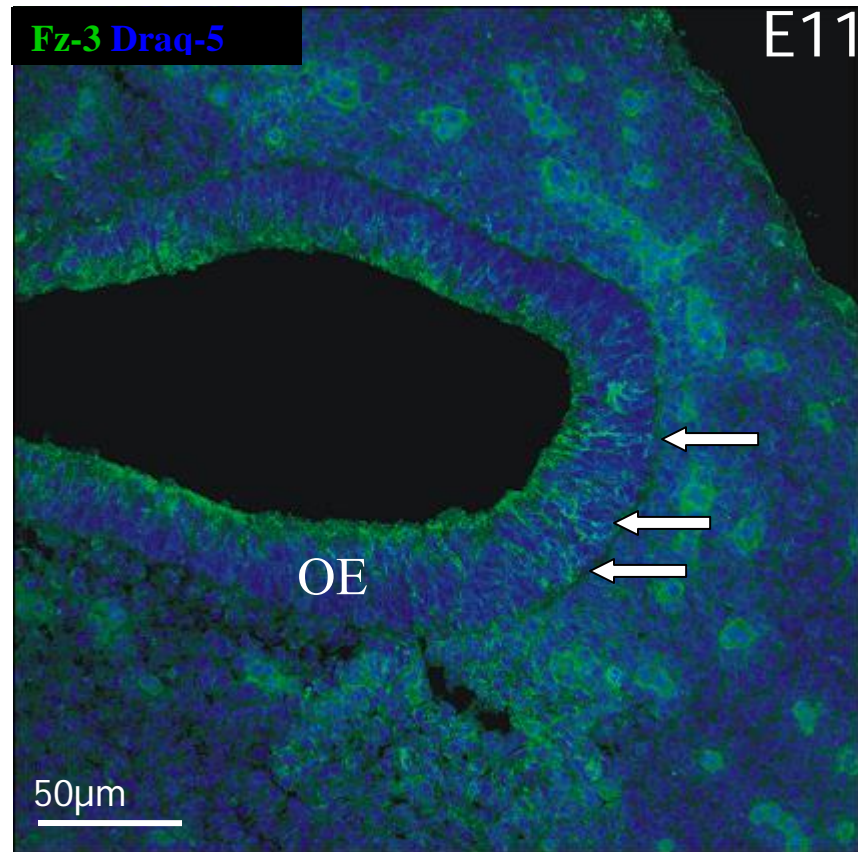


Figure 9: The developing olfactory epithelium of an E11 mouse shown here is stained for Fz-3 (green) and DRAQ5 (blue) for nuclear staining. In this projection Fz-3 is clearly expressed in a small number of cells in the dorsal pit of the olfactory epithelium (arrows). The cells stained are near the telencephalic vesicle. OE=olfactory epithelium

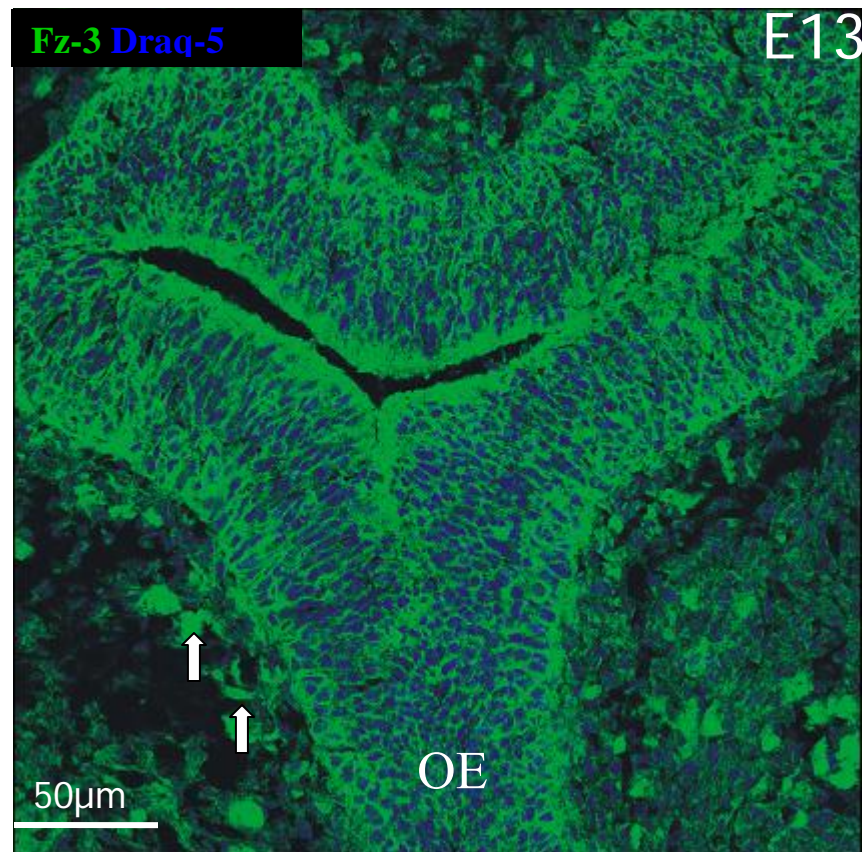


Figure 10: Shown here is the olfactory epithelium of an E13 mouse with clear staining of Fz-3 (green) and DRAQ5 (blue) for nuclear staining. In this single confocal section Fz-3 expression has dramatically increased, staining all cells in the olfactory epithelium. In addition, axon bundles are clearly stained with Fz-3. Stained axons (arrows) are seen exiting the epithelium and entering axon bundles. OE=olfactory epithelium

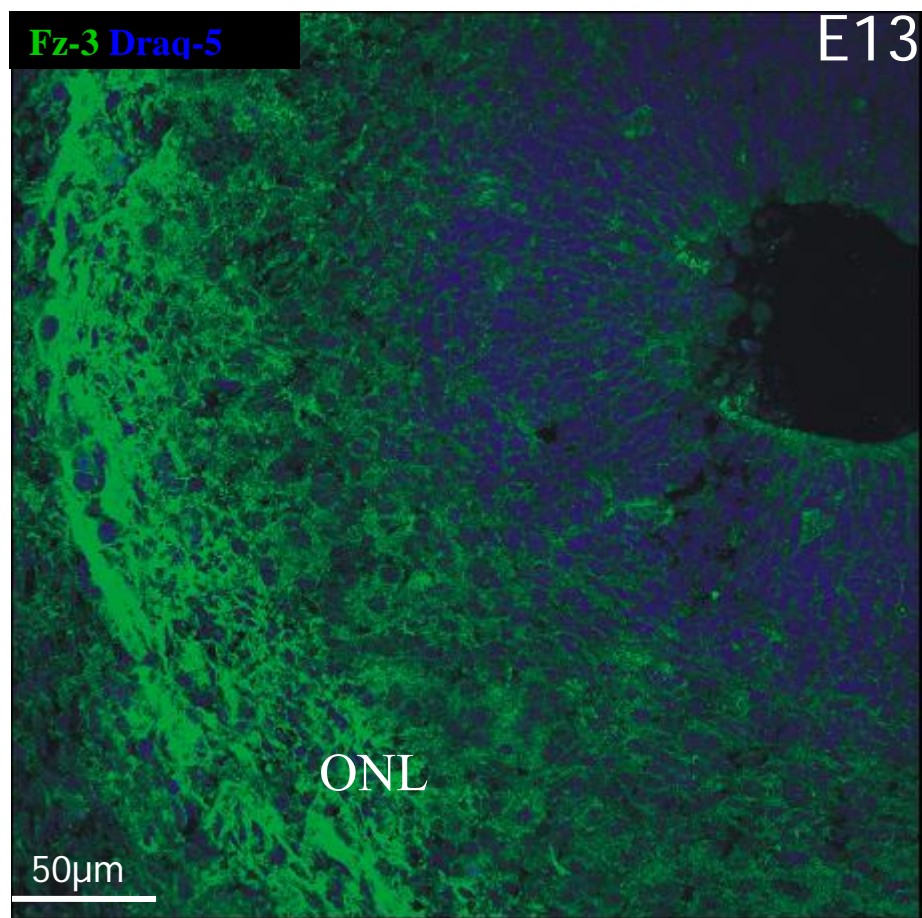


Figure 11: Shown here is the developing olfactory bulb stained with Fz-3 (green) and DRAQ-5 (blue). The presumptive olfactory nerve layer is stained heavily for Fz-3. The inner layers of the bulb are not stained. Additionally, staining is also seen on the edge of the ventricle of the developing olfactory bulb. ONL=olfactory nerve layer.

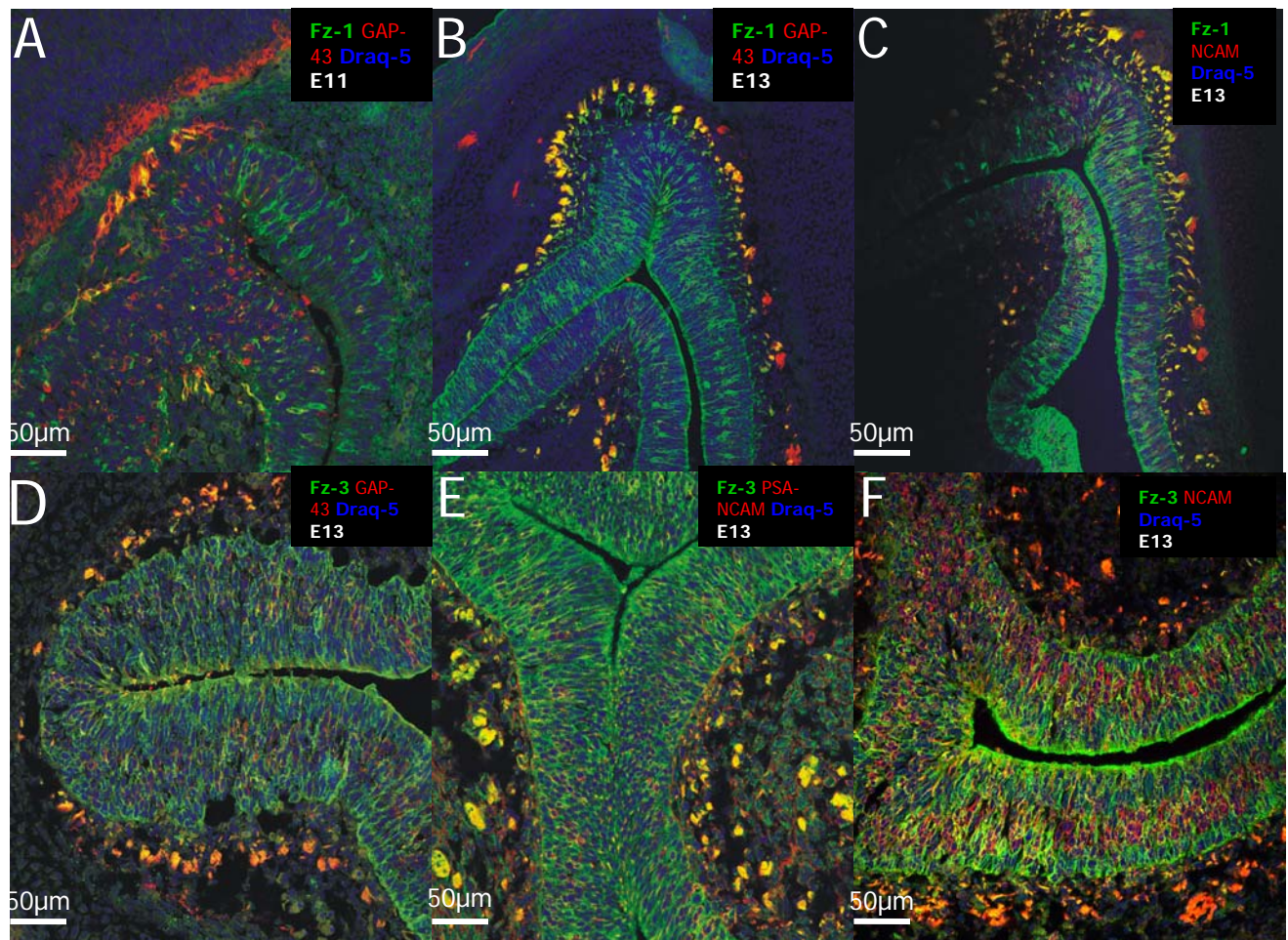


Figure 12: Several double stainings are shown which show co-localization of either Fz-1 or Fz-3 with other markers including Growth Associated Protein-43 (GAP-43), Neuronal Cell Adhesion Molecule (NCAM), or Polysialic Acid Neuronal Cell Adhesion Molecule (PSA-NCAM). In all stainings Fz-1 or Fz-3 heavily stain the olfactory epithelium and axon bundles that are exiting. **A.)** Fz-1 and GAP-43 show colocalization in axons exiting the olfactory epithelium in an E11 mouse. **B.)** Fz-1 and GAP-43 in an E13 mouse. Axon bundles show clear colocalization with both markers. Interestingly, some GAP-43+ axon bundles are Fz-1 negative but all Fz-1 positive axon bundles are also GAP-43+. **C.)** Fz-1 also shows colocalization with NCAM in axon bundles surrounding the Olfactory Epithelium. Once again some NCAM+ axon bundles are Fz-1 negative but all Fz-1 positive axon bundles are also NCAM+. **D.)** Fz-3 and GAP-43 in an E13 mouse. Axon bundles show clear colocalization with both markers. It is evident that few GAP-43+ axon bundles are Fz-3 negative but all Fz-3 positive axon bundles are also GAP-43+. **E.)** Fz-3 and PSA-NCAM are shown here with colocalization in axon bundles and cell bodies in an E13 mouse. The majority of axons are stained with both markers and axons that are only stained with PSA-NCAM are rare. **F.)** Fz-3 is shown colocalizing with NCAM in heavily NCAM+ but some are also Fz-3 positive. There are no axon bundles that are Fz-3 positive but NCAM negative.

Discussion

In previous work, olfactory sensory neuron targeting has been shown to be divided into three separate and equally important phases. First, axons exit the olfactory epithelium and extend their processes into the telencephalon. Next, these axons enter through the telencephalon and form the presumptive olfactory nerve layer. This phase lasts a significant amount of time since axons are also forming into bundles that are directed to the same general region of the developing olfactory bulb. Finally, once the axons have coalesced into bundles of like type they extend into dendrites of mitral and tufted cells and begin the formation of what will be the future glomeruli. In previous mouse work of the same laboratory (data not shown), the first OSN axons have been shown to exit the olfactory epithelium around E9 and first contact the bulb and form the primitive olfactory nerve layer around E12.

The current work focused on determining the expression of two receptors hypothesized to be involved in OSN targeting on E10, 10.5, 11 and 13. These receptors were found to be expressed as early as E10-10.5, where minimal expression was noted, and continued to show an explosion of expression throughout the time period studied. Additionally, the presumptive olfactory nerve layer was noted to be heavily stained on E13 with both Fz-1 and Fz-3, strengthening the hypothesis that these new neurons entering the developing olfactory bulb express these two receptors. We have therefore shown that these receptors are expressed during the time of OSN development. It is likely that the upregulation of these proteins at this specific time is closely linked to their function in OSN targeting. However, it will be necessary, and in fact is currently being tested in the present lab, to determine whether these receptors are the same players used in adult neurogenesis and neuronal targeting. These studies should include methods of

lesioning of the adult olfactory epithelium and subsequent study of new OSNs development. Finally, it will be important to note whether OSNs express all or have a limited expressivity of types of Frizzled receptors depending on the age and development of the animal. This will help better characterize whether certain Frizzled proteins are performing similar functions at different time points in development.

Markers of OSNs have been used in previous studies to determine the level of maturity that is held by a specific OSN. Some of the most common markers used include Neuronal Cell Adhesion Molecule (NCAM) as a general marker for OSNs, and Olfactory Marker Protein (OMP) to label OSN as mature and fully functional neurons. In addition, markers such as Growth Associated Protein-43 (GAP-43) and Polysialic Acid Neuronal Cell Adhesion Molecule (PSA-NCAM) have been used to label immature and growing OSNs. In the current study an interesting and unexpected result was observed. Both Frizzled-1 and Frizzled-3 colocalize with markers of both mature and immature OSN markers. Additionally, not all neurons expressing the markers of maturity or immaturity co localized with Frizzled-1 and Frizzled 3 but all Frizzled-1 and Frizzled-3 expressing neurons colocalized with both types of markers. There are a few likely explanations for this current finding. First, and most likely, there maybe some OSNs that never expressed Frizzled-1 or Frizzled-3 but could have expressed a different type of Frizzled receptor. It may be that certain subtypes of Frizzled receptors function with certain specific Olfactory Receptors in a cooperative manner. Secondly, it may be that some OSNs do not use Frizzleds as their targeting mechanism and express a similar but different family of receptors to accomplish the same function of the Frizzled receptors. Finally, it is plausible that the expression of these receptors has been turned off in some of these

mature neurons and that if we were to assess these neurons later in development, no Frizzled receptors would co-localize with mature OSN markers. This unexpected result will have to be looked into further since it would yield a better understanding of the Frizzled family of receptor temporal expression pattern.

In the current study Frizzled-1 and Frizzled-3 have been shown to be expressed early in mouse embryonic development. These proteins seem to be upregulated and expressed more heavily on OSNs during the onset and throughout the period that is essential for proper glomerular targeting. It is therefore likely that these proteins are highly involved in some aspect of OSNs intricate pathway from the olfactory epithelium into the glomerular layer of the developing olfactory bulb. Interestingly, these proteins are on neurons that express both markers of mature and immature OSNs. This suggests that these proteins have functions in both developing and mature OSNs. A question that is essential is whether these functions are the same. In addition to our hypothesis that these proteins are involved in neuronal targeting they may also have a role in stabilizing and maintaining correct glomerular targeting. In addition, another function of these proteins could be the modulation of synaptic strength by varying the degree of synaptic adhesion. Understanding the role of these proteins in the mature OSN and in adult life is essential to unlocking the questions that still exist about these proteins function in the developing olfactory system.

Further study is warranted that should involve a variety of approaches. First, the same experiments involved here should be extended to other members of this family to answer questions if different members of this family have same or differing functions. In addition, it would be interesting to determine if one neuron is expressing multiple types

of this family of receptors. Secondly, in vitro studies of olfactory epithelium and molecular ligands for these receptors should be tried in order to strengthen the hypothesis that these receptors are truly involved in neuronal targeting. Finally, experimental designs involving knockout models of these two proteins or in this family of proteins during the stages of olfactory neuronal outgrowth and targeting is necessary in order to determine if glomerular targeting is preserved without these genes.

The importance of solving this mystery of OSN targeting is essential and can have broad implications for both basic science and clinical research. Developing an understanding of both embryonic and adult neurogenesis and targeting has wide-ranging applicability to the treatment of debilitating neurological diseases and accidental trauma that occurs to the neurological system.

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