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Olfactory sensory axon growth and branching is influenced by Sonic hedgehog

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Abstract

Olfactory sensory neuron (OSN) axons extend from the olfactory epithelium to the olfactory bulb without branching until they reach their target region, the glomerulus. In this report we present evidence to support the involvement of *sonic hedgehog* in promoting rat olfactory sensory axons to branch and to enter into the glomerulus. Sonic hedgehog (Shh) protein is detected in the glomeruli of the olfactory bulb whereas its transcript is expressed in the mitral and tufted cells, suggesting that Shh in the glomeruli is produced by mitral and tufted cells. In primary OSN cultures, Shh-N peptide promotes olfactory axon branching. When Shh function is neutralized *in vivo* by its antibody, growth of newly generated OSN axons into the glomeruli is vastly reduced.

Keywords

trophic factor; olfactory bulb; glomerulus; axon branching

Introduction

Olfactory sensory neurons (OSNs) express one out of 1000 odorant receptor gene choices (Chess et al., 1994; Malnic et al., 1999; Rawson et al., 2000). Those that express the same receptor are distributed widely in the olfactory epithelium (OE), although they may be restricted to broadly defined zones (Ressler et al., 1993; Vassar et al., 1993). Despite their relatively wide distribution, their axons converge onto the same glomerulus in the olfactory bulb (OB) (Ressler et al., 1994; Vassar et al., 1994; Mombaerts et al., 1996). Several lines of evidence supported the notion that the odorant receptors play an instructive role in axon convergence and targeting (Mombaerts, 1996; Wang et al., 1998; Gogos et al., 2000). Once having reached a glomerulus the axons must enter, branch, find synaptic sites and stop growing (Halász and Greer, 1993; Klenoff and Greer, 1998; Kasowski et al., 1999). It is conceivable, and even likely, that these last steps of OSN maturation are regulated by mechanisms different from those that regulate axon convergence and targeting.

Axonal terminal branching is the first step of synaptic patterning in many developing neural pathways. In some systems, neurotrophins are shown to promote axonal arborization in the target region. For example, nerve growth factor (NGF) regulates collateral sprouting of cutaneous sensory nerves in the skin (Diamond et al., 1992), and in the *Xenopus* visual system, brain derived neurotrophic factor (BDNF) stimulates extensive branching of the optic nerve axons within the optic tectum (Cohen-Cory and Fraser, 1995). The recently

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identified *slit2* gene has been shown to be involved in promoting axon branching *in vitro* and may serve as a positive regulator for interstitial branching of spinal sensory neuron axons *in vivo* (Wang et al., 1999).

In this paper we examine the effects of Sonic hedgehog (Shh) as a regulator of axon entry into the glomerulus and of axon branching. Shh plays several essential roles in multiple aspects of vertebrate neural development (Goodrich and Scott, 1998; Marti and Bovolenta, 2002; Sanchez-Camacho and Bovolenta, 2008). It is a secreted signaling molecule generated by auto-proteolytic cleavage of its precursor protein and modified with a cholesterol group on the active N-terminal peptide (Shh-N) (Porter et al., 1995). Shh is known to regulate the behavior of retinal ganglion cell growth cones and therefore plays an important role in the patterning of neuronal connections in the visual system (Trousse et al., 2001; Sanchez-Camacho and Bovolenta, 2008).

Here we provide *in vivo* and *in vitro* evidence that Shh may be involved in two aspects of OSN axon development: the regulation of axon entry into glomeruli, and the branching of OSN axons after they enter. The spatial and temporal expression patterns of *shh* suggest that OB mitral and tufted cell dendrites participate in permitting OSN axons to enter the glomeruli and to branch in the target region. Neutralization of Shh function prevents axons from entering the glomeruli and branching.

Results

Shh expression in the developing olfactory bulb

Shh protein expression was examined in the rat olfactory system at different developmental ages, from E12 fetuses to adults. No Shh immunoreactivity was found in the OE throughout development, at all ages examined. However, Shh immunoreactivity was first detected in the OB at E19 as fibrous staining restricted to a few clusters only in the rostral portion of the OB (Fig. 1A). More intense, punctate staining was also observed within the fibrous immuno-positive cluster (Fig. 1C). The morphology and the location of the Shh immunoreactivity indicate that it was found in the earliest appearing glomeruli (Bailey et al., 1999). As development proceeded, immunoreactivity became more abundant as glomeruli became recognizable structures (Bailey et al., 1999; Treloar et al., 1999). At P0, more Shh immunoreactive clusters appeared in the rostral portion of the OB (Fig. 1A). At P2, Shh immunoreactivity was observed in the caudal portion of the OB (Fig. 1B). The shape of the Shh immuno-positive cluster is sometimes not globular, which is consistent with the fact that OSN axons and the dendrites of mitral and tufted neurons and interneurons are still organizing to form proto-glomeruli at this stage (Bailey et al., 1999; Treloar et al., 1999) (Fig. 1C, D). At P15, when mature glomeruli can be positively identified, Shh immunoreactivity was localized within the glomerular structure (Fig. 1F). Immunoreactivity appeared to peak around P25 and decreased to a very low level in the adult OB (Fig. 1G, H). The intensity of Shh immunoreactivity showed no gradient along either the dorsal-ventral or rostral-caudal axis at any age examined.

The “fibrous” immunoreactivity we observed in proto-glomeruli and glomeruli could be due to expression in one or more of the following: mitral and tufted cell dendrites, OSN axons or the dendrites of periglomerular neurons. All of these elements enter the glomerulus. In order to distinguish among these we investigated the expression of *shh* transcript to pinpoint the cell types that were responsible for Shh immunostaining in the glomeruli. By *in situ* hybridization, we detected *shh* transcripts in the mitral cell layer of the OB. Low levels of *shh* *in situ* signal were also present in the internal plexiform layer and the granule cell layer of the OB. Both mitral and tufted cells exhibited high levels of *shh* expression (Fig. 2). To further validate *shh* expression in the OB, we performed RT-PCR analysis (Fig. 2E). *shh*

transcripts were detected in the OB along with its receptor *smoothed (smo)*, co-receptor *patched-1 (ptc-1)* and transcription factors *gli-1*, *gli-2* and *gli-3*. Though *shh* signaling pathway members *ptc-1*, *smo*, and *gli-1* and *gli-2* were expressed in the OE, *shh* transcripts, along with *gli-3* were absent in the OE by RT-PCR analysis (Fig. 2E)

Shh promotes olfactory axon branching *in vitro*

To examine Shh function in regulating olfactory sensory axon growth and branching, we established a dissociated primary OSN culture system (Chen et al., 2008). We grew OSNs at a low density on a layer of cortical astrocytes for 24 hours, with and without addition of Shh-N. Under our culture conditions, most of OSNs extended their processes without attaching to each other, allowing the identification of individual neurons and their axons. The morphology of OSNs was examined by β -tubulin immunostaining. OSNs identified by β -tubulin type III immunostaining were bipolar and resembled their *in vivo* morphology in the control culture (Fig. 3A). OSNs can be cultured for several days and maintain their bipolar morphology (Chen et al., 2008). In this study, we investigated the effect of Shh on OSN axon morphology over 24 hours *in vitro*. After 24 hours in culture, OSN axons extended over distances without forming extensive branches. The average number of branch points of the OSN axons was 0.6 under control culture conditions (branch points represent the number of times the axon bifurcates, SE = 0.02, n = 35). When Shh-N peptide was added to the culture, olfactory axons exhibited significantly more branches than those of the control culture (Fig. 3B, C). The average number of branch points was 2.2 (SE = 0.03, n = 45, $p < 0.001$) in the Shh-N culture. The total length of the axons is slightly longer, but not statistically significant, in the Shh-N culture (150.5 μ m, SE = 1.29) than that of the control (127.3 μ m, SE = 1.27, $p < 0.06$, Fig. 3D). We did not detect qualitative changes in OSN numbers in our cultures. These data showed that Shh-N promoted axon branching *in vitro*.

OSNs were cultured on a bed layer of cortical astrocytes to obtain morphology close to that of *in vivo* with minimal branching. To investigate the possibility that the branching effect induced by Shh-N was a secondary effect through cortical astrocytes, we tested whether cortical astrocytes react to Shh-N signal under our culture conditions. If cortical astrocytes react to Shh-N, there would be an up-regulation of *ptc-1* transcripts in astrocytes by RT-PCR. We did not observe up-regulation of *ptc-1* transcripts in cortical astrocyte with or without Shh-N treatment (data not shown). Therefore, the morphological changes in OSN axons are likely a direct effect of Shh-N.

Blocking Shh function *in vivo*

To study the role of Shh in the glomeruli, we applied 5E1 hybridoma cells that secrete a function blocking antibody (IgG1) to neutralize Shh function in the OB. 5E1 hybridoma cells were implanted under the pial surface close to the midline just behind the OB in P0 rats. In each animal, the implanted hybridoma cells were found as a restricted tumor at five days after implantation (data not shown). Five days after the implantation, OBs were examined by GAP43 and OMP immunoreactivity to evaluate the distribution of immature (GAP43-positive) and mature (OMP-positive) olfactory axons (Verhaagen et al., 1989; Bailey et al., 1999; Treloar et al., 1999). 1B7 hybridoma cells, which secrete antibody (IgG1) against trinitrophenyl, were injected in the same manner into a group of control animals of the same age. At 5 day post-implantation for both control 1B7 and 5E1 hybridoma, laminar cyto-structure of the olfactory bulb appeared to be normal. We did not observe changes in the cell density of all layers of the olfactory bulb and glomerular sizes are comparable between the control 1B7- and 5E1-implanted animals.

In the OBs from control animals, GAP43-positive olfactory axons were distributed throughout the olfactory nerve layer and within the glomeruli of the OB. This indicated that

most glomeruli received ingrowth of immature OSN axons (Fig. 4A). OMP immunostaining was distributed throughout the olfactory nerve layer and was present in all of the glomeruli detected in the control OB as well, indicating the presence of mature OSN axons (Fig. 4B). In animals implanted with 5E1 hybridoma cells, GAP43-like immunoreactivity was also detected throughout the olfactory nerve layer. Moreover, staining appeared stronger in the olfactory nerve layer in some regions compared to comparable regions in control animals. However, a large percentage of glomeruli (80%, 218 out of 272 glomeruli) demonstrated low or undetectable levels of GAP43-like immunoreactivity, and only 20% of the glomeruli (54 out of 272 glomeruli analyzed) exhibited immunostaining at the comparable level as that of the adjacent nerve layer (Fig. 4C). We did not observe any distinct spatial distribution for glomeruli lacking or exhibiting low levels of GAP43 immunostaining along the rostral-caudal or dorsal-ventral axis of the OB. A quantitative measurement of GAP43 staining intensity was collected from the glomerular region and compared to their adjacent nerve layer region (see method section for detail). In the control OBs, the intensity of GAP43 immunostaining in the nerve layer ($I_{NL} = 124.7$, $SD = 12.3$) was slightly stronger than that in the glomeruli ($I_{GL} = 82.4$, $SD = 7.9$, $p < 0.005$) and the GAP43 staining intensity ratio between the nerve layer and the glomerular layer was 1.5 (Fig. 4F). In 5E1-implanted animals, GAP43 staining intensity was higher in the olfactory nerve layer ($I_{NL} = 169.3$, $SD = 22.6$) and much lower in the glomerular layer ($I_{GL} = 35.2$, $SD = 5.1$) compared to the nerve layer ($p < 0.005$). The ratio of staining intensity in 5E1 animals ($I_{NL}/I_{GL} = 4.8$) represents a dramatic decrease in GAP43-positive olfactory axons in the glomeruli. The GAP43 staining intensity is more variable between different locations in 5E1-implanted OB than that of the controls, demonstrated by their standard deviations of staining intensity. However, no spatially restricted distribution of such differences was observed. This evidence suggested that, when Shh function blocking antibody is present in the OB for five days (Fig. 4E), many immature olfactory axons did not enter the glomeruli. OMP immunostaining intensity was also analyzed in both control 1B7-implanted and 5E1-implanted olfactory bulb (Fig. 4D). No difference in the staining intensity ratio was observed between the control 1B7-implanted OB ($I_{NL}/I_{GL} = 1.21$, $SD = 0.37$) and 5E1-implanted OB ($I_{NL}/I_{GL} = 1.16$, $SD = 0.26$; $p > 0.1$). Thus, the Shh blocking antibody may not have interfered with axons that had entered the glomerulus before the hybridoma was implanted.

Discussion

The formation of glomerular structure in the OB is a highly regulated process. Here we report the expression of Shh in the glomeruli during early postnatal stages. Shh is able to regulate olfactory sensory axon terminal branching. When Shh function is blocked during early postnatal stages, olfactory axons were seen accumulating outside the glomerular structure, suggesting that Shh is required for promoting olfactory axon entry into this region.

We showed in this report that *shh* mRNA is expressed by mitral and tufted cells in the OB. Shh protein, most likely transported through mitral/tufted cell dendrites, was detected in the early appearing protoglomeruli during development and persisted in mature glomeruli into adulthood. However, contributions from the other cell types cannot be completely ruled out because we also detected low levels of *shh* transcripts in the astrocytes or juxtglomerular neurons in the glomerular layer. Cultured OSNs, after exposing to Shh protein, exhibit more axonal branches than the controls. These data, taken together, suggest that Shh is involved in growth and branching of olfactory axon terminals and their entry into OB glomeruli.

Shh is an important signaling protein in neural development

Hedgehog function is not restricted to nearby targets by the cholesterol modification tying it to cell membranes (Porter et al., 1996), but is also involved in long-range target activation.

For example, the observation of long cytoplasmic processes (cytonemes) produced by target cells makes it plausible for Hh-N to act via direct cell-cell interaction even when the cell bodies are separated by substantial distances (Ramirez-Weber and Kornberg, 1999). In addition, the discovery of *Drosophila tout-velu* suggests that transcellular trafficking of cholesterol-modified Hh-N may occur and probably involves proteoglycans (The et al., 1999). Hedgehog can also be transported through axons to signal over distances. In the *Drosophila* visual system, photoreceptors in the eye imaginal disc transmit Hh through their axons to reach the target ganglion, the lamina. Hedgehog secreted by photoreceptor axons triggers neurogenesis to control the number of target neurons (Huang and Kunes, 1996; Huang and Kunes, 1998). Our observations in the olfactory system suggest that Shh protein is transported via mitral and tufted cell dendrites to the glomerulus where it interacts with olfactory axon terminals. Further investigation is needed to confirm this hypothesis.

Shh plays multiple roles during neural development (Marti and Bovolenta, 2002). Shh is known to influence the behavior of chick retinal ganglion cell growth cones. Ectopic expression of *shh* along the visual pathway alters the trajectory of the retinal ganglion cell axons. In addition, providing Shh-N coated beads suppresses neurite outgrowth from retinal explants. It appears that the Shh-mediated growth cone arrest is dependent on the intracellular level of cyclic AMP (cAMP). The presence of Shh-N *in vitro* induces a decrease in the level of cAMP inside the growth cone. In this study we have shown that the Shh seems to promote the growth and the branching of the olfactory sensory axons. Whether or not this phenomenon is also mediated through changes in cAMP levels in the growth cone remains to be tested. Changes in cAMP or cGMP signaling pathways can elicit opposite effects in growth cone behavior (Song et al., 1998). It is conceivable that the olfactory sensory axons react to the Shh signal in a fashion opposite that of the retinal ganglion cells.

Shh function in glomerular formation

Glomerular formation has been studied in both invertebrates and vertebrates but some of the molecular signals involved in this process are not clear. Most investigators agree that glomerular formation is initiated by an interaction between OSN axons and the OB (Oland et al., 1988; Valverde et al., 1992; Oland and Tolbert, 1998; Bailey et al., 1999; Treloar et al., 1999). Morphological changes during glomerular initiation have been investigated in detail in rats. From the reports of both Treloar et al. (1999) and Bailey et al. (1999), OSN axons first coalesce into proto-glomeruli at E19. The presence of glial cells is required in early glomerular formation (Oland et al., 1988). The proto-glomeruli develop into mature glomeruli in early postnatal life in rodents. The formation of glomeruli may follow a rostro-caudal gradient within the OB. In our experiments, the temporal and spatial expression of Shh correlates with this possible developmental sequences of glomerular formation. Multiple cell types are involved in the formation of glomeruli during postnatal development (Bailey et al., 1999; Treloar et al., 1999; Blanchart et al., 2006). Though Shh-N peptide regulated OSN axon growth behavior *in vitro*, blocking Shh function postnatally for 5 days did not appear to alter the size of the glomeruli in the olfactory bulb. During postnatal development, in addition to the ingrowth of OSN axons, several developmental events are progressing simultaneously. Developmental events contributing to the formation of glomeruli include olfactory axon terminal arborization within the glomeruli, ingrowth of immature olfactory sensory axons, maturation of innervated olfactory axons (from GAP43-positive to OMP-positive), ingrowth of periglomerular neuron dendrites and elaboration of mitral cell dendritic tufts within the glomeruli (Halász and Greer, 1993; Malun and Brunjes, 1996; Klenoff and Greer, 1998; Lee et al., 2008). Therefore, short-term decreased GAP43-positive axon ingrowth may not necessarily alter the sizes of the existing glomeruli. It is reasonable to speculate that if Shh function is blocked for longer period of time, glomerular size may be altered.

Mitral and tufted cells are not considered essential for the initiation of glomerular formation. In moths, no obvious disruption was observed in the initiation of glomerular formation when mitral-like cells were surgically removed during development. (Oland and Tolbert, 1998). This appears to be true in mammals as well. In *Tbr-1* knockout mice, the mitral and tufted cells fail to develop in the OB. Even in the absence of mitral and tufted cells, olfactory axons expressing the same odorant receptor are able to converge and enter the bulb at their “appropriate” positions (Bulfone et al., 1998). Although these studies provide evidence that mitral and tufted cells are dispensable for the initiation of OSN axon convergence and proto-glomerular formation, they do not rule out the possibility that the mitral/tufted cells plays a role to assist the target recognition and initiation of synapse formation.

Mitral and tufted cells express semaphorin 3A (Sema 3A), which has been shown to have a chemorepellent function (i.e., it inhibits axonal extension and induces growth cone collapse) (Giger et al., 1998). The receptor of Sema 3A, neuropilin-1, is expressed by primary olfactory neurons (Kawamaki et al., 1996). Kobayashi et al. (1997) have done *in vitro* experiments to demonstrate that chick OSN axons collapse when Sema 3A is present in the culture (Kobayashi et al., 1997). These findings strongly support the notion that the mitral/tufted cells participate in influencing the growth behavior of OSN axons in the OB.

This study provides evidence that Shh is part of the molecular complex that regulates OSN axon growth into the glomerulus. When Shh function is blocked between P0 to P5, we observed severely reduced ingrowth by immature olfactory axons into glomeruli. Between P0-P5, olfactory axons from different maturation stages normally can be found in the glomeruli. Some will have entered the glomeruli already and will be essentially mature (i.e., they had entered the glomerulus before P0 and had stopped growing); these would be OMP positive and GAP43 negative. Axons from more recently generated neurons will have just entered the glomerulus and would still be GAP43 positive and OMP negative. And some, from even more recently generated neurons, will still be growing toward their target glomerulus and would be GAP43 positive and OMP negative. This finding that inhibiting Shh function results in an accumulation of immature OSN axons in the nerve layer and decreases the number of OSN terminal branches in the glomeruli, combined with our knowledge in the expression of growth inhibitory molecules, together argue that Shh participates in gating axon entry into glomeruli either directly, as an attractant, or indirectly, by disinhibiting the repellent.

In addition to Sema 3A and Shh, several other molecules may participate in regulation of growth and synapse formation in the OB. For example, some may act to prevent OSN axon growth beyond the glomerular layer (Gonzalez and Silver, 1994; Kafitz and Greer, 1998). These growth regulating signals could be produced by mitral/tufted cells, juxtglomerular neurons and/or glial cells. Axonal entry into glomeruli and branching may involve complex integration of both positive and negative signals. Our studies suggest that Shh may serve as one of the signals in this process.

A characteristic behavior of OSN axons when they enter a glomerulus is to form branches. Target-derived factors play important roles in axon branching and terminal arborization in multiple systems (Diamond et al., 1992; Cohen-Cory and Fraser, 1995; Wang et al., 1999). Our *in vitro* evidence supports the notion that Shh is one of the signals involved in OSN axon branching. When OSNs were treated with Shh-N *in vitro* we observed a significant increase in the numbers of axon branch points. It is not yet clear why the OSN axons failed to enter the glomeruli when Shh function was blocked. It is possible that Shh provides the general target recognition signal to help OSN axons innervate the glomerular region. It is also possible that Shh serves as a growth promoting signal to the OSN axons. We did observe a slight increase in OSN axon elongation when Shh peptide is provided *in vitro*.

Therefore, stalling of the immature OSN axons in the nerve layer could be caused by disturbance of the delicate balance between inhibiting and promoting cues in the glomeruli for OSN axons. When one of the promoting cues was missing, a negative growth environment was created, and OSN axons remained within the nerve layer. Further study is needed to investigate these hypotheses.

Experimental Procedures

Immunocytochemistry and RNA in situ hybridization

Pregnant Sprague-Dawley rats were used in this study. The date the copulation plug was found was defined as embryonic day 0 (E0). All animals used for immunocytochemistry and in situ hybridization were perfusion fixed with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS, pH 7.4) and were subsequently post-fixed by immersion in the same fixative overnight. To obtain frozen sections, fixed tissues were cryoprotected with 30% sucrose overnight, embedded in O.C.T. compound and sectioned at a thickness of 14 μ m. To obtain paraffin sections, fixed tissues were washed with PBS, dehydrated through a ascending series of ethanol, embedded in paraffin and sectioned at a thickness of 10 μ m as described previously (Weiler and Farbman, 1997).

Immunocytochemistry was done essentially as described (Gong and Shipley, 1995). Briefly, sections were post-fixed with 4% paraformaldehyde for 15 min at room temperature. After three rinsing steps, sections were blocked with 5% normal horse serum followed by incubating with primary antibodies overnight at 4°C. Antibody against Shh (C-18, Santa Cruz, CA) was used at 4 μ g/ml; monoclonal anti-GAP43 was used at 1:1000 (Sigma, St. Louis, MO); monoclonal anti- β -tubulin isotype III was used at 1:400 (Sigma), goat polyclonal anti-OMP was used at 1:1000 (kindly provided by F. L. Margolis). After washing, sections were incubated with biotinylated secondary antibodies (Jackson ImmunoResearch). Immuno-signals were amplified by VECTASTAIN ABC kit (Vector Lab) and detected by peroxidase substrate DAB.

RNA in situ hybridization was performed on 14 μ m frozen sections of the olfactory tissue. Riboprobes were made with FITC-labeled ribonucleotide mix (Boehringer, Indianapolis, IN). *shh* cDNA-containing plasmids (generously provided by A. P. McMahon) were linearized with EcoRI and antisense riboprobes were transcribed with T7 RNA polymerase. In situ hybridization was done essentially as described (Gong et al., 1999). Briefly, 14 μ m frozen sections were post-fixed with 4% paraformaldehyde. After rinsing with PBS and acetylation, sections were incubated in hybridization solution without probe for 1 hour (hybridization solution: 50% formamide, 5 $\times$ SSC, 100 μ g/ml tRNA, 500 μ g/ml herring sperm DNA and 50 μ g/ml of heparin). Hybridization was carried out at 65°C overnight with probe concentration of 0.5 ng/ μ l. Sections were washed with 50% formamide and 5 $\times$ SSC at 70°C twice for 15 minutes each, followed by 0.2 $\times$ SSC at 70°C, and further washed with PBS plus 0.1% Tween-20 at room temperature for 45 minutes. Hybridized probes were detected by alkaline phosphatase-conjugated antibody against FITC (1:1000, Boehringer), and the signals were developed by 4-nitro blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). To illustrate cytostructure of the OB, adjacent sections were counterstained by Nissl stain following the established protocol (McCasland and Graczyk, 2000).

Olfactory sensory neuron culture

OSNs obtained from E21 rats were cultured on a cortical astrocyte feeder layer in Waymouth's MB 752/1 medium containing N2 supplements (GIBCO) and 100 μ g/ml gentamicin (WayN2 medium).

Astrocytes were harvested from E21 rat cerebral cortex. After dissociation with 0.2% trypsin and 500 U/ml DNase I in CMF-HBSS/HEPES for 15 minutes at 37°C, cells were triturated and filtered through 80 µm nylon membrane. Cortical cells were then plated in a 75 cm² flask (10⁷ cells per flask) and cultured in DMEM plus 10% Fetal Bovine Serum (FBS) until confluent. The flasks were then shaken on a rotatory platform at 275 rpm overnight at 37°C to remove loosely attached neurons. To eliminate contaminating fibroblasts, astrocyte cultures were then treated with 200 mM cytosine-β-D-arabinofuranoside (Ara-C) for 48 hours and subsequently washed and recovered in DMEM plus 10% FBS for 24 hours. Pure cortical astrocytes were replated on 12 mm round cover slips for the olfactory neuronal cultures.

Olfactory epithelial sheets were dissected from the septum region in the nasal cavity. After treatment with 2 mg/ml Dispase and 500U/ml DNase I for 15 minutes at 35°C, lamina propria and connective tissue were separated from the OE using tungsten needles. The olfactory epithelial sheets were then incubated in suspension for 2 hours in WayN2 medium. Cells in the OE were treated with 0.05% trypsin with 0.5 mM EDTA for 10 minutes at 37°C followed by adding 10% FBS to stop trypsin activity. After trituration, cells were filtered through 20 µm nylon mesh. Olfactory neurons were plated at a density of 10⁵ cells/cm² in 24 well plates (Corning) on the cortical astrocyte bed layer in WayN2 medium. Shh-N peptide (B & D Systems) was added to the olfactory neuronal culture at a concentration of 0.2 µg/ml. Control and Shh-N peptide containing cultures were fixed with ice cold 4% paraformaldehyde after 24 hours at 37°C and immunostained with an antibody against isotype III β-tubulin to identify olfactory neurons. The length of the neurites was measured using Scion Image software. Axonal process and their branches are measured and added together to obtain total axon length of each OSN. Each time the axon bifurcates is defined as a branch point. The number of branch points of each OSN was analyzed and compared between control and Shh-N treated populations.

Injection of hybridoma cells

The 5E1 hybridoma cell line which secretes functional blocking antibody (IgG1) against Shh-N was made by J. Ericson et. al. and obtained from the Developmental Studies Hybridoma Bank (Ericson et al., 1996). The 1B7.11 cell line which produces antibody (IgG1) reactive with 2,4,6-trinitrophenyl (TNP) was used as a control (obtained from American Type Culture Collection). Both 5E1 and 1B7 cells were harvested with 0.05% trypsin in Dulbecco's Modified Eagle Medium (DMEM, GIBCO BRL, Grand Island, NY) for 2-3 min at 37°C. After washing with DMEM, cells were diluted with DMEM and 500 U/ml DNase I to a density of 5×10⁵ cells/µl. Using a Hamilton syringe with a 33 gauge needle, hybridoma cells in a volume of 2-3 µl (~10⁶ cells) were implanted into P0 rats. The injections were aimed under the pial surface at the midline around the caudal end of the OB. P0 rats were injected with either 5E1 (n = 7) or 1B7 (n = 8) hybridoma cells and sacrificed at 5 days post-implantation to examine their effects on olfactory axon distribution. Animals were perfusion-fixed as described above and processed for GAP43 and OMP immunostaining to visualize axons from immature and mature olfactory neuron axons respectively (Chen et al., 2005).

Image analysis

The staining intensity of GAP43 in both control and 5E1-implanted animals was measured using Photoshop software. Grayscale, darkfield images of GAP43 immunostained OB sections were obtained. Every 8th of the serially sectioned OB were selected. In each selected section, an area was selected within each glomerulus and a same sized area in the nerve layer next to the selected glomerulus using Marquee tool and the mean value of the luminosity was obtained from the histogram and was documented as GAP43 staining

intensity. Using this method, we measured the staining intensity of GAP43 and OMP in the glomeruli and the adjacent olfactory nerve layer. Same area size was chosen to ensure that we compare the same value from the same number of pixels. We measured 272 glomeruli and their adjacent nerve layer from three 5E1-implanted animals and 202 glomeruli and their adjacent nerve layer from two control animals. Student t-test was performed to determine statistical significance.

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Figure 1.

Sonic hedgehog is expressed in the early appearing glomeruli. Sagittal sections of the OB are shown with the rostral tip pointing to the right. Shh protein is first detected in the rostral part of the OB at E19 coincidental with the formation of the early appearing protoglomeruli (A). More Shh staining is detected in the developing glomeruli at P2 (B). High magnification images show that the Shh staining is fibrous (arrowheads, C, D). Arrows in A and B indicate Shh-positive clusters shown in C and D respectively. Shh expression is developmentally regulated. The level of Shh increases as the expansion of the glomeruli continues from P0 (A), P15 (B) to P25 (C). The intensity of Shh staining appears to peak

around P25 and then declines till P65 (D), which is considered as adult. Arrows point to Shh-positive glomeruli in A-D; Bar = 200 μm in A, B; 20 μm in C, D; 100 μm in E-F.

Figure 2.

shh transcript is present in the mitral and tufted cells. *shh* transcripts were detected by in situ hybridization in the mitral cell layer and, at lower levels, in other layers of the OB at P2 (A). Control was performed on P2 OB sections using a sense probe (B). Higher magnification image demonstrates that *shh* transcripts were present in the mitral and tufted cells (arrows in C). The adjacent section was counterstained by cresyl violet based Nissl staining to demonstrate the cyto-architecture of the OB (D). (E) Transcripts of *shh*, its receptor *smo*, co-receptor *ptc-1*, signaling pathway members *gli-1*, *gli-2* and *gli-3* were all detected in the OB by RT-PCR. No *shh* transcripts were detected in the OE. Bar = 200 μ m in A, B; 50 μ m in C, D.

Figure 3. Shh-N peptide promotes olfactory axon branching *in vitro*. OSNs were cultured at a low density on the feeder layer of cortical astrocytes. The morphology of the olfactory receptor neurons was examined by β -tubulin III immunostaining 24 hours after the treatment of Shh-N peptide and compared to the control. More axonal branches were observed in Shh-N culture (B) compared to that of the control culture which contains olfactory receptor neurons bearing bipolar morphology (A). The average number of branch points in the Shh-N culture was 2.2 (SE = 0.03, n = 45) compared to that of the control culture which was 0.6 (SE = 0.02, n = 35, $p < 0.001$). The total length of the axon in the Shh-N culture was 150.5 μ m (SE = 1.29) compared to that of the control which was 127.3 μ m (SE = 1.27, $p < 0.06$).

Figure 4.

Blocking Shh function *in vivo* interferes with the entry of immature olfactory axons into glomeruli. 5E1 hybridoma cells, which produce functional blocking antibody against Shh, are injected behind the OB at P0. 1B7 hybridoma cells, which produce antibody against trinitrophenyl, are injected into P0 rats as controls. OB sections were examined for the distribution of GAP43 (A,C) and OMP (B,D) immunoreactivity in the nerve layer (NL) and the glomerular layer (GL) at P5. After blocking Shh function for 5 days, GAP43 staining is significantly lower in the glomeruli (circled with dotted line, in C) compared to uniform staining in the glomeruli in the control animal (A). GAP43 staining appears to be higher in the nerve layer in 5E1-implanted animals than that of control. The distribution of OMP immunostaining appears similar between 5E1-implanted (D) and control animals (B). Presence of the monoclonal antibody in 5E1-injected OB was validated by western blotting. Both heavy and light chain of mouse IgG was detected in the rat OB (E). GAP43 staining intensity was quantified and significant reduction in glomerular expression was observed in 5E1-implanted OB (F). Bar = 100 μ m.