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Reconstructing Smell

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Abstract

Odorant signal transduction and neurogenesis are fundamental properties of the olfactory epithelium. Many preparations have been used to elucidate some of the mechanisms underlying these properties. In this article, we briefly review these research areas and describe some of the techniques used to obtain the data. We focus specifically on the cell-culture paradigm and the data obtained from various immortal cell lines in their attempts to reconstruct the olfactory epithelium in vitro.

Index Entries: Olfactory epithelium; odorant receptor; olfactory receptor neuron; neurogenesis; cell lines; cell culture.

Introduction

The olfactory epithelium is the prime focus for two areas of experimental research. The first concerns the mechanisms that exist for discrimination of odor quality. This includes the physiology of the olfactory receptor neuron, the molecular events surrounding odorant binding, the factors that affect neuronal projections to the olfactory bulb, and the interpretations that can be made following olfactory receptor neuron activation. The second area of research reflects a unique property of the olfactory epithelium, namely its ability to undergo continual neurogenesis. This postnatal neurogenesis, and the accessibility of the olfactory epithelium for in vitro culture, provides an ideal opportunity to investigate neuronal development and regeneration. This article summarizes the molecular events underlying olfactory signal transduction and olfactory neurogenesis before focusing on the different preparations of the olfactory epithelium and how they can be used to further our understanding of olfactory neurobiology.

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Olfactory Signal Transduction

Over the last two decades, the application of molecular biological techniques to neuroscience has exploded. Specifically, in olfaction, we have seen cloning of the members of the signal-transduction cascade (Fig. 1), including the odorant

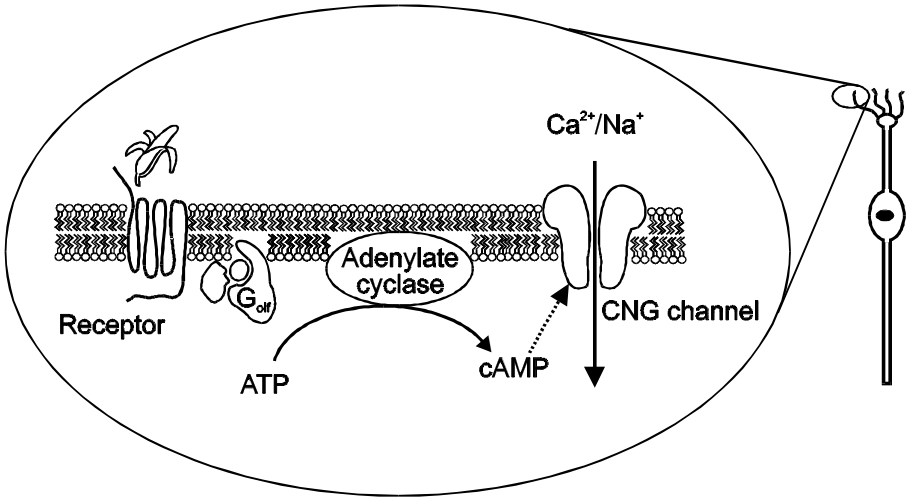

Fig. 1. Olfactory signal transduction. In the cilia of an olfactory receptor neuron, an odorant binds to a 7-transmembrane-spanning domain odorant receptor. Binding of the odorant to the receptor leads to activation and dissociation of the Gαolf G-protein subunit from the βγ complex. The activated Gαolf stimulates the Type III adenylate cyclase, which converts ATP into cAMP. In turn, cAMP opens the cyclic-nucleotide gated (CNG) ion channel and cation entry through the ion channel causes the cell to depolarize. Depolarization results in the generation of an action potential that is propagated along the axon to a synapse in the olfactory bulb.

receptors, Gαolf, a type III adenylate cyclase, and multiple subunits of the olfactory cyclic-nucleotide gated (CNG) ion channel *(1–7).* It is believed that one member of the odorant-receptor gene family is expressed in a single olfactory-receptor neuron *(8)* and following odorant binding to this receptor, activation of the G-protein-mediated signal-transduction pathway ensues. Activation of this pathway results in an increase in cAMP that in turn activates the cation-permeable CNG channel and causes depolarization *(9–11).* This excitation is carried along the axon of the olfactory-receptor neuron to the point of the first synapse in the olfactory bulb, where all neurons expressing a single receptor species converge on a small number of glomeruli *(12,13).*

In addition to the main olfactory organ, distinct receptor families (V1Rs and V2Rs) have been shown to be expressed in neurons of the vomeronasal organ, and each family of receptors appears to be associated with a separate signal-transduction mechanism *(14–16).* In direct contrast to the main olfactory system, these neurons project to 10–20 glomeruli in the accessory olfactory bulb, *(17,18)* and have recently been reviewed *(19,20).*

The combinatorial conundrum that underlies comprehension of the olfactory-receptor code and discrimination of odor quality is enormous. The odorant receptor gene family is thought to number ~1000 members in mammals and believed to be capable of discriminating somewhere in the region of 100,000 odors. In fish, these numbers may be reduced by a factor of 10 *(21).* However, it is only recently, almost 10 years after the initial cloning of these putative receptors, that any functional evidence supporting a role in odorant detection has been described. These pieces of evidence, and the experiments through which they were obtained, have been recently reviewed by Mombaerts *(22).* Briefly, they include the identification of 1) diacetyl as a ligand for the *Caenorhabditis elegans* odr-10 receptor in behavioral studies; *(23),* 2) octyl aldehyde as a ligand for the rat 17 receptor following adenovirus-mediated transfection in vivo *(24)*; 3) receptors that respond to carvone, or citronellal following the transient overexpression of chimeric mouse receptors *(25);* and 4) a

goldfish receptor for basic amino acids from an expression cloning strategy *(26).* Additionally, single-cell reverse transcription polymerase chain reaction (RT-PCR) has indicated that single receptors can be obtained from single olfactory receptor neurons and from prior physiological experiments putative ligands can be ascribed to these receptors *(8,27).* What stands out from these experiments is that most receptors that make up the olfactory-receptor gene family are still orphans. Clear structure/function relationships are absent and this makes our understanding of how molecular events at the olfactory epithelium can be integrated into a conscious description of odor quality technically challenging.

One explanation for the absence of an abounding number of reports has been summarized by studies by McClintock and coworkers *(28).* They have constructed numerous chimeric receptors from the odorant receptors U131 and OR5 and the β_2 adrenergic receptor that have either N- or C-terminal green fluorescent protein (GFP) tags. These data demonstrate that when these receptors, or some of their chimeric constructs, are expressed in heterologous systems, the receptors are retained by the endoplasmic reticulum. As discussed previously, this trafficking problem has been overcome in a few cases. Most notably this has been demonstrated by some receptors that have been tagged at their 5′ end with leader sequences of either rhodopsin or serotonin receptors *(25,29).* Recently however, the U131 receptor, which could not be expressed by McClintock et al., has been expressed. The cells used in this study were a conditionally immortal culture of olfactory-receptor neurons *(30)* and will be discussed again in subsequent paragraphs.

Olfactory Neurogenesis, Development, and Regeneration

The olfactory receptor neuron is a bipolar cell. Cilia are exposed to the atmosphere in the mucus covering the olfactory epithelium and are thought to be where odorants bind to receptors. The cilia are attached to the dendritic knob that projects onto the neuronal cell body. Distal from the cell body is the axon that projects through the cribiform plate into the olfactory bulb. The olfactory epithelium is composed of three major cell types and these are distributed in layers through the epithelium (Fig. 2). Underneath the mucus layer, and through which the dendritic knob of the olfactory receptor neuron projects, are the sustentacular cells or supporting cells. These cells are thought to support the function or survival of olfactory receptor neurons by metabolizing odorants, the removal of foreign particles or by maintenance of the mucus fluidity on the nasal epithelium *(31,32).* Below the sustentacular cells are the cell bodies of the olfactory receptor neurons that have migrated up from the layer of basal cells at the bottom of the olfactory epithelium. The basal cells of the olfactory epithelium can be subdivided into two populations, horizontal and globose. Typically, the globose basal cells are situated more superficially in the epithelium than the horizontal cells. The horizontal cells stain more darkly with toluidine blue and stain positive for cytokeratin, in contrast to the globose basal cells, which are only lightly stained with toluidine blue and are negative for cytokeratin *(33,34).* Progeny of both populations of cells incorporate ^{3}H-thymidine *(35,36)* and have been shown to migrate towards the apical surface of the epithelium. Of these two populations, the globose basal cells are thought to be the neuronal precursors. The reasons for this are that the cells found in closest proximity to newly generated neurons (designated immediate neuronal precursors in *[33]*) are cytokeratin-negative, and that these cells disappear with a similar time course to the appearance of neuron-specific tubulin immunoreactivity in olfactory cultures in vitro. Subsequently, a retroviral labeling technique has demonstrated a direct pathway from the globose basal cells to mature olfactory receptor neurons *(37).*

There are numerous factors that influence the state of the olfactory epithelium. Because

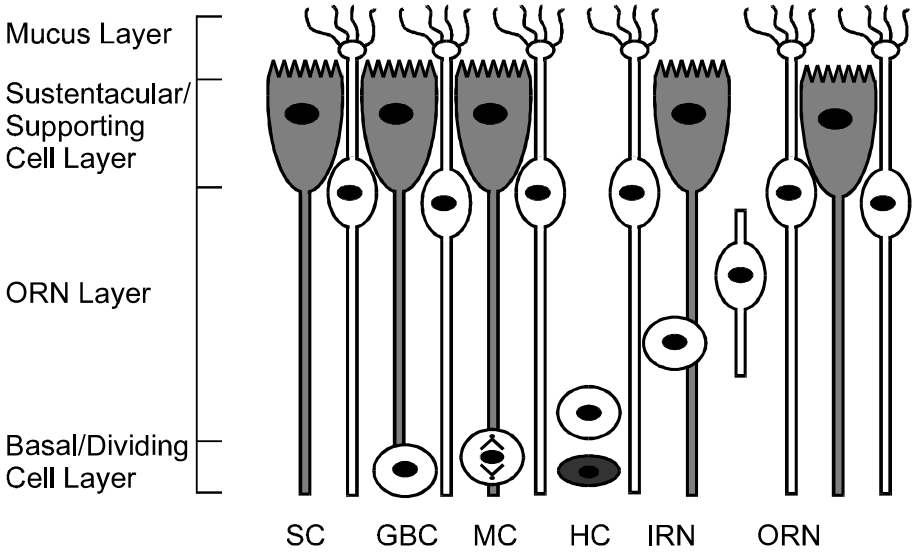

Fig. 2. A schematic diagram of the olfactory epithelium. Globose basal cells (GBC), situated at the base of the olfactory epithelium, are thought to be the precursors of olfactory receptor neurons (ORN, clear cells). In the process of neurogenesis, globose basal cells are thought to undergo mitotic cell division (MC, mitotic cell) and to become immature neuron precursors/immature receptor neurons (IRN). These cells then mature into olfactory receptor neurons. Coincident with maturation, the cell body of the neuron migrates apically through the epithelium. The second basal cell, the horizontal cell is shown (HC, darkest cell) as well as several sustentacular or supporting cells (SC, mid-tone cells)

the olfactory epithelium is exposed to the environment, there is a continual loss of cells, arguably akin to the sloughing of skin. This loss requires continual replenishment and it has been proposed that the half-life of an olfactory-receptor neuron is between 30 and 90 d, depending upon the surrounding conditions *(38,39).* Some of the factors that have been reported to be involved in olfactory neurogenesis include nerve growth factor (NGF), epithelial growth factor (EGF), transforming growth factor α (TGF-α), brain-derived neurotrophic factor (BDNF), neurotrophin-3, leukemia inhibitory factor (LIF), and others. For each of the factors listed, the presence of the factor itself, a cognate receptor, and a physiological function have been described in the olfactory epithelium *(40–54).* In order to assess the function of these factors and to delineate the processes of neurogenesis and regeneration in the olfactory epithelium, two categories of experiments have been used. These involve: 1) fairly drastic manipulations of the olfactory system in vivo, or 2) the development of primary or even immortal cell cultures.

In Vivo Manipulations of the Olfactory Epithelium

To investigate the processes of neurogenesis and apoptosis in the adult animal, several different paradigms have been developed. These include nares occlusion, lavage of the olfactory epithelium with zinc sulfate or detergent, olfactory-nerve transection, and olfactory bulbectomy. In each of these experimental procedures, neuronal turnover is enhanced, providing a physiological correlate for in vitro data. A detailed review has been published recently in which these tech-

niques are compared *(55)* and the information will only be summarized briefly here.

The olfactory bulb is required for survival of olfactory receptor neurons *(56,57).* Removing the bulb causes a hyperinduction of the olfactory receptor neuron life-cycle, in which there is a peak of apoptosis 2 d after surgery. Associated with the degeneration of the olfactory epithelium, there is an increased proliferation of neuronal precursors, reaching a peak 5–6 d after bulbectomy *(47).* This sequence of events suggests a time frame during which the level of expression of factors relevant to neurogenesis and olfactory receptor neuron survival may be increased, facilitating their identification. This increased proliferation has also been used to promote survival in a primary olfactory-receptor-neuron culture from adult animals *(58).*

Olfactory receptor neuron precursors can be induced to regenerate synchronously following ablation of the olfactory epithelium with a detergent or zinc sulfate lavage. The time course for this paradigm is longer than with olfactory bulbectomy and occurs over at least 6 wk *(59,60).* However, it preserves the olfactory bulb and allows the restoration of synaptic connections. Olfactor nerve transection similarly preserves the olfactory bulb and can be used, for example, for investigating factors that are involved in neurogenesis in the olfactory epithelium *(61)* and the role that olfactory receptor neurons play in olfactory-bulb development *(62).*

The fourth in vivo preparation is nares occlusion and has been used extensively by Brunjes and coworkers *(63).* This protocol involves the reversible closure of a naris in either rats or mice but, as these animals are obligate nose breathers, it is crucial that the closure is unilateral. In these experiments, cellular changes in the olfactory epithelium and olfactory bulb in response to alterations in ambient odor levels can be assessed.

Olfactory Cell Culture

Cell-culture systems form the other major paradigms for investigations of olfactory neuronal function. Many cultures are isolated acutely and have provided much data for physiological studies. Of these, most have been obtained in enzyme-free conditions, *(64–67)* although enzymic digestions of the epithelia with trypsin, papain, or collagenase have been used successfully *(68–73).* These methods of cell isolation are very useful for preparing cells for either fluorescence imaging or patch-clamp electrophysiology, but can be characterized by low yields, suspect viability, and abnormal cellular morphology. For the longer-term cultures, in which cells are needed either for neurogenesis or growth-factor investigations, typically enzymes are used.

Collagenase, a collagenase/hyaluronidase mix, or a collagenase/trypsin mix have been used for the generation of olfactory receptor-neuron cultures from mature or early neonatal rats *(74,43,44).* In the embryonic (E14.5-15.5) mouse preparation, used by Calof and coworkers, a trypsin-pancreatin solution is used for the dissociation of cells *(33).* These protocols generate an increased number of cells compared to the enzyme-free preparations, providing opportunity for the developmental studies to be performed.

Different media and substrates have been used in these preparations. Generally, standard substituted minimum essential media are used but Ronnett et al. *(43)* have used a media containing D-valine. Fibroblasts, being of mesenchymal origin, lack the D-amino acid oxidase that is present in cells originating in the neuroectoderm. The nominal removal of L-valine apparently inhibits fibroblast survival and growth *(75)* allowing preferential survival of the olfactory receptor neuron. To enhance neuronal adhesion, cell-culture materials have been pretreated with laminin, concanavalin A, fibronectin, or poly-D-lysine hydrobromide followed by laminin and fibronectin *(43,74,52,33).* These preparations enhance olfactory receptor neuron adherence and may benefit the cultures additionally, as glial cells do not adhere efficiently to laminin-coated culture dishes *(43).* The other technique that has been reported to improve olfactory receptor neuron culture is the

growth of cells on feeder layers of cortical astrocytes. Increased longevity in culture of olfactory receptor neurons from embryonic rats has been described *(76)*, as have increases in survival, cell division, and differentiation of olfactory cultures in the presence of astrocytes *(77)*.

Significant advances in our understanding of the olfactory system have been obtained using these preparations, specifically in relation to the processes and control of neurogenesis in the olfactory epithelium *(33,50,78)*. Additionally, these cultures have been used for biochemical studies that demonstrate secondary responses that occur following odorant stimulation. These include a delayed elevation of cGMP and phosphorylation of the cAMP response element binding protein after 2 and 30 min, respectively *(79,80)*. However, for neurogenesis and growth-factor studies, cellular heterogeneity exists that may complicate the data obtained. In one example, fibroblast growth factor (FGF) has been reported both to promote and delay neuronal differentiation, though the reasons for this are not entirely clear *(50,52)*. Similarly, the age of the animal from which cells are derived appears to affect the survival of the olfactory receptor neurons. When the olfactory epithelium was isolated from mature animals, the number of cells with immunoreactivity for neuron-specific tubulin and olfactory marker protein, a marker of mature olfactory receptor neurons, decreased rapidly with time *(74)*. In cultures obtained from early postnatal animals, temporal survival may be improved *(81)* but is longest when tissue is removed from embryonic animals *(33)*.

Immortal Olfactory Cell Lines

To provide a homogeneous supply of cells, specific attempts have been made to create immortal cell lines *(30,82–84)*. Additionally, there are reports of spontaneous transformations giving rise to immortal lines in primary cultures of the olfactory epithelium *(85–87)* and of an olfactory neuroblastoma cell line *(88)*. The neuroblastoma cell line is interesting because, despite the absence of immunolocalization for any olfactory-specific markers, once the cells are treated with TGF-α, the cells can respond to odorants. However, to our knowledge, this is the only report describing responses, function, or any other data from these cells.

The spontaneously transformed cells described by Coon et al. *(85)* were established after explanting rat olfactory epithelium into culture dishes containing Matrigel. By Western blot, these cells, (sensory neuron in vitro facsimile; SNIF), expressed neuron-specific enolase and GAP-43, but neither glial fibrillary acidic protein or olfactory-marker protein. The cells were able to synthesize carnosine from ^{3}H-alanine and cAMP levels were increased over controls in the presence of citralva ($10^{-7}M$). However, the cAMP levels in SNIF cells were decreased after treatment with high concentrations of odorant.

The other olfactory receptor neurons in which spontaneous transformations have been observed were isolated from adult human cadavers *(86)* and from aborted human fetuses *(87)*. The human cadaver work again used Matrigel as the substrate for the tissue fragments and after cloning, the cells were able to respond to an odorant mixture as measured both by cAMP assay and fluorimetry with Indo-1. However, immunocytochemical data revealed that non-neuronal markers such as GFAP could be detected in the same cells as neuronal-specific markers. Explanations for these data are that the precursor isolated may be multipotent, or, given the absence of olfactory-marker protein, a sign of incomplete differentiation. Either way, this has wide-ranging implications for our understanding of the processes of olfactory neurodevelopment. Data obtained from cells arising from the spontaneous transformation in human fetuses look extremely promising *(87)*. Cells express olfactory markers, including olfactory marker protein (OMP), as well as neuronal markers. The cells respond to odorants, with increases in cAMP levels, and are biochemically active. These cells have been used in several studies, in which they have been shown to secrete gonadotrophin-releasing hormone and to

express odorant receptors *(89,90).* Additionally, basic fibroblast growth factor (bFGF) supports neurogenesis in these cells *(91).* It will be interesting to investigate how these cells continue to proliferate and still maintain the expression of a differentiated phenotype.

In the last 12 mo, two different groups have reported the derivation of conditionally immortal olfactory receptor cell lines *(30,84).* These cell lines represent a significant advance over the cell lines described previously *(82,83)* as there is a degree of control that can be maintained over cell proliferation and differentiation. The first transformed cell lines *(82)* were derived from a transgenic mouse in which the Simian Virus 40 Large Tumor Antigen is expressed under control of the regulatory elements of the OMP gene and from n-myc transformed primary cultures *(83).* To prepare the n-myc transformed cells, adult mice were subjected to a bilateral bulbectomy 4 d prior to removal of the olfactory epithelia. As described previously, bulbectomy causes a biphasic response in the olfactory epithelium, an initial period of cell death that is followed by a marked upregulation of olfactory neurogenesis. It was during this period of neurogenesis that the cells were isolated, and, as dividing cells, could be infected with a retrovirus containing the n-myc oncogene. In the cell lines obtained in this manner, Northern analysis revealed the presence of mRNA encoding olfactory markers. Additionally, immunolocalization of olfactory-specific proteins was described but expression of OMP was not observed, nor were functional data available.

In the first study *(82)*, when transformed olfactory cells were cloned from a mouse in which the immortalizing oncogene SV40 TAg was expressed, these cells expressed GAP-43, and underwent morphological changes in response to "differentiating" agents. However, these cells were apparently relatively "immature" as they did not express mRNA for either of $G\alpha_{olf}$ or ACIII. They did express the TAg mRNA transcript as expected, but did not express OMP transcripts despite evidence that the same transcriptional start sites were used for the TAg expression in culture as OMP *in situ.* The absence of OMP expression is a feature that has plagued olfactory cell-culture preparation *(92).* Although the precise role of OMP is unknown, recent studies indicate a role for OMP in signal transduction/odor detection *(93).*

Expression of OMP has been overcome in the most recently described olfactory receptor-neuron cell lines *(30,84).* Both of these cell lines were conditionally immortalized with a temperature-sensitive mutant (tsA58) of the SV40 large T antigen *(95).* Olfactory-derived odorant receptor activatable (Odora) cells were created from dissociated rat pup olfactory-receptor neurons by retroviral infection *(30)*, whereas the cell lines (such as 3NA12) from Barber et al. *(84)* were obtained from primary cultures of the olfactory epithelium of the H-2Kb-tsA58 transgenic mouse *(96).* In this mouse, the SV40 TAg is flanked at its 5′ end with an interferon-inducible promotor that effectively increases transcription of the tsA58 at the permissive temperature (33°C) but not at nonpermissive temperatures (37°C). This has been demonstrated in a variety of tissues *(97–99)* and increasing numbers of investigators are turning to the H-2Kb-tsA58 transgenic mouse for the derivation of immortal cell lines *(100).*

Both the 3NA12 and odora cell lines express all neuronal and mature olfactory markers in nonpermissive ("differentiated") conditions. Odora cells express basal-cell markers such as GBC-1 and Sus-4 *(101)* at permissive (proliferative) temperatures, whereas all of the olfactory markers, except OMP, were detected in 3NA12 cells at permissive temperatures. In odora cells, these data may reflect a progression from an immature to a mature phenotype as cells move from permissive to nonpermissive culture conditions. The same is true for 3NA12 cells, although the starting point for these cells may be somewhat more mature/differentiated. This is supported by the ability of 3NA12 cells to respond to odorants in both permissive and nonpermissive conditions. The odorant responses that have been seen in 3NA12 cells (that single cells respond to multiple odorants) indicate that multiple receptors may be expressed or alternatively, that the receptors

expressed are broadly tuned. This evidence is not consistent with the hypothesis suggesting specific odorant receptor expression is predetermined at a genetic level. In odora cells, no endogenous odorant receptors activity could be detected, but following transient transfection with the U131 receptor, surface expression and responsiveness to the odorant n-enanthic acid can be observed. It is here that these two cell lines may have significant value. As stated earlier, odorant receptor expression has been extremely difficult and at least in odora cells, odorant receptors can be expressed. The low level of individual receptor expression in 3NA12 cells should also permit functional data from cloned receptors to be obtained.

Olfactory-Slice Cultures

The final preparation of the olfactory epithelium is one in which an in vitro slice culture is established *(45,102).* This technique was pioneered by Farbman and associates *(45).* In this technique, 400-µm thick slices of the olfactory epithelium can be grown in the absence or presence of dissociated olfactory-bulb neurons *(103).* In a variation of this technique, the olfactory epithelium and olfactory bulb can be removed simultaneously and placed close to each other in culture *(104).* In these two preparations, olfactory receptor neurons extend numerous axons, form functional synapses with the olfactory bulb, and modulate the function of the olfactory-bulb cells. Consequently, these processes, and factors that regulate them, can be investigated.

Concluding Remarks

Understanding the olfactory epithelium comes in two parts. Recently, much has been made of how smell can be "deconstructed" and in doing so, the molecular basis of olfaction has been revealed. This process has been exciting as the largest seven transmembrane-spanning, G-protein-coupled receptor family has been identified, as has the signal-transduction pathway that is activated following odorant/receptor binding. Some elucidation of the structure and function of the odorant receptors has been achieved and it has been made apparent that these receptors have an additional role in axon guidance. However, we remain far from total comprehension of the process of odor-quality discrimination. The second part refers to the neurogenic capability of the olfactory epithelium. With exposure to the environment, neurons in the olfactory epithelium are in a continual process of death and rebirth. The very accessibility of these cells makes the olfactory epithelium a prime option for investigators looking to study neurogenesis. In this review, we have delineated some of the preparations that are useful for investigations relevant to these areas and have focused on the attempts to use primary and immortal cell cultures to reconstruct the olfactory epithelium in vitro. It is hoped that these techniques will assist in the search for a better understanding of olfactory function.

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References

1. Buck L. and Axel, R. (1991) A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* **65,** 175–187.
2. Jones D. T. and Reed, R. R. (1989) Golf: an olfactory neuron specific-G protein involved in odorant signal transduction. *Science* **244,** 790–795.
3. Bakalyar H. A. and Reed R. R. (1990) Identification of a specialized adenylyl cyclase that may mediate odorant detection. *Science* **250,** 1403–1406.

4. Dhallan R. S., Yau K. W., Schrader K. A., and Reed, R. R. (1990) Primary structure and functional expression of a cyclic nucleotide-activated channel from olfactory neurons. *Nature* **347,** 184–187.
5. Liman E. R. and Buck, L. B. (1994) A second subunit of the olfactory cyclic nucleotide-gated channel confers high sensitivity to cAMP. *Neuron* **13,** 611–621.
6. Bonigk W., Bradley, J., Muller F., Sesti F., Boehoff I., Ronnett G. V., et al. (1999) The native rat olfactory cyclic nucleotide-gated channel is composed of three distinct subunits. *J. Neurosci.* **19,** 5332–5347.
7. Bradley J., Li J., Davidson N., Lester H. S., and Zinn K. (1994) Heteromeric olfactory cyclic nucleotide-gated channels: a subunit that confers increased sensitivity to cAMP. *PNAS* **91,** 8890–8894.
8. Malnic B., Hirono J., Sato T., and Buck L. B. (1999) Combinatorial receptor codes for odors. *Cell* **96,** 713–723.
9. Reed R. R. (1992) Signaling pathways in odorant detection. *Neuron* **8,** 205–209.
10. Shepherd G. M. (1994) Discrimination of molecular signals by the olfactory receptor neuron. *Neuron* **13,** 771–790.
11. Buck L. B. (1996) Information coding in the vertebrate olfactory system *Ann. Rev. Neurosci.* **19,** 517–544.
12. Ressler K. J., Sullivan S. L., and Buck L. B. (1994) Information coding in the olfactory system: evidence for a stereotyped and highly organized epitope map in the olfactory bulb. *Cell* **79,** 1245–1255.
13. Mombaerts P., Wang F., Dulac C., Chao S. K., Nemes A., Mendelsohn M., et al. (1996) Visualizing an olfactory sensory map. *Cell* **15,** 675–686.
14. Dulac C. and Axel R. (1995) A novel family of genes encoding putative pheromone receptors in mammals. *Cell* **83,** 195–206.
15. Herrada G. and Dulac C. (1997) A novel family of putative pheromone receptors in mammals with a topographically organized and sexually dimorphic distribution. *Cell* **90,** 763–773.
16. Matsunami H. and Buck L. B. (1997) A multigene family encoding a diverse array of putative pheromone receptors in mammals. *Cell* **90,** 775–784.
17. Rodriquez I., Feinstein P., and Mombaerts P. (1999) Variable patterns of axonal projections of sensory neurons in the mouse vomeronasal system. *Cell* **97,** 199–208.
18. Belluscio L., Kolfactory epitheliumntges G., Axel R., and Dulac C. (1999) A map of pheromone receptor activation in the mammalian brain. *Cell* **97,** 209–220.
19. Buck L. B. (2000) The molecular architecture of odor and pheromone sensing in mammals, *Cell* **100,** 611–618.
20. Leinders-Zufall T., Lane A. P., Puche A. C., Ma W., Novotny M. V., Shipley M. T., and Zufall F. (2000) Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature* **405,** 792–796.
21. Ngai J., Dowling M. M., Buck L., Axel R., and Chess A. (1993) The family of genes encoding odorant receptors in the channel catfish. *Cell* **72,** 657–666.
22. Mombaerts P. (1999) Seven-transmembrane proteins as odorant and chemosensory receptors. *Science* **286,** 707–711.
23. Sengupta P., Chou J. H., and Bargmann C. I. (1996) odr-10 encodes a seven transmembrane domain olfactory receptor required for responses to the odorant diacetyl. *Cell* **84,** 899–909.
24. Zhao H., Ivic L., Otaki J. M., Hashimoto M., Mikoshiba K., and Firestein S. (1998) Functional expression of a mammalian odorant receptor. *Science* **279,** 237–242.
25. Krautwurst D., Yau K.-W., and Reed R. R. (1998) Identification of ligands for olfactory receptors by functional expression of a receptor library. *Cell* **95,** 917–926.
26. Speca D. J., Lin D. M., Sorensen P. W., Isacoff E. Y., Ngai J., and Dittman A. H. (1999) Functional identification of a goldfish odorant receptor. *Neuron* **23,** 487–498.
27. Touhara K., Sengoku S., Inaki K., Tsuboi A., Hirono J., Sato T., et al. (1999) Functional identification and reconstitution of an odorant receptor in single olfactory neurons. *Proc. Natl. Acad. Sci. USA* **96,** 4040–4045.
28. McClintock T. S., Landers T. M., Gimelbrant A. A., Fuller L. Z., Jackson B. A., Jayawickreme C. K., and Lerner M. R. (1997) Functional expression of olfactory-adrenergic receptor chimeras and intracellular retention of heterologously expressed olfactory receptors. *Brain. Res. Mol. Brain Res.* **48,** 270–278.
29. Wellerdieck C., Oles M., Pott L., Korsching S., Gisselmann G., and Hatt H. (1997) Functional expression of odorant receptors of the zebrafish Danio rerio and of the nematode C. elegans in HEK293 cells. *Chem. Senses* **22,** 467–476.

30. Murrell J. R. and Hunter D. D. (1999) An olfactory sensory neuron line, odora, properly targets olfactory proteins and responds to odorants. *J. Neurosci.* **19,** 8260–8270.
31. Dahl A. R., (1988) Metabolism and olfaction, in *Molecular Neurobiology of the Olfactory System,* Margolis F. L. and Getchell, T. V., eds., Plenum Press, New York, pp. 51–70.
32. Getchell M. V., Zielinski B., and Getchell T. V. (1988) Odorant and autonomic regulation of secretion in the olfactory mucosa, in *Molecular Neurobiology of the Olfactory System,* Margolis F. L. and Getchell, T. V. eds., Plenum Press, New York, pp 71–98.
33. Calof A. L. and Chikaraishi D. M. (1989) Analysis of neurogenesis in a mammalian neurolfactory epitheliumpithelium: proliferation and differentiation of an olfactory neuron precursor in vitro. *Neuron* **3,** 115–127.
34. Schwartz Levey M., Chikaraishi D. M., and Kauer J. S. (1991) Characterization of potential precursor populations in the mouse olfactory epithelium using immunocytochemistry and autoradiography. *J. Neurosci.* **11,** 3556–3564.
35. Hinds J. W., Hinds P. L., and McNelly N. A. (1984) An autoradiographic study of the mouse olfactory epithelium: evidence for long-lived receptors. *Anat. Rec.* **210,** 375–383.
36. Camara C. G. and Harding J. W. (1984) Thymidine incorporation in the olfactory epithelium of mice: early exponential response induced by olfactory neurectomy. *Brain Res.* **308,** 63–68.
37. Caggiano M., Kauer J. S., and Hunter D. D. (1994) Globose basal cells are neuronal progenitors in the olfactory epithelium: a lineage analysis using a replication-incompetent retrovirus. *Neuron* **13,** 339–352.
38. Monti Graziadei G. A. and Graziadei P. P. (1979) Studies on neuronal plasticity and regeneration in the olfactory system: morphologic and functional characteristics of the olfactory sensory neuron, in *Neural Growth and Differentiation,* Meisami E. and Brazier M. A. B., eds., Raven Press, New York, pp. 373–396.
39. Graziadei P. P. and Monti Graziadei G. A. (1980) Neurogenesis and neuron regeneration in the olfactory system of mammals. III. Deafferentation and reinnervation of the olfactory bulb following section of the fila olfactoria in rat. *J. Neurocytol.* **2,** 145–162.
40. Miwa T., Uramoto N., Ishimaru T., Furukawa M., Shiba K., and Morjizumi T. (1998) Retrograde transport of nerve growth factor from olfactory bulb to olfactory epithelium. *Neuroreport* **9,** 153–155.
41. Balboni G. C., Zonefrati R., Repice F., Barni T., and Vannelli G. B. (1991) Immunohistochemical detection of EGF and NGF receptors in human olfactory epithelium. *Boll. Soc. Ital. Biol. Sper.* **67,** 901–906.
42. Aiba T., Mori J., and Nakai Y. (1993) Nerve growth factor (NGF) and its receptor in rat olfactory epithelium. *Acta. Otolaryngol. Suppl. (Stockh)* **506,** 37–40.
43. Ronnett G. V., Hester L. D., and Snyder S. H. (1991) Primary culture of neonatal rat olfactory neurons. *J. Neurosci.* **11,** 1243–1255.
44. Mahanthappa N. K. and Schwarting G. A. (1993) Peptide growth factor control of olfactory neurogenesis and neuron survival in vitro: roles of EGF and TGF-beta s. *Neuron* **10,** 293–305.
45. Farbman A. I. and Buchholz J. A. (1996) Transforming growth factor-alpha and other growth factors stimulate cell division in olfactory epithelium in vitro. *J. Neurobiol.* **30,** 267–280.
46. Buckland M. E. and Cunningham A. M. (1998) Alterations in the neurotrophic factors BDNF, GDNF and CNTF in the regenerating olfactory system. *Ann. NY Acad. Sci.* **855,** 260–265.
47. Holcomb J. D., Mumm J. S., and Calof A. L. (1995) Apoptosis in the neuronal lineage of the mouse olfactory epithelium: regulation in vivo and in vitro. *Dev. Biol.* **172,** 307–323.
48. Deckner M. L., Frisen J., Verge V. M., Hokfelt T., and Risling M. (1993) Localization of neurotrophin receptors in olfactory epithelium and bulb. *Neuroreport* **5,** 301–304.
49. Roskams A. J., Bethel M. A., Hurt K. J., and Ronnett G. V. (1996) Sequential expression of Trks A, B, and C in the regenerating olfactory neurolfactory epitheliumpithelium. *J. Neurosci.* **16,** 1294–1307.
50. DeHamer M. K., Guevara J. L., Hannon K., Olwin B. B., and Calof A. L. (1994) Genesis of olfactory receptor neurons in vitro: regulation of progenitor cell divisions by fibroblast growth factors. *Neuron* **13,** 1083–1097.
51. Goldstein B. J., Wolozin B. L., and Schwob J. E. (1997) FGF2 suppresses neuronogenesis of a cell line derived from rat olfactory epithelium. *J. Neurobiol.* **33,** 411–428.
52. MacDonald K. P., Murrell W. G., Bartlett P. F., Bushell G. R., and Mackay-Sim A. (1996) FGF2 promotes neuronal differentiation in explant cultures of adult and embryonic mouse olfactory epithelium. *J. Neurosci. Res.* **44,** 27–39.

53. Farbman A. I. (1994) Developmental biology of olfactory sensory neurons. *Semin. Cell. Biol.* **5,** 3–10.
54. Plendl J., Stierstorfer B., and Sinowatz F. (1999) Growth factors and their receptors in the olfactory system. *Anat. Histol. Embryol.* **28,** 73–79.
55. Murray R. C. and Calof A. L. (1999) Neuronal regeneration: lessons from the olfactory system. *Semin. Cell Dev. Biol.* **10,** 421–431.
56. Costanzo R. M. and Graziadei P. P. C. (1983) A quantitative analysis of changes in the olfactory epithelium following bulbectomy in hamster. *J. Comp. Neurol.* **215,** 370–381.
57. Carr VMcM and Farbmann A. I. (1992) Ablation of the olfactory bulb up-regulates the rate of neurogenesis and induces precocious cell death in olfactory epithelium. *Exp. Neurol.* **115,** 55–59.
58. Liu N., Shields C. B., and Roisen F. J. (1998) Primary culture of adult mouse olfactory receptor neurons. *Exp. Neurol.* **151,** 173–183.
59. Harding J. W., Getchell T. V., and Margolis F. L. (1978) Denervation of the primary olfactory pathway in mice. V. Long-term effect of intranasal ZnSO4 irrigation on behavior, biochemistry and morphology. *Brain. Res.* **140,** 271–285.
60. Burd G. D. (1993) Morphological study of the effects of intranasal zinc sulfate irrigation on the mouse olfactory epithelium and olfactory bulb. *Microsc. Res. Tech.* **24,** 195–213.
61. Miwa T., Moriizumi T., Sakashita H., Kimura Y., Donjo T., and Furukawa M. (1993) Transection of the olfactory nerves induces expression of nerve growth factor receptor in mouse olfactory epithelium. *Neurosci. Lett.* **155,** 96–98.
62. Burd G. D. and Sein V. (1998) Influence of olfactory innervation on neurogenesis in the developing olfactory bulb of the frog, Xenopus laevis. *Ann. NY Acad. Sci.* **855,** 270–273.
63. Brunjes P. C. (1994) Unilateral naris closure and olfactory system development. *Brain Res. Brain Res. Rev.* **19,** 146–160.
64. Kleene S. J. and Gesteland R. C. (1983) Dissociation of frog olfactory epithelium. *J. Neurosci. Methods* **9,** 173–183.
65. Ivanova T. T. and Caprio J. (1993) Odorant receptors activated by amino acids in sensory neurons of the channel catfish Ictalurus punctatus. *J. Gen. Physiol.* **102,** 1085–1105.
66. Firestein S., Picco C., and Menini A. (1993) The relation between stimulus and response in olfactory receptor cells of the tiger salamander. *J. Physiol. (Lond)* **468,** 1–10.
67. Leinders-Zufall T., Greer C. A., Shepherd G. M., and Zufall F. (1998) Imaging odor-induced calcium transients in single olfactory cilia: specificity of activation and role in transduction. *J. Neurosci.* **18,** 5630–5639.
68. Kleene S. J. and Gesteland R. C. (1981) Dissociation of frog olfactory epithelium with N-ethylmaleimide. *Brain Res.* **229,** 536–540.
69. Maue R. A. and Dionne V. E. (1987) Preparation of isolated mouse olfactory receptor neurons. *Pflugers Arch.* **409,** 244–250.
70. Nakamura T. and Gold G. H. (1987) A cyclic nucleotide-gated conductance in olfactory receptor cilia. *Nature* **325,** 442–444.
71. Sato T., Hirono J., Tonoike M., and Takebayashi M. (1991) Two types of increases in free Ca^{2+} evoked by odor in isolated frog olfactory receptor neurons. *Neuroreport* **2,** 229–232.
72. Rawson N. E., Gomez G., Cowart B., Brand J. G., Lowry L. D., Pribitkin E. A., and Restrepo D. (1997) Selectivity and response characteristics of human olfactory neurons. *J. Neurophysiol.* **77,** 1606–1613.
73. Tareilus E., Noe J., and Breer H. (1995) Calcium signals in olfactory neurons. *Biochim. Biophys. Acta* **1269,** 129–138.
74. Vargas G. and Lucero M. T. (1999) A method for maintaining odor-responsive adult rat olfactory receptor neurons in short-term culture. *Chem. Senses.* **24,** 211–216.
75. Gilbert S. F., Oster-Granite M. L., Kirby W., Durie D., Lyons D., and Greene E. (1986) Selective culture medium enhances survival of neuroblasts from postnatal rodent brain. *Brain Res. Bull.* **16,** 853–860.
76. Chuah M. I., David S., and Blaschuk O. (1991) Differentiation and survival of rat olfactory epithelial neurons in dissociated culture. *Dev. Brain Res.* **60,** 123–132.
77. Pixley S. K. (1992) CNS Glial cells support in vitro survival, division, and differentiation of olfactory neuronal progenitor cells. *Neuron* **8,** 1191–1204.
78. Mumm J. S., Shou J., and Calof A. L. (1996) Colony-forming progenitors from mouse olfactory epithelium:Evidence for feedback regulation of neuron production. *Proc. Natl. Acad. Sci. USA* **93,** 11,166–11,172.
79. Moon C., Sung Y. K., Reddy R., and Ronnett G. V. (1999) Odorants induce the phosphorylation of the cAMP response element binding protein in olfactory receptor neurons. *Proc. Natl. Acad. Sci. USA* **96,** 14,605–14,610.

80. Moon C, Jaberi P., Otto-Bruc A., Baehr W., Palczewski K., and Ronnett G. V. (1998) Calcium-sensitive particulate guanylyl cyclase as a modulator of cAMP in olfactory receptor neurons. *J. Neurosci.* **18,** 3195–3205.
81. Cunningham A. M., Manis P. B., Reed R. R., and Ronnett G. V. (1999) Olfactory receptor neurons exist as distinct subclasses of immature and mature cells in primary culture. *Neuroscience* **93,** 1301–1312.
82. Largent B. L., Sosnowski R. G., and Reed R. R. (1993) Directed expression of an oncogene to the olfactory neuronal lineage in transgenic mice. *J. Neurosci.* **13,** 300–312.
83. MacDonald K. P., Mackay-Sim A., Bushell G. R., and Bartlett PF (1996) Olfactory neuronal cell lines generated by retroviral insertion of the n-myc oncogene display different developmental phenotypes. *J. Neurosci. Res.* **45,** 237–247.
84. Barber R. D., Jaworsky D. E., Yau K. W., and Ronnett G. V. (2000) Isolation and in vitro differentiation of conditionally immortalized murine olfactory receptor neurons. *J. Neurosci.* **20,** 3695–3704.
85. Coon H. G., Curcio F, Sakaguchi K., Brandi M. L., and Swerdlow R. D. (1988) Cell cultures of neuroblasts from rat olfactory epithelium that show odorant responses. *Proc. Natl. Acad. Sci. USA* **86,** 1703–1707.
86. Wolozin B., Sunderland T., Zheng B. B., Resau J., Dufy B., Barker J., Swerdlow R., and Coon H. (1992) Continuous culture of neuronal cells from adult human olfactory epithelium. *J. Mol. Neurosci.* **3,** 137–146.
87. Vannelli G. B., Ensoli F., Zonefrati R., Kubota Y., Arcangeli A., Becchetti A., et al. (1995) Neuroblast long-term cell cultures from human fetal olfactory epithelium respond to odors. *J. Neurosci.* **15,** 4382–4394.
88. Gomez G., Restrepo D., Rawson N., Lowry L. D., Keane W. M., and Rothstein J. L. (1996) Induction of differentiation of human olfactory neuroblastoma cells into odorant-responsive cells. *Neuroscience* **74,** 567–577.
89. Barni T., Maggi M., Fantoni G., Granchi S., Mancina R., Gulisano M., et al. (1999) Sex steroids and odorants modulate gonadotropin-releasing hormone secretion in primary cultures of human olfactory cells. *J. Clin. Endocrinol. Metab.* **84,** 4266–4273.
90. Kubota Y., Sakano H., Ensoli F., Thiele C., and Vannelli G. B. (1994) Odorant receptor genes expressed in a human olfactory neuroblast cell line. *J. Cell. Biochem.* **18B**(Suppl.), 182.
91. Ensoli F., Fiorelli V., Vannelli B., Barni T., De Cristofaro M., Ensoli B., and Thiele C. J. (1998) Basic fibroblast growth factor supports human olfactory neurogenesis by autocrine/paracrine mechanisms. *Neuroscience* **86,** 881–993.
92. Margolis F. L. (1988) Molecular cloning of olfactory-specific gene products, in *Molecular Neurobiology of the Olfactory System,* Margolis F. L. and Getchell T. V., eds., Plenum Press, New York, pp. 237–265.
93. Buiakova O. I., Baker H., Scott J. W., Farbman A., Kream R., Grillo M., et al. (1996) Olfactory marker protein (OMP) gene deletion causes altered physiological activity of olfactory sensory neurons. *Proc. Natl. Acad. Sci. USA* **93,** 9858–9863.
94. Youngentob S. L. and Margolis F. L. (1999) OMP gene deletion causes an elevation in behavioral threshold sensitivity. *Neuroreport* **10,** 15–19.
95. Jat P. S., Cepko C. L., Mulligan R. C., and Sharp P. A. (1986) Recombinant retroviruses encoding simian virus 40 large T antigen and polyomavirus large and middle T antigens. *Mol. Cell. Biol.* **6,** 1204–1217.
96. Jat P. S., Noble M. D., Ataliotis P., Tanaka Y., Yannoutsos N., Larsen L., and Kioussis D. (1991) Direct derivation of conditionally immortal cell lines from an H-2K^b-tsA58 transgenic mouse. *Proc. Natl. Acad. Sci. USA* **88,** 5096–5100.
97. Chambers T. J., Owens J. M., Hattersley G., Jat P. S., and Noble M. D. (1993) Generation of osteoclast-inductive and osteoclastogenic cell lines from the H-2K^b-tsA58 transgenic mouse. *Proc. Natl. Acad. Sci. USA* **90,** 5578–5582.
98. Whitehead R. H., VanEeden P. E., Noble M. D., Ataliotis P., and Jat P. S. (1993) Establishment of conditionally immortalized epithelial cell lines from both colon and small intestine of adult H-2Kb-tsA58 transgenic mice. *Proc. Natl. Acad. Sci. USA* **90,** 587–591.
99. Barber R. D., Woolf A. S., and Henderson R. M. (1997) Potassium conductances and proliferation in conditionally immortalized renal glomerular mesangial cells from the H-2K^b-tsA58 transgenic mouse. *Biochim. Biophys. Acta* **1355,** 191–203.
100. Noble M., Groves A. K., Ataliotis P., Ikram Z., and Jat P. S. (1995) The H-2K^b-tsA58 transgenic mouse: a new tool for the rapid generation of novel cell lines. *Transgenic Res.* **4,** 215–225.

101. Goldstein B. J. and Schwob J. E. (1996) Analysis of the globose basal cell compartment in rat olfactory epithelium using GBC-1, a new monoclonal antibody against globose basal cells. *J. Neurosci.* **16,** 4005–4016.
102. Gong Q., Liu W. L., Srodon M., Foster T. D., and Shipley M. T. (1996) Olfactory epithelial organotypic slice cultures: a useful tool for investigating olfactory neural development. *Int. J. Dev. Neurosci.* **14,** 841–852.
103. Puche A. C. and Shipley M. T. (1999) Odor-induced, activity-dependent transneuronal gene induction in vitro: mediation by NMDA receptors. *J. Neurosci.* **19,** 1359–1370.
104. Kanaki K, Sato K., and Kashiwayanagi M. (2000) Functional synapse formation between rat olfactory receptor neurons and olfactory bulb neurons in vitro. *Neurosci. Lett.* **285,** 76–78.