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Morphological and molecular features of the mammalian olfactory sensory neuron axons: What makes these axons so special?

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Abstract

The main organization and gross morphology of the mammalian olfactory primary pathway, from the olfactory epithelium to the olfactory bulb, has been initially characterized using classical anatomical and ultrastructural approaches. During the last fifteen years, essentially thanks to the cloning of the odorant receptor genes, and to the characterization of a number of molecules expressed by the olfactory sensory neuron axons and their environment, significant new insights have been gained into the understanding of the development and adult functioning of this system. In the course of these genetic, biochemical and neuroanatomical studies, however, several molecular and structural features were uncovered that appear somehow to be unique to these axons. For example, these axons express odorant receptors in their terminal segment, and transport several mRNA species and at least two transcription factors. In the present paper, we review these unusual structural and molecular features and speculate about their possible functions in the development and maintenance of the olfactory system.

Introduction

In adult mammals, most of the sensory information is transmitted to the central nervous system through presumably stable axonal tracts, almost all of which are developed during early embryogenesis. A notable exception is the olfactory nerve connecting the olfactory sensory epithelium (OE) to the olfactory bulb (OB). Following its early development during embryogenesis, this axonal tract is subject to continuous remodeling throughout life due to the renewal of the olfactory sensory neurons (OSNs, Crews & Hunter, 1994; Farbman, 1994; Gogos *et al*., 2000; Graziadei & Monti Graziadei, 1979, Huard *et al*., 1998; Mackay-Sim & Kittel. 1991). The adult neurogenic process has a series of important features, among them the fact that developing OSN axons are growing out of the OE toward their target in the OB, while older axons are degenerating. Due to this permanent renewal of OE to OB connections, one can predict that the adult olfactory axonal tracts and their cellular and extracellular matrix environments should have molecular and structural features distinct from most of the other adult axonal tracts. These features are expected to be the persistence to some extent of those embryonic properties that allow the growth of axons, their guidance towards a specific target, as well as their synaptogenesis onto appropriate bulbar neurons. As we discuss in this review, several such embryonic features are still present in the adult olfactory system. However, as we will point out, a series of unique properties of the OSN axons which do not seem at first glance to be simply related to these developmental events, are in some instances also retained by mature OSN axons, already functionally connected to their targets.

We will first describe the organization and the main characteristics of the primary olfactory tract and OSN axonal projections from the OE to the OB mouse. Then we will discuss the molecular features of the developing and mature OSN axons and of their cellular and extracellular environment, highlighting aspects most

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probably related to the growth of new axons originating from newborn OSNs. The discussion will then be focused on possible functions of some of the unique molecular features of the OSN axons, in particular their ability to transport subsets of mRNAs and proteins (*i.e.* transcription factors) that are generally not observed in the axonal compartment of other mature neurons of the vertebrate nervous system.

Anatomical elements of the olfactory sensory neuron projections: From the olfactory epithelium to the olfactory bulb

In adult mammals, the OSN cell bodies are found in the olfactory mucosa (Fig. 1), a sensory organ serving to detect odors that is located in the nasal cavity (Graziadei, 1971; Menco & Morrison, 2003). This tissue comprises two main layers, the OE and the lamina propria. The OE contains several cell types (Frisch, 1967; Holbrook *et al*., 1995; Shipley *et al*., 1995), the sustentacular cells, the glandular cells (Bowman glands), horizontal and basal stem cells that are neuronal progenitors, and the numerous OSN bipolar cell bodies (more than 2 million OSNs in mice, Mori *et al*., 1999). The lamina propria, which lies underneath the OE, contains blood vessels, special glial cells called olfactory ensheathing cells (OECs, see below and Raisman, 1985) as well as small diameter bundles of axons originating from the OSN cell bodies. These axonal bundles coalesce to form larger ones that exit the lamina propria (Fig. 1). Assemblies of these large

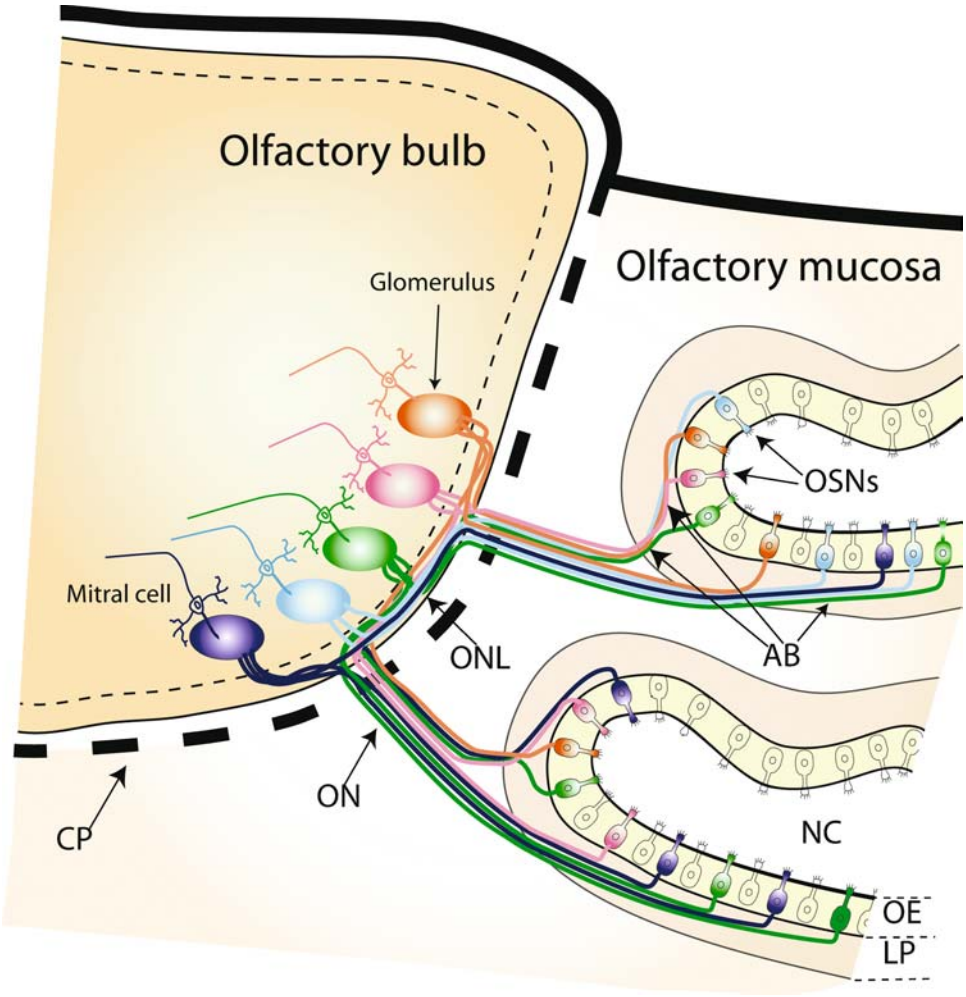

Fig. 1. Organization of the mouse main olfactory system. The olfactory sensory neurons (OSNs) located in the olfactory mucosa project into glomeruli of the olfactory bulb (OB), in which they contact bulbar neurons such as mitral cells. Each OSN expresses one odorant receptor (OR) gene (schematized by the color code on the figure), and all axons from the OSNs expressing the same OR converge into a few glomeruli (see correspondence between the colored axons and the colored glomeruli). On their way from the olfactory epithelium (OE) to the OB, OSNs axons assemble into axon bundles (AB) in the lamina propria (LP), before they coalesce into branches of the olfactory nerve (ON) that cross the cribriform plate (CP). OSN axon bundles remain heterotypic until they reach the olfactory nerve layer (ONL) of the olfactory bulb. In this layer, the OSN axons rearrange in an homotypic way and they converge into their target glomeruli. NC: nasal cavity.

bundles constitute the branches of the olfactory nerve that cross the cribriform plate of the ethmoid bone at multiple points before connecting the OB (Fig. 1). Each OSN axon then courses within the most peripheral part of the bulb called olfactory nerve layer (ONL) before entering a glomerulus in which it branches and connects to apical dendrites of bulbar principal cells (mitral and tufted cells, Fig. 1), as well as periglomerular cells (Bailey *et al*., 1999; Shepherd & Greer, 2003; Shepherd, 1972; Shipley *et al*., 1995). These initial projections are subject to postnatal refinement, which requires sensory input during critical periods (Zhao & Reed, 2001; Zou *et al*., 2004).

This apparent simple organization, revealed by classical anatomical investigations, hides a very complex projection pattern. Indeed, the cloning of the odorant receptor (OR) encoding genes (Buck & Axel, 1991) and the analysis of their expression patterns in mouse (Chess *et al*., 1994; Mombaerts *et al*., 1996; Ressler *et al*., 1994; Vassar *et al*., 1993; Wang *et al*., 1998) demonstrated that there are about 1,000 OR-encoding genes in the rodents rat and mouse (Zhang & Firestein, 2002), and that each of these OR genes is expressed in one of four zones of the OE numbered I to IV from the dorsal to the ventral OE, according to the Buck (Ressler *et al*., 1993) nomenclature (for reviews see Mombaerts, 2001; Mori *et al*., 1999; O'Leary *et al*., 1999; Reed, 2004). Strong evidence supports the idea that, at least in mammals (in other species there are some exceptions---most current reference: Goldman *et al*., 2005, and references therein), each OSN expresses only one OR gene out of this large repertoire (Belluscio & Katz, 2001; Lewcock & Reed, 2004; Serizawa *et al*., 2000; Serizawa *et al*., 2003; Serizawa *et al*., 2004; Shykind *et al*., 2004). Interestingly, although the neurons expressing the same OR gene are widely scattered in their OE zone (a given OR can be expressed by several thousands OSNs in mouse), they all converge onto a few glomeruli of the olfactory bulbs (Fig. 1). In general, they form one or two glomeruli per hemi-bulb, each OB having a bilateral symmetry with respect to the glomeruli organization. This complex organization imposes an enormous constraint on every axon growing from the OE to the OB, during embryogenesis as well as in the adult. Indeed, due to the very wide distribution of the OSNs expressing a given OR, to the high number of distinct types of OSNs (as many as there are OR genes) and to the high density of OSNs within the OE, the proximal axonal bundles within the lamina propria are extremely heterogeneous in terms of OR identity (Fig. 1). However, at some point, these axons rearrange in a way that allows all axons originating from a given type of OSN (characterized by the expression of a given OR gene) to converge onto the few correct glomeruli. As we shall see, the high heterogeneity of the axon bundles is maintained throughout the course of the OSN axons until they reach the outer ONL (Fig. 1) of the OB (Royal & Key, 1999; Treloar *et al*., 2002). As they reach this layer, the previously intermingled axons defasciculate and rearrange and converge onto selected glomeruli (Fig. 1). This process seems in many instances to be facilitated by the formation, within the ONL, of homotypic bundles of axons expressing the same OR gene, even though this homotypic fasciculation is not a prerequisite for correct targeting (Treloar *et al*., 2002).

These axonal rearrangements, homotypic fasciculations and convergences of axons are rather unique to the olfactory system. In other sensory systems, such as the visual system, the topographic map is closely conserved from the periphery to the central centers by maintaining the spatial relationships between the axons along their course (O'Leary *et al*., 1999). This singularity probably relies on important structural and molecular properties of both the OSN axons and their environment.

Structural features of the OSN axonal tracts: Axons and axon bundle arrangements

OSN axons are unmyelinated fibers of a very small diameter along their entire course towards the OB (about 0.2 μm in mouse, Griff *et al*., 2000). As they grow, they exit the OE layer by crossing its basal lamina, and enter the lamina propria where they co-assemble in small bundles engulfed by olfactory ensheathing cell (OEC) processes (Farbman & Squinto, 1985). The fine organization of these axonal bundles has been recently documented, using ultrastructural approaches on the rat olfactory mucosa and olfactory nerve (Field *et al*., 2003; Li *et al*., 2005). The more proximal bundles (typical diameter is about 5 μm) typically contain a significant amount of space unoccupied by axons, and a few hundreds of axons oriented in various directions. The simplest bundles are generally wrapped by a single OEC, delineated externally by a basal lamina. Further distally from their origin, the OSN axons become packed at very high density within bundles that become larger. These larger bundles have diameters up to 20 μm and contain several thousand OSN axons. They are limited externally by two or three OECs having peripheral processes that enwrap the bundle of axons, as well as processes penetrating into the bundles and intermingle with the axons. At this level, the OSN axons are tightly packed and fully occupy the space between the processes of the OEC, and they are all aligned with the axis of the olfactory nerve. Within an olfactory nerve branch as a whole, which is constituted by the assembly of several such large bundles, each bundle is surrounded by its basal lamina and thus physically separated from neighboring bundles. In addition, at the level of the olfactory nerve, surrounding olfactory nerve fibroblasts further enwrap these assemblies of large bundles.

Thus, as they contact the OB, the OSN axons arrive as tight groups of a few thousands axons, which all originate from a same local area of the OE. Such an

organization, with physically isolated bundles of axons all along their course within the lamina propria and the olfactory nerve, precludes the topographic rearrangement of axons before their arrival in the bulb external layer. In line with these ultrastructural observations, a combination of molecular and morphological approaches showed that the axons from OSNs expressing a given OR gene are intermingled with many other axons which do not express this OR in the axonal bundles within the olfactory nerve (Royal & Key, 1999; Treloar *et al*., 2002, and for review see Key & St John, 2002). Furthermore, the thousands of axons arising from all of the dispersed OSNs expressing a given OR gene are probably conveyed within a number of physically isolated axonal bundles. Hence, their convergence onto a small number of specific glomeruli requires the disorganization of these bundles, defasciculation of the OSN axons, and their extensive rearrangement and sorting before they fasciculate in an homotypic way and converge onto a glomerulus. During embryogenesis, these crucial events probably occur during a "waiting period" in a transient tissue aggregate called the migratory mass that contacts the developing OB, in which the axonal growth cones pause before they penetrate the OB (Bailey *et al*., 1999; Valverde *et al*., 1992; Whitesides & LaMantia, 1996). In the postnatal and adult mouse, the sorting of growing axons takes place between the outer and the inner ONL of the OB (Treloar *et al*., 2002).

Molecular correlates of the primary olfactory tract's structural organization : Sequential OSN axon interactions with their successive environments

The structural organization of the primary olfactory tract depends on sequential cellular and molecular interactions between growing OSN axons and their physical environments (Key & St John, 2002; Lin & Ngai, 1999), as well as on odor-evoked activity (Zhao & Reed, 2001; Zou *et al*., 2004). In both the embryo and the adult organism, the OSN environment comprises mainly other OSN axons (being mostly mature axons in the adult olfactory system), OECs and extracellular matrix (ECM). Therefore, the growth and navigation pattern of each developing OSN axon, including its integration and fasciculation with other axons in a given bundle, its defasciculation upon arrival in the outer ONL, and the subsequent sorting, are sequentially controlled by local cues expressed by OECs (*i.e.* membrane-attached or secreted molecules) or present in the ECM (*i.e.* glycoproteins or guidance factors). Numerous molecules having adhesion or guidance activities are expressed in the primary olfactory tract (for reviews see Brunjes & Greer, 2003; Key & St John, 2002; Mori, 1993; O'Leary *et al*., 1999).

Early in development, the OSN axons cross the fronto-nasal mesenchyme by following the migration of OECs en route from the OE to the rostral telencephalon. This developing olfactory nerve pathway coincides with sub-domains of the fronto-nasal mesenchyme co-expressing both laminin and heparan sulfate proteoglycans, two ECM molecules possibly produced by the OECs themselves (Treloar *et al*., 1996). The OSN axon growth is probably partly restricted to this pathway by the presence, in the adjacent fronto-nasal mesenchyme, of high levels of chondroitin sulfate proteoglycans (Tisay & Key, 1999; Treloar *et al*., 1996), which are known to inhibit the migration of OECs in vitro (Tisay & Key, 1999). It appears therefore, that the initial guidance of the OSN axons depends to some extend on the migration of the OECs. Additional repulsive and attractive cues are also involved in the early growing of the nascent OSN axons toward the bulb. It remains however unclear whether this initial guidance of OSN axons is controled by identical factors in different vertebrate species. In rodents, several lines of evidence indicate that the diffusible protein Netrin 1 may promote the outgrowth of the nascent OSN axons toward the OB primordium by acting as an attractant through the DCC ("deleted in colorectal cancer") receptor expressed by OSNs (Astic *et al*., 2002). In zebrafish, Robo/Slit repulsive signalling clearly plays an important role in this process (Miyasaka *et al*., 2005a). Loss and gain of function experiments showed that Robo2, a receptor of Slit proteins, is expressed transiently in the olfactory placode where it steers early olfactory axons toward the OB primordium. This function is permitted by the surrounding expression of the Slit repulsive cues, preventing the misrouting of the growing OSN axons, and favoring their tight association in bundles.

The fasciculation of OSN axons per se relies at least on axon-axon and axon-ECM interactions involving adhesion molecules and their associated signal transduction pathways. The Robo2 transmembrane protein itself might be involved in this process, since it also has homophilic binding activity (Hivert *et al*., 2002; Miyasaka *et al*., 2005a). Other members of the immunoglobulin superfamily are also likely candidates to participate in OSN axon fasciculation. For example, L1 and N-CAM 180 are strongly expressed on the OSN axons, all along their paths (Gong & Shipley, 1996; Miragall *et al*., 1988; Whitesides & LaMantia, 1996). Functional analyses documented defects in OSN axon fasciculation in various genetic contexts including Robo2 mutation in zebrafish (Miyasaka *et al*., 2005a), and N-CAM-180 or galectin-1 gene inactivation in mice (Puche *et al*., 1996; Treloar *et al*., 1997). Interestingly, in all three cases, this fasciculation defect is correlated with the formation of aberrant glomeruli, suggesting that these molecules also play a role later on in the sorting of these axons in the outer ONL and glomeruli. Alternatively, this formation of a map of abnormal glomeruli may originate from defects in OSN axon fasciculation as they travel

from the OE toward the OB. The transient tight fasciculation of OSN axons may indeed be required for their propper rearrangement and targeting as they enter the OB. In line with this hypothesis is the observation that in the Robo2 zebrafish mutants, OSN axons that fail to properly fasciculate enter into the OB at abberant positions, which may constitute abnormal environments in terms of guidance cues that ultimately re-route these axons to ectopic sites in the OB (Miyasaka *et al*., 2005a).

The sorting and glomerular targeting of the OSN axons taking place in the migratory mass (embryo) or outer ONL (postnatal and adult mice) of the bulb probably involve chemoattractive and chemorepulsive molecules and their receptors expressed by OSNs, subsets of OECs (located for example in the outer ONL) and/or bulbar neurons. Although the expression patterns of many such molecules have been at least partially documented in the olfactory system, their precise functions in the patterning of the OSN axonal projections are not yet fully understood (Astic *et al*., 2002; Cloutier *et al*., 2004; Giger *et al*., 1996; Kobayashi *et al*., 1997; Pasterkamp *et al*., 1998; Zhang *et al*., 1996). Developmentally expressed chemorepulsive cues and their receptors have a variety of functions in restricting the OSN projections to the glomerular layer itself, or to specific domains within this layer. Thus far, such functions have been characterized for only a few molecules, including ephrin-As and their EphA receptors, as well as the class 3 semaphorins (*i.e*. Sema3A) and some of the semaphorin receptors (neuropilin 1 and 2). Below we will focus our discussion on the functions of these molecules in the targeting of OSN axons to their ultimate targets.

Several ephrin-As and Bs, as well as their respective receptors, the EphAs and Bs, are expressed by OSNs and/or their target cells in the OB in a dynamic manner during the embryonic development of the olfactory system (St John *et al*., 2000; St John & Key, 2001; Zhang *et al*., 1996; Zhang *et al*. 1997). Interestingly, it has been shown that OSNs expressing different ORs express different levels of ephrin-As on their axons, suggesting that ephrin-As might participate in controlling the targeting of the OSN axons (Cutforth *et al*., 2003). The function of ephrin-As in this system has been addressed using gene targeting or overexpression approaches (Cutforth *et al*., 2003). Deletion of ephrin-A5 and ephrin-A3 genes shifts selected glomeruli (*i.e*. the P2 glomeruli) posteriorly, whereas overexpression of ephrin-A5 in P2 OR-expressing OSNs shifts the corresponding glomeruli anteriorly, demonstrating that altering the level of expression of ephrin-As by OSNs leads to significant modifications of the glomerular map.

Sema3A is expressed not only by OSNs and mitral cells, but also by a subset of OECs located in the rostral and ventral outer ONL, two OB domains avoided by neuropilin-1 (a receptor of Sema3A) expressing OSN axons (Schwarting *et al*., 2000; Williams-Hogarth *et al*., 2000). Interestingly, Sema3A knockout (ko) mice have defects in the sorting of the OSN axons, leading to an ectopic projection (*i.e*. in the medio-ventral OB), as well as abnormal locations and numbers of P2 OR glomeruli (Crandall *et al*., 2000; Taniguchi *et al*., 2003; Schwarting *et al*., 2000; Schwarting *et al*., 2004). The Sema3A/Neuropilin 1 system therefore, seems to allow the partial segregation of subsets of OSN axons such as the Neuropilin 1 positive axons projecting to the Sema3A negative area of the OB. The observation of aberrant P2 glomeruli formation in Sema3A ko mice also suggests that Sema3A participates in the sorting of P2 axons in the external ONL of the OB. The function of Sema3A may somehow be slightly different in other vertebrates. In the chick embryo for example, expression during the waiting period of a dominant negative Neuropilin 1 that blocks Sema3A-mediated signaling in OSN axons induces them to enter the telencephalon prematurely and to overshoot the glomerular layer (Renzi *et al*., 2000). Interestingly, another Neuropilin receptor to class 3 semaphorins, Neuropilin 2, probably has very similar functions in rodents. Neuropilin 2 is expressed by a subset of OSNs localized throughout the OE. These OSNs project to a restricted set of glomeruli located mostly in the rostral tip and the ventroposterior region of the OB, two areas that partially overlap with the Neuropilin 1-positive domains described above. Using a knock in strategy in which the tau-GFP coding sequence was inserted into the Neuropilin 2 locus, Walz *et al*. (2002) demonstrated that the Neuropilin 2 loss of function in mice did not apparently modify the location of the GFP-positive OSNs. However, these mice are characterized by a striking overshoot of the GFP-labeled OSN axons beyond their glomeruli into the external plexiform layer. This result indicates that Neuropilin 2 is required for a subset of OSNs to confine their axon terminals to appropriate glomeruli. So far, the Neuropilin 2 ligand involved in this process is not identified, but three candidates, all expressed transiently or continuously in both mitral and granule cells, are suspected to play this role: Sema3B, Sema3C and Sema3F (Cloutier *et al*., 2004; Giger *et al*., 1998; Walz *et al*., 2002).

The guidance cues described above are not the only molecules participating in the guidance of OSN axons within the OB. Numerous studies also implicated carbohydrates in this process, most of them being linked to unidentified membrane proteins (Crandall *et al*., 2000; Dowsing *et al*., 1997; Key & Akeson, 1993; Lipscomb *et al*., 2003; Puche & Key, 1996; Storan *et al*. 2004). A "glycocode" hypothesis has even been envisaged, based on studies showing that subsets of OSN axons expressing specific surface carbohydrates project to topographically invariant groups of glomeruli (Key & St John, 2002). According to this hypothesis, these surface carbohydrates would allow the OSN axons to become

progressively sorted into smaller and smaller fascicles as they course through the ONL, hence participating in the glomerular targeting. In line with this view, β1, 3-*N*-acetylglucosaminyltransferase 1 glycosylation is required for axon pathfinding and proper glomerular formation in the mouse's primary olfactory pathway (Henion *et al*., 2005). Although surface carbohydrates and their associated glycocode probably play an important role in restricting the projection of subsets of axon types to subdomains of the OB, it is unlikely that such a glycocode is sufficient to specify the full and precise glomeruli map formation. First, in order to fully establish the primary olfactory projection glomerular map, the OSN axons would need to present at their surface a very large - possibly combinatorial - repertoire of sugars. Yet, we do not know if a large enough repertoire exist in this system. Second, this mechanism would require the coordinate expression of a given OR gene with the surface expression of the corresponding proper set of sugars, which would probably necessitate very complex genetic and biochemical regulation and interplay.

One current view (John & Key, 2005) is that the glycocode-dependent sorting of the OSN axons may be a necessary prerequisite for the subsequent specific targeting of the OSN axons to their proper glomeruli, which itself depends on the OR gene expressed by the OSNs (see below).

From the examples described above, one can view the functions of the ECM, adhesion and surface molecules, guidance cues and their receptors as a means to restrict and target the projections of groups of OSN axons to subdomains of the OB, along the antero-posterior and medio-lateral axes of the OB, as well as within its depth (hence avoiding OSN axon projections beyond the glomerular layer, *i.e*. into the external plexiform layer). Given the high number of cues expressed in this system (for reviews see Key & St John, 2002; Mori, 1993; O'Leary *et al*., 1999), as well as their possible functional interactions (*i.e*. NP1-L1 interactions, Castellani *et al*., 2000), it is likely that a large number OB domains arise from the combinatorial expression of ligands by subsets of neurons, glial cells, or OECs located in the OB (Au *et al*., 2002) and their receptors expressed by subsets of OSN axons. Interestingly, the expression of some of these cues may be restricted to one or several zones of the OE (or OB). For example, the olfactory cell adhesion molecule OCAM is expressed in zones II to IV of the OE (Yoshihara *et al*., 1997), and it may participate in the control of the projections of the OSN axons to the corresponding zones II to IV of the OB. In addition, continuous gradients of expression of genes encoding guidance cues or morphogenes, as well as proteins upstream or downstream in their pathways (*i.e*. transcription factors or proteins belonging to intracellular transduction cascades) may be involved in the control of the OSN axon targeting to subdomains of the OB along the dorso-ventral axis. Such an expression in dorso-ventral gradients, correlating with the zonal topography of the sensory map, have recently been observed for several genes (Norlin *et al*., 2001). Of particular interest, the homeobox gene Msx1 and the neuropilin 2 gene are expressed in a graded way (high ventral to low dorsal) in the basal OSN precursor cells and OSNs, respectively. The Msx1 gene may play a role in the regulation of the differential production, along the ventro-dorsal axis, of OSN subtypes having distinct guidance response properties. The neuropilin 2 receptor may well be one of the guiding response determinants expressed differentially along the ventro-dorsal axis.

Whether such a graded ventro-dorsal expression of guidance molecules also exists in the OB is presently unknown. However, a recent study aimed at characterizing the spatial patterns of gene expression in the OB suggests a complex map with multiple and partially overlapping domains of differential gene expression by subsets of cells located in different layers of the OB (Lin *et al*., 2004).

The molecular mechanisms for zonal or graded expression of transcription factors or guidance cues in the OE, as well as the projection of OSN axons to selected domains of the OB that express different genes remain to be characterized. The identification of classic adhesive and guidance cues expressed in this system is now actively pursued in several laboratories using loss or gain of function strategies in mice. This effort will undoubtedly tell us more about the guidance and projection of subsets of molecularly distinct OSN axons to their proper OB target. However, this knowledge may not be sufficient to comprehensively explain the precise olfactory primary map, which involves the formation in the OB of about a thousand distinct and spatially invariant glomeruli per hemi-bulb. This precise map most probably relies on the recruitment of additional mechanisms that thus far appear unique to the OSN axons in the sense that they have not been described in other well-studied sensory systems such as the visual or somatosensory systems. We will examine below a series of molecular features of the OSN axons that may be highly relevant to this fine-tuning of OSN axon targeting, which allows all the OSN axons expressing a given OR to converge on specific glomeruli.

Unique molecular features of the OSN axons and their functional significance

One of the most striking features of the OSN axons is their ability to fasciculate homotypically upon arrival in the outer ONL (Treloar *et al*., 2002), a mechanism which may favor the co-targeting of a large number of OSNs expressing the same OR gene to single glomeruli. We will see that the late aspects of homotypic fasciculation and targeting depend on the recruitment of a unique process involving the OR protein

itself. This astonishing guidance process may be further linked to other unusual molecular features of OSNs axons. Indeed, as compared to most other axons of the mammalian nervous system, they contain molecules which are generally not expected to be present in the axonal compartment, such as selected populations of mRNAs and transcription factors. In addition, despite their common identity as OSN axons, they appear to constitute a highly heterogeneous population in term of expression of a series of molecular markers, such as membrane-associated proteins like glycoproteins and ORs. While this high level of molecular heterogeneity has often been proposed to be somehow related to the convergence of OSN axons into their target glomeruli, the presence in these axons of transcription factors and mRNAs has remained enigmatic. In the following section, we will review some molecular features of the OSN axons, and speculate about their possible functions.

ODORANT RECEPTORS AND THEIR FUNCTION IN THE HOMOTYPIC FASCICULATION OF OSN AXONS

The homotypic fasciculation of OSN supposes, theoretically, that the 1,000 categories of OSN axons characterized by the expression of a single OR gene also express, in a coordinate way, at least 1,000 distinct unique molecular features. What then could be the nature of these axonal molecular features? A molecular model explaining the glomeruli map formation involves the ORs themselves (reviewed in O'Leary *et al*., 1999; Mombaerts, 2001; Reed, 2004). According to this view, the OSN axons expressing the same OR gene recognize each other and hence tend to fasciculate together when they encounter, thanks to direct or indirect interactions between their ORs present at the surface of their growth cone and axon membranes (Singer *et al*., 1995). The beauty of this hypothesis is its parsimony: it requires no coordinated expression of the individual OR genes with any other specific surface molecules, nor any precise prepatterning of the OB glomeruli map in the OB (Feinstein & Mombaerts, 2004). The first lines of evidence for such a guidance function of the OR genes were obtained with OR gene knock-out experiments in mice, in which the tauLacZ expressing sequence was inserted in the targeted locus (Wang *et al*., 1998). In these mice, the LacZ expressing axons, assumed to correspond to an homogenous population of OSN neurons with respect to their OR identity, failed to fasciculate and to converge onto a single glomerulus. These early experiments were however recently re-interpreted in light of evidence demonstrating that individual developing OSN are not programmed irreversibly to express a given OR. In fact, it now seems that during their early differentiation the OSN select randomly one OR gene locus out of the full repertoire of OR alleles (including the OR pseudogenes loci). More importantly, they have the ability to switch from one OR gene locus to another one. The frequency of this switch is very low when the first selected locus encodes a functional OR, but it is very high when the first OR gene locus selected encodes a non-functional OR protein (Lewcock & Reed, 2004; Serizawa *et al*., 2003; Shykind *et al*., 2004). Therefore, the apparent absence of convergence of the LacZ expressing OSN axons in the described above OR ko mice can be interpreted as the result of a switch to a locus expressing a functional OR in all the OSN having first chosen the targeted LacZ locus. Since the second locus is randomly selected, the LacZ-expressing axons correspond to a heterogenous population of OSNs in terms of OR expression. Compelling evidence of this was obtained by knock-in experiments demonstrating that replacing the coding sequence of an OR at a given locus by that of another OR altered the positions of the corresponding glomeruli (Bozza *et al*., 2002; Mombaerts *et al*., 1996; Mombaerts, 2001; Wang *et al*., 1998). More recently, a contextual model for axonal sorting of the OSN axons into glomeruli, in which the OR proteins mediate the homotypic interactions between like axons, has been proposed (Feinstein & Mombaerts, 2004; Feinstein *et al*., 2004). This model is based on evidence obtained from genetically manipulated mice and proposes that the precise positioning of a given glomerulus within the OB is controlled not only by the OR identity of the OSN axons, but also by the OR expression level, as well as the ORs expressed by neighbouring axons (Feinstein & Mombaerts, 2004).

The contextual model for axonal sorting gained substantial histochemical support in 2004 with three independent reports documenting the presence of OR proteins in the OSN axonal tracts, in particular in the ONL and in the glomeruli (Barnea *et al*., 2004; Feinstein *et al*., 2004; Strotmann *et al*., 2004). From a functional point of view, the localization of the MOR256-17 and MOR28 OR proteins uncovered by Strotmann *et al*. (2004) and Barnea *et al*. (2004) strikingly correlates with the bulbar domains in which the OSN axons rearrange and homotypically fasciculate. This observation supports the supposition that OR proteins participate in the late homotypic fasciculation process. Interestingly, these authors detected no or very little OR protein in more proximal parts of the OSN axons, *i.e*., in the olfactory nerve (Barnea *et al*., 2004; Strotmann *et al*., 2004). This absence of OR immunoreactivity in the olfactory nerve could have several explanations. The protein could be concentrated in distal parts of the axon only, due to its fast transport or higher turnover in proximal parts of the axon. Alternatively, the OR protein may be present all along the axon, but its concentration may be too low to allow the labeling of individual axons embedded in bundles of axons expressing other ORs. As a result, only the homotypic bundles of axons, all located in the ONL and glomeruli, would exhibit sufficient amounts of OR to allow immunohistochemical detection. Another

possibility would be that the ORs may be homogeneously present all along the axon, but that their epitopes recognized by the antibodies used are masked in proximal parts of the axons, due to protein-protein interactions. In the latter case, one could then speculate that the OR protein may not be functional until the axon reaches the outer ONL. Further studies are needed to determine whether or not the OR protein is truly absent (or present but not functional) in the proximal parts of the OSN axons. From a functional point of view, the absence of OR protein along the OSN axons from their origin to the outer ONL may be critical to avoid a premature homotypic fasciculation of the OSN axons within the olfactory nerve, which may impair the proper initial packing of OSN axons in bundles, or their correct sorting in the outer ONL.

Together, the above data definitively demonstrate that the OR genes play critical roles in the guidance of the OSN axons, and provides strong genetic and histochemical evidence for a control of the fine positioning of the glomeruli by the OR proteins themselves, even though the most critical biochemical evidence (*i.e.* the demonstration of protein-protein interactions between OR located at the surface of OSN axons) is still awaited.

mRNAs, but no translational machinery in mature axons?

The presence of mRNAs in the OSN axons was revealed soon after the cloning of the OR genes. In situ hybridization aimed at localizing the OR mRNAs showed that, in the OB, each OR mRNA was concentrated inside a few glomeruli, providing the first demonstration for the convergence of all OSNs expressing a given OR gene (Ressler *et al*., 1994; Vassar *et al*., 1994). In parallel, the mRNA encoding the olfactory marker protein (OMP), a cytosolic/nucleoplasmic protein specifically and highly expressed by all OSNs (Koo *et al*., 2005; Rogers *et al*., 1987), was also observed in the OSN axons and present in the glomeruli (Wensley *et al*., 1995). Since OMP is expressed late in the maturation process of the OSNs and thereafter remains expressed until their death, OSN axons can transport mRNAs even when mature and functionally connected to their targets. At that time, these observations were unexpected, because mRNAs were generally considered to be excluded from the axonal compartment of mature vertebrate neurons. In fact, the only other system in which mRNAs were previously observed in adult and mature axons is the adult hypothalamo-neurohypophyseal system. Hypothalamic vasopressin and oxytocin secretory neurons indeed transport several RNAs including neuropeptide-encoding mRNAs in their axons, down to the posterior lobe of the pituitary (Landry & Hökfelt, 1998; Mohr *et al*., 1991; Trembleau *et al*., 1994; Trembleau *et al*., 1995). In this latter system, however, no evidence for the translation of these mRNAs in the axonal compartment has been obtained, and their function in this neuronal projection remains enigmatic.

More recently, evidence for the presence of mRNAs in developing axons and in particular in their growth cones have been provided by direct visualization using *in situ* hybridization (Bassell *et al*., 1998; Zhang *et al*., 1999), or by indirectly documenting the inhibition of growth cone responses to guidance cues by local actions of inhibitors of protein synthesis (Campbell & Holt, 2001; Ming *et al*., 2002). These studies clearly demonstrate that growing axons contain the translational machinery, as well as transport mRNAs in their growth cone, where these mRNAs are locally translated in response to local cues (Bassell *et al*., 1998; Brittis *et al*., 2002; Campbell & Holt, 2001). In addition, local translation may also occur in developing axon terminals during synaptogenesis, where it may play a role in neurotrophin-dependent synaptic potentiation (Zhang & Poo, 2002). More generally, local translation of mRNAs in distal compartments of neurons (*i.e.* postsynaptic domains of dendrites or axonal growth cones) allows for rapid expression and sorting of selected proteins in response to specific signals (for review see Kelleher *et al*., 2004; Steward & Schuman, 2003).

As described above, the adult olfactory primary pathway contains three classes of axons with respect to their differentiation status: growing axons, mature axons and degenerating axons. In light of the data concerning the axonal localization of mRNAs, one may distinguish these classes of OSN axons. Growing OSN axons contain mRNAs and the translational machinery in their growth cones, and may use local translation of axonal mRNAs for their guidance. In support of this, we recently obtained evidence for the presence of ribosomal proteins and the initiation factor eIF4E in OSN axon growth cones (Nedelec *et al*., unpublished data).

Morphological and immunohistochemical studies did not provide any strong evidence for the presence of significant ribosomal immunoreactivity nor ribosomes in mature OSN axons (Greer & Margolis, 1997; Kafitz *et al*., 1998; Nedelec *et al*., unpublished data). Furthermore, the functional significance of the presence of mRNAs in the mature axons is not well understood. However, we should be cautious about these negative data. Local translation of importin β mRNA has been observed following nerve crush of the adult sensory neuron axons of the sciatic nerve which were previously considered to be devoid of mRNAs and translational machinery (Hanz *et al*., 2003; Zheng *et al*., 2001). Thus these mature axons have the ability to translate mRNAs, at least in response to injury. The recent description of "ribosomal periaxoplasmic plaque domains" in myelinated axons of lumbar spinal nerve root in rat and rabbit further indicates that, at least in

certain axons, ribosomes may be arranged in special structures which cannot be identified as bona fide ribosomes at the electron microscope level (Koenig *et al*., 2000). Whether such ribosomal domains, as well as elements of the membranous machinery allowing the sorting of membrane-embedded proteins such as OR exist in OSN axons remains to be established. Further electron microscopic and high-resolution immunocytochemical investigations may help clarify this point. Should this machinery be present, it will then be important to determine if the two mRNAs known to be present in mature OSN axons (OMP and OR mRNAs) or other unknown mRNAs are translated in these axons and under what conditions. The functional significance of this putative local translation of mRNAs in mature axons remains unclear.

As mentioned above, the local translation of importin mRNA in injured sciatic axons is followed by retrograde transport of putative nuclear localization signal (NLS)-bearing proteins back to the cell nucleus (Hanz *et al*., 2003). Irrespective of whether importin proteins or mRNAs are present in OSN axons, it is interesting to note that several bona fide transcription factors bearing NLS have been identified in the OSN axons.

TRANSCRIPTION FACTORS REMOTE FROM THE CELL NUCLEUS

Two transcription factors have been identified in OSN axons, NFATp (now called NFATC2, http: / /www.gene.ucl.ac.uk/ nomenclature/ genefamily/ NFAT/NFAT.shtml) and Emx2 (Ho *et al*., 1994; Nedelec *et al*., 2004). NFATC2, initially identified in the context of *T* cell stimulation, was immunolocalized in OSN axons within the OB glomeruli, suggesting that this transcription factor is transported all along the axon, including the axon terminal (Ho *et al*., 1994). However, since NFATC2 has not been co-localized with maturation markers like GAP43 and OMP, it is not known whether NFATC2 is expressed in all OSNs, and whether it is present in developing axons, mature axons or both categories. Furthermore, nothing is known about its subcellular compartmentalization and function in these axons. Interestingly, an important function has recently been assigned to NFATC proteins in other developing neurons. Graef *et al*. (2003) showed that neurotrophins and netrins stimulate the outgrowth of embryonic axons by triggering calcineurin-dependent NFATC-nuclear translocation (Graef *et al*., 2003). In light of recent data showing that the classical nuclear import pathway mediated by importins is functional from the synapse to the nucleus (Hanz *et al*., 2003; Thompson *et al*., 2004), it is tempting to speculate that axonal NFATC2 may have a retrograde function in the olfactory system. Upon activation in the axon terminal (*i.e*. by signals activating the dephosphorylation of NFATC2), NFATC2 could be retrogradely transported to the neuronal cell body, and then translocated into the nucleus where it may regulate the expression of specific target genes. Further studies are needed to determine whether such a regulated retrograde signaling exists and, if so, whether this putative signaling pathway plays a role in physiological events taking place in the OSNs (*i.e*. olfactory axon growth, synapse formation or survival of OSNs). One should note here that the retrograde transport of activated signals in the OSN axon plays an important role in the induction of apoptosis. A recent report documented that upon NMDA-mediated excitotoxic death of OSN target neurons in the OB, caspase 8 is activated in the OSN axons and associated with the retrograde motor protein complex dynactin $p150^{Glued}$/dynein, before retrograde transported to the OSN cell body to induce apoptosis (Carson *et al*., 2005). It will be important to determine whether this retrograde pathway dependent on dynactin $p150^{Glued}$ is also involved in the retrograde transport of other molecules, like NFATC2. More generally, it would be interesting to identify the regulatory processes triggering pro-apoptotic or putative anti-apoptotic retrograde signaling occuring in the OSN terminal.

Emx2, a homeodomain transcription factor, has also been localized in the OSN axons (Nedelec *et al*., 2004). This protein was detected in the axons of both developing and mature (OMP-expressing) OSNs (Nedelec *et al*., 2004 and unpublished data). Emx2 is present throughout the whole axon from its proximal to its more distal domains, including the axon terminals. As we proposed for NFATC2, Emx2 may function as a retrograde signal. Alternatively, Emx2 may have local functions in the axonal compartment, related to the transport of mRNAs or to their translation in the OSN axons. Indeed, subcellular fractionation of the OB showed that Emx2 is present in high-density particle-containing fractions and that it interacts with the eukaryotic translational initiation factor 4E (eIF4E). These particles may correspond to granules transporting mRNAs or protein complexes involved in local translation in axons. HOXA9, another homeodomain protein, is known to regulate the translation of selected mRNAs in myeloid cells, through a direct interaction with eIF4E (Topisirovic *et al*., 2005). Our observations are compatible with a similar role of Emx2 in regulating the translation of mRNAs (Richter & Sonenberg, 2005) within the OSN axons, possibly during their transport from the OSN cell body to the axonal terminal.

DO MATURE OSN AXONS RETAIN JUVENILE CHARACTERISTICS?

The characteristics of OSN axons described above may be considered juvenile, retained by OSNs throughout their full life which lasts about 3 months in mice (Mackay-Sim & Kittel, 1991; Gogos *et al*., 2000),

well beyond the early stages of differentiation, axonal growth and synaptogenesis in the OB. Indeed, a substantial amount of axonal RNA has been generally considered as a property of growing axons only; as maturation proceeds, the axonal transport of RNAs is dramatically decreased in cultured neurons, possibly due to the specific exclusion of RNA translocation from axonal processes (Kleiman *et al*., 1994). The presence of OR proteins on terminal segments of mature OSN axons can also be interpreted as a juvenile feature, since the axonal OR has a guidance function allowing the targeting of growing axons onto their proper glomeruli (see above). Finally, the axonal localization of NFATC2 may be viewed as a juvenile property since neuritic localization of transcription factors belonging to the same family has been observed in cultured developing neurons (Graef *et al*., 2003).

Interestingly, previous studies reported that mature OSN axons have immature molecular features with respect to the molecular composition of their cytoskeleton. For example, throughout their life OSNs keep expressing the microtubule-associated protein MAP1B (also called MAP5), which is usually abundant in extending axons but subsequently down-regulated upon neuronal maturation (Viereck *et al*., 1989). Similarly, OSNs express vimentin and peripherin as their intermediate filament proteins during their growth and differentiation. But as they become mature, OSNs keep expressing vimentin rather than neurofilament proteins as most other neurons do (Chien *et al*., 1998; Schwob *et al*., 1986). Vimentin is localized in OSN axons into the OB, but does not extend into their glomerular terminals (Gorham *et al*., 1991). Interestingly, a new function has recently been assigned to vimentin. Upon sciatic nerve injury, soluble forms of vimentin are produced in the axoplasm where they link pErk to importin β. These interactions lead to the retrograde transport of activated MAP kinases in injured sensory axons (Perlson *et al*., 2005). It is thus tempting to speculate that the continued expression of vimentin in OSNs allows it to participate in a retrograde signaling pathway between axon distal regions and neuronal cell bodies.

The functional significance of other juvenile molecular characteristics described above remains unclear. One can speculate that they may favor axonal growth and sorting of neurons generated throughout adult life. For example, the persistent OR expression on terminal segments of the OSN axons probably provides a guidance cue for the OSN growth cones arriving in the adult ONL, allowing them to grow homotypically on mature axons expressing the same OR and to reach their proper glomeruli. Should the translation of the OR mRNAs occur in developing or mature terminal segments of OSN axons, such a local production of OR may contribute to the presence of this guidance cue on the axon surface in the ONL and glomeruli.

Whatever the significance of these juvenile features, the latter examples illustrate that the expression of juvenile biochemical characteristics by the adult OE and its OB projection is not solely due to the presence of growing neurons in these tissues. They may be related more globally to the ongoing neurogenic processes taking place in this system, which perhaps requires that not only the growing neurons themselves, but also the mature ones to have functional properties usually restricted to early developmental stages.

Conclusions and future directions

During the last decade, thanks to the cloning of the mammalian OR genes, a giant step forward has been achieved in the understanding of the functional diversity of the OSNs and the neuroanatomical organization of their projections onto the OB, giving profound insights into the basic principles of information processing in the olfactory system.

In parallel to the genetic, anatomical and biochemical analyses performed in mouse, many other studies have been conducted in various other animal models from different phyla, including the fly. Interestingly, the basic logic and anatomical organization of the primary olfactory projections are strikingly similar across species from drosophila to mouse. In both cases indeed, numerous OSN axons converge onto selected glomeruli in a brain area, in which they establish synapses with a principal cell dendrite (Hildebrand & Shepherd, 1997). This conserved cytoarchitecture raises an important question: do these species use the same molecular mechanisms to build similar structures and circuits ? Several lines of evidence suggest that a simplistic extrapolation from one phyla to another one should not be made, because distinct molecular adaptations may have differentially arisen and evolved in phylogenetically distant species (Strausfeld & Hildebrand, 1999). For example, unlike the mouse OSNs, drosophila OSNs can co-express several OR genes, and the targeting of their axon onto their proper glomeruli does not involve the ORs (Dobritsa *et al*., 2003; Goldman *et al*., 2005; Hallem & Carlson, 2004; Neuhaus *et al*., 2005). Whenever we compare the olfactory system of drosophila to the one of mouse, we should keep in mind the fact that a striking difference between their olfactory system resides in the number of glomeruli into which the OSN projections segregate. Whereas there are only 43 glomeruli in the antennal lobe of drosophila (Ramaekers *et al*., 2005), there are about 1800 glomeruli in the mouse OB (Rogers & Firestein, 2001). Establishing and maintaining a map of 1800 glomeruli, despite the OSN renewal occuring in mouse, may require specific and additive cooperative molecular mechanisms not required for the construction of a less complex olfactory map. The recruitment of OR proteins to guide the OSN axons may thus have occured in species

in which maps with high number of glomeruli were emerging.

Significant differences may also exist in the OSN axon guidance mechanisms between different vertebrate species belonging to different classes, such as mouse and zebrafish. While fish have a single olfactory epithelium, many terrestrial vertebrates have two distinct and anatomically separated olfactory systems (main: olfactory mucosa; and accessory: vomeronasal organ) (Dulac & Torello, 2003; Miyasaka *et al*., 2005b). Thus, ancestral molecular mechanisms involved in the initial OSN axon growth may have been recruited for controlling the developement on either the main or accessory system in rodents. This might be the case for the Robo-Slit repulsive signaling, which guides the OSN axons from the olfactory placode toward the OB in zebrafish (Miyasaka *et al*., 2005a), and seems to have been recruted in mammals to control the targeting of basal vomeronasal organ axons (Knoll *et al*., 2003).

Here, we attempted to highlight several unexpected molecular features of the mammalian OSN axons. Among them, the presence in mature axons of certain mRNAs and transcription factors, one of them potentially having translational regulatory functions, seems unique to these sensory axons. These observations raise a number of fundamental questions. One key question, which still remains unsolved, is that of the functions of the mRNAs transported in the OSN growing and mature axons. Are these mRNA translated locally in growing axons? Does cue-dependent translational regulation play a role in the guidance of OSN growth cones? Do the mature axons, having established synaptic contacts with bulbar cells, also translate the mRNAs they transport? If yes, what is the function of this local translation? What are the signals regulating this translation occuring in axon terminals? And last but not least, what is the function of the two transcription factors so far identified in the OSN axons? In the case of Emx2, is its function related to axonal translation regulation? Alternatively, is the function of these axonal transcription factors similar to that of NFATC in developing neurites, *i.e*. to signal retrogradely from the axon terminal to the nucleus? If so, in what context do such retrograde signals exert their functions? Is it during axonal development and synaptogenesis or does it participate in information processing in mature neurons? Overall, are the described above molecular features related to the renewal of this population of neurons throughout life, to their relatively short life span, to the singular way these axons project onto the olfactory bulb, or to the processing of olfactory information within the olfactory system?

The current development of efficient approaches involving genetic, anatomical, biochemical, electrophysiological, *in vitro* and *in vivo* cell imaging techniques and their combinations will help to address more specifically these fundamental questions, and to unravel the still unsolved mysteries of the development and physiology of olfactory primary projections in mice.

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