Int. J. Dev. Biol. 49: 193-200 (2005)
doi: 10.1387/ijdb.041933fl

Experimental study of early olfactory neuron differentiation and nerve formation using quail-chick chimeras

FABRICE L. LALLOUÉ* and †CHRISTIANE S. AYER-LE LIÈVRE[1]

SHU Psychiatrie du Sujet Âgé, CH Esquirol, Limoges, France and
[1]Homéostatie Cellulaire et Pathologie, Faculty of Medicine, Limoges, France

ABSTRACT For the formation of a functional olfactory system, the key processes are neuronal differentiation, including the expression of one or the other olfactory receptors, the correct formation of the nerve and organization of periphero-central connections. These processes take place during embryonic development starting from early stages. Consequently, avian embryos afford an attractive model to study these mechanisms. Taking advantage of species-specific equipment of olfactory receptors genes in different bird species, interspecific avian chimeras were set up by grafting early chick olfactory placodes in same stage quail embryos. Their analysis was performed using different complementary approaches. *In situ* hybridisation using probes to different chick olfactory receptor (COR) genes indicated that the choice of expression of an olfactory receptor by a neuron is independent of the environment of the olfactory placode and of interactions with the central nervous system. Futhermore, a chick olfactory receptor gene subgroup (*COR3*), absent in the host genome, was expressed by neurons from the graft. The question was then raised of the consequences of such heterospecific differentiation on axonal projections and fiber convergence. The DiI labeling of olfactory fibres in chimeras revealed anomalies in the formation of the nerve from the chick graft. In agreement with the hypothesis of olfactory receptor (OR) involvement in axonal guidance and periphero-central synapse organisation, the presence of migrating cells and axonal fibres from the graft, expressing foreign ORs and having different interactions with the host environment than the host fibres and migrating cells, might explain these anomalies.

KEY WORDS: *olfactory development, olfactory receptor, differentiation, nerve formation, avian chimera, DiI labelling*

Introduction

The sense of olfaction is based on a wide diversification of the sensory neurons linked i)to their expression of olfactory receptor molecules (OR) chosen from a large repertoire of genes, ii)to the adequate organization of periphero-central synapses and iii) to the modulation and integration at the central nervous system level of the sensory signals received by the central neurons. The first two processes take place primarily during embryonic development which make it difficult to investigate.

However, bird embryos afford an attractive model in which the morphogenesis of the peripheral olfactory system is now fairly well known (Ayer- Le Lièvre *et al.*, 1995, for review). The sensory organ, the olfactory epithelium lining the deeper recess of the nasal cavity and a unique nasal concha on each side, originates in the olfactory placode, a thickening of the anterior facial ectoderm which appears during the 3rd day of incubation (ED2-3 in the chick and quail, equivalent to ED9 in mouse). Its presumptive area is originally located close to that of the olfactory bulb in the external aspect of the anterior neural fold before the closure of the neural tube (Yntema, 1955; Couly and Le Douarin, 1988; Ayer-Le Lièvre *et al.*, 1995).

Soon, after their formation, the olfactory placodes invaginate in the underlying mesectoderm to constitute a finger-like primordium of the nasal cavity (Croucher and Tickle, 1989). Its most external

Abbreviations used in this paper: COR, chicken olfactory receptor; DAB, diaminobenzidine; DiI, carbocyanine DiI; ED, embryonic day; NBT/BCIP, 4-nitro blue tetrazolium chloride and 5-bromo 4-chloro indolyl phosphate; OB, olfactory bulb; OE, olfactory epithelium; ON, olfactory nerve; OR, olfactory receptor; ORN, olfactory receptor neuron; PCR, polymerisation chain reaction; POD, peroxydase.

***Address correspondence to:** Dr. Fabrice Lalloué. SHU psychiatrie du sujet âgé, CH Esquirol, 15, rue du Dr Macland, 87025 Limoges, France. Fax: +33-5-5543-1148. e-mail: lalloue.fabrice@wanadoo.fr

0214-6282/2005/$25.00

Printed in Spain
www.intjdevbiol.com

part will give rise to the nasal or respiratory epithelium and its deeper part to the olfactory epithelium (Farbman, 1992, for review). The later rapidly thickens while neuroblasts start to differentiate. The first axonal fibres reach the presumptive olfactory bulb area of the anterior prosencephalon during the 4th day of incubation and are responsible of the induction of the olfactory bulb (Van Campenout, 1936). The olfactory bulb starts bulging out at ED7 and periphero-central functional synaptogenesis does not start before ED8-9 in the chick.

This step of the first axonal outgrowth is closely linked but slightly anterior to the differentiation of olfactory neurons which is dependant of the expression of olfactory receptor (OR) genes.

OR genes have been first cloned and sequenced in 1991 (Buck and Axel, 1991). They are members of the superfamily of 7 transmembrane domain receptors coupled to G-proteins and they proved later to be involved in odor discrimination (Zhao *et al.*, 1998; Krautwurst *et al.*, 1998). The magnitude of this OR gene family has been evaluated to be in the order of one thousand of different olfactory receptor genes in mammals (Buck and Axel, 1991; Parmentier *et al.*, 1992, Lancet, D. and Ben Arie, N., 1993, Rouquier *et al.*, 1998, Mombaerts, 1999) while in fish (Ngai *et al.*, 1993; Barth *et al.*, 1996) and chick (Leibovici *et al.*, 1996, Ayer - Le Lièvre *et al.*, 1995) this repertoire would be about one hundred genes. From such a large repertoire each olfactory neuron (ORN) would only express a limited number of OR genes. These genes are classified in subfamilies and subgroups. OR belonging to a same subgroup present sequence homologies higher than 90% which means that their RNAs will cross-hybridize with the same riboprobes even under high stringency conditions. In birds, 13 OR genes have been sequenced first (Leibovici *et al.*, 1996; Nef *et al.*, 1996). It is currently known that 40 to 50% of OR genes are dispatched between three subgroups only: *COR2*, *COR3* and *COR4* with only 56% similarity of sequence from one subgroup to another (Leibovici *et al.*, 1996; Ladjali-Mohamedi *et al.*, personnal communication).

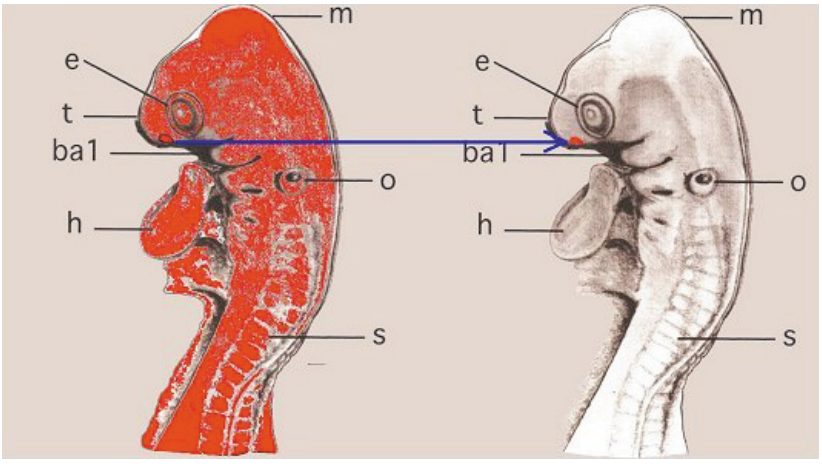

Fig.1. Microsurgery technique. *After dissection, the olfactory placode of a chick embryo (in red) is grafted to replace the excised quail olfactory placode respecting its orientation. This operation needs three steps: excision of the quail olfactory placode, removal of chick olfactory placode with or without removal of the underlying mesectoderm using a trypsin solution and graft of this placode onto the quail embryo. Abbreviations: e, eye; o, otic placode; m, mesencephalon; t, telencephalon; h, heart; s, somites; ba1, first branchial arch.*

An important question which arises about these differentiation events concerns the mechanisms involved in the choice made by a differentiating olfactory neuron to express one or another OR gene either at the genetic or chromosomal level or through interactions of the developing neurons with their environment or other cell types.

Considering the early OR gene expression in olfactory neurons and in placodal cells migrating along the same route as ORN axonal fibers, a dual role has been proposed for the ORs: as odorant molecule receptors in sensory olfactory cilia and as guidance molecules in the developing olfactory system (Singer *et al.*, 1995; Leibovici *et al.*, 1996).

In order to address the question of the mechanisms involved in OR gene expression and olfactory nerve formation, an experimental model has been set up in bird embryos taking advantage of the *in vivo* accessibility of their olfactory system for microsurgery and of the knowledge accumulated about their OR gene repertoires. A comparison of the quail and chick repertoires indicated that one chick subgroup (*COR3*) is absent from the quail genome, while the two others are also represented in this species (Ladjali –Mohamedi *et al.*, personal communication).

This observation permitted the use of chick/quail embryonic chimeras in which the quail olfactory placode was replaced by a chick placode, on order to check the role of environment and cell-cell interactions in the choice of COR expression by a differentiating olfactory neuron and in olfactory nerve formation.

Results

Expression of the COR3b *gene in chick-quail chimeras*

The embryos were grafted at embryonic day 3 (stage 17-18 of H&H and 14-15 of Zacchei)(Fig.1). and sacrificed 3 to 11 days later. Consequently this study concerned chimeric embryos between ED 7 (stage 24 of Zacchei) and ED14 (stage 31) (Quail embryos hatch around day 16). Such chimeras were performed to analyze the expression of *COR3b* gene by the chick graft under the control of a quail environment from which the *COR3b* gene is absent. The quails used for these experiments were always tested for the absence of *COR3* in their genome (Data not shown).

In situ hybridisation was used to check the presence of *COR3b* mRNAs in E7 to E14 chimeric embryos. Viability of operated embryos was about 20%, death was mainly due to bleeding at the level of the graft. For the 50 embryos in which the operation had been successful, alternate serial sections performed in the OE region were treated for *in situ* hybridisation (Fig.2, 3) or immuno-cytochemistry (Fig.2).

The extent of the chick graft was always checked using immuno-cytochemistry with QCPN antibodies. The DAB immunostaining of quail nuclei provided a general brown staining of the quail host tissue while the chick graft tissue appeared unstained (Fig. 2B,D,F).

The structure of both operated and contro-lateral epithelia was normal. However, the shape of the nasal cavity was generally different from normal animals, being slightly hypertrophied on the side of the graft,

thus showing a similar morphology to that of a normal chick embryo of the same stage but the thickness of olfactory epithelium was unchanged. On the contrary in quail/quail and chick/chick grafted embryos the morphology of the nasal cavity, olfactory epithelium and nerve was identical on both sides and to that of control non-operated embryos of the same species.

Since the operations were performed only on the right side, the contro-lateral (left) olfactory epithelium could be used as control. *COR3b* mRNAs were detected in the ipsilateral olfactory epithelium indicating that neurons from the chick graft differentiated and expressed *COR3b* gene in a quail environment whereas labeled cells were absent from the olfactory epithelium of the control side. Nevertheless, on both sides olfactory receptor neurons expressed COR2 and 4 (Fig.2E). The same results were obtained whether the underlying chick mesectoderm was left in or excluded from the chick graft. In order to study the involvement of centro-peripheral interactions on olfactory neurons expressing *COR3b* gene, the levels of COR3b expression in chimeric quail embryos and normal chick embryos of corresponding stages control as control were compared before and after synaptogenesis between ED 7 (stage 24 of Zacchei) and ED 14 (stage 31 of Zacchei) and chick embryos of corresponding stages as control (Fig.2, 3).

At embryonic day ED 7, the morphology of the nasal cavity was still normal on both sides. *COR3b* mRNAs were detected in the olfactory epithelium derived from the chick graft (fig.3B) in a small population of *COR3b* -positive cells (one or two cells) mostly located basally where neuroblast cell bodies are located. The number of positive cells was higher in the control chick embryo (Fig.3A) than in the olfactory epithelium from the chick graft in the chimera (Fig.3B). At this stage, the first neurons are differentiating and sending their axons together with migrating neuroblasts from the placodal epithelium to the anterior telencephalon, but epithelio-bulbar synapses are not yet formed (Ayer-Le-Lièvre *et al.*, 1995). A few *COR3b* positive migrating cells were also observed along the olfactory nerve between the olfactory epithelium and the olfactory bulb presumptive area of the anterior prosencephalon on the operated side but not on the contro- lateral side.

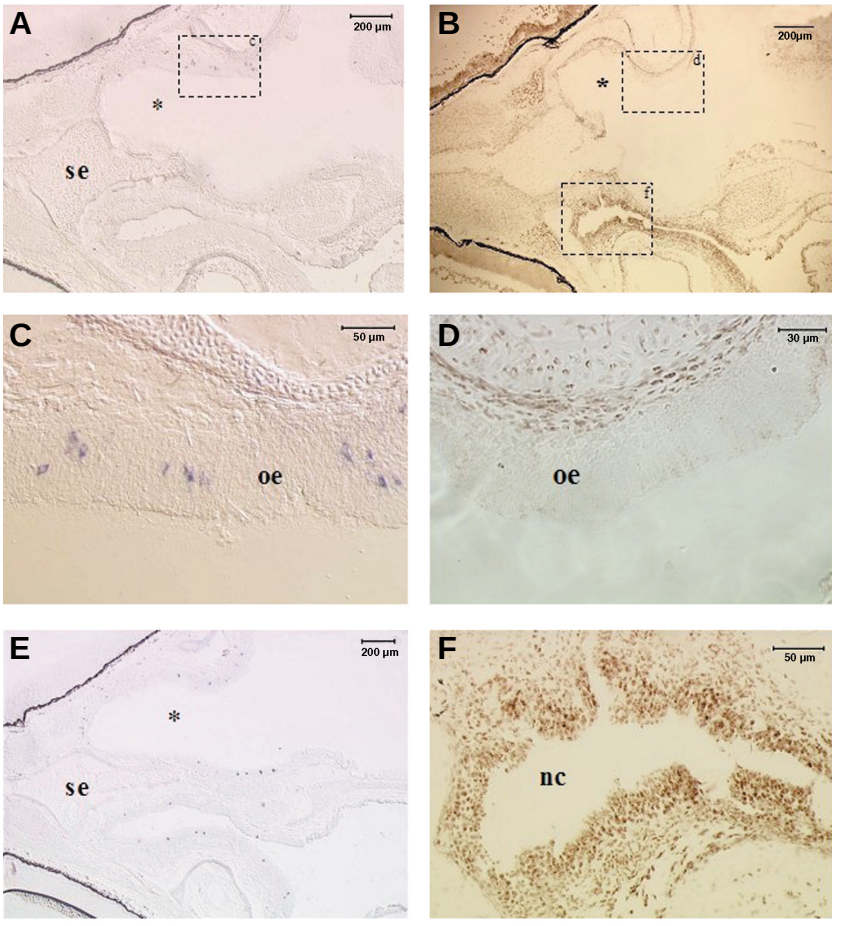

Fig. 2. Comparison of *COR* gene expression in ipsi- and contro-lateral sides of ED14 chimeras. (A) In situ *hybridisation using a* COR3b *riboprobe on a coronal section of the olfactory region of an ED14 chimera. Labeled cells were detected in the grafted (*) chick olfactory epithelium (oe) but not in the contro-lateral oe of the quail host.* **(B)** *QCPN immunoreactivity on the next serial section of the chimera produced a general brown nuclear labeling of the head tissues except for the right epithelium (*) developed from the chick graft.* **(C)** *Higher magnification of (A) showing the perinuclear labeling of* COR3 *expressing cells in the chick graft.* **(D,F)** *Higher magnifications of (B) showing the absence of the nuclear QCPN immuno-reaction on cells of the right oe, confirming its origin from the grafted chick placode (D) and its presence on cells of the quail oe of the chimera (F).* **(E)** In situ *hybridisation using a mix of antisense riboprobes for COR2 and COR4 confirmed that genes from these two OR subgroups were expressed in cells of both contro- and ipsi-lateral sides. Abbreviations: se, septum; nc, nasal cavity; oe, olfactory epithelium. The Nomarski interferential contrast system was used for figures A,C,E.*

At ED8-10, during the beginning of bulbar synaptogenesis, some chick neurons still expressed *COR3b* gene in the quail environment. These cells were always randomly distributed but their position in the depth of the olfactory epithelium (OE) became more medial. The level of COR gene expression increased markedly between ED8 (Fig.3C) and ED10 (Fig.3E) in normal chick embryos (Leibovici *et al.*, 1996; Ayer-Le Lièvre *et al.*, 1995), but not in grafted chick OEs(Fig.3D, F). In the OE of the quail embryos, sections hybridized with *COR3b* antisense riboprobes lacked positive cells (fig.3F). The same was observed when a sense riboprobe was used as control in the OE of grafted embryos (data not shown).

At ED 13- 14, the shape of the nasal cavity was modified in chimeras. On the grafted side, the nasal cavity was dilated and lined with the chick OE which is larger than the normal quail OE (Fig.3H, 2A, B, E). The quail host OE was consequently reduced and slightly compressed on the other side. The number of positive cells and intensity of labeling were higher than in stage ED8-9 and reached the same level as in control chick embryos.

DiI labeling and formation of the olfactory nerve in chimeras

In order to visualize the projections of the olfactory nerve fibres and their target zones on the olfactory bulb, a tiny crystals of DiI was placed into the olfactory pit of formaldehyde fixed embryos (stage 29-30 of H&H). The olfactory region of the placode derived epithelium

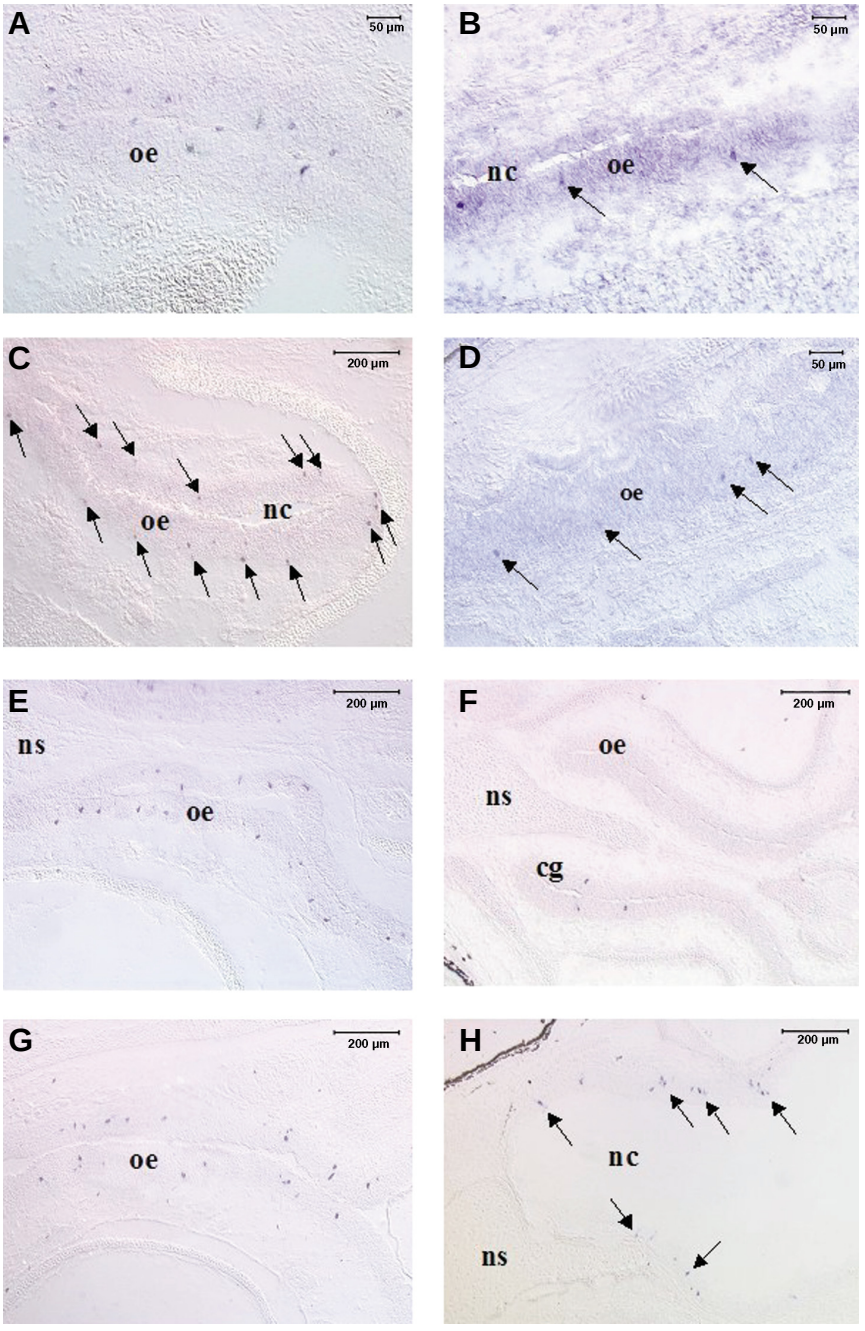

Fig.3. Comparison of *COR3* expression in chick olfactory epithelium of control chick and chimeric embryos between ED6 and ED14. *In chick olfactory epithelium grafted in quail, whatever the stage of development, the neurons were differentiated and some of them expressed a* COR3b *gene (B,D,F,H) as visible after* in situ *hybridisation. At ED6, the level of expression of* COR3b *gene appeared higher in a control chick embryo* **(A)** *than in the epithelium of chimeras* **(B)** *where the number of positive cells (arrows) was around 2 or 3 cells per section. At ED8, the number of labeled cells (arrows) was still higher in the chick control* **(C)** *than in the grafted epithelium of the chimeras* **(D)**, *although in this one, it was slightly increased. Two days later, ED10, just after the beginning of synaptogenesis, the level of* COR3 *expression clearly increased in the control chick epithelium* **(E)** *but not in the chick graft (cg) of the chimeras* **(F)**. *At ED14, two days before quail hatching, the nasal ipsilateral cavity was hypertrophied* **(H)** *compared to that of the quail host but the number of cells expressing* COR3 *gene (arrows) had increased to become similar to that of the control chick epithelium* **(G)**. *Abbreviations: oe, olfactory epithelium; cg, chick graft; ns, nasal septum; nc, nasal cavity.*

was restricted to the multilayered epithelium covering the superior concha and the olfactory nerve was lying along the roof, the posterior, the ventro-lateral walls and the upper part of the nasal septum. For this reason it was not necessary to dissect the nasal septum of the embryos to observe labeling with DiI. In the chick embryo, at E6.5, anterograde transport of DiI labeled the olfactory fibres which constitute the olfactory nerve from the crystal site area to the prosencephalon. Axons from the olfactory receptor neurons assembled to form the olfactory nerve (ON) and reached their target in the anterior telencephalon at the presumptive site of the olfactory bulb (OB) primordium (Fig.4A, B).

In chick control embryos, the fibres rapidly converge and follow a specific way (Fig.4A, B). The same experiments was performed on quail embryos from equivalent ED 6 (stage 22 of Zacchei). There was no difference in the migration pathway of the olfactory nerve fibres from OE to olfactory bulb between both species (Fig.4A, C). When observed at higher magnification, the fibres were notß modified in shape (Fig.4B, D). The development of the olfactory nerve was similar in quail and chick, although the head is already smaller in quail than in chick.

In the heterospecific chick-quail chimeras, we placed a DiI crystal on both sides in order to compare the development of ON in normal and experimental conditions. In the host quail OE, the same characteristics of migration as in control quail embryos were observed (Fig.4C, E). However, on the grafted side, although the axonal projections of *COR3b* neurons reached the quail prosencephalon, the route followed by the ON fibres was abnormal (Fig.4E, F). The nerve fibres formed separated fascicles and the olfactory nerve seemed to lose its homogeneity (Fig.4F). In fact, part of the olfactory fibres failed to converge with the main group of olfactory nerve fibres (Fig.4F) and reached the prosencephalon in a different zone (more lateral) of the prosencephalon after crossing the normal nerve route below the main bundle of olfactory fibres. Alternatively, the presence of axons from the chick graft in a quail environment or the expression by some of them of *COR* genes absent from the quail host genome might be responsible for these modifications of the axonal pathway of a subpopulation of nerve fibres.

Discussion

The early choice of expression of an OR by a sensory neuron is independent of periphero-central connections and of interactions with the tissular environment of the placode.

COR expression starts early in placodal cells (ED 5) in chick, more than 2 days before the first periphero-central synapses are established. This

favours the idea that COR expression choice is not influenced by functional periphero-central synapses either in chick or in mammals (Ayer-Le Lièvre *et al.*, 1995; Strotmann *et al.*, 1995). However, the last authors suggested that diffusible factors might govern the expression of a specific OR gene by an olfactory neuron (Strotmann *et al.*, 1995). Actually, after synaptogenesis between ED8 and ED10, the level of expression of COR genes increases suggesting that it might be controlled by the olfactory bulb (Leibovici *et al.*, 1996; Mathonnet *et al.*, 2002). This hypothesis was also supported by the results of experiments of disruption of olfactory periphero-central connections by axotomy *in ovo* which resulted in a drastic decrease in the expression of OR. (Leibovici *et al.*, 1996). Consequently, a role for central nervous system and tissular environment in OR expression and terminal differentiation had to be considered. In favor of the hypothesis according to which prosencephalon might play a role in the olfactory neuron differentiation at early stages prior to synaptogenesis is the fact that a few periphero-central connections organize early in development. Periphero-central connections before ED8 in the chick are characterised by the outgrowth of sensory axons from a few placodal neurons which start to reach, around ED3, their central target, i.e., the presumptive bulbar region of the anterior telencephalon without making yet functional synapses and one day before migration of placodal neurons begins along the same pathways. Thus, there are cellular connections between both peripheral and central olfactory primordia before the first expression of OR can be detected. Conversely it has been knownfor long that olfactory peripheral fibres are involved in bulbar formation from the anterior prosencephalon (Van Campenout, 1936; Farbman, 1992, for review). Considering the existence of such connections, they might have a role in olfactory neuron differentiation This point was tested here in the chick/ quail chimera model. In such chimeras before synaptogenesis, two types of influences could be expected: either COR expression in chick neurons would have been restricted to the choice of genes present in the host genome, or the bulb would have prevented *COR3* expressing ORNs from differentiating, or surviving, eventually by blocking the transfer of survival factors to these cells or by sending apoptosis or cell death signals. However, the expression of a chick specific gene, *COR3,* by a chick ORN, derived from a chick placode grafted into a quail embryo, confirms that the central nervous system does not specify a given receptor choice by a population of sensory neurons. It was also shown here that the olfactory bulb of chimeras does not exert any negative selection and that survival cues seem to be available to the ORNs without consideration for the OR genes expressed by the olfactory neurons.

The same is also true after synaptogenesis. The presence of *COR3* expressing neurons was maintained at least up to one and a half day before host hatching time. The number and the level of *COR3* expressing neurons were clearly increased after 10 days as they are in normal chick embryos. Since, the level of expression depends on interactions with bulbar neurons through the transmission of neuronal growth factors necessary for neuronal survival and differentiation maintenance (Mathonnet *et al.*, 2001; Mathonnet *et al.*, 2002), this increased level of expression also indicates that such functional periphero-central connections were established between *COR3*+ chick olfactory neurons and the quail bulbar neurons.

Thus, these results clearly prove for the first time that the early choice of an OR expression by a neuron is independent from periphero-central interactions and does not interfere with its subsequent survival capabilities whatever the developmental stage of the embryos.

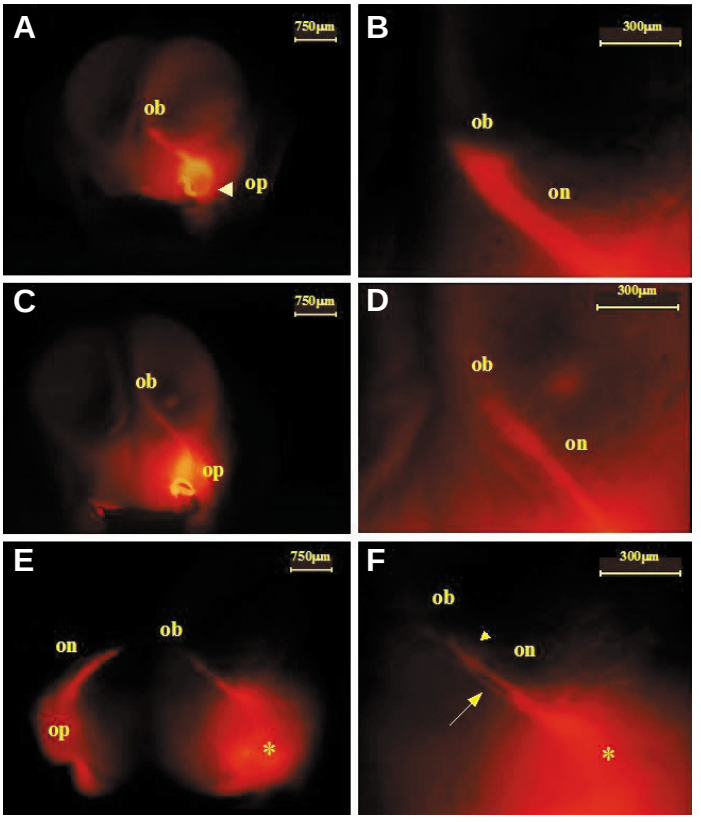

Fig.4. DiI labeling of the olfactory nerve (ON) in normal and chimeric embryos at E6,5. (A) *Low magnification of left ON in a chick embryo after dissection of the facial ectoderm and mesenchyme.* **(B)** *Higher magnification of ON in chicken showing that it consists of a bundle of olfactory fibres which converge to the olfactory bulb (ob).* **(C)** *Low magnification of olfactory placodal epithelium (op) and ON in normal quail embryo after dissection of the facial mesenchyme.* **(D)** *High magnification of quail ON and OB, showing the homogeneous bundle of fibres constituting the ON.* **(E)** *Low magnification of a facial view of olfactory DiI labeling in a chimera allowing the comparison of the quail normal ON on the left and the ON from chick graft (*****) on the right.* **(F)** *A high magnification of the latter shows that some fibres from the chick graft present a defasciculation (arrow) forming a separate bundle which crosses the route of the olfactory nerve below the main bundle to reach a more lateral part (arrowhead) of the telencephalon. ob, olfactory bulb primordium area of the telencephalon; on, olfactory nerve; op, olfactory placodal epithelium.*

Interestingly, this observation is in agreement with the amazing adaptability and phylogenetic evolution of olfactory capabilities. Thus any new OR gene produced by duplications and mutations, a process recognized in primates for example (Brand-Arpon *et al.*, 1999) would be expressed in neurons as long as they could connect functionally with the central second order neurons and could afford new odorant detection capabilities eventually supporting a positive selection of the new OR gene.

Furthermore analysis of the quail chick chimeras point to a specific influence of the sensory epithelium on the mesenchymal environment. The chick epithelium develops to a size

corresponding to that of the control chick, hence larger than the quail host epithelium. The quail mesenchyme produces a cartilaginous concha adapted to the size of the grafted epithelium. This is an agreement with previous observations concerning the adaptation of the size of the skull to that of the brain in quail/chick chimeras (Schowing, 1968; Le Lièvre, 1978).

The complementary series of grafting experiments with and without the underlying mesectoderm of the olfactory placode demonstrated also that the tissular environment of placodes, outgrowing axons and migrating neurons is not involved either in the choice of OR expression.

Consequently this choice is intrinsically determined in the placodal cells themselves and could be originally at random. However, the general dispersion of olfactory neurons expressing the same OR subgroup and the absence of large clones of neurons expressing a single OR in bird embryo olfactory epithelium (Leibovici *et al*, 1996) might still reflect the existence of some kind of lateral inhibition between placodal differentiated neurons and their neighboring differentiating neuroblasts to prevent the expression of the same OR.

The questions of the establishment of functional peripheral-central synapses of fibres from neurons expressing an exogenous OR and the maintenance of their function at postnatal stages remains opened and currently under screening.

COR expression and olfactory nerve formation

It has been previously shown in mammals that most axonal fibres from neurons expressing the same OR converge on a limited number of glomeruli in the olfactory bulb allowing the organisation of complex topographic maps of odorant sensitivity on the bulb (Mombaerts *et al*., 1996; Malnic *et al*., 1999)

The abnormalities of the nerve formation observed in the avian chimeras may provide some indications on the mechanisms responsible for the axonal guidance'and convergence of sensory fibres on glomeruli for synaptogenesis with second order central bulbar neurons.

The role of extracellular molecules such as semaphorins had been evoked (Tessier-Lavigne and Goodman, 1996 for review; De Castro *et al*., 1999; Pasterkamp *et al*., 1999). However, the diversity of signals or their combinations used for the guidance of ORN axons should then be of the same order of magnitude as the OR repertoire. Thus, considering the wide variety of OR genes, particularly in rodents where it has been evaluated to be about one thousand, the implication of olfactory receptors themselves as one of the key factors of axonal guidance cues has been evoked several times (Gierer, 1998; Wang *et al*., 1998; Leibovici *et al*., 1996). An hypothetical role in olfactory axonal guidance has been attributed to COR expressing sub-population of placodal migrating cells based on possible interactions between the same OR or OR belonging to the same subgroup expressed both by the outgrowing axons and migrating cells (Singer, 1995; Leibovici *et al*., 1996; Dreyer *et al*., 1998). A possible mechanism is based on a computerized analysis of OR sequences (Singer, 1995) showing that sequence variation of the transmembrane domains, supposed to be the specific interaction site of odorants and OR, is reflected by sequence variations in the extracellular loops. Consequently, possible homophilic interactions by their extracellular loops have been hypothesised for OR expressed on axonal fibers and cells, notably between ORN growth cones and target cells in the bulb (Kubick *et al*., 1997; Dreyer, 1998).

As confirmed here in bird chimeras, the expression of COR outside the olfactory neurons is restricted to cells migrating from the placode to the anterior prosencephalon along with sensory axonal fibres and the expression of COR mRNA in such cells stops around E8 before the beginning of synaptogenesis. Thus, the main role of OR in the nerve formation would be restricted to early stages between E5 and E8 corresponding to axonal guidance up to the bulbar primordium.

The originality of chick-quail chimeras lies in the fact that *COR3* genes are absent from the quail genome and can only be expressed in cells originating in the chick grafted placode. Furthermore, in chimeras, the growth cones from neurons of the grafted placode express an OR which could not be represented in glomerular cells of the olfactory bulb. This situation is different from that of transgenic mice, in which the complete genomic DNA from every cell including olfactory placodes, facial mesenchyme and CNS has been modified after the electroporation of a targeting vector into E14 embryonic stem (ES) cells (Momtbaerts *et al*. 1996; Bozza *et al* 2002). In the chick/quail chimeras, *COR3* expressing cells, which must have migrated from the chick grafted placode,.are not present in the OB primordium but adjacent to it. Thus they can specify restricted areas of this structure to be used as target for the *COR3* +growing axons. Each COR subgroup would eventually interact with specific combinations of extracellular molecules to define the migration pathway of the migrating cells expressing the corresponding COR subgroup. Indeed *COR3* positive cells were observed along the olfactory nerve, suggesting a major role of OR expressing migrating neurons in the signalisation of pathways, which allow the olfactory axons to reach their target on the OB primordium, eventually using homophilic interactions. Possibly in the quail host environment, the foreign CORs expressed by chick neuroblasts do not find their specific combination of extracellular molecules along the main nerve pathway. This would explain why, in chimeras, anomalies of the olfactory nerve formation with divergent nerve fibre fascicles were observed, which probably correspond to axon bundles from neurons expressing exogenous COR in the quail host embryo.

In order to assess the role of the expression of COR exogenous genes in the phenomenon of axonal guidance, it will be interesting to develop a new experimental model to confirm and obtain more precisions about this hypothesis.

Materials and Methods

Animals and embryos

Fertilized chicken and quail eggs (Gallus gallus Linné and Coturnix coturnix japonica) from commercial sources (Commercial label chicken heterozygous for the naked neck gene, couvoir du Faget, Lot, France; caille de Chanteloup, Ille et Villaine, France) were incubated at 38ºC in a humidified atmosphere. Embryos were staged according to the developmental time table of Hamburger and Hamilton (H&H, 1951) for the chicken and the table of Zacchei (Zacchei, 1961) for the quail. Control and operated embryos were sacrificed at different stages, their head frozen to be sectioned for *in situ* hybridisation or immuno-cytochemistry. In order to check the absence of *COR3* gene in each quail host genome, DNA preparation from a piece of tissue have been tested by PCR using *COR3* specific primers.

Microsurgery

Chicken–quail chimera system (Fig.1). Chicken and quail eggs at E3,5 were used at homologous embryonic stages, namely stage 17-18 of Hamburger and Hamilton and stage 14-15 of Zacchei.

The graft involved several steps: First, the quail olfactory placode was excised *in ovo* using sickle shaped microscalpels made from a sewing needle honed on an Arkansas stone fragment. The operation was performed under a dissection microscope (Leica).

Second, the olfactory placode from a chick embryo was dissected *ex ovo* including the lining mesectoderm; alternately the chicken head was cut off and the placode dissected with microscalpels in a solution of pancreatin (Life technology-GIBCObrl) diluted 1/4 in a Tyrode solution without Ca^{2+} and Mg^{2+}, in order to completely remove the adjacent underlying mesectoderm. Finally, the chick olfactory placode was transplanted isotopically into the operated quail embryo. After the graft, the eggs were returned to the incubator until sacrifice. As controls a few homeografts of quail on quail and chick on chick were also performed (data not shown).

In situ *hybridisation*

Heads from ED5.5 to ED14 chimeras were cut off, frozen and sectioned (para-sagittal or coronal sections) on a cryostat (CM3050, LEICA, France) at -25ºC. Coronal cryosections (12 µm thick) were collected onto superfrost Plus Slides (Polylabo, France) and treated for hybridisation as described by Leibovici *et al.* (1996). Clones encoding the complete chicken olfactory receptor 3b (*COR3b*) and the 3'end deleted COR2 and COR4 cDNA provided by M. Leibovici were used as template to generate riboprobes. *COR2*, *COR3*, *COR4* were subcloned in pGEM 4Z (Promega, France).

Digoxigenin labeled sense or antisense riboprobes (cRNA) were produced by *in vitro* transcription with T7 or Sp6 RNA polymerase (Roche Molecular Biology, France) from linearized full length COR2, *COR3*, COR4 clones. After a fixative step with PFA 4%, pre-hybridisation, hybridisation, washing and immunological detection conditions were as described by Schaeren-Wiemers and Gerfin-Moser (1993). Hybridisation was visualized using 4-nitroblue tetrazolium chloride and 5-bromo- 4-chloro-3 indolyl phosphate (NBT/BCIP/Roche Molecular Biology, France) which yields a blue precipitate and the sections were mounted using «immu-mount» (Shandon, USA). Sections were observed with a Nomarski interferential contrast system.).without any background staining

Immunocytochemistry.

Serial 8 µm cryosections from heads of normal and chimeric embryos were fixed in Carnoy's fluid or PFA 4%, immunostained with the QCPN (Developmental Studies Hybridoma Banks, Iowa) antibody which recognizes a quail nuclear antigen. The monoclonal antibody QCPN was prepared in mice and the second antibody was an anti-mouse Ig conjugated with horseradish peroxidase (anti-mouse-POD, Sigma, France). The immunostaining was revealed using DAB substrate (Diaminobenzidine, Roche Molecular Biochemistry, France). Quail cell nuclei were thus stained in brown (Fig.2B, F).

Fluorescent tracer: carbocyamine dye (Di I)

Tracing of neuronal projections was performed in normal and chimeric embryos, after fixation, by immersion in formalin 1% or 4% (Sigma, France) during one to two hours at room temperature or over-night at 4ºC.

After complete fixation, a small crystal of Di I (Carbocyanine DiI, Interchim, Montluçon, France) was inserted into the nasal pit with microscalpels under a dissection microscope. The embryos were then stored in 1% formalin at room temperature allowing DiI to diffuse along the olfactory nerve during two to four days.

Embryos were studied and photographed using a fluorescence dissection microscope (MZFL III, Leica) equipped with the appropriate filter, Di I: 540-600 nm (Fig.4).

Acknowledgments

Financial support was provided by grants from Conseil Régional du Limousin and CNRS.

References

AYER-LE LIÈVRE, C., LAPOINTE, F. AND LEIBOVICI, M. (1995).£Avian olfactory neurogenesis. *Biol. Cell* 84: 25-34.

BARTH, A.L., JUSTICE, N.J. AND NGAI, J. (1996) Asynchronous onset of odorant receptor expression in the developing zebrafish olfactory system. *Neuron* 16: 23-34.

BRAND-ARPON V., ROUQUIER S., MASSA H., DE JONG P.J., FERRAZ C., IOANNOU P.A., DEMAILLE J.G., TRASK B.J. AND GIORGI D. (1999) A genomic region encompassing a cluster of olfactory receptor genes and a myosin light chain kinase (MYLK) gene is duplicated on human chromosome regions 3q13-q21 and 3p13. *Genomics*. 56: 98-110.

BUCK, L.B. AND AXEL, R. (1991). A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65: 175-187.

BOZZA T, FEINSTEIN P, ZHENG C, MOMBAERTS P. (2002) Odorant receptor expression defines functional units in the mouse olfactory system. *J. Neurosci.* 22: 3033-3043.

CAMPENHOUT. VAN E. (1936) Développement du système nerveux crânien chez le poulet. *Arch. Biol,* 48: 611-665.

COULY, G. AND LE DOUARIN, N. M. (1988). The fate map of the cephalic neural primordium at the presomitic to the 3-somite stage in the avian embryo. *Development* 103: 101-113.

CROUCHER, S.J. AND TICKLE, C. (1989). Characterization of epithelial domains in the nasal passages of chick embryos: spatial and temporal mapping of a range of extracellular matrix and cell surface molecules during development of the nasal placode. *Development*. 106: 493-509.

DE CASTRO F., HU L, DRABKIN H., SOTELO C., CHEDOTAL A. (1999) Chemoattraction and chemorepulsion of olfactory bulb axons by different secreted semaphorins. *J. Neurosci*. 19: 4428-36.

DREYER, W.J. (1998). The area code hypothesis revisited: olfactory receptors and other related transmembrane receptors may function as the last digits in a cell surface code for assembling embryos. *Proc. Natl. Acad. Sci. USA* 95: 9072-9077.

FARBMAN, A.I. (1992). «Cell biology of olfaction» In *Developmental and cell biology Series* (P.W. Barlow, D. Bray, P.B. Green and J.M.W. Slacks, Eds.), Cambridge Univ. Press, Cambridge.

GIERER, A. (1998). Possible involvement of gradients in guidance of receptor cell axons towards their target position on the olfactory bulb. *Eur. J. Neurosci*. 10: 388-391.

HAMBURGER, V. AND HAMILTON, H.L. (1951). A series of normal stages in the development of the chick embryos. *J. Morphol.* 88: 49-92.

KRAUTWURST D., YAU K.W., REED R.R. (1998) Identification of ligands for olfactory receptors by functional expression of a receptor library. *Cell*. 95: 917-926.

KUBICK, S., STROTMANN, J. ANDREINI, I. AND BREER, H. (1997). Subfamily of olfactory receptors characterised by unique structural features and expression patterns. *J. Neurochem*. 69: 465-475.

LANCET, D. AND BEN-ARIE, N. (1993) Olfactory receptors. *Curr. Biol*. 3: 668-674.

LEIBOVICI, M., Lapointe, F., Aletta, P. and Ayer-Le Lievre, C. (1996). Avian olfactory receptors: differentiation of olfactory neurons under normal and experimental conditions. *Dev. Biol*. 175: 118-131.

LE LIEVRE C.S. (1978) Participation of neural crest-derived cells in the genesis of the skull in birds. *J. Embryol. Exp. Morphol*. 47: 17-37.

MALNIC B., HIRONO J., SATO T., BUCK L.B. (1999) Combinatorial receptor codes for odors. *Cell*. 96: 713-23.

MATHONNET M., COMTE I., LALLOUE F., AYER-LE LIEVRE C. (2001). Insulin-like growth factor I induced survival of axotomized olfactory neurons in the chick. *Neurosci. Lett.* 308: 67-70.

MATHONNET M., LALLOUE F., PETIT B., COMTE I., LEBOUTET M.J. AND AYER-LE LIEVRE C. (2002). Differential responses of olfactory neurons to axotomy at embryonic and postnatal stages. *Neuroscience*. 109: 207-17.

MOMBAERTS, P., WANG, F., DULAC, C., CHAO, SK., NEMES, A., MENDELSOHN, M., EDMONDSON, J. AND AXEL, R. (1996). Visualizing an olfactory sensory map. *Cell* 87: 675-686.

MOMBAERTS P. (1996) Targeting olfaction. *Curr. Opi. Neurobiol.* 6: 481-486.

MOMBAERTS P. (1999). Odorant receptor genes in humans. *Curr. Opin. Genet. Dev.* 9: 315-320.

NEF, S., ALLAMAN, I., FIUMELLI., H, DE CASTRO, E. AND NEF, P. (1996). Olfaction in birds: differential embryonic expression of nine putative odorant receptor genes in the avian olfactory system. *Mech. Dev.* 55: 65-77.

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.

PARMENTIER, M., LIBERT, F., SCHURMANS, S., SCHIFFMANN, S., LEFORT, A., EGGERICKX, D., LEDENT, C., MOLLEREAU, C., GERARD, C., PERRET, J., GROOTEGOED, A. AND VASSART, G. (1992). Expression of members of the putative olfactory receptor gene family in mammalian germ cells. *Nature* 355: 453-455.

PASTERKAMP R.J, RUITENBERG M.J, VERHAAGEN J. (1999) Semaphorins and their receptors in olfactory axon guidance.*Cell Mol. Biol.* 45: 763-79.

ROUQUIER, S., FRIEDMAN, C., DELETTRE, C., VAN DEN ENGH, G., BLANCHER, A., CROUAU-ROY, B., TRASK, BJ. AND GIORGI, D. (1998). A gene recently inactivated in human defines a new olfactory receptor family in mammals. *Hum. Mol. Genet.* 7: 1337-45.

SCHAEREN-WIEMERS, N. AND GERFIN-MOSER, A. (1993). A single protocol to detect of various types and expression levels in neural tissue and cultured cells: in situ hybridisation using digoxigenin- labeled cRNA probes. *Histochemistry* 100: 431-440.

SCHOWING J. (1968) Demonstration of the inductive role of the brain in osteogenesis of the embryonic skull of the chicken *J. Embryol. Exp. Morphol.* 19: 83-94.

SINGER, M.S., SHEPHERD, G.M. AND GREER, C.A. (1995). Olfactory receptors guide axons. *Nature* 377: 19-20.

STROTMANN, J., WANNER, I., HELFRICH, T. AND BREER, H. (1995). Receptor expression in olfactory neurons during rat development: in situ hybridisation studies. *Eur. J. Neurosci.* 7: 492-500.

TESSIER-LAVIGNE M, GOODMAN C.S. (1996) The molecular biology of axon guidance. *Science* 274: 1123-33.

VASSAR, R., NGAI, J. AND AXEL, R. (1993). Spatial segregation of odorant receptor expression in the mammalian olfactory epithelium. *Cell* 74: 309-318.

WANG, F., NEMES, A., MENDELSOHN, M. AND AXEL, R. (1998). Odorant receptors govern the formation of a precise topographic map. *Cell* 93: 47-60.

YNTEMA, C L. (1955) Ear and nose, In: «Analysis of Development», B H Miller, P A Weiss and V Hamburger eds, W B Saunders Company, Philadelphia & London, pp. 415-426.

ZACCHEI, A.M. (1961). Lo sviluppo embrionale della quaglia giapponese (coturnix coturnix japonica T.S.).*Arch. ital. di Anat. e di Embriol*, 66: 36-62.

ZHAO, H., IVIC, L., OTAKI, JM., HASHIMOTO, M., MIKOSHIBA, K. AND FIRESTEIN, S. (1998). Functional expression of a mammalian odorant receptor. *Science* 279: 237-242.