Role of the neural crest in face and brain development.

Nicole Le Douarin, José M Brito, Sophie Creuzet

▶ To cite this version:

Nicole Le Douarin, José M Brito, Sophie Creuzet. Role of the neural crest in face and brain development.. Brain Researchearch Reviews / Brain Research Brain Research Rev; BRAIN RESEARCH BRAIN RESEARCH REVIEWS, 2007, 55 (2), pp.237-47. 10.1016/j.brainresrev.2007.06.023 . hal-00180605

HAL Id: hal-00180605
https://hal.science/hal-00180605

Submitted on 19 Oct 2007

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

This article has been published in *Brain Res Rev. 2007 vol. 55(2); pp 237-47*
doi : 10.1016/j.brainresrev.2007.06.023; Elsevier
The publication is available online at
http://www.sciencedirect.com/

A Century of Neuroscience Discovery:
Reflecting on the 1906 Nobel Prizes To Golgi and Cajal
Brain Research Reviews - Special Issue

ROLE OF THE NEURAL CREST IN FACE AND BRAIN DEVELOPMENT

Nicole M. LE DOUARIN, José M. BRITO and Sophie CREUZET

Institut de Neurobiologie—Alfred Fessard
Laboratoire de Développement, Evolution et Plasticité du Système Nerveux
Avenue de la Terrasse, 91198 Gif-sur-Yvette, France
Tel: 33 1 69 82 41 33 – Fax: 33 1 69 82 41 32

Keywords : *Craniofacial development; Endoderm; Prosencephalon; Shh; Fgf8; Quail-chick chimeras.*
Abbreviations *: ANR, anterior neural ridge; BA: branchial arch; E, embryonic day; NC, neural crest; NCC neural crest cells; NT, neural tube; r, rhombomere; ss: somite stage; ZLI, zona limitans intra-thalamica.*

Abstract :

Since the time of Ramon y Cajal, very significant progress has been accomplished in our knowledge of the fate of the early neural primordium. The origin of the peripheral nervous system from the transient and pluripotent embryonic structure, the neural crest has been fully deciphered through the use of appropriate cell marking techniques. Most of the pioneer work in this field was carried out in lower vertebrates up to 1950 and later on in the avian embryo. New techniques which allow the genetic labelling of embryonic cells by transgenesis are now applied in mammals and fish.

One of the highlights of neural crest studies was its paramount role in head and face morphogenesis. Work pursued in our laboratory for the last fifteen years or so has analysed at both cellular and molecular levels the contribution of the NCC to the construction of the facial and cranial structures. Recently, we have found that the cephalic neural crest plays also a key role in the formation of the fore- and mid-brain.

Introduction

The neural crest (NC) was discovered by Wilhem His in 1868 in the chick in which he called it *Zwischenstrang*, meaning that it appears just after the closure of the neural tube (NT) as a "band of particular material lying between the NT and the superficial ectoderm" (quoted from Sven Horstadius in "the Neural Crest", 1950). W.His noticed that the cells forming the NC rapidly started to migrate away from the neural primordium and, as seen under the microscope, aggregated laterally to the neural tube to form the dorsal root ganglia. Hence, the term of "ganglionic crest" also attributed to this structure.

Investigations carried out mainly in lower vertebrates at the turn of the XXth century revealed that the NC was the source of many other derivatives. Apart from the components of the peripheral nervous system and melanocytes, it was shown to yield cells participating in the formation of the facial skeleton. The first mention that "some mesenchymal cells" of the head are of NC origin was from Katschenko in 1888 in selacians. This notion was extended to teleosts and birds by Goronowitsch (1892; 1893). Julia Platt then claimed that NC-derived cells contributed to the cartilage of the visceral arches and to the dentine of the teeth (1893; 1897). She coined the term of *mesectoderm* to designate these cells which were different in origin from the rest of the body mesenchyme which is derived from the mesodermal germ layer and that she called *mesendoderm*. The term *mesectoderm* or *ectomesenchyme* have subsisted until now to designate the mesenchyme derived from the NC, thus of ectodermal origin.

These seminal observations were not readily accepted at the end of the XIXth century since the germ layer theory developed by von Baer (1828) was still powerful. According to the latter, the skeletal tissue was considered to be exclusively derived from the mesoderm.

However, the contribution of the mesectoderm to the head skeleton was further confirmed during the first half of the XXth century. A thorough account of the investigations carried out on this problem essentially on the amphibian embryos can be found in the monograph written by Horstadius in 1950. The work of Pierre Chibon in France (1964) based on the use of a cell-marking technique using tritiated thymidine ([^{3}H]TdR) to label neural crest cells (NCC), fully confirmed the results of the previous investigations. This technique has also been used in the chick embryo by Jim Weston in 1963. This was the first attempt to follow the migration of NCC in higher vertebrates by means of an efficient cell-marking technique (see le Douarin, 1982, and references therein). Labelling cells with [^{3}H]TdR to investigate the fate of the cephalic NCC in the chick embryo was first undertaken by Malcolm C. Johnston (1966) and later on by Drew Noden (1975; 1976; 1978a, b), The demonstration that the entire facial skeleton and most of the skull were formed by cells of ectodermal origin was brought about after the quail-chick chimera system was devised by one of us (Le Douarin, 1969; 1973a; 1973b; 1974; 1976). This technique which provides a stable cell labelling allowed to demonstrate that the NC is the only source of the cells forming the facial skeleton. Moreover, it led to the construction of a very precise fate map of the cephalic NC derivatives (Le Lièvre et Le Douarin, 1975; Le Lièvre, 1978; Couly et al., 1993; Couly et al., 1996; Köntges and Lumsden, 1996).

1. The role of the cephalic neural crest in the formation of craniofacial structures

Although in fish the NC of the entire neural axis is at the origin of mesectodermal derivatives (see Le Douarin, 1982, and Le Douarin and Kalcheim, 1999, for references), in higher vertebrates, the ability of the NCC to form skeletal tissues is not uniformly distributed along the antero-posterior axis. Yielding cartilage and endochondral bone appears as a ground property of the skeletogenic NC corresponding to the domain located between the mid-diencephalic level to rhombomere 8 (r8) included. However, within this domain, the capacity of NCC to form membrane bones (e.g. skull bones) is restricted to its most rostral part corresponding to NCC of the nasofrontal and maxillo-mandibular processes. The rostral NC which forms the craniofacial skeleton is designated FSNC (for Facial Skeletogenic NC; **Fig. 1A,B**).

These different domains exhibit distinct molecular traits. The caudalmost domain of the cephalic NC (from r4 down to r8) expresses *Hox* genes of the first four paralogous groups (Hunt et al., 1991; Prince and Lumsden, 1994; Köntges and Lumsden, 1996; Couly et al., 1996). Rostrally, the FSNC (from mid-diencephalon down to r2) does not express any Hox genes (it is referred to as *Hox*-negative). At the edge of both, NCC from r3 exhibit a versatile *Hox* gene status according to the environment into which they migrate (Graham et al., 1993; 1994; Creuzet et al., 2004). The cells exiting from r3 level and contributing to the first branchial arch (BA1) lose their *Hoxa2* expression, whereas those migrating to BA2 maintain it (**Fig. 1C-F**).

2. The Hox-negative cephalic NC is the only domain able to yield the facial skeleton

If the entire *Hox*-negative NC domain is removed, no facial structures develop (Couly et al., 2002; Creuzet et al., 2002; **Fig. 2A,B**). Moreover, if grafted to the anterior domain, *Hox*-expressing NCC cannot substitute for the *Hox*-negative ones (Couly et al., 1998; 2002; **Fig. 2C,D**). In this experimental situation, however, the *Hox*-positive cells migrate within the fore-mid-brain area and maintain their *Hox* gene expression at least up to embryonic day 6 (E6). These *Hox*-positive cells differentiate into neural derivatives, yield some connective-like cells but do not differentiate into cartilage and bones (see Fig. 4 in Couly et al., 2002). By contrast, any fragment (about a third of the normal *Hox*-negative NC domain) of the *Hox*-negative NC can regenerate a normal face if left in situ (or if grafted from a quail to a chick embryo following the excision of the entire host *Hox*-negative NC; Couly et al., 2002; **Fig. 2E,F**). Thus, the *Hox*-negative NCC behave as an "equivalence group". Since any small region of this area is able to yield the same derivatives as the whole area itself. One can then conclude that NCC do not possess the information to pattern each of the skeletal elements forming the facial skeleton. This information has thus to come from the environment in which the facial skeleton develops.

3. Inhibition of the development of skeletal tissues by Hox gene expression in the rostral NC domain

Another question raised by these experiments was whether forced expression of Hox genes in the rostral NCC exerts an inhibition on facial skeleton development. This possibility was tested in experiments where *Hox* gene expression was experimentally targeted to the FSNC by electroporation of *Hoxa2*, *Hoxa3* or *Hoxb4* constructs into the pre-migratory NCC. Expression of these genes was found to preclude the development of the facial skeleton (Creuzet et al., 2002; **Fig. 2G-M**).

4. Role of the foregut endoderm in patterning the facial skeleton

The next step of our investigation was to look for a source for the information able to pattern the facial skeleton from the *Hox*-negative NCC. It has been known for long that the interaction between NCC and the pharyngeal endoderm is critical for branchial skeleton development. Experiments performed in amphibians, chick and mouse, involving explants of NC-derived mesenchyme, showed that the mesenchyme of crest origin can differentiate into cartilage only if co-cultured or co-grafted in contact with pharyngeal endoderm (Epperlein and Lehmann, 1975; Hall and Armstrong, 1980; Graverson and Hall, 1987; Seufert 1990). In contrast, the differentiation of other NC derivatives such as melanocytes, ganglia, nerves lined by Schawnn cells, does not depend on the presence of endoderm (Epperlein, 1978). Moreover, organ culture of chick mandibular ectomesenchyme at E3, after removal of the ectodermal

epithelium, does not prevent chondrogenesis to occur, while membrane bone formation does not take place in these explants (Takahashi et al, 1991).

In vivo, mutation of the *van gogh* (*vgo*) gene in zebrafish prevents the segmentation of the pharyngeal endoderm and consequently the formation of pharyngeal pouches. Pharyngeal cartilages differentiate and fuse to form a single piece of cartilage. Thus, during embryonic development, formation of the visceral cartilages directly depends on the segmentation of pharyngeal arches (Piotrowski and Nüsslein-Volhard, 2000). Therefore, the pharyngeal endoderm has at least two roles: first on NCC differentiation into cartilage and second in BA segmentation a process critical for patterning the branchial cartilages.

Furthermore, disruption of the *Nodal* pathway by mutation of *casanova* (*cas*) gene, completely hampers endoderm development in zebrafish and results in the absence of all the branchial cartilages. In absence of endoderm, the cephalic NCC fail to migrate ventrally and the expression of chondrogenic markers is down-regulated (Alexander et al., 1999 and David et al., 2002). Moreover, repression by morpholinos of Fgf3 production by the pharyngeal endoderm results in defects in the posterior arch derivatives, without affecting the first and second BAs (David et al; 2002). Hence, the endoderm is essential for development of the BAs. It is a source of growth factors which display different antero-posterior distribution and have specific functions in the ontogeny of the visceral skeleton.

Another set of evidence on the role of the pharyngeal endoderm of facial skeleton development was provided by experiments carried out in our laboratory in chick embryos. Ablation of antero-posterior stripes of foregut endoderm at 5-6 somite stage (5-6ss) resulted in the loss of skeletal pieces specific of the level of ablation (Couly et al., 2002). This is in agreement with previous observation that the presence of the pharyngeal endoderm is critical for the migration of the NCC leading to the development of the branchial arches (Alexander et al., 1999). In addition, it was possible to demonstrate that orthotopic grafts of the same stripes of quail ventral foregut endoderm into an intact chick embryo induced the duplication of the bone rudiments that normally develop at this level (Couly et al., 2002). Thus, graft of the endoderm of the anterior foregut of 5-6ss quail embryos (see zone II/III; **fig. 3A**) at the presumptive level of BA1 ectoderm (Couly et al., 1990) leads to the formation of an extra-Meckel's cartilage in addition to the endogenous lower jaw (**Fig. 3B,C**). It is noteworthy that the extra-cartilage tissue was exclusively made up by endogenous NCC of the chick host embryo (see Couly et al., 2002, fig. 9C). The role of the quail endoderm was only to induce the formation of this extra bone. Moreover, changing the antero-posterior (AP) and dorso-ventral (DV) orientation of the grafted endodermal stripes modified the polarity of the supernumerary cartilage elements (see Couly et al., 2002, fig.10). Conversely, the grafts of the ventral foregut endoderm were not able to induce BA1-type cartilages or bones in the posterior BAs (in which the endoderm, NCC and surrounding tissues express Hox genes) (Couly et al., 2002). One can therefore conclude that the anterior ventral endoderm of the 5-6ss embryo foregut is able to trigger the skeletogenic developmental programme of the lower jaw when it is in the context of BA1.

The nature of the signal through which this programme is initiated was the subject of our recent investigation.

5. Role of Shh in triggering the development of the lower jaw.

The disruption of *Shh* gene in the mouse by Chiang et al (1996) revealed the paramount role of this morphogen in the development of the vertebrate head and particularly of its craniofacial skeleton. In *Shh*-/- mutant embryos, the expression of several genes fails to be activated in the developing BA1. Such is the case for *Fgf8*, *Bmp4*, *Barx1*, *Gsc*, *Sox9* and *Twist* (Moore-Scott and Manley, 2005; Washington-Smoak et al., 2005; Yamagishi et al., 2006). The source of the Shh morphogen playing a role in the induction and/or maintenance of expression of these genes was not known until recently.

We were able to show that Shh produced by the anterior foregut endoderm is critical for lower jaw development (Brito et al., 2006). In chick embryos the expression of *Shh* in the foregut endoderm starts at about 4-5ss in the most rostral part of the endoderm which is in close contact with the prechordal plate (**Fig. 3D,E**). At 6ss, *Shh* is expressed in the ventral part of the endoderm down to the level of the prosencephalon-mesencephalon boundary. *Shh* expression in the foregut endoderm remains restricted to its anterior ventral domain up to 16ss. Later on, *Shh* transcripts are present along the whole pharyngeal ventro-lateral endoderm from which BA1 endodermal pouches eventually form. Excision of the forehead (including the

complete area of the anterior foregut endoderm producing Shh) at 5-6ss prevented the expression of *Shh* in the first branchial pouch endoderm. The NCC however colonized BA1 but failed to survive and the absence of lower jaw ensued (**Fig. 3F,F'**). At E3-4, expression of *Fgf8*, *Bmp4* and *Pitx1* in the oral ectoderm did not occur. The same was true for *Pitx1* in the mesenchyme of BA1.

We decided to try and substitute the normal source of Shh production by providing the decapitated embryos with Shh-soaked beads as indicated in **figure 3I**. This was followed by the expression of *Shh* in the endoderm of the first branchial pouch and by the survival of NCC which had colonized BA1. The development of a lower jaw was rescued as seen at E11 (**Fig. 3J,K**). Expression of *Pitx1, Bmp4* and *Fgf8* was restored in BA1 (see Brito et al., 2006, **fig 3N,Q**). Although, at these early stages, Shh is produced by various other sources (ventral neuroectoderm, notochord and facial ectoderm), the most important one for BA1 development is the ventral foregut endoderm.

On the same line, the culture of E3 chick embryo BA1 in which the endoderm had been removed, did not show expression of *Fgf8* in the ectoderm of BA1. Interestingly, treatment of these cultures with Shh-soaked beads was able to restore this expression (Haworth et al., 2004; 2006).

In conclusion, the foregut endoderm is a source of morphogens crucial for the development of BA1 derivatives. Among those, Shh plays a key role in the survival of the NCC which have migrated to BA1 and in the induction of several downstream gene activities critical for their further developmental programme.

6. NCC and *Fgf8* expression from ectoderm : a reciprocal signalling process

As described above, excision of the FSNC results in the agenesis of the facial skeleton. In addition, it promotes a strong impairment of brain development (**Fig. 2B,D**). Removal of the FSNC is accompanied by the nearly complete absence of *Fgf8* expression in BA1 ectoderm and in the anterior neural ridge (ANR; **Fig. 4B**). In FSNC-ablated embryos, defects are significantly corrected by implantation of Fgf8-soaked beads either laterally to the presumptive ANR or on the bilateral BA1 ectodermal presumptive territories. In these cases, the upper and lower jaw skeleton develops, closure of the NT takes place and the overall development of the brain tends to normalize (**Fig. 4F**).

The results of these Fgf8-rescue experiments raised the question of the origin of the NCC responsible for regeneration of the face. Replacing r3 in chick FSNC-deprived embryos by its quail counterpart showed that Fgf8 supply strongly stimulates the r3-derived NCC growth. These cells migrate massively rostrally and colonize BA1 as can be seen by staining r3-derived NCC by the quail specific antibody QCPN (**Fig. 4G,H**). In addition, the cells which have invaded BA1 become *Hoxa2*-negative whereas those which have migrated into BA2, remain *Hoxa2*-positive (see Creuzet et al., 2004, fig. 1W-Z).

This is in agreement with the fact that when high expression of *Hoxa2* is experimentally imposed to BA1-NCC (Creuzet et al., 2002), no differentiation of skeletal tissue ensues. Moreover, this result shows that maintenance of expression of this gene depends upon cues arising from the environment. These cues are clearly missing in BA1.

In experiments where the excision of FSNC includes r3, it is seen that NCC from r4 do not migrate to BA1 even when Fgf8 beads are present. These experiments demonstrate the strong regeneration capacities and the high degree of plasticity displayed by the pre-otic NCC and the absence of such properties in post-otic NCC.

7. In vivo silencing of FGF8 gene in BA1

The above described experiments suggest a chemo-attractive role of Fgf8 in directing the migratory flux of NCC. In order to further explore this problem, double strand RNA designed from the Fgf8 c-DNA (Pekarik et al., 2003) was introduced by electroporation into the superficial ectoderm of the presumptive BA1 territory on the right side of 5-6ss chick embryos, the contra-lateral side being the control. The aim of this inhibition of Fgf8 protein synthesis was to see whether the migration of NCC was disturbed on the experimental side. When the embryos were observed at E2.5 (25ss) after whole-mount immunostaining of NCC by the HNK1 monoclonal antibody (Abo and Balch, 1981; Tucker et al., 1984; Vincent and Thiery, 1984) which decorates migratory NCC, the experimental side was strikingly devoid of NCC (Creuzet et al., 2004), showing that *Fgf8* expression by the ectodermal component of BA1 is critical for its colonization

by NCC. Whether this effect is related to the stimulation of NCC survival and proliferation or to a chemo-attractive process remains to be investigated.

8. NCC signal regulates FGF8 expression in the superficial and neural ectoderm of the ANR

The previously described experiments showed that removal of the anterior (Hox-negative) NCC at pre-migratory stages strongly precludes *Fgf8* expression in ANR ectoderm. In order to know if this effect can be mediated exclusively by the FSNC, we replaced the Hox-negative NCC domain by the neural fold corresponding to r4-r6 from 5-6ss quail embryos (**Fig. 4I**). The results observed from E2 to E11 showed that the NCC from the *Hox*-positive domain had normally migrated. At E2.5, they had invaded the space between the prosencephalic neural epithelium and the superficial ectoderm (**Fig. 4J-L**), where they were mixed up with chick mesodermal cells as in normal development (see Etchevers et al., 1999, fig. 7 and Etchevers et al., 2001, Figs. 2 and 3). Interestingly, the superficial ectoderm and the neural epithelium corresponding to the ANR exhibited abundant *Fgf8* transcripts (while NCC did not; **Fig. 4M**). At E6, the head morphology of these embryos was characterized by the dorsal closure of the brain, a rudimentary BA1 and a strongly reduced forebrain (**Fig. 4N**). At E8, the lower jaw skeleton had further developed (**Fig. 4O,P**). At E11, the lower jaw skeleton was nearly complete, the occipital and otic regions of the skull of mesodermal origin (see Couly et al., 1993) were present. In contrast, the nasal skeleton was absent as well as the frontal and parietal bones (**Fig. 4Q**).

The interpretation of this experiment was that NCC, whether originating from the anterior *Hox*-negative domain (as in normal development) or from a more caudal (*Hox*-positive) area of the neural axis exert a positive effect on Fgf8 production by the superficial and neural ectoderm of the ANR. The embryos subjected to this operation are therefore in a situation comparable to those in which the normal source of Fgf8 was replaced by exogenous Fgf8-soaked beads. In both cases, r3-derived NCC invade BA1 (**Fig. 4K**) and develop into a lower jaw (**Fig. 4O-Q**; Creuzet et al. 2004).

9. The role of the NC in patterning the pre-otic brain.

During embryogenesis, regionalisation of the neural plate and the neural tube is controlled by secreted molecules of the Shh, Fgf, and Wnt families which are endowed with morphogenetic properties. These morphogens were shown to be involved in the regulation of pre-otic brain development. They are produced from signalling centres (the ANR, the isthmus, the dorsal midline, the Zona Limitans Intrathalamica -ZLI-, and the floor plate) that act on brain regionalisation. These centres regulate the expression of definite transcription factors that control the identity of thevarious domains of the neural epithelium (Martinez et al., 1999; McMahon and Bradley, 1990; Houart et al., 1998; Reifers et al., 1998; Vieira et al., 2005). These signalling centres are considered as "secondary brain organizers". Distinct sets of signals are produced by each of them : Shh by the basal plate and the ZLI, Wnt by the dorsal midline, Fgf8 by the ANR and the isthmus.

In FSNC-deprived embryos, the brain remains wide open all along the resected territory and the cephalic vesicles (tel,- di-, and mesencephalon) are no longer distinguishable. The prosencephalic and mesencephalic exencephaly is accompanied by the complete loss of the dorso-medial markers, *Wnt1* and *Wnt8b* (**Fig. 4R,S**). In the telencephalic primordium, expression of *Emx2*, *Pax6* and *Dlx2* is severely reduced (**Fig. 4U,V**). Moreover, a striking dorsalization of *Shh* transcript distribution increases the prosencephalic basal plate at the expense of the alar plate. At this level, *Otx2* expression is up-regulated as if the diencephalic alar plate was posteriorized. Long-term structural defects in the dorsal diencephalic midline ensue, together with agenesis of the thalamic and pre-tectal nuclei (Creuzet et al., 2006).

In the embryos subjected to FSNC removal and further supplemented FGF8-soaked beads at the level of the ANR, the morphogenesis of the brain is mostly rescued. As mentioned above, exogenous FGF8 stimulates the progression of NCC (from the edge of the excised territory, i.e. r3) to the forehead. Our data show that NCC migrating rostrally are necessary for the neural tube of the mid- and fore-brain to close. Moreover, the deployment of NCC restores the antero-posterior division of the cephalic vesicles and rescues the growth of the prosencephalic alar plate as shown by the expression of genes normally detected dorsally (*Wnt1*, *Wnt8b*, *Emx2*, *Pax6*, *Otx2*; **Fig. 4T,W**). These data suggest that NCC also repress the Shh ventral signalling arising from the basal plate (Creuzet et al., 2006).

Concluding remarks

Altogether, our data support the emerging picture that the cephalic NC coordinates the formation of craniofacial structures and the development of the anterior encephalic vesicles. The ventral foregut endoderm is essential for the development of the facial skeleton since it provides the facial skeletogenic neural crest cells (FSNC) with a survival signal mediated by Shh (**Fig. 5**). Shh also induces the expression of a set of genes which are critical for patterning the facial structures. These genes are expressed either in the ectoderm (*Bmp4*, *Fgf8*) or in the mesectoderm (*Pitx1*) of the facial processes. Fgf8 is itself crucial for the migration, further survival and proliferation of Hox-negative NCC (**Fig. 5**).We have discovered a novel role for the Hox-negative NCC on brain development (Creuzet et al., 2006). Removal of the FSNC (from mid-diencephalon down to r2 included) results in exencephaly at the level of fore- and mid-brain. The neural tube does not close dorsally and most of the alar plate-derived structures do not develop. If the lack of Fgf8 in the ANR is compensated by an exogenous source of Fgf8, NCC from r3 migrate rostrally and mostly rescue brain development. In addition, the NC is at the origin of (still unknown) signals that trigger and maintain the expression of *Fgf8* in the ANR ectoderm. Fgf8 of ANR origin exerts a positive effect on the patterning of the basal plate in the di- and mesencephalon as attested by the restriction of *Shh* expression to the ventral midline and ZLI when exogenous Fgf8 is provided to FSNC-ablated embryos. On the other hand, the NC has a direct effect on patterning the development of alar and roof plates in the di- and mesencephalon. It is also essential for the normal forward extension of the telencephalon.

Acknowledgements :

Work in the authors' laboratory is supported by the Centre National de la Recherche Scientifique, Association pour la Recherche sur le Cancer (grant n°3929). J.B. is a recipient of a fellowship from la Fondation pour la Recherche Médicale.

References :

Abo, T., Balch, C.M., 1981. A differentiation antigen of human NK and K cells identified by a monoclonal antiboby (HNK-1). J. Immunol. 127, 1024-1029.

Alexander, J., Rothenberg, M., Henry, G.L., Stainier D.Y., 1999. casanova plays an early and essential role in endoderm formation in zebrafish. Dev. Biol. 215, 343-357.

Baer, K.E. von, 1828. Ueber Entwicklungsgeschichte der Tiere. Bornträger, Köningsberg.

Brito, J.M., Teillet, M.A., Le Douarin, N.M., 2006. An early role for sonic hedgehog from foregut endoderm in jaw development: ensuring neural crest cell survival, Proc. Natl. Acad. Sci. U S A, 103, 11607-11612.

Chiang, C., Litingtung, Y., Lee, E., Young, K.E., Corden, J.L., Westphal, H., Beachy, P.A., 1996. Cyclopia and defective axial patterning in mice lacking Sonic hedgehog gene function. Nature 383, 407-413.

Chibon, P., 1964. Analyse par la méthode de marquage nucléaire à la thymidine tritiée des dérivés de la crête neurale céphalique chez l'Urodèle Pleurodèles waltii. C. R. Acad. Sci. 259, 3624-3627.

Couly, G., Le Douarin, N.M., 1990. Head morphogenesis in embryonic avian chimeras: evidence for a segmental pattern in the ectoderm corresponding to the neuromeres. Development 108, 543-558.

Couly, G.F., Coltey, P.M., Le Douarin, N.M., 1993. The triple oprigin of skull in higher vertebrates : a study in quail-chick chimeras. Development 117, 409-429.

Couly, G.F., Grapin-Botton, A., Coltey, P.M., Le Douarin, N.M., 1996. The regeneration of the cephalic neural crest, a problem revisited : the regenerating cells originate from the contralateral or form the anterior and posterior neural fold. Development 122, 3393-3407.

Couly, G.F., Grapin-Botton, A., Coltey, P.M., Le Douarin, N.M., 1998. Determination of the identity of the derivatives of the cephalic neural crest : incompatibility between Hox gene expression and lower jaw development. Development 125, 3445-3459.

Couly, G., Creuzet, S., Bennaceur, S., Vincent, C., Le Douarin, N.M., 2002. Interactions between Hox-negative cephalic neural crest cells and the foregut endoderm in patterning the facial skeleton in the vertebrate head. Development 129, 1061-1073.

Creuzet, S., Couly, G., Vincent, C., Le Douarin, N. M., 2002. Negative effect of Hox gene expression on the development of neural crest derived facial skeleton. Development 129, 4301-4313.

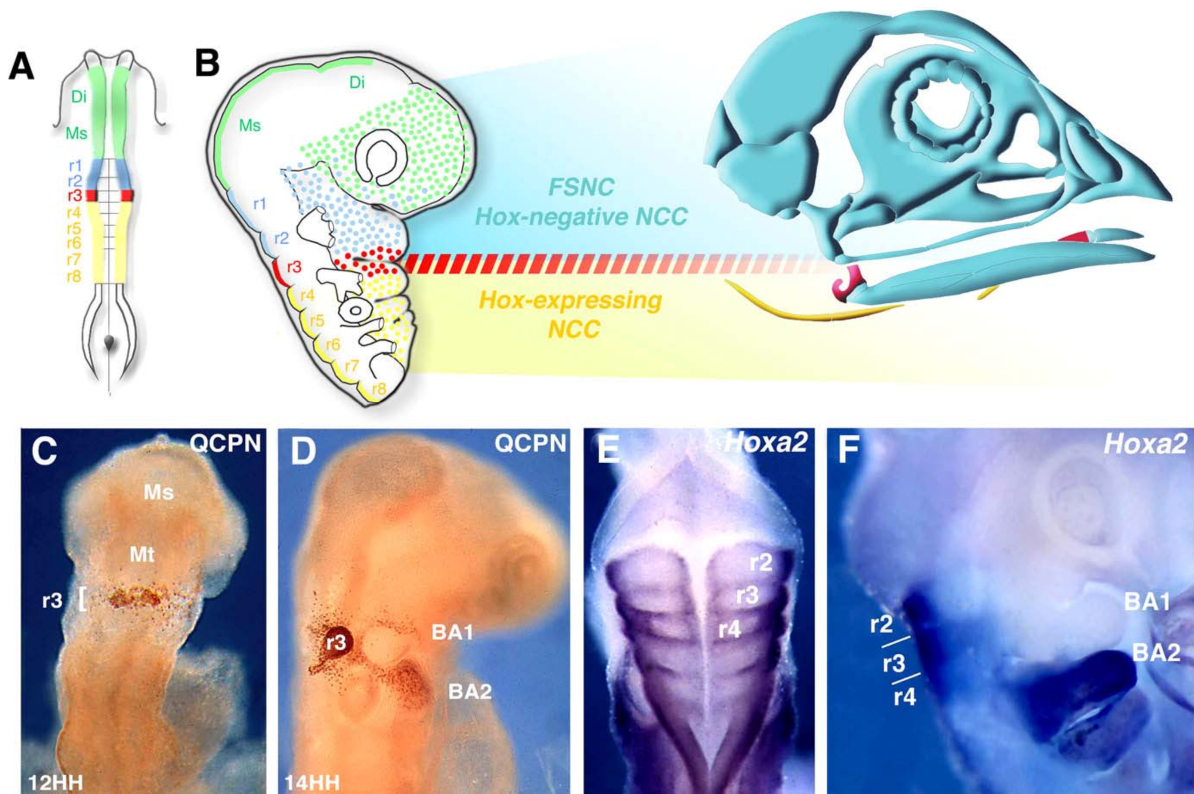
Creuzet, S., Schuler, B., Couly, G., Le Douarin, N.M., 2004. Reciprocal relationships between Fgf8 and neural crest cells in facial and forebrain development. Proc. Natl. Acad.Sci. USA. 101, 4843-4847.

Creuzet, S., Martinez, S., Le Douarin ,N.M., 2006. The cephalic neural crest exerts a critical effect on forebrain and midbrain development. Proc. Natl. Acad.Sci. USA. 103, 14033-14038.
David, N.B., Saint-Etienne, L., Tsang, M., Schilling, T.F., Rosa, F.M., 2002. Requirement for endoderm and FGF3 in ventral head skeleton formation. Development 129, 4457-4468.
Epperlein, H.H., 1978. The ectomesenchymal-endodermal interaction system of Triturus alpestris in tissue culture: morphological and histochemical characterization of developing neural derivatives. Differentiation 11, 109-123.
Epperlein, H.H., Lehmann, R., 1975. The ectomesenchymal-endodermal interaction-system (EEIS) of Triturus alpestris in tissue culture. I. Observations of visceral cartilage. Differentiation 4, 159-174.
Etchevers, H.C., Couly, G., Vincent, C., Le Douarin, N.M., 1999. Anterior cephalic neural crest is required for forebrain viability. Development 126, 3533-3543.
Etchevers, H.C., Vincent, C., Le Douarin, N.M., Couly, G.F., 2001. The cephalic neural crest provides pericytes and smooth muscle cells to all blood vessels of the face and forebrain. Development 128, 1059-1068.
Goronowitsch, N., 1892. Die axiale and die laterale Kopfmentamerie des Vögelembryonen. Die Rolle der sog. "Ganglienleisten" im aufbaue der Nervenstämme. Anat. Anz. 7, 454-464.
Goronowitsch, N., 1893. Weiters über die ektodermale Entstehung von Skeletanlagen im Kopfe der Wirbeltiere. Morphol. Jahrb. 20, 425-428.
Graham, A., Heyman, I., Lumsden, A., 1993. Even-numbered rhombomeres control the apoptotic elimination of neural crest cells from odd-numbered rhombomeres in the chick hindbrain. Development 119, 233-245.
Graham, A., Francis-West, P., Brickell, P., Lumsden, A.,1994. The signalling molecule BMP4 mediates apoptosis in the rhombencephalic neural crest. Nature 372, 684-686.
Graveson, A.C., Armstrong, J.B., 1987. Differentiation of cartilage from cranial neural crest in the axolotl (Ambystoma mexicanum). Differentiation 35, 16-20.
Hall, B.K., 1980. Tissue interactions and the initiation of osteogenesis and chondrogenesis in the neural crest-derived mandibular skeleton of the embryonic mouse as seen in isolated murine tissues and in recombinations of murine and avian tissues. J. Embryol. Exp. Morphol. 58, 251-264.
Haworth, K.E., Healy, C., Morgan, P., Sharpe, P.T., 2004. Regionalisation of early head ectoderm is regulated by endoderm and prepatterns the orofacial epithelium. Development, 131, 4797-4806.
Haworth, K.E., Wilson, J.M., Grevellec, A., Cobourne, M.T., Healy, C., Helms, J.A., Sharpe, P.T., Tucker, A.S., 2006. Sonic hedgehog in the pharyngeal endoderm controls arch pattern via regulation of Fgf8 in head ectoderm. Dev. Biol. In press.
His, W., 1868. Untersuchungen über die erste Anlage des Wirbeltierleibes. Die erste Entwicklung des Hühnchens im Ei.F.C.W. Vogel, Leipzig.
Houart, C., Westerfield, M., Wilson, S.W. 1998. A small population of anterior cells patterns the forebrain during zebrafish gastrulation. Nature 391, 788-792.
Hörstadius, S.,1950. The Neural Crest : its Properties and Derivatives in the Light of Experimental Research. Oxford University Press.
Hunt, P., Gulisano, M., Cook, M., Sham, M. H., Faiella, A., Wilkinson, D., Boncinelli, E., Krumlauf, R., 1991. A distinct Hox code for the branchial region of the vertebrate head. Nature 353, 861-864.
Johnston, M.C., 1966. An autoradiographic study of the migration and fate of cranial neural crest cells in the chick embryo. Anat. Rec. 156, 143-156.
Katschenko, N., 1888. Zur Entwicklungsgeschichte des selachierembryos. Anat. Anz., 3, 445-467.
Köntges, G., Lumsden, A., 1996. Rhombencephalic neural crest segmentation is preserved throughout craniofacial ontogeny. Development 122, 3229-3242.
Le Douarin, N.M., 1969. Particularités du noyau interphasique chez la Caille japonaise (Coturnix coturnix japonica). Utilisation de ces particularités comme "marquage biologique" dans les recherches sur les interactions tissulaires et les migrations cellulaires au cours de l'ontogenèse. Bull. Biol. Fr. Belg. 103, 435-452.
Le Douarin, N.M., 1973a. A biological cell labelling technique and its use in experimental embryology. Dev. Biol. 30, 217-222.
Le Douarin, N.M., 1973b. A Feulgen-positive nucleolus. Exp. Cell Res. ***77****, 459-468.*
Le Douarin, N.M., 1974. Cell recognition based on natural morphological nuclear markers. Med. Biol. 52, 281-319.
Le Douarin, N., 1976. cell migration in early vertebrate development studied in interspecific chimeras. In Embryogenesis in Mammals, Ciba Foundation Symposium 40, 71-101. Excerpta Medica, Amsterdam.
Le Douarin, N., 1982. The Neural Crest. Cambridge University Press, Cambridge.
Le Douarin, N.M., Kalcheim, C., 1999. The Neural Crest (second edition). Cambridge University Press, New York.
Le Lièvre, C.S., 1978. Participation of the neural crest-derived cells in the genensis of the skull in birds. J. Embryol. Exp. Morphol. 47, 17-37.
Le Lièvre, C.S., Le Douarin, N.M., 1975. Mesenchymal derivatives of the neural crest: analysis of chimaeric quail and chick embryos. J. Embryol. Exp. Morphol. 34, 125-154.

McMahon, A.P., Bradley, A., 1999. Wnt-1 (int-1) proto-oncogene is required for development of a large region of the mouse brain. Cell 62, 1073-1085.
Martinez, S., Crossley, P.H., Cobos, I., Rubenstein, J.L., Martin, G.R. , 1999. FGF8 induces formation of an ectopic isthmic organizer and isthmocerebellar development via a repressive effect on Otx2 expression. Development 126, 1189-1200.
Moore-Scott, B.A., Manley, N.R., 2005. Differential expression of Sonic hedgehog along the anterior-posterior axis regulates patterning of pharyngeal pouch endoderm and pharyngeal endoderm-derived organs. Dev. Biol. 278, 323-335.
Noden, D.M., 1975. An analysis of migratory behavior of avian cephalic neural crest cells. Dev. Biol. 42, 106-130.
Noden, D.M., 1976. Cytodifferentiation in heterotopically trasnplanted neural crest cells. J. Gen. Physiol. 68, 13a.
Noden, D.M., 1978a. The control of avian cephalic neural crest cytodifferentiation. I. Skeletal and connective tissues. Dev. Biol. 67, 296-312.
Noden, D.M., 1978b. The control of avian cephalic neural crest cells cytodifferentiation. II. Neural tissues, Dev. Biol. 67, 313-329.
Pekarik, V., Bourikas, D., Miglino, N., Joset, P., Stoekli, E. 2003. Screening for gene function in chicken embryo using RNAi and electroporation. Nature Biotech. ***21****, 93-96.*
Prince, V., Lumsden, A. 1994. Hoxa-2 expression in normal and transposed rhombomeres : independent regulation in the neural tube and neural crest. Development 120, 911-923.
Piotrowski, T., Nusslein-Volhard, C., 2000. The endoderm plays an important role in patterning the segmented pharyngeal region in zebrafish (Danio rerio). Dev. Biol. 225, 339-356.
Platt, J., 1893. Ectodermic origin of the cartilage of the head. Anat. Anz. 8, 506-509.
Platt, J., 1897. The development of the cartilaginous skull and the branchial and hypoglossal musculature in Necturus. Morphol. Jahrb.25, 377-464.
Reifers, F., Bohil, H., Walsh, E.C., Crosseley, P.H., Stainer, D.Y., Brand, M. 1998. Fgf8 is mutated in zebrafish acerebellar (ace) mutants and is required for maintenance of midbrain-hinbrain boundary development and somitogenesis. Development 125, 2381-2395.
Seufert, D.W., Hall, B.K., 1990. Tissue interactions involving cranial neural crest in cartilage formation in Xenopus laevis (Daudin). Cell Differ. Dev. 32, 153-165.
Takahashi, Y., Bontoux, M., Le Douarin, N.M., 1991. Epithelio--mesenchymal interactions are critical for Quox 7 expression and membrane bone differentiation in the neural crest derived mandibular mesenchyme. Embo J. 10, 2387-2393.
Tucker G.C., Aoyama, H., Lipinski, M., Tursz, T., Thiery, J.P., 1984. Identical reactivity of monoclonal antibodies HNK-1 ans NC-1: conservation in vertebrates on cells derived from the neural primordium and on some leucocytes. Cell Differ. 14, 223-230.
Vieira, C.,Garda, A.L., Shimamura, K., Martinez, S., 2005. Thalamic development induced by Shh in teh chick embryo. Dev.Biol. 284, 351-363.
Washington Smoak, I., Byrd, N.A., Abu-Issa, R., Goddeeris, M.M., Anderson, R., Morris, J., Yamamura, K., Klingensmith, J., Meyers, E.N., 2005. Sonic hedgehog is required for cardiac outflow tract and neural crest cell development, Dev. Biol. 283, 357-372.
Vincent, M., Thiery, J.P., 1984. A cell surface marker for neural crest and placodal cells : further evolution in peripheral and central nervous sytem. Dev. Biol. 103, 468-481.
Weston, J., 1963. A radiographic analysis of the migration and localization of trunk neural crest cells in the chick, Dev. Biol. 6, 279-310.
Yamagishi, C., Yamagishi, H., Maeda, J., Tsuchihashi, T., Ivey, K., Hu, T., Srivastava, D., 2006. Sonic hedgehog is essential for first pharyngeal arch development. Pediatr. Res. 59, 349-354.

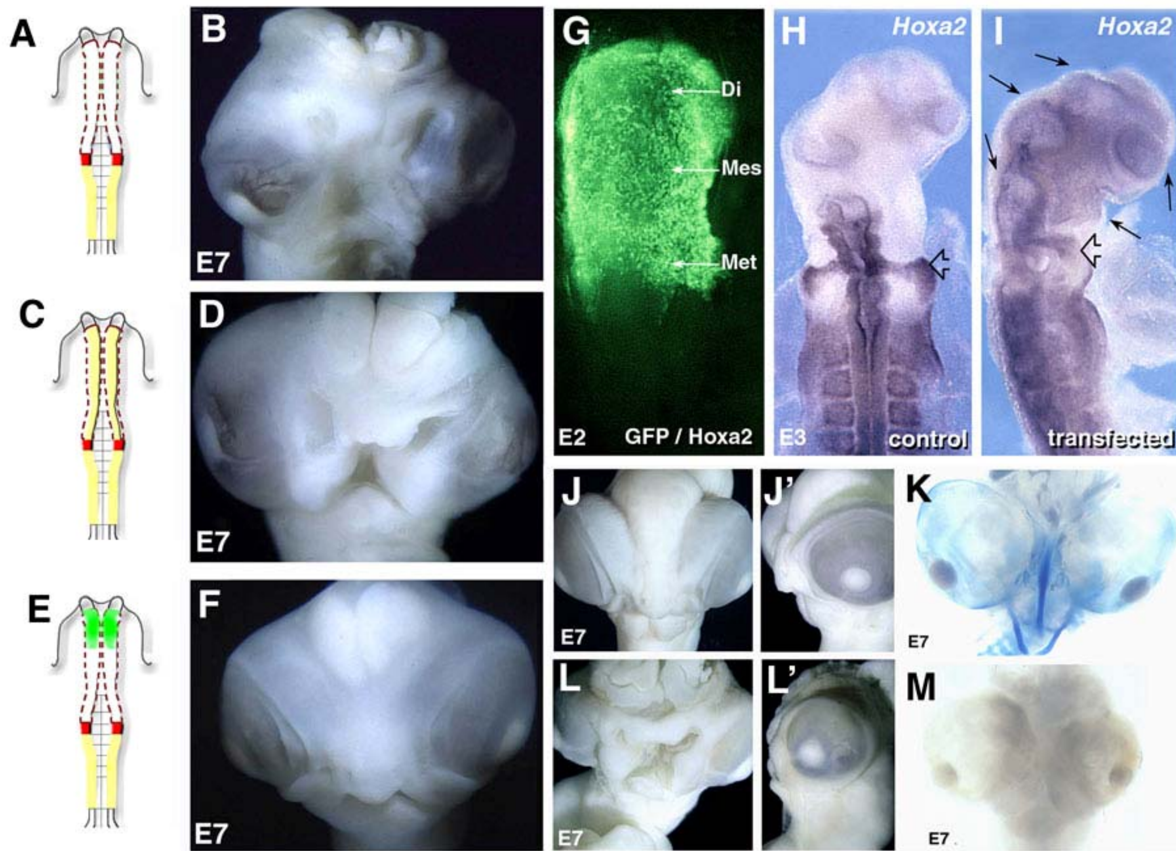
Figures and legends

Figure 1 : Skeletogenic NC and craniofacial development.



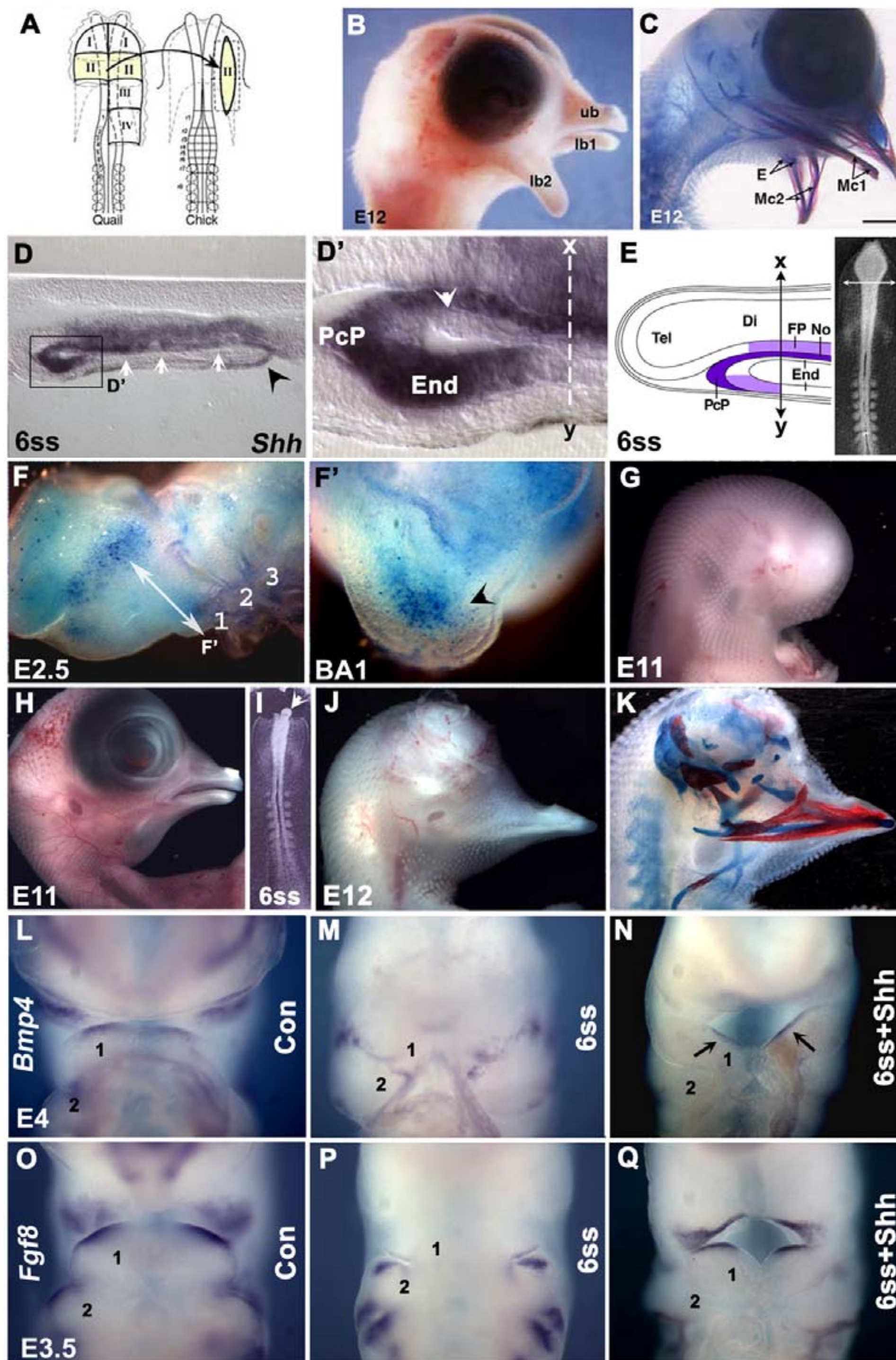
(**A**) In a 5ss chick embryo, the cephalic NC is divided into an anterior *Hox*-negative domain (from mid-diencephalon down to mesencephalon, in green; from r1 to r2, in blue; this domain is referred to as FSNC for facial skeletogenic NC) and a posterior, *Hox*-positive one (in yellow). Rostral to the mid-diencephalon no NCC are ever produced from the neural fold. (**B**) Colonization of facial processes and BAs by the skeletogenic NCC. The anterior *Hox*-negative NCC form skeletal elements of the skull vault, the upper face and jaws. By contrast, the posterior *Hox*-expressing NCC (in yellow) form of the hyoid structure. At the edge of these two NCC domains, r3 NC (in red) is a transition area; these cells participate in BA1 and BA2, as shown in C. (**C**) r3-NCC (here replaced by their quail counterparts and evidenced by QCPN Mab) migrate in BA1 and BA2. (**D**) Their Hox-status obey the environment in which they migrate.

Figure 2 :



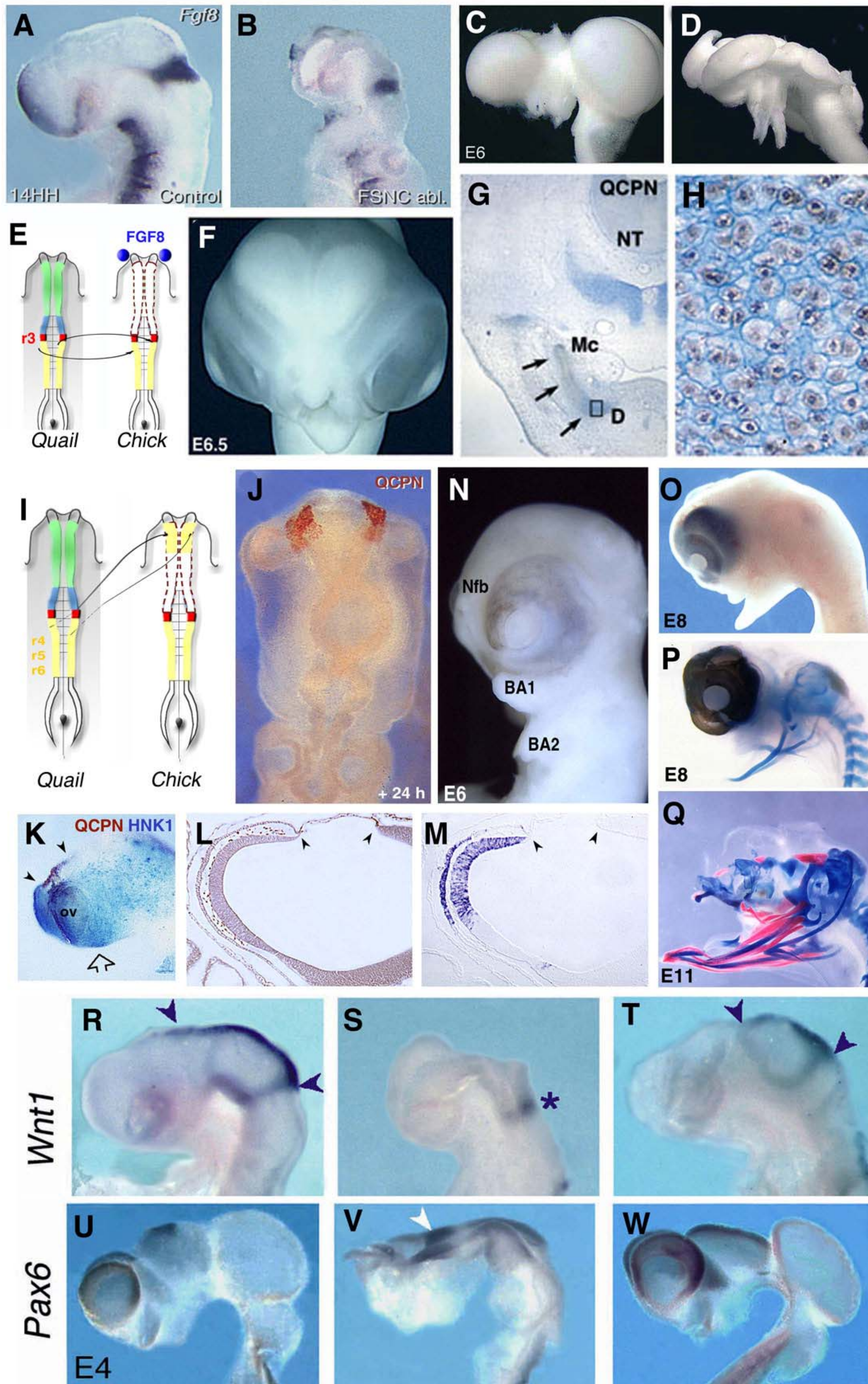
Skeletogenic capacities of the cephalic NCC and the expression of Hox genes.Removal of the *Hox*-negative FSNC in early chick neurula (dotted lines) abolishes the development of facial structures and results in brain malformation in E7 operated embryo (**B**). (**C**)Replacement of FSNC in 5ss chick embryo by a fragment of *Hox*-expressing NC taken from a stage-matched quail embryo severely hampers head morphogenesis at E7: these cells are able to fill the facial processes with mesenchymal cells but fail to yield facial structures (**D**). (**E**) Following removal of whole FSNC (as in A), implantation of only a fragment of the FSNC (from either di-, mes- or anterior rhombencephalic level) restores normal development of face and forebrain at E7 (**F**). (**G**) *In ovo* co-electroporation of *Hoxa2* retroviral and GFP constructs in the entire *Hox*-negative NF ; ectopic expression of the GFP reporter gene in the FSNC migrating tranfected cells (**G**) along with the forced expression of *Hoxa2* in the forehead territory (arrows ; **I;** compare with the normal pattern of Hoxa2 expression in **H**; arrowhead). At E7, compared to the morphology (**J,J'**) and the formation of facial skeletic elements (Alcian blue staining; **K**) in control embryos, *Hoxa2* transgenic embryo exhibits severe defects in facial development (**L, L'**), and facial skeleton (**M**), ressembling those in B and D.

Figure 3:



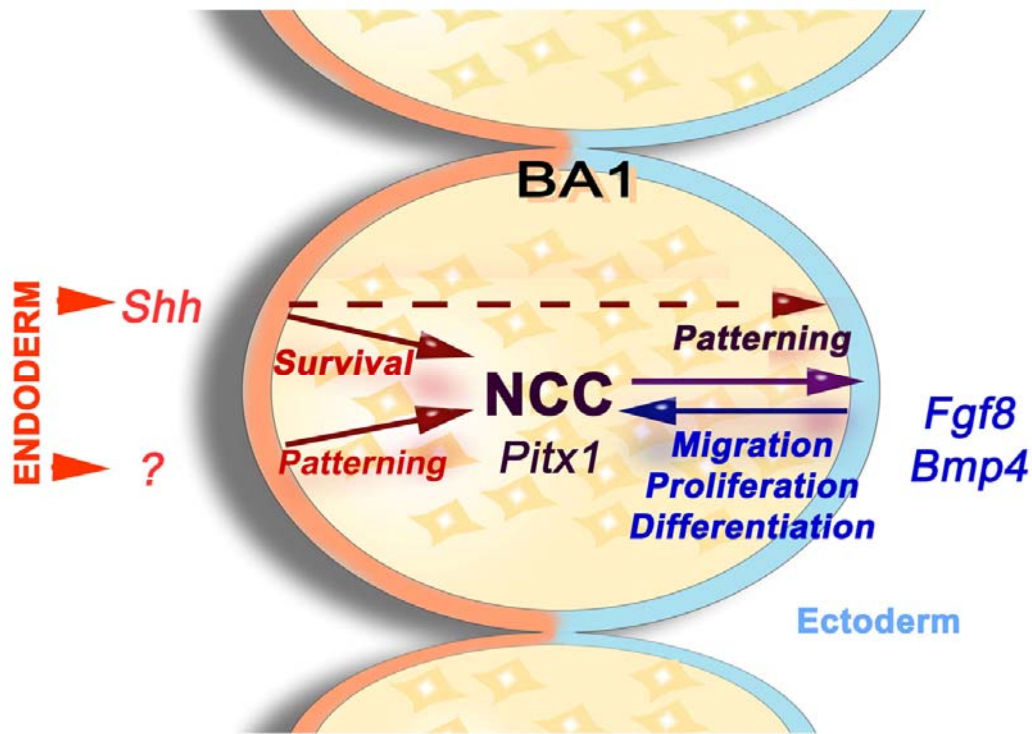
Grafts of bilateral stripes of quail foregut endoderm duplicate the lower jaw skeleton. Two stripes of zone II quail ventral endoderm are positioned laterally under the ectoderm as represented in A (dorsal view). (B) This operation leads to the appearance of a supernumerary lower beak (lb2 in B at E12) with two Meckel's cartilages (Mc2 in C) and corresponding membrane bones; two entoglossum (E) are present under and lateral by to the host Meckel's cartilages (Mc1); ub: upper beak. (D,D') Sagittal section (50   m) of a 6ss chick embryo showing *Shh* expression, in midline cells (notochord and floor plate), precordal plate (PcP), in the anterior ventral foregut endoderm (End), and more posteriorly, in the anterior intestinal portal (black arrowhead). Shh transcripts are absent in the dorsal foregut endoderm (white arrowhead) (D') and in most of the ventral foregut endoderm (white arrow). The PcP the anterior most source of Shh at this stage, is in continuities with the rostral ventral endoderm (D'). (E) Schematic representation of *Shh* expression and the anterior level of the embryos forehead excision (x-y indicates the level of) in chick embryos at 6ss in D', E. Floor plate (FP); endoderm (End); notochord (No); telencephalon (Tel); diencephalons (Di). (F,F') Whole-mount cell death analysis on chick embryos by Nile Blue Sulfate (NBS) staining. F' shows the level of cross section of BA1 in F and shows dying mesenchymal cells stained with NBS (black arrowhead) after forehead excision at 6ss. Morphology (G,H,J) and skeletal (K) analyses of E11-12 embryos using alcian blue staining for cartilage and alizarin red for bone. (G) Absence of uppper and lower beak in an embryo with forehead excised at 6ss. (J,K) E12 embryo in which the forehead was excised at 6ss and which was treated with Shh-bead (I, white arrowhead): the lower beak with cartilages and bones corresponding to mandible and maxilla are present. (L-Q) Whole-mount *in situ* hybridization in E3.5-4.5 chick control and operated embryos. *Fgf8* and *Bmp4* mRNAs are present in the oral epithelium and ectoderm of BA1 in control (L,O) but not in embryos whose forehead was excised at 6ss (M,P). (N,Q) Embryos whose forehead was excised at 6ss and replaced by a Shh-bead show patterns of expression of those genes (black arrows for *Bmp4*) and a well developed BA1 (1), in contrast to non-treated embryos which exhibit a reduced sized BA1 and missexpression of these genes (M,P). BA1(1); BA2(2).

Figure 4 :



Fgf8 expression in 24ss control (**A**) and FSNC-deprived (**B**) embryos showing the loss of *Fgf8* expression in ANR as a consequence of FSNC ablation. Morphology of E6 brains isolated from control (**C**) and FSNC-deprived (**D**) embryos showing an extended exencephaly in operated compared to stage-matched control. In FGF8-treated FSNC-ablated embryos, r3-NC is replaced by its quail counterpart (**E)**. At E6.5, the development of facial and cephalic structures is rescued (**F**). (**G**) r3-derived NCC are the sole source of regenerating cells to form a mandibular skeleton (arrows) which is entirely made up of quail cells (**H**). (**I**) Implantation of r4-r6 NC in FSNC excised embryos at the diencephalic level. (**J**) Whole-mount QCPN Mab immunodetection (in brown) at E2 reveals the presence of graft-derived cells at the diencephalic level. (**L**) The presence of r4-r6 NCC which have migrated in the nasofrontal bud (arrow) between the prosencephalic neuroepithelium and the overlaying ectoderm stimulate the expression of Fgf8 in the superficial and neural ectoderm (**M**). Restoring Fgf8 expression in the forehead directs the migration of NCC emanating from r3 towards BA1 (here evidenced in blue; HNK1 Mab immunodetection). (**N**) At E6, the development of the nasal processes is atrophied while an BA1 has developed. (**O**) At E8, these embryos fail to develop an upper beak but show a fully developed lower jaw (**P**) in which the cartilaginous components of the mandible are evidenced. (**Q**) At E11, the complete set of cartilages and dermal bones in the lower jaw have developed while the skeletal differentaition of the skull and upper is severly perturbed. Gene expression in cephalic neuroepithelium in control (**R,U**) , FSNC-ablated (**S,V**), and FGF8-treated FSNC-ablated (**T,W**) embryos. At E2, *Wnt1* which is normally expressed along the dorsal mesencephalic and thalamic midline (**R**; arrowheads), is lost in absence of FSNC (except at the level of the isthmus (**S**; asterix) but is restored when NCC progression is stimulated by FGF8 in ANR (**T**; arrowheads). At E4.5, Pax6 expression in pallium and dorsal diencephalon (**U**) is reduced in the telencephalon, in absence of FSNC (**V**). Treating with FGF8 rescues normal pattern of *Pax6* expression in dorsal prosencephalic vesicles (**W**).

Figure 5:



Schematic summary of the molecular pathways regulating the development of BA1 derivatives. The ventral foregut endoderm exerts an effect on FSNC by inducing the formation of extra-Meckel's cartilages (Couly et al., 2002). This effect is mediated (partly) by Shh which triggers the survival of the NCC and re-establishes the normal patterning of gene expression in BA1 ectoderm. Presence of NCC in BA1 is critical for expression of Fgf8 in BA1 ectoderm. Fgf8 has a positive effect on NCC migration and proliferation (Creuzet et al., 2004).