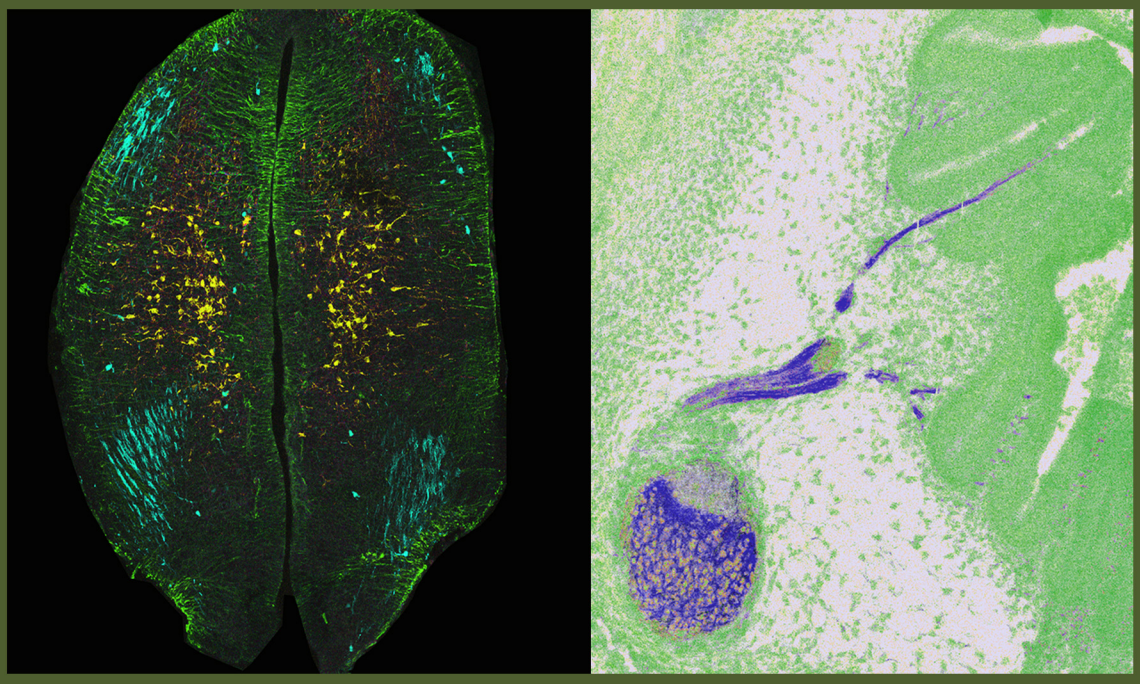
Departamento de Bioloxía Celular e Ecoloxía
Area de Bioloxia Celular
Facultade de Bioloxía

Development and regionalization of the telencephalon and peripheral associated systems in the shark *Scyliorhinus canicula*



Doctoral Thesis

Idoia Quintana Urzainqui
Santiago de Compostela, 2013

UNIVERSIDADE DE SANTIAGO DE COMPOSTELA
DEPARTAMENTO DE BIOLOXÍA CELULAR E ECOLOXÍA
FACULTADE DE BIOLOXÍA

PROGRAMA DE DOCTORADO INTERUNIVERSITARIO EN NEUROCIENCIA
(UNIVERSIDAD DE SANTIAGO DE COMPOSTELA, UNIVERSIDAD DE VIGO,
UNIVERSIDAD DE A CORUÑA)

Development and regionalization of the telencephalon and peripheral associated systems in the shark *Scyliorhinus canicula*

MEMORIA

que para la obtención del Título de Doctor por la Universidad de Santiago de Compostela presenta

IDOIA QUINTANA URZAINQUI

Santiago de Compostela, 2013

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Santiago de Compostela, 2013

ISABEL RODRÍGUEZ-MOLDES REY, CATEDRÁTICA DEL ÁREA DE BIOLOGÍA CELULAR Y EVA CANDAL SUÁREZ, PROFESORA CONTRATADA DOCTORA DEL DEPARTAMENTO DE BIOLOGÍA CELULAR Y ECOLOGÍA DE LA UNIVERSIDAD DE SANTIAGO DE COMPOSTELA,

CERTIFICAN:

Que la presente memoria titulada "Development and regionalization of the telencephalon and peripheral associated systems in the shark *Scyliorhinus canicula*", que para optar al Grado de Doctor en Biología presenta Dña. IDOIA QUINTANA URZAINQUI, ha sido realizada bajo nuestra dirección. Y considerando que constituye trabajo de tesis, autorizamos su presentación al Comisión Académica correspondiente.

Y para que así conste, expedimos el presente certificado en Santiago de Compostela, a 3 de Junio de 2013.

La Doctoranda | Las Directoras de la Tesis

Fdo: Idoia Quintana Urzainqui | Fdo.: Isabel Rodríguez-Moldes Rey | Fdo.: Eva Candal Suárez

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

A realización desta Tesis Doctoral foi posible grazas á financiación dos seguintes proxectos e actividades de investigación:

- Título do proxecto: Formación del patrón del encéfalo en condrictios (peces cartilaginosos)
  Entidade financiadora: Ministerio de Educación y Ciencia- Dirección General de Investigación-FEDER (BFU2007-61154)
- Título do proxecto: Buscando la condición ancestral de la organización cerebral de gnatóstomos: regionalización, migración, proyecciones y asimetrías en el cerebro en desarrollo de un tiburón.
  Entidad financiadora: Ministerio de Ciencia e Innovación- Dirección General de Investigación-FEDER (BFU2010-15816)
- Título do proxecto: Control de la expresión génica in ovo durante el desarrollo del sistema nervioso de peces. Identificación de los mecanismos conservados evolutivamente
  Entidade financiadora: Dirección Xeral de I+D - Xunta de Galicia (10PXIB200051PR)
- Consellería de Economía – Programa xeral de Consolidación e estruturación das unidades de investigación do sistema galego de I+D+I. INCITE09ENA200048ES (Año 2009)
- Consellería de Economía e Industria – Programa xeral de Consolidación e estruturación das unidades de investigación do sistema galego de I+D+I. IN845B-2010/159 (Año 2010).
- Conselleria de Cultura, Educación e Ordenación Universitaria – Proxectos Plan Galego IDT- Consolidación e Estruturación de Unidades de Investigación competitivas (GPC

Parte da realización desta Tesis foi posible grazas á concesión de dous contratos de investigación asociados ós proxectos de investigación BFU2007-61154 e BFU2010-15816.

A estadía de investigación na Universidade de Edimburgo foi financiada por The Company of Biologists (Travelling fellowships).

A adquisición de material biolóxico foi financiada pola European Community- Research Infrastructure Action baixo o programa específico FP7 “Capacities” (ASSEMBLE 227799 grant agreement no. 227799). Embrións e xuvenís tamén foron amablemente facilitados polo Acuarium Finisterrae, A Coruña, España; Acuario de O Grove, Pontevedra, España; Acuario de Gijón, Asturias, España.

A dispoñibilidade de sondas dos diferentes xenes foi posible grazas á colaboración coa Dra. Sylvie Mazan, coordinadora do programa “EST sequencing project” (financiado por Génoscope, Evry, Francia).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Y como es de bien nacido ser agradecido ahí van mis agradecimientos a todos aquellos que han estado ahí todos estos años.

Gracias…

Por supuesto en primer lugar a mis directoras de tesis. A Isa, que me dio la oportunidad de iniciarme en la ciencia, me abrió puertas, me mostró el camino y el entusiasmo y luchó por mí cuando las cosas se pusieron difíciles. Eternamente agradecida. A Eva, por haberme ayudado tanto, por tu paciencia y consejos y por ser cojonuda (con perdón). Para mí eres más que una jefa.

A mis compañeros del área. A Iván y Antón, que me enseñaron los primeros pasos en este mudillo, estuvieron y están siempre dispuestos a ayudarme y guiarme en lo que sea, por las risas echadas con vosotros. A Verona, que siempre estuvo dispuesta a ayudar en cualquier cosilla. A Susana, por enseñarme los misterios de las in situs. A Sol, porque empezamos, recorrimos y acabamos juntas esta aventura, por todo lo compartido. A Gabi, por ser nuestro "McGiver" y traer el siglo XXI al laboratorio. A Maru, Nuria y Blanca, por las risas, los "corrillos" y por darle vidilla al lab. Especial mención para Nuri por su trabajo como asesora artístico-técnica. A Davichín, Dani y a Pablo por ser unos tíos majos y hacerme soltar una carcajada siempre que aparecen. A los genéticos, Diego, Luis y Xoana que siempre que lo he necesitado me han echado un cable.

A los profes. A Ramón por estar siempre cerca y tener solución a cualquier duda inimaginable. Por tu interés y apoyo. Por ser una fuente de sabiduría inagotable. A Fátima por las incontables risas, por tus chistes clásicos y por supuesto por haber hecho mis in situs más rápidas y hermosas. A Miguel, por hacernos reír tanto, por ser ese gran personaje que te alegra el día. A Celina por echarnos un cable con la gestión. A Sole, Suso y Manolo.

A Fran que puso su arte a mi disposición de manera altruista (y por los bocatas de nocilla). Mil gracias.

No quiero olvidarme de Merche, esa gran artista del confocal con la que también me he echado alguna que otra risa. Gracias por hacer que mis fotos luzcan mucho más chulas.

A Espe, Carlos, Fina y compañía, por hacer esos deliciosos desayunos y por ser tan buena gente.

A mis niñas, las superurbis. Porque conservar a este nivel tus amigas de la infancia no tiene precio. Por haber estado ahí siempre que lo he necesitado y por tener la certeza de que seguiréis ahí toda la vida. En especial a mi Sariña. Porque como me dijiste un día, sobran las palabras.

A las petardis santiaguesas. Susana, Sara, Nuelia, Antía, Berta, Rebe y Cris. Por aguantar mis innumerables chapas, por las fiestas, por el apoyo, por los ánimos. A Susana, porque si tengo que enumerar aquí las veces que me has ayudado tendría que añadir un anexo. No os quiero nada…

A MIS PADRES. Porque sois mis pilares. Porque me habéis dado siempre mucho más de lo que teníais. Por haberme apoyado en todas y cada una de mis decisiones y por hacérmelo todo más fácil.

Y por último por supuestísimo ao meu pequeno. Por ser mi media castaña, por cuidarme tanto, por hacerme reír todos los días, por hacerme feliz.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

A mis padres

A Becho

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Contents

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

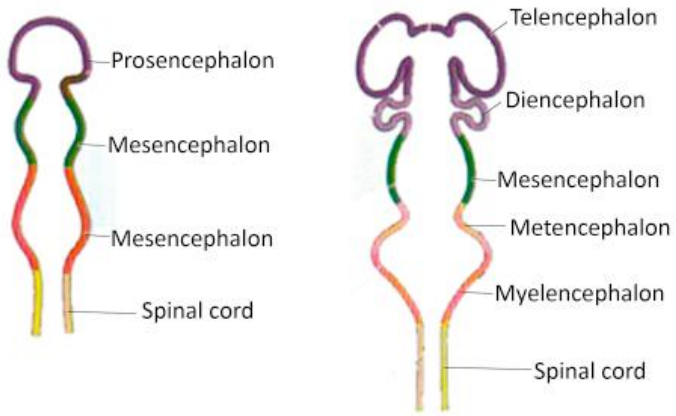
General introduction

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The prosencephalon and the prosomeric model.

The anterior region of the early neural tube present three dilatations or primary vesicles from rostral to caudal: prosencephalon (or forebrain), mesencephalon (or midbrain) and rhombencephalon (or hindbrain), which become subdivided into secondary vesicles as development proceeds. The prosencephalon gives rise to the telencephalon and the diencephalon, the mesencephalon remains as a single vesicle and the rombencephalon becomes subdivided into the metencephalon and the myelencephalon (Fig.1).

Figure 1. Regional specification of the developing brain. Primary and secondary vesicles. Modified from Purves 2001



Two main models have been proposed to explain the organization of the central nervous system: the columnar model (Herrick 1913) and the neuromeric model (Gilland and Baker 1993; Rubenstein et al. 1994; Puelles and Rubenstein 2003).

Briefly, the columnar model maintains that the central nervous system can be subdivided into longitudinal zones (roof plate, alar plate, basal plate and floor plate) separated by ventricular sulci, as the sulcus limitans of His (His 1904) that marks the alar-basal boundary (for an extensive review, see Puelles et al. 2012). Although these divisions are evident in the spinal cord and rhombencephalon they are not completely recognizable at mesencephalic and prosencephalic levels.

The neuromeric model was proposed as an alternative to the columnar model. It is based on the recognition of the bent longitudinal axis of the forebrain with respect to midbrain and hindbrain, and defines the primary anteroposterior and dorsoventral divisions. The neuromeric model holds that the neural tube is divided in longitudinal columns but also in transverse domains or segments. Each longitudinal and transverse segmental division is specified by genetic fate determinants and constitutes an independent compartment with individual developmental fates. These units were called neuromeres (reviewed in Puelles et al. 1987) and were readily identified in both the rhombencephalon and the mesencephalon of vertebrates. The prosomeric model represents the neuromeric interpretation of the prosencephalon. According to its last statement (Puelles and Rubenstein 2003), the prosencephalon embraces two transverse protosegments: the diencephalon (caudally) and the secondary prosencephalon (rostrally). The diencephalon contains three prosomeres called p1, p2 and p3 from caudal to rostral. The secondary prosencephalon give rise to specialized histogenetic territories as the telencephalon (dorsally) or the hypothalamus (ventrally). How these territories match the prosomeric model has largely been a matter of debate. In its updated version (Puelles et al. 2012; see Fig. 2) it is stated that the secondary prosencephalon is divided in two prosomeres (see Fig. 2): a caudal one (hp1) that includes the peduncular hypothalamus (ventrally) and the evaginated telencephalon (dorsally); and a rostral one (hp2) that includes the terminal hypothalamus (ventrally) and the non-evaginated telencephalon (dorsally). Since the alar-basal boundary roughly divided in half the hypothalamus (red line in Fig. 2) the telencephalon and part of the hypothalamus are alar derivatives while the remaining part of the hypothalamus is derived from the basal plate.

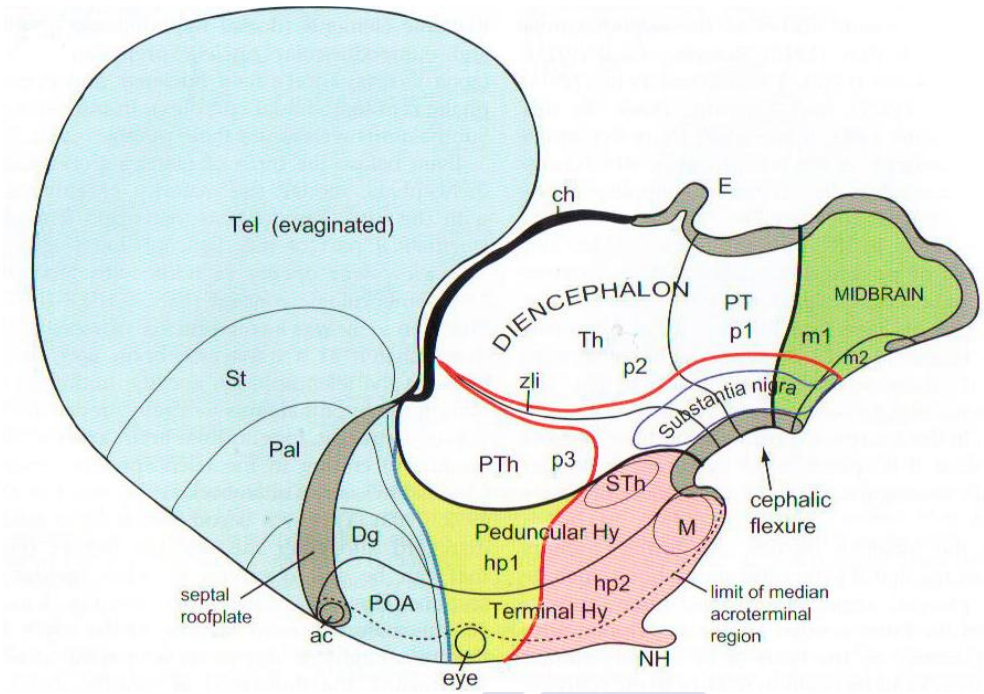


Figure 2. The secondary prosencephalon according to the prosomeric model. Red line represents the alar-basal limit. From Puelles et al. 2012

In all vertebrates, the evaginated telencephalon becomes subdivided from early stages of embryonic development into two main territories: pallium (dorsal) and subpallium (ventral), each of them characterized by the expression of different subsets of developmental regulatory genes. Pallial compartment typically expresses *Tbr1* and *Emx* genes while genes belonging to the Distal-less (*Dlx*) family are characteristically expressed in the subpallium (Smith-Fernández et al. 1998; Puelles et al. 2000; Murakami et al. 2001; Bachy et al. 2002; Brox et al. 2003; Brox et al. 2004; Medina et al. 2004; Wullimann and Mueller 2004; Mueller and Wullimann 2009). Generally, developmental regulatory genes exhibit highly conserved expression patterns along evolution and thus serve as powerful tools for the identification of homolog structures between different species and animal groups (Puelles and Medina 2002). Under this rationale, comparative studies about the telencephalon carried out in different species of vertebrates have demonstrated the existence of certain histogenetic subdivisions within pallial and

subpallial territories. At least four different sectors (medial, dorsal, lateral and ventral) have been defined in the pallium of all tetrapods (reviewed in Medina and Abellán 2009). Some of them were tentatively described in non-tetrapod vertebrates as lamprey and zebrafish, representatives of agnathans (jawless vertebrates) and modern bony fishes, respectively, based on the expression of developmental genes (Murakami et al. 2001; Wullimann and Mueller 2004) but, as far as we know, similar approach has not been implemented in cartilaginous fishes. On the other hand, the developing mammalian subpallium is broadly subdivided into three principal territories: the lateral ganglionic eminence (LGE), the medial ganglionic eminence (MGE) and the preoptic area (PO) (Medina and Abellán 2012). Homolog territories have also been characterized from birds to teleost (Puelles et al. 2000; Medina et al. 2011). Furthermore, two complex structures formed by a mixture of pallial and subpallial derivatives were described in the telencephalon of most tetrapods (Moreno et al. 2009; Medina et al. 2011) these are the septum (at rostral levels of the medial telencephalic walls) and the amygdaloid complex (in the lateral telencephalic walls).

Despite certain number of revisions have provided information about the regionalization of the prosencephalon in different groups of vertebrates on the basis of the comparative analysis of the patterns of expression of developmental regulatory genes (see Medina 2008a; Moreno and González 2011), a profound lack of information exists at the phyogenetic position of cartilaginous fishes, which represent a key group to understand the transition from agnathans to gnatosthomes. Thus, the study of the telencephalon in cartilaginous fishes would be extremely useful to complete the evolutionary scheme of telencephalic development and will shed light into the ancestral condition of this region along vertebrate phylogeny.

Importance of cartilaginous fishes in evolutionary and developmental studies.

Cartilaginous fishes or chondrichthyans represent a monophyletic group and an ancient lineage of the jawed vertebrates or gnathostomes. This group includes two major radiations that diverged over 350 million years ago (Fig.3): Holocephala (chimaeras), which present non-articulated jaws and Elasmobranchs (sharks, skates and rays), characterized by possessing articulated jaws. Chondrichthyans are considered the sister group of gnathostomes osteichthyans, which in turn embraces two main groups:

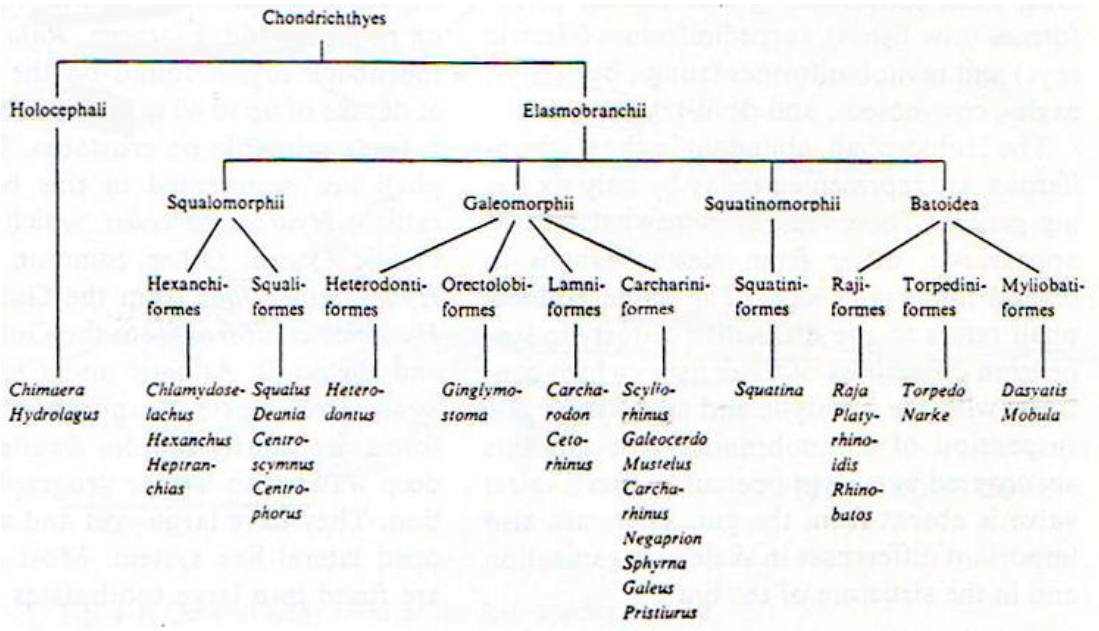


Figure 3. Clasification of cartilaginous fishes. From Compagno (1977)

actynopterigians (ray-finned fishes) including bony fishes as zebrafish and sarcopterygians (lobe-finned fishes) from which all tetrapods derive (Fig. 4). Thus cartilaginous fishes occupy a phylogenetic position as an out-group to all living gnatostomes which makes them essential to assess the ancestral condition of vertebrate brain organization. Despite their ancient origin, the brain of cartilaginous fishes are less simple in organization than previously supposed and some elasmobranchs possess a brain-body weight ratio comparable to that of birds and mammals and that exceeds that of

most other non-mammalian species (Ebbeson and Northcutt 1976; Bauchot et al. 1976; Northcutt 1977, 1978; reviewed in Smeets 1998).

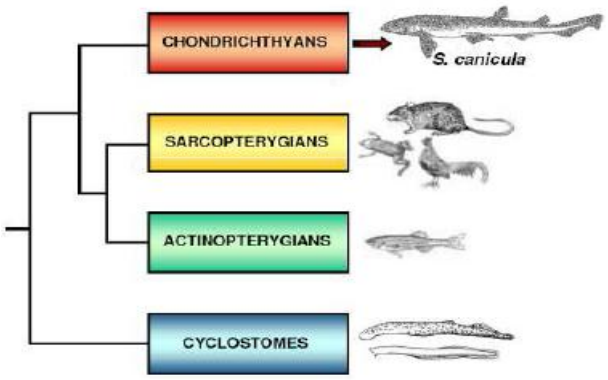


Figure 4. Phylogenetic position of chondrichthyans among gnathostomes. From Coolen et al. (2009)

The species used in this study, the lesser spotted dogfish *Scyliorhinus* canicula, belongs to the Family *Scyliorhinidae*, Genus *Scyliorhinu*s, Order *Charchariniformes*, SuperOrder *Galeomorphii* (Fig. 3). In the last years, this species has been used as the main elasmobranch model in evolutionary developmental (evo-devo) studies (reviewed in Coolen et al. 2009) as it presents several advantages. *S.canicula* is easily reared in captivity and abundant embryonic material can be obtained throughout the year. It is an oviparous species and the eggs are easily maintained under laboratory conditions until hatching. The transparency of the eggs permits the staging of the specimens (according to Ballard et al. 1993) and the selection of the required developmental stages for particular experimental approaches. The relative big size of the embryos and the protracted embryonic period (between five and twelve months depending on the water temperature) allows a thorough analysis of different developmental processes evidencing details that can be neglected in other species of rapid development.

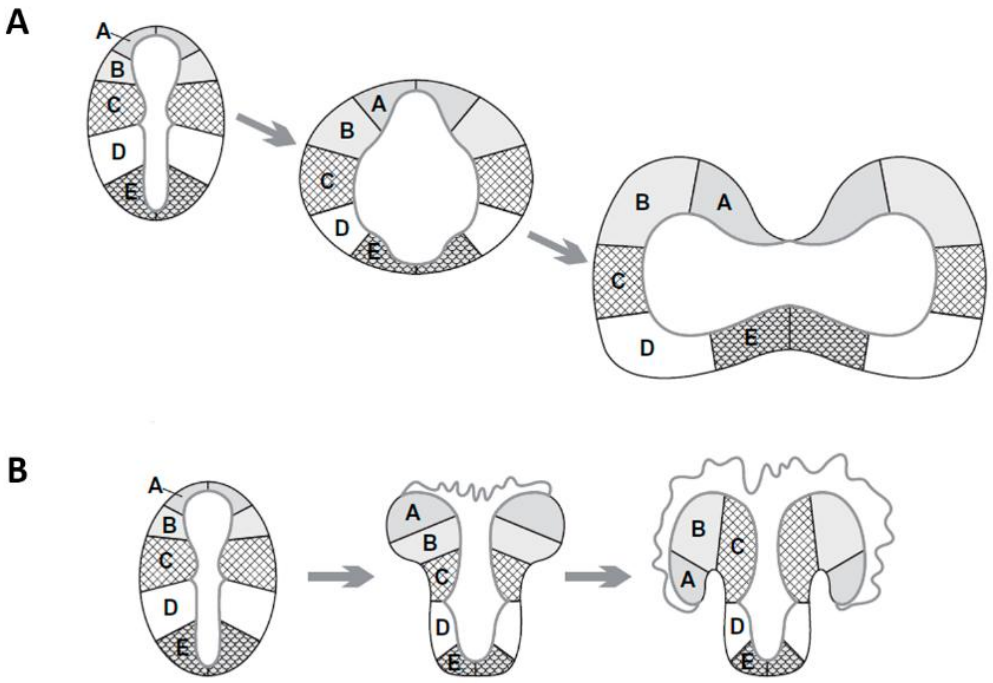


Figure 5. Morphogenetic processes that give rise to the telencephalon in different vertebrates. **A**: evagination (cartilaginous fishes and sarcopterygians). **B:** eversion (actinopterygians). Letters refer to different histogenetic subdivisions. From Butler and Hodos (2005)

On the other hand, the telencephalon of cartilaginous fishes develops, as tetrapods, by a morphogenetic process known as evagination (Fig. 5A) that involves an enlargement of the central lumen of the neural tube to form the telencephalic ventricles, followed by an outward expansion of the telencephalic walls. This mode of growth constitutes an advantage for comparative studies with respect to that of bony fishes, since the telencephalon of most actinopterygians (including zebrafish) develops by eversion (Fig. 5B). This process of eversion implies that part of the roof of the neural tube becomes thin and elongates and that the pallium bends outward (reviewed in Nieuwenhuys 2009), which reverses the topography of the pallial areas in actinopterygians.

The telencephalon of cartilaginous fishes.

The telencephalon of cartilaginous fishes can be divided on three main parts from caudal to rostral: the telencephalon impar (the non-evaginated region of the telencephalon enclosing an unpaired ventricle), the evaginated telencephalic hemispheres (divided in a dorsal pallial and a ventral subpallial part) and the olfactory bulbs (reviewed in Smeets 1998) that in these animals become physically separated from the rest of the telencephalon by peduncles or stalks of different lengths depending on the species (See Butler and Hodos 2005).

Traditionally, the telencephalon of cartilaginous fishes was thought to be mainly implicated in olfactory processing (Edinger 1908; Johnston 1911; Bäckström 1924; Ariëns Kappers et al 1936; Williams 1973; Kuhlenbeck 1977) and was therefore considered to be a rhinencephalon. This classic conception changed with the publication of studies which revealed that a restricted area of the forebrain receives olfactory fibers (Ebbeson and Heimer 1970; Bruckmoser 1973; Bruckmoser and Dieringer 1973; Vesselkin and Kovacevic 1973; Northcutt 1978; Ebbeson 1980; Smeets 1983; Hofman and Northcutt 2008, 2012; Yáñez et al. 2011).

Numerous studies about the telencephalic organization in cartilaginous fishes were carried out through the twentieth century based on normal material and classic techniques as Golgi stains (Johnston 19 11; Holmgren 1922; Bäckström 1924; Gerlach 1947; Faucette 1969a,b; Ebbeson and Heimer 1970; Ebbeson and Schroeder 1971; Kuhlenbeck 1973,1977; Schroeder and Ebbeson 1974; Northcutt 1978,1981; Ebbeson 1980; Smeets et al. 1983; Smeets 1990; Manso and Anadón 1993). On the basis of these and other studies,

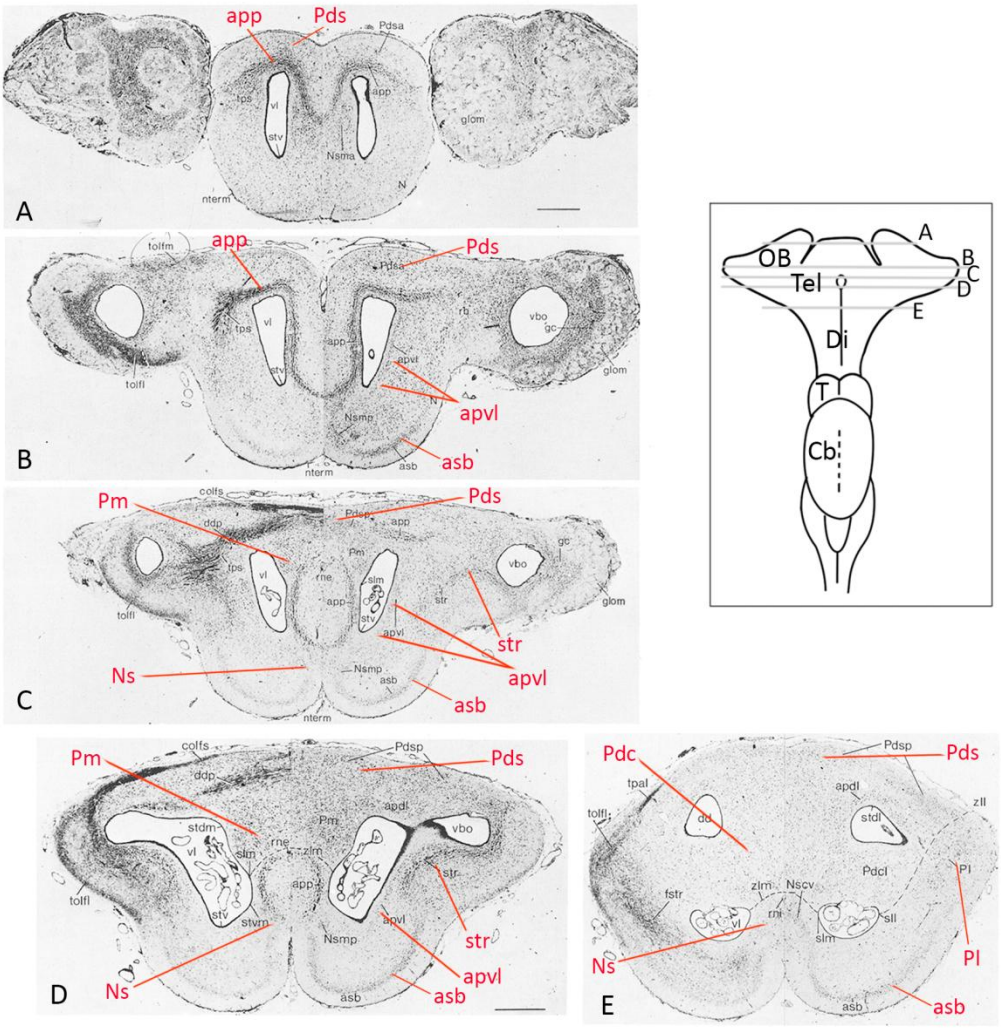


Figure 6. Principal cell masses of the telencephalon of *S.canicula* showed in transverse sections. Scale bar: 1mm. Modified from Smeets et al. (1983). Abbreviations: app, *area periventricularis pallialis*; asb, *area superficialis basalis*; apvl, *area periventricularis ventrolateralis*; OB, olfactory bulb; Di, diencephalon; Cb, cerebellum; Ns, *nucleus septalis*; Pd, *pallium dorsalis*; Pdc, *pallium dorsalis centralis*; Pl, *pallium lateralis*; Pm, *pallium medialis*; str, *striatum*; T, tectum; Tel, telencephalon.

main cell masses, nuclear organization and fiber pathways of the mature forebrain of representative species of four different groups of chondrichthyans were compiled by Smeets et al. (1983). Briefly, the principal cell masses defined in the pallium (the dorsal part of the telencephalon) are: *area periventricularis pallialis* (app), *pallium dorsalis* (Pd), *pallium dorsalis centralis* (Pdc), *Pallium medialis* (Pm), *Pallium lateralis* (Pl);

while those described in the subpallium (the ventral part of the telencephalon) are: *area superficialis basalis* (asb*), area periventricularis ventrolateralis* (apvl), *nucleus septalis* (Ns) and *striatum* (str) (see Fig.6). Most of this nomenclature is still maintained, however the homologies of these telencephalic nuclei with those of other vertebrates are still a matter of debate.

The improvement of immunohistochemical techniques and the development of antibodies against different neuroactive substances increasingly revealed the complexity of the telencephalon in elasmobranchs. Some of these studies aimed to further characterize the cell masses identified in classical studies and described the distribution of antibodies against catecholamines (Meredith and Smeets 1987; Northcutt et al. 1988; Stuesse et al. 1994; Carrera et al. 2012), serotonin (Ritchie et al. 1983; Nothcutt et al. 1988; Yamanaka et al. 1990; Carrera et al. 2008a) neuropeptide Y (Vallarino et al. 1988, Chiba and Honma 1992); substance P (Rodríguez-Moldes et al. 1993); enkephalins (Northcutt et al. 1988; Vallarino et al. 1994; Sueiro 2003) or somatostatin (Chiba et al. 1989). These works also allowed the proposal of more accurate homologies between these cell masses and those of other vertebrates. However, while this information was very helpful for certain neuroanatomical comparisons, it did not clarify the identity of most nuclei and even produced some controversies. What can be learnt from this is that immunohistochemical approaches in adult specimens are not enough to define corresponding areas in different species.

Recently, information about the regionalization of the developing telencephalon in *S.canicula* obtained by means of the analysis of the expression pattern of GABA or the transcription factor Pax6, allowed to roughly describe pallial and subpallial divisions (Ferreiro-Galve et al. 2008; Carrera et al. 2008b; Rodríguez-Moldes 2009). The use of gene expression patterns has revealed as an essential tool to define the embryonic origin

of specific regions of the adult brain (reviewed in Joyner and Sudarov 2012). This approach allows identifying functional subdivisions of the developing brain in a manner independent of landmarks such as morphology and cytoarchitecture and, therefore, it eases the identification of homologous subdivisions across vertebrates, regardless of the morphogenetic changes occurring up to adulthood. The definition of pallial and subpallial subdivisions in *S. canicula* under a genoarchitectonic (or neural genoarchitecture, i.e., descriptions of neural structure in terms of discrete gene expression patterns: Puelles and Ferrán 2012) approach and the identification of their derivatives in the postnatal telencephalon would be extremely useful to carry out reliable comparisons of the adult elasmobranch telencephalic regions with other vertebrates.

The telencephalon and associated peripheral systems

As mentioned above, it is currently accepted that the telencephalon comprises a non-evaginated part, and an evaginated part which gives rise to the telencephalic hemispheres and the olfactory bulbs. The olfactory bulbs represent the primary olfactory centers, that is, the region of the central nervous system where peripheral olfactory axons make contact.

Cartilaginous fishes are animals with highly developed sense of smell and therefore they present relative large olfactory structures easily accessible for different experimental approaches. Despite the existence of various works about adult olfactory organization in elasmobranchs (Ferrando et al. 2007, 2009, 2010) little is known about its development. Recently Ferreiro-Galve et al. (2012) performed a study of the development of the olfactory system of *S.canicula* on the basis of Pax6 expression, which demonstrates the first evidence in vertebrates of cells expressing this transcriptional factor along olfactory fibers. However, they did not investigate their possible identity. Apart from this work,

there is a complete lack of studies about the development of the peripheral olfactory system in elasmobranchs.

Besides the olfactory system, the terminal nerve (or *nervus terminalis*) system is another peripheral component directly related to the telencephalon. It was observed and studied for the first time in adult cartilaginous fishes (Fritsch 1878; Locy 1905) and subsequently described in most vertebrate groups (reviewed in Von Bartheld 2004). Despite its rather generalized presence in vertebrates, there are differences in the course and entry of the nerve, as well as in the structure of the system. In osteichthyans (tetrapods and bony fishes) the terminal nerve is closely associated with the olfactory system while in most of the elasmobranchs studied they are anatomically separated (Demski and Fields 1988). A recent tract-tracing study revealed that in adults of *S.canicula*, their central connections do not overlap (Yáñez et al. 2011). There are also notable differences in the location of the cell bodies of the terminal nerve (reviewed in Butler and Hodos 2005; Whitlock 2004). While in lampreys, the cells identified as belonging to the terminal nerve lie along the olfactory nerve, in teleosts most of the cells bodies migrate into the brain. In other vertebrates including elasmobranchs, the cells of the terminal nerve form peripheral ganglia that remain in adult animals, the terminal nerve ganglia, although they are also located along the distal part of the nerve itself (see Butler and Hodos 2005). Considerable variability also exists between different cartilaginous fishes in relation to the course and entry of this nerve (Locy 1905; Johnston 1911; Bäckström 1924). The evolutionary meaning of these differences is not known. The function of this system is also still a matter of debate, although it has been mainly associated to reproductive physiology and behavior (Demski 1989; Oka 1992; Yamamoto et al. 1997). As far as we are concerned, the study of the embryonic development of the terminal nerve system in elasmobranch has been totally ignored.

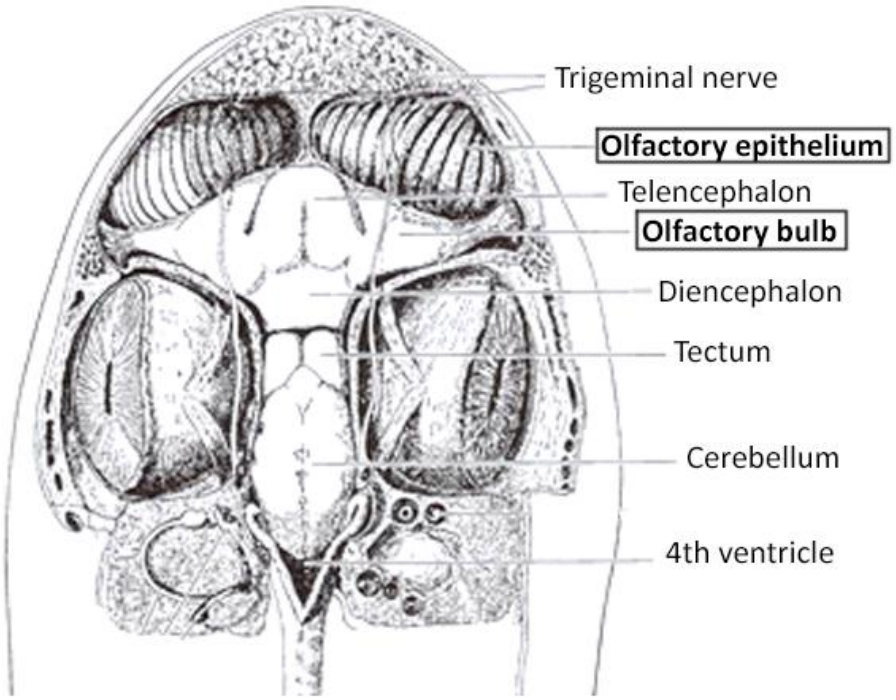


Figure 7. Dorsal view of brain and sensory organs of a juvenile Scyliorhinus canicula. Adapted from Storch and Welsch (2001).

The relative large size of the olfactory (see Fig. 7) and terminal nerve structures in elasmobranchs with respect to that of other vertebrates and the fact that, only in this animal group, the terminal nerve courses separately from the olfactory nerve (Smeets 1998; Yáñez et al. 2011) make them excellent models to study the anatomy and development of these systems and its relation with the telencephalon.

The basal ganglia

The lateral and medial ganglionic eminences (LGE and MGE, respectively) are the embryonic precursors of the striatal and pallidal components of the basal ganglia, respectively. The basal ganglia system, however, embraces other forebrain and midbrain structures as the subtalamic nuclei, the substantia nigra or the ventral tegmental area. This system has an important role in the control of motor behavior (Reiner et al. 1998; Smeets et al. 2000; Medina 2008b; Reiner 2010) and it has been extensively studied due to its

implication in human neurological diseases as Parkinson´s disease (particularly the mesencephalic catecholaminergic input to the striatum) (Alexander and Crutcher 1990; DeLong 1990; Heimer et al. 1995). Although some features of basal ganglia circuitry vary among vertebrate groups, certain common traits are recognized in all tetrapods (including the presence of striatopallidal systems in the telencephalon; a striatal descending projection towards a mesencephalic area through direct and indirect pathways; or a strong dopaminergic input to the striatum from nuclei located in the basal plate of mesencephalic and diencephalic zones; (reviewed in Reiner et al. 1998; Smeets et al. 2000) and some of them are also recognized in non-tetrapods (Rink and Wullimann 2001, 2002; Stephenson-Jones et al. 2012). Despite various studies were focused on the characterization of the basal ganglia in adult cartilaginous fishes, most equivalences have been proposed mainly based on immunohistochemical criteria. Particularly, there still exists controversy since different telencephalic territories have been proposed to represent the striatal and pallidal homologous (Smeets et al. 1983; Northcutt et al. 1988; Meredith and Smeets 1987; Smeets et al. 2000; Sueiro 2003; Carrera et al. 2012). Therefore, connectional and developmental studies are needed to unequivocally define the basal ganglia homolog structures of cartilaginous fishes

Tangential migrations in the telencephalon

The establishment of subdivisions, nuclei and connections give rise to a complex and intricately organized adult brain, which require highly regulated developmental mechanisms. Besides the correct spatio-temporal expression of key developmental genes, other important factor that leads to the accurate formation of functional brain structures is the cellular migration. Two main classes of cell migration are distinguished in the nervous system: radial and tangential (Marín and Rubenstein 2003). The former is the process by which postmitotic cells migrate associated to the radial glia away from the ventricle. It

establishes the basic pattern of the neural tube and derived structures. In opposition to radial migration, tangential migration has been defined as the migrating process taking place along a different axis to that demarcated by radial glia (Marín and Rubenstein 2003). Tangential migration acts as a strong developmental mechanism to increase neuronal complexity of brain circuits by allowing the dispersion of multiple neuronal types (Marín and Rubenstein 2003; Mione et al. 2008). Most tangential migrating neurons of the telencephalon emerge from the ganglionic eminences of the subpallium (LGE and MGE) (Marín and Rubenstein 2003; Wu et al. 2011) and invade different telencephalic domains, where they mainly give rise to interneurons. Telencephalic tangential migrations have been described from mammals to bony fishes (Cobos et al. 2001; Tuorto et al. 2003; Marin and Rubenstein 2003; Métin et al. 2007; Moreno et al. 2008; Mueller et al. 2008; Wullimann 2009) although their routes of migration were only well detailed in amniotes (Cobos et al. 2001; Corbin et al. 2001). In the telencephalon of *S. canicula*, the study of the development of the GABAergic system allowed the identification of subpallial derived neurons in the pallium, which suggested the existence of tangential migratory pathways (Carrera et al. 2008b). However, a more detailed analysis of these potential migrations in S. canicula would be of great interest to shed light into the evolution of tangential migratory routes in the telencephalon.

REFERENCES

Alexander GE, Crutcher MD (1990) Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci 13:266–271

Äriens Kappers CU, Huber GC, Crosby EC (1936) The comparative anatomy of the nervous system of vertebrates, including man, vol 1. Mac Millan , New York

Bachy I, Berthon J, Rétaux S (2002) Defining pallial and subpallial divisions in the developing Xenopus forebrain. Mech Dev 117:163–172

Bäckström K (1924) Contributions to the forebrain morphology in selachians. Acta Zool 5:123–240

Ballard WW, Mellinguer J, Lechenault H (1993) A series of normal stages for development of Scyliorhinus canicula, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). 267:318–336

Bauchot R, Platel R, Ridet JM (1976) Brain-body weight relationships in Selachii. Copeia 1976:305–309

Brox A, Puelles L, Ferreiro B, Medina L (2003) Expression of the genes GAD67 and Distal-less-4 in the forebrain of Xenopus laevis confirms a common pattern in tetrapods. J Comp Neurol 461:370–393

Brox A, Puelles L, Ferreiro B, Medina L (2004) Expression of the genes Emx1, Tbr1, and Eomes (Tbr2) in the telencephalon of Xenopus laevis confirms the existence of a ventral pallial division in all tetrapods. J Comp Neurol 474:562–577

Bruckmoser P (1973) Beziehungen zwischen struktur und function in der evolution des telencephalon. Verh Dtsh Zool Ges 66:219-229

Bruckmoser P, Dieringer N (1973) Evoked potentials in the primary and secondary olfactory projection areas of the forebrain in Elasmobranchia. J Comp Physiol 87:65-74

Butler AB, Hodos W (2005) Comparative vertebrate neuroanatomy. Evolution and adaptation. 2nd ed. Wiley-Liss, Hoboken, NJ

Carrera I, Ferreiro-Galve S, Sueiro C, Anadón R, Rodríguez-Moldes I (2008b) Tangentially migrating GABAergic cells of subpallial origin invade massively the pallium in developing sharks. Brain Res Bull 75:405–9

Carrera I, Molist P, Anadón R, Rodríguez-Moldes I (2008a) Development of the serotoninergic system in the central nervous system of a shark, the lesser spotted dogfish *Scyliorhinus canicula*. J Comp Neurol 511:804–31

Carrera I, Anadón R, Rodríguez-Moldes I (2012) Development of tyrosine hydroxylase-immunoreactive cell populations and fiber pathways in the brain of the dogfish Scyliorhinus canicula: new perspectives on the evolution of the vertebrate catecholaminergic system. J Comp Neurol 520:3574–603

Chiba A, Honma Y (1992) Distribution of neuropeptide Y-like immunorreactivity in the brain and hypophysis of the cloudy dogfish, *Scyliorhinus torazame*. Cell tissue Res 268:453-461

Chiba A, Honma Y, Ito S, Honma S (1989) Somatostatin-immunoreactivity in the brain of the gummy shark, *Mustelus manazo* Bleeker, wih special regard to the hypothalamo-hypophiseal system. Biomed Res 10:1-12

Cobos I, Puelles L, Martínez S (2001) The avian telencephalic subpallium originates inhibitory neurons that invade tangentially the pallium (dorsal ventricular ridge and cortical areas). Dev Biol 239:30–45

Compagno, LJV (1977) Phyletic relationships of living sharks and rays. Am Zool 17:303-322

Coolen M, Menuet A, Chassoux D, Compagnucci C, Henry S, Leveque L, Da Silva C, Gavory F, Samain S, Wincker P, Thermes C, D'Aubenton-Carafa Y, Rodriguez-Moldes I, Naylor G, Depew M, Sourdaine P, Mazan S (2009) The dogfish Scyliorhinus canicula, a reference in jawed vertebrates. Emerging model organisms. A laboratory manual. Cold Spring Harbor, New York, pp 431–446

Corbin JG, Nery S, Fishell G (2001) Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. Nat Neurosci 4:1177-1182

DeLong MR (1990) Primate models of movement disorders of basal ganglia origin. Trends Neurosci 13:281–285

Demski LS (1989). Pathways for GnRH control of elasmobranch reproductive physiology and behavior. J Exp Zool 252, 4–11

Demski LS, Fields RD (1988) Dense-cored vesicle-containing components of the terminal nerve of sharks and rays. J Comp Neurol 278:604-614

Ebbeson SOE, Northcutt RG (1976) Neurology of anamniotic vertebrates. In: Masterton RB, Bitterman ME, Campbell CBG, Hotton N (eds) Evolution of brain and behavior in vertebrates. Erlbaum, Hilsdale, pp 155–146

Ebbeson SOE, Heimer L (1970) Projections of the olfactory tract fibers in the nurse shark (Ginglymostoma cirratum). Brain Res 17:47-55

Ebbeson SOE, Schroeder D (1971) Connections of the nurse shark's telencephalon. Science 173:254-256

Ebbeson SOE (1980) On the organization of the telencephalon in elasmobranchs. In: Ebbeson SOE (ed) Comparative neurology of the telencephalon. Plenum, New York, pp 1-16

Edinger L (1908) Corlesungen über den bau der nervösen zentralorgane des menschen und der tiere. Vogel, Leipzig

Faucette JR (1969a) The olfactory bulb and medial hemisphere wall of the rat-fish, Chimaera. J Comp Neurol 137:377-406

Faucette JR (1969b) The accessory olfactory bulbs and the lateral telencephalic wall of the rat-fish, Chimaera. J Comp Neurol 137:407-432

Ferrando S, Bottaro M, Gallus L, Girosi L, Vacchi M, Tagliafierro G (2007) First detection of olfactory marker protein (OMP) immunoreactivity in the olfactory epithelium of a cartilaginous fish. Neurosci Lett 413:173-176

Ferrando S, Gambardella C, Ravera S, Bottero S, Ferrando T, Gallus L, Manno V, Salati AP, Ramoino P, Tagliafierro G. (2009) Immunolocalization of G-protein alpha subunits in the olfactory system of the cartilaginous fish Scyliorhinus canicula. Anat Rec 292:1771-1779

Ferrando S, Gallus L, Gambardella C, Ghigliotti L, Ravera S, Vallarino M, Vacchi M, Tagliafierro G (2010) Cell proliferation and apoptosis in the olfactory epithelium of the shark Scyliorhinus canicula. J Chem Neuroanat 40:293-300

Ferreiro-Galve S, Candal E, Rodriguez-Moldes I (2012) Dynamic expression of Pax6 in the shark olfactory system: Evidence for the presence of Pax6 cells along the olfactory nerve pathway. J Exp Zool (Mol Dev Evol) 318:79-90

Ferreiro-Galve S, Carrera I, Candal E, Villar-Cheda B, Anadón R, Mazan S, Rodríguez-Moldes I (2008) The segmental organization of the developing shark brain based on neurochemical markers, with special attention to the prosencephalon. Brain Res Bull 75:236–40

Fritsch G (1878). Untersuchungen über den feineren Bau des Fischgehirns mit besonderer Berücksichtigung der Homologien bei anderen Wirbelthierklassen. Berlin: Guttmann 1–94

Gerlach J (1947) Beiträge zur vergleichenden morphologie des selachierhirnes. Anat Anz 96:79-165

Gilland E, Baker R (1993) Conservation of neuroepithelial and mesodermal segments in the embryonic vertebrate head. Acta Anat 148:110-123

Heimer L, Zahm DS, Alheid GF (1995) Basal ganglia. In: Paxinos GF (ed) The rat nervous system. San Diego, Academic Press, pp579-628.

Herrick CJ (1913) Anatomy of the brain. In: The reference handbook of the medical sciences. Word, New York, pp 274-342

His W (1904) Die entwicklung des menschlichen gehirns wahrend der ersten monate. Hirzel Leipzig, Germany

Hofmann MH, Northcutt RG (2008) Organization of major telencephalic pathways in an elasmobranch, the thornback ray Platyrhinoidis triseriata. Brain Behav Evol 72:307–325

Hofman MH, Northcutt RG (2012) Forebrain organization in elasmobranchs. Brain Behav Evol 80:142-151

Holmgren N (1922) Points of view concerning forebrain morphology in lower vertebrates. J Comp Neurol 34:391-440

Johnston JB (1911) The telencephalon of selachians. J Comp Neurol 21:1-113

Joyner AL, Sudarov AM (2012) Genetic neuroanatomy. In: Watson C, Paxinos G, Puelles L (eds). The mouse nervous system. Academic press, Elsevier, pp 36-51

Kuhlenbeck H (1973) Overall morphological pattern. In: Kuhlenbeck H (ed) The central nervous system of vertebrates, vol 3/II. Karger, Basel

Kuhlenbeck H (1977) Derivatives of the prosencephalon: diencephalon and telencephalon. In: Kuhlenbeck H (ed). The central nervous system of vertebrates, vol 5/I, Karger, Basel

Locy WA (1905). On a newly recognized nerve connected with the forebrain of selachians. Anat Anz. 26, 33–123

Manso MJ, Anadón R (1993) Golgi study of the telencephalon of the small-spotted dogfish Scyliorhinus canicula L. J Comp Neurol 333:485-502

Marin O, Rubenstein JL (2003) Cell migration in the forebrain. Annu Rev Neurosci 26:441–483

Medina, L (2008a) Evolution and embryological development of forebrain. In: Binder MD, Hirokawa N (eds) Encyclopedic Reference of Neuroscience. Springer-Verlag, pp 1172-1192

Medina L (2008b) Basal ganglia: evolution. In: Squire LR (ed) Encyclopedia of Neuroscience. Amsterdam, Elsevier, pp 67-85

Medina L, Abellán A (2009) Development and evolution of the pallium. Semin Cell Dev Biol 20:698–711

Medina L, Abellán A (2012) Subpallial structures. In: Watson C, Paxinos G, Puelles L (eds). The mouse nervous system. Academic press, Elsevier, pp 173-220

Medina L, Bupesh M, Abellán A (2011) Contribution of Genoarchitecture to Understanding Forebrain Evolution and Development, with Particular Emphasis on the Amygdala. Brain Behav Evol 78:216–36

Medina L, Legaz I, Gonzalez G, De Castro F, Rubenstein JL, Puelles L (2004) Expression of Dbx1, Neurogenin 2, Semaphorin 5A, Cadherin 8, and Emx1 distinguish ventral and lateral pallial histogenetic divisions in the developing mouse claustroamygdaloid complex. J Comp Neurol 474:504–523

Meredith GE, Smeets WJ (1987). Immunocytochemical analysis of the dopamine system in the forebrain and midbrain of Raja radiata: evidence for a substantia nigra and ventral tegmental area in cartilaginous fish. J Comp Neurol 265: 530-548

Métin C, Alvarez C, Moudoux D, Vitalis T, Pieau C, Molnár Z (2007) Conserved pattern of tangential neuronal migration during forebrain development. Development 134:2815–2827

Mione M, Baldessari D, Deflorian G, Nappo G, Santoriello C (2008) How neuronal migration contributes to the morphogenesis of the CNS: insights from the zebrafish. Dev Neurosci. 30:65-81

Moreno N, González A (2011) The Non-Evaginated Secondary Prosencephalon of Vertebrates. Front Neuroanat 5:12

Moreno N, González A, Rétaux S (2008) Evidences for tangential migrations in Xenopus telencephalon: developmental patterns and cell tracking experiments. Dev Neurobiol 68:504–520

Moreno N, González A, Rétaux S (2009) Development and evolution of the subpallium. Semin Cell Dev Biol 20:735–43

Mueller T, Wullimann MF (2009) An evolutionary interpretation of teleostean forebrain anatomy. Brain Behav Evol 74:30–42

Mueller T, Wullimann MF, Guo SU (2008) Early teleostean basal ganglia development visualized by zebrafish GAD67 gene expression. J Comp Neurol 507:1245–1257

Murakami Y, Ogasawara M, Sugahara F, Hirano S, Satoh N, Kuratani S (2001) Identification and expression of the lamprey Pax6 gene: evolutionary origin of the segmented brain of vertebrates. Development 128:3521–3531

Nieuwenhuys R (2009) The forebrain of actinopterygians revisited. Brain Behav Evol. 73:229-52

Northcutt RG (1977) Elasmobrach central nervous system organization and its possible evolutionary significance. Am Zool 17:411–429

Northcutt RG (1978) Brain organization in the cartilaginous fishes. In: Hodgson ES, Mathewson RF (eds) Sensory biology of sharks, skates and rays. US Government Printing Office, Washington D, pp 117-193

Northcutt RG (1981) Evolution of the telencephalon in non-mammals. Annu rev Neurosci 4:301-350

Northcutt RG, Reiner A, Karten HJ (1988) Immunohistochemical Study of the Telencephalon of the Spiny Dogfish , Squalus acanthias. J Comp Neurol 277:250–267

Oka Y (1992) Gonadotropin-releasing hormone (GnRH) cells of the terminal nerve as a model neuromodulator system. Neurosci Lett 142, 119–122

Puelles L, Amat JA, Martinez-de-la-Torre M (1987) Segment-related, mosaic neurogenetic pattern in the forebrain and mesencephalon of early chick embryos: I. Topography of AChE-positive neuroblasts up to stage HH18. J Comp Neurol 266:247-68

Puelles L, Ferran JL (2012) Concept of neural genoarchitecture and its genomic fundament. Front Neuroanat 6:47

Puelles L, Kuwana E, Puelles E, Bulfone A, Shimamura K, Keleher J, Smiga S, Rubenstein JL (2000) Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. J Comp Neurol 424:409–38

Puelles L, Martínez-de-la-Torre M, Bardet S, Rubenstein JLR (2012) Hypothalamus. In: Watson C, Paxinos G, Puelles L (eds). The mouse nervous system. Academic press, Elsevier, pp 221-312

Puelles L, Medina L (2002) Field homology as a way to reconcile genetic and developmental variability with adult homology. Brain Res Bull 57: 243–255

Puelles L, Rubenstein JL (2003) Forebrain gene expression domains and the evolving prosomeric model. Trends Neurosci 26:469–476

Purves D, Augustine GJ, Fitzpatrick D, Katz LC, LaMantia AS, McNamara JO (2001) Invitación a la neurociencia. Editorial Médica Panamericana, Madrid.

Reiner A, Medina L, Veenman CL (1998) Structural and functional evolution of the basal ganglia in vertebrates. Brain res 28:235–285

Reiner A (2010) The conservative evolution of the vertebrate basal ganglia. In: Steiner H, Tseng KY (eds.) Handbook of Basal Ganglia Structure and Function. San Diego, Academic Press, pp 29-62

Rink E, Wullimann MF (2001) The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). Brain res 889:316–330

Rink E, Wullimann MF (2002) Connections of the ventral telencephalon and tyrosine hydroxylase distribution in the zebrafish brain (Danio rerio) lead to identification of an ascending dopaminergic system in a teleost. Brain Res Bull 57:385–387

Ritchie TC, Livinston CA, Hughes MG, McAdoo DJ, Leonard RB (1983) The distribution of serotonin in the CNS of an elasmobranch fish: Immunocytochemical

and biochemical studies in the atlantic stingray, Dasyatis sabina. J Comp Neurol 213:414–125

Rodríguez-Moldes I (2009) A developmental approach to forebrain organization in elasmobranchs: new perspectives on the regionalization of the telencephalon. Brain Behav Evol 74:20-9

Rodríguez-Moldes I, Manso MJ, Becerra M, Molist P, Anadón R (1993) Distribution of substance P-like immunoreactivity in the brain of the elasmobranch *Scyliorhinus canicula*. J Comp Neurol 335:228-244

Rubenstein JL, Martínez S, Shimamura K, Puelles L (1994) The embryonic vertebrate forebrain: the prosomeric model. Science 266:578–580

Schroeder DM, Ebbeson SOE (1974) Nonolfactory telencephalic afferents in the nurse shark (Ginglymostoma cirratum). Brain Behav Evol 9:121–155

Smeets WJAJ (1983) The secondary olfactory connections in two chondrichthyans, the shark Scyliorhinus canicula and the ray Raja clavata. J Comp Neurol 218:334–344

Smeets WJAJ (1990) The telencephalon of cartilaginous fishes. In: Jones EG, Peters A (eds) Cerebral cortex, vol 8A: comparative structure and evolution of cerebral cortex, part I. Plenum, New York, pp3–30

Smeets WJAJ (1998) Cartilaginous fish. In: Nieuwenhuys R, Ten Donkelaar HJ, Nicholson C (eds). The central nervous system of vertebrates, vol 1. Springer-Verlag, Berlin, pp 551-654

Smeets WJAJ, Marín O, González A (2000) Evolution of the basal ganglia: new perspectives through a comparative approach. J Anat 196:501–517

Smeets WJAJ, Nieuwenhuys R, Roberts BL (1983) The central nervous system of cartilaginous fishes. Structure and functional correlations. Berlin, Springer

Smith-Fernández A, Pieau C, Repérant J, Boncinelli E, Wassef M (1998) Expression of the Emx-1 and Dlx-1 homeobox genes define three molecularly distinct domains in the telencephalon of mouse, chick, turtle and frog embryos: implications for the evolution of telencephalic subdivisions in amniotes. Development 125:2099–2111

Stephenson-Jones M, Ericsson J, Robertson B, Grillner S (2012) Evolution of the basal ganglia; Dual output pathways conserved throughout vertebrate phylogeny. J Comp Neurol 520:2957–2973

Storch V, Welsch U (2001) Curso práctivo de zoología de Kütental. Ariel Practicum, Barcelona, pp 399-410

Stuesse SL, Cruce WL, Northcutt RG (1994) Localization of catecholamines in the brains of Chondrichthyes (cartilaginous fishes). In: Smeets WJAJ, Reiner A (Eds) Philogeny and development of catecholamine systems in the CNS of vertebrates. Cambridge University Press pp, 21-47

Sueiro C (2003) Estudio inmunohistoquímico de los sistemas gabaérgicos del sistema nervioso central de peces elasmobranquios y su relación con sistemas catecolaminérgicos y peptidérgicos. Doctoral thesis, University of Santiago de Compostela, Spain

Tuorto F, Alifragis P, Failla V, Parnavelas JG, Gulisano M (2003) Tangential migration of cells from the basal to the dorsal telencephalic regions in the chick. Eur J Neurosci 18:3388-3393

Vallarino M, Bucharles C, Facchinetti F, Vaudry H (1994) Immunocytochemical evidence for the presence of met-enkephalin and leu-enkephalin in distinct neurons in the brain of the elasmobranch fish *Scyliorhinus canicula*. J Comp Neurol 347:585-597

Vallarino M, Danger JM, Fasolo A, Pelletier G, Saint-Pierre S, Vaudry H (1988) Distribution and characterization of neuropeptide Y in the brain of an elasmobranch fish. Brain Res 448:67-76

Vesselkin NP, Kovacevic N (1973) Nonolfactory telencephalic afferent projections in elasmobranch fishes. Zh Evol Ion Biokhim Fiziol 9:585-592

Von Bartheld CS (2004). The terminal nerve and its relation with extrabulbar olfactory projections: lessons from lampreys and lungfishes. Microsc Res Tech 65:13-24

Williams HT (1973) The telencephalon of the newborn dogfish shark, Squalus acanthias. J Hirnforsch 14:261-285

Whitlock KE (2004) Development of the nervus terminalis: origin and migration. Microsc Res Tech 65:2-12

Wu S, Esumi S, Watanabe K, Chen J, Nakamura KC, Nakamura K, Kometani K, Minato N, Yanagawa Y, Akashi K, Sakimura K, Kaneko T, Tamamaki N (2011) Tangential migration and proliferation of intermediate progenitors of GABAergic neurons in the mouse telencephalon. Development 138:2499–2509

Wullimann MF (2009) Secondary neurogenesis and telencephalic organization in zebrafish and mice: a brief review. Integr Zool 41:123-133

Wullimann MF, Mueller T (2004) Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. J Comp Neurol 475:143–162

Yamamoto N, Oka Y, Kawashima S. (1997). Lesions of gonadotropin releasing hormone-immunoreactive terminal nerve cells: effects on the reproductive behavior of male dwarf gouramis. Neuroendocrinology 65:403–412

Yamanaka S, Honma Y, Ueda S, Sano Y (1990) Immunohistochemical demonstration of serotonin neuron system in the central nervous system of the japanese dogfish, Scyliorhinus torazame (Chondrichthyes). J Hirnforsch 31:385–397

Yáñez J, Folgueira M, Köhler E, Martínez C, Anadón R. (2011) Connections of the terminal nerve and the olfactory system in two galeomorph sharks: an experimental study using a carbocyanine dye. J Comp Neurol 519:3202–17

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Rationale and Aims

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

The vertebrate telencephalon represents one of the most complex structures in the nervous system in terms of morphology, organization, function and genetic specification. Indeed, the telencephalon is probably the zone of the central nervous system that underwent more drastic structural changes throughout evolution, which can be perceived in adult vertebrates as highly variable morphologies and sizes. Still, it exhibits a remarkable degree of conservation regarding to genetic specification and developmental patterning. Despite the abundant information obtained in the last decade about the development of the olfactory system and the regionalization of the telencephalon in different groups of vertebrates, a considerable lack of information exists about cartilaginous fishes, which represent a fundamental group to understand the transition from jawless (agnathans) to jaw (gnatosthomes) vertebrates. Therefore, the scarcity of genoarchitechtonic studies in basal vertebrates hampers the completion of the evolutionary scheme for telencephalic development.

In order to fill the gap of knowledge about the development of the telencephalon in basal vertebrates and to shed light into the ancestral condition of this region along vertebrate phylogeny we have carried out a combination of anatomical and molecular methods on embryos and postnatal specimens of the small shark *Scyliorhinus canicula*, the current model organism representative of cartilaginous fish. The present thesis contains a corpus of results obtained after applying different experimental approaches (ranging from immunohistochemical, *in situ* hybridization to tract-tracing techniques) to explore the telencephalon and peripheral systems related to it in sharks. Together, the present results provide the first comprehensive work about the development and structure of the anterior-most part of the brain in a cartilaginous fish.

This thesis is structured in two sections:

Section I (Chapters 1 and 2) deals with the characterization of the development of the peripheral systems related with the telencephalon, i.e. olfactory and terminal nerve systems.

Section II (Chapters 3, 4 and 5) concerns the study of different aspects of the development and regionalization of the telencephalon proper.

The specific aims of this thesis are:

1) To investigate the early formation of the peripheral olfactory system, characterizing different cell types of the olfactory epithelium and those associated to the olfactory nerve. Particularly, we aim to discern the phenotype of an intriguing population of Pax6 expressing cells in this system to shed light on its possible roles. The results of this study are presented in **Chapter 1** entitled **Development of the peripheral olfactory system. Insights on Pax6 neurons migrating along olfactory nerve**.

2) The study of the developing peripheral olfactory system revealed a neuronal subpopulation associated to olfactory fibers that seemed to bypass the olfactory bulb and head towards more anterior levels. In order to test if this population would form part of the terminal nerve system, we perform a detailed characterization of its development examining the spatial-temporal relationship with the peripheral olfactory system. The results of this study are presented in **Chapter 2** entitled **Development of the terminal nerve system**.

3) To identify the main histogenetic fields and their subdivisions in the telencephalon of *S.canicula*. To this end we analyze the distribution of well-known markers that have been proved to show specific expression in different

telencephalic territories in other vertebrates. The results of this study are presented in **Chapter 3** entitled **Genoarchitechtonic study of the telencephalon**.

4) The analysis of gene expression in the subpallium (Chapter 3) pointed to a particular region of the basal telencephalon to represent the homologous of the striatum in elasmobranchs, disagreeing with former proposals about the identity of this structure in cartilaginous fishes. To provide connectional data that would help to accept or discard this new suggested homology we analyze the connections of this subpallial zone by means of tract tracing experiments in juveniles. We also study the relationship of these connections with the dopaminergic cell groups of diencephalon and mesencephalon. The results of this study are presented in **Chapter 4** entitled **Pilot study of basal ganglia connectivity**.

5) To identify embryonic tangential migratory routes between supballium and pallium in *S.canicula* and their possible homologies with those of other vertebrates. We carry out a comprehensive analysis of well-known expression markers for tangential migrations of subpallial origin. We also explore if these routes are conserved in adult specimens. The results of this study are presented in **Chapter 5** entitled **Evidences of tangential migrations in the telencephalon. Possible homologies and divergencies**.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Chapter 1

Development of the peripheral olfactory system. Insights on Pax6 neurons migrating along the olfactory nerve

Quintana-Urzainqui I, Rodríguez-Moldes I, Candal E (2012) Developmental, tract-tracing and immunohistochemical study of the peripheral olfactory system in a basal vertebrate: insights on Pax6 neurons migrating along the olfactory nerve. Brain Struct Funct DOI: 10.1007/s00429-012-0486-2

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

The olfactory system is an excellent model for studying several developmental aspects of the nervous system including cell migration, tract formation and guidance as well as adult neurogenesis. Its organization is well conserved in vertebrates and consists of the olfactory epithelium, which contains the olfactory receptor neurons (ORNs), the olfactory nerve composed of the axons from ORNs and supporting elements, and the olfactory bulb, the region of the telencephalon where olfactory axons make contact with central neurons, that is, the primary olfactory center.

The olfactory organ (where olfactory epithelium is placed) originates from a cephalic placode, the olfactory placode, which gives rise to olfactory and non-olfactory epithelia. Several studies have described the emergence of different cell populations from the olfactory placode and/or the olfactory epithelium and heading for telencephalic walls (Filogamo and Robecchi 1969; Schwanzel-Fukuda and Pfaff 1989; Wray et al. 1989; Drapkin and Silverman 1999;Schwanzel-Fukuda 1999; Puche and Baker 2007; Treloar et al. 2009; Blanchart et al. 2011). Although the placodal origin of these populations has been traditionally accepted, it is still a matter of discussion and the neural crest is considered as an alternative source for some of them (Whitlock 2004; Barraud et al. 2010; Forni et al. 2011; Katoh et al. 2011). Regardless of their origin, several cell populations have been described traveling along this pathway closely related to developing olfactory axons which seemed to serve as tracks for their migration (Mendoza et al. 1982; Farbman and

Squinto 1985; Valverde et al. 1992, 1993; Pellier and Astic 1994; Pellier et al. 1994; De Carlos et al. 1996; Whitlock and Westerfield 1998; Fornaro et al.2001, 2003; Balmer and LaMantia 2005; Miller et al. 2010; Blanchart et al. 2011; Maier and Gunhaga 2009). Some of these migrating cell populations are related to olfactory function. Among them, the olfactory ensheathing cells (OECs) represent a specific type of olfactory glia that ensheathes groups of unmyelinated olfactory axons and also plays an important role in guidance (Doucette 1984, 1990; Norgren et al. 1992; Chuah and West 2002; Franssen et al. 2007). This population exhibits antigenic and morphological characteristics of both astrocytes and Schwann cells and typically expresses the astrocyte-specific marker glial fibrillary acid protein (GFAP) (Barber and Lindsay 1982; Smithson and Kawaja 2009; Pellitteri et al. 2010; Forni et al. 2011). Neurons expressing olfactory receptor genes and the olfactory marker protein (OMP, a typical marker of some types of ORNs) have also been observed during development along the course of the olfactory nerve, where they have been proposed to act as "guidepost" cells for olfactory axons in their migration toward the olfactory bulb (Valverde et al. 1993; Conzelmann et al. 2002). Other migrating populations associated to the developing olfactory nerve do not appear directly related to olfactory functions. This is the case of a population apparently implicated in neocortical development (De Carlos et al. 1996), and the population of LHRH (luteinizing hormone-releasing hormone)/GnRH (gonadotropin-releasing hormone) expressing cells that are part of the terminal nerve system (Wray et al. 1989; Whitlock 2004).

Because of its key phylogenetic position as a representative species of the most ancient radiation of jawed vertebrates, the elasmobranch *Scyliorhinus canicula* (lesser spotted dogfish) has become a very suitable fish model in studies of vertebrate evolution and development (Coolen et al. 2009). Moreover, as other cartilaginous fishes, it possesses a highly developed sense of smell that is easily accessible to different experimental approaches, and represents an important emerging model for olfactory development studies. The structure and ultrastructure of the adult olfactory bulb and olfactory epithelium have been described in sharks and rays (elasmobranchs), including *S. canicula* (olfactory epithelium/ placode: Theisen et al. 1986; Takami et al.1994; Ferrando et al. 2006, 2007a, 2009, 2010; Schluessel et al. 2008; olfactory bulb: Dryer and Graziadei, 1993, 1996) but, although some genetic data in early embryos are available (Sauka-Spengler et al. 2001; O'Neill et al. 2007) studies on the development of the olfactory system are really scarce (Fishelson and Baranes 1997;Ferrando et al. 2007b; Ferreiro-Galve et al. 2012a). As far as we know, no axonal tracing studies of the olfactory system have been performed in developing cartilaginous fishes so far.

Pax6 is a transcription factor conserved from invertebrates to vertebrates defined by the presence of two highly conserved DNA-binding motifs: a paired domain (PD) in its N-terminus and a paired-like homeodomain in the middle, which bind to distinct DNA consensus sequences. Pax6 acts in crucial developmental processes in the central nervous system, eyes, nose, pancreas, and

pituitary gland (reviewed in Osumi et al. 2008). It is, indeed, a pleiotropic player in development as it participates in multiple aspects such as control of proliferation and cell fate (Marquardt et al. 2001; Simpson and Price 2002; Philips et al. 2005; Oron-Karni et al. 2008; Osumi et al. 2008), patterning and boundary formation (Haubst et al. 2004) and development of axonal pathways (Jones et al. 2002; Pratt and Price 2006; Nomura et al. 2007).Studies of loss of function and Pax6 mutants highlighted this gene as necessary for the normal development of the olfactory system (Hogan et al. 1986; Grindley et al. 1995; Anchan et al 1997; Jimenez et al. 2000). The expression and possible functions of this transcription factor have been largely studied in the olfactory system of vertebrates, where it appears to be involved in the development of the olfactory placode and olfactory epithelium, the generation of specific interneuron subtypes in the postnatal olfactory bulb, positioning and axon guidance of neurons within the olfactory bulb and migration and alignment of olfactory cortex neurons (reviewed in Nomura et al. 2007). Furthermore, Pax6 expressing cells have been first described along the course of the olfactory nerve in *S.canicula* (Ferreiro-Galve et al. 2012a), although the nature of these cells and their relation to the olfactory nerve development was not determined. The characterization of the phenotype of these cells is crucial to ascertain the involvement of Pax6 in the development of the dogfish olfactory nerve.

In this study, we have analyzed the development of the olfactory system in the shark *S. canicula* with two aims: 1) to identify the early formation of the olfactory

epithelium and primary olfactory projections in a cartilaginous fish, characterizing the different cell types of the olfactory epithelium and those associated to the developing olfactory nerve; and 2) to discern the phenotype of Pax6 cells at the olfactory epithelium and olfactory nerve, in order to shed light on their possible role(s). To meet these goals we have implemented tract-tracing techniques in combination with immunohistochemistry with markers for Pax6, proliferating cells (PCNA, proliferative cell nuclear antigen), glial cells (GFAP, glial fibrillary acidic protein), early postmitotic neurons (HuC/D), migrating immature neurons and their growing fibers (DCX, doublecortin) and mature ORNs and primary tracts (protein $G\alpha_0$). This study represents the first detailed analysis of the development of the olfactory system in an elasmobranch species and provides important information about the basic developmental processes that take place during the formation of olfactory structures in basal vertebrates, thus helping to understand how the vertebrate olfactory system has evolved. Unraveling the details of the olfactory development in cartilaginous fish is crucial, not only for comparative purposes (as they are among the vertebrates with highly developed sense of smell) but also to shed light on how the relations between peripheral sense organs and central olfactory structures are established.

MATERIAL AND METHODS

Experimental animals

Some embryos of the lesser spotted dogfish (Scyliorhinus canicula) were supplied by the Marine Biological Model Supply Service of the CNRS UPMC Roscoff Biological Station (France) and the Estación de Bioloxía Mariña da Graña (Galicia, Spain). Additional embryos and juveniles were kindly provided by the Aquaria of Gijón (Asturias, Spain), O Grove (Pontevedra, Spain) and Finisterrae (A Coruña, Spain). Embryos were staged by their external features following Ballard et al. (1993). For more information about the relationships of the embryonic stages, body size, gestation and birth, see Table 1 in Ferreiro-Galve et al. (2010). Fifty-six embryos from stages 21 to 34 and five juvenile dogfish were used. Eggs from different broods and juveniles were raised in seawater tanks in standard conditions of temperature (15-16ºC), pH (7.5-8.5) and salinity (35g/L). Adequate measures were taken to minimize animal pain or discomfort. All procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by the Spanish Royal Decree 53/2013 for animal experimentation, and were approved by the Ethics Committee of the University of Santiago de Compostela.

Tissue processing

Embryos were deeply anesthetized with 0.5% tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO) in seawater and separated from the yolk before fixation in 4% paraformaldehyde (PFA) in elasmobranch´s phosphate buffer [EPB: 0.1 M phosphate buffer (PB) containing 1.75% urea, pH 7.4] for 48-72 h depending on the stage of development. Embryos from stage 32 onwards and juveniles were deeply anesthetized with MS-222 and then perfused intracardially with elasmobranch Ringer's solution (see Ferreiro-Galve et al., 2012b) followed by 4% PFA in EPB. The brains with the olfactory organs attached were removed and postfixed in the same fixative for 24-48 hours at 4ºC. Subsequently, they were rinsed in phosphate buffered saline (PBS), cryoprotected with 30% sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (18-20 µm thick) were obtained in sagittal, horizontal or transverse planes on a cryostat and mounted on Superfrost Plus (Menzel-Glasser, Madison, WI) slides.

Immunohistochemistry

For heat-induced epitope retrieval, sections were pre-treated with 0.01 M citrate buffer (pH 6.0) for 30 min at 95ºC, and allowed to cool for 20-30 min at room temperature (RT). Sections were rinsed twice in 0.05 M Tris-buffered saline (TBS; pH 7.4) for 5 min each, and incubated overnight at RT with the following primary antibodies (see Table 1): mouse monoclonal anti-human HuC/HuD

(HuC/D), rabbit and goat polyclonal anti-Pax6, rabbit and goat polyclonal anti-doublecortin (DCX), rabbit polyclonal anti-glial fibrillary acidic protein (GFAP), mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) and rabbit polyclonal anti-$G\alpha_0$ protein. Sections were rinsed twice in 0.05 M Tris-buffered saline (TBS) pH 7.4 for 5 min each, and incubated in the appropriate fluorescent dye-labeled secondary antibody (see Table 1) for 2 h at RT. All dilutions were made with TBS containing 15% normal donkey serum (Millipore, Billerica, MA) 0.2% Triton X-100 (Sigma) and 2% bovine serum albumin (BSA, Sigma). All incubations were carried out in a humid chamber. Sections were then rinsed in TBS for 30 min and in distilled water (twice for 30 min). Sections were then allowed to dry for 2 h at 37ºC, and mounted in MOWIOL 4-88 Reagent (Calbiochem, MerkKGaA, Darmstadt, Germany). Additional information about the primary and secondary antibodies is included in Table 1.

Double and triple immunofluorescence

Double and triple immunolabeling were performed on alternate series of sections, which were incubated overnight at RT with cocktails of primary antibodies mixed at optimal dilutions and subsequently detected by using mixtures of appropriate secondary antibodies. All incubations were carried out in a humid chamber and processed as described above. Double immunofluorescence with primary antibodies raised in the same species was performed as described in Tornehave et al. (2000).

Controls and specificity of the antibodies

Control experiments were carried out by omitting the primary or secondary antiserum in the incubations. No immunostaining was detected in any case.

Mouse monoclonal anti-HuC/D has been shown to specifically label neuronal cells in zebrafish, birds and humans (Marusich et al. 1994; Ekström and Johansson 2003; Soukkarieh et al. 2007; Vellema et al. 2010). The same antibody labeled different types of neurons in the spinal cord of embryos and juveniles of the lesser spotted dogfish (Sueiro et al. 2004).

The rabbit polyclonal and the goat polyclonal Pax6 antibodies were raised against peptides derived from the C-terminus of the mouse and human peptide respectively. Multiple sequence alignment (Corpet 1988) of Pax6 (GenBank NP_000271.1), Pax6-5a (GenBank NP_001595.2), andPax6-ΔPD (GenBank AAL40860) showed that the C-terminus of the protein recognized by the rabbit antibody is identical in the three Pax6 isoforms (Ferreiro-Galve et al. 2012b). The pattern of immunostaining was identical using both antibodies. The specificity of the immunoreaction of both Pax6 antibodies in the retina and brain of *S. canicula* was previously tested in our laboratory by preadsorbing the primary antibodies with the antigenic Pax6 peptide (NB100-2913PEP; Novus Biologicals, Littleton, CO) used for generation of the NB100-2913 antiserum. For both antibodies, the immunostaining was completely abolished in sections treated with primary

antibodies at working dilution and preadsorbed with the blocking peptide (see Ferreiro-Galve et al. 2012 b).

The rabbit polyclonal and goat polyclonal anti-DCX antibodies recognized a single band of approximately 45 kDa (manufacturer's technical information). In our hands, results obtained with both antibodies were identical. We tested the specificity of the immunoreaction for both DCX antibodies in the brain of *S. canicula* by preadsorbing the primary antibodies with the antigenic DCX peptide (sc-8066P; Santa Cruz Biotechnology, Santa Cruz, CA). The immunostaining was completely abolished in *S. canicula* sections treated with primary antibodies at working dilution after preadsorption with the blocking peptide at a concentration of 10 mg/mL at 4º C for 24 hours.

The polyclonal anti-GFAP antibody is a purified immunoglobulin fraction of rabbit antiserum generated to bovine spinal cord GFAP. This antibody has been previously used as an immunohistochemical marker for olfactory ensheathing cells (Katoh et al. 2011) and as a glial marker in *S.caninula* (Wasowicz et al. 1999; Sueiro et al. 2007)

The monoclonal anti-PCNA recognizes a protein of 36 kDa corresponding to the acidic non-histone auxiliary protein of DNA polymerase, according to the manufacturer. This antibody specifically labels proliferating cells in the brain and olfactory epithelium of *S. canicula* (Rodríguez-Moldes et al. 2008; Ferrando et al. 2010).

The $G\alpha_0$ (K-20) antibody is an affinity purified rabbit polyclonal antibody raised against a peptide mapping within a highly divergent domain of the trimeric G protein subunit $G\alpha_0$ of rat origin. In Western Blots of protein homogenates of the olfactory organ of *S. canicula*, this antiserum recognizes a single band of approximately 42kDa (Ferrando et al. 2009). In *S. canicula*, this antibody has been previously used to label olfactory receptor neurons and axon bundles in the *fila olfactoria* (Ferrando et al. 2009).

In vitrotract-tracing techniques

A total number of 10 animals ranging from stage 28-embryos to juveniles were used. The experiments were performed under *in vitro* whole-brain conditions. All specimens were deeply anaesthetized in seawater containing 0.5% MS-222. Animals were then immersed in (stages 28 and 31) or perfused transcardially with (stage 32 onwards) 30 ml of ice-cooled elasmobranch Ringer's solution containing1mM glucose, which was oxygenated with an oxygen injector aerator to a pH of 7.4. After the animal decapitation, the brains were isolated by removing the overlying cartilage skull and skin and transferred to fresh Ringer's solution to proceed with the immediate application of the tracer. We applied neurobiotin (Vector Laboratories, Burlingame, CA), an amino derivative of biotin used as an intracellular label for neurons, whose transport efficiency in retrograde labeling has been proved (Barreiro-Iglesias et al., 2008). The tracer was dissolved in distilled water until saturation and re-crystallized at the tip of an entomological

needle (00) according to Morona et al. (2005) and the application was carried out manually or using a micromanipulator (Narishige MN-151, Japan) under a stereomicroscope. The olfactory epithelium and the olfactory bulb were accessed by vertical penetrations and all applications were made unilaterally. After tracer application, brains were maintained for 2-3 days at 8ºC in continuously oxygenated elasmobranch Ringer's solution containing 1mM glucose, and then fixed for 2 days in 4% paraformaldehyde in EPB. The tissue was cryoprotected and sectioned as explained above. Neurobiotin was visualized by incubating the sections with fluorescein isothiocyanate (FITC)-labeled Avidin D (Vector Laboratories; diluted 1:1000 in PBS containing 0.2% Triton X-100) in a humid chamber for 2.5h at 37ºC. The slides were rinsed in TBS, then in distilled water, dried for 30 min at 37ºC, and mounted in MOWIOL.

Combined tract-tracing and immunohistochemistry

For combined tract-tracing and immunohistochemistry, the tracer was applied and the tissue was processed as described above. Primary antibody solutions and Avidin B were simultaneously incubated and revealed and mounted as previously explained.

Bisbenzimide staining

Fluorescent counterstaining of cell nuclei was carried out by immersing the samples in 0.5µg/mL bisbenzimide (Sigma) in TBS for 5 seconds. The labeling was

visualized with an UV filter coupled to a spectral confocal laser scanning microscope TCS-SP2 (Leica, Wetzlar, Germany).

Apoptosis detection

We assessed cell death in the olfactory system of *S. canicula* with the TUNEL technique using the NeuroTACS II *in situ* apoptosis detection kit (Trevigen, Inc., Gaithersburg, MD; catalog number 4823-30-K). Sections were treated according to the manufacturer to detect DNA fragments generated by apoptosis using a highly purified terminal deoxynucleotidyl transferase enzyme (TdT) and to reveal the incorporated nucleotides using a horseradish peroxidase system. Positive and negative controls were performed. For positive control, sections treated with TACS-Nuclease™ (Trevigen) to generate DNA breaks in every cell showed pale staining in most of cells after the application of the apoptosis detection kit. No labeling was observed when TdT was not included in the reaction.

Image acquisition and analysis

Double labeled sections were analyzed and photographed with theTCS-SP2 scanning microscope with a combination of blue and green excitation lasers. Stacks of confocal images were acquired separately for each laser channel with steps of 0.8 or 2 µm along the z-axis and collapsed images were obtained from an average of 12 optical sections with the LITE software (Leica). Some sections were photographed with a epifluorescence photomicroscope Olympus AX70

fitted with an Olympus DP70 color digital camera. Sections of apoptosis experiments were photographed with an Olympus BX51 microscope equipped with an Olympus DP71 color digital camera. Photographs were adjusted for brightness and contrast and plates were prepared using Adobe Photoshop CS4 (Adobe, San Jose, CA). Images were not otherwise modified.

RESULTS

We have identified the key events that take place during the development of the olfactory system in the lesser spotted dogfish (*Scyliorhinus canicula*) and we have framed them into the context of the three developmental periods that were established on the basis of tract-tracing experiments and the expression of various immunohistochemical markers (see Fig. 1 and Table 2). These events and developmental periods constitute a useful reference for comparative developmental studies of the olfactory system as they can be consistently identified in different vertebrates (see discussion). The first period (Fig. 1A, stages 20 to 24) is marked by the onset of neurogenesis and the observation of the first postmitotic neurons in the olfactory placode, which seem to pioneer the olfactory pathway across the mesenchyme. The second period (Fig. 1B, stages 25 to 30) is characterized by profuse neuronal migration along the olfactory fibers. During the third period (Fig. 1C, stage 31 to hatching), the mature structure of the olfactory system components is progressively acquired. Consequently, we have termed these periods pioneer, migratory and maturation periods, respectively.

First period: Pioneer period (stages 20 to 24)

The olfactory placode derives from the ectoderm on the ventrolateral surface of the head of the early embryo and becomes morphologically visible at stage 20 (Sauka-Spengler et al. 2001), which corresponds to the beginning of the first period. At this very early stage, most placodal cells showed PCNA immunoreactivity but immunolabeling for markers of differentiation was not observed (data not shown). Shortly after (stage 21) the olfactory placode was visibly thickened and a few early postmitotic neurons were already detected by means of HuC/D immunoreactivity (arrowhead in Fig. 2A,A'). Some of the earliest HuC/D-immunoreactive (ir) neurons seemed to delaminate from the inner region of the placodal epithelium, extending short processes in the mesenchyme (arrow in Fig. 2A-A'). The earliest delaminated neurons also showed immunoreactivity to DCX, a marker for migrating immature neurons (Fig. 2A,A''). Because of their early emergence from the olfactory placode epithelium, we identified these cells as pioneer neurons, a transient class of neurons that prefigure the primary olfactory pathway before the expression of olfactory receptor genes and the outgrowth of olfactory axons (Whitlock and Westerfield 1998). Later in development (stage 22),the first signs of invagination of the olfactory placode took place. At this stage, DCX-ir axons of first pioneer neurons contacted with the surface of the telencephalon (not shown). During stages 22 to 24 the thickened placodal epithelium invaginated to form the olfactory pit (Fig. 1A). Most cells throughout the olfactory pit neuroepithelium continued showing PCNA immunoreactivity

(Fig. 2B) although some postmitotic immature neurons (DCX-ir) whose axons reached the surface of the telencephalon were also observed (arrowhead in Fig. 2B). These axons of olfactory pit cells contacting the telencephalon represent the first evidence of the olfactory nerve primordium.

Second period: Migratory period (stages 25 to 30)

At stage 25 (Fig. 1B, 2C), the number of early postmitotic (HuC/D-ir) neurons of the olfactory pit epithelium increased. They were mainly located in a neurogenic region found in the medial/deeper zone of the developing olfactory epithelium where HuC/D-ir cells mostly occupied its basal part, close to the mesenchyme. At this stage, strongly DCX-ir fibers belonging to HuC/D-ir cells were observed exiting the neurogenic region (red arrows in Figs. 2C,C',C'') and contributing to the primordial olfactory nerve. Concurrently, new subsets of HuC/D-ir and DCX-ir cells delaminated from this region of the olfactory pit and entered the adjacent mesenchyme in apposition to the developing olfactory nerve (arrowheads in Fig. 2C). Shortly after (stage 26), a stream of neurons was observed extending from the neurogenic region of the olfactory pit toward the surface of the telencephalon (Figs. 2D-F). At stage 28 (Fig. 1B, 2G-I), the invagination of the olfactory pit continued and the primordial olfactory epithelium expanded laterally to form a sac divided in two recesses with a common narrow entrance. As a result of these movements, the neurogenic region became situated in the medial part of the olfactory pit. The spatial extension of the neurogenic region within the

olfactory pit was concurrent with an increasing number of postmitotic HuC/D-ir and DCX-ir immature neurons (Fig. 2G-I), which remained located in the basal part of the neuroepithelium (asterisk in Fig. 2I). There was also a marked increase in the number of olfactory fibers in the olfactory nerve, and of neurons migrating along it as revealed by their DCX (Figs. 2G,H) and HuC/D immunoreactivity (Fig. 2I), respectively. At stage 29the olfactory epithelium began to fold forming the first characteristic lamellae (Fig. 1B, 2J,K) and the neurogenic region of the epithelium extended from medial to lateral aspects of the organ, as revealed the distribution of HuC/D-ir cells (see direction of arrows in Fig. 2J). At this stage, the primordial olfactory bulbs (pOB in Fig. 1B) became recognizable as small protrusions that emerged at the lateral walls of the telencephalon. Of note is the observation of a cluster of HuC/D-ir cells near the olfactory nerve-olfactory bulb contact region, which deviates from the olfactory pathway toward more anterior regions near the telencephalic wall (arrowhead in Fig. 2K). Although more studies are necessary to demonstrate it, we are persuaded that this branched population form part of the developing terminal nerve and ganglia. Also at this stage, the first evidence of the presence of olfactory glia in the olfactory nerve was obtained by means of GFAP immunohistochemistry (Figs. 1B,2L). Glial processes (GFAP-ir) were arranged surrounding bundles of DCX-ir cells, characterized as migrating immature neurons, and DCX-ir fibers(Fig. 2L). Colocalization of GFAP and DCX in the same processes was ruled out after analyzing 1µm-confocal sections (see inset in Fig. 2L).

Third period: Maturation period (stages 31 to 34/prehatching)

At stage 31, the olfactory epithelium became further folded (Fig. 1C) and neurogenesis extended to the entire epithelium. Cells immunoreactive to HuC/D (Fig. 3A) and DCX (Fig. 3B) appeared throughout the epithelium, but the scattered distribution of these markers indicates that the stratification of neurons in the epithelium has not yet occurred. The structure of the olfactory nerve was similar to that observed in previous stages, i.e., bundles of DCX-ir cells (migrating neurons)and fibers appeared surrounded by glial processes (GFAP-ir) (Figs. 3C,D),although the nerve fascicles were thicker than in previous developmental stages. In the olfactory bulb, first protoglomeruli were noticed by their intense immunoreactivity to the Gα0-protein (Fig. 3E). The whole primary projection at this stage was evidenced by massive application of neurobiotin to the olfactory epithelium (Fig.3F). In these experiments, the olfactory bundles ended in two rather compact protoglomerular fields surrounded by HuC/D neurons of the bulb.

As development proceeded (stage 32), secondary epithelial lamellae extended perpendicular to the primary laminae (Fig. 1C) and stratification of olfactory neurons through the height of the epithelium became evident as shown with different neuronal markers (Figs. 3G,H). Proliferating (PCNA-ir) cells were found throughout the olfactory epithelium, though PCNA immunoreactivity was more intense at the basal part (not shown). Cells immunoreactive to HuC/D (Fig. 3G) and DCX (Fig. 3H) occupied most of the epithelium, except the apical layer that eventually will contain sustentacular (non-neuronal) cells. Some DCX-ir cells

showed bipolar morphology with an apical dendrite that reached the apical surface (arrows in Fig. 3H). The same morphology was observed in cells that showed immunoreactivity to Gα0-protein, especially in the apical dendrite ending in an olfactory knob (arrowheads in Fig. 3I) and in those that we have identified as the first maturing ORNs. At this stage, the density of HuC/D-ir cells along olfactory fibers was notably reduced in relation to previous stages (Figs. 3J,K) and they disappeared at the end of this stage (Fig. 3L). As development proceeded, there was an increase in the number of mature ORNs (mORNs), as revealed by their intense Gα0-protein immunoreactivity. Similar bipolar neurons were retrogradely labeled after massive application of neurobiotin in the olfactory bulb (Fig. 3M). These ORNs showed apical dendrites of variable lengths depending of the location of the perikaryon in the epithelium. The density of mORNs showing strong $G\alpha_0$ immunoreactivity increased in postnatal specimens with respect to embryos (Figs. 3N,O). At juvenile stages, $G\alpha_0$-ir cells that showed immunoreactivity to HuC/D and DCX were relatively abundant (large arrows in Figs. 3N,O). Of note, DCX immunoreactivity was observed in the apical processes of maturing (young) ORNs that showed low expression for Gα0-protein (large arrow in Figs. 3O,O',O''), while it was absent in mature ORNs that expressed high levels of Gα0-protein (arrowhead in Fig. 3O,O',O'').

Characterization of Pax6 cell populations in the peripheral olfactory system

The presence and distribution of Pax6-expressing cells in developing and adult olfactory system of the lesser-spotted dogfish has been recently reported by some of us (Ferreiro-Galve et al., 2012a), who presented the first evidence in vertebrates of strings of Pax6-expressing cells extending along the developing olfactory nerve. In the present study, we have not only confirmed the existence of such cells and extended the search into earlier developmental stages to shed light on its origin but more importantly, we have characterized their nature through different developmental stages.

At stage 21, Pax6 was expressed throughout the olfactory placode but not in cells adjacent to the placode (Fig. 4A), which showed HuC/D immunoreactivity and appeared to delaminate from the neurogenic region (see above, first period).The expression of Pax6 immunoreactivity in this neurogenic region appeared to be lower than in the surrounding ectoderm (Fig. 4A). Conversely, during the second period (Figs. 4B-I) Pax6 immunoreactivity was intense in a subset of cells located in the neurogenic area of the olfactory epithelium (arrows in Fig. 4B), as well as in a subset of migrated cells (arrowheads in Fig. 4B) that were in apposition to the DCX-ir fibers of the developing olfactory nerve (see also Figs. 4C,D).

To assess the nature of these Pax6-ir cells, we applied neurobiotin to the olfactory epithelium of stage 28 embryos, which allowed the labeling of a few

developing olfactory neurons per specimen and their respective axons (Fig. 4E). Experiments combining tract-tracing and DCX immunohistochemistry showed that neurobiotin-labeled axons were also immunoreactive to DCX (Fig. 4E). Other experiments with different immunomarkers revealed that some neurobiotin-labeled cells in the olfactory epithelium were double Pax6-ir/HuC/D-ir neurons (arrowhead and detail in Fig. 4F) that extended their axons toward the telencephalon (open arrowheads in Fig. 4F). Moreover, we observed migrated Pax6-ir neurons associated to these neurobiotin-labeled axons that did not contain the tracer (arrow in Fig. 4F), which suggests that they were not contacting with the olfactory epithelium surface at the moment of the tracer application. The density of Pax6-ir cells closely associated to the DCX-ir axons of the olfactory nerve continued being high at stage 29 (Fig. 4G) but decreased from this stage onwards. As reported above, glial cells showing GFAP immunoreactivity were firstly detected at stage 29 and, as expected, colocalization between Pax6 and this glial marker was not observed (Fig. 4H), which ruled out a glial nature for the Pax6-ir cells associated to the olfactory nerve. Interestingly, while numerous Pax6-ir neurons were observed along the olfactory nerve, the number of these cells did not increase in the periphery of the olfactory bulb in a perceptible way. Instead, Pax6-ir neurons associated to the olfactory nerve seemed to accumulate distally in the olfactory path, close to the olfactory nerve–olfactory bulb junction (Fig. 4I) and they were absent along which could represent the terminal nerve pathway (Figs. 4I,J).

At the beginning of the maturation period (stage 31 to 34), a notable increase in the density of Pax6-ir cells was observed throughout the olfactory epithelium (Fig. 4K) but the density of Pax6-ir cells associated to the olfactory nerve notably diminished, as compared with previous developmental stages (Fig. 4K). Of note, Pax6-ir cells in the periphery of the olfactory bulb appeared to form corridors between the bundles of olfactory axons (see arrows in Fig. 4L). Interestingly, a number of apoptotic cells was detected at this stage at the entrance of the olfactory bulb (Fig. 4M), but not along the course of the olfactory nerve.

From stage 32 onwards the olfactory epithelium became clearly stratified and strongly Pax6-ir cells became progressively restricted to the basal part of the neuroepithelium (Figs. 5A-C), although weakly Pax6-ir cells were also observed at basal and intermediate levels. In the basal olfactory epithelium, weak Pax6-ir cells showed low levels of PCNA immunoreactivity (open arrows in Fig. 5C) and probably correspond to early postmitotic cells, while intense Pax6-ir cells were PCNA-negative but DCX-immunoreactive (short arrows in Figs. 5B, C)and they could represent immature/differentiating ORNs. Weak Pax6-ir cells were also observed at intermediate levels of the epithelium (arrowheads in Figs. 5B, C).By their morphology (roundish cells with prominent nuclei), position (middle third of the epithelium), and the weak levels of the Gα0-protein (arrowheads in Fig. 5B), they could correspond to maturing ORNs. However, Pax6 expression was absent from fully mature ORNs that were identified by neurobiotin labeling after application of the tracer to the olfactory bulb (Fig. 5A) or by their intense

immunoreactivity to the Gα0-protein (large arrows in Fig. 5B). It is noteworthy that, despite the increase in density of Pax6-ir cells in the olfactory epithelium throughout this period, the density of Pax6-ir cells along the olfactory nerve and in the vicinity of the olfactory bulb became progressively reduced (Figs. 5D,E).

DISCUSSION

This study describes for the first time in cartilaginous fish the development of the olfactory nerve and characterizes the nature of some cells observed in the developing olfactory epithelium and associated to the olfactory nerve, particularly, the intriguing Pax6-expressing population that was recently observed along the olfactory pathway during the development of *S. canicula* (Ferreiro-Galve et al., 2012a) Our results also provide information about the main events that take place during the development of the olfactory system in vertebrates.

Main events of the development of the peripheral olfactory system

The slow development of the lesser-spotted dogfish and the relative large size of embryonic structures were particularly advantageous to analyze developmental processes in the peripheral components of the olfactory system, namely the olfactory epithelium and olfactory nerve. This led us to establish three morphogenetic periods characterized by the appearance of pioneer neurons along the olfactory pathway (pioneer period), the massive migration of cells along the olfactory fibers (migratory period), and the acquisition of the mature organization (maturing period) (see Table 2). These periods constitute a useful

framework for comparative research on the development of the olfactory system as the main events considered can be reliably recognized in different groups of vertebrates (mouse: Wray et al., 1989; Mori, 1993; Schwanzel-Fukuda and Pfaff, 1989; Schwanzel-Fukuda, 1999; Balmer and LaMantia, 2005; Ikeda et al., 2007; Treloar et al., 2009; Gokoffski et al., 2010; Miller et al., 2010; Blanchart et al., 2011; García-González and de Castro, 2011; rat: Valverde et al., 1993; De Carlos et al., 1996; chick: Norgren et al., 1992; Drapkin and Silverman, 1999; Fornaro et al., 2001; Maier et al., 2011; zebrafish: Whitlock and Westerfield, 1998; Whitlock, 2004; Yanicostas et al., 2009). Similarities or novelties observed with respect to other vertebrates may serve as a cue to understand the development and evolution of the olfactory system.

The early events of the olfactory system development include the formation of the olfactory placode and its invagination to form the olfactory pit and the centralward growing of olfactory axons to form the olfactory nerve (see Table 2). Important neurogenic processes take place during these early events, such as the differentiation of the first neurons in the placodal epithelium and the growth of first olfactory axons toward the forebrain, which eventually will join in fascicles to form the olfactory nerve. First evidence of neuronal differentiation in the dogfish olfactory placode was seen at stage 20-21, when the earliest neurons appeared to delaminate from the placode and enter the mesenchyme extending short processes prior to the onset of other migratory events or the establishment of the olfactory nerve. We have identified these early cells as pioneer neurons of the

olfactory pathway. Similar precocious neuronal populations have been reported to play a pioneer role in other species (zebrafish: Whitlock and Westerfield 1998; chick: Fornaro et al. 2001; human: Bystron et al. 2006; mouse: Ikeda et al. 2007), where they appear involved in axon guidance from the olfactory placode to the olfactory bulb region of the telencephalon (Whitlock and Westerfield 1998). It is interesting to note that in dogfish embryos at stage 24, shortly after the appearance of pioneer neurons, early postmitotic neurons located within the olfactory epithelium extended their axons (DCX-ir) to contact with the telencephalon, which represent the earliest evidence of olfactory projections. These first contacts between fibers arising from the neuroepithelium and the telencephalon have been considered by other authors as a landmark for the morphological identification of the developing olfactory nerve (Drapkin and Silverman 1999; Maier et al. 2011). As in *S. canicula*, the emergence of olfactory axons from the olfactory pit in rat and chick also appeared delayed with respect to early neuronal differentiation (De Carlos et al. 1996; Drapkin and Silverman 1999).

During the second period, the olfactory nerve of *S. canicula* appeared to be highly cellular, as migrating neurons (DCX and HuC/D immunoreactive) were observed along olfactory fibers. As the density of these cells progressively increased, achieving its maximum at stages 29-30 (the end of the second period) and decreased until they disappear at late stage 32, when the basic mature morphology of the olfactory system is established, the second period has been

characterized by the massive neuronal migrations along the olfactory nerve. This second wave of neurons migrating outside the olfactory epithelium was also described in other vertebrates and has been commonly referred as the migratory mass. The most frequently described cells in this migratory mass in a variety of vertebrates include olfactory ensheathing cells (OECs), neurons expressing olfactory receptor genes and the olfactory marker protein (OMP neurons) and LHRH/GnRH positive neurons (see introduction). Nevertheless, there is no strict definition for this term and some authors include pioneer neurons in this concept (Miller et al. 2010). We showed that pioneer neurons observed in *S. canicula* during the first period, before the outgrowth of olfactory fibers from the epithelium, are phenotypically distinct from the population of migrating cells observed along developing olfactory axons during the second and third developmental periods, as only these cells show Pax6 immunoreactivity (see below). Accordingly, we will use the term migratory mass to refer only to the later.

A crucial observation of this study refers to the GFAP-ir population surrounding olfactory axons that was observed in *S. canicula* as early as at stage 29, and which we have identified as OECs. As indicated above, the migratory mass in mammals include OECs that can be readily identified as they express GFAP and wrap bundles of axons (Barber and Lindsay 1982; Smithson and Kawaja 2009; Pellitteri et al. 2010; Forni et al. 2011; Higginson and Barnett 2011). In *Xenopus*, GFAP immunoreactivity, likely expressed by ensheathing glia, has been described along the olfactory nerve from tadpoles to adults (Huang et al. 2005) though the

relation between these cells and the olfactory nerve was not cleared by the authors. In teleosts, some markers for mammalian olfactory ensheathing cells also stain the olfactory pathway (Lazzari et al. 2013) but whether a glial ensheathing structure is present in the olfactory nerve of bony fishes is not well known. Present results firstly reporting cells with immunohistochemical and structural characteristics of OECs in a cartilaginous fish indicate that ensheathing glia is already present in the most ancient radiation of jawed vertebrates.

It has been suggested that bundling of fibers is a property of glial cells (Drapkin and Silverman 1999) and that OECs provide essential growth and guidance cues for ORNs axons (Forni et al. 2011). However, in *S. canicula*, the migratory mass and several olfactory projections bundled together into a single nerve before the ensheathing glia can be detected by means of GFAP immunohistochemistry. Nerve bundling also precedes the expression of glial markers in mouse (Miller et al. 2010) and chick (Drapkin and Silverman 1999). The possibility that immature glia could be present along the olfactory nerve before it expresses GFAP has been considered (Drapkin and Silverman 1999), which is consistent with the finding that the migratory mass in mouse contain undifferentiated cells that are a source of OECs (Blanchart et al. 2011). To our knowledge, whether the immature glia actually guides axons toward the olfactory bulb has not been addressed so far. Moreover, the possibility that other cell types contribute to the signaling required for the establishment and guidance of the

olfactory pathway before the maturation of the ensheathing glia, cannot be ruled out (see discussion below).

Pax6 in the olfactory system: Characterization and possible roles

Studies in mammals have demonstrated that the expression of *Pax6* gene is indispensable for the normal formation of the olfactory placode, differentiation of the olfactory epithelium and development of the olfactory bulb (reviewed in Nomura et al. 2007). These roles are not exclusive of mammals as the expression of this gene has been demonstrated in components of the developing olfactory system of a basal vertebrate, the shark *S. canicula* (Ferreiro-Galve et al. 2012 a). Interestingly, that study presented the first evidence in vertebrates of Pax6-expressing cells located along the course of the olfactory nerve, but the nature of such cells was not determined. In the present study, we demonstrate the neuronal nature of Pax6-expressing cells present in different components of the peripheral olfactory system such as the olfactory placode and epithelium, and along the olfactory nerve. Moreover, we show the existence of different subpopulations of Pax6 cells, which could be playing different roles during development.

Pax6 in the olfactory placode and epithelium during development

Different levels of Pax6 expression were detected by means of immunohistochemistry, ranging from a weak and diffuse expression observed at very early stages in cells of the olfactory placode, to a very strong expression that

clearly delimitated the nuclei of olfactory epithelium cells from the second developmental period onwards. The relative levels of Pax6 protein in a cell have been correlated to differential functions in the regulation of proliferation and differentiation processes (Hsieh and Yang 2009; Sansom et al. 2009; Ferreiro-Galve et al. 2012 b).

In this study, we show that during the first period (early placode formation), weak Pax6 expression extends throughout the highly proliferating olfactory placode, but it is excluded from the neurogenic region, which contains the first postmitotic (though immature) neurons. This fact suggests a possible implication of Pax6 in maintaining the proliferative state of most placodal cells and supports the assumption that it is necessary to maintain low Pax6 protein levels for S phase re-entry, whereas either rapid accumulation or complete downregulation of Pax6 protein levels during the G2/M phase may be required to specify diverse neuronal fates (Hsieh and Yang 2009).

During the second period, intense and nuclear Pax6-immunoreactivity was observed first in a subset of postmitotic neurons in the olfactory epithelium, and then it extended from medial to distal parts following the spatio-temporal expansion of neurogenesis, which was visualized by means of neuronal postmitotic markers as HuC/D and DCX. However, not all postmitotic cells in the olfactory epithelium were Pax6-ir. In the chick retina, Hsieh and Yang (2009) showed that, upon the influence of unidentified cues, subsets of progenitors alter their Pax6 level in preparation for cell cycle exit, so that postmitotic neurons

achieve and maintain distinct levels of Pax6, ranging from Pax6 negative (in specific cell types) to strongly Pax6-ir (in others). We thus performed double- and triple-labeling assays to determine whether Pax6 expression was related to particular (neuronal or non-neuronal) cell types within the mature epithelium. The mature olfactory epithelium of *S. canicula* has already been described by Ferrando et al. (2010) and consists of three main cell types: basal cells, including globose-like and horizontal-like progenitor cells involved in neuroepithelial renewal and located at the base of the epithelium; sensory cells, including numerous ORNs and quite rare crypt neurons (CNs), located at the middle and upper third of the olfactory epithelium, respectively; and sustentacular cells supporting both epithelial and glial functions, also localized in the upper third. A further cell type has been described in the olfactory epithelium of adult *S. canicula*, the light stained cells (LSCs) for which a role in ionic regulation has been suggested (revised in Ferrando et al. 2006, 2010). In this study, we have characterized the transition from proliferating progenitors to mature ORNs in the postnatal olfactory epithelium attending to the sequential expression of different markers (see Fig. 6). Numerous intense PCNA-ir cells were found in the basal part of the epithelium, though scattered weak PCNA-ir cells were also found within the middle and the apical parts of the epithelium. However, PCNA was absent from cells with round clear nucleus localized in the middle zone of the olfactory epithelium, which can be identified as maturing ORNs in the basis of their morphology. These results are consistent with that reported in the adult olfactory

epithelium of *S. canicula* (Ferrando et al. 2010), where PCNA has been found in basal cells (probably globose-like basal cells) and sustentacular (non-neuron) cells, but not in ORNs. We found that weakly Pax6-ir basal cells were also weakly PCNA-ir, which supports the idea that keeping low Pax6 protein levels is necessary for maintaining the proliferative state of cells (Hsieh and Yang 2009). Pax6 has also been described in basal progenitor cells of the olfactory epithelium of the adult mouse (Guo et al. 2010). On the other hand, intense Pax6-ir cells in *S. canicula* were mainly located at basal levels where they codistribute but not colocalize with PCNA-ir progenitor cells, which again supports the hypothesis that rapid accumulation of Pax6 protein levels may be required to specify diverse neuronal fates (Hsieh and Yang 2009). Most of these postmitotic Pax6-ir cells were identified as immature neurons as they also expressed DCX, which has been used as a marker of immature ORNs in the olfactory epithelium of mouse (Murdoch and Roskams 2007). In addition, weak Pax6-ir cells were observed in the middle part of the epithelium. These cells coexpressed low levels of DCX but additionally showed weak levels of the $G\alpha_0$-protein, which has been largely used as a marker for ORNs (Ferrando et al. 2009). We thus identified these weak Pax6-ir cells as maturing ORNs because of their morphology (roundish cells with prominent nuclei; Ferrando et al. 2007a,b), location (middle third of the epithelium) and the presence of low levels of $G\alpha_0$-protein in their apical processes. Finally, Pax6 expression was absent from fully mature ORNs, which lack DCX and show intense immunoreactivity to Gα0-protein, as reported in adults

(Ferrando et al. 2009). These results strongly suggest that Pax6 is expressed in postmitotic maturing cells belonging to the ORN lineage. In fact, studies in birds demonstrated that olfactory precursors are capable of expressing Pax6 (and *Dlx3*) even when grafted to the trunk and that some of these graft-derived cells eventually differentiate as Hu-positive neurons (Bhattacharyya and Bronner-Fraser 2008). In mouse, Pax6 expression was mainly described in basal progenitors and apical non-neuronal sustentacular cells (Davis and Reed 1996; Behrens et al. 2000), although recent works showed an additional Pax6 expression in a reduced number of neuron-committed Mash1-expressing cells (Gokoffski et al. 2010; Guo et al. 2010). These observations differ from that observed in *S.canicula* (Ferreiro-Galve et al. 2012a and present results), in which Pax6 was strikingly expressed in high numbers of cells of the ORN lineage throughout the olfactory epithelium. Whether this difference could be related to different rates of ORN renewal between species remains elusive. Further studies in other vertebrate groups will be needed to clarify this issue.

Pax6 in the migratory mass

While Pax6 expression was never detected in pioneer neurons of the olfactory pathway (present results), an intense Pax6 expression was observed in a subset of neurons within the migratory mass, which further supports the idea that the pioneer cells and the migratory mass represent different cell populations. To elucidate the identity of the Pax6-positive cells of the migratory mass, we

analyzed their spatio-temporal pattern of expression as they migrate from the olfactory epithelium to the olfactory bulb. Although Pax6-ir cells appeared to delaminate from the epithelium, a neural crest origin cannot be discarded. Observations from transgenic mice and cell tracing studies in chick revealed the presence of neural crest-derived cells in the olfactory epithelium, which were able to give rise to all cell types found in the olfactory epithelium and presented morphologic and antigenic properties identical to placode-derived cells (Katoh et al. 2011). In the dogfish migratory mass, Pax6-ir cells were observed in apposition to olfactory axons and they accumulated near the olfactory nerve–olfactory bulb junction (stages 29 to 31) at the point where the supposed terminal nerve migrating cells appeared to branch off (see Chapter 2). As indicated above, different cell types including OECs, OMP-expressing cells and terminal nerve cells are known to migrate along olfactory fibers during development as part of the migratory mass. Pax6-ir cells have not been described so far within the migratory mass of vertebrates, so we aimed to know whether these cells could belong to any of the cell populations described in the literature. In this work we proved that Pax6 cells did not colocalize with the glial marker GFAP and expressed the neuronal marker HuC/D. Accordingly, we dismissed their glial nature and ruled out the possibility that they represent OECs. We consider very unlikely that Pax6-ir migrating cells were terminal nerve cells, as we did not detect any Pax6-positive cell in the clusters of branched neurons heading for anterior telencephalon that we tentatively identified as belonging to the developing terminal nerve system.

However, more studies are necessary to confirm the nature of the deviated population (Chapter 2). Instead, these cells seemed to head for the vicinity of the olfactory bulb. However, Pax6-expressing cells in the olfactory bulb of *S. canicula* are scarce and mostly restricted to the granular layer (Ferreiro-Galve et al. 2012a). Pax6-ir cells were not detected between the entrance of the olfactory nerve and the olfactory bulb granule layer, which strongly suggests that these cells did not migrate into the granule layer and supports the assumption that Pax6-ir granule cells originate in the telencephalic hemispheres (Franco et al. 2001; Kohwi et al. 2005). Although downregulation of Pax6 expression at the entrance of the olfactory bulb cannot be discarded, the presence of numerous apoptotic cells in the same region (present results), suggests that Pax6 cells could undergone programmed cell death at this point. However, colocalization experiments with apoptotic markers will be needed to confirm this assumption.

Although Pax6 cells have not been described in the migratory mass of other vertebrates, we do not consider that they represent a derived feature of *Scyliorhinus canicula*, or of basal gnathostomes. They do not form part of any specialized structure as those present in the olfactory system of some vertebrates, as the Grueneberg ganglion of mouse (Roppolo et al. 2006) or the accessory olfactory organ of the sea lamprey (Ren et al. 2009). On the contrary, Pax6-ir cells are located in regions of the olfactory system that show highly conserved structural and developmental features along evolution, as the olfactory nerve. Therefore, similar cells may have been overlooked in studies of other vertebrates,

perhaps because they form a transient population that would be more easily detectable in vertebrates with protracted development and large embryonic brains as *S. canicula*. Moreover, most studies of Pax6 expression are based on in situ hybridization techniques, which give a lower cell labeling than immunohistochemical methods, although these Pax6 cells have been clearly demonstrated with in situ hybridization (Ferreiro-Galve et al. 2012a). Other possibility is that equivalent migrating neurons are present in the migratory mass of other vertebrates but they express other gene/s that play a similar role than *Pax6* in the shark. Possible candidates are *Dlx3* and *Dlx5*, which, together with Pax6 appear to act as molecular guideposts for olfactory placode precursors at different developmental stages in the chick (Bhattacharyya and Bronner-Fraser 2008).Various studies have proposed a guidance role for some migrating cells associated to olfactory nerve fibers. In mouse, extraepithelial cells expressing a particular olfactory receptor gene have been described closely associated with outgrowing axons of maturing ORNs that express the same receptor type (Conzelmann et al. 2002). It has been suggested the possibility that the extraepithelial cells might be acting as guideposts or transient targets for exactly those outgrowing axons equipped with the same olfactory receptor type (Conzelmann et al. 2002). These authors were not able to follow the fate of this population as it was no longer detectable after a certain developmental stage. In rat, OMP-expressing cells located along the course of the olfactory axons seemed to disappear during development (Valverde et al. 1993).These cells may represent

a group of intervening neurons between the ORNs and the olfactory bulb that serve as hints for olfactory axons to reach their targets. De Carlos et al. (1996) also described cells that detached from the olfactory epithelium and navigated along the course of olfactory fibers, which they identified as the OMP-ir neurons described by Valverde et al. (1993). Several lines of evidence suggest that the Pax6 migrating neurons observed in shark may have a role in the guidance of outgrowing olfactory axons. First, Pax6-ir cells in the migratory mass do not contact the epithelium as they were not labeled after application of neurobiotin in the olfactory epithelium, but rather they were apposed to axons that emerge from Pax6-positive cells anchored to the olfactory epithelium, what highly reminds the scenario observed by Conzelmann et al. (2002). The fact that Pax6 in the olfactory epithelium was specifically expressed by neurons committed to the ORN lineage strongly support this hypothesis. Studies with early markers of ORNs might provide direct evidence that Pax6-ir migrating cells within the shark migratory mass correspond to the ORN-expressing cells described by others. Second, the number of Pax6-ir cells decreases in the olfactory nerve coinciding with the maturation of the olfactory ensheathing glia, whose role in assistance to growing olfactory axons is largely accepted. The possibility that mature OECs could substitute Pax6-expressing cells in their guidance role as development proceeds, cannot be ruled out. In addition, the scarce number of Pax6-ir cells observed at the entrance of the olfactory bulb were found to form corridors between arriving olfactory axons, which further suggests a signaling role for these

neurons. Finally, many transcription factors have dual roles in both forebrain morphogenesis and development of axonal pathways (Pratt and Price 2006). Indeed, Pax6 seems to regulate genes implicated in axon guidance including *Sema5A* and *Sema3C* during development of various brain regions (Jones et al. 2002). There is also strong evidence that Pax6 regulates cell-cell adhesion during brain morphogenesis, which is probably important in mechanisms of axon guidance (Pratt and Price 2006). Nonetheless, further functional experiments will be needed to ascertain the precise role of Pax6-ir neurons along outgrowing axons of the shark olfactory pathway.

REFERENCES

Anchan RM, Drake DP, Haines CF, Gerwe EA, LaMantia AS (1997) Disruption of local retinoid-mediated gene expression accompanies abnormal development in the mammalian olfactory pathway. J Comp Neurol 379:171-184

Ballard WW, Mellinguer J, Lechenault H(1993) A series of normal stages for development of *Scyliorhinus canicula*, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). J Exp Zool 267:318-336

Balmer CW, LaMantia AS (2005) Noses and neurons: induction, morphogenesis, and neuronal differentiation in the peripheral olfactory pathway. Dev Dyn 234:464-481.

Barber PC, Lindsay RM (1982) Schwann cells of the olfactory nerves contain glial fibrillary acidic protein and resemble astrocytes. Neuroscience 7:3077-3090

Barraud P, Seferiadis AA, Tyson LD, Zwart MF, Szabo-Rogers HL, Ruhrberg C, Liu KJ, Baker CV (2010) Neural crest origin of olfactory ensheathing glia. Proc Nat Acad Sci U S A 107:21040-21045

Barreiro-Iglesias A, Villar-Cerviño V, Anadón R, Rodicio MC (2008)Descending brain-spinal cord projections in a primitive vertebrate, the lamprey: cerebrospinal fluid-contacting and dopaminergic neurons. J Comp Neurol 511:711-723

Behrens M, Venkatraman G, Gronostajski RM, Reed RR, Margolis FL(2000) NFI in the development of the olfactory neuroepithelium and the regulation of olfactory marker protein gene expression. Eur J Neurosci 12:1372-1384

Bhattacharyya S, Bronner-Fraser M (2008) Competence, specification and commitment to an olfactory placode fate. Development 135:4165-4177

Blanchart A, Martín-López E, De Carlos JA, López-Mascaraque L (2011) Peripheral contributions to olfactory bulb cell populations (migrations towards the olfactory bulb). Glia 59:278-292

Bystron I, Rakic P, Molnár Z, Blakemore C (2006)The first neurons of the human cerebral cortex. Nat Neurosci 9:1-7

Chuah MI, West AK (2002) Cellular and molecular biology of ensheathing cells. Microsc Res Tech 58:216-227

Conzelmann S, Levai O, Breer H, Strotmann J (2002) Extraepithelial cells expressing distinct olfactory receptors are associated with axons of sensory cells with the same receptor type Cell Tissue Res 307:293-301

Coolen M, Menuet A, Chassoux D, Compagnucci C, Henry S, Lévèque L, Da Silva C, Gavory F, Samain S, Wincker P, Thermes C, D'Aubenton-Carafa Y, Rodríguez-Moldes I, Naylor G, Depew M, Sourdaine P, Mazan S (2009) The dogfish *Scyliorhinus canicula*, a reference in jawed vertebrates. In: Behringer RR, Johnson AD, Krumlauf RE, editors. Emerging model organisms. A laboratory manual. Vol. 1. Cold Spring Harbor, NY: CSHL Press pp, 431-446

Corpet F (1988) Multiple sequence alignment with hierarchical clustering. Nucleic Acids Res 16:10881-10890

Davis JA, Reed RR (1996) Role of Olf-1 and Pax-6 transcription factors in neurodevelopment.J Neurosci 16:5082-5094

De Carlos JA, Lopez-Mascaraque L, Valverde F (1996) Early olfactory fiber projections and cell migration into the rat telencephalon. Int J Dev Neurosci 14:853-866

Doucette R (1984) The glial cells in the nerve fiber layer of the rat olfactory bulb. Anat Rec 210:385-391

Doucette R (1990) Glial influences on axonal growth in the primary olfactory system. Glia 3:433-449

Drapkin PT, Silverman AJ (1999) Development of the chick olfactory nerve. Dev Dyn 214:349-360

Dryer L, Graziadei PP (1993) A pilot study on morphological compartmentalization and heterogeneity in the elasmobranch olfactory bulb. Anat Embryol 188:41-51

Dryer L, Graziadei PP (1996) Synaptology of the olfactory bulb of an elasmobranch fish, *Sphyrna tiburo*. Anat Embryol 193:101-114

Ekström P, Johansson K (2003) Differentiation of ganglion cells and amacrine cellsin the rat retina: correlation with expression of HuC/D and GAP-43 proteins.Brain Res Dev Brain Res 145:1-8

Farbman AI, Squinto LM (1985) Early development of olfactory receptor cell axons. Brain Res 351:205-213

Ferrando S, Bottaro M, Gallus L, Girosi L, Vacchi M, Tagliafierro G (2006) Observations of crypt neuron-like cells in the olfactory epithelium of a cartilaginous fish. Neurosci Lett 403:280-282

Ferrando S, Bottaro M, Gallus L, Girosi L, Vacchi M, Tagliafierro G (2007a) First detection of olfactory marker protein (OMP) immunoreactivity in the olfactory epithelium of a cartilaginous fish. Neurosci Lett 413:173-176

Ferrando S, Bottaro M, Pedemonte F, De Lorenzo S, Gallus L, Tagliafierro G (2007b) Appearance of crypt neurons in the olfactory epithelium of the skate *Raja clavata* during development. Anat Rec 290:1268-1272

Ferrando S, Gambardella C, Ravera S, Bottero S, Ferrando T, Gallus L, Manno V, Salati AP, Ramoino P, Tagliafierro G. (2009) Immunolocalization of G-protein alpha subunits in the olfactory system of the cartilaginous fish *Scyliorhinus canicula*. Anat Rec 292:1771-1779

Ferrando S, Gallus L, Gambardella C, Ghigliotti L, Ravera S, Vallarino M, Vacchi M, Tagliafierro G (2010) Cell proliferation and apoptosis in the olfactory epithelium of the shark *Scyliorhinus canicula*. J Chem Neuroanat 40:293-300

Ferreiro-Galve S, Candal E, Rodriguez-Moldes I (2012a) Dynamic expression of Pax6 in the shark olfactory system: Evidence for the presence of Pax6 cells along the olfactory nerve pathway. J Exp Zool (Mol Dev Evol) 318:79-90

Ferreiro-Galve S, Rodríguez-Moldes I, Anadón R, Candal E (2010) Patterns of cell proliferation and rod photoreceptor differentiation in shark retinas. J Chem Neuroanat 39:1-14

Ferreiro-Galve S, Rodríguez-Moldes I, Candal E (2012b) Pax6 Expression during retinogenesis in sharks: Comparison with markers of cell proliferation and neuronal differentiation. J Exp Zool(Mol Dev Evol) 318:91-108

Filogamo G, Robecchi MG (1969) Neuroblasts in the olfactory pits in mammals. Acta Anat Suppl 56:182-187

Fishelson L, Baranes A (1997) Ontogenesis and cytomorphology of the nasal olfactory organs in the Oman shark, *Iago omanensis* (Triakidae), in the Gulf of Aqaba, Red Sea. Anat Rec 249:409-421

Fornaro M, Geuna S, Fasolo A, Giacobini-Robecchi MG (2001) Evidence of very early neuronal migration from the olfactory placode of the chick embryo. Neuroscience 107:191-197

Fornaro M, Geuna S, Fasolo A, Giacobini-Robecchi MG (2003) HuC/D confocal imaging points to olfactory migratory cells as the first cell population that expresses a post-mitotic neuronal phenotype in the chick embryo. Neuroscience 122:123-128

Forni PE, Taylor-Burds C, Melvin VS, Williams T, Wray S (2011) Neural crest and ectodermal cells intermix in the nasal placode to give rise to GnRH-1 neurons, sensory neurons, and olfactory ensheathing cells. J Neurosci 31:6915-6927

Franco MD, Pape MP, Swiergiel JJ, Burd GD (2001) Differential and overlapping expression patterns of X-dll3 and Pax-6 genes suggest distinct roles in olfactory system development of the African clawed frog *Xenopus laevis*. J Exp Biol 204:2049-2061

Franssen EHP, de Bree FM, Verhaagen J (2007) Olfactory ensheathing glia: Their contribution to primary olfactory nervous system regeneration and their regenerative potential following transplantation into the injured spinal cord. Brain Res Rev 56:236-258

García-González D, de Castro F (2011) How is the sense of smell connected? Cellular and molecular mechanisms guiding the development of the synaptic connections from the nose to the cortex (I). Rev Neurol 52:477-488

Gokoffski KK, Kawauchi S, Wu H, Santos R, Hollenbech PLW, Lander AD, Calof AL (2010) Feedback regulation of neurogenesis in the mammalian olfactory epithelium: new insights from genetics and systems biology. In: Menini A (ed): The neurobiology of olfaction. Boca Raton, FL, CRC Press, pp 241-265

Grindley JC, Davidson DR, Hill RE (1995) The role of Pax-6 in eye and nasal development. Development 121:1433-1442

Guo Z, Packard A, Krolewski RC, Harris MT, Manglapus GL, Schwob JE (2010) Expression of pax6 and sox2 in adult olfactory epithelium. J Comp Neurol 518:4395-4418

Haubst N, Berger J, Radjendirane V, Graw J, Favor J, Saunders GF, Stoykova A, Götz M (2004) Molecular dissection of Pax6 function: the specific roles of the paired domain and homeodomain in brain development. Development 131:6131-6140

Higginson JR, Barnett SC (2011) The culture of olfactory ensheathing cells (OECs)—a distinct glial cell type. Exp Neurol 229:2-9

Hogan BL, Horsburgh G, Cohen J, Hetherington CM, Fisher G, Lyon MF (1986) Small eyes (Sey): a homozygous lethal mutation on chromosome 2 which affects the differentiation of both lens and nasal placodes in the mouse. J Embryol Exp Morphol 97:95-110

Hsieh Y-W, Yang X-J (2009) Dynamic Pax6 expression during the neurogenic cell cycle influences proliferation and cell fate choices of retinal progenitors. Neural Dev 4:32

Huang Q, Zhao S, Gaudin A, Quennedey B, Gascuel J (2005) Glial fibrillary acidic protein and vimentin expression in the frog olfactory system during metamorphosis. Neuroreport 16:1439-1442

Ikeda K, Ookawara S, Sato S, Ando Z-ichi, Kageyama R, Kawakami K (2007) Six1 is essential for early neurogenesis in the development of olfactory epithelium. Dev Biol 311:53-68

Jiménez D, García C, de Castro F, Chedotal A, Sotelo C, de Carlos JA, Valverde F, López-Mascaraque L (2000) Evidence for intrinsic development of olfactory structures in Pax-6 mutant mice. J Comp Neurol 428:511-526

Jones L, López-Bendito G, Gruss P, Stoykova A, Molnár Z (2002) Pax6 is required for the normal development of the forebrain axonal connections. Development 129:5041-5052

Katoh H, Shibata S, Fukuda K, Sato M, Satoh E, Nagoshi N, Minematsu T, Matsuzaki Y, Akazawa C, Toyama Y, Nakamura M, Okan H (2011) The dual

origin of the peripheral olfactory system: placode and neural crest. Mol Brain 4:34

Kohwi M, Osumi N, Rubenstein JLR, Alvarez-Buylla A (2005) Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. J Neurosci 25:6997-7003

Lazzari M, Bettini S, Franceschini V (2013) Immunocytochemical characterization of olfactory ensheathing cells in fish. Brain Struct Funct 218:539-49

Maier E, Gunhaga L (2009) Dynamic expression of neurogenic markers in the developing chick olfactory epithelium. Dev Dyn 238:1617-1625

Maier E, Nord H, Von Hofsten J, Gunhaga L (2011) A balance of BMP and Notch activity regulates neurogenesis and olfactory nerve formation. PLoS One 6:13

Marquardt T, Ashery-Padan R, Andrejewski N, Scardigli R, Guillemot F, Gruss P (2001) Pax6 is required for the multipotent state of retinal progenitor cells. Cell 105:43-55

Marusich MF, Furneaux HM, Henion PD, Weston JA (1994) Hu neuronal proteins areexpressed in proliferating neurogenic cells. J Neurobiol 25:143-155

Mendoza AS, Breipohl W, Miragall F (1982) Cell migration from the chick olfactory placode: a light and electron microscopic study. J Embryol Exp Morphol 69:47-59

Miller AM, Treloar HB, Greer CA (2010) Composition of the migratory mass during development of the olfactory nerve. J Comp Neurol 518:4825-4841

Mori K (1993) Molecular and cellular properties of mammalian primary olfactory axons. Microsc Res Tech 24:131-141

Morona R, Moreno N, López JM, Muñoz M, Ten Donkelaar HJ, González A (2005) Calbindin-D28k immunoreactivity in the spinal cord of *Xenopus laevis* and its participation in ascending and descending projections. Brain Res Bull 66:550-554

Murdoch B, Roskams AJ (2007) Olfactory epithelium progenitors: insights from transgenic mice and in vitro biology. J Mol Histol 38:581-599

Nomura T, Haba H, Osumi N (2007) Role of a transcription factor Pax6 in the developing vertebrate olfactory system. Dev Growth Differ 49:683-690

Norgren RB Jr, Ratner N, Brackenbury R (1992) Development of olfactory nerve glia defined by a monoclonal antibody specific for Schwann cells. Dev Dyn 194:231-238

Oron-Karni V, Farhy C, Elgart M, Marquardt T, Remizova L, Yaron O, Xie Q, Cvekl A, Ashery-Padan R (2008) Dual requirement for Pax6 in retinal progenitor cells. Development 135:4037-4047

Osumi N, Shinohara H, Numayama-Tsuruta K, Maekawa M (2008) Concise review: Pax6 transcription factor contributes to both embryonic and adult neurogenesis as a multifunctional regulator. Stem Cells 26:1663-1672

O'Neill P, McCole RB, Baker CV (2007) A molecular analysis of neurogenic placode and cranial sensory ganglion development in the shark, *Scyliorhinus canicula*. Dev Biol 304:156-181

Pellier V, Astic L (1994) Histochemical and immunocytochemical study of the migration of neurons from the rat olfactory placode. Cell Tissue Res 275:587-598

Pellier V, Astic L, Oestreicher AB, Saucier D (1994) B-50/GAP-43 expression by the olfactory receptor cells and the neurons migrating from the olfactory placode in embryonic rats. Brain Res Dev Brain Res 80:63-72

Pellitteri R, Spatuzza M, Stanzani S, Zaccheo D (2010) Biomarkers expression in rat olfactory ensheathing cells. Front Biosci 2:289-298

Philips GT, Stair CN, Young Lee H, Wroblewski E, Berberoglu MA, Brown NL, Mastick GS (2005) Precocious retinal neurons: Pax6 controls timing of differentiation and determination of cell type. Dev Biol 279:308-321

Puche AC, Baker H (2007) Olfactory cell derivation and migration. J Mol Histol 38:513-515

Ren X, Chang S, Laframboise A, Green W, Dubuc R, Zielinski B (2009) Projections from the accessory olfactory organ into the medial region of the olfactory bulb in the sea lamprey (*Petromyzon marinus*): a novel vertebrate sensory structure?. J Comp Neurol 516:105-116

Rodríguez-Moldes I, Ferreiro-Galve S, Carrera I, Sueiro C, Candal E, Mazan S, Anadón R (2008) Development of the cerebellar body in sharks: spatiotemporal relations of Pax6 expression, cell proliferation and differentiation. Neurosci Lett 432:105-110

Roppolo D, Ribaud V, Jungo VP, Lüscher C, Rodriguez I (2006) Projection of the Grüneberg ganglion to the mouse olfactory bulb. Eur J Neurosci 23:2887-2894

Sansom SN, Griffiths DS, Faedo A, Kleinjan D-J, Ruan Y, Smith J, Van Heyningen V, Rubenstein JL, Livesey FJ (2009) The level of the transcription factor Pax6 is essential for controlling the balance between neural stem cell self-renewal and neurogenesis. PLoS Genet 5:16

Sauka-Spengler T, Baratte B, Shi L, Mazan S (2001) Structure and expression of an Otx5-related gene in the dogfish *Scyliorhinus canicula*: evidence for a conserved role of Otx5 and Crxgenes in the specification of photoreceptors. Dev Genes Evol 211:533-544

Schluessel V, Bennett MB, Bleckmann H, Blomberg S, Collin SP (2008) Morphometric and ultrastructural comparison of the olfactory system in elasmobranchs: the significance of structure-function relationships based on phylogeny and ecology. J Morphol 269:1365-1386

Schwanzel-Fukuda M (1999) Origin and migration of luteinizing hormone-releasing hormone neurons in mammals. Microsc Res Tech 44:2-10

Schwanzel-Fukuda M, Pfaff DW (1989) Origin of luteinizing hormone-releasing hormone neurons. Nature 338:161-164

Simpson TI, Price DJ (2002) Pax6; a pleiotropic player in development. Bioessays 24:1041-1051

Smithson LJ, Kawaja MD (2009) A comparative examination of biomarkers for olfactory ensheathing cells in cats and guinea pigs. Brain Res 1284:41-53

Soukkarieh C, Agius E, Soula C, Cochard P (2007) Pax2 regulates neuronal-glial cell fate choice in the embryonic optic nerve. Dev Biol 303:800-813

Sueiro C, Carrera I, Ferreiro S, Molist P, Adrio F, Anadón R, Rodríguez-Moldes I. (2007) New insights on saccus vasculosus evolution: a developmental and immunohistochemical study in elasmobranchs. Brain Behav Evol 70:187-204

Sueiro C, Carrera I, Molist P, Rodríguez-Moldes I, Anadón R (2004) Distribution and development of glutamic acid decarboxylase immunoreactivity in the spinal cord ofthe dogfish *Scyliorhinus canicula* (elasmobranchs). J Comp Neurol 478:189-206

Takami S, Luer CA, Graziadei PP (1994) Microscopic structure of the olfactory organ of the clearnose skate, *Raja eglanteria*. Anat Embryol 190:211-230

Theisen B, Zeiske E, Breucker H (1986) Functional morphology of the olfactory organs in the spiny dogfish (*Squalus acanthias* L.) and the small-spotted catshark (*Scyliorhinus canicula* (L.). Acta Zool 67:73-86

Tornehave D, Hougaard DM, Larsson L (2000) Microwaving for double indirect immunofluorescence with primary antibodies from the same species and for staining of mouse tissues with mouse monoclonal antibodies. Histochem Cell Biol 113:19-23

Treloar HB, Ray A, Dinglasan LA, Schachner M, Greer CA (2009) Tenascin-C is an inhibitory boundary molecule in the developing olfactory bulb. J Neurosci 29:9405-9416

Valverde F, Heredia M, Santacana M (1993) Characterization of neuronal cell varieties migrating from the olfactory epithelium during prenatal development in the rat. Immunocytochemical study using antibodies against olfactory marker protein (OMP) and luteinizing hormone-releasing hormone (LH-RH). Dev Brain Res 71:209-220

Valverde F, Santacana M, Heredia M (1992) Formation of an olfactory glomerulus: morphological aspects of development and organization. Neuroscience 49:255-275

Vellema M, van der Linden A, Gahr M (2010) Area-specific migration and recruitmentof new neurons in the adult songbird brain. J Comp Neurol 518:1442-1459

Wasowicz M, Ward R, Repérant J (1999) An investigation of astroglial morphology in *Torpedo*and *Scyliorhinus*. J Neurocytol 28:639-653

Whitlock KE (2004) Development of the nervus terminalis: origin and migration. Microsc Res Tech 65:2-12

Whitlock KE, Westerfield M (1998) A transient population of neurons pioneers the olfactory pathway in the zebrafish. J Neurosci 18:8919-8927

Wray S, Nieburgs A, Elkabes S (1989) Spatiotemporal cell expression of luteinizing hormone-releasing hormone in the prenatal mouse: evidence for an embryonic origin in the olfactory placode. Brain Res Dev Brain Res 46:309-318

Yanicostas C, Herbomel E, Dipietromaria A, Soussi-Yanicostas N (2009) Anosmin-1a is required for fasciculation and terminal targeting of olfactory sensory neuron axons in the zebrafish olfactory system. Mol Cell Endocrinol 312:53-60

ABBREVIATIONS

iORN	immature olfactory receptor neuron
mORN	mature olfactory receptor neuron
Mes	mesenchyme
OB	olfactory bulb
OE	olfactory epithelium
ON	olfactory nerve
OP	olfactory placode
OPit	olfactory pit
ORN	olfactory receptor neuron
pOB	primordial olfactory bulb
pOE	primordial olfactory epithelium
protoglom	protoglomerular fields
st	stage
Tel	telencephalon
TN	terminal nerve
v	telencephalic ventricle

Table 1 Primary and Secondary antibodies

Primary antibody	Source	Working dilution	Secondary antibody	Source	Working dilution
HuC/D	Monoclonal mouse anti-HuC/D Molecular Probes, Eugene, OR Catalog number: A-21271	1:100	546-conjugated donkey anti-rabbit (DAR^{546})	Molecular probes Catalog number: A10040	1:100
Pax6	Polyclonal rabbit anti-Pax6 Covance, Emeryville, CA Catalog number: PRB-278P	1:200	546-conjugated donkey anti-mouse (DAM^{546})	Molecular probes Catalog number: A10036	1:200
Pax6	Polyclonal goat anti-Pax6 Novus Biologicals, Littleton, CO Catalog number: NB100-2913	1:100	488-conjugated donkey anti-rabbit (DAR^{488})	Molecular probes Catalog number: A21206	1:100
DCX	Polyclonal rabbit anti-DCX Cell Signaling Technology, Beverly, MA. Catalog number: 4604	1:300	488-conjugated donkey anti-mouse (DAM^{488})	Molecular probes Catalog number: A21202	1:100
DCX	Polyclonal goat anti-DCX Santa Cruz Biotechnology, Santa Cruz, CA Catalog number: sc-8066	1:100	488-conjugated donkey anti-goat (DAG^{488})	Molecular probes Catalog number: A11055	1:100
GFAP	Polyclonal rabbit anti-GFAP Dako, Glostrup, Denmark Catalog number: Z 0334	1:500	633-conjugated donkey anti-goat (DAG^{633})	Molecular probes Catalog number: A21082	1:150
PCNA	Monoclonal mouse anti-PCNA Sigma, St. Louis, MO Catalog number: P8825	1:300	633-conjutated donkey anti-mouse (DAM^{647})	Molecular probes Catalog number: A31571	1:100
$G\alpha_0$	Polyclonal rabbit anti-$G\alpha_0$ Santa Cruz Biotechnology, Santa Cruz, CA Catalog number: sc-387	1:400			

Table 2 Key events during the development of the peripheral components of *S.canicula* olfactory system.

	FIRST PERIOD St20-St24 (Pioneer period)	**SECOND PERIOD St25-St30 (Migratory period)**	**THIRD PERIOD St31-St34/prehatching (Maturation period)**
OE	Formation of the OP First neuronal precursors (establishment of neurogenic region) Indentation of the OP Formation of the OPit	Invagination of the OPit Expansion of the neurogenic region (St29) First folds (primary *lamellae*)	Stratification Secondary *lamellae* Mature morphology (St32)
ON	Pioneering axons and neurons	Massive neuronal migrations along ON First evidence of glia ensheathing ON(St29)	Increase in thickness of nerve fascicles Gradual disappearance of migrating neurons

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Fig.1 Schematic drawings representing the main key events of the development of peripheral components of the lesser spotted dogfish olfactory system. Dots represent immature postmitotic neurons (and respective projections represented as grey lines). Thick black lines in b and c represent glial processes surrounding the olfactory nerve. For abbreviations, see list. Scale bars: 100 µm (**A**); 150µm (**B**); 250 µm (**C**)

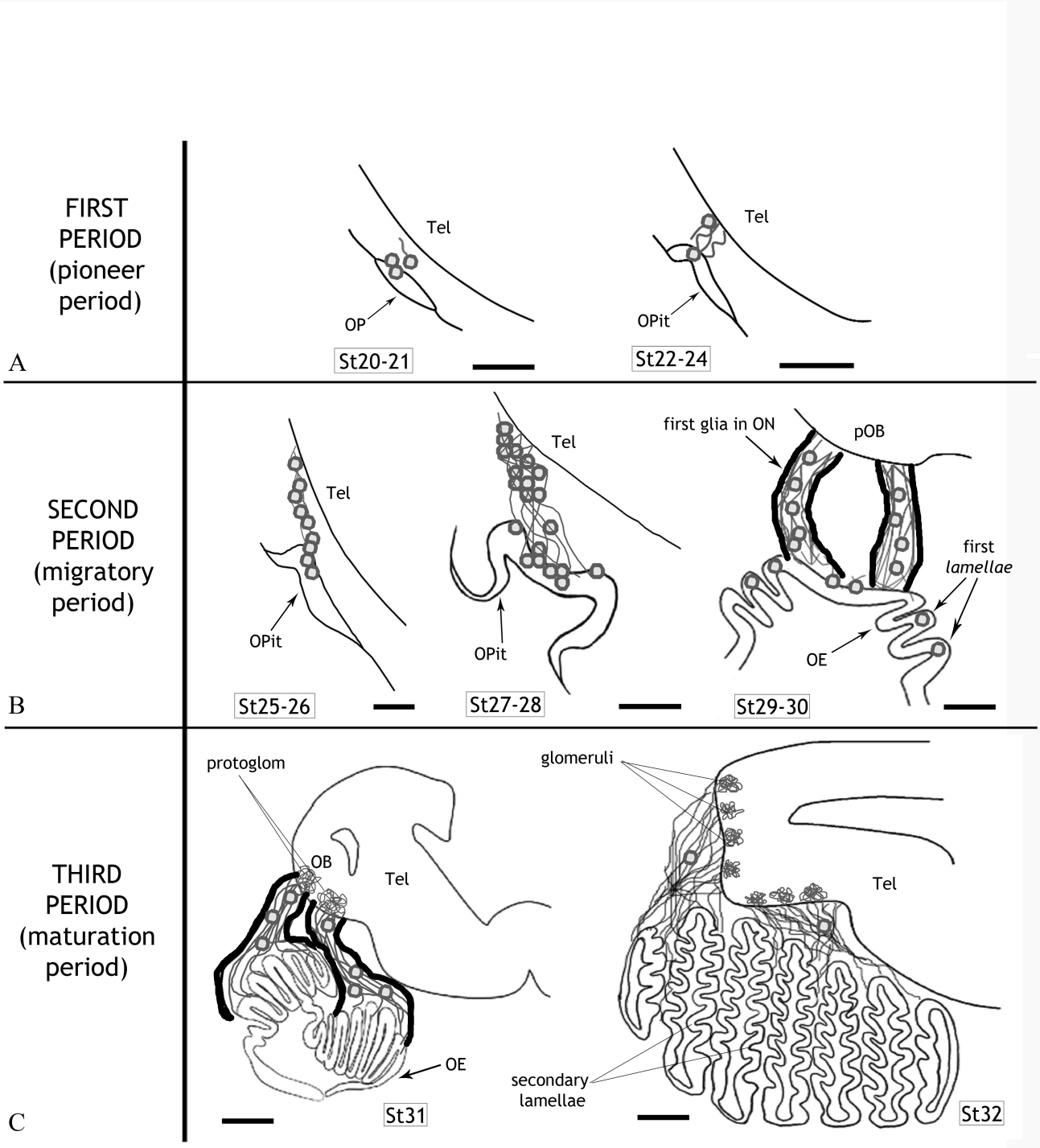

FIRST
PERIOD
(pioneer
period)
A
Tel
OP
St20-21
Tel
OPit
St22-24
SECOND
PERIOD
(migratory
period)
B
Tel
OPit
St25-26
Tel
OPit
St27-28
first glia in ON
pOB
first
lamellae
OE
St29-30
THIRD
PERIOD
(maturation
period)
C
protoglom
OB
Tel
OE
St31
glomeruli
Tel
secondary
lamellae
St32

Figure1

Fig.2 Sagittal sections of embryos at stages 21 and 24 (first period, A,B) and 25, 26, 28 and 29 (second period, C-L) showing the early development of the olfactory placode, olfactory pit and olfactory nerve. **A-A'':** Detail of the olfactory placode with immature postmitotic HuC/D-ir neurons (arrowhead), some of them delaminated from the olfactory placode and extending short DCX-ir processes across the mesenchyma (arrow). **B:** Lateral section of the olfactory pit of a stage-24 embryo, where most cells showed PCNA immunoreactivity. Note also DCX-ir fibers emerging from cells located within the epithelium (dotted line) and extending to the telencephalon (arrowhead), probably establishing their first contacts. **C-C'':** Section of the olfactory pit to show the neurogenic region defined by its HuC/D expression. Some double HuC/D- and DCX-ir cells (arrowheads) were observed associated to DCX-ir fibers from neurons anchored to the epithelium (red arrow). **D-F:** Sequence of consecutive sections showing a string of HuC/D-ir cells extending across the mesenchyme between the olfactory pit and the telencephalon. **G-I:** Adjacent sections of the olfactory epithelium of a stage-28 embryo to show the increased number of DCX-ir fibers (G,H) and HuC/D-irneurons (I) along the developing olfactory nerve with respect to previous stages. Asterisk in I shows the basal position of HuC/D-ir postmitotic immature neurons within the olfactory epithelium. **J-L**: Sections of the olfactory epithelium and olfactory nerve of stage-29embryos to show the epithelium organized in primary lamellae and the medial to lateral expansion of the neurogenic region within the olfactory epithelium (in J, arrows indicate the direction of the expansion); a cluster of HuC/D-ir cells at the level of the olfactory nerve–olfactory bulb junction that branched off from the olfactory pathway (arrowhead) towards more anterior telencephalic levels (K); and GFAP-ir glial processes that surrounded the DCX-ir fibers of the olfactory nerve and strings of DCX-ir somata identified asimmature migrating neurons (L). The inset in L is a detail of a single confocal section (1µm thick) to show that there was no colocalization between both markers. For abbreviations, see list. Scale bars: 50µm (**A**); 100µm (**B,C,K,L**); 150µm (**D-J**)

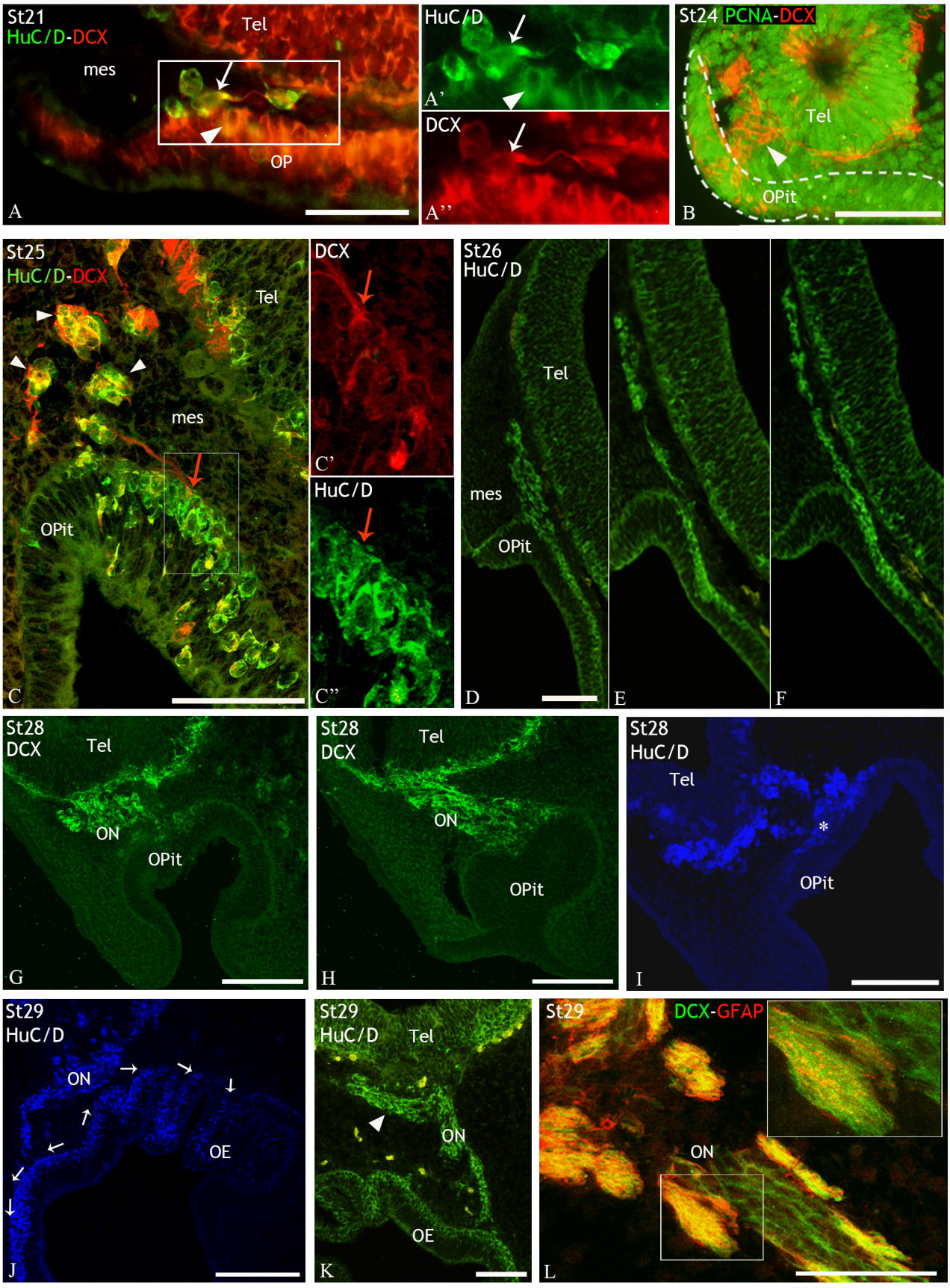

St21
HuC/D-DCX
Tel
mes
OP
A
HuC/D
A'
DCX
A''
St24 PCNA-DCX
Tel
OPit
B
St25
HuC/D-DCX
Tel
mes
OPit
C
DCX
C'
HuC/D
C''
St26
HuC/D
Tel
mes
OPit
D
E
F
St28
DCX
Tel
ON
OPit
G
St28
DCX
Tel
ON
OPit
H
St28
HuC/D
Tel
*
OPit
I
St29
HuC/D
ON
OE
J
St29
HuC/D
Tel
ON
OE
K
St29
DCX-GFAP
ON
L

Figure 2

Fig.3 Sections of embryos at stages 31-34 (third period, A-L), and of juveniles (M-O) to show the late development of the olfactory epithelium and olfactory nerve, and the early development of the olfactory bulb. **A,B:** Details of lamellae of the olfactory epithelium at stage31 to show the scattered distribution of HuC/D-ir (A) and DCX-ir (B) neurons. Note in B, fascicles of DCX-ir fibers exiting the olfactory epithelium. **C,D:** Double DCX/GFAP immunofluorescence in sections across the olfactory nerve at stage 31 to show the ensheathing GFAP-ir glial fibers around DCX-ir migrating neurons and fibers. Bisbenzimide counterstaining of cell nuclei is showed in c. Insets in c and d are details to show the absence of colocalization between DCX and GFAP. **E,F:** Transverse sections of the telencephalon at stage 31 to show the first evidence of protoglomerular fields, revealed by $G\alpha_0$ immunohistochemistry (E) and tract-tracing experiments (F). Note the absence of HuC/D-ir neurons within protoglomerular fields and the presence of $G\alpha_0$ immunoreactive and anterogradely labeled fibers, respectively. **G-I:** Sections through olfactory lamellae at stage 32 to show the stratification of cells in the epithelium. Note that HuC/D-ir and DCX-ir perikarya were absent from apical layer (G,I) and that some DCX-ir cells (arrows in H) presented a bipolar morphology with a dendrite directed towards the apical surface of the epithelium, indicated with asterisk. The inset in I is a detail to show that the dendrites of most neurons contain the ORN marker $G\alpha_0$ (arrows). **J-L:** Transverse sections to comparatively show the decreasing density of HuC/D-ir neurons (white arrows) along olfactory nerves as the development of stage-32 embryos proceeds. K is a detail of the area squared in J. The primary olfactory projection was revealed by its immunoreactivity to the $G\alpha_0$ protein. **M:** Transverse sections of the olfactory epithelium of a prehatching embryo showing cells immunoreactive to the protein $G\alpha_0$ (ORNs) that were retrogradely labeled after application of neurobiotin in the olfactory bulb (arrows). **N,O:** Transverse sections through the olfactory epithelium of juveniles showing the intense $G\alpha_0$ immunolabeling of mature ORNs (small arrows), especially in the apical dendrite (arrowhead in N,O,O'). Arrowhead in O'' points to the DCX immunonegative dendrite of a mature ORN (intensely $G\alpha_0$-ir). Large arrows in O-O'' indicate a maturing ORN that shows weak levels of both DCX and $G\alpha_0$ protein. Asterisks in A-C, G-I, and m indicate the apical surface of the olfactory epithelium. For abbreviations, see list. Scale bars: 75μm (**A**); 50μm (**B,G**); 300μm (**C**); 150μm (**D**); 200μm (**E,F,J,K,L**); 25μm (**H,M,O**); 100μm (**I**); 10μm (**N**)

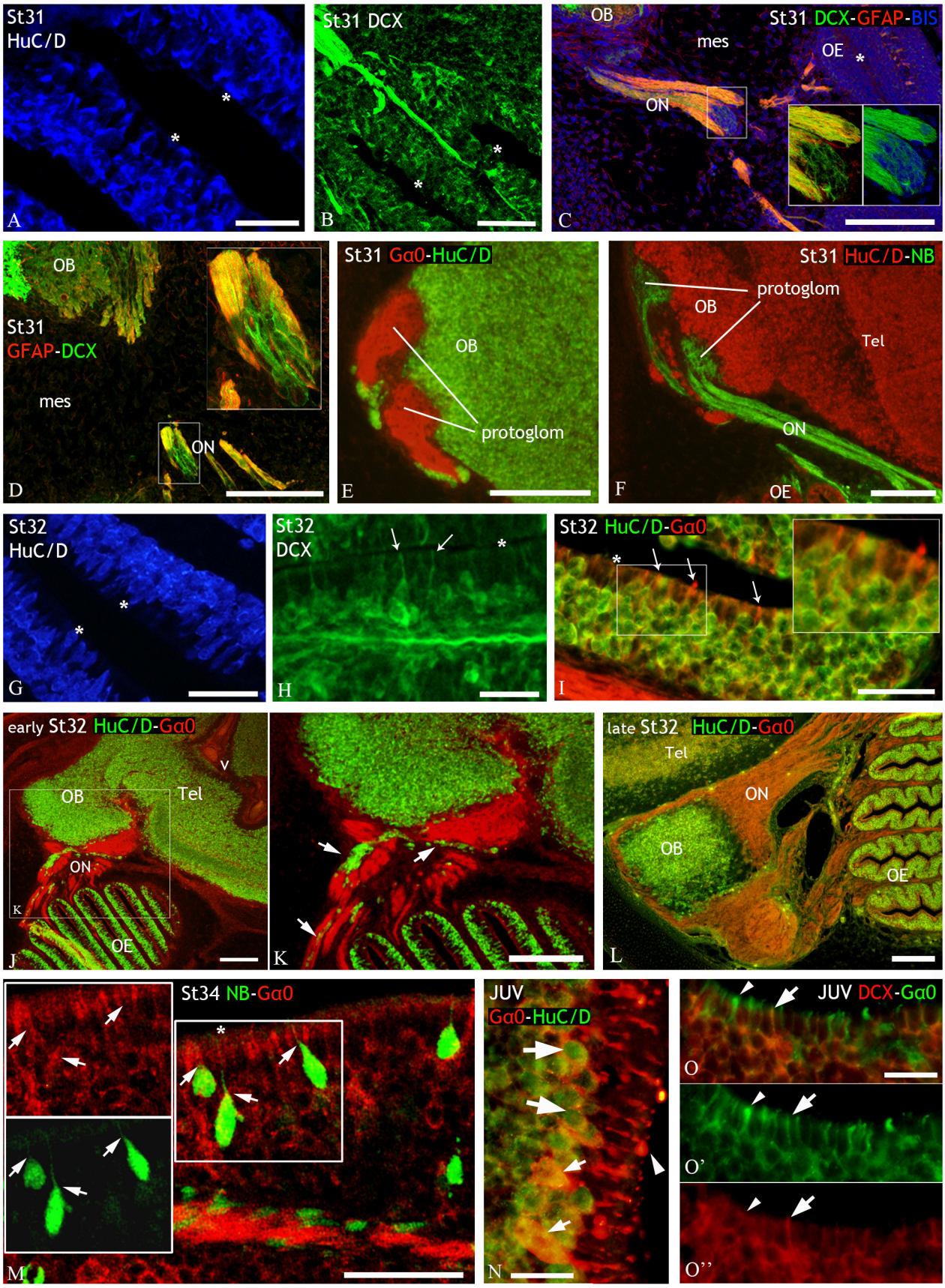

St31
HuC/D
A
St31 DCX
B
OB
mes
St31 DCX-GFAP-BIS
OE
ON
C
St31
GFAP-DCX
OB
mes
ON
D
St31 Gα0-HuC/D
OB
protoglom
E
St31 HuC/D-NB
protoglom
OB
Tel
ON
OE
F
St32
HuC/D
G
St32
DCX
H
St32 HuC/D-Gα0
I
early St32 HuC/D-Gα0
V
OB
Tel
ON
K
OE
J
K
late St32 HuC/D-Gα0
Tel
ON
OB
OE
L
St34 NB-Gα0
M
JUV
Gα0-HuC/D
N
JUV DCX-Gα0
O
O'
O''

Figure 3

Fig.4 Pax6 expression in relation to the components of the olfactory system during development**.** Sagittal sections through the head of embryos at stages 21 (first period, A), 25-30 (second period, B-J) and 31 (third period) (K-M). **A:** In stage-21 embryos, Pax6 expression was observed throughout the olfactory placode, being faint in the neurogenic region from which HuC/D-ir pioneer cells delaminated. **B:** Section of the olfactory pit at stage 25 to show high levels of Pax6 expression in a subset of HuC/D-ir neurons in the neurogenic region (arrows) and the mesenchyme (arrowheads). This section is parallel to that of Figure 2C. **C,D:** Adjacent sections of the olfactory epithelium of a stage-28 embryo to show high numbers of Pax6-ir cells neighboring DCX-ir fibers that invaded the mesenchyme. **E,F**: Adjacent sections of a stage-28 embryo after application of neurobiotin into the olfactory epithelium, to show anterogradely-labeled cells (arrowheads) and their outgrowing axons (open arrowheads). Inset in E: Detail to show that neurobiotin-labeled fibers were DCX immunoreactive. The inset in F shows that some of these neurobiotin-labeled cells (maturing ORNs) were Pax6-ir (arrowhead). Note in f Pax6/HuC/D-ir cells (immature neurons) along unlabeled olfactory axons (arrow). **G:** Section of the olfactory epithelium of a stage-29 embryo to show the increased number of Pax6-ir cells within the olfactory epithelium and some Pax6-ir cells in apposition to the DCX-ir olfactory fibers. **H:** Detail of the olfactory nerve of a stage-29 embryo double labeled for GFAP and Pax6 to show that there were no colocalization between these markers. **I:** Section across the olfactory nerve-olfactory bulb junction at stage 29 (compare with Figure 2K). Pax6 (HuC/D-ir) neurons seemed to accumulate at this point as they never were detected along the terminal primordium. **J:** Section of the head showing the string of terminal HuC/D-ir cells apposed to the ventral part of the telencephalic hemisphere. Inset: detail of the squared area to show Pax6 immunonegativity of primordial terminal ganglion cells. **K:** Section of a stage-31 embryo to show the increased number of Pax6-ir cells within the olfactory epithelium and the scarce number of Pax6-ir cells within the olfactory nerve, identified by the DCX immunoreactivity, as detailed in the inset. **L:** Section of the olfactory bulb of a stage-31 embryo to show that Pax6-ir cells formed corridors along the entrance of bundles of neurobiotin-labeled olfactory axons in the olfactory bulb (arrows). **M:** Section of the olfactory bulb of a stage-31 embryo to show apoptotic (TUNEL positive) cells at the entrance of the olfactory bulb, which mirrored the position of Pax6 cells at the same stage. Scale bars: 100μm (**A,I,M**); 150μm (**C,D,K,L**); 50μm (**B,H**); 75μm (**E-G,J**)

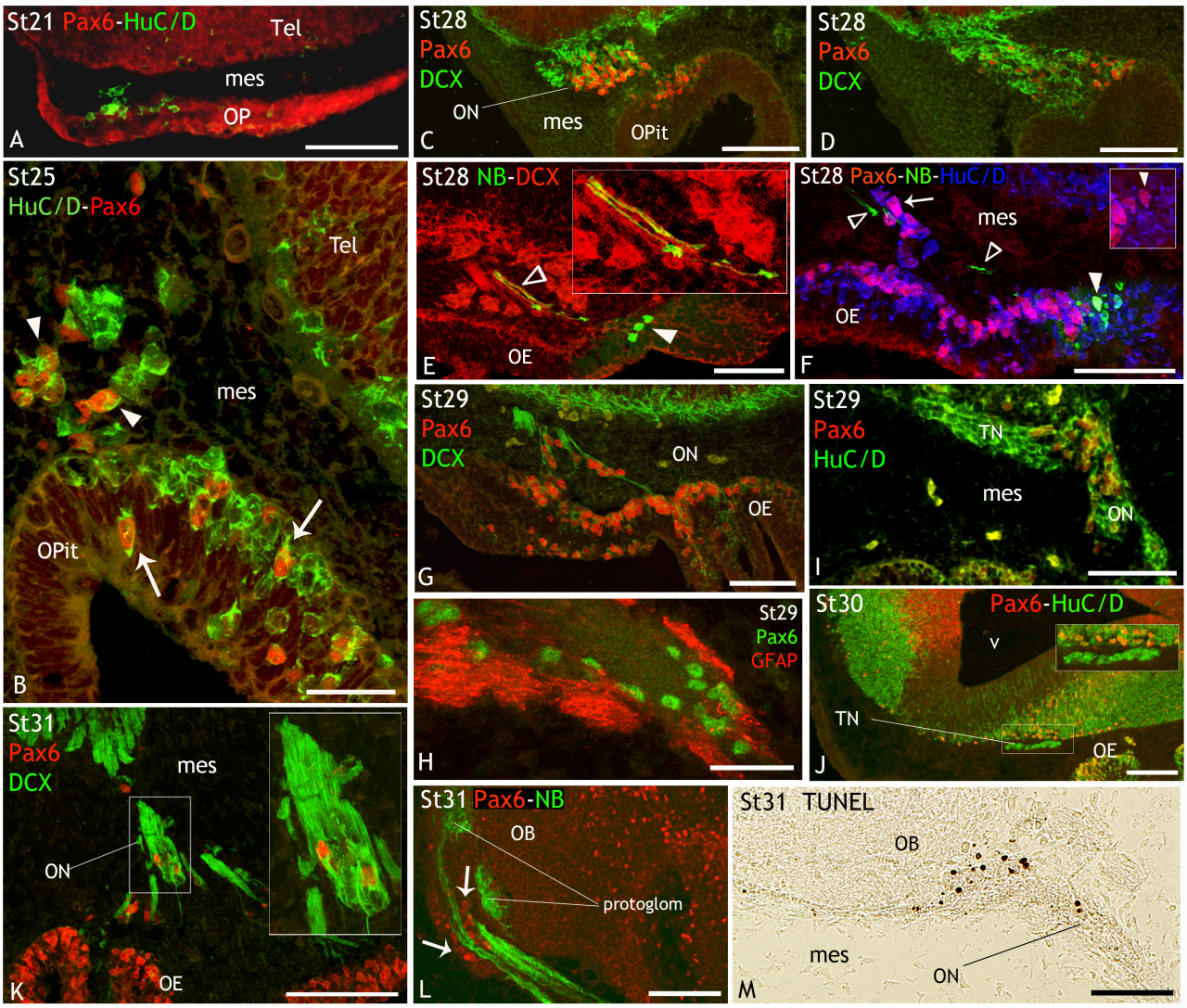

St21 Pax6-HuC/D
Tel
mes
OP
A
St28
Pax6
DCX
ON
mes
OPit
C
St28
Pax6
DCX
D
St25
HuC/D-Pax6
Tel
mes
OPit
B
St28 NB-DCX
OE
E
St28 Pax6-NB-HuC/D
mes
OE
F
St29
Pax6
DCX
ON
OE
G
St29
Pax6
HuC/D
TN
mes
ON
I
St29
Pax6
GFAP
H
St30
Pax6-HuC/D
v
TN
OE
J
St31
Pax6
DCX
mes
ON
OE
K
St31 Pax6-NB
OB
protoglom
L
St31 TUNEL
OB
mes
ON
M

Figure 4

Fig.5 Pax6 expression during late third period (stages 32 to 34) (A,B,D,E) and juvenile (C). **A-C:** Sections of the olfactory epithelium to show the changes in distribution of Pax6-ir cells as the epithelium became stratified. Note that Pax6 cells progressively adopted a more basal position as development proceeded (compare A and B). The inset in a shows that Pax6 immunoreactivity was absent from retrogradely labeled cells (mature ORNs) after application of neurobiotin to the olfactory bulb. Mature ORNs were also identified by its intense $G\alpha_0$ immunoreactivity (large arrows in B; note also the absence of Pax6 expression in these cells). Arrowheads in B and C indicate cells at intermediate olfactory epithelium levels with weak immunoreactivity to Pax6 and $G\alpha_0$, probably representing early maturing ORNs. Open arrows in C point to basal cells with weak immunoreactivity to Pax6 and PCNA, probably early postmitotic cells; short arrows in C indicate PCNA-negative basal cells with intense immunoreactivity to Pax6 and DCX, possible immature/differentiating ORNs. **D,E:** Sections across olfactory axons to show the scarcity of Pax6-ir cells along the olfactory nerve during stage 32 (D), and their absence in stage 33 (E). Asterisks indicate the apical surface of the olfactory epithelium. see list for abbreviations. Scale bars: 100μm (**A**); 75μm (**B**); 40μm (**C**); 300μm (**D,E**)

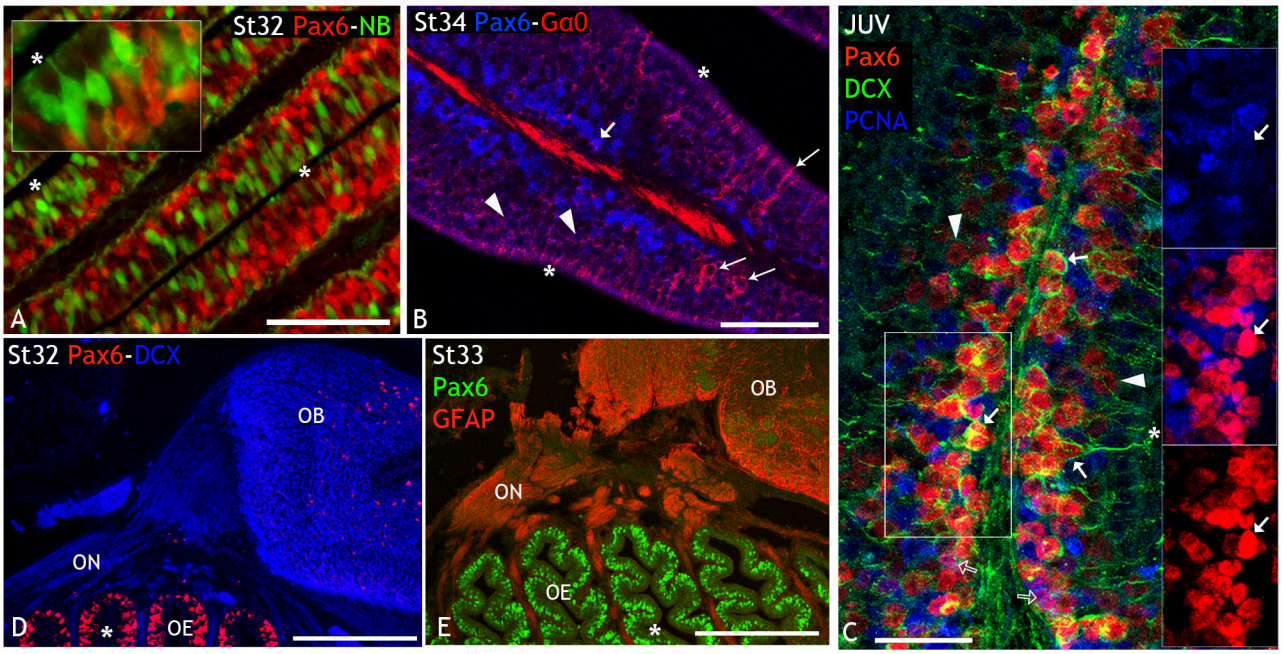

St32 Pax6-NB
A
St34 Pax6-Gα0
B
JUV
Pax6
DCX
PCNA
C
St32 Pax6-DCX
OB
ON
OE
D
St33
Pax6
GFAP
OB
ON
OE
E

Figure 5

Fig.6 Sequence of markers expressed during the maturation of the ORN lineage. A model relating the sequence of maturation of the ORN lineage with the changes observed along the development in the intensity of labeling of the markers used.

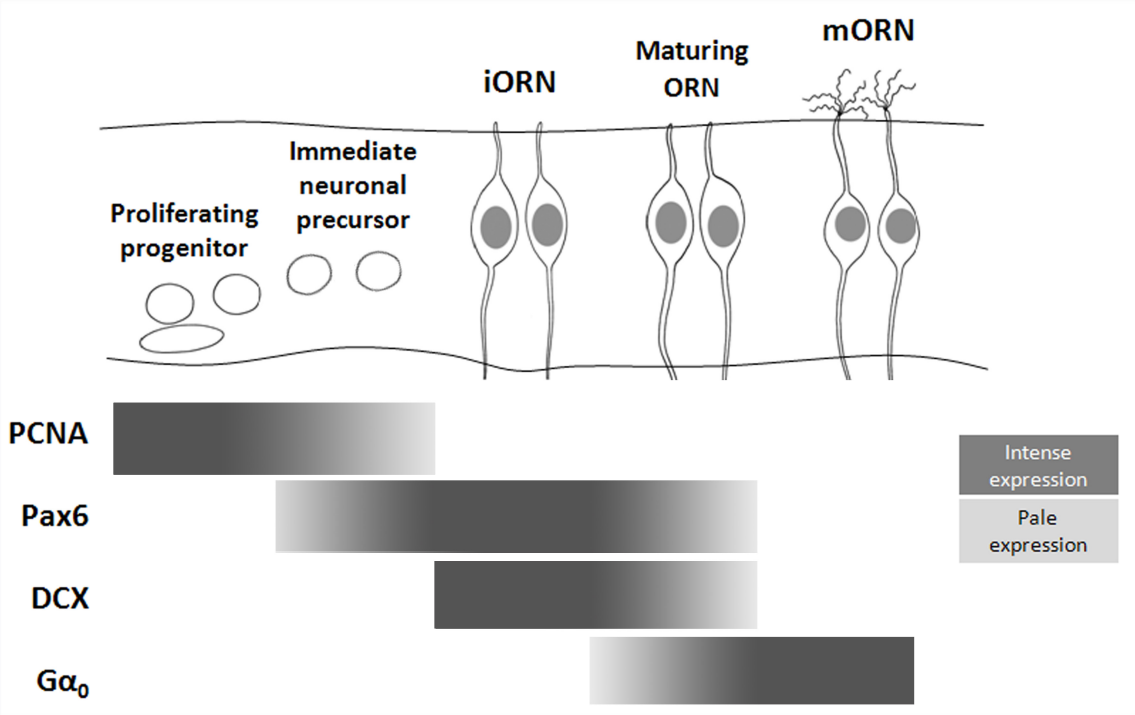

mORN
Maturing ORN
iORN
Immediate neuronal precursor
Proliferating progenitor
PCNA
Pax6
DCX
Gα0
Intense expression
Pale expression

Figure 6

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Chapter 2

Development of the terminal nerve system

Quintana-Urzainqui, Candal E, Rodríguez-Moldes I. Development of the terminal nerve system in *Scyliorhinus canicula* (on preparation to be submitted to Brain Behavior and Evolution).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

The terminal nerve, or cranial nerve zero, was first described in an elasmobranch species (*Galeorhinus galeus*) by Fritsch (1878) as a "supernumerary nerve" belonging to the olfactory system. In 1899, Locy described it in *Squalus acanthias* as the "accessory olfactory nerve" separated from the olfactory system. In 1905, this author carried out a study in 27 species of elasmobranchs and coined the term "*Nervus Terminalis*" after observing that this nerve consistently entered to the brain through the *lamina terminalis*. Throughout the twentieth century, the terminal nerve was progressively documented in most vertebrate groups from lampreys to mammals (reviewed in Von Bartheld 2004), although its presence in lampreys and birds has been a matter of debate (see Butler and Hodos 2005).

The exact function of the terminal nerve has not been completely elucidated so far, though it has been mainly associated to reproductive physiology and behavior (Demski 1989; Oka 1992, Yamamoto et al. 1997). Its developmental origin is also under discussion and the olfactory placode (Murakami et al. 1992; Northcutt and Muske 1994) or, alternatively, the neural crest (Von Bartheld and Baker 2004; Whitlock 2004; Forni et al. 2011; Katoh et al. 2011) have been proposed as its embryonic sources. In most vertebrates studied hitherto, neurons of the terminal nerve system migrate out of its peripheral origin, i.e. the olfactory placode or the neural crest, and enter the brain along a migration route associated to olfactory fibers (Schwanzel-Fukuda and Pfaff 1989; Tobet et al.

1997; Drapkin and Silverman 1999; Schwanzel-Fukuda 1999). These terminal neurons form part of the heterogeneous group of migrating cells along the olfactory pathway called the "migratory mass" (Wray et al. 1989; Whitlock 2004).

Markers as gonadotropin-releasing hormone (GnRH) or luteinizing hormone-releasing hormone (LHRH) have been traditionally used to identify terminal nerve components (Schwanzel-Fukuda and Pfaff 1989; Wray et al. 1989; Mulrenin et al. 1999; Schwanzel-Fukuda 1999; Forlano et al. 2000; Behrens and Wagner 2004; Biju et al. 2005; Chiba 2005; Abraham et al. 2008; Wray 2010), with the exception of lampreys that do not appear to contain GnRH. Nevertheless a variety of neuroactive peptides as Phe-Met-Arg-Phe-NH2 peptide (FMRF-amide peptide or FMRF-amide) or neuropeptide tyrosine (NPY) have been reported to be consistently expressed in different subpopulations of cells belonging to the terminal nerve of different vertebrate species (Ekström et al. 1988; Pinelli et al. 2000; Castro and Becerra 2001; Jadhao 2001; Rastogi et al. 2001; Pinelli et al. 2004; Mousley et al. 2006; for a review see Von Bartheld 2004) thus representing reliable markers of this system.

Numerous studies about the adult structure and function of the terminal nerve have been performed in elasmobranchs (Bullock and Northcutt 1984; Demski et al. 1987; Demski and Schwanzel-Fukuda 1987; Chiba et al. 1991; Wu et al. 1992; White and Meredith 1993; Chiba 2000; Forlano et al. 2000; Moeller and Meredith 2010; Yáñez et al. 2011), but the study of its development has been largely ignored in this animal group. Understanding the embryonic origin of the terminal

nerve in elasmobranchs will provide information about the evolutionary origin of this cranial nerve.

The developmental study performed in Chapter 1 allowed to established three developmental periods for olfactory development in the shark *Scyliorhinus canicula* on the basis of the appearance of the first postmitotic neurons derived from the olfactory placode across the mesenchyme (first "*pioneer*" period), the profuse neuronal migration along olfactory fibers (second "*migratory*" period) and the progressively achieved maturity in the olfactory structures (third "*maturation*" period). In that study we have also described a transient, heterogeneous cell population associated to the olfactory nerve (ON), the "migratory mass", from which a cluster of neurons seemed to deviate near the point of entrance to the olfactory bulb (OB) pointing to the possibility that they could belong to the developing terminal nerve system. In order to perform a more detailed characterization of this deviated population, we have analyzed the development of this potential terminal nerve system in the shark *S. canicula* with the aim of discriminating between olfactory components and non-olfactory components related to the terminal nerve system. We also investigated the spatial relationship between these two systems during embryogenesis. To meet these goals we have implemented tract-tracing techniques in combination with immunohistochemistry with reliable markers for components of the terminal nerve system (FMRF-amide) and also with the markers used in the previous chapter for characterizing components of the developing olfactory system as

Pax6, glial cells (GFAP, glial fibrillary acidic protein), early postmitotic neurons (HuC/D), migrating immature neurons and their growing fibers (DCX, doublecortin) and mature ORNs and primary tracts (protein $G\alpha_0$). This work represents the first developmental study of the terminal nerve system in an elasmobranch species and complements information of previous studies about the formation of olfactory structures in *S. canicula*, thus helping to understand how the vertebrate terminal nerve and olfactory systems have evolved.

MATERIAL AND METHODS

Experimental animals

Some embryos of the lesser spotted dogfish (Scyliorhinus canicula) were supplied by the Marine Biological Model Supply Service of the CNRS UPMC Roscoff Biological Station (France) and the Estación de Bioloxía Mariña da Graña (Galicia, Spain). Additional embryos and juveniles were kindly provided by the Aquaria of Gijón (Asturias, Spain), O Grove (Pontevedra, Spain) and Finisterrae (A Coruña, Spain). Embryos were staged by their external features following Ballard et al. (1993). For more information about the relationships of the embryonic stages, body size, gestation and birth, see Table 1 in Ferreiro-Galve et al. (2010). Forty-five embryos from stages 21 to 34 and three juvenile dogfish were used. Eggs from different broods and juveniles were raised in seawater tanks in standard conditions of temperature (15-16ºC), pH (7.5-8.5) and salinity (35g/L). Adequate measures were taken to minimize animal pain or discomfort. All

procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by the Spanish Royal Decree 53/2013 for animal experimentation, and were approved by the Ethics Committee of the University of Santiago de Compostela.

Tissue processing

Embryos were deeply anesthetized with 0.5% tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO) in seawater and separated from the yolk before fixation in 4% paraformaldehyde (PFA) in elasmobranch´s phosphate buffer [EPB: 0.1 M phosphate buffer (PB) containing 1.75% urea, pH 7.4] for 48-72 h depending on the stage of development. Embryos from stage 32 onwards and juveniles were deeply anesthetized with MS-222 and then perfused intracardially with elasmobranch Ringer's solution (see Ferreiro-Galve et al., 2012) followed by 4% PFA in EPB. The brains with the olfactory organs attached were removed and postfixed in the same fixative for 24-48 hours at 4ºC. Subsequently, they were rinsed in phosphate buffered saline (PBS), cryoprotected with 30% sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (18-20 µm thick) were obtained in sagittal, horizontal or transverse planes on a cryostat and mounted on Superfrost Plus (Menzel-Glasser, Madison, WI) slides.

Immunohistochemistry

For heat-induced epitope retrieval, sections were pre-treated with 0.01 M citrate buffer (pH 6.0) for 30 min at 95ºC, and allowed to cool for 20-30 min at room temperature (RT). Sections were rinsed twice in 0.05 M Tris-buffered saline (TBS; pH 7.4) for 5 min each, and incubated overnight at RT with rabbit polyclonal anti-FMRF-amide (FMRF) (see Table 1). Sections were rinsed twice in 0.05 M Tris-buffered saline (TBS) pH 7.4 for 5 min each, and incubated in the 546-conjugated donkey anti-rabbit (DAR^{546}) (see Table 2) for 2 h at RT. All dilutions were made with TBS containing 15% normal donkey serum (Millipore, Billerica, MA) 0.2% Triton X-100 (Sigma) and 2% bovine serum albumin (BSA, Sigma). All incubations were carried out in a humid chamber. Sections were then rinsed in TBS for 30 min and in distilled water (twice for 30 min). Sections were then allowed to dry for 2 h at 37ºC, and mounted in MOWIOL 4-88 Reagent (Calbiochem, MerkKGaA, Darmstadt, Germany).

Double immunolabeling was performed on alternate series of sections, which were incubated overnight at RT with cocktails of primary antibodies (mouse anti-HuC/D + rabbit anti-DCX; mouse anti-HuC/D + rabbit anti-Pax6; goat anti-DCX + rabbit anti-GFAP; mouse anti-HuC/D + rabbit anti-protein $G\alpha_0$; mouse anti-HuC/D + rabbit anti-FMRF) mixed at optimal dilutions and subsequently detected by using mixtures of appropriate secondary antibodies. All incubations were carried out in a humid chamber and processed as described above.

Complete information about the antibodies, immunogens, producer, catalog number, host, nature and dilution of the primary antibodies used here were indicated in Table 1. Data about secondary antibodies used in this study were included in Table 2.

In vitro tract-tracing techniques

Seven embryos ranging from stage 30 to 33 were used. The experiments were performed under *in vitro* whole-brain conditions. Specimens were deeply anaesthetized in seawater containing 0.5% MS-222. Animals were then immersed in ice-cooled elasmobranch Ringer's solution containing 1mM glucose, which was oxygenated with an oxygen injector aerator to a pH of 7.4. After the animal decapitation, the brains were isolated by removing the overlying tissue and transferred to fresh Ringer's solution to proceed with the immediate application of the tracer. We applied neurobiotin (Vector Laboratories, Burlingame, CA), an amino derivative of biotin used as an intracellular label for neurons, whose transport efficiency in retrograde labeling has been proved (Barreiro-Iglesias et al. 2008). The tracer was dissolved in distilled water until saturation and re-crystallized at the tip of an entomological needle (00) according to Morona et al. (2005) and the application was carried out manually or using a micromanipulator (Narishige MN-151, Japan) under a stereomicroscope. The olfactory epithelium (OE) was accessed by vertical penetrations. After tracer application, brains were maintained for two days at 8ºC in continuously oxygenated elasmobranch

Ringer's solution containing 1 mM glucose, and then fixed for 2 days in 4% paraformaldehyde in EPB. The tissue was cryoprotected and sectioned as explained above. Neurobiotin was visualized by incubating the sections with fluorescein isothiocyanate (FITC)-labeled Avidin D (Vector Laboratories; diluted 1:1000 in PBS containing 0.2% Triton X-100) in a humid chamber for 2.5h at 37ºC. The slides were rinsed in TBS, then in distilled water, dried for 30 min at 37ºC, and mounted in MOWIOL.

Combined tract-tracing and HuC/D immunohistochemistry

For combined tract-tracing and immunohistochemistry, the tracer was applied and the tissue was processed as described above. Primary antibody (mouse anti-HuC/D) was incubated overnight as described above. Then, secondary antibody (DAM^{546}) and Avidin B solutions were simultaneously incubated and mounted as previously explained.

Image acquisition and analysis

Double labeled sections were analyzed and photographed with theTCS-SP2 scanning microscope with a combination of blue and green excitation lasers. Stacks of confocal images were acquired separately for each laser channel with steps of 0.8 or 2 µm along the z-axis and collapsed images were obtained from an average of 12 optical sections with the LITE software (Leica). Some sections were photographed with an epifluorescence photomicroscope Olympus AX70 fitted with an Olympus DP70 color digital camera. Photographs were adjusted for

brightness and contrast and plates were prepared using Adobe Photoshop CS4 (Adobe, San Jose, CA). Images were not otherwise modified.

RESULTS

The main events that take place during the development of the olfactory system in the lesser spotted dogfish (*Scyliorhinus canicula*) have been framed into three developmental periods established on the basis of tract-tracing experiments and the expression of various immunohistochemical markers (Chapter 1). In the present Chapter, these developmental events in the olfactory system were spatially and temporally related to the development of an extra-bulbar, non-olfactory projection. We have observed and characterized a migratory stream of peripheral neurons and its associated nerve fibers extending towards the basal region of the telencephalic hemispheres that may represent the primordium of the terminal nerve and ganglia.

First period (*pioneer period*) (stages 20 to 24)

During this early phase of development, no peripheral populations were observed besides that of the pioneer neurons, described in Chapter 1 as the transient class of neurons that prefigure the primary olfactory pathway.

Second period (*migratory period*) (stages 25 to 30)

During this period, a heterogeneous population, i.e. the migratory mass, migrates towards the OB across the mesenchyme in close apposition to the olfactory fibers (see Chapter 1).

At stage 28, we detected for the first time a peripheral population of closely arranged HuC/D-ir/DCX-ir cells located near the surface of the telencephalic walls (Figs. 1A,B). DCX-ir fibers arising from this cell cluster did not course to the OB but bordered the telencephalic walls (arrows in Figs. 1A,B) before entering at the basal telencephalon (arrowhead in Figs. 1A,B). As development proceeded, these HuC/D-ir cells became progressively arranged in clusters that gradually extended from the vicinity of the OB (arrow in Figs. 1C,D) and along the course of DCX-ir fibers (outlined in Fig.1D). Finally, we also detected DCX fibers entering into the basal telencephalon (arrowhead in Fig. 1E).

The analysis of sagittal sections revealed that this HuC/D-DCX cell population was intermingled to some extent with that belonging to the migratory mass (see Chapter 1) in a location close to the ON-OB junction (Figs. 1F-I). Moreover, the former apparently branched off the latter at this level and headed rostrally (arrows in 1F-I). The fact that this novel population, as observed in stage 28, seemed to by-pass the OB and projected to rostral telencephalon led us to consider that it belongs to the developing terminal nerve. Olfactory and presumed terminal nerve populations were easily discernible by their different GFAP and Pax6 expression. Unlike the ON, the terminal nerve component did not

show any Pax6-ir neuron (Figs. 1F,G) nor any GFAP-ir glial component ensheathing migrating neuronal clusters (Fig. 1H-I).

Third period (*maturation period*) (stage 31 to hatching)

To further explore the spatial relationship between the ON and this peripheral population proposed to represent the embryonic terminal nerve system, we performed double labeling to discern between the terminal nerve component (on the basis of its HuC/D immunoreactivity) and the olfactory projection which specifically showed Gα0 protein immunoreactivity (Figs. 2A-G). Both systems were visualized in a series of transverse sections in early stage-31embryos. At caudal levels the whole length of the olfactory projection was evident (Figs. 2A-C) and some neurons belonging to the migratory mass (arrows in 2A-C) were observed near the ON-OB junction. More rostrally, the deviated HuC/D cluster was detected heading towards rostral telencephalic regions (arrowheads in 2D-G), as observed at previous stages.

We additionally revealed the olfactory projection by massive application of the tracer neurobiotin in the OE and combined it with HuC/D immunohistochemistry, which yielded identical results (Figs. 2H-I).

In late stage-31 embryos, a subpopulation of neurons within the deviated HuC/D peripheral population began to express FMRF-amide peptide (Fig. 2J), which is a reliable marker of the terminal nerve system. As development proceeded, clusters were progressively associated in ganglia that contained a

subpopulation of cells immunoreactive to FRMF-amide peptide, which were especially evident from stage 32 onwards (Fig. 2K,L and Fig.3B) and thus were clearly recognized as the characteristic terminal nerve ganglia.

DISCUSSION

Although the terminal nerve or cranial nerve zero was first described in an elasmobranch (Fritsch 1878) and there exists abundant literature about this nerve in adult elasmobranchs (Bullock and Northcutt 1984; Demski et al. 1987; Demski and Schwanzel-Fukuda 1987; Chiba et al. 1991; Wu et al. 1992; White and Meredith 1993; Chiba 2000; Forlano et al. 2000; Moeller and Meredith 2010; Yáñez et al. 2011), the study of its development has been absolutely overlooked. As far as we are concerned, this work represents the first description of the development of the terminal nerve in a cartilaginous fish.

We have observed a mass of peripheral cells and fibers extending towards the basal telencephalon of *S. canicula* that represents the terminal nerve system primordium (see Fig. 3). This mass shows similarities with the location of ganglia and nerve course, which were experimentally demonstrated in tract-tracing studies in adults of this species (Yáñez et al. 2011). Our results in *S. canicula* show that a group of neurons segregated from the migratory mass project their axons towards subpallial levels of the telencephalic hemispheres. The neurons of the migratory mass appear to accumulate at the ON-OB junction and then, a subset of these cells branched off forming cell clusters along the terminal nerve. This observation is in agreement with that described in other vertebrates where the

neurons of the terminal nerve appear as a series of cell bodies strung out along the ventral telencephalon and first evident near the site of ON entry (reviewed in Whitlock 2004). Indeed, it is widely accepted that terminal nerve neurons (mostly identified by their GnRH expression) migrate within the migratory mass along with other cell types before they turn off toward the basal telencephalon (Schwanzel-Fukuda 1999; Schwanzel-Fukuda and Pfaff 1989; Valverde et al. 1993).

During the maturation period, we have shown as cells of these clusters began to show immunoreactivity to FMRF-amide from stage 31. The FMRF-amide immunoreactivity has been used to identify a subpopulation of terminal nerve ganglion cells in adult elasmobranchs including *Scyliorhinus* species (Demski and Schwanzel-Fukuda 1987; Chiba et al. 1991; Chiba 2000). Our observations of FMRF-ir cells in juveniles matched with that described in adults of *S. torazame* by Chiba's group and validate our identification of clusters of FMRF-amide peptide positive cells in embryos from stage 31 onwards as the primordial terminal nerve ganglia. These results were supported by the fact that segregated cells from the migratory mass never expressed Gα0, a specific marker of the olfactory system. As we lack of early specific markers for terminal nerve cells (before early stage 31), we can only attribute a terminal nerve identity to those cells observed beyond the bifurcation point. Whether the neurons of the terminal nerve originate in the placode or the neural crest remains a matter of debate (for review see Von Bartheld 2004 and Whitlock 2004) and is beyond the scope of this paper to identify their origin.

Despite in adult elasmobranchs the terminal nerve and the olfactory system appear to be more clearly separated than in any other vertebrate group studied so far (Yáñez et al. 2011), we have shown that the components of both systems share a common developmental origin in the migratory mass, just as happens in other vertebrates. The observation of this feature in a basal vertebrate reveals the existence of a basic pattern, mainly evident during development, which may be considered as a highly conserved trait along evolution.

REFERENCES

Abraham E, Palevitch O, Ijiri S, Du SJ, Gothilf Y, Zohar Y (2008) Early development of forebrain gonadotrophin-releasing hormone (GnRH) neurones and the role of GnRH as an autocrine migration factor. J Neuroendocrinol 20:394–405

Ballard WW, Mellinguer J, Lechenault H (1993) A series of normal stages for development of Scyliorhinus canicula, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). J Exp Zool 267:318–336

Barreiro-Iglesias A, Villar-Cerviño V, Anadón R, Rodicio MC (2008) Descending brain-spinal cord projections in a primitive vertebrate, the lamprey: cerebrospinal fluid-contacting and dopaminergic neurons. J Comp Neurol 511:711–723

Behrens U, Wagner HJ (2004) Terminal nerve and vision. Microsc Res Tech 65:25–32

Biju KC, Gaikwad A, Sarkar S, Schreibman MP, Subhedar N (2005) Ontogeny of GnRH-like immunoreactive neuronal systems in the forebrain of the Indian major carp, Cirrhinus mrigala. Gen Comp Endocrinol 141:161–171

Bullock TH, Northcutt RG (1984) Nervus terminalis in dogfish (Squalus acanthias, Elasmobranchii) carries tonic efferent impulses. Neurosci Lett 44:155–160

Butler AB, Hodos W (2005) Comparative vertebrate neuroanatomy. Evolution and adaptation. 2nd ed. Wiley-Liss, Hoboken, NJ

Castro A, Becerra M, Anadón R, Manso MJ (2001) Distribution and development of FMRFamide-like immunoreactive neuronal systems in the brain of the brown trout, Salmo trutta fario. J Comp Neurol 64:43–64

Chiba A (2005) Neuropeptide Y-immunoreactive (NPY-ir) structures in the brain of the gar Lepisosteus oculatus (Lepisosteiformes, Osteichthyes) with special regard to their anatomical relations to gonadotropin-releasing hormone (GnRH)-ir structures in the hypothalamus and the terminal nerve. Gen Comp Endocrinol 142:336–346

Chiba A (2000) Immunohistochemical cell types in the terminal nerve ganglion of the cloudy dogfish, Scyliorhinus torazame, with special regard to neuropeptide Y/FMRFamide-immunoreactive cells. Neurosci Lett 286:195–198

Chiba A, Oka S, Honma Y (1991) Immunocytochemical distribution of FMRFamide-like substance in the brain of the cloudy dogfish, Scyliorhinus torazame. Cell Tissue Res 265:243–250

Demski LS (1989) Pathways for GnRH control of elasmobranch reproductive physiology and behavior. J Exp Zool 252: 4–11

Demski LS, Fields RD (1988) Dense-cored vesicle-containing components of the terminal nerve of sharks and rays. J Comp Neurol 278:604–614

Demski LS, Schwanzel-Fukuda M (1987) The terminal nerve (nervus terminalis): structure, function, and evolution. Introduction. Ann N Y Acad Sci 519:ix–xi

Demski LS, Fields RD, Bullock TH, Schreibman MP, Margolis-Nunno H (1987) The terminal nerve of sharks and rays: electron microscopic, immunocytochemical, and electrophysiological studies. Ann N Y Acad Sci 519:15–32

Drapkin PT, Silverman AJ (1999) Development of the chick olfactory nerve. Dev Dyn 214:349–360

Ekström P, Honkanen T, Ebbesson SO (1988) FMRFamide-like immunoreactive neurons of the nervus terminalis of teleosts innervate both retina and pineal organ. Brain Res 460:68–75

Ferreiro-Salve S, Rodríguez-Moldes I, Candal E (2012) Pax6 expression during retinogenesis in sharks : comparison with markers of cell proliferation and neuronal differentiation. J Exp Zool 318: 91–108

Ferreiro-Galve S, Rodríguez-Moldes I, Anadón R, Candal E (2010) Patterns of cell proliferation and rod photoreceptor differentiation in shark retinas. J Chem Neuroanat 39:1–14

Forlano PM, Maruska KP, Sower SA, King JA, Tricas TC (2000) Differential distribution of gonadotropin-releasing hormone-immunoreactive neurons in the stingray brain: functional and evolutionary considerations. Gen Comp Endocrinol 118:226–48

Forni PE, Taylor-Burds C, Melvin VS, Williams T, Wray S (2011) Neural crest and ectodermal cells intermix in the nasal placode to give rise to GnRH-1 neurons, sensory neurons, and olfactory ensheathing cells. J Neurosci 31:6915–6927

Fritsch G (1878) Untersuchungen über den feineren bau des fischgehirns mit besonderer berücksichtigung der homologien bei anderen wirbelthierklassen. Berlin: Guttmann 1–94

Jadhao AG (2001) Localization of molluscan cardioexcitatory tetrapeptide in the brain of African Cichlid fish (Haplochromis burtoni) revealed by immunocytochemistry. Neurosci Lett 303:103–106

Katoh H, Shibata S, Fukuda K, Sato M, Satoh E, Nagoshi N, Minematsu T, Matsuzaki Y, Akazawa C, Toyama Y, Nakamura M, Okano H (2011) The dual origin of the peripheral olfactory system: placode and neural crest. Mol Brain 4:34

Locy WA (1899) New facts regarding the development of the olfactory nerve. Anat Anz 16:273–290

Locy WA (1905) On a newly recognized nerve connected with the forebrain of selachians. Anat Anz 26:33–123

Moeller JF, Meredith M (2010) Differential co-localization with choline acetyltransferase in nervus terminalis suggests functional differences for GnRH isoforms in bonnethead sharks (*Sphyrna tiburo*). Brain Res 1366: 44-53

Morona R, Moreno N, López JM, Muñoz M, Ten Donkelaar HJ, González A (2005) Calbindin-D28k immunoreactivity in the spinal cord of *Xenopus laevis* and its participation in ascending and descending projections. Brain Res Bull 66:550–554

Mousley A, Polese G, Marks NJ, Eisthen HL (2006) Terminal nerve-derived neuropeptide y modulates physiological responses in the olfactory epithelium of hungry axolotls (*Ambystoma mexicanum*). J Neurosci 26:7707–7717

Mulrenin EM, Witkin JW, Silverman AJ (1999) Embryonic development of the gonadotropin-releasing hormone (GnRH) system in the chick: a spatio-temporal analysis of GnRH neuronal generation, site of origin, and migration. Endocrinology 140:422–433

Murakami S, Kikuyama S, Arai Y (1992) The origin of the luteinizing hormone-releasing hormone (LHRH) neurons in newts (Cynops pyrrhogaster): the effect of olfactory placode ablation. Cell Tissue Res 269: 21–27

Northcutt RG, Muske LE (1994) Multiple embryonic origins of gonadotropin-releasing hormone (GnRH) immunoreactive neurons. Dev Brain Res 78:279–290

Oka Y (1992) Gonadotropin-releasing hormone (GnRH) cells of the terminal nerve as a model neuromodulator system. Neurosci Lett 142:119–122

Pinelli C, Aniello BD, Polese G, Rastogi RK (2004) Extrabulbar olfactory system and nervus terminalis FMRFamide immunoreactive components in Xenopus laevis ontogenesis. J Chem Neuroanat 28:37–46

Pinelli C, D'Aniello B, Sordino P, Meyer DL, Fiorentino M, Rastogi RK (2000) Comparative immunocytochemical study of FMRFamide neuronal system in the brain of *Danio rerio* and *Acipenser ruthenus* during development. Brain Res Dev Brain Res 119:195–208

Rastogi RK, D'Aniello B, Pinelli C, Fiorentino M, Di Fiore MM, Di Meglio M, Iela L (2001) FMRFamide in the amphibian brain: a comprehensive survey. Microsc Res Tech 54:158–172

Schwanzel-Fukuda M (1999) Origin and migration of luteinizing hormone-releasing hormone neurons in mammals. Microsc Res Tech 44:2–10

Schwanzel-Fukuda M, Pfaff DW (1989) Origin of luteinizing hormone-releasing hormone neurons. Nature 338:161–164

Tobet SA, Sower SA, Schwarting GA (1997) Gonadotropin-releasing hormone containing neurons and olfactory fibers during development: from lamprey to mammals. Brain Res Bull 44:479–486

Valverde F, Heredia M, Santacana M (1993) Characterization of neuronal cell varieties migrating from the olfactory epithelium during prenatal development in the rat. Immunocytochemical study using antibodies against olfactory marker protein (OMP) and luteinizing hormone-releasing hormone (LH-RH). Brain Res Dev Brain Res 71:209–220

Von Bartheld CS, Baker CVH (2004) Nervus terminalis derived from the neural crest? A surprising new turn in a century-old debate. Anat Rec B New Anat 278:12–13

Von Bartheld CS (2004) The terminal nerve and its relation with extrabulbar olfactory projections: lessons from lampreys and lungfishes. Microsc Res Tech 65:13-24

White J, Meredith M (1993) Spectral analysis and modelling of ACh and NE effects on shark nervus terminalis activity. Brain Res Bull 31:369–374

Whitlock KE (2004) Development of the Nervus Terminalis : Origin and Migration. Microsc Res Tech 12:2–12

Wray S (2010) From nose to brain: development of gonadotrophin-releasing hormone-1 neurones. J Neuroendocrinol 22:743–753

Wray S, Nieburgs A, Elkabes S (1989) Spatiotemporal cell expression of luteinizing hormone-releasing hormone in the prenatal mouse: evidence for an embryonic origin in the olfactory placode. Brain Res Dev Brain Res 46:309–318

Wu CC, Yoshimoto M, Ito H (1992) The selachian terminal nerve. Kaibogaku Zasshi J Anat 67:317–332

Yamamoto N, Oka Y, Kawashima S (1997) Lesions of gonadotropin releasing hormone-immunoreactive terminal nerve cells: effects on the reproductive behavior of male dwarf gouramis. Neuroendocrinology 65:403–412

Yáñez J, Folgueira M, Köhler E, Martínez C, Anadón R (2011) Connections of the terminal nerve and the olfactory system in two galeomorph sharks: an experimental study using a carbocyanine dye. J Comp Neurol 519:3202–3217

ABBREVIATIONS

OB	olfactory bulb
OE	olfactory epi thelium
ON	olfactory nerve
OPit	olfactory pit
St	stage
Tel	telencephalon
TN	terminal nerve
v	ventricle

TABLE 1. Primary antibodies

Antibody	Immunogen	Source	Working dilution
HuC/D	Human HuD peptide (QAQRFRLDNLLN-C)-Keyhole Limpet Hemocyanin (KLH) conjugate	Monoclonal mouse anti-HuC/D Molecular Probes, Eugene, OR Catalog number: A-21271	1:100
Pax6	Peptide QVPGSEPDMSQYWPRLQ derived from the C-terminus of the mouse Pax6 protein	Polyclonal rabbit anti-Pax6 Covance, Emeryville, CA Catalog number: PRB-278P	1:200
DCX	Synthetic peptides corresponding to amino acids 48–69 (sequence GHFDERDKTSRNMRGSRMNGLP) and amino acids 380–402 (sequence LRKHKDLYLPLSLDDSDSLGDSM) of human doublecortin	Polyclonal rabbit anti-DCX Cell Signaling Technology, Beverly,MA. Catalog number: 4604	1:300
DCX	A peptide mapping at the C-terminus of doublecortin of human origin	Polyclonal goat anti-DCX Santa Cruz Biotechnology, Santa Cruz, CA Catalog number: sc-8066	1:100
GFAP	Purified bovine spinal cord GFAP	Polyclonal rabbit anti-GFAP Dako, Glostrup, Denmark Catalog number: Z 0334	1:500
FMRF	Synthetic FRMF-amide peptide coupled to bovine thyroglobulin	Polyclonal rabbit anti-FMRF Incstar Corp. Stillwater Catalog number: 20091	1:1,000
$G\alpha_0$	Peptide (105-124) mapping within a highly divergent domain of Gα0 of rat origin	Polyclonal rabbit anti-G α_0 Santa Cruz Biotechnology, Santa Cruz, CA Catalog number: sc-387	1:400

TABLE 2. Secondary antibodies

Secondary antibody	Comercial suppliers	Dilution
546-conjugated donkey anti-rabbit (DAR^{546})	Molecular probes Catalog number: A10040	1:100
488-conjugated donkey anti-mouse (DAM^{488})	Molecular probes Catalog number: A21202	1:100
488-conjugated donkey anti-goat (DAG^{488})	Molecular probes Catalog number: A11055	1:100
633-conjutated donkey anti-mouse (DAM^{647})	Molecular probes Catalog number: A31571	1:100
546-conjugated donkey anti-mouse (DAM^{546})	Molecular probes Catalog number: A10036	1:200

Fig. 1 Sections of embryos during the second (migratory) period showing the spatial distribution of the developing terminal nerve. **A,B:** Transverse sections across telencephalon of a stage 28-embryo showing the HuC/D-DCX immunoreactive cell population whose fibers enter the telencephalon (arrowhead) after coursing in close apposition to the rostral telencephalic walls (arrows). **C-E:** Parallel transverse sections of the telencephalon from caudal (C) to rostral (E) showing the migratory mass (arrows) and the deviated population forming neuronal clusters (outlined in D) and projecting DCX-ir fibers to the rostral telencephalon. Arrowhead points to the region where DCX-ir fibers enter the telencephalon. **F-I**: Sagittal sections of embryos at stages 29 and 30 at the level of the olfactory nerve-olfactory bulb junction to show the point where the rostrally-projected HuC/D cell population branches off from the migratory mass (arrows) to form the terminal nerve. This deviated population does not contain Pax6 immunoreactive cells (F,G) nor GFAP-positive glial tubes surrounding the neuronal clusters (H,I), in contrast to the olfactory projection. For abbreviations, see list. *Scale bars*: 100 µm (**A,B,E,G-I**); 200 µm (**C,D,F**).

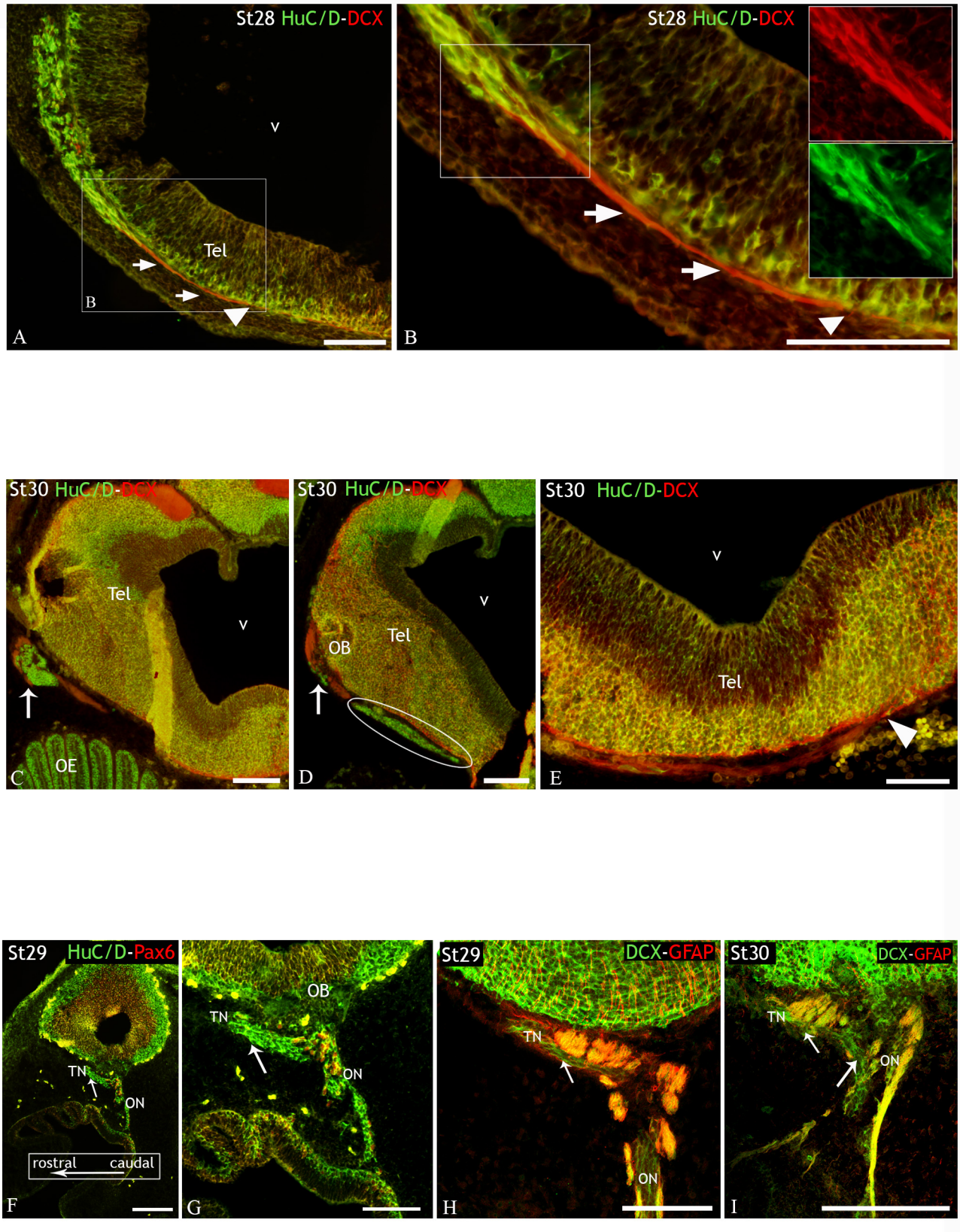

St28 HuC/D-DCX
v
Tel
B
A
St28 HuC/D-DCX
B
St30 HuC/D-DCX
Tel
v
OE
C
St30 HuC/D-DCX
v
OB
Tel
D
St30 HuC/D-DCX
v
Tel
E
St29 HuC/D-Pax6
TN
ON
rostral caudal
F
OB
TN
ON
G
St29 DCX-GFAP
TN
ON
H
St30 DCX-GFAP
TN
ON
I

Figure 1

Fig. 2 Sections of embryos showing the terminal nerve at different developmental stages during the third (maturating) period and in juveniles. **A-G**: Series of transverse sections across the telencephalon and peripheral olfactory system of a stage-31 embryo from caudal (A) to rostral (G) to show the spatial relationship between the olfactory system (Gα0-protein positivity in A-D), the migratory mass (arrows in A-C) and the HuC/D immunoreactive clusters of cells identified as belonging to the developing terminal system (arrowheads in D-G). **H**,**I**: Olfactory projection revealed by means of neurobiotin application to the olfactory epithelium combined with immunohistochemistry for HuC/D also served to study spatial relationship of both systems and yielded identical results than those showed in figures A-G. Arrows in H and arrowheads in I point, respectively, the migratory mass and the developing terminal nerve. **J**: From late stage 31, a subset of cells belonging to the peripheral HuC/D clusters began to express FMRF-amide peptide, a classical marker of terminal nerve system. **K**,**L**: The observed clusters were posteriorly associated in ganglia expressing FMRF-amide peptide and recognized as the classical terminal nerve ganglia. For abbreviations, see list. *Scale bars*: 500 µm (**A-I**); 300 µm (**J**); 100 µm (**K,L**).

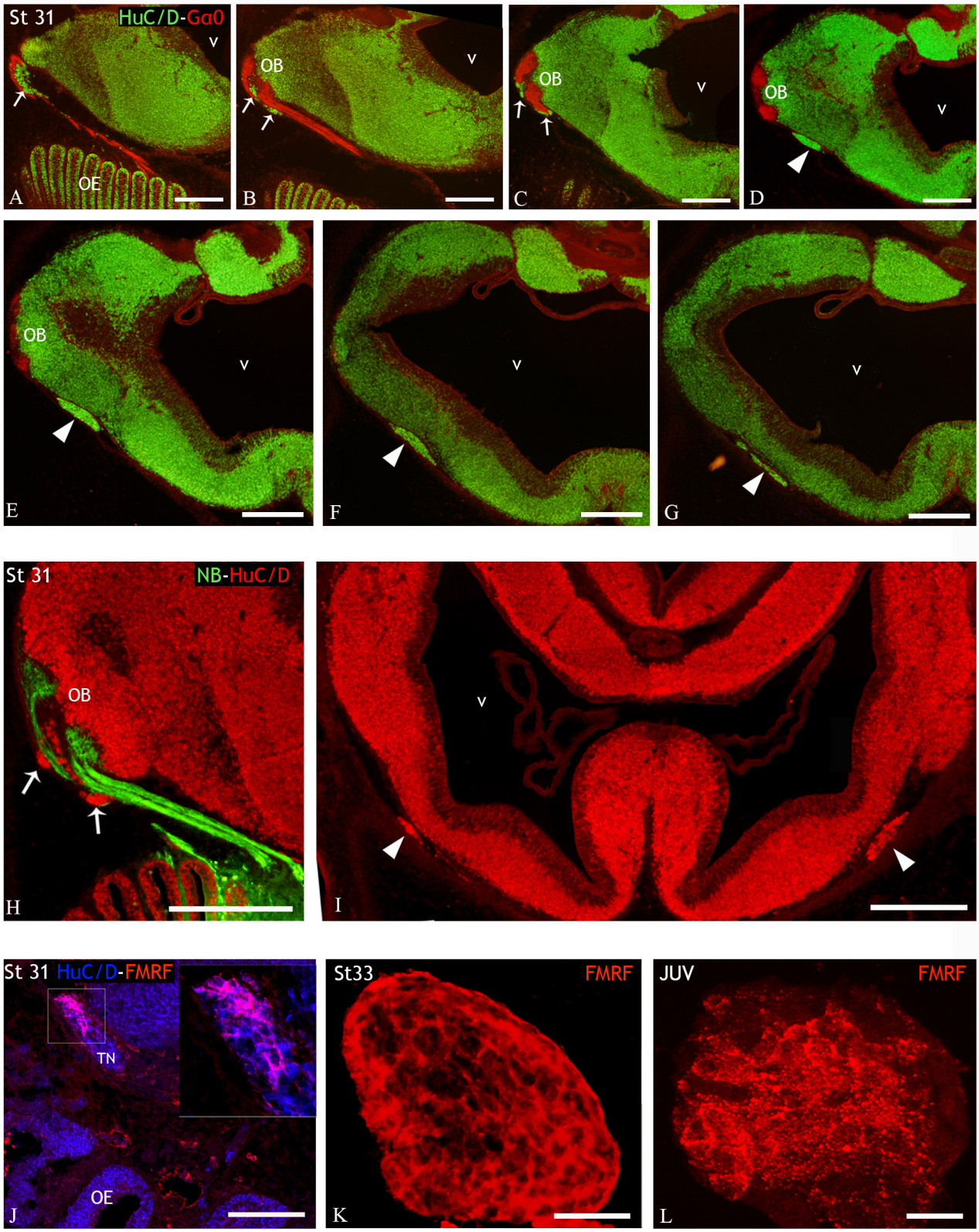

St 31
HuC/D-Gα0
v
OE
A
OB
B
C
D
E
F
G
NB-HuC/D
H
I
St 31 HuC/D-FMRF
TN
J
St33
FMRF
K
JUV
L

Figure 2

Fig. 3 Schematic representations of the telencephalon showing the spatial relationship between olfactory and terminal nerve components during characteristic stages of second (A) and third (B) periods of development. (**A**) shows a transverse section while (**B**) offers an external view of the telencephalic structure at stage 32. For abbreviations, see list.

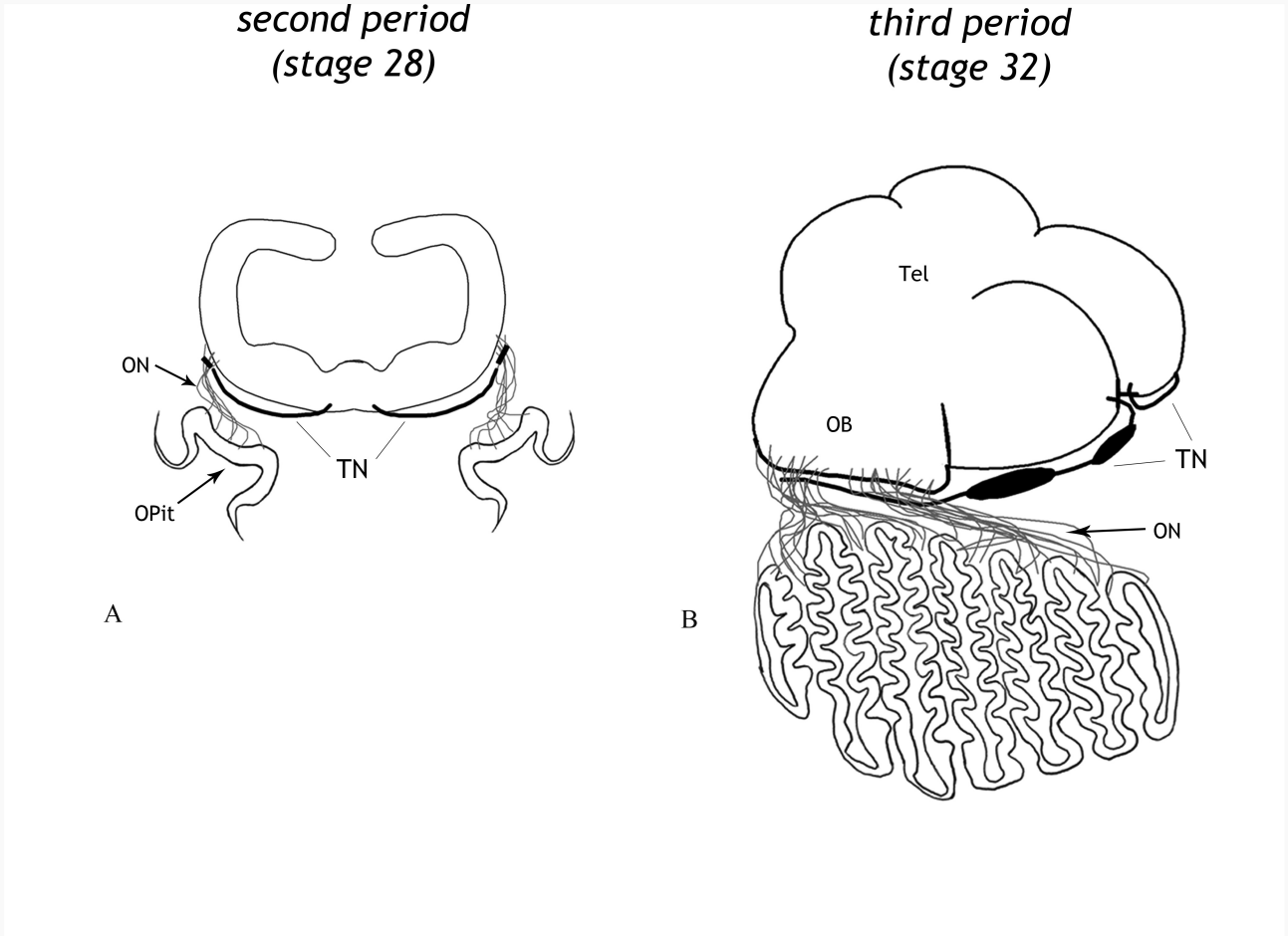

second period
(stage 28)
third period
(stage 32)
ON
TN
OPit
A
Tel
OB
TN
ON
B

Figure 3

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Chapter 3

Genoarchitechtonic study of the telencephalon of *Scyliorhinus canicula*

Some results of the present work are published in Quintana-Urzainqui I, Sueiro C, Carrera I, Ferreiro-Galve S, Santos-Durán G, Pose-Méndez S, Mazan S, Candal E, Rodríguez-Moldes I (2012) Contributions of developmental studies in the dogfish *Scyliorhinus canicula* to the brain anatomy of elasmobranchs: insights on the basal ganglia Brain Behav Evol 80:127-41.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

The definition of different progenitor regions of the brain of vertebrates has been classically based on anatomical landmarks, such as sulci and bulges. However, morphological boundaries not always coincide with molecular limits and histogenetic compartments and often mislead correct interpretations about brain subdivisions. The analysis of the expression of key regulatory genes on different species during embryogenesis under the neuromeric model (the prevailing topological segmental model of regionalization; see general introduction), suggests the existence of particular histogenetic domains that correspond to specific nuclear derivatives in the adult brain (Puelles et al. 2012), and thus represents a more reliable tool to establish homologous brain fields between different vertebrates. The prosencephalon is the rostral-most portion of the brain and, according to the prosomeric model (the neuromeric model for the prosencephalon) it is divided in two transverse protosegments: the diencephalon (caudally) and the secondary prosencephalon (rostrally) (Puelles and Rubenstein 2003; Puelles et al. 2004). The secondary prosencephalon embraces histogenetic territories as the hypothalamus, the optic vesicles and the telencephalon. The alar-basal boundary roughly divides in half the hypothalamus, so that the telencephalon, the optic vesicles and a part of the hypothalamus represent alar plate derivatives while the remaining part of the hypothalamus is a basal derivative.

The adult telencephalon is one of the regions of the vertebrate brain presenting higher morphological and functional variability between different groups. Nevertheless, it shows a great degree of conservation in terms of specification, signaling and gene patterning during embryonic development (Striedter 1997; Reiner et al. 1998; Puelles et al. 2000; Moreno et al. 2009). Developmental studies about the telencephalon have proved to be extremely useful to define its basic histogenetic domains and to identify homolog structures between different vertebrates (Bulfone et al. 1993; Striedter 1997; Smith-Fernández et al. 1998; Lavdas et al. 1999; Sussel et al. 1999; Puelles et al. 2000; Stoykova et al. 2000; Bachy et al. 2001; Hevner et al. 2001; Puelles 2001; Puelles and Medina 2002; Moreno et al. 2008a). The telencephalon of all vertebrates becomes subdivided during development in two main territories: the pallium (dorsal) and the subpallium (ventral), separated by the pallial-subpallial boundary (PSB; reviewed in Medina and Abellán 2009; Moreno et al. 2009). The existence of these subdivisions has been corroborated in different vertebrate species by the combinatorial expression of developmental regulatory genes, as *Pax6, Emx* and *Tbr1* (expressed in the pallium, where glutamatergic cells are produced) or those belonging to the Distal-less gene family (typically expressed in the subpallium and associated to the production of GABAergic cells) (Smith-Fernández et al. 1998; Puelles et al. 2000; Murakami et al. 2001; Bachy et al. 2002; Brox et al. 2003, 2004; Marín and Rubenstein 2003; Medina et al. 2004; Wullimann and Mueller 2004; Mueller and Wullimann 2009; Sugahara et al. 2013).

Although pallial and subpallial compartments are present in all vertebrates studied so far, it is unclear whether all subdivisions within these main territories are present in all vertebrate groups. Briefly, the subpallium can be subdivided in different territories from mammals to teleost fishes on the basis of differential gene expression: lateral ganglionic eminence (LGE) or striatal domain, that show expression of the *Dlx2* gene; medial ganglionic eminence (MGE) or pallidal domain, in which the *Dlx2* and *Nkx2.1* genes are expressed; and preoptic area (PO) or non-evaginated telencephalon, which show combined expression of *Dlx2, Nkx2.1* and *Shh* (Puelles et al. 2000; Marín and Rubenstein 2003; Medina et al. 2011). On the other hand, all tetrapods present at least four different sectors within the pallium (medial, dorsal, lateral and ventral pallium) attending to differential distribution of genetic markers as *Tbr1, Emx1* or *Lhx9* (Puelles et al. 2000; Medina and Abellán 2009). Despite the occurrence of some of these pallial compartments has been suggested in bony fishes (Wullimann and Mueller, 2004; Alunni et al. 2004; Menuet et al. 2007), the scenary in agnathans is far from clear (Murakami et al. 2001; reviewed in Moreno et al. 2009; Sugahara et al. 2013).

The regionalization/organization of the telencephalon of adult cartilaginous fishes has been characterized by means of morphological, histochemical and tract-tracing experiments (Smeets et al. 1983; Reiner and Carraway 1985; Meredith and Smeets 1987; Northcutt et al. 1988; Smeets 1990; Stuesse et al. 1991; Stuesse and Cruce 1992; Manso and Anadón, 1993; Smeets et al. 2000; Sueiro, 2003; Hofmann and Northcutt 2008, 2012; Rodríguez-Moldes, 2009;

Carrera et al. 2012). Nevertheless, there is a big controversy regarding the accurate identification of the different telencephalic regions. Consequently, developmental gene expression analyses are absolutely needed to shed light into this topic. Recent developmental studies in *S. canicula* have tentatively delimited the PSB by using GABA/GAD and Pax6 as markers (Carrera et al. 2008; Ferreiro-Galve et al. 2008; Rodríguez-Moldes, 2009), although more complete studies about combinatorial gene expression are lacking.

Taking advantage of these studies and that the lesser-spotted dogfish *S. canicula* has demonstrated to be a crucial model for developmental studies by providing insights into the commonality of apparently diverse developmental mechanisms (Coolen et al. 2009; Gillis and Shubin 2009), we have attempted a developmental approach using GAD (glutamic acid decarboxylase, the synthetizing enzyme of GABA) and Pax6 immunohistochemistry in combination to some genetic markers to try to identify the main subdivisions of the telencephalon in this species. The markers we used in this study have been selected because of their well-known specific expression in different telencephalic domains: *Pax6, Tbr1, Emx1* and *Lhx9* are pallial markers and key factors in dorsoventral telencephalic specification (Medina and Abellán 2009), while GAD, *Dlx2* and *Nkx2.1* are specifically expressed in the subpallium and have served to demilit the LGE and MGE (see above). Shh has been reported to be expressed in the preoptic compartment of the subpallium and *Otp* has been described to be expressed in the alar part of the hypothalamus (Moreno and González 2011),

therefore representing a useful markers to delimit the telencephalic-hypothalamic boundary.

In the present work we analyze the development of the telencephalon of *S. canicula* (with special attention to the evaginated telencephalon) in selected developmental stages in order to identify the basic histogenetic fields homologous to those observed in other vertebrates. This study represents a baseline for future comparative studies and a useful framework of reference for Chapters 4 and 5. Most information came from analyses in stages 31 and 32 of *S. canicula*, chosen as representatives of the transition from the achievement of the morphological differentiation of main forebrain centers to the onset of forebrain maturation (second and third forebrain developmental periods according Rodríguez-Moldes, 2009). Because a similar histogenetic sequence can be roughly recognized in other vertebrates, these results may provide a useful tool to carry out more reliable comparisons between homologous regions of cartilaginous fishes and other vertebrates.

MATERIAL AND METHODS

Experimental animals

Some embryos of the lesser spotted dogfish (*S. canicula*) were supplied by the Marine Biological Model Supply Service of the CNRS UPMC Roscoff Biological Station (France) and the Estación de Bioloxía Mariña da Graña (Galicia, Spain). Additional embryos were kindly provided by the Aquaria of Gijón (Asturias,

Spain), O Grove (Pontevedra, Spain) and Finisterrae (A Coruña, Spain). Embryos were staged by their external features according to Ballard et al. (1993). For more information about the relationship of the embryonic stages with body size, gestation and birth, see Table 1 in Ferreiro-Galve et al. (2010). Thirty-seven embryos from stages 28 to 34 were used in this study. Eggs from different broods were raised in seawater tanks in standard conditions of temperature (15-16ºC), pH (7.5-8.5) and salinity (35g/L). Adequate measures were taken to minimize animal pain or discomfort. All procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by the Spanish Royal Decree 53/2013 for animal experimentation and were approved by the Ethics Committee of the University of Santiago de Compostela.

Tissue processing

Embryos were deeply anesthetized with 0.5% tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO) in seawater and separated from the yolk before fixation in 4% paraformaldehyde (PFA) in elasmobranch's phosphate buffer [EPB: 0.1M phosphate buffer (PB) containing 1,75% urea, pH 7.4] for 48-72 h depending on the stage of development. Embryos from stage 32 onwards were deeply anesthetized with MS-222 and then perfused intracardially with elasmobranch Ringer´s solution (see Ferreiro-galve et al. 2012) followed by 4% PFA in EPB. Brains were removed and postfixed in the same fixative for 24-48 h at 4ºC.

Subsequently, they were rinsed in phosphate buffere saline (PBS), cryoprotected with 30% sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (18-20 µm thick) were obtained in transverse planes on a cryostat and mounted on to Superfrost Plus (Menzel-Glasser, Madison, WI, USA) slides.

Single and double immunofluorescence

For heat-induced epitope retrieval, sections were pre-treated with 0.01 M citrate buffer (pH 6.0) for 30 min at 95ºC and allowed to cool for 20–30 min at room temperature (RT). Sections were then rinsed twice in 0.05 M Tris-buffered saline (TBS; pH 7.4) for 5 min each and incubated overnight with the primary antibody (polyclonal sheep anti-Glutamic acid decarboxylase 65/67, GAD65/67, provided by Dr. E. Mugnaini, dilution 1:30000; polyclonal rabbit anti-Pax6, Covance, Emeryville, CA, dilution 1:200; polyclonal rabbit anti-Sonic Hedgehog, Shh, Sta Cruz Biotechnology, Santa Cruz, CA, dilution 1:100). Appropriate fluorescent dye-labeled donkey secondary antibodies (546-conjugated donkey anti-rabbit, Molecular Probes, Eugene, OR, dilution 1:100; 633-conjugated donkey anti-sheep, Molecular Probes, Eugene, OR, dilution 1:100) were incubated for 2 h at RT in the dark. For double immunofluorescence experiments, cocktails of primary antibodies were mixed at optimal dilutions and subsequently detected by using mixtures of appropriate secondary fluorescent antibodies. Sections were rinsed in distilled water (twice for 30 min), allowed to dry for 2 h at 37 ºC and

mounted in MOWIOL 4-88 Reagent (Calbiochem, MerkKGaA, Darmstadt, Germany). All dilutions were made with TBS containing 15 % donkey normal serum (Millipore, Billerica, MA) 0.2 % Triton X-100 (Sigma) and 2 % bovine serum albumin (BSA, Sigma).

In situ hybridization on slides

We applied in situ hybridization for *Dlx2, Nkx2.1, Tbr1, Emx1, Lhx9* and *Otp* probes. *Emx1* probe was used and analyzed in early stages of *S. canicula* development (Derobert et al. 2002). These probes were selected from a collection of *S. canicula* embryonic cDNA library (mixed stages, S9 to 22), submitted to high throughput EST sequencing (coord. Dr. Sylvie Mazan at the Station Biologique de Roscoff, France). cDNA fragments, kindly provided by Dr. Mazan, were cloned in pSPORT vectors. Cloned fragments were amplified by PCR and then purified using a High Pure kit (Roche). Sense and antisense digoxigenin-UTP-labeled *Dlx2, Nkx2.1 Tbr1, Emx1, Lhx9* and *Otp* (*ScDlx2*, *ScNkx2.1, ScTbr1, ScEmx1, ScLhx9 and ScOtp*) were synthesized directly by transcription in vitro. *In situ* hybridization was performed on cryostat sections of stages 29 to prehatching embryos following standard protocols (Coolen et al. 2007). Briefly, sections were permeabilized with proteinase K, hybridized with sense or antisense probes overnight at 65 ºC and incubated with the alkaline phosphatase-coupled anti-digoxigenin antibody (1:2000, Roche Applied Science, Manheim, Germany) overnigh at 4ºC. The color reaction was performed in the presence of BM-Purple (Roche). Finally, sections

were dehydrated and coverslipped. Control sense probes did not produce any detectable signal.

Image acquisition and analysis

Light field images were obtained with an Olympus BX51 microscope equipped with an Olympus DP71 color digital camera. Fluorescent sections were analyzed and photographed with the TCS-SP2 scanning microscope with a combination of blue and green excitation lasers. Stacks of confocal images were acquired separately for each laser channel with steps of 0.8 or 2 µm along the z-axis and collapsed images were obtained from an average of 12 optical sections with the LITE software (Leica). Some sections were photographed with an epifluorescence photomicroscope Olympus AX70 fitted with an Olympus DP70 color digital camera. Photographs were adjusted for brightness and contrast and plates were prepared using Adobe Photoshop CS4 (Adobe, San Jose, CA).

RESULTS

In this study we have analyzed the boundaries and basic subdivisions of the telencephalon of *S. canicula* by means of the combinatorial analysis of the expression patterns of some proteins and key developmental regulatory genes. We present data from two main developmental periods (stage 31 and 32) as representatives of early and late states of telencephalic development. Stage 31 corresponds with a period in which basic regionalization has occurred that is

easily comparable with key embryonic stages of other vertebrates (mouse: E12.5; chick: E6.5-E7/ HH30, approximately). During stage 32, important morphological changes take place and the basic mature structure of the telencephalon is achieved. Therefore, the cytoarchitecture and organization of the telencephalon from stage 32 onwards is highly similar to that observed in juvenile and adults. Information from stages 33 and 34 (prehatching) specimens is not included as the gene expression patterns observed during this late period are identical to that obtained in stage 32 embryos.

Principal boundaries and histogenetic subdivisions of the evaginated telencephalon of *S. canicula*

Pallial-subpallial boundary (PSB)

Previous studies of our group intended to describe the PSB at different embryonic stages in *S. canicula* by analyzing Pax6 and GAD markers separately (Carrera et al. 2008; Ferreiro-Galve et al. 2008). These studies allowed to define this limit at ventricular levels but were insufficient to clearly identify its position at the intermediate and marginal zones of the telencephalic walls. In order to define this boundary accurately we have used double labeling with Pax6 and GAD and extended the comparison to other genetic markers.

At embryonic stage 29, GAD expression exhibits a sharp dorsal limit that demarcates the position of the PSB (Figs. 1A,A''). Intense Pax6 expression occupies the ventricular region of the entire pallium while progressively weaker

Pax6 expression is also detected within the dorsal-most subpallial zone (see Figs. 1A,A'). At stage 31, the intermediate and marginal zones become wider and the ventricular region occupies less than one third of the total thickness of the telencephalic walls (see Figs. 1B-B''). Intense Pax6 expression was detected in the ventricular zone of the pallium (Figs. 1B,B'). As previously described (Ferreiro-Galve et al. 2008), the ventral limit of this ventricular expression represents the landmark of the PSB at this developmental period. However, the PSB is not so easily traceable in the intermediate zone. Based on the apparent dispersion of pallial (Pax6-positive) cells, it seems that the pallial territory in the intermediate zone extends ventrally with respect to its ventricular limit (see broken line in Fig. 1B') which coincides with the limit of expression of GAD at this level (see broken line in Fig. 1B'',C). By comparing GAD (Fig. 1C) versus Pax6 (Fig. 1G) and *Tbr1* (a well-known pallial marker) (Fig. 1H) expression, we were able to confirm the position of the PSB. Of note, this limit was parallel to a stream of Pax6-expressing cells in the intermediate and marginal zone of the telencephalic walls (arrows in Fig. 1G) that was previously reported by Ferreiro-Galve (2010) as a subpallial cell population. The present results demonstrate that the disposition of this stream of Pax6 cells is coincident with the ventral border of the *Tbr1* domain (see broken line in Figs. 1G,H and red dots in Fig. 1K).

Pallial and subpallial subdivisions

Early development (stage 31)

Subpallium

The entire subpallial territory was characterized by the expression of *Dlx2* which, at this developmental period, showed identical distribution to that of GAD (Figs. 1C,D). Some streams of GAD/*Dlx2* positive cells were observed at pallial zones and interpreted as possible palliopetal migrating cells from the subpallium (Carrera et al. 2008; arrows in Figs. 1C,D; see Chapter 5). The transcriptional factor *Nkx2.1* was expressed in the ventricular zone of a conspicuous protrusion in the most medial region of the subpallium (Fig. 1E), which defines the extension of the pallidal compartment of the subpallium, that is, the homolog in *S. canicula* of the MGE (see Figs.1E,F). Therefore, the remaining territory of the subpallium (*Nkx2.1* negative, GAD/*Dlx2* positive) has been identified as the striatal subdivision, i.e., the homolog in *S. canicula* of the LGE (see Fig. 1F).

Pallium

The combinatorial expression pattern of Pax6, *Tbr1, Emx1* and *Lhx9* allowed us to tentatively divide the pallium of *S. canicula* early embryos in different histogenetic regions: (1) the most medial part of the pallium, mainly defined by the strong *Lhx9* expression (Figs. 1I,K); (2) *Emx1* was expressed in a restricted medial region of the *Lhx9* domain occupying ventricular and marginal zones (Figs.

1J,K); (3) adjacent to medial territory, a domain was found that contained Pax6 cells not only at the ventricular zone but also at intermediate and marginal levels (Figs. 1G,K); none of the other markers were expressed in this region; (4) laterally to the former, a region was identified that strongly expressed *Tbr1* (Figs. 1H,K) and *Emx1* (Figs. 1J,K) and showed weak expression of *Lhx9* (Figs. 1I,K); (5) the most ventral part of the pallium corresponds to the region that showed weak expression of *Tbr1* (arrow in Fig. 1H) and very weak expression of *Lhx9* (arrow in Fig.1I); this region was negative for *Emx1* (arrow in Fig. 1J).

Main findings about pallial regionalization have been summarized in Fig. 1K. A summary of the pallial and subpallial subdivisions proposed is showed in Fig. 1L.

Late development (stage 32).

At stage 32, discrete histogenetic domains could be still identified by the same developmental genes described above. As exposed above, during this stage the basic cytoarchitecture of the telencephalon was easily comparable with that of juvenile and adult specimens; therefore analyzing pallial and subpallial derivatives during this period should shed light into the identification of adult structures in *S. canicula*.

Subpallial derivatives

From stage 32 on, the subpallial territory could be still delineated by means of the expression of *Dlx2* (broken line in Fig. 2A), though *Dlx2* was also widely

dispersed across pallial regions, particularly at the level of the olfactory bulbs (Fig. 2A; see also Carrera et al., 2008 and Chapter 5). Of note, the expression of *Dlx2* was weaker in the most medial protrusion of the subpallium. *Nkx2.1* was expressed in the ventricular zone of this medial protrusion demarcating the extension of the MGE (broken line in Fig. 2B) as at previous stages of embryogenesis, although some dispersion of *Nkx2.1*-positive cells was detected in pallial and striatal zones (arrows in Fig. 2B; see also Chapter 5). Thus, combined expression of these markers allowed us to accurately define striatal and pallidal derivatives in late embryos (Fig. 2C).

Pallial derivatives

As in previous developmental stages, different regions can be identified on the basis of the expression of *Lhx9, Tbr1* and *Emx1* (Figs. 3D-G): (1) the most medial domain experimented a patent growth with respect to the previous developmental stage and formed two evident protrusions that showed a strong expression of *Lhx9* (arrows in Fig. 2D, E). This region appeared negative for *Tbr1* (Fig. 2F); (2) just dorsal to the former, we observed a domain with intense expression of *Emx1* (Fig. 2G,H) but not of *Lhx9* or *Tbr1*; (3) laterally to the former, a region negative for the three markers was observed (Figs. 2H,I); (4) weak expression of *Emx1* was found in the adjacent lateral domain, which additionally expressed *Tbr1* (arrows in Figs. 2F, G; Fig. 2H); (5) the most ventral part of the pallium showed strong expression of both *Lhx9* (arrow heads in Figs. 2D, E; Fig.

2H) and *Tbr1* (arrowhead in Fig. 2F; Fig. 2H); this domain was adjacent to the *Dlx2*-expressing subpallial domain (compare with Fig. 2A); (6) *Tbr1* expression was strong in the glomerular layer of the olfactory bulb (asterisks in Fig.2F). These findings are summarized in Fig. 2I.

Because Pax6-positive cells were broadly dispersed across the telencephalon from stage-32 embryos (Ferreiro-Galve 2010) onwards, we have not used this marker to define pallial subdivisions in late embryos.

Delimitation of the preoptic area

The preoptic area represents a subdivision of the subpallium that corresponds with the non-evaginated telencephalon (for a review, see Medina 2008). This domain has been characterized in different vertebrates by the combined expression of *Nkx2.1, Dlx2* and *Shh* (see introduction above). Therefore, we aimed to delimit the preoptic compartment in the telencephalon of *S. canicula* on the basis of the expression of these developmental genes.

The preoptic area limits anteriorly with the pallidal compartment. Both structures show ventricular expression of the transcriptional factor *Nkx2.1*, but only the preoptic region expresses *Shh*. Thus we have delineated the pallidal-preoptic boundary based on their different expression of Shh (see Figs. 3A-F). The Shh positive domain was considered as the preoptic compartment (Figs. 3B,D,F). The comparison of the expression patterns of Shh and *Nkx2.*1 reveals the boundary between both territories (broken line in Fig. 3A-D). At more caudal

levels, the territory of the preoptic area extended along the region related to the anterior commissure (Fig. 3E,F).

The posterior limit of the preoptic region (preoptic-hypothalamic limit) corresponds with the frontier between the telencephalon and hypothalamus (alar part). This boundary has been defined as the interface of territory expressing *Dlx2* (that corresponds to the preoptic part of the subpallium) and that expressing *Otp* (that corresponds to the alar hypothalamus) (reviewed in Medina 2008). We have thus analyzed the expression pattern of these genes to tentatively define the telencephalon-hypothalamic boundary. At anterior levels (Figs. 3G,H), *Dlx2* is expressed in a broad domain representing the caudal part of the preoptic area (Fig. 3H); scattered *Otp*-positive cells were detected in this region (Fig. 3H), which could represent migrating cells originated in the hypothalamus (see discussion below). More caudally (Figs. 3I,J) *Dlx2* defines a neat boundary probably corresponding to the onset of the hypothalamic territory (see broken line in Fig. 3I). *Otp* expression is slightly complementary at this level probably due to the quantity of immigrant hypothalamic cells entering in the preoptic area. Still, it is possible to trace the boundary in relation to *Dlx2* distribution (see broken line in Fig. 3J). As we analyze more caudal sections, *Otp* expression clearly complements that of *Dlx2*, unmistakably defining the telencephalic-hypothalamic boundary (Figs. 3K-N).

DISCUSSION

Identification of the main telencephalic boundaries and subdivisions in *S. canicula*. Comparison with gene expression patterns in other vertebrates.

The analysis of the combined expression of key developmental genes used in this study allowed us to recognize the main boundaries and telencephalic domains in the brain of *S. canicula* as have been described in tetrapods.

Pallial-subpallial boundary (PSB)

The PSB represents a key anatomical landmark with important roles in dorsoventral patterning (Fishell et al. 1993; Chapouton et al. 1999; Marín and Rubenstein 2003; Carney et al. 2006). Its position has been addressed in different vertebrates by the analysis of the distribution of pallial and subpallial markers such as Pax6, *Tbr1*, GAD, Dlx2, Dbx1 or Sp8 (Ekström and Ohlin 1995; Melendez-Ferro et al. 2002; Wullimann and Rink 2002; Carney et al. 2006; Moreno et al. 2010). In *S. canicula* the position of the PSB cannot be correlated with evident anatomical landmarks. Previous studies in this species have described the PSB by analyzing Pax6 and GAD markers separately (Carrera et al. 2008; Ferreiro-Galve et al. 2008). These studies allowed to define the boundary at ventricular levels but leaded to misinterpret its position at the intermediate and marginal zone of the telencephalic walls. By analyzing the combined expression of subpallial (GAD/*Dlx2*) and pallial (*Lhx9/Tbr1*) markers we have redefined the location of this boundary (present results). Of note, from stage 31 onwards, a string of Pax6

expressing cells was shown to serve as a reliable landmark for the PSB position at intermediate levels, as in other vertebrates (Puelles et al. 2000; Torreson et al. 2000; Yun et al. 2001; Wullimann and Mueller 2004; Tole et al. 2005; Carney et al. 2006; Flames et al. 2007).

Subpallial subdivisions

Lateral ganglionic eminence (LGE)

The LGE or developing striatum is defined by the expression of distal-less genes as *Dlx2* (described in all vertebrates studied including lampreys : Myojin et al. 2001; Neidert et al. 2001; reviewed in Moreno et al. 2009), some subdomains expressing Pax6 (Bulfone et al. 1993; Flames et al. 2007; Stoykova et al. 2000) and the absence of *Nkx2.1* expression (reviewed in Moreno et al. 2009). The dorsal part of the LGE has been shown to present a characteristic distribution of Pax6-positive cells forming a stream on its marginal zone (Puelles et al. 2000; Stenman et al. 2003; Wullimann and Mueller 2004; Carney et al. 2006; Flames et al. 2007). Under these criteria we have defined a *Dlx2*-positive and *Nkx2.1*-negative area in the subpallium that we propose as the equivalent to the LGE or striatal homolog of *S. canicula*. Although the ventricular region of the proposed LGE shows weak Pax6 expression during early development, as in other vertebrates (Flames et al. 2007), this feature does not seem to be present at posterior stages of embryogenesis in *S. canicula*. In fact, from stage 30, Pax6 is absent from the entire ventricular zone of the subpallium (Ferreiro-Galve 2010 and present results),

which might be interpreted as a main difference with other vertebrates. On the other hand, the proposed LGE area was bordered by a conspicuous string of Pax6 cells (those that extended along the proposed PSB, see above) which may correspond to the migratory stream of Pax6 cell observed in other vertebrates delimiting the LGE territory (Puelles et al. 2000; Campbell 2003; Wullimann and Mueller 2004; Carney et al. 2006). Although the ventricular zone of the pallium appears to be the potential source for these Pax6 cells, more studies are needed to know if this is the only source as reported in *Xenopus* (Moreno et al. 2008b) or if they may derive in part from the dorsal subdivision of the LGE as reported in amniotes (Puelles et al. 2000; Flames et al. 2007; Abellán and Medina 2009; Moreno et al. 2010; Bupesh et al. 2011; Medina et al. 2011). Whatever its origin, this stream of Pax6 cells is a useful landmark in *Scyliorhinus* of the dorsolateral margin of the LGE equivalent.

Medial ganglionic eminence (MGE)

The MGE or developing pallidum has been characterized in other vertebrates by Dlx1/2/5, strong *Nkx2.1* and lack of Pax6 expression (Bulfone et al. 1993; Sussel et al. 1999; Puelles et al. 2000; Flames et al. 2007; Moreno et al. 2009). The region showing these features in the subpallium of *S. canicula* corresponded with a medial protrusion with strong *Nkx2.1* expression at the ventricular zone. Hence, the limit between LGE and MGE in this species can be exactly correlated with a

ventricular sulcus in the ventromedial telencephalon that can be also recognized in late embryos and postnatal specimens.

In teleost fishes, the position of the MGE derivatives is not clear and the possibility that pallidal cells are intermingled with those of the striatum has been discussed (Alunni et al. 2004; Wullimann and Mueller 2004), while in agnathans *Nkx2.1* is absent from the telencephalon and a pallidal compartment could not be defined on these terms (Ogasawara et al. 2001; Osório et al. 2005; Sugahara et al. 2013; reviewed in Moreno et al. 2009 and Medina et al. 2011). The situation in sharks seems closer to that described in tetrapods where clearly separated striatal and pallidal domains were observed (present results). Nevertheless, the characterization of a pallidal territory in lamprey based on connectivity, neurochemistry and electrophysiological properties has led to consider the origin of the pallidum in the agnathan lineage (Stephenson-Jones et al. 2012). However, to our knowledge, the fact that *Nkx2.1* is not expressed on the lamprey subpallium implies that cartilaginous fishes represent the most basal vertebrates that express this gene in the telencephalon and emphasizes the importance of the developmental study of *S. canicula* to assess how basal ganglia should evolve.

Preoptic area (PO)

Recent segmental models of brain development contemplate the non-evaginated region of the telencephalon anterior to the optic chiasm, namely the preoptic region, as part of the subpallium (reviewed in Moreno et al. 2009;

Medina et al. 2011; Puelles et al. 2012). It represents the posterior-most area of the telencephalon limiting anteriorly with the MGE and posteriorly with the alar hypothalamus, forming the preoptic-hypothalamic border region close to the chiasmatic region (Moreno and González 2011). This area has been defined in all tetrapods by the ventricular expression of *Nkx2.1* and *Shh* (Flames et al. 2007; Moreno and González 2011), though the presence of scattered *Otp*-expressing cells was additionally described in the turtle (Moreno et al. 2010). We have delimited a region with these features in the non-evaginated part of the embryonic telencephalon of *S. canicula*, which thus represent the primordial preoptic region. An equivalent compartment has been described in terms of *Shh* and *Dlx* expression in teleost fishes (Scholpp et al. 2006; Menuet et al. 2007) and amphibians (Moreno et al. 2008c; Domínguez et al. 2010). However, in lampreys (jawless fish) the expression of *Nkx2.1* or *Shh* in the telencephalon could not be demonstrated (Murakami et al. 2005; Osório et al. 2005; Sugahara et al., 2013), which implies that both pallidal (see above) and preoptic regions might emerge during the transition from agnatans to gnatostomes, as has been previously suggested (Osório and Rétaux 2008; reviewed in Moreno and González 2011).

The posterior limit of the preoptic subdivision (the preoptic-hypothalamic boundary) was assessed by the combined expression of *Otp* and *Dlx2*. In all vertebrate groups examined so far, the alar hypothalamus expresses *Otp* and lacks *Dlx2* in a very conserved pattern, while the preoptic area (when present) exhibit *Dlx2*-expressing cells and only scattered *Otp*-expressing cells (Bardet et al.

2008; Medina 2008; Moreno et al. 2010; Moreno and González 2011). In *S. canicula*, the interface between the expressions of these two markers has served as a reliable landmark to delimit this boundary.

Pallial subdivisions.

The subdivisions of the pallium have been established on the basis of differential expression of key regulatory genes in most vertebrates (reviewed in Medina et al. 2011). The pallium of all vertebrates studied so far is defined as the dorsal telencephalic region rich in *Emx1, Pax6* and negative to Distal-less genes as *Dlx2* (Puelles et al. 2000; Murakami et al. 2001; Wullimann and Mueller 2004; Medina 2008; Medina and Abellán 2009; Moreno et al. 2009). In tetrapods, four major domains were described in the pallium, including medial, dorsal, lateral and a novel ventral pallial subdivision (reviewed in Medina and Abellán 2009).

The recently identified ventral pallial domain is defined as the ventral most aspect of the pallium (in direct contact to the PSB) in which *Emx1* is not expressed (Fernandez et al. 1998; Puelles et al. 2000; Bachy et al. 2002; Brox et al. 2003, 2004). This region was previously included within the territory of the lateral pallium, but it is now considered to represent a distinct radial pallial domain that gives rise to the ventral part of the piriform cortex, claustral complex and pallial amygdala in mammals (Medina and Abellán 2009). The ventral pallium of tetrapods typically expresses *Lhx9* in the mantle zone (Moreno et al. 2004; García-López et al. 2008; Abellán et al. 2009). An equivalent ventral pallium was

tentatively identified in non-tetrapods as zebrafish and lamprey as a pallial region poor in *Emx1* (Murakami et al. 2001; Wullimann and Mueller 2004; reviewed in Medina and Abellán 2009). Nevertheless, the existence and precise extent of this territory must be corroborated by more detailed gene expression studies (Wullimann and Mueller 2004). In our study we present data which strongly support the existence of a ventral pallial domain in the brain of *S. canicula* (number 5 in Figs. 1K,L), since it showed the main features described in tetrapods (that is, *Tbr1* and *Lhx9* expression and absence of *Emx1*-expression), which allows to accurately delimit the extent of this domain and represents the first solid description of a ventral pallium in non-tetrapod vertebrates.

On the other hand, the territory immediately dorsal to the proposed ventral pallium that express *Emx1* and *Tbr1* appears as a good candidate to represent the lateral pallium of *S. canicula* (number 4 in Figs. 1K,L) since these features have been used to define this region in other vertebrates (reviewed in Medina and Abellán 2009). However, further data would be needed to confirm this assumption.

The medial pallium of mouse and chicken has been delimited by the specific expression of *Lhx9* (Rétaux et al. 1999; Bulchand et al. 2003; Abellán et al. 2009). In this study, we have observed that the medial-most subdivision of the pallium of *S. canicula* intensely expressed *Lhx9* throughout development, so we propose this domain to represent the medial pallial compartment (number 1 in Figs. 1K,L),

homolog to that described in other vertebrates. Although *Lhx9* gene was studied in the developing brain of the medaka fish (Alunni et al. 2004), no clear conclusion was drawn about the delimitation of the medial pallial compartment in teleost fishes.

The possibility that the *Emx1* positive medial region included in the *Lhx9* medial territory (number 2 in Figs. 1 K,L) represent an additional domain will be discussed in the context of its possible derivative in next section.

The region lying between the proposed lateral and medial pallial sectors, which does not express any of the genes analyzed in this study with the exception of Pax6 (that extends in the intermediate and marginal zones), should topographically correspond to the dorsal pallium of *S. canicula* (number 3 in Figs. 1K,L), the homolog region to the dorsal pallium of tetrapods. However, its identification by means of conserved gene expression patterns turns out more difficult to achieve than for other pallial compartments, probably due to the great variability of this region throughout the vertebrate phylogeny. Still, the fact that it receives a *Dlx2*-expressing stream of cells from the subpallium and that it expresses Reelin in a subpial position (see Chapter 5), as in mammals (Gadisseux et al. 1992; Meyer et al. 1998; Lavdas et al. 1999; Corbin et al. 2001; Wichterle et al. 2001; Morante-Oria et al. 2003; Antypa et al. 2011), support our suggestion.

Finally, it is interesting to highlight the most striking difference found in the expression pattern of the markers analyzed in *S. canicula* with respect to other

vertebrates. That is the stream of Pax6 cells observed in the marginal zone of the pallium (proposed dorsal pallium) at stage 31. To our knowledge, there is no report of such stream in the pallium of any other vertebrate analyzed. Although Pax6 is a conserved gene along phylogeny, some important differences regarding its expression pattern have been also reported in other vertebrates. For instance, most of the zebrafish pallium lacks Pax6 expression (Wullimann and Rink 2001), in contrast to that observed in the pallium of other vertebrate groups. However, both structures undeniably represent homologous regions. Despite some specific differences, the analysis of the distribution of conserved genes is a powerful tool to identify homologous territories between different animal groups. However, discordant data must be taken into account as they may serve as cues to understand the diversification of the brain plan in each animal group (Wullimann and Mueller 2004).

Pallial and subpallial derivatives. Implications in the interpretation of the identity of adult structures in elasmobranchs

The analysis of the expression of the selected genes during late development served us to shed light into the characterization of some adult structures whose identity in elasmobranchs have largely been a matter of controversy.

Subpallium/basal ganglia

In elasmobranchs, the existence of basal ganglia containing striatal and pallidal subdivisions is currently accepted although the territories that have been

proposed for representing these adult structures are still a matter of debate (for a thoughtful review about this issue see Chapter 4). Based on histochemical criteria, different authors have proposed different telencephalic domains in cartilaginous fishes as the striatal homologs of mammals. Smeets and colleagues (1983) labeled as striatum the cell mass situated in the ventrolateral walls of the telencephalic hemispheres (Smeets' striatum) (see Fig. 4). Northcutt et al. (1988) considered the ventral periventricular part of the subpallium, (located ventrally to the Smeets' striatum and called area *periventricularis ventrolateralis*, (APVL, see Fig. 4) as the proper striatum in *Squalus*. Posterior studies proposed both regions as belonging to the striatal subdivision (Sueiro 2003). Alternative, Carrera et al. (2012) considered the whole mediobasal telencephalon including the so termed *area superficialis basalis* (ASB) and the APVL (see Fig. 4) as the telencephalic basal ganglia, including pallidal and striatal components. Most of the equivalences proposed so far were based on histochemical criteria which obviously have brought confusion to this issue. Studies based on the expression of conserved developmental genes are necessary before unequivocally accept the identification of basal ganglia structures in this vertebrate group. On the basis of the present study, we hypothesize that the main derivatives of the LGE territory (identified on the basis of *Dlx2* expression and the lack of *Nkx2.1*) correspond, at least in part, to the adult ASB and APVL regions (see Fig. 4). This is an adventurous and innovative proposal as it opposes to the currently followed classical view that has considered the shark ASB as the pallidum homologous (Smeets et al. 1983; Reiner and

Carraway 1985; Northcutt et al 1988; Sueiro 2003; Carrera et al., 2012). However, based on the present genoarchitectonic data we consider the pallidal sector as the region exhibiting ventricular *Nkx2.1* expression which was observed occupying the medial protrusion of the subpallial compartment and corresponds to the caudal part of a zone classically considered the septal region (asterisk in Fig. 4). Our proposal is supported by the results of tract-tracing experiments further addressed in Chapter 4.

Finally, it is interesting to highlight that the ASB (one of the structures proposed as LGE derivative) is a vast structure that occupies almost the whole basal telencephalic zone. Despite it is usually conceived as a morphological unit, it probably presents a complex inner organization with specialized subzones. For instance, it is highly probably that the laterodorsal ASB may be part of an amygdala-like structure (possibly representing the subpallial component of the amygdaloid complex equivalent) as it exhibits intense expression of Pax6 and it is in direct contact with the proposed ventral pallium.

Pallium

The region identified as the ventral pallium in early embryos was easily recognized in late embryos (number 5 in Fig. 4A). It corresponds with the cell mass located in the ventrolateral walls of the telencephalic hemispheres, which have been classically labeled as the Smeets´ striatum (Smeets et al. 1983) in the adult telencephalon (see above and Fig. 4B). Here we show that this region

unequivocally expresses pallial markers as *Lhx9* and *Tbr1* throughout development and thus cannot be considered as part of the subpallium (Fig. 4A). As exposed above, the ventral pallial subdivision gives rise to the ventral part of the piriform cortex, claustral complex and pallial amygdala in mammals (Puelles et al. 2004; Medina and Abellán 2009). Therefore, this region likely represents the homolog of some of these structures in elasmobranchs.

The medial pallium of late embryos was clearly identified as two evident structures protruding in the medial region of the dorsal telencephalon which become fused in the medial plane and exhibit intense expression of *Lhx9* (number 1 in Fig. 4A). The medial pallial derivatives in other vertebrates mainly correspond to the hippocampal formation (reviewed in Medina and Abellán 2009). Consequently, the adult structures derived from this region probably represent the homolog in sharks of the hippocampal region or at least its ancestral form (MP in Fig. 4B). The hippocampus in mammals and birds is characterized by its complex layered structure and for being one of the few regions that retain proliferation capabilities in the adult brain. The adult medial pallium of sharks does not show signs of lamination as in other amniotes, although it has been described as one of the discrete regions where adult proliferation is present in *S. canicula* telencephalon (see Chapter 5).

Finally, adult derivatives from the dorsal and lateral pallial compartments could not be exactly characterized with the markers used in the present study although

their position can be postulated (DP and LP in Fig. 4). Of note, the *Emx1* positive medial domain (number 2 in Fig. 4A), defines a specific territory that correlates with the position of the regions classically termed *pallium dorsalis superficialis* (PDS in Fig. 4) and *pallium dorsalis centralis* (Pdc). Despite the classical nomenclature termed these regions as "dorsalis" it seems that these domains emerge as a subdomain of the *Lhx9* positive medial pallium (see Figs. 1J,L) and adopts a dorsal position in late embryos. However, further genetic, hodologic and functional studies would be necessary to shed light into the identity of these adult structures in cartilaginous fishes.

Final considerations

Despite some differences have been found which might correspond with specific features of cartilaginous fishes, our results imply that the basic genetic program for telencephalic development is already present in the most basal extant gnatostome and that, therefore, this developmental program is highly conservative throughout evolution.

REFERENCES

Abellán A, Legaz I, Vernier B, Rétaux S, Medina L (2009) Olfactory and amygdalar structures of the chicken ventral pallium based on the combinatorial expression patterns of LIM and other developmental regulatory genes. J Comp Neurol 516:166–186

Abellán A, Medina L (2009) Subdivisions and derivatives of the chicken subpallium based on expression of LIM and other regulatory genes and markers of neuron subpopulations during development. J Comp Neurol 515:465–501

Alunni A, Blin M, Deschet K, Bourrat F, Vernier P, Rétaux S (2004) Cloning and developmental expression patterns of Dlx2, Lhx7 and Lhx9 in the medaka fish (Oryzias latipes). Mech Dev 121:977–983

Antypa M, Faux C, Eichele G, Parnavelas JG, Andrews WD (2011) Differential gene expression in migratory streams of cortical interneurons. Eur J Neurosci 34:1584–1594

Bachy I, Berthon J, Rétaux S (2002) Defining pallial and subpallial divisions in the developing Xenopus forebrain. Mech Dev 117:163–172

Bachy I, Vernier P, Retaux S (2001) The LIM-homeodomain gene family in the developing Xenopus brain: conservation and divergences with the mouse related to the evolution of the forebrain. J Neurosci 21:7620–7629

Ballard WW, Mellinguer J, Lechenault H (1993) A series of normal stages for development of Scyliorhinus canicula, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). 267:318–336

Bardet SM, Martinez-de-la-Torre M, Northcutt RG, Rubenstein JL, Puelles L (2008) Conserved pattern of OTP-positive cells in the paraventricular nucleus and other hypothalamic sites of tetrapods. Brain Res Bull 75:231–235

Brox A, Puelles L, Ferreiro B, Medina L (2003) Expression of the genes GAD67 and Distal-less-4 in the forebrain of Xenopus laevis confirms a common pattern in tetrapods. J Comp Neurol 461:370–393

Brox A, Puelles L, Ferreiro B, Medina L (2004) Expression of the genes Emx1, Tbr1, and Eomes (Tbr2) in the telencephalon of Xenopus laevis confirms the existence of a ventral pallial division in all tetrapods. J Comp Neurol 474:562–577

Bulchand S, Subramanian L, Tole S (2003) Dynamic spatiotemporal expression of LIM genes and cofactors in the embryonic and postnatal cerebral cortex. Dev Dyn 226:460–469

Bulfone a, Puelles L, Porteus MH, Frohman MA, Martin GR, Rubenstein JL (1993) Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. J Neurosci 13:3155–3172

Bupesh M, Abellán A, Medina L (2011) Genetic and experimental evidence supports the continuum of the central extended amygdala and a mutiple embryonic origin of its principal neurons. J Comp Neurol 519:3507–3531

Campbell K (2003) Dorsal-ventral patterning in the mammalian telencephalon. Curr Opin Neurobiol 13:50–56

Carney RSE, Alfonso TB, Cohen D, Dai H, Nery S, Stoica B, Slotkin J, Bregman BS, Fishell G, Corbin JG (2006) Cell migration along the lateral cortical stream to the developing basal telencephalic limbic system. J Neurosci 26:11562–11574

Carrera I, Anadón R, Rodríguez-Moldes I (2012) Development of tyrosine hydroxylase-immunoreactive cell populations and fiber pathways in the brain of the dogfish Scyliorhinus canicula: new perspectives on the evolution of the vertebrate catecholaminergic system. J Comp Neurol 520:3574–3603

Carrera I, Ferreiro-Galve S, Sueiro C, Anadón R, Rodríguez-Moldes I (2008) Tangentially migrating GABAergic cells of subpallial origin invade massively the pallium in developing sharks. Brain Res Bull 75:405–409

Chapouton P, Gärtner A, Götz M (1999) The role of Pax6 in restricting cell migration between developing cortex and basal ganglia. Development 126:5569–5579

Coolen M, Menuet A, Chassoux D, Compagnucci C, Henry S, Leveque L, Da Silva C, Gavory F, Samain S, Wincker P, Thermes C, D'Aubenton-Carafa Y, Rodriguez-Moldes I, Naylor G, Depew M, Sourdaine P, Mazan S (2009) The dogfish Scyliorhinus canicula, a reference in jawed vertebrates. Emerging model organisms. A laboratory manual. Cold Spring Harbor, New York, pp 431–446

Corbin JG, Nery S, Fishell G (2001) Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. Nat Neurosci 4 Suppl:1177–1182

Derobert Y, Plouhinec JL, Sauka-Spengler T, Le Mentec C, Baratte B, Jaillard D, Mazan S (2002) Structure and expression of three Emx genes in the dogfish Scyliorhinus canicula: functional and evolutionary implications. Dev Biol 247:390-404

Domínguez L, González A, Moreno N (2010) Sonic hedgehog expression during Xenopus laevis forebrain development. Brain Res 1347:19–32

Ekström P, Ohlin LM (1995) Ontogeny of GABA-immunoreactive neurons in the central nervous system in a teleost, gasterosteus aculeatus L. J Chem Neuroanat 9:271–288

Fernandez a S, Pieau C, Repérant J, Boncinelli E, Wassef M (1998) Expression of the Emx-1 and Dlx-1 homeobox genes define three molecularly distinct domains in the telencephalon of mouse, chick, turtle and frog embryos:

implications for the evolution of telencephalic subdivisions in amniotes. Development 125:2099–2111

Ferreiro-Galve S (2010) Brain and retina regionalization in sharks: study based on the spatiotemporal expression pattern of Pax6 and other neurochemical markers. Doctoral Thesis, University of Santiago de Compostela, Spain

Ferreiro-Galve S, Carrera I, Candal E, Villar-Cheda B, Anadón R, Mazan S, Rodríguez-Moldes I (2008) The segmental organization of the developing shark brain based on neurochemical markers, with special attention to the prosencephalon. Brain Res Bull 75:236–240

Ferreiro-Galve S, Rodríguez-Moldes I, Anadón R, Candal E (2010) Patterns of cell proliferation and rod photoreceptor differentiation in shark retinas. J Chem Neuroanat 39:1–14

Ferreiro-Galve S, Rodriguez-Moldes I, Candal E (2012) Pax6 expression during retinogenesis in sharks: comparison with markers of cell proliferation and neuronal differentiation. J Exp Zool 318:91-108

Fishell G, Mason CA, Hatten ME (1993) Dispersion of neural progenitors within the germinal zones of the forebrain [published erratum appears in Nature 1993 May 20;363(6426):286]. Nature 362:636–638

Flames N, Pla R, Gelman DM, Rubenstein JL, Puelles L, Marín O (2007) Delineation of multiple subpallial progenitor domains by the combinatorial expression of transcriptional codes. J Neurosci 27:9682–9695

Gadisseux JF, Goffinet AM, Lyon G, Evrard P (1992) The human transient subpial granular layer: an optical, immunohistochemical, and ultrastructural analysis. J Comp Neurol 324:94–114

García-López M, Abellán A, Legaz I, Rubenstein JL, Puelles L, Medina L (2008) Histogenetic compartments of the mouse centromedial and extended amygdala based on gene expression patterns during development. J Comp Neurol 506:46–74

Gillis JA, Shubin NH (2009) The evolution of gnathostome development: Insight from chondrichthyan embryology. Genesis 47:825-841

Hevner RF, Shi L, Justice N, Hsueh Y, Sheng M, Smiga S, Bulfone A, Goffinet AM, Campagnoni AT, Rubenstein JL (2001) Tbr1 regulates differentiation of the preplate and layer 6. Neuron 29:353–366

Hofmann MH, Northcutt RG (2008) Organization of major telencephalic pathways in an elasmobranch, the thornback ray Platyrhinoidis triseriata. Brain Behav Evol 72:307–325

Hofmann MH, Northcutt RG (2012): Forebrain organization in elasmobranchs. Brain Behav Evol 80:142-151

Lavdas a a, Grigoriou M, Pachnis V, Parnavelas JG (1999) The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J Neurosci 19:7881–7888

Manso MJ, Anadón R (1993) Golgi study of the telencephalon of the small-spotted dogfish Scyliorhinus canicula . J Comp Neurol 333: 485-502

Marín O, Rubenstein JL (2003) Cell migration in the forebrain. Annu Rev Neurosci 26:441–483

Marín O, Rubenstein JLR (2003) Cell migration in the forebrain. Annu Rev Neurosci 26:441–483

Medina, L (2008) Evolution and embryological development of forebrain. In: Binder MD, Hirokawa N (eds) Encyclopedic Reference of Neuroscience. Springer-Verlag, pp 1172-1192

Medina L, Abellán A (2009) Development and evolution of the pallium. Semin Cell Dev Biol 20:698–711

Medina L, Bupesh M, Abellán A (2011) Contribution of Genoarchitecture to Understanding Forebrain Evolution and Development, with Particular Emphasis on the Amygdala. Brain Behav Evol 78:216–236

Medina L, Legaz I, Gonzalez G, De Castro F, Rubenstein JL, Puelles L (2004) Expression of Dbx1, Neurogenin 2, Semaphorin 5A, Cadherin 8, and Emx1 distinguish ventral and lateral pallial histogenetic divisions in the developing mouse claustroamygdaloid complex. J Comp Neurol 474:504–523

Melendez-Ferro M, Perez-Costas E, Villar-Cheda B, Abalo XM, Rodríguez-Muñoz R, Rodicio MC, Anadón R (2002) Ontogeny of gamma-aminobutyric acid-immunoreactive neuronal populations in the forebrain and midbrain of the sea lamprey. J Comp Neurol 446:360–376

Menuet A, Alunni A, Joly JS, Jeffery WR, Rétaux S (2007) Expanded expression of Sonic Hedgehog in Astyanax cavefish: multiple consequences on forebrain development and evolution. Development 134:845–855

Meredith GE, Smeets WJ (1987) Immunocytochemical analysis of the dopamine system in the forebrain and midbrain of Raja radiata: evidence for a substantia nigra and ventral tegmental area in cartilaginous fish. J Comp Neurol 265:530–548

Meyer G, Soria JM, Martínez-Galán JR, Martín-Clemente B, Fairén A (1998) Different origins and developmental histories of transient neurons in the marginal zone of the fetal and neonatal rat cortex. J Comp Neurol 397:493–518

Morante-Oria J, Carleton A, Ortino B, Kremer EJ, Fairén A, Lledo PM (2003) Subpallial origin of a population of projecting pioneer neurons during corticogenesis. Proc Natl Acad Sci U S A 100:12468–12473

Moreno N, Bachy I, Rétaux S, González A (2004) LIM-homeodomain genes as developmental and adult genetic markers of Xenopus forebrain functional subdivisions. J Comp Neurol 472:52–72

Moreno N, Domínguez L, Rétaux S, González A (2008c) Islet1 as a marker of subdivisions and cell types in the developing forebrain of Xenopus. Neuroscience 154:1423–1439

Moreno N, González A (2011) The Non-Evaginated Secondary Prosencephalon of Vertebrates. Front Neuroanat 5:12

Moreno N, González A, Rétaux S (2009) Development and evolution of the subpallium. Semin Cell Dev Biol 20:735–743

Moreno N, González A, Rétaux S (2008b) Evidences for tangential migrations in Xenopus telencephalon: developmental patterns and cell tracking experiments. Dev Neurobiol 68:504–520

Moreno N, Morona R, López JM, González A (2010) Subdivisions of the turtle Pseudemys scripta subpallium based on the expression of regulatory genes and neuronal markers. J Comp Neurol 518:4877–4902

Moreno N, Rétaux S, González A (2008a) Spatio-temporal expression of Pax6 in Xenopus forebrain. Brain Res 1239:92–99

Mueller T, Wullimann MF (2009) An evolutionary interpretation of teleostean forebrain anatomy. Brain Behav Evol 74:30–42

Murakami Y, Ogasawara M, Sugahara F, Hirano S, Satoh N, Kuratani S (2001) Identification and expression of the lamprey Pax6 gene: evolutionary origin of the segmented brain of vertebrates. Development 128:3521–3531

Murakami Y, Uchida K, Rijli FM, Kuratani S (2005) Evolution of the brain developmental plan: Insights from agnathans. Dev Biol 280:249–259

Myojin M, Ueki T, Sugahara F, Murakami Y, Shigetani Y, Aizawa S, Hirano S, Kuratani S (2001) Isolation of Dlx and Emx gene cognates in an agnathan species, Lampetra japonica, and their expression patterns during embryonic and larval development: conserved and diversified regulatory patterns of homeobox genes in vertebrate head evolution. J Exp Zool 291:68–84

Neidert AH, Virupannavar V, Hooker GW, Langeland JA. Lamprey Dlx genes and early vertebrate evolution (2001) Proc Natl Acad Sci 98:1665–1670

Northcutt RG, Reiner A, Karten HJ (1988) Immunohistochemical Study of the Telencephalon of the Spiny Dogfish , Squalus acanthias. J Comp Neurol 277:250–267

Ogasawara M, Shigetani Y, Suzuki S, Kuratani S, Satoh N (2001) Expression of thyroid transcription factor-1 (TTF-1) gene in the ventral forebrain and endostyle of the agnathan vertebrate, Lampetra japonica. Genesis 30:51–58

Osório J, Mazan S, Rétaux S (2005) Organisation of the lamprey (Lampetra fluviatilis) embryonic brain: insights from LIM-homeodomain, Pax and hedgehog genes. Dev Biol 288:100–112

Osório J, Rétaux S (2008) The lamprey in evolutionary studies. Dev Genes Evol 218:221–235

Puelles L (2001) Thoughts on the development, structure and evolution of the mammalian and avian telencephalic pallium. Philos Trans R Soc Lond B Biol Sci 356:1583–1598

Puelles L, Kuwana E, Puelles E, Bulfone A, Shimamura K, Keleher J, Smiga S, Rubenstein JL (2000) Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. J Comp Neurol 424:409–438

Puelles L, Martínez S, Marínez-de-la-Torre M, Rubenstein JLR (2004) Gene maps and related histogenetic domains in the forebrain and midbrain. In: Paxinos G (editor). The Rat Nervous System. Elsevier, Amsterdam pp 3-25

Puelles L, Medina L (2002) Field homology as a way to reconcile genetic and developmental variability with adult homology. Brain Res Bull 57:243–55

Puelles L, Martínez-de-la-Torre M, Bardet S, Rubenstein JLR (2012) Hypothalamus. In: Watson C, Paxinos G, Puelles L (eds). The mouse nervous system. Academic press, Elsevier, pp 221-312

Puelles L, Rubenstein JL (2003) Forebrain gene expression domains and the evolving prosomeric model. Trends Neurosci 26:469–476

Reiner A, Carraway RE (1985) Phylogenetic conservatism in the presence of a neurotensin-related hexapeptide in neurons of globus pallidus. Brain Res 341:365–371

Reiner A, Medina L, Veenman CL (1998) Structural and functional evolution of the basal ganglia in vertebrates. Brain Res 28:235–285

Rétaux S, Rogard M, Bach I, Bach I, Failli V, Besson MJ (1999) Lhx9: a novel LIM-homeodomain gene expressed in the developing forebrain. J Neurosci 19:783–793

Rodríguez-Moldes I (2009) A developmental approach to forebrain organization in elasmobranchs: new perspectives on the regionalization of the telencephalon. Brain Behav Evol 74:20-29

Scholpp S, Wolf O, Brand M, Lumsden A (2006) Hedgehog signalling from the zona limitans intrathalamica orchestrates patterning of the zebrafish diencephalon. Development 133:855–864

Smeets WJ (1990) The telencephalon of cartilaginous fishes. In: Jones EG, Peters A (eds) Comparative structure and evolution of cerebral cortex, part I. Cerebral cortex. Plenum Press, New York, pp 3-30

Smeets WJAJ, Marín O, González A (2000) Evolution of the basal ganglia: new perspectives through a comparative approach. J Anat 196:501–517

Smeets WJAJ, Nieuwenhuys R, Roberts BL (1983) Telencephalon. The central nervous system of cartilaginous fishes. Structure and functional correlations. Springer-Verlag, Berlin Heidelberg New York, pp 122–147

Smith-Fernández A, Pieau C, Repérant J, Boncinelli E, Wassef M (1998) Expression of the Emx-1 and Dlx-1 homeobox genes define three molecularly distinct domains in the telencephalon of mouse, chick, turtle and frog embryos: implications for the evolution of telencephalic subdivisions in amniotes. Development 125:2099–2111

Stenman J, Yu RT, Evans RM, Campbell K (2003) Tlx and Pax6 co-operate genetically to establish the pallio-subpallial boundary in the embryonic mouse telencephalon. Development 130:1113–1122

Stephenson-Jones M, Ericsson J, Robertson B, Grillner S (2012) Evolution of the basal ganglia; Dual output pathways conserved throughout vertebrate phylogeny. J Comp Neurol 520:2957–2973

Stoykova A, Treichel D, Hallonet M, Gruss P (2000) Pax6 modulates the dorsoventral patterning of the mammalian telencephalon. J Neurosci 20:8042–8050

Striedter GF (1997) The telencephalon of tetrapods in evolution. Brain Behav Evol 49:179–213

Stuesse SL, Cruce WL (1992) Distribution of tyrosine hydroxylase, serotonin, and leu-enkephalin immunoreactive cells in the brainstem of a shark, Squalus acanthias. Brain Behav Evol 39:77–92

Stuesse SL, Cruce WL, Northcutt RG (1991) Localization of serotonin, tyrosine hydroxylase, and leu-enkephalin immunoreactive cells in the brainstem of the horn shark, Heterodontus francisci. J Comp Neurol 308:277–29

Sueiro C (2003) Estudio inmunohistoquímico de los sistemas gabaérgicos del sistema nervioso central de peces elasmobranquios y su relación con sistemas catecolaminérgicos y peptidérgicos. Doctoral thesis, University of Santiago de Compostela, Spain

Sugahara F, Murakami Y, Adachi N, Kuratani S (2013) Evolution of the regionalization and patterning of the vertebrate telencephalon: what can we learn from cyclostomes? Curr Opin Genet Dev doi: 10.1016/j.gde.2013.02.008

Sussel L, Marín O, Kimura S, Rubenstein JL (1999) Loss of Nkx2.1 homeobox gene function results in a ventral to dorsal molecular respecification within the basal telencephalon: evidence for a transformation of the pallidum into the striatum. Development 126:3359–3370

Tole S, Remedios R, Saha B, Stoykova A (2005) Selective requirement of Pax6, but not Emx2, in the specification and development of several nuclei of the amygdaloid complex. J Neurosci 25:2753–2760

Toresson H, Potter SS, Campbell K (2000) Genetic control of dorsal–ventral identity in the telencephalon: opposing roles for Pax6 and Gsh2. Development 127:4361–4371

Wichterle H, Turnbull DH, Nery S, Fishell G, Alvarez-Buylla A (2001) In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. Development 128:3759–3771

Wullimann MF, Mueller T (2004) Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. J Comp Neurol 475:143–162

Wullimann MF, Rink E (2001) Detailed immunohistology of Pax6 protein and tyrosine hydroxylase in the early zebrafish brain suggests role of Pax6 gene in development of dopaminergic diencephalic neurons. Brain Res Dev Brain Res 131:173–191

Wullimann MF, Rink E (2002) The teleostean forebrain: a comparative and developmental view based on early proliferation, Pax6 activity and catecholaminergic organization. Brain Res Bull 57:363–370

Yun K, Potter S, Rubenstein JL (2001) Gsh2 and Pax6 play complementary roles in dorsoventral patterning of the mammalian telencephalon. Development 128:193–205

ABBREVIATIONS

APVL	*area periventricularis ventrolateralis*
ASB	*area superficialis basalis*
Cb	cerebellum
Di	diencephalon
DP	dorsal pallium
Hy	hypothalamus
LGE	lateral ganglionic eminence
LP	lateral pallium
Mes	mesencephalon
MGE	medial ganglionic eminence
MP	medial pallium
OB	olfactory bulb
P	pallium
PDS	pallium dorsalis superficialis
PO	preoptic area
PSB	pallial-subpallial boundary
Rh	rhombencephalon
Sp	subpallium
Str. Smeets	Smeets´ striatum
Tel	telencephalon
VP	ventral pallium

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Fig.1 Principal boundaries and subdivisions of the evaginated telencephalon during early development. **A,B:** Transverse sections showing the differential distribution of Pax6 and GAD immunoreactivities. The position of the pallial-subpallial boundary is inferred by means of Pax6 and GAD double immunofluorescence at stages 29 (A-A'') and 31 (B-B''). **C-F:** Parallel sections showing GAD (C), *Dlx2* (D) and *Nkx2.1* (E) expression. Combined expression patterns of these substances clearly delimit lateral and medial ganglionic eminence domains (illustrated in F). **G-K:** Parallel sections showing Pax6 (G), *Tbr1* (H), *Lhx9* (I), *Emx1* (J) expression. The combination of these markers allows us to define five different territories within the pallium (numbers in K). **L:** Summary of the expression patterns of all markers analyzed in this study and the proposed subdivisions. The level of the section is indicated in the small squared drawing. Scale bars: 300 μm

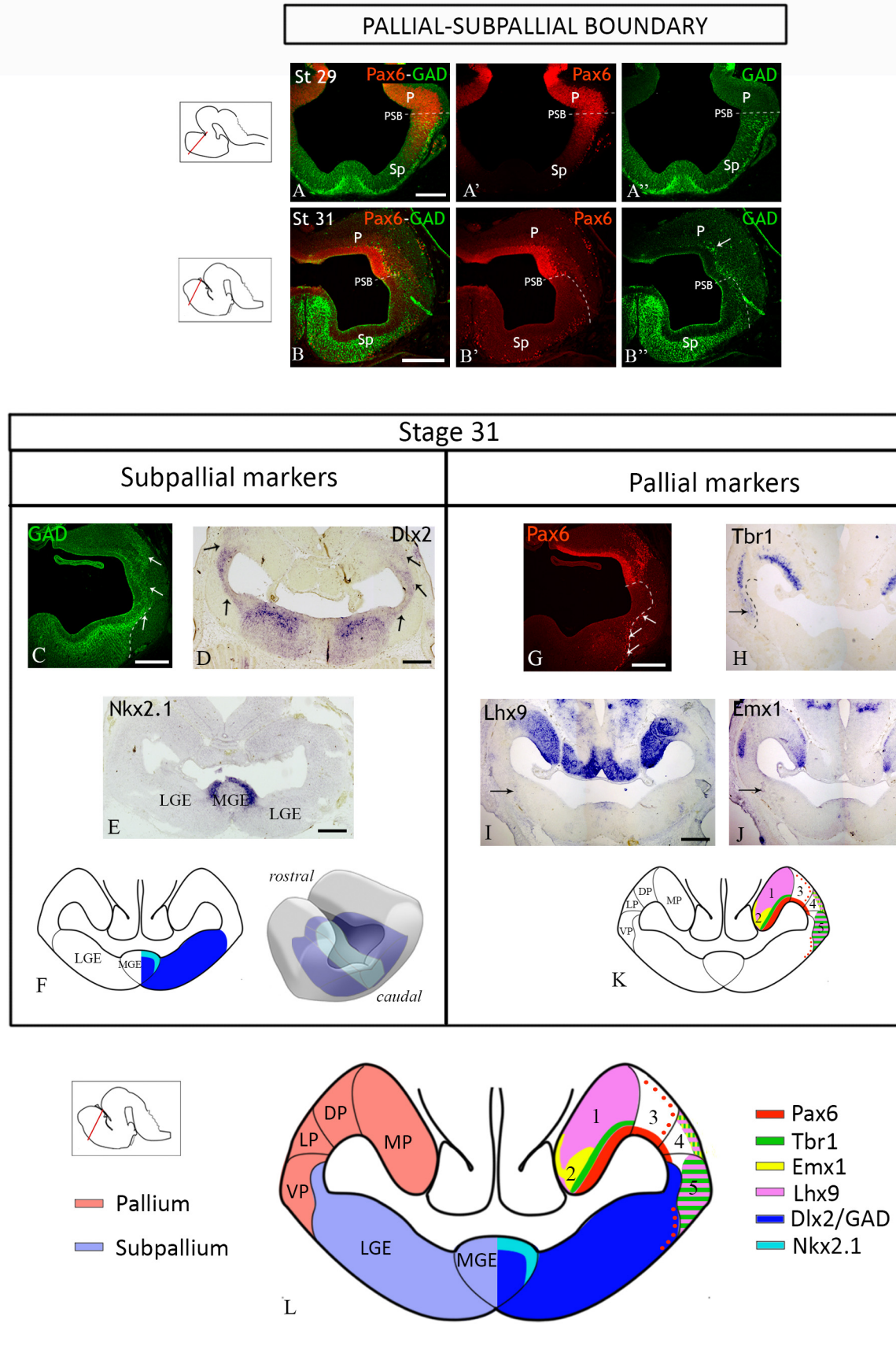

PALLIAL-SUBPALLIAL BOUNDARY
St 29
Pax6-GAD
Pax6
GAD
P
PSB
Sp
A
A'
A''
St 31
B
B'
B''
Stage 31
Subpallial markers
Pallial markers
GAD
C
Dlx2
D
Pax6
G
Tbr1
H
Nkx2.1
LGE
MGE
E
Lhx9
I
Emx1
J
rostral
caudal
F
DP
LP
MP
VP
K
Pallium
Subpallium
L
Pax6
Tbr1
Emx1
Lhx9
Dlx2/GAD
Nkx2.1

Figure 1

Fig.2 Principal subdivisions of the evaginated telencephalon during late development. **A-C:** Parallel sections showing *Dlx2* (A) and *Nkx2.1* (B) expression. Combined expression patterns of these substances delimit lateral and medial ganglionic eminence domains (illustrated in C). **D-H:** Parallel sections showing *Lhx9* (D,E), *Tbr1* (F) and *Emx1* (G) expression. The comparative analysis of the expression of these substances served us to tentatively define some pallial subdivisions (numbers in H). **I:** Summary of the expression patterns of all markers analyzed here and the proposed subdivisions. Acronyms in grey indicate that the positions of these regions are preliminary proposals. The level of the section is indicated in the small squared drawing. Scale bars: 300 μm

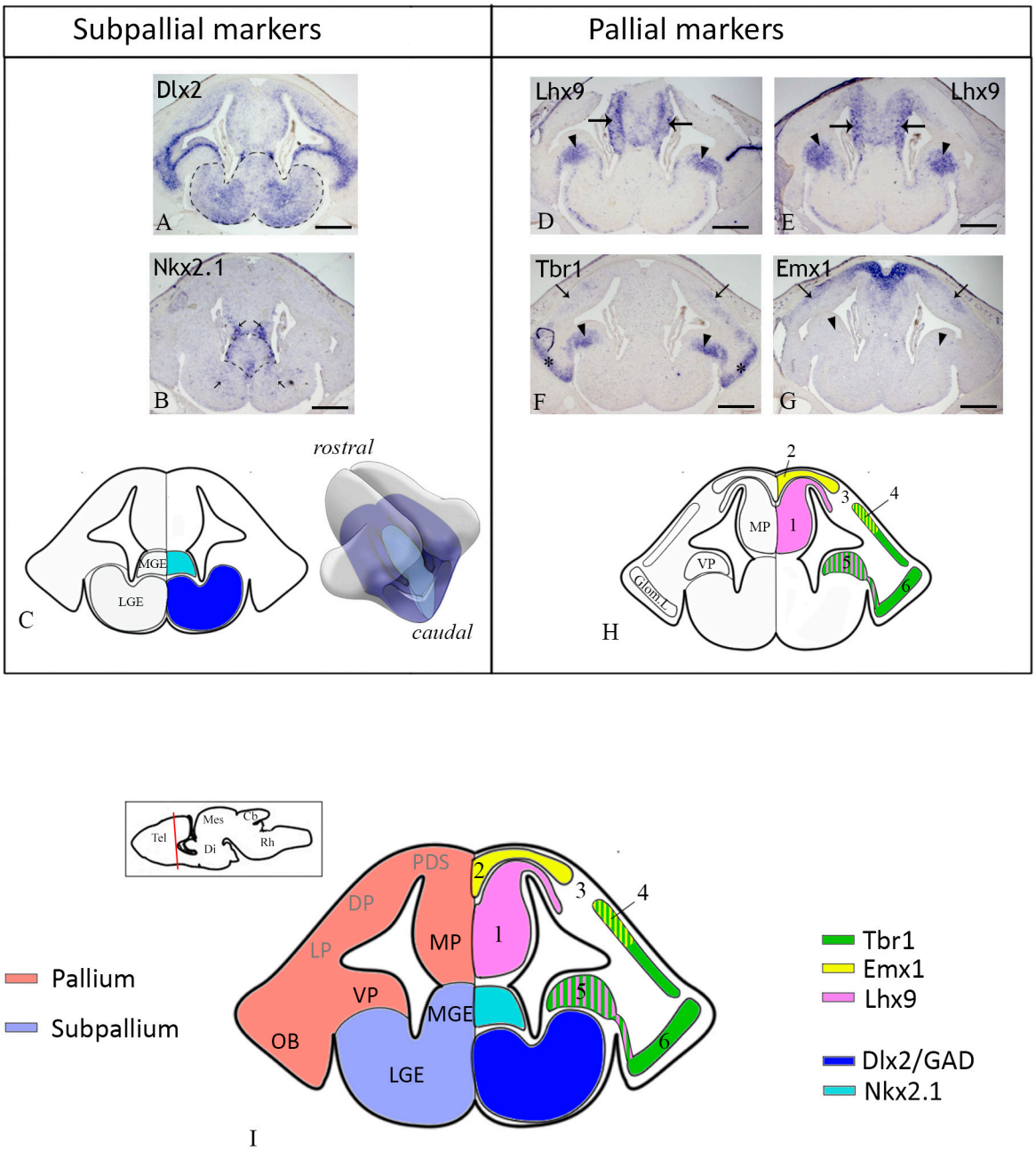

Subpallial markers
Pallial markers
Dlx2
A
Nkx2.1
B
Lhx9
D
Lhx9
E
Tbr1
F
Emx1
G
rostral
caudal
MGE
LGE
C
2
3
4
MP
1
VP
GlomL
6
H
Tel
Mes
Cb
Di
Rh
PDS
DP
LP
MP
VP
MGE
OB
LGE
1
2
3
4
5
6
I
Pallium
Subpallium
Tbr1
Emx1
Lhx9
Dlx2/GAD
Nkx2.1

Figure 2

Fig.3 Transverse sections showing anterior and posterior limits of the preoptic area (non-evaginated telencephalon) at stage 31. **A-F**: Sections through the caudal telencephalon at equivalent rostro-caudal levels showing the limit between the medial ganglionic eminence and the preoptic area by means of *Nkx2.1* (A,C,E) and Shh (B,D,F). **G-N:** Parallel sections across the caudal telencephalon and rostral hypothalamus to show the preoptic-hypothalamic limit by means of *Dlx2* (G,I,K,M) and *Otp* (H,J,L,N) in stage 31 embryos. Acronyms in grey indicate that the positions of these regions are preliminary proposals. The rostro-caudal level of each section is indicated in the squared drawings with a red line. For abbreviations, see list. Scale bars: 300 µm

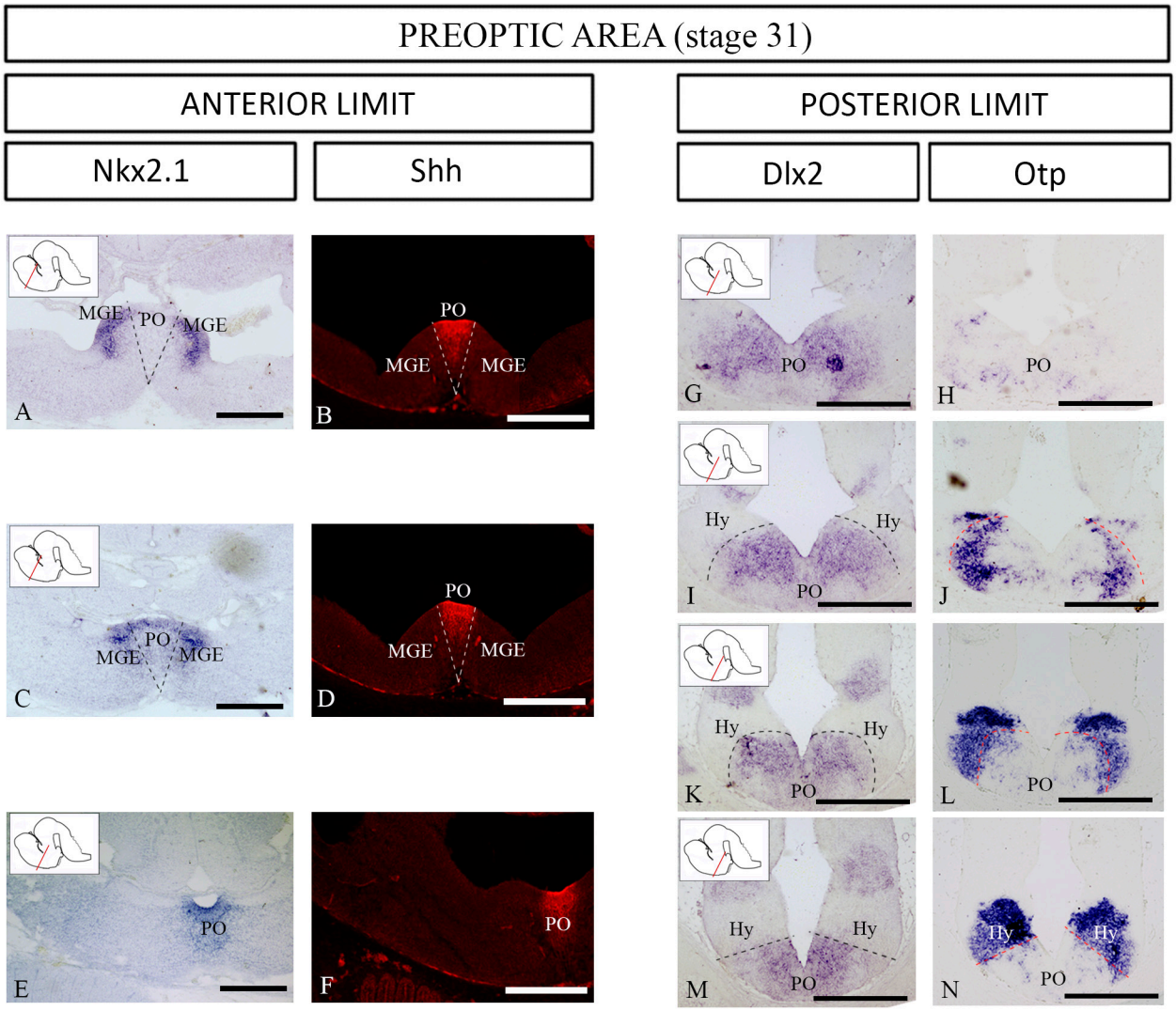

PREOPTIC AREA (stage 31)
ANTERIOR LIMIT
POSTERIOR LIMIT
Nkx2.1
Shh
Dlx2
Otp
MGE
PO
MGE
A
PO
MGE
MGE
B
PO
G
PO
H
Hy
Hy
PO
I
J
PO
MGE
MGE
C
PO
MGE
MGE
D
Hy
Hy
PO
K
PO
L
Hy
Hy
PO
E
PO
F
Hy
Hy
PO
M
Hy
Hy
PO
N

Figure 3

Fig.4. Main territories in the late *S. canicula* embryo defined on the basis of the expression of conserved genes (A) and their corresponding structures in adults (B). For abbreviations, see list.

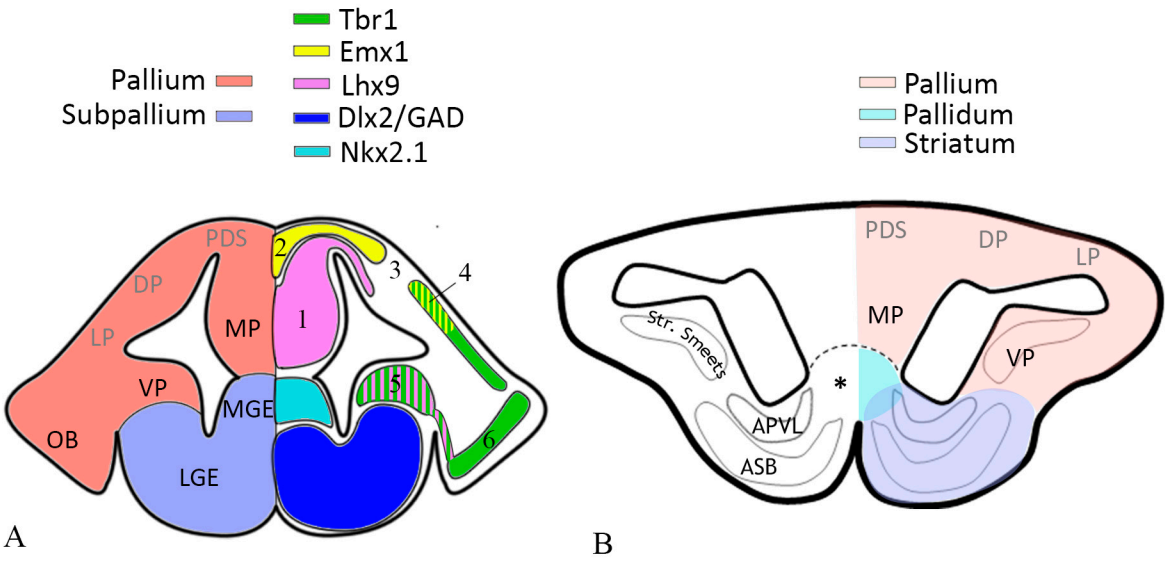

Pallium
Subpallium
Tbr1
Emx1
Lhx9
Dlx2/GAD
Nkx2.1
PDS
DP
LP
MP
VP
MGE
LGE
OB
1
2
3
4
5
6
A
Pallium
Pallidum
Striatum
PDS
DP
LP
MP
VP
Str. Smeets
*
APVL
ASB
B

Figure 4

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Chapter 4

Pilot study of basal ganglia connectivity

Some results of the present work are published in Quintana-Urzainqui I, Sueiro C, Carrera I, Ferreiro-Galve S, Santos-Durán G, Pose-Méndez S, Mazan S, Candal E, Rodríguez-Moldes I (2012) Contributions of developmental studies in the dogfish Scyliorhinus canicula to the brain anatomy of elasmobranchs: insights on the basal ganglia Brain Behav Evol 80:127-41.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

The basal ganglia are a group of nuclei located at the basal (subpallial) part of the telencephalon of mammals that are involved in the control of the motor behavior (Reiner et al. 1998; Smeets et al. 2000; Medina 2008; Reiner 2010). In fact, most of our knowledge about basal ganglia comes from the study of neurodegenerative human syndromes causing motor disorders as Parkinson's or Huntington's disease (Alexander and Crutcher 1990; DeLong 1990; Heimer et al. 1995). In a restrictive sense, the term basal ganglia refer to the striatal and pallidal components of the basal telencephalon that develop from the lateral and medial ganglionic eminences (LGE and MGE), respectively. Usually, other forebrain and midbrain structures are also included (as the subtalamic nucleus, substantia nigra or ventral tegmental area) as they form part of the basal ganglia circuitry. Although some features of basal ganglia vary among different vertebrate groups, it seems that all tetrapods exhibit some common recognizable traits (reviewed in Reiner et al. 1998; Smeets et al. 2000): 1) presence of striatopallidal systems in the telencephalon derived from embryonic structures (LGE and MGE) specified by the same subpallial transcriptional factors; 2) striatal projection towards a diencephalic-mesencephalic area (substantia nigra pars reticulata, SNr) through direct and indirect (via globus pallidus and subtalamic nucleus) pathways; 3) cortical and thalamic inputs to the striatum (probably related to the expansion of the cortex in reptiles and mammals); and 4) strong dopaminergic input to the

striatum from nuclei located in the basal plate of mesencephalic and diencephalic zones.

In all amniotes, the striatum receives catecholaminergic input mainly from basal diencephalic and mesencephalic cell populations [substantia nigra pars compacta (SNc, A9 catecholaminergic group) and the ventral tegmental area (VTA, A10 group)]. In amphibians, the catecholaminergic neurons projecting to the subpallium forms a continuous field along the rostrocaudal axis of the basal plate from rostral regions of the posterior tubercle, where the main catecholaminergic cell group of the diencephalon is located, to the mesencephalon (Marín et al. 1997a,b). Despite differences in the location and organization of these subpallial-projecting centers in tetrapods, their homology and functional equivalence is generally accepted. Some of these general features described for tetrapods have also been recognized in non-tetrapods as bony fishes or lampreys (Pombal et al. 1997a,b; Rink and Wullimann 2001, 2002; Mueller et al. 2008; Stephenson-Jones et al. 2012). However, the absence of mesencephalic catecholaminergic cells and its presence only in more rostral diencephalic groups as the posterior tubercle represent a main difference in the basal ganglia organization in these animals (reviewed in Reiner et al. 1998; Smeets and González 2000; Medina 2008; Reiner 2010).

In cartilaginous fishes, the existence of basal ganglia with striatal and pallidal subdivisions is currently accepted, although they have not been unambiguously identified. In fact, the correspondence among the various territories proposed for

being the adult striatal and pallidal homologous between different elasmobranch species is still controversial. Smeets and colleagues (1983) labeled as striatum the cell mass situated in the protrusion of the ventrolateral walls of the telencephalic hemispheres, where a dense plexus of TH immunoreactive fibers was noted (Smeets et al. 2000). This region could be equivalent to the 'striatal ridge' of Northcutt (1978) or striatal swellings of Holmgren (1922). Instead, Northcutt and colleagues (Northcutt et al. 1988) considered the striatal zone in *Squalus* as the region called *area periventricularis ventrolateralis* (APVL), which corresponds to the ventral part of the subpallium that occupies a periventricular position, just medial to the striatum labeled by Smeets (Smeets' striatum from here on) and internally to the region called *area superficialis basalis* (ASB). By its position, the APVL has been compared with the nucleus accumbens or ventral striatum (Smeets 1990). Also in the skate *Raja radiata,* the striatum has been identified as the area with the densest catecholaminergic innervation of the basal forebrain located internally to the ASB (Meredith and Smeets 1987; Smeets et al. 2000). Previous studies in our group led to propose the mentioned regions, APVL and Smeets' striatum as belonging to the striatal subdivision (Sueiro 2003). On the other hand, the ASB, which has been defined as the extensive U-shaped cell mass that occupies the outer ventral zone of the subpallium, has been considered as the pallidum by other authors (Reiner and Carraway 1985; Northcutt et al. 1988; Carrera et al. 2012), although histochemical characteristics of the olfactory tubercle (ventral striatum) have been also recognized (Northcutt et al. 1988). In

Squalus and *Raja*, the connectivity of Smeets' striatum, APVL and ASB with anatomical and functionally related centers in the diencephalic and rostral mesencephalic tegmentum has been tentatively defined on the basis of immunohistochemical data (Meredith and Smeets, 1987; Northcutt et al. 1988; Smeets 1998). In addition, it has been claimed that neurons in the tegmentum of diencephalon and mesencephalon belonging to the posterior tubercle nucleus and ventral tegmental area-substantia nigra (VTA/SN) are the source of the catecholaminergic innervation of the striatum (Meredith and Smeets 1987; Northcutt et al. 1988; Smeets 1990; Stuesse et al. 1991; Stuesse and Cruce 1992; Hofmann and Northcutt 2008; Carrera et al. 2012). Most of the equivalences and projections proposed so far are based on immunohistochemical criteria. Therefore, connectional and developmental studies based on the expression of conserved genes are necessary before unequivocally accepting the identification of striatal and pallidal structures in elasmobranchs.

The analysis of subpallial developmental gene expression in late embryos and prehatching specimens (Chapter 3) pointed to the ASB-APVL region to represent the homologous of the striatal compartment while the pallidal subdivision seemed to be located in the medial protrusion of the subpallium classically considered as part of the *nucleus septalis*. In contrast, the Smeets' striatum was found to express pallial markers (and not subpallial markers). These genoarchitectonic evidences did not support any of the proposals based on immunohistochemical criteria, thus representing a innovative interpretation of the

elasmobranch basal ganglia. To further characterize the ASB-APVL region and provide connectional data which help to accept or discard the proposal that it would represent the striatal compartment, we analyzed the connections of this region by means of tract-tracing experiments. We also examine their relationship with catecholaminergic cell groups of diencephalon and mesencephalon by means of immunohistochemistry for tyrosine hydroxylase (TH), the rate-limiting enzyme of catecholaminergic synthesis, combined with the visualization of the tracer.

MATERIAL AND METHODS

Experimental animals

Specimens were maintained in seawater tanks in standard conditions of temperature (15-16ºC), pH (7.5-8.5) and salinity (35g/L). Adequate measures were taken to minimize animal pain or discomfort. All procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by the Spanish Royal Decree 53/2013 for animal experimentation, and were approved by the Ethics Committee of the University of Santiago de Compostela.

In vitro tract-tracing techniques

Three juveniles of 14, 16 and 18 cms were used. The experiments were performed under *in vitro* whole-brain conditions. All specimens were deeply

anaesthetized in seawater containing 0.5% tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO). Animals were then perfused transcardially with 30 ml of ice-cooled elasmobranch´s phosphate buffer [EPB; 0.1 M phosphate buffer (PB) containing 1.75% urea] containing 1mM glucose, which was oxygenated with an oxygen injector aerator to a pH of 7.4. After animal decapitation, brains were isolated by removing the overlying cartilage skull and skin and transferred to fresh Ringer's solution to proceed with the immediate application of the tracer. We applied Neurobiotin (Vector Laboratories, Burlingame, CA), an amino derivative of biotin used as an intracellular label for neurons. This tracer has been mainly used as a retrograde tracer (Barreiro-Iglesias et al. 2008), although it also appeared to be transported anterogradely (Huang et al. 1992). The tracer was dissolved in distilled water until saturation and re-crystallized at the tip of an entomological needle (00) according to Morona et al. (2005) and the application was carried out manually or using a micromanipulator (Narishige MN-151, Japan) under a stereomicroscope. The ASB-APVL region was accessed by vertical penetrations and all applications were made unilaterally. After tracer application, brains were maintained for three days at 8ºC in continuously oxygenated elasmobranch Ringer's solution containing 1 mM glucose, and then fixed for two days in 4% paraformaldehyde (PFA) in EPB. Subsequently, they were rinsed in phosphate buffered saline (PBS), cryoprotected with 30% sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (20 µm thick) were

obtained in transverse planes on a cryostat and mounted on Superfrost Plus (Menzel-Glasser, Madison, WI) slides. Sections were pretreated with H_2O_2 to eliminate the endogenous peroxidase activity and then incubated with the preformed Avidin:Biotinylated enzyme Complex (Vectastain ABC system, Vector Laboratories) following the procedure indicated by manufacturers. The tracer was then visualized with 0.25 mg/mL diaminobenzidine tetrahydrochloride (DAB, Sigma) in PBS pH 7.4 with 2.5 mg/mL nickel ammonium sulfate and 0.00075% H_2O_2. All dilutions were made with PBS containing 0.2% Triton X-100 (Sigma) and 2 % bovine serum albumin (BSA, Sigma). Sections were then dehydrated and coverslipped.

Combined tract-tracing and TH immunohistochemistry

For combined tract-tracing and fluorescence immunohistochemistry parallel series of sections of the traced specimens were processed as described above. Primary antibody monoclonal mouse anti-Tyrosine Hydroxylase (TH; Millipore, Billerica; diluted 1:500) was incubated overnight at room temperature. Then, secondary antibody 488-conjugated donkey anti-mouse (DAM^{488}; Molecular probes; diluted 1:100) and rhodamine-labeled Avidin D (Vector Laboratories; diluted 1:1000) solutions were simultaneously incubated in a humid chamber for 2h at 37ºC. All dilutions were made with PBS containing 15 % donkey normal serum (Millipore, Billerica, MA) 0.2 % Triton X-100 (Sigma) and 2 % bovine serum albumin (BSA, Sigma). Slides were rinsed in PBS, then in distilled water, dried for

30 min at 37ºC, and mounted in MOWIOL 4-88 Reagent (Calbiochem, MerkKGaA, Darmstadt, Germany).

Image acquisition and analysis

Light field sections were photographed with an Olympus BX51 microscope equipped with an Olympus DP71 color digital camera. Double labeled fluorescent sections were analyzed and photographed with the TCS-SP2 scanning microscope with a combination of blue and green excitation lasers. Stacks of confocal images were acquired separately for each laser channel with steps of 0.8 or 2 µm along the z-axis and collapsed images were obtained from an average of 12 optical sections with the LITE software (Leica). Some sections were photographed with an epifluorescence photomicroscope Olympus AX70 fitted with an Olympus DP70 color digital camera. Photographs were adjusted for brightness and contrast and plates were prepared using Adobe Photoshop CS4 (Adobe, San Jose, CA). Images were not otherwise modified.

RESULTS

In order to evaluate if the ASB-APVL region may correspond with the striatal subdivision of the basal ganglia in *S.canicula*, we applied the tracer Neurobiotin to this structure in postnatal specimens to investigate its connections.

The recrystallized tracer was applied to the ASB-APVL region at the level indicated in figure 1 (arrowheads in Figs. 1A-C,G). Note that Smeets' striatum was

not affected (Figs. 1C,G). Some retrogradelly labeled cells were observed in the pallium, however since the experimental procedure often produced damage at this level, we could not obtain reliable results. Rostrally to the application site, we consistently detected retrogradely labeled cells in subpallial regions corresponding to the area superficialis basalis (mainly in its inner part) but not in the Smeets' striatum (Figs. 1C,G). Also consistent was the detection of retrogradely labeled cells caudal to the application site of the tracer, extending along the tegmentum of the diencephalon and mesencephalon. They were tentatively identified as belonging to the posterior tubercle nucleus (PT in Figs. 1D,E,H,I), substantia nigra (SN in Figs. 1E,F,I,J) and ventral tegmental area (VTA in Fig. 1F,J). Of note, a dense plexus probably representing terminal fields was also observed particularly in the region surrounding the posterior tubercle and SN (Figs. 1H,I).

With the aim of examining the relation of these subpallial projecting groups with the known catecholaminergic centers of the diencephalon and mesencephalon in *S.canicula* previously described by Carrera et al. (2012), we performed fluorescence immunohistochemistry for TH, combined with the visualization of the tracer. As exposed above, the retrogradely labeled centers at diencephalic-mesencephalic zones were consistently observed in the three specimens studied, however, we only unambiguously detected colocalization of labeled cells with TH in one specimen showed in Figure 2 (this issue will be discussed below). TH-positive cells containing the tracer were detected within the

catecholaminergic groups belonging to the VTA and SN (Figs. 2A,B), evidencing the existence of subpallial-projecting dopaminergic cells within these groups. Analysis of confocal sections confirmed that TH and Neurobiotin were present in the same cell bodies (arrows in Figs. 2D-D'',E-E''). On the contrary, we did not detect any double labeled cell in the catecholaminergic posterior tubercle group (Figs. 2A,C). In fact, cells and fibers labeled with the tracer tentatively identified as belonging to the PT (Fig. 1), were not exactly located within this catecholaminergic group. Instead, they were placed in an adjacent and inner position (see arrows in Figs. 2A,C). Actually, this Neurobiotin labeled group was mainly comprised of terminal fields, some of them appearing as buttons surrounding immunonegative somata (arrows in Fig. 2A'). Moreover, this group seemed to intermingle to a certain extent with the SN group at caudal levels (see arrrowheads in Figs. 1I,2A).

DISCUSSION

Technical considerations

Because of the low number of specimens used, this study must be considered preliminary. However, the consistency of some results obtained allows us to draw interesting conclusions. After applying the tracer Neurobiotin in the same subpallial region in the three juvenile specimens (the ASB-APVL), we consistently labeled cells at the same nuclei of the basal diencephalon and mesencephalon, i.e. in the posterior tubercle and in the SN and VTA, evidencing the reliability of

these results. However, retrogradelly labeled catecholaminergic cells were only unequivocally detected in one of the three specimens studied. We attribute this difference to the quantity of tracer employed (massive application resulted in strong damage of subpallial structures) as well as to the exact rostro-caudal point of application. We successfully labeled double Neurobiotin-TH-positive cells when the tracer was applied covering most of the rostro-caudal extent of the ASB. When the labeling was in the caudal ASB zone, the same diencephalic-mesencephalic centers were revealed, but no catecholaminergic cell were Neurobiotin-positive. These evidences point to the existence of a rostro-caudal zonation of the ASB-APVL dismissing the conception of this structure as a functional homogenous entity.

ASB-APVL fulfills some requirements to be considered the striatum of cartilaginous fishes

The ASB-APVL region presents some classical features described for the striatum of vertebrates.

Developmental data

Previous developmental data about the expression of subpallial genes as *Dlx2* and *Nkx2.1* helped to delimit the striatal and pallidal embryonic subdivisions in *S.canicula* subpallium, i.e the LGE and MGE homologs (Chapter 3). According to that study, main adult derivatives from the LGE seemed to correspond to the ASB-APVL region. Conversely, the Smeets' striatum was found to express pallial

markers. The fact that the ASB-APVL region is derived from the embryonic LGE homolog in *S.canicula* strongly supports that it represents the striatum. On the other hand, the MGE territory was restricted to the medial protrusion in the subpallium of late embryos and prehatching specimens. Indeed, Nkx2.1 was never observed at the level of the ASB, which strongly support our assumption that the ASB does not represent the pallidum of elasmobranchs (as previously supposed see introduction).

ASB-APVL region receives input from catecholaminergic VTA/SN group (nigrostriatal pathway homolog

The existence of inputs from catecolaminergic groups in the diencephalon and mesencephalon is a classical feature to define the striatum and has been reported in all vertebrate groups studied so far. The retrogradelly labeled catecholaminergic centers described in this work were identified as belonging to the SN/VTA catecolaminergic group. This study is the first evidence of this catecholaminergic connection by means of tract-tracing experiments combined with immunohistochemistry for TH in an elasmobranch species. Moreover, it represents additional evidence favoring the conception of the ASB-APVL as the striatum of elasmobranchs.

ASB-APVL projects to a PT-adjacent zone proposed as a substantia nigra reticulata-like region

The experiments performed in this study also revealed a dense terminal field in a region close to the posterior tubercle nucleus indicating that a descending

pathway from the ASB-APVL may project to this region. After comparing the position of this terminal field with TH expression in the diencephalon we recognized that it only partially overlapped with the catecholaminergic group of the posterior tubercle but also extended caudally towards the catecholaminergic group of the SN. Interestingly, the labeled terminal field did not contain cells or fibers positive for TH. The main direct striatal output target located in the diencephalic-mesencephalic zone is the substantia nigra pars reticulata (SNr) described in tetrapods (Kokoros and Northcutt 1977; Vesselkin et al. 1980; Wilczynski and Northcutt 1983; Marín et al. 1997b; Marín et al. 1998; Smeets et al. 2000; Roth et al. 2004; Medina 2008) and recently also claimed to be present in agnathans (Stephenson-Jones et al. 2012). Typically, this region does not contain dopaminergic neurons (Marín et al. 1997b; Marín et al. 1998) and it is located in a position related to the SNc (Smeets et al. 2000). We thus consider that the terminal field described in the present work does not form part of the catecholaminergic group of the posterior tubercle but might represent a SNr of elasmobranchs. The fact that it receives a direct projection from the subpallium along with its topographic relation with the SN catecholaminergic group (supposed SNc) (Reiner et al. 1998; Smeets et al. 2000) point to that it may be the case. Consequently, the descending labeled connection may be homologous to the basal ganglia striatonigral pathway (direct pathway) described in other vertebrates which typically expresses GABA and Substance P (Smeets et al. 2000). In elasmobranchs, some authors (Northcutt et al. 1988; Rodriguez-Moldes et al.

1995) described a substance-P pathway probably descending from basal telencephalon towards diencephalic and mesencephalic zones compatible with the location of the proposed descending projection described in the present study. Similarly, the description of GABA (Carrera 2008) and GAD (Sueiro 2003) fiber networks and terminals at diencephalic and mesencephalic levels in *S.canicula* juveniles also support our results. Tract-tracing experiments combined with labeling for these substances would be of great interest to confirm the phenotype of this descending pathway.

These evidences point to the existence of a dual SN (SNc and SNr) also in elasmobranchs.

ASB-APVL versus other regions classically considered as the striatum of elasmobranchs

Despite there exist abundant literature about the characterization of the basal ganglia in different species of adult elasmobranchs (Reiner and Carraway 1985; Northcutt et al. 1988; Medina and Reiner 1995; Reiner et al. 1998; Smeets et al. 2000), most of them are only based on immunohistochemical data and there is no consensus about what subpallial structure represents the striatum (see introduction). Developmental and connectional studies must be taken into consideration to establish a reliable homology of the basal ganglia subdivisions in the telencephalon of elasmobranchs.

As exposed above, developmental data pointed to the ASB-APVL region to represent the striatum since it is the main derivative of the embryonic LGE homolog of *S.canicula*. Moreover, these genetic expression data showed that the striatum proposed by Smeets et al. (Smeets et al. 1983) actually expressed pallial markers, dismissing the possibility that this structure could even belong to the subpallium. Connectional studies carried out in the present work support the conclusions drawn from genetic developmental experiments. The fact that we obtained a clear catecholaminergic input from a SN/VTA region by means of tracing experiments affecting only the ASB-APVL area and not to Smeets' striatum, suggests that the former represents the striatum to the detriment of the latter. However, further connectional studies focused on the Smeets' striatum must be performed to confirm our proposal. On the other hand, the projections revealed in this study are highly compatible with striatal connections. We consider that the possibility that the region labeled would include a pallidal structure can be discarded as no typical pallidal connections were revealed (i.e. no centers were labeled at dorsal thalamic zones), which represents an additional argument against considering the ASB as the pallidum of elasmobranchs.

In summary, developmental and connectional data support the ASB-APVL to represent the striatal subdivision of the basal ganglia. However, we do not conceive this structure as a homogeneous unit (see above). We consider that it exhibits a rostrocaudal and mediolateral specialization. That is, the intermediate part (the region labeled in this work) may represent the striatum while more

medial and more lateral regions could be related to pallidal and amigdalar structures, respectively (see Chapter 3). Based on our results, it seems that the caudal region of the ASB-APVL receives input from diencephalic-mesencephalic centers but it does not come from catecholaminergic neurons. However, more studies would be necessary to confirm this assumption.

Posterior tubercle catecholaminergic group does not seem to project to the subpallium in elasmobranchs

In amphibians, catecholaminergic neurons which are the origin of the dopaminergic input of the striatum are not restricted to the SN/VTA centers (as occurs in amniotes), but extend more anteriorly into the posterior tubercle (Gonzalez and Smeets 1991; González and Smeets 1994; Marín et al. 1997a; Marín et al. 1998; Hoke et al. 2007). Lampreys and bony fishes completely lack catecholaminergic cells in the basal midbrain and the ascending dopaminergic system to the basal telencephalon seems to emerge exclusively from neurons of the posterior tubercle nucleus (Meek and Joosten 1993; Pierre et al. 1997; Pombal et al. 1997; Rink and Wullimann 2001,2002; Anadón et al. 2002; Huesa et al. 2006). Some authors have hypothesized that all these ascending dopaminergic system could be homogenized among various vertebrates despite being originated in different positions (Rink and Wullimann 2001) as they seem to be used in the same context in all vertebrate groups. Thus, lampreys and bony fishes could have secondarily lost midbrain projecting centers. On the other hand, elasmobranchs

exhibit a catecholaminergic group in the posterior tubercle but also in the basal midbrain equivalent to SN and VTA (Meredith and Smeets 1987; Northcutt et al. 1988; Smeets 1990; Stuesse et al. 1991; Stuesse and Cruce 1992; Carrera et al. 2012). Carrera et al. (2012) proposed all these centers as a continuous catecholaminergic cell band from hypothalamus to midbrain and suggested that subpallial innervation may be originated from them. In this work, we observed that the catecholaminergic groups of the VTA/SN project to the subpallium while that of the posterior tubercle appears not to do it. Therefore, they might represent different entities with different functions. This notion is further supported by the fact that both catecholaminergic groups emerge at very different moments during embryonic development (Carrera et al. 2012). In summary, the scenario in *S.canicula* seems to be more similar to that observed in amniotes, where VTA/SN centers are the main source of the dopaminergic input to the striatum, than that observed in other anamniotes as lampreys or bony fishes.

REFERENCES

Alexander GE, Crutcher MD (1990) Functional architecture of basal ganglia circuits: neural substrates of parallel processing. Trends Neurosci 13:266–271

Anadón R, Rodríguez-Moldes I, González A (2002) Tyrosine hydroxylase immunoreactive neurons in the forebrain of the trout: organization, cellular features and innervation. Brain Res Bull 57:389–392

Barreiro-Iglesias A, Villar-Cerviño V, Anadón R, Rodicio MC (2008) Descending brain-spinal cord projections in a primitive vertebrate, the lamprey: cerebrospinal fluid-contacting and dopaminergic neurons. J Comp Neurol 511:711–723

Carrera I (2008) Desarrollo de los sistemas gabaérgico y aminérgicos en el sistema nervioso central de peces cartilaginosos. Doctoral Thesis, University of Santiago de Compostela, Spain

Carrera I, Anadón R, Rodríguez-Moldes I (2012) Development of tyrosine hydroxylase-immunoreactive cell populations and fiber pathways in the brain of the dogfish Scyliorhinus canicula: new perspectives on the evolution of the vertebrate catecholaminergic system. J Comp Neurol 520:3574–3603

DeLong MR (1990) Primate models of movement disorders of basal ganglia origin. Trends Neurosci 13:281–285

Gonzalez A, Smeets WJ (1991) Comparative analysis of dopamine and tyrosine hydroxylase immunoreactivities in the brain of two amphibians, the anuran Rana ridibunda and the urodele Pleurodeles waltlii. J Comp Neurol 303:457–477

González A, Smeets WJ (1994) Distribution of tyrosine hydroxylase immunoreactivity in the brain of Typhlonectes compressicauda (Amphibia, Gymnophiona): further assessment of primitive and derived traits of amphibian catecholamine systems. J Chem Neuroanat 8:19–32

Heimer L, Zahm DS, Alheid GF (1995) Basal ganglia. In: Paxinos GF (ed) The rat nervous system. San Diego, Academic Press, pp579-628

Hofmann MH, Northcutt RG (2008) Organization of major telencephalic pathways in an elasmobranch, the thornback ray Platyrhinoidis triseriata. Brain Behav Evol 72:307–325

Hoke KL, Ryan MJ, Wilczynski W (2007) Functional coupling between substantia nigra and basal ganglia homologues in amphibians. Behav Neurosci 121:1393–1399

Holmgren N (1922) Points of view concerning forebrain morphology of lower vertebrates. J Comp Neurol 34: 391-460

Huang Q, Zhou D, DiFiglia M (1992) Neurobiotin, a useful neuroanatomical tracer for in vivo anterograde, retrograde and transneuronal tract-tracing and for in vitro labeling of neurons. J Neurosci Methods 41:31–43

Huesa G, Anadón R, Yáñez J (2006) Topography and connections of the telencephalon in a chondrostean, Acipenser baeri: an experimental study. J Comp Neurol 497:519–541

Kokoros JJ, Northcutt RG (1977) Telencephalic efferents of the tiger salamander Ambystoma tigrinum tigrinum (Green). J Comp Neurol 173:613–628

Marín O, Smeets WJ, González A (1998) Basal ganglia organization in amphibians: chemoarchitecture. J Comp Neurol 392:285–312

Marín O, Smeets WJ, González A (1997a) Basal ganglia organization in amphibians: catecholaminergic innervation of the striatum and the nucleus accumbens. J Comp Neurol 378:50–69

Marín O, González A, Smeets WJ (1997b) Basal ganglia organization in amphibians: efferent connections of the striatum and the nucleus accumbens. J Comp Neurol 380:23–50

Medina L, Reiner A (1995) Neurotransmitter organization and connectivity of the basal ganglia in vertebrates: implications for the evolution of basal ganglia. Brain Behav Evol 46:235–258

Medina L (2008) Basal ganglia: evolution. In: Squire LR (ed) Encyclopedia of Neuroscience. Amsterdam, Elsevier, pp 67-85

Meek J A B Joosten HWJA (1993) Tyrosine hydroxylase-immunoreactive cell groups in the brain of the teleost fish Gnathonemus petersii. J Chem Neuroanat 6:431–446

Meredith GE, Smeets WJ (1987) Immunocytochemical analysis of the dopamine system in the forebrain and midbrain of Raja radiata: evidence for a substantia nigra and ventral tegmental area in cartilaginous fish. J Comp Neurol 265:530–548

Morona R, Moreno N, López JM, Muñoz M, Ten Donkelaar HJ, González A (2005) Calbindin-D28k immunoreactivity in the spinal cord of Xenopus laevis and its participation in ascending and descending projections. Brain Res Bull 66:550–554

Mueller T, Wullimann MF, Guo S (2008) Early teleostean basal ganglia development visualized by zebrafish Dlx2a, Lhx6, Lhx7, Tbr2 (eomesa), and GAD67 gene expression. J Comp Neurol 507: 1245–1257

Northcutt RG (1978) Brain organization in the cartilaginous fishes. In: Hodgson ES, Mathewson RF (eds) Sensory biology of sharks, skates, and rays. Arlington, pp 117–193

VA: Office of Naval Research, Department of the Navy.

Northcutt RG, Reiner A, Karten HJ (1988) Immunohistochemical study of the telencephalon of the spiny dogfish , Squalus acanthias. J Comp Neurol 277:250–267

Pierre J, Mahouche M, Suderevskaya EI, Repérant J, Ward R (1997) Immunocytochemical localization of dopamine and its synthetic enzymes in the central nervous system of the lamprey Lampetra fluviatilis. J Comp Neurol 380:119–135

Pombal MA, El Manira A, Grillner S (1997a) Afferents of the lamprey striatum with special reference to the dopaminergic system: a combined tracing and immunohistochemical study. J Comp Neurol 386:71–91

Pombal MA, El Manira A, Grillner S (1997b) Organization of the lamprey striatum –transmitters and projections. Brain Res 766:249–254

Reiner A (2010) The conservative evolution of the vertebrate basal ganglia. In: Steiner H, Tseng KY (eds.) Handbook of Basal Ganglia Structure and Function. San Diego, Academic Press, pp 29-62

Reiner A, Carraway RE (1985) Phylogenetic conservatism in the presence of a neurotensin-related hexapeptide in neurons of globus pallidus. Brain res 341:365–371

Reiner A, Medina L, Veenman CL (1998) Structural and functional evolution of the basal ganglia in vertebrates. Brain res 28:235–285

Rink E, Wullimann MF (2001) The teleostean (zebrafish) dopaminergic system ascending to the subpallium (striatum) is located in the basal diencephalon (posterior tuberculum). Brain res 889:316–330

Rink E, Wullimann MF (2002) Connections of the ventral telencephalon and tyrosine hydroxylase distribution in the zebrafish brain (Danio rerio) lead to identification of an ascending dopaminergic system in a teleost. Brain Res Bull 57:385–387

Rodriguez-Moldes I, Manso MJ, Becerra M, Molist P, Anadon R (1995) Distribution of substance P-like immunoreactivity in the brain of the elasmobranch Scyliorhinus canicula. J Comp Neurol 353:419–437

Roth G, Mühlenbrock-Lenter S, Grunwald W, Laberge F (2004) Morphology and axonal projection pattern of neurons in the telencephalon of the fire-bellied toad Bombina orientalis: an anterograde, retrograde, and intracellular biocytin labeling study. J Comp Neurol 478:35–61

Smeets WJ (1990) The telencephalon of cartilaginous fishes. In: Jones EG, Peters A, (eds) Cerebral cortex: Comparative structure and evolution of cerebral cortex, part I. New York, Plenum Press, pp 3-30

Smeets WJAJ (1998) Cartilaginous fish. In: Nieuwenhuys R, Ten Donkelaar HJ, Nicholson C (eds). The central nervous system of vertebrates, vol 1. Springer-Verlag, Berlin, pp 551-654

Smeets WJAJ, Marín O, González A (2000) Evolution of the basal ganglia: new perspectives through a comparative approach. J Anat 196:501–517

Smeets WJ, González A (2000) Catecholamine systems in the brain of vertebrates: new perspectives through a comparative approach. Brain Res Brain Res Rev 33:308-379

Smeets WJAJ, Nieuwenhuys R, Roberts BL (1983) Telencephalon. The central nervous system of cartilaginous fishes. Structure and functional correlations. Springer-Verlag, Berlin Heidelberg New York, pp 122–147

Stephenson-Jones M, Ericsson J, Robertson B, Grillner S (2012) Evolution of the basal ganglia; Dual output pathways conserved throughout vertebrate phylogeny. J Comp Neurol 520:2957–2973

Stuesse SL, Cruce WL (1992) Distribution of tyrosine hydroxylase, serotonin, and leu-enkephalin immunoreactive cells in the brainstem of a shark, Squalus acanthias. Brain Behav Evol 39:77–92

Stuesse SL, Cruce WL, Northcutt RG (1991) Localization of serotonin, tyrosine hydroxylase, and leu-enkephalin immunoreactive cells in the brainstem of the horn shark, Heterodontus francisci. J Comp Neurol 308:277–292

Sueiro C (2003) Estudio inmunohistoquímico de los sistemas gabaérgicos del sistema nervioso central de peces elasmobranquios y su relación con sistemas catecolaminérgicos y peptidérgicos. Doctoral Thesis. University of Santiago de Compostela, Spain

Vesselkin NP, Ermakova T V, Kenigfest NB, Goiković M (1980) The striatal connections in frog Rana temporaria: an HRP study. Journal fur Hirnforschung 21:381–392

Wilczynski W, Northcutt RG (1983) Connections of the bullfrog striatum: afferent organization. J Comp Neurol 214:321–332

ABBREVIATIONS

APVL	*area periventricularis ventrolateralis*
ASB	*area superficialis basalis*
OT	optic tectum
PT	posterior tubercle
SN	substantia nigra
Str. Smeets	striatum proposed by Smeets
VTA	ventral tegmental area

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Fig.1 Drawings of transverse and sagittal sections and photographs of transverse sections of the brain of *S.canicula* showing labeled cell bodies and fibers after Neurobiotin application to the APVL-ASB region. **A:** Schematic drawing of a transverse section across the telencephalon to show the main regions proposed to be the striatum (Str. Smeets, APVL, ASB) and the point of application of the tracer (arrowhead). **B:** Sagittal representation of the brain of *S.canicula* showing the level of each photograph (red lines) and the point of application of the tracer (arrowhead). **C:** Section across the telencephalon at the level of the point of application of the tracer (arrowhead). **D-F:** Section across diencephalic and mesencephalic zones showing labeled regions. Rectangles indicate the region zoomed in H,I and J. **G:** Detail of figure C. **H-J:** Details of figures D,E,F. For abbreviations, see list. Scale bars: 500 µm

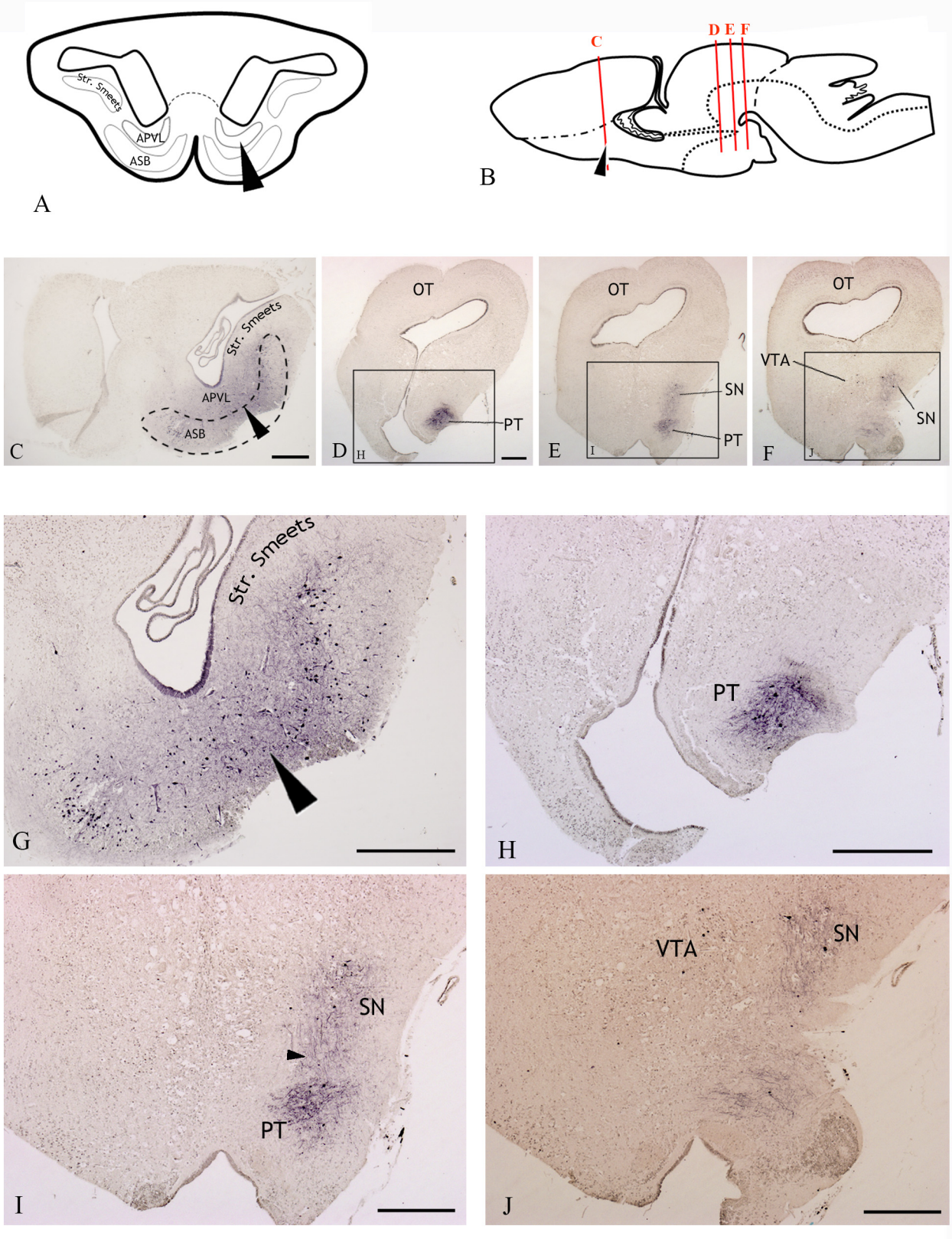

A
Str. Smeets
APVL
ASB
B
C
D E F
C
Str. Smeets
APVL
ASB
D
OT
PT
H
E
OT
SN
PT
I
F
OT
VTA
SN
J
G
Str. Smeets
H
PT
I
SN
PT
J
VTA
SN

Figure 1

Fig.2 Fluorescence (A) and confocal (B-E) photographs of transverse sections showing the distribution of the tracer in the diencephalon and mesencephalon combined with immunohistochemistry for TH **A:** Catecholaminergic groups of the diencephalon and midbrain codistribute and/or colocalize with retrogradely labeled cells and fibers. Insets show regions zoomed in A′ and B′. **A'**: Detail of the Neurobiotin positive group of fibers showing terminal buttons surrounding immunonegative somata (arrows).**B:** Confocal photograph corresponding to the region squared in A to show the region of VTA and SN catecholaminergic groups. **C:** Section across the region of the posterior tubercle catecholaminergic group to show its spatial relationship with the Neurobiotin positive group at this level. **D-D'':** Detail of the inset indicated in B to show the colocalization of some Neurobiotin positive cells with TH (arrows) in the VTA group. E-E'': Detail of the inset indicated in B showing the colocalization of Neurobiotin positive cells and TH (arrows). For abbreviations, see list. Scale bars: 500 µm (A,C); 250 µm (D); 100 µm (E).

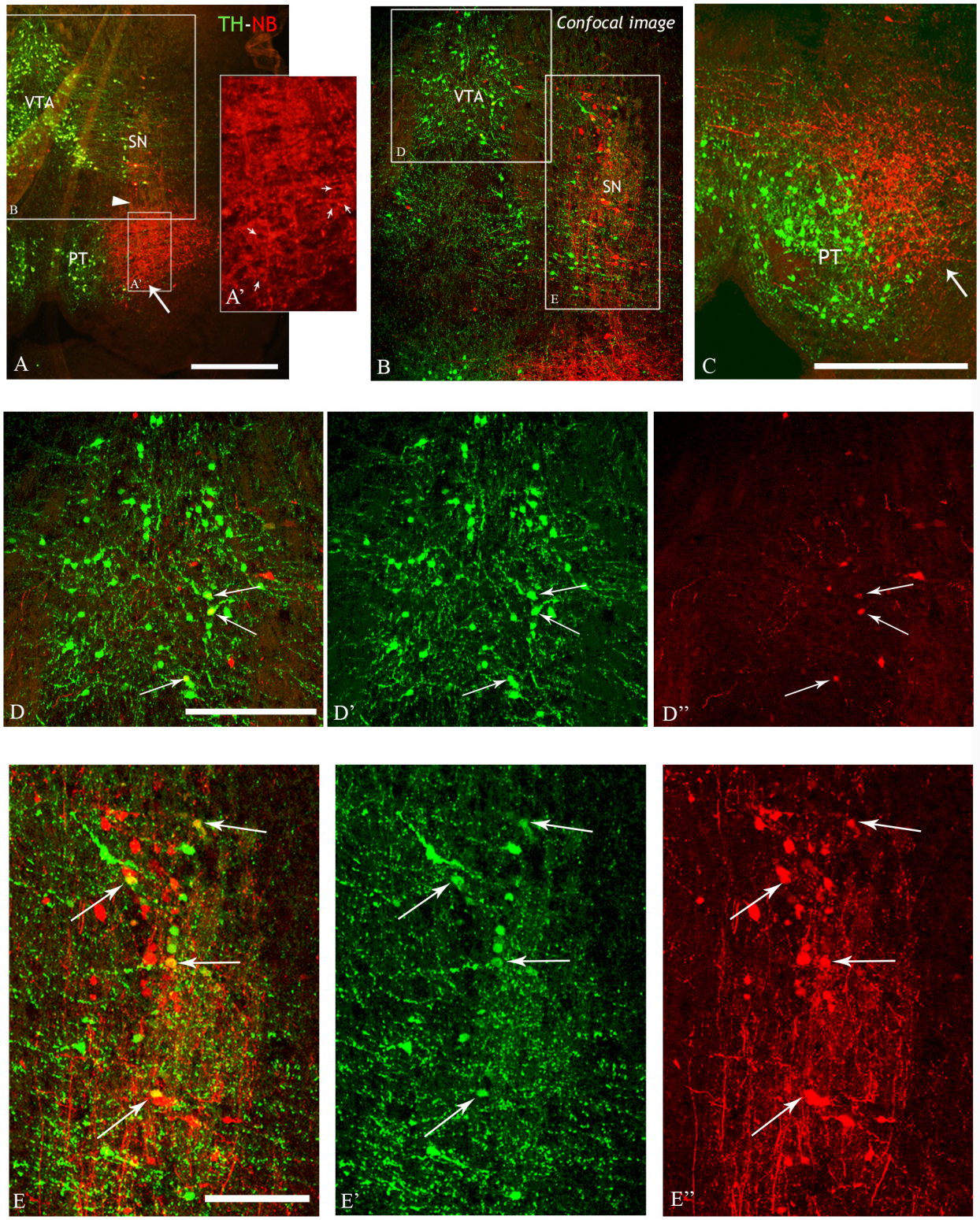


Figure 2

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Chapter 5

Evidences of tangential migrations in the telencephalon. Possible homologies and divergencies

Quintana-Urzainqui I, Rodríguez-Moldes I, Candal E. Evidences of tangential migrations in the telencephalon of a basal vertebrate. Possible homologies and divergencies (on preparation to be submitted to Developmental Biology).

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCTION

The vertebrate telencephalon is a complex structure that shows great morphological and size variability between different animal groups. However, it exhibits a remarkable degree of conservation regarding to genetic specification and developmental patterning (Puelles et al. 2000; Puelles and Rubenstein 2003; Medina and Abellán 2009; Moreno et al. 2009). It is broadly subdivided into a pallial (dorsal) domain, and a subpallial (ventral) domain that includes the ventral region of the evaginated telencephalic vesicles and the non-evaginated telencephalon. Roughly, in mammals, the principal structures derived from the pallium are the neocortex and hippocampus originated from dorsal and medial pallial compartments, respectively (Puelles et al. 2000; reviewed in Medina and Abellán 2009). In turn, the evaginated subpallium is subdivided in striatum and pallidum which emerge from two embryonic protrusions called the lateral and medial ganglionic eminences (LGE and MGE), respectively.

The cytoarchitectonics of telencephalic domains depends on a variety of tangential cell migrations take place correctly. Tangential migration acts as strong developmental mechanism to increase neuronal complexity of brain circuits by allowing the dispersion of multiple neuronal types. Embryonic subpallial structures as the LGE and the MGE are the origin of most of the tangentially migrating neurons of the telencephalon (reviewed in Marín and Rubenstein 2003; Wu et al. 2011), which travel long distances towards their final locations in diverse

telencephalic zones where they give rise to interneurons. The subpallial markers GAD and *Dlx2* have been widely used as reliable markers of the cell populations that migrate tangentially from the ganglionic eminences (Anderson 1997; Lavdas et al. 1999; Anderson et al. 2001; Corbin et al. 2001; Wichterle et al. 2001), as most of these cells are GABAergic and express genes belonging to the *Dlx* family since their early specification (near the ventricular region of the subpallium) until their arrival to diverse telencephalic domains.

Telencephalic tangential migrations have been reported with these and other markers during embryonic development from mammals to fishes, including cartilaginous fishes (De Carlos et al. 1996; Cobos et al. 2001; Marín and Rubenstein 2003; Tuorto et al. 2003; Métin et al. 2007; Carrera et al. 2008; Moreno et al. 2008; Mueller et al. 2008; Wullimann 2009), although the specific routes of migration and end targets of these pathways have only been well-documented in amniotes with genetic and transplantation studies (Cobos et al. 2001; Corbin et al. 2001). Some of the best characterized tangential routes include cell populations that migrate from the MGE towards the neocortex and hippocampus, from the MGE to the LGE, or from the LGE to the olfactory bulb (reviewed in Corbin et al. 2001). Of these, the latter has been proposed to be the embryonic precursor of the rostral migratory stream (RMS) found in adults (Pencea and Luskin 2003). The RMS has been extensively studied in mammals as it forms part of one of the few neurogenic systems in the adult brain (reviewed in Alvarez-Buylla and García-Verdugo 2002; Ming and Song 2011). In this system, progenitor cells born in the

subventricular zone of the telencephalic lateral ventricles migrate along a tangential path into the olfactory bulb, where they differentiate into granular or periglomerular cells, generally adopting GABAergic or dopaminergic phenotypes under the influence of Pax6 (Alvarez-Buylla and García-Verdugo 2002; Hack et al. 2005; Kohwi et al. 2005; Adolf et al. 2006; Brill et al. 2008; Whitman and Greer 2009; de Chevigny et al. 2012). Cells along the RMS move through a unique mechanism of tangential migration called "chain migration" (Lois and Alvarez-Buylla 1994; Alvarez-Buylla 1997). This peculiar kind of migration consists of neuroblasts closely associated to each other forming "chains" of migrating cells ensheathed by glial cells which form a tubular structure. Routes to the olfactory bulb in adults have also been reported in some non-mammalian vertebrates from birds to teleosts (Font et al. 2002; Adolf et al. 2006; Grandel et al. 2006; Kaslin et al. 2008; Barker et al. 2011; Kishimoto et al. 2011), although chain migration-like mechanisms have not been described in these species.

Comparative cross-species studies of the embryonic development have provided a new perspective to understand evolutionary changes. The species we used in this study, the lesser-spotted dogfish *Scyliorhinus canicula*, represents a key piece in evolutionary developmental (Evo-Devo) studies (see Coolen et al. 2009; Compagnucci et al. 2013).

In the telencephalon of *S. canicula*, the study of the development of the GABAergic system allowed the identification of subpallial derived neurons in the

pallium, which suggested the existence of tangential migratory pathways (Carrera et al. 2008; Rodríguez-Moldes et al. 2009). Moreover, the genoarchitectonic study carried out in Chapter 3 has shown that striatal and pallidal subdivisions, with similar embryological origin and genetic specification to those of tetrapods, are present in this shark. These developmental studies have given first evidence in *S. canicula* supporting the existence of equivalents to the lateral and medial ganglionic eminences (LGE and MGE) of mammals.

With the aim of identifying derived and/or evolutionary conserved tangential migratory routes, we perform a thoughtful analysis of the spatiotemporal pattern of distribution of immunoreactivity to GAD and the expression pattern of *Dlx2* (GAD and *Dlx2* expression, from here onwards), which have been widely used as reliable markers of tangentially migrating neurons. To identify the domains of origin of tangential migrations in the subpallium and the end targets of these routes, we have correlated the expression of GAD and *Dlx2* with that of *Nkx2.1* (MGE marker) and *Tbr1* (pallial marker). Additionally, we have investigated if these routes are maintained postnatally and if they are related to adult neurogenic niches. With this aim, we have correlated the presence of these routes in adults with the expression of PCNA and PH3 (cell proliferation markers), DCX (neuroblast migration marker), Reelin (an extracellular glycoprotein implied in neuronal migration and positioning processes in the developing brain) and GFAP (a marker for quiescent proliferating radial glia-like cells in the subventricular zone of the lateral ventricles in mammals). The expression of Pax6 and the presence of

dopaminergic cells in the olfactory bulbs have been further investigated in order to look for possible homologies with the RMS described in mammals.

The results obtained from the analysis of the telencephalic development in such an ancient species will shed light into the evolution of the telencephalon and the tangential migratory routes that take place within it.

MATERIAL AND METHODS

Experimental animals

Some embryos of the lesser spotted dogfish (*Scyliorhinus canicula*) were supplied by the Marine Biological Model Supply Service of the CNRS UPMC Roscoff Biological Station (France) and the Estación de Bioloxía Mariña da Graña (Galicia, Spain). Additional embryos and juveniles were kindly provided by the Aquaria of Gijón (Asturias, Spain), O Grove (Pontevedra, Spain) and Finisterrae (A Coruña, Spain). Adult specimens (40-60 cm in length) were provided by a local fisherman. Embryos were staged by their external features according to Ballard et al. (1993). For more information about the relationship of the embryonic stages with body size, gestation and birth, see Table 1 in Ferreiro-Galve et al. (2010). Thirty-three embryos from stages 28 to 34, five juveniles and four adult specimens were used in this study. Eggs from different broods and juveniles were raised in seawater tanks in standard conditions of temperature (15-16ºC), pH (7.5-8.5) and salinity (35g/L). Adequate measures were taken to minimize animal pain

or discomfort. All procedures conformed to the guidelines established by the European Communities Council Directive of 22 September 2010 (2010/63/UE) and by the Spanish Royal Decree 53/2013 for animal experimentation, and were approved by the Ethics Committee of the University of Santiago de Compostela.

Tissue processing

Embryos were deeply anesthetized with 0.5% tricaine methane sulfonate (MS-222; Sigma, St. Louis, MO) in seawater and separated from the yolk before fixation in 4% paraformaldehyde (PFA) in elasmobranch's phosphate buffer [EPB: 0.1M phosphate buffer (PB) containing 1.75% of urea, pH 7.4] for 48-72 h depending on the stage of development. Embryos from stage 32 onwards, juveniles and adults were deeply anesthetized with MS-222 and then perfused intracardially with elasmobranch Ringer´s solution (see Ferreiro-Galve et al. 2012a) followed by 4% PFA in EPB. Brains were removed and postfixed in the same fixative for 24-48 h at 4ºC. Subsequently, they were rinsed in PB saline (PBS), cryoprotected with 30% sucrose in PB, embedded in OCT compound (Tissue Tek, Torrance, CA), and frozen with liquid nitrogen-cooled isopentane. Parallel series of sections (18-20 µm thick) were obtained in transverse or sagittal planes on a cryostat and mounted on to Superfrost Plus (Menzel-Glasser, Madison, WI, USA) slides. Some sections were counterstained with haematoxylin–eosin to facilitate identification of different cell domains.

Single and double immunohistochemistry

Immunoenzyme staining

For heat-induced epitope retrieval, sections were pre-treated with 0.01 M citrate buffer (pH 6.0) for 30 min at 95ºC and allowed to cool for 20–30 min at room temperature (RT). Residual avidin/biotin activity was removed by incubation with the avidin/biotin blocking kit (Vector, Burlingame, CA). To eliminate endogenous peroxidase, sections were also pre-treated with 3% H_20_2, rinsed twice in 0.05 M Tris-buffered saline (TBS; pH 7.4) for 5 min each and incubated overnight with the primary antibody (see Table 1). Sections were rinsed twice in TBS for 5 min each and incubated in the appropriate biotinilated secondary antibody (see Table 1) for 1 h, followed by incubation with the preformed avidin-biotinylated horseradish peroxidase complex (Vector Laboratories) for 30 min. The immunoreaction was finally developed, either with 0.25 mg/ml diaminobenzidine tetrahydrochloride (DAB; Sigma) containing 2.5 mg/ml nickel ammonium sulfate and 0.00075% H_2O_2 (blue precipitate), or with SIGMAFAST™ 3,3′-DAB tablets (brown precipitate). All dilutions were made with TBS containing 15 % goat normal serum (Millipore, Billerica, MA) 0.2 % Triton X-100 (Sigma) and 2 % bovine serum albumin (BSA, Sigma). Finally, sections were dehydrated, mounted and coverslipped. All incubations were carried out in a humid chamber at RT. Additional information about the primary and secondary antibodies we used is included in Table 1.

Some sections were processed for double immunoenzyme staining being sequentially incubated firstly with anti-DCX and treated as above for yielding a blue precipitate in the revealing procedure, and then were incubated with anti GFAP and revealed to obtain a brown precipitate.

Immunofluorescence

Heat-induced epitope retrieval and incubation of primary antibodies (see Table 1) for single immunohistochemistry were performed as described above. Appropriate fluorescent dye-labeled donkey secondary antibodies (see Table 1) were incubated for 2 h at RT in the dark. For double immunofluorescence experiments, cocktails of primary antibodies were mixed at optimal dilutions and subsequently detected by using mixtures of appropriate secondary fluorescent antibodies. Sections were rinsed in distilled water, allowed to dry and mounted in MOWIOL 4-88 Reagent (Calbiochem, MerkKGaA, Darmstadt, Germany).

Control and specificity of the antibodies

The specificity of the antibodies against GAD, TH, DCX and Pax6 in the brain of the lesser spotted dogfish was previously confirmed by Western blot or preadsorption test (Sueiro et al. 2004; Carrera et al. 2012; Chapter 1). The same GAD antiserum has allowed the identification of subpallial derived GABAergic cells in the developing pallium of *S. canicula* (Carrera et al. 2008). The polyclonal anti-GFAP antibody has been previously used as a glial marker in the olfactory system and brain of *S. canicula* (Wasowicz et al. 1999; Sueiro et al. 2007; Chapter

1) and the monoclonal anti-PCNA specifically labels proliferating cells in the brain and olfactory epithelium of this species (Rodríguez-Moldes et al. 2008; Ferrando et al. 2010; Chapter 1).

The mouse monoclonal anti-reelin antibody (clone 142; Millipore, Billerica, MA; catalog number MAB5366) was raised against the aminoacids 164-189 of the protein. According to manufactures, it recognizes three bands of approximately 400kDa (full length protein), 300kDa and 180kDa (cleavage products). The specificity of the antibody in fishes was confirmed by Western blot analysis that reported three equivalent bands in dogfish *(S. canicula),* sturgeon *(Acipenser baeri)* and brown trout *(Salmo trutta fario)* brain extracts (Pérez-Costas 2002; Pérez-Costas et al. 2002; Candal et al. 2005). This antibody has been previously found to be expressed in subsets of pallial neurons in diverse vertebrate species from fish to mammals (Pérez-García et al. 2001).

The polyclonal rabbit anti-phospho-histone-H3 (ser 10) antiserum (anti-PH3; Upstate BioTechnology) has been widely used as a mitotic marker, since H3 phosphorylation is associated with chromosome condensation and dynamics during mitosis (Hendzel et al. 1997; McManus and Hendzel 2006; Carney et al. 2007). This antibody has been previously used as a mitotic marker in the retina of *S. canicula* (Ferreiro-Galve et al. 2010).

In situ hybridization on slides

We applied in situ hybridization for *Dlx2, Nkx2.1* and *Tbr1*. These probes were selected from a collection of *S. canicula* embryonic cDNA library (mixed stages, S9 to 22), submitted to high throughput EST sequencing (coord. Dr. Sylvie Mazan at the Station Biologique de Roscoff, France). cDNA fragments, kindly provided by Dr. Mazan, were cloned in pSPORT vectors. Cloned fragments were amplified by PCR and then purified using a High Pure kit (Roche). Sense and antisense digoxigenin-UTP-labeled *Dlx2, Nkx2.1* and *Tbr1* probes (*ScDlx2*, *ScNkx2.1* and *ScTbr1*) were synthesized directly by transcription in vitro. *In situ* hybridization was performed on cryostat sections of stages 29 to prehatching embryos following standard protocols (Coolen et al. 2007). Briefly, sections were permeabilized with proteinase K, hybridized with sense or antisense probes overnight at 65 ºC and incubated with the alkaline phosphatase-coupled anti-digoxigenin antibody (1:2000, Roche Applied Science, Manheim, Germany) overnigh at 4ºC. The color reaction was performed in the presence of BM-Purple (Roche). Finally, sections were dehydrated and coverslipped. Control sense probes did not produce any detectable signal.

3D reconstructions

RECONSTRUCT™ software (Fiala 2005) was used for three-dimensional reconstruction of different migration routes from serial sections of embryos at

different developmental stages (stages 29, 31 and 32). The obtained 3D objects were then illustrated with Photoshop CS4 (Adobe, San Jose, CA).

Image acquisition and analysis

Light field images were obtained with an Olympus BX51 microscope equipped with an Olympus DP71 color digital camera. Fluorescent sections were analyzed and photographed with the TCS-SP2 scanning microscope with a combination of blue and green excitation lasers. Stacks of confocal images were acquired separately for each laser channel with steps of 0.8 or 2 µm along the z-axis and collapsed images were obtained from an average of 12 optical sections with the LITE software (Leica). Some sections were photographed with an epifluorescence photomicroscope Olympus AX70 fitted with an Olympus DP70 color digital camera. Whole brains were studied and photographed with an Olympus SZX12 stereo microscope fitted with an Olympus DP12 color digital camera. Photographs were adjusted for brightness and contrast and plates were prepared using Adobe Photoshop CS4 (Adobe, San Jose, CA).

RESULTS

The telencephalon of the lesser spotted dogfish deserves special attention regarding its topology with respect to that of the olfactory bulbs (see Fig. 1). Early in development, the rostral parts of the prosencephalon bulge out laterally and the olfactory bulbs are formed later as an outgrowth of the lateral walls of the

expanded portions (arrows in Fig.1A,B), which is in contrast with the rostral mode of insertion found in other chondrichthyans (Smeets 1983) and in most vertebrates (Butler and Hodos 2005). During the formation of the olfactory bulbs, another rostrally and laterally directed evagination occurs which cause the emergence of the telencephalic hemispheres (arrowheads in Fig. 1B). As part of the evagination process the dorsal parts of the hemispheric walls undergo inversion (arched arrows in Fig. 1A) and, in *Scyliorhinus*, meet each other completely in the median plate (arched arrows in Fig. 1B). Later in development, the olfactory bulbs separate from the hemispheres and become interconnected by short stalks (arrows in Figs. 1B, D-F). In adults, the lateral telencephalic ventricles remain wide (Figs. 1C,F) and connected by a ventricular narrowing to the olfactory bulb ventricles (asterisks in Figs. 1C,F).

***Dlx2*, as GAD, is expressed in streams of cells at various telencephalic locations, which may emerge from the shark equivalents to the ganglionic eminences.**

In order to identify divergent and evolutionary conserved tangential migratory routes in the telencephalon of *S. canicula*, we have analyzed the distribution pattern of *Dlx2* and GAD during embryogenesis, two markers that have been largely used to identify tangentially migrating neurons. Streams of cells expressing GAD or GABA in the embryonic telencephalon of *S.canicula* have been previously described by Carrera et al. (2008), who suggested that these cells

followed tangential routes to the pallium, similar to those described in other vertebrates. Previously, we demonstrated that GAD-immunoreactive (-ir) cells show identical distribution to *Dlx2*-expressing cells during early development (Chapter 3). In this work, we focus on identifying the original location of these cells (i.e., where they emerge from) and what telencephalic domains they occupy as development proceeds by analyzing the spatial and temporal pattern of cells expressing these subpallial markers.

Early development (stages 28 to 31)

At stage 28-29, GAD expression was mainly restricted to the subpallial part of the telencephalon (Fig. 2; see also Carrera et al. 2008), though scarce GABAergic cells showing typical morphology of tangentially migrating cells have been previously described beyond the pallial-subpallial boundary (Carrera et al. 2008). At stage 31, streams of GAD cells were clearly distinguished across different pallial regions (Fig. 3A-C; see grey arrows in Fig. 3H) and seemed to branch in two different routes: one subventricular (white arrows in Figs. 3A-C; red and yellow streams in Fig. 3H) and another subpial (empty arrow in Fig. 3B; green stream in Fig. 3H) as previously described by Carrera et al. (2008). These incipient streams of GAD were also *Dlx2*-positive (Figs. 3D-F) and continuous with the expression found in the LGE of *S.canicula* (asterisks in Figs. 3A,B,D,E; blue territory in Fig. 3H), although contributions from the MGE cannot be discarded. The GAD/*Dlx2* subventricular stream became subdivided into two different paths adjacent to the

ventricle surface, one oriented towards the olfactory bulbs ventricles (hereafter *lateral subventricular stream* ; *LS* in Figs. 3A,D; yellow stream in Fig. 3H) and the other oriented to the subventricular zone of rostral telencephalon (hereafter *rostral subventricular stream*; *RS* in Figs. 3B,C,E,F; red stream in Fig. 3H). Of note, this rostral stream is also continuous with a domain expressing GAD and *Dlx2* in the rostral subpallium (Figs. 3C,F; black arrows in Fig. 3H) which could correspond to the rostral-most part of the LGE or to the prospective septal region. In turn, the GAD/*Dlx2* subpial stream extends up to the marginal zone of an unidentified pallial region (empty arrows in Figs. 3B,E; green stream in Fig. 3H) where the glycoprotein Reelin is specifically expressed (empty arrow in Fig. 3G).

Late development (stages 32 to prehatching)

During stage 32, the basic morphology of the brain is progressively achieved. At this stage, the GAD/*Dlx2* subpial stream was no longer recognizable. In contrast, cells along the rostral and lateral subventricular streams continued expressing *Dlx2* (*RS* and *LS* in Figs. 4A,B,D,E). No substantial changes were observed with respect to the rostral subventricular stream at this stage (Fig. 4A,B). As in previous developmental stages, *Dlx2*-expressing cells belonging to the *lateral stream* were particularly apparent in the ventral subventricular zone of the lateral ventricles (arrows in Fig. 4,D,E) that connect the telencephalon to the incipient granular layer of the olfactory bulb (asterisks in Fig. 4E). At this stage,

Dlx2-expressing cells were additionally observed in the dorsal subventricular zone of the lateral ventricle (empty arrows in Fig. 4D,E; compare with Fig. 3A,D).

In late stage 32 embryos, the walls of the medial pallium become continuous with that of the MGE (compare medial zone of Fig. 4D versus 4E and Fig. 4H versus 4I). At the same point, an additional domain of *Dlx2*-expressing cells was observed in the medial pallium which was continuous with that of the MGE territory (arrowheads in Fig. 4E). These cells also expressed *Nkx2.1* (arrowheads in Fig. 4I), a classical marker of the MGE and its derivatives in mammals, which clearly demarcates the MGE in *S. canicula* at previous stages (Chapter 3; Fig. 4H). As MGE derivatives (*Nkx2.1* expressing cells) were found in the medial pallium, hereafter we will refer to this *Nkx2.1* stream-like domain as the *medial stream* (*MS* in Fig. 4E,I).

At the same stage, an additional population of *Nkx2.1*-expressing cells was observed out of the MGE occupying the LGE territories (arrows in Figs. 4I-K). Again, for simplicity, as MGE derivatives were found in the prospective striatal territory, we will refer to this dispersion as *pallido-striatal stream.*

The rostral and lateral subventricular streams, together with the medial and pallido-striatal streams were fully maintained in late embryos (stage 33) (Figs. 4C,F,G,J,K). Yet, the rostral subventricular stream showed an apparent dispersion from the subventricular zone to an internal layer (arrows in Figs. 4C).

All streams are illustrated in Fig. 4K.

Lateral subventricular stream is related to the olfactory bulb and exhibits characteristics of a RMS-like route.

Dlx 2-positive embryonic tangential paths to the olfactory bulb emerging first from the LGE and eventually also from the SVZ of the pallium have been described in mammals (Porteus et al. 1994; Tucker et al. 2006). They seem to be maintained in the adult and probably give rise to the RMS (Pencea and Luskin, 2003). The sequential analysis throughout development of cells belonging to this stream revealed a clear continuity with the olfactory bulb. This fact led us to investigate if the lateral subventricular stream observed by us in *S. canicula* may correspond to the embryonic precursor of a RMS-like structure in the adult brain. With this goal, we aimed at analyzing the expression patterns of some markers that have been typically used to identify this route in different vertebrate species. In mammals, the RMS constitutes a neurogenic niche of cells that born in the subventricular zone of the lateral ventricles, where they express proliferation markers (such as PCNA and PH3), neuronal migration markers (as DCX) and transit-amplifying progenitor markers (as *Dlx2* and *Pax6*). While migrating, these cells form cell chains that become ensheathed by glial (GFAP-ir) cells. As they arrive to the olfactory bulb, they generally adopt GABAergic or dopaminergic phenotypes under the influence of the transcription factor Pax6 (Hack et al. 2005; Kohwi et al. 2005; Adolf et al. 2006), which may act in combination with *Dlx2* (Brill et al. 2008; de Chevigny et al. 2012).

Accordingly, we have first investigated the presence of PCNA-ir and PH3-ir within the lateral subventricular stream bordering the lateral and olfactory bulb ventricles (illustrated in Fig. 5A). In stage-32 and prehatching embryos, the lateral ventricles leading to the OBs as well as the ventricle of the olfactory bulb itself showed a notably thicker proliferating ventricular zone than other telencephalic regions (asterisks in Figs. 5B-D). The same pattern was maintained in adult specimens (Fig. 5E), which indicates that this ventricular zone strongly retains cells with proliferative capacity even in the adulthood. Furthermore, PCNA positive cells showing lengthened nuclei compatible with the morphology of migrating cells (that is, lengthened cells with their large axis oriented parallel to the ventricular rim) were observed subventricularly both in late embryos and in adults (arrows in Figs. 5F,G).

To explore if these PCNA positive cells were migrating neuroblasts, we analyzed the expression of DCX, a microtubule associated protein related with neurogenesis, neuronal migration and synaptogenesis, which is notably expressed by cells along the RMS (Alvarez-Buylla and García-Verdugo, 2002; Brown et al. 2003; Curtis et al. 2007; Balthazart et al. 2008; Brill, 2008; Brill et al. 2008; Mendoza-Torreblanca et al. 2008; Guerrero-Cázares et al. 2011; Ming and Song, 2011; Hodge et al. 2012; Plachez and Puche, 2012). In juveniles of *S. canicula*, the lateral subventricular stream exhibited the highest density of DCX-ir cells of the telencephalon, which showed the same distribution as PCNA-ir cells (Figs. 5H-H''). DCX processes were also oriented parallel to the ventricular surface (arrows in

Figs. 5H'') and they were mainly located in a subventricular position (arrowheads in Figs. 5H,I). In the juvenile (results not shown) and adult olfactory bulb, DCX was strongly expressed by numerous neuronal prolongations in the olfactory glomeruli (Figs. 5J,K) suggesting that formation of new connections and neuronal migration occur also in the olfactory bulb. Hence, the lateral stream seems to contain cells presenting active signs of proliferation, migration and synaptogenesis, even in adults.

To investigate if glial structures were associated to the *S.canicula* lateral route, we analyzed the distribution of GFAP immunoreactivity throughout development. We observed some glial (GFAP-ir) processes in direct contact with the ventricular surface (open arrows in Fig. 5I) which has been defined as a typical feature of quiescent proliferating radial glia-like cells present in the subventricular zone of the lateral ventricles in mammals (reviewed in Alvarez-Buylla and Lim, 2004; Dhaliwal and Lagace 2011; Ming and Song 2011). However, in contrast to mammals, we did not observed any evidence of a glial sheath-like structure related to migrating neurons along postnatal lateral subventricular stream by means of GFAP immunohistochemistry, which is a classical marker of ensheathing glia in other vertebrates (Lois et al. 1996; Kam et al. 2009; Nityanandam et al. 2012).

It has been proved that *Dlx2* and *Pax6* are expressed in transit-amplifying progenitors and neuroblasts in the adult neurogenic subependymal zone of

mouse, which derives from the embryonic dorsal and ventral subventricular zone of the telencephalon (reviewed in Brill 2008). Eventually, *Pax6* acts in combination with *Dlx2* to promote the commitment of olfactory bulb interneurons towards a dopaminergic phenotype (Brill et al. 2008; de Chevigny et al. 2012). We have therefore studied the expression of Pax6 in relation to that of *Dlx2* along the lateral subventricular stream and also in the olfactory bulb of *S. canicula*, both during embryonic development and in the adulthood. We detected a Pax6-positive stream of cells within the *Dlx2*/GAD-expressing lateral subventricular stream (arrow in Fig. 5L). Pax6-ir cells in this stream co-expressed the neuronal migrating marker DCX (arrows in Fig. 5M). To examine if those migrating Pax6 and *Dlx2* neurons would be related to future dopaminergic interneurons of the olfactory bulb, we additionally analyzed the expression of the enzyme tyrosine hydroxylase (TH) , the rate-limiting enzyme of dopamine synthesis. We observed TH-ir cells in the lateral subventricular stream (including the olfactory bulbs ventricles region) of *S. canicula* from late stage 32-embryos (Fig. 5N) up to adulthood (Fig. 5O). Co-localization with Pax6 was detected in some olfactory bulb cells (Figs. 5P,P'). These results are in agreement with those carried out by some of us in *S.canicula* describing Pax6-ir granular cells (Ferreiro-Galve et al. 2012b) and TH-ir (Sueiro 2003; Carrera et al. 2012) granular and periglomerular cells in the olfactory bulb. To sum up, our data suggest that in *S. canicula*, as in other vertebrates, migrating neurons expressing Pax6 and *Dlx2* within the stream

towards the olfactory bulb correspond at least in part with future dopaminergic granular and periglomerular neurons in the mature olfactory bulb.

Characterization of the rostral and medial streams as embryonic precursors of potential adult neurogenic systems

Besides the lateral subventricular stream, other two cell streams were observed across pallial zones. We then seek to know if they could represent the embryonic precursors of adult neurogenic systems.

As reported above, the *Dlx2*-expressing *rostral subventricular stream* extended between the subpallium and the pallial part of the rostralmost telencephalon from early embryonic stages onwards (see location in Fig. 6A). In the rostral pallium of late embryos, the domain containing *Dlx2*-expressing cells extended up to a conspicuous layer adjacent to the subventricular zone (see Figs. 6B,C). Of note, we observed that this layer is placed immediately bellow a domain that contain cells expressing *Tbr1* (Figs. 6B',C'), a specific pallial marker characteristically expressed in glutamatergic projection neurons (Englund et al. 2005; Berninger et al. 2007; Hodge et al. 2012). Thus, this pattern strongly reminds the characteristic arrangement of complex circuits of projection neurons (*Tbr1*) and interneurons (*Dlx2*). The existence of a complex layered cell organization in the adult brain was also evidenced by means of hematoxilin-eosin staining (Fig. 6D,D').

Some features indicate that the *rostral subventricular stream* could represent the embryonic precursor of an adult neurogenic system. PCNA-positive cells were abundant in the rostral telencephalic ventricular zone of late embryos (Fig. 6E). While the density of proliferating cells gradually decreased during postnatal development, this zone still exhibited a population of ventricular proliferating cells (Fig. 6F). Subventricular DCX expression (Figs. 6G,H) and some GFAP-ir processes (Fig. 6G,G') were also observed in the ventricular zone. TH-ir cells and fibers were observed in the subventricular zone and seemed to extend to the adjacent inner cell layers (Figs. 6I,J).

The *medial stream*, in turn, extended between the MGE and the medial pallium from stage 32 onwards (see location in Fig. 6K), at the time when the walls of the medial pallium become fused with that of the MGE. In contrast with that observed in the rostral pallium, no layered organization was observed in the medial pallium. However, the density of PCNA-positive cells was higher in the ventricular zone of the medial pallium than in other telencephalic regions during prenatal stages (arrows in Figs. 6L,M). Although in adult brains the number of PCNA cells was moderate here (arrow in Fig. 6N), it seemed to be one of the discrete three regions where telencephalic proliferation is detectable in adults (see discussion). Moreover, we observed subventricular DCX staining in this zone together with the strongest ventricular expression of GFAP-positive processes in contact with the ventricular surface (Figs. 6O-Q), which point to the presence of a significant number of potential stem cells.

DISCUSSION

Tangential neuronal migration occurs along different axes to that demarcated by radial glia and allows the increase of the cellular complexity of brain circuits during development. Interneuron tangential migration emerging from the basal telencephalon has been proved to be highly dependent on *Dlx* genes, as *Dlx1/2* mutants lack subpallial-derived tangential migrations due to a cell autonomous defect (Anderson et al. 2001; Anderson 1997). Together with *Dlx2*, cell populations that migrate tangentially have been commonly reported to express GABA (Lavdas et al. 1999; Anderson et al. 2001; Corbin et al. 2001; Wichterle et al. 2001). Indeed, streams of tangentially migrating cells identified by cell trackers or demonstrated by grafting experiments are located in places accurately predicted by inmunohistochemical or hybridization studies aimed to detect GABA and *Dlx2* expression patterns (Stühmer et al. 2002; Métin et al. 2007). Hence, the presence of GAD and *Dlx2*-expressing cells out of the subpallium as a result of the upregulation of these markers within progressively far-off domains can be firmly discarded.

Under this rationale, we performed a deep analysis of the spatio-temporal pattern of distribution of GAD and *Dlx2,* which pointed to the existence of five embryonic tangential migratory routes originated in the subpallial compartment of *S.canicula* (see Table 2 and Fig. 7). Four of them are palliopetal streams. Of these, one represents a transient stream towards the subpial zone of the pallium

that was detected only during a short period of embryonic development. The other three were maintained in the adult and reached pallial domains that correspond with the only regions in the telencephalon that present features of potential adult neurogenic niches (see Fig. 7). In all three cases, supposed migration occurs in two main phases: 1) cells migrate tangentially from the ganglionic eminences to the subventricular zone of the corresponding pallial region and 2) once there, some cells become integrated within pallial structures away from the ventricle. In addition, a *pallido-striatal stream* of *Nkx2.1*-expressing cells was observed out of the MGE (the prospective pallidal territory) invading the LGE (the prospective striatal territory. We discuss here whether these routes are conserved along evolution or they may represent specializations of the shark telencephalon.

The embryonic tangential subpial stream is directed towards the dorsal pallium

The transitory stream of subpallial *Dlx2*-expressing and GAD-ir cells we observed at stage 31 seemed to reach a subpial zone whithin the pallium where the glycoprotein Reelin was specifically expressed. In mammals, a similar transient migration has been described, and it is the origin of subpial granule neurons that invade the marginal zone of the dorsal pallium to become integrated in the layer I of the developing neocortex (Gadisseux et al. 1992; Meyer et al. 1998; Lavdas et al. 1999; Corbin et al. 2001; Wichterle et al. 2001; Morante-Oria et al. 2003), where

they expressed GABA and Reelin (Meyer et al. 1998; Lavdas et al. 1999; Antypa et al. 2011). Although neocortex is a mammalian novelty, it has been proved that the tangential migration of interneurons is an astonishingly conserved process along evolution (Cobos et al. 2001; Métin et al. 2007; Carrera et al. 2008; Moreno et al. 2008; Mueller et al. 2008; Wullimann 2009). These facts led us to propose that the *subpial stream* found in *S. canicula* may be homologous to that described in mammals and that the Reelin-ir region in the pallium where this stream ends may represent the dorsal pallium (see Figs. 7A,B and Table 2), the region that, in mammals, give rise to the neocortex (see introduction). Most authors argue that the origin of this stream in mammals resides in the MGE (Lavdas et al. 1999; Corbin et al. 2001; Wichterle et al. 2001) although the retrobulbar and LGE origin have also been considered (Gadisseux et al. 1992; Meyer et al. 1998; Jiménez et al. 2002). In our hands, the expression of *Dlx2* in the pallium was visibly continuous with that found in the LGE. Although *Nkx2.1*-expressing cells (i.e., of MGE origin) were not observed in the pallium, contributions from the MGE to the *subpial stream* could not be discarded since, in mammals, it has been described that this gene is downregulated as the future cortical neurons migrate to the cortex (Marín et al 2000). Further studies will be needed to discriminate the precise origin of cells belonging to the subpial stream in *S.canicula*.

The lateral stream to the olfactory bulbs and the RMS of mammals. Evolutionary implications

We described here an embryonic *lateral subventricular stream* that emerged from the LGE of *S. canicula* and reached the olfactory bulb (see Table 2 and Figs. 7A,B). This stream was maintained during adulthood around the telencephalic lateral ventricles, the olfactory bulb ventricles and the ventricular narrowing that connects them (see Fig. 7C). Migratory routes from the LGE to the olfactory bulb during embryonic development have been described from lampreys to mammals (Porteus et al. 1994; Cobos et al. 2001; Corbin et al. 2001; Meléndez-Ferro et al. 2002; Stenman et al. 2003; Tucker et al. 2006; Long et al. 2007), and evidences were given in mammals that this embryonic route gives rise to the adult RMS (Wichterle et al. 2001; Pencea and Luskin 2003; Stenman et al. 2003; Tucker et al. 2006). On this basis, the migratory stream to the olfactory bulbs we observed in adult sharks could be interpreted as the ancestral form of the RMS in vertebrates. This statement is further supported by the fact that a it expresses a specific sequence of typical RMS markers (Figure 8; see Ming and Song, 2011; de Chevigny et al. 2012) was observed in sharks along this route: PCNA in the ventricular and subventricular zone (proliferation), where cells showed morphological features of migratory cells; GFAP in the ventricular zone (activation of radial glia-like cells) and *Dlx2* in the subventricular zone (proliferation of transient amplifying cells); *Dlx2* and DCX in the subventricular zone along the stream (tangential migration) and in the olfactory bulb (radial migration); and TH

and *Pax6* along the stream and in the olfactory bulb (synaptic integration and maturation of granular or periglomerular cells).

Of note, we did not observe any hint of glial (GFAP-ir) tubes surrounding migrating neuroblasts, which suggests that *chain migration* as described in mammals, does not take place in *S.canicula*. As far as we know, glial tubes or of chain migration-like processes have not been reported in non-mammalian vertebrates. Instead, neuroblasts were usually described as running subventricularly (present study and Garcia-Verdugo et al. 1989; Byrd and Brunjes 2001; Font et al. 2002; Adolf et al. 2006; Kaslin et al. 2008; Barker et al. 2011). The shift from a subventricular mode of cell migration to a *chain migration* process could be related to the closure of the ventricles of the olfactory bulbs and the narrowing that connects them to the lateral ventricles of the telencephalon. This closure might occur independently during evolution in different animal groups from fishes to mammals. In agreement with the fact that ontogeny recapitulates phylogeny, the closure of this ventricular communication has been also reported during the embryonic development of rodents and humans (Humprey 1940; Sanai et al. 2007; Sanai et al. 2004; Allen Brain Atlas data portal, http://.brain-map.org). As far as the subventricular path becomes disrupted, this closure involves the separation of the olfactory bulbs from its source of cell renewal in the lateral ventricles. Remarkably, in mammals, this chain-migrating source of cells seems to follow the remnant left behind by a shrunken ventricle (Kam et al. 2009; Guerrero-Cázares et al. 2011). In this context, *chain migration* in mammals could emerge as

a solution to provide the olfactory bulb with new cells despite the lack of ventricular connection. To our knowledge, if it holds true for other vertebrate species with closed olfactory bulb ventricles remain unexplored.

Rostral stream. Possible specialization of Cartilaginous fish?

The *Dlx2*-expressing *rostral subventricular stream* emerged from the subpallium and headed for the subventricular zone of the rostral-most pallial region. The expression of *Dlx2* in the pallium was manifestly continuous with that found in the LGE, but contributions from the MGE cannot be discarded despite *Nkx2.1*-expressing cells (i.e., of MGE origin) were not observed in the rostral pallium (see discussion above). Tract-tracing experiments would be useful to accurately discriminate the region from which this migratory stream emerges. Eventually, cells belonging to the *Dlx2*-expressing domain in the rostral subventricular zone of the pallium seem to integrate into a discrete cell layer. We did not found any comparable migration in the literature (see Table 2), thus we propose that this route might be a shark specialization.

As the olfactory system is highly elaborated in elasmobranchs, the particular forebrain organization of these animals could be morphologically correlated with special features of the olfactory system (Hoffmann and Northcutt 2012). Indeed, the *rostral subventricular stream* of sharks appears to be related with the establishment of a prominent u-shaped cell layer called *area periventricularis pallialis*, which receives secondary olfactory tracts via the *tractus olfactorius*

medialis (Smeets 1983, 1998). Morphological comparison among different Chondricthyans suggests a correlation between the arrival of olfactory fibers to this complex (layered) area and the formation of the rostral bulges of the telencephalic hemispheres found in some species of cartilaginous fishes, including *S. canicula*. On the other hand, the *rostral subventricular stream* appears to represent the embryonic precursor of an adult neurogenic system as PCNA-ir cells (ventricular), DCX-ir cells (subventricular) and GFAP-ir cell processes (subventricular) were found in the rostral pallium during postnatal development (see Fig.7C) TH-immunoreactive cells and fibers were observed in the subventricular zone and adjacent inner layers, which suggest that, as occurs in the olfactory bulbs, this subventricular stream could be the source of dopaminergic neurons in the adult.

The medial stream and its relation with the hippocampus

The *medial stream* extended from the MGE towards the medial pallium and expressed the *Nkx2.1* gene, which further confirmed its pallidal origin. Interestingly, this domain corresponds with one of the three ventricular regions where adult proliferation takes place in the telencephalon of *S.canicula* (see Fig.7C). This stream may represent a conserved trait during evolution since in mouse, a route has been described emerging from the caudal region of MGE toward the medial pallium, i.e. the hipocampal primordium (DeDiego et al. 1994; Pleasure et al. 2000; Corbin et al. 2001; see Table 2). Moreover, it is well known

that the hippocampus is one of the few adult neurogenic structures of the mammalian brain (Doetsch 2002; Kempermann 2012; LaDage et al. 2011). In other vertebrate species, proliferation is almost constitutive along lateral ventricles and discrete areas in the pallium that show hot spots of proliferation during adulthood have been interpreted as hippocampal domains (for a review see Kaslin et al. 2008). That is the case of the dorso-medial cortex in reptiles (Lanuza et al. 2002; Ramirez-Castillejo et al. 2002; Rodríguez et al. 2002) or the lateral portion of dorsal telencephalon in teleost fish (Rodríguez et al. 2002; Broglio et al. 2005; Zupanc et al. 2005; Grandel et al. 2006; Adolf et al. 2006). On this basis, we tentatively identify the medial pallial domain that contains subpallial-derived *Nkx2.1*-expressing cells as the homolog in sharks of the primordial hippocampus. Though the typical complex layered structure of the hippocampus should appear later in evolution (probably in reptiles; Srivastava et al. 2009), it seems that the existence of a tangential pathway towards this region and the presence of cell proliferation during adulthood are conserved traits in gnathosotme vertebrates.

Pallido-striatal stream. An evolutionary conserved trait

The fact that *Nkx2.1*-positive cells appear in the LGE mantle during late embryonic development is probably due to the integration of cells of pallidal (MGE) origin in the developing striatum (LGE). Colonization of the LGE by cells originated in the MGE seems to be a rather conserved phenomenon in evolution.

Some authors have attributed this migration to future striatal interneurons in species such distant as frog (Moreno et al. 2008) or mouse (Marín et al. 2000). As far as we know this result represents the first report of such trait in fishes. Moreover, this process appears to be already present in sharks, animals belonging to the most basal gnatostome group that prensent a clear Nkx2.1-expressing MGE (Chapter 3), which implies that this migration might appear simultaneously to the evolutionary emergence of the MGE.

REFERENCES

Adolf B, Chapouton P, Lam CS, Topp S, Tannhäuser B, Strähle U, Götz M, Bally-Cuif L (2006) Conserved and acquired features of adult neurogenesis in the zebrafish telencephalon. Dev biol 295:278–293

Alvarez-Buylla A (1997) Mechanism of migration of olfactory bulb interneurons. Semin Cell Dev Biol 8:207–213

Alvarez-Buylla A, Garcia-Verdugo JM (2002) Neurogenesis in adult subventricular zone. J Neurosci 22:629–634

Alvarez-Buylla A, Lim DA (2004) For the long run: maintaining germinal niches in the adult brain. Neuron 41:683–686

Anderson SA, Marín O, Horn C, Jennings K, Rubenstein JL (2001) Distinct cortical migrations from the medial and lateral ganglionic eminences. Development 128:353–363

Anderson SA (1997) Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. Science 278:474–476

Antypa M, Faux C, Eichele G, Parnavelas John G, Andrews WD (2011) Differential gene expression in migratory streams of cortical interneurons. Eur J Neurosci 34:1584–1594

Ballard WW, Mellinguer J, Lechenault H (1993) A series of normal stages for development of *Scyliorhinus canicula*, the lesser spotted dogfish (Chondrichthyes: Scyliorhinidae). J Exp Zool 267:318-336

Balthazart J, Boseret G, Konkle ATM, Hurley, LL, Ball, GF (2008) Doublecortin as a marker of adult neuroplasticity in the canary song control nucleus HVC. Eur J Neurosci 27:801–817

Barker JM, Boonstra R, Wojtowicz JM (2011) From pattern to purpose: how comparative studies contribute to understanding the function of adult neurogenesis. Eur J Neurosci 34:963–77

Berninger B, Guillemot F, Götz M (2007) Directing neurotransmitter identity of neurones derived from expanded adult neural stem cells. Eur J Neurosci 25:2581–2590

Brill MS (2008) Regionalization of adult neurogenesis : The role of the transcription factors Dlx2 and Pax6 in the murine subependymal zone. Doctoral thesis. Ludwig-Maximilians-Universität München, Germany.

Brill MS, Snapyan M, Wohlfrom H, Ninkovic J, Jawerka M, Mastick GS, Ashery-Padan R, Saghatelyan A, Berninger B, Götz M (2008) A dlx2- and pax6-dependent transcriptional code for periglomerular neuron specification in the adult olfactory bulb. J Neurosci 28:6439–6452

Broglio C, Gómez a, Durán E, Ocaña FM, Jiménez-Moya F, Rodríguez F, Salas C (2005) Hallmarks of a common forebrain vertebrate plan: specialized pallial areas for spatial, temporal and emotional memory in actinopterygian fish. Brain Res Bull 66:277–281

Brown JP, Couillard-Després S, Cooper-Kuhn CM, Winkler J, Aigner L, Kuhn HG (2003) Transient expression of doublecortin during adult neurogenesis. J Comp Neurol 467:1–10

Butler AB, Hodos W (2005) Comparative vertebrate neuroanatomy. Evolution and adaptation. 2nd ed. Wiley-Liss, Hoboken, NJ

Byrd CA, Brunjes PC (2001) Neurogenesis in the olfactory bulb of adult zebrafish. Neuroscience 105:793–801.

Candal EM, Caruncho HJ, Sueiro C, Anadón R, Rodríguez-Moldes I (2005) Reelin expression in the retina and optic tectum of developing common brown trout. Dev Brain Res 154:187-197

Carney RSE, Bystron I, López-Bendito G, Molnár Z (2007) Comparative analysis of extra-ventricular mitoses at early stages of cortical development in rat and human. Brain Struct Funct 212:37–54.

Carrera I, Anadón R, Rodríguez-Moldes I (2012) Development of tyrosine hydroxylase-immunoreactive cell populations and fiber pathways in the brain

of the dogfish Scyliorhinus canicula: new perspectives on the evolution of the vertebrate catecholaminergic system. J Comp Neurol 520:3574–3603

Carrera I, Ferreiro-Galve S, Sueiro C, Anadón R, Rodríguez-Moldes I (2008) Tangentially migrating GABAergic cells of subpallial origin invade massively the pallium in developing sharks. Brain Res Bull 75:405–409

Cobos I, Puelles L, Martínez S (2001) The avian telencephalic subpallium originates inhibitory neurons that invade tangentially the pallium (dorsal ventricular ridge and cortical areas). Dev Biol 239:30–45

Compagnucci C, Debiais M, Coolen M, Fish J, Griffin JN, Bertocchini F, Minoux M, Rijli FM, Borday-Birraux V, Casane D, Mazan S, Depew MJ (2013) Pattern and polarity in the development and evolution of the Gnatostome jaw: both conservation and heterotopy in the branchial arches of the shark, *Scyliorhinus canicula*. Dev Biol 377:428-448

Coolen M, Sauka-Spengler T, Nicolle D, Le-Mentec C, Lallemand Y, Da Silva C, Plouhinec JL, Robert B, Wincker P, Shi DL, Mazan S (2007) Evolution of axis specification mechanisms in jawed vertebrates: insights from a chondrichthyan. PLoS One 2, 2:e374

Coolen M, Menuet A, Chassoux D, Compagnucci C, Henry S, Lévèque L, Da Silva C, Gavory F, Samain S, Wincker P, Thermes C, D'Aubenton-Carafa Y, Rodríguez-Moldes I, Naylor G, Depew M, Sourdaine P, Mazan S (2009) The dogfish

Scyliorhinus canicula, a reference in jawed vertebrates. In: Behringer RR, Johnson AD, Krumlauf RE, editors. Emerging model organisms. A laboratory manual. Vol. 1. Cold Spring Harbor, NY: CSHL Press pp, 431-446

Corbin JG, Nery S, Fishell G (2001) Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. Nat Neurosci 4:1177–1182

Curtis MA, Kam M, Nannmark U, Anderson MF, Axell MZ, Wikkelso C, Holtås S, Van Roon-Mom WMC, Björk-Eriksson T, Nordborg C, Frisén J, Dragunow M, Faull RLM, Eriksson PS (2007) Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension. Science 315:1243–1249

De Carlos JA, López-Mascaraque L, Valverde F (1996) Dynamics of cell migration from the lateral ganglionic eminence in the rat. J Neurosci 16:6146-6156

De Chevigny A, Core N, Follert P, Wild S, Bosio A, Yoshikawa K, Cremer H, Beclin C (2012) Dynamic expression of the pro-dopaminergic transcription factors Pax6 and Dlx2 during postnatal olfactory bulb neurogenesis. Front Cell Neurosci 6:6

DeDiego I, Smith-Fernández A, Fairén A (1994) Cortical cells that migrate beyond area boundaries: characterization of an early neuronal population in the lower intermediate zone of prenatal rats. Eur J Neurosci 6:983–997

Dhaliwal J, Lagace DC (2011) Visualization and genetic manipulation of adult neurogenesis using transgenic mice. Eur J Neurosci 33:1025–1036

Doetsch F (2002) Challenges for Brain Repair : Insights from Adult Neurogenesis in birds and mammals. Brain Behav Evol 58:306-322

Englund C, Fink A, Lau C, Pham D, Daza RA, Bulfone A, Kowalczyk T, Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia, intermediate progenitor cells, and postmitotic neurons in developing neocortex. J Neurosci 25:247–251

Ferrando S, Gallus L, Gambardella C, Ghigliotti L, Ravera S, Vallarino M, Vacchi M, Tagliafierro G (2010) Cell proliferation and apoptosis in the olfactory epithelium of the shark Scyliorhinus canicula. J Chem Neuroanat 40:293–300

Ferreiro-Galve S, Candal E, Rodríguez-Moldes I (2012b) Dynamic expression of pax6 in the shark olfactory system : evidence for the presence of pax6 cells along the olfactory nerve pathway. J Exp Zool 318:79-90

Ferreiro-Galve S, Rodríguez-Moldes I, Candal E (2012a) Pax6 expression during retinogenesis in sharks : comparison with markers of cell proliferation and neuronal differentiation. J Exp Zool 318:91-108

Ferreiro-Galve S, Rodríguez-Moldes I, Anadón R, Candal E (2010) Patterns of cell proliferation and rod photoreceptor differentiation in shark retinas. J Chem Neuroanat 39:1–14

Fiala JC (2005) Reconstruct: a free editor for serial section microscopy. J Microsc 218:52–61

Font E, Desfilis E, Pérez-Cañellas MM, García-Verdugo JM (2002) Neurogenesis and neuronal regeneration in the adult reptilian brain. Brain Behav Evol 58:276–295

Gadisseux JF, Goffinet AM, Lyon G, Evrard P (1992) The human transient subpial granular layer: an optical, immunohistochemical, and ultrastructural analysis. J Comp Neurol 324:94–114

Garcia-Verdugo JM, Llahi S, Ferrer I, Lopez-Garcia C (1989) Postnatal neurogenesis in the olfactory bulbs of a lizard. A tritiated thymidine autoradiographic study. Neurosci Lett 98:247–252

Grandel H, Kaslin J, Ganz J, Wenzel I, Brand M (2006) Neural stem cells and neurogenesis in the adult zebrafish brain: origin, proliferation dynamics, migration and cell fate. Dev Biol 295:263–277

Guerrero-Cázares H, Gonzalez-Perez O, Soriano-Navarro M, Zamora-Berridi G, García-Verdugo JM, Quinoñes-Hinojosa A (2011) Cytoarchitecture of the

lateral ganglionic eminence and rostral extension of the lateral ventricle in the human fetal brain. J Comp Neurol 519:1165–1180

Hack MA, Saghatelyan A, De Chevigny A, Pfeifer A, Ashery-Padan R, Lledo PM, Götz M (2005) Neuronal fate determinants of adult olfactory bulb neurogenesis. Nat Neurosci 8:865–872

Hendzel MJ, Wei Y, Mancini MA, Van Hooser A, Ranalli T, Brinkley BR, Bazett-Jones DP, Allis CD (1997) Mitosis-specific phosphorylation of histone H3 initiates primarily within pericentromeric heterochromatin during G2 and spreads in an ordered fashion coincident with mitotic chromosome condensation. Chromosoma 106:348–360

Hodge RD, Kahoud RJ, Hevner RF (2012) Transcriptional control of glutamatergic differentiation during adult neurogenesis. Cell Mol Life Sci 69:2125–2134

Hofmann MH, Northcutt RG (2012) Forebrain organization in elasmobranchs. Brain Behav Evol 80:142–151

Humphrey T (1940) The development of the olfactory and the accessory olfactory formations in human embryos and fetuses. J Comp Neurol 73:431–468

Jiménez D, López-Mascaraque LM, Valverde F, De Carlos JA (2002) Tangential migration in neocortical development. Dev Biol 244:155–169

Kam M, Curtis M a, McGlashan SR, Connor B, Nannmark U, Faull RL (2009) The cellular composition and morphological organization of the rostral migratory stream in the adult human brain. J Chem Neuroanat 37:196–205

Kaslin J, Ganz J, Brand M (2008) Proliferation, neurogenesis and regeneration in the non-mammalian vertebrate brain. Philos Trans R Soc Lond B Biol Sci 363:101–122

Kempermann G (2012) New neurons for "survival of the fittest". Nat Rev Neurosc 13:727–736

Kishimoto N, Alfaro-Cervello C, Shimizu K, Asakawa K, Urasaki A, Nonaka S, Kawakami K, Garcia-Verdugo JM, Sawamoto K (2011) Migration of neuronal precursors from the telencephalic ventricular zone into the olfactory bulb in adult zebrafish. J Comp Neurol 519:3549–3565

Kohwi M, Osumi N, Rubenstein JLR, Alvarez-Buylla A (2005) Pax6 is required for making specific subpopulations of granule and periglomerular neurons in the olfactory bulb. J Neurosci 25:6997–7003

LaDage LD, Roth TC, Pravosudov VV (2011) Hippocampal neurogenesis is associated with migratory behaviour in adult but not juvenile sparrows (Zonotrichia leucophrys ssp.). Proc Biol Sci 278:138–143

Lanuza E, Novejarque A, Moncho-Bogani J, Hernández A, Martínez-García F (2002) Understanding the basic circuitry of the cerebral hemispheres: the case of lizards and its implications in the evolution of the telencephalon. Brain Res Bull 57:471–473

Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG (1999) The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J Neurosci19:7881–7888

Lois C, Alvarez-Buylla A (1994) Long-distance neuronal migration in the adult mammalian brain. Science 264:1145–1148

Lois C, García-Verdugo JM, Alvarez-Buylla A (1996) Chain migration of neuronal precursors. Science 271:978–981

Long JE, Garel S, Alvarez-Dolado M, Yoshikawa K, Osumi N, Alvarez-Buylla A, Rubenstein JL (2007) Dlx-dependent and -independent regulation of olfactory bulb interneuron differentiation. J Neurosci 27:3230–3243

Marín O, Anderson SA, Rubenstein JL (2000) Origin and molecular specification of striatal interneurons. J Neurosci 20:6063–6076

Marín O, Rubenstein JL (2003) Cell migration in the forebrain. Annu Rev Neurosci 26:441–483

McManus KJ, Hendzel MJ (2006) The relationship between histone H3 phosphorylation and acetylation throughout the mammalian cell cycle. Biochem Cell Biol 84:640–657

Medina L, Abellán A (2009) Development and evolution of the pallium. Semin Cell Dev Biol 20:698–711

Meléndez-Ferro M, Pérez-Costas E, Villar-Cheda B, Abalo XM, Rodríguez-Muñoz R, Rodicio MC, Anadón R (2002) Ontogeny of gamma-aminobutyric populations in the forebrain and midbrain of the sea lamprey. J Comp Neurol 376:360–376

Mendoza-Torreblanca JG, Martínez-Martínez E, Tapia-Rodríguez M, Ramírez-Hernández R, Gutiérrez-Ospina G (2008) The rostral migratory stream is a neurogenic niche that predominantly engenders periglomerular cells: in vivo evidence in the adult rat brain. Neurosci Res 60:289–299

Métin C, Alvarez C, Moudoux D, Vitalis T, Pieau C, Molnár Z (2007) Conserved pattern of tangential neuronal migration during forebrain development. Development 134:2815–2827

Meyer G, Soria JM, Martínez-Galán JR, Martín-Clemente B, Fairén A (1998) Different origins and developmental histories of transient neurons in the marginal zone of the fetal and neonatal rat cortex. J Comp Neurol 397:493–518

Ming G-L, Song H (2011) Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron 70:687–702

Morante-Oria J, Carleton A, Ortino B, Kremer EJ, Fairén A, Lledo PM (2003) Subpallial origin of a population of projecting pioneer neurons during corticogenesis. Proc Natl Acad Sci U S A 100:12468–12473

Moreno N, González A, Rétaux S (2008) Evidences for tangential migrations in Xenopus telencephalon: developmental patterns and cell tracking experiments. Dev Neurobiol 68:504–520

Moreno N, González A, Rétaux S (2009) Development and evolution of the subpallium. Semin Cell Dev Biol 20:735–743

Mueller T, Wullimann MF, Guo SU (2008) Early teleostean basal ganglia development visualized by zebrafish GAD67 gene expression. J Comp Neurol 507:1245–1257

Nityanandam A, Parthasarathy S, Tarabykin V (2012) Postnatal subventricular zone of the neocortex contributes GFAP+ cells to the rostral migratory stream under the control of Sip1. Dev Biol 366:341–356

Pencea V, Luskin MB (2003) Prenatal development of the rodent rostral migratory stream. J Comp Neurol 463:402–418

Pérez-Costas E (2002). Expresión y distribución de reelina en el sistema nervioso central de la lamprea de mar. Doctoral thesis, University of Santiago de Compostela, Spain

Pérez-Costas E, Meléndez-Ferro M, Santos Y, Anadón R, Rodicio MC, Caruncho HJ (2002) Reelin immunoreactivity in the larval sea lamprey brain. J Chem Neuroanat 23:211-221

Pérez-García CG, González-Delgado FJ, Suárez-Solá ML, Castro-Fuentes R, Martín-Trujillo JM, Ferres-Torres R, Meyer G (2001) Reelin-immunoreactive neurons in the adult vertebrate pallium. J Chem Neuroanat 21:41-51

Plachez C, Puche AC (2012) Early specification of GAD67 subventricular derived olfactory interneurons. J Mol Histol 43:215–221

Pleasure SJ, Anderson S, Hevner R, Bagri A, Marín O, Lowenstein DH, Rubenstein JL (2000) Cell migration from the ganglionic eminences is required for the development of hippocampal GABAergic interneurons. Neuron 28:727–740

Porteus MH, Bulfone A, Liu JK, Puelles L, Lo LC, Rubenstein JL (1994) Dlx-2 , Mash-l , and Map-2 expression and bromodeoxyuridine incorpkation define molecularl and distinct cell population & in the embryonic mouse forebrain. J Neurosci14:6370–6383

Puelles L, Kuwana E, Puelles E, Bulfone A, Shimamura K, Keleher J, Smiga S, Rubenstein JL (2000) Pallial and subpallial derivatives in the embryonic chick and mouse telencephalon, traced by the expression of the genes Dlx-2, Emx-1, Nkx-2.1, Pax-6, and Tbr-1. J Comp Neurol 424:409–438

Puelles L, Rubenstein JL (2003) Forebrain gene expression domains and the evolving prosomeric model. Trends Neurosci 26:469–476

Ramirez-Castillejo C, Nacher J, Molowny A, Ponsoda X, Lopez-Garcia C (2002) PSA-NCAM immunocytochemistry in the cerebral cortex and other telencephalic areas of the lizard Podarcis hispanica: differential expression during medial cortex neuronal regeneration. J Comp Neurol 453:145–156

Rodríguez F, López JC, Vargas JP, Broglio C, Gómez Y, Salas C (2002) Spatial memory and hippocampal pallium through vertebrate evolution: insights from reptiles and teleost fish. Brain Res Bull 57:499–503

Rodríguez-Moldes I, Ferreiro-Galve S, Carrera I, Sueiro C, Candal E, Mazan S, Anadón R (2008) Development of the cerebellar body in sharks: spatiotemporal relations of Pax6 expression, cell proliferation and differentiation. Neurosci Lett 432:105–110

Rodríguez-Moldes I (2009) A developmental approach to forebrain organization in elasmobranchs: new perspectives on the regionalization of the telencephalon. Brain Behav Evol 74:20-29

Sanai N Tramontin AD, Quiñones-Hinojosa A, Barbaro NM, Gupta N, Kunwar S, Lawton MT, McDermott MW, Parsa AT, Manuel-García Verdugo J, Berger MS, Alvarez-Buylla A (2004) Unique astrocyte ribbon in adult human brain contains neural stem cells but lacks chain migration. Nature 427:740–744

Sanai N, Berger MS, Garcia-Verdugo JM, Alvarez-Buylla A (2007) Comment on "Human neuroblasts migrate to the olfactory bulb via a lateral ventricular extension". Science 318:393

Smeets WJAJ (1998) Cartilaginous fish. In: Nieuwenhuys R, Ten Donkelaar HJ, Nicholson C (eds). The central nervous system of vertebrates, vol 1. Springer-Verlag, Berlin, pp 551-654

Smeets WJ (1983) The secondary olfactory connections in two chondrichthians, the shark Scyliorhinus canicula and the ray Raja clavata. J Comp Neurol 218:334–344

Srivastava UC, Maurya RC, Chand P (2009) Cyto-architecture and neuronal types of the dorsomedial cerebral cortex of the common Indian wall lizard, Hemidactylus flaviviridis. Arch Ital Biol 147:21–35

Stenman J, Toresson H, Campbell K (2003) Identification of two distinct progenitor populations in the lateral ganglionic eminence: implications for striatal and olfactory bulb neurogenesis. J Neurosci 23:167–74

Stühmer T, Puelles L, Ekker M, Rubenstein JLR (2002) Expression from a Dlx gene enhancer marks adult mouse cortical GABAergic neurons. Cereb Cortex 12:75–85

Sueiro C (2003) Estudio inmunohistoquímico de los sistemas gabaérgicos del sistema nervioso central de peces elasmobranquios y su relación con sistemas catecolaminérgicos y peptidérgicos. Doctoral Thesis. Universidade de Santiago de Compostela, Spain

Sueiro C, Carrera I, Ferreiro S, Molist P, Adrio F, Anadón R, Rodríguez-Moldes I (2007) New insights on Saccus vasculosus evolution: a developmental and immunohistochemical study in elasmobranchs. Brain Behav Evol 70:187–204

Sueiro C, Carrera I, Molist P, Rodríguez-Moldes I, Anadón R (2004) Distribution and development of glutamic acid decarboxylase immunoreactivity in the spinal cord of the dogfish Scyliorhinus canicula (elasmobranchs). J Comp Neurol 478:189–206

Tucker ES, Polleux F, LaMantia A-S (2006) Position and time specify the migration of a pioneering population of olfactory bulb interneurons. Dev Biol 297:387–401

Tuorto F, Alifragis P, Failla V, Parnavelas JG, Gulisano M (2003) Tangential migration of cells from the basal to the dorsal telencephalic regions in the chick. Eur J Neurosci 18:3388-3393

Wasowicz M, Ward R, Repérant J (1999) An investigation of astroglial morphology in torpedo and scyliorhinus. J Neurocytol 28:639–653

Whitman MC, Greer CA (2009) Adult neurogenesis and the olfactory system. Prog Neurobiol 89:162-175

Wichterle H, Turnbull DH, Nery S, Fishell G, Alvarez-Buylla A (2001) In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. Development 128:3759–3771

Wu S, Esumi S, Watanabe K, Chen J, Nakamura KC, Nakamura K, Kometani K, Minato N, Yanagawa Y, Akashi K, Sakimura K, Kaneko T, Tamamaki N (2011) Tangential migration and proliferation of intermediate progenitors of GABAergic neurons in the mouse telencephalon. Development 138:2499–2509

Wullimann MF (2009) Secondary neurogenesis and telencephalic organization in zebrafish and mice: a brief review. Integr Zool 4:123–133

Zupanc GKH, Hinsch K, Gage FH (2005) Proliferation, migration, neuronal differentiation, and long-term survival of new cells in the adult zebrafish brain. J Comp Neurol 488:290–319

ABBREVIATIONS

Cb	cerebellum
Di	diencephalon
Glom	glomerulus
LGE	lateral ganglionic eminence
LS	lateral stream
Mes	mesencephalon
MGE	medial ganglionic eminence
MS	medial stream
OB	olfactory bulb
OT	optic tectum
P	pallium
PSB	pallial-subpallial boundary
Rh	rhombencephalon
RS	rostral stream
Sp	subpallium
Tel	telencephalon
v	ventricle

Table I. Primary and secondary antibodies used. IF, immunofluorescence; IE, immunoenzyme staining

Primary Antibody	Source	Working dilution
GAD (Glutamic acid decarboxylase)	Polyclonal sheep anti-GAD65/67 Provided by Dr. E. Mugnaini	1:30000 (IF)
DCX (Doublecortin) Cat. no. 4604	Polyclonal rabbit anti-DCX Cell Signaling Technology, Beverly, MA.	1:500 (IE) 1:300 (IF)
DCX (Doublecortin) Cat. no. sc-8066	Polyclonal goat anti-DCX Santa Cruz Biotechnology, Santa Cruz, CA.	1:100 (IF)
GFAP (glial fibrillary acidic protein) Cat. no. Z0334	Polyclonal rabbit anti-GFAP Dako, Glostrup, Denmark;	1:500
RELN (Reelin; clone 142) Cat.no. MAB5366	Monoclonal mouse anti-RELN Millipore, Billerica, MA	1:150 (IF)
PCNA (Proliferating cell nuclear antigen) Cat. no. P8825	Monoclonal mouse anti-PCNA Sigma, St. Louis, MO	1:200 (IE) 1:300 (IF)
Pax6 Cat. no. PRB-278P	Polyclonal rabbit anti-Pax6 Covance, Emeryville, CA	1:200 (IF)
PH3 (Phospho-histone-H3) Cat.no. 06-570	Polyclonal rabbit anti- PH3 Upstate BioTechonogy	1:400
TH (Tyrosine Hydroxylase) Cat.no. MAB318	Monoclonal mouse anti-TH Millipore, Billerica, MA	1:1000 (IE) 1:500 (IF)
Secondary antibody	**Source**	**Working dilution**
GARb (Biotinilated goat anti-rabbit)	Dako Glostrup, Denmark	1:500
GAMb (Biotinilated goat anti-mouse)	Dako Glostrup, Denmark	1:500
DASh^{633} (633-conjugated donkey anti-sheep)	Molecular Probes Eugene, OR	1:100
DAM^{488} (488-conjugated donkey anti-mouse)	Molecular Probes Eugene, OR	1:100
DAR^{546} (546-conjugated donkey anti-rabbit)	Molecular Probes Eugene, OR	1:100
DAG^{488} (488-conjugated donkey anti-goat)	Molecular Probes Eugene, OR	1:100

Table 2. Summary of the different streams related to embryonic stages of development and markers used as diagnostics to characterize their pathways. The proposed homologies with mammalian routes are indicated at the right column. Abbreviations: sub, subventricular; v, ventricular.

	Developmental stages	Route		Origin	To	Markers	Features of adult neurogenic system	Proposed homology
EARLY DEVELOPMENT	St.29 to 32	Subpial stream		LGE MGE ?	Dorsal pallium	*Dlx2*/GAD Reelin	--	Embryonic tangential route to the dorsal pallium
	St.29 on	Subventricular	***Ls** (lateral stream)*	LGE	OB	*Dlx2*/GAD DCX Pax6 TH	PCNA, subDCX vGFAP Pax6 TH	Mammalian rostral migratory stream
			***Rs** (rostral stream)*	LGE MGE ?	Rostral pallium	*Dlx2*/GAD	PCNA subDCX vGFAP TH	Not found (elasmobranch specialization)
LATE DEVELOPMENT	St.32 on	***Ms**(medial stream)*		MGE	Medial pallium	*Dlx2* *Nkx2.1*	PCNA subDCX vGFAP	Tangential stream to the hippocampus
	St.32 on	Pallido-striatal stream		MGE	LGE	*Nkx2.1*	-	Tangential route of future striatal interneurons

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Fig.1. Gross morphology of the embryonic and adult telencephalon of *S.canicula*. Some specific features are shown, as the lateral emergence of the olfactory bulbs (arrows), the characteristic rostral protrusion (arrowheads) and the existence of an opened ventricle between olfactory bulb and lateral ventricles (asterisks). **A,B:** Schematic drawings of the surface of the early and late developing telencephalon. Arched arrows show the medial convergence of the telencephalic walls. **C:** Drawing of horizontal, sagittal and transverse sections of prehatching-juvenile *S.canicula* brain. The level of the transverse sections is indicated with red lines. **D,E:** Lateral and dorsal views of the adult telencephalon. **F**: Schematic representation of a horizontal section across the adult telencephalon. For abbreviations, see list. *Scale bars*: 5mm

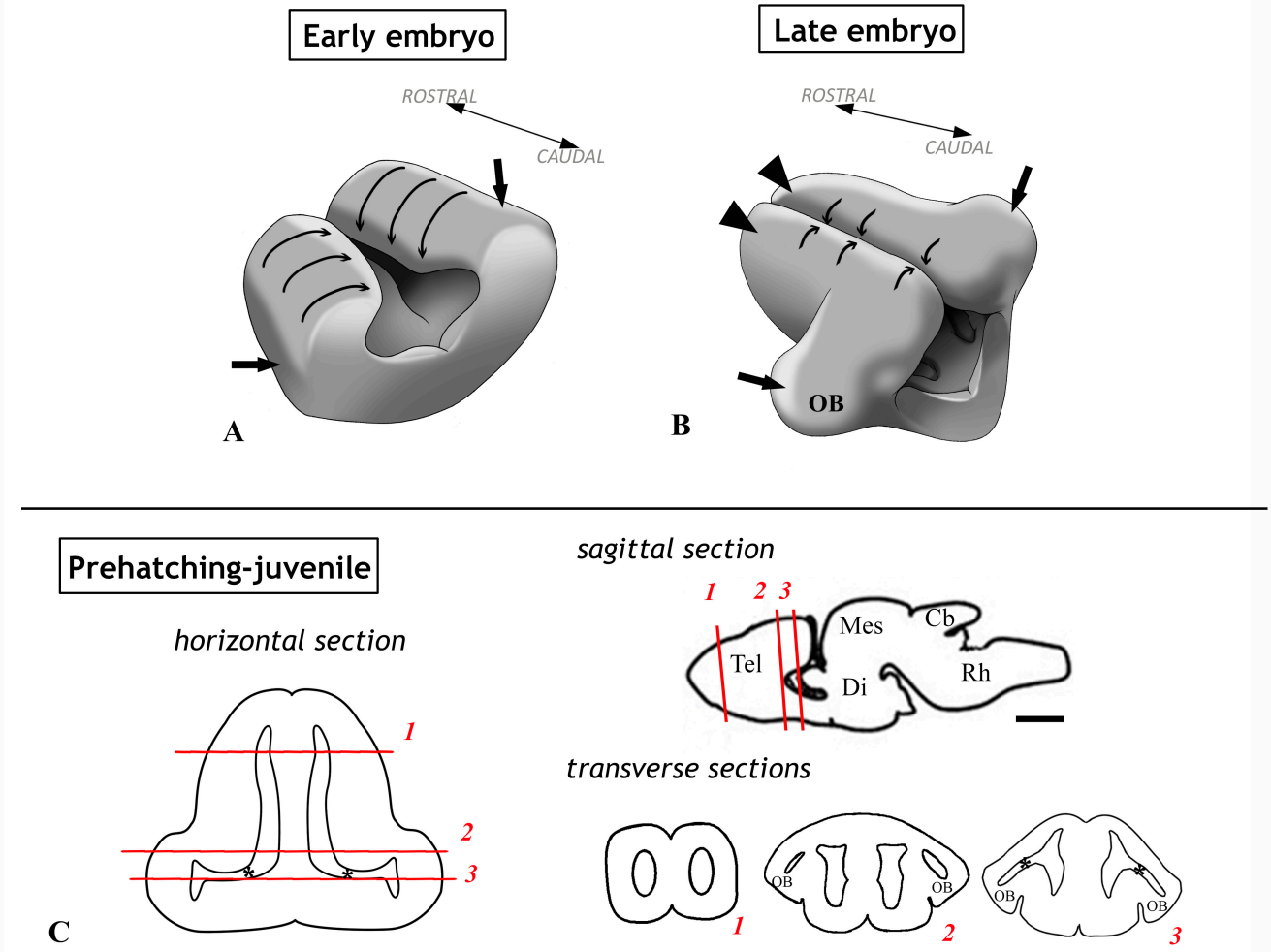

Early embryo
ROSTRAL
CAUDAL
A
Late embryo
ROSTRAL
CAUDAL
OB
B
Prehatching-juvenile
horizontal section
1
2
3
C
sagittal section
1 2 3
Tel
Mes
Cb
Di
Rh
transverse sections
OB
OB
OB
OB
1
2
3
Adult
Frontal-lateral view
Cb
OB
Tel
OT
D
Dorsal view
OB
OB
Tel
E
horizontal section
OB
OB
V
F

Figure 1

Fig.2. GAD telencephalic expression at stage 28-29 is restricted to the subpallial territory. **A,B:** Sagittal (A) and transverse (B) sections. **C:** Three-dimensional representation of the external morphology of the telencephalon (grey) and the expression domain of GAD (blue) at this stage. For abbreviations, see list. *Scale bars*: 200 µm.

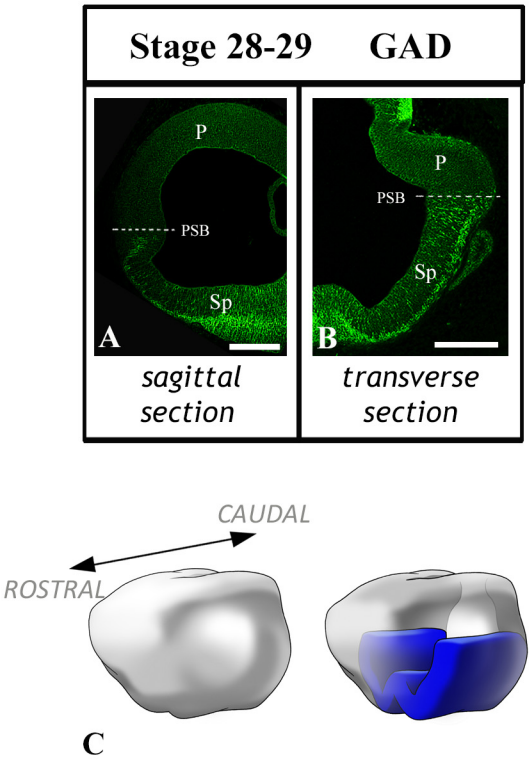

Stage 28-29 GAD
P
PSB
Sp
A
P
PSB
Sp
B
sagittal section
transverse section
CAUDAL
ROSTRAL
C

Figure 2

Fig.3. Streams of subpallial cells observed in the pallium at stage 31. **A-G:** Transverse sections of the telencephalon showing the expression of GAD (A,B,C), *Dlx2* (D,E,F) and Reelin (G). Note that GAD and *Dlx2* distribution was identical at this stage (A-F) and depicted the trajectory of three proposed tangential palliopetal streams one of which ended in a pallial region where reelin was specifically expressed (G). Diagrams on the left show the level of the sections. Asterisks indicate the position of the lateral ganglionic eminence. **H:** Three-dimensional representation of the external morphology of the telecephalon (grey) and an internal view showing the distribution of the subpial, lateral and rostral streams (green, yellow and red, respectively). Blue territory corresponds to the subpallium. For abbreviations, see list. *Scale bars*: 300µm.

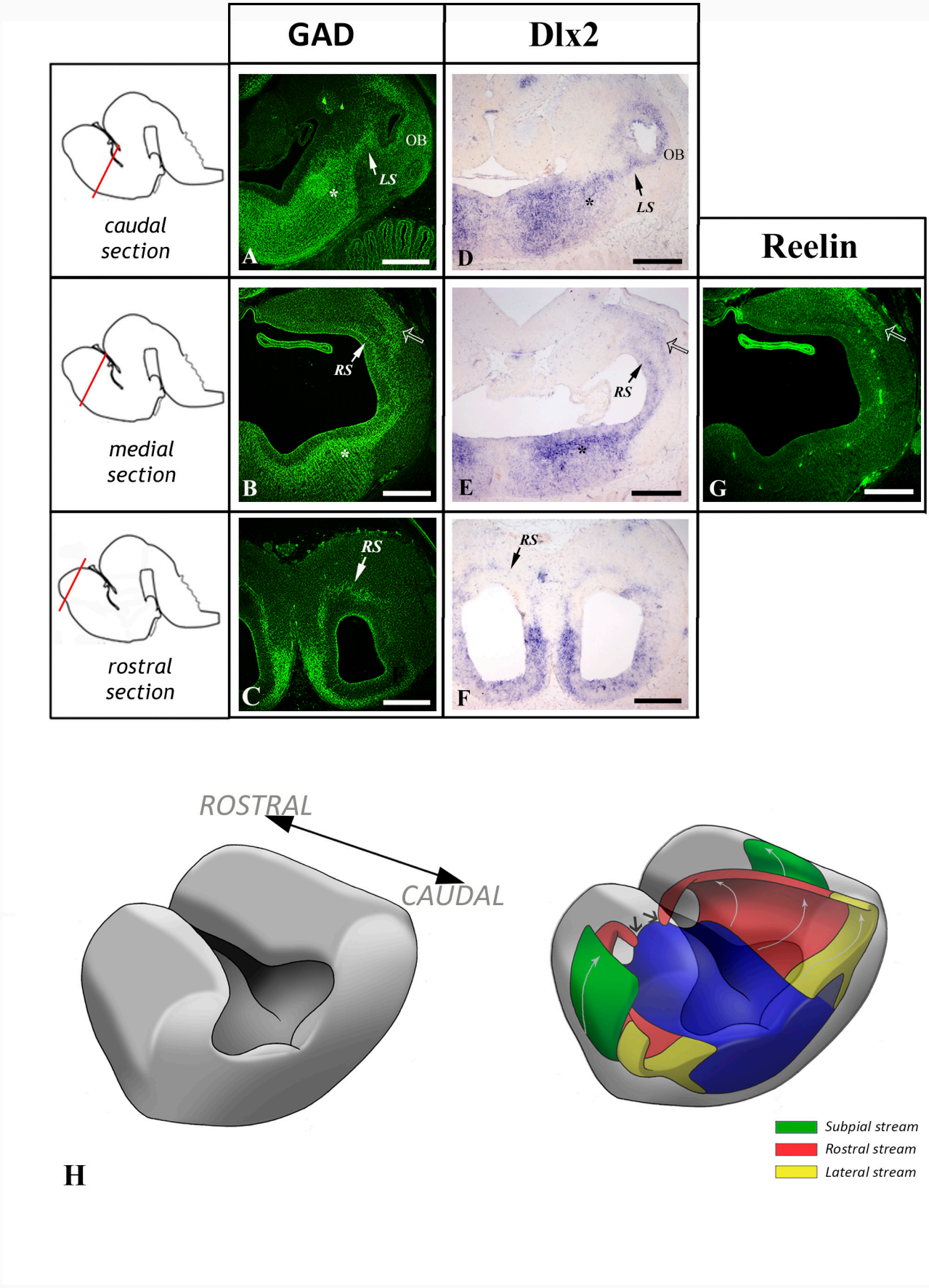

GAD
Dlx2
Reelin
caudal section
medial section
rostral section
OB
LS
RS
A
B
C
D
E
F
G
ROSTRAL
CAUDAL
Subpial stream
Rostral stream
Lateral stream
H

Figure 3

Fig.4. Tangential streams during late development in *S.canicula* telencephalon. *Dlx2* expression still depicted rostral (*RS* in A-C) and lateral (*LS* in D-F) routes. Two additional routes apparently emerging from MGE were evident by means of *Nkx2.1 in situ* hybridization (H-J): the medial stream towards the medial pallium (*MS* in G-J) and an apparent dispersion toward LGE territories (I,J). Diagrams on the top show the level of the sections. (K) Three-dimensional representation of the external morphology of the telencephalon in the late embryo (grey) and an internal view showing the distribution of the described streams (red, yellow, light blue and white arrows). For abbreviations, see list. *Scale bars*: 500µm.

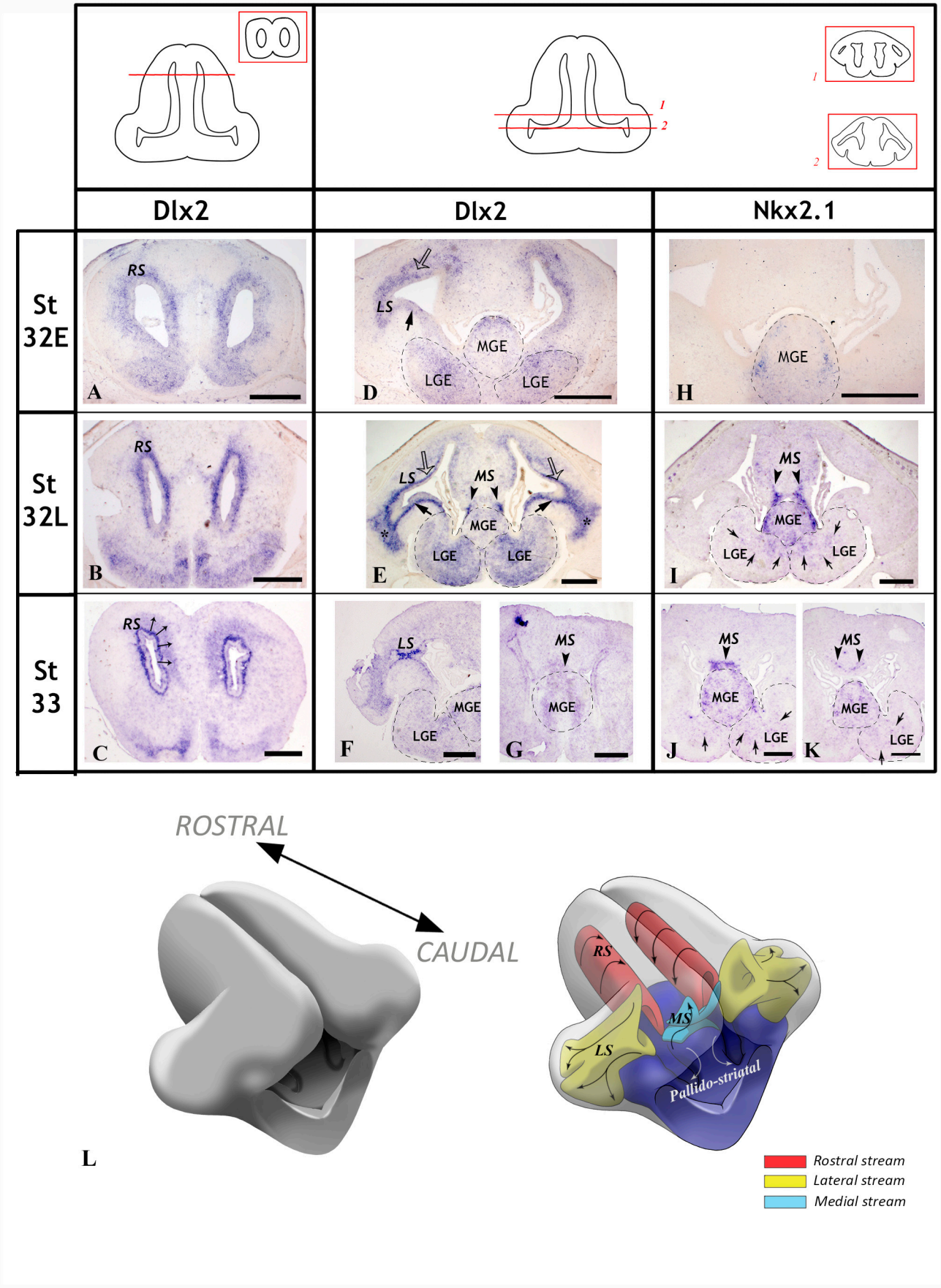

Dlx2
Dlx2
Nkx2.1
St 32E
St 32L
St 33
RS
LS
MS
MGE
LGE
A
B
C
D
E
F
G
H
I
J
K
L
ROSTRAL
CAUDAL
Pallido-striatal
Rostral stream
Lateral stream
Medial stream

Figure 4

Fig.5. Lateral stream towards the olfactory bulb exhibits features of a RMS-like route. **A:** Schematic representation of a horizontal section across the telencephalon illustrating the position of the lateral stream in the mature brain (red points). **B-G:** Ventricles leading to the olfactory bulbs contain abundant cells expressing proliferating markers as PCNA and PH3 from late development (B-D) till adulthood (E). Some PCNA-positive cells exhibit lengthened morphology in the subventricular zone (arrows in F,G). **H-I:** Along with PCNA, DCX is highly expressed in fibers oriented parallel to the ventricular surface (arrows in H'') and also in a subventricular position (arrowheads in H, I). GFAP-positive processes in contact with the ventricular surface were also noticed here (open arrows in I). **J,K:** DCX-positive processes are abundant in the glomeruli of the olfactory bulb of adults. **L,M:** Pax6 is expressed in cells at the lateral stream during early development (arrow in L) colocalizating with DCX (arrows in M). **N-P':** TH expression was specially noted in cells located alongthe lateral stream from from stage-32 embryos (N) and became very evident in adult specimens (O). Colocalization of TH with Pax6 was detected in neurons of the olfactory bulb from embryonic stage 32 (P,P'). For abbreviations, see list. *Scale bars*: 500µm (B-E,J,K,O); 100µm (F,G,I,P'); 150µm (H); 300µm (L); 50µm (M); 200µm (N,P).

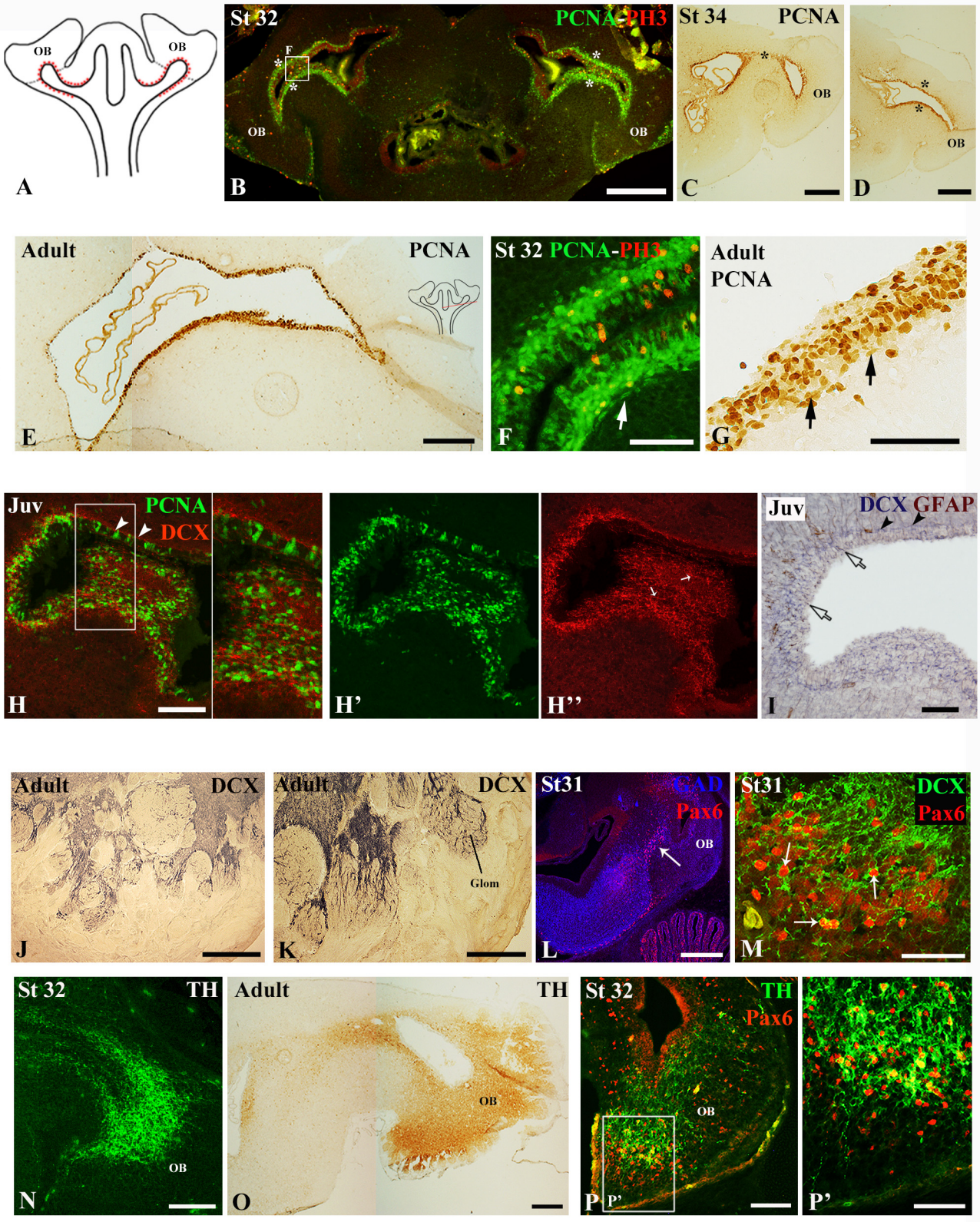

A
OB
OB
St 32
PCNA-PH3
F
OB
OB
B
St 34
PCNA
OB
C
OB
D
Adult
PCNA
E
St 32 PCNA-PH3
F
Adult
PCNA
G
Juv
PCNA
DCX
H
H'
H''
Juv
DCX GFAP
I
Adult
DCX
J
Adult
DCX
Glom
K
St31
GAD
Pax6
OB
L
St31
DCX
Pax6
M
St 32
TH
OB
N
Adult
TH
OB
O
St 32
TH
Pax6
OB
P
P'
P'

Figure 5

Fig. 6. Pallial structures derived from rostral and medial streams during late development and adulthood show characteristics of adult neurogenic zones. **A:** Schematic representation of a horizontal section across the telencephalon illustrating the position of the rostral proliferating zone (red points). **B,C:** *Dlx2* expression in the rostral telencephalon during late development showing the apparent dispersion of cells at stage 32 from the subventricular region towards an inner zone (arrows in B), which exhibits a layering arrangement at stage 33 (broken line in C). (B',C') Parallel sections to B-C showing *Tbr1* expression. Note the additional layer (broken line) located immediately above the *Dlx2*-positive layer (compare B,C versus B',C'). **D,D':** Hematoxylin-eosin staining also showed a patent layered structure in the adult telencephalon at the same rostral position. **E,F:** Proliferating cells in the ventricular rostral zone of late embryos (E) and adults (F). **G,H:** Subventricular DCX and GFAP processes are also present in this rostral zone. **I,J:** TH-positive cells and fibers are also abundant in the rostral ventricular zone and adjacent inner layers. **K:** Schematic representation of a horizontal section across the telencephalon illustrating the position of the medial proliferating zone (red points). **L-N:** Markers of cell proliferation are expressed in cells in the ventricular region of the medial pallium from late embryos (arrows in L,M) to adults (arrow in N). **O-Q:** DCX-positive elements and GFAP processes contacting with the ventricular surface are present in the medial pallium of postnatspecimens. *Scale bars*: 500µm (B-F,H,I,L,M); 100µm (D', G, P,Q); 250µm (J,N,O).

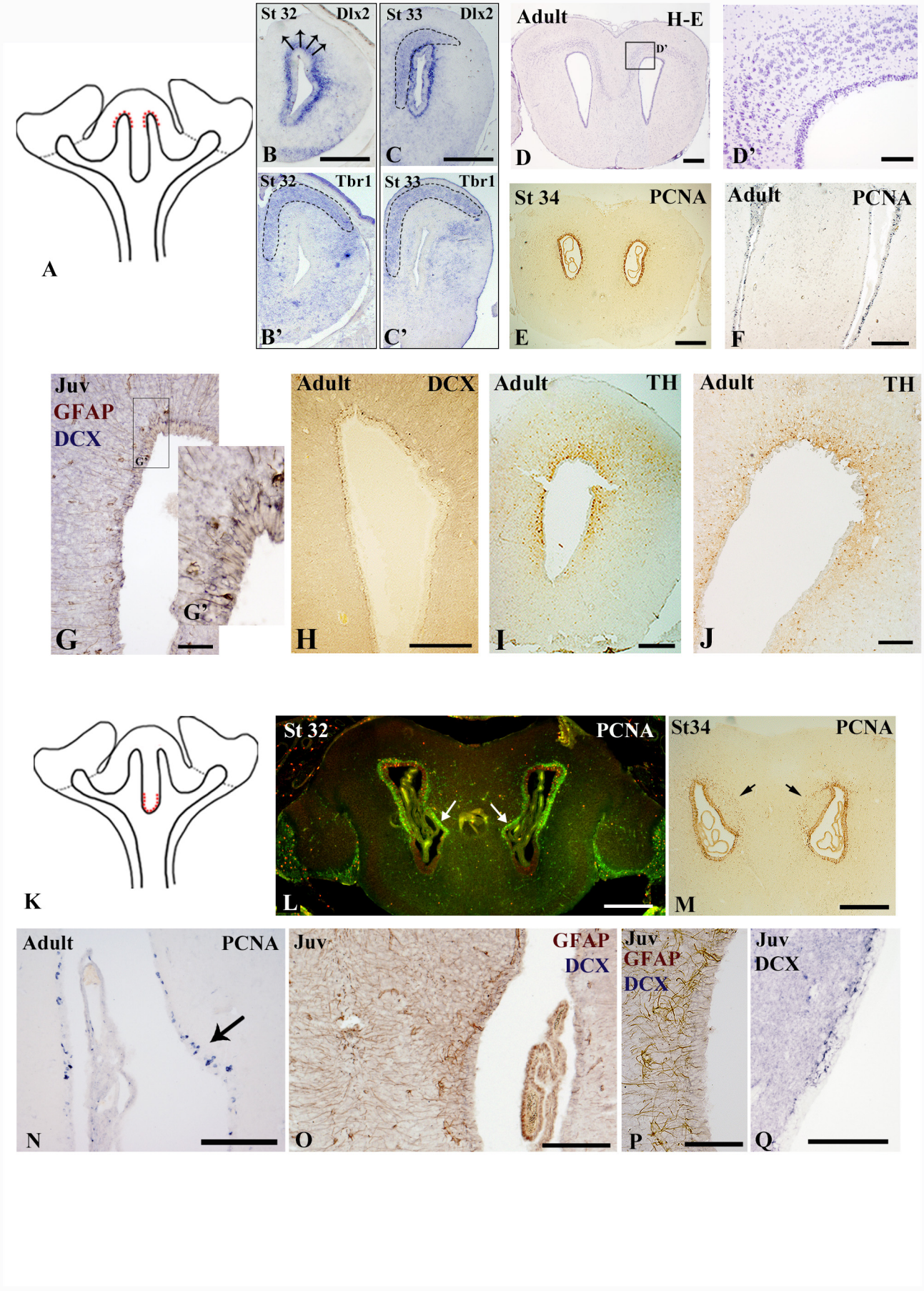
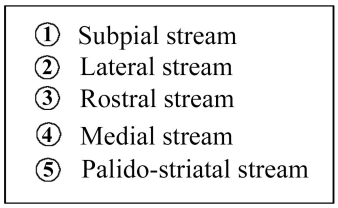
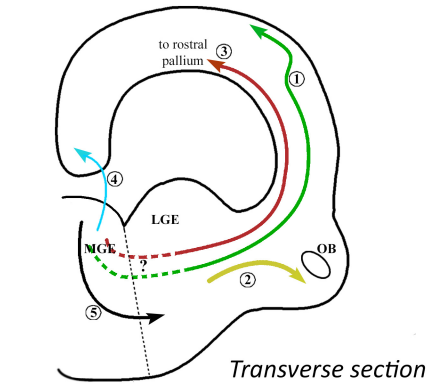

A
St 32 Dlx2
B
St 33 Dlx2
C
Adult H-E
D'
D
D'
St 32 Tbr1
B'
St 33 Tbr1
C'
St 34 PCNA
E
Adult PCNA
F
Juv
GFAP
DCX
G'
G
Adult DCX
H
Adult TH
I
Adult TH
J
K
St 32 PCNA
L
St34 PCNA
M
Adult PCNA
N
Juv GFAP DCX
O
Juv GFAP DCX
P
Juv DCX
Q

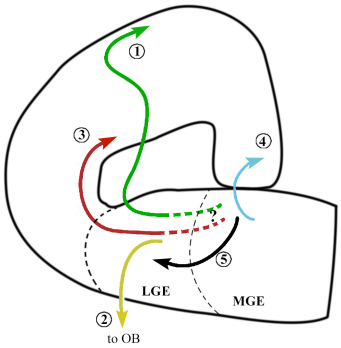
Figure 6

Fig.7. Schematic drawings of the proposed embryonic routes for telencephalic tangential migrations (A,B) and the proliferative regions of the postnatal telencephalon (C). **A,B:** Pointed lines and question mark indicate that the MGE origin in these routes is considered but could not be determined in the present work. **C:** Some of the routes are directed towards the only telencephalic pallial regions where adult cell proliferation (red points) seems to be maintained (indicated with their corresponding numbers).

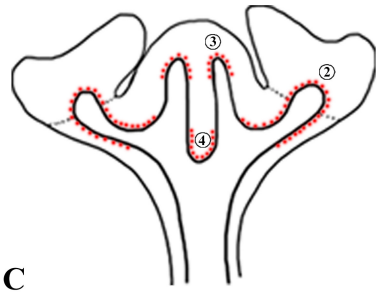

① Subpial stream
② Lateral stream
③ Rostral stream
④ Medial stream
⑤ Palido-striatal stream


to rostral pallium
③
①
④
LGE
MGE
OB
②
⑤
Transverse section

A


①
③
④
⑤
LGE
MGE
②
to OB
Sagittal section

B


③
②
④

C

Figure 7

Fig. 8. Summary of spatial and temporal sequence of cells expressing different markers along the lateral stream in S.canicula compared with the developmental stages during adult mammalian subventricular zone neurogenesis proposed by Ming and Song (2011). Adapted from Ming and Song (2011). For abbreviations, see list

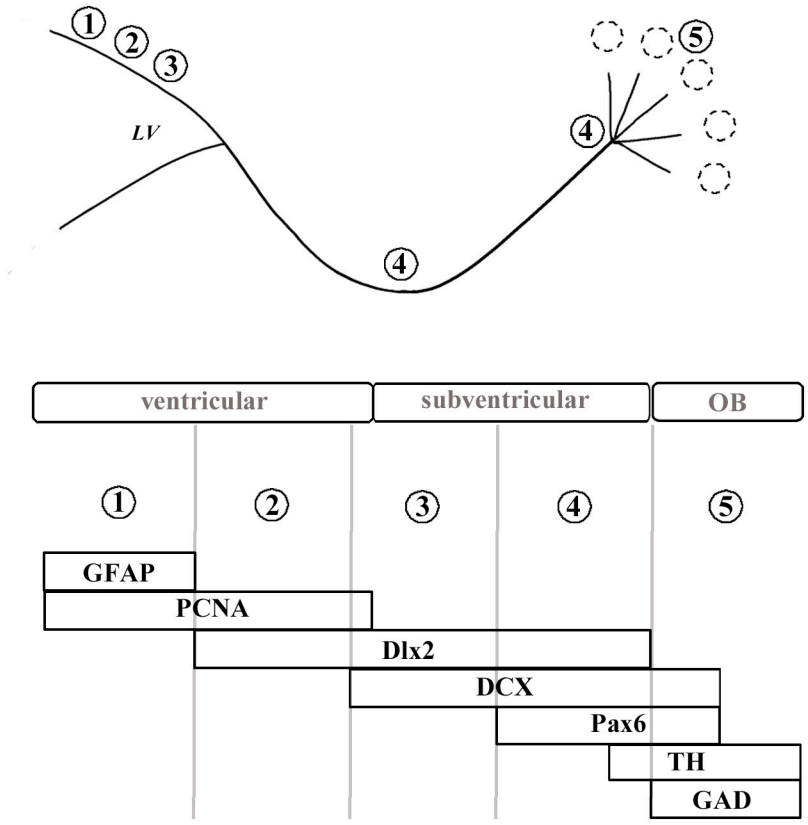

1
2
3
LV
4
5
4
ventricular
subventricular
OB
1
2
3
4
5
GFAP
PCNA
Dlx2
DCX
Pax6
TH
GAD
1 Quiescent radial-glia like cell
2 Transient amplifying cell
3 Neuroblast
4 Migrating neuroblast/immature neuron
5 Interneurons

Figure 8

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Discussion

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Despite in the last decade some studies have been focused on the development of the cartilaginous fishes brain (Chiba et al. 2002; Sueiro et al. 2003; O'Neill et al. 2007; Carrera et al. 2008; Ferreiro-Galve et al. 2008; Rodríguez-Moldes et al. 2011), this thesis represents the first monographic study of the telencephalon and peripheral systems associated to it employing a variety of experimental approaches. We performed immunohistochemical, tract-tracing and *in situ* hybridization techniques in an elasmobranch species, the lesser spotted dogfish *Scyliorhinus canicula*. We present a thoughtful characterization of these structures from early embryonic development to adulthood, which intend to fill a gap in the knowledge about vertebrate telencephalon evolution and, particularly, about its development.

A previous study from our group (Ferreiro-Galve et al. 2012) established the general events of the developing peripheral olfactory system in *S.canicula* by means of Pax6 expression pattern analysis. In the present thesis, we have extended that work by analyzing additional embryonic stages, additional immunohistochemical markers and performing tract-tracing experiments. We detected an early population of neurons that seemed to delaminate from the olfactory placode and migrate toward the telencephalon before the olfactory nerve was formed, which suggested that these cells play a pioneering role in the peripheral olfactory pathway. These pioneer olfactory neurons have been also described in other vertebrates (zebrafish: Whitlock and Westerfield 1998; chick: Fornaro et al. 2001; human: Bystron et al. 2006; mouse: Ikeda et al. 2007) but our

work represents the first evidence in a cartilaginous fish of this key event in the initial formation of the olfactory pathway. Ferreiro-Galve et al (2012) described some Pax6 cells in the olfactory epithelium and along the fibers of the olfactory nerve which have never been reported in any other vertebrate species so far. This fact led us to characterize the nature of these Pax6 cells to shed light on their identity and possible roles. In the olfactory epithelium, we evidenced that Pax6 is specifically and transiently expressed during the maturation of cells belonging to the olfactory receptor neurons lineage, representing the first evidence of the implication of this transcriptional factor in the formation of olfactory receptors. We also evidenced for the first time that Pax6 cells along the olfactory fibers were migrating neurons representing a subpopulation of cells within the migratory mass and that they eventually formed transient corridors at the entrance of the olfactory bulb. Studies focused on the migratory mass have been performed in other vertebrates, however none of them described Pax6 neurons within it. We postulate that these Pax6 migrating neurons may be acting as guidepost cells for axons of the olfactory receptor neurons navigating towards the olfactory bulb, as these cells exhibit some of the features described for guidepost cells (Conzelman et al. 2002). This is an innovative hypothesis that must be confirmed with further studies. Still, it raises interesting questions about Pax6 implication in axon guidance processes. Additionally, we described as some of the Pax6 negative neurons within the migratory mass branched off this route at certain point and head toward rostral telencephalic regions. This led us to investigate if they could

be related with the embryonic component of the terminal nerve system. This system had been broadly studied in adult elasmobranchs (Bullock and Northcutt 1984; Demski et al. 1987; Demski and Schwanzel-Fukuda 1987; Chiba et al. 1991; Wu et al. 1992; White and Meredith 1993; Chiba 2000; Forlano et al. 2000; Moeller and Meredith 2010; Yáñez et al. 2011) but its development remained completely unexplored. We performed a deep characterization of this intriguing group of neurons and found that they projected their axons to a basal region of the telencephalic hemispheres. We described as this initial group of migrating neurons became progressively arranged in clusters coursing along their own fibers and began to express FMRF-amide peptide, a specific marker of the terminal nerve system. Eventually, they formed the typical ganglia of the terminal nerve system described in adults which confirmed that they effectively belonged to this system. This study is the first description of the development of the terminal nerve in a cartilaginous fish and provides interesting data about its origin and spatio-temporal relationship with the olfactory system during embryogenesis. Together, our results pointed to a shared origin of the olfactory and terminal nerve systems in the olfactory epithelium.

While performing this study a number of questions arose that deserved to be addressed. What is the identity of the entrance territory of the terminal nerve? How is the telencephalon regionalized in elasmobrachs? We realized that the developmental studies available were not enough to interpret the anatomy of the developing telencephalon of cartilaginous fishes and that even the adult

nomenclature for telencephalic structures was a bit confusing. In order to shed light on the telencephalic development and regionalization of sharks as well as on the identification of some adult structures, we aimed at exploring this zone of the central nervous system with different experimental methodologies. Firstly, we analyzed the distribution pattern of key regulatory genes involved in regional specification of the telencephalon. Selected genes were proved to be conserved between different vertebrate species, thus serving as reliable markers of telencephalic subdivisions. We successfully delineated the pallial-subpallial boundary not only at ventricular levels (as previously described by Carrera et al. 2008; Ferreiro-Galve et al. 2008) but also along its entire extent in the intermediate and marginal zones. This delimitation paved the way to clarify the identity of some telencephalic nuclei. Furthermore, we carried out the first description of telencephalic embryonic subdivisions in a cartilaginous fish under a genoarchitectonic approach. In the pallium, we proposed the existence of four territories comparable to those observed in tetrapods: medial, dorsal, lateral and ventral pallium. In the subpallium, the equivalents of lateral and medial ganglionic eminences, the embryonic precursors of the striatal and pallidal components of the basal ganglia, were identified. Additionally, a preoptic area compartment was described in the caudal subpallium. Our results indicate that the basic genetic program for telencephalic development was already present in the most basal extant gnathostome evidencing that this program is highly conservative throughout evolution. Moreover, comparisons of genoarchitectonic data from

late and prehatching embryos with the adult morphology allowed us to clarify the identity of some telencephalic structures in the adult telencephalon.

The establishment of the basic map of telencephalic subdivisions carried out in this thesis will be very useful as a frame for subsequently interpretations of the telencephalic anatomy. In fact, it served us as a starting point for deeper studies about telencephalic development processes and anatomy. For instance, the identification of the embryonic precursors of the basal ganglia in the subpallium (lateral and medial ganglionic eminences equivalents) allowed us to follow their development and identify their derivatives. We found that the classic definition of basal ganglia territories in elasmobranchs was highly controversial and that any of the proposals coincided with the conclusions drawn from our genetic study. Therefore, we decided to carry out additional experiments which could support or discard out hypothesis. We focused on the proposed striatal domain, the regions termed *area periventricularis ventrolateralis* (APVL) and *area superficialis basalis* (ASB) by classic studies. We characterized this subpallial basal region by analyzing its connections by means of tract-tracing experiments and analyzed the relation of these connections with the catecholaminergic cell groups of the diencephalon and mesencephalon previously described by Carrera et al. (2012). We demonstrated that the region proposed by us as the striatum (ASB/APVL) showed a connectional profile highly compatible with a striatal structure. It receives catecholaminergic inputs from the diencephalic-mesencephalic groups and appears to be the source of a non catecholaminergic descending projection.

These projections are greatly reminiscent to the nigrostriatal and striatonigral connections described in the basal ganglia of other vertebrates and represent the first evidence of the existence of these basal ganglia pathways in elasmobranchs. Moreover, these evidences support our premise that the region proposed by us actually represents the striatum of elasmobranchs.

Besides representing the basal ganglia precursors, lateral and medial ganglionic eminences are well-known structures for being the origin of most tangential migrations of the telencephalon (reviewed in Marín and Rubenstein 2003; Wu et al. 2011). These migrations have been described in several representative species of different vertebrate groups, including the shark *S. canicula* (Carrera et al, 2008). However, their routes and specific pathways have only been well documented in mammals and birds (Cobos et al. 2001; Corbin et al. 2001). The subpallial markers GAD and *Dlx2* have been widely used as reliable markers of the cell populations that migrate tangentially from the ganglionic eminences, as most of these cells are GABAergic and express genes belonging to the Dlx family since their early specification until their arrival to diverse telencephalic domains (Anderson 1997: Lavdas et al. 1999; Anderson et al. 2001; Corbin et al. 2001; Wichterle et al. 2001). We performed a detailed analysis of the spatio-temporal distribution of GAD and *Dlx2* in the embryonic telencephalon of *S. canicula* with the aim of identifying potential tangential migratory pathways. Our results pointed to the existence of five embryonic migratory routes directed towards different telencephalic zones. Comparison of our results with those of

other vertebrates indicated that some of these routes seemed to be equivalent to tangential routes described in mammals while other seemed to be a derived feature of elasmobranchs. Moreover, some of these routes seemed to reach pallial zones that corresponded with the only regions that presented features of potential adult neurogenic niches. Of these, the tangential route directed towards the olfactory bulb was undoubtedly recognized in postnatal and adult specimens as a proliferative system, probably equivalent to the rostral migratory stream of mammals. The observation of this system in such a basal vertebrate led us to propose a hypothesis about the evolution of the rostral migratory stream along vertebrate phylogeny involving a ventricular closure event. Besides the interesting evolutionary implications, the existence of these systems in *S.canicula* paves the way to the identification and investigation of adult neurogenic niches in elasmobranchs.

The existence of palliopetal migrations in the telencephalon of *S.canicula* could be predictable from previous works. However, the colonization of the pallium by subpallial cells observed in this thesis resulted way more massive than expected. We described as regions considered classical pallial nuclei have a subpallial embryonic origin and they probably represent groups of inhibitory interneurons that contribute to the formation of complex structures in the pallium. A good example is the olfactory bulb. Our results demonstrate that it is a pallial derivative that receives a remarkable contribution from subpallial zones that form the granular layer of this structure. Similarly, other regions of the telencephalon

showed a similar stratified pattern, with an inner layer formed by subpallial originated cells, and an outer layer exhibiting pallial markers. This is the case of the *area periventricularis pallialis*, which seems to be a more important and complex structure than previously thought.

In conclusion, our results highlight the importance of the studies about morphogenesis and development in cartilaginous fishes to unravel the evolution of the telencephalon of vertebrates. Furthermore, this study also evidences the suitability of *S.canicula* for the study of the developmental dynamics of the telencephalic hemispheres as well as ofolfactory and terminal nerve systems

REFERENCES

Anderson SA (1997) Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. Science 278:474–476

Anderson SA, Marín O, Horn C, Jennings K, Rubenstein JL (2001) Distinct cortical migrations from the medial and lateral ganglionic eminences. Development 128:353–363

Bullock TH, Northcutt RG (1984) Nervus terminalis in dogfish (Squalus acanthias, Elasmobranchii) carries tonic efferent impulses. Neurosci Lett 44:155–160

Bystron I, Rakic P, Molnár Z, Blakemore C (2006) The first neurons of the human cerebral cortex. Nat Neurosci 9:1-7

Carrera I, Ferreiro-Galve S, Sueiro C, Anadón R, Rodríguez-Moldes I (2008) Tangentially migrating GABAergic cells of subpallial origin invade massively the pallium in developing sharks. Brain Res Bull 75:405–409

Carrera I, Anadón R, Rodríguez-Moldes I (2012) Development of tyrosine hydroxylase-immunoreactive cell populations and fiber pathways in the brain of the dogfish Scyliorhinus canicula: new perspectives on the evolution of the vertebrate catecholaminergic system. J Comp Neurol 520:3574–3603

Chiba A (2000) Immunohistochemical cell types in the terminal nerve ganglion of the cloudy dogfish, Scyliorhinus torazame, with special regard to neuropeptide Y/FMRFamide-immunoreactive cells. Neurosci Lett 286:195–8

Chiba A, Oka S, Honma Y (1991) Immunocytochemical distribution of FMRFamide-like substance in the brain of the cloudy dogfish, Scyliorhinus torazame. Cell Tissue Res 265:243–250

Chiba A, Oka S, Saitoh E (2002) Ontogenetic changes in neuropeptide Y-immunoreactive cerebrospinal fluid-contacting neurons in the hypothalamus of the cloudy dogfish, Scyliorhinus torazame (Elasmobranchii). Neurosci Lett 329:301-304

Cobos I, Puelles L, Martínez S (2001) The avian telencephalic subpallium originates inhibitory neurons that invade tangentially the pallium (dorsal ventricular ridge and cortical areas). Dev Biol 239:30–45

Conzelmann S, Levai O, Breer H, Strotmann J (2002) Extraepithelial cells expressing distinct olfactory receptors are associated with axons of sensory cells with the same receptor type. Cell Tissue Res 307:293-301

Corbin JG, Nery S, Fishell G (2001) Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. Nat Neurosci 4:1177–1182

Demski LS, Fields RD, Bullock TH, Schreibman MP, Margolis-Nunno H (1987) The terminal nerve of sharks and rays: electron microscopic, immunocytochemical, and electrophysiological studies. Ann N Y Acad Sci 519:15–32

Demski LS, Schwanzel-Fukuda M (1987) The terminal nerve (nervus terminalis): structure, function, and evolution. Introduction. Ann N Y Acad Sci 519:ix–xi

Ferreiro-Galve S, Candal E, Rodríguez-Moldes I (2012) Dynamic expression of Pax6 in the shark olfactory system: evidence for the presence of Pax6 cells along the olfactory nerve pathway. J Exp Zool B Mol Dev Evol 318:79-90

Ferreiro-Galve S, Carrera I, Candal E, Villar-Cheda B, Anadón R, Mazan S, Rodríguez-Moldes I (2008) The segmental organization of the developing shark brain based on neurochemical markers, with special attention to the prosencephalon. Brain Res Bull 75:236–240

Ferreiro-Galve S, Carrera I, Candal E, Villar-Cheda B, Anadón R, Mazan S, Rodríguez-Moldes I (2008) The segmental organization of the developing shark brain based on neurochemical markers, with special attention to the prosencephalon. Brain Res Bull 75:236-240

Forlano PM, Maruska KP, Sower SA, King JA, Tricas TC (2000) Differential distribution of gonadotropin-releasing hormone-immunoreactive neurons in the stingray brain: functional and evolutionary considerations. Gen Comp Endocrinol 118:226–48

Fornaro M, Geuna S, Fasolo A, Giacobini-Robecchi MG (2001) Evidence of very early neuronal migration from the olfactory placode of the chick embryo. Neuroscience 107:191-197

Ikeda K, Ookawara S, Sato S, Ando Z-ichi, Kageyama R, Kawakami K (2007) Six1 is essential for early neurogenesis in the development of olfactory epithelium. Dev Biol 311:53-68

Lavdas AA, Grigoriou M, Pachnis V, Parnavelas JG (1999) The medial ganglionic eminence gives rise to a population of early neurons in the developing cerebral cortex. J Neurosci19:7881–7888

Marin O, Rubenstein JL (2003) Cell migration in the forebrain. Annu Rev Neurosci 26:441–483

Moeller JF, Meredith M (2010) Differential co-localization with choline acetyltransferase in nervus terminalis suggests functional differences for GnRH isoforms in bonnethead sharks (*Sphyrna tiburo*). Brain Res 1366: 44-53

O'Neill P, McCole RB, Baker CV (2007) A molecular analysis of neurogenic placode and cranial sensory ganglion development in the shark, Scyliorhinus canicula. Dev Biol 304:156-181

Rodríguez-Moldes I, Carrera I, Pose-Méndez S, Quintana-Urzainqui I, Candal E, Anadón R, Mazan S, Ferreiro-Galve S (2011) Regionalization of the shark hindbrain: a survey of an ancestral organization. Front Neuroanat 5:16

Sueiro C, Carrera I, Rodríguez-Moldes I, Molist P, Anadón R (2003) Development of catecholaminergic systems in the spinal cord of the dogfish Scyliorhinus canicula (Elasmobranchs). Brain Res Dev Brain Res 142:141-150

White J, Meredith M (1993) Spectral analysis and modelling of ACh and NE effects on shark nervus terminalis activity. Brain Res Bull 31:369–374

Whitlock KE, Westerfield M (1998) A transient population of neurons pioneers the olfactory pathway in the zebrafish. J Neurosci 18:8919-8927

Wichterle H, Turnbull DH, Nery S, Fishell G, Alvarez-Buylla A (2001) In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. Development 128:3759–3771

Wu CC, Yoshimoto M, Ito H (1992) The selachian terminal nerve. Kaibogaku Zasshi J Anat 67:317–332

Wu S, Esumi S, Watanabe K, Chen J, Nakamura KC, Nakamura K, Kometani K, Minato N, Yanagawa Y, Akashi K, Sakimura K, Kaneko T, Tamamaki N (2011) Tangential migration and proliferation of intermediate progenitors of GABAergic neurons in the mouse telencephalon. Development 138:2499–2509

Yáñez J, Folgueira M, Köhler E, Martínez C, Anadón R (2011) Connections of the terminal nerve and the olfactory system in two galeomorph sharks: an experimental study using a carbocyanine dye. J Comp Neurol 519:3202–3217

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

Resumen

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

INTRODUCCIÓN

La región anterior del tubo neural presenta tres dilataciones o vesículas primarias: rombencéfalo (cerebro posterior), mesencéfalo (cerebro medio) y prosencéfalo (cerebro anterior), que se subdividen en vesículas secundarias durante el desarrollo embrionario. Las vesículas prosencefálicas secundarias son el diencéfalo y el llamado prosencéfalo secundario que engloba al hipotálamo y al telencéfalo. Este último representa la vesícula más anterior del encéfalo que, en el caso de mamíferos, da lugar a importantes estructuras complejas como el córtex, el hipocampo, los ganglios basales o los bulbos olfativos. La presente tesis se centra en el estudio del telencéfalo así como de sus sistemas periféricos asociados (sistema olfativo periférico y sistema del nervio terminal) de un pez cartilaginoso, la pintarroja *Scyliorhinus canicula* (*S.canicula*).

Los peces cartilaginosos ocupan una posición filogenética como grupo externo al resto de gnatóstomos (vertebrados con mandíbula) lo que los hace esenciales para el estudio de la condición ancestral de la organización del telencéfalo y estructuras periféricas asociadas de vertebrados. A pesar de su posición basal en el árbol evolutivo de los gnatóstomos, presentan cerebros complejos y bien desarrollados que son generalmente más grandes que los de agnatos (peces no mandibulados como la lamprea) o los de peces óseos. La especie utilizada para este estudio (*S.canicula*) es un elasmobranquio que se ha posicionado como el principal modelo de peces cartilaginosos para estudios sobre el desarrollo

embrionario porque presenta numerosas ventajas. Se cría fácilmente en cautividad y permite obtener material embrionario abundante a lo largo del año. Se trata de una especie ovípara y los huevos pueden mantenerse fácilmente bajo condiciones de laboratorio hasta la eclosión. El tamaño relativamente grande de los embriones y un período embrionario prolongado (entre cinco y doce meses dependiendo de la temperatura del agua) permiten un análisis pormenorizado de los diversos eventos que ocurren a lo largo del desarrollo, evidenciando detalles que pueden ser obviados en otras especies de desarrollo más rápido.

Esta tesis está estructurada en dos partes.

La Sección I (Capítulos 1 y 2) se centra en la caracterización del desarrollo de los sistemas periféricos asociados al telencéfalo, es decir, el sistema olfativo y el sistema del nervio terminal. Los bulbos olfativos son los centros olfatorios primarios, esto es, constituyen la región del sistema nervioso central que recibe aferencias directas del sistema olfativo periférico. El sistema del nervio terminal (o *nervus terminalis*) constituye otro componente periférico directamente asociado al telencéfalo. Este sistema se describió y estudió por primera vez en peces cartilaginosos. Posteriormente fue descrito en especies representativas de la mayoría de grupos animales vertebrados. La función precisa de este sistema todavía es objeto de estudio aunque parece tener un papel importante en la fisiología y comportamiento de la reproducción. El tamaño relativamente grande de los sistemas olfativo y terminal en peces cartilaginosos con respecto a otros

vertebrados, junto con el hecho de que sólo en este grupo animal el nervio terminal discurre separadamente del nervio olfativo, hace de estos animales excelentes modelos para el estudio de la anatomía y desarrollo de ambos sistemas así como de su relación con el telencéfalo.

La Sección II (Capítulos 3, 4 y 5) engloba el estudio de diferentes aspectos del desarrollo y de la regionalización del propio telencéfalo. Al igual que ocurre en tetrápodos, el telencéfalo de los peces cartilaginosos se desarrolla mediante un proceso morfogenético conocido como evaginación que implica el crecimiento del lumen central del tubo neural para formar los ventrículos telencefálicos, seguido de una expansión de las paredes telencefálicas. Por el contrario, el telencéfalo de la mayoría de los peces óseos (incluyendo su principal modelo, el pez cebra) se desarrolla por eversión, lo que implica que la región del techo del tubo neural sobre el lumen central se estrecha y alarga, y que la parte dorsal del telencéfalo (palio) se dobla hacia afuera. En consecuencia, el proceso de eversión revierte la topografía de las áreas paliales en peces óseos. Por tanto, el hecho de que el telencéfalo de peces cartilaginosos se desarrolle por evaginación, como en tetrápodos, representa una gran ventaja en estudios comparativos con respecto a peces óseos. A pesar de todo ello, el estudio del telencéfalo de peces cartilaginosos ha sido ampliamente ignorado. Esta tesis pretende llenar parte del vacío de conocimiento que concierne al desarrollo y la organización del telencéfalo del tiburón.

CAPÍTULO 1

Desarrollo del sistema olfativo periférico. Evidencias de la existencia de neuronas Pax6 migrando a lo largo del nervio olfativo

El sistema olfativo representa un modelo excelente para el estudio de diversos aspectos del desarrollo del sistema nervioso como la neurogénesis o los mecanismos de crecimiento y guía axónica. Descubrimientos importantes en este campo han surgido de estudios comparativos. En este capítulo analizamos los eventos clave en el desarrollo del sistema olfativo del tiburón *Scyliorhinus canicula* mediante la combinación de métodos inmunohistoquímicos y de trazado neuronal.

Este trabajo tiene dos objetivos principales: 1) identificar la formación temprana del epitelio olfativo y de las proyecciones primarias olfativas en un pez cartilaginoso, caracterizando los diferentes tipos celulares del epitelio y aquéllos asociados al nervio olfativo en desarrollo; y 2) definir el fenotipo de las células Pax6 que se habían descrito previamente en el epitelio y nervio olfativo de *S. canicula* para arrojar luz sobre su(s) posible(s) función(es). Para cumplir dichos objetivos hemos aplicado técnicas de trazado neuronal en combinación con técnicas inmunohistoquímicas usando marcadores para Pax6, células proliferantes (PCNA, antígeno nuclear de proliferación celular), células gliales (GFAP, proteína acídica fibrilar glial), neuronas jóvenes postmitóticas (HuC/D), neuronas

migratorias inmaduras y sus axones (DCX, doublecortin) y células receptoras olfativas y sus tractos primarios (proteína Gα0).

Los acontecimientos analizados en este estudio durante el desarrollo del sistema olfativo periférico fueron enmarcados en el contexto de tres períodos de desarrollo, establecidos en base a los experimentos de trazado neuronal e inmunohistoquímicos realizados. El primer período (estadíos 20 a 24) está marcado por el inicio de la neurogénesis y la observación de las primeras neuronas postmitóticas en la placoda olfativa. El segundo período (estadíos 25 a 30) se caracteriza por la aparición de migraciones celulares abundantes a lo largo de las fibras olfativas. Durante el tercer período (estadío 31 hasta la eclosión), la estructura madura del sistema olfativo se va adquiriendo progresivamente. En consecuencia, hemos denominado a estos períodos pionero, migratorio y de maduración, respectivamente. Dichos acontecimientos y períodos de desarrollo constituyen una referencia de mucha utilidad para estudios comparativos del desarrollo del sistema olfativo ya que pueden identificarse en los diferentes vertebrados.

Durante el primer período, describimos por primera vez en un pez cartilaginoso una población temprana de neuronas pioneras HuC/D-inmunorreactivas que parecen delaminarse del epitelio olfativo en desarrollo y migrar hacia el telencéfalo, antes incluso de que el nervio olfativo hubiese aparecido. Durante el segundo periodo, otra población transitoria, llamada masa

migratoria, fue detectada en estrecha aposición al nervio olfativo. Esta población contiene células gliales olfativas envolventes (GFAP positivas) y neuronas HuC/D positivas, algunas de las cuales parecen desviarse y dirigirse hacia una región distinta del bulbo olfativo. Asimismo, demostramos que las células Pax6 positivas detectadas a lo largo del nervio en *S.canicula* son neuronas inmaduras de la masa migratoria (HuC/D y DCX positivas) y que no forman parte del nervio terminal. Además aportamos evidencias de que estas neuronas parecen originarse en el epitelio olfativo. Teniendo en cuenta que las neuronas Pax6 en el epitelio olfativo muestran características de neuronas receptoras olfativas y que las neuronas Pax6 inmaduras de la masa migratoria se disponen en forma de pasillo en la zona donde el nervio entra en el bulbo olfativo, proponemos que estas neuronas pueden estar cumpliendo un papel señalizador para los axones en crecimiento de las neuronas olfativas receptoras hacia el bulbo olfativo. Se necesitan estudios en otros vertebrados para averiguar si la presencia de estas neuronas Pax6 es una característica específica de peces cartilaginosos o forma parte del patrón general de vertebrados.

CAPÍTULO 2

Desarrollo del sistema del nervio terminal

El nervio terminal fue descrito por primera vez hace más de un siglo en una especie del grupo de peces elasmobranquios. A lo largo del siglo XX, fue progresivamente detectado en numerosas especies de vertebrados de peces a humanos. Sin embargo, sus funciones, origen embrionario o su relación con el sistema olfativo son temas que a día de hoy siguen siendo objeto de discusión. Aunque existe una literatura considerable sobre el sistema del nervio terminal en elasmobranquios adultos, el estudio de su desarrollo ha sido ampliamente ignorado. En otros vertebrados, se han descrito células del nervio terminal formando parte de la masa migratoria desde el epitelio olfativo hacia el telencéfalo y asociadas a las fibras olfativas primarias. Hasta el momento no se ha determinado si este proceso también ocurre en peces cartilaginosos.

En este capítulo, analizamos el desarrollo del sistema del nervio terminal en *S.canicula* dentro del contexto de los tres períodos descritos para el desarrollo olfativo en el capítulo 1. El principal objetivo de este estudio fue discriminar entre componentes olfativos y no olfativos relacionados con el sistema del nervio terminal. Asimismo, investigamos la posible relación entre estos dos sistemas durante el desarrollo. Para ello, hemos utilizado técnicas de trazado neuronal en combinación con inmunohistoquímica para marcadores de componentes del sistema del nervio terminal (FMRF-amida) así como para marcadores de

componentes del sistema olfativo en pintarroja como Pax6, células gliales (GFAP, proteína acídica fibrilar glial), neuronas postmitóticas tempranas (HuC/D), neuronas inmaduras y sus elongaciones (DCX, doblecortina) y neuronas receptoras olfativas y sus tractos primarios (proteína Gα0).

Este trabajo representa el primer estudio del desarrollo del nervio terminal en un pez cartilaginoso. En este estudio proporcionamos herramientas que permiten distinguir el nervio terminal del sistema olfativo en desarrollo. Igualmente, establecemos la relación espacio-temporal de ambos sistemas durante la embriogénesis. Describimos cómo ambos componentes parecen compartir un origen común en la masa migratoria descrita en el Capítulo 1. Por último, localizamos el punto en el que ambos sistemas se separan en un lugar adyacente a la unión nervio olfativo-bulbo olfativo.

CAPÍTULO 3

Estudio genoarquitectónico del telencéfalo

Durante las últimas décadas, el telencéfalo de especies representativas de diferentes grupos de vertebrados ha sido mapeado en el contexto de la genoarquitectura y del modelo prosomérico. En todos los vertebrados, el telencéfalo se subdivide en dos territorios: el palio (dorsal) y el subpalio (ventral). El palio forma parte del telencéfalo evaginado y, en tetrápodos, se ha dividido en al menos cuatro regiones diferentes: palio medial, dorsal, lateral y ventral. El subpalio consiste principalmente en una parte evaginada, que incluye las eminencia ganglionar lateral (LGE) y medial (MGE), y una parte no evaginada (o telencéfalo impar) denominada área preóptica. Se ha recopilado importante información acerca de la dinámica de expresión génica en los diferentes territorios telencefálicos, lo que ha servido como un arma potente para el establecimiento de homologías. Nuestro conocimiento sobre la evolución del desarrollo del telencéfalo basado en datos genoarquitectónicos se enriquecería considerablemente con información sobre vertebrados basales como los peces cartilaginosos. La escasez de información sobre la genoarquitectura del telencéfalo en desarrollo de peces cartilaginosos nos llevó a estudiar el patrón de expresión de determinados genes durante el desarrollo de *S.canicula*. Los marcadores que hemos utilizado en este estudio han sido seleccionados por su bien conocida expresión en dominios telencefálicos específicos. Pax6, Tbr1, Emx1

y Lhx9 son marcadores paliales y factores claves en la especificación dorsoventral del telencéfalo. GAD (glutamic acid decarboxylase, la enzima de síntesis del neurotransmisor GABA), Dlx2 y Nkx2.1 se expresan de manera específica en el subpalio y han servido para delimitar la LGE y la MGE. Shh (Sonic hedgehog) se expresa en el compartimento preóptico y Otp ha sido descrito como marcador de la parte alar del hipotálamo, por lo tanto representan marcadores muy útiles para definir el límite telencefálico-hipotalámico. La expresión combinada de estos marcadores nos ha permitido identificar ciertos límites (incluyendo el límite palial-subpalial y el telencefálico-hipotalámico) y caracterizar por primera vez en tiburones algunos territorios embriológicos, como las estructuras equivalentes a las eminencias ganglionares lateral y medial del subpalio de otros vertebrados. Asimismo, mostramos evidencias de la existencia de un palio ventral en tiburones, una subdivisión que no había sido identificada previamente en peces cartilaginosos. Además, hemos seguido a lo largo del desarrollo los derivados de algunos de los territorios telencefálicos definidos sobre la base de expresión de genes, lo que nos permitió proponer homologías fiables en *S. canicula* de las regiones del telencéfalo adulto identificadas en otros vertebrados. Estos resultados proporcionan una buena base para realizar comparaciones entre regiones homólogas de peces cartilaginosos y otros vertebrados.

A pesar de que encontramos contadas diferencias en los patrones de expresión (como la existencia de una corriente Pax6 positiva a niveles paliales), nuestros resultados indican que el programa genético básico para el desarrollo

del telencéfalo está presente en los vertebrados que ocupan la posición filogenética más basal en el árbol de los gnatóstomos y que, por tanto, este programa de desarrollo está altamente conservado a lo largo de la evolución.

CAPÍTULO 4

Estudio piloto de la conectividad de los ganglios basales

Los ganglios basales son un grupo de núcleos localizados en la parte basal (subpalio) del telencéfalo de mamíferos relacionados con el control del comportamiento motor. En sentido estricto, el término ganglios basales se refiere a los componentes estriatal y palidal del telencéfalo basal que se desarrollan durante el período embrionario a partir de las eminencias lateral y medial (LGE y MGE), respectivamente. A menudo, en este concepto también se incluyen otras estructuras como el núcleo subtalámico, sustancia negra o área tegmental ventral dado que forman parte del circuito de los ganglios basales. Aunque ciertas características varían entre los distintos grupos de vertebrados, parece que todos los tetrápodos presentan ciertos rasgos comunes como: 1) la presencia de sistemas estriatopalidales en el telencéfalo derivados de estructuras embrionarias (LGE, MGE) especificadas por factores de transcripción subpaliales; 2) una proyección estriatal hacia áreas mesencefálicas y diencefálicas (substancia negra reticulada) por medio de las vías directa e indirecta; o 3) una fuerte innervación dopaminérgica al estriado desde núcleos localizados en la placa basal de ciertas zonas diencefálicas y/o mesencefálicas (tubérculo posterior, sustancia negra compacta y área tegmental ventral). En peces cartilaginosos, actualmente se acepta la existencia de ganglios basales con subdivisiones estriatal y palidal, aunque no se han identificado de forma inequívoca. El análisis de la expresión

génica durante el desarrollo del telencéfalo (Capítulo 3), nos ha permitido identificar una región particular del subpalio basal de *S. canicula* como el origen probable del estriado de embriones tardíos. En este capítulo analizamos las conexiones de esta zona subpalial por medio de experimentos de trazado neuronal con el objetivo de profundizar en la caracterización de esta región basal y comprobar si corresponde al estriado de peces cartilaginosos. Hemos elegido como trazador la Neurobiotina por su eficacia y versatilidad demostrada tanto en el transporte anterógrado como retrógrado. Además examinamos la relación de las conexiones con los grupos celulares catecolaminérgicos del diencéfalo y mesencéfalo por medio de detección inmunohistoquímica para la enzima tirosina hidroxilasa (TH; la enzima limitante de la síntesis de catecolaminas) combinada con la visualización del trazador.

Demostramos que la región estudiada recibe aferencias de células localizadas en el área tegmental ventral y sustancia negra de pintarroja, algunas de las cuales son catecolaminérgicas. Por el contrario, el grupo dopaminérgico del tubérculo posterior no parece proyectar esta región subpalial. Los experimentos llevados a cabo en este estudio también revelaron un denso campo terminal en una región relacionada espacialmente con el grupo dopaminérgico de la sustancia negra. Dicho campo terminal resultó no ser catecolaminérgico. Por lo tanto proponemos que esta proyección descendiente puede ser equivalente a la proyección nigroestriatal de otros vertebrados, y que la región que recibe esta innervación debe corresponder a la sustancia negra reticulada de peces cartilaginosos. El

circuito revelado con estas técnicas soporta la hipótesis planteada de que la región del subpalio trazada corresponde, efectivamente, al estriado de peces cartilaginosos.

En definitiva, este trabajo representa la primera evidencia de la existencia de células catecolaminérgicas en el mesencéfalo de elasmobranquios que proyectan al subpalio y sugiere la existencia de un circuito de los ganglios basales equivalente al descrito en tetrápodos.

CAPÍTULO 5

Evidencias de migraciones tangenciales en el telencéfalo. Posibles homologías y divergencias

La migración neuronal tangencial se define, en contraposición a la migración radial, como el proceso migratorio que tiene lugar a lo largo de un eje diferente al demarcado por la glía radial. Mientras la migración radial establece el marco citoarquitectónico del encéfalo, la migración tangencial incrementa la complejidad celular de los circuitos cerebrales permitiendo la dispersión de múltiples tipos celulares. La existencia de poblaciones migratorias tangenciales que emergen de las eminencias medial y lateral (MGE y LGE) durante el desarrollo embrionario parece ser una característica conservada a lo largo de la evolución de los vertebrados ya que se ha descrito desde peces a mamíferos. Sin embargo, poco se sabe sobre el recorrido específico y los destinos finales de estas rutas en vertebrados no mamíferos.

Los marcadores subpaliales GAD y Dlx2 han sido ampliamente utilizados como marcadores fiables de la poblaciones que migran tangencialmente desde las eminencias ganglionares, ya que la mayoría de estas células son GABAérgicas y expresan genes pertencientes a la familia Dlx desde su especificación temprana (cerca de la región ventricular del subpalio) hasta su destino en diferentes dominios telencefálicos. Algunas de las rutas tangenciales mejor caracterizadas incluyen poblaciones que migran desde la MGE hacia el neocórtex e hipocampo,

desde la MGE a la LGE o desde la LGE hacia el bulbo olfativo. De entre todas ellas, la última ha sido propuesta como el precursor embrionario de la ruta migratoria rostral (RMS por sus siglas en inglés) descrita en mamíferos adultos. La RMS ha sido ampliamente estudiada en mamíferos porque se trata de uno de los pocos nichos neurogénicos del cerebro adulto. En el telencéfalo de *S.canicula*, el estudio del desarrollo del sistema GABAérgico permitió identificar neuronas derivadas del subpalio en zonas paliales, lo que llevó a proponer la existencia de rutas migratorias tangenciales. Sin embargo, el recorrido exacto de dichas rutas no fue completamente definido. Los resultados obtenidos en el Capítulo 3 de esta tesis mostraron que las divisiones estriatal y palidal de *S.canicula* tenían un origen embriológico y una especificación genética equivalente a la de tetrápodos, aportando evidencias de la existencia de territorios equivalentes a la LGE y MGE de mamíferos.

En este capítulo nos propusimos identificar las rutas migratorias tangenciales del telencéfalo de la pintarroja así como su carácter derivado o conservado evolutivamente. Para ello, llevamos a cabo un análisis detallado del patrón de distribución espacio-temporal de GAD y Dlx2. También relacionamos el patrón de expresión de estas sustancias con el de Nkx2.1 (marcador de MGE) y Tbr1 (marcador palial), con el fin de identificar los dominios de origen de las rutas así como sus posibles destinos. Asimismo investigamos si las rutas se mantenían en el período postnatal y si estaban relacionadas con posibles nichos neurogénicos adultos. Para ello, analizamos la expresión de marcadores como PCNA o PH3

(proliferación celular), DCX (neuroblastos en migración), Reelina (una glicoproteína extracelular implicada en la migración y el posicionamiento neuronal en el cerebro embrionario) y GFAP (marcador de células proliferantes quiescentes tipo glía radial).

Los resultados obtenidos en este capítulo apuntan a la existencia de cinco posibles rutas tangenciales originadas en el compartimento subpalial de *S.canicula*. Cuatro de ellas son corrientes paliopetales. De ellas, una representa una corriente transitoria que parece estar dirigida hacia la zona subpial del palio y fue detectada sólo durante un corto período del desarrollo embrionario. Las otras tres parecen mantenerse en el adulto y alcanzar dominios paliales que se corresponded con las únicas regiones del telencéfalo que presentan características inmunohistoquímicas compatibles con nichos neurogénicos adultos. La comparación de nuestros resultados con los obtenidos en otros vertebrados indica que algunas de las rutas observadas parecen ser equivalentes a las descritas en mamíferos, aunque también ponen de manifiesto la existencia de rutas específicas o derivadas en elasmobranquios, como la corriente que parece dirigirse hacia dominios paliales rostrales. Asimismo, también aportamos datos que apoyan que la corriente que se dirige a los bulbos olfativos origina, en el adulto, una estructura con características de nicho neurogénico equivalente a la RMS de mamíferos. Por otra parte, el análisis de las regiones paliales que contienen células derivadas del subpalio, junto con los resultados genoarquitectónicos obtenidos previamente (capítulo 3), nos permitió demarcar

ciertas regiones como el palio dorsal y el palio medial. Finalmente, el hecho de que algunas de las rutas caracterizadas terminen en regiones telencefálicas en las que la proliferación celular parece mantenerse en el adulto abre el camino a la futura identificación de posibles nichos neurogénicos en el cerebro de peces cartilaginosos.

Conclusions

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. Three morphogenetic periods have been established during the development of the peripheral olfactory system of the dogfish *S. canicula* characterized by the appearance of pioneer neurons along the olfactory pathway (pioneer period); the massive migration of cells along olfactory fibers (migratory period); and the acquisition of the mature organization (maturing period). These periods constitute a useful framework for comparative studies about olfactory system development as they can be reliably recognized in different groups of vertebrates.

2. We have characterized the sequence of markers expressed during the maturation of the olfactory receptor neuron lineage on the basis of changes observed along the development in the intensity of labeling of the markers used. Thus, we reported that the transition from proliferating progenitor to mature olfactory receptor neuron in *S.canicula* involves the sequential expression of PCNA, Pax6, DCX and $G\alpha_0$-protein.

3. We report for the first time in a basal vertebrate the existence of a wave of transient migratory cells (the migratory mass) coursing along olfactory fibers and ensheathed by olfactory glia, similar to that described in other vertebrates, thus revealing a common pattern in the development of olfactory system in vertebrates.

4. The study of the phenotype of Pax6 cells located along the olfactory nerve during development revealed that these cells are young neurons migrating toward the olfactory bulb. Although studies in other species are needed before determining if this represents a common trait of vertebrates, we propose that they play a role as guidepost cells for the outgrowing axons of the olfactory receptor neurons anchored to the olfactory epithelium.

5. The characterization of the development of the terminal nerve by means of immunohistochemical methods combined with tract-tracing experiments allowed the definition of the spatial and temporal relationship of this nerve with the olfactory system during embryogenesis, demonstrating their shared developmental origin in the migratory mass, as occurs in other vertebrates.

6. The analysis of the combined expression of key developmental genes allowed us to recognize the main telencephalic subdomains in the brain of *S.canicula* as have been described in other gnatostomes, demonstrating a common pattern of telencephalic specification in vertebrates. Specifically, the position of the pallial-subpallial boundary was identify by means of comparative expression of markers as Pax6, Tbr1, Dlx2 and GAD. In the pallium, four histogenetic territories,were defined on the basis of the differential expression of Pax6, Tbr1, Emx1 and Lhx9. These are medial, dorsal, lateral and ventral pallium.

7. Three subpallial subdivisions, representing the lateral and medial ganglionic eminences and the preoptic area of cartilaginous fishes were defined by the combined expression of Dlx2, Nkx2.1 and Otp. The fact that cartilaginous fishes present Nkx2.1 and Shh in the subpallium, a feature absent in lampreys, implies that both pallidal and preoptic regions might emerge during the transition from agnatans to gnatostomes.

8. Tract-tracing experiments combined with immunohistochemistry for catecholaminergic cells performed in juveniles of *S.canicula* provided valuable data that support the homology of the a*rea superficialis basalis-area periventricularis ventrolateralis* as the proper striatum in the dogfish. The results of these experiments revealed nigrostriatal and striatonigral-like connections probably equivalent to those described in the basal ganglia of other vertebrates. However, the catecholaminergic group of the posterior tubercle does not seem to be the source of an ascendent projection to the subpallium in *S.canicula*, as previously believed.

9. The analysis of the spatiotemporal pattern of distribution of GAD, Dlx2 and Nkx2.1 expression pointed to the existence of five embryonic tangential migratory routes originated in the ganglionic eminences that might represent telencephalic tangential migratory routes. Comparison with other vertebrates

revealed that some of the routes observed *Scyliorhinus* may be equivalent to those described in mammals.

10. Some of the migratory routes characterized in this shark seem to be directed towards discrete pallial regions that retain neurogenic markers during adulthood, which paves the way for the identification of adult neurogenic niches in the telencephalon of sharks. In fact, the migratory stream that seems to reach the olfactory bulb gives rise to an adult proliferative system probably equivalent to the rostral migratory stream of mammals. The presence of this system in sharks reveals its evolutionary conservation and highlights its functional importance in the vertebrate brain.

Conclusiones

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

1. Se han establecido tres períodos morfogenéticos a lo largo del desarrollo del sistema olfativo periférico de la pintarroja *S. canicula* caracterizados por la aparición de neuronas pioneras a lo largo de la vía olfativa (período de células pioneras); la migración masiva de células a lo largo de las fibras olfativas (período de migración); y la adquisición de la organización madura (período de maduración). Estos períodos constituyen una referencia para estudios comparativos ya que se reconocen fácilmente en diferentes grupos de vertebrados.
2. Hemos caracterizado la secuencia de marcadores expresados durante la maduración del linaje de neuronas olfativas receptoras en base a los cambios observados a lo largo del desarrollo en la intensidad de los marcadores utilizados. De esta manera, hemos descrito que la transición desde progenitores proliferantes hasta neuronas receptoras olfativas maduras en S. canicula conlleva la expresión secuencial de PCNA, Pax6, DCX y proteína $G\alpha_{0.}$
3. Hemos descrito por primera vez en un vertebrado basal, la existencia de una ola de migración transitoria (la masa migratoria) cursando a lo largo de las fibras olfativas y envueltas por glía olfativa, de manera similar a lo observado en otros vertebrados, lo que revela un patrón común en el desarrollo del sistema olfativo conservado a lo largo de la evolución.
4. El estudio del fenotipo de las células Pax6 dispuestas a lo largo del nervio olfativo durante el desarrollo reveló que estas células son neuronas jóvenes

que migran hacia el bulbo olfativo. Aunque estudios en otras especies son necesarios antes de determinar si estas células representan una característica común de vertebrados, proponemos que puedan estar desempeñando un papel como células guía para los axones de las neuronas receptoras olfativas ancladas en el epitelio olfativo.

5. La caracterización del desarrollo del nervio terminal mediante métodos inmunohistoquímicos combinados con experimentos de trazado neuronal permitieron la observación de la relación espacio-temporal de este nervio con el sistema olfativo durante el período embrionario. De este modo, demostramos que ambos sistemas tienen un origen embriológico común en la masa migratoria, al igual que ocurre en otros vertebrados.

6. El análisis de la expresión combinada de genes del desarrollo nos permitió reconocer los principales subdominios telencefálicos en el cerebro *de S. canicula*, del mismo modo que ha sido descrito para otros gnatóstomos, demostrando la existencia de un patrón común de especificación telencefálica en vertebrados. En concreto, hemos identificado la posición del límite palial-subpalial por medio de la expresión comparada de marcadores como *Pax6, Tbr1, Dlx2* y GAD. En el palio, hemos identificado cuatro territorios histogenéticos en base a su expresión diferencial de Pax6, *Tbr1, Emx1* y *Lhx9*, estos son palio medial, dorsal, lateral y ventral.

7. Hemos definido tres subdivisiones subpaliales que representan las eminencias ganglionares lateral y medial y el área preóptica de peces

carilaginosos por medio de la expresión combinada de *Dlx2, Nkx2.1* y *Otp*. El hecho de que los peces cartilaginosos presenten Nkx2.1 y Shh en el subpalio, característica ausente en lampreas, implica que ambas regiones palidal y preóptica debieron emerger durante la transición de agnatos a gnatostomos.

8. Por medio de experimentos de trazado neuronal combinado con inmunohistoquímica para células catecolaminérgicas en juveniles de *S. canicula* obtuvimos interesantes resultados que apoyan la homología del a*rea superficialis basalis-area periventricularis ventrolateralis* al estriado de pintarroja. Estos resultados revelaron conexiones probablemente equivalentes a las nigroestriatales y estriatonigrales descritas en los ganglios basales de otros vertebrados.

9. El análisis del patrón espaciotemporal de la distribución la expresión de GAD, *Dlx2* y *Nkx2.1* nos permitió proponer la existencia de cinco rutas de migración tangencial originadas en las eminencias ganglionares. La comparación con otros vertebrados reveló que alguna de estar rutas debe ser equivalentes a las descritas en mamíferos.

10. Algunas de las rutas de migración caracterizadas en *S.canicula* parecen estar dirigidas hacia regiones paliales que retienen marcadores de neurogénesis en el adulto, lo que abre el camino a la identificación de nichos neurogénicos adultos en el telencéfalo de tiburones. De hecho, la corriente migratoria que parece colonizar el bulbo olfativo da lugar a un sistema

proliferativo adulto probablemente equivalente a la ruta migratoria rostral de mamíferos. La presencia de este sistema en tiburones revela su conservación evolutiva y pone de manifiesto su importancia funcional en el cerebro de vertebrados.

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA

USC
UNIVERSIDADE
DE SANTIAGO
DE COMPOSTELA