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## Evolution of the craniate brain : patterning and regionalisation of the lamprey developing forebrain.

Joana Vaz Pato Osorio, Joana Osório

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Ecole Doctorale Gènes, Génomes, Cellules

# Thèse de Doctorat

présentée par  
Joana Vaz Pato Osório

pour obtenir le grade de  
Docteur de l'Université Paris-Sud 11  
Spécialité : Neurosciences

**Evolution of the craniate brain :  
patterning and regionalisation of the  
lamprey developing forebrain**

soutenue le 28 septembre 2007 devant le jury composé de :

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*To my parents Maria da Glória and Eduardo*

*To the memory of Cina and Luís*



*Un replicant quand il naît, il est physically formé comme un gars de 40 ans,  
mais son brain... comment on dit? son computer brain? son cerveau, il faut  
qu'il absorbe tout ce qu'il y a autour, qu'il voie les couleurs, qu'il touche  
comment sont les choses, il est aware...*

Jean-Claude Van Damme





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# Abbreviations

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ANR	anterior neural ridge
AP	antero-posterior
bHLH	basic helix-loop-helix
Bmp	bone morphogenetic protein
CNE	conserved non-coding element
CNS	central nervous system
Dhh	desert hedgehog
dpf	day(s) post-fertilisation
DV	dorso-ventral
Fgf	fibroblast growth factor
GABA	$\gamma$ -aminobutyric acid
GFP	green fluorescent protein
Hh	hedgehog
Ihh	indian hedgehog
ISH	<i>in situ</i> hybridisation
IsO	isthmic organiser
LGE	lateral ganglionic eminence
Lhx	LIM-homeobox
LIM-hd	LIM-homeodomain
MGE	medial ganglionic eminence
ML	medio-lateral
Mya	million years ago
p1,p2,...	prosomere 1, prosomere 2,...
RT-PCR	reverse transcription-polymerase chain reaction
Shh	sonic hedgehog
WGD	whole-genome duplication
Wnt	wingless-int
ZLI	zona limitans intrathalamica

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# Résumé

La lamproie devient un modèle de plus en plus populaire en biologie du développement et de l'évolution (evo-devo), grâce à sa position phylogénétique particulière. Ce modèle a d'ores et déjà fourni des informations importantes sur les caractéristiques ancestrales des craniates, mais également sur l'apparition de nouveautés au moment de la transition agnathe/gnathostome (Kuratani et al., 2002).

Dans ce travail, nous avons étudié les mécanismes de spécification et d'organisation génétique du cerveau chez l'embryon de lamproie. En utilisant les techniques de clonage par RT-PCR et d'hybridation *in situ*, nous avons isolé et étudié le patrons d'expression de gènes des familles *LIM-à-homéoboîte* (*Lhx*), *Pax* et *Hedgehog*. Les résultats principaux sont les suivants : (1) les gènes *Lhx* et *Pax* révèlent un grand degré de conservation des mécanismes de "patterning" des parties antérieure et postérieure du cerveau entre cyclostomes et gnathostomes ; néanmoins, quelques différences importantes ont été observées, particulièrement au niveau du télencéphale ; ces différences sont probablement corrélées avec les changements majeurs subis par cette région au moment de la transition agnathe/gnathostome ; (2) l'analyse de l'expression des gènes *Lhx* et son interprétation montrent que le cerveau antérieur de lamproie présente une organisation prosomérique évidente ; cette organisation est ainsi un caractère propre aux vertébrés ; (3) l'analyse de l'expression du gène *LfHh* met en évidence une possible origine moléculaire de cette organisation neuromérique du cerveau (Osorio et al., 2005) ; (4) en outre, l'expression d'au moins un de ces gènes *Lhx* persiste tout au long de la période larvaire (non-embryonnaire), ce qui montre l'utilité de ces gènes comme marqueurs pour suivre le développement et la morphogenèse du cerveau au cours du temps (Osorio et al., 2006).

Ce type d'organisation pseudosegmentaire du cerveau est limité aux gna-

thostomes : chez l'amphioxus et les ascidies, où l'expression de *Hh* est absente de la région la plus rostrale du tube neural, on n'observe pas de neuromères. Il est important de noter que *Hh* n'est pas exprimé dans le télencéphale ventral embryonnaire de lamproie, et que *Nkx2.1*, un marqueur de la subdivision pallidale du sous-pallium des gnathostomes, n'est pas non plus exprimé à ce niveau (Ogasawara et al., 2001; Osorio et al., 2005; Uchida et al., 2003). Cette différence suggère que des modifications dans la signalisation *Hh* de la ligne médiane ventrale peuvent avoir été des moteurs de l'évolution du cerveau antérieur.

Nous avons cherché à étudier l'origine de ces différences à la lumière de l'évolution moléculaire de la famille multigénique *Hedgehog*. Il est probable que l'ancêtre des chordés ait possédé un gène *Hh* unique. L'amphioxus (céphalocordé) présente un seul gène *Hh*, alors qu'il y en a deux chez les ascidies (urochordés), probablement issus d'une duplication spécifique dans cette lignée. Il y a trois groupes de gènes *Hedgehog* chez les gnathostomes : *Sonic*, *Indian* et *Desert*.

Nous avons criblé une librairie d'ADN génomique de *L. fluviatilis*, visant à identifier des cosmides contenant le gène *Hh*. Les résultats mettent en évidence l'existence de deux gènes *Hedgehog* chez la lamproie. Une analyse phylogénétique préliminaire suggère une duplication indépendante des gènes *Hedgehog* dans la lignée de la lamproie, au sein du groupe *Sonic/Indian*. Ces résultats ouvrent une nouvelle vision sur l'évolution de cette famille de gènes chez les chordés.

Enfin, dans le but d'étudier le rôle des modifications de l'organisation génique et du contrôle de l'expression des gènes au cours de l'évolution du cerveau chez les chordés, nous avons également isolé le gène *Lhx9* de roussette (*Scyliorhinus canicula*) à partir d'une librairie d'ADN génomique de cette espèce.

**Mots clés :** Cerveau ; Evolution ; Agnathe ; Cyclostome ; Gnathostome ; Lamproie ; Roussette ; Expression génique ; "Patterning" ; Neuromère ; Cerveau antérieur ; LIM-homéoboîte ; Lhx ; LIM-homéodomaine ; Pax ; Hedgehog ; Duplication génique ; Région régulatrice

# Abstract

Lampreys are becoming increasingly popular in evolutionary developmental biology, due to their phylogenetic position. They provide insights into craniate ancestral characteristics, but also into the emergence of novelties at the important cyclostome/gnathostome evolutionary split (Kuratani et al., 2002).

This work was focused on the study of the genetic specification and organisation of the embryonic lamprey brain. By RT-PCR cloning and *in situ* hybridisation we were able to isolate and study the expression pattern of genes from the *LIM-homeobox* (*Lhx*), *Pax* and *Hedgehog* families. The main results are as follows: (1) *Lhx* and *Pax* genes reveal a strikingly similar forebrain and hindbrain patterning between cyclostomes and gnathostomes; some important differences are nevertheless observed, especially at the level of the telencephalon, a region where major changes took place at the cyclostome/gnathostome division; (2) the interpretation of the expression pattern of *Lhx* genes shows that the lamprey forebrain presents a clear prosomeric organisation, which is thus a truly craniate character; (3) analysis of the *LfHh* gene expression reveals the possible molecular origin of this neuromeric brain pattern (Osorio et al., 2005); (4) finally, *Lhx* gene expression persists through the larval (non-embryonic) period, making these genes useful markers to follow brain development and morphogenesis (Osorio et al., 2006).

This brain organisation is restricted to gnathostomes: in amphioxus and ascidians, where the expression of *Hh* is absent from the anteriormost part of the neural tube, no neuromeres are observed. Importantly, *Hh* is not expressed in the embryonic lamprey ventral telencephalon, and *Nkx2.1*, a marker of the pallidal subdivision of the gnathostome-specific subpallium, is not expressed in the embryonic lamprey subpallium (Ogasawara et al., 2001; Osorio et al., 2005; Uchida et al., 2003). This difference points out the possible role of the *Hh* ventral midline signalling as a powerful motor of forebrain evolution.

We next sought to investigate the origin of these differences in the light of the molecular evolution of the *Hedgehog* multigene family. It is likely that the chordate ancestor possessed a unique *Hh* gene. The cephalochordate amphioxus has still a single *Hh* gene, while there are two in ascidians, originated by a urochordate-specific duplication. There are three groups of *Hh* genes in gnathostomes (*Sonic*, *Indian* and *Desert*).

We have screened a cosmid genomic DNA library of *L. fluviatilis* aiming at identifying cosmids containing the *Hh* gene. The results strongly suggest the existence of two *Hedgehog* genes in lamprey. A preliminary phylogenetic analysis suggests an independent duplication of the lamprey *Hedgehog* genes within the *Sonic/Indian* group. These findings open a new vision on the evolution of this gene family within chordates.

Finally, with the purpose of studying the role of changes in gene organisation and control of gene expression in the evolution of the chordate brain, we have also isolated the *Lhx9* gene from a dogfish (*Scyliorhinus canicula*) BAC genomic library.

**Keywords:** Brain; Evolution; Agnathan; Cyclostome; Gnathostome; Lamprey; Dogfish; Gene expression; Patterning; Neuromere; Forebrain; LIM-homeobox; Lhx; LIM-homeodomain; Pax; Hedgehog; Gene duplication; Enhancer

# Résumé détaillé

## Introduction

Le terme “evo-devo” (biologie du développement et de l’évolution) a été défini comme l’étude de la comparaison des processus ontogénétiques des différentes espèces afin d’essayer d’identifier les relations ancestrales entre les organismes et l’évolution de ces processus (Goodman and Coughlin, 2000). Hall (2000) a identifié cinq questions importantes pour les études d’evo-devo, à savoir : (1) l’origine et l’évolution du développement embryonnaire ; (2) comment les modifications du développement et des processus ontogénétiques mènent à la production de nouveautés ; (3) la plasticité adaptative du développement au cours de l’évolution des espèces ; (4) quel est l’impact de l’écologie sur le développement pour moduler les changements évolutifs ; et (5) la base ontogénétique de l’homoplasie et de l’homologie. Des structures ou processus sont définis comme homologues s’ils ont été issus d’une caractéristique ancestrale commune. Il est important de distinguer les caractéristiques homologues des caractéristiques homoplastiques (ou analogues), qui sont le résultat de l’évolution convergente et ont différentes origines embryonnaires et/ou évolutives. Les premiers biologistes “evo-devo” ont commencé à utiliser les patrons d’expression de gènes du développement de différentes espèces pour essayer de comprendre comment les différents groupes d’espèces ont évolué.

Dans le travail discuté dans cette thèse, nous avons concentré notre attention sur l’étude de l’évolution du système nerveux central des craniates. Les craniates constituent un des trois taxa principaux des chordés, les deux autres étant les urochordés (ex : les ascidies) et les céphalochordés (ex : l’amphioxus). Les craniates sont divisés en deux groupes, les cyclostomes (lamproies et myxines) et les gnathostomes (les craniates à mâchoire), qui ont divergé il y a plus de 500 millions d’années.

Sa position phylogénétique fait de la lamproie un important modèle evo-devo pour identifier des caractéristiques ancestrales des craniates, mais aussi pour comprendre l'émergence de nouveautés au moment de la transition évolutive agnathe(sans mâchoire)/gnathostome (Kuratani et al., 2002). Le cerveau est particulièrement intéressant à cet égard. En effet, l'acquisition de mâchoires liée à un nouveau style de vie doit avoir été associée à des modifications de sa structure et de ses fonctions, encore largement inconnues. Des organisations neuronales différentes ont probablement été liées à de nouveaux comportements adaptatifs. L'étude de l'organisation du cerveau de lamproie embryonnaire devrait fournir des pistes importantes sur le développement et l'évolution du cerveau des craniates, sur les nouveautés qui ont émergé et évolué au sein des gnathostomes.

Une interprétation largement acceptée de l'organisation du cerveau embryonnaire des gnathostomes postule la présence de domaines présentant des frontières morphologiques et génétiques bien définies tout au long des axes transversal et longitudinal du cerveau (Puelles et Rubenstein, 1993, 2003). Ces unités segmentaires, appelées neuromères (prosomères pour le cerveau antérieur), ont été observées chez tous les gnathostomes, et fournissent un cadre pour des analyses comparatives. Pombal et Puelles (1999) ont montré qu'une analyse prosomérique peut être aussi appliquée au cerveau de lamproie adulte. N'observant pas une telle organisation prosomérique chez les céphalochordés, cette dernière est donc une caractéristique spécifique des craniates (Holland et Holland, 1999 ; Mazet et Shimeld, 2002).

Notre connaissance du cerveau de lamproie en développement est encore assez incomplète, mais les données disponibles nous permettent d'avoir une image où des similitudes et des différences claires par rapport aux gnathostomes peuvent être reconnues. Les patrons d'expression de gènes tels que *Pax6* (Derobert et al., 2002b; Murakami et al., 2001), *Otx* (Tomsa and Langeland, 1999), *Dlx*, *Emx* (Myojin et al., 2001; Neidert et al., 2001), *Pitx* (Boorman and Shimeld, 2002), *Nkx2.1/TTF-1* (Ogasawara et al., 2001) sont conservés dans le cerveau postérieur et moyen, dans les prosomères p1 et p2, et dans l'hypothalamus. Cette conservation est également présente, jusqu'à à un certain degré, dans la région antérieure à la frontière p2/p3, ce qui a permis de dessiner une première carte de la régionalisation moléculaire dans le cerveau antérieur embryonnaire de lamproie. Cette carte inclut des subdivisions du télencéphale et la délimitation de la frontière di-télencéphalique (Murakami et al.,

2001; Myojin et al., 2001). Une différence frappante entre les lamproies et les gnathostomes est l'absence de l'expression du gène *Nkx2.1* dans le télencéphale des lamproies, ce qui soutient l'idée de l'absence d'un pallidum chez ces animaux (Weigle et Northcutt, 1999).

Dans nos travaux nous avons cherché à étudier les mécanismes à la base de l'organisation du cerveau antérieur des craniates en isolant et en étudiant l'expression d'importants gènes de "patterning" et de régionalisation du cerveau de lamproie en développement. Les résultats ont été ensuite comparés aux données disponibles chez d'autres espèces de chordés, dans une perspective evo-devo. Nous avons également commencé à investiguer certaines différences de mécanismes de l'organisation de cerveau, suggérées par des études d'expression génique, à la lumière de l'évolution moléculaire. Des changements dans les régions codantes ou régulatrices — tout particulièrement ces dernières — peuvent avoir des conséquences importantes dans les fonctions des protéines et dans la régulation de la synchronisation et de la spécificité tissulaire de l'expression des gènes. L'étude de l'évolution des familles multigéniques est particulièrement important dans ce contexte, parce qu'elle peut fournir un regard plus détaillé sur les processus qui, après duplication de gènes, peuvent finalement mener à la diversification morphologique et à la spéciation.

Le chapitre 2 de cette thèse fournit une analyse de l'expression de gènes de régionalisation et "patterning" du cerveau au cours du développement embryonnaire et larvaire de la lamproie. Une étude, encore en cours, sur l'évolution de la structure des gènes et de la régulation de l'expression génique chez la lamproie et la roussette est discutée dans le chapitre 3.

## Organisation moléculaire du cerveau de lamproie en développement

### Organisation du cerveau embryonnaire de lamproie (*Lampetra fluviatilis*) : étude de l'expression des gènes *LIM-à-homéoboîte*, *Pax* et *Hedgehog*

Les facteurs de transcription *LIM-à-homéoboîte* (*Lhx*) sont impliqués dans des processus du développement neuronal tels que le guidage axonal, la spécification des phénotypes des neurotransmetteurs et la régionalisation globale du cerveau (Bach, 2000; Hobert and Westphal, 2000; Retaux and Bachy, 2002).



Ces gènes et leurs fonctions sont fortement conservés, et ils ont été employés comme marqueurs prosomériques dans plusieurs espèces-clé parmi les craniates (Alunni et al., 2004; Bachy et al., 2001; Moreno et al., 2004; Retaux et al., 1999). Jusqu'à aujourd'hui, ces études comparatives ont été seulement réalisées chez des gnathostomes.

Par clonage par RT-PCR et hybridation *in situ* nous avons pu isoler et étudier les patrons d'expression de deux gènes *Lhx*, *LfLhx15* et *LfLhx29* (représentants des groupes de paralogues *Lhx1/5* et *Lhx2/9* des gnathostomes, respectivement) dans le cerveau de la lamproie *Lampetra fluviatilis*. Les gènes *LfPax3/7*, *LfPax6* et *LfNkx2.1* ont été également employés pour identifier des frontières importantes dans le cerveau antérieur. En outre, pour comprendre davantage comment une organisation neuromérique est mise en place dans le cerveau embryonnaire de lamproie, nous avons isolé *LfHh*, gène appartenant à la famille *Hedgehog*, qui code pour des molécules de signalisation.

Ce projet a été fait en collaboration avec Sylvie Mazan (Laboratoire d'Immunologie et d'Embryologie Moléculaires à l'Institut de Transgénose à Orléans, France), et les résultats ont été publiés en novembre 2005 dans le journal *Developmental Biology* (Osorio et al., 2005). Je remercie sincèrement l'aide de Jean-Louis Plouhinec dans l'analyse phylogénétique.

Les résultats principaux de ce travail sont les suivants : (1) l'expression des gènes *Lhx* et *Pax* révèle des similarités remarquables dans le "patterning" du cerveau antérieur et postérieur entre cyclostomes et les gnathostomes. On observe néanmoins quelques différences intéressantes, particulièrement au niveau du télencéphale, région où des changements majeurs se sont produits et où beaucoup de nouveautés ont émergé à la transition agnathe/gnathostome ; (2) l'analyse de l'expression de *LfLhx15* et *LfLhx29* et son interprétation montrent que le cerveau antérieur de lamproie présente une organisation prosomérique claire, qui est ainsi un caractère craniate-typique ; (3) l'analyse de l'expression du gène *LfHh* met en évidence l'origine moléculaire possible de cette organisation neuromérique du cerveau.

Les différences dans l'expression de *Hh/Shh* peuvent en effet expliquer des différences importantes dans l'organisation du système nerveux central parmi les chordés. Aussi bien chez les cephalochordés que chez les urochordés, *Hh* n'est exprimé dans aucune région du cerveau antérieur. Dans ces deux groupes, il existe une région qui exprime *Nkx2.1* et qui partage certaines caractéristiques avec l'hypothalamus des craniates (Moret et al., 2005; Venkatesh et al., 1999) ;

cependant, cette région n'exprime pas *Hh*. L'expression de *Hh* dans le cerveau antérieur d'un ancêtre des craniates a été probablement associée avec l'apparition d'un hypothalamus de type craniate et d'une organisation prosomérique. Chez les craniates, la plaque du plancher et la zona limitans intrathalamica (ZLI) sont des centres de signalisation exprimant *Hh* jusqu'à des phases relativement tardives du développement embryonnaire. L'expression sous-palliale de *Shh* et de *Nkx2.1* est exclusive des gnathostomes, et est susceptible d'avoir joué un rôle important dans l'apparition d'un télencéphale gnathostome-spécifique. Parmi les chondrichthyens (poissons cartilagineux), *Shh* a été isolé chez la roussette (Tanaka et al., 2002) et d'autres espèces (Dahn et al., 2007), mais une analyse détaillée de son expression dans le cerveau n'a jamais été réalisée.

En réunissant ces différentes données ainsi que d'autres résultats, il est possible d'avoir une meilleure image du cerveau antérieur du craniate ancestral sur la base des patrons d'expression de gènes de développement. D'après nos connaissances actuelles, nous pouvons émettre l'hypothèse que ce cerveau antérieur aurait : (1) un diencéphale prosomérique, avec au moins trois prosomères identifiables ; (2) la ZLI exprimant *Hh/Shh* comme un centre organisateur ; un hypothalamus de type craniate, exprimant *Nkx2.1* et *Hh/Shh* ; (4) une région télencéphalique, avec un pallium *Pax6*-positif, et un sous-pallium *Dlx*-positif. Le télencéphale lui-même est une nouveauté des craniates, car une structure similaire est absente chez l'amphioxus et les ascidies (Holland and Holland, 1999; Wada and Satoh, 2001), mais est présente chez les myxines et les lamproies (Nieuwenhuys and Nicholson, 1998; Reiner et al., 1998).

### **Expression dynamique du gène *Lhx15* au cours du développement larvaire de la lamproie marine *Petromyzon marinus***

Chez le xénope, les gènes *Lhx* se sont révélés très utiles pour suivre la morphogénèse des noyaux du cerveau au cours du temps, due à leur expression persistante dès l'embryogenèse au stade adulte (Moreno et al., 2004, 2005). Nous avons analysé l'expression de *Lhx15* dans des larves de lamproie (*Petromyzon marinus*) de différents âges, afin de mieux caractériser la base moléculaire de l'identité régionale dans le cerveau de lamproie. Ce travail a été la première étude d'un patron d'expression génique dans le cerveau non-embryonnaire de lamproie en développement. Ce projet a été fait en collaboration avec Manuel Pombal et Manuel Megías (équipe Neurolam, Université de Vigo, Espagne), et

les résultats ont été publiés en octobre 2006 dans le journal *Gene Expression Patterns* (Osorio et al., 2006).

Dans cette étude, nous avons montré que l'expression du gène *Lhx15* persiste tout au long de la période larvaire, faisant de ce gène — et probablement d'autres gènes *Lhx* — un marqueur très utile pour suivre le développement et la morphogenèse du cerveau aussi chez la lamproie. En outre, nous avons mis en évidence la conservation de l'expression de ce gène dans la moelle épinière, la notochorde et le système urogénital. L'ensemble de ces résultats montre que *Lhx15* est exprimé dans les mêmes tissus endomésodermiques que le gène *Lhx1* des gnathostomes, et illustrent un cas de grande conservation spatiale et temporelle de l'expression de ce groupe de gènes au cours de l'évolution des craniates.

## Evolution de la régulation de l'expression génique dans le cerveau

Dans ce chapitre nous présentons un projet qui vise l'étude du rôle potentiel des changements de l'organisation génique et du contrôle de l'expression des gènes au cours de l'évolution du cerveau des chordés. Nous avons employé des représentants de deux groupes clé parmi les craniates, les cyclostomes (lamproie) et les chondrichthyens (roussette), où ces aspects ont été jusqu'ici mal étudiés. L'isolement et l'étude initiale du gène *Hh* de lamproie et du gène *Lhx9* de roussette seront décrits et les résultats discutés. A un niveau plus fonctionnel, des injections de la protéine Shh de souris dans le sous-pallium de la lamproie ont constitué une approche expérimentale afin de tester l'existence d'une possible conservation de la fonction de cette protéine et de la compétence du tissu de cette région du cerveau antérieur entre les cyclostomes et les gnathostomes.

## Evolution de la structure, fonction et régulation des gènes *Hedgehog*

Nous avons discuté précédemment le rôle des gènes *Hedgehog/Sonic hedgehog* au cours de l'évolution du cerveau antérieur. À la recherche d'une meilleure compréhension de ce scénario évolutif, nous avons initié un projet ayant pour but l'étude des mécanismes génétiques à la base de l'acquisition et de la perte

de fonction(s) des gènes *Hedgehog* au cours de l'évolution. Nous étions particulièrement intéressés par les apparitions de nouveaux domaines d'expression de *Hh/Shh* dans certains groupes, et par ses conséquences sur le "patterning", la régionalisation et l'acquisition de la spécification cellulaire dans le cerveau antérieur.

**Essai de phénotypage de la signalisation Shh qui est présente chez les gnathostomes sur des embryons de lamproie** Nous avons essayé de déterminer si nous pourrions partiellement induire une expression gnathostome-typique des gènes "pallidales" dans le sous-pallium de la lamproie par l'application exogène de la forme soluble (déjà clivée) de la protéine Shh de souris. Dans cette approche *in vivo*, nous avons injecté cette protéine dans le sous-pallium d'embryons de lamproie, et avons en suite examiné si la protéine avait un effet sur l'expression du gène *Nkx2.1*. Nous avons observé un développement normal des embryons, ce qui prouve que le traitement n'est pas nuisible au développement des animaux. L'ARNm du gène *Nkx2.1* est détecté dans des régions où il s'exprime habituellement, c.-à-d. l'hypothalamus et l'endostyle. On n'a observé aucune expression ectopique du gène dans le sous-pallium. Un certain nombre de cellules exprimant *Nkx2.1* a été détecté dans les régions diencéphaliques non-hypothalamiques (prethalamus et thalamus), qui sont des régions ectopiques d'expression pour ce gène. Lorsque nous injectons la plus forte concentration de protéine Shh, les niveaux d'expression de *Nkx2.1* semblent être plus faibles. Cependant, le petit nombre d'embryons examinés (n=10 pour chaque traitement) ne permet pas une analyse plus précise.

Nous pouvons néanmoins conclure que, dans nos conditions, les injections de Shh sont incapables d'induire l'expression de *Nkx2.1* dans le sous-pallium. Ceci peut être dû à une grande variété de facteurs, tels que l'absence de récepteurs appropriés ou d'autres facteurs agissant sur la compétence du tissu, à une divergence plus grande que prévue entre les protéines de souris et de lamproie, ou à un choix inadéquat de la fenêtre de développement utilisée.

**Isolement du gène *Hh* chez la lamproie** Dans le but d'étudier les changements évolutifs associés aux divergences entre cyclostomes et gnathostomes et entre chondrichthyens et osteichthyens, nous avons essayé d'isoler *Hh/Shh* chez la lamproie (*Lampetra fluviatilis*) et chez la roussette (*Scyliorhinus canicula*). Nous avons réalisé le criblage de bibliothèques génomiques de ces

deux espèces, mais nous avons isolé ce gène uniquement chez la lamproie. Nous avons cherché à répondre à deux questions, à savoir : (1) quelle est l'organisation génomique du gène *Hh* chez la lamproie et quelles informations peut-elle nous donner sur l'évolution de la famille de gènes *Hedgehog* ; et (2) quelles modifications dans sa régulation pourraient être impliqués dans l'apparition d'un patron d'expression *Shh* typique des gnathostomes.

Le projet d'isoler et d'étudier le gène *Hh* de la lamproie a commencé par mon séjour de deux mois (de mi-septembre à mi-novembre 2006) au laboratoire de Marc Ekker à l'Université d'Ottawa (Canada). Ce laboratoire a exécuté l'analyse moléculaire et fonctionnelle détaillée de plusieurs familles importantes de gènes chez les craniates, et plus particulièrement de ses régions régulatrices (Amores et al., 1998; Hukriede et al., 1999; Knapik et al., 1998; Zerucha and Ekker, 2000). Je suis reconnaissante à Marc Ekker pour son accueil chaleureux dans le laboratoire et pour nos discussions fructueuses. Je remercie Ashish Maurya de sa collaboration dans ce travail, et Gary Hatch pour son aide utile.

Ce travail a commencé par le crible d'une librairie de cosmides contenant de l'ADN génomique de *Lampetra fluviatilis* (librairie No. 55 de RPDZ) dans le but d'identifier des cosmides contenant le gène *Hh*. Nous avons réussi à identifier plusieurs clones positifs en utilisant le fragment d'ADNc de *LfHh* que nous avons précédemment isolé comme sonde (Osorio et al., 2005). Quatre des cinq clones commandés contiennent au moins le premier exon du gène désiré, identifié aussi bien par séquençage que par Southern blot. Il est important de noter que les séquences et les profils de digestion de ces quatre clones peuvent être classés dans deux groupes distincts : la séquence jusqu'ici disponible des clones 1 à 3 est remarquablement différente de celle du clone 4 ; la sonde permettant d'identifier la région ayant la plus haute similitude entre les différentes séquences. Ces résultats soulèvent la possibilité de l'existence de deux gènes *Hedgehog* chez la lamproie.

### **Evolution de la structure et expression géniques : le cas du gène *Lhx9* chez la roussette**

La roussette (*Scyliorhinus canicula*) appartient au groupe des chondrichthyens, le groupe-frère des osteichthyens (actinoptérygiens et sarcoptérygiens). La petite taille de ce requin et le fait qu'on le trouve souvent dans les eaux peu profondes près du rivage ont été des raisons importantes expliquant l'intérêt

des embryologistes pour cette espèce.

Nous avons voulu étudier la question du rapport entre l'organisation génique et la régulation de l'expression des gènes, et le rôle de ces aspects au cours de l'évolution du cerveau chez les craniates. Nous avons concentré notre attention sur un gène *LIM-à-homéoboîte*, *Lhx9*, à cause de son rôle crucial dans le développement de la partie dorsale du cerveau antérieur mais aussi par rapport aux nombreuses données d'expression et/ou structure génomique dont nous disposons chez un grand nombre d'espèces, tels que la drosophile (apterous, Lu et al., 2000; Rincon-Limas et al., 1999), l'actinoptérygien medaka (Alunni et al., 2004), la souris (Retaux et al., 1999), le xénope (Bachy et al., 2001) et la lamproie (Osorio et al., 2005). Chez tous les gnathostomes étudiés, le patron d'expression de *Lhx9* s'est révélé remarquablement utile pour comprendre la régionalisation du cerveau antérieur. En outre, des comparaisons interspécifiques ont mis en évidence l'intérêt de ce marqueur pour étudier l'évolution palliale. Jusqu'ici, aucun gène *Lhx* n'a été étudié chez un chondrichthyen.

*Lhx9* et son paralogue *Lhx2* ont été générés par duplication génique, probablement chez l'ancêtre des gnathostomes. L'expression diencéphalique des deux gènes dans le thalamus et l'hypothalamus est commune à tous les craniates, aussi bien que l'expression de *Lhx9* (*Lhx29* chez la lamproie) dans l'éminence préthalamique. Dans le télencéphale du xénope et de la souris, le domaine d'expression de *Lhx9* est plus restreint que celui de *Lhx2*. L'expression de l'orthologue de lamproie, *Lhx29*, couvre la totalité du télencéphale (pallium et sous-pallium), alors que chez la souris l'expression de *Lhx9* est exclusivement palliale (Retaux et al., 1999), et *Lhx2* est exprimée dans l'ensemble du télencéphale (Porter et al., 1997). Une inversion partielle du patron d'expression de *Lhx9* et de *Lhx2* dans le télencéphale a été observée chez le xénope par comparaison avec la souris (Bachy et al., 2001). Ces observations indiquent un lien entre des différences significatives dans l'expression de *Lhx9* et des différences majeures dans l'organisation du cerveau antérieur. Souvent, l'apparition de nouveautés est corrélée avec un événement de duplication génique, car la redondance ainsi créée peut faciliter la fixation de mutations autrement délétères. Ceci peut être l'explication pour le "shuffling" de l'expression de *Lhx2* et *Lhx9* dans la glande pinéale.

Dans le but d'étudier l'évolution moléculaire de la famille multigénique *LIM-à-homéoboîte*, nous avons réalisé le crible d'une librairie génomique de

BACs afin d'isoler le gène *Lhx9* de la roussette *S. canicula*. Ce projet comporte l'analyse de l'organisation et de l'expression de ce gène et la recherche d'éléments non-codants conservés qui pourrait avoir un rôle d'enhancer. Cette approche doit être plus facile chez la roussette que chez la lamproie. En effet, la roussette est plus proche, du point de vue phylogénétique, d'autres craniates dont le génome est entièrement séquencé. Nous avons employé une sonde *Lhx9* hétérologue (de l'actynopterygien *Astyanax mexicanus*) pour réaliser le crible de cette librairie. Un clone positif a été identifié par Southern blot et par PCR, et est actuellement en cours de séquençage au centre de séquençage Genoscope (Evry, France).

## Conclusions

L'étude du patron d'expression de gènes des familles *LIM-à-homéoboîte*, *Pax* et *Hedgehog* dans le cerveau de lamproie en développement a révélé une grande conservation des mécanismes de "patterning" et de régionalisation du cerveau antérieur. D'importantes innovations chez les craniates incluent l'établissement de la zona limitans intrathalamica comme organisateur secondaire qui exprime *Hh/Shh*. Ses propriétés de signalisation peuvent être à la base de l'organisation prosomérique du cerveau antérieur.

Les nouveautés chez les gnathostomes incluent une expression de *Shh* et *Nkx2.1* dans la région du télencéphale ventral. Cette région correspond au pallidum chez l'adulte, la région fonctionnelle où les interneurones palliaux GABAergiques et les neurones cholinergiques sous-palliaux sont spécifiés, et d'où ils migrent vers le pallium. Pour cette raison, la présence du pallidum est probablement corrélée avec des différences majeures d'intégration et de traitement d'information dans le télencéphale.

Chez la lamproie, comme chez le xénope, l'identité régionale des prosomères diencephaliques est maintenue tout au long des stades larvaires, ce qui est démontré par l'expression non-embryonnaire du gène *Lhx15*. Nos études mettent également en évidence la possibilité d'employer ce marqueur pour suivre le développement de régions et populations neuronales spécifiques au cours du temps.

L'étude des gènes *Hh* a suggéré l'existence de deux gènes de cette famille chez la lamproie, ce qui est un changement par rapport aux idées précédentes sur l'évolution de cette famille multigénique chez les chordés. L'isolement du

gène *Lhx9* chez la roussette a montré que la duplication à l'origine des gènes *Lhx9* et *Lhx2* a probablement eu lieu avant la divergence entre les chondrichthyens et les osteichthyens.

De plus amples analyses moléculaires et d'expression génique seront maintenant entreprises dans le but d'approfondir nos connaissances sur l'histoire des duplications de gènes et des modifications de la régulation génique qui peuvent être à la base des différences observées au sein des craniates et entre les craniates et les autres chordés.





# Chapter 1

## Introduction

In evolutionary developmental biology, the comparison of developmental processes in different species is used to understand evolution. **Evo-devo** aims to determine the ancestral relationship between organisms and how developmental processes evolved (Goodman and Coughlin, 2000, an introduction to a special *PNAS* issue on evo-devo). Hall (2000) identified five important questions addressed in evo-devo studies, namely: (1) the origin and evolution of embryonic development; (2) how modifications of development and developmental processes lead to the production of novel features; (3) the adaptive plasticity of development in life-history evolution; (4) how ecology impacts on development to modulate evolutionary change; and (5) the developmental basis of homoplasy and homology. Structures or processes are defined as homologous if they were inherited from a common ancestor. It is important to distinguish homologous from homoplastic, (or analogous) features, which are the result of convergent evolution from different origins. The first evo-devo biologists began using developmental gene expression patterns of individual organisms to explain how groups of organisms evolved.

The **developmental-genetic toolkit** consists of genes whose products control the development of a multicellular organism. Differences in deployment of toolkit genes affect the body plan and the number, identity, and pattern of body parts. The toolkit is highly conserved across animal phyla. The majority of toolkit genes code for components of signalling pathways, which include transcription factors, cell adhesion proteins, cell surface receptor proteins, and secreted morphogens. Their function is highly correlated with their spatial and temporal expression patterns (True and Carroll, 2002).

One of the fundamental principles at the base of the process of evolution through development is the principle of **modularity**. Modularity allows three processes to alter development: dissociation, duplication and divergence, and co-option. Since the modules are on all levels from molecular to organismal, these principles operate at all levels of development (Raff and Raff, 2000). The process of dissociation includes heterochrony (the shift in the relative timing of two developmental processes during embryogenesis) and allometry (different parts of the organism grow at different rates). In the process of co-option, pre-existing units can be recruited for new functions (Raff and Raff, 2000; True and Carroll, 2002). In some cases, it happens that the function of a given module seems to be conserved, but the structures where the module is active are not homologous (e.g. the cascade triggered by *Pax6* involved both in insect and craniate eye development; reviewed in Cook, 2003).

In the work discussed in this thesis, we have focused our attention on the study of the evolution of the craniate central nervous system (CNS). The Craniates constitute one of the three main Chordate taxa, the other two being the Urochordates (or tunicates, e.g. ascidians) and the Cephalochordates (e.g. amphioxus). Craniates are split in two groups: the Cyclostomes (lampreys and hagfish) and the Gnathostomes (jawed craniates), which diverged more than 500 million years ago. The phylogenetic relationships between the three groups of chordates, and between lampreys, hagfish and gnathostomes are still a controversial issue, and will be discussed further in the Introduction. We have sought to investigate the mechanisms underlying the organisation of the craniate forebrain by isolating and studying the expression of important brain patterning and regionalisation genes during lamprey development. The results were then compared to available data from other chordate species, in an evo-devo perspective.

We have also started to investigate some of the differences in the mechanisms of brain organisation, as revealed by gene expression studies, in the light of molecular evolution. Coding or regulatory sequence changes, especially the latter, may have important consequences in protein function and in the regulation of the timing and tissue-specificity of gene expression. The study of the evolution of multigene families is of particular importance in this context, as it provides a closer look into the processes which, after gene duplication, may ultimately lead to morphological diversification and speciation.

## 1.1 The CNS of craniates

### 1.1.1 The neural tube

After fertilisation of the egg by the sperm, the early embryo starts the process of **cleavage**, through which an increasing larger number of cells is generated. The cells at this stage are called blastomeres, and an embryo undergoing such a process is called a **blastula**. Later, blastomeres start to change their position relative to one another, in a combination of movements (which usually involve epiboly, invagination, involution, ingression and delamination) which marks the important stage known as gastrula. During **gastrulation**, three distinct germ layers appear, the outer ectoderm, the inner endoderm and the intermediate mesoderm. These three germ layers possess distinct properties, but have influence on each other, for example through the production of diffusible molecular signals which will affect cells from another germ layer. These patterning processes will establish a dorso-ventral (DV), an antero-posterior (AP) and a medio-lateral (ML) polarity in the future neural tissue, and this is already evident at gastrula stage (Fig. 1.2 A).

Evidence for early neural patterning came first from the experiments of Hans Spemann and his students on the frog *Xenopus laevis*. The inductive properties of the dorsal blastopore lip of gastrulating embryos had already been recognised by Spemann and Hilde Mangold (Spemann and Mangold, 1924), and this region had been named the gastrula **organiser**. The definitive identification of the capacity of this region to induce a secondary neural plate came from studies using the Einsteck method of transplantation by Bruno Geinitz, another of Spemann's students (Geinitz, 1925). By this method, developed by Spemann and Otto Mangold, a transplant is introduced into the blastocoel of the gastrula through a small opening, and later adheres to the inner layer of the ventrolateral ectoderm, where it can act upon the overlying tissue, inducing the formation of an ectopic neural plate ([www.devbio.com](http://www.devbio.com), the Companion Website to the book *Developmental Biology* by Scott F. Gilbert, 8<sup>th</sup> ed.). Following studies using this and other techniques further clarified the sequence of events that causes the tissue which will later become the CNS to be differentially patterned along its antero-posterior (AP) axis. Some of these techniques included the use of "Keller sandwiches", thus named in honour of Ray Keller, who first developed their microsurgical isolation (Keller and Danilchik, 1988). These sandwiches are isolations of the tissue above the

blastopore lip in its full depth, and extend dorsally up to the animal cap, including the non-involuting and the involuting marginal zones. They can be grown in culture out of the embryo, which makes possible the observation of the behaviour of each zone, the analysis of converging and extending cell populations at the single-cell level, and the study of planar signals that may be sent by the dorsal lip of the blastopore to nearby ectoderm as part of neural induction. Keller et al. (1992) have provided evidence for the involvement of planar signals in the induction of the convergent extension of the hindbrain and spinal cord ectoderm.

In chordates, organogenesis is initiated when the mid-dorsal ectodermal cells acquire a neural identity, distinct from the non-neural identity of the neighbouring ectodermal cells (Gilbert, 1997). The process of formation of the neural tube is called **neurulation**, and an embryo undergoing such changes is called a neurula. The craniate neural tube forms from two tubes that develop independently, by distinct morphogenetic and molecular processes. An anterior (or primary) tube extends from the brain to the cervicothoracic region, and a more posterior tube develops later in the lumbar and tail region. The anterior tube forms via “primary neurulation” from an epithelial cell sheet (the neural plate) (reviewed in Colas and Schoenwolf, 2001). In contrast, the posterior tube forms from the tail bud via “secondary neurulation” in which there is a transformation of a solid rod of mesenchymal cells into an epithelial tube (reviewed in Griffith et al., 1992; Lowery and Sive, 2004, Fig. 1.1). The definitions of epithelial and mesenchymal tissues used in this description are those given by Lowery and Sive (2004): epithelial tissues are defined as an organised and contiguous sheet of cells held together by junctional complexes, while the mesenchymal tissue is defined as a loosely associated group of cells.

In primary neurulation, the columnar cells that form the neural plate are generated from the dorsal ectoderm of the gastrulated embryo. Bone morphogenetic protein (Bmp) signalling, present in the non-neural ectoderm, is inhibited in the neural plate. The neural plate folds, producing the neural groove. The hinge point in the middle of the groove corresponds to the floor plate, the future ventral midline of the neural tube (Gilbert, 1997; Lowery and Sive, 2004). At the end of neurulation, the lateral edges of the neural tube (the neural folds) fuse in the dorsal midline, the future roof plate, and the neural tube segregates from the non-neural epithelium. This type of neurulation involves a complex set of cell movements that include epithelial columnarisation, mi-

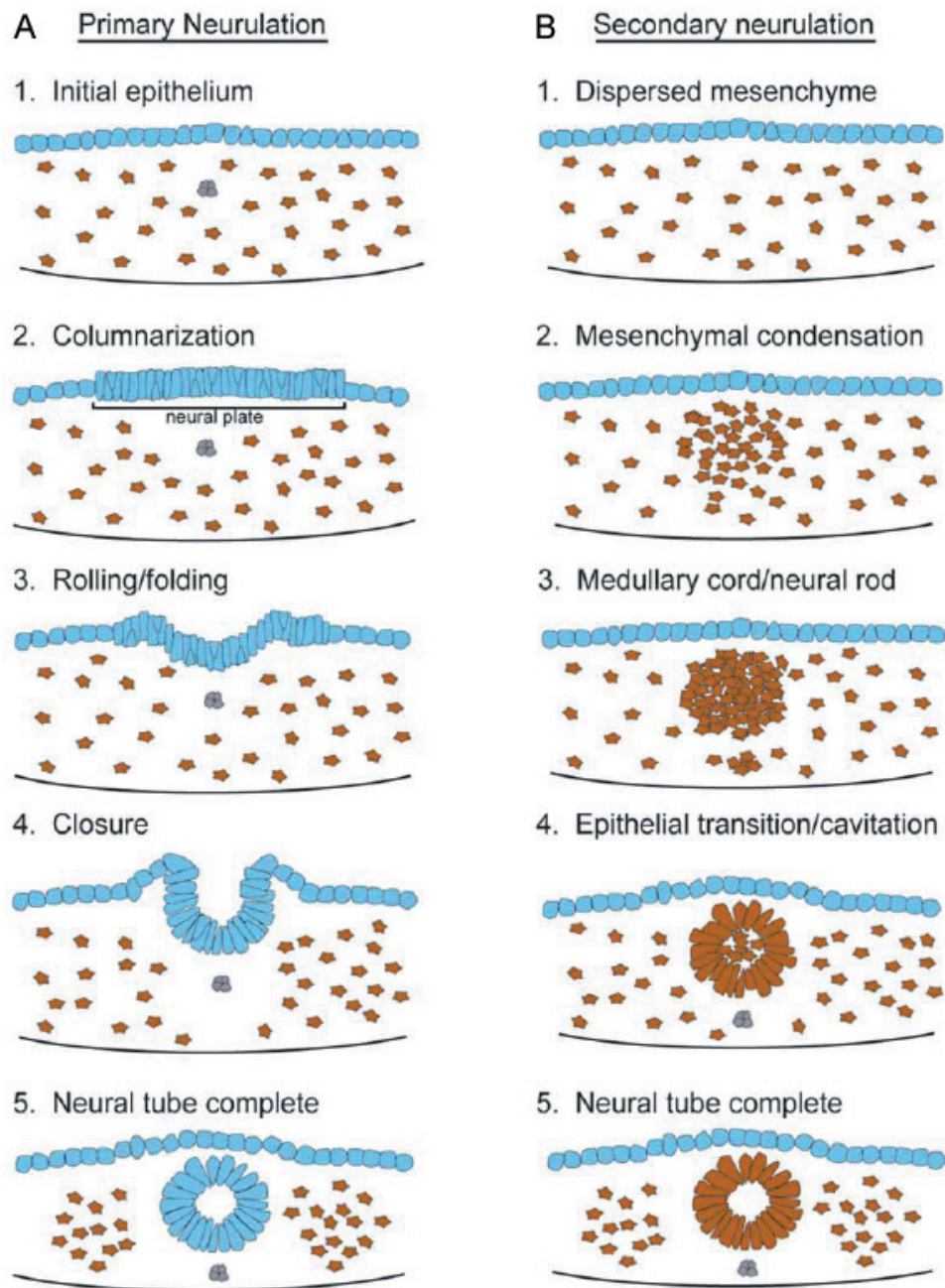


FIGURE 1.1: The neural tube may form by two different mechanisms. (A) Primary neurulation involves columnnarisation of an existing epithelium, and then rolling or folding the epithelium (blue). (B) secondary neurulation is characterised by condensation of mesenchyme (brown) to form a rod, which then undergoes an epithelial transition to form the neural tube. From Lowery and Sive (2004).

gration, intercalation, and convergent extension (Colas and Schoenwolf, 2001; Davidson and Keller, 1999). In birds, amphibians and mammals, it was long recognised that anterior parts of the neural tube are formed by primary neurulation, more posterior parts by secondary neurulation (Gilbert, 1997), but neurulation in teleost fish (and lampreys, see Damas, 1944) was thought to be exclusively secondary. This was principally because the teleost trunk neural tube initially forms a solid rod (the neural keel) that later develops a lumen. However, some authors now think that this description is not accurate, since the teleost neural tube derives from an epithelial sheet that folds. According to these authors, neurulation in teleosts best fits the description of primary neurulation (Lowery and Sive, 2004). Neurulation in amphioxus and ascidians follows the general chordate pattern, developing from a dorsal neural plate, which rolls up to form the neural tube (reviewed in Holland and Holland, 1999; Meinertzhagen et al., 2004). However, neurulation in amphioxus is a two-phase process: first, there is a rapid epidermal overgrowth over the neural plate, which later slowly rolls into a neural tube (Holland and Holland, 1999).

The process of patterning of the neural tube along its DV, AP and ML axis (Fig. 1.2 A and B) will eventually result in its differentiation: at a regional level, distinct cell populations will arise, and will give rise to the future functional units of the brain; at a cellular level, cell differentiation processes will generate different types of neurons and glial cells (Gilbert, 1997).

Neural crest cells are a particular population of cells which originates at the interface between the neural plate and the adjacent non-neural ectoderm. Their neural crest fate is specified by a network of molecular factors that ultimately promotes an epithelial-to-mesenchymal transition, delamination, migration and subsequent terminal differentiation. Neural crest cells will give rise to pigment cells, much of the peripheral nervous system, the craniofacial skeleton and many other cell types (reviewed in Sauka-Spengler and Bronner-Fraser, 2006). Thought to be an exclusively craniate characteristic, recent studies in ascidians have identified a cell population that shares similarities with craniate neural crest cells (Jeffery et al., 2004).

The patterning of the neuroepithelium is a result of the activity of signalling centres. The **dorso-ventral (DV) axis** of the neural tube is established under the influence of signals produced by the dorsal and ventral midlines. These patterning centres produce molecules with antagonistic effects: the roof plate is a source of dorsalising signals such as Bone Morphogenetic Proteins

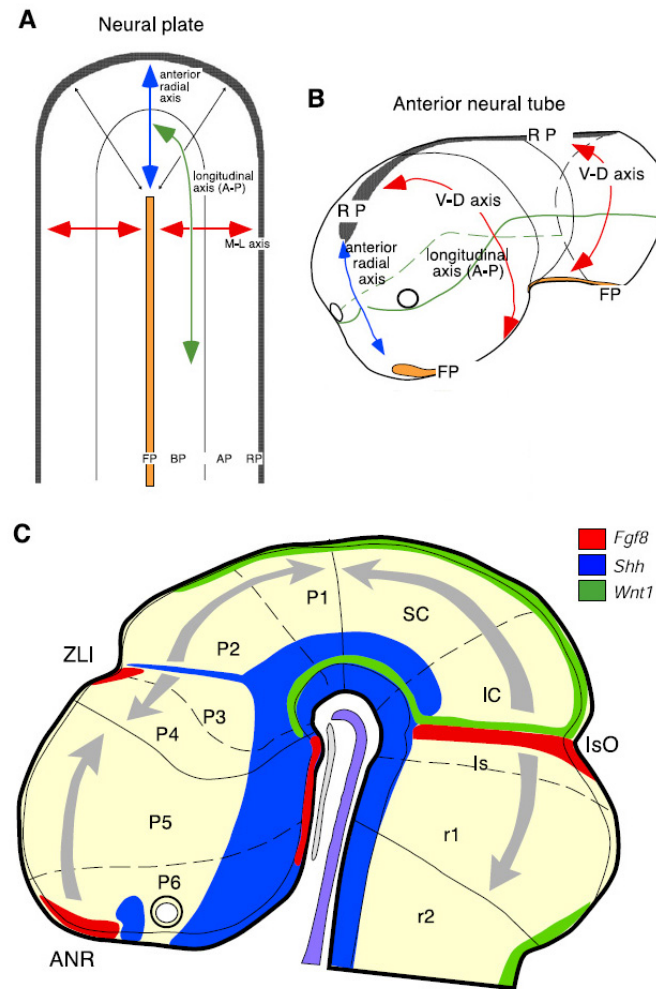


FIGURE 1.2: (A) Schematic illustration of the longitudinal and medio-lateral (M-L) axes at the neural plate stage of embryonic development. The latter polarity is already defined during the formation of the node and primitive streak at gastrula stages. Red arrows indicate medio-lateral signalling which takes place. The blue arrow represents the planar/radial signals from the node to the adjacent tissue and the green arrow represents the antero-posterior axis. (B) Subsequent to neurulation, the medio-lateral axis (in the neural plate) becomes the ventro-dorsal axis (now in the tube). Each region (segment) of the anterior neural tube will have a floor plate (FP), basal plate (BP), alar plate (AP) and roof plate (RP). (C) General schematic representation of the expression patterns of some of the principal genes codifying for signalling molecules based on the prosomeric model. T, telencephalon; D, diencephalon; M, mesencephalon; R, rhombencephalon; os, optic stalk primordium; fp, floor plate in the anterior neural tube of craniates and the topological location of the secondary organisers; ANR, anterior neural ridge; IC, inferior colliculus; Is, isthmus; IsO, isthmic organizer; P1–P6, prosomeres; r1–r2, rhombomeres; SC, superior colliculus; ZLI, zona limitans intrathalamica. From Echevarria et al. (2003).



(e.g. *Bmp4* and *Bmp7*), Wnts (Wingless-Int) and possibly retinoic acid, while the floor plate and the underlying chordal and epichordal mesoderm secrete molecules with a ventralising effect such as Sonic Hedgehog (Shh). Four main longitudinal zones of the neural tube are commonly recognised: the floor, basal, alar and roof plates, with the sulcus limitans separating the basal from the alar plate (Fig. 1.2). In all gnathostomes, these domains are characterised by the expression of *Shh* (floor plate), *Nkx2.2* (ventro-lateral domain at the basal-alar boundary) and *Noggin* and *Wnt1* (roof plate) (reviewed in Echevarria et al., 2003).

The neural tube will also be patterned along its **antero-posterior (AP) axis**. At the anteriormost region of the neural tube, the brain will form. In amniotes, several AP signalling centres generate fields of organisation in their zone of influence, regulating the growth, patterning and regionalisation of the brain (Bally-Cuif and Wassef, 1995; Houart et al., 1998; Lumsden and Krumlauf, 1996; Ohkubo et al., 2002; Shimogori et al., 2004). These centres are now commonly described as “secondary organisers”, a name derived from the “primary” gastrula organiser (the Spemann organiser in the frog). The most important AP secondary organisers in the brain are the anterior neural ridge (ANR), the zona limitans intrathalamica (ZLI) and the isthmus organiser (IsO) (Fig. 1.2; for a review, see Echevarria et al., 2003). The signalling proteins which are secreted by these organisers belong to the Wnt (Wingless-Int), Bmp (Bone Morphogenetic Protein), Fgf (Fibroblast Growth Factor) or Shh (Sonic Hedgehog) families.

The activity of these signalling centres will result in the differential expression of regionalisation genes, among which homeobox genes are numerous (Fig. 1.5), which will in turn affect neurogenesis and connectivity. Important factors involved in these latter processes are the products of bHLH (basic helix-loop-helix) genes<sup>1</sup>. The acquisition of regional identity during embryonic development, as seen by the expression pattern of regionalisation genes, is thus tightly linked to the future function of the different brain areas.

A clear external evidence of the AP patterning of the neural tube is the appearance, at a relatively late stage of neurogenesis, of a series of bulges and constrictions throughout this axis. These bulges correspond to the main future brain subdivisions, the forebrain, classically divided in telencephalon and diencephalon, the midbrain or mesencephalon, and the hindbrain, or rhomben-

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<sup>1</sup>For a study on genes involved in neurogenesis, see appendix A.

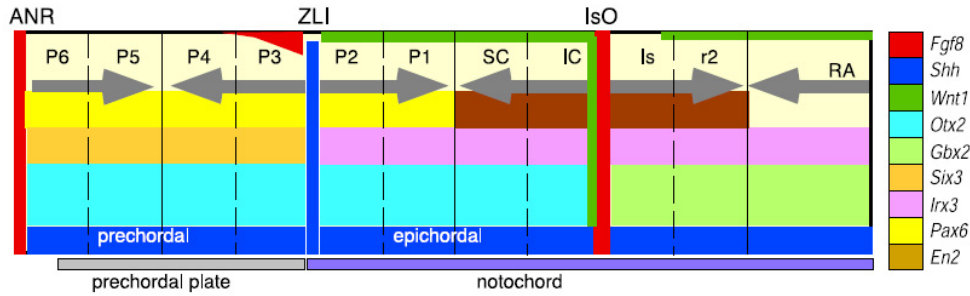


FIGURE 1.3: Schematic diagram representing a hypothetical model of action of secondary organisers. Based on the prosomeric model, each organiser would be located at the boundary of the expression domains of two principal specific genes and at a distance of four prosomeres/neuromeres from each other. Their molecular effects would be perceived in the adjacent rostral and caudal segments. Thus, the gray arrows represent the orientations of signalling and inductive effects from each organiser on the neighbouring tissue. IC, inferior colliculus; Is, isthmus; p1–p6, prosomeres 1–6; r2, rhombomere 2; RA, retinoic acid; SC, superior colliculus. From Echevarria et al. (2003).

cephalon; and to the spinal cord.

The ANR acts upon the rostralmost part of the brain. Studies in mouse and chick have shown that this centre expresses *Fgf8* and *Fgf15*, and that *Shh* is expressed in a region close to the ANR (Fig. 1.2 and Fig. 1.3). The ZLI is a neuroepithelial site in the diencephalon with important morphogenetic properties. Both the neural regions located posteriorly and anteriorly to the ZLI are under the influence of this patterning centre. In gnathostomes, the ZLI expresses *Shh*, in a domain which is continuous with the floor plate *Shh* expression domain, starting as a small ventral expression and extending dorsally during embryogenesis. In this manner, a thin finger-shaped domain (as seen sagittally) is formed, and acts in the neighbouring anterior and posterior brain tissue (Fig. 1.2 and Fig. 1.3). Furthermore, the triangle defined by the expression of *Shh*, *Wnt1* and *Fgf8* at the dorsal region of the ZLI may represent the source of secreted factors which control proliferation, regionalisation and polarity. The isthmus organiser (IsO) is located in the isthmus territory, which includes the mid-hindbrain boundary. *Fgf8* has been shown to be one of the principal effector molecules underlying the morphogenetic activity of the isthmus. Other important factor in this region is *Wnt1*, expressed at the isthmus-mesencephalic boundary. The mid-hindbrain boundary is defined by the juxtaposition of *Gbx2* (posterior) and *Otx1/2* (anterior) expression do-

mains (Fig. 1.2 and Fig. 1.3). A complex cascade of factors expressed in this region, or in either its rostral or caudal areas, is crucial to the correct morphogenesis of the midbrain and hindbrain regions (reviewed in Echevarria et al., 2003).

The neural tube of craniates, especially its anteriormost region, is subject to very important morphogenetic deformations. One of these deformations is the ventral bending of the anterior portion of the neural tube and the concomitant “overgrowth” of the anterior alar plate, the future telencephalon. Because of this deformation, the rostro-caudal axis and, consequently, the sulcus limitans, will no longer be co-linear to the apparent AP axis (compare B and C in Fig. 1.2; see also Fig. 1.6). It is important to bear the topology of the brain in mind when comparing different species. Through this thesis, the mentions of AP and rostro-caudal axis will always make reference to the true axis of the brain, not the apparent one.

The telencephalon is the rostralmost portion of the brain, originating exclusively from the alar and roof plates of the neural tube. It is divided in two regions which have fundamentally different structures and functions. In each of these regions, the differential expression of regulatory genes will generate distinct neuronal populations, which can migrate from their original location and populate other regions of the brain (Fig. 1.4). The dorsal telencephalon corresponds to all the pallial<sup>2</sup> areas (that is, the cortical regions in mammals) while the ventral telencephalon corresponds to the subpallial structures composed of the basal ganglia. In gnathostomes the pallium is classically divided into four regions, the medial, dorsal, lateral and ventral pallia (Butler and Hodos, 1996). The subpallium is composed of three main subdivisions, the lateral and medial ganglionic eminences (LGE and MGE, respectively) and the telencephalic stalk. The LGE will give rise to striatal components of the subpallium, the MGE will become the pallidum, and the main cholinergic nuclei of the basal forebrain will have their origin in the telencephalic stalk.

From a neurophysiological point of view, the cognitive capabilities of craniates rely on the organisation and expansion of the pallial and subpallial areas of the telencephalon. One of the major challenges in this research field is to understand how important novelties and increased diversity were generated in the forebrain of craniates through evolution. All the differences and sim-

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<sup>2</sup>The word “pallium” means “canopy”, an adequate description of the appearance of this region in mammals.

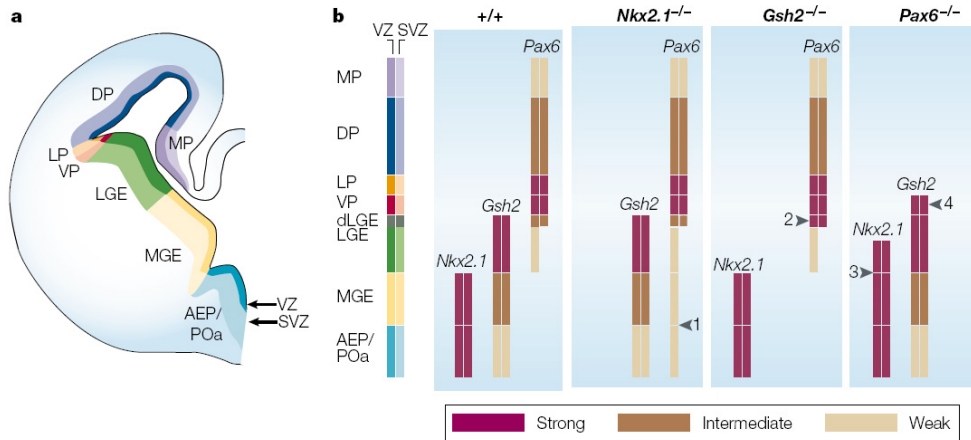


FIGURE 1.4: Telencephalic subdivisions and expression of regulatory genes. Interactions between regulatory genes contribute to the generation of subpallial progenitor domains in the telencephalon. (a) A coronal hemisection through a brain at embryonic day 14.5, showing in different colours the distinct progenitor cell domains of the telencephalon. (b) The expression of *Nkx2.1*, *Gsh2* and *Pax6* is required to define independent progenitor cell populations in the lateral ganglionic eminence (LGE) and medial ganglionic eminence (MGE). Interactions between these genes define boundaries between the different progenitor zones. In *Nkx2.1* mutants, *Pax6* expression is expanded ventrally into the MGE and anterior entopeduncular area (AEP) (arrowhead 1). In *Gsh2* mutants, *Pax6* expression is expanded ventrally into the dorsal LGE (dLGE), along with other pallial markers (arrowhead 2). Finally, in *Pax6* mutants, *Nkx2.1* expression is expanded dorsally into the LGE (arrowhead 3) and *Gsh2* expression is expanded dorsally into the ventral pallium (VP) (arrowhead 4). DP, dorsal pallium; LP, lateral pallium; MP, medial pallium; POa, anterior preoptic area; SVZ, subventricular zone; VZ, ventricular zone. From Marin and Rubenstein (2001).

ilarities which can be observed between species must be linked to differences and similarities in the developmental genetic processes which build and shape the forebrain. The position of the telencephalic and diencephalic embryonic signalling centres is adequate to their probable role in the control of morphogenesis, growth, patterning and subsequent neuronal organisation of the forebrain of all craniates. Thus, variations in signalling centres during embryonic development may have been a motor of craniate forebrain evolution.

### 1.1.2 From early patterning to brain regionalisation

During the process of patterning of the neural tube, neural and glial cells will differentiate and acquire specific cell fates. Discrete borders will be formed throughout the neuraxis, and distinct brain regions will appear. The nature of

these borders and the spacial definition of the regions they separate have been perceived and interpreted in many different ways. In general, we can think of a brain region as a structure specified by a unique combination of molecular factors, composed by a mixture of cell populations which is more homogeneous within the region than when compared with those of neighbouring regions, and that will develop into a future functional brain unit. According to Kiecker and Lumsden (2005), a region will be considered a true compartment if it consists of polyclonally-related cells that do not mix with cells from neighbouring compartments.

We have described above the influence of secondary organisers in the establishment of regional and cellular identity in the brain. Diffusible signals emitted from these sources (“morphogens”) will have a concentration-dependent effect in the neighbouring tissue, creating distinct domains characterised by the expression of different regionalisation genes. The delimitation of future functional regions of the brain often corresponds to the boundaries of mutually-exclusive expression domains of two or more of these genes at a given embryonic stage (e.g. the border between *Otx* and *Gbx* expression domains defines the mid-hindbrain boundary, see Fig. 1.3. For this reason, the comparison of brain regionalisation gene expression patterns has been used to establish homologies between different species. However, the question of how exactly does a region appear is still a riddle. How do the cells respond to the positional information given by the morphogen? In other words, how do the cells perceive a continuous gradient which will be transformed into discrete domains with well-defined limits?

In a recent review by Ashe and Briscoe (2006), several molecular mechanisms that may underlie morphogen signalling are discussed (see Fig. 1.5). In particular, cross repression has been demonstrated in the case of ventral Shh signalling in the craniate neural tube, which has a role in the specification of different ventral-to-dorsal neuronal cell fates by distinct homeodomain transcription factors (Gritli-Linde et al., 2001). Note that several of these mechanisms probably cooperate to create each patterning effect (Ashe and Briscoe, 2006).

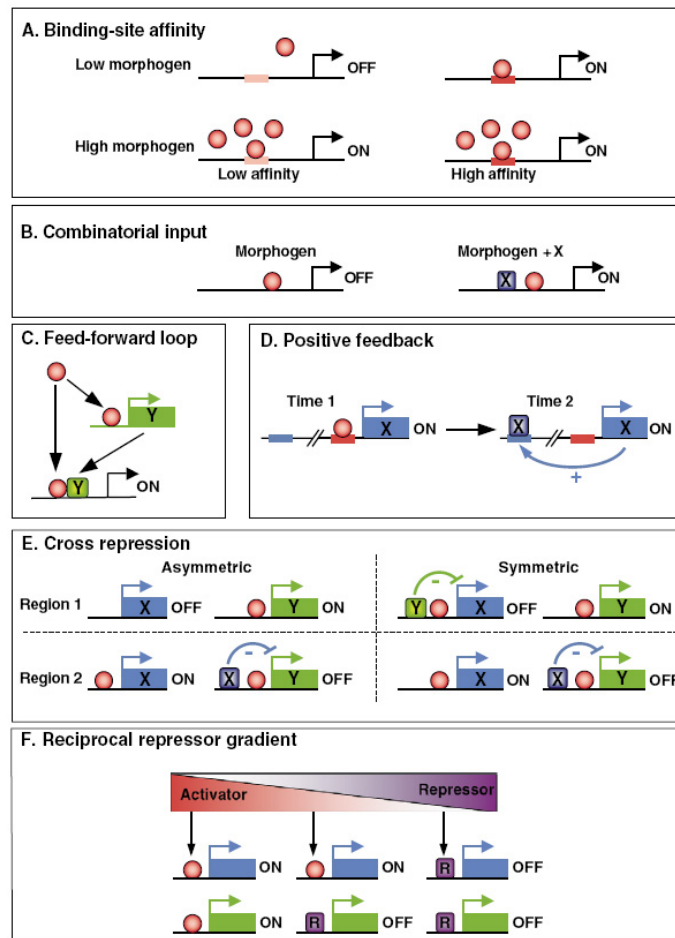


FIGURE 1.5: Strategies employed to interpret graded signals. (A) Binding-site affinity. The number and affinity of transcription factor binding sites determine threshold responses. Low amounts of transcriptional effector are sufficient to bind to and activate transcription from high-affinity binding sites; lower-affinity binding sites require larger amounts of transcriptional effector. (B) Combinatorial inputs. The integration of multiple positive and/or negative inputs with the transcriptional effector of the morphogen establishes a threshold response. Other regulatory elements (X) can also determine the response of a target gene. (C) Feed-forward loop. A regulatory circuit in which the transcriptional effector activated by the morphogen controls the expression of a second regulator (Y); the combination of the two regulate the transcription of a target gene. (D) Positive feedback. A gene (X) induced by the morphogen autoregulates to enhance its own expression. (E) Cross repression. Repressive interactions between morphogen-regulated genes (X and Y) establish discrete changes in gene expression. Repressive interactions can be asymmetric (for example ventral dominance in the *Drosophila* neuroectoderm) or symmetric, resulting in reciprocal cross repression (for example in the craniate neural tube). (F) Reciprocal repressor gradient. The transcriptional effector sets up an inverse transcriptional repressor gradient that is interpreted by target genes. The ratio of repressor (R) to activator defines the threshold response of target genes, depending on the binding sites present in the enhancer. From Ashe and Briscoe (2006).

### 1.1.3 On the prosomeric model

The process of patterning will lead to the acquisition of regional identity. The early embryonic regionalisation as seen through the study of the expression pattern of regionalisation genes, morphology and neurochemical differentiation gives already an indication of the future functional properties of each domain. At the level of the forebrain, several models of organisation have been proposed throughout the last two centuries. The first description of brain segmentation comes from von Baer (1828); the morphological recognisable brain segments were later named **neuromeres** by Orr (1887). Since then, neuromeres have been identified based on morphological, gene expression and cell lineage restriction features (discussed in Candal, 2002).

The idea of neuromery implies a rostro-caudal succession of transverse independent units sharing the same dorso-ventral organisation. This type of organisation was long recognised for the hindbrain, where these neural units are called rhombomeres. The prosomeric model (Puelles and Rubenstein, 1993) extends this notion to the entire brain. All the brain neuromeres (prosomeres, mesomeres and rhombomeres) are composed of the same longitudinal zones: the floor, basal, alar and roof plates. The first version of the model proposed the existence of 4 diencephalic (p1-p4) and 2 telencephalic (p5-p6) prosomeres (Fig. 1.6). In terms of gene expression, the model relies on the limits of the expression domains of regionalisation genes belonging to very conserved families such as *Tbr*, *Pax*, *Dlx*, *Otx*, *Wnt*, *Emx*, *Lhx*<sup>3</sup>, *Gbx* and others (see Fig. 1.5).

In a later version of the model (Puelles and Rubenstein, 2003), the forebrain is divided into a neuromeric caudal prosencephalon (diencephalon proper), with three prosomeres (p1-p3), and an unsegmented secondary prosencephalon (telencephalon and hypothalamus). As a reason for this change, the authors invoke the many problems in recognising true, complete, ventro-dorsal prosomeric borders within the secondary prosencephalon due to the high deformation present in this region and the existence of patterning effects here that are not found elsewhere. However, several regions with specific patterning “singularities” can be nonetheless recognised in the secondary prosencephalon (Puelles and Rubenstein, 2003).

Even if it may be an oversimplification of brain architecture, the power of

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<sup>3</sup>*Lhx1/5* and *Lhx2/9* are expressed in alternative prosomeres in the diencephalon.

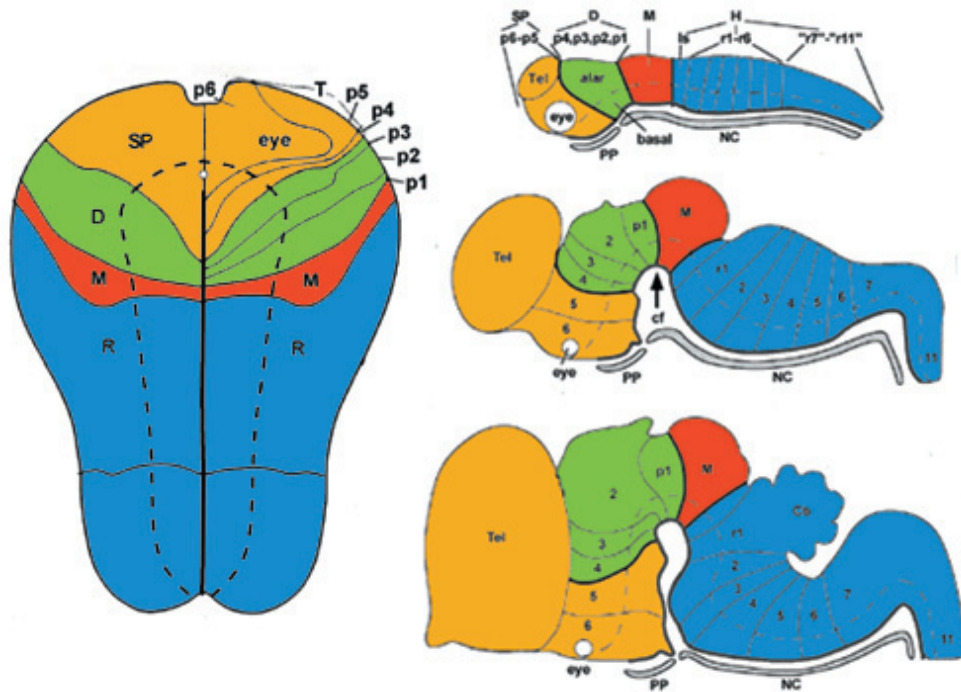


FIGURE 1.6: The prosomeric model. (A) Fate map of the neural plate in the frog *Xenopus*. The floor plate is represented as a thick midline line. The basal-alar boundary appears as a dashed line. The zones that will originate the major future brain regions are indicated in different colours. These regions are the secondary prosencephalon (with the eye field and telencephalon), diencephalon, midbrain, rhombencephalon and spinal cord. At right, prospective prosomeres postulated by the model. (B) Schemata illustrating the axial bending and transversal segmentation of the rostral neural tube in relation to the underlying parts of the axial mesoderm (prechordal plate and notochord). The floor plate induced and maintained by the axial mesoderm is marked as a thicker black line in the floor of the neural tube. The alar-basal boundary (sulcus limitans) appears as a dashed line. Transverse lines mark interneuromeric boundaries in the hindbrain (rhombomeres 1–6 and pseudorhombomeres 7–11) and forebrain (prosomeres 1–6). The cephalic flexure (arrow marked cf in the middle drawing) progressively separates the midbrain and diencephalon from the rigid axial mesoderm, causing a corresponding deformation of all the longitudinal and transverse boundaries in the area. Adapted from Puelles (2001).



the prosomeric model lays in its usefulness for comparative analysis between different species, as it stresses the topological relationships between the different forebrain regions regardless of secondary deformation of the neural tissue (structural homology). The model has been applied to virtually all major craniate groups, revealing a high conservation of the general brain “construction plan” (*Bauplan*) within this taxon<sup>4</sup>. This forebrain organisation is absent from non-craniate chordates (Holland and Holland, 1999; Mazet and Shimeld, 2002; Wada and Satoh, 2001).

Although the idea of a topologically comparable forebrain regionalisation is widely accepted, and has been implicitly or explicitly used through the last century to establish homologies between brain regions of different species, the prosomeric interpretation has met some criticism. The main problems are the ideas of **neuromery** (the neuromeres as metameric structures) and **compartmentalisation**. The notion of neuromery is stronger than that of **segmentation**, the patterning of the structure into distinct and complete serially arranged segments transversal to the AP axis. A neuromeric organisation implies that all the segments are serially homologous, that is, they share a common DV patterning (in this case, they are divided into four longitudinal zones). It was also proposed that neuromeres could be compartments, i.e. developmental units that consist of polyclonally-related cells that do not mix with cells from neighbouring compartments (Mathis et al., 1999). The boundaries of a compartment would be thus clonal restriction limits.

The idea of neuromery is controversial partly because some authors do not recognise that the division into the four classical longitudinal zones is a strong enough argument to support the idea of true forebrain “neuromere metamery”, accepted for the more clearly repetitive hindbrain. Furthermore, for these authors the notions of segmentation and compartmentalisation are also linked. The hindbrain would have a truly metameric organisation because there is compartmentalisation and segmentally reiterative neuronal architecture, associated with the nested expression of *Hox* genes. In the forebrain, the proposed neuromeres would be much more diverse (reviewed in Kiecker and Lumsden, 2005).

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<sup>4</sup>Among the ideas that have been put forward to explain the evolutionary conservation of brain organisation at a very particular developmental moment is the thought that the coordination of gene expression is correlated to the coordination of neurogenesis, a process which would be under high selective pressure. The work presented in the appendix A is related to this topic.

The proven utility of the model in the comparison of homologous structures in evo-devo studies — because it reflects, anatomically and molecularly, the plan of organisation of the brain — is a strong argument in favour of its application. The need of a common language among neurobiologists and the uniformisation of brain nomenclature in different species is another argument for its use. Through this thesis, the use of the prosomeric nomenclature will not imply a strict notion of “segmentation”, and the words “border” or “limit” will be given preference to “boundary”, except in the cases where cell-lineage restriction boundaries are more widely accepted.

## 1.2 Mechanisms of evolution

During the last decade, the field of evolutionary biology has been enriched with a number of powerful molecular tools (genome sequencing, microarrays, functional genomic analysis...) that can be used to understand the generation and maintenance of genetic and morphological diversity. Fig. 1.7 is a schematic representation of the three modes of gene evolution that are most commonly invoked to explain the generation of diversity: gene duplication, regulatory sequence expansion and diversification, and alternative isoform expression (reviewed in Carroll, 2005). The discussion of other important processes of molecular evolution, such as epigenetic changes, will not be developed here.

What is the relative contribution of the different modes of gene evolution in the evolution of anatomy? In general, it is expected that mutations with greater pleiotropic effects will have more deleterious effects on organismal fitness and will be a less common source of variation in form than mutations with less widespread effects. In animals, the capacity to generate tolerable, heritable variation (“evolvability”) depends on redundancy (which reduces constraint on change by circumventing or minimising the potentially deleterious effects of some mutations) and compartmentalisation (by uncoupling variation in one process from variation in another, pleiotropy is decreased). So, the relative contribution to anatomical variation of the processes of gene evolution mentioned above will depend on the pleiotropic effect of the mutations which they typically generate (Carroll, 2005; Kirschner and Gerhart, 1998). The relative contribution of gene duplication and coding and regulatory sequence changes in evolution remains an open question (discussed in Meyer and Van de Peer,

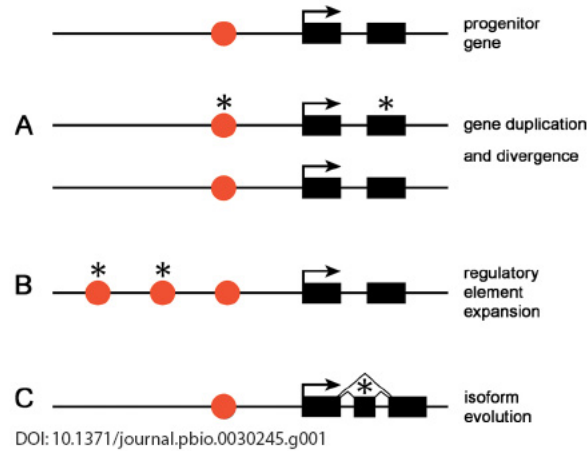


FIGURE 1.7: Different modes of gene evolution increase the diversity of gene function and minimise pleiotropy. The function of a progenitor gene with the simple structure of one *cis*-regulatory element (red circle) and a pair of exons (black rectangles) can be expanded and diversified in several ways. (A) Gene duplication followed by mutations (asterisks) in either coding or regulatory sequences of the initially identical paralogs will produce genes that may be expressed in different ways or proteins with distinct functions, while the original function can be maintained. (B) An expansion in the number of *cis*-regulatory elements by any of a number of means (transposition, rearrangement, duplication, point mutation) can expand the number of tissues in which the gene is active, but preserves the original function. (C) The evolution of a new exon and splicing sites creates the potential for alternative forms of a protein to be made. Mutations in alternative exons (asterisks) need not affect the original function of the protein. From Carroll (2005).

2005). However, there is a tendency to agree that the evolution of anatomy occurs primarily through changes in regulatory sequences (Carroll, 2005; Wray, 2007). In the following sections we will discuss in more detail the effects of gene duplication and regulatory sequence change in evolution.

### 1.2.1 Gene duplications and evolution

In his very influential book *Evolution by Gene Duplication*, published in 1970, Susumo Ohno has argued that “allelic mutations of already existing gene loci cannot account for major changes in evolution” and that gene duplication might be a more important mechanism in evolution than natural selection (“Natural selection merely modified, while redundancy created”). Gene redundancy would allow previously deleterious mutations to be retained in one of the duplicates, eventually leading to the acquisition of novel functions, while

the other duplicate would retain the original functions (Ohno, 1970). Ohno's ideas were confirmed by recent functional genomic analyses in *Drosophila* and yeast (Gu et al., 2004; reviewed in Li et al., 2005).

Ohno has stressed the role of duplication in the acquisition of novel functions (neofunctionalisation). However, the fate of duplicated genes can also be nonfunctionalisation (or gene loss, the most frequent event; Lynch and Conery, 2000) or subfunctionalisation (a split of the original functions). The role of these mechanisms and their combinations in speciation has been recently reviewed by Taylor et al. (2001). In the case of subfunctionalisation, versions of the Duplication – Degeneration – Complementation (DCC) model were proposed by Force et al. (1999), who emphasised the subfunctionalisation of regulatory elements of genes and by Hughes (1994), who emphasised protein sequence divergence (discussed at length in the PhD thesis of Aoife McLysaght, 2001). Curiously, the duplication of a developmentally regulated gene seems more likely to be advantageous than the duplication of a gene that is not involved in development (Gu et al., 2004).

It is important to mention, however, that some authors argue that duplication *per se* is not the major factor in evolution, and this for two main reasons: first, the estimated rate of gene duplication is about once per gene per 100 million years; and second, the relative infrequency of gene duplication is documented by the actual histories of key developmental regulatory gene families (Carroll, 2005; Meyer and Van de Peer, 2005).

**Gene/genome duplications** Duplication events in genomes can be divided into two broad classes: whole-genome duplications (WGD), in which the total chromosome complement of an organism is doubled, and segmental duplication, in which segments of a genome, or even single genes, are duplicated (Eichler and Sankoff, 2003). Genome duplications have been associated, albeit controversially, to major evolutionary transitions, including the evolution of multicellularity, bilateral symmetry and the evolution of craniates. In fact, larger genomes might facilitate the functional diversification of genes, lead to larger gene families and thereby permit more complex interactions and gene networks to evolve (reviewed in Meyer and Van de Peer, 2005; Taylor et al., 2001).

The distinction between genome and gene duplication, the latter being clearly the most common duplication event (Meyer and Van de Peer, 2005), can

emerge from the study of multigene families. The most well-studied example of duplication and gene loss are *Hox* genes (reviewed in Hoegg and Meyer, 2005), and the fragmentation, reduction, or expansion of *Hox* gene clusters in many animals was correlated with important morphological changes in evolution (Lemons and McGinnis, 2006). Several multigene families have been studied in relation to brain development and evolution, especially among homeobox genes (reviewed in Holland and Takahashi, 2005). Good examples are *Dlx* genes (Panganiban and Rubenstein, 2002) and *Emx* genes (Derobert et al., 2002b). In the latter case, the careful study of the representatives of this family in a chondrichthyan has led to the correct understanding of the orthology relationships of *Emx* genes in craniates.

Within this context, a keen interest has been raised about the outcome of sequencing projects, which will hopefully provide enough raw material to understand molecular evolutionary scenarios currently difficult to interpret. The field of phylogenomics is indeed in permanent expansion (Delsuc et al., 2005).

**WGD in deuterostomes** A popular hypothesis postulates that two rounds of whole-genome duplication (WGD) may have occurred in a gnathostome ancestor (2R hypothesis; Amores et al., 1998; Garcia-Fernandez and Holland, 1994; Holland et al., 1994; Ohno, 1970; Skrabanek and Wolfe, 1998). Two main arguments support this theory: the “one-to-four” rule and the observation that paralogous genes are clustered in a similar fashion. The “one-to-four” rule stemmed initially from the comparison of *Hox* gene clusters in craniate and non-craniate species (e.g. amphioxus). While all the studied non-craniates have a single *Hox* cluster, four *Hox* clusters are now observed in tetrapods (Fig. 1.8). Early in the evolution of deuterostomes, the ancestral possibly cephalochordate-like genome was converted to two (1R), after the first duplication, and then to four genomes after the second (2R) genome duplication. Further observations have found that clusters of other genes also remain linked, often even in the same gene order, on different chromosomes. Several authors agree that this synteny cannot not be easily explained by numerous individual gene duplication events, since those events would not maintain syntenic relationships across distantly related genomes (for reviews on WGD events in deuterostomes, see Hoegg and Meyer, 2005; McLysaght, 2001; Meyer and Van de Peer, 2005; Panopoulou and Poustka, 2005).

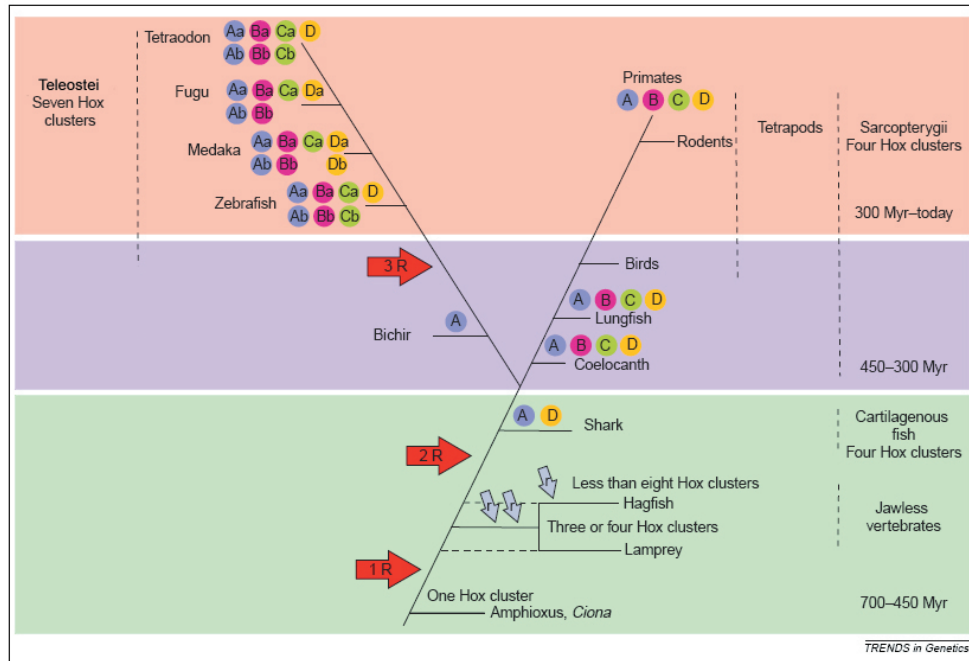


FIGURE 1.8: Whole-genome duplications in the craniate lineage, based on either complete genome analysis or *Hox* cluster number. Each circle is equivalent to a *Hox* cluster with each cluster coloured differently. The arrangement of circles does not represent the arrangement of clusters in the genome. Arrows indicate where WGDs have occurred. The only definitely proven WGD is the teleost fish-specific 3R WGD. Evidence for 1R or 2R WGDs is provided by numerous paralogs and by many quadruplicate regions in the human genome. Recent data from jawless craniates indicate that additional WGDs occurred after their divergence from the gnathostome (grey arrows). There is some controversy over the monophyly of jawless craniates (broken lines). The time windows given are estimates. Bichir (*Polypterus senegalus*) has one *HoxA* cluster, whereas teleosts have two, which have undergone 3R. The existence of one *HoxA* and one *HoxD* cluster (which implies that *HoxB* and *HoxC* should be present) in shark (*Heterodontus francisci*) places the 2R duplication before the emergence of cartilaginous fishes. Adapted from Panopoulou and Poustka (2005), where references of *Hox* gene cluster studies on each of these species can also be found.

While most authors accept the probable existence of one round of WGD in an ancestral gnathostome, controversy remains on the number of duplication events (1 R or 2R hypothesis). Some authors accept only one genome-doubling event, while others have taken a neutral position, arguing that current data neither support nor reject the 2R hypothesis (see McLysaght, 2001; Meyer and Van de Peer, 2005; Panopoulou and Poustka, 2005). More recent, less controversial, is the teleost “Fish-Specific Genome Duplication” (FSGD or 3R; reviewed in Meyer and Van de Peer, 2005) (see Fig. 1.8).

Relatively recent molecular analysis places both lamprey and hagfish in the group of Cyclostomes, the sister-group of Gnathostomes (jawed craniates) (see section 1.3.1 of this chapter for a discussion on the phylogenetic relationships of these groups). When considering the 2R hypothesis, opinions diverge; the most widely accepted scenario proposes that one of these duplications has occurred in the ancestor of all craniates, while a second one is gnathostome-specific. Additional duplications may have taken place within the cyclostome group. These ideas stem mainly from the study of *Hox* gene clusters in hagfish (Stadler et al., 2004) and lamprey (Force et al., 2002; Fried et al., 2003; Irvine et al., 2002; Sharman and Holland, 1998).

Lamprey has at least four *Hox* clusters (Force et al., 2002; Irvine et al., 2002). One study suggests that at least one *Hox*-cluster duplication occurred before the divergence of gnathostome and jawless craniates, whereas an independent cluster duplication occurred in the lamprey lineage, after it diverged from the gnathostome lineage (Force et al., 2002). Hagfish might have up to seven *Hox* clusters (Stadler et al., 2004). Two of them are homologous to mammalian *Hox* clusters, which also supports the hypothesis that at least one *Hox*-cluster duplication occurred in the ancestor of gnathostomes and cyclostomes. According to the authors, their results also suggest that additional independent *Hox* cluster/gene duplication events have occurred in the hagfish lineage. The two *Hox* clusters isolated so far from cartilaginous fish are homologous to the mammalian *HoxA* and *HoxD* (Prohaska et al., 2004), placing the second “2R” duplication before the divergence of cartilaginous fishes (Fig. 1.8; see Panopoulou and Poustka, 2005).

### 1.2.2 Regulatory sequences and evolution

There is an association between gain, loss or modification of morphological traits and changes in gene regulation during development (reviewed in Carroll, 2005; Wray, 2007). Ohno proposed that the duplication of regulatory genes and their control regions must have contributed greatly to the evolution of craniates (Ohno, 1970). But his book focused exclusively on the evolution of new proteins and did not consider the potential of non-coding, regulatory sequences in evolutionary diversification. Today, many authors agree that the evolution of anatomy occurs primarily through changes in regulatory sequences. Four aspects have been invoked to support this statement: (i) empirical studies of the evolution of traits and of gene regulation in development, (ii) the rate of gene duplication and the specific histories of important developmental gene families, (iii) the fact that regulatory proteins are the most slowly evolving of all classes of proteins, and (iv) theoretical considerations concerning the pleiotropy of mutations (Carroll, 2005).

The tissue-specific and temporal control of gene expression, particularly of genes encoding the regulatory proteins that shape pattern formation and cell differentiation in animals, is typically governed by arrays of discrete regulatory elements, in regions that flank coding regions (5' or 3') and lie within introns. These elements are under higher selective pressure than the neighbouring regions, and for this reason they have been highly conserved through evolution (Dickmeis and Muller, 2005). Conserved non-coding, potentially regulatory, regions have been termed Conserved Non-coding Elements (CNEs), or Conserved Regulatory Elements (CREs) when its regulatory function has been demonstrated.

**Characteristics of CNEs** CNEs were thought to be a typical craniate characteristic, but have been recently detected also in flies and worms (Vavouri et al., 2007). These regulatory elements have been found preferentially associated to transcription factors or genes involved in development (*trans-dev* genes; Bejerano et al., 2004). They are under a strong selective constraint, which contributes to the maintenance of synteny, as there are sometimes elements regulating multiple genes or elements within other genes. We can thus expect genomic regions rich in *trans-dev* genes to be more conserved than other regions in the genome. The relationship between long range enhancers and the



maintenance of synteny has been supported by recent genomic comparative studies (e.g. Goode et al., 2005). The maximum known distance between a regulatory element and a gene is 1.2 Mb upstream from the transcription unit (*FOXC1*, Davies et al., 1999; reviewed in Kleinjan and van Heyningen, 2005). Another notable example of a very long range enhancer is the one that controls *Shh* gene expression during limb development (1 Mb from the transcription start, Lettice et al., 2003).

CNE-rich genomic regions are often poor in transposable elements and RNA genes, and the genes are sparsely distributed. For this reason, and due to their evolutionary conservation, they have been called **stable gene deserts** (reviewed in Ovcharenko et al., 2005).

**CNE detection and use in phylogeny** The conservation of regulatory CNEs, which can be as small as 6 bp long, is probably due to their high specificity to bind a particular transcription factor. However, they are also (as we have seen in the previous section) prone to be lost or modified after gene duplication, leading to subfunctionalisation or neofunctionalisation. The study of CNEs may be an important source of information for understanding the role of these processes in evolution.

Phylogenetic footprinting<sup>5</sup> has been used to identify CNEs by comparing homologous sequences from different species (Fig. 1.9). In *Hox* clusters, for example, novel CNEs have been detected by phylogenetic footprinting. *Hox* clusters are compact, which makes it easier to make interspecies comparisons based on alignment, being this is another reason for their establishment as a model for craniate genome evolution (reviewed in Hoegg and Meyer, 2005). Screens of highly conserved elements have also been performed in large genomic regions, including around the *Shh* gene (Goode et al., 2003, 2005; Woolfe et al., 2005). Certain types of conserved elements were used to estimate evolutionary rates, while duplicated CNEs have been employed to study the genomic radius of long-range enhancer action (Vavouri et al., 2006).

It is worth to note, however, that not all regulatory sequences are evolutionarily conserved. For example, *cis*-acting sequences around the *RET* gene, conserved in mammals but not in fish, are able to reproduce patterns of *RET*

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<sup>5</sup>Phylogenetic footprints are short blocks of non-coding DNA sequences ( $\geq 6$  bp) which are conserved in taxa that have an additive evolutionary time of at least 250 million years (Hoegg and Meyer, 2005).

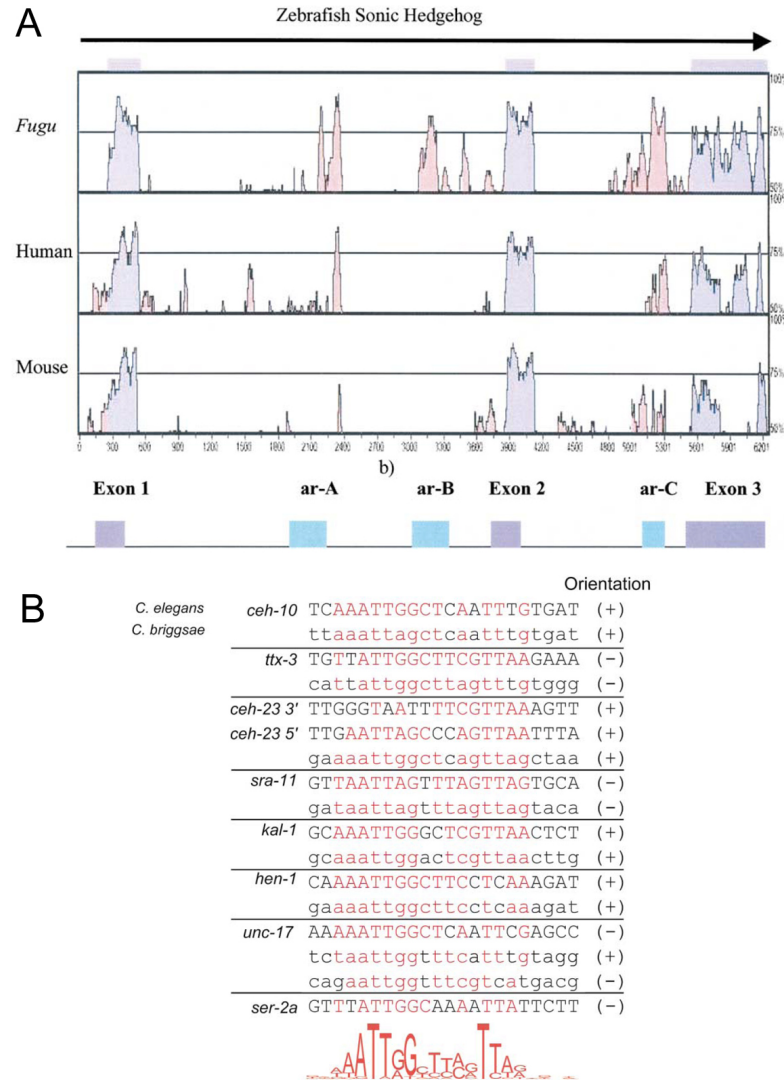


FIGURE 1.9: Phylogenetic footprinting. (A) Vista plots of the *Shh* gene, using zebrafish as the base sequence for a comparison with *Fugu*, human and mouse. This plot shows the location of two main regions of conserved non-coding sequence between all species, and also identifies another conserved region in zebrafish and *Fugu*. The diagram below the plots represents the positions of the exons and the location of the activator regions (ar) found by Muller et al. (1999). From Goode et al. (2003). (B) The AIY cis-Regulatory Motif, identified by multispecies sequence comparison in nematodes. Capital letters correspond to *C. elegans* sequences, lower case to *C. briggsae* sequences. The sequence alignment defines a position weight matrix (PWM) that is represented by a sequence logo (Schneider and Stephens, 1990). From Wenick and Hobert (2004).

expression in zebrafish embryos (Fisher et al., 2006; reviewed in Elgar, 2006). Other methods than sequence similarity can be employed for the detection of regulatory sequences sharing the same role (reviewed in Dickmeis and Muller, 2005). New, more performant, software is being constantly developed, which could help to resolve the problem of detecting cryptic CNEs (e.g. Tracker, Prohaska et al., 2004).

Other puzzling finding was that some very conserved CNEs were found to have quite divergent functions. This is the case of the central nervous system *Shh* enhancers, which drive the expression of the gene in different regions of mouse and zebrafish brain and spinal cord (Epstein et al., 1999; Ertzer et al., 2006; Jeong and Epstein, 2003; Jeong et al., 2006; Muller et al., 1999), and which will be discussed in more detail later in this chapter.

These studies taken together show that the conservation of sequence and function of regulatory elements are not always correlated. The frequency and evolutionary implications of these disparities are still largely unknown, but the subject remains a promising research domain.

**Evolutionary significance of CNEs** There are numerous examples of the direct consequences of evolution at a particular gene locus in the gain, loss, or modification of morphological traits, whether that evolution is associated to a duplication event or not. Known examples are the trichome, bristle and pigmentation pattern in fruit flies; the flower coloration, architecture and branch patterns in plants; limb and axial diversity in gnathostomes; and dietary changes during human evolution (reviewed in Carroll, 2005; Wray, 2007).

Fig. 1.10 illustrates two examples of subtle differences in gene regulation among different populations or species of insects and craniates. Expression of the *yellow* pigmentation gene of *Drosophila* is controlled by several different *cis*-regulatory elements. Differences in the activity of selected elements underlie differences in pigment patterns between species (Fig. 1.10 A; Gompel et al., 2005). Similarly, the expression of the *Pitx1* gene of gnathostomes is inferred to be controlled by multiple elements. In pelvic-reduced freshwater stickleback fish, *Pitx1* expression is absent from the pelvic region. This was suggested to occur due to a selective loss of activity of the hindlimb regulatory element (Fig. 1.10 B; Shapiro et al., 2004).

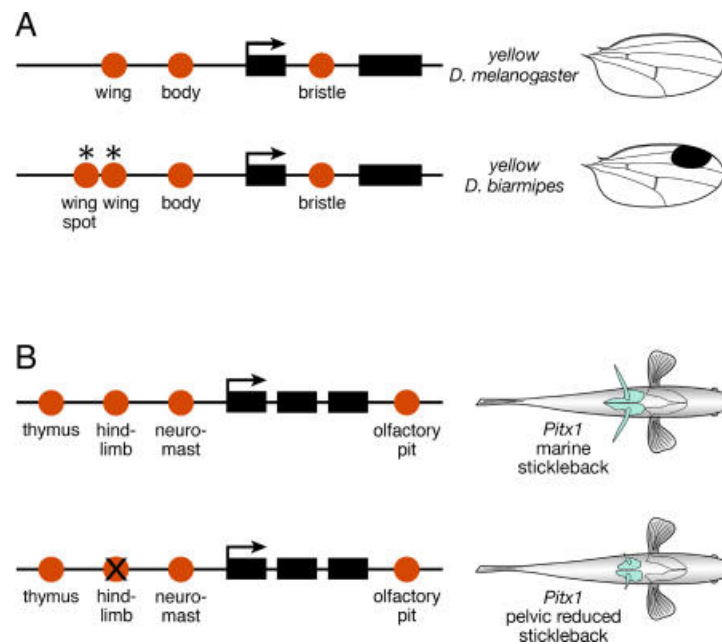


FIGURE 1.10: Differences of gene regulation among two different populations or species. (A) Regulation of *yellow* and pigmentation pattern differences in *Drosophila*. (B) Loss of *Pitx1* hindlimb enhancer and pelvic reduction in stickleback fish. Adapted from Carroll (2005).

**Evolution of gene regulatory networks** Development of the animal body plan is controlled by large gene regulatory networks, and hence evolution of body plans must depend upon changes in the architecture of these networks (Davidson and Erwin, 2006; Vavouri et al., 2007). The relationship between gene duplication and/or evolution of regulatory regions and the evolution of gene networks is still poorly known (Li et al., 2005). Studies in *E. coli* and *S. cerevisiae* indicate a role of gene duplication in the growth of gene networks (Teichmann and Babu, 2004). However, the effect of duplication in genes that occupy different positions in the network (Li et al., 2005), and the potential role of duplication in the co-option of gene networks is largely unknown. Co-option has been an important factor in evolution (reviewed in Raff and Raff, 2000; True and Carroll, 2002). In many cases, gene networks have been co-opted to previously non-existing organs or processes: in some transgenesis experiments where the activity of a particular enhancer was tested in a different species, it was observed that the enhancer was capable of driving the gene's expression in structures which are absent from the animal to which the enhancer originally belonged.

### 1.2.3 *Hedgehog* genes: a case study

The structure (number of exons, exon-intron splice sites) and the coding sequence of *Hedgehog* genes has been highly conserved during evolution. The phylogenetic relationships of Hedgehog proteins are represented in Fig. 1.11. Within chordates, only one representative from this multigene family has been identified so far in amphioxus (Shimeld, 1999) and lamprey (Uchida et al., 2003; Osorio et al., 2005). There are two *Hedgehog* genes in ascidians, arising from an independent duplication in the ascidian lineage (Takatori et al., 2002, *Ci-hh2* expression is represented in Fig. 3.1). In osteichthyans, there are three families of *Hedgehog* genes, named *Sonic*, *Desert* and *Indian*, and this is probably the situation also in chondrichthyans. It is worth noting that a specific genome duplication has occurred in the teleost ancestor, being at the origin of the double number of *Hedgehog* genes found in this group (Fig. 1.11).

In zebrafish (Ertzer et al., 2006; Muller et al., 1999) and mouse (Epstein et al., 1999; Jeong and Epstein, 2003; Jeong et al., 2006), functional studies have shown that different conserved non-coding elements (CNEs) drive *Shh* expression in specific regions of the central nervous system. Some of these CNEs are present in all jawed craniates studied so far (puffer fish, zebrafish, mouse, human) (Goode et al., 2003, 2005), while others are teleost-specific (e.g. intronic enhancer ArB in zebrafish and puffer fish; Goode et al., 2003). There are other *Shh* intronic enhancers (e.g. mouse SBE1) which may be mammalian-specific (reviewed in Dickmeis and Muller, 2005, see also Fig. 1.13 and Fig. 1.12).

In the mouse CNS, The elements SBE1, SBE2, SBE3 and SBE4 (SBE for Sonic Brain Enhancer, each about 500–1000 bp long) drive *Shh* expression in the forebrain (Jeong et al., 2006). SBE1, located in the second intron, controls expression in the mesencephalon and ZLI. SBE2, SBE3 and SBE4, located in the 5' UTR, control expression in the ventral diencephalon and telencephalon. SFPE1 and SFPE2 control floor plate expression. The most distant of the mouse enhancers, SBE3, is at more than 400 bp from the transcription start, but the distance of these elements varies greatly between species and is probably an important factor in transcriptional control (Jeong et al., 2006, see Fig. 1.12). The detailed study of the zebrafish *Shh* enhancers by Ertzer et al. (2006) shows an intriguing structural but not functional conservation of enhancer sequences when compared to the mouse (Fig. 1.13). Clearly, much more research

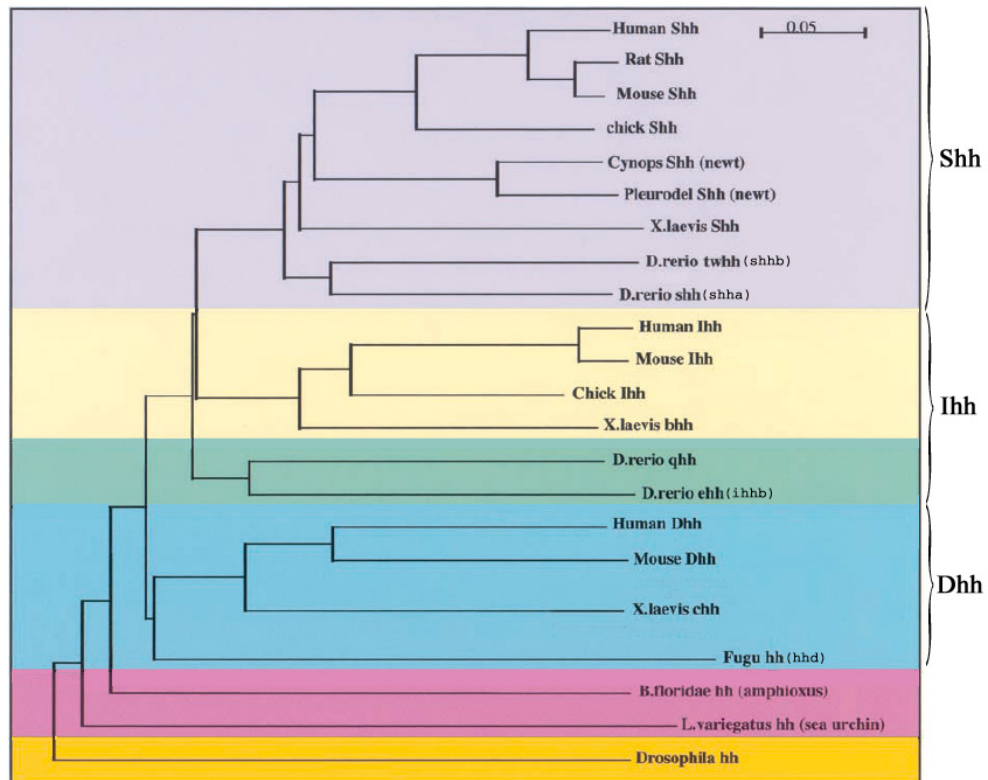


FIGURE 1.11: Phylogenetic relationship of members of the Hh protein family from different craniate species, cephalochordates and *Drosophila* (adapted from Borycki 2001). Amniote Hhs fall into three distinct subgroups: Sonic (lilac), Indian (yellow), and Desert (blue). The zebrafish Ehh (Currie and Ingham 1996) and Qhh (T. Qiao and P.W. Ingham, unpubl.) are divergent members of the Indian subgroup (green). Figure adapted from Ingham and McMahon (2001). After this tree was published, a *D. rerio* homologue of *F. rubripes* hhd (Dre dhh) has been isolated and characterised (Avaron et al., 2006). There are two groups of Actynopterygian *Indian Hedgehog* genes. The qhh sequence indicated in the tree was never published to this date, but might correspond to the Dre ihha isolated by Avaron and collaborators (Avaron et al., 2006). *Fugu rubripes* homologues of *Danio rerio* shha (Fru hhc), ihhb (Fru hhb), and ihha (Fru hha), have also been identified (Gellner and Brenner, 1999; Avaron et al., 2006).

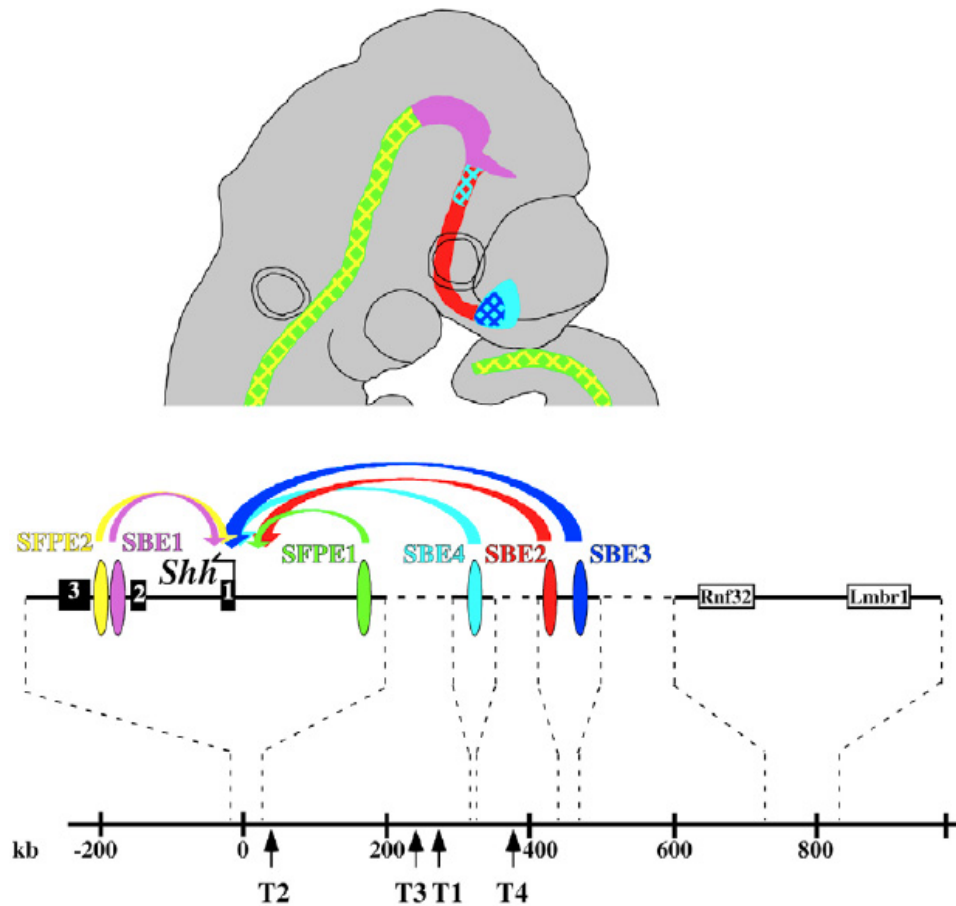


FIGURE 1.12: *Shh* expression in the CNS is controlled by multiple regulatory modules. Schematic view of *Shh* expression in the CNS, colour-coded to depict the distinct regulatory elements governing *Shh* transcription along the AP axis of the mouse neural tube. Hatched patterns in the floor plate of the spinal cord and hindbrain, in the p3 domain of the diencephalon and in the subventricular zone of the telencephalon represent the sites of *Shh* expression regulated by more than one enhancer. Solid patterns in the ventral midbrain, diencephalon and telencephalon represent sites of *Shh* expression controlled by single regulatory elements. The location of the six CNS enhancers with respect to the *Shh* transcription start site is also indicated. From Jeong et al. (2006).

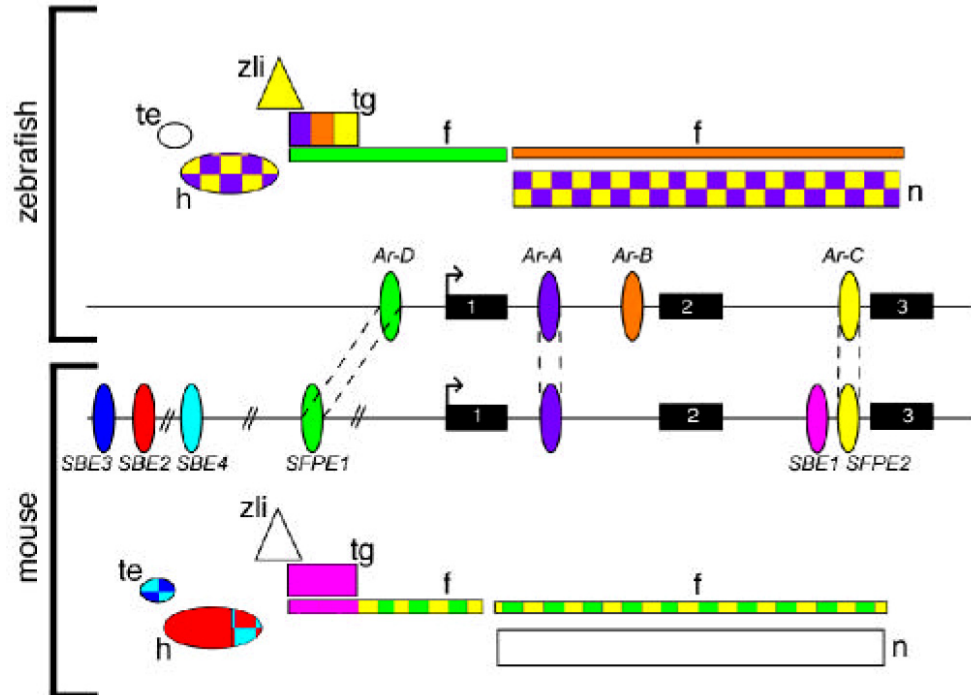


FIGURE 1.13: Comparison of the *shh* enhancer regions driving expression in the central nervous system and the notochord in zebrafish and mouse. Zebrafish *shh* enhancers ar-A, ar-D, and ar-C share sequence similarity with regions in the *Shh* locus of mouse, the latter two being named SFPE1 and SFPE2 in the mouse, respectively. While the SFPE1/ar-D regions share functional properties by mediating both floor plate expression, the SFPE2/ar-C enhancers show differing activities in zebrafish and mouse, giving mostly floor plate in mouse (Epstein et al., 1999) and mostly notochord in zebrafish (Muller et al., 1999). Moreover, the ar-C activity in the ZLI and hypothalamus of the zebrafish embryo is not mediated by SFPE2 of mouse *Shh*. Rather four distinct sequences (SBE1 to -4) direct reporter gene expression in the diencephalon of the mouse (Epstein et al., 1999; Jeong et al., 2006). While all three enhancer regions ar-A, -B, -C can have activity in the tegmentum of the zebrafish midbrain, a distinct enhancer region in intron 2 (SBE1) mediates expression in the mouse midbrain. Moreover, Epstein and co-workers did not detect reporter activity in the mouse *shh* intron 1 despite the fact that ar-A is strongly conserved and mediates notochord expression in zebrafish. f: floor plate, h: hypothalamus, n: notochord, tg: tegmentum, zli: zona limitans intrathalamica, te: telencephalon. From Ertzer et al. (2006).



will be needed before the mechanisms of this very complex regulation are fully understood.

## 1.3 The lamprey in evolutionary studies

### 1.3.1 Phylogeny

The phylum Chordata includes three major taxa, the Cephalochordates (e.g. the amphioxus), the Urochordates (e.g. the ascidians) and the Craniates. Until recently, the Cephalochordates were considered the closest relatives of Craniates, but recent studies have provided strong support for the existence of a monophyletic group composed by the Urochordates and the Craniates (Delsuc et al., 2006). In Fig. 1.14 a simplified phylogenetic tree of the deuterostomes is shown. Important known evolutionary novelties within the different groups are indicated. However, many characteristics thought, at a given moment, to be exclusive of a certain group were later detected in other groups. Two examples are the cranial motoneurons (Dufour et al., 2006) and neural crest-like cells (Jeffery et al., 2004), believed to be craniate novelties and now detected also in ascidians.

Although, as we will see, similarities can be found in the patterning of the anterior neural tube between Cephalochordates, Urochordates and Craniates, the question of the emergence of the Craniate brain and its evolution within the clade cannot be tackled without including in the study all of the main Craniate taxa. The great majority of the so called “model species” among Craniates belong to the Osteichthyans, a subgroup of Gnathostomes which includes ray-finned fishes and tetrapods (amphibians, diapsids, mammals, birds). Two comparatively less studied key groups among the Craniates are the Chondrichthyans (cartilaginous fishes), which are the sister-group of the Osteichthyans, and the Cyclostomes (lampreys and hagfish) (Fig. 1.14)<sup>6</sup>.

Until very recently, hagfish embryos were impossible to obtain, posing obvious problems to study embryonic development in this group. From the end of the 19<sup>th</sup> century, where three independent researchers had procured and studied some hagfish embryos, only three fertilised eggs were obtained during the whole 20<sup>th</sup> century, the latest in 1969. It was only in 2007 that the feat

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<sup>6</sup>The majority of this thesis discusses studies undertaken in lampreys (cyclostomes). A section of chapter 3 describes a study in the dogfish (a chondrichthyan).

was repeated (Ota et al., 2007). For a comprehensive review of the history of hagfish embryology, see Ota and Kuratani (2006). For more than 100 years, lampreys have thus remained the only non-gnathostome craniate species where developmental studies were feasible.

The correct assessment of the phylogenetic relationships between the three groups of craniates — lampreys, hagfish and gnathostomes — is still a matter of debate. The opinions diverge towards two possible evolutionary scenarios, schematised in Fig. 1.14. In the first scenario, the ancestral jawless (agnathan) craniate would have given rise to the sister-groups of gnathostomes (jawed craniates) and to cyclostomes (the name means “round-mouth”), the latter including the extant groups of lampreys (Hyperoartia, Petromyzontidea) and hagfish (Hyperotreti, Myxinoidea) and many fossil groups (Forey and Janvier, 1993). In the second hypothesis, based mainly on morphological and palaeontological studies, lampreys and gnathostomes would be more closely related, and therefore would both belong to the group Vertebrata, being the hagfish an outgroup. Molecular analysis of mitochondrial, nuclear ribosomal RNA genes and nuclear protein-coding genes (Delarbre et al., 2000, 2002; Kuraku et al., 1999; Kuraku and Kuratani, 2006; Lee and Kocher, 1995; Mallatt and Sullivan, 1998; Stock and Whitt, 1992) have strengthened the first hypothesis, which for that reason will be the one applied throughout this thesis. The difficulty of resolving the phylogenetic relationships among Craniates is related to the long evolutionary age of the split between Cyclostomes and Gnathostomes (about 500 Mya, Kuraku and Kuratani, 2006), and to the fact that hagfish and lamprey lineages have diverged shortly after that split (470–390 Mya, Kuraku and Kuratani, 2006).

The successive remodellings of the craniate phylogenetic tree have given origin to different choices of nomenclature, which may cause some problems when studying this subject. Being the lack of jaws an ancestral character, it cannot be used *per se* to define a monophyletic group, and some authors object to the use of the term “agnathan” (however, the same might also hold true for the term “cyclostome”). Another source of confusion is the name “vertebrate”, adequate to describe both gnathostomes and lampreys, and with the advantage of its widespread use in common and scientific language, but which may seem awkward when discussing hagfish. To avoid confusion with the theory of the vertebrates as a monophyletic group including only lampreys and gnathostomes, the terms “cyclostomes” and “craniates” will be consistently

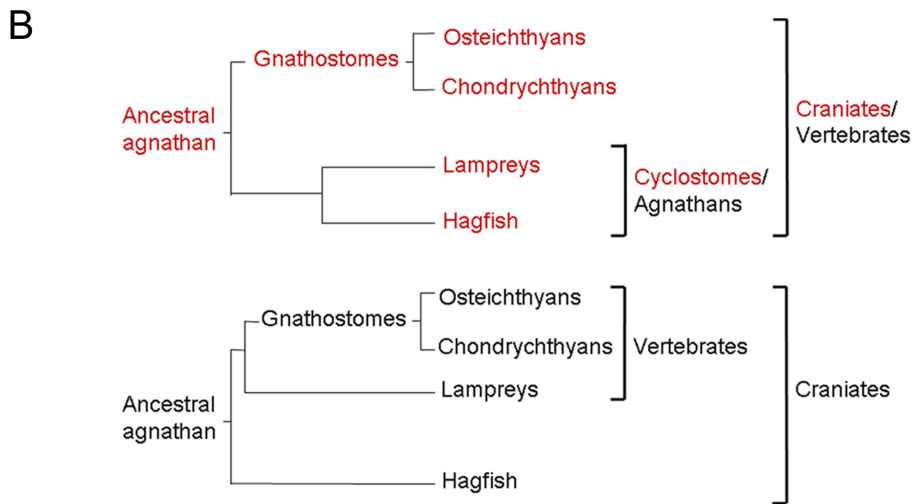
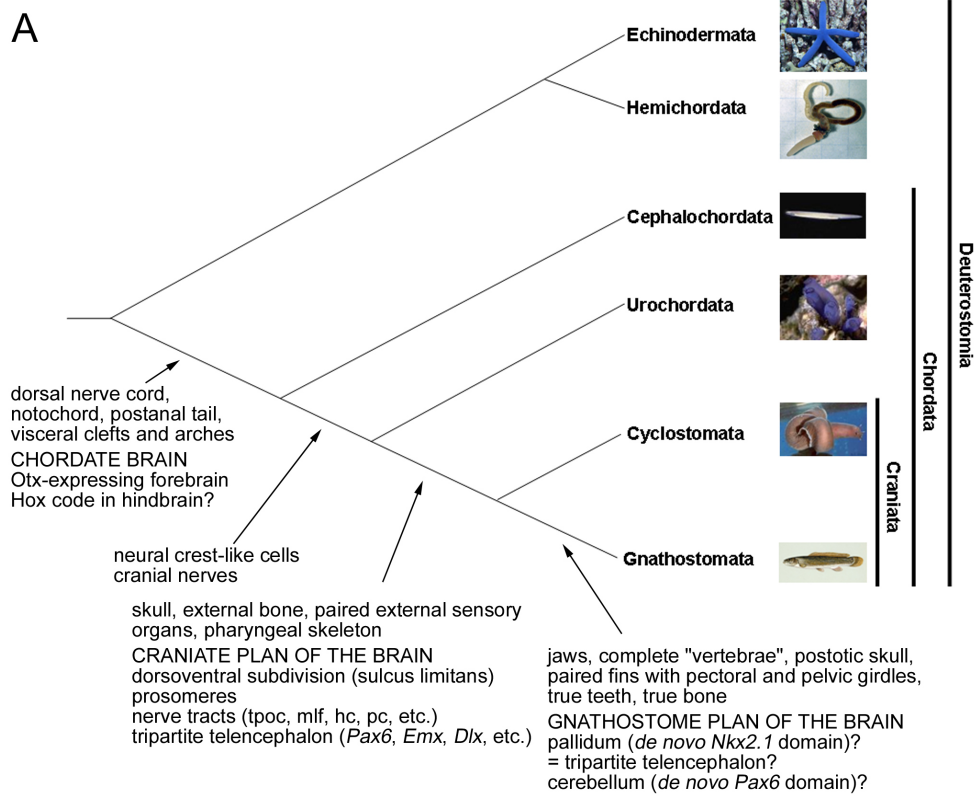


FIGURE 1.14: The lamprey phylogenetic position. (A) Chordate phylogenetic tree where important evolutionary novelties are indicated. Adapted from Dufour et al. (2006); Gans and Northcutt (1983); Forey and Janvier (1993); Jeffery et al. (2004); Murakami et al. (2001, 2005); Neidert et al. (2001). Photos from the Tree of Life web project ([www.tolweb.org/tree/](http://www.tolweb.org/tree/)). (B) Debate on lamprey phylogeny: two alternative hypothesis on the evolution of craniates, where the terms used in this thesis are indicated in red.

used throughout this text.

As referred in the precedent section, one or two events of whole genome duplication (1R or 2R hypothesis) are thought to have taken place in an ancestor of all gnathostomes. At least one of these events may have taken place prior to the cyclostome/gnathostome split. One lamprey (*Petromyzon marinus*) sequenced genome (International Sequencing Consortium, [www.intlgenome.org](http://www.intlgenome.org)) is currently being assembled. The analysis of hagfish and lamprey genomes is of primary importance to resolve the issue of the number and position in evolution of these duplications. This analysis will also provide information on the fate of coding and regulatory regions after gene duplication which can be related to morphological changes within the craniate taxon. In particular, it will hopefully shed light into the genetic mechanisms underlying the generation of novelties in the development of the nervous system, especially those related to the patterning of the anteriormost neural tube region, where striking differences are evident when comparing Cephalochordates, Urochordates and Craniates.

### 1.3.2 Ecology and embryology

The multi-volume book *The Biology of Lampreys* (Hardisty and Potter, 1971a), which remains to this day an important reference on lamprey studies, includes chapters on lamprey ecology, embryology and neurophysiology. Lampreys are aquatic animals, with eel-like bodies. There are about 40 species of lampreys, living exclusively in the temperate zones of both hemispheres. Many, but not all, adult lampreys are predacious, using their round sucking mouth to attach to the bodies of fish. Then, they rasp the tissue with a tongue-like structure to open a wound through which they can suck the blood and tissue fragments from their prey. Predacious lamprey species are typically anadromous, with a fresh water larval stage, where they are filter feeders, and a salt water post-metamorphic stage. However, species like the brook lamprey (*Lampetra planeri*) never prey upon fish, reproducing and dying in fresh water shortly after metamorphosis (see Hardisty and Potter, 1971b; Nieuwenhuys and Nicholson, 1998, for more detailed descriptions).

The typical life cycle of anadromous lampreys is shown in Fig. 1.15. During the spawning season, adult lampreys migrate to shallow-water streams, build a nest using their sucking mouths, reproduce and die. The eggs develop into

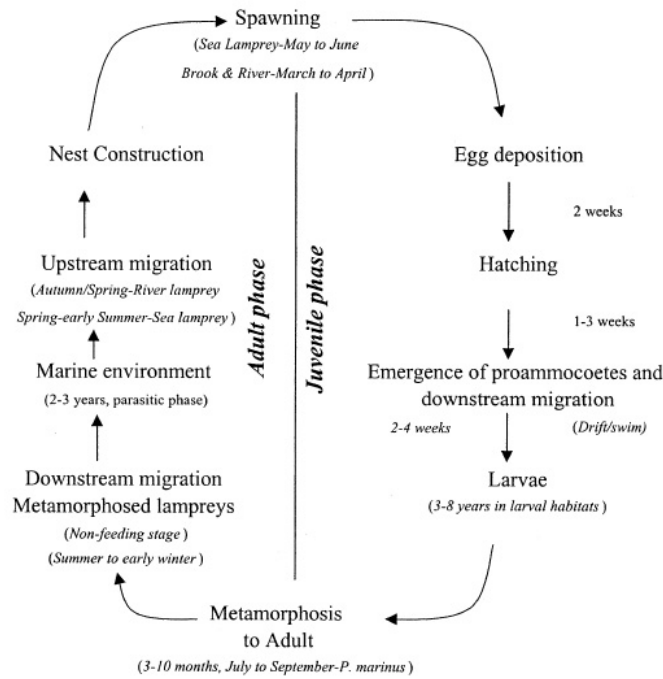


FIGURE 1.15: General life cycle of anadromous lampreys. From Kelly and King (2001).

larvae, which are so different from the adults that they were once seen as a separate species, *Ammocoetes branchialis*, and lamprey larvae are sometimes still called ammocoetes for this reason. After an initial “prolarval” stage, where they absorb the yolk, they become filter feeding larvae. The larval period is very long, usually not less than five years, and may last up to 18 years (Beamish and Potter, 1975). After metamorphosis, the adult period starts, and may last for one to four years.

As the embryonic development of the lamprey is relatively long, most researchers use either the developmental table of Piavis (1971) or that of Tahara (1988), built for *Petromyzon marinus* and for *Lampetra reissneri*, respectively, to stage embryos of other species. Piavis divided the developing lampreys into pre-hatching embryos and post-hatching prolarvae. In this work, we have used Tahara’s table, where the stages are numbered from fertilisation on, considering that the “embryonic-type” development ends only when the animals have consumed the yolk and start to feed. Piavis prolarval stage 1 (hatching) corresponds to Tahara stage 24. A classical detailed histological description of lamprey embryonic development is the beautiful atlas drawn by H. Damas

(Damas, 1944, see Fig. 1.2 D for an example).

### 1.3.3 Genome organisation

The size of lamprey genomes varies from 40% to 70% of the human genome size, according to the species and the method of analysis used. Hagfish genomes tend to be larger, from 65% to 130% of the human genome size (Animal Genome Size Database, [www.genomesize.com](http://www.genomesize.com)). This gives some practical advantage to the choice of *Petromyzon marinus* as the first cyclostome genome to be sequenced<sup>7</sup>. However, the genome organisation may be quite different between lampreys and gnathostomes: lamprey genomes, contrarily to hagfish genomes, have a much higher GC content (Kuraku and Kuratani, 2006), and some evidence shows that the introns may be in general much larger (Marc Ekker's laboratory, personal communication), which increases the difficulty of genomic and genetic studies.

### 1.3.4 Lampreys and gnathostomes, similarities and differences

Lampreys are unique in having a single median dorsal "nostril" (the nasohypophyseal opening) in the head. Their skin is naked and slimy, and they have seven gill openings extending behind the eyes. The sucker which surrounds the mouth is strengthened by a ring-shaped annular cartilage and bears numerous horny denticles. The eyes possess a lens, but no intrinsic eye muscles for accommodation. The extrinsic eye muscles are as in extant gnathostomes, except for the superior oblique muscle, which is attached posteriorly in the orbit, instead of anteriorly. The labyrinth has two vertical semicircular canals, a blind endolymphatic duct, and a number of large ciliated sacs which play a role in equilibrium (Philippe Janvier, Tree of Life web project). Lampreys have dorsal and caudal unpaired fins, which are strengthened by numerous, thin cartilaginous radials associated with radial muscles. The brain has a very poorly developed cerebellum but large optic lobes. The spinal cord is flattened, almost ribbon-shaped, yet thicker than that of hagfish (*idem*). The organisation of the brain in the embryonic and adult lamprey will be the subject of the last section of this chapter. In this section, I have chosen to mention two examples which reflect the particular phylogenetic position of cyclostomes and its

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<sup>7</sup>The preliminary assembly of the *P. marinus* genome is already available at Pre!Ensembl ([www.pre.ensembl.org/Petromyzon\\_marinus/index.html](http://www.pre.ensembl.org/Petromyzon_marinus/index.html)). It consists principally in a series of small, unlinked contigs.

interest in molecular evolutionary studies. The first of these examples shows how certain genetic mechanisms involved in the formation of skeletal tissues may be more conserved than previously thought; the second is a study on the immune system of cyclostomes which revealed how very different immunity strategies were generated through the course of evolution.

Lampreys are devoid of a mineralised skeleton, although traces of globular calcified cartilage may occur in the endoskeleton. The skull of lampreys is, like that of hagfish, made up of cartilaginous plates and bars, but it is more complex and includes a true cartilaginous braincase. The gills, although enclosed in muscularised pouches in the adult, are supported by unjointed gill arches, which form a “branchial basket”. The gill arches lie externally to the gill filaments and associated blood vessels. Lampreys possess, like hagfish, a very large notochord but, in addition, there are small cartilaginous dorsal arcualia (basidorsals and interdorsals) (P. Janvier, Tree of Life). It was previously thought that one of the major differences involved in the evolution of the skeleton among craniates was the presence of a collagenous extracellular matrix in the cartilage of gnathostomes, and its absence in cyclostomes (Wright et al., 2001). The cartilage and bone that form the skeleton of gnathostomes contain high levels of type II and type I collagen, while the cartilaginous skeletons of lampreys and hagfish would be noncollagenous, containing instead the elastin-like proteins lamprin and myxinin (Wright et al., 2001). Non-collagenous cartilages would be also present in the cephalochordates (amphioxus) and in many other non-chordate groups, although collagen-containing cartilages were also found among these groups. However, recent results have shown that type II collagen is present in lamprey (Zhang et al., 2006) and hagfish (Zhang and Cohn, 2006) cartilage, indicating that type II collagen-based cartilage evolved earlier than previously recognised. These authors have also shown that amphioxus possesses an ancestral clade A fibrillar collagen (*ColA*) gene that is expressed in the notochord. Their results suggest that the duplication and diversification of *ColA* genes at the chordate-craniate transition may underlie the evolutionary origin of craniate skeletal tissues.

An example that highlights the evolution of different adaptive defence mechanisms among craniates is a study on the lamprey and hagfish immune systems. It was recently discovered that hagfish and lamprey, which do not have immunoglobulins, TCR or MHC genes, have unique forms of anticipatory immunity, remarkably different from the recombination-activating gene(RAG)-

mediated adaptive immunity of gnathostomes (for a review on the mechanisms of immunity across animals, see Litman et al., 2005). Lampreys (Pancer et al., 2004) and hagfish (Pancer et al., 2005), have variable lymphocyte receptors (VLR) composed of leucine-rich repeats, which are generated by somatic rearrangement. It is likely that the cyclostome VLR receptors derive from an ancestral gene, which was later duplicated in the hagfish lineage (Pancer et al., 2005). VLR rearrangements, as RAG-mediated rearrangements of gnathostome antibody genes, lead to a very high number of possible different combinations; in lamprey,  $10^{13}$  unique receptors may be generated (Alder et al., 2005). It was demonstrated that lamprey lymphocytes are clonally selected upon antigen stimulation (Alder et al., 2005). These results show that two remarkably different forms of antigen recognition have evolved independently in the two craniate sister-groups.

### 1.3.5 The lamprey as an evo-devo model

The study of lampreys has started to attract the attention of evo-devo biologists more than a decade ago, and the promises offered by the lamprey as an evo-devo model were the subject of a review by Kuratani et al. (2002). Gene expression, immunocytochemical and sequence analysis are frequently performed techniques, while more functional studies are now at their beginnings<sup>8</sup>. This difficult start has been mainly due to the limited period in which live lamprey embryos are available, only a few weeks per year (although different species reproduce at different times). While an exhaustive list of the major contributions to the field is beyond the scope of this thesis, a few examples of studies where promising technical advances were made will be mentioned.

**Jaws** The question of the emergence of the gnathostome jaw is closely associated to the acquisition of a new feeding behaviour, and to the presence of novel features related to the perception of and response to environmental stimuli. As the jaw is one of the most obvious external morphological differences between cyclostomes and gnathostomes, it was one of the first characteristics to be investigated by evo-devo biologists. A large number of anatomical and gene expression studies (Cohn, 2002; Horigome et al., 1999; Kuratani et al., 2002; Kuratani, 2005; McCauley and Bronner-Fraser, 2003; Neidert et al., 2001;

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<sup>8</sup>An attempt to modify gene expression by injecting the mouse Shh protein in the lamprey brain is described in chapter 3.



Ogasawara et al., 2000; Shigetani et al., 2002), have provided insight into the mechanisms of tissue patterning, cell specification and migration and morphogenetic movements underlying the formation of the lamprey head and jaw (reviewed in Shigetani et al., 2005). One of the main theories that have been put through to explain the emergence of the jaw is the occurrence of a **heterotopic epithelial-mesenchymal shift** in the head of the gnathostome ancestor. In gnathostomes the jaw originates from the mandibular arch. Importantly, genes involved in late specification of the mandibular arch are expressed with similar patterns in the oral regions of chick and lamprey embryos. However, morphological comparisons indicate that apparently orthologous homeobox genes are expressed in different subdivisions of the ectomesenchyme in the two species. Therefore, the homology and gene expression of the oral region are uncoupled during the transition from agnathan to gnathostome. The authors conclude that a heterotopic shift of tissue interaction was involved in the evolution of the jaw (Shigetani et al., 2002). This study was also the first to report ectopic gene expression in the lamprey by application of protein-coated beads. In this experiment, FGF8 and BMP4 impregnated beads were applied to the lamprey embryo, inducing the ectopic up-regulation of putative target genes, *Dlx1/6* and *MsxA* (Shigetani et al., 2002).

**Neural crest** Isolation, expression and phylogenetic analysis of lamprey homologues of known neural crest markers (Horigome et al., 1999; Kuratani et al., 2004; McCauley and Bronner-Fraser, 2002, 2003, 2004, 2006; Meulemans and Bronner-Fraser, 2002; Meulemans et al., 2003; Morikawa et al., 2001; Neidert et al., 2001; Ogasawara et al., 2000; Tomsa and Langeland, 1999) has revealed a conservation of the general mechanisms of neural crest cell specification and migration in craniates. However, assessment of the expression of more neural crest cell markers would be necessary to know the extent of conservation of the neural crest regulatory network present in gnathostomes (reviewed in Sauka-Spengler and Bronner-Fraser, 2006). A recent study on the *SoxE* gene represents an important technical advance, as it was the first to report a successful experiment of morpholino oligonucleotide injection in lamprey embryos. The *SoxE* gene is expressed in the neural crest cells which invade branchial arches in a similar way in both lamprey and gnathostomes (McCauley and Bronner-Fraser, 2006). This observation raises the possibility that one or more *SoxE* genes might have an ancestral role in neural crest and pharyngeal arch

development. To address this question, the authors have injected two different morpholino antisense oligonucleotides to disrupt *SoxE1* translation. By stage 26, knock-down of SoxE1 protein led to profound defects in the pharyngeal arches, similar to effects reported in *Xenopus* (Spokony et al., 2002), which supports the homology of pharyngeal elements between lamprey and gnathostomes (McCauley and Bronner-Fraser, 2006).

**Skeletal muscle** The expression of many skeletal muscle specific genes is now known in lamprey (reviewed in Kusakabe and Kuratani, 2005). As in amphioxus and gnathostomes (but not in ascidians) the paraxial postotic mesoderm of lampreys is segmented in myotomes. Contrarily to amphioxus, the lamprey head mesoderm never develops into cephalic myotomes, and a primarily unsegmented mesoderm may be a pan-craniate feature (Kuratani et al., 1999). In the first transgenesis experiment reported in the lamprey, eggs were injected with constructs in which a sequence encoding the green fluorescent protein (GFP) had been placed downstream of either a virus promoter or 5' regulatory regions of medaka (a teleost fish) actin genes. Reporter gene expression was recorded for more than a month starting two days after injection. Although the expression patterns were highly mosaic and differed among individuals, GFP was expressed predominantly in the striated muscles of lamprey embryos when driven by the 5' upstream regions of the medaka muscle actin genes. This suggests that a pan-craniate muscle-specific gene regulatory mechanism may have evolved before the cyclostome/gnathostome divergence (Kusakabe et al., 2003). On the other hand, this study has also shown that some of the most rostral postotic myotomes and the hypobranchial muscle grow rostrally and cover the cranial region, which does not occur in gnathostomes (Kusakabe et al., 2003).

### 1.3.6 The lamprey brain: history and state of the art

The comparison of the expression domains of genes involved in brain development in representative species of Cephalochordates, Urochordates and Craniates has led to the idea of a common **tripartite organisation** of the ancestral chordate brain (e.g. Takahashi and Holland, 2004). These serial gene expression patterns are shared to a certain extent with hemichordates (Lowe et al., 2003) and arthropods, which has led to the rather controversial idea

of an urbilaterian origin of the tripartite brain (Hirth et al., 2003). In the anterior region of the neural tube of both ascidians and craniates, these three regions are: (1) an *Otx*-expressing domain (which corresponds to the forebrain and midbrain of craniates); (2) a central region expressing *Pax2/5/8* genes; and (3) a more posterior region expressing *Hox* genes (the craniate hindbrain and spinal cord). In amphioxus, *Pax2/5/8* expression is not detected in an intermediate domain between the anterior *Otx*-expressing and the posterior *Hox*-expressing regions (Kozmik et al., 1999).

Due to the long divergence time between the three chordate taxa, the establishment of unequivocal homologies (a notion that implies a common evolutionary origin) within the neural tube has been a difficult task. A pertinent example is the re-evaluation of the homologies of the anterior neural tube subdivisions in ascidians when compared to craniates thanks to the analysis of *Phox2* gene expression (Dufour et al., 2006).

Despite the presence of similarities in the organisation of the anterior neural tube between the three main chordate groups, the craniate brain has a very distinctive type of regionalisation not present in cephalochordates and urochordates. This craniate-type brain organisation (the craniate brain *Bauplan*) appears under the influence of unique patterning centres, specific secondary organisers<sup>9</sup>. Great similarities between the lamprey brain and that of gnathostomes were revealed by anatomical, hodological, immunohistochemical and gene expression studies, and highlighted by the application of the prosomeric model to the lamprey adult brain (Pombal and Puelles, 1999, Fig. 1.16 A). This high degree of conservation was not necessarily apparent in a first approach, as the lamprey brain morphology may look quite different from that of known gnathostome brains (Fig. 1.16 B).

**Organisation of the adult brain** The adult lamprey brain is very small, one of the smallest among craniates, as a proportion of total body mass — but within the size range of some teleost fish (reviewed in Wullimann and Vernier, 2006). The large olfactory bulbs are a very conspicuous feature, as well as the well developed pineal and parapineal organs, which connect to the brain via long stalks. The relative size of these organs reflects the importance

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<sup>9</sup>One of the molecules secreted by these patterning centres is the Hh/Shh protein. Experiments aimed at testing the role of this protein in lamprey brain development are described in chapter 3. The isolation and study of the lamprey *Hh* gene are also described in the same chapter.

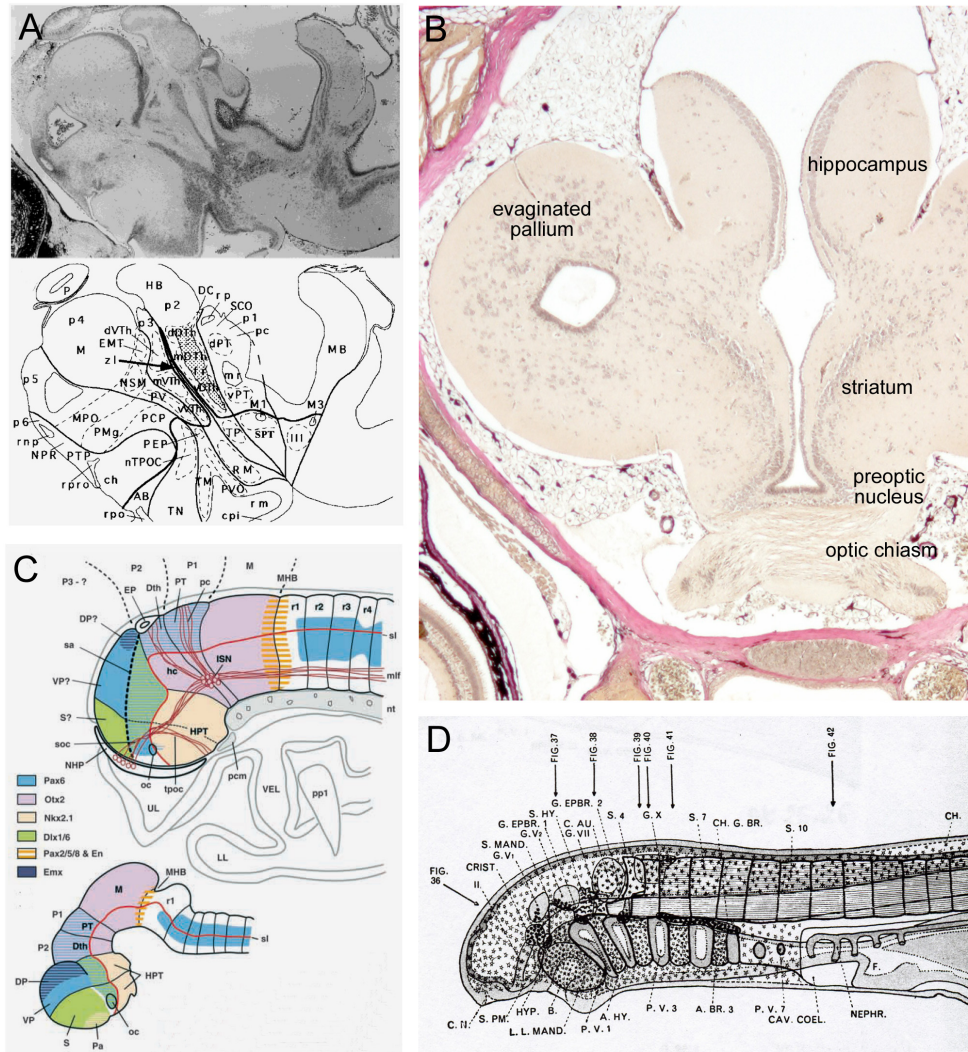


FIGURE 1.16: The lamprey embryonic and adult forebrain. (A) The prosomeric model applied to the adult lamprey brain by Pombal and Puelles (1999); a cresyl violet coloration of a sagittal section is shown as an example of their material. (B) Histology of the lamprey forebrain as seen by a section through the late larval brain (van Gieson coloration, Gabe (1968), Frank Bourrat, unpublished). (C) Expression of regionalisation genes in the lamprey embryonic forebrain compared to mouse (Murakami et al., 2001). (D) Histological drawing of a stage 24 embryo (Damas, 1944).

of the olfaction and the regulation of the circadian rhythm that are of major importance for these animals. The lamprey telencephalon is partly evaginated into two vesicles, but the non-evaginated middle portion is rather large, including parts of both the pallium and the subpallium. All of the lamprey axons are unmyelinated, as those from hagfish and cephalochordates, and this is likely to be an ancestral chordate characteristic (reviewed in Nieuwenhuys and Nicholson, 1998). The majority of the neural cell bodies in the lamprey brain are concentrated around the ventricular walls. This pattern of organisation, characterised by a small degree of migration from the zone where the neurons develop, has appeared several times in evolution, being present in different taxa, and has been referred to as laminar (Butler and Hodos, 1996). Lampreys possess three types of giant cells, the Müller and Mauthner cells of the hindbrain (the latter also present in some gnathostomes) and the giant interneurons of the spinal cord. Curiously, they have mixed or dual synapses which have both electrical and chemical modes of transmission (Nieuwenhuys and Nicholson, 1998). These animals possess several circumventricular organs with a neurohemal function (e.g. Tsuneki, 1986)<sup>10</sup>.

A rudimentary cerebellum, absent from hagfish, has been recognised in the lamprey. However, this region is devoid of Purkinje cells and cerebellar nuclei, as well as components of the rhombic lip-derived cerebellar and pre-cerebellar systems (reviewed in Nieuwenhuys and Nicholson, 1998; Murakami et al., 2005). The absence of *Pax6* expression in the rhombic lip of the developing brain has been related to the absence of these latter components (Murakami et al., 2005).

Classical histological techniques have started to be applied to the lamprey brain more than a century ago. More recently, immunohistochemical, tract-tracing (HRP, DiI, etc.), electrophysiological and behavioural studies have provided a clearer picture of the organisation and functioning of the lamprey central nervous system. Notable advancements have been made using the lamprey spinal cord as a model to study the regeneration of nerves after injury, the respiration and the swimming behaviour (e.g. Gravel et al., 2007; Grillner and Wallen, 2002; McClellan, 1994; reviewed in Rovainen, 1982, 1996).

In the book *The Central Nervous System of Vertebrates*, the chapter dedicated to the description of the lamprey CNS (Nieuwenhuys and Nicholson, 1998)

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<sup>10</sup>See appendix B for a review on the evolution of circumventricular and other neurohemal organs.

is a good survey of the model, even if much has been found after its publication. The chapter contains an extensive list of the localisation of neurotransmitters and other neuronal substances in the lamprey brain. More recent data on fore-brain neurochemical distribution, proliferation and connectivity, or data not mentioned in this list, include the work of Abalo et al. (2005); Auclair et al. (2004); De Andres et al. (2002); de Arriba and Pombal (2007); El Manira et al. (1997); Melendez-Ferro et al. (2001); Menard et al. (2007); Perez-Costas et al. (2004); Pflieger and Dubuc (2004); Pombal and Puelles (1999); Pombal et al. (1997a,b, 2001, 2002, 2006); Reed et al. (2002); Robertson et al. (2006, 2007); Root et al. (2005); Vidal Pizarro et al. (2004); Weigle and Northcutt (1999). Neuronal phenotype distribution and fiber connectivity in the lamprey brain have also been reviewed by Wullimann and Vernier (2006) in a chapter of the recently published book *Evolution of Nervous Systems*.

**Connectivity** The **sensory systems** (primary sensory and higher integrative) in the lamprey brain share many similarities with those of gnathostomes. The pallium and the thalamus (previously called dorsal thalamus) are the major sensory integrative centres. The pallium receives massive inputs from the olfactory bulb, but also from the thalamus (Polenova and Vesselkin, 1993). This shows that the sensory information that reaches the telencephalon is not only of olfactory nature, as previously thought. The thalamus is a higher centre where sensory information is processed. It receives inputs from the retina and tectum, and from the octavolateral area and reticular formation (hindbrain). The correlation of sensory impulses from the specialised sensory organs and from the spinal cord occurs at the level of the reticular formation and at the level of the tectum. In the most rostral part of the rhombencephalic alar plate, a special somatosensory zone, the octavolateral area, receives electroreceptive and mechanoreceptive sensory inputs and is also connected to the labyrinth. The organisation in three distinct nuclei, with the three types of sensory information separated, is common to the one found in cartilaginous fishes, bony fishes and urodeles (reviewed in Nieuwenhuys and Nicholson, 1998; Wullimann and Vernier, 2006).

The **integrative and motor systems** of the lamprey brain are also similar to those found in other craniates. The optic tectum of both lampreys and hagfish shares various inputs and outputs with those of gnathostomes. One important difference in the organisation of these systems in cyclostomes

is the absence (hagfish) or rudimentary nature (lamprey) of the cerebellum. The main pre-motor integrative centre of cyclostomes seems to be the reticular formation. As in other craniates, all parts of the reticular formation, as well as vestibular and sensory trigeminal nuclei, give rise to descending spinal projections. However, they both lack the red nucleus, which is probably related to the absence of extremities (reviewed in Nieuwenhuys and Nicholson, 1998; Wullimann and Vernier, 2006).

**Neurotransmitter distribution** The dopaminergic system of lampreys includes retinal, olfactory bulb, preoptic and some subpallial cells (Pombal et al., 1997a). This shows that subpallial dopamine cells occur ancestrally in craniates, being present in cyclostomes, chondrichthyans and actinopterygians; they were lost in tetrapods, and their presence in mammals appears to be due to convergent evolution. There are dopamine cells in the posterior tuberculum and hypothalamus, as seen in chondrichthyans and actinopterygians; a midbrain expression resembles that of chondrichthyans and non-teleost actinopterygians. Noradrenergic cells in lampreys are present in the brainstem, in a group that corresponds to the locus coeruleus (in lamprey, nucleus reticularis medius) and which projects to the telencephalon (Pombal et al., 1997a). Serotonin cell groups are found in the raphe nuclei, posterior tuberculum and hypothalamus (as in hagfish), and also in the pretectum, as seen in some gnathostomes (Pierre et al., 1992). Histaminergic neurons in lamprey are located in the hypothalamus and, unlike in gnathostomes, at the mid-hindbrain boundary (Brodin et al., 1990). Cholinergic systems in the lamprey brain also exhibit many ancestral characteristics. There are populations of cholinergic neurons in the motor nuclei of cranial nerves and in the nucleus isthmi, there is possibly a secondary viscerosensory population and a small cholinergic group is also found in the basal forebrain (Pombal et al., 2001). The distribution of all neurotransmitters has been reviewed in Wullimann and Vernier (2006). For a review on the comparison of cholinergic systems between the different craniate groups see Rodriguez-Moldes et al. (2002).

Finally, Robertson et al. (2007) have recently reported the distribution of GABA ( $\gamma$ -aminobutyric acid) in the lamprey adult brain. GABA is the main inhibitory neurotransmitter in the craniate brain, and is among the first to be expressed during development. GABAergic cell groups were organised virtually in the same manner in *L. fluviatilis* and *P. marinus*, the two species

studied, and their organisation throughout the brain is remarkably similar to that of other classes of craniates. In the forebrain, GABAergic neurons were detected in the olfactory bulb, the pallium and the subpallium, and also in the hypothalamus and the pretectum. However, an important difference between lampreys and gnathostomes is the timing of appearance of GABAergic neurons in the pallium: in lampreys, pallial GABAergic cells appear remarkably late (Melendez-Ferro et al., 2002a), which suggests that these cells have a different origin and/or are specified or migrate from their place of origin at a different time in development (heterochrony).

**Embryonic and larval development** The embryonic development of the CNS in lampreys involves the formation of a neural keel (Damas, 1944). This type of neurulation was classified as primary by Lowery and Sive (2004; see Fig. 1.17 B). In *Lampetra reissneri* (Tahara, 1988), the neural groove forms on the fifth day post-fertilisation (dpf), in the middle of the neural plate (stage 17). The neural folds start to contact and fuse in the dorsal midline by stage 20 (6<sup>1</sup>/<sub>4</sub> dpf). A median region of the dorsal ectoderm gradually detaches itself from the surface to form a cord of solid cells (Nieuwenhuys and Nicholson, 1998). This region, the neural rod, will increase in size at its anteriormost part. Finally, at stage 22 (8 dpf) a longitudinal slit appears in the nerve cord and the neural tube is formed. When hatching (stage 24), the anterior neural tube is already relatively well developed, and the fore- mid- and hindbrain become discernible due to the formation of the epyphysis and the folding of the cerebral commissure (Fig. 1.2 D; Tahara, 1988).

The study of a number of genes expressed during embryonic development in lamprey has provided insight into the mechanisms of brain and head patterning and organisation, and neural crest cell specification and migration. The expression patterns of representatives of the *Otx* (Tomsa and Langeland, 1999; Ueki et al., 1998), *Emx* (Myojin et al., 2001), *Pax6* (Derobert et al., 2002a; Murakami et al., 2001), *Dlx* (Myojin et al., 2001; Neidert et al., 2001), *Fgf8/17* (Shigetani et al., 2002), *Hh* (Osorio et al., 2005; Uchida et al., 2003), *Nkx2.1* (Ogasawara et al., 2001), *Lhx1/5* (Osorio et al., 2005), *Lhx2/9* (Osorio et al., 2005), *Pax2/5/8* (McCauley and Bronner-Fraser, 2002), *Pax3/7* (McCauley and Bronner-Fraser, 2002; Osorio et al., 2005), *Bmp2/4* (McCauley and Bronner-Fraser, 2004; Shigetani et al., 2002), *Pax1/9* (Ogasawara et al., 2000), *Hox* (Cohn, 2002; Murakami et al., 2004; Takio et al., 2007),



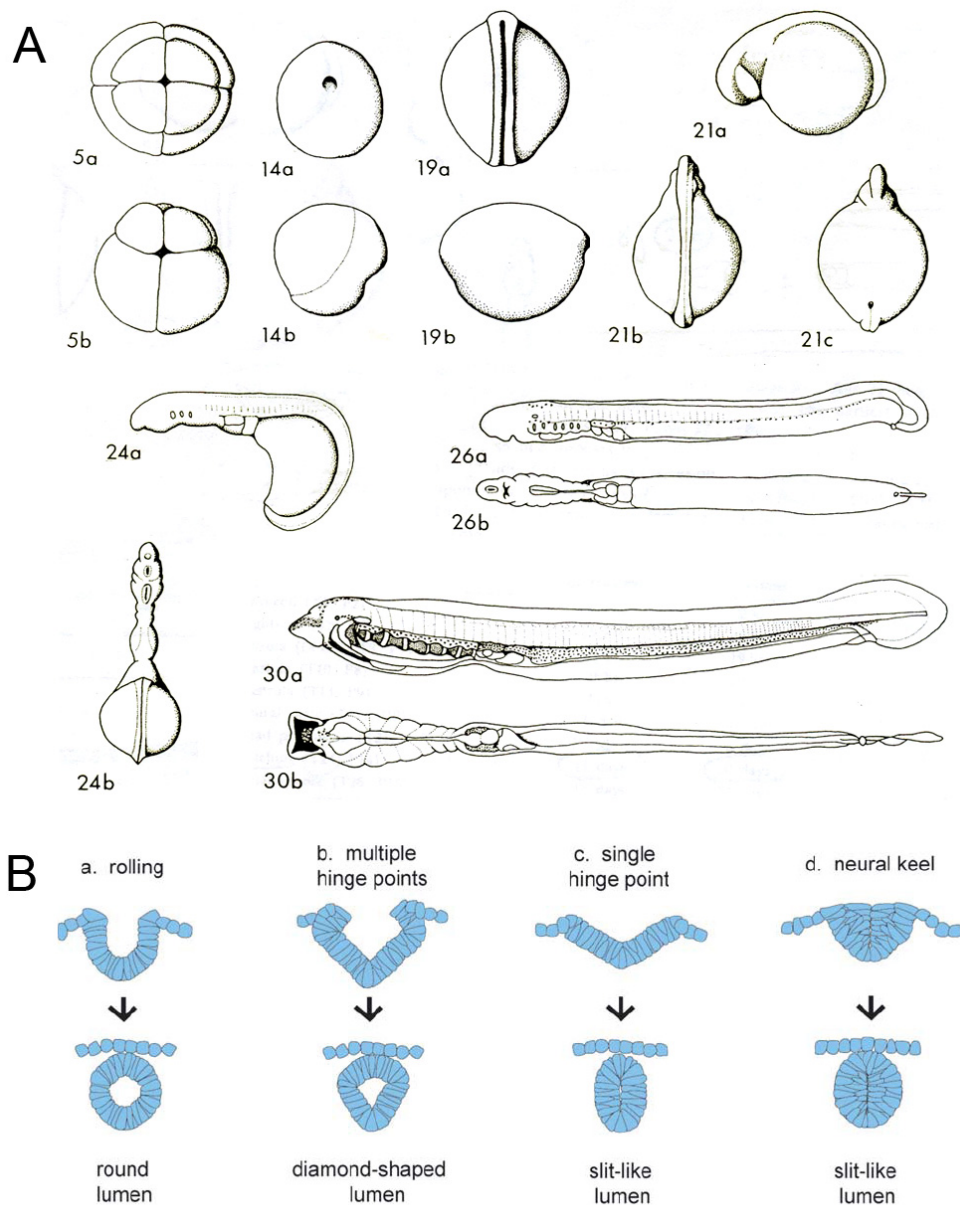


FIGURE 1.17: Lamprey embryonic development. (A) Examples of different stages of *Lampetra reissneri*'s embryonic development, from the developmental table of Tahara (1988): st 5, eight-cell stage; st 14, hemi-circular blastopore, gastrula III; st 19, elevation of neural folds, neurula III; st 21, head protrusion II; st 24, nasal pit, hatching; st 26, melanophore, tailbud II; st 30, completion of digestive tract, earliest ammocoete larva. (B) Variations of primary neurulation. The neural tube is shown at the open stage (top row) and after initial closure (bottom row). The initial flat neuroepithelium may roll smoothly into a tube (a), bend sharply at one (c) or more (b) hinge points, or form a solid rod of cells (d). From Lowery and Sive (2004). The lamprey neural tube forms as in (d).

*Krox20* (Murakami et al., 2004) and *Eph* (Murakami et al., 2004) families have been particularly important to understand brain development and evolution<sup>11</sup>. Some of these genes and members of the *HNK-1* (Morikawa et al., 2001), *Id* (Meulemans et al., 2003), *AP-2* (Meulemans and Bronner-Fraser, 2002), *SoxE* (McCauley and Bronner-Fraser, 2006), *Tbx1* (Sauka-Spengler et al., 2002), Brachyury (Sauka-Spengler et al., 2003), *Pitx* (Boorman and Shimeld, 2002; Uchida et al., 2003) and *Msx* (Shigetani et al., 2002) groups have also been used to investigate the evolution of the neural crest and adenohypophysis, the process of gastrulation and the emergence of jaws.

The analysis of the expression patterns of genes involved in lamprey brain development, and comparisons with ascidians and amphioxus, has revealed a high degree of conservation of the brain organisation between lampreys and gnathostomes. The presence of neuromeres throughout the neuraxis, and of a telencephalon with a *Pax6*-expressing pallium and a *Dlx*-expressing subpallium are distinctive features of the craniate brain (reviewed in Murakami et al., 2005). Fig. 1.16 C is a schematic comparison of the basic forebrain organisation between lamprey and mouse, based on the expression patterns of some of the genes mentioned above. The absence of a *Hh* and *Nkx2.1*-expressing region in the subpallium has been correlated to the absence of a pallidum (the mammalian MGE; Murakami et al., 2005; Osorio et al., 2005), a region which cannot be recognised in adult lampreys (Nieuwenhuys and Nicholson, 1998).

In the hindbrain, the different reticulospinal neurons develop from specific neuromeres both in lampreys and gnathostomes, and lamprey homologues of *Krox20* and *Eph* are similarly expressed in rhombomeres 3 and 5. The coupling of the rhombomeric organisation of the hindbrain and the rhombomeric-specific distribution of reticulospinal neurons is probably a craniate novelty (Murakami et al., 2005). On the other hand, it is important to note that the hindbrain Hox code is not correlated to the rhombomeric borders in lamprey, and while in gnathostomes the motor nuclei of cranial nerves develop in register with the rhombomeric boundaries, this is not so in the lamprey. A striking case is that of the transition between the trigeminal (V) and facial (VII) motoneurons. In gnathostomes, this transition occurs at the r3-r4 border, while in lamprey it occurs instead in the middle of r4, where it coincides

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<sup>11</sup>For our contribution to the study of the lamprey brain organisation by analysing the expression pattern of regionalisation and patterning genes in the developing lamprey brain, see chapter 2.

with the rostral expression boundary of *Hox3* (Murakami et al., 2004). This shows that the association between the rhombomeres and the development of motoneurons (defined by the Hox code) has only appeared in gnathostomes after the split from the lineage leading to lampreys (Murakami et al., 2005). These findings can be seen as a challenge to the definition of rhombomeres as seen from gene expression patterns, and raise important questions about the nature of the hindbrain “compartments” in lampreys and gnathostomes.

Data on the neuroanatomical organisation, connectivity, and distribution of neurotransmitters and related substances in the brain of embryos and/or larval lampreys are gradually accumulating. Anatomical, tract-tracing and immunocytochemical studies in developing lampreys of different species<sup>12</sup> (Abalo et al., 2005; Cheung et al., 1991; De Andres et al., 2002; de Miguel et al., 1990; Frontini et al., 2003; Gonzalez et al., 1999; Kuratani et al., 1998; Laframboise et al., 2007; Melendez-Ferro et al., 2002a,b, 2003; Perez-Costas et al., 2002; Pierre-Simons et al., 2002; Tobet et al., 1996; Vidal Pizarro et al., 2004; Villar-Cheda et al., 2002, 2005, 2006; Yanez and Anadon, 1994; Yanez et al., 1993; Zaidi et al., 1998; Zielinski et al., 2000) have provided a better understanding of the development of the lamprey forebrain, and also of the visual (eye, pineal complex), olfactory and hypophyseal systems. While many of these studies show a conservation of the way the CNS is formed between lampreys and gnathostomes, some of them have revealed important differences, such as the lack of tangential migration in the lamprey brain, or differences in the timing of appearance of pallial GABAergic neurons.

**Proliferation** In a recent important analysis of neuronal proliferation and differentiation in the brain of embryonic, larval and adult lamprey (*Petromyzon marinus*), proliferation has been correlated to lamprey brain morphogenesis (Villar-Cheda et al., 2006). These authors have shown that differences in the thickness and appearance of the ventricular zone (the portion of the neural tube closest to the ventricle), as well as the presence of proliferation discontinuities, correspond to distinct neuroanatomical regions.

Even if the general histogenesis of the lamprey brain follows the general craniate pattern (Nieuwenhuys and Nicholson, 1998), this study has also highlighted the differences of proliferation in the lamprey brain when compared to

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<sup>12</sup>To our knowledge, the first gene expression study in the brain of larval animals is the study of *Lhx15* expression described in chapter 2.

other craniate brains. Some of these differences are probably related to the particular life cycle of these animals, which comprises an embryonic/prolarval period, a very long larval phase, a metamorphosis event and the adult life. In lampreys, the presence of conspicuous late-proliferating regions during larval life and metamorphosis is in remarkable contrast to the limited proliferative activity in the adult brain. The study of neuronal differentiation markers has also shown that there is little, if any, tangential migration of lamprey neurons. Furthermore, while postembryonic neurogenesis in teleosts appears to be rapidly restricted to specialised small areas, neurogenesis in the lamprey fore-brain and midbrain is widespread throughout most of the ventricular surface over a rather long developmental period. Analysis of both PCNA (proliferating cell nuclear antigen) and BrdU (5'-bromo-2-deoxyuridine) has revealed that the cell cycle in lamprey is very long, explaining both the slow growth of the brain and the presence of an extensive ventricular zone for many years. All of these characteristics show a striking contrast to the proliferation patterns of the teleost brain (e.g. Wullimann and Puelles, 1999; Wullimann and Knipp, 2000).

## 1.4 Aim

The aim of this thesis is to discuss the emergence of the craniate brain and its evolution within the two extant craniate sister-groups, Cyclostomata and Gnathostomata. Emphasis will be given to the study of developmental genes, their expression pattern through time and the mechanisms of its regulation. The discussion will be based on the results of our research work on lamprey (and dogfish), which will be compared to data from other chordate groups. Chapter 2 provides an analysis of brain regionalisation and patterning gene expression in embryonic and larval lampreys. Chapter 3 examines an ongoing study on the evolution of gene number, structure and gene expression regulation in lamprey and dogfish.



## Chapter 2

# Molecular organisation of the developing lamprey forebrain

In the first part of this chapter, we discuss an analysis of the genetic specification and organisation of the embryonic lamprey brain. By RT-PCR cloning and *in situ* hybridisation we were able to isolate and study the expression pattern of two *LIM-homeobox* genes, *LfLhx15* and *LfLhx29* (homologues to gnathostome *Lhx1/5* and *Lhx2/9* groups of paralogs, respectively) in the brain of the river lamprey *Lampetra fluviatilis*. The genes *LfPax3/7*, *LfPax6* and *LfNkx2.1* were also used to identify important boundaries in the forebrain. In addition, to further understand how neuromeric organisation might be set up in the embryonic lamprey brain, we have isolated *LfHh*, a member of the Hedgehog family of signalling molecules.

The main results are as follows: (1) *Lhx* and *Pax* genes reveal strikingly similar forebrain and hindbrain patterning between cyclostomes and gnathostomes; some interesting differences are nevertheless observed, especially at the level of the telencephalon, a region where major changes took place and many novelties emerged at the agnathan/gnathostome transition; (2) expression analysis of *LfLhx15* and *LfLhx29* and its interpretation shows that the lamprey forebrain presents a clear prosomeric organisation, which is thus a truly craniate character; (3) analysis of the *LfHh* gene expression reveals the possible molecular origin of this neuromeric brain pattern.

This project was made in collaboration with Sylvie Mazan from the “Immunologie et Embryologie Moléculaires” laboratory at the Institut de Transgénése at Orléans (France), and the results have been published in November

2005 in the journal *Developmental Biology* (Osorio et al., 2005). This article is presented in the following section of this chapter, with some comments and minor modifications. I sincerely thank the help of Jean-Louis Plouhinec on the phylogenetic analysis.

In *Xenopus*, *Lhx* genes have proven useful to follow the morphogenesis of brain nuclei, due to their persistent expression from embryogenesis to the adult stage (Moreno et al., 2004, 2005). In the second part of this chapter we present an analysis of *Lhx15* expression on larval sea lampreys (*Petromyzon marinus*) of different ages in order to characterise the molecular basis of regional identity in the lamprey brain. This was the first developmental gene expression pattern study ever performed in the non-embryonic lamprey brain.

We have shown that *Lhx15* gene expression persists through the larval period, making this gene — and probably other *Lhx* genes — useful markers to follow brain development and morphogenesis in lamprey as well. Furthermore, we provide evidence for the evolutionary conservation of the expression of this gene in the spinal cord, notochord and urogenital system. Taken together, these results show that *Lhx15* is expressed in the same endomesodermal tissues as the gnathostome *Lhx1*, and illustrate a case of very high spatial and temporal gene expression conservation throughout Craniate evolution.

This project was made in collaboration with Manuel Pombal and Manuel Megias from the Neurolam group at the University of Vigo (Spain), and the results have been published in October 2006 in the journal *Gene Expression Patterns* (Osorio et al., 2006). This article is presented in the second part of this chapter.

In gnathostomes, genes of the *Hedgehog* gene family are involved in numerous developmental processes. In the third section of this chapter, a comparative description of the embryonic expression of the lamprey *Hh* gene in non-neural tissues is presented.

## 2.1 Organisation of the lamprey (*L. fluviatilis*) embryonic brain: insights from *Lhx*, *Pax* and *Hedgehog* genes

### 2.1.1 Introduction

Lampreys are jawless craniates, belonging to the Cyclostomata, the sister-group of gnathostome craniates. Their phylogenetic position makes them an invaluable model of study in evolutionary developmental biology to gain new insight into the craniate ancestral characteristics, but also to understand the emergence of novelties at important evolutionary transitions, such as the agnathan/gnathostome transition (Kuratani et al., 2002).

The brain is particularly interesting in this respect. Indeed, the acquisition of jaws associated with a change in lifestyle must have had a yet largely unknown influence on its structure and functions, with neuronal organisations being modified to generate new adaptive behaviours. The study of the embryonic organisation of the lamprey brain should thus reveal important information on the development and evolution of the craniate brain, on the novelties which have emerged with jawed craniates, and how such a complex structure evolved in the gnathostome lineage. It is currently accepted that the embryonic gnathostome brain is organised into transverse domains, presenting well-defined morphological and gene expression boundaries along its transverse and longitudinal axes (Puelles and Rubenstein, 1993, 2003). These segmental units, called neuromeres (in the forebrain, prosomeres), are conserved among gnathostome craniates, and have provided a framework for comparative analyses. Pombal and Puelles (1999) have shown that a prosomeric analysis can be applied to the adult lamprey brain. The fact that such a prosomeric organisation is not observed in ascidians and amphioxus makes it a specific craniate characteristic (Holland and Holland, 1999; Mazet and Shimeld, 2002).

Our knowledge of the developing lamprey forebrain is still very incomplete, but accumulating data allow us to draw a picture where clear similarities and differences can be recognised from the gnathostomes. The expression patterns of genes such as *Pax6* (Derobert et al., 2002a; Murakami et al., 2001), *Otx* (Tomsa and Langeland, 1999), *Dlx*, *Emx* (Myojin et al., 2001; Neidert et al., 2001), *Pitx* (Boorman and Shimeld, 2002), *Nkx2.1/TTF-1* (Ogasawara et al., 2001) are conserved in the hindbrain, midbrain, prosomeres p1 and p2, and



hypothalamus. This conservation may also be present to a certain degree in the region anterior to the p2/p3 boundary, leading to the tentative delineation of telencephalic subdivisions, and of the di-telencephalic border (Murakami et al., 2001; Myojin et al., 2001). One striking difference when compared to gnathostomes is the absence of expression of *Nkx2.1* in the telencephalon of lampreys, which raises the question of whether the lamprey possesses a pallidum or not (Weigle and Northcutt, 1999).

*LIM-homeobox* (*Lhx*) transcription factors are involved in neuronal developmental processes such as axonal pathfinding, neurotransmitter phenotype specification and overall regionalisation (Bach, 2000; Hobert and Westphal, 2000; Retaux and Bachy, 2002). These genes and their functions are highly conserved, and they have been used as prosomeric markers in several key species of craniates (Alunni et al., 2004; Bachy et al., 2001; Moreno et al., 2004; Retaux et al., 1999). These comparative studies have been only performed, so far, in gnathostomes. In this paper, we report the isolation and expression patterns of two *Lhx* genes, *LfLhx15* and *LfLhx29* (homologues to gnathostome *Lhx1/5* and *Lhx2/9* groups of paralogs, respectively) in the brain of the river lamprey *L. fluviatilis*. We also isolated *LfPax3/7*, which we used together with *LfPax6* to identify important boundaries in the forebrain. In addition, to get some clues on how neuromeric organisation might be set up in embryonic lamprey brain, we isolated *LfHh*, a member of the Hedgehog family of signalling molecules. Expression analysis provides new insights into the identification of the main subdivisions of the lamprey embryonic brain, and clearly shows a strikingly high degree of conservation between cyclostome and gnathostome brains. Some interesting differences are nevertheless observed, especially at the level of the telencephalon. Implications of these findings are discussed in terms of molecular and structural evolution of the forebrain.

### 2.1.2 Materials and methods

#### Embryos

*Lampetra fluviatilis* embryos were obtained from in vitro fertilisation and kept in oxygenated tap water at 18°C. They were staged according to the table established by Tahara (1988) for *L. reissneri*, a species that is closely related to *L. fluviatilis*. For *in situ* hybridisation, embryos were fixed overnight in MEMFA (4% formaldehyde, 0.1M MOPS (pH 7.4), 1mM MgSO<sub>4</sub>, 2mM EGTA), then

dehydrated in methanol, and stored at  $-20^{\circ}\text{C}$  in 100% methanol.

### Reverse transcription-polymerase chain reaction (RT-PCR) cloning

Total RNA extraction was performed on embryos from stage 19 to 29 using the Trizol Reagent method (Invitrogen). First strand cDNA was obtained by reverse transcription (Roche) and used as template for PCR reactions (Qiagen). A 376 bp fragment of *LfLhx15* cDNA was amplified using degenerate primers in forward (sequence encoding NCFTCMM/VC; nested DENKFFVCK) and reverse (QVWFNQRR) orientations. A 160 bp fragment corresponding to the homeobox of *LfLhx29* was obtained using degenerate primers in forward (MRTSFKHHQL) and reverse (WFQNARAK) directions. A 276 bp fragment of the *LfHh* cDNA was obtained with primers in forward (AYKQFMPN) and reverse (EGWDEDGH) directions. A 1.2 kb full-length *LfNkx2.1* cDNA was amplified using primers designed on the *L. japonica* sequence. The amplified PCR fragments were sub-cloned into the pGEM-T Easy vector (Promega). A 333 bp fragment coding for the sequence comprised between the paired domain (forward, IFAWEI) and the homeodomain (reverse, YPDVFA) of *LfPax37* was amplified and sub-cloned into the pTZ18R vector. GenBank accession numbers are: *LfLhx29*, DQ002014; *LfLhx15*, DQ002012; *LfHh*, DQ002013; *LfPax37*, DQ054789; *LfNkx2.1*, DQ060244.

### Phylogenetic analysis

The *Pax3/7*, *Lhx1/5*, *Lhx2/9*, *Hh* and *Nkx2.1* sequences used for comparison with *L. fluviatilis* sequences were retrieved from Genbank. Sequence alignments were carried out with MUSCLE (Edgar, 2004) and refined by visual inspection. Phylogenetic trees and bootstrap proportions were computed from unambiguously aligned sequence segments using two reconstruction algorithms: Neighbour-Joining, using Mega (Kumar et al., 2004) with the Poisson correction model, and Maximum-Likelihood, using PhyML (Guindon and Gascuel, 2003) with the JTT-f/invariant+gamma model of sequence evolution.

### Whole-mount *in situ* hybridisation

Digoxigenin-labeled antisense riboprobes against *LfLhx15*, *LfLhx29*, *LfPax37*, *LfPax6* (Derobert et al., 2002a) and *LfHh* mRNAs were synthesised and used for *in toto in situ* hybridisation according to the protocol described in Bachy

et al. (2001) with minor modifications. Embryos were progressively re-hydrated, then treated with proteinase K (10  $\mu\text{g}/\text{ml}$ , 45 min) and with PBT-glycin (2 mg/ml). Embryos were then fixed with MEMFA for 20 min before pre-hybridisation (2–4 hr at 65°C). Hybridisation was carried on overnight at 70°C in a 50% formamide hybridisation medium containing 100–300 ng/ml of DIG-labelled RNA probe. Specimens were incubated with alkaline phosphatase (AP)-conjugated anti-digoxigenin Fab fragments (diluted 1:4000; Roche Diagnostics, Mannheim, Germany) at 4°C overnight. Alkaline phosphatase activity was detected with NBT/BCIP (Roche Diagnostics, Mannheim, Germany). For *in toto* histological observation, embryos were fixed in 4% paraformaldehyde, washed, and cleared with a 1 benzyl alcohol:2 benzyl benzoate solution. Some embryos were dehydrated in ascending ethanol after fixation, embedded in paraffin and sectioned with a Leica microtome at 8–12  $\mu\text{m}$ . Photographs were taken on a Nikon microscope equipped with a DXM-1200 camera.

### 2.1.3 Results

#### Phylogenetic analysis of *Lampetra fluviatilis* (*Lf*) *Lhx15*, *Lhx29* and *Pax37* genes

Degenerate RT-PCR was used to isolate the *Lampetra fluviatilis* homologs of the gnathostome *Lhx1/5*, *Lhx2/9* and *Pax3/7* gene groups, and led to the identification of one gene within each group. As these genes are each unambiguous representatives of these groups, but could not be clearly assigned to any of the classes found inside these groups in gnathostomes, they were named *LfLhx15*, *LfLhx29* and *LfPax37*, respectively.

***LfLhx15*** The isolated *LfLhx15* partial cDNA sequence encoded the region comprised between the LIM2 domain and the homeodomain. It thus includes the linker domain, which is the most variable sequence among LIM-homeodomain proteins. Alignment with available chordate Lhx1 and Lhx5 proteins showed that the lamprey sequence is virtually identical to its counterparts over the LIM2 and homeodomain sequences, thus confirming its identity (Fig. 2.2 A). Over the linker region, the Lhx1 and Lhx5 classes found in gnathostomes exhibit selectively conserved residues, which provide hallmarks of each class. The lamprey sequence could not be clearly assigned either to the Lhx1 class or to the Lhx5 class on the basis of these distinctive features

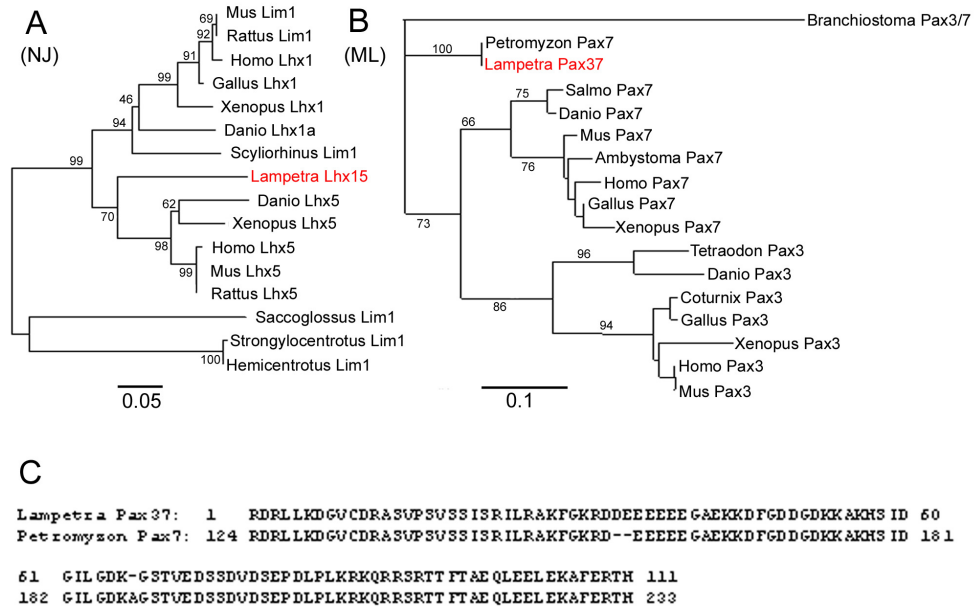


FIGURE 2.1: Phylogenetic trees of Lhx1/5 and Pax3/7 families, and alignment of Pax37 lamprey peptides. (A) Neighbour-Joining (NJ) phylogenetic tree showing the orthology relationship of LfLhx15 (red) with available Lhx1 and Lhx5 sequences from deuterostomes (*Saccoglossus kowalevskii* is a hemichordate, *Hemicentrotus pulcherrimus* and *Strongylocentrotus purpuratus* are two echinoderms). A monophyletic group containing LfLhx15 and the gnathostome Lhx5 sequences was obtained in some reconstructions, albeit with a low statistical support (bootstrap value (BP) = 70% with Neighbour-Joining (NJ) analysis). However, this result was not confirmed using other reconstruction algorithms (Maximum-Likelihood (ML), data not shown). (B) Maximum-Likelihood (ML) phylogenetic tree showing the orthology relationship of LfPax37 (red) with available Pax3 and Pax7 sequences from gnathostomes and cephalochordates (*Branchiostoma floridae*). With phylogenetic analyses using NJ and ML algorithms, the lamprey sequences emerged as a sister group of a monophyletic group containing the gnathostome Pax3/7 sequences, but again with relatively low statistical supports (BP = 73% with ML, 46% in NJ, not shown). (C) Protein alignment of *Lampetra* LfPax37 (Osorio et al., 2005) and *Petromyzon* Lamp-Pax7 (McCauley and Bronner-Fraser, 2002). From Osorio et al. (2005), supplementary data.

(4 Lhx1 and 8 Lhx5 specific residues, respectively, red or blue on Fig. 2.2 A). Phylogenetic analyses also remained inconclusive as to the relationships between the lamprey gene and the gnathostome Lhx1 and Lhx5 classes (Fig. 2.1 A).

**LfLhx29** The PCR strategy used to amplify *LfLhx29* led to the isolation of a cDNA fragment spanning positions 5 to 56 of the homeodomain. Comparison of the deduced amino acid sequence with those of the different osteichthyan LIM-homeodomain (LIM-hd) groups unambiguously confirmed the assignment of the resulting sequence to the Lhx2/9 class, with only two differences with the mouse Lhx2 and Lhx9 sequences (Fig. 2.2 B). The relationships of the lamprey sequence with the Lhx2 and Lhx9 classes found in gnathostomes could not be addressed in this case, since the two classes display identical sequences over the isolated fragment.

**LfPax37** To identify *Pax3/7* related sequences in *Lampetra fluviatilis*, we amplified a fragment encoding the C-terminal residues of the paired domain, N-terminal residues of the homeodomain, and linker sequence located between them. The deduced amino-acid sequence could be unambiguously aligned with the Pax3 and Pax7 sequences characterised in osteichthyans, and showed a particularly high level of identity with the *Petromyzon marinus* LampPax7 sequence reported by McCauley and Bronner-Fraser (2002) (97% identity at amino-acid level, see Fig. 2.1 C). After phylogenetic analyses, and as in the case of LfLhx15, we considered that the relationships of the lamprey sequences with the gnathostome Pax3 and Pax7 orthology classes remain unresolved (Fig. 2.1 B).

### **Expression patterns of *Lhx* and *Pax* genes in the embryonic lamprey brain**

The expression patterns of the isolated genes were studied between stage 19 and 28, and most results are presented at stage 24 and 26 for the purpose of easy comparison with previous related studies.

***Pax* genes: *LfPax6* and *LfPax37* as AP and DV patterning cues**  
The developmental expression pattern of *Pax6* has been previously reported

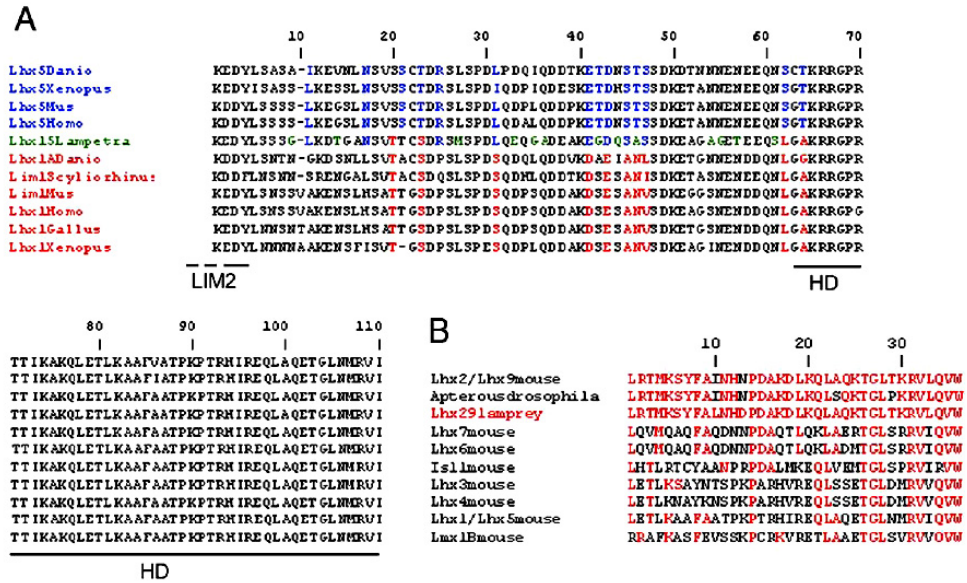


FIGURE 2.2: Sequence analysis of the isolated genes. (A) LfLhx15 amino acid sequence alignment with gnathostome Lhx1/Lhx5 sequences. Signature amino acids for the Lhx5 and Lhx1 orthology classes are in blue and red, respectively. They were defined as amino acid positions which are found in all members of one class but never in the other class. Amino acids that are found only in *Lampetra* are in green. The position of the LIM2 domain and the homeodomain (HD) are indicated. (B) LfLhx29 amino acid sequence alignment with mouse and *Drosophila* LIM-hd sequences belonging to the Lhx2/Lhx9, Lhx6/Lhx7, Islet, Lhx3/Lhx4, Lhx1/Lhx5 and Lmx classes, showing unambiguously that LfLhx29 belongs to the Lhx2/Lhx9 class.

in two lamprey species, *Lampetra japonica* (Murakami et al., 2001) and *Lampetra fluviatilis* (Derobert et al., 2002a). Consistent with these studies, *LfPax6* showed prominent forebrain expression, including the pallial telencephalon and all the diencephalon dorsal to the sulcus limitans (alar plate), together with a strong rhombencephalic expression excluding the roof and floor plates. As expected, *LfPax6* was also present in the optic vesicle and optic stalk, and in the epiphysis (pineal gland) at later stages. *LfPax6* expression pattern displayed sharp ventral and posterior borders, thus providing us a good definition of the pallio-subpallial and diencephalic-mesencephalic boundaries, respectively (Fig. 2.3 A, Fig. 2.4 A).

The previously reported expression of *Petromyzon marinus LampPax-7* in the dorsalmost aspect of the neural tube with the exception of the forebrain, within the somites, and in the trigeminal placode and developing ganglion fits with the idea that *Pax3/7* genes are markers of placode and neural crest

cells (McCauley and Bronner-Fraser, 2002). We also observed these expression territories for the related *L. fluviatilis* *LfPax37* (Fig. 2.3 B, Fig. 2.4 B, and Fig. 2.5). In addition, a prominent hybridisation signal was also detected at all stages studied (st.23 to 27) along the dorsal aspect of the entire forebrain including the telencephalon (Fig. 2.3 B, Fig. 2.4 B and Fig. 2.5 C–D–E). Moreover, this dorsal expression domain was continuous, but two adjacent territories separated by a sharp boundary could be observed: a caudalmost territory characterised by a wide alar *LfPax37* expression, and a rostral domain where *LfPax37* transcripts were restricted to a thin midline band (Fig. 2.3 B, Fig. 2.4 B). This specific forebrain expression pattern is shared by *Pax3/Pax7* in gnathostomes, in which the limit between the two domains corresponds to the p1/p2 boundary. *LfPax37* therefore allowed us to accurately identify the p1/p2 boundary in the lamprey. Of note, *LfPax37* was not expressed in the pineal gland (Fig. 2.5 D).

***Lhx* genes: *LfLhx15* and *LfLhx29* as prosomeric markers** The types of expression patterns observed for *Lhx* factors were different from those shown by *Pax* genes, and almost exclusively restricted to the nervous system (Fig. 2.3 C–D, Fig. 2.4 C–D; and Fig. 2.6). Here we analyse these patterns from rostral to caudal parts of the brain.

*LfLhx15* presented a prominent and complex expression in the forebrain (Fig. 2.3 C, Fig. 2.4 C). Comparison with *LfPax6* and analysis on sections showed that *LfLhx15* was present in the pallial but not in the subpallial part of the telencephalon (Fig. 2.4 C and Fig. 2.6 C). Interestingly, the *LfLhx15* differential expression levels may allow to distinguish two pallial subdivisions: a highly-expressing dorsal division, and a low expressing ventral division (Fig. 2.4 C and Fig. 2.6). *LfLhx15* was also expressed at modest levels in the hypothalamus, and at high levels in several regions of the alar and basal plates in the diencephalon, including (1) a diencephalic area closely adjacent to the telencephalon, and probably including the tel-diencephalic border, (2) a domain which, after comparison with *LfPax6* (compare Fig. 2.3 A and Fig. 2.3 C; Fig. 2.4 A and Fig. 2.4 C) and *Dlx* in other lamprey species (Murakami et al., 2001; Myojin et al., 2001), probably corresponds to the ventral thalamus/p3, (3) a thick comma-shaped domain, predominantly located in the basal plate, highly reminiscent of the *Lhx1/Lhx5* patterns previously observed in *Xenopus* (Bachy et al., 2001), which encircles the dorsal thalamus/p2, and, importantly,

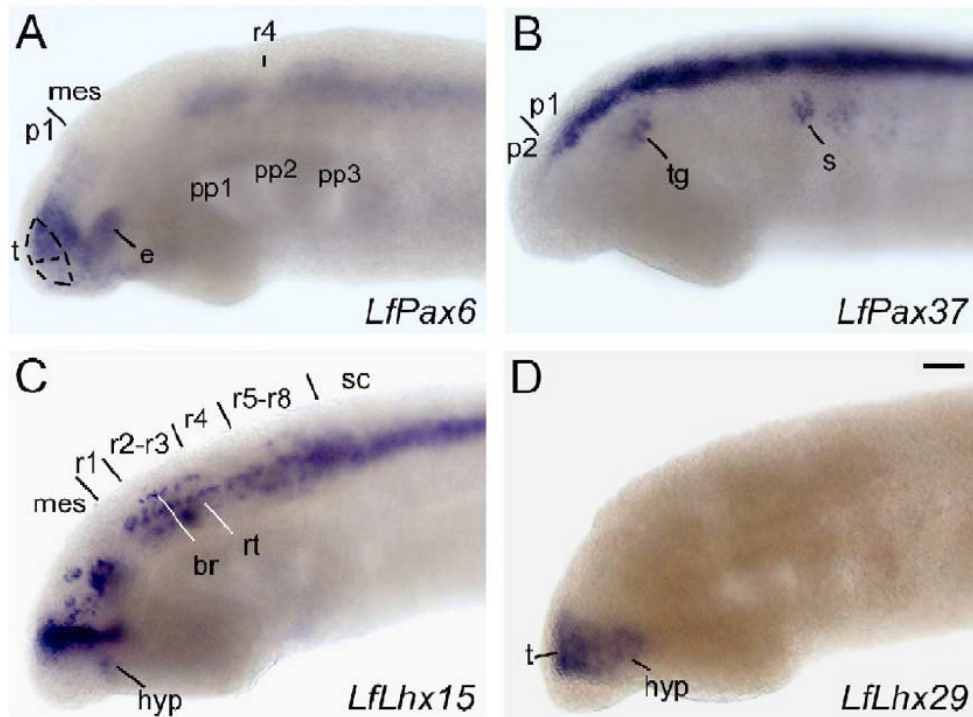


FIGURE 2.3: *In toto* expression patterns of *LfPax6*, *LfPax37*, *LfLhx15* and *LfLhx29* at stage 24. Whole-mount lateral views of embryos hybridised with the indicated probe. Anterior is left, dorsal is up. In panel A, the dotted line delineates the pallial and subpallial parts of the telencephalon, as deduced from *LfPax6* expression. The hindbrain reticulospinal (rt) and branchial (br) motoneurons are indicated. eye; hyp, hypothalamus; mes, mesencephalon; p1/p2, prosomeres 1,2; pp, pharyngeal pouch; r1–8, rhombomere 1 to 8; s, somite; sc, spinal cord; t, telencephalon; tg, trigeminal ganglion. Scale bar, 100  $\mu\text{m}$ .

also expresses *LfHh* (see below), and (4) a band of expression at the border of the mesencephalon, identified as pretectum/p1. Notably, the appearance and formation of these diencephalic domains could be clearly followed between st.24 and st.26 (compare Fig. 2.3 C and Fig. 2.4 C; Fig. 2.6 A and Fig. 2.6 B). *LfLhx15* was absent in the midbrain, but was prominent again posterior to the midbrain/hindbrain boundary with a clear rhombomeric pattern in the hindbrain. Indeed, after comparison with *LfPax6*, which is specifically absent in r4 at st.24 and therefore is a good marker of this rhombomere (Fig. 2.3 A), the two discontinuous longitudinal stripes of *LfLhx15* expression probably correspond to rhombomeric-specific variations of expression in two columns



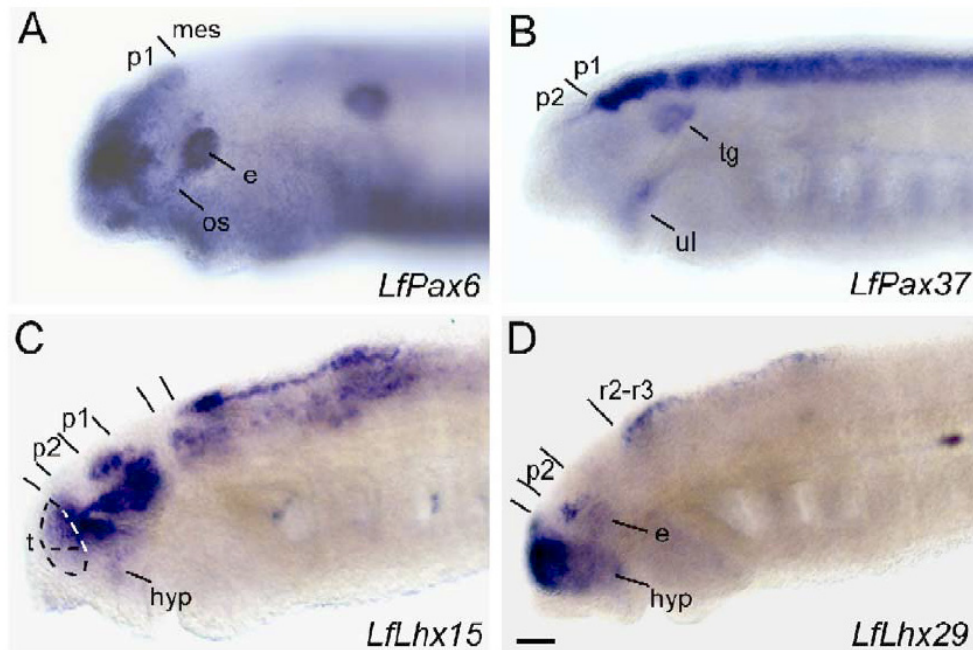


FIGURE 2.4: *In toto* expression patterns of *LfPax6*, *LfPax37*, *LfLhx15* and *LfLhx29* at stage 26. Whole-mount lateral views of embryos hybridised with the indicated probe. Anterior is left, dorsal is up. In panel C, the dotted line delineates the pallial and subpallial parts of the telencephalon, as deduced from *LfLhx15* expression (compare with Fig. 2.3 A). e, eye; hyp, hypothalamus; mes, mesencephalon; os, optic stalk; p1/p2, prosomeres 1,2; rn, rhombomere n; t, telencephalon; ul, upper lip. Scale bar, 100  $\mu\text{m}$ .

of motoneurons (Fig. 2.3 C, Fig. 2.4 C)<sup>1</sup>. In summary, *LfLhx15* outlined a developmental neuromeric organisation of the river lamprey forebrain and hindbrain (summarised in Fig. 2.8 B–C).

*LfLhx29* pattern was mainly restricted to the anteriormost part of the brain, where its expression level increased significantly between st.24 and st.26 (Fig. 2.3 D, Fig. 2.4 D and Fig. 2.6 D–F). *LfLhx29* transcripts were then detected in the entire telencephalon (pallial and subpallial divisions), the pineal gland, the hypothalamus, and, similar to *LfLhx15*, in the di-telencephalic junction area. The ventral thalamus was free of expression, whereas the dorsal

<sup>1</sup>The pattern of rhombencephalic *Lhx15* expression observed in larvae (Osorio et al., 2006) contradicts this hypothesis. In these much larger brains it was clear that, while one of these bands of expression, located in the basal plate, includes the interpeduncular nucleus and the reticular formation, the other is located in the alar plate and corresponds to the octavolateral nuclei, which are involved in the transmission of sensorial information to the brain.

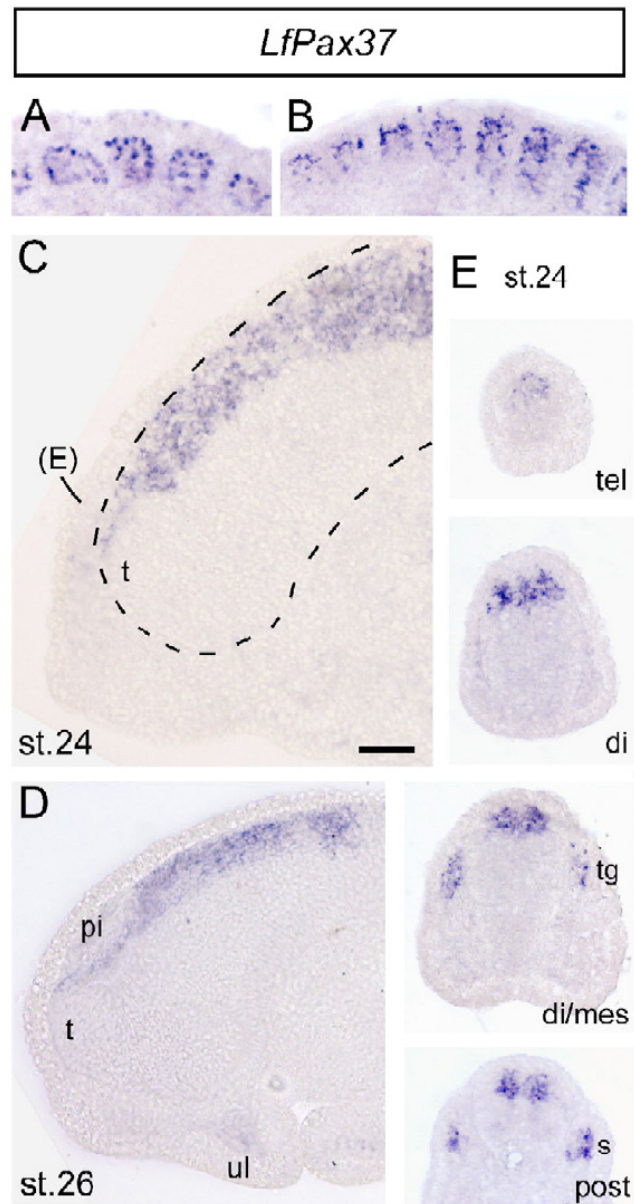


FIGURE 2.5: Expression of *LfPax37* in the developing brain and somites. Panels A, B show *LfPax37* expression in somites at stages 19/20 (A) and 22/23 (B), on saggital sections. Panels C–E show expression of *LfPax37* in the developing brain, analysed in saggital (C, D) or transverse (E) sections at the indicated level (tel, telencephalon; di, diencephalon; mes, mesencephalon; post, posterior neural tube) and at the indicated stage through the embryonic brain. In panel C, the dotted line delineates the shape of the forebrain, and the orientation of the sections presented in panel E is indicated. pi, pineal gland; s, somite; t, telencephalon; tg, trigeminal ganglion; ul, upper lip. Scale bar, 50  $\mu\text{m}$ .

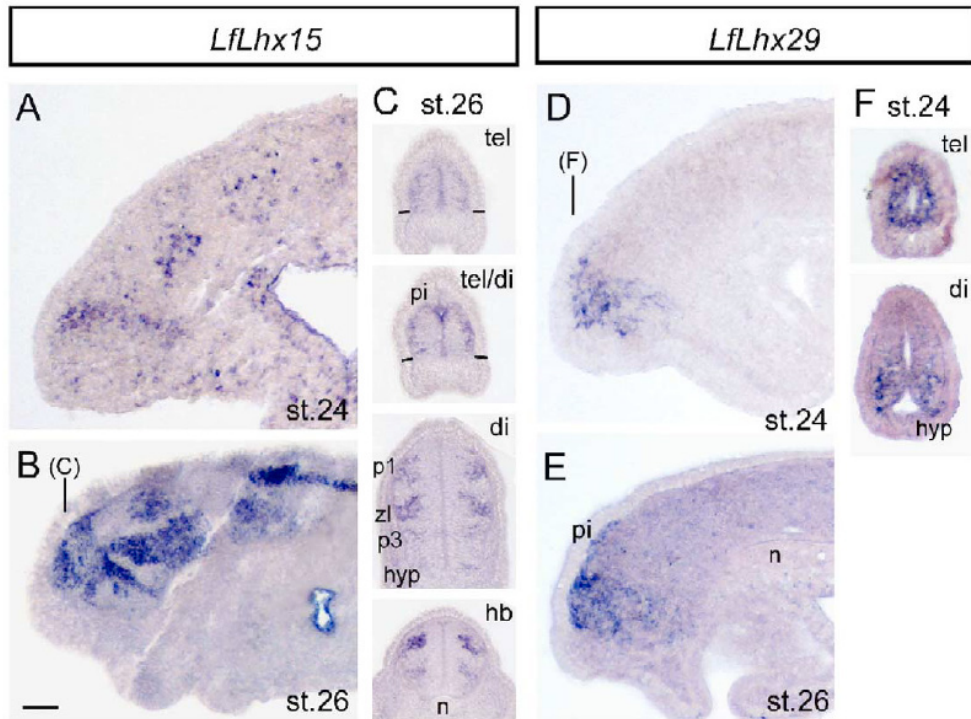


FIGURE 2.6: Expression of *LfLhx15* and *LfLhx29* in the developing brain. Expression of *LfLhx15* and *LfLhx29* analysed in saggital (A, B, D, E) or transverse (C, F) sections at the indicated level (tel, telencephalon; di, diencephalon; hb, hindbrain) and at the indicated stage through the embryonic brain. The orientations of the sections presented in panels C, F are indicated in panels B, D, respectively. In panel C, the black lines indicate the limit between the pallial and subpallial telencephalon, and the prosomeric expression of *LfLhx15* is clearly observable. hyp, hypothalamus; n, notochord; pi, pineal; p1/p3, prosomeres 1,3; zl, zona limitans. Scale bar, 50  $\mu\text{m}$ .

thalamus/p2 expressed *LfLhx29*, therefore giving rise to alternate bands of *LfLhx29/LfLhx15* expression along the diencephalic rostro-caudal axis (Fig. 2.4 D; summarised in Fig. 2.8 D). *LfLhx29* was also expressed in the developing eye. Finally, at st.26, *LfLhx29* was expressed in the hindbrain, possibly in the same dorsalmost column as *LfLhx15*. Taken together, the expression patterns of the two *Lhx* and the two *Pax* family members suggested the existence of transverse domains with distinct genetic specification in the developing diencephalon of the lamprey. This prompted us to investigate how such a prosomeric organisation could be set up in the cyclostome embryo, and to search for potential upstream organising genes, such as those from the *Hedgehog* family.

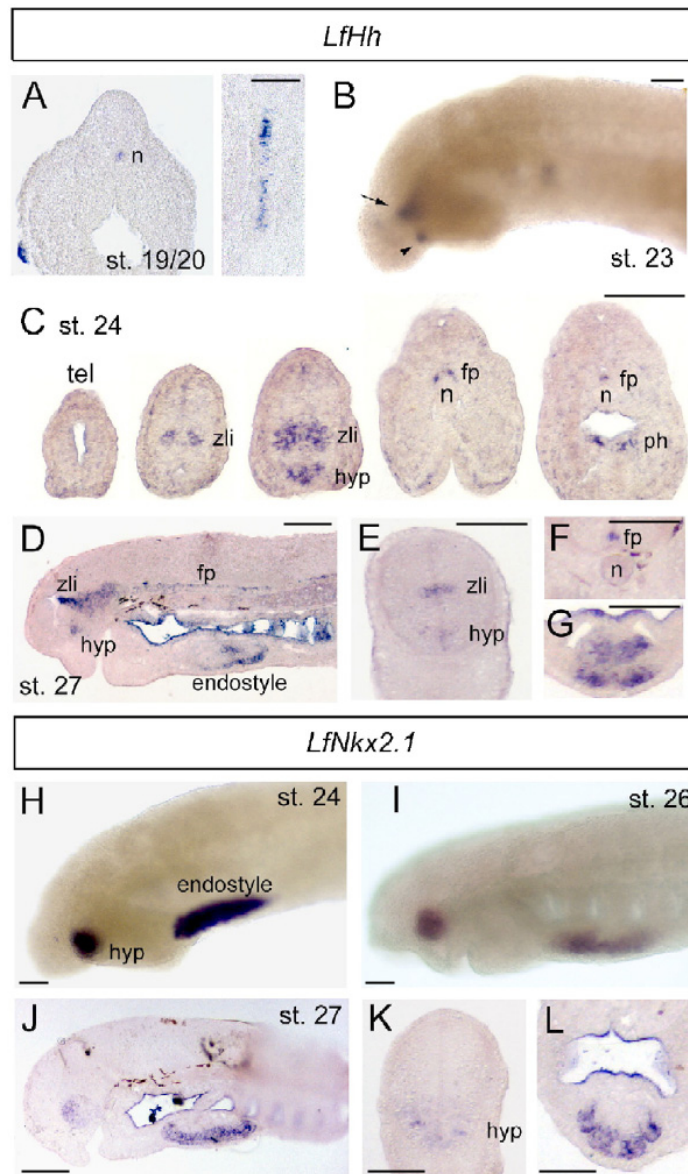


FIGURE 2.7: Expression of *LfHh* and *LfNkx2.1* in the developing embryo. (A–G) Expression of *LfHh* on sections and in toto (B) at different stages (indicated) of development. In panel B, the arrow points to the ZLI, and the arrowhead indicates the hypothalamic expression. Panel C presents transverse rostro-caudal sections encompassing all the *LfHh* expression domains. Panels D, E show a sagittal and a transverse section at stage 27, respectively. Panels F, G show close-up views of the floor plate and the endostyle expressions on coronal sections. (H–L) Expression of *LfNkx2.1* in toto (H,I) and on sections (J–L) at the indicated stages. Put side by side panels J to D, E to K, and G to L for a comparison of the two gene expression patterns. fp, floor plate; hyp, hypothalamus; n, notochord; ph, pharynx; tel, telencephalon; zli, zona limitans intrathalamica. Scale bar, 100  $\mu\text{m}$ .

**Hedgehog: *LfHh* as a cue for the emergence of a patterned brain**

Three orthology classes have been identified so far in the *Hedgehog* family in gnathostomes: *Indian (Ihh)*, *Desert (Dhh)*, and *Sonic (Shh) Hedgehog*. To isolate orthologous *L. fluviatilis* sequences, we used primers degenerated from *Ihh*, *Dhh* and *Shh* gnathostome sequences, taking advantage of the high conservation of Hedgehog protein motifs in gnathostomes. This strategy led to the amplification of a 276 bp cDNA fragment encoding the HH signal motif. Over this fragment, the deduced amino acid sequence was identical to a *Lampetra japonica* Hedgehog related sequence recently identified, LjHh (Uchida et al., 2003). This confirmed the assignment of the *L. fluviatilis* sequenced fragment to the *Hedgehog* family. In order to examine the relationships of this partial protein sequence with the 3 gnathostome classes, we included it into an alignment containing all the craniate Hedgehog sequences characterised so far. This alignment was used for a phylogenetic analysis using NJ and ML algorithms (Osorio et al., 2005, supplementary data, not shown). No robust support for the orthology of *LfHh* with either one of the three Hh subfamilies could be obtained<sup>2</sup>, but the data reported below showed that *LfHh* shares important expression features with *Shh*.

At early stages (st.19–20) *LfHh* was prominently expressed throughout the notochord (Fig. 2.7 A). Later, as shown at st.23 to 27, expression became apparent in the floor plate of the neural tube, in some discrete areas of the forebrain, in the ventral aspect of the pharynx, and in the endostyle derived from it (Fig. 2.7 B to G). Unlike its gnathostome homologs, at these stages, *LfHh* was not expressed as a continuous domain stretching along the ventral midline from the most anterior part of the hypothalamus to the spinal cord, with the intervening finger-shaped zona limitans intrathalamica(ZLI). Rather, *LfHh* forebrain expression pattern consisted in two separate domains (Fig. 2.7 B and D): (1) a (small) hypothalamic domain, and (2) a large and robust comma-shaped domain in the diencephalon, showing continuity with floor plate expression all along the neural tube (Fig. 2.7 C, D and F). At this level, the *LfHh* diencephalic expression was very similar to the *LfLhx15* expression territory, which is located in the basal plate and alar-basal boundary at the level of the p2/p3 junction, and might therefore correspond to the lamprey equivalent of the gnathostome ZLI. Notably, unlike in its gnathostome coun-

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<sup>2</sup>A project aiming at identifying *Hh* genes in lamprey, and the orthology analysis which followed, is presented in chapter 3.

terpart, at this stage, the *LfHh* “ZLI” domain did not send a finger-shaped extension far within the alar plate, and was not restricted to a thin line of one/two cell diameters (Fig. 2.7 C and E). While these expression features are strongly reminiscent of those displayed by *Shh* in osteichthyans, an important difference was observed at rostral levels: whatever the stage examined (up to st.29 ammocoete larvae, not shown) we could not detect any *LfHh* signal in the basal telencephalon (see st.27, Fig. 2.7 D), suggesting a lack of ventralising activity in the lamprey forebrain, a feature which is profoundly different from the situation in gnathostomes.

In *L. japonica*, the homeobox gene *Nkx2.1* is not expressed in the basal telencephalon (Ogasawara et al., 2001), in contrast to the situation reported in osteichthyans. Since *Nkx2.1* is known to be under the control of *Shh* signalling in osteichthyans, we sought to investigate whether *Nkx2.1* was also absent from the telencephalon of *L. fluviatilis*. Using primers designed on the *L. japonica* sequence, we isolated a full-length cDNA for its *L. fluviatilis* orthologue, termed *LfNkx2.1*. *LfNkx2.1* expression pattern showed unambiguously that this gene was strongly expressed in the hypothalamus and endostyle (Fig. 2.7 H–L) of the developing *L. fluviatilis*, but not in the ventral telencephalon at the studied stages 22 to 29 (Fig. 2.7 H–J).

#### 2.1.4 Discussion

We have isolated and studied the expression patterns of members of the *Lhx*, *Pax*, *Hh*, and *Nkx2.1* families in the developing river lamprey nervous system. The main results are as follows: (1) *Lhx* and *Pax* genes reveal highly similar forebrain and hindbrain patterning between cyclostomes and gnathostomes, (2) expression analysis of *LfLhx15* and *LfLhx29* and its interpretation shows that the lamprey forebrain presents a clear prosomeric organisation, which is thus a truly craniate character, and (3) analysis of the *LfHh* gene reveals the possible molecular origin of this neuromeric brain pattern.

#### Lamprey members of the *Pax*, *Lhx*, and *Hh* families

As the only representative of cyclostome craniates where developmental studies are technically possible (hagfish embryos are almost inaccessible<sup>3</sup>), lampreys

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<sup>3</sup>A recent review describes the history of hagfish embryological studies, and discusses recent attempts to obtain hagfish embryos both in the wild and in laboratory (Ota and Ku-

belong to the only available sistergroup of gnathostomes, essential to identify characters of ancestral craniates. They are becoming popular to study early craniate evolution (Kuratani et al., 2002), and to address the question whether gene duplication events correlate with craniate origins. Among few examples, we know that lampreys and gnathostomes underwent independent duplications of a tandem pair of *Dlx* genes that was present in the craniate ancestor (Neiderert et al., 2001)<sup>4</sup>. Likewise, their last common ancestor probably had only a single *Hox* gene cluster which was subsequently duplicated independently in the two lineages (Fried et al., 2003; see also Force et al., 2002; Sharman and Holland, 1998). Here we have characterised in *L. fluviatilis* members of five additional gene families, *Pax3/7*, *Lhx1/5*, *Lhx2/9*, *Hh*, and *Nkx*. A single gene could be identified in each case, while multigene families are present in all gnathostomes studied thus far. In this respect, it must be noted that the RT-PCR approach we used is not an exhaustive screening method, and that the existence of other family members in lampreys is still a possibility. The amino acid sequence of isolated genes was always very closely related to the one characterised in other lamprey species when available, either *P. marinus* (*Pax3/7*), or *L. japonica* (*Hh*, *Nkx2.1*). In contrast, these lamprey sequences cannot be clearly assigned to either of the orthology classes described in jawed craniates. In their most straightforward interpretation, these data suggest that the multigene families, which characterise jawed craniates (Panopoulou et al., 2003; Robinson-Rechavi et al., 2004), may have emerged after the split between cyclostomes and gnathostomes. A more exhaustive characterisation of the gene content in lampreys but also other cyclostomes, relying on genomic data and taking synteny arguments into account, will be important to reach definitive conclusions as to the chronology of craniate duplication events and their possible correlation with the emergence of craniate characteristics. Even though the relationship between lamprey and gnathostome genes remain unresolved, lamprey genes provide insights on the evolution of expression features of the studied families.

For instance, we report an expression of the lamprey *Pax3/7* family in

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ratani, 2006). This research group has successfully obtained hagfish embryos at the pharyngula stage, and published a pioneer molecular and embryological study on the evolution of the neural crest (Ota et al., 2007).

<sup>4</sup>New analyses currently performed at Marc Ekker's and Shigeru Kuratani's laboratories will hopefully soon provide new information on the organisation of *Dlx* gene clusters in lamprey.

the dorsal midline of the entire neural tube, including the forebrain, for the first time. In chick, both *Pax3* and *Pax7* are expressed in the entire length of the tube at early stages, with a clear distinction at the p1/p2 boundary (Matsunaga et al., 2001). In mouse, *Pax3* and *Pax7* are present in the alar and/or the roof plate, along the entire axis (Stoykova and Gruss, 1994). Thus, although not always the same and with temporal variations, at least one paralog of the *Pax3/7* group is expressed in the telencephalic dorsal midline of craniates, illustrating a probable expression shuffling between paralogs during evolution in this crucial signalling centre for patterning the pallium. The expression of *LfPax37* reported here might indicate that the cyclostome pallium shares more common features with the gnathostome pallium than could have been expected from neuroanatomical studies: indeed, the lamprey forebrain exhibits a poorly evaginated telencephalon with large olfactory bulbs, and ambiguities remain regarding homologies with gnathostome subdivisions (Nieuwenhuys and Nicholson, 1998).

Similarly, the *LfLhx15* pattern is globally highly similar to the combined expressions of the two gnathostome paralogs, suggesting that the family expression territories are largely conserved between cyclostomes and jawed craniates. However, this comparison also suggests that some new expression domains have been added in the latter: we did not detect *LfLhx15* expression in the tectum or in the dorsal part of the ventral thalamus, where *Lhx1* and/or *Lhx5* are transcribed in tetrapods (Moreno et al., 2004).

*LfLhx29* also shows prominent telencephalic, eye, and dorsal thalamic expressions which are reminiscent of those reported in gnathostome model organisms, suggesting that they might correspond to ancestral features of the family in craniates (Alunni et al., 2004; Bachy et al., 2001; Moreno et al., 2004; Retaux et al., 1999). Again, we cannot exclude the possibility of other *Lhx2/Lhx9* family members in lamprey. Nevertheless, the expression of *Lhx2/9* genes in the pineal gland may provide another example of expression shuffling: *OlLhx9* and *mLhx9* are expressed in medaka fish and mouse pineal, respectively, whereas *xLhx2* but not *xLhx9* is expressed in *Xenopus* pineal gland (Retaux et al., 1999; Bachy et al., 2001; Alunni et al., 2004). It would be interesting to analyse the *Lhx2/9* group in chondrichthyans and in diapsids, together with genomic analyses, to understand the process and timing of this shuffling which implies generally poorly understood mechanisms (Locascio et al., 2002).

Finally, orthologues of *Lhx2/9* have been characterised in very distant



groups. *C. elegans ttx-3* is expressed in the AIY neuron involved in thermoregulatory function (Altun-Gultekin et al., 2001; Hobert et al., 1997), and *Drosophila apterous* is expressed in the brain and eyes (Lu et al., 2000; Rincon-Limas et al., 1999). Besides pineal expression, the gnathostome *Lhx2* has also prominent expression and function in the eye (Porter et al., 1997). Thus, the *Lhx2/9/apterous LIM-homeobox* members show evolutionary conserved expression (and function?) in organs related to phototransduction. Interestingly, an evolutionary link has been suggested between thermo-sensation (the function where *ttx-3* is involved) and phototransduction (Satterlee et al., 2001)<sup>5</sup>.

### Neuromeric organisation of the embryonic lamprey brain

**Four diencephalic prosomeres** We relied on *LfPax6* to determine the mesencephalic/diencephalic boundary, on *LfPax3* to assess the p1/p2 border, on the alternate banded patterns of *LfLhx15* and *LfLhx29* to delineate the p2 (dorsal thalamus) and p3 (ventral thalamus, or prethalamus) presumptive domains, on the expression of *LfHh* to hypothesise the existence of a “zona limitans-like” in the lamprey diencephalon, and on the combined expressions of *LfPax6*, *LfLhx15* and *LfLhx29* to identify telencephalic subdivisions. This led us to propose the interpretation presented in Fig. 2.8 B, where we recognise four prosomeric divisions in the diencephalic forebrain, and where the global conservation of the organisation when compared to jawed craniates is surprisingly high (see Fig. 2.9). In their interpretation of the developmental plan of the lamprey brain, Murakami and collaborators (Murakami et al., 2001) could not resolve prosomeric divisions rostral to p2, due to the lack of markers. Here, we can clearly identify p2 and p3, and also the tel-diencephalic border, by *Lhx* expression (Fig. 2.4 C and D). In addition, between p3 and the telencephalon, we could then identify a subdivision that is closely associated to the telencephalon but clearly distinct from it morphologically, and which — surprisingly — expresses both *LfLhx15* and *LfLhx29*. We tentatively call it p4, as it may represent the equivalent of the eminentia thalami<sup>6</sup>, being contiguous but distinct from the medial pallium. In the diencephalon of *Xeno-*

<sup>5</sup>The isolation of the *Lhx9* gene in the dogfish (*Scyliorhinus canicula*) is described in chapter 3, where the idea of a function shuffling between members of the *Lhx2/9* group is also further discussed.

<sup>6</sup>See also this chapter’s general discussion in section 2.4. Note that this region, known for a long time as the eminentia thalami, is now called prethalamic eminence.

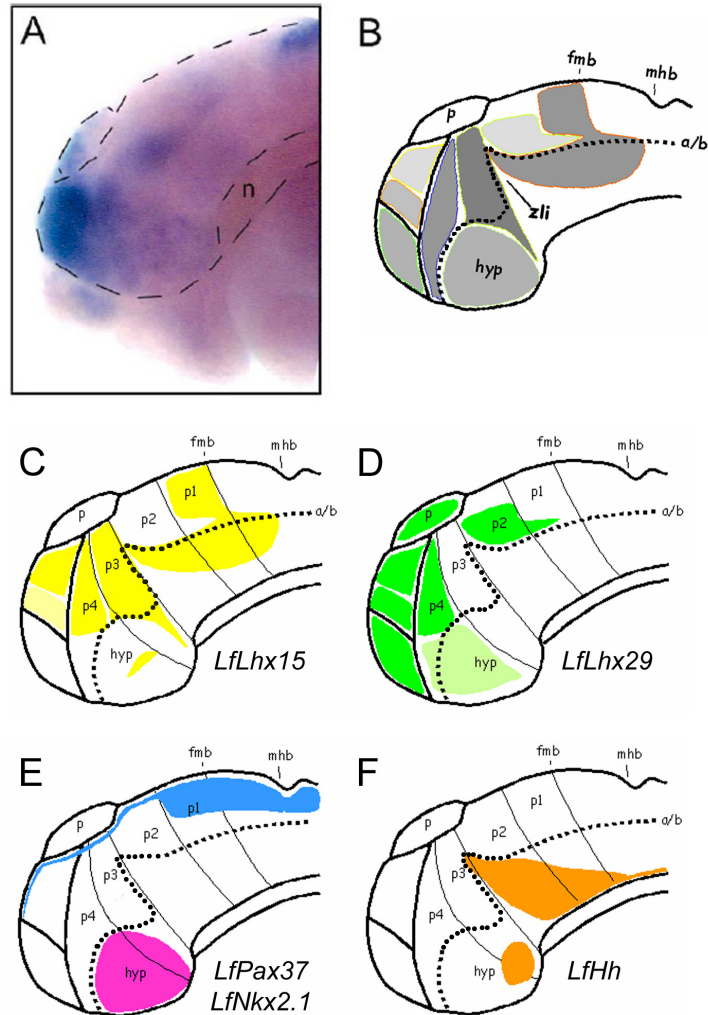


FIGURE 2.8: Organisation of the embryonic lamprey forebrain. Summary illustration and interpretation of the expression data presented in the paper. In panel A, a photomicrograph of a stage 24 embryo hybridised with *LfLhx29* without benzylbenzoate clearing is presented as this gene clearly highlights the anatomical and neuromeric organisation of the embryonic lamprey brain. Panel B shows a composite drawing where the main expression domains of the studied genes are compiled in different shades of grey, allowing the identification of the p1/p2 boundary (*LfPax37*), p2/p3 boundary (*LfLhx29* and *LfLhx15*), ZLI (*LfLhx15* and *LfHh*), p4 prosomere (*LfLhx29* and *LfLhx15*), telencephalic/diencephalic border (*LfLhx29* and *LfPax6*) and 3 telencephalic subdivisions (*LfLhx29*, *LfLhx15* and *LfPax6*). Panels C–F show the detailed interpretation of expression patterns of the five main genes studied in the present paper. The thick dotted line indicates the alar/basal boundary (a/b), and thin lines indicate the tentative prosomeric borders. mhb, mid–hindbrain boundary; fmb, fore–midbrain boundary; n, notochord.

*pus*, *xLhx1/xLhx5* and *xLhx2/xLhx9* present a strict alternation of expression. The co-expression of *LfLhx15* and *LfLhx29* in p4 might thus indicate that this subdivision is symptomatic of the progressive establishment of a neuromeric brain in basal craniates, by setting up and organising the patterned expression of developmental regulators. Interestingly, although a recent variation of the prosomeric model only recognises three diencephalic prosomeres in amniotes (Puelles and Rubenstein, 2003), the first prosomeric map of the adult lamprey brain clearly identified p1 to p4 (Pombal and Puelles, 1999). Our observations and interpretations in the embryonic brain would fit well with the older version of the prosomeric model, and also with the claim of Pombal and Puelles (1999) that the minimal morphogenetic deformation of the forebrain renders easy the observation of prosomeric boundaries in lampreys.

**A zona limitans intrathalamica expressing *LfHh*** Our conclusions on the neuromeric nature of the embryonic lamprey forebrain indicate that this major innovation in the way to build a forebrain arose in the common ancestor of craniates. Indeed, neither cephalochordates nor urochordates show such a patterned organisation of their anterior central nervous system (Holland and Holland, 1999; Mazet and Shimeld, 2002)(Fig. 2.9). This prompted us to search for upstream organising activities, and more specifically to ask whether a zona limitans intrathalamica (ZLI) with Hedgehog signalling activity was present in the developing lamprey diencephalon. A ZLI has not always been recognised in lampreys (see discussion of Pombal and Puelles, 1999), and the identification of a secondary organiser of this type in this species is thus of major interest to understand forebrain evolution. The *LfHh* pattern reported here is highly supportive of the existence of such a signalling centre. However, this *LfHh* pattern is different from that of gnathostomes (Fig. 2.9). Indeed, *LfHh* is expressed as patches in the hypothalamus and inter-thalamic regions, but not as a continuous band at the anterior ventral midline — which is the prototypical *sonic hedgehog* (*Shh*) pattern in other craniates. The mechanisms of ZLI formation begin to be deciphered (Kiecker and Lumsden, 2004; Larsen et al., 2001; Zeltser, 2005): the ZLI differentiates and progresses dorsally under the influence of *Shh*-dependent signals from the basal plate, and gets restricted to a narrow line by inhibitory signals from the dorsal diencephalon (Zeltser, 2005). Interestingly, we found here that the lamprey ZLI was rather large, suggesting that the inhibitory constraining mechanisms emanating from the

dorsal thalamus might not be in place or might be shifted later in development in lamprey embryos, and illustrating how the progressive establishment of this signalling centre might be set up by recruitment of gene cascades during evolution. In addition, the identification of the *LfHh* expressing, comma-shaped domain as the ZLI is reinforced by the finding that it also expresses *LfLhx15*. This finding places *Hh* as a potential upstream gene for the LIM-homeobox factor *Lhx1/5*, and is similar to our previous findings in *Xenopus* (Bachy et al., 2001) and mouse (unpublished observations) where *Lhx1* and *Lhx5* clearly label the ZLI — although in these two species they are expressed as very thin bands of one/two cell diameters<sup>7</sup>.

Finally, contrarily to gnathostomes, *LfHh* was not expressed in the ventral telencephalon (Fig. 2.9). *Shh* is known to control the expression of the homeobox gene *Nkx2.1* (Pabst et al., 2000) and vice-versa (Sussel et al., 1999), and *Nkx2.1* is itself crucial for the development of the hypothalamus and the pallidal part of the basal ganglia (Sussel et al., 1999). As *Nkx2.1* expression seems repeatedly absent from the ventral forebrain in two species of lampreys, *L. japonica* (Ogasawara et al., 2001) and *L. fluviatilis* (our results) we can postulate that the *de novo* expression of *Shh* and *Nkx2.1* in the basal telencephalon of gnathostomes was required for the emergence of a new brain structure: the pallidum (see also Murakami et al., 2005). Of note, we also found expression of both *LfHh* and *LfNkx2.1* in the developing endostyle, the future thyroid organ of the lamprey, suggesting that this developmental regulatory “cassette” was indeed recruited independently in several regions of the embryo. However and importantly, we cannot exclude the possibility that *Hh* and *Nkx2.1* were actually expressed in the ventral forebrain of the ancestor of cyclostomes and gnathostomes, and that this “cassette” has been selectively lost in lampreys.

**A segmented hindbrain with emerging LIM-hd code** During development, the craniate hindbrain is subdivided along its antero-posterior axis into a series of segmental bulges called rhombomeres, which in turn generate a repeated pattern of rhombomere-specific neurons. Among these, the reticulospinal neurons are involved in the modulation of visceral and somatic spinal motor responses. They form discrete groups with specific antero-posterior and medio-lateral locations in the hindbrain, and they establish stereotypic

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<sup>7</sup>A project aimed at testing the potential role of the Shh protein to change neuronal specification is outlined in the perspectives of this chapter (section 2.5).

topographic axonal connections towards the spinal cord. Lampreys have reticulospinal neurons, which are involved in swimming behaviour. A rhombomeric organisation of reticulospinal neurons exists in lampreys, as deduced from segmented patterns of regulatory gene expression and from the study of hindbrain connectivity (Murakami et al., 2004). For example, Mauthner cells were found in rhombomere 4 (r4), suggesting that a r4-specific program to generate this neuronal type has been conserved in evolution. On the other hand, factors of the *Lhx* family are largely implicated through a so-called “LIM-hd code” in the establishment of proper topographical connections of the spinal and cranial motoneurons (Shirasaki and Pfaff, 2002; Tsuchida et al., 1994; Varela-Echavarría et al., 1996). The beginnings of a similar code were reported recently in the reticular formation of *Xenopus* (Moreno et al., 2005), and in chick and mouse reticulospinal neurons (Cepeda-Nieto et al., 2005). There, *Lhx1* and/or *Lhx5* (together with *Lhx3/4*) are expressed in rhombomeric patterns in two columns of ipsi- and contra-laterally projecting reticulospinal neurons. Our data show that these two columns of neurons also segmentally express *Lhx15* in lampreys. Moreover, the more dorsally-located branchial motoneurons of the lamprey hindbrain also express *Lhx15* and *Lhx29* in specific rhombomeric patterns<sup>8</sup>. Thus, although only one paralog might exist in lamprey for each *Lhx* subgroup, the expressions of *LfLhx15* and *LfLhx29* highlight the segmental organisation of the cyclostome hindbrain, and reveal the premises of an emerging LIM-hd code of neuronal specification and axonal pathfinding in the brain of a basal craniate.

**Hh/Shh: a motor of forebrain evolution?** In cephalochordates (amphioxus) and urochordates (tunicates), comparative molecular studies have recognised a cerebral/sensory vesicle which expresses *Otx* and which could correspond to diencephalic (di) and midbrain (mb) regions, together with a hindbrain and a spinal cord expressing *Hox* genes (reviewed in Holland and Holland, 1999; Wada and Satoh, 2001). In amphioxus as well as ascidians, the cerebral/sensory vesicle expresses *Nkx2.1*, and an equivalence with the craniate hypothalamus has been proposed (Moret et al., 2005; Venkatesh et al., 1999). On the other hand, cephalochordates and urochordates lack a telen-

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<sup>8</sup>After our studies in the larval brain (Osorio et al., 2006), we know now that this dorsal expression probably corresponds to the population of sensory neurons projecting to the octavolateral nuclei.

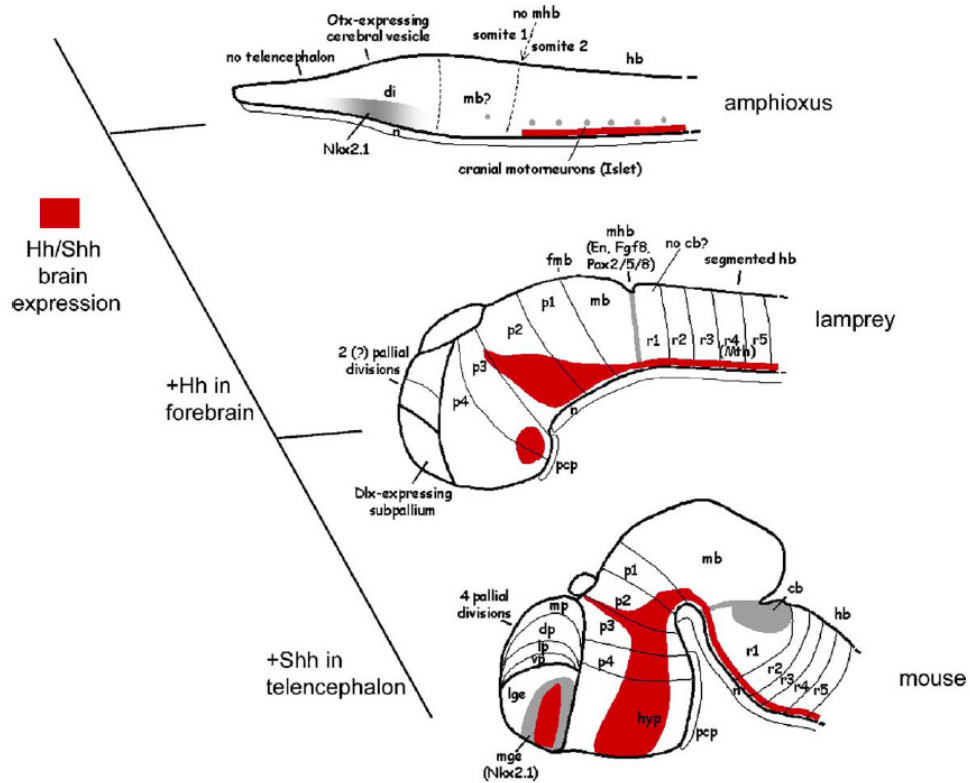


FIGURE 2.9: *Hh/Shh* and evolution of brain patterning among chordates. A highly simplified tree represents the organisation of the brain in amphioxus, lamprey and mouse and highlights the potential crucial role of *Hh/Shh* midline signalling as a “motor” of forebrain evolution. The amphioxus drawing is compiled from Holland and Holland (1999), Jackman et al. (2000) and Wada and Satoh (2001). The expression of *Hh/Shh* in the neural tube is drawn in pink, and the major innovations in brain patterning which are discussed in text are in grey. Note that, in amphioxus, the notochord (n) extends to the anterior tip of the neural tube and that a prechordal plate (pcp) is a craniate-specific character. The existence of four pallial divisions in amniotes has been proposed by Puelles et al. (2000). See text for details. cb, cerebellum; dp/lp/mp/vp, dorsal/lateral/medial/ventral divisions of the pallium; di, diencephalon; fmb, fore–midbrain boundary; hb, hindbrain; hyp, hypothalamus; lge/mge: lateral and medial ganglionic eminences; mb, midbrain; mhb, mid–hindbrain boundary; Mth, Mauthner cell; p1–p4, prosomeres 1 to 4; r1–5: rhombomeres 1 to 5.

cephalon and a mhb secondary organiser (Holland and Holland, 1999; Wada and Satoh, 2001). Moreover, their hindbrain is not segmented (lack of overt morphological boundaries and segmental gene expression), although cell-type segmentation can be recognised with genes such as *Islet* which marks cranial motoneurons in amphioxus (Jackman and Kimmel, 2002; Jackman et al., 2000, Fig. 2.9). In summary, even though the global rostro-caudal order of regulatory gene expression is roughly conserved among chordates, major differences in patterning mechanisms can be recognised in craniates, one of them being the presence of secondary organisers. In this respect, the expression of *Hh* in cephalochordates and urochordates is fundamentally different from craniates: in *Ciona*, *Ci-hh2* is expressed in the ventral nerve cord – but only at posterior levels, and is not expressed in the notochord (Takatori et al., 2002); in amphioxus, *AmphiHh* is expressed in the notochord (which runs to the tip of the cerebral vesicle), and, again, only in the posterior aspect of the ventral neural tube (Shimeld, 1999). Thus, *Hh* probably does not play a direct role in the regionalisation of the anterior nervous system in these species<sup>9</sup> (Fig. 2.9).

By contrast, in lampreys and jawed craniates, the patterning of the central nervous system shares many similarities. Major innovations within this group include a segmented hindbrain (Murakami et al., 2004, this study), a mid-hindbrain boundary organising centre (for a review, see Murakami et al., 2005), and a regionally-organised forebrain which develops according to a prosomeric pattern (this study, Fig. 2.9). The latter is strikingly correlated to the expression of *Hh/Shh* in the ventral aspect of the anterior brain, including the emergence of a ZLI secondary organising centre. Finally, the expression of *Shh* (and *Nkx2.1*) in the ventral telencephalon of osteichthyans seems to have allowed the emergence of a novel subpallial division, the MGE<sup>10</sup> (see also Murakami et al., 2005). Taken together, these observations suggest that important changes in *Hh/Shh* midline signalling at the urochordate/craniate and cyclostome/gnathostome splits have been a driving force for forebrain evolution.

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<sup>9</sup>For more on the roles of Hh signalling in the development and evolution of chordates, see the general discussion of this chapter (section 2.4).

<sup>10</sup>The embryonic region which will give rise to the pallidum.

## 2.2 Dynamic expression of the *LIM-homeobox* gene *Lhx15* through larval brain development of the sea lamprey (*Petromyzon marinus*)

### 2.2.1 Introduction

Multiple roles of the *LIM-homeobox* (*Lhx*) gene family of transcription factors in the development of the craniate nervous system have been identified. They are crucial in the regionalisation and patterning of the neural tube, and in the establishment of neuronal phenotypes (reviewed in Hobert and Westphal, 2000; Shirasaki and Pfaff, 2002; Subramanian et al., 2003; Hunter and Rhodes, 2005). Studies on several representatives of the major craniate groups – mouse, *Xenopus*, axolotl, medaka and lamprey (Bachy et al., 2001; Alunni et al., 2004; Moreno et al., 2004; Showalter et al., 2004; Moreno et al., 2005; Osorio et al., 2005) – have shown a high degree of structural and functional conservation of this family throughout evolution (Rincon-Limas et al., 1999).

The expression patterns of *Lhx* genes have been previously analysed in the embryonic lamprey (*Lampetra fluviatilis*) brain (Osorio et al., 2005). This study and earlier findings on other patterning genes (Ueki et al., 1998; Tomsa and Langeland, 1999; Ogasawara et al., 2000; Murakami et al., 2001; Myojin et al., 2001; Neidert et al., 2001; Ogasawara et al., 2001; Boorman and Shimeld, 2002; Derobert et al., 2002a), are consistent with the idea of a prosomeric organisation of the lamprey brain, first proposed in adults by Pombal and Puelles (1999). As in other craniates, the lamprey representatives of the *Lhx1/5* and *Lhx2/9* paralog groups, respectively, named *LfLhx15* and *LfLhx29*, are expressed in a complementary fashion in the dorsal part of diencephalic prosomeres (Osorio et al., 2005). Furthermore, these genes are co-expressed in the hypothalamus, prethalamic eminence and telencephalic pallium, but not in the subpallium, where *Lhx15* mRNA is absent. Interestingly, *Lhx15* expression levels are also distinct in the dorsal and ventral aspects of the pallium.

Lampreys undergo a very long non-embryonic period of development (more than 5 years; Beamish and Potter, 1975) preceding metamorphosis. Knowledge on brain gene expression patterns throughout this particular larval period is virtually absent, although data on neurotransmitter distribution and neuroanatomy are available (Weigle and Northcutt, 1999; Zielinski et al., 2000; De Andres et al., 2002; Melendez-Ferro et al., 2002a,b; Perez-Costas et al.,



2002; Pierre-Simons et al., 2002; Villar-Cheda et al., 2002; Melendez-Ferro et al., 2003; Vidal Pizarro et al., 2004). In *Xenopus*, *Lhx* genes have proven useful to follow the morphogenesis of brain nuclei, due to their persistent expression from embryogenesis to the adult stage (Moreno et al., 2004, 2005). In order to characterise the molecular basis of regional identity in the brain of lamprey larvae, we undertook an analysis of *Lhx15* expression on animals of different ages. We will follow the nomenclature of Pombal and Puelles (1999), including the modifications introduced in the simplified prosomeric model recently proposed by Puelles and Rubenstein (2003).

### 2.2.2 Materials and methods

#### Animals

Sea lamprey (*Petromyzon marinus*) larvae of several sizes (34–108 mm) were captured in early summer in the Miño river (Northwest of Spain). Animals were kept in aerated fresh water prior to experimentation. Procedures conformed to European Community Guidelines on animal experimentation. After anaesthesia (0.4 mg/ml MS222, Sigma Chemical Co., St. Louis, MO), the heads were cut and fixed overnight in 4% paraformaldehyde, dehydrated in methanol and stored at  $-20^{\circ}\text{C}$  in 100% methanol. After re-hydration, the brains were dissected out and processed for *in situ* hybridisation. River lamprey (*Lampetra fluviatilis*) embryos and prolarvae were processed as previously described (Osorio et al., 2005).

#### Whole-mount *in situ* hybridisation

A digoxigenin-labelled antisense riboprobe against *Lampetra fluviatilis Lhx15* mRNA (*LfLhx15*, GenBank Accession No. DQ002012) was synthesised and used for *in toto in situ* hybridisation. This probe recognises specifically regions of *Lhx15* expression in both lamprey species when hybridised at very high stringency, ensuring that the hybridisation signals are strictly specific. *In situ* hybridisation was carried out as described in Osorio et al. (2005). Samples were progressively re-hydrated, treated with proteinase K (10  $\mu\text{g}/\text{ml}$ , 45 min), and fixed again with MEMFA (0.1M MOPS, 2 mM EGTA, 1 mM  $\text{MgSO}_4$  and 3.7% formaldehyde) for 20 min before pre-hybridisation (2–4 h at  $65^{\circ}\text{C}$ ). Hybridisation was carried out overnight at  $70^{\circ}\text{C}$  in a 50% formamide hybridisation medium containing 1  $\mu\text{g}/\text{ml}$  of DIG-labelled RNA probe. Specimens

were incubated with alkaline phosphatase (AP)-conjugated anti-digoxigenin Fab fragments (diluted 1:1500; Roche Diagnostics, Mannheim, Germany) at 4°C. Alkaline phosphatase activity was detected with NBT/BCIP (Roche Diagnostics, Mannheim, Germany). For histological observation, specimens were dehydrated in ascending ethanol after fixation, embedded in paraffin and sectioned in either frontal, sagittal or horizontal planes with a Leica microtome at 10–12  $\mu\text{m}$ . Photographs were taken on a Nikon microscope equipped with a DXM-1200 camera.

### 2.2.3 Results and discussion

#### Conservation

In the telencephalon of young larvae (representative sections of a 34 mm larva are shown in Fig. 2.10), *Lhx15* mRNA is detected in the outer regions of olfactory bulbs (OB, Fig. 2.10 A), probably corresponding to the mitral cell layer, and in the medial pallium (MP, Fig. 2.10 B and C). Strong expression is also detected in the preoptic area (Poa, Fig. 2.10 B), in the magnocellular preoptic nucleus (PMg, Fig. 2.10 B), as well as in prosomere p3, which includes the prethalamus (PTh, Fig. 2.10 D and E, previously named ventral thalamus; for the new prosomeric nomenclature see Puelles and Rubenstein, 2003) and the dorsally located prethalamic eminence (PE, Fig. 2.10 D; previously named thalamic eminence of the p4 prosomere, see Pombal and Puelles, 1999 and Osorio et al., 2005). The rostral limit of this new p3 subdivision has been defined by the expression of several genes, including *Dlx* and *Pax6*, which also label this boundary in the lamprey brain (Murakami et al., 2001; Neidert et al., 2001; Derobert et al., 2002a; Osorio et al., 2005). On the other hand, *Lhx15* expression is absent in the thalamus (p2) (Th, Fig. 2.10 D–F, previously called dorsal thalamus). Posteriorly, *Lhx15* transcripts are present in the pretectal region (p1), but not in the midbrain (PT, MB, Fig. 2.10 D and E). Ventrally, the strong hypothalamic expression in the tuberal and mammillary nuclei (TN, TM, Fig. 2.10 D–F) is continuous caudally with the mammillary area (M, Fig. 2.10 G).

In the hindbrain, two bands of expression extend posteriorly, parallel one to another. One is located in the basal plate and is divided in two dorso-ventral (latero-medial) populations, the most ventral one showing the strongest *Lhx15* expression (Fig. 2.10 H–L). This thick band clearly includes the interpeduncu-

lar nucleus and the reticular formation, which is organised in three rostrocaudal nuclei, i.e., anterior, intermediate and posterior reticular nuclei. Most of the cells in this longitudinal band, if not all, seem to express *Lhx15*. Of note, the rostral cholinergic cell group described by Pombal et al. (2001) seems to be also included in this *Lhx15*-expressing, rostralmost part of the isthmus. The other *Lhx15*-expressing band is located dorsally, in the hindbrain alar plate, extending from the isthmic region to the caudal hindbrain (Fig. 2.10 H–L). The single population identified rostrally segregates into three nuclei in the middle that finally join together again caudally. This pattern is probably due to the arrangement of the octavolateral area into the dorsal, medial and ventral octavolateral nuclei (dn, mn and vn, Fig. 2.10 K; (see Nieuwenhuys and Nicholson, 1998). These nuclei receive primary afferents mostly from the octaval nerve and the anterior and posterior lateral line nerves. In the caudal hindbrain, the *Lhx15* expression reaches the level of the spinal cord, thus including the dorsal column nucleus (dcn, Fig. 2.10 L). The presence of *Lhx15* expression in the octavolateral system and dorsal column nucleus is thus comparable to that reported in *Xenopus* (Moreno et al., 2005). The negative gap between these two bands of *Lhx15* expression is occupied by the visceromotor column, which is rostrocaudally composed of the trigeminal (Fig. 2.10 I), facial (Fig. 2.10 J), glossopharyngeal (Fig. 2.10 K) and vagus (Fig. 2.10 L) motor nuclei. According to the scarce cell migration exhibited by the young larval lamprey brain, most of the *Lhx15* expression is located close to the ventricle in the mantle zone, with the exception of the olfactory bulbs.

Thus, the *Lhx15* expression pattern resembles that observed in the embryonic and prolarval lamprey brain (Fig. 2.11 A; Osorio et al., 2005), showing the persistence of expression and the usefulness of this marker to follow particular structures through development. As in prolarvae, the expression of this gene is detected in the pallium, prosomeres p1 and p3 (including the prethalamus eminentia and prethalamus), hypothalamus and two conspicuous longitudinal bands in the hindbrain. In larvae, a pallial expression corresponding to the olfactory bulbs is now clearly discernible. Furthermore, the organisation of prethalamus and hypothalamus nuclei is clearly visible. Interestingly, no *Lhx15* expression is detected in the thalamus (p2) and midbrain, showing that the prosomeric specificity of this gene is maintained.

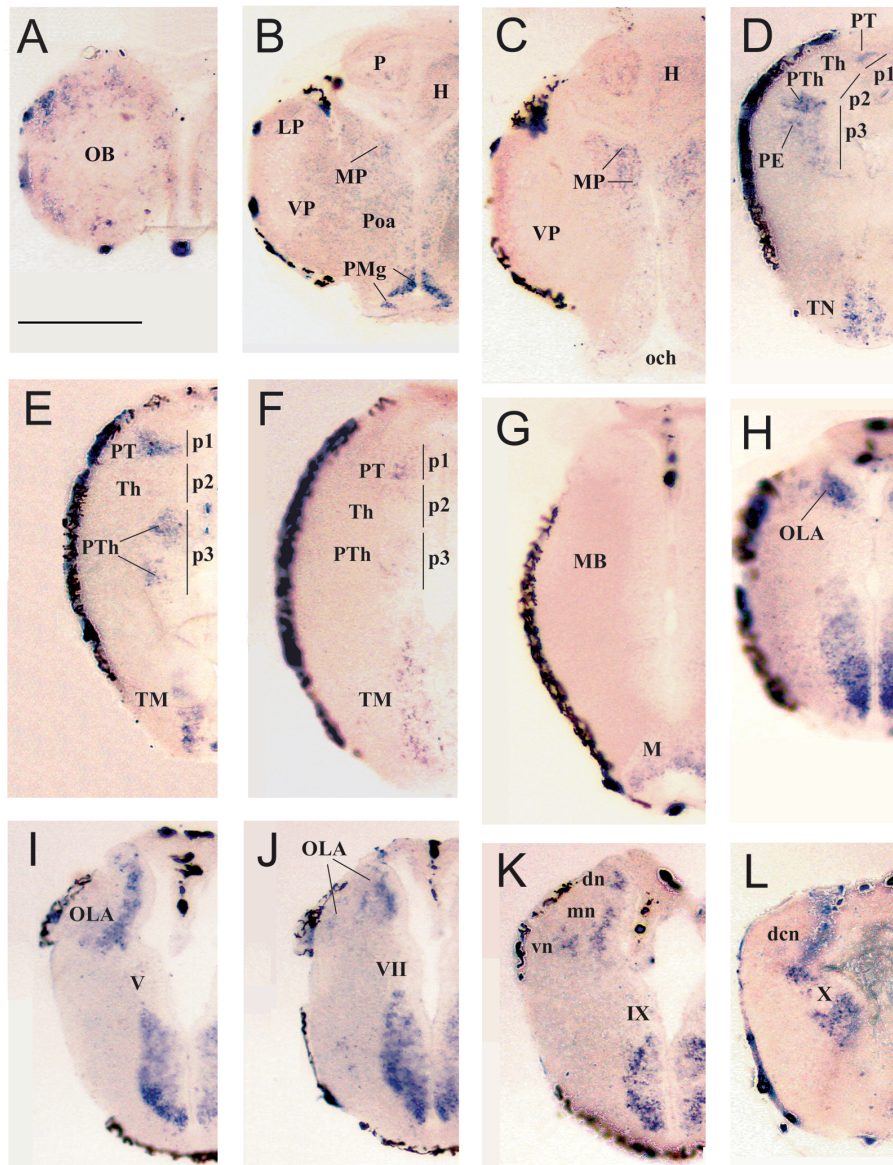


FIGURE 2.10: Expression of *Lhx15* in a young larva. Expression of *Lhx15* in a 34 mm larva, analysed in transverse sections, rostral (A) to caudal (L). dcn, dorsal column nucleus; dn, dorsal nucleus of the octavolateral area; H, habenula; IX, glossopharyngeal nucleus; LP, lateral pallium; M, mammillary area; MB, midbrain; mn, medial nucleus of the octavolateral area; MP, medial pallium; OB, olfactory bulb; och, optic chiasma; OLA, octavolateral area; p1–3, prosomeres 1–3; P, pineal; PE, prethalamic eminentia; PMg, magnocellular preoptic nucleus; Poa, preoptic area; PT, preteetum; PTh, prethalamus; Th, thalamus; TN, tuberal nucleus; TM, tuberomammillary nucleus; V, trigeminal motor nucleus; VII, facial motor nucleus; VP, ventral pallium; vn, ventral nucleus of the octavolateral area; X, vagal motor nucleus. Scale bar, 100  $\mu\text{m}$ .

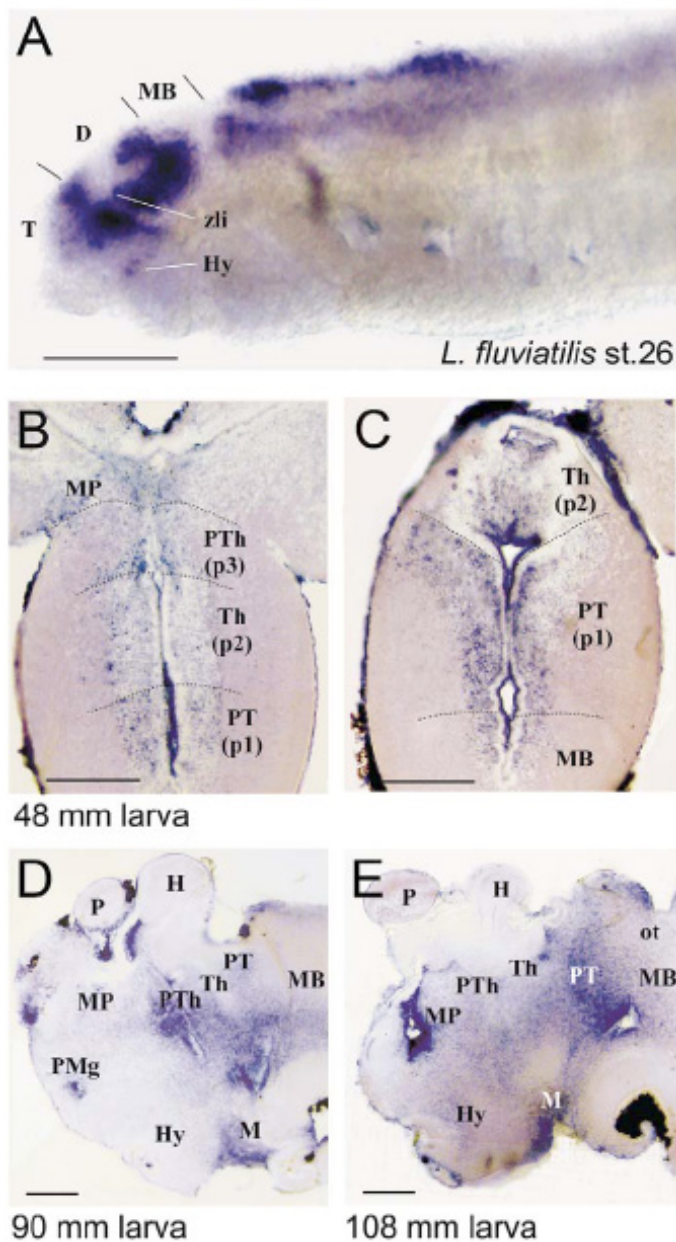


FIGURE 2.11: Expression of *Lhx15* through development. Comparative expression of *Lhx15* in the developing brain: (A) in toto *Lampetra fluviatilis* stage 26 embryo, for comparison; (B and C) horizontal sections of a 48 mm larval forebrain, where B is more ventral than C; (D and E) sagittal sections of 90 mm (D) and 108 mm (E) larval brains, at a medial level. D, diencephalon; H, habenula; Hy, hypothalamus; M, mammillary area; MB, midbrain; MP, medial pallium; ot, optic tectum; p1–3, prosomeres 1–3; P, pineal; PMg, magnocellular preoptic nucleus; PT, pretectum; PTh, prethalamus; T, telencephalon; Th, thalamus; zli, zona limitans intrathalamica. Scale bar, 100  $\mu\text{m}$ .

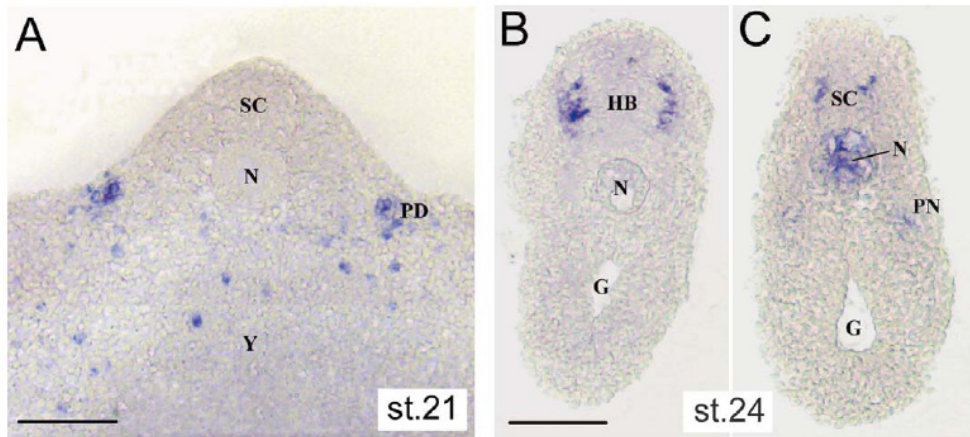


FIGURE 2.12: Expression of *Lhx15* in lamprey embryos and prolarvae. Expression of *Lhx15* in neural and non-neural tissues of *Lampetra fluviatilis* embryos and prolarvae: (A) transverse section of a stage 21 embryo; (B and C) transverse sections of a stage 24 prolarva, where B is rostral to C. G, gut; HB, caudal hindbrain; N, notochord; PD, pronephric duct; PN, pronephros; SC, spinal cord; Y, yolk. Scale bar, 100  $\mu\text{m}$ .

### Dynamics

The expression of *Lhx15* was then followed in a series of increasingly older animals. Fig. 2.11 shows representative examples taken from 48 mm (Fig. 2.11 B and C), 90 mm (Fig. 2.11 D) and 108 mm (Fig. 2.11 E) larvae, to be compared with expression at embryonic stage 26 (Fig. 2.11 A) and the young larva shown in Fig. 2.10. In these older larvae, *Lhx15* expression became more widespread and diffuse, and prosomeric borders were less clear, although some regions never expressed this gene (like the dorsalmost part of p2, Fig. 2.11 D and E). Notably, older larvae seem to express *Lhx15* in the ventral portion of the thalamus (Th, Fig. 2.11 D and E), whereas this region is strictly *Lhx15*-negative in younger animals. This situation resembles that reported for *Xenopus*, where *Lhx1*-expressing cells are detected in the ventral portion of the thalamus of late larvae and adult animals, while in embryos this factor is always absent in p2 (Moreno et al., 2004).

In conclusion, the *Lhx15* pattern in larval lampreys shows both conserved and dynamic features when compared to the embryonic/prolarval expression pattern, and strengthens the idea that *Lhx* factors are useful markers to follow brain morphogenesis.

### Embryonic expression of *Lhx15* outside the brain

The data obtained so far show that *Lhx15* expression in the lamprey embryonic and larval brain is remarkably similar to that of its gnathostome homologs, *Lhx1* and *Lhx5* (Osorio et al., 2005; present paper). We next sought to investigate if this similarity is maintained in the other embryonic structures where the expression of these two genes has been reported, namely the spinal cord (*Lhx1* and *Lhx5*), the urogenital system (*Lhx1*) and the notochord (*Lhx1*).

In the spinal cord, *Lhx15* expression is absent at stage 21, but strong at stage 24 (SC, Fig. 2.12 A and C). While in the hindbrain there was always more than one longitudinal stripe of *Lhx15* mRNA in each rhombomere (Osorio et al., 2005), these stripes seem to converge to a single lateral one in the spinal cord. This organisation resembles what was observed in zebrafish, *Xenopus*, mouse and chick embryos for both *Lhx1* and *Lhx5* (Barnes et al., 1994; Fujii et al., 1994; Taira et al., 1994; Toyama et al., 1995; Sheng et al., 1997; Toyama and Dawid, 1997), although the number of stripes in the spinal cord varies with the species and paralog member. In larvae, *Lhx15*-positive labelling is detected in the columns of spinal motoneurons, in lateral interneurons and in edge cells (data not shown).

At early embryonic stages (st.21, Fig. 2.12 A), high levels of *Lhx15* mRNA are detected in the pronephros and pronephric duct (PD) primordia. A weak labelling still remains at stage 26 (data not shown). At hatching stage (st.24), the rostralmost part of the urogenital system (PN, Fig. 2.12 C) shows only a faint expression when compared to the caudalmost ducts, where this gene is still strongly expressed (data not shown). The presence of *Lhx1* transcripts in the urogenital system has been reported in mouse (Barnes et al., 1994; Fujii et al., 1994), *Xenopus* (Taira et al., 1994), and zebrafish (Toyama and Dawid, 1997) embryos.

A transient labelling of the posterior notochord is observed at stage 24 (N, Fig. 2.12 C), while both at stage 21 (Fig. 2.12 A) and stage 26 (data not shown) this structure is free of *Lhx15* transcripts. We were not able to detect labelling in the rostralmost notochord (Fig. 2.12 B). A similar transient expression of *Lhx1* in the notochord takes place in zebrafish and *Xenopus* embryos (Taira et al., 1994; Toyama and Dawid, 1997), although, in *Xenopus*, the protein persists much longer than the mRNA (Karavanov et al., 1998).

Some *Lhx15*-positive cells were detected scattered through the yolk mass,

both at stage 21 (Y, Fig. 2.12 A) and at stage 24 (data not shown). At this latter stage, some label was also observed in the cells around the yolk. Taken together, these results show that *Lhx15* is expressed in the same endomesodermal tissues as the gnathostome *Lhx1*, and illustrate a case of very high spatial and temporal gene expression conservation throughout Craniate evolution.

### 2.3 Expression of *Hh* in non-neural tissues

In gnathostomes, the members of the *Hedgehog* gene family are expressed in a variety of tissues during embryonic development, including non neural tissues. They are involved in the development of the eye, heart, limb, gut, bone and cartilage, and also in angiogenesis and in the establishment of left-right asymmetry in many species (reviewed in Ingham and McMahon, 2001). In cephalochordates and urochordates, the expression of these genes is almost exclusive to the neural tube, with the exception of the pharyngeal endoderm in amphioxus. A thorough description of *Hh* expression in lamprey non-neural tissues is lacking, even if previous studies have reported the presence of *Hh* transcripts in the endostyle (Osorio et al., 2005; Uchida et al., 2003).

Lamprey *Hh* gene expression was detected in several non-neural tissues in post-neurula embryos. By Tahara stage 19–20, the gene is not only expressed in the notochord but also in the prechordal plate, the ventral pharyngeal epithelium and in the somites (Fig. 2.13 A–C). By stage 24, when the brain expression is clearly visible, and the gene is no longer expressed in the notochord, *Hh* mRNA is detected in the branchial arches, somitic mesoderm, pharynx and mouth epithelium, and in what Damas (1944) has called the “cheek processes” (Fig. 2.13 D–E). The gene is also conspicuously expressed in the endostyle, the larval structure which gives rise to the thyroid after metamorphosis (as seen at stage 27–28 in Fig. 2.13 F and H). The possible expression of *Hh* in the developing tail bud of the lamprey, important to understand similarities and differences in limb development between lampreys and gnathostomes, has not yet been assessed. In the following paragraphs, we will analyse these results focusing specifically on the development and evolution of the prechordal plate and endostyle/thyroid.

**Prechordal plate** The prechordal plate is the axial mesoderm above the foregut that is rostral to the developing notochord and that underlies the fore-



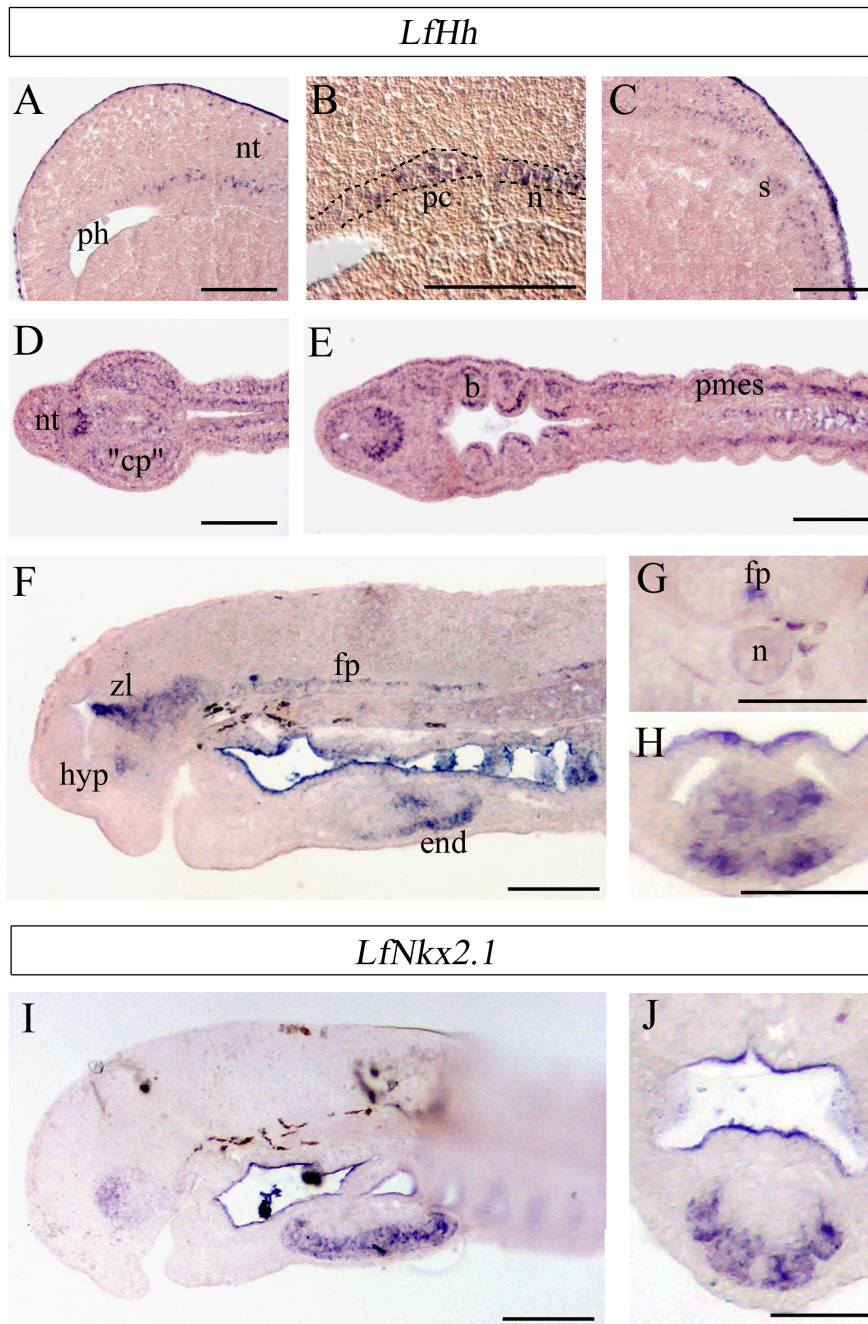


FIGURE 2.13: *Hh* expression outside the brain in *L. fluviatilis* embryos. (A–C) sagittal sections of st 19–20 embryos, where B is a high magnification of a selected zone in A. (D–E) horizontal sections of st 24 embryos, where D is more ventral than E. (F–J) sagittal (F and I) and coronal (G–H and J) sections of st 27 embryos, from Osorio et al. (2005). Compare the *Hh* expression at st 27 (F–H) with the *Nkx2.1* expression (I–J). “cp”, “cheek processes”; end, endostyle; fp, floor plate; hyp, hypothalamus; n, notochord; nt, neural tube; pc, prechordal plate; ph, pharynx; pmes, paraxial mesoderm; s, somite; zl, zona limitans. Scale bar, 100  $\mu\text{m}$ .

brain. Both the prechordal plate and the notochord are organisers, sources of patterning signals with an effect in the development of the adjacent neuroectoderm and paraxial mesoderm (reviewed in Rubenstein et al., 1998). Grafts of prechordal plate cells can induce ectopic forebrain structures in gnathostomes (Pera and Kessel, 1997). The prechordal plate (often called the head organiser) and the notochord (the trunk/tail organiser) express both common and distinct signals. The level and timing of expression of patterning genes in these two structures may also vary. The prechordal plate is not a definitive structure, becoming smaller with time; its dividing cells produce mesoderm which contributes to the notochord.

The prechordal plate is present in both lampreys and gnathostomes, but is absent from amphioxus and ascidians. In what probably remains to this date the most influential histological atlas of lamprey embryogenesis, Damas (1944) had described the prechordal plate as distinct from the notochord from a very early stage and during the whole period of embryonic development. The prechordal plate can be recognised as an independent entity at stage 21, and at stage 22 it grows laterally to both sides to form the premandibular mesoderm. By stage 24 the premandibular mesoderm grown from the prechordal plate penetrates a rostral subpopulation of trigeminal crest cells (Kuratani et al., 1999).

How is this structure specified, and what are its evolutionary origins? Neidert et al. (2000) have observed that the expression of *gooseoid*, a prechordal plate-specific gene, is not maintained in the anterior axial mesoderm in amphioxus. A transient expression of *Brachyury* genes in the anterior axial mesoderm of most craniates (including lampreys, dogfish and zebrafish, but not mice), seems to be correlated with the presence of the prechordal plate (Sauka-Spengler et al., 2003). In craniates and amphioxus alike, *Brachyury* genes are more stably expressed through the notochord, but in amphioxus this expression persists evenly until the rostral tip of the body (Holland et al., 1995). These observations led Sauka-Spengler et al. (2003) to conclude that the transient expression of *Brachyury* in the anterior axial mesoderm is an ancestral craniate feature. These differences are probably associated with the emergence of the prechordal plate, a region with unique properties, in the craniate group.

Our results, showing the expression of lamprey *Hh* in the prechordal plate (Fig. 2.13 A–B), further strengthen the idea of the similarity of this structure

with the gnathostome prechordal plate. Notably, *Hh* is also expressed in the mandibular and/or premandibular mesoderm (see “cheek processes” in Fig. 2.13 D). At stage 24 it is difficult to distinguish the two cell populations; in the latter case, they are derived from the prechordal plate. At this stage, the other main population of cells in the “cheeks” is a trigeminal crest cell population. From our observations, we cannot rule out the possibility that those cells also express *Hh*. It was demonstrated that *Shh* expression in neural crest cells is important in cranial facial morphogenesis (e.g. Ahlgren and Bronner-Fraser, 1999). Further histological analysis is necessary to provide a more detailed description of *Hh* expression in particular head mesoderm cell populations.

**Endostyle** The endostyle is a pharyngeal organ found in urochordates, cephalochordates and lampreys. In lampreys, this larval organ seems to be converted into a functioning thyroid at metamorphosis. For this reason, and based on gene expression studies (e.g. *thyroid peroxidase*; also *thyroid transcription factor*, *Nkx2.1/TTF1*), this organ has been proposed as a thyroid gland homologue (see, among others, Hiruta et al., 2005; Ogasawara et al., 2000).

The regulatory network involved in thyroid organogenesis has been well characterised in gnathostomes. Part of this network may be highly conserved, being common to all chordates (Hiruta et al., 2005). However, some molecular pathways for gnathostome thyroid development might have been recruited from an adjacent region of the pharynx (Mazet, 2002).

None of the *Hedgehog* genes is expressed in the thyroid of gnathostomes, cephalochordates or urochordates. Parlato et al. (2004) have ruled out the possibility of a role of *Shh* in the specification of thyroid precursors in mouse. In mouse, as in all gnathostomes, the thyroid primordium migrates from the pharyngeal endoderm. These authors have proposed that the distribution of *Shh* in a regional-specific pattern along the AP axis of the endoderm could be in part responsible for this migration, through a mechanism involving *Foxe1*. *Shh* seems to have, however, an indirect role in thyroid morphogenesis in both mouse (Alt et al., 2006; Fagman et al., 2004) and zebrafish (Alt et al., 2006). In fact, defects in *Shh* signalling will affect vasculogenesis, and in particular, the development of the ventral aorta in zebrafish and of the carotid arteries in mouse. These structures are adjacent to the thyroid and seem to have an important role in its correct morphogenesis.

The genetic specification of the lamprey endostyle is still poorly known. *Pax8* is a gene involved in gnathostome thyroid development, and belongs to the *Pax2/5/8* paralogy group; *Pax-2*, the lamprey single known representative of *Pax2/5/8* genes, is expressed in the endostyle (McCauley and Bronner-Fraser, 2002). Another characteristic thyroid gene, *Nkx2.1* (*TTF1*), is also expressed throughout the endostyle, from the anteriormost to the posterior-most parts (Fig. 2.13 I). In lamprey, the endostyle is composed of two medial and two lateral cylinders. *Nkx2.1* expression is restricted to the outermost cell layers of the cylinders (Fig. 2.13 J; Ogasawara et al., 2001; Osorio et al., 2005). The expression of *Nkx2.1* in lamprey resembles that in ascidians, leading Ogasawara et al. (2001) to propose that the basic architecture and gene expression mechanisms of the endostyle are conserved among chordates. Unique features observed in amphioxus would have been introduced independently (given the current phylogenetic view of chordates, this “chordate” endostyle might have not been even present in the amphioxus ancestor).

In the light of these results, the expression of the lamprey *Hh* gene in the endostyle (Fig. 2.13 F and H) is somehow surprising. The lamprey seems to be the only craniate where a gene from the *Hedgehog* family is expressed in the presumptive thyroid. Curiously, the transcription of this gene seems to increase in an anterior to posterior gradient. *Hh* and *Nkx2.1* are coexpressed in the outer regions of the lateral and medial cylinders, but *Hh* is also expressed in the most internal zones, at least in the intermediate endostyle. These results are difficult to interpret, but analysis of orthologues of other thyroid-specific genes (e.g. *FoxE*, *Hhex*,...) would be important to understand to which point the molecular cascade involved in thyroid development is shared between lampreys and other chordates.

## 2.4 Discussion

### 2.4.1 Origin and evolution of the craniate brain

From the results presented in this chapter it is possible to draw a picture of the ancestral craniate forebrain based on gene expression patterns. Such a picture is depicted in Fig. 2.14. Summarising our current knowledge, this ancestral brain would have: (1) a prosomeric diencephalon, with at least three recognisable prosomeres; (2) a ZLI organising centre, expressing *Hh/Shh*; a

craniate-type hypothalamus, expressing both *Nkx2.1* and *Hh/Shh*; (4) a telencephalic region, with a *Pax6*-expressing pallium, and a *Dlx*-expressing subpallium. The telencephalon itself is a craniate novelty, as a counterpart structure does not exist in amphioxus and ascidians (Holland and Holland, 1999; Wada and Satoh, 2001), but is present in hagfish and lamprey (Nieuwenhuys and Nicholson, 1998; Reiner et al., 1998).

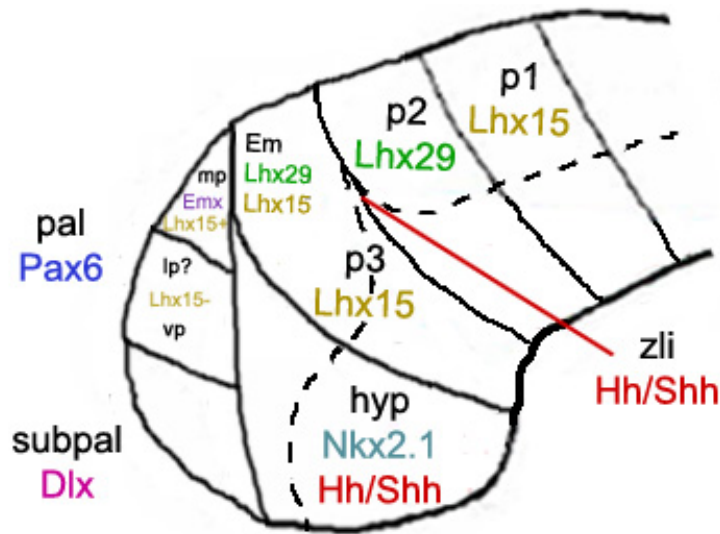


FIGURE 2.14: A hypothetical schematic view of the ancestral craniate brain. Em, prethalamic eminence; hyp, hypothalamus; lp, lateral pallium; mp, medial pallium; p1–3, prosomeres 1–3; pal, pallium; subpal, subpallium; vp, ventral pallium; zli, zona limitans intrathalamica; dashed line, sulcus limitans.

**How many prosomeres?** In an article published in 2005 we have proposed that a dorsal diencephalic region adjacent to the pallium which expresses both *Lhx29* and *Lhx15* could be the lamprey homologue of the gnathostome prethalamic eminence (Osorio et al., 2005, see also section 2.1). In the *Xenopus* and mouse diencephalon, *Lhx1/Lhx5* and *Lhx2/Lhx9* present a strict alternation of expression (Bachy et al., 2001; Moreno et al., 2004; Retaux et al., 1999). The co-expression of *Lhx29* and *Lhx15* could be an ancestral character. We can look at this aspect in the light of the much-debated variations of the prosomeric model. An older version of the model has proposed the existence of 6 prosomeres, the prethalamic eminence corresponding to the p4 prosomere (Puelles and Rubenstein, 1993). In our case, the *Lhx* dorsal diencephalic gene expression would then respect strict prosomeric boundaries. In the first pro-

someric interpretation of the adult lamprey brain, Pombal and Puelles (1999) have identified these 6 prosomeres. However, the updated version of this model accepts the existence of only three “true” prosomeres (Puelles and Rubenstein, 2003). The region where both *Lhx15* and *Lhx29* are co-expressed would correspond to the dorsal portion of p3 (prethalamus), which, in this new interpretation, also includes the prethalamic eminence in its dorsal aspect. In this case, the expression of *Lhx29* does not respect a prosomeric boundary (see Fig. 2.14), which seems to be also the situation of the *Lhx* expression in some regions of the lamprey basal plate.

Even if the lamprey brain shares many elements of a common organisation with the brain of gnathostomes, these two groups are separated by a rather long evolutionary time. It is quite possible that the genetic specification of the forebrain sub-regions differs in many aspects. For example, the position in the neuraxis occupied by the ZLI, one of the secondary organisers which is likely to orchestrate the regionalisation of the forebrain, seems different in these two groups. It is possible to speculate that this actually reflects existing differences in the role of the ZLI as a morphogenetic border, and also in the way it contributes to the patterning of the adjacent neuroepithelium.

**Pallial subdivisions** The mammalian pallium is divided in four main regions, named medial, dorsal, lateral and ventral pallia, the latter contacting the striatum over the pallial-subpallial boundary. Homologies of these regions have been proposed and discussed for a variety of animal models. In mouse, an opposing gradient of *Pax6* and *Emx1* expression has an important role in the specification of these four regions, and expression patterns in other gnathostomes indicate that this role was probably conserved. In lamprey, *Emx* is expressed in a small dorsal region in the telencephalon. This led to the suggestion that the role of *Emx* genes in telencephalic regionalisation and patterning is an ancestral feature of craniates (Murakami et al., 2001). The expression of *Emx* genes in dogfish (a chondrichthyan) corroborates this idea (Derobert et al., 2002b). This *Emx*-expressing region could correspond to the *Lhx15* high-expression region we have reported in Osorio et al. (2005), being the *Lhx15* low-expression region devoid of *Emx* expression. Our results support the conclusion of the existence of at least two recognisable pallial subdivisions in the developing lamprey brain.

Is it possible to establish a comparison between these regions and the four

recognised mammalian pallial subdivisions? Pombal and Puelles (1999) were not able to identify with confidence a dorsal pallium in lampreys. This observation indicated that the *Emx*-expressing region, or at least part of it, could be the medial pallium, homologous to the mammalian hippocampus. Northcutt and Wicht (1997) had already recognised in the lamprey telencephalon a region comparable to the gnathostome limbic system. They have also observed, however, that the pallial regions thought to be homologous to the cortex and hippocampus are poorly developed in lamprey, and that lampreys have a very small dorsal thalamus sending fibers to the pallium (reviewed in Murakami et al., 2005).

The large ventral *Emx* and *Lhx15* expression-poor subdivision is a potential ventral pallium, a region which is present in most extant craniates. The ancestral nature of the ventral pallium may be explained by the fact that this region has several areas receiving olfactory inputs, and both lampreys and fish have relatively large olfactory bulbs and massive olfactory projections to the pallium, and this was likely to be the ancestral condition (reviewed in Medina et al., 2005).

**The pallidum: function and role in evolution** A major novelty in the telencephalon of gnathostomes is the presence of a region, specified by *Shh* and *Nkx2.1*, which is called medial ganglionic eminence (in the embryo), pallidum or globus pallidus (in the adult). In mouse, most of the GABA ( $\gamma$ -aminobutyric acid)ergic interneurons which populate the pallium through tangential migration have their origin in this region (reviewed in Marin and Rubenstein, 2001). The specification of these neurons seems to happen through similar molecular mechanisms in most of the other osteichthyans. The GABAergic interneurons provide a very high level of integrative capacity to the pallium, and their appearance was probably associated with the development of new abilities and the diversification of the pallium.

While in sharks (chondrichthyans) there is an anatomically recognisable pallidum, in hagfish a comparable region is absent (reviewed in Reiner et al., 1998). In lampreys, a region rich in substance P fibers was proposed to have a pallidal identity to some extent (Reiner et al., 1998), but a true pallidum seems to be lacking (Nieuwenhuys and Nicholson, 1998). This absence has been correlated to the absence of the regulatory network involving *Hh/Shh* and *Nkx2.1* expression in the subpallium (Osorio et al., 2005; Uchida et al.,

2003; Murakami et al., 2005). Murakami et al. (2005) have proposed that the *Nkx2.1* knock-out mouse, where the MGE fails to develop (Sussel et al., 1999), can be seen as a phenocopy of the cyclostome state.

*Nkx2.1* expression in the MGE has a dual action: it contributes to the migration of GABAergic neurons to the pallium via activation of *Lhx6* expression (Alifragis et al., 2004), and it induces the acquisition of a cholinergic subpallial phenotype by activating *Lhx7*. *Lhx7* has two functions: it represses GABA expression at early stages and, later in development, it induces choline acetyltransferase (ChAT) expression (Bachy and Retaux, 2006; Zhao et al., 2003). Our attempts to isolate the lamprey ortholog(s) of *Lhx6/7* by RT-PCR were unsuccessful. It is possible that these genes, which have very restricted MGE-specific expression patterns in the gnathostome brain (even if they are also expressed in the preoptic area and in the hypothalamus), might even not exist in this cyclostome.

A puzzling question remains, nevertheless: even if no GABAergic neurons are detected at early stages in the lamprey dorsal telencephalon, they appear, in small number, at later stages (Melendez-Ferro et al., 2002a). Furthermore, there is evidence of cholinergic cells in the adult lamprey subpallium (Pombal et al., 2001). These findings may indicate a heterochrony of the neurogenesis and/or migration of the future pallial GABAergic interneurons if the lamprey is compared to gnathostomes. However, an alternative hypothesis is that most of the GABAergic cells observed in the lamprey pallium, abundant at adult stage (Robertson et al., 2007), are not interneurons, but pyramidal neurons which project outside the cortex (Menard et al., 2007; Robertson et al., 2006, 2007). In the cortices of amniotes, pyramidal neurons are glutamatergic, but the striatum, amygdala and pallidum contain GABAergic projection neurons. The pallial areas of the lamprey containing GABAergic projection neurons may thus be analogous to these structures (Menard et al., 2007). The gene network controlling the specification and possible migration of GABAergic neurons in the lamprey brain, probably quite different from that of gnathostomes, remains to be found.



### 2.4.2 *Lhx*, *Pax* and *Hh* genes and chordate evolution

#### *Lhx1/5* gene family evolution

The embryonic and larval expression of the *Lhx15* gene in the lamprey brain are similar to those of both *Lhx1* and *Lhx5* from gnathostome, while in the tissues outside the brain we have shown that the lamprey *Lhx15* expression pattern closely resembles that of gnathostome *Lhx1* genes (Osorio et al., 2006). While in ascidians the expression of the single representative of the *Lhx1/5* group is still unknown, a recent paper reported the isolation and study of an amphioxus gene, named *AmphiLim1/5* (Langeland et al., 2006). Fig. 2.15 is an updated version of the orthology relationships of Lhx1/5 proteins, including an amphioxus (*Branchiostoma floridae*) and an ascidian (*Ciona intestinalis*) sequences, and the sequences used to build the tree from Fig. 2.1. Note that the bootstrap values do not allow definitive conclusions about the correct relationships between the sequences from urochordates, cephalochordates, hemichordates and echinoderms.

The phylogenetic analysis of what seems to be the single protein of this family in amphioxus has placed it as an outgroup of Lhx1 and Lhx5 gnathostome proteins. This amphioxus *Lhx15* gene is expressed in the ectoderm at the blastula stage (as the gnathostome *Lhx5*), and in the mesendoderm of the gastrula organiser (as the gnathostome *Lhx1*<sup>11</sup>). At later stages, it is expressed in the urogenital system, as the lamprey *Lhx15* and the gnathostome *Lhx1* genes, in the CNS and in some other amphioxus-specific epidermal and mesodermal tissues. Curiously, the expression is absent from the notochord, being instead detected in the hindgut. The authors hypothesise that the expression of *Lhx1* or *AmphiLim1/5* genes in a continuous domain from the region of the dorsal blastopore to the tail bud (whether it is a notochordal or a hindgut expression) supports the concept that the chordate tail bud, and at least part of the gene network operating there, derives from the organiser during development.

The phylogenetic tree of Fig. 2.15 seems to place the lamprey peptide within the Lhx5 group. However, the short length of the sequences used, the morphology of the tree (presence of long branches), and the fact that slightly different topologies are found when other algorithms of tree construction are used (e.g. Minimum Evolution), suggest some caution with this interpretation. If true, this may be a case of function shuffling: the function of *Lhx1* in the

<sup>11</sup>It is noteworthy that *Lhx1*<sup>-/-</sup> mice are headless (Shawlot and Behringer, 1995).

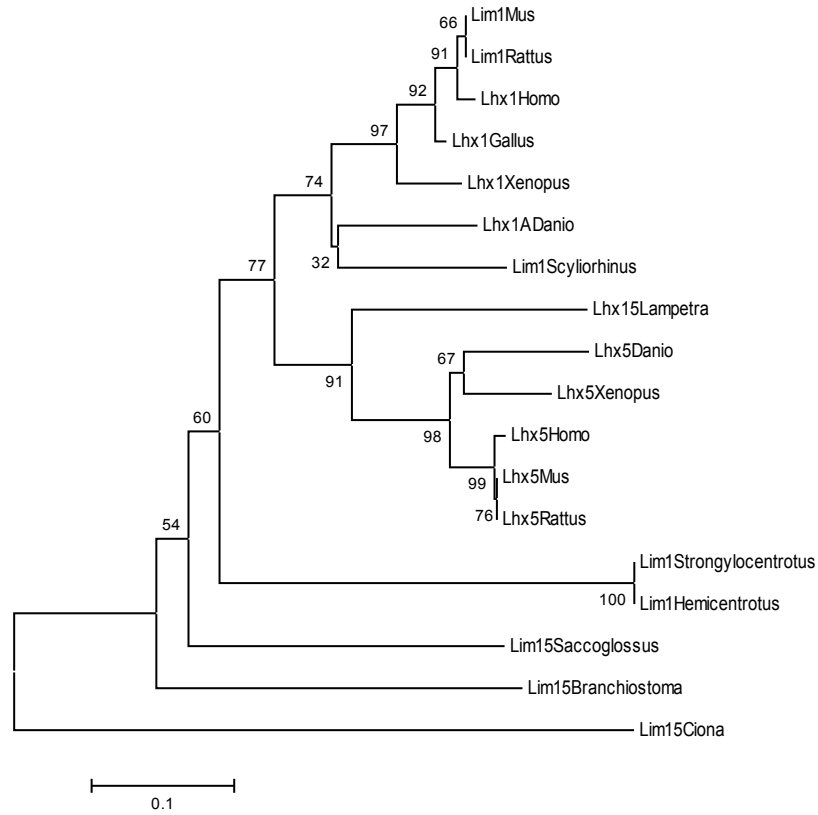


FIGURE 2.15: Neighbour-Joining phylogenetic tree of Lhx1/5 proteins including amphioxus (*Branchiostoma floridae*) and ascidian (*Ciona intestinalis*) sequences.

gnathostome notochord, urogenital system, tail bud and organiser (the latter not known in lamprey), would be, in the lamprey, taken by *Lhx5*. We still ignore if another *Lhx1/5* gene is present in lampreys or not.

Divergent homologues of *Lhx1/5* genes have been isolated in an enteropneust hemichordate, where the gene is expressed in a region of the developing epidermis that might correspond to developing nerve cells (Lowe et al., 2003), and in sea urchins, where the gene is expressed in the early gastrula in a position that indicates a possible role in the organiser (Kawasaki et al., 1999). These results show that some of the roles of this gene subfamily might be very ancient in evolution, but they also particularly highlight a functional conservation that is specific to chordates.

### *Pax3/7* genes in neural crest development

An alignment of the *L. fluviatilis Pax3/7* fragment and the corresponding *P. marinus Pax7* predicted aminoacid sequences has shown the quasi-identity of the two peptides (Fig. 2.1 C). A phylogenetic analysis of the Pax3/7 family did not provide clear information about the orthology relationships between the different proteins (Fig. 2.1 B). A gene from this family was isolated in the lamprey *P. marinus*, and was assigned to the *Pax7* group (McCauley and Bronner-Fraser, 2002). Curiously, the expression of this gene in the developing brain differs from that of *L. fluviatilis Pax3/7*. While the latter has a clear telencephalic midline expression domain (Fig. 2.5), the reported *P. marinus Pax7* expression pattern does not include a similar domain (McCauley and Bronner-Fraser, 2002). Results from a large-scale *in situ* hybridisation screen using probes synthesised from a cDNA library of *P. marinus* have confirmed the absence of telencephalic expression of the *Pax7* gene (see perspectives, section 2.5). It is possible that two genes of this group exist in lamprey, or, less likely, that we are in presence of an event of gain/loss of expression domains for the same gene within the lamprey group. Additional molecular studies in lamprey and hagfish, including large-scale sequencing, will be necessary to solve these recurrent orthology problems, to clarify events of gene and genome duplication in the craniate lineage, and to eventually resolve the long-lasting debate on the phylogenetic relationships between the different groups of craniates.

*Pax3/7* genes are important for placode and neural crest development in gnathostomes, and the lamprey member of this family is expressed in the dorsal neural tube, somites and trigeminal placode (McCauley and Bronner-Fraser, 2002; Osorio et al., 2005), which suggests a similar function. The amphioxus homologue of *Pax3/7* genes, *AmphiPax3/7*, is first expressed in bilateral anteroposterior stripes along the edges of the neural plate (Holland et al., 1999). This and other studies in amphioxus and ascidians (e.g. Jeffery et al., 2004) suggest that cell populations that eventually gave rise to definitive neural crest might have been present in an ancestral chordate.

The conservation of some of the mechanisms of neural crest transcriptional regulation in craniates was demonstrated by a technically challenging experiment where *SoxE1* morpholino injections in lamprey gave rise to abnormal branchial arch development (McCauley and Bronner-Fraser, 2006). A pioneer

molecular and embryological study in a hagfish species, *Eptatretus burgeri*, showed recently that a *Pax3/7* gene is present in these animals (Ota et al., 2007). This gene is expressed in the dorsal neural tube and somites of developing embryos, as in the other craniates. Expression of hagfish *Pax3/7* and other markers, HNK-1 immunohistochemistry and histological observations support the idea that the hagfish neural crest is specified by molecular mechanisms which are common to all craniates. The authors further suggest that the neural crest probably existed as a population of delaminating and migrating cells in the common craniate ancestor. The expression of this gene in the brain of hagfish, which would be of particular interest for our studies, was not described in this article.

### **Hh signalling and body patterning evolution**

Expression of at least one *hedgehog* gene in the ventral neural tube has been observed in gnathostomes, lampreys (Osorio et al., 2005; Uchida et al., 2003), urochordates (Takatori et al., 2002) and cephalochordates (Shimeld, 1999) alike. In craniates and cephalochordates, *hedgehog* gene expression was also detected in the notochord. This conservation highlights the evolutionary old role of Hedgehog signalling in neural tube dorso-ventral patterning. Furthermore, *AmphiHh*, the only amphioxus *Hedgehog* gene, is also expressed in the left side of the pharyngeal endoderm, suggesting its involvement in the establishment or regulation of left/right asymmetry in a similar way to the gnathostome *Shh* and *Ihh* genes (Shimeld, 1999). If true, this would indicate another ancient role of this gene family.

On the other hand, *Hh/Shh* expression in the brain seems to be a craniate novelty. The expression of *Hh/Shh* in the ZLI and hypothalamus is probably related to the very different brain organisation, in terms of regionalisation, neuronal specification and migration that we observe in this group when compared to urochordates (Osorio et al., 2005). The differences in shape and dorsal extent of the proposed ZLI in lampreys, when compared to gnathostomes, and the fact that the hypothalamic expression of *Hh* is small and discontinuous from the floor plate domain in lamprey may be an indication of more subtle differences between these groups. It is therefore interesting to investigate if changes in *Hedgehog* gene regulatory regions could possibly account for some

of these expression differences<sup>12</sup>.

The co-localisation of *Lhx15* and *Hh/Shh* in the ZLI of both lampreys and gnathostomes has led us to suggest the possibility that *Hh* might be upstream of *Lhx15* in a genetic cascade involved in the establishment of specific neuronal phenotypes. We have started to investigate this possibility by an ectopic expression assay in mouse explants in culture (see section 2.5).

The analysis of *Hh* expression in non-neural tissues highlights a remarkable conservation of expression pattern, and possible function, between lamprey *Hh* and the *Shh* gene from gnathostomes. An exception is the thyroid, where no *Hedgehog* gene expression was detected in gnathostomes. In a hypothetical evolutionary scenario, the additional *Hedgehog* genes of gnathostomes, originated by duplication after the cyclostome-gnathostome split, would have become involved with more divergent developmental processes, such as gonad development and peripheral nerve sheath formation (*Dhh*), or unique functions in angiogenesis, hematopoiesis, chondrogenesis and gut development (*Ihh*) (for a review, see Ingham and McMahon, 2001). However, this scenario should be considered with caution, as, among all gnathostome *Hedgehog* genes, *Dhh* is the most similar to the *Drosophila Hh*, which could indicate a slower rate of divergence.

## 2.5 Perspectives

**Study of the organising role of *Shh* in the zona limitans intrathalamica by overexpression** Several recent studies have focused on the possible role of *Shh* expression in the determination of the ZLI as an organising centre in the gnathostome embryo (reviewed in Echevarria et al., 2003; Kiecker and Lumsden, 2005). Our personal observations in the lamprey show that a similar expression domain of *Hh* exists in the cyclostome embryo, although this hypothetical “ZLI” domain seems slightly larger and its dorsalmost tip does not approach the roof plate as close as the gnathostome *Shh* domain. It may be that the gnathostome ZLI does not share all of its properties with this lamprey “ZLI”. The co-expression of *Hh* and *Lhx15* in its dorsal portion allowed us to hypothesise that *Hh/Shh* may act upstream of this latter gene. *Lhx* genes are known to be involved both in patterning broad regions in the brain and in establishing neuronal phenotypes. The induction of the expression of an *Lhx*

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<sup>12</sup>This is the subject of most of the following chapter (chapter 3).

gene by Shh has been already documented in the case of *Islet-1*. In fact, Shh acts in collaboration with neurotrophin 3 to induce *Islet-1* expression in the murine spinal cord, conditioning in this way a motoneuron phenotype (Dutton et al., 1999). A similar cascade could be set in place in the ZLI, being a *Lhx* gene in an intermediate position between the morphogen and the determination of a neuronal phenotype. If true, it is possible that this cascade may be acting also in cyclostomes, being thus a pan-craniate characteristic.

As functional experiments are very difficult to conduct in developing lampreys, we decided to approach this question by increasing the levels of Shh in different regions of the mouse embryonic brain. We hoped in this way to be able to observe a link between the presence of Shh and the expression of *Lhx* genes, namely *Lhx1* and *Lhx5*. As an ultimate goal, we would like to understand the effects of this hypothetical molecular cascade in the choice of a specific neuronal phenotype.

Two different techniques were used. In the first technique, small beads (AffiGel blue beads or AG 1-X2 resin, Bio-Rad) coated with the mouse amino-terminal Shh protein (R&D Systems) were inserted into the mouse explant tissue, which was then cultivated for up to 48 hours. Fig. 2.16 A shows the detection of GABA by immunohistochemistry, the bead appearing to have a small effect on the trajectories of GABAergic neuronal fibers. In the same set of experiments, Fig. 2.16 B shows an apparent overexpression of *Lhx1*, assessed by *in situ* hybridisation, in the experimental side of the explant.

The second technique involves the overexpression or misexpression of *Shh* by microinjection of expression vectors in embryonic brain explants in culture, followed by a focal electroporation. After 24 to 48 hours, the signal of the reporter GFP (green fluorescent protein) may be amplified by immunohistochemistry and the *Lhx* mRNA detected by *in situ* hybridisation. Possible changes of neuronal specification can be also investigated by immunohistochemistry assays, using neuronal phenotype markers such as GABA. Fig. 2.16 C shows an example of a brain slice injected with a vector where the expression of a green fluorescent protein (AFP) is driven by the chicken  $\beta$ -actin promoter. After 48 hours of explant culture, the focal restriction of the GFP reporter expression is clearly visible. This technique will therefore allow the study of possible changes in neuronal specification after modification of ZLI Shh signalling. The preliminary experiments already undertaken using these two techniques are encouraging and will be pursued in our laboratory.

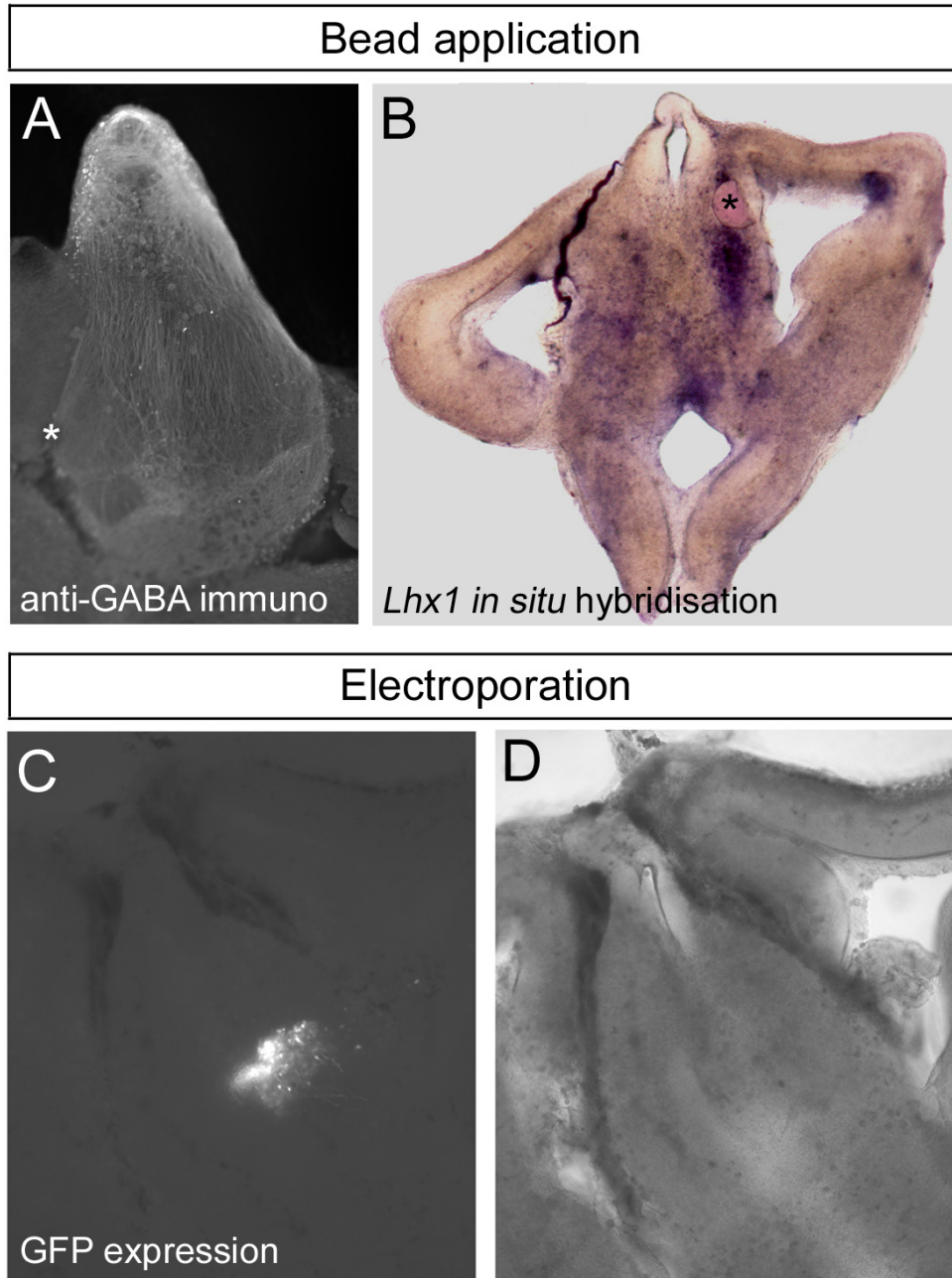


FIGURE 2.16: Mouse explant culture experiments. (A) anti-GABA immunofluorescence after 400 ng/ $\mu$ l Shh-coated AffiGel blue bead application, and 48 hours culture. (B) *Lhx1* ISH after 400 ng/ $\mu$ l Shh-coated AG 1-X2 bead application and 48 hours culture. (C) GFP expression, 48h culture after injection of pCAGGS-AFP. (D) Bright field photo of the explant in C. Arrow indicates bead position in B.

Thanks are due to Bertrand Mollé for teaching me the explant culture and injection protocol, and to Paula Alexandre, Isabelle Bachy and Marion Wassef (Brain Regionalisation team, Development and Evolution of the Nervous System laboratory, Ecole Normale Supérieure, Paris) for the demonstration of the bead protocol in chicken embryos.

**Large scale sequencing and analysis of lamprey cDNAs** The lamprey is an emergent model of study, to which many of the molecular tools existing for other species were not available at the beginning of this doctoral project. As a consequence, the early stages of the work described in this thesis were spent adapting standard techniques to this new model. A promising new source of information has begun to be developed in 2004, when a consortium of several laboratories, directed by Sylvie Mazan (Institut de Transgénése, Orléans), was created with the purpose of constructing and sequencing several cDNA libraries of lamprey (*Lampetra fluviatilis* and *Petromyzon marinus*) from three different sources (embryos; larva; adult brain and pineal gland), and of dogfish (*Scyliorhinus canicula*). I participated in the construction of these libraries, being the sequencing part taken in charge by the company Genoscope.

The people taking part in this project come from the laboratories of Sylvie Mazan (head of the consortium), Didier Casane and Sylvie Rétaux. At the CGM laboratory (CNRS Gif-sur-Yvette), Claude Thermes, Yves D'Aubenton and Emna Marrakchi have been responsible for the bioinformatics aspect of the project. Since September 2006, the consortium has received approximately 150,000 lamprey sequences. In our group, lamprey clones corresponding to genes suspected to be involved in forebrain development are now systematically analysed through large scale *in situ* hybridisation (Fig. 2.17). This high throughput approach will soon provide us with a more general picture of forebrain evolution among Craniates.



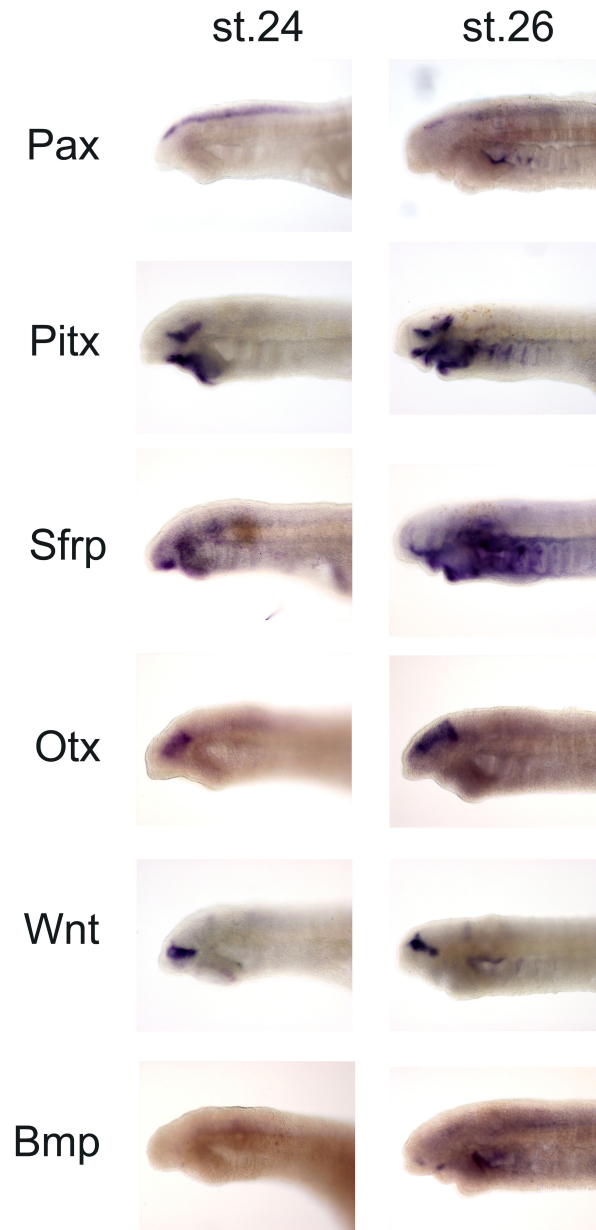


FIGURE 2.17: Large-scale analysis by *in situ* hybridisation of the expression pattern of genes involved in the brain development of *Petromyzon marinus*. Only the large family to which each gene belongs is indicated, as the precise orthology relationship within each family are currently under analysis. Among the expression patterns shown, note, in particular, that of *Pax* (the same gene isolated by McCauley and Bronner-Fraser, 2002, and called *Pax-7*). This gene is clearly not expressed in the telencephalon, in contrast to the *Pax3/7* gene isolated in *Lampetra fluviatilis* (Osorio et al., 2005). Figure courtesy of Adèle Guérin and Sylvie Rétaux.

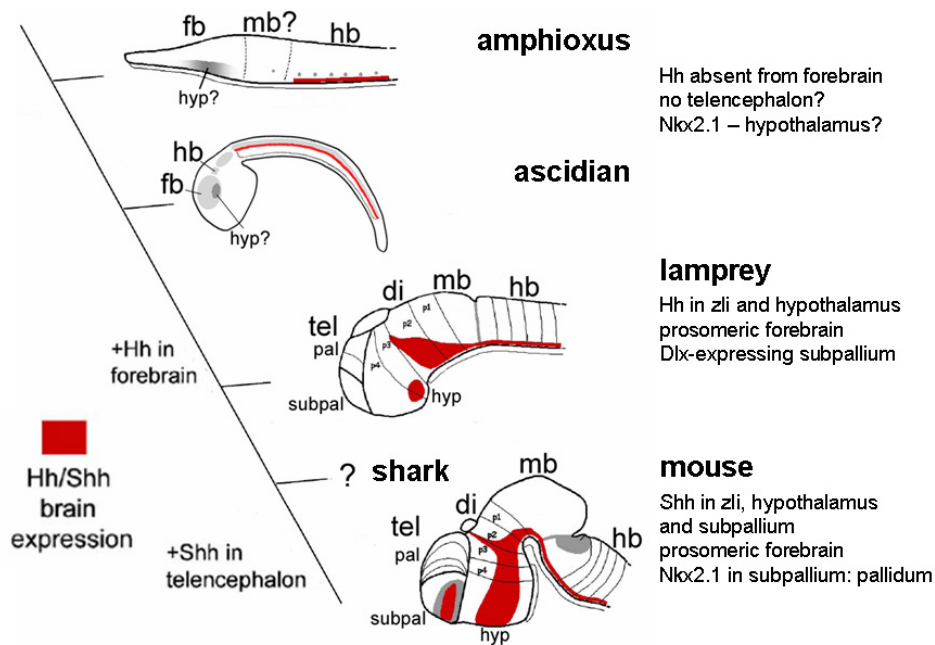
## Chapter 3

# Evolution of gene expression regulation in the brain

In the Introduction chapter we have mentioned some of the processes involved in the generation of evolutionary novelty (e.g. mutations, translocations, duplications, changes in gene regulation and epigenetic changes). In this chapter we present a project aimed at studying the potential role of changes in gene organisation and control of gene expression in the evolution of the chordate brain. We have used representatives of two major craniate groups, the Cyclostomes (lamprey) and the Chondrichthyans (dogfish), where these aspects have been so far poorly studied. We describe the isolation and initial study of the lamprey *Hh* and dogfish *Lhx9* genes, undertaken with this purpose. At a functional level, the injections of the mouse Shh protein in the lamprey subpallium were a tentative approach to the question of a possible conservation of protein function and tissue competence in the basal forebrain of cyclostomes and gnathostomes.

### 3.1 Evolution of the structure, function and regulation of *Hedgehog* genes

In the previous chapter we have discussed the implication of *Hedgehog/Sonic hedgehog* genes in forebrain evolution. Fig. 3.1 summarises our current views on the differences of *Hh/Shh* expression which may account for major differences of CNS organisation within chordates. Within chondrichthyans, *Shh* has been isolated from dogfish (Tanaka et al., 2002) and other species (Dahn et al.,

FIGURE 3.1: *Hh/Shh* and chordate brain evolution.

2007), but a detailed expression analysis in the brain is lacking.

In search of a better understanding of this evolutionary scenario, we have started a project aimed at the study of the genetic mechanisms underlying the acquisition and loss of function(s) of *Hedgehog* genes through evolution. We were particularly interested in the emergence of new *Hh/Shh* expression domains and its consequences on forebrain patterning and regional and cellular specification.

Hh proteins, coded by *Hh* genes, are composed of a short signal peptide, followed by a N-terminal domain (HH signal; Fig. 3.2 A and B), which is the active signalling molecule, and of a less conserved C-terminal domain (Hint domain; Fig. 3.2 A and C) responsible for the auto-cleavage of the pre-protein. The active N-ter domains of Hedgehog proteins have been extremely well conserved through evolution (Fig. 3.2 B). The lamprey N-ter domain fragment predicted from the cDNA we have isolated (Osorio et al., 2005) does not clearly group with either one of the three known gnathostome Hh subfamilies, but we have also shown that *LfHh* shares important expression features with *Shh*. Knowing this, we have undertaken an experiment aimed at mimicking the gnathostome Shh effects in the lamprey subpallium (described in section 3.1.1).

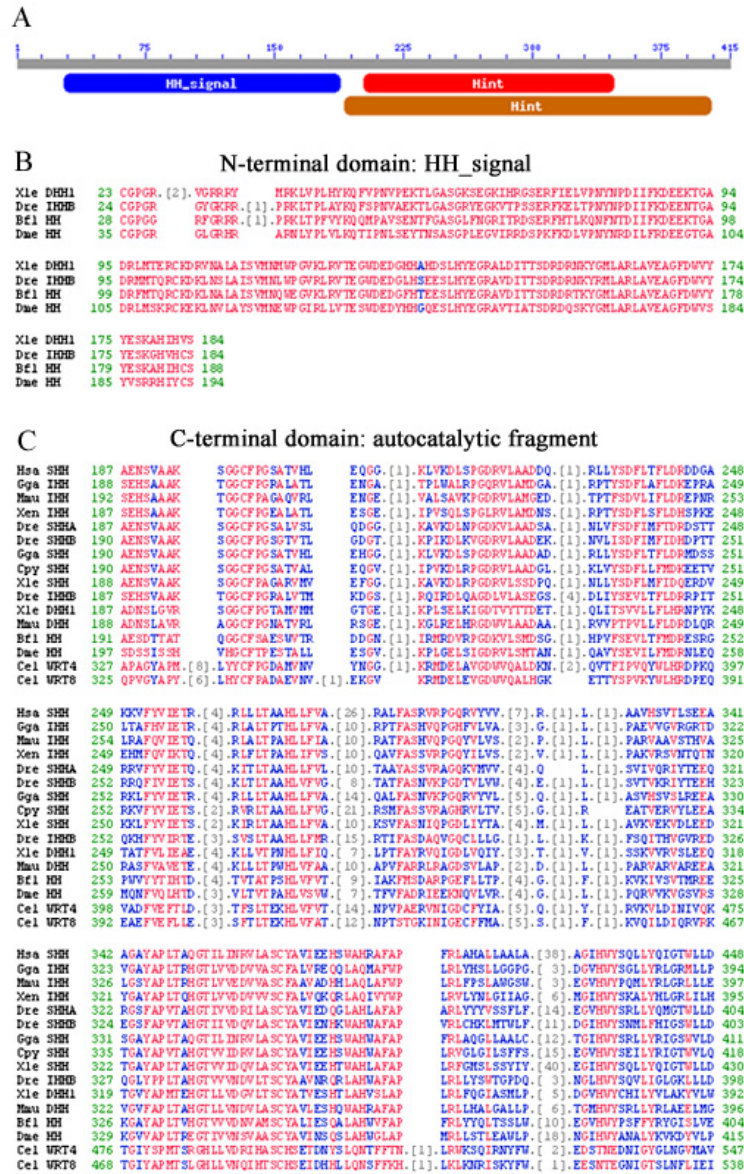


FIGURE 3.2: Conserved Hedgehog active domains. (A) General structure of Hedgehog proteins, as exemplified by the Hedgehog protein of the amphioxus *Branchiostoma belcheri*. HH\_signal, Hedgehog amino-terminal signalling domain; Hint (red), Hedgehog/Intein domain, found in Hedgehog proteins as well as proteins which contain inteins and undergo protein splicing; Hint(brown), conserved Hint module of Hedgehog proteins, not including inteins. The mature signalling forms of Hedgehog proteins are the N-terminal fragments (B), which are covalently linked to cholesterol at their C-termini. This modification is the result of an autoprocessing step catalysed by the C-terminal fragments (C). Adapted from the online NCBI Conserved Domain Database (CDD) (Marchler-Bauer and Bryant, 2004; Marchler-Bauer et al., 2007).

With the goal of investigating major evolutionary changes in the Cyclostome/Gnathostome and Chondrichthyan/Osteichthyan transitions, we have tried to isolate *Hh/Shh* in both lamprey (*Lampetra fluviatilis*) and dogfish (*Scyliorhinus canicula*). We have screened genomic libraries of these two species, but we have been able to find this gene only in lamprey. We sought to answer two related questions, namely: (1) what is the genomic organisation of the *Hh* gene in lamprey and what information can it give us on the evolution of the *Hedgehog* gene family; and (2) what types of regulatory changes might be involved in the emergence of a gnathostome-like *Shh* expression pattern (see 3.1.2).

The project of isolating and studying the *Hh* gene of the river lamprey *Lampetra fluviatilis*, described in section 3.1.2, has begun with my two month stay (from mid-September to mid-November 2006) at the laboratory of Marc Ekker at the University of Ottawa (Canada). This laboratory has performed a detailed molecular and functional analysis of several important craniate gene families, having become a reference in the study of regulatory regions and their evolutionary history (Amores et al., 1998; Hukriede et al., 1999; Knapik et al., 1998; Zerucha and Ekker, 2000). In particular, they have worked extensively on *Dlx* genes (which have an important role in forebrain development), (Ghanem et al., 2003; Park et al., 2004; Quint et al., 2000; Zerucha and Ekker, 2000; Zerucha et al., 2000), *Hox* genes (Amores et al., 1998; Prince et al., 1998), and, more recently, on keratin genes (Krushna Padhi et al., 2006). Although they are most known for their studies in zebrafish, they have also worked with other animal models, notably the lamprey. I am grateful to Marc Ekker for his warm welcome in his laboratory and for fruitful discussions. I thank Ashish Maurya for his collaboration in this work, and Gary Hatch for his helpful assistance.

### 3.1.1 A trial to phenocopy gnathostome *Shh* signalling in lamprey embryos

We have tried to determine if we could partially induce a gnathostome-typical expression of pallidal genes in the lamprey subpallium by exogenous application of the soluble (already cleaved) form of the mouse *Shh* protein. In this *in vivo* approach, we have injected the mouse protein in the lamprey subpallium, and then tested its possible effects on the expression of *Nkx2.1*.

### Materials and Methods

Living lamprey embryos are available once a year, during the spawning season. On the season of 2006, murine Shh protein (amino-terminal Shh protein, R&D Systems) at two different concentrations (100 ng/ $\mu$ l and 400 ng/ $\mu$ l) was microinjected at the base of the forebrain on one side of stage 24 *P. marinus* embryos, previously anaesthetised in 0.2 mg/ml MS222. A 0.1% solution (1 $\mu$ g/ml) of BSA (bovine serum albumine) was similarly injected in control embryos. Phenol red was added to protein solutions for better visibility. To stabilise the embryos while performing the injections, a solution of 4% methylcellulose was used. The embryos were let to develop in this solution, at the bottom of a petri dish filled with water. After 24 hours of development at 18°C, embryos were fixed in MEMFA (4% formaldehyde, 0.1M MOPS (pH 7.4), 1mM MgSO<sub>4</sub>, 2mM EGTA) overnight at 4°C and then dehydrated in a graded series of methanol. They were kept at -20°C in 100% methanol until assessment for *Nkx2.1* expression by *in situ* hybridisation, which was carried out as described in Osorio et al. (2005). Photos of whole-mount and sectioned embryos (8  $\mu$ m paraffin sections) were taken as in Osorio et al. (2005).

### Results and discussion

We observed a normal development of the embryos, showing that the animals recovered well from the treatment. The results show that *Nkx2.1* mRNA is detected in the usual places of expression, i.e. the hypothalamus and the endostyle. No ectopic expression of the gene was observed in the subpallium (Fig. 3.3, Fig. 3.4). Some *Nkx2.1*-expressing cells were detected in non-hypothalamic diencephalic regions (prethalamus and thalamus), which are ectopic expression areas for this gene (Fig. 3.4). The levels of *Nkx2.1* seemed to be lower in the case of the highest concentration of Shh protein injected (Fig. 3.3). However, the small number of embryos tested (n=10 for each treatment) prevents a more accurate analysis.

We can nevertheless conclude that, in our conditions, the injections of Shh are incapable of inducing the expression of *Nkx2.1* in the subpallium. This may be due to a large variety of factors, such as the absence of adequate receptors or other aspects of tissue competence, to a larger-than-expected divergence between the mouse and lamprey proteins, or to an inadequate choice of the developmental window.

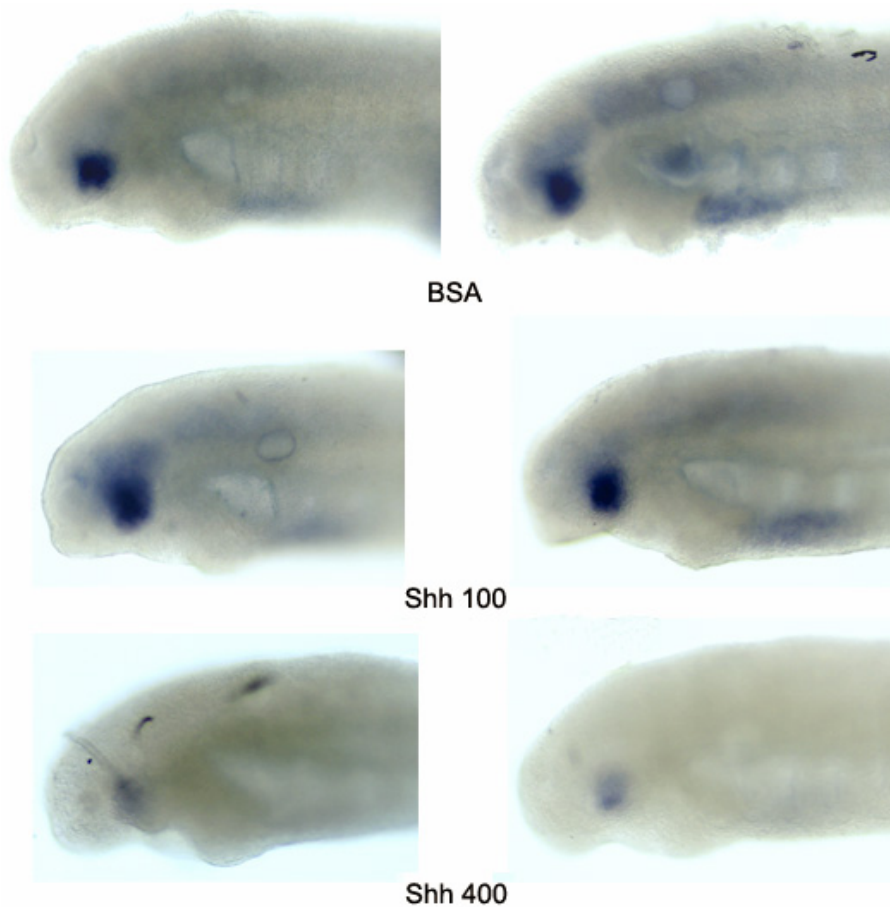


FIGURE 3.3: Whole-mount photos of *Nkx2.1* *in situ* hybridisation of *P. marinus* embryos injected with the murine Shh protein. From top to bottom: control BSA injected embryos; embryos injected with 100 ng/ $\mu$ l of Shh; embryos injected with 400 ng/ $\mu$ l of Shh.

It is worth noting that there is evidence in mouse for both *Nkx2.1* regulation by Shh (Gulacsi and Anderson, 2006; Pabst et al., 2000; Watanabe et al., 2005; Xu et al., 2005) and *Shh* dependence on *Nkx2.1* expression (Nery et al., 2001; Sussel et al., 1999). Jeong et al. (2006) have identified a Nkx2 binding site in the *Shh* enhancer which drives the expression of *Shh* in the mouse subpallium (SBE3). A mutation in this binding site completely abolishes *Shh* subpallial expression. These studies suggest a double interaction of Shh and Nkx2.1 in the specification of the gnathostome pallidum. The precise origin, regulation, maintenance and timing of such a loop are yet to be clarified.

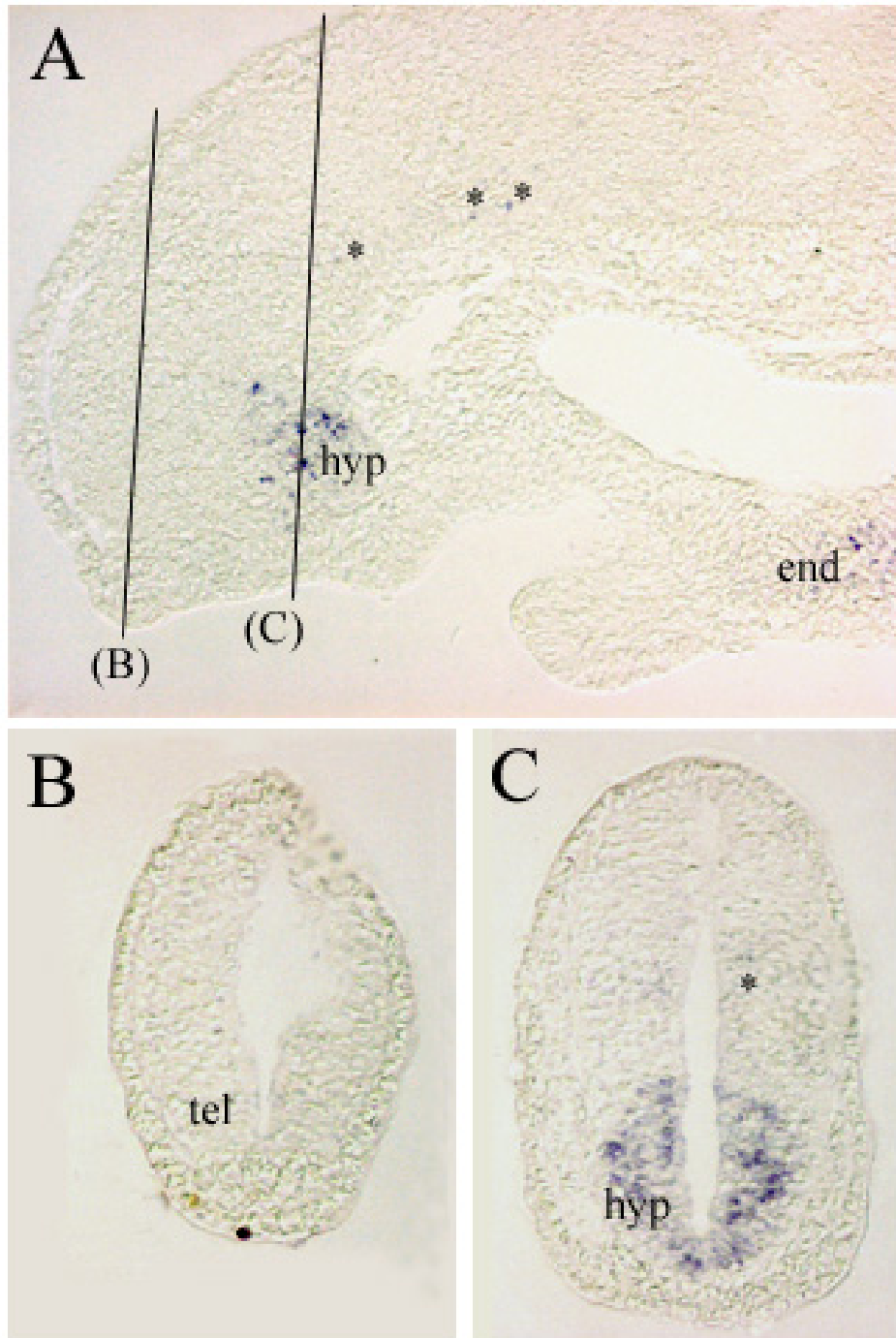


FIGURE 3.4: Sections of *Nkx2.1* *in situ* hybridisation of *P. marinus* embryos injected with 400 ng/ $\mu$ l of mouse Shh protein. A, sagittal section; B and C, coronal sections at telencephalic and diencephalic levels, respectively. hyp, hypothalamus; tel, telencephalon; asterisks, *Nkx2.1* ectopic sites of expression.



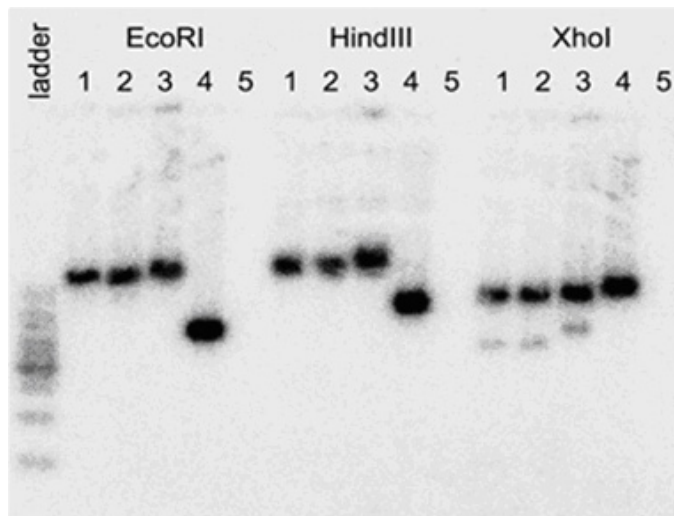


FIGURE 3.5: Southern blot of *Hh*-containing cosmids digested by EcoRI, HindIII and XhoI restriction enzymes, using the *hedgehog* probe from the cosmid genomic library screen. The size of the positive band in the EcoRI and HindIII digestions is remarkably different between cosmids number 1 to 3 and cosmid number 4. The absence of a signal for cosmid number 5 indicates that it is a false positive.

### 3.1.2 Isolating the *Hh* gene in lamprey (*L. fluviatilis*)

#### Results and discussion

We have screened a cosmid genomic DNA library of the river lamprey *Lampetra fluviatilis* (RPDZ library No. 55) with the aim to identify cosmids containing the *Hh* gene. We were able to identify several positive clones using the *LfHh* cDNA fragment we had previously isolated (Osorio et al., 2005) as a probe. Four of the five clones ordered contain at least the first exon of the desired gene, identified both by sequencing and Southern blot (Fig. 3.5).

Importantly, the sequences and digestion patterns of these four clones are divided in two distinct groups: the so far available sequence of clones 1 to 3 is remarkably different from that of clone 4, the similarity being higher in the region recognised by the probe. The Southern blot of Fig. 3.5 illustrates well the difference between the two groups. These results raise the possibility of the existence of two *Hedgehog* genes in lamprey.

To investigate this idea we have started the progressive sequencing of the exons and introns in the two different groups of cosmids. So far, we have sequenced approximately 4 kb of cosmid 3 and 3 kb of cosmid 4.

As the sea lamprey (*Petromyzon marinus*) genome was under sequencing,

we have searched the yet incomplete database for sequence similarity to known *Hedgehog* genes. We were able to identify two sequences (named PmseqA and PmseqB, 718 and 815 bp long, respectively) corresponding to the first exon of *Hh* flanked by fragments of the 5' UTR and of the first intron. A sequence alignment has shown that *L. fluviatilis* cosmid 4 sequence and *P. marinus* sequence A are identical, while *L. fluviatilis* cosmid 1, 2 and 3 sequences and *P. marinus* sequence B are highly similar (Fig. 3.6). A new analysis will be soon performed, using the partially assembled *P. marinus* genome sequence.

The organisation of *Hedgehog* genes is highly conserved. As in *Drosophila*, chordate *Hedgehog* genes are relatively small, and possess 3 exons in all species studied except ascidians (where they have four exons). In all of the other groups, the two introns are within the sequence coding for the N-ter active domain. The newly identified lamprey sequences suggest a conservation of this organisation also in cyclostomes (e.g. conservation of the first splice site, Fig. 3.6).

The possibility of the existence of two *Hedgehog* genes in lampreys opens a new vision on the evolution of this gene family within chordates. We sought to answer the question of whether a duplication of the ancestral *Hh* gene occurred in the lamprey lineage (similarly to what happened to the same gene in the ascidian lineage) or if each gene can be assigned to one of the *Hedgehog* families of gnathostome genes (*Sonic*, *Desert* and *Indian hedgehog* families), which would imply an event of duplication previous to the cyclostome-gnathostome split. With this goal, we have undertaken a preliminary phylogenetic analysis using the predicted aminoacid sequences from the first exon of *P. marinus* putative *Hedgehog* genes and of *Hedgehog* family members from other species (Fig. 3.7). This analysis suggests an independent duplication of the lamprey *Hedgehog* genes within the *Sonic/Indian* group. The hypothesis of an earlier pan-craniate duplication which would have been at the origin of the *Desert* and *Sonic/Indian* groups remains open. If true, this hypothesis would mean that the absence of lamprey homologues of *Desert hedgehog* genes is due to gene loss. An alternative scenario would be a gnathostome-specific duplication of *Hedgehog* genes into the *Shh/Ihh* and *Dhh* groups, and a rapid divergence rate of the latter. An argument against this latter hypothesis is the fact that *Dhh*, among all gnathostome *Hedgehog* genes, is the closest in sequence to the *Drosophila Hh* gene.

In order to identify possible functional differences between the two genes

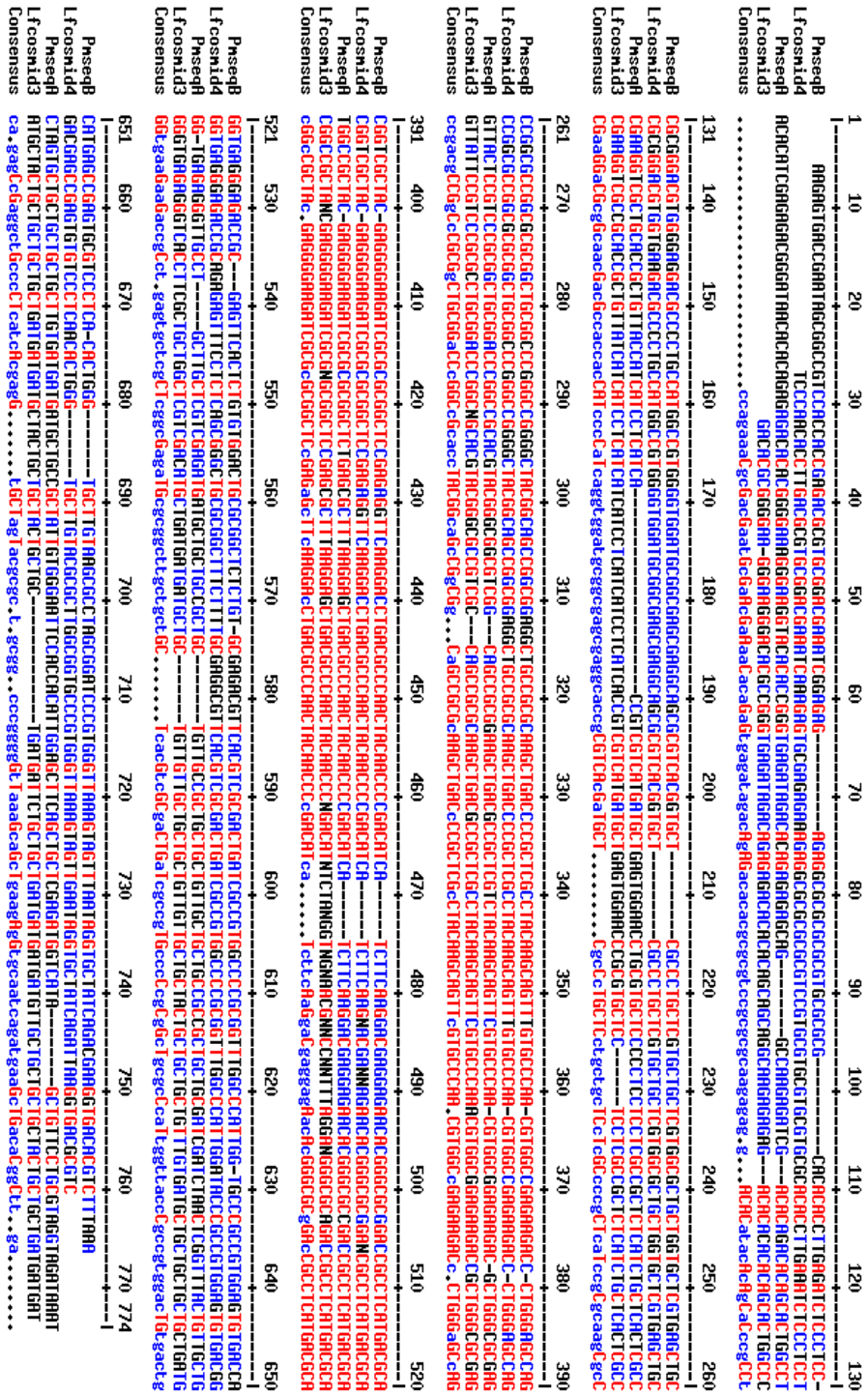


FIGURE 3.6: Preliminary alignment of a 709 bp sequence including the putative first exon of *Hedgelyog* genes of *Lampetra fluviatilis* (cosmids 3 and 4) and *Petromyzon marinus* (sequences A and B). 477 bp 5' and 232 bp 3' to the exon-intron splice site were aligned. The predicted span of the exon is from positions 279 to 521 in this graph.

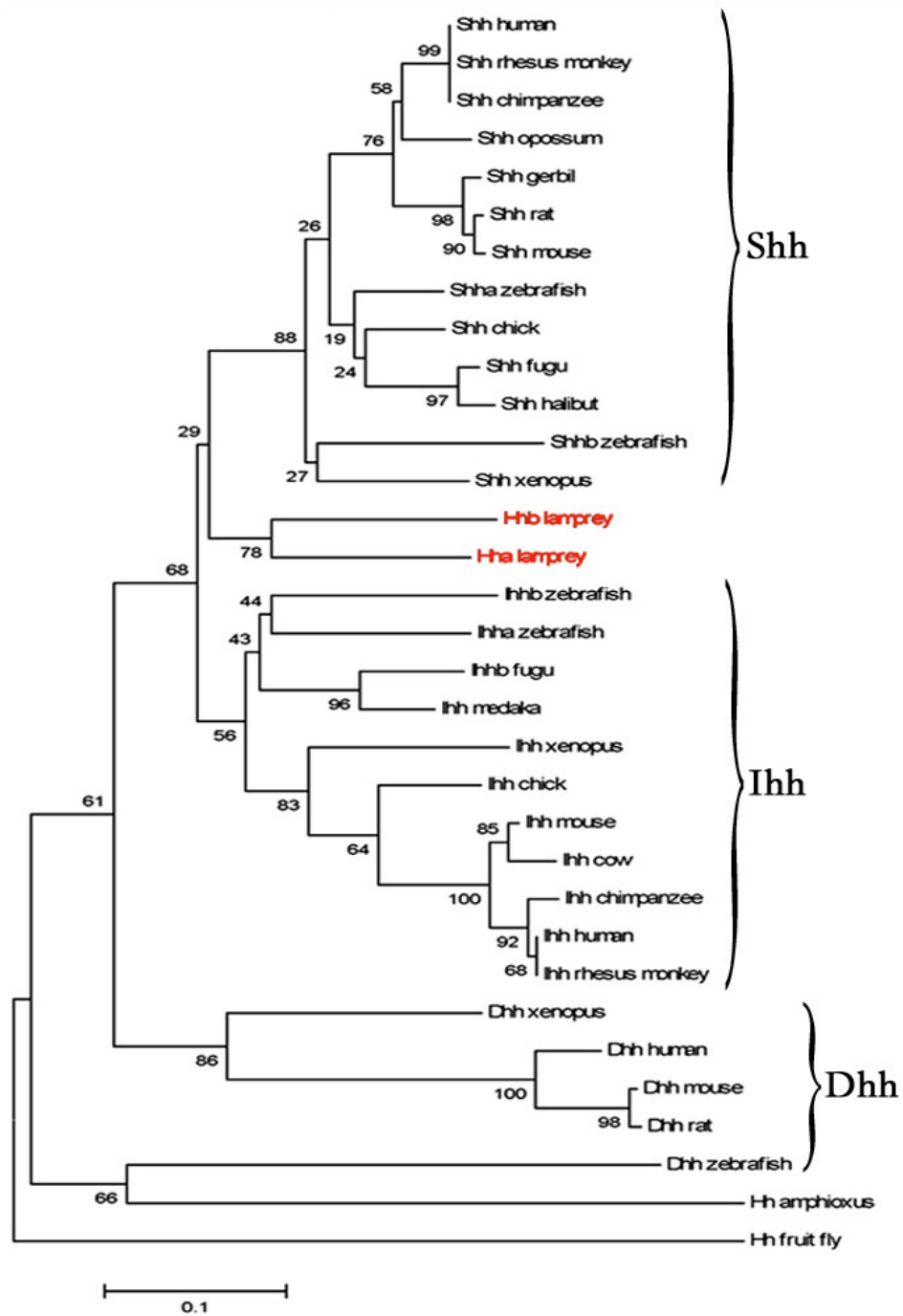


FIGURE 3.7: Preliminary Minimum Evolution phylogenetic tree of Hedgehog proteins, using the aminoacid sequence coded by the first exon. The *Xenopus* sequences used are from *X. tropicalis*. *Drosophila* hedgehog was chosen as an outgroup.

during *L. fluviatilis* embryogenesis, we have designed two intronic probes for *in situ* hybridisation. Based in our sequencing results, we have selected two different sequences, 895 bp and 618 bp long, from the first *hedgehog* intron of cosmid 3 and cosmid 4, respectively. These regions were then amplified by PCR. Sub-cloning, probe synthesis and purification and whole-mount *in situ* hybridisation were carried out as described in (Osorio et al., 2005), with minor modifications. However, the ISH experiment was unsuccessful, due to high background staining (more likely to happen when using probes designed to recognise introns), which prevented a clear interpretation of the results.

## Perspectives

### Sequence analysis

- Complete sequencing of the coding region of the two hypothetical lamprey genes and phylogenetic analysis.
- Assessment of the presence of high homology intronic non-coding sequences (potential regulatory regions), especially those conserved across gnathostomes (e.g. a conserved enhancer in the second intron of mouse and zebrafish *Shh*).
- Full sequencing of the 5' regions of the *Hh* gene from the two different groups of cosmids by the company Genoscope (Evry, France) and test for the presence of potential regulatory sequences in these regions.
- Identification of known protein binding sites in the gene (e.g. Nkx).

**Functional analysis of *Hh/Shh* regulatory sequences** A comparative functional study of medaka and lamprey *Shh/Hh* putative regulatory elements will be undertaken, using transgenesis in medaka fish. This analysis will start by testing if the lamprey constructs described below are capable of driving the GFP expression when injected in medaka, and, if so, if the territories of expression correspond to those of *Hedgehog* genes.

Working with Ashish Maurya, we have started to make the first constructs to be used in transient expression assays in medaka fish, using the technique of homologous recombination in *E. coli*. The objective was to insert an EGFP-ampicillin<sup>res</sup> cassette, already contained in a plasmid, into the first *Hh* exon of the two different cosmids identified. We have designed primers containing

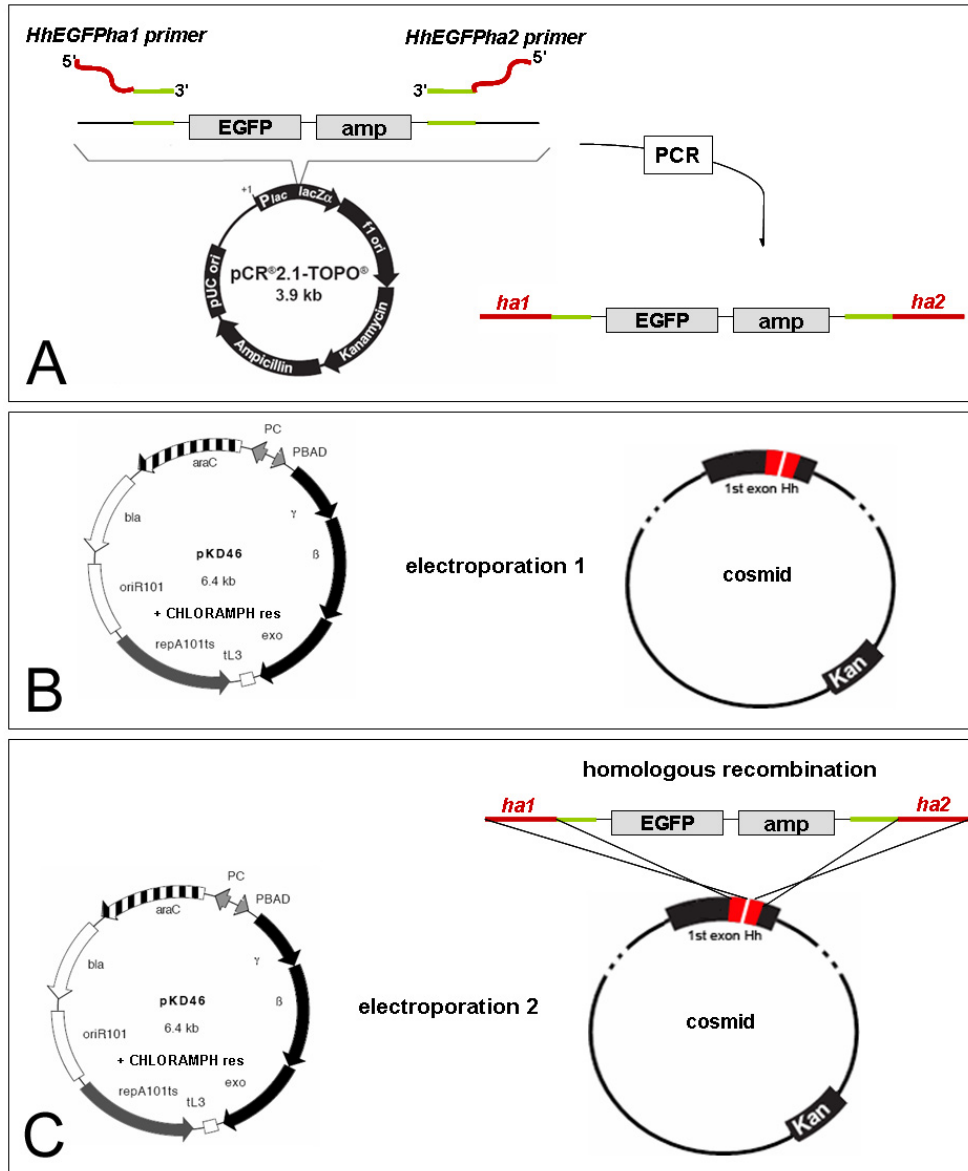


FIGURE 3.8: Schematic view of the homologous recombination protocol.

both the sequences flanking the cassette and two homology arms for the target *Hh* sequence. By PCR, it was possible to amplify the cassette. Two of the clones (3 and 4) were transfected by electroporation with a plasmid containing the phage Red locus genes, which promotes homologous recombination. The clones were grown in a context where the recombination-promoting genes can be expressed and electroporated with the EGFP-ampicillin<sup>res</sup> cassette (Fig. 3.8). The success of the homologous recombination process is currently being tested. If ready, these constructs will be then injected in medaka fish. One of the long-term goals of this project is to obtain two stable transgenic lines of fish where the GFP would be expressed under the control of each sequence.

**Hedgehog genes in dogfish** Knowledge on the number, genomic organisation and expression of *Hedgehog* genes in Chondrichthyans would be of major importance to understand early events in the evolution of Gnathostomes (Fig. 3.1). We have tried to isolate the *Shh* gene from a dogfish (*S. canicula*) genomic BAC library (the one used to isolate *Lhx9*, see section 3.2), without success. New attempts to isolate this gene by a similar approach would probably require the use of a different library, as it is likely that this particular one did not cover the whole dogfish genome (as shown by attempts to isolate other genes, Didier Casane and Sylvie Mazan laboratories, personal communication). Were new attempts successful, a project similar to the lamprey *Hh* project described above could be then undertaken. As with lamprey, the dogfish *Hh* project would be focused on the study of gene organisation and identification of regulatory regions, whose function could be potentially tested by transgenesis.

## 3.2 Evolution of gene structure and expression regulation: the case of dogfish *Lhx9*

### 3.2.1 Introduction

The lesser-spotted dogfish (*Scyliorhinus canicula*) belongs to the Chondrichthyans (cartilaginous fishes), the sister-group of Osteichthyans (Actinopterygians, or ray-finned fishes, and Sarcopterygians). The small size of this shark, and the fact that it is often found in shallow waters close to the shore, are some of the main reasons for which it has attracted attention from embryologists.

Even if knowledge of genomic organisation and gene expression data in Chondrichthyans is still clearly lagging behind that of Osteichthyans, this group is raising a growing interest in fundamental and biomedical research. The ongoing sequencing project of the little skate (*Raja erinacea*) genome is an important advancement in the field.

The chondrichthyan forebrain shares a common basic organisation with that of osteichthyans. As one would expect, the forebrain of cartilaginous fishes shows a prosomeric organisation. This organisation suggests a conservation of the fundamental mechanisms of patterning and regionalisation in gnathostomes, which has been confirmed by gene expression studies (e.g., in the dogfish: *Pax6*, Derobert et al., 2002a; *Emx*, Derobert et al., 2002b). The telencephalon of cartilaginous fishes forms by evagination, as in all other gnathostomes except teleosts, where it forms by eversion (Butler and Hodos, 1996). The evagination process, present also in lampreys, is likely to be an ancestral characteristic. In the dorsal telencephalon of chondrichthyans, medial, dorsal and lateral pallial subdivisions were identified (Wullimann and Vernier, 2006). The subpallium contains well defined septal, striatal and pallidal areas (Reiner et al., 1998). This feature, which probably reflects the ancestral gnathostome condition, is not present in teleosts, where the “pallidal” and “striatal” neurons intermingle (Alunni et al., 2004).

Immunohistochemistry and tract-tracing studies have provided information about connectivity and neurotransmitter distribution in the chondrichthyan brain (reviewed in Reiner et al., 1998; Wullimann and Vernier, 2006). At the level of the sensory systems, these studies have shown that information from most of the sensory pathways (and not only the olfactory projections) eventually reaches the telencephalon. The integrative and motor centres are highly similar to those of osteichthyans. Chondrichthyans possess a well developed cerebellum. Information about neurotransmitter distribution can be found in Smeets and Reiner (1994); Stuesse et al. (1991); Stuesse and Cruce (1991) and Rodriguez-Moldes et al. (2002). Curiously, there are pallial cholinergic cells in chondrichthyans, otherwise only seen in mammals (Rodriguez-Moldes et al., 2002).

Genes from the *Lhx* family are important at multiple levels of CNS development, from region identity to neuronal specification (Bach, 2000; Hobert and Westphal, 2000; Retaux and Bachy, 2002). We have focused our attention on one of these genes, *Lhx9*, due to its crucial role in dorsal forebrain



development and the availability of expression and/or genomic structure data in a large number of species, including *Drosophila* (*apterous*, Lu et al., 2000; Rincon-Limas et al., 1999), the actinopterygian medaka (Alunni et al., 2004), mouse (Retaux et al., 1999), *Xenopus* (Bachy et al., 2001) and lamprey (Osorio et al., 2005). In all gnathostomes studied, the expression pattern of *Lhx9* has been remarkably useful to understand the regionalisation of the forebrain. Furthermore, interspecies comparisons have highlighted the interest of this marker when studying pallial evolution. No *Lhx* gene has been studied in a chondrichthyan so far.

Among other regions, *Lhx9* is expressed in the medial pallium of mouse (Retaux et al., 1999) and medaka (Alunni et al., 2004). This region, which corresponds to the mammalian hippocampus, is characterised by the expression of the gene *Emx*, and an *Emx*-expressing region in the telencephalon may have appeared very early in evolution (Derobert et al., 2002a; Murakami et al., 2005). Dogfish has a medial pallium expressing *Emx* (Derobert et al., 2002a). To further investigate the similarity of this region to the medial pallium of osteichthyans, it would be important to assess the expression of other medial pallial genes such as *Lhx9*. Curiously, *Lhx9* is not expressed in the medial pallium of the frog *Xenopus laevis* (Bachy et al., 2001).

*Lhx9* and its paralog *Lhx2* were generated by gene duplication, probably in an ancestral gnathostome. The diencephalic expression of both genes in the thalamus and hypothalamus is common to all craniates, as well as the expression of *Lhx9* (*Lhx29* in the lamprey) in the prethalamic eminence. In the telencephalon of *Xenopus* and mouse, *Lhx9* expression is more restricted than that of *Lhx2*. The expression of the lamprey orthologue, *Lhx29*, covers the entire telencephalon (pallium and subpallium), while in mouse the expression of *Lhx9* is exclusively pallial (Retaux et al., 1999), and *Lhx2* is expressed throughout the telencephalon (Porter et al., 1997). A partial inversion of the expression pattern of *Lhx9* and *Lhx2* in the telencephalon was reported in *Xenopus* when compared to mouse (Bachy et al., 2001). These observations indicate a link between significant differences of *Lhx9* expression and major differences in forebrain organisation.

Often, the appearance of variety is correlated to a gene duplication event, as the redundancy then created may facilitate the fixation of mutations otherwise deleterious. This may be the explanation for the *Lhx2/Lhx9* shuffling in the pineal gland (Fig. 3.9).

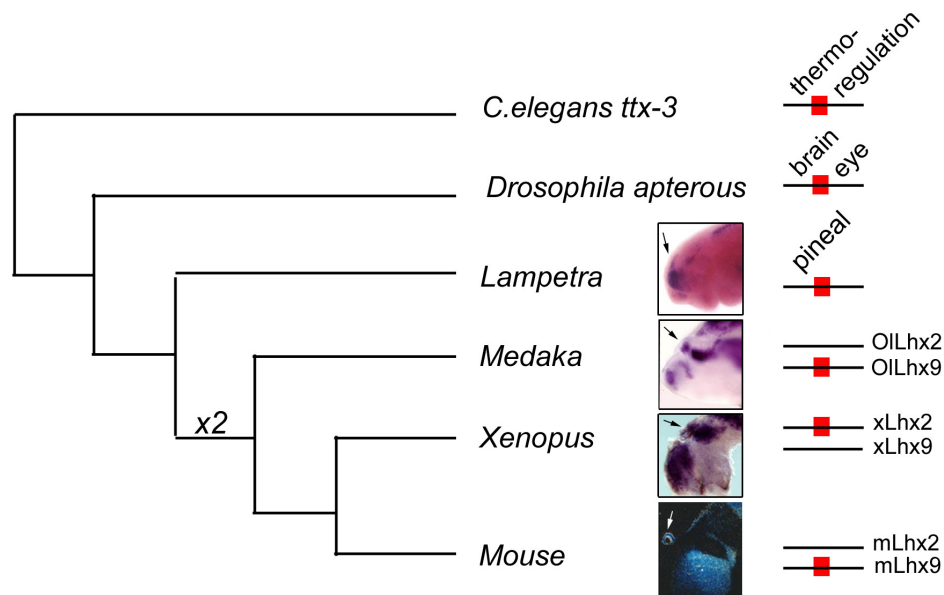


FIGURE 3.9: Shuffling between *Lhx2/9* family members in the pineal gland. A simplified tree schematises the expression of *Lhx2/9* in the pineal gland of craniates, and the expression of their orthologs in other groups. The photographs illustrate the expression of either *Lhx2* or *Lhx9* in the indicated species, as described in the present paper and in Retaux et al. (1999) for mouse, Bachy et al. (2001) for *Xenopus*, and Alunni et al. (2004) for medaka fish. On the right, the red square symbolises which paralog is expressed, and in which structures the orthologues are found in distantly related species. In *C. elegans*, *ttx-3* is expressed in the AIY neuron involved in thermoregulatory function (Altun-Gultekin et al., 2001; Hobert et al., 1997). In *Drosophila*, *apterous* is expressed among other regions in the brain and eyes (Lu et al., 2000; Rincon-Limas et al., 1999). Besides pineal expression, the gnathostome *Lhx2* gene has also prominent expression and function in the eye (Porter et al., 1997). Thus, the *Lhx2/9/apterous* Lhx members show evolutionary conserved expression (and function?) in organs related to phototransduction. Interestingly, an evolutionary link has been suggested between thermo-sensation (the function where *ttx-3* is involved) and phototransduction (Satterlee et al., 2001). Figure and legend courtesy of Sylvie R etaux.

An interesting feature of the *Lhx9* gene is the presence of an isoform, *Lhx9 $\alpha$* , generated by alternative splicing. The protein coded by this isoform lacks the DNA-recognition helix of the homeodomain, which had led to the suggestion that *Lhx9 $\alpha$*  could function as an endogenous dominant-negative form of *Lhx9* during development (Failli et al., 2000). These two isoforms have different effects in neuronal differentiation and different biochemical properties (Molle et al., 2004). The alternative splicing of the *Lhx9* is present at least in mouse, chick, zebrafish and medaka (Ensembl, [www.ensembl.org](http://www.ensembl.org)), which suggests a conserved role in brain development.

The presence of highly conserved non-coding elements (CNEs) associated with the control of gene expression is a typical feature of transcription factors and genes involved in embryonic development (*trans-dev* genes). These elements are often found at a large distance from the gene they regulate, which contributes to the conservation of synteny (e.g. the region around the *Shh* gene, Goode et al., 2005). These very conserved CNE-rich regions with a low density of protein coding-sequences and repetitive elements were named **stable gene deserts** (for a recent review on the evolution and classification of vertebrate gene deserts, see Ovcharenko et al., 2005). In the case of the *Lhx9* gene, the synteny of a large chromosomal region around the gene is indeed conserved in mouse, human, rat, dog and chicken, and is particularly coding-sequence poor (Ensembl).

Wanting to gain insight into the molecular evolution of the *Lhx* multigene family, we have screened a genomic BAC library with the purpose of isolating the *S. canicula* *Lhx9* gene. The project involves the analysis of the organisation and expression of this gene and the search for conserved non coding sequences with a potential enhancer function. This approach might be easier in the dogfish than in the lamprey, due to the dogfish phylogenetic position, closer to other craniates with already fully sequenced genomes.

### 3.2.2 Experimental approach

#### Materials and Methods

The consortium of laboratories interested in the development of lamprey and dogfish (see section 2.5) has promoted the construction of a BAC dogfish genomic library by the company Genoscope. We have used a heterologous *Lhx9* probe (from the actynopterygian *Astyanax mexicanus*) to screen this library.

One positive clone was identified by Southern blot and PCR, and is currently being sequenced at the Genoscope Sequencing Centre (Evry).

From this BAC we have synthesised an antisense *Lhx9* RNA probe for *in situ* hybridisation. A region of 1311 bp, mostly intronic, was amplified by PCR, using primers which recognise the beginning and the end of the homeobox of *Lhx9* genes. This fragment was used to synthesise an RNA probe, which will be used for *in situ* hybridisation on dogfish embryos of several stages.

### Results

In the mouse, the *Lhx9* gene possesses 6 exons, spanning a genomic region of 10 kb (Failli et al., 2000). We have currently access to a sequence length of 50 kb of the chosen dogfish BAC, for an expected final sequence of 150 kb. The available sequence allows us to know the position of the gene in the BAC, which is expected to contain the complete dogfish *Lhx9* gene. In the known sequence we have identified four exons of the gene, which correspond to the third, fourth, fifth and sixth mouse exons. The incomplete sequence is also sufficient to unambiguously assign this clone to the Lhx9 group, distinct from its paralogy group Lhx2. This confirms, as expected, that the gene duplication event which gave rise to *Lhx9* and *Lhx2* has indeed occurred in a gnathostome ancestor.

A preliminary sequence analysis suggests the possibility of the presence of alternative splicing, the end sequence of the potential Lhx9 $\alpha$  isoform being exactly identical to the mouse one. This would place the possible evolutionary origin of the two Lhx9 isoforms further back in time.

The size of the known introns does not differ significantly from those in mouse. If the gene size is approximately the same in the two species, 3 or 4 kb of additional sequence would be enough to reach the start codon of the gene. A large 5' upstream sequence will be thus available for CNE search by comparison to other species whose genomes are available online.

### Perspectives

***Lhx* genes in dogfish brain development** After the BAC sequencing is complete, we will try to identify dogfish *Lhx9* regulatory sequences and complete the study of this gene organisation in this species (intron-exon organisation, size of introns, possible sites of alternative splicing). Multi-species

alignment and identification of highly conserved regions will be used to identify hypothetic regulatory sequences. Homologous or heterologous transgenesis can then be performed to test the potential enhancer activity of these regions in *Lhx9* expression. A possible long-term expansion of this project would involve the mutation of these regions followed by further functional tests, and the analysis of other *Lhx* genes.

Note that if several independent but overlapping *Lhx9* BACs were available, an enhancer trap assay similar to those designed by Spitz et al. (2003) and Jeong et al. (2006) in their studies of *HoxD* and *Shh* control regions, respectively, could be a more efficient strategy to test large genomic intervals for the presence of enhancers.

### 3.3 Discussion

#### 3.3.1 Whole-genome duplications and the dawn of craniates

The hypothesis of one or two rounds of whole-genome duplications (WGD) in an ancestral craniate (1R or 2R) were based primarily in studies of gene content and number of certain gene clusters between protostomes and cephalochordates when compared to osteichthyans (e.g. *Hox* gene clusters, reviewed in Hoegg and Meyer, 2005; Panopoulou and Poustka, 2005). The exact timing of these events (e.g. 2R before or after the cyclostome/gnathostome split) is still a matter of debate, even after the analysis of *Hox* gene clusters in cyclostomes (hagfish, Stadler et al., 2004; lamprey, Force et al., 2002; Fried et al., 2003; Irvine et al., 2002). The idea of a WGD in an ancestral teleost (3R or FSGD) stemmed from similar comparative approaches between teleosts and sarcopterygians (reviewed in Meyer and Van de Peer, 2005). Recent studies have shown that non-teleost fishes have diverged before the FSGD (Hoegg et al., 2004). Cyclostomes and chondrichthyans are relatively less studied groups, but extremely relevant to clarify the molecular processes behind these evolutionary transitions, and their potential for the creation of novelty. They are also highly informative to assess the correct orthology relationships between gene family members, which was clearly demonstrated, for example, in the studies of the *Otx* (Germot et al., 2001; Plouhinec et al., 2003) and *Emx* (Derobert et al., 2002b) genes, where the dogfish sequences were included.

Interested in the evolution of the brain, and most particularly of the fore-

brain (a highly diverse structure among craniates), we have isolated *LfHh* and *ScLhx9*, both members of multigene families with important roles in brain development. Even if large-scale genomic events such as genome duplications cannot be studied by analysing a small number of genes, the accumulating data on a variety of gene families will help to confirm or dismiss their existence, and to correctly place their timing in evolutionary history. Their study will also shed light on the mechanisms of gene retention, gene loss and changes in gene regulation within each particular animal group.

### 3.3.2 Two *Hh* genes in lamprey

Duplicate genes are an important tool to study genome duplication events and the evolution of coding and non-coding regions. Our preliminary results indicate that two genes of the *Hedgehog* family might be present in lampreys (see 3.1.2). The phylogenetic analysis of the putative first exon of *Hedgehog* genes suggests an independent duplication of the lamprey *Hedgehog* genes within the *Sonic/Indian* group. Previous trees, where the predicted peptides encoded by *L. fluviatilis* (Osorio et al., 2005, supplementary data) and *P. marinus* (Uchida et al., 2003) cDNA fragments were used, have also assigned these two sequences to the same group. The *Desert* and *Sonic/Indian* groups were probably originated by an earlier pan-craniate duplication, being *Dhh* most closely related to the *Drosophila hedgehog* gene (see Ingham and McMahon, 2001). A lamprey *Dhh* homologue may exist, still undetected, or have been lost. An alternative scenario would be a gnathostome-specific duplication of *Hedgehog* genes into the *Sonic/Indian* and *Desert* groups.

The specific lamprey duplication of *Hedgehog* genes may have had still unknown consequences at the sub- or neofunctionalisation levels. Alternatively, is there a possibility that one of the lamprey *Hh* sequences detected corresponds to a pseudogene? While PmseqA and Lfcosmid3 are almost identical, there is a larger difference between PmseqB and Lfcosmid4. This sequence difference between the two species of lamprey might indicate the presence of a nonfunctional gene. This hypothesis can be fully tested only when the complete sequences of the two putative genes are analysed.

Previous results of *in situ* hybridisation with a probe synthesised from the lamprey *Hh* cDNA have revealed an expression pattern which is highly similar to the gnathostome *Shh* expression pattern (Osorio et al., 2005). This has led

us to propose the scenario discussed in chapter 2, where the functions of *Hh* and *Shh* in brain development would be globally similar, excepting those involved in ventral forebrain specification. This pattern contrasts with the *Dhh* and *Ihh* patterns, which are not expressed in the brain (see review by Ingham and McMahon, 2001), even if *Ihh* expression was recently detected in the chick spinal cord (Aglyamova and Agarwala, 2007). The fact that *Shh* expression is more widespread (Ingham and McMahon, 2001) resembles a typical “Ohno-mechanism”, where an expression divergence occurs after duplication (specifically for one of the paralogs, in Ohno’s original idea). *Ihh* has roles in vasculogenesis and hematopoiesis, heart, gut, eye and skeletal development, and regulation of left-right asymmetry. *Shh* expression is important in all of these processes as well, even if their functions are not always redundant. *Dhh*, on the other hand, is the sole *hedgehog* involved in gonad and peripheral nerve development (reviewed in Ingham and McMahon, 2001). The accelerated regulatory evolution of duplicate genes could have been the basis for the acquisition of these new *Dhh* expression domains.

### 3.3.3 Conserved non-coding sequences in lamprey and dogfish

Even more than changes in coding sequences, changes in gene expression regulatory regions may have played a major role in evolution (Carroll, 2005; Wray, 2007). It is likely that these regions are under high selective constraint, and conserved non-coding elements (CNEs) are often more conserved than coding regions themselves (Dermitzakis et al., 2005; Woolfe et al., 2005). The majority of the CNEs identified controls the expression of transcriptional regulators and genes involved in early development (*trans-dev* genes, e.g. Woolfe et al., 2005). Although at first CNEs were thought to be absent outside of vertebrates, a recent study has identified a set of CNEs sharing sequence characteristics, but not identity, between nematodes, flies and humans. CNEs thus represent a very unusual class of sequences that are extremely conserved within specific animal lineages yet are highly divergent between lineages (Vavouri et al., 2007).

So far, none of the CNEs identified among other species was found in lamprey or dogfish. However, a recent important study has identified two distinct enhancers which drive mouse *Otx2* expression in the anterior neurectoderm (AN) and forebrain/midbrain (FM), and which are conserved in all tetrapods, but also in skates and coelacanth (Kurokawa et al., 2006). Curiously, these

authors have also shown that the AN enhancer has been lost in the teleost lineage, the FM enhancer having acquired an AN activity. In lamprey, the first attempts to identify CNEs have been unsuccessful so far (e.g. *Dlx* genes, Marc Ekker's laboratory, personal communication). This is probably related to the evolutionary distance between the animals compared, and to the smallness of these elements, but also to the particularities of the lamprey genome — abundance of highly repetitive motifs in non-coding regions, high GC content and long introns. It is also worth noting that, as mentioned in chapter 1, not all regulatory sequences are evolutionarily conserved, even within gnathostomes (Fisher et al., 2006; reviewed in Elgar, 2006). A recent article by Dickmeis and Muller (2005) reviews the current methods used to identify conserved regulatory elements of developmental genes by comparative genomics, including new attempts to detect conserved features beyond simple sequence similarities.

The genes we have isolated, *LfHh* and *ScLhx9*, are both developmentally expressed in a structure — the brain — which underwent dramatic changes during chordate evolution. After their sequencing is complete, we will investigate the presence/absence of CNEs conserved within gnathostomes which may control the expression of these genes in the brain. After functional tests are performed, we will try to correlate differences in gene regulation with the acquisition/loss of gene expression domains and with changes in the timing of gene expression.

**Lamprey *Hh*** Are the gnathostome *Shh* enhancers conserved in the lamprey *Hh* gene? If so, are the distances from the transcription start smaller or larger than in gnathostomes, and does this have an effect in gene expression? If existent, do the conserved enhancers have a similar function? Are we capable of detecting “hidden”, non-conserved enhancers (for example in introns) with similar functions?

The evolutionary distance between lamprey and gnathostomes poses obvious problems for CNEs search. Alignment-based long-range CNEs search is facilitated by the fact that long range enhancers are often found in syntenic regions. In the case of *Shh*, the synteny is maintained across human, mouse and *Fugu* (Goode et al., 2005). However, the distances from the *Shh* long-range enhancers to the transcription start vary between species, and may be important for expression differences (Jeong et al., 2006). It would be interesting to know what the situation may be in the regulation of the lamprey *Hh*



gene expression.

If the lamprey and gnathostome sequences are similar enough to detect highly conserved non-coding sequences with the methods currently available, we may find the answer to the very speculative question of the existence of any potentially active sequence which would resemble the SBE3 mouse enhancer. This enhancer drives *Shh* expression in the mouse subpallium (Jeong et al., 2006), and would obviously not have the same function in lamprey.

An intriguing observation is the conservation of enhancer structure but not of its function, while the overall expression pattern of the gene is conserved. This discrepancy has been notably reported for *sonic hedgehog*, where the conserved enhancers drive the gene expression in distinct regions of the CNS of mouse and zebrafish (Fig. 1.13). Although it is possible that regulatory motifs evolve slowly by gradual mutations, duplication enhances the chances of divergence of these motifs between the two paralogs, due to an initial redundancy (reviewed in Taylor et al., 2001). We could speculate that the *Shh* regulatory differences in mouse and zebrafish could be related to the additional whole-genome duplication of teleost fish. The initial redundancy of the two duplicate genes (now *shha* and *shhb*, also known as *tiggy-winkle hedgehog*) in an ancestral fish might have permitted a complex regulatory switch that resulted in the actual correlation between particular enhancers and sites of expression. In the brain, *shhb* is expressed in the same sites as *shha*, although at lower levels and often in a subset of cells within each *shha*-expressing region (Ekker et al., 1995; Scholpp et al., 2006). Even if the expression of these two genes is very similar, its timing is rather different. Furthermore, the *shhb* mRNA is completely absent from the notochord (Ekker et al., 1995). Identification and functional analysis of the *shhb* brain enhancers and additional studies in non-teleost craniates would be necessary to test this hypothesis, which predicts that the mouse situation would be closer to the ancestral state.

After the release of the lamprey genome, currently under sequencing, we will have a better notion of frequency of repeated elements, typical intergenic distances and size of introns. Most importantly, we will be able to search CNEs by whole-genome comparisons, as what has been done between zebrafish and mouse (McEwen et al., 2006), currently two of the most distant craniates with a fully sequenced genome.

An alternative to enhancer search by sequence comparison will be to test functionally, by heterologous transgenesis in a gnathostome, large non-coding

regions of lamprey genes, in search for a conserved functional (although not sequence) conservation. For the lamprey *Hh*, the intronic sequences would be given priority, as they are known to possess functionally active regulatory elements in both mouse and zebrafish, being some of them conserved in all studied mammals and fish (human, mouse, zebrafish, *Fugu*; Goode et al., 2003).

**Dogfish *Lhx9*** Feasibility of searching for CNEs with a regulatory function will be higher, using standard phylogenetic footprinting methods, in the case of dogfish than in lamprey, given its closer proximity to gnathostomes and the fact that many of these techniques have been specially developed bearing in mind the genomic characteristics of this group. The successful detection of CNEs in another chondrichthyan, the skate (Kurokawa et al., 2006), fully confirms this idea.

The identification of regions regulating *Lhx9* expression in dogfish and their functional test will be important contributions to further understand the mechanisms of pallial regionalisation and its evolution within gnathostomes. They will also be important to gain insight into the evolution of the *Lhx2/Lhx9* gene group, and into possible function shuffling events. In the future, we expect to extend this genetic analysis to the lamprey *Lhx29* gene.



## Chapter 4

# Conclusions

The study of the expression pattern of genes of the *LIM-homeobox*, *Pax* and *Hedgehog* families in the developing lamprey brain has revealed a striking conservation of the mechanisms of forebrain patterning and regionalisation. Craniate novelties are the establishment of the *Hh*-expressing zona limitans intrathalamica as a secondary organiser, whose signalling properties might orchestrate the prosomeric organisation of the forebrain.

Gnathostome innovations include a *Shh*- and *Nkx2.1*-expressing region in the ventral telencephalon. This region corresponds to the adult pallidum, where GABAergic interneurons and subpallial cholinergic neurons are specified and from where they migrate. The presence of the pallidum is, for this reason, probably correlated with major differences of information integration and processing in the telencephalon.

In lamprey as in *Xenopus* the regional identity of diencephalic prosomeres is maintained in larval stages, as demonstrated by the non-embryonic expression of the *LIM-homeobox* gene *Lhx15*. Our study also highlights the possibility of using this marker to follow the development of specific regions and neuronal populations through time.

The study of *Hh* genes at a genomic level has suggested the existence of two *Hedgehog* genes in lamprey, a finding that changes our previous views in the evolution of this multigene family within chordates. The isolation of the *LIM-homeobox* gene *Lhx9* in dogfish has shown that the duplication of *Lhx9* and *Lhx2* has probably taken place prior to the divergence between chondrichthyans and osteichthyans.

Further molecular and gene expression analysis will be now undertaken

with the goal of gaining insight into the history of gene duplications and changes in gene regulation which underlie important differences within craniates and between craniates and urochordates.

## Appendix A

# Neurogenesis in the mouse brain

After the broad regionalisation of the craniate brain is completed, a set of molecular cues will ultimately define neurogenetic regions. Neurons from the proliferating zone will be specified to a certain neuronal phenotype while migrating to the differentiation zone. From their original location they can then migrate and populate other regions of the brain.

The *Mash1*, *Neurogenin1/2* and *NeuroD* bHLH genes are known to be implicated in the process of neurogenesis. The complementary expression pattern of these genes during a specific period of forebrain development, when massive neurogenesis is taking place, and its association with specific neuronal phenotypes seems highly conserved during craniate evolution.

During my PhD I had the opportunity of working on this subject with Mario Wullmann, then at the DEPSN lab. Our project was to analyse in more detail the expression of bHLH genes in the mouse embryonic brain, and compare this data with the body of work he and his colleagues had already published on zebrafish and *Xenopus*.

Our results show the extremely high evolutionary conservation of expression patterns (e.g. almost complementary expression of *Mash1* versus *Neurogenin2* and *NeuroD*) and coordination of expression timing of these genes.

An interesting question remains if and to which extent this conservation is present in the lamprey brain, and how exactly is the timing of the molecular specification of neurogenesis in Cyclostomes.

The work presented next constitutes the current form of an article to be soon completed.

**Phylogenic expression of the bHLH genes  
*Neurogenin2*, *NeuroD* and *Mash1* in the mouse  
embryo**

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**Key words:** Cash, cortex, eminentia thalami, Dlx, Mash, Neurogenin, NeuroD, pallium, proneural genes, subpallium, ventral thalamus, Xash, Zash, zona limitans intrathalamica

**Abstract**

Recent detailed reports in two amniote model animals, zebrafish and African clawed frog *Xenopus laevis*, revealed highly comparable early forebrain expression patterns of basic helix-loop-helix (bHLH) genes relevant for neurogenesis (*atonal* homologues, i.e., neurogenins/*NeuroD* and *achaete-scute* homologues, i.e., *Zash1a/Xash1*) during a particular stage of development (zebrafish: 3d; frog: stage 48). Furthermore, forebrain GABA cell developmental patterns are positively correlated to *Zash1a/Xash1* expression in frog and zebrafish. In the embryonic mouse telencephalon (E12.5-13.5), homologous bHLH gene expression and GABA/GAD65/GAD67 cell patterns are highly similar to the situation in these amniote models. In particular, subpallial *Mash1* expression is part of a neurogenetic pathway involving downstream regulatory genes (e.g., *Dlx1/2*, *Nkx2.1*, *Lhx6*) that guide the development of GABA/GAD cells,

different from the pathway in the cortex leading to glutamatergic cells involving *Neurogenin1/2*, *NeuroD* and different downstream genes (e.g., *Emx1/2*, *Tbr1*, *Lhx9*). In the frog and zebrafish, the discussed bHLH and other regulatory genes, as well as GABA/GAD cells are also expressed in a complementary way in most of the remaining forebrain beyond the telencephalon. Therefore, we describe in the mouse brain these bHLH gene expression patterns in more detail than previously done to fill some of the existing gaps in the identification of expression domains, especially outside of the telencephalon. Clearly, there is almost perfect correlation of *Mash1* gene expression with GABA/GAD cell distribution and complementarity of territories expressing *Mash1* versus neurogenins/*NeuroD* in the mouse brain. Thus, the situation resembles much that in frog and zebrafish. The data in three vertebrate model species continue to support our previous notion of a “phylotypic stage” of neurogenesis during vertebrate forebrain development.

## Introduction

The roles of various basic helix-loop-helix (bHLH) genes in central and peripheral nervous system neurogenesis (for example *Drosophila atonal* homologues, such as neurogenins/*NeuroD*, and *Drosophila achaete-scute* homologues, such as *Mash1*) have been well established in several vertebrate neurogenetic model animals, such as the mouse or rat, the chicken, the African clawed frog *Xenopus laevis* or the zebrafish. In particular, the ventricularly located central nervous expression in proliferative neural cells of *Neurogenin1*, *Neurogenin2* and *Neurogenin3* of murine mammals (Ma et al., 1996, Sommer et al., 1996), of *Neurogenin1* and *Neurogenin2* in the chicken (von Frowein et al., 2002), of *Neurogenin-related-1* in the frog *Xenopus* (Lee et al., 1995; Ma et al., 1996) and of *neurogenin1* in the zebrafish (Blader et al., 1997; Korzh et al. 1998) has been demonstrated in these neurogenetic model animals to be earlier than and to promote the subsequent activity of *NeuroD* which is expressed in early postmitotic neuronal cells. The *NeuroD* gene is expressed downstream of all the above neurogenin homologues or paralogues later in the same cells when they sit more remote from the ventricular lining in a migrated position. In murine mammals, *Neurogenin1* and *2* have largely overlapping expression domains (but see Table A.1 and text below for some exceptions) covering most brain territories. However, the murine *Neurogenin3* is expressed in an ad-



ditional limited domain within the hypothalamus. The murine *Mash1* gene (Johnson et al., 1990; Lo et al., 1991; Guillemot and Joyner, 1993; Ma et al., 1997; Horton et al., 99; Torii et al., 1999; Casarosa et al., 1999; Fode et al., 2000, Parras et al., 2002) and its homologues in zebrafish (*Zash1a*; Allende and Weinberg, 1994), chicken (*Cash1*; Jasoni et al., 1994, von Frowein et al., 2002) and *Xenopus* (*Xash1*; Ferreira et al., 1993, 1994) are generally expressed in different central nervous cell populations than neurogenins/*NeuroD*.

These pioneer reports focussed on particular central nervous locations, such as the telencephalon or spinal cord, and used various molecular genetic techniques to reveal important functional aspects of differential bHLH gene activity. Apparently, neurogenins/*NeuroD*, on the one hand, and *Mash1*, on the other hand, act in different neurogenetic pathways in the murine brain (Johnson et al., 1990; Lo et al., 1991; Guillemot and Joyner, 1993; Ma et al., 1997, Horton et al., 99; Torii et al., 1999; Casarosa et al., 1999; Fode et al., 2000, Parras et al., 2002), with the exception of the mouse sensory olfactory epithelium where both *Neurogenin1* and *Mash1* are expressed in the same cells (Cau et al., 2002). Furthermore, cortical *Neurogenin1* activity partially depends on *Neurogenin2* and both neurogenins act inhibitory on *Mash1* expression in the cortex (Fode et al., 2000). *Mash1* mutant mice show severe morphological disturbance of ventral telencephalic development due to reduced proliferation and lack of subsequent differentiation (Horton et al., 1999). Thus, the mutual spatial exclusive activity of neurogenins/*NeuroD* in the cortex and *Mash1* in the embryonic basal ganglia (medial and lateral ganglionic eminences; MGE/LGE) during neurogenesis has been emphasised in the mouse brain and many details regarding the involvement of downstream genes that control neuronal differentiation in these two telencephalic regions are meanwhile known (see Wullmann and Mueller, 2004b; Mueller et al., 2006 for recent discussions). The spatial difference in activity of these two neurogenetic pathways in the telencephalon is directly related to the embryonic generation of glutamatergic versus  $\gamma$ -aminobutyric acid (GABA)-ergic neuronal phenotypes in the cortex and ganglionic eminences, respectively (Casarosa et al., 1999; Fode et al., 2000; Marín et al., 2000; Parnavelas, 2000; Bertrand et al., 2002; Parras et al., 2002; Schuurmans and Guillemot, 2002; Ross et al., 2003; Chapouton and Bally-Cuif, 2004; Schuurmans et al., 2004). However, no attempt has been made to give a full account of these murine central nervous bHLH gene expression domains. More often than not, additional expression domains — if fortuitously

captured in a photograph — were not identified neuroanatomically (see Table A.1 for detailed analysis) and, thus, have remained elusive to the uninitiated. As the mouse is the most important model vertebrate for studies in neurogenesis and neuronal phenotype differentiation, the unambiguous identification of mouse bHLH gene expression domains outside the telencephalon is a necessary prerequisite for further studies in this direction.

Recent studies in the frog *Xenopus* (Wullimann et al., 2005) and the zebrafish (Mueller and Wullimann, 2003; 2005; see for more literature there and in discussion below) have provided a complete account on brain expression patterns of *neurogenin1* (*Neurogenin-related-1* in *Xenopus*) and *NeuroD* (as well as on *Zash1a* and *b* in the zebrafish) in combination with a description of early neural proliferation (using PCNA and/or BrdU) during beginning secondary neurogenesis (3d zebrafish; stage 48 frog) in these two amniote model species. Especially the studies in the zebrafish revealed that there is complementarity in forebrain expression of *neurogenin1* / *NeuroD* domains, on the one hand, and *Zash1a* domains, on the other hand, beyond that noted in the pallium (cortex) and subpallium (basal ganglia) before in the mouse or rat. This complementarity also correlates perfectly with recently described early GABA cell populations in the zebrafish brain which exclusively develop in *Zash1a* domains in the forebrain, but not in *neurogenin1* / *NeuroD* domains (Mueller et al., 2006). Also, early zebrafish GABA cell distribution fits the pattern seen in embryonic GABA/glutamic acid decarboxylase (GAD65, GAD67) cell development in the mouse (Katarova et al., 2000). Moreover, expression patterns of the *Neurogenin-related-1* / *NeuroD* (Wullimann et al., 2005) and *Xash1* genes (Ferreiro et al., 1993; 1994) in *Xenopus* also nicely conform to the patterns seen in zebrafish and mouse. Furthermore, presence of early GABA cells may be recognised in the *Xenopus* subpallium, ventral thalamus (prethalamus) and preoptic region, as well as the absence of such cells in pallium and dorsal thalamus (Roberts et al., 1987; Barale et al. 1998; see also Table A.2). Together, this strongly suggests that a common pattern of early, spatially exclusive generation of glutamatergic and GABAergic neuronal phenotypes is common to the forebrains of zebrafish and mouse/rat — and likely all vertebrates — during a particular stage of beginning secondary neurogenesis which precedes the later intermingling of neuronal phenotypes by long-distance tangential or other types of migration. This stage has been suggested to represent kind of a phylotypic (or class typic) stage of vertebrate forebrain neurogenesis (Wullimann

et al., 2005; Mueller et al., 2006).

The present study re-examines in detail the expression domains of *Neurogenin2*, *NeuroD* and *Mash1* in the embryonic mouse brain in order to provide an explicit molecular neuroanatomy of relevant bHLH gene expression domains. The mouse is the primary vertebrate model for neurogenesis and future studies in neurogenesis relating to neurochemical phenotypes beyond the telencephalon will depend on such a detailed analysis. Furthermore, the data presented here also shed light on comparative aspects between model systems and, thus, brain evolution.

## Material and Methods

### *In situ* hybridisation

Mouse brains (E11.5, E12.5, E13.5) were dissected out of the embryos, and either parasagittally cut into two halves or the dorsal part of the cortex of one side was removed to allow for an opening in the lateral ventricles. For *in situ* hybridisation, embryos were fixed overnight in 4% paraformaldehyde (PFA), then progressively dehydrated in methanol, and stored at  $-20^{\circ}\text{C}$  in 100% methanol. Digoxigenin-labeled antisense riboprobes against *Neurogenin1*, *Neurogenin2*, *NeuroD* and *Mash1* mRNAs were synthesised and used for wholemount *in situ* hybridisation according to standard protocols. Brains were progressively re-hydrated, then treated with proteinase K ( $10\mu\text{g}/\text{ml}$ , 10 min) and with PBT-glycin (2 mg/ml). Embryos were then fixed with 4% PFA — 0.2% glutaraldehyde for 20 min before pre-hybridisation (2 hr at  $65^{\circ}\text{C}$ ). Hybridisation was carried out overnight at  $65^{\circ}\text{C}$  in a 50% formamide hybridization medium containing 100–300 ng/ml of DIG-labelled RNA probe. Specimens were incubated with alkaline phosphatase (AP)-conjugated anti-digoxigenin Fab fragments (diluted 1:4000; Roche Diagnostics, Mannheim, Germany) at  $4^{\circ}\text{C}$  overnight. Alkaline phosphatase activity was detected with NBT/BCIP (Roche Diagnostics, Mannheim, Germany). Brains dehydrated in 80 % ethanol after fixation were observed in toto and photographs were taken. Some brains were dehydrated in ascending ethanol, then butanol-1, embedded in paraffin and sectioned with a Leica microtome at  $10\mu\text{m}$ .

## Terminology

Neuroanatomical designations for the zebrafish brain are taken from the atlas of early zebrafish brain development (Mueller and Wullimann, 2005), which is based on publications by Wullimann and Puelles (1999), Mueller and Wullimann (2002, 2003), Wullimann and Mueller (2004a,b). Neuroanatomical designations for the *Xenopus* brain are from Wullimann et al., 2005, and terms for the mouse brain have been adopted from Katarova et al. (2000).

## Photomicrograph production

Photomicrographs have been shot with a NIKON Digital Camera DXM 1200. Digital photoprocessing — including sharpening, color and brightness adjustments as well as merging — has been carried out with Adobe Photoshop. Picture arrangements and designation labeling have been done with Corel Draw.

## Results

Mouse brains whose ages cover the period of phylotypic proneural gene expression (stages E11.5, E12.5, E13.5) were *in situ* hybridised (wholemounds or wholemounts sagittally cut into two parts, see Materials and Methods) for *Ngn2*, *NeuroD* or *Mash1* transcripts. A representative example for each gene is shown in Fig. A.1. It also reflects our impression of what stage yielded the best result for each gene. Some specimens were subsequently paraffin embedded and sectioned for visualisation and corroboration of expression domains (Fig. A.2, Fig. A.3, Fig. A.4, Fig. A.5, Fig. A.6), the section plane being transverse (especially for the midbrain) or — taking the anteriorposterior axis into account — horizontal (forebrain, hindbrain).

### *Neurogenin2*

Generally, *Neurogenin2* (*Ngn2*) expression domains lie in a broad band from ventricle towards periphery, leaving the most pially lying tissue free of transcripts. Distinct *Ngn2* expression domains in the secondary forebrain between E11.5 and E13.5 are present in the olfactory bulb, all parts of the cortex (ventral, lateral, dorsal and medial pallium/cortex) and in the eminentia thalami. It has to be noted that the strong oval signal in the cortex seen in Fig. A.1

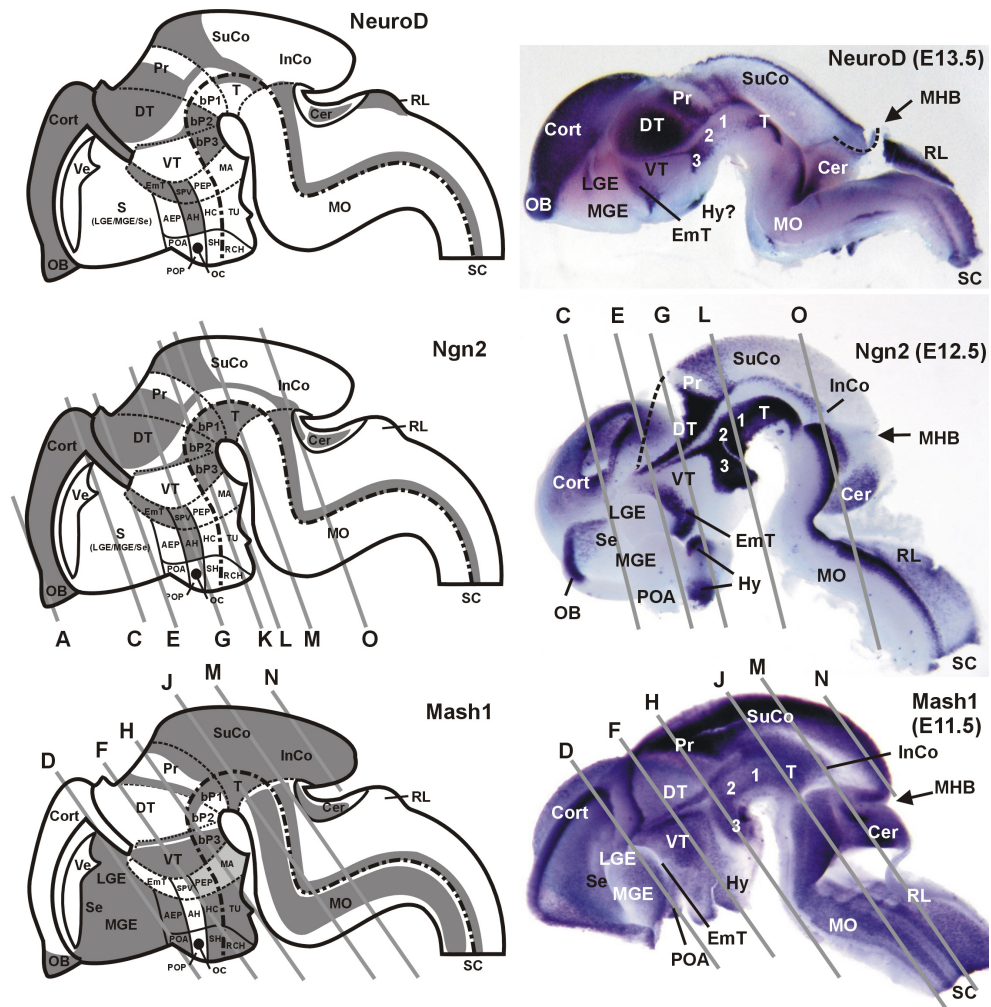


FIGURE A.1: Wholemount mouse brains (right panels) *in situ* hybridised for (A) *NeuroD* (E13.5), (B) *Neurogenin2* (E12.5), (C) *Mash1* (E11.5). Interpretative schemata are shown in left panels. Grey lines indicate section levels shown in Fig. A.2, Fig. A.3, Fig. A.4, Fig. A.5, Fig. A.6. Note that *Ngn2* and *NeuroD* have largely corresponding expression domains (except for the rhombic lip), and that *Mash1* has complementary expression domains to *Ngn2/NeuroD* in most of the forebrain. For abbreviations, see list.

is an artefact at the rim of an opening that has been made to allow for better penetration of the probe; the situation for the cortex is visualised in the sectioned material (Fig. A.2, Fig. A.3, Fig. A.4). In contrast, medial and lateral ganglionic eminences (MGE/LGE), septum and preoptic region are free of *Neurogenin2* expression (Fig. A.1, Fig. A.2, Fig. A.3, Fig. A.4). There is a gradient from stronger expression in the anterior cortex to weaker expression in the posterior cortex.

A massive diencephalic *Neurogenin2* expression domain is seen in the alar plate dorsal thalamus (Fig. A.1, Fig. A.2, Fig. A.3, Fig. A.4; tissue is partially lost in wholemount shown in Fig. A.1, the true extent of dorsal thalamic expression domain is indicated by a stippled line and is visualised in its entirety in sectioned material, see Fig. A.2, Fig. A.3, Fig. A.4 G-J). The *Ngn2* domain in the pretectum appears less dense, i.e., labelled cells are more spaced and more remote from the ventricle compared to those in the thalamus. Thus, these pretectal *Ngn2* positive cells are much better seen in the sectioned material. These diencephalic alar plate expression domains contrast with a clear absence of expression in the alar plate ventral thalamus (prethalamus).

In the posterior diencephalic basal plate there is another strong *Ngn2* expression domain (Fig. A.1, Fig. A.2, Fig. A.3, Fig. A.4), covering basal prosomeres 1 through 3, i.e., basal P1 (bP1; basal pretectal prosomere or synencephalon), basal P2 (bP2; dorsal thalamic prosomere or posterior parencephalon), and basal P3 (bP3; ventral thalamic prosomere or anterior parencephalon). There is a sharp, transverse boundary of expression in the basal plate towards the more anterior *Neurogenin2*-free hypothalamus. From the diencephalic basal plate expression domain a conspicuous, spear-shaped domain extends dorsally at the bP2/bP3 boundary zone, representing a transversely oriented domain. This spear-shaped extension seemingly corresponds to the zona limitans intrathalamica (or a region directly adjacent to it; see discussion) and it is separated by an expression-free gap from the alar plate (dorsal) thalamic domain (Fig. A.1, Fig. A.2, Fig. A.3, Fig. A.4). The hypothalamus is free of *Ngn2* expression, with the exception of a band of expression anterior to the eminentia thalami and prethalamus. This *Ngn2*-domain lies in the alar plate part of the mouse hypothalamus and starts out at the third ventricle directly contiguous with the *Ngn2*-positive domain of the eminentia thalami and extends then, anterolaterally to the (*Ngn2*-negative) prethalamus, into

the periphery of the hypothalamic neural tube wall (Fig. A.2, Fig. A.3, Fig. A.4 F-G).

In the midbrain, a first expression domain is present in the anterior part of the superior colliculus, some dispersed *Ngn2* positive cells in the more posterior superior colliculus are also visualised in the sectioned material. These labelled cells in the superior colliculus are always migrated to some degree away from the ventricle. A second expression domain is located at the ventricle and forms a longitudinal stripe that runs along the entire midbrain roof (alar plate), finally broadening into the inferior colliculus at its posterior pole (Fig. A.1). Separate from those alar plate midbrain domains, an additional *Neurogenin2* expression domain is seen in the basal plate midbrain tegmentum, which lies directly posteriorly adjacent to the diencephalic basal plate domains mentioned above. Its expression is clearly seen in the wholemount specimen shown in Fig. A.1. In the sectioned material, its expression is less strong and may be already downregulated. Caudal to the tegmentum, there is a clear gap of *Neurogenin2* expression in the isthmus. However, in the rhombencephalon, a longitudinal stripe of expression is again present in the medulla oblongata. This domain lies more dorsally than the midbrain tegmental one, and is clearly within the rhombencephalic alar plate, which is obvious in the sectioned material (Fig. A.2, Fig. A.3, Fig. A.4 N-Q). This medullary expression seems thicker in dorsoventral extent in the anterior rhombencephalon (rhombomere 1) than more posteriorly. Another distinct *Neurogenin2* domain is present in the cerebellum.

### ***NeuroD***

The expression pattern of *NeuroD* in the mouse is only described here from wholemount material. *NeuroD* expression in the mouse brain follows closely that of *Neurogenin2*. In accordance with the literature, our preliminary sectioned material also showed in many places that *NeuroD* expressing cells lie more remote from the ventricle than *Ngn2* positive cells (e.g. in the cortex), indicating that *NeuroD* is expressed in postmitotic mantle cells. Between E11.5 and 13.5, *NeuroD* is expressed in the olfactory bulb, all parts of cortex, and the eminentia thalami, but not in medial and lateral ganglionic eminences (MGE/LGE), and not in septum and preoptic region, which are all free of expression. The alar plate diencephalon shows strong expression domains in the

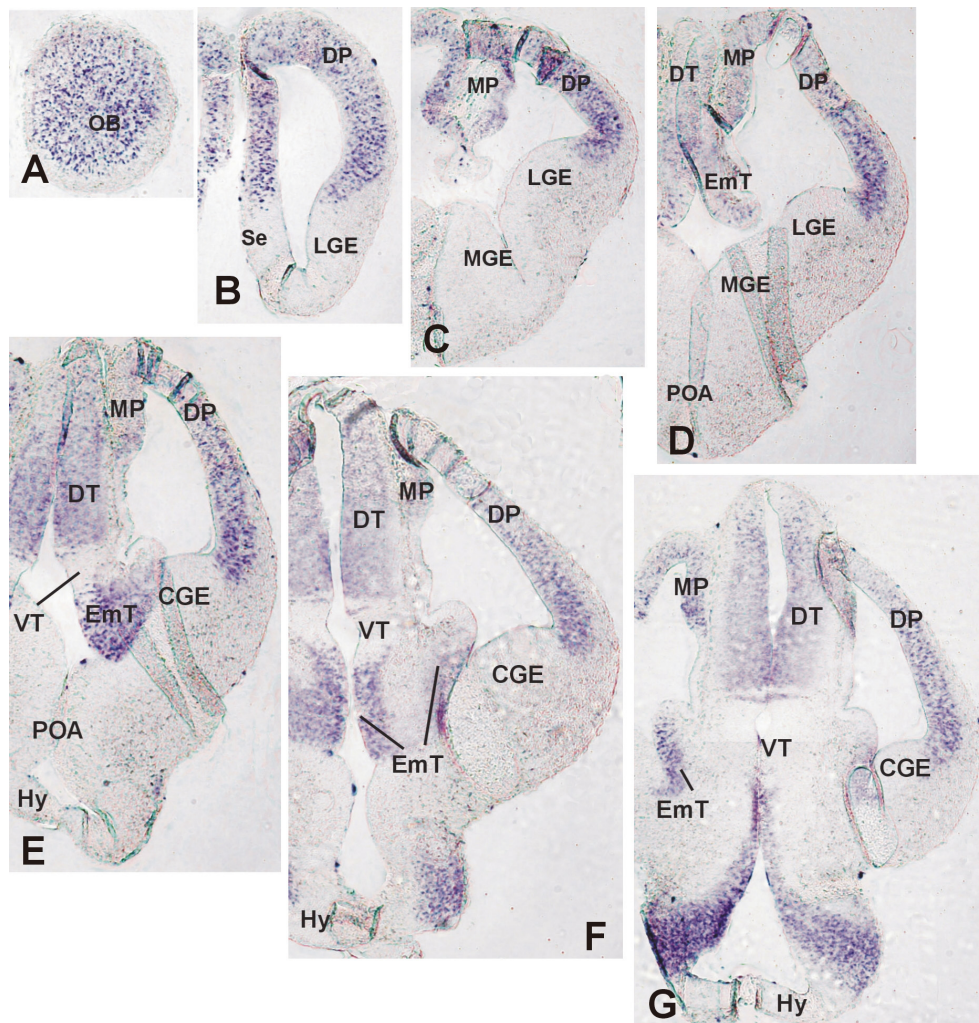


FIGURE A.2: Transverse sections of a mouse brain *in situ* hybridised for *Neurogenin2* at E12.5, Plate 1. A whole mount view of expression domains as well as levels of sectioning are indicated in Fig. A.1.



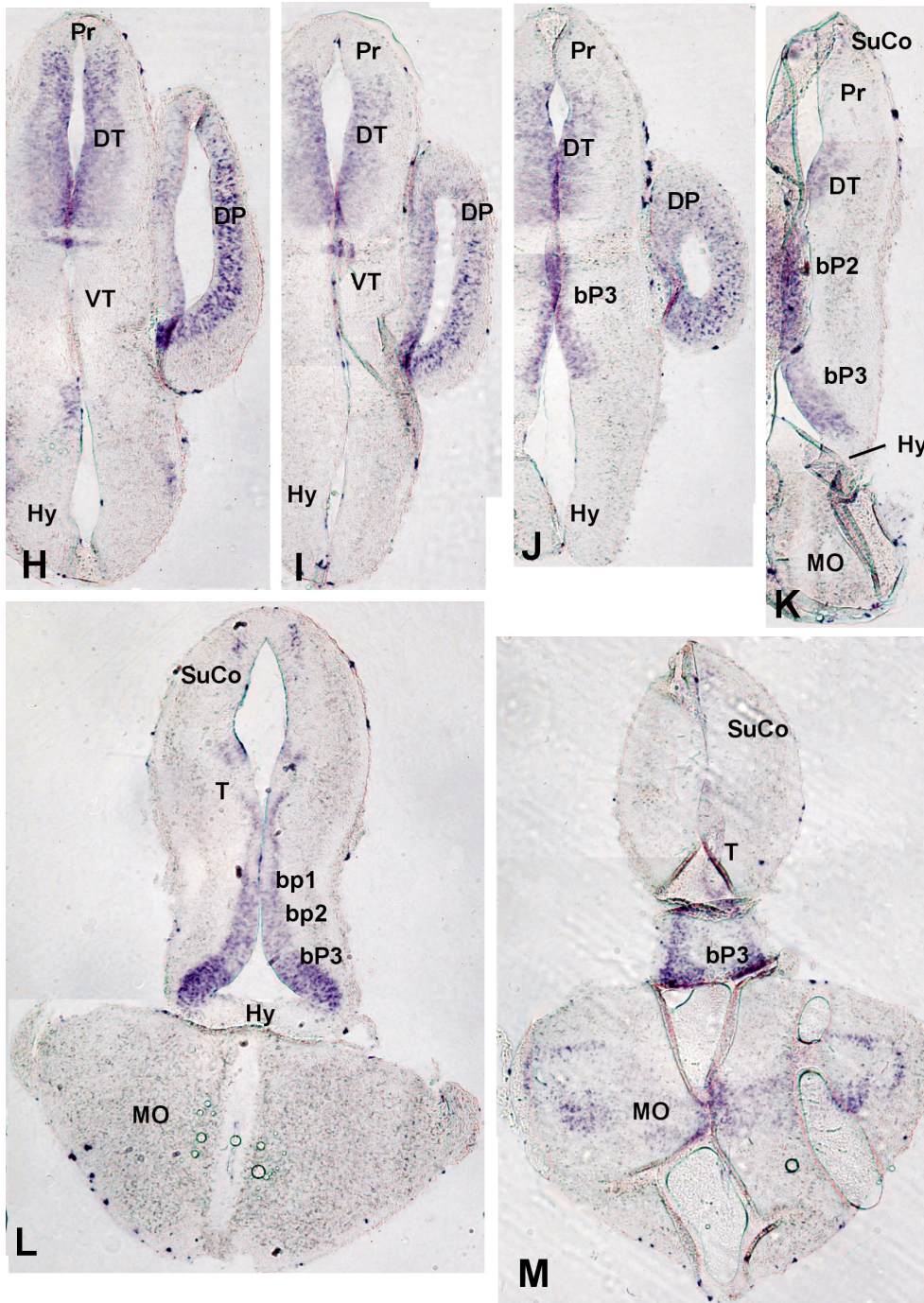


FIGURE A.3: Transverse sections of a mouse brain *in situ* hybridised for *Neurogenin2* at E12.5, Plate 2. A whole mount view of expression domains as well as levels of sectioning are indicated in Fig. A.1.

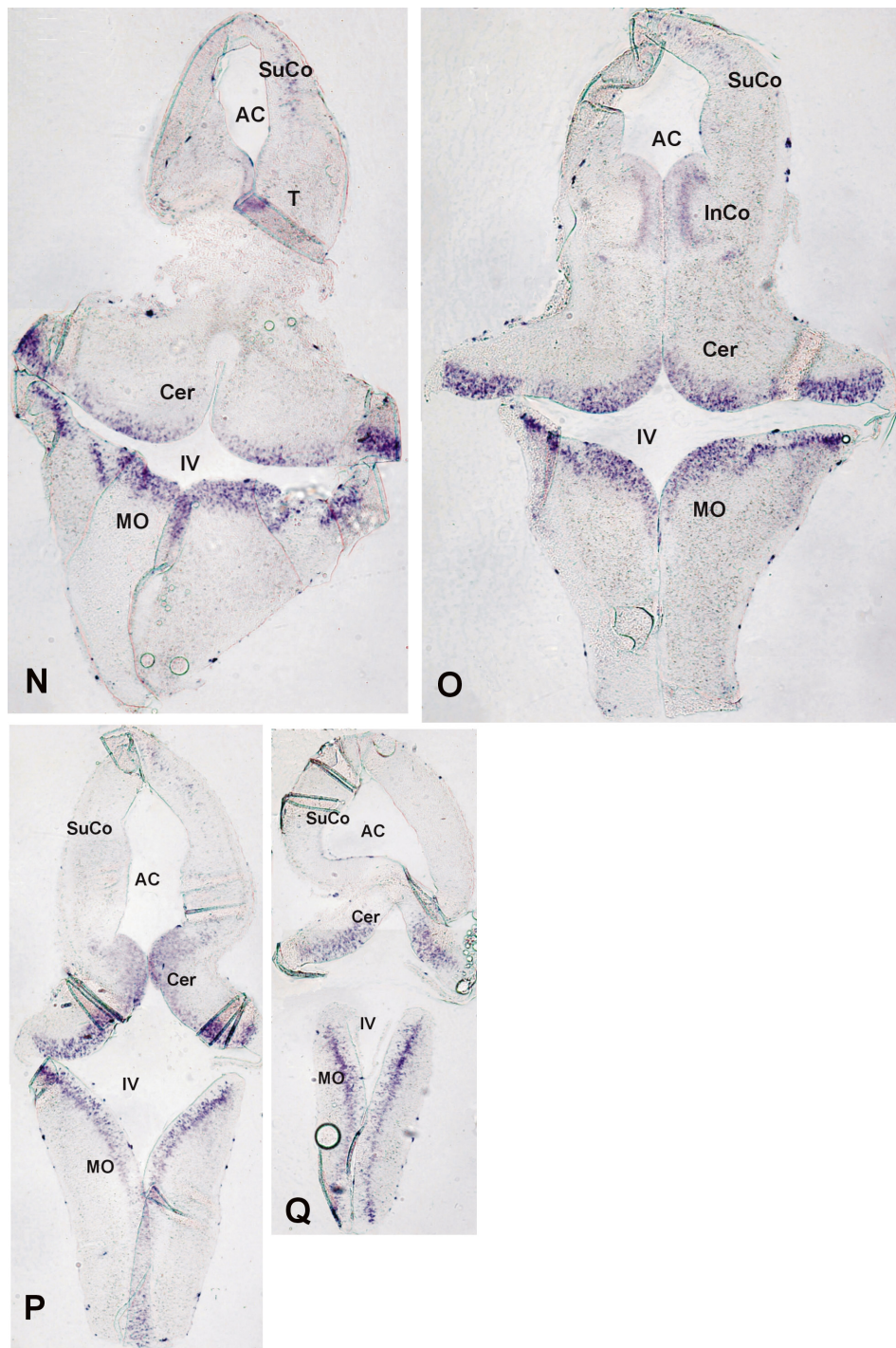


FIGURE A.4: Transverse sections of a mouse brain *in situ* hybridised for *Neurogenin2* at E12.5, Plate 3. A whole mount view of expression domains as well as levels of sectioning are indicated in Fig. A.1.

dorsal thalamus and pretectum, but none in the ventral thalamus (prethalamus). The basal plate posterior diencephalon also shows expression in at least two parts (bP2 and bP3) while bP1 seems to be expression-free. The more anteriorly lying hypothalamus is free of expression. We could not establish with the wholemount material whether there is a ventriculopially extending band of *NeuroD* expression in the alar hypothalamus similar to the situation for *Ngn2*. However, the spear-shaped transverse expression at the bP2/bP3 boundary in the general region of the zona limitans intrathalamica also shows up in *NeuroD*, similarly as in *Neurogenin2 in situ* preparations.

In the midbrain, there is a basal plate expression in the tegmentum. The midbrain roof shows an anterior as well as a longitudinal expression, similar to the *Ngn2* situation. In addition, there appears to be a *NeuroD* expression domain in the midline roof plate of the midbrain and at E13.5, a strong expression domain is seen in the most posterior part of the midbrain roof (not shown in Fig. A.1). Again similar to *Neurogenin2*, a gap of *NeuroD* expression is seen in the isthmus. An alar plate *NeuroD* rhombencephalic expression is present; it starts out thick in rhombomere 1 and thins out more posteriorly. At E13.5, a strong expression domain is seen in the cerebellum and, additionally and different from *Ngn2*, in the rhombic lip.

### ***Mash1***

Generally, *Mash1* expression domains lie in a broad band close to the ventricle, leaving the mantle layer free of transcripts, similar to the situation for *Ngn2* regarding the ventriculopial distribution. The anteroposterior regional distribution of *Mash1* secondary forebrain expression patterns between 11.5 and 13.5 days essentially complement those of *Ngn2/NeuroD*, with the exception of the olfactory bulb, where *Mash1* positive cells are also seen in the central part. However, all parts of cortex as well as the eminentia thalami are completely free of *Mash1* expression. As mentioned above for the wholemount brain showing *Ngn2* expression, there is a strong, but artefactual, oval signal in the cortex representing the rim of an artificially opened hole to allow for better penetration of the probe; again, the real situation for the *Mash1* expression in the cortex is visualised in the sectioned material (Fig. A.5, Fig. A.6 A-E). In contrast to the structures just listed as negative, the lateral and medial ganglionic eminences (LGE, MGE), and the preoptic region show a strong signal



for *Mash1* transcripts. Similarly, a *Mash1*-negative dorsal thalamus contrasts with a strongly positive ventral thalamus (prethalamus) and positive (basal plate) hypothalamus (Fig. A.5, Fig. A.6 F-H). Notably however, the area of the alar hypothalamus that is positive for *Ngn2* (see above) remains unstained in *Mash1* preparations (E 11.5, Fig. A.5, Fig. A.6 E), adding to the picture of complementarity.

Turning to the remaining parts of the diencephalon, namely the alar plate pretectum and basal plate portions of prosomeres 1 through 3, they apparently also express *Mash1* (as *Ngn2*), which, naturally, does not imply a co-localisation on the cellular level. In addition, it is also clear that the spear-shaped transverse *Mash1* expression domain in or close to the zona limitans intrathalamica (Fig. A.1, Fig. A.5, Fig. A.6 F) does exist, and as in the case of *Ngn2* and *NeuroD*, it grades from a transverse orientation into a longitudinal expression of bP2/1 posteriorly and bP3 anteriorly (Fig. A.1).

The midbrain displays a strong *Mash1* expression in the superior colliculus that is continued into the inferior colliculus. In addition to the expected ventricular *Mash1* expression in the alar midbrain (superior and inferior colliculus), there is a conspicuous peripheral, strongly stained stripe of *Mash1* expression beginning in the inferior colliculus and extending into the transition zone towards the cerebellum (Fig. 3 K-L). There is also a distinct *Mash1* domain in the mesencephalic tegmentum and cerebellum. In the medulla oblongata, there are two longitudinal stripes of expression (Fig. A.1), one in the alar plate and one in the basal plate in accordance with what is shown in the literature already (e.g. Gaufo et al., 2000).

## Discussion

### Detailed mouse *Neurogenin1/2/NeuroD* and *Mash1* patterns: an update

The basic expression patterns of the bHLH genes *Neurogenin1/2* and *NeuroD* as well as *Mash1* and their roles in neurogenesis in the murine brain (including eye), spinal cord and peripheral nervous system have been established previously (Lo et al., 1991; Ma et al., 1996; Ma et al. 1997; Sommer et al., 1996; see Table A.1 for later citations). Recent detailed reports on homologous gene expression patterns in two additional vertebrate model organisms, namely the zebrafish *Danio rerio* (Mueller and Wullimann, 2003; Mueller and Wullimann,

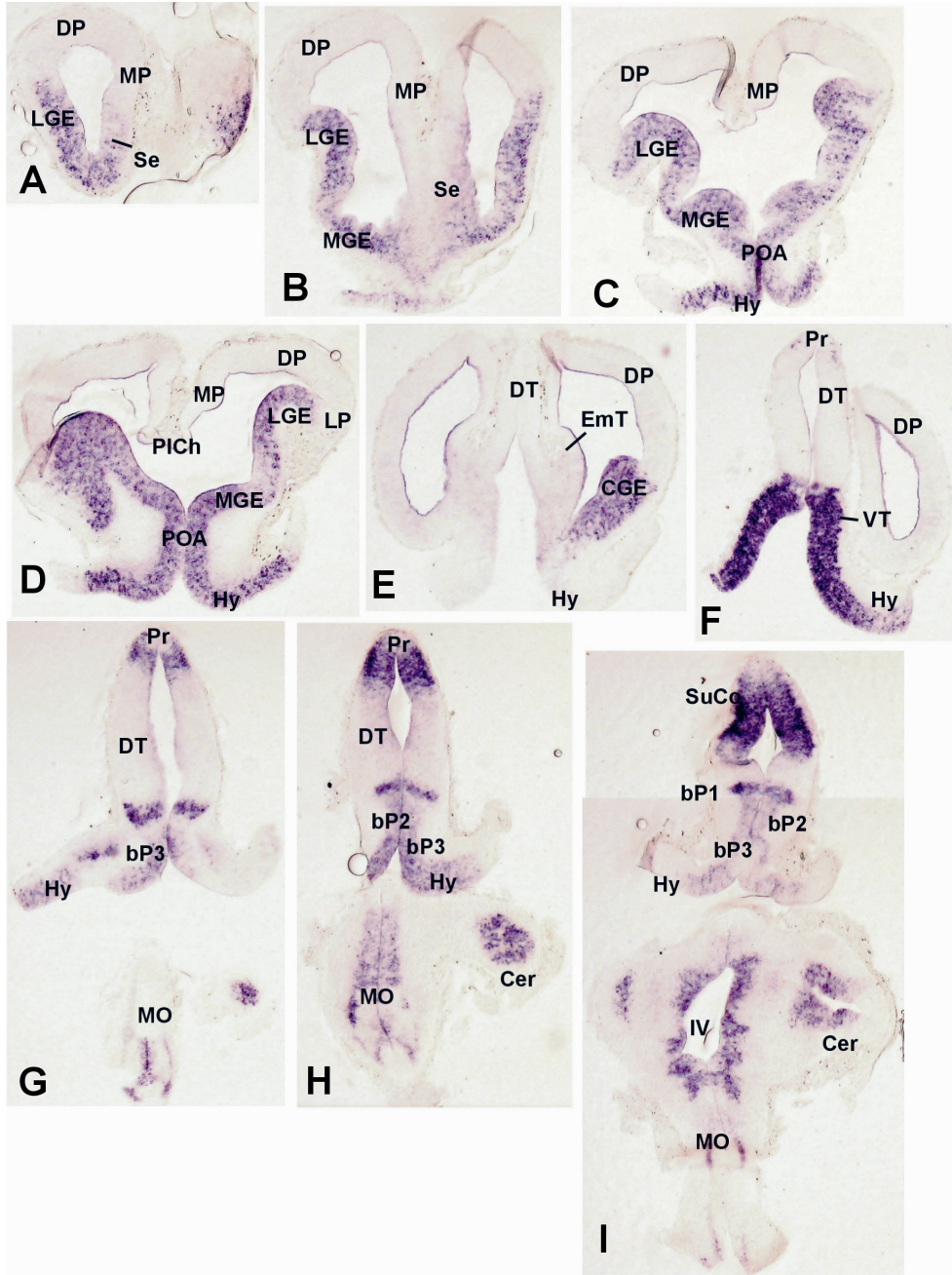


FIGURE A.5: Transverse sections of a mouse brain *in situ* hybridised for *Mash1* at E11.5, Plate 1. A whole mount view of expression domains as well as levels of sectioning are indicated in Fig. A.1.

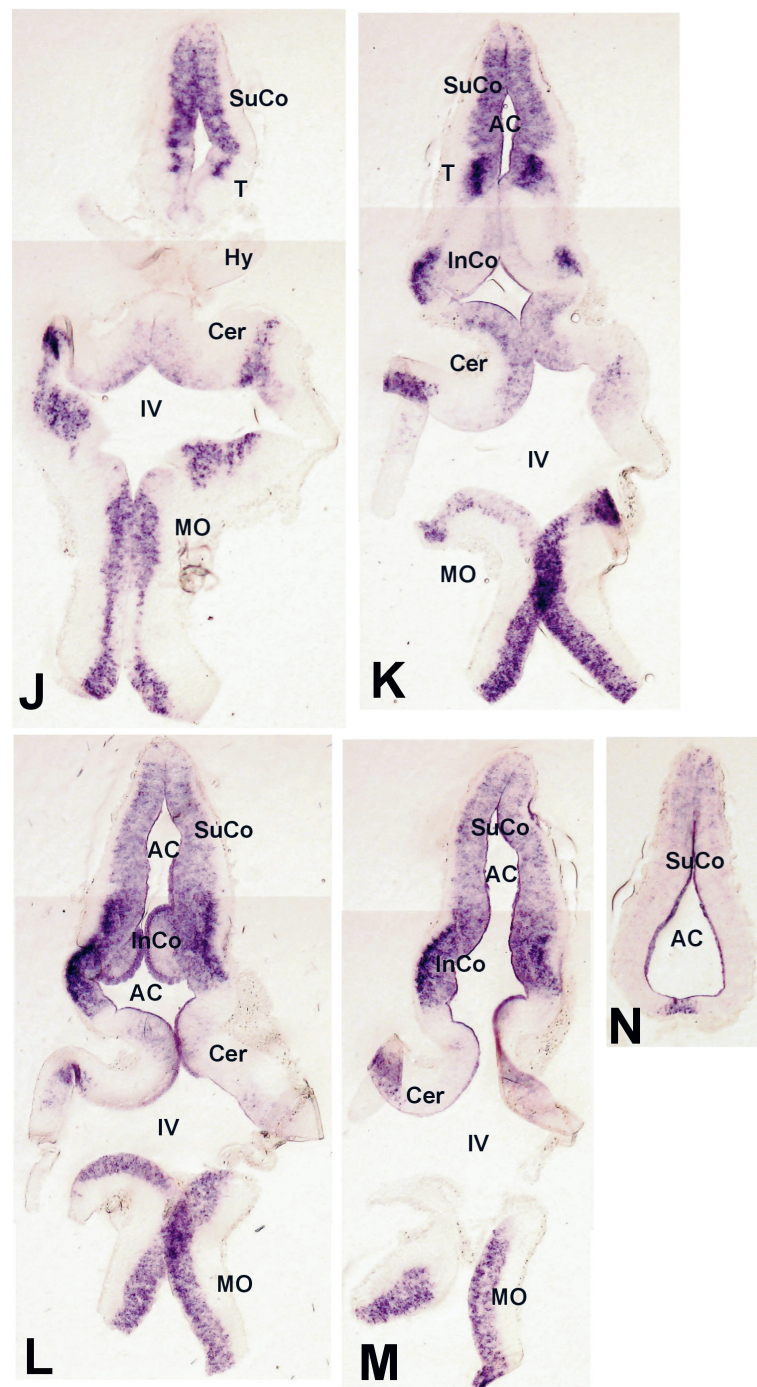


FIGURE A.6: Transverse sections of a mouse brain *in situ* hybridised for *Mash1* at E11.5, Plate 2. A whole mount view of expression domains as well as levels of sectioning are indicated in Fig. A.1.

2005) and the African clawed frog *Xenopus laevis* (Wullimann et al., 2005) call for a re-examination in the mouse, as some expression domains seen in these two amniote species have either not been noted before or have not been explicitly identified in the mouse or rat (see Introduction and Tables A.1 and A.2 for details). Therefore, our more extended description of mouse bHLH gene expression confirms and complements earlier accounts and allows for an improved comparative interpretation with the amniote model systems recently investigated.

The initial description in the mouse brain (Sommer et al., 1996) already noted the prominent cortical expression of both *Neurogenin1/2* (ventricular zone) and *NeuroD* (mantle zone), as well as the absence of expression of those genes in the MGE/LGE. Similarly, the regionally correlated expression of those three genes in the mouse eminentia thalami (depicted, but unidentified in Sommer et al., 1996), dorsal thalamus, basal diencephalon (“subthalamus” of Sommer et al., 1996) and midbrain tegmentum (except for *Neurogenin1*), as well as in the superior colliculus (anterior part of midbrain roof) and medulla oblongata has also been documented in this pioneer study. However, we report here in addition correlated *Neurogenin2* and *NeuroD* expression in the mouse olfactory bulb, pretectum, zona limitans intrathalamica (ZLI; or directly adjacent to it, see below), as well as in the cerebellum. We furthermore explicitly identify the eminentia thalami as a *Neurogenin2* and *NeuroD* positive region. Further, we demonstrate for the first time a correlated expression of *Neurogenin2* and *NeuroD* in the alar plate hypothalamus. We also clarify the extent of gene expression domains in the basal diencephalon (basal P1 through P3; see next section), midbrain tegmentum and midbrain roof. In our preparations, the longitudinal expression domains of the two genes in the midbrain roof extend along its entire length, including superior and inferior colliculi. While there is at most a weak expression of neurogenins in the rhombic lip, *NeuroD* is strongly expressed there at E13.5.

For *Mash1*, expression domains have been unambiguously described in the following areas of the murine brain: retina (and olfactory epithelium), olfactory bulb, MGE/LGE, preoptic region, hypothalamus, ventral thalamus, pretectum, midbrain roof, medulla oblongata and cerebellum (Lo et al., 1991; Guillemot and Joyner, 1993; Ma et al. 1997; Horton et al., 1999; for later citations see Table A.1). In addition, the present contribution clarifies the spear-shaped expression domain in or near the zona limitans intrathalamica,

in the three basal plate diencephalic regions (bP1, bP2, bP3), and the midbrain tegmentum.

### Comparative implications

Studies in zebrafish (Mueller and Wullimann, 2002; 2003; 2005) and *Xenopus* (Wullimann et al., 2005) have revealed a common pattern of early bHLH gene expression that can now be tested in more detail with respect to the mouse. The relevant data are summarised in Table A.2 and Fig. A.7.

**Mouse-Zebrafish** There are some interesting differences between the mouse and the zebrafish. For example in the mouse (Sommer et al., 1996), *Neurogenin2* is expressed in the retina (but not in the olfactory epithelium) while *Neurogenin1* is expressed in the olfactory epithelium (but not in the retina), whereas zebrafish *neurogenin1* is expressed in both sensory organs (Mueller and Wullimann, 2003, 2005). Furthermore, whereas both *Neurogenin1* and *Neurogenin2* are expressed in the anterior part of the mouse medulla oblongata, only *Neurogenin2* has been said to extend into the posterior medullary part. However, in the caudally adjacent ventral mouse spinal cord, both genes are again expressed. In contrast, the zebrafish brain shows an uninterrupted anteroposterior medullary *Neurogenin1* expression that extends into the spinal cord. These specific differences of bHLH gene expression in the two model systems are consistent with the fact that an orthologue of the murine *Neurogenin2* has not been identified in the zebrafish (Blader et al., 1997; 2003; 2004; Korzh et al., 1998, Korzh and Strähle, 2002) and may indicate that a zebrafish *Neurogenin2* paralogue has been lost in this species, with *Neurogenin1* assuming a more inclusive central nervous expression pattern. This likely loss is also indirectly supported by the fact that another paralogous gene, *Neurogenin3*, is indeed found in the zebrafish with a corresponding limited hypothalamic expression (Wang et al., 2001), as seen in the mouse (Sommer et al., 1996). However, Mueller and Wullimann (2003; 2005) failed to detect a correlated *NeuroD* expression in the zebrafish hypothalamus. In the mouse, this small hypothalamic *NeuroD* expression is much weaker in strength compared to other domains (Sommer et al., 1996) and — if similarly present in the zebrafish — this may explain why it has escaped detection.

Another point of interest is our detection of strong, but regionally limited expression of *Neurogenin2* (and possibly *NeuroD*) in the very rostral, alar



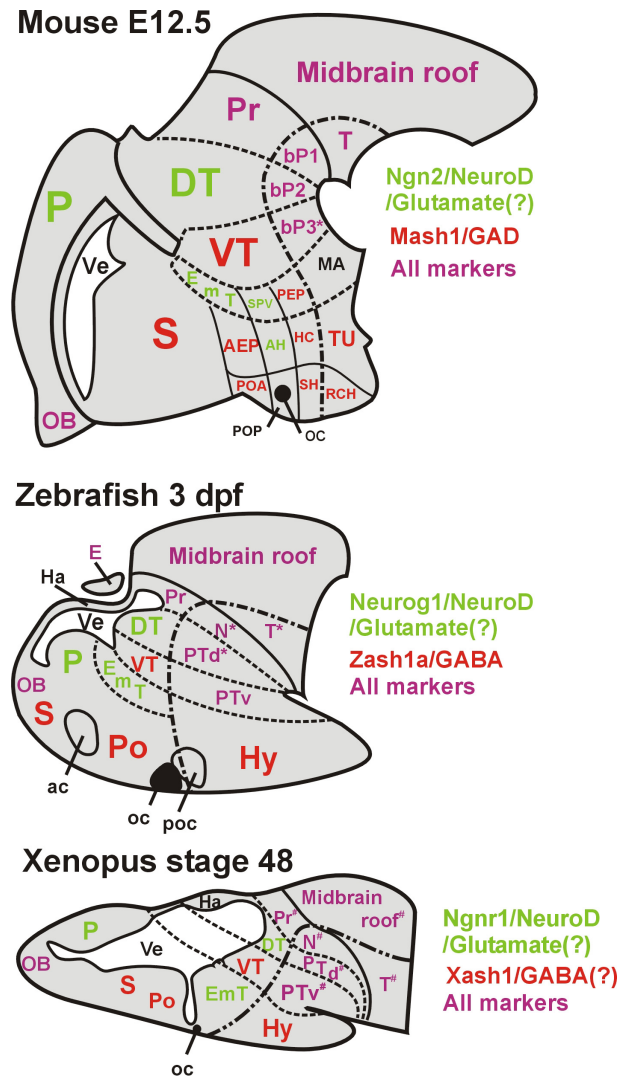


FIGURE A.7: Expression of bHLH genes involved in forebrain secondary neurogenesis during the phylotypic (or class typical) stage in major vertebrate model animals reveals complementarity of neurogenins/*NeuroD* (pallium/cortex, dorsal thalamus, eminentia thalami) versus *Mash1*/*Zash1a*/*Xash1* (subpallium, ventral thalamus, preoptic region, hypothalamus) positive forebrain territories as well as correlation of the latter with GABA/GAD cell development. (A) Mouse (after Katarova et al., 2000; present study). Note that the fine resolution of *Mash1*/*Ngn2* expression indicated in the hypothalamus is a hypothetical interpretation of our data). (B) Zebrafish (after Wullmann and Mueller, 2002; Mueller and Wullmann, 2003; Mueller et al. 2006). (C) *Xenopus laevis* (after Roberts et al., 1987; Ferreiro et al., 1993; 1994; Barale et al., 1996; Wullmann et al., 2005). ?: indicates that the distribution of glutamate has not been directly established. \*: *Zash1a* (PTd, N, T) or *neurog1/NeuroD* (N) in zebrafish, and GAD in mouse not reported (compare Tables A.1 and A.2). ‡: *Xash1* and/or GABA/GAD in *Xenopus* not demonstrated (see Table A.2). For abbreviations see list.

plate hypothalamus, with a corresponding absence of *Mash1* expression. In the zebrafish, this alar hypothalamic area may correspond to the cell masses emanating ventral (or rostral) to the eminentia thalami (on the opposite side of the lateral forebrain bundle) towards the periphery (Wullimann and Mueller, 2004a). This is in sharp contrast to the rest of the hypothalamus in both mouse and zebrafish, where *Mash1* is strongly — but neurogenins are not — expressed. It is highly interesting that Katarova et al. (2000) reported a correlated gap of GAD expression in this alar part of the mouse hypothalamus.

Of particular interest is our observation here that the basal plate of the mouse mesencephalon (midbrain tegmentum), as well as all basal plate portions of prosomeres 1 through 3 (i.e., basal pretectal, dorsal thalamic, and ventral thalamic neuromeres, bP1 through 3) have a strong *Neurogenin1/2* and *NeuroD* expression, with sharp boundaries towards the hypothalamus anteriorly, towards the isthmus posteriorly, and dorsally towards the alar-basal plate boundary. This confirms in retrospect the previous similar description of a *neurogenin1/NeuroD* expression pattern in the zebrafish basal plate of prosomeres P2 and P3 (i.e., dorsal part of the posterior tuberculum, PTd, and ventral part of the posterior tuberculum, PTv) and the interpretation of these basal plate diencephalic regions as being separate from the “true” hypothalamus (the latter being free of *Neurogenin1/2* and *NeuroD* gene expression in both zebrafish and mouse, except for a very limited, weak *NeuroD* expression in the mouse correlated with *Neurogenin3*, and for the alar hypothalamic *Ngn2* expression, see above). Whereas in the mouse both the *Neurogenin1/2* and the *NeuroD* domains in the midbrain tegmentum are contiguous with the anterior basal plate diencephalic domains (P1 through P3), the corresponding expression domains are segmentally or neuromerically organised in the zebrafish, with separate domains in basal P2 (PTd) and P3 (PTv). However, in the zebrafish, no separate *neurog1/neuroD* domain has been noted in basal P1 (N), but there is again a distinct expression domain in the zebrafish midbrain tegmentum of these genes (Mueller and Wullimann, 2003; 2005).

Furthermore, an alar plate expression of *Neurogenin1/2* and *NeuroD* is seen in both mouse and zebrafish dorsal thalamus (P2) and pretectum (P1), but such expression domains are absent in the ventral thalamus (prethalamus) and preoptic region in both species. Interestingly, there is a thin bilateral, transversely oriented, spear-shaped expression both of *Neurogenin1/2* and *NeuroD* in the mouse thalamus sandwiched between the dorsal thalamic ex-

pression domains (lying posteriorly) and the expression-free ventral thalamus (lying anteriorly). These transverse spear-shaped expression domains resemble very much the typical shape and position of gene expression domains (such as sonic hedgehog) documented in the zona limitans intrathalamica (ZLI), which represents a boundary and signalling centre critical for establishing differences between dorsal and ventral thalamus (Larsen et al., 2001; Zeltser et al., 2001; Kiecker and Lumsden, 2004). Interestingly, in or close to the ZLI, also expression of *Mash1* has been noted (Miyoshi et al., 2004), which is otherwise expressed in the ventral, but not in the dorsal thalamus. Furthermore, the homeobox-containing *Pitx2* gene has been implicated in the differentiation of GABAergic cells and has been reported to be expressed in the ZLI, flanked by GAD-positive (GABAergic) cells anteriorly and posteriorly (Martin et al., 2002). Furthermore, GAD-positive cells have been reported in or close to the mouse ZLI (Katarova et al., 2000), very similar to GABA cell distribution in ventral thalamus and ZLI in the zebrafish (Mueller et al., 2006). It will be crucial in the future to exactly demarcate these spatially highly complex expression patterns of bHLH genes *Neurogenin1/2/NeuroD* as well as *Mash1* (or its homologues) with respect to the ZLI. As already noted above, pallial (cortical) versus subpallial expression patterns of bHLH genes are highly comparable in zebrafish and mouse.

In summary, it is clear that both the mouse and zebrafish forebrains exhibit comparable major areas exclusively positive for neurogenins/*NeuroD*, i.e., pallium, eminentia thalami, dorsal thalamus, which differ in location from *Mash1* (*Zash1a*, respectively) positive areas, i.e., subpallium, preoptic region, ventral thalamus and hypothalamus (Fig. A.1, Fig. A.2, Fig. A.3, Fig. A.4, Fig. A.5, Fig. A.6). In addition, in both mouse and zebrafish brain, there are corresponding GABAergic cell populations which are restricted to *Mash1/Zash1* domains. In contrast, in both the mouse and zebrafish brain, mid- and hindbrain (and the olfactory bulb, alar plate prepectum, and basal parts of prosomeres 1 through 3), both neurogenins/*NeuroD* and *Mash1/Zash1a* are expressed in the same brain regions, but also in different cell populations. Accordingly, GABA cell populations are generally present in these mixed locations too. Together, the expression domains of neurogenins/*NeuroD* and *Mash1* (*Zash1a*) bHLH factors cover all regions of the early mouse/zebrafish brain.

**Mouse-*Xenopus*** A recent report on *Neurogenin-related-1* and *NeuroD* expression in the African clawed frog *Xenopus laevis* (stage 48) largely conforms to the neuroanatomical pattern of homologous gene expression domains just outlined for mouse and zebrafish (Wullimann et al., 2005), for example regarding forebrain complementarity of bHLH gene expression. Interestingly, a *Neurogenin-related-1/NeuroD* expression has been noted in the ZLI (Wullimann et al., 2005), similar to the situation in the mouse. Also, *Neurogenin-related-1* is expressed in both developing retina and olfactory epithelium (Schlosser and Northcutt, 2000), as is *Neurogenin1* in the zebrafish. Furthermore, the medullary expression appears uninterrupted into the spinal cord (Wullimann et al., 2005). Although both *Neurogenin-related-1a* and *1b* genes have been identified in *Xenopus* (Ma et al., 1996), their expression has been reported to be identical (incl. olfactory epithelium and eye). This further supports the notion that the mutual exclusive expression of murine *Neurogenin1* and *Neurogenin2*, for example in either retina or olfactory epithelium, respectively (compare with Table A.1), is a derived situation.

Early *Xash1* expression has been reported (Ferreiro et al., 1993, 1994) in the diencephalon (probably ventral thalamus of stage 22) and somewhat later in the ventral telencephalon (probably subpallium of stage 27), supporting the nested complementary expression of bHLH genes discussed above in zebrafish/mouse. However, complementarity of *Xash1* forebrain expression patterns (as discussed above for zebrafish/mouse) to those of *Neurogenin-related-1/NeuroD* have not been fully addressed (see Table A.2, Fig. A.2, Fig. A.3, Fig. A.4) and remain to be shown in detail. Similarly, reports on GABA-immunoreactive cells in the *Xenopus* embryo (Roberts et al., 1987) and tadpole (Barale et al., 1996) remain incomplete in comparison to those discussed above for zebrafish/mouse. A subpallial GABA cell cluster (“rostral forebrain neurons”) may be recognised in the late *Xenopus* embryo. In the (postembryonic) tadpole, GABA cell distribution indicates that preoptic and ventral thalamic populations leave a gap of unstained cells in between, possibly representing the eminentia thalami. Both *Xash1* and GABA cell patterns clearly are in need of detailed reconsideration in *Xenopus*.

**bHLH gene expression and GABA/GAD cell distribution in embryonic mouse/rat**

Retina	Ngn1	-	Sommer et al 96
	Ngn2	+	Sommer et al 96
	NeuroD	+	Sommer et al 96
	Mash1	+	Guillemot and Joyner 93
	GABA/GAD	+	Katarova et al 00
Olfactory epithelium	Ngn1	+	Sommer et al 96, Ma et al 97, Cau et al 02
	Ngn2	-	Sommer et al 96
	NeuroD	+	Sommer et al 96
	Mash1	+	Guillemot and Joyner 93, Ma et al 97, Cau et al 02
	GABA/GAD	+	Katarova et al 00
Olfactory bulb	Ngn1	+	Ma et al 97
	Ngn2	+	this study
	NeuroD	+	this study
	Mash1	+	Ma et al 97
	GABA/GAD	+	Katarova et al 00
Pallium (Cortex)	Ngn1	+	Sommer et al 96, Ma et al 96, 97, Fode et al 00
	Ngn2	+	Sommer et al 96, Fode et al 00, Parras et al 02
	NeuroD	+	Sommer et al 96, Parras et al 02
	Mash1	-	Lo et al. 91, Ma et al 97, Horton et al 99, Torii et al 99, Fode et al 00, Parras et al 02
	GABA/GAD	-	Casarosa et al 99, Katarova et al 00, Parras et al 02
Subpallium(MGE/LGE)	Ngn1	-	Sommer et al 96, Ma et al 97, Fode et al 00
	Ngn2	-	Sommer et al 96, Fode et al 00, Parras et al 02
	NeuroD	-	Sommer et al 96, Parras et al 02
	Mash1	+	Lo et al. 91, Guillemot and Joyner 93, Ma et al 97, Horton et al 99, Torii et al 99, Fode et al 00, Parras et al 02
	GABA/GAD	+	Casarosa et al 99, Katarova et al 00, Parras et al 02

Preoptic region	Ngn1	-	Ma et al. 97 <sup>6</sup>
	Ngn2	-	this study
	NeuroD	-	this study
	Mash1	+	Lo et al. 91 un, Ma et al 97 <sup>6</sup> , Horton et al 99, Torii et al 99 <sup>11</sup> , Fode et al 00 un, this study
	GABA/GAD	+	Fode et al 00 un, Katarova et al 00
Eminentia thalami	Ngn1	+	Sommer et al 96 un, Ma et al. 97 <sup>7</sup>
	Ngn2	+	Sommer et al 96 un, this study
	NeuroD	+	Sommer et al 96 un, this study
	Mash1	-	Ma et al 97 <sup>7</sup> , Fode et al 00 un, this study
	GABA/GAD	-	Fode et al 00 un, Katarova et al 00
Alar hypothalamus	Ngn1	?	---
	Ngn2	+	this study
	NeuroD	?	this study
	Mash1	-	this study
	GABA/GAD	-	Katarova et al. 00
Rest of hypothalamus	Ngn1	-	Ma et al. 97
	Ngn2	-	this study
	NeuroD	+ <sup>1</sup>	Sommer et al. 96. this study
	Mash1	+	Lo et al. 91 un, Torii et al 99, this study
	GABA /GAD	+	Katarova et al 00
Pretectum	Ngn1	?	---
	Ngn2	+	this study
	NeuroD	+	this study
	Mash1	+	Lo et al. 91 un, Fode et al 00 un, this study
	GABA /GAD	+	Fode et al 00 un, Katarova et al 00
Dorsal thalamus	Ngn1	+	Sommer et al 96, Fode et al 00
	Ngn2	+ <sup>2</sup>	Sommer et al 96, Fode et al 00, this study
	NeuroD	+	Sommer et al 96, this study
	Mash1	-	Horton et al 99, Torii et al 99, Fode et al 00, this study
	GABA /GAD	-	Fode et al 00, Katarova et al 00

«ZLI»	Ngn1	+	Sommer et al 96 <b>un</b> , this study
	Ngn2	+	Sommer et al 96 <b>un</b> , this study
	NeuroD	+	Sommer et al 96 <b>un</b> , this study
	Mash1	+	this study
	GABA/GAD	+	Katarova et al 00
Ventral thalamus	Ngn1	-	Ma et al. 97 <sup>7</sup>
	Ngn2	-	this study
	NeuroD	-	this study
	Mash1	+	Lo et al. 91 <sup>8</sup> , Ma et al. 97 <sup>7</sup> , Horton et al 99, Torii et al 99, Fode et al 00, this study
	GABA /GAD	+	Fode et al 00, Katarova et al 00
bP1	Ngn1	?	---
	Ngn2	+	this study
	NeuroD	?	this study
	Mash1	+	Lo et al. 91 <b>un</b> , this study
	GABA /GAD	+	Katarova et al 00
bP2	Ngn1	?	---
	Ngn2	+	this study
	NeuroD	+	this study
	Mash1	+	Lo et al. 91 <b>un</b> , this study
	GABA/GAD	+	Katarova et al 00
bP3	Ngn1	+	Sommer et al 96 <sup>4</sup>
	Ngn2	+	Sommer et al 96 <sup>4</sup> , this study
	NeuroD	+	Sommer et al 96 <sup>4</sup> , this study
	Mash1	+	Lo et al. 91 <b>un</b> , this study
	GABA/GAD	-	Katarova et al 00
Tegmentum	Ngn1	+	Sommer et al 96 <sup>5</sup>
	Ngn2	+	Sommer et al 96, this study
	NeuroD	+	Sommer et al 96, this study
	Mash1	+	Lo et al. 91 <b>un</b> , this study
	GABA /GAD	+	Katarova et al 00

Midbrain roof (SuCo/InCo)	Ngn1	+	Sommer et al 96, Ma et al 97
	Ngn2	+	Sommer et al 96, this study
	NeuroD	+	Sommer et al 96, this study
	Mash1	+	Lo et al. 91, Guillemot and Joyner 1993, Ma et al 97, this study
Cerebellum	GABA /GAD	+	Katarova et al 00
	Ngn1	+	Sommer et al 96 <b>un</b>
	Ngn2	+	Sommer et al 96 <b>un</b> , this study
	NeuroD	+	Sommer et al 96 <b>un</b> , this study
Medulla oblongata	Mash1	+	Lo et al. 91 <sup>9</sup> , this study
	GABA/GAD	+	Katarova et al 00
	Ngn1	+ <sup>3</sup>	Sommer et al 96
	Ngn2	+	Sommer et al 96, this study
Rhombic lip	NeuroD	+	Sommer et al 96, this study
	Mash1	+	Lo et al. 91 <sup>10</sup> , this study
	GABA /GAD	+	Katarova et al 00
	Ngn1	-	---
	Ngn2	-	this study
	NeuroD	+	this study
	Mash1	-	this study

1: restricted, weak expression correlated with *Ngn3*. 2: more laterally extending than *Ngn1*. 3: *ngn1* restricted to anterior medulla oblongata. 4: identified as “subthalamus”. 5: noted absence of expression in tegmentum. 6: identified as part of “hypothalamus”. 7: identified as part of “thalamus”. 8: identified as “band in diencephalon”. 9: identified as “metencephalon”. 10: identified as “myelencephalon”. 11: identified as “hypothalamus”. **un**: unidentified depiction in photograph.

TABLE A.1: bHLH gene expression and GABA/GAD cell distribution in embryonic mouse/rat.



		Mouse/Rat <sup>1</sup>	Zebrafish <sup>2</sup>	Xenopus <sup>3</sup>
Olfactory bulb	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	+	+	?
	GABA	+	+	?
Pallium	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	-	-	-
	GABA	-	-	-
Subpallium (i.e., Striatum/ Pallidum/Septum)	Ngn1/2 (neurog1, ngn1r)	-	-	-
	<u>NeuroD</u>	-	-	-
	Mash1 (Zash1a, Xash1)	+	+	+
	GABA	+	+	+
Preoptic region	Ngn1/2 (neurog1, ngn1r)	-	-	-
	<u>NeuroD</u>	-	-	-
	Mash1 (Zash1a, Xash1)	+	+	?
	GABA	+	+	+
Eminentia thalami	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	-	?	?
	GABA	-	-	?
Hypothalamus	Ngn1/2 (neurog1, ngn1r)	-	-	-
	<u>NeuroD</u>	-	-	-
	Mash1 (Zash1a, Xash1)	+	+	+
	GABA	+	+	?
Pretectum	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	+	+	?
	GABA	+	+	?
Dorsal thalamus	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	-	-*	-
	GABA	-	-	-
Ventral thalamus	Ngn1/2 (neurog1, ngn1r)	-	-	-
	<u>NeuroD</u>	-	-	-
	Mash1 (Zash1a, Xash1)	+	+	+
	GABA	+	+	+

bP1 (N)	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	+	?	+
	GABA	+	+	?
bP2 (PTd)	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	+	?	+
	GABA	+	+	?
bP3 (PTv)	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	+	+	+
	GABA	-	+	?
Tegmentum	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	+	-	+
	GABA	+	+	?
Midbrain roof	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	+	+	+
	GABA	+	+	?
Cerebellum	Ngn1/2 (neurog1, ngn1r)	+	-	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	+	+	?
	GABA	+	+	?
Medulla oblongata	Ngn1/2 (neurog1, ngn1r)	+	+	+
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	+	+	+
Rhombic lip	Ngn1/2 (neurog1, ngn1r)	-	-	-
	<u>NeuroD</u>	+	+	+
	Mash1 (Zash1a, Xash1)	?	?	?

1: For detailed citations see Table 1. 2 : Wullimann and Mueller 2002, Mueller and Wullimann, 2003, Mueller et al., 2006. 3 : Roberts et al., 1987, Ferreiro et al., 1993; Barale et al., 1996, Wullimann et al., 2005. \* very few cells, probably migrating in from ventral thalamus. ?: not demonstrated

TABLE A.2: Interspecies comparison of bHLH gene expression.

## Conclusion

Together, these data on complementary forebrain bHLH gene expression (i.e., neurogenins/*NeuroD* versus *ash1* homologues (as well as additional different downstream genes) in *Xenopus*, zebrafish and mouse strongly suggest a common pattern of early, spatially exclusive generation of glutamatergic versus GABAergic neuronal phenotypes during a particular stage of beginning secondary neurogenesis preceding the later intermingling of neuronal phenotypes by long-distance tangential or other types of migration. In contrast, both neurogenins/*NeuroD* and *ash1* homologues are expressed in mid- and hindbrain, as well as in some forebrain regions (olfactory bulb, pretectum, basal plate of P1 through P3), although also there in different cell populations. This has been suggested to represent kind of a phylotypic (or class typic) stage of vertebrate forebrain secondary neurogenesis (Wullimann et al., 2005, Mueller et al., 2006). A summary of corresponding gene expression domains and GABA/GAD cell distribution of all three species is given in Table A.2 and Fig. A.7.

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## Abbreviations

ac	anterior commissure
AC	aquaeductus cerebri
AEP	anterior entopeduncular area
AH	anterior hypothalamus
bP1	basal plate of prosomere 1
bP2	basal plate of prosomere 2
bP3	basal plate of prosomere 3
Cer	cerebellum
CGE	caudal ganglionic eminence
Cort	Cortex
DB	diagonal band of Broca
DP	dorsal pallium (Isocortex)
DT	dorsal thalamus (thalamus)
E	epiphysis
EmT	eminencia thalami
GABA	$\gamma$ -aminobutyric acid
GAD	glutamic acid decarboxylase
Hy	hypothalamus
Ha	habenula
HC	hypothalamic cell cord
InCo	inferior colliculus
LGE	lateral ganglionic eminence
LP	lateral pallium (olfactory cortex)
MA	mammillary hypothalamus
MGE	medial ganglionic eminence
MHB	midbrain-hindbrain boundary
MO	medulla oblongata
MP	medial pallium (hippocampus)
N	region of the nucleus of medial longitudinal fascicle
OB	olfactory bulb
oc	optic chiasma
P	pallium
pc	posterior commissure
PEP	posterior entopeduncular area
PCh	plexus chorioideus



Po	preoptic region
POA	anterior preoptic area
poc	postoptic commissure
POP	posterior preoptic area
Pr	pretectum
PTd	dorsal part of posterior tuberculum
PTv	ventral part of posterior tuberculum
RCH	retrochiasmatic hypothalamus
RCT	rostral cerebellar thickening (valvula)
RL	rhombic lip
S	subpallium
SC	spinal cord
Se	septum
SH	suprachiasmatic area
SPV	supraoptic/paraventricular area
SuCo	superior colliculus
T	midbrain tegmentum
TU	tuberal hypothalamus
Ve	telencephalic brain ventricle
VT	ventral thalamus (prethalamus)
ZLI	zona limitans intrathalamica
IV	fourth brain ventricle

## Appendix B

# Evolution of circumventricular and other neurohemal organs

Circumventricular organs (CVOs) are specialised neurohemal structures located along the midline of the craniate brain. Some CVOs have been very conserved during evolution, and are present in all craniates studied, including the lamprey. A few neurohemal organs in non-vertebrate groups were found to possess important similarities to particular craniate CVOs, to which they may be homologous.

As a student at the DEPSN laboratory I had the opportunity to contribute to a review that discusses the cellular organisation, development and evolution of these organs. This project was co-ordinated by Jean-Stéphane Joly from the “Morphogenèse du Système Nerveux des Chordés” research group.

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Invited article

# Windows of the brain: Towards a developmental biology of circumventricular and other neurohemal organs

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## Abstract

We review the anatomical and functional features of circumventricular organs in vertebrates and their homologous neurohemal organs in invertebrates. Focusing on cyclostomes (lamprey) and urochordates (ascidians), we discuss the evolutionary origin of these organs as a function of their cell type specification and morphogenesis.

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**Keywords:** Neurohypophysis; Neural gland; *otp*; Lamprey; *Ciona*; Tanycytes; Radial glia

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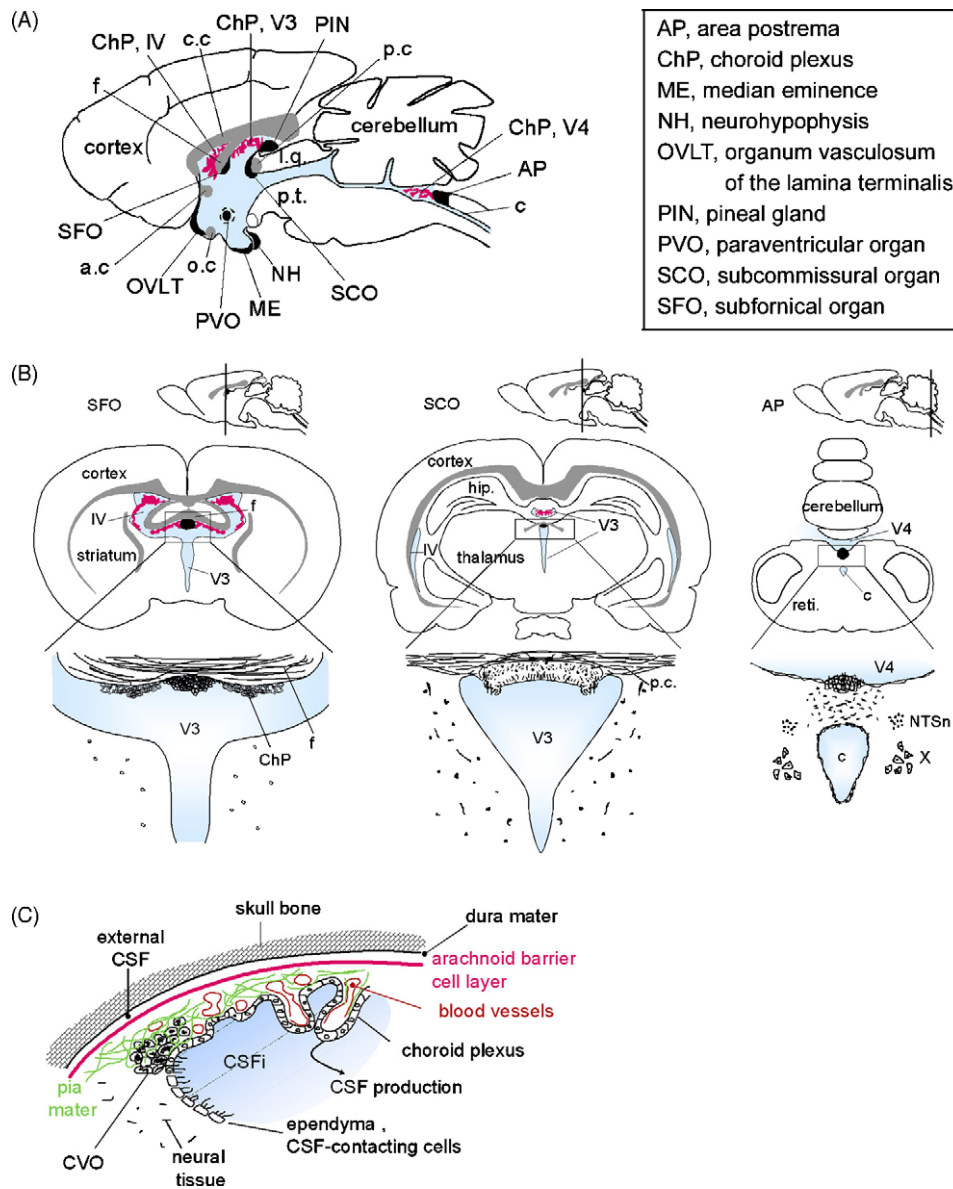
## 1. Introduction

Circumventricular organs (CVOs) are neurohemal organs (i.e. brain regions in which the brain–blood barrier is permeable) located along the midlines of vertebrate brain ventricles (Fig. 1). They have many essential functions, including the transduction of information between the blood, neurons and cerebrospinal

fluid (CSF), and CSF secretion. Recent results have highlighted the importance of CVOs in various developmental and adult functions, suggesting that more attention should be paid to these organs. They display exceptionally high levels of cell diversity, with a range of cell types extending from modified glial cells retaining a capacity to proliferate in adulthood to highly diversified sensory neurons. We provide a detailed description of a typical CVO, the median eminence (ME). We then review CVOs in different vertebrates, comparing these organs with invertebrate neurohemal organs, which are not called CVOs because the brains of invertebrates usually consist of ganglions

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AP, area postrema  
 ChP, choroid plexus  
 ME, median eminence  
 NH, neurohypophysis  
 OVLT, organum vasculosum of the lamina terminalis  
 PIN, pineal gland  
 PVO, paraventricular organ  
 SCO, subcommissural organ  
 SFO, subfornical organ

Fig. 1. Anatomical distribution and structure of the main brain CVOs. (A) The nine CVOs recognised in the mammalian brain are represented on a sagittal median view of a cat brain. Colour code: the CVOs are shown in black, the choroid plexuses in red, the axonal commissures in grey, the ventricular liquid in blue, and the brain tissues in white. The PVO is circled with a dotted line because it is bilateral, on either side of the third ventricle. Note that all other CVOs are located on the midline, in close association with axonal tracts. A key to the nine organs is provided in the box on the right. Anatomical abbreviations: lq, quadrigeminal lamina; pc, posterior commissure; cc, corpus callosum; f, fornix; oc, optic chiasm; cm, corpus mammilari; pt, posterior tuberculum; ac, anterior commissure; cc, central canal. (B) CVOs are associated with the ventricular liquid. Three examples are given (SFO, SCO and AP). For each, gross antero-posterior position is given on a sagittal view (top) and on a coronal section (middle) of a rodent brain, showing the midline position of these organs. Bottom: histological appearance (redrawn and adapted from Bollner et al. [92]). Anatomical abbreviations: str, striatum; lv, lateral ventricle, 3rd, 4th V: third and fourth ventricle; thal, thalamus; ret, reticular formation; cb, cerebellum. (C) Principle interactions between brain tissues and external fluids. CVOs are ideally placed to sense and release molecules from and into the blood and CSF (cerebrospinal fluid). Note that the pia matter/arachnoid tissue constitutes the connective tissue of CVOs. The CSF itself is produced by the choroid plexuses.

without ventricles. Very little is known about CVO development. We therefore also describe a few recent advances in vertebrate research concerning the patterning mechanisms along the brain midline underlying the organogenesis of CVOs. Finally, we consider the evolutionary origin of CVOs, by looking at their presence in two chordate groups – cyclostomes and urochordates – and by analysing *Otp*, an essential determinant of neurosecretory cell types.

## 2. A brief overview of mammalian CVOs, focusing on the median eminence and neurohypophysis

The anterior pituitary (adenohypophysis, pars distalis) has received considerable attention in recent years and is one of the organs for which genetic networks, morphogenesis, and cell types have been studied in the most detail (see Author et al., in this issue).

In contrast, much less attention has been paid to a set of specialised structures appearing on the surfaces of the ventricle of the central nervous system (CNS), collectively known as circumventricular organs. However, CVOs have been shown to play essential regulatory roles in diverse physiological functions, including body fluid homeostasis in particular [1–4].

CVOs have morphological or molecular characteristics that distinguish them from the rest of the nervous system, and they display a remarkably high level of cellular diversity, an extraordinary high capillary content and permeability. In most CVOs, the glial cells of the brain capillaries are more loosely apposed, creating relatively large perivascular spaces, and there are no tight junctions between endothelial cells (Fig. 2). These features result in most CVOs having fenestrated capillaries, making it possible for high-molecular weight and polar substances to pass through them. These morphological features favour exchanges between the perivascular space and the blood. CVOs have therefore been referred to as “windows of the brain” [2]. CVOs are associated with many functions, ranging from homeostasis to processes relating to the most complex cognitive functions [5,6]. The proposed functions of CVOs include the maintenance of body fluid balance, growth, blood pressure, biological rhythms, sleep, temperature, respiration, energy balance, the mediation of immune responses, pain modulation, protection against ingested toxic substances, taste aversions, reproduction, parental behaviour and lactation [2]. A particular type of CVO, the choroid plexuses, plays a key role in human health, and has been associated with migraine, ageing and neurodegeneration [7–12].

In mammals, nine organs are commonly recognised as CVOs: the pineal gland (PIN), subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), paraventricular organ (PVO), median eminence, neurohypophysis (NH), subcommissural organ (SCO), area postrema (AP) and choroid plexus (ChP) (Fig. 1A and B) [1,2].

Grouping these mammalian organs together under the generic title of “CVO” is of heuristic value but tends to mask their diversity. These organs have previously been classified into two separate categories: sensory and secretory CVOs, with secretory activities classically viewed as the main function of these organs. However, although sensory functions were initially attributed to three CVOs (the SFO, OVLT and AP) [1], most CVOs display both sensory and secretory functions, making it difficult to categorise them on the basis of function. The PIN is sometimes excluded from the standard CVO category, because its action is endocrine in nature, requires no neural input for signalling, and does not translate any blood-borne signal into the CNS. The SCO is not particularly permeable and does not have fenestrated capillaries, and may therefore also be considered not to be a standard CVO. Its function is the secretion of Reissner fibres, composed of SCO-spondin, a complex modular glycoprotein [13]. These fibres extend into the lumen of the central canal. It has been suggested that they are involved in the mechanosensory detection of movement in the vertebral column or in the regulation of CSF flux [13]. ChPs are only occasionally considered to be CVOs. Like other CVOs, ChPs are located in the vicinity of the ciliated ependymal layer covering the ventricle walls (Figs. 1C, 2B,C). They have fenestrated capillaries but

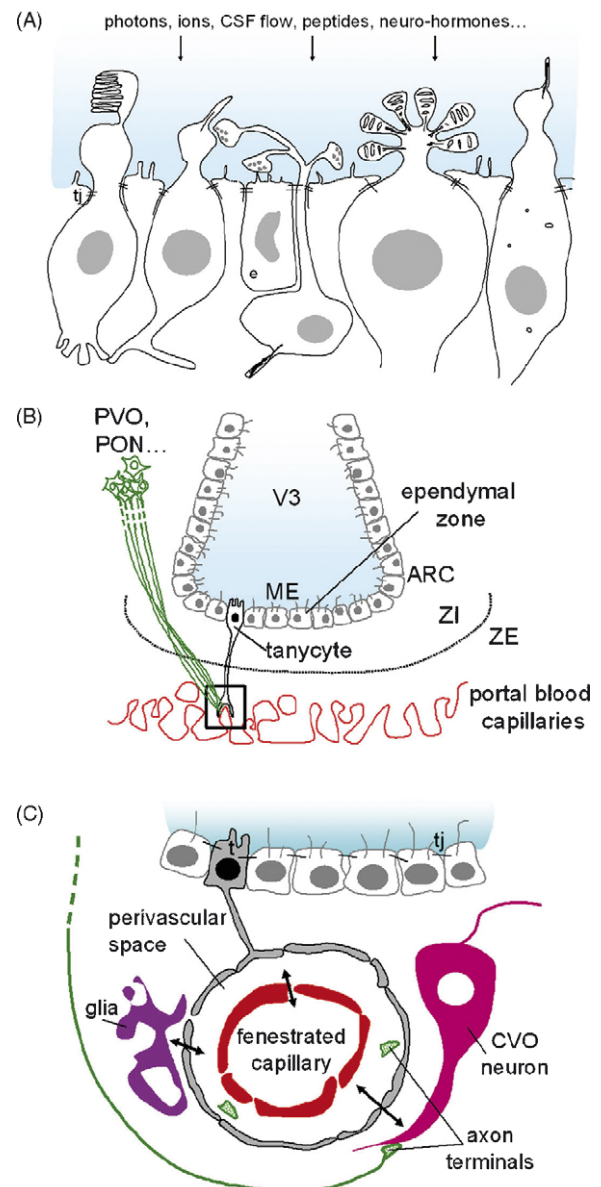


Fig. 2. A diversity of cell types and contact specialisations in CVOs. (A) Redrawn and adapted from Vigh and Vigh-Teichmann [17]. Various types of CSF-contacting cells are represented. From left to right, a pineal CSF-contacting cell, a ciliated CSF-contacting neuron, a coronet cell with distinctive morphological differentiation, a CSF-contacting neuron of the vascular sac. Note, in the middle, the nerve terminals able to release their neurohormonal content directly into the CSF, or to establish synapses with neighbouring CSF-contacting cells or ependymal cells (e). Also note the existence of ciliated intra-ependymal cells. (B) Organisation of the median eminence (ME). The ME ependymal zone constitutes the floor of the third ventricle (V3) and can be subdivided into subzones (ZI and ZE, internal and external zone). Cytoplasmic extensions of specialised tanycytes reach and ensheath the capillaries of the hypothalamo-hypophyseal portal system (red). The ME is very rich in axonal fibres, which convey massive innervation from various hypothalamic nuclei (green). (C) Focus on cell interactions and fluid exchanges in the ME. Black double arrows indicate the diffusion of small molecules and neurohormones through the blood-brain barrier. Neurons and glia of the ME receive axonal efferences and information diffusing from fenestrated capillaries. Note also the presence of axon terminals (green dotted elements), which release their content directly into the perivascular space delineated by tanycyte (t) cytoplasmic extensions. By contrast, no diffusion is observed from ependymal cells, which establish non-permeable tight junctions (tj).



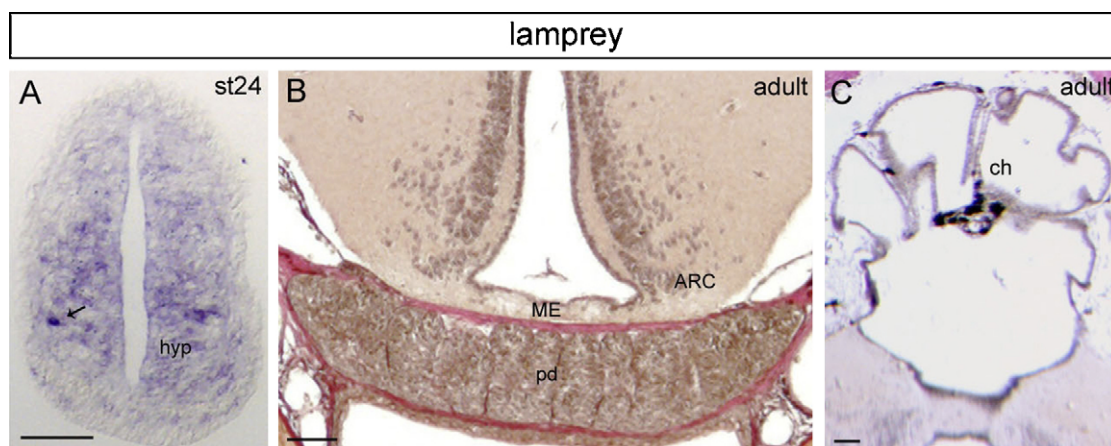


Fig. 3. CVOs in lamprey, developmental and anatomical elements. (A) Expression of *Otp* in the developing *Petromyzon marinus* (sea lamprey) hypothalamus. Transverse section of stage 24 embryos after *in situ* hybridisation. Note the strong *Otp* expression in cells located deep in the neuroepithelium (arrow). (B) The lamprey median eminence (ME). Transverse section through the adult brain and adenohypophysis of a *Lampetra fluviatilis* (river lamprey), also showing the arcuate nucleus (ARC); pd, pars distalis of the adenohypophysis. Courtesy of Franck Bourrat. (C) The lamprey dorsal mesencephalic choroid plexuses (ch) are well developed. Scale bars: (A) 50  $\mu\text{m}$ , (B and C) 100  $\mu\text{m}$ .

do not contain neural tissue. Their major role is the production of CSF, regulated by various neuropeptides [11]. Recent studies [14] have suggested that greater attention should be paid to CSF, as new evidence suggests that its flow along the ventricles of the adult mammalian brain may direct the migration of newly generated neurons towards their destination in the olfactory bulb.

The median eminence has recently been classified as a sensory CVO in addition to its well-known secretory function. This hypothalamic region is tightly interconnected to the adjacent bilateral arcuate nucleus (ARC, Fig. 2B, see also Fig. 3 in lamprey). The neurons of the ME and ARC not only share the same projection patterns, but also contain GnRH, NPY and AGRP. It has therefore been suggested that the ARC and ME should be viewed as an arcuate–median eminence complex (AMC) [15].

The AMC is best known as the terminal field of neuroendocrine neurons, which release their secretory products into the portal vasculature for delivery to the anterior pituitary gland. It receives numerous inputs from diverse brain structures (Fig. 2B and C) and other sensory CVOs [2,15]. Most of the axons of the magnocellular and parvocellular neurosecretory nuclei projecting to neurohemal areas (in the so-called pars intermedia of the NH and ME, but also in the vascular sac in fish) do not terminate directly on vessels. Instead, they form neurohormonal nerve terminals attached to the basal lamina of the external and vascular surface of the brain tissue (Fig. 2C). The neuropeptides released from these terminals initially enter the perivascular space; they then enter the blood vessels by diffusion [16,17].

The AMC also has sensory functions. These functions are illustrated by the histological distribution of peptide hormone-binding sites in the ME [18] and the presence of large numbers of receptors for almost all known circulating metabolically active hormones, including leptin, insulin, glucocorticoid, and ghrelin in the ME and the arcuate nucleus [15]. The axons of the CSF-contacting neurons terminate throughout the CNS, not exclusively in the AMC or neighbouring hypothalamic nuclei. Thus, the chemical information thought to be taken up by CSF-

contacting neurons from the ventricular CSF may influence the function of these areas of the CNS [17].

### 3. Little is known about the developmental biology of mammalian CVOs

Almost nothing is known about how CVOs differentiate at specific sites along the ventricles and how this evolutionary conserved pattern is generated in vertebrates. The topographic location of CVOs across the roof, alar, basal, and floor plates of the neural tube suggests a diversity of specification mechanisms. Interestingly, a common feature of all CVOs is differentiation along the midline—close to the main signalling centres involved in patterning of the developing brain.

The vertebrate hypothalamus contains a particularly large number of CVOs (Fig. 1A). A number of developmental genes expressed in the hypothalamic anlagen are known in many species, but the regional or cellular specification of CVOs *per se* has not been investigated in many developmental biology studies. *Nkx2.1*, expressed throughout almost the entire presumptive hypothalamus, is a homeodomain gene critical for its regional specification [19]. *Brn2* (a POU domain factor) [20], *Sim1* (a bHLH factor) [21], *Gsh1* [22] and *otp* [23] have all been implicated in the specification of cell lineages secreting peptides and hormones in neuroendocrine hypothalamic nuclei. For example, in *Sim1* knock-out mice, magnocellular neurons of the paraventricular (PVN), supraoptic (SON), and anterior periventricular nucleus (aPV) display impaired terminal differentiation and do not produce CRH, AVP, OT, TRH or SS. Similarly, no GnRH is produced in the AMC of *Gsh1*<sup>-/-</sup> animals [22]. In chicken, the expression of two genes of the *Wnt* family of signalling molecules is also correlated with the prospective location of the PVO [24]. Many early signalling pathways may therefore be involved in the specification of these organs, and of other brain regions.

Several studies on CVOs developing from the roof and alar plates of the neural tube have focused on the SCO and ChP. Both the SCO and the ChP are strongly affected in mice in which *Rfx3* is inactivated [25]. This transcription factor, which regulates ciliogenesis, is strongly expressed in the embryonic dorsal midline of the neural tube, along which the SCO and ChP develop. *Rfx3*<sup>-/-</sup> mice have SCO ependymal cell specification defects. They do not express SCO-spondin, a conspicuous SCO marker, and they have a much reduced abnormal ChP, resulting in hydrocephalus.

One recent and original study on formation of the lateral septal organ (LSO, a sauropsid-specific telencephalic CVO not shown in Fig. 1) in chickens investigated the mechanisms involved in the molecular specification of this organ, based on the idea that the fundamental development mechanisms patterning the telencephalic subpallium may also be involved in the positioning and formation of CVOs [26]. Indeed, the authors found that what appears to be a larger than expected “LSO complex”, extending along the entire wall of the telencephalic lateral ventricle, develops at the boundary between the future striatum and the future pallidum. This complex seems to be specified molecularly by a combination of *Dlx*-positive and *Nkx2.1*-negative expression patterns, similar to that observed during the subdivision of adjacent neuroepithelial cells.

Consistent with this idea that CVOs are patterned by combinations of genes similar to those patterning the neighbouring neuroepithelium, the SCO develops from dorsal midline cells with a distinctive anteroposterior identity—a diencephalic prosomere 1 identity (P1 or pretectum). Transgenic mice ectopically expressing *Engrailed-1* in their dorsal diencephalon (including P1) have no SCO [27]. This is probably due to the specific down-regulation of *Wnt1* in the P1 dorsal midline, leading to the disappearance of cells destined to become SCO cells. These mice also display abnormal differentiation of their third ventricle ChP and hydrocephalus [27].

Similarly, for dorso-ventral patterning, it has been shown that genes such as *Msx1*, which is expressed at the diencephalic dorsal midline, are required for SCO development [28]. Conversely, in the telencephalon, *Emx1/2* expression is observed not in midline cells, but in the adjacent neuroepithelium, restricting the presumptive ChP territory to the midline [29]. Indeed, the forced expression of *Emx1* in the telencephalic dorsal midline prevents the acquisition of a choroidal cell fate, due to interference with Wnt dorsal midline signalling pathways. This notion is also supported by the lack of telencephalic plexuses in mouse *extra-toes* mutants carrying a mutation in the *Gli3* gene, which encodes a Wnt regulator that also mediates responses to Shh signals [30,31]. These data highlight the midline origin of CVOs and ChP and show that the development of these organs is strongly dependent on the extensively studied ventral and dorsal midline signalling pathways.

#### 4. Exceptional diversity of CVO cell types – a link to adult neurogenesis?

CVOs display exceptional diversity in terms of cell types (Fig. 2A). These cell types include CSF-contacting neurons,

which are highly variable in terms of their cellular morphology and molecular signature. Unlike other brain neurons, they are not isolated from the CSF (or blood), and are highly sensitive to CSF composition, as shown by electrophysiological experiments [6]. They play a special role in the uptake, transformation and emission of non-synaptic signals mediated by the internal and external CSF and intercellular fluid of the brain. They are located at various distances from the ventricular lumen (ependymal, hyperependymal, proximal or distal neurons) but remain connected to the CSF [17]. Most are ciliated and sensitive to CSF flow, and some have very unusual morphologies: coronet cells resemble mechanoreceptors of the lateral line organ, whereas spinal CSF-contacting neurons may be sensitive to pressure. These neurons are found mostly in teleost and shark sacchi vasculosi – structures located close to the hypothalamus [32] – and in ascidian tadpoles [33]. Other unusual CSF-contacting neurons found in hypothalamus include deep brain photoreceptors. These photoreceptors are thought to be present in the brains of fish expressing *Rx* genes [34]. They contain various opsins and other compounds of the phototransduction cascade. They may be involved in regulating circadian and reproductive responses to light.

Astrocytic glial cells have been shown to play important roles in controlling both synaptic transmission and neurosecretion [35,36]. These cells interact with neurons and are in close contact with vascular endothelial cells [37]. The extent and distribution of this relationship have led to the suggestion that glial and endothelial cells form “gliovascular units” contributing to the architecture of CVOs, forming the AMC for example [38].

CVOs also contain modified ependymogial cells [39]. These cells are particularly well developed in the ME of the hypothalamus, where they are known as tanycytes [40] (Fig. 2B and C). They are involved in the control of neurosecretion.

Xu et al. [41] recently showed that there are neural progenitor cells in the ependymal layer of the third ventricle in adult rats, some of which may be derived from tanycytes. This suggests a possible role for some tanycytes in neurogenesis [41]. These tanycytes retain the morphological features of embryonic radial glial cells. Radial glial cells, which extend processes from subependymal regions to the pial surface, are abundant during mammalian brain development. These cells are considered to correspond to a transient phase of astrocyte maturation. Radial glial cells have been shown to function as primary precursors in the adult avian brain and in the developing mammalian CNS, giving rise to neurons and/or glia, depending on the age and region of the brain analysed [42]. It is increasingly accepted that adult neural stem cells are astrocytes derived from radial glia [42,43]. Thus, radial glial cells seem to be the source of all brain neurons, both during development and in adults [44–46].

Radial glial cells persist in the brains of other vertebrates, including birds [47] and fish [48,49]. In birds, neurogenesis is associated with marked seasonal diversity in singing behaviour. Fish brains are known to display persistent growth during adulthood, but the mechanisms underlying this growth remain poorly understood [50,51]. The persistence of radial glia in the fish adult CNS is thought to be related to continuing neurogenic capacity [52]. Several studies have documented, by BrdU incorporation,

the presence of a number of periventricular proliferative areas in the adult brain of different fish species [50,51,53–57]. Over time, newly generated cells in zebrafish brain move away from the ventricles; this suggests that they migrate and ultimately differentiate into neurons [52,54].

In teleosts, astroglia develop from ependymal tanycytes and radial glial cells, which are abundant in CVOs such as the ME [58–61]. Strong glial marker expression has been observed in some CVOs of the mullet, *Chelon labrosus* – such as the AP, the organum vasculosum hypothalami and the saccus vasculosus – but not in the cells of the SCO [62]. In the hypothalamus, the proliferative cells are located in the dorsal zone of the periventricular hypothalamus [57] and in the mediobasal hypothalamus. The hypothalamus contains a complex system of ventricular recesses, including the lateral and posterior recesses, both of which are bordered by very large numbers of strongly aromatase B-immunoreactive radial cells. A recent study documented two unique, and probably linked features of the fish brain: active neurogenesis and the presence of aromatase in radial glial cells, which appear to be progenitor cells in the brains of adult fish [63].

What function does adult neurogenesis in these ventricular regions serve? It remains unclear whether it is related to hypothalamus-specific functions or used for more general cognitive purpose. In other words, we do not know whether the newly generated cells in these regions are needed locally, for cell renewal in CVOs, or whether they contribute to the production of neurons for other regions of the brain.

## 5. Evolution of CVOs I: other vertebrate and protostomian neurohemal organs

A vast study [64] described about 18 different CVOs in 31 species belonging to various groups of vertebrates, from cyclostomes to mammals. The NH, ME, SCO and PIN may be the oldest such organs, because they were found in almost all vertebrate species examined.

Vertebrate CVOs are highly diverse. The morphology of organs that are clearly homologous varies considerably between species. In particular, the ME is closely apposed to the NH in fish, whereas the ME and NH are clearly separated in birds and crocodiles.

In some cases, it is hard to identify clear relationships between the CVOs of different vertebrate groups. For example, the saccus vasculosus is found in almost all chondrichthyans and actinopterygians, but has no apparent counterpart in amniotes. Its absence in amniotes is thought to be secondary. Its presence in fish seems to be correlated with the salinity of the water. This organ is reduced or absent in freshwater fish, whereas it is well developed and folded in euryhaline species. Lamprey may have a saccus dorsalis (Fig. 3C), which may correspond to extreme development of the fourth ventricle choroid plexus. Other dorsal mesencephalic structures are also present in fish [64]: the tectal organ of teleosts and the midline ridge formation in chondrichthyans [65,66].

So, do other bilaterian animals have neurohemal organs? The situation in insects and other protostomians is not dealt with here,

as it has been the subject of a recent review [67] and of another review in this issue. As reported in these papers, clusters of neurosecretory cells send their axons to the annelid pericapsular organ, a neurohemal structure at the ventral surface of the brain. The pericapsular organ is composed of a glial sheath, a layer of epithelial cells and blood vessels. Specialised neurosecretory cell endings are clustered next to glia and among epithelial cells, suggesting that the pericapsular organ represents a site of active neuropeptide release, like the vertebrate ME. In molluscs, neurosecretory cells and neurohemal structures located in the glial sheath of the nervous system have been described.

According to Hartenstein [67], the prevailing view assumes the integration of specialised epithelial cells into the epidermis in multicellular animals. In cnidarians, neurosecretory cells are observed as epithelial cells in the epidermis and gastrodermis, but also as scattered neurons. These animals have modified cilia, which receive stimuli. Cnidarians seem to possess almost all the neuropeptides present in chordates and arthropods [68].

When scientists first became interested in CVOs, all nine organs listed above were investigated and their neurosecretory activity in the blood and CSF was assessed. The CVOs were quickly classified as unique structures, due to their neurosecretory properties, leading to the suggestion that CVOs (especially the ME and NH) secrete substances into the bloodstream. Immunocytochemical and biochemical studies then identified a long list of hormonal and neurotransmitter-like factors in sensory CVOs. Neuropeptides and chemical substances found in sensory CVOs include adrenocorticotrophic hormone (ACTH), angiotensin, endothelin, gonadotropin-releasing hormone (GnRH), enkephalins, neuropeptide Y, somatostatin, substance P, thyrotrophic-releasing hormone (TRH), VIP, oxytocin, vasopressin, adrenaline, noradrenaline, acetylcholine, dopamine, serotonin, aspartate, GABA, glycine, and glutamate. Some of these substances may be concentrated in sensory CVOs due to extraction from the blood, but most are probably synthesised locally for export, or transported into CVOs from other brain regions by axons.

As concluded by Vigh et al. [16], the “ventricular CSF-contacting neurons and associated ependymal and glial cell types found in neurohemal organs may represent the phylogenetically oldest component detecting the internal fluid milieu of the brain”. The neurohormonal terminals on the external surface of the brain also represent an ancient form of non-synaptic signal transmission [16].

## 6. Evolution of CVOs II: the situation in cyclostomes and urochordates

### 6.1. Cyclostomes

It has been suggested [64] that the phylogenetically oldest vertebrate CVOs are the NH (intermediate lobe and neural lobe), the ME, the SCO and the PIN. These organs have been identified in all the main groups of vertebrates, including cyclostomes (lampreys and hagfish, although the PIN is not found in hagfish). An OVLT was also observed in both cyclostome groups, while the parapineal, saccus dorsalis and ChP are absent from hagfish



but present in lampreys. No PVO can be identified in lampreys by routine histology, but the presence of such a structure has been suggested, based on histochemical data (immunoreactivity to dopamine, serotonin, histamine, neurotensin,  $\beta$ -endorphin and photoreceptor proteins) and the concentration of large numbers of CSF-contacting neurons in a slightly concave area of the third ventricle wall [69].

CSF-contacting neurons in cyclostomes are exclusively intraependymal, with no evidence of distal CSF-contacting or intercellular fluid-contacting neurons such as those found in other groups of vertebrates [16]. This led Vigh and Vigh-Teichmann [17] to suggest that this feature of cyclostomes reflects the ancestral situation. In the hagfish hypothalamus, CSF-contacting neurons are found in the lower part of the ventricle and in the preoptic, infundibular and lateral infundibular recesses, but not in the subhabenular recess [70]. In lamprey, magnocellular and parvicellular CSF-contacting neurons are found in the preoptic nucleus. CSF-contacting neurons have been detected in many hypothalamic regions, including the preoptic nucleus, NH and presumptive PVO.

The lamprey hypothalamo-pituitary axis can be divided into anterior and posterior regions. The anterior region corresponds to the ME and is separated from the pars distalis of the adenohypophysis by a layer of connective tissue (Fig. 3B). There is no trace of a portal system in this animal group, although it has been suggested that some neurohormones and monoamines freely cross the connective tissue layer to reach the adenohypophysis [71,72].

A large number of the larger (magnocellular) neurons of the preoptic nucleus of lampreys are immunoreactive for vasotocin, and fibres derived from these cells form a tract that innervates the NH and ME. Some of these fibres split from the main tract and innervate the marginal stratum of the postoptic recess, the probable origin of the ME [69]. Other neurons of the preoptic nucleus containing gonadotropin-releasing hormone, growth hormone, prolactin and met-enkephalin, also project to the NH, showing that the (probably evolutionarily ancient) preoptico-pituitary neurosecretory system is well developed in the lamprey.

Although the architecture of the neurosecretory system in lampreys is relatively well understood, no data are available concerning the early molecular interactions responsible for its organisation. In mammals, *otp* (*Orthopedia*, see also below) plays a critical role in the proliferation, migration and differentiation of many lineages of the hypothalamic neurosecretory system [23]. In *Otp*<sup>-/-</sup> mice, the corresponding peptide is absent and no axon outgrowth to the ME and NH is observed, probably due to progenitor proliferation and migration defects (reviewed in [73]). In fish, *Otp1* is also expressed in the neuroepithelial mantle layer of the preoptic region and in clusters of cells in the posterior tuberculum (hypothalamic posterior basal plate). These clusters progressively fuse along the midline of the basal diencephalon during development [74]. *Otp1* expression along the midline is regulated by a combination of midline signals (Hedgehog, Fgf8 and Nodal). Double labelling with TH, mutant analysis and morpholino injections have clearly shown that *Otp* is involved in the determination of a subgroup of catecholaminergic neurons in the posterior tuberculum, and

possibly in the preoptic area [74]. Dopamine (DA) is the principal neuroactive catecholamine in the CNS of lampreys, and neither adrenaline nor noradrenaline has been detected in the hypothalamus [75]. During development, DA-immunoreactivity is detected in the mantle area of the posterior tuberculum and in the dorsal hypothalamic nucleus at early stages and in the preoptic nucleus and other hypothalamic nuclei at later stages. The NH displays abundant innervation from DA-immunoreactive fibres [76]. DA-immunoreactive CSF-contacting cells are detected in the PVO of adults [75]. We have found that *Otp* is expressed in the hypothalamus of the sea lamprey before any DA is detected (Fig. 3A). Expression is uniform through the neuroepithelium, except for a very small number of cells with strong *Otp* expression in the deep mantle zone. The distribution of these cells resembles that of the *Otp*-positive neurons of zebrafish, suggesting these cells may be the precursors of some of the DA-positive cells of the lamprey hypothalamus. These data provide additional evidence for the ancient origin of these complex circuitries in vertebrates.

## 6.2. Urochordates

If we are to understand the evolutionary origin of CVOs, it is important to identify their homologues in non-vertebrate chordates. In the cephalochordate *Branchiostoma belcheri*, a ventral evagination of the neural tube is thought to be homologous to the infundibular NH [77]. Another infundibular organ in *Branchiostoma lanceolatum* is thought to be homologous to the SCO [78]. In the near future, it will be exciting to investigate the genes expressed in these organs. This work will accelerate with the completion of whole genome sequences and EST data for several cephalochordate species (e.g. the *Branchiostoma floridae* genome sequence is available at <http://genome.jgi-psf.org/Brafl1/Brafl1.home.html>).

Ascidians, the largest class of the Tunicata/Urochordata subphylum, are also closely related to vertebrates. They include *Ciona intestinalis*, a cosmopolitan species that has attracted considerable attention in the broader scientific community [33,79]. Ascidians have an assortment of cells and organs closely associated with the nervous system. It has been suggested that the larval ocellus of ascidians is homologous to the median eye (pineal organ) of vertebrates [80]. It is also possible that a third population of cells located on the latero-ventral side of the sensory vesicle is homologous to the deep brain photoreceptors of vertebrates (Takehiro Kusakabe, personal communication). These cells are located next to coronet cells similar to those found in the saccus vasculosus of fish [81,82]. However, it is not always easy to identify invertebrate counterparts of vertebrate organs. We focus here specifically on two of these organs: the neural gland and the ciliated/neurohypophyseal duct (Fig. 4A and B). This duct opens into the oral cavity, via a thickened region called the ciliated funnel. Its homology to the pituitary gland is discussed by Toro and Varga, and in the foreword of this issue. Below, we briefly summarise the data that have led us to conclude that the neural gland is homologous to a vertebrate dorsal CVO rather than the pituitary gland, and that the homology between the ascidian neurohypophyseal duct and

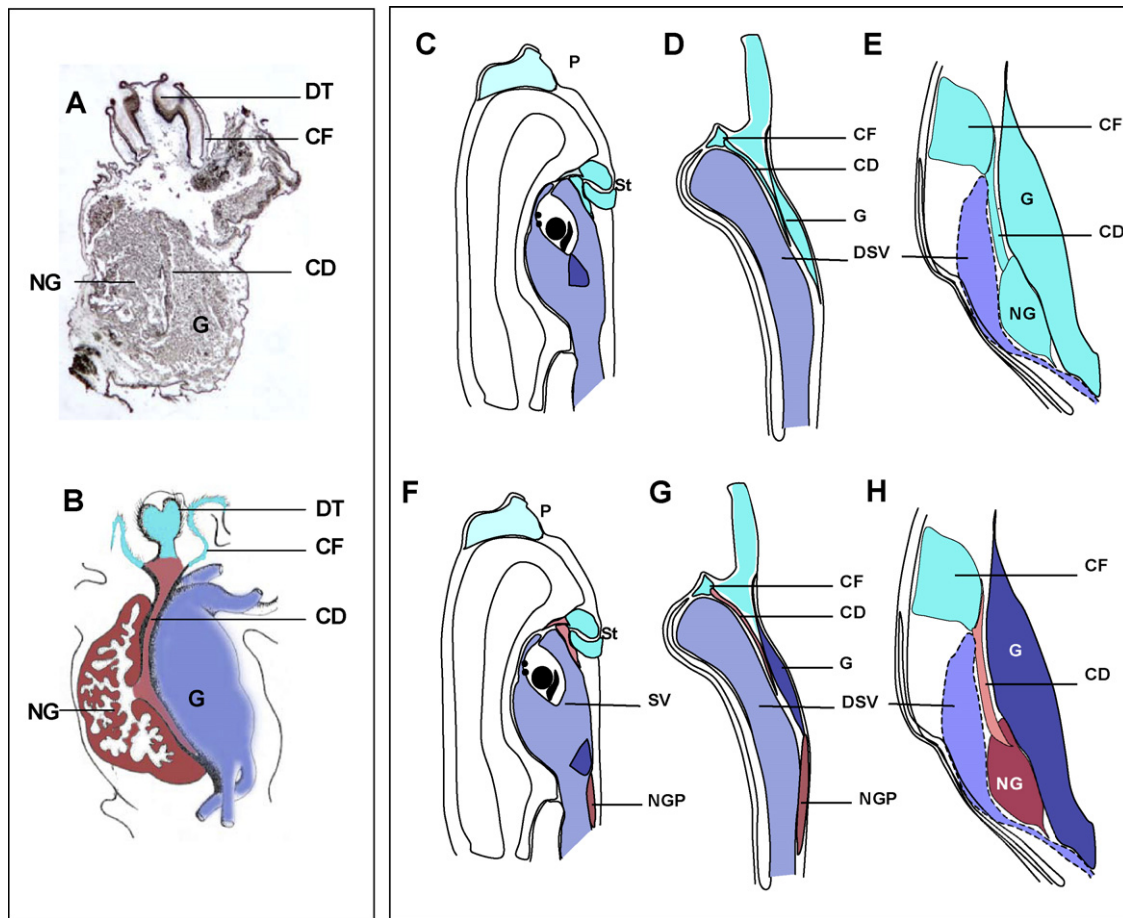


Fig. 4. Morphogenesis and organisation of ascidian neural complex from larval to adult stages. (A) Histology of the neural complex and (B) diagram of the neural complex. Cerebral ganglion (G, blue), and the neural gland, a spongy organ apposed to it (NG, red). It contains a cavity, in contact with seawater through the ciliated/neurohypophyseal duct (CD, dark pink). This duct opens into the oral cavity, via a thickened region called the ciliated funnel (CF, light blue), containing a bulb named the dorsal tubercle (DT, light blue). Anterior to the top. Ventral to the left. (C–E) Diagrams illustrating the hypothesis summarised by Manni [96]: the whole neural complex is derived from the neurohypophyseal/ciliated duct, which is itself derived from the anterior placodes and stomodeum (St); P, palps. Colour coding is as in (B). Legends as in (F–H) below. (F–H) Diagrams illustrating alternative hypotheses concerning the developmental origin of the different organs of the neural complex. The ciliated funnel remains at the anterior tip of the CNS, in front of the sensory vesicle (SV), which later degenerates (degenerating sensory vesicle (DSV)). The ciliated duct is derived from a region located between the sensory vesicle and stomodeum at larval stages that is thought to be homologous to the neurohypophysis. In this diagram, we illustrate an alternative hypothesis: the neural gland is derived from a population of cells dorsal to the neural tube (neural gland primordium: NGP, dark red) as it is the case for the hindbrain choroid plexuses in vertebrates. (A–H) Sagittal views of larval, metamorphosing juvenile and adult neural complex. Anterior to the top. In (D) and (G), only the dorsal part of the animal, around the pharynx, is represented. In (E) and (H), detail of the neural complex is shown, in the same orientation as (D) and (G). (C and F). Larva 3 h after hatching. (D and G) Side view of the dorsal part of a stage I, IV protostigmata juvenile (day 3). (E and H) A stage V protostigmata juvenile (day 8) (stages as described in [96]).

the vertebrate neurohypophysis requires additional support from further studies.

The neural gland is a spongy sac with a folded epithelium consisting of phagocytic cells with pseudopodia, microvilli and glycogen reserves [83,84]. Its function has long remained enigmatic. It was thought to be an endocrine gland [85,86], possibly homologous to the vertebrate adenohypophysis [87,88]. However, recent molecular data have provided no clear evidence for homology between the neural and pituitary glands [89].

Ruppert [84] proposed an alternative hypothesis, according to which the principal role of the neural gland is the regulation of blood volume. He provided evidence that the ciliated duct generates a unidirectional influx of seawater into the neural gland. The neural gland thus serves as an interface between seawater and blood, just as the CVOs serve as an interface between CSF and

blood in vertebrates. Both types of organ display high levels of exchange activity. Based on cytological data, the ascidian neural gland has long been thought to consist of macrophage-like cells [90,91] protecting against compounds or microorganisms present in seawater. Similarly, the vertebrate choroid plexuses contain numerous macrophages and dendritic cells, providing the first line of defence for the brain, via the neuroimmune system.

We recently showed that several GPCRs (G protein-coupled receptors) including somatostatin, vasopressin and CRH receptors are expressed in the neural gland [81]. These receptors for neuropeptides involved in osmoregulation constitute molecular signatures clearly relevant to those found in CVOs, as described above. We also recently showed that the molecular signatures of choroid plexuses are expressed in the neural gland (Hélène

Auger, unpublished data). In contrast, very few genes encoding conserved neuropeptides have been identified (e.g. tachykinins) [92,93], despite recent extensive *in silico* searches [81,94,95]. The isolation of peptides by methods not based on homology would demonstrate the high level of divergence of these genes in this animal group.

However, as it seems unlikely that the very small neural gland can regulate blood volume in a 10 cm long animal (the size of *Ciona* adults), we suggest that the main function of this gland is local regulation of the composition and volume of the liquid surrounding the cerebral ganglion. The ganglion and nerves are girdled by a connective sheath, which may isolate them from the mantle, in which no osmoregulation occurs. Studies are still underway to determine the precise role of this gland in osmoregulation.

Neural gland development is also a major subject of debate. Moreover, this debate is not recent as this extract from Wiley's publication [86] suggests: "Observations made by Herdman on *Ascidia mammillata*, confirm Julin's discovery that in this species the neural gland, besides having the usual duct running anteriorly to communicate with the dorsal tubercle, has also a number of short funnel-shaped apertures into the peribranchial cavity. He adds that in two specimens examined by him the duct of the hypophysis had no opening in the pharynx, the dorsal tubercle being entirely absent. Herdman therefore suggested that the dorsal tubercle and neural gland represent originally distinct structures. This view, which receives only the slenderest support from the fact intended to establish it, is obviously untenable in the light of what has been said above as to the origin of these structures".

More recently [96], it has been suggested that the neural gland, like the pituitary gland, is of placodal origin (Fig. 4A and B). This placode probably initially grows posteriorly and dorsally, to give rise to the ependymal ciliated duct [97]. The ventral part of the neural tube is believed to degenerate completely during ascidian metamorphosis [86]. It has therefore been suggested that this dorsal placodal epithelium generates the whole neural complex, including the neural gland and ganglion (Fig. 4C–E).

However, this suggestion is based on very old data [86] and on a study by Takamura (2002), using a neural antibody, which showed that the neural gland and ganglion develop dorsally to the degenerating sensory vesicle. Moreover, the neural gland primordium seems to form behind the ganglion primordium, and although it is dorsal to the degenerating sensory vesicle – like the neural gland – its slightly more ventral position leads to a final location of the neural gland ventral to the ganglion and nerves (Fig. 4E and H). The position of the neural gland primordium, posterior to the sensory vesicle, is distant from the anterior placode [98], and seems to be apposed to a region in which a hindbrain-like primordium has been identified [99]. In vertebrates, the choroid plexus of the fourth ventricle also develops from a thin sheet of cells, the tela choroidea, located at the top of the hindbrain roof. Thus, the position of the neural gland primordium in ascidians may be consistent with that of the choroid plexus primordia at the roof of the vertebrate hindbrain [81].

The ciliated duct is another organ for which homology must be considered with caution. This duct, also known as the neurohypophyseal canal, is located dorsal to the sensory vesicle at the larval stage. It is thought to give rise to either the ganglion or the neural gland, hence the name "neurohypophyseal canal" conferred on this structure by Wiley [86]. "Thus the neural tube, of which the neurohypophyseal canal, so called on account of its later destiny, is merely a continuation, opens in the stomodeum". This term was first used [86] to refer not to the currently known vertebrate neurohypophysis, but this dorsal neural tube region. In a similar way to what is observed in *Botryllus*, this region is thought to result from an evagination of the sensory vesicle, from which the ciliated duct and neural gland are derived. This hypothesis is based on the observation that this dorsal evagination of the CNS connects to an invagination of the stomodeum, just as the diencephalic infundibulum of mammals joins the stomodeal evagination (Rathke's pouch, the precursor of the pituitary gland). This hypothetical homology is potentially interesting but should be treated with caution as further support is required before it can be accepted unequivocally. Many questions remain to be addressed:

- Which cell types in or around the ciliated duct indicate its neurohemal nature? As in the case of the neural gland, ependymal, endothelial and conjunctive cell types in the vicinity of the ciliated duct are clearly similar to those seen in neurohemal organs. In adult ascidians, the presence of CSF contacting-like neurons would provide additional support for homology to CVOs.
- Which neurosecretory neurons in the ascidian central nervous system indicate neurohypophyseal-like nuclei? Giant neurosecretory neurons were first identified in the cerebral ganglion on the basis of histochemical staining [100]. The magnocellular nature of these neurons indicates possible homology to the NH, but the precise location of the ends of their terminals remains to be studied.
- What molecular signatures of NH are found in the ciliated duct? Many studies have reported neuropeptide immunoreactivity in the cerebral ganglion, specifically in the regions bordering the blood sinus apposed to the neural gland [92]. Interestingly, some cells of the ciliated duct itself have also been tested positive [92]. As in the neural gland, receptors for neuropeptides are expressed in the ciliated duct [81,93]. This indicates a clear relationship to CVOs, but not specifically to NH.

With the aim assigning a neurohypophyseal nature to the ciliated duct, we have searched *in silico* for orthologues of the neurohypophyseal specific neuropeptides oxytocin and vasopressin in the *Ciona* genome. Queries with sequences found in protostomian or deuterostomian species did not lead to the identification of such orthologues (Jean-Stéphane Joly, unpublished data). Although not specifically found in the vertebrate neurohypophysis, the most relevant neuropeptide identified in ascidians is GnRH [101–103]. The presence of GnRH in ascidians provides evidence consistent with homology between the ciliated duct and the ME, in which GnRH is secreted into the portal sys-

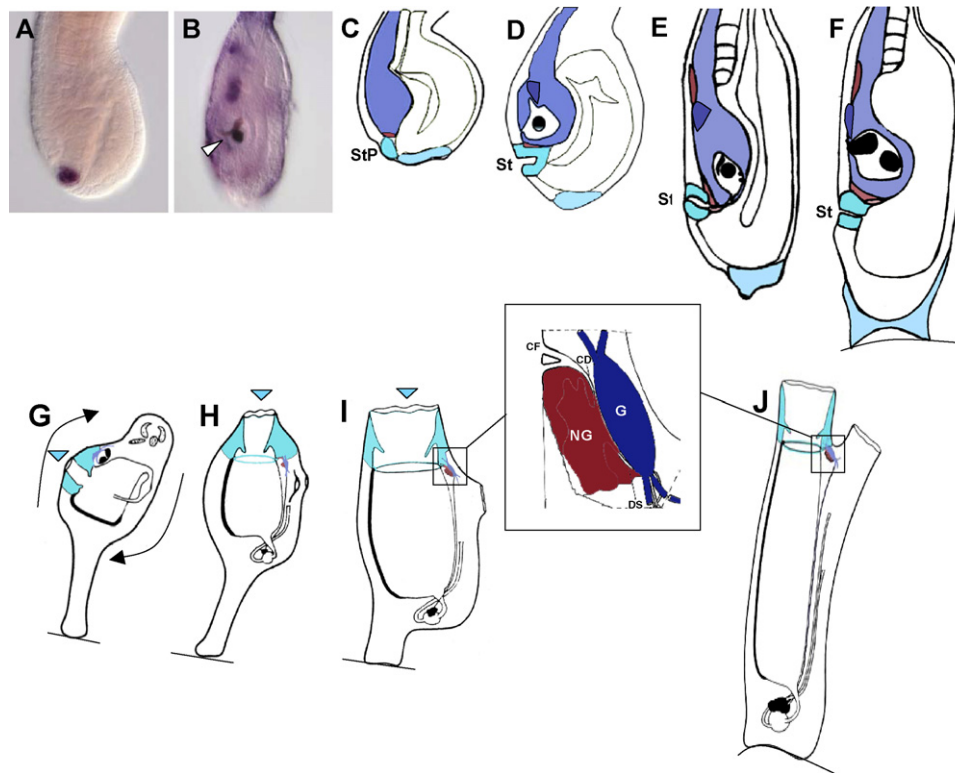


Fig. 5. *Ci-otp* gene expression and ascidian morphogenesis. (A and B) Pattern of expression of the *otp* gene at the mid-tail bud and larval stages (3 h post-hatching). This gene is expressed at the anterior tip of the CNS at the tail bud stage. The white arrowhead in B indicates *otp* expression in the ciliated duct dorsal to the sensory vesicle at this stage. Dorsal to the left. Posterior to the top. (A) Courtesy of F. Moret. (B) Courtesy of Y. Jaszczyszyn. (C–F) Diagrammatic representation of the metamorphosis of ascidian larva based on *Ciona intestinalis* development (adapted from [92]). In (E and F), the primordia of the neurohypophyseal duct and neural gland are indicated with same colour code as in Fig. 4. StP, stomodeum primordium; St, stomodeum; DS, dorsal strand. See Fig. 4 for other legends. (G–J) Rotation of the animal after settlement is a well-known developmental event in the ascidian *Ciona intestinalis*, and can be seen here to start during embryonic and larval development. Detail of the neural complex is given in the panel between (I) and (J). The stomodeal placode moves from an anterior location to form a dorsal stomodeum (refer to Christiaen et al. in this issue). Rotation continues during metamorphosis, resulting in the oral siphon being located at a site very different from that of its embryonic primordium. The blue arrowhead indicates the position of the oral siphon. Location of the CNS (blue), stomodeum (light blue), neural gland (red), ciliated duct (dark pink) and endoderm. The positions of the neural gland and neurohypophysis primordia indicated here remain speculative. Anterior at bottom, dorsal to the left. (G) Rotation of the body axis (stage 3a). (H) First ascidian stage. (I) Second ascidian stage. (J) Adult (as described by Chiba et al. [94]).

tem for delivery to the pituitary gland but not *sensu stricto* to the neural or intermediate part of the NH. It will be interesting in the future to investigate further the distribution of GnRH-producing cells during development, at larval stages and during metamorphosis. It is also possible that the ventral sensory vesicle does not only degenerate, but also differentiates some populations with hypothalamic/neurohypophyseal-like properties. This is consistent with the production of GnRH peptides in the ventral sensory vesicle of larvae (Takehiro Kusakabe, personal communication). Interestingly, this region of the ascidian sensory vesicle undergoes patterning events similar to those observed in the vertebrate hypothalamus and neurohypophysis. According to this hypothesis, the neurohypophysis primordia would be ventral to the sensory vesicle at late tailbud stages.

However, *otp* – encoding a major, but not exclusive, factor in NH development (see above) – is expressed dorsally rather than ventrally in the sensory vesicle of larvae (Fig. 5B). In early tail bud embryos, *otp* is expressed at the anterior and ventral tip of the CNS, in a position similar to that of the NH primordium of vertebrates at early stages [104]. This pattern of *otp* expression indicates that this anterior region of the sensory vesicle may fol-

low the dorsal movement observed during ascidian development (see Christiaen et al. in this issue). This observation is linked to a global rotation of the animal after settlement to reposition the mouth (Fig. 5D–K). Whether these early anterior and late dorsal *otp* expression domains are related by cell lineage remains to be verified.

Despite observed topological concordances between the positions of the ascidian and vertebrate organ primordia, lineage analyses during metamorphosis in ascidians are required to produce a reliable description of the ontogeny of this complex set of organs. The embryonic origins of these organs must be clearly assigned, particularly at late stages, at which lineages cannot be inferred based on published information [105–109]. The development of modern imaging methods is likely to provide a major impetus to these studies.

## 7. Conclusion

We are just beginning to understand the diversity, functions, and ontogeny of the vertebrate circumventricular organs and their homologous neurohemal organs in non-vertebrates. It is



clear that attempts to identify homology between cyclostome or ascidian and vertebrate organs have so far met with only limited success. In future studies, imaging techniques should provide data with higher spatial and temporal resolution and genomic data should provide us with a more reliable basis for comparing the molecular signatures of cell types. Such studies may lead us to conclude that vertebrate CVOs and the invertebrate organs they resemble have a common origin within the bilaterian phylum. Alternatively, some non-vertebrates may have their own types of CVOs, generated by a convergence of function, with no clear homology to vertebrate CVOs. Whatever the final conclusion, renewed interest in these organs is bound to provide a more comprehensive understanding of their functions, development, and evolution in chordates.

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