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Houda Hamdi-Rozé

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Par

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Etude du rôle de la voie NOTCH dans la régulation de *Shh* au cours du développement du cerveau antérieur chez les vertébrés

Thèse présentée et soutenue à Rennes, le 29 Novembre 2019

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See You Space Cowboy...

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LISTE DES ABREVIATIONS

ACTH : Adreno CorticoTrophic Hormone

ADH : Hormone anti-diurétique

ADN : Acide désoxyribonucléique

bHLH : basic Helix-Loop-Helix

bHYP : cellules basales hypothalamiques

CGH : Hybridation génomique comparative

CRE : Cyclization Recombinase

DAPT : -[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester

DSL : Delta – Serrate - Lag-2

EV : Endoderme Viscéral

EVA : Endoderme Viscéral Antérieur

FSH : Hormone Folliculo-Stimulante

GH : Growth Hormon, Hormone de croissance

GPLD : Génétique des Pathologies Liées au développement

HH : Hamburger-Hamilton

HPE : Holoprosencéphalie

HuC/D : Antigènes Hu C et D

KO : Knock-Out

LH : Hormone Lutéinisante

L/L : lox/lox

MLF: Faisceau Médio-Longitudinal

MSH : melanocytes stimulating hormone

MTH : Tractus Mamillothalamique

NECD : Domaine extracellulaire de NOCTH

NICD : Domaine intracellulaire de NOCTH

nmesV : noyau du tractus descendant mésencéphalique du nerf trijumeau

nMTT : nucleus of the Mamillo-Tegmental Tract. Noyau du Tractus Mamillo-Tegmentaire

nTPOC : nucleus of the Tract of the Postoptic Commissure. Noyau du Tractus de la Commissure Post-Optique

TSH : thyreocytes stimulating hormon

VLT : Tractus Longitudinal Ventral

LISTE DES GENES

ASCL1 : Achaete-scute complex homolog 1
BMP : Bone Morphogenetic Protein
BOC : BOC cell adhesion associated, oncogene regulated
CDON : Cell adhesion associated, oncogene regulated
CHGA : Chromogranin A
CHRD : Chordin
CNOT1 : CCR4-NOT transcription complex subunit 1
DHCR7 : 7-Dehydrocholesterol Reductase
DKK1 : Dickkopf WNT signaling pathway inhibitor 1
DISP1 : Dispatched homolog 1
DLL1 : Delta-like 1
FGF : Fibroblast Growth Factor
FGFR : Fibroblast Growth Factor Receptor
FOXA2 : Forkhead box A2
FOXH1 : Forkhead box H1
GAS1 : Growth Arrest-Specific 1
GLI : GLI family zinc finger 1
HES5 : Hairy and enhancer of split 5
HEY1 : Hairy/enhancer-of-split related with YRPW motif 1
KIF7 : Kinesin Family member 7
MAML : Mastermind like
NHLH1 : Nescient helix loop helix 1
NKX2.1 : NK2 homeobox 1
NOG : Noggin
PTCH1 : Patched 1
RALDH : Retinaldehyde DésHydrogénase
RBPJ : Recombination signal binding protein for immunoglobulin kappa J region
SBE2 : SHH Brain Enhancer 2
SCUBE2 : Signal peptide, CUB domain and EGF like domain containing 2
SHH : Sonic Hedgehog

SIX3 : Sine oculis-related homeobox 3
SKI : Skinny hedgehog
SMAD : SMAD family member
SMO : Smoothened, frizzled class receptor
STMN2 : Stathmin 2
SUFU : SUFU negative regulator of hedgehog signaling
TAGLN3 : Transgelin 3
TBX : T-box transcription factor
TDGF1 : Teratocarcinoma-Derived Growth Factor 1
TGF-Beta : Transforming Growth Factor Beta
TGIF : TGFB-induced factor homeobox 1
WNT : Wingless-type MMTV integration site family
ZIC2 : Zinc finger protein of the cerebellum 2

Nomenclature génétique officielle utilisée

	Humain	Souris, Poulet
Nom du gène	Sonic Hedgehog	Sonic Hedgehog
Symbole du gène	<i>SHH</i>	<i>Shh</i>
Symbole de la protéine	SHH	SHH
Voie de signalisation	SHH	SHH

AVANT-PROPOS

L'équipe de "Génétique des Pathologies Liées au Développement" (GPLD) travaille sur le développement cérébral embryonnaire et les pathologies qui y sont associées. Elle s'intéresse en particulier à l'holoprosencéphalie (HPE), une malformation congénitale due à un défaut de clivage du cerveau antérieur. La dérégulation spatio-temporelle de la voie de signalisation SHH est à l'origine de cette pathologie.

Grâce au centre de référence Rennais pour cette pathologie, l'équipe a accès à une importante cohorte de patients atteints d'HPE. Des études génétiques ont été menées afin d'établir un diagnostic moléculaire pour ces patients. Mais les mutations retrouvées n'expliquent que 30% des cas d'HPE d'origine génétique (*Mercier et al., 2011, Dubourg et al., 2018*). L'objectif de notre équipe est d'identifier de nouveaux gènes candidats pour l'HPE et d'étudier les mécanismes moléculaires engagés.

Mon travail de thèse a porté sur l'étude de l'implication de la voie NOTCH dans cette pathologie (*Dupé et al., 2011*). Les étapes précoces du développement cérébral sont très conservées chez les vertébrés. J'ai donc utilisé 2 modèles animaux (poulet et souris) pour caractériser la fonction de cette voie au cours du développement précoce du cerveau; et plus particulièrement son impact dans le développement de l'axe hypothalamo-hypophysaire.

Dans mon introduction, j'exposerai tout d'abord les différentes phases du développement cérébral chez les vertébrés. Je détaillerai ensuite le développement de l'axe hypothalamo-hypophysaire. Je consacrerai une partie de mon introduction spécifiquement à la voie NOTCH, nouvellement impliquée dans l'HPE. Je définirai ensuite l'HPE et l'état des connaissances actuelles sur la survenue de cette pathologie, en y incluant la voie NOTCH. J'exposerai enfin les nouvelles théories sur les modes de transmission de cette pathologie et notamment le multigénisme.

Les résultats présentés dans ce manuscrit décrivent le rôle de la voie NOTCH au cours de la neurogenèse précoce dans l'hypothalamus. Ils montrent également que la voie NOTCH est impliquée dans le maintien de l'expression de *Shh* dans le prosencéphale ventral. Ces travaux ont également permis de générer un modèle souris développant une forme mineure d'holoprosencéphalie (ou microforme).

INTRODUCTION

INTRODUCTION

I- LE DEVELOPPEMENT DU CERVEAU ANTERIEUR CHEZ LES VERTEBRES

Les anomalies du développement menant à une HPE ont lieu très précocement lors de l'embryogénèse. Afin de comprendre ce contexte, les principales étapes du développement précoce du cerveau antérieur et de l'axe hypothalamo-hypophysaire sont détaillées dans les paragraphes qui suivent.

A/ Développement du cerveau vertébré

L'embryogénèse précoce est très conservée chez les vertébrés. De nombreuses études ont été menées chez l'embryon de souris, de poulet, de xénope et de zebrafish. Elles ont permis, entre autre, de caractériser les mécanismes moléculaires impliqués au cours du développement précoce du cerveau des vertébrés. Pour l'essentiel, ces mécanismes peuvent être transposés au développement cérébral humain.

Après la fécondation, le zygote subit une série de divisions cellulaires jusqu'à constituer la morula. Les cellules externes de la morula se compactent et forment une paroi étanche : le trophoctoderme. Celui-ci entoure une cavité emplie de liquide (le blastocèle) et un amas cellulaire qui se développera en embryon (l'embryoblaste) (*Figure 1A*). A ce stade, l'embryoblaste est constitué de deux feuillets : l'épiblaste, à l'origine des tissus embryonnaire, et le pré-endoderme, qui donnera les tissus extra-embryonnaires (*Chazaud et al., 2006 ; Plusa et al., 2008*). La nidation dans l'utérus se fait côté bouton embryonnaire ce qui définit le premier axe d'organisation de l'embryon : l'axe proximo-distal (*Rossant & Tam, 2004 ; Mesnard et al., 2006 ; Rivera Perez et al., 2003*).

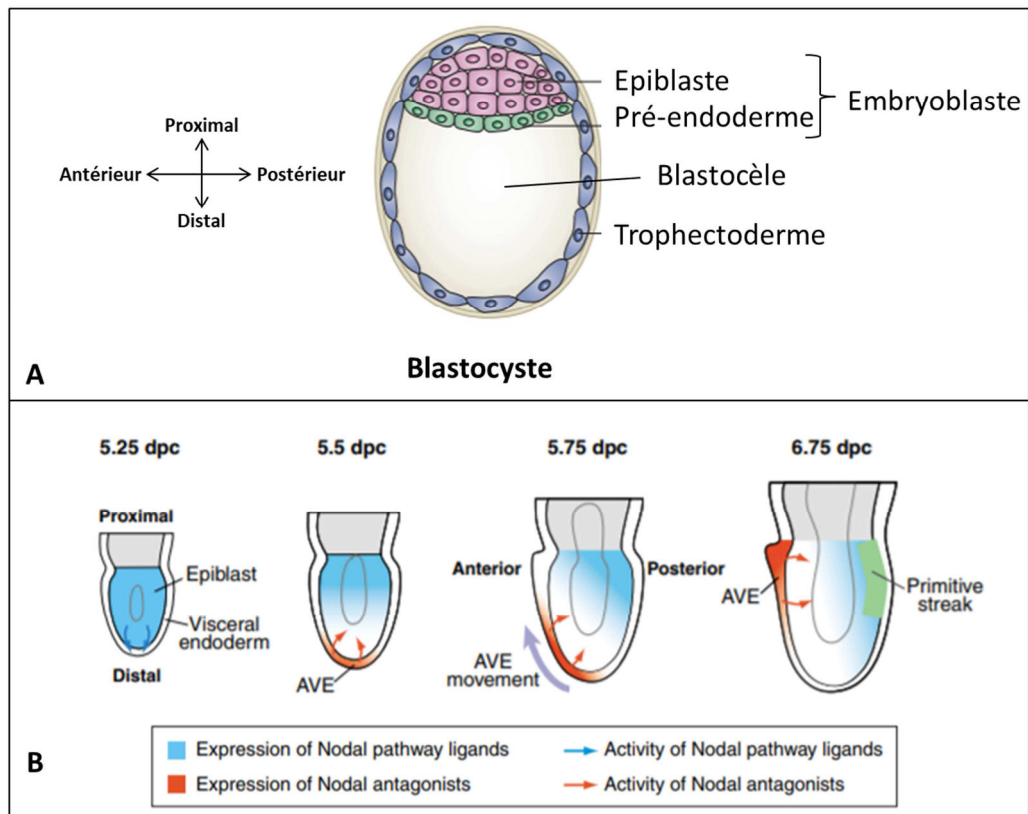


Figure 1 : Processus de Gastrulation chez la souris. (A) Le blastocyste est constitué de l'embryoblaste (épiblaste et pré-endoderme) et du trophoctoderme qui entoure le blastocèle. (B) Après invagination de l'épiblaste dans le blastocèle, le pré-endoderme se différencie en endoderme viscéral et endoderme pariétal. La voie Nodal et ses antagonistes entrent alors en jeu pour induire l'endoderme viscéral antérieur (AVE pour Anterior Visceral Endoderm), qui migre en position proximo-antérieur et permet la mise en place de la ligne primitive dans la partie postérieure. dpc = days post conception, correspondant au nombre de jours post-fécondation pour l'embryon de souris. (Adapté d'Arnold & Robertson; 2009 (A) et de Shen; 2007 (B)).

Lors de la pré-gastrulation, l'invagination de la partie proximale du trophoctoderme va repousser les cellules de l'épiblaste vers le pôle distal. A ce stade, l'épiblaste est entouré par l'endoderme viscéral embryonnaire (EV embryonnaire, qui dérive du pré-endoderme), et en contact, dans sa partie proximale, avec l'ectoderme extra-embryonnaire (dérivé du trophoctoderme).

Différentes voies de signalisation rentrent en jeu pour mettre en place l'organisation de l'embryon. NODAL, un membre de la superfamille des facteurs de croissance TGF-Beta, joue un rôle central dans ce processus en régulant l'expression de nombreux facteurs de transcription.

Des anomalies antérieures ont été trouvées chez des chimères de souris fabriquées avec des cellules normales et mutantes pour *Nodal*, dans lesquelles l'endoderme viscéral était composé exclusivement de cellules mutantes *Nodal* (Varlet et al., 1997a).

Ainsi, un gradient NODAL proximo-distal permet la mise en place de l'endoderme viscéral (Ben Haim et al., 2006) qui va ensuite acquérir une identité antérieure et migrer pour se retrouver face à

l'épiblaste antérieur présomptif (*Takaoka et al., 2011 ; Rossant & Tam, 2004 ; Camus et al., 2006 ; Figure 1B*). Ce tissu est appelé EVA (Endoderme Viscéral Antérieur) chez la souris et l'homme, hypoblaste chez le poulet. Il a une origine extra-embryonnaire et est indispensable à la spécification du neurectoderme antérieur. L'axe antéro-postérieur est ainsi défini dans l'embryon (*Varlet et al., 1997a*). Il est intéressant de noter que les embryons de souris hétérozygotes pour les mutations *Nodal* et son effecteur *Smad2* présentent des défauts dans la région antérieure qui s'apparentent à une cyclopie (*Nomura & Li, 1998*).

1) Gastrulation

Au début de la gastrulation, l'embryon de souris possède donc un axe antéro-postérieur (ou rostro-caudal). Sous l'influence de la voie de signalisation NODAL, les cellules de l'épiblaste migrent vers le pôle postérieur (*Rossant & Tam, 2004*). L'épiblaste va s'épaissir, d'abord dans la partie caudale puis le long de l'axe médian ou « ligne médiane » pour constituer la ligne primitive (*Figure 1B, 6.75 dpc*). Celle-ci est composée du nœud primitif (ou nœud de Hensen chez le poulet) dans sa partie antérieure et du sillon primitif dans la partie postérieure. Les cellules de l'épiblaste vont pousser peu à peu l'endoderme viscéral pour le remplacer par ce qui sera appelé l'endoderme définitif. Il est à l'origine des systèmes digestifs et pulmonaires. Une partie des cellules de l'épiblaste va s'infiltrer via le nœud primitif entre l'épiblaste et l'endoderme pour migrer selon l'axe antéro-postérieur. Elles se détachent progressivement et forment la plaque préchordale et la notochorde (*Figure 2A,B ; Sulik et al., 1994*). En position postérieure, ces cellules subissent une transition épithélio-mésenchymateuse et forment un nouveau feuillet embryonnaire, le mésoderme, à l'origine des os, des muscles squelettiques et du cœur. Enfin, les cellules de l'épiblaste restées en surface vont se différencier en ectoderme, à l'origine des futurs tissus neuraux et épidermiques. L'embryon est donc, à ce stade, constitué de trois feuillets : l'ectoderme, le mésoderme et l'endoderme.

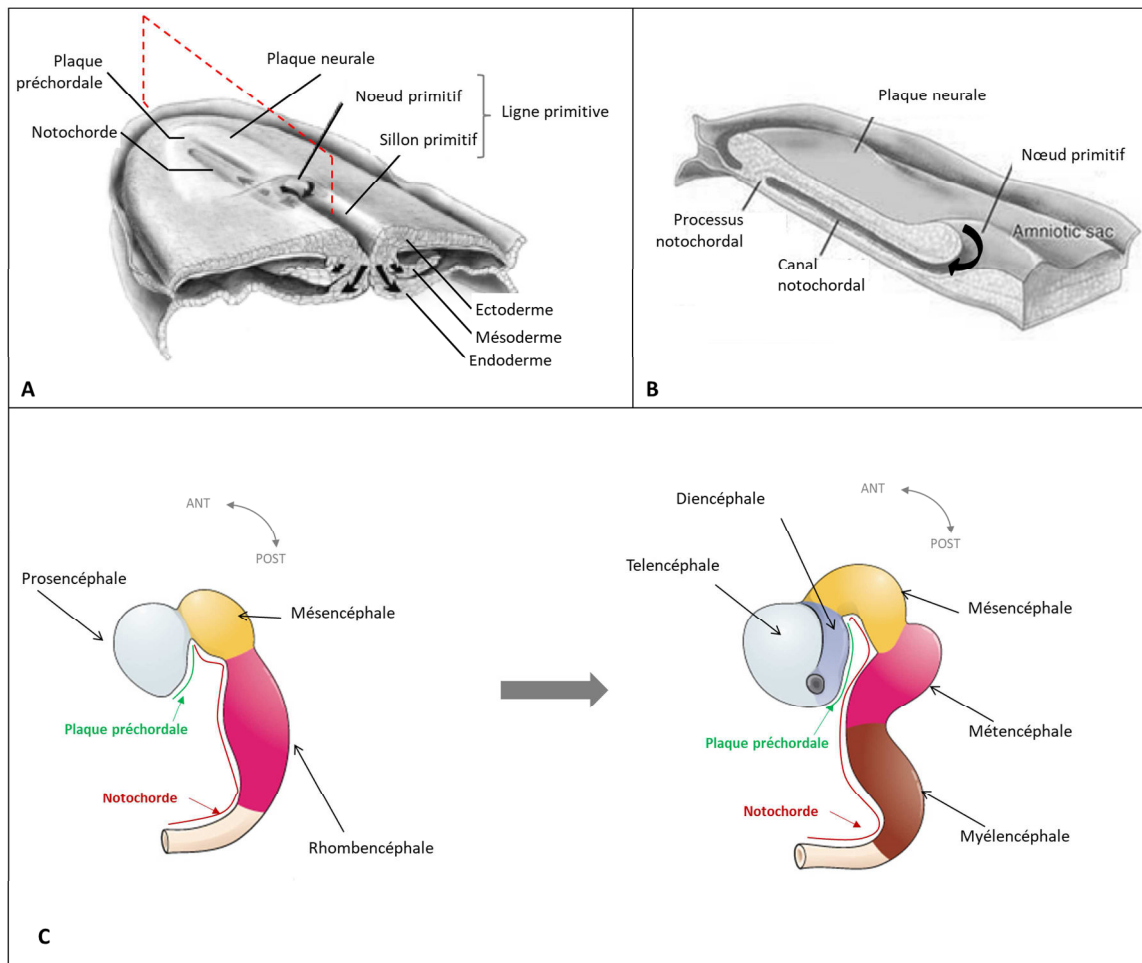


Figure 2 : Ligne primitive et formation de la notochorde chez l'homme. (A) Lors de la gastrulation, les cellules de l'épiblaste migrent via le nœud primitif (flèches noires) pour former la notochorde et la plaque préchordale. Sous l'induction de la notochorde sous-jacente, l'ectoderme s'épaissit et forme la plaque neurale. Les flèches au premier plan représentent les mouvements d'invagination des cellules de l'épiblaste primitif pour former les trois feuilletts embryonnaires. (B) Section de (A) selon le rectangle pointillé rouge. (selon *Dias & Partington, 2004*). (C) Positionnement de la notochorde et de la plaque préchordale lors de la segmentation du tube neural, au stade 3 puis 5 vésicules (cf *infra*, paragraphe I-A/2)d.). Ces deux structures sécrètent différentes molécules, et notamment SHH, qui vont influencer le développement du tube neural ventral. ANT : antérieur ; POST : postérieur.

a. Mécanismes moléculaires et formation de la ligne primitive

La voie de signalisation NODAL est indispensable à la mise en place de l'EVA chez la souris (*Brennan et al., 2001*). Une absence de signalisation NODAL induit un défaut de formation de ligne médiane et une absence de gastrulation (*Conlon et al., 1994*). *Nodal* est exprimé dans la partie caudale de l'ectoderme primitif où il permet l'induction de la ligne primitive (*Shen, 2007*). Puis les antagonistes de cette voie (LEFTY et CERBERUS) sont produits dans la partie rostrale de la ligne primitive pour inhiber la voie NODAL et induire une identité antérieure. *TGIF1* et *TGIF2* sont également nécessaires au processus de gastrulation en inhibant l'activité NODAL (*Power et al., 2010*). Il a été montré qu'une activité NODAL excessive au niveau distal perturbait la mise en place de l'axe antéro-postérieur et

entraînait l'apparition de multiples lignes primitives (*Perea Gomez et al., 2002 ; Bertocchini et al., 2004; Bertocchini and Stern, 2002*).

b. La plaque préchordale et la notochorde :

La plaque préchordale et la notochorde sont des centres organisateurs indispensables au bon développement de la ligne médiane. Ces deux structures sont transitoires et vont permettre la régionalisation du tube neural (*Figure 2C*). L'ablation de la plaque préchordale chez l'embryon de poulet induit l'absence de tissus du cerveau antérieur ventral et la fusion des vésicules optiques (*Pera & Kessel, 1997*).

La notochorde et la plaque préchordale expriment toutes les deux la protéine Sonic Hedgehog (SHH), qui va maintenir viable les cellules de la chorde et leur permettre d'acquérir leur identité (*Aoto et al., 2009*). SHH va également diffuser et induire la plaque neurale ventrale. Les rats ayant subi une ablation de la plaque préchordale et les souris *Shh*^{-/-} présentent un phénotype similaire : défaut de ligne médiane et fusion des vésicules optiques (*Aoto et al., 2009*). C'est donc la molécule SHH, sécrétée par la plaque préchordale, qui est responsable de l'induction de la région ventrale du cerveau antérieur. L'expression de *Shh* au niveau de la plaque préchordale est dépendante de la voie de signalisation NODAL (*Muller et al., 2000 ; Rohr et al., 2001 ; Lowe et al., 2001*). L'activité NODAL dans la plaque préchordale intervient, chez le poisson zèbre, la souris et le poulet, via la voie de signalisation canonique, mettant en jeu les protéines TDGF1, SMAD et FOXH1 (*Chu et al., 2005 ; Vincent et al., 2003 ; Yamamoto et al., 2001 ; Ellis et al., 2015*). Cependant, il a été montré chez le poulet que la régulation de SHH par la voie NODAL se faisait par une voie non-canonique faisant intervenir le précurseur de NODAL, PRO-NODAL, et le récepteur FGFR3 (*Ellis et al., 2015*).

2) Neurulation

Lors de la neurulation, sous l'induction de la notochorde, l'ectoderme s'épaissit et forme la plaque neurale le long de la ligne médiane. Les bords de cette plaque s'épaissent peu à peu et se surélèvent jusqu'à former les bourrelets neuraux qui délimitent la gouttière neurale (Figure 3), puis le tube neural. La partie caudale du tube neural constituera la moelle épinière.

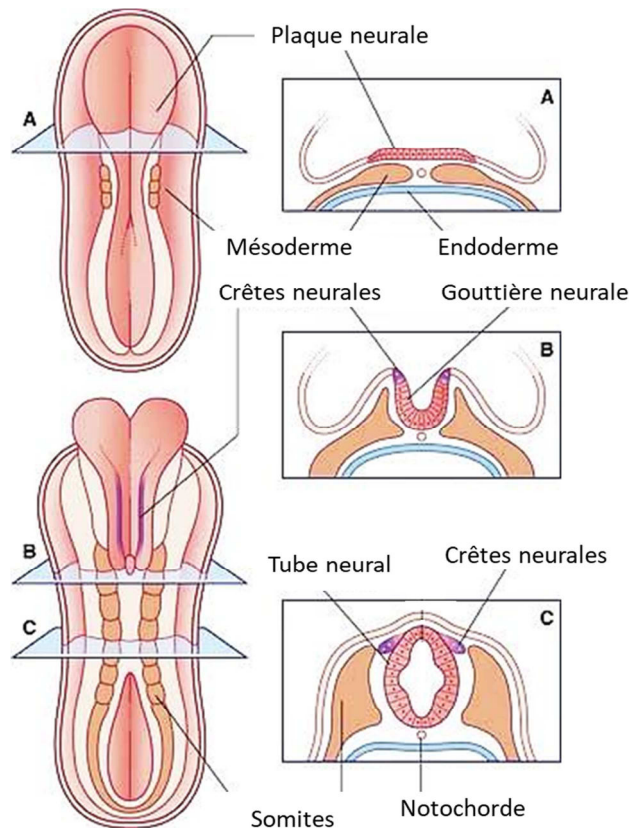


Figure 3 : Formation de la gouttière puis du tube neural. Sous l'induction de la notochorde, l'ectoderme s'épaissit le long de la ligne médiane et forme la gouttière neurale, dont les bords se rejoignent pour former le tube neural, d'abord au milieu du tube neural puis vers les régions antérieures et postérieures (selon <https://neupsykey.com/embryology/>).

a. Régionalisation antéro-postérieure du tube neural :

Différentes voies de signalisation interviennent dans l'acquisition de l'identité neurale et l'induction des tissus antérieurs. Les facteurs caudalisants sécrétés depuis la notochorde dans la partie postérieure de l'embryon sont les facteurs des voies FGF, WNT et de l'acide rétinoïque (ainsi que NODAL chez le Xénope et le poisson-zèbre), tandis que des antagonistes de WNT, FGF et NODAL inhibent leur action dans la partie antérieure de l'embryon (Saint-Jeannet et al., 1997 ; Tuazon and Mullins, 2015 ; Stern et al., 2005). Le tissu neural acquiert une identité antérieure sauf s'il est soumis

à ces facteurs caudalisants (Wilson & Houart, 2004). La future plaque neurale antérieure se protège de ces facteurs d'une part de façon physique, grâce à la migration cellulaire qui éloigne le tissu neural de la ligne primitive, et d'autre part en étant soumis à des molécules inhibant ces mêmes facteurs. Il se crée donc des gradients de concentration antéro-postérieurs notamment pour la voie WNT (Nordström et al., 2002). L'inhibition de *Wnt1* par SIX3 permet au neurectoderme d'acquérir une identité antérieure et ainsi donner le prosencéphale (Lagutin et al., 2003).

b. Régionalisation dorso-ventrale

La régionalisation dorso-ventrale dépend principalement de 3 signaux : SHH, BMP et WNT. SHH est sécrétée par la corde et diffuse vers la région ventrale du tube neural (le plancher neural) pour induire sa propre expression. Au niveau de la région antérieure, d'autres facteurs s'additionnent à l'action de SHH, tels que FGF8 qui active la synthèse de ZIC2, indispensable à la ventralisation du cerveau au niveau de la ligne médiane (Fernandes & Hebert, 2008 ; Hayhurst et al., 2008). En effet, chez la souris, la perte de fonction de *Zic2* entraîne un défaut de régionalisation ventrale et de sévères malformations du cerveau antérieur (Warr et al., 2008). De même, une déficience de *Fgf8* entraîne une hypoplasie des structures de la ligne médiane (Okada et al., 2008).

SHH induit également dans la plaque neurale la synthèse de facteurs spécifiques des populations neurales ventrales. Ainsi, à E9.5 chez la souris, SHH induit l'expression de *Nkx2.1* dans la partie la plus ventrale du prosencéphale. *Nkx2.1* est d'ailleurs considéré comme étant le premier marqueur spécifique de l'hypothalamus. Le gradient SHH serait également à l'origine de l'expression des facteurs de transcription *Gsx2* et *Nkx6.2*, spécifiques des populations neurales du cerveau ventral (Sousa & Fichell, 2010).

De manière opposée, au niveau dorsal, les voies WNT et BMP vont permettre l'activation de gènes dorsalisants comme *Bmp4* (Hu et al., 2004 ; Bertrand and Dahmane, 2006). Ces voies permettent l'établissement d'une identité dorsale opposée au gradient SHH (Figure 4).

Enfin, une autre voie de signalisation intervient dans la régulation de SHH et dans la régionalisation dorso-ventrale du tube neural, celle de l'acide rétinoïque.

L'acide rétinoïque est produit par les enzymes RALDH (retinaldéhyde déshydrogénases), et plus particulièrement RALDH2 au niveau du développement cérébral précoce. Chez les souris mutantes *Raldh2*^{-/-}, on observe une perturbation des voies de signalisation FGF et SHH ainsi qu'une diminution

des domaines d'expression de *Nkx2.1* et de *Gli3* (Ribes et al., 2005). En découle un défaut de morphogénèse du cerveau antérieur.

La régionalisation dorso-ventrale du cerveau antérieur implique donc des interactions complexes entre plusieurs voies de signalisation. C'est un défaut de régulation de ces voies conduisant à une diminution de l'activité SHH qui va entraîner une holoprosencéphalie.

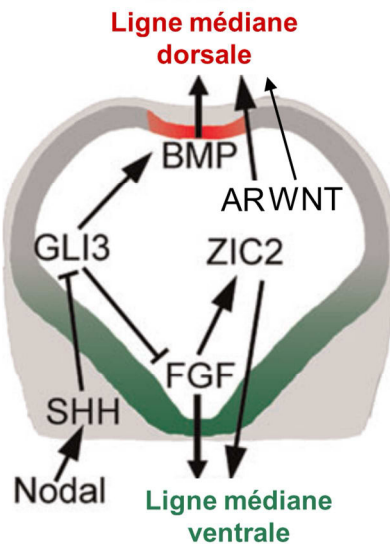


Figure 4 : Les voies de signalisation impliquées dans la régionalisation du tube neural. Lors de la neurulation, les voies SHH, NODAL et FGF induisent la ventralisation du tube neural, alors que BMP, WNT et l'acide rétinoïque (AR) agissent comme des facteurs dorsalisants (selon *Fernandes & Hébert, 2008*).

c. Mécanismes moléculaires mis en jeu dans la régionalisation du tube neural

i. La voie Sonic Hedgehog :

1. Synthèse, maturation et sécrétion de SHH

La voie de signalisation SHH est mise en jeu entre des cellules sécrétrices et des cellules réceptrices. La protéine SHH est synthétisée dans les cellules sécrétrices sous forme de précurseur qui subit plusieurs modifications post-transcriptionnelles : tout d'abord un clivage auto-protéolytique et l'ajout d'un groupement cholestérol à la partie N-terminale (N-SHH) (*Mann & Beachy, 2004*). Le domaine C-terminal (C-SHH) est adressé au protéasome puis dégradé. Puis un groupement palmitate est ajouté au peptide N-SHH-Cholestérol du côté N-ter, ajout catalysé par l'enzyme SKI (skinny hedgehog). Après maturation et translocation à la membrane, N-SHH peut être sécrétée par la cellule via l'un des mécanismes suivants (*Figure 5*):

- Sous forme monomérique, grâce à la protéine transmembranaire DISP1 et à la protéine excrétée SCUBE2 qui fixent toutes les deux le groupement cholestérol et permettent l'externalisation de SHH (*Tukachinsky et al., 2012*) ;

- Sous la forme d'un multimère soluble qui peut agir à distance de la cellule sécrétrice (*Zeng et al., 2001*)

- Sous forme de lipoprotéines. SHH interagit avec les chaînes héparane-sulfate des glypicanes membranaires, qui eux même recrutent des lipoprotéines circulantes qu'ils mettent en contact avec SHH via leurs groupements glycosaminoglycanes. Après intégration de SHH dans ces vésicules, les glypicanes sont clivés au niveau du GPI (GlycosylPhosphatidyInositol) et la lipoprotéine est libérée (*Panakova et al., 2005 ; Eugster et al., 2007*).

- Par exocytose. Des particules entourées de membranes plasmiques (donc assimilables à des exosomes) et contenant des molécules SHH et des molécules d'acides rétinoïque ont été identifiées au niveau des structures ventrales chez la souris, dont la sécrétion est induite par la voie FGF (*Tanaka et al., 2005*)

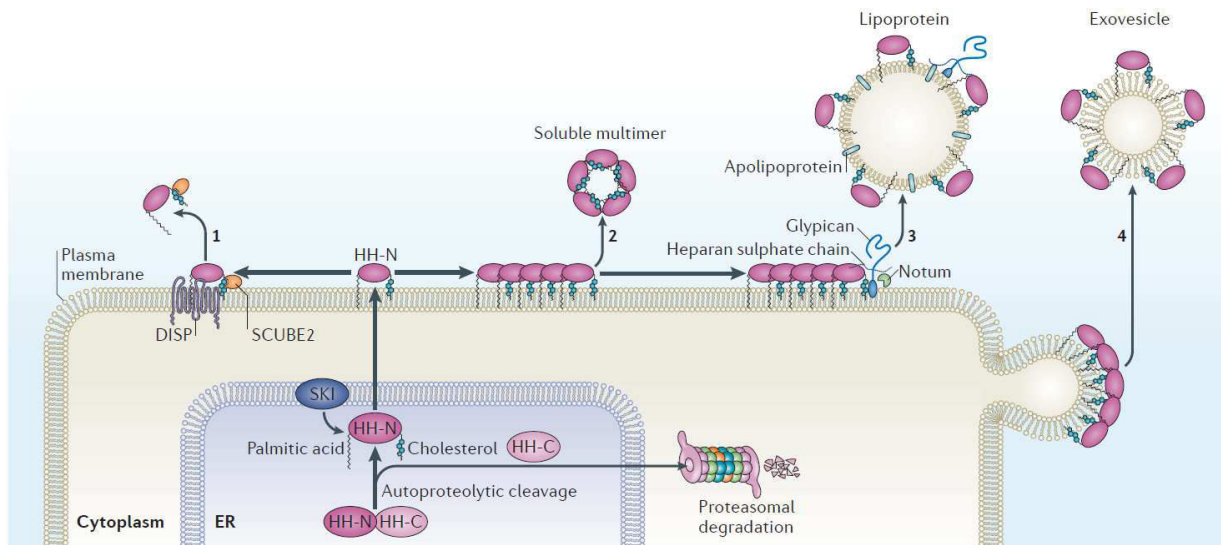


Figure 5 : Maturation et différents processus de sécrétion de SHH. Les processus sont représentés par des numéros : 1) sécrétion sous forme monomérique ; 2) sécrétion sous forme de multimère soluble ; 3) sécrétion sous forme de lipoprotéine ; 4) exocytose. ER : réticulum endoplasmique (Briscoe & Théron, 2013).

2. Réception du signal

SHH se fixe au niveau du récepteur PTCH1 sur la cellule réceptrice (Figure 6). En l'absence de SHH, PTCH1 est accumulé à la base du cil primaire, un organe qui se situe à la membrane cellulaire et dont la structure est maintenue par des microtubules. PTCH1 inhibe alors l'activité de SMO, la protéine permettant la transduction du signal SHH (Denef et al., 2000). Lors de la fixation de SHH à PTCH1 et à ses corécepteurs (CDON, BOC et GAS1), l'inhibition de SMO est levée et celui-ci migre au niveau de la membrane du cil primaire. Ceci libère le facteur de transcription GLI du complexe SUFU/KIF7/GLI et permet leur translocation nucléaire. Les effecteurs GLI peuvent ainsi activer les gènes cibles de la voie SHH.

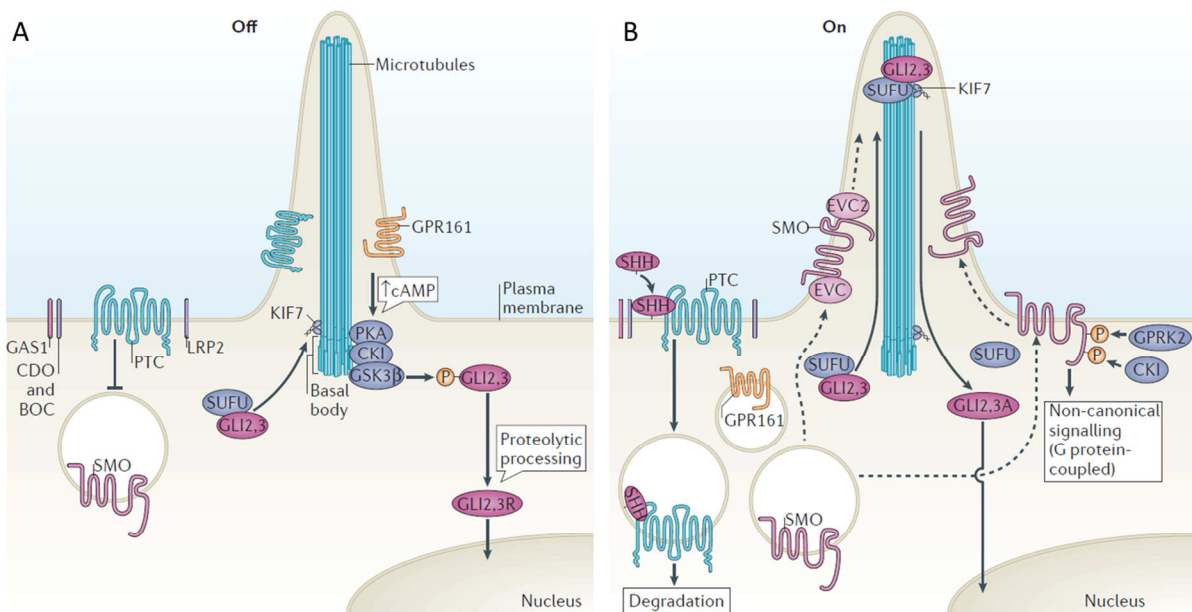


Figure 6 : Réception de SHH et transmission du signal. (A) En l'absence de SHH (situation « Off »), PTCH1 s'accumule à la base du cil primaire et inhibe SMO, l'empêchant d'activer les GLI. (B) La liaison de SHH à son récepteur PTCH1 (situation « On ») entraîne la libération de SMO, la migration de celui-ci le long du cil primaire et la relocalisation des GLI. (Briscoe & Thérond, 2013).

ii. La voie NODAL :

Au cours de la gastrulation la voie NODAL met en jeu des ligands de la superfamille des TGF-Beta (NODAL chez l'homme, la souris et le poulet, Cyclops chez le poisson zèbre), qui se lie à des récepteurs transmembranaires à activité sérine/thréonine kinase via le corécepteur TDGF1 (Cripto chez le poisson zèbre (*Gritsman et al., 1999 ; Chu et al., 2005*)). Cette liaison induit une transduction du signal via SMAD2/3 et SMAD4 qui forment des complexes notamment avec FOXH1 et activent la transcription des gènes cibles (*Figure 7 ; Chu et al., 2004 ; Dunn et al., 2004*).

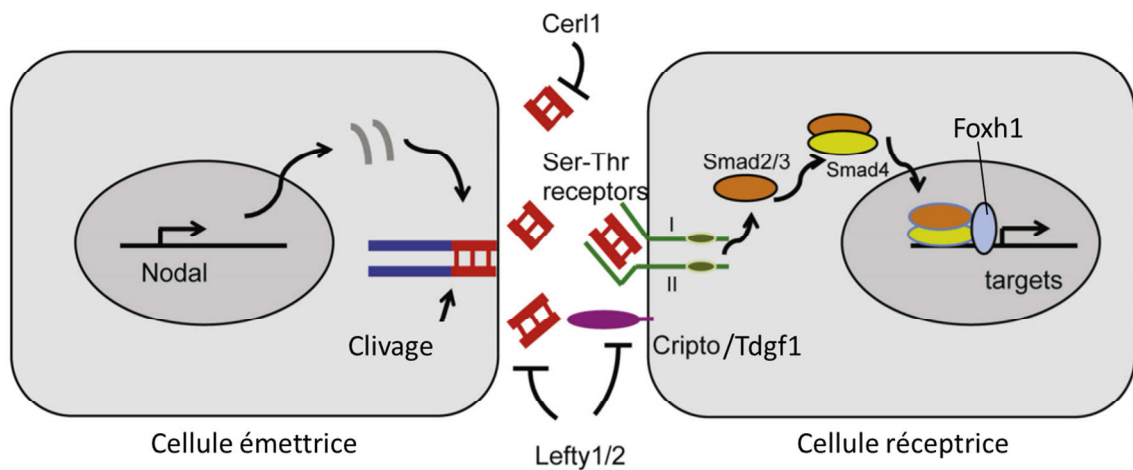


Figure 7 : Cascade d'activation de la voie NODAL. (Adapté de *Robertson, 2014*)

iii. La voie des BMPs :

Les BMPs sont également des ligands de la super-famille des TGF β . De façon similaire à la voie NODAL, les BMPs se lient à des récepteurs de type I et II à activité sérine/thréonine kinase (*Figure 8*). S'en suit une phosphorylation des récepteurs de type I par les récepteurs de type II. La transduction du signal est ensuite assurée par les facteurs SMAD1/5/8 qui se complexent avec SMAD4 avant de pouvoir être transloqués dans le noyau pour l'activation des gènes cibles. (*Liu & Niswander, 2005*). Le cofacteur SMAD4 et les régulateurs SMAD6/7 étant communs aux voies NODAL et BMP, une compétition peut avoir lieu entre les deux voies et intervenir dans leur régulation (*Katsu et al., 2013*).

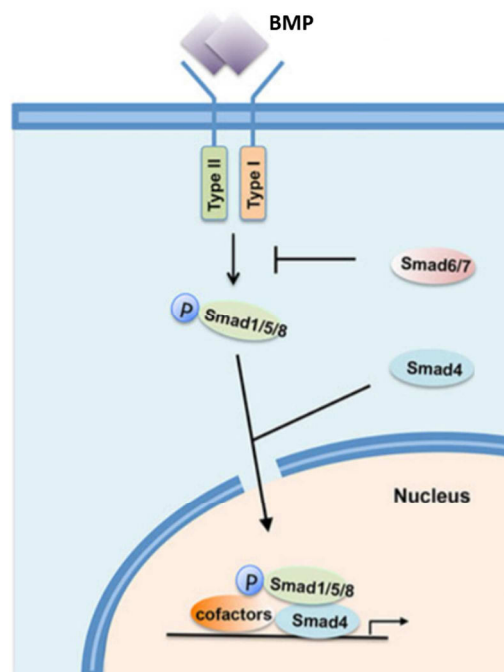


Figure 8 : Cascade d'activation de la voie des BMPs. (Adapté de *Liu et al., 2018*)

iv. La voie FGF :

La voie des FGF est impliquée dans le maintien du signal SHH dans la plaque préchordale (Ellis et al., 2015) et dans le développement de la neurohypophyse.

L'activation de la voie FGF dépend de la liaison des ligands FGF (1-18) à leurs récepteurs FGFR (1-4), qui activent la cascade moléculaire via un domaine cytoplasmique tyrosine kinase (Figure 9 ; Dorey & Amaya, 2010). La transduction du signal peut s'effectuer via 4 processus : la cascade RAS–RAF–MAPK, la cascade PI3K–AKT, les protéines STAT (signal transducers and activators of transcription) ou les protéines PLC γ (phospholipase Cy). Les gènes cibles de la voie sont principalement des facteurs de croissance impliqués dans les processus de survie, prolifération ou encore différenciation cellulaire. La spécificité de la réponse induite dépend de la combinaison FGF-FGFR.

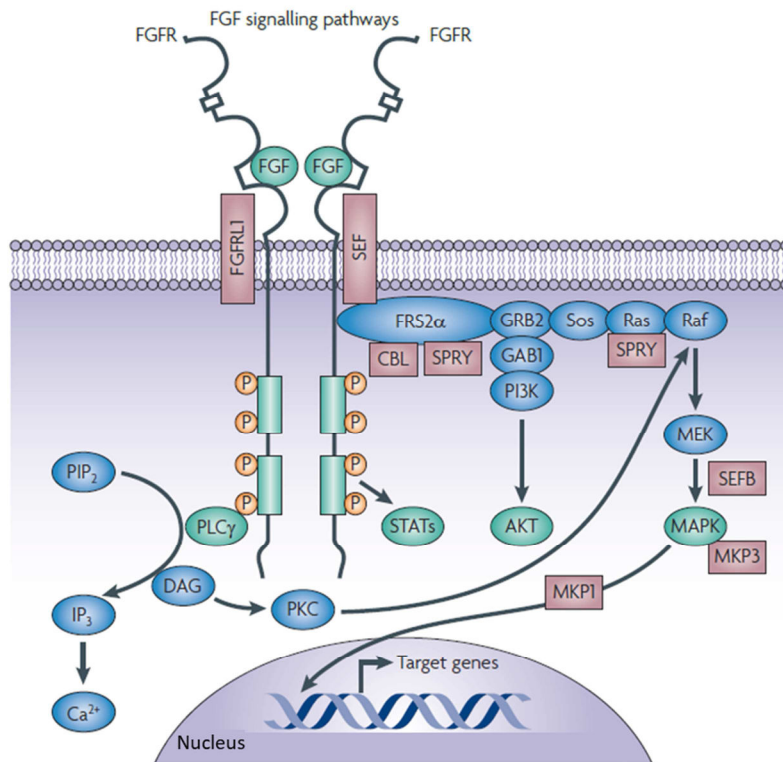


Figure 9 : Voie FGF et transduction du signal. La liaison des ligands FGF aux récepteurs FGFR induit l'auto-phosphorylation de leurs domaines tyrosine-kinases. Ceci entraîne l'activation de voies RAS–RAF–MAPK, PI3K–AKT, STAT et PLC γ . (Turner & Grose, 2010).

d. Segmentation du tube neural :

Simultanément aux processus de régionalisation, le tube neural subit des transformations morphologiques : il se segmente le long de l'axe antéro-postérieur. La partie antérieure du tube neural va se diviser en trois vésicules cérébrales primitives : le prosencéphale (ou cerveau antérieur), le mésencéphale (ou cerveau moyen) et le rhombencéphale (ou cerveau postérieur) (Figure 10).

Le Prosencéphale se divise par la suite en 2 entités :

- o Prosencéphale secondaire puis télencéphale, qui donnera par la suite les hémisphères cérébraux, les vésicules optiques, l'hypothalamus et la neurohypophyse
- o et diencéphale, d'où découleront le thalamus et l'épiphyse

Le Mésencéphale sera impliqué principalement dans les fonctions motrices

Le Rhombencéphale sera à l'origine du cervelet, du pons et du bulbe rachidien

Nous nous concentrerons ici sur le développement du cerveau antérieur (prosenéphale), et plus particulièrement sur la région ventrale à l'origine de l'hypothalamus.

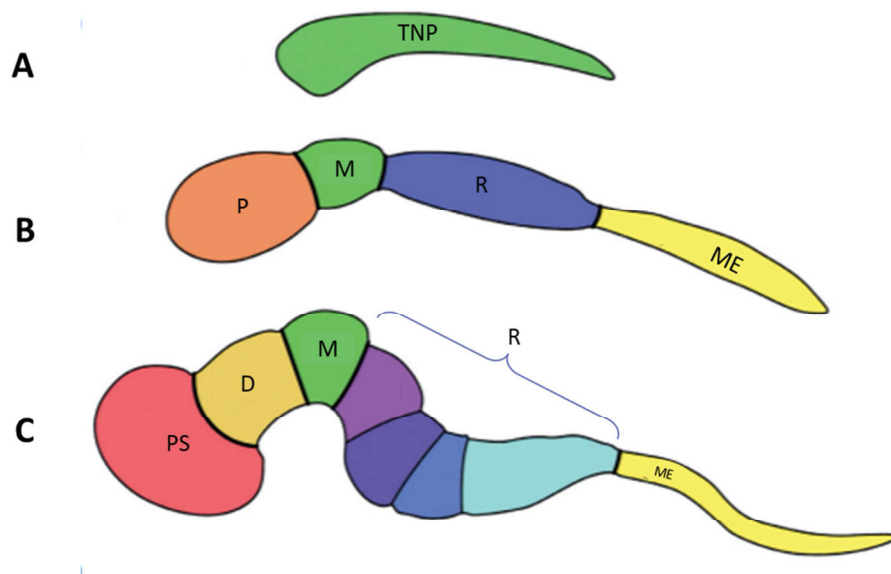


Figure 10 : Segmentation du tube neural. (A) le tube neural primaire (TNP) ne comporte pas de subdivisions. (B) Il se divise ensuite en 3 vésicules primaires : Prosencéphale (P), Mésencéphale (M) et Rhombencéphale (R). (C) Le Prosencéphale se divise en Prosencéphale secondaire (PS, qui donnera par la suite le télencéphale) et en diencéphale (D). Le mésencéphale reste à ce stade inchangé. Le rhombencéphale commence à se segmenter en sous-régions qui se diviseront ensuite en rhombomères. ME : Moelle Epinière. (adapté de *Puelles et al., 2013*).

B/ L'hypothalamus :

L'hypothalamus est une structure très conservée chez les vertébrés, qui contrôle de nombreuses fonctions physiologiques (rythme circadien, appétit, soif...). En étroite connexion avec une glande appelée l'hypophyse, ils constituent ensemble l'axe hypothalamo-hypophysaire, régulateur des fonctions endocriniennes de l'organisme.

1) Développement de l'hypothalamus

Plusieurs théories s'affrontent dans la littérature pour expliquer le développement précoce de l'hypothalamus (*Figure 11*) :

- La théorie en colonnes : la plus ancienne, elle se base sur l'observation anatomique des structures. Dans cette théorie émise par Herrick (1910) et soutenue par Swanson (2012), le diencephale est divisé en 4 « colonnes » ou régions. La partie la plus antérieure du tube neural se situe au milieu du télencéphale et l'axe antéro-postérieur divise le tube neural de façon quasi-arbitraire, constituant la limite pour définir les parties ventrales et dorsales du tube neural.

Cette théorie définit l'hypothalamus comme étant la partie la plus ventrale du diencephale, limité dans sa partie antérieure par le télencéphale et dans sa partie postérieure par le mésencéphale (Alvarez-Bolado and Swanson, 1996). Il est situé, selon l'axe antéro-postérieur, « sous » le thalamus (d'où son nom). L'hypothalamus trouve son origine dans une population cellulaire du plancher basal antérieur nommée RDVM (Rostral Diencephalic Ventral Midline, cellules de la ligne médiane rostrale du diencephale). Cependant, cette théorie peine à concilier les définitions anatomiques des structures cérébrales et les domaines d'expression des différents gènes (décrits depuis) mis en jeu dans leur développement.

- La théorie prosomérique (révisée), selon laquelle les différentes structures du tube neural sont divisées en prosomères (prosomères télencéphaliques, prosomères diencephaliques, mésomères, rhombomères) est défendue principalement par Puelles (*Puelles et al., 2012 ; Puelles et al., 2013 ; Puelles, 2019 ; Puelles and Rubenstein, 2015 ; Nieuwenhuys and Puelles, 2016*).

Dans cette conception, les axes d'observation sont partiellement modifiés et l'extrémité rostrale du tube neural se retrouve sous l'aire préoptique (*Figure 11*). Elle définit une région basale et une région alaire, de part et d'autre de l'axe rostro-caudal. Cette théorie permet de positionner la notochorde et la plaque préchordale tout le long de la région ventrale du tube neural. L'hypothalamus est situé ici dans le prosencéphale secondaire, au niveau des parties alaire et basale.

Cette définition plus moderne de l'architecture cérébrale se base notamment sur les domaines d'expression génique et suggère que les cellules hypothalamiques antérieures découlent de

progéniteurs de la région alaire, et que les cellules tubéro-mamillaire découlent de progéniteurs de la région basale. Ceci suppose donc que les cellules hypothalamiques ne proviennent donc pas toutes des mêmes progéniteurs.

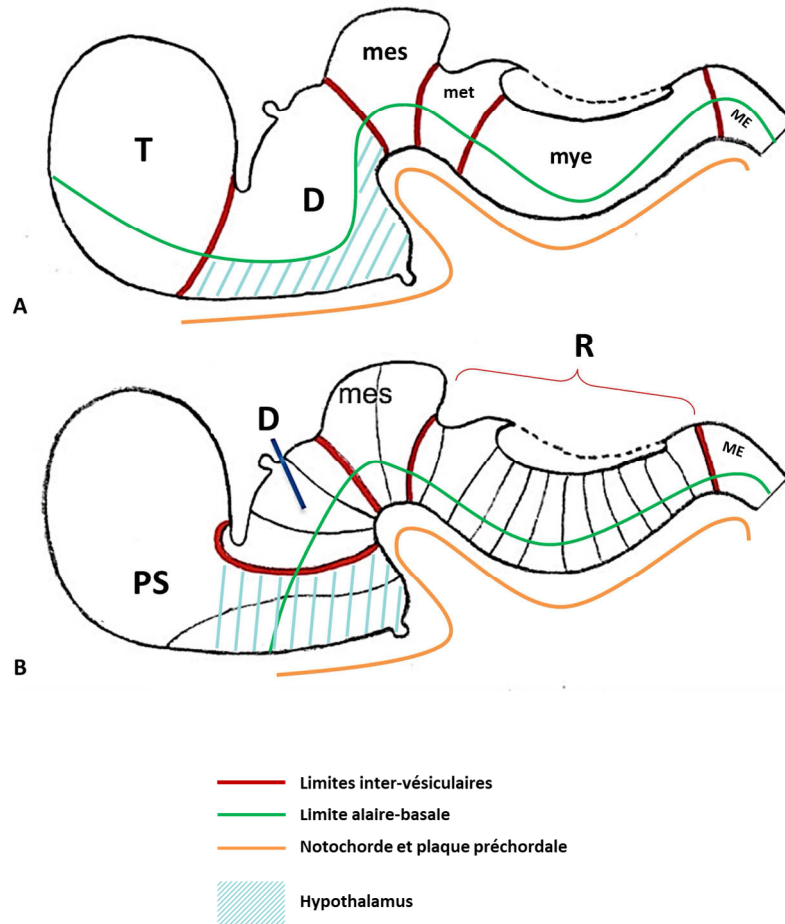


Figure 11 : Segmentation du tube neural : différences entre la théorie en colonnes (A) et prosomérique (B). Ces deux théories décrivent différemment la segmentation du tube neural, notamment dans la partie antérieure. Selon la théorie en colonne, l'hypothalamus constitue la partie ventrale du diencéphale (sous la limite alaire-basale). Selon la théorie prosomérique, l'hypothalamus fait partie du prosencéphale secondaire, une partie étant dans la partie basale et l'autre dans la partie alaire. T : télencéphale ; D : diencéphale ; mes : mésencéphale ; met : métencéphale ; mye : myélocéphale ; ME : moelle épinière ; PS : prosencéphale secondaire ; R : rhombencéphale. (Adapté de *Nieuwenhuys & Puelles, 2016*).

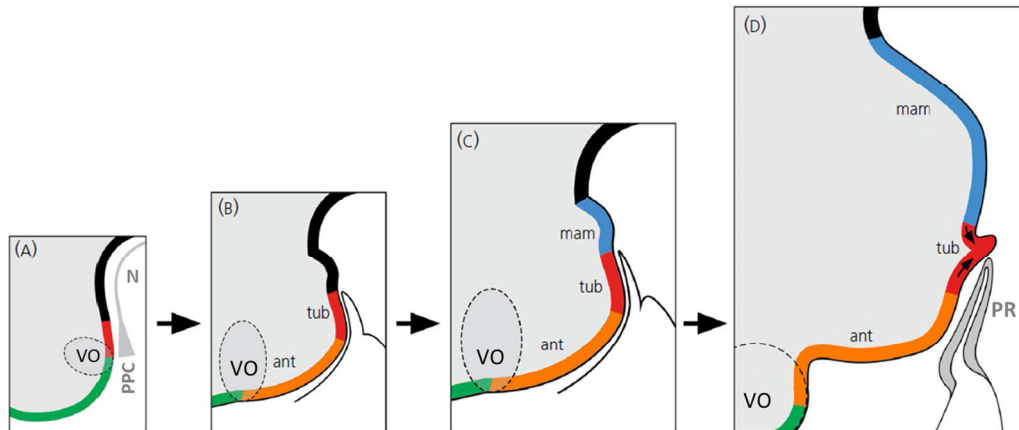


Figure 12 : Développement de l'hypothalamus selon la théorie anisotrope. (A) Les premières cellules de l'hypothalamus, les cellules basales hypothalamiques (bHyp, en rouge) sont adjacentes au télencéphale (vert). Elles permettent d'abord l'émergence des cellules de l'hypothalamus antérieur (B, orange) puis des cellules de l'hypothalamus mamillaire (C, bleu). Les cellules bHyp se différencient en hypothalamus tubulaire (rouge) et s'évagine en infundibulum, qui va au contact de la poche de Rathke (D). VO : vésicule optique ; PPC : plaque préchordale ; N : notochorde ; ant : antérieur ; tub : tubulaire ; mam : mamillaire ; PR : poche de Rathke. (Fu et al., 2019)

- La théorie anisotrope, qui a émergé très récemment, est défendue par l'équipe de Marysia Placzek (Fu et al., 2019). Elle reprend les deux premières théories, en y ajoutant le concept d'anisotropie, c'est-à-dire de l'influence de la direction sur le développement des cellules de l'hypothalamus. Dans cette théorie, on retrouve la notion de RDVM, donc de cellules progénitrices au niveau du diencéphale ventral qui donnent naissance à un pool de cellules basales hypothalamiques (bHyp). Une partie de ces cellules bHyp va migrer en position rostrale et se différencier pour donner l'hypothalamus antérieur (Figure 12). Une deuxième partie des progéniteurs bHyp va ensuite migrer en position postérieure pour donner l'hypothalamus mamillaire. Et enfin, les cellules restantes vont se différencier et donner l'hypothalamus tubulaire et, en s'étendant ventralement, donner l'infundibulum (futur lien entre l'hypothalamus et l'hypophyse).

2) Neurogenèse dans l'hypothalamus

Le cerveau est constitué de différentes populations neuronales, définies par la position du corps cellulaire et les projections axonales. Les progéniteurs se différencient en neurones, qui vont ensuite émettre des axones se projetant à des endroits précis en suivant un guidage axonal (Figure 13, Ware & Schubert, 2011). Chez le poulet, les tout premiers neurones apparaissent dans l'hypothalamus au stade HH13, au niveau de la région antérieure. Ce sont les nTPOC (neurones du tractus de la commissure post-optique), ils projettent leurs axones vers la frontière diencephale-mésencéphale. Quelques heures plus tard, au stade HH16, une nouvelle population neuronale est détectée au niveau de l'hypothalamus postérieur, les nMTT (neurones du tractus mamillo-tegmental), dont les axones rejoignent ceux des nTPOC dans le tractus longitudinal ventral et s'étendent jusqu'à la moelle épinière. Ces neurones ont la même organisation chez la souris (Mastick & Easter, 1996 ; Ware et al., 2015).

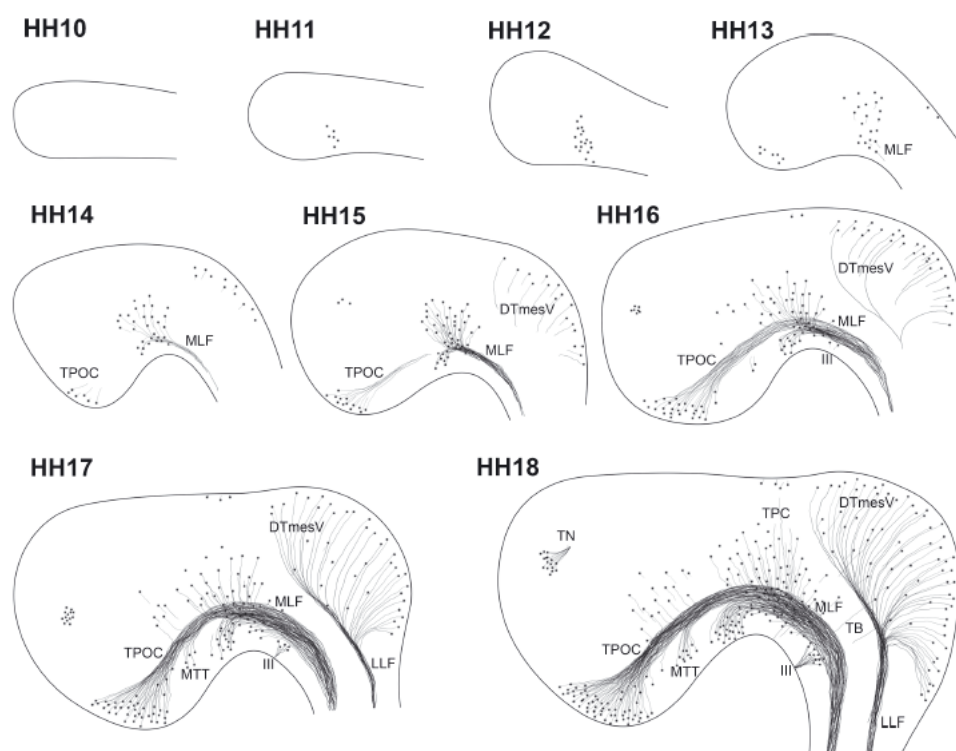


Figure 13 : Développement des neurones et de leurs axones chez le poulet. Les premiers neurones sont induits à HH11 : les nMLF (neurones du faisceau médio-longitudinal). A HH13, les nTPOC (neurones du tractus de la commissure post-optique) apparaissent et les nMLF projettent leurs premiers axones en position caudale. HH14 : Les nTPOC débutent leur projection axonale et les nDTmesV (noyaux du tractus descendant du noyau mésencéphalique du trijumeau) apparaissent au niveau du mésencéphale. Ensuite arrivent les nTN (noyaux des nerfs terminaux) à HH15 puis les noyaux des nerfs oculomoteurs (III), les nMTT (noyaux du tractus mamillo-tegmental, dans l'hypothalamus mamillaire) et les nTPC (noyaux du tractus de la commissure postérieure) à HH16. Les derniers neurones à se développer sont ceux des nTB (noyaux tecto-bulbaires) et des nLLF (noyaux du faisceau latéro-longitudinal). Tous ces neurones projettent leurs axones en position postérieure, et se constitue ainsi notamment le tractus ventro-longitudinal, qui rassemble les axones des nTPOC, nMTT, nMLF et neurones occulo-moteurs. (Ware & Schubert, 2011)

3) Rôle de SHH dans l'hypothalamus en développement

Les cellules de la ligne médiane rostrale précédemment induites par *Shh* acquièrent une identité ventrale grâce aux expressions coordonnées de *Shh* et *Bmp7* (Dale et al., 1997 ; Figure 14). Au niveau de la plaque neurale ventrale, SHH induit sa propre synthèse ainsi que celle de *Nkx2.1*. *Bmp7* induit également l'expression de *Nkx2.1*, qui va lui-même maintenir l'expression de *Shh* au sein de l'hypothalamus. *Nkx2.1* est considéré comme le marqueur ventral de l'hypothalamus en développement (Dale et al., 1997; Manning et al., 2006; Pera and Kessel, 1998). La perte de signalisation SHH entraîne une absence de structures ventrales au niveau du prosencéphale (Chiang et al., 1996 ; Ishibashi & McMahon, 2002).

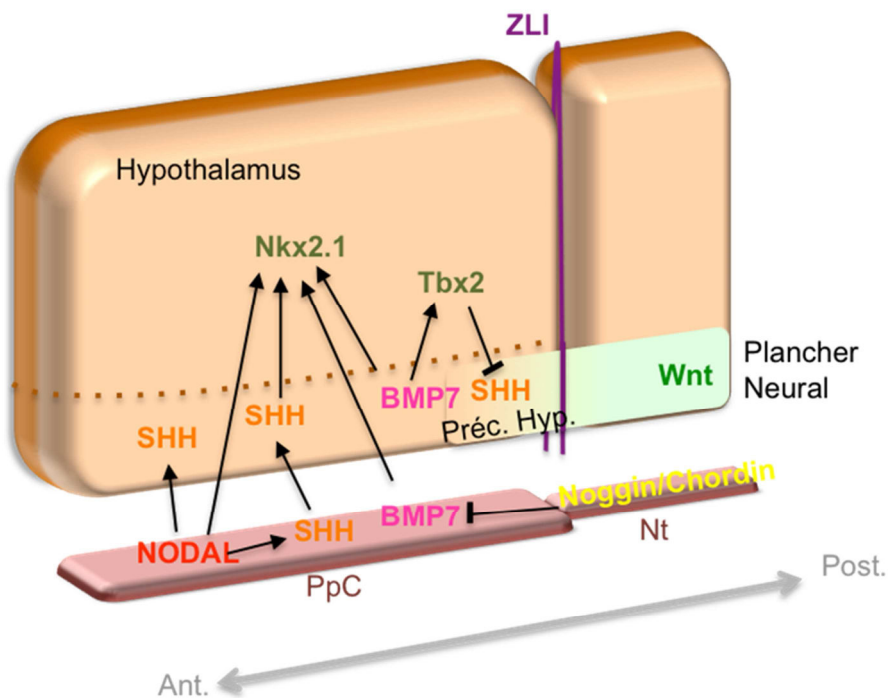


Figure 14 : Mécanismes moléculaires mis en jeu à l'induction de l'hypothalamus. L'action coordonnée de SHH, BMP7 et les antagonistes des BMP (CHORDIN et NOGGIN), est nécessaire à l'induction des précurseurs hypothalamiques (Préc. Hyp.). De même, NODAL, SHH et BMP7 sont essentiels à l'expression de *Nkx2.1* dans le tube neural ventral pour le développement de l'hypothalamus. Un gradient de signalisation antéro-postérieur des Wnt permet d'établir la limite postérieure des précurseurs hypothalamiques. PpC : plaque préchordale ; Nt : Notochorde ; ZLI : *zona limitans intra thalamica*

BMP7 induit sa propre expression dans les cellules de la plaque neurale ventrale via BMP4 et les répresseurs transcriptionnels TBX2/TBX3 (Manning et al., 2006 ; Xie & Dorsky, 2017). BMP7, dans la plaque neurale, va ensuite induire l'expression de *Fgf10*. Se forme alors un pool de cellules exprimant transitoirement *Shh/Bmp/Fgf10* et qui constitue l'hypothalamus présomptif (Figure 15). Dans le modèle anisotrope, une partie de ces cellules migre dans la partie rostrale. Elles sécrètent à la fois *Shh* et *Fgf10*, prolifèrent et se séparent en deux groupe : un groupe antérieur exprimant *Shh* et un groupe postérieur exprimant uniquement *Fgf10*. Dans ce groupe, *Shh* est réprimé par les facteurs de transcription de la famille des TBX (TBX2 chez le poulet, TBX3 chez la souris) (Manning et al., 2006 ; Trowe et al., 2013 ; Fu et al., 2017). Les cellules *Shh*-positives vont constituer l'hypothalamus antérieur. Parallèlement, un pool de cellules *Fgf*-positives migre vers le pôle postérieur et subit l'action du facteur de transcription *Lhx5*. Celui-ci maintient l'inhibition de *Shh* via *Tbx3* et permet aux cellules de se différencier en hypothalamus mamillaire (Heide et al., 2015). Enfin, les cellules *Fgf*-positives restantes constitueront l'hypothalamus tubulaire et l'infundibulum (Figure 12 Figure 15).

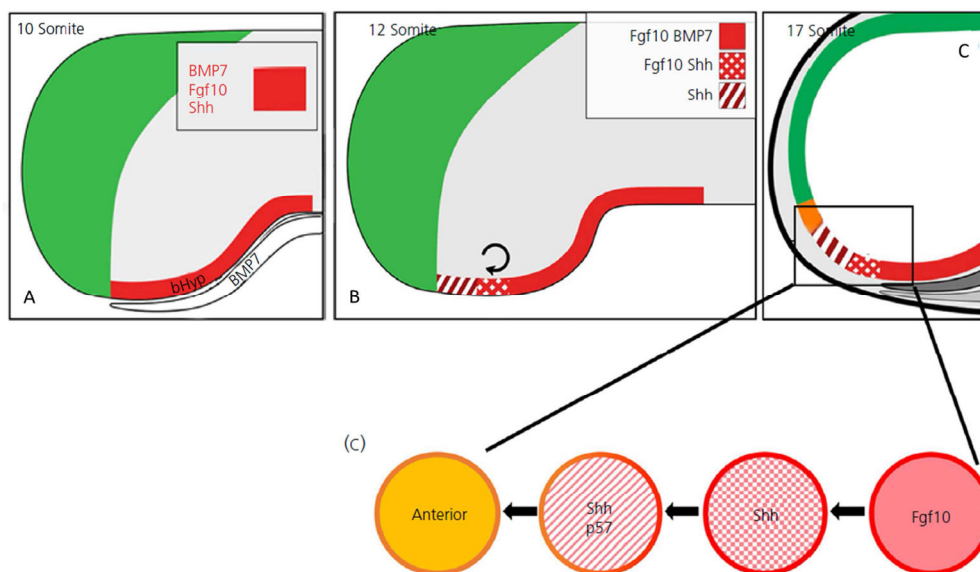


Figure 15 : Prolifération et différenciation des progéniteurs dans l'hypothalamus chez le poulet. (A) Au stade 10 somites et sous l'influence de BMP7 dans la plaque préchordale, les progéniteurs hypothalamiques donnent naissance à des cellules basales (bHyp) exprimant *Bmp7*, *Fgf10* et *Shh*. (B) Au stade 12 somites, un pool de cellules migre en position antérieure pour exprimer *Shh* et *Fgf10*, puis uniquement *Shh*. Le pool *Shh*⁺/*Fgf10*⁺ a une capacité de prolifération (flèche noire). (C) Au stade 17 somites, les cellules en position antérieure expriment *Shh* et sont de plus en plus différenciées (p57=marqueur de différenciation). Les cellules en position postérieure n'expriment que *Fgf10* et *BMP7*, *Shh* étant inhibé via l'action des TBX. (selon Fu et al., 2019)

Bien que *Shh* et *Bmp7* soient exprimés au niveau de toute la notochorde, cela n'entraîne pas pour autant une différenciation des cellules de la ligne médiane ventrale caudale en précurseurs hypothalamiques. Cette différence est due à l'expression d'inhibiteurs de BMP7 (CHORDIN et NOGGIN) par la notochorde, avec une limite antérieure correspondant à la limite postérieure de l'hypothalamus. En effet, en l'absence des antagonistes des BMP au niveau de la notochorde chez le modèle souris, l'activité BMP est augmentée, provoquant une désorganisation du tissu (*Bachiller et al., 2000 ; Anderson et al., 2002*).

Une autre voie intervient dans la délimitation postérieure de l'hypothalamus : la voie WNT. Comme démontré chez le poisson-zèbre, un gradient d'inhibition de la voie WNT semble déterminer le destin cellulaire hypothalamique (*Kapsimali et al., 2004*). En effet, l'utilisation d'un antagoniste de la voie WNT (WIF : Wnt inhibitory factor) est suffisante pour restaurer l'expression de *Tbx2* au niveau d'explants où la signalisation BMP est bloquée. Les auteurs suggèrent que la voie des BMP pourrait inhiber l'activité WNT au niveau de l'hypothalamus. Par ailleurs, la déficience pour les antagonistes de la voie WNT chez la souris provoque des défauts de spécification du cerveau antérieur incluant l'hypothalamus (*Hoch et al., 2009*).

C/ L'hypophyse

L'hypophyse est une glande (appelée également glande pituitaire) qui, sous le contrôle de l'hypothalamus, dirige les fonctions endocrines de l'organisme. Elle est située sous l'hypothalamus et y est reliée par la tige pituitaire ou infundibulum. L'hypophyse est divisée en deux lobes :

- le lobe antérieur ou adénohypophyse a pour origine l'ectoderme oral. Elle sécrète l'hormone de croissance (Growth hormone, GH), la prolactine, les hormones gonadotropes (LH, FSH), l'ACTH qui agit sur les cortico-surrénales, la MSH (melanocytes stimulating hormon) et la TSH qui agit sur la thyroïde. On y retrouve également des cellules progénitrices pour l'expansion et le renouvellement cellulaire post-natal (*Andoniadou et al., 2013 ; Rizzoti et al., 2013*).

- le lobe postérieur ou neurohypophyse provient du tissu hypothalamique (neuroectoderme). Elle permet la sécrétion de l'ocytocine (hormone intervenant lors de l'accouchement et en post-natal) et de la vasopression ou ADH (hormone anti-diurétique, qui régule le volume sanguin).

Lors de la neurulation, la région de l'ectoderme oral qui se trouve juste sous le diencephale ventral, en avant de la plaque préchordale, s'épaissit et donne la placode hypophysaire. Elle s'invagine ensuite pour donner la poche de Rathke, en regard direct des cellules hypothalamiques ventrales (*Figure 16*, pour revues *Rizzoti, 2015 ; Burbridge et al., 2016*). La poche se détache ensuite progressivement, en laissant d'abord un pédicule pharyngo-hypophysaire qui disparaît petit à petit.

Lors de la formation de l'hypothalamus tubulaire, celui-ci s'évagine dans la région qui jouxte la poche de Rathke, pour donner d'abord l'infundibulum puis la neurhypophyse (*Figure 16*, *Pearson et al., 2011 ; Tsai et al., 2011*). L'hypothalamus tubulaire est dépourvu d'activité SHH, par inhibition via *Tbx2/Tbx3*, ce qui permet la mise en place de l'infundibulum (*Trowe et al., 2013*). Il a été montré récemment que l'activité SHH présente dans l'hypothalamus antérieur intervient également dans le développement de l'adénohypophyse. En effet, l'induction de la poche de Rathke nécessite des signaux de l'hypothalamus ventral mitoyen : tout d'abord *Bmp4*, qui initie l'évagination, puis *Fgf8*, qui, via les facteurs de transcription *Lhx3/4*, permet l'établissement de la poche définitive (*Takuma et al., 1998*). Les facteurs de transcription *Lhx3* et *Lhx4* sont donc indispensables à la mise en place des progéniteurs de la poche de Rathke (*Sheng et al., 1996 ; Sheng et al., 1997*). Or, il a été montré que le signal SHH émanant de l'hypothalamus antérieur était nécessaire à l'expression de *Lhx3/Lhx4* dans la poche de Rathke chez la souris et le poulet (*Carreno et al., 2017 ; Fu et al., 2017*).

L'équipe de Carreno a inhibé spécifiquement *Shh* dans l'hypothalamus antérieur grâce à une CRE recombinase exprimée dans les cellules *Hesx1*⁺ (Carreno et al., 2017). Cette inhibition induit une perte d'expression de *Lhx3* et *Lhx4* dans la poche de Rathke ; il en résulte une aplasie de l'hypophyse. Par ailleurs, chez des souris où *Shh* est inhibé spécifiquement dans l'hypothalamus grâce à une CRE recombinase couplée à SBE2 (enhancer de *Shh* dans l'hypothalamus), on constate une anomalie de l'évagination de l'infundibulum ainsi qu'une poche de Rathke dysmorphique (Zhao et al., 2012). Dans ce modèle, l'absence de *Shh* dans l'hypothalamus antérieur entraîne un élargissement du domaine d'expression de *Fgf10* et *Bmp4* qui, habituellement restreints à l'hypothalamus tubulaire, se retrouvent ici exprimés dans l'hypothalamus antérieur. La modification de la limite d'expression entre *Shh* et *Bmp4/Fgf10* semble être à l'origine des malformations de l'hypophyse. Cette équipe a également montré que l'expression de *Shh* dans l'hypothalamus était régulée par SOX2 et SOX3, des facteurs de transcriptions qui agissent sur SBE2. Enfin, ces deux modèles de mutants conditionnels montrent une perte d'expression de *Shh* dans l'hypothalamus entraînant une dysplasie septo-optique, une pathologie regroupant des anomalies optiques (des yeux ventralisés), des anomalies hypophysaires et une microcéphalie, et qui se rapproche de l'HPE.

En conclusion, l'activité SHH est indispensable à l'induction initiale de la région ventrale du prosencéphale. Ensuite, le contrôle de l'activité SHH dans cette région est impliqué dans la spécification des différents domaines de l'hypothalamus (antérieur, tubéral et mammillaire) (Mathieu et al., 2002 ; Dale et al., 1997) et également, à distance, dans la différenciation de l'adénohypophyse.

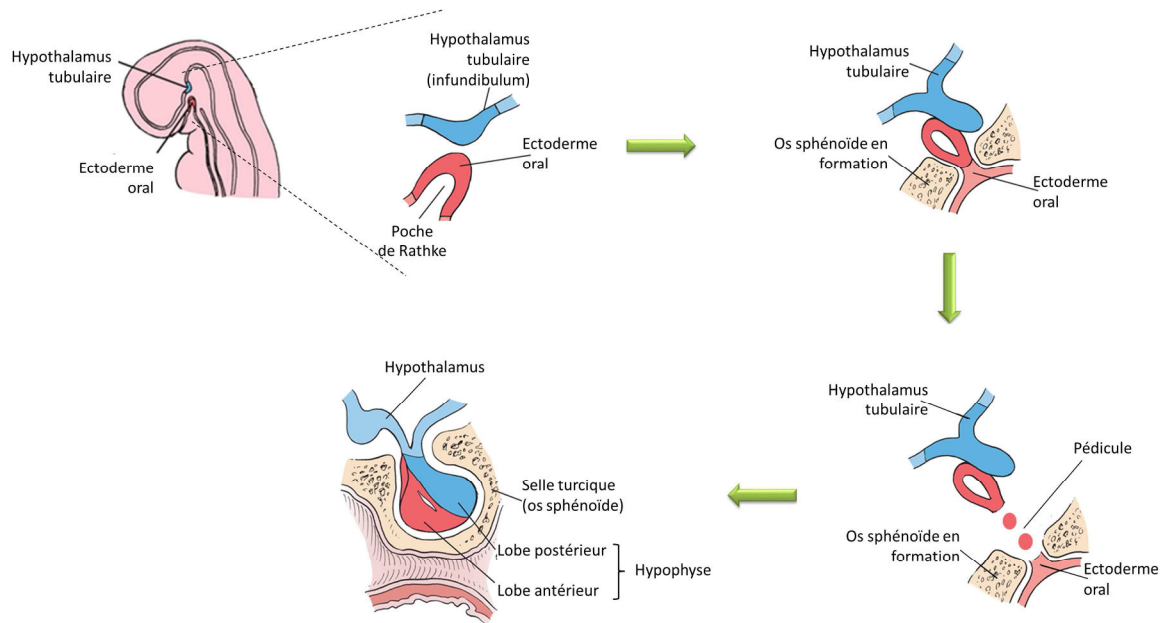


Figure 16 : Formation de l'hypophyse chez les vertébrés. L'hypophyse a une double origine embryonnaire : le neurectoderme (évagination de l'hypothalamus tubulaire, appelé l'infundibulum, qui donnera le lobe postérieur) et l'ectoderme oral qui, en s'invaginant, donne la poche de Rathke à l'origine du lobe antérieur. La poche de Rathke se détache petit à petit de l'ectoderme oral, en laissant d'abord un pédicule qui finit par disparaître et laisser la place à l'os sphénoïde pour se développer et former la selle turcique (chez l'humain), qui accueillera l'hypophyse. (Adapté de *Ross & Pawlina, 2006*).

D/ La voie NOTCH

La signalisation NOTCH est une voie très conservée. Son rôle au cours de la somitogenèse et la neurogenèse a été très étudié (*Hori et al., 2013 ; Wahi et al., 2016*). Des études de génétique chez des patients ont permis d'incriminer la voie NOTCH dans la survenue de l'HPE (*Dupé et al., 2011*). Cependant le rôle de cette voie au cours du développement précoce du cerveau n'est pas décrit.

1) Cascade moléculaire :

L'activité de la voie NOTCH nécessite une interaction physique entre deux cellules voisines, par l'intermédiaire de l'interaction entre un ligand de la famille des DSL (**Delta** et **Serrate** chez la drosophile, **Lag-2** chez *C. Elegans*) et le récepteur transmembranaire NOTCH (*Artavanis-Tsakonas et al., 1995*).

Chez les vertébrés, il existe quatre récepteurs Notch différents (NOTCH-1 à NOTCH-4), tous composés d'une partie intracellulaire (le NICD : *Notch Intra-Cellular Domain*), d'une courte portion transmembranaire et d'un domaine extracellulaire (NECD : *Notch Extra-Cellular Domain*). L'interaction ligand-récepteur entre les protéines DELTA (*Dll1, Dll3, Dll4*) ou JAGGED (1 et 2) et NOTCH entraîne le clivage de la portion intracellulaire par une gamma-sécrétase (*Figure 17*). Le NICD ainsi libéré migre dans le noyau, où il se lie à RBPJ (Recombination signal Binding Protein for immunoglobulin kappa J region). RBPJ est une molécule qui, en absence du NICD, forme un complexe avec des répresseurs transcriptionnels et des histones désacétylées, complexe inhibant les gènes cibles *HES* (hairy enhancer of split) et *HEY* (hairy/enhancer-of-split related with YRPW motif). L'interaction NICD/RBPJ permet donc le désassemblage de ce complexe répresseur et le recrutement de l'activateur MAML (*Mastermind-L*), induisant la transcription des gènes *HES* et *HEY* (*Sasaki et al., 2011*). Chez les mammifères, il existe 7 membres de la famille *HES* (*HES1-7*) et trois membres de la famille *HERP* (*HEY1, HEY2 ET HEYL*). Ce sont principalement des répresseurs transcriptionnels de la famille bHLH (basic helix-loop-helix) et ils sont essentiels à l'activité de la voie NOTCH. Par exemple, en l'absence d'*Hes1* et *Hes5*, l'activité de la voie NOTCH est fortement diminuée dans le système nerveux central d'embryon de souris (*Kageyama and Ohtsuka, 1999*).

Les facteurs *HES* et *HEY* vont ensuite réprimer notamment la transcription des gènes proneuraux (*ASCL1* et *Neurogénines*), ce qui a pour effet de maintenir les cellules progénitrices dans un état indifférencié (*Davis & Turner, 2001*).

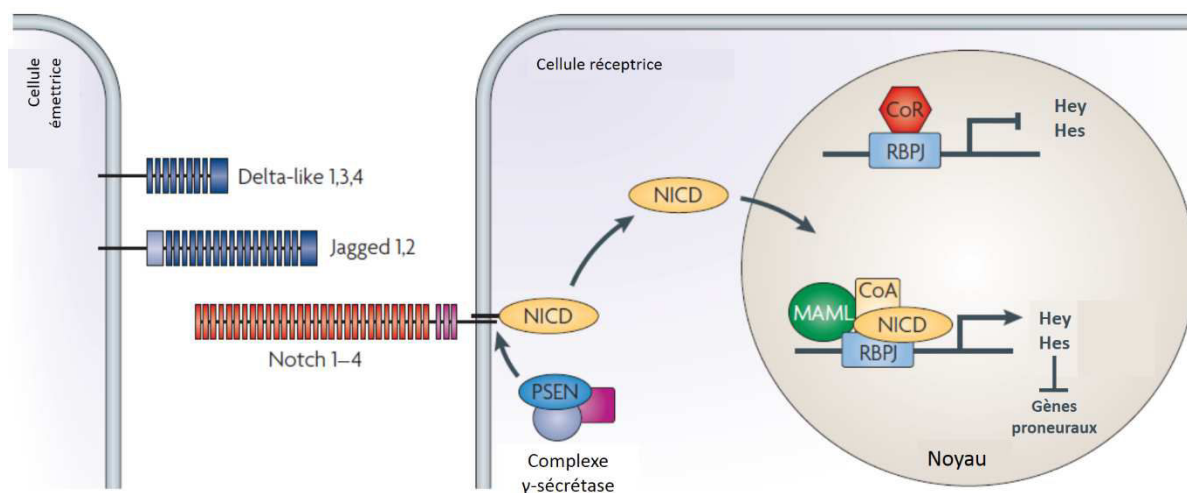


Figure 17 : Voie de signalisation NOTCH. Deux cellules voisines interagissent par l'intermédiaire des ligands DLL ou JAG et des récepteurs transmembranaires NOTCH. Cette interaction induit la protéolyse de NOTCH par un complexe γ -sécrétase et la libération de la partie intracellulaire, le NICD. Celui-ci est transloqué dans le noyau et se lie à la molécule RBPJ, ce qui permet le désassemblage d'un complexe répresseur et la liaison de RBPJ avec l'activateur MAML et d'autres co-activateurs. Ce complexe induit la transcription des gènes *HES/HEY*, des répresseurs de la transcription des gènes proneuraux (*ASCL1* et *Neurogénines*) (selon *High & Epstein, 2008*).

2) Rôle au cours de la neurogenèse :

L'activité NOTCH est décrite très tôt au cours du développement (E3.5 chez la souris) puisqu'elle intervient dans la formation du trophoctoderme (Rayon *et al.*, 2014). Cependant, les embryons inhibés pour la voie NOTCH sont similaires aux embryons sains au stade E8 (Souilhol *et al.*, 2006), mais ils ne survivent que jusqu'au stade E9.5 avec des défauts majeurs de la somitogénèse, de la neurogenèse et du développement cardiaque (Oka *et al.*, 1995). Cependant, des expériences de surexpression de la voie NOTCH, ont montré un rôle potentiel lors de la gastrulation ; d'une part en induisant une spécification en neurectoderme de l'épiblaste, et d'autre part en inhibant la voie NODAL dans la ligne primitive et en l'activant dans l'ectoderme antérieur et le mésoderme (Souilhol *et al.*, 2015). La voie NOTCH semble ainsi intervenir dans la spécification des feuillets embryonnaires en favorisant le neurectoderme par rapport à l'endomésoderme chez les mammifères et en jouant un rôle dans le développement de la ligne médiane dorsale chez les mammifères et chez le poulet (Favarolo & Lopez, 2018).

Une cellule souche neurale est un progéniteur indifférencié et pluripotent capable d'autorenouvellement. Cette cellule souche se différencie ensuite en cellule neuroépithéliale à forte capacité de prolifération. Elle donnera ensuite une cellule neurale qui peut se différencier en neurone immature post-mitotique ou progéniteur neural intermédiaire. Après migration, les neurones deviennent matures et les progéniteurs intermédiaires se divisent de façon asymétrique pour se renouveler et proliférer, avant de se différencier en neurones matures.

Au cours de ce processus de différenciation des neurones, le rôle de la voie NOTCH est très bien décrit (Zhang *et al.*, 2017). Elle doit maintenir les cellules souches neurales dans un état indifférencié, en inhibant l'expression des gènes proneuraux. Les souris mutantes pour *Notch1* et *Rbpj* entraînent l'inhibition de l'expression d'*Hes5* ; ce qui induit une neurogenèse précoce (De la Pompa *et al.*, 1997). Il a été montré par exemple chez la souris que *Notch1* maintenait les précurseurs neurales dans un état indifférencié au sein du neuroépithélium du cervelet et contrôlait leur différenciation entre E10 et E12.5 (Lutolf *et al.*, 2002; Stump *et al.*, 2002). L'inactivation des gènes proneuraux (i.e. *Ascl1*, *Neurog1/2*), principales cibles des facteurs HES/HEY, a engendré des défauts de neurogenèse (Bertrand *et al.*, 2002; Castro & Guillemot, 2011). L'ensemble de ces études a permis de mettre en évidence une boucle de régulation entre les acteurs de la voie NOTCH et les gènes proneuraux (Figure 19).

3) Inhibition latérale :

La voie NOTCH fonctionne via un processus nommé inhibition latérale (*Sjöqvist & Andersson, 2019*). Au sein d'un cluster de cellules exprimant les gènes proneuraux, une cellule va se différencier en neurone et exprimer les ligands DELTA. Ceux-ci permettent d'activer la voie NOTCH dans les cellules voisines, et de les maintenir donc à un état de progéniteur (*Figure 18*). Ce mode d'action repose sur une autorégulation qui déséquilibre l'expression des ligands et récepteurs entre cellules voisines. Là où la voie NOTCH sera suractivée, l'expression des ligands sera diminuée et réciproquement. Ce phénomène permet d'établir un choix binaire via l'inhibition d'un programme de différenciation. Il explique les profils d'expression en « poivre et sel » lors d'hybridations *in situ* qui ciblent les acteurs de cette voie de signalisation.

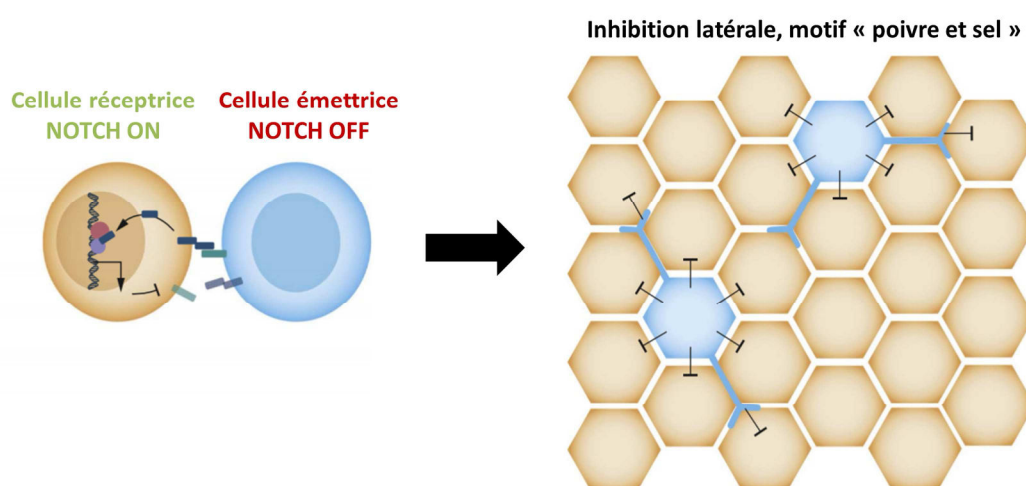


Figure 18 : Processus d'inhibition latérale. Au sein de la voie de signalisation NOTCH, une cellule émettrice (bleue) exprimera les ligands DELTA ou JAG qui se lieront au récepteur NOTCH sur la cellule réceptrice (beige) et y activeront la voie NOTCH. Au sein d'un groupe de cellule exprimant des gènes proneuraux, une cellule va se différencier en neurone. Au cours de la détermination neurale, la cellule isolée inhibera l'expression des gènes proneuraux dans les cellules voisines pour les maintenir à l'état de progéniteur. Cette action peut se produire à une certaine distance grâce à l'émission de filopodes par la cellule émettrice.

4) Des nouvelles cibles de la voie NOTCH :

Pour étudier la voie NOTCH, mon équipe de recherche a utilisé un modèle poulet inhibé pour cette voie. Pour cela, et afin de s'affranchir de la redondance des ligands et récepteurs, nous avons ciblé la gamma-sécrétase, impliquée dans le clivage du NICD et la transduction du signal. Nous avons cultivé des embryons de poulet *ex ovo* dans du DAPT (N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester), un inhibiteur de la gamma secrétase. Cette technique, appelée « roller-culture », nous permet de contrôler précisément le stade auquel on inhibe la voie NOTCH, ainsi que la durée et l'intensité de cette inhibition.

Par analyse transcriptomique de cerveaux antérieurs d'embryons de poulets traités au DAPT, l'équipe a mis en évidence de nouvelles cibles de la voie NOTCH (Ratié *et al.*, 2013). Ainsi, les gènes *Chga* (Chromogranin A, protéine sécrétoire neuroendocrine), *Chrdl1* (Chordin-like 1, antagoniste de BMP) et *Tagln3* (Transgelin 3, protéine associée aux microtubules) notamment, n'avaient jamais été impliqués dans le développement du cerveau antérieur, ni caractérisés précédemment comme cibles de la voie NOTCH. Une étude *in silico* des promoteurs a permis d'émettre l'hypothèse de l'existence d'une boucle de régulation responsable du contrôle de l'expression de ces gènes (Figure 19).

Ces expérimentations ont également montré que la voie NOTCH était impliquée dans la neurogenèse précoce de l'hypothalamus chez l'embryon de poulet. En effet, les acteurs de la voie NOTCH sont présents de façon précoce dans le prosencéphale ventral et leurs domaines d'expression se superposent aux expressions de *Shh* et *Nkx2.1* dans l'hypothalamus antérieur, suggérant un lien entre la voie NOTCH et la voie SHH.

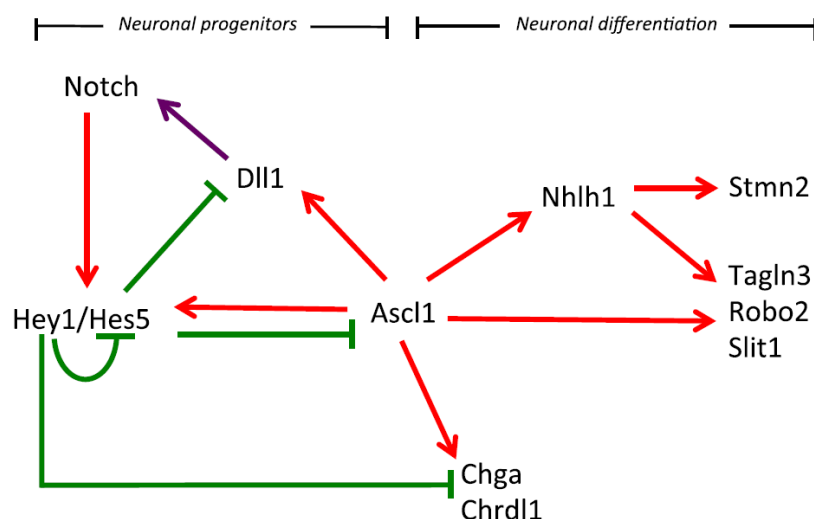


Figure 19 : Hypothèse de la boucle de régulation impliquant la voie NOTCH dans le développement de l'hypothalamus. Cette boucle a été établie par l'équipe grâce aux résultats des études menées chez le poulet, à une analyse *in silico* et à des données de la littérature. NOTCH active la transcription des gènes *Hes5* et *Hey1*, qui inhibent la transcription de *Ascl1*, afin de conserver les cellules progénitrices. *Ascl1*, le nœud de cette boucle, induit la transcription de nombreux gènes impliqués dans la différenciation neuronale. Flèches rouges : activation ; lignes barrées vertes : inhibition ; flèche violette : activation du récepteur NOTCH par son ligand DLL1 (Ratié *et al.*, 2013; Ratié *et al.*, 2014).

II- L'HOLOPROSENCEPHALIE

L'holoprosencéphalie (HPE, OMIM #236100) est une pathologie du développement du cerveau antérieur. Elle est caractérisée par un défaut de clivage du prosencéphale entre le 18^{ème} et le 28^{ème} jour de gestation chez l'humain. Ce défaut primaire induit des malformations cérébrales et faciales de sévérité variable. Sa prévalence est estimée à 1/250 produits de conception et à 1/10000 naissances vivantes (Orioli & Castilla, 2010 ; Yi et al., 2019).

A/ Classification morphologique de l'HPE :

L'HPE se caractérise par un large spectre phénotypique et a d'abord été classée en 3 types définis selon le degré de clivage des hémisphères cérébraux (Demyer & Zeman, 1963 ; Hahn & Barnes, 2010) (Figure 20).

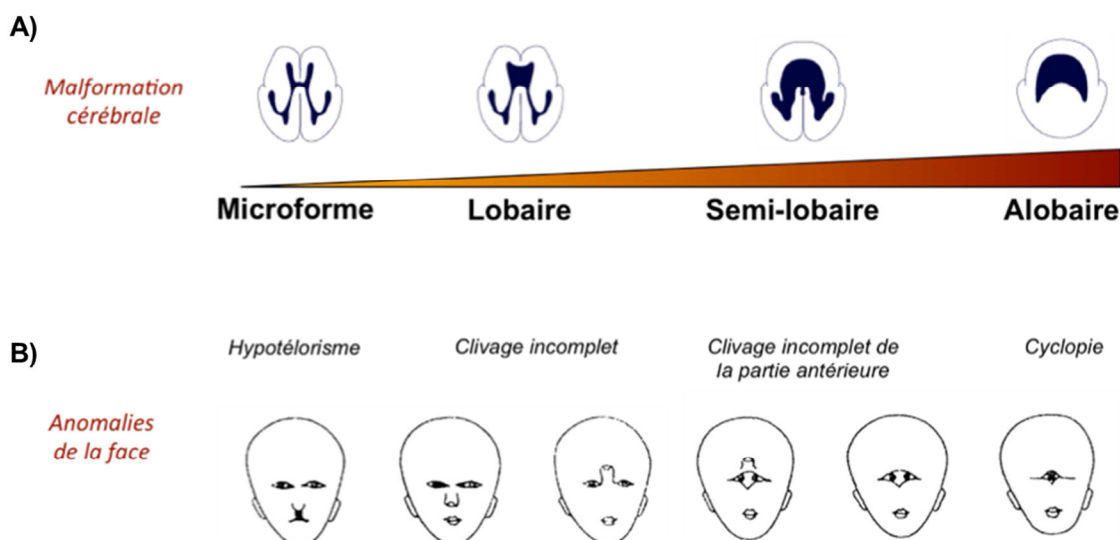


Figure 20 : Les différentes formes d'holoprosencéphalie (HPE). (A) L'HPE provient d'un défaut de clivage du prosencéphale. Il en résulte un spectre de malformations cérébrales, (B) qui sont associées à des anomalies de la ligne médiane de la face allant de l'hypotélorisme à la cyclopie (selon Nishimura & Okamoto, 1976).

- HPE alobaire : La forme la plus sévère et la plus caractéristique de l'HPE, avec une absence totale de division des hémisphères cérébraux. On y retrouve une fusion ou une absence des annexes cérébrales telles que le corps calleux, le thalamus ou l'hypothalamus. Les anomalies de la face sont souvent sévères, allant d'un probocis (trompe entre les orbites oculaires) à une ethmocéphalie avec hypotélorisme grave, voire une cyclopie. Chez le modèle animal, cette forme est associée à une inactivation totale de la voie SHH (Chiang et al., 1996 ; Mercier et al., 2013).

- L'HPE semi-lobaire, définie par la présence d'un ventricule cérébral unique avec présence d'une scissure intrahémisphérique visible au niveau postérieur. Elle s'accompagne d'annexes cérébrales (bulbes olfactifs) peu ou mal développées et de nombreuses malformations de la face. Cette forme est également associée à une réduction sévère de la voie SHH (*Cordero et al., 2004*).

- L'HPE lobaire, où le cerveau est toujours constitué de deux hémisphères distincts, mais avec une fusion persistante des lobes frontaux au niveau ventral et parfois fusion voire absence des annexes cérébrales. Ce type d'HPE s'accompagne d'anomalies faciales modérées à type de hypotélorisme ou incisive médiane unique.

- Une quatrième catégorie est maintenant définie : la syntélocéphalie ou MIHV pour « middle interhemispheric variant of holoprosencephaly » (variante interhémisphérique modérée de l'holoprosencéphalie). Cette forme est caractérisée par une fusion des lobes frontaux postérieurs et pariétaux, avec des anomalies faciales absentes ou discrètes.

-Enfin, les microformes, pour lesquelles les anomalies sont plus légères : microcéphalie, fente palatine ou incisive médiane unique (*Hahn et al., 2010*). Chez ces patients, le cerveau ne semble présenter aucune malformation typique de l'HPE, mais des anomalies subtiles de ligne médiane peuvent être observées.

Cette pathologie, dans sa forme sévère, permet rarement la survie du fœtus, et si c'est le cas, l'enfant décède au cours de la première année de vie. A l'opposé, les patients porteurs d'une microforme ont une espérance de vie identique à celle de la population générale (*Solomon et al., 2010*).

Des malformations de l'axe hypothalamo-hypophysaire ont été décrites dans l'HPE. Il est admis que le défaut de spéciation de la ligne médiane entraîne un défaut de dissociation des noyaux hypothalamiques. Ainsi, les patients HPE peuvent souffrir de diabète insipide, de dérégulation du contrôle de la température corporelle et d'un déséquilibre électrolytique pouvant entraîner des convulsions (*Solomon et al., 2010*). Il a été montré que la sévérité du diabète insipide était corrélée au degré de non-séparation des lobes de l'hypothalamus chez les patients (*Hahn et al., 2005*). Il a également été montré que des malformations de l'hypophyse étaient présentes dans des microformes d'HPE présentant une incisive médiane unique et une fente palatine (*Kjaer et al., 2001*). Ces malformations hypophysaires peuvent entraîner un retard de croissance et des troubles endocriniens.

A ce jour, il n'existe pas d'étude épidémiologique recensant les formes mineures qui permettraient de décrire leur proportion dans la population et l'étendue du phénotype. Néanmoins, les dysmorphies légères retrouvées dans ces formes sont souvent associées à une déficience intellectuelle et/ou des retards de développement et il est donc indispensable de mieux en caractériser la physiopathologie afin d'en améliorer le diagnostic et la prise en charge.

B/ Étiologies de l'HPE :

L'HPE est une pathologie qui peut être causée par plusieurs facteurs : quatre étiologies principales ont été définies (Figure 21). Notre cohorte locale ainsi que des publications internationales (Dubourg et al., 2018 ; Roessler et al., 2018 ; Cohen, 2006) nous ont permis de définir les proportions de répartitions :

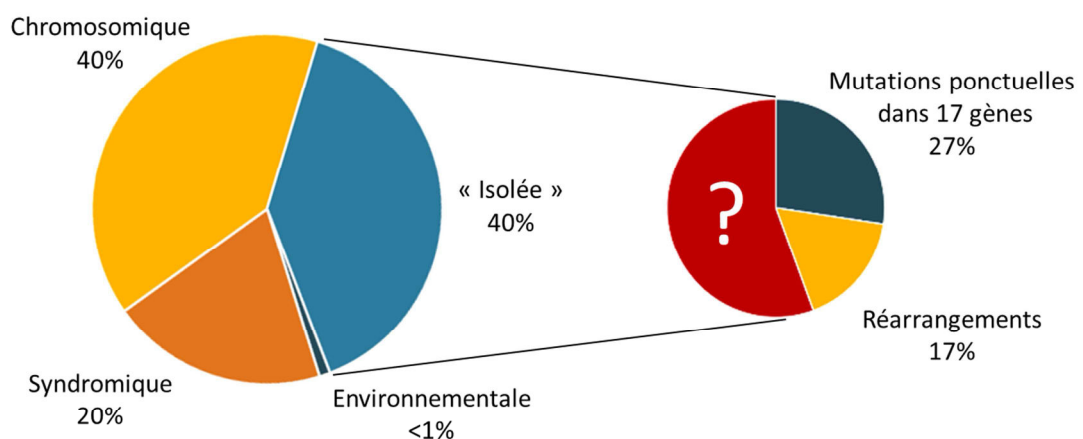


Figure 21: Etiologies de l'HPE.

1) Causes environnementales (<1% ?) :

L'holoprosencéphalie pourrait avoir une origine exogène. Mais la contribution de l'environnement dans l'apparition de l'HPE n'a jamais été estimée par une étude épidémiologique. Cependant, des travaux sur les modèles animaux ont montré l'implication de l'alcool comme cause directe d'apparition de la maladie et comme facteur aggravant en association avec une anomalie génétique (Coulter et al., 1993 ; Su et al., 2009 ; Hong & Krauss, 2012). De même, l'exposition à des produits tératogènes (acide rétinoïque, antiépileptiques, antibiotiques, acide salicylique, statines, certaines hormones) peut entraîner l'apparition d'HPE (Miller et al., 2010 ; Kotzot et al., 1993 ; Orioli et al.,

2000 ; Edison et al., 2004 ; Edison et al., 2005 ; Lammer et al., 1985 ; Corona-Rivera et al., 2010). Certaines pathologies maternelles comme le diabète (Barr et al., 1983) ou des infections à Cytomégalovirus (Byrne et al., 1987) favorisent également l'apparition de l'HPE. Une revue récente de la littérature (Summers et al., 2018), incluant principalement des études menées sur le registre national Américain des malformations congénitales, confirme une corrélation entre diabète maternel et apparition de l'HPE. Cette revue suggère également une plus grande proportion d'HPE lors de grossesses gémellaires ou chez des fœtus de sexe féminin, sans pour autant pouvoir l'expliquer.

2) HPE Syndromiques (20%)

L'HPE peut être associée à d'autres pathologies et s'intégrer dans des syndromes polymalformatifs :

- Le syndrome de Hartsfield qui associe une HPE et une ectrodactylie, et impliquant souvent une mutation de *FGFR1* (Simonis et al., 2013 ; Hong et al., 2016) ;

- Le syndrome de Smith-Lemli-Opitz, dû à une mutation dans le gène *DHCR7*, intervenant dans le métabolisme du cholestérol, et caractérisé par des anomalies congénitales multiples, un déficit intellectuel et des troubles comportementaux (Caruso et al., 2004 ; Weaver et al., 2010) ;

- Le syndrome de Pallister-Hall, caractérisé par des mutations dans *GLI3*. Ce syndrome associe une HPE avec des hamartomes hypothalamiques, une dysfonction hypophysaire, une polydactylie centrale et des malformations viscérales (Verloes et al., 1992) ;

- Le syndrome CHARGE, associant des malformations et des déficits sensoriels, et dû à une mutation dans le gène *CHD7* dans 2 cas sur 3 (Lin et al., 1990 ; Sanlaville & Verloes, 2007) ;

- etc...

Récemment, des mutations ont été retrouvées chez des patients HPE sur *KTMD2*, un gène habituellement associé au syndrome Kabuki associant retard de croissance, déficit intellectuel et malformations faciales caractéristiques, sans qu'aucun de ces signes cliniques ne soit retrouvé chez les patients HPE (Tekendo-Ngongang et al., 2019).

3) HPE chromosomiques (40%)

Les principales anomalies chromosomiques associées à des HPE sont les trisomies 13 ou 18 et les triploïdies (Epstein et al., 1988 ; Lin et al., 2007 ; Petracchi et al., 2011).

4) Formes isolées, non chromosomiques-non syndromiques (40%)

Enfin, dans 40% des cas, l'HPE n'est ni chromosomique ni syndromique et les nombreux cas familiaux suggèrent qu'ils auraient une origine génétique (Cohen, 2006 ; Krauss, 2007). Ces formes isolées sont celles sur lesquelles se concentrent les travaux de mon équipe d'accueil.

Au CHU de Rennes, notre cohorte comportant plus de 2400 individus (cas index et apparentés) nous a permis d'impliquer 17 gènes dans l'HPE, dans lesquels ont été identifiées des mutations ponctuelles (27% des cas) ou des réarrangements de grande taille (17%) (Mercier et al., 2011 ; Dubourg et al., 2018) (Tableau 1).

Tableau 1 : Liste des gènes impliqués dans l'HPE et le pourcentage de variants retrouvés dans chacun d'eux au sein de notre cohorte Rennaise (Dubourg et al., 2018)

Chromosome	Gene	NM	%
7	SHH	000193.2	5.4
13	ZIC2	007129.3	5.2
2	GLI2	005270.4	3.2
2	SIX3	005413.3	3.0
10	FGF8	033163.3	2.5
8	FGFR1	023110.2	2.0
1	DISP1	032890.3	1.2
6	DLL1	005618.3	1.2
18	TGIF1	170695.2	0.9
10	SUFU	016169.3	0.4
1	STIL	001048166.1	1 case/375
9	GAS1	002048.2	0
3	TDGF1	003212.3	0
11	CDON	016952.4	0
8	FOXH1	003923.2	0
10	NODAL	018055.4	0
3	BOC	001301861.1	Not tested

La seule cohorte équivalente est celle du Pr Maximilian Muenke (National Human Genome Research Institute, National Institutes of Health, Bethesda, Etats-Unis). La démarche diagnostique utilisée se rapproche de la nôtre (analyse en NGS de trios sur le même panel de gènes) et les résultats retrouvés sont similaires : 25 à 30% de diagnostics positifs, ZIC2, SHH, SIX3 et FGFR1 étant les gènes le plus souvent mutés (Roessler et al., 2018). La principale différence avec la cohorte rennaise se trouve

dans l'interprétation des variants: l'équipe du Pr. Muenke a pour hypothèse de travail une transmission de mutations « drivers » avec gènes modificateurs, là où nous préférons parler de transmission multigénique avec accumulation de variants hypomorphes (*cf. infra*).

Les gènes analysés chez les patients interviennent tous dans le développement du cerveau antérieur et appartiennent à plusieurs voies de signalisation, la principale étant la voie SHH. Comme décrit précédemment, cette voie est fortement impliquée dans le développement du prosencéphale ventral, et l'induction et le maintien de l'expression du gène *SHH* sont régulés par de nombreuses voies de signalisation (NODAL, FGF,...) et de nombreux facteurs de transcription (ZIC2, SIX3...)

Plus récemment, des cas d'HPE ont été rapportés par d'autres équipes avec des variants dans les gènes *STAG2*, *SMC1A*, *SMC3* et *RAD21*, appartenant au complexe de la cohésine. Ce complexe pourrait être impliqué dans la régulation de l'expression de *ZIC2*, *GLI3*, *SMAD3* et *FGFR1* (*Kruszka et al., 2019a*).

Enfin, des cas d'HPE ont récemment été associés à des variants dans le gène *CNOT1* (CCR4-NOT Transcription Complex, Subunit 1) un régulateur de la transcription des ARNs messagers dont les variants semblent avoir un impact sur l'expression de *SHH* (*Kruszka et al., 2019b ; De Franco et al., 2019*).

C/ Mécanismes moléculaires mis en jeu dans l'HPE

1) La voie de signalisation Sonic Hedgehog (SHH) :

Nous savons que la voie de signalisation *SHH* régule le développement du cerveau, et qu'un défaut de fonctionnement de SHH ou des composants de sa cascade conduit à la formation d'HPE. Le gène *SHH*, situé en 7q36 chez l'homme, code pour le ligand activateur de cette voie. Environ 200 variants dans le gène *SHH* ont été publiés et liés à l'HPE, soit 5 à 10% des cas d'origine génétique (Roessler et al., 2009a, Mercier et al., 2011, Dubourg et al., 2018).

Des variants ont également été retrouvés au niveau de différents acteurs de la voie SHH, comme *PTCH1* (Ming et al., 2002), *DISP1* (Roessler et al., 2009b), *GAS1* (Pineda-Alvarez et al., 2012), *CDON* (Hong & Kross, 2012) et *GLI2* (Roessler et al., 2003) mais dans des proportions plus faibles (Tableau 1).

La protéine SHH fait partie de la famille des morphogènes : ce sont des molécules qui sont sécrétées localement et agissent à distance pour induire la croissance et le modelage d'un tissu donné. Cette action à distance suppose donc une sensibilité des cellules réceptrice à la concentration de morphogène, et donc une importance des gradients de concentration (Briscoe & Small, 2015). De plus, il a été montré que le moment et la durée pendant laquelle les cellules sont soumises à un morphogène a un impact sur la réponse induite (Dessaud et al., 2007 ; van Boxtel et al., 2015).

Il a été montré au niveau du tube neural que les neurones se différencient en fonction de leur durée d'exposition à SHH et de sa concentration (Dessaud et al., 2007 ; Ribes et al., 2010). Ainsi, les cellules du tube neural qui sont soumises à une action forte, transitoire, à une étape précoce du développement (gastrulation et somitogénèse précoce) se différencient en neurones du plancher basal. Si cette action de SHH se prolonge, les cellules se différencient en progéniteurs neuronaux p3.

Ce mode d'action attribué au morphogène SHH peut expliquer l'hétérogénéité phénotypique retrouvée dans l'holoprosencéphalie. En effet, la mise en place des structures du tube neural ventral est étroitement liée à la concentration de SHH, on peut donc supposer qu'une perturbation plus ou moins importante du gradient et/ou du stade du développement entrainera des phénotypes plus ou moins sévères. Ainsi, une inhibition totale du signal SHH, chez des souris knockout *Shh*^{-/-} ou chez des modèles animaux avec ablation de la plaque préchordale (Pera & Kessel, 1997 ; Aoto et al., 2009), entraîne des HPE sévères. Par ailleurs, il a été montré, chez l'embryon de poulet une corrélation entre l'intensité de l'inhibition du signal SHH et la sévérité du phénotype HPE (Mercier et al., 2013 ; Cordero et al., 2004). Enfin, lorsqu'on inactive *Shh* spécifiquement dans certains tissus, à l'aide par

exemple de Cre-recombinases, le phénotype observé est moins sévère. C'est le cas lorsque *Shh* est inactivé spécifique dans la région rostrale de l'hypothalamus (RDVM), les anomalies sont restreintes à une hypoplasie du cerveau (ou microcéphalie) et à une dysmorphie de l'axe hypothalamo-hypophysaires (*Zhao et al., 2012 ; Carreno et al., 2017*).

2) La voie NODAL :

Des mutations induisant une holoprosencéphalie ont été retrouvées dans le gène *NODAL* lui-même, et dans certains cofacteurs comme *FOXH1* et *TDGF1* (*Roessler et al., 2008 ; De la Cruz et al., 2002*).

Des mutations sont aussi décrites dans *TGIF*, un répresseur transcriptionnel modulant les gènes intervenant dans la dorsalisation notamment par interaction avec la voie NODAL (*Gripp et al., 2000 ; Wotton & Taniguchi, 2018*).

Les mutations de gènes appartenant à cette voie restent cependant très rares chez les patients. Les études réalisées avec les modèles animaux suggèrent que ceci est probablement dû au rôle primordial de cette voie de signalisation lors de la gastrulation. L'hypothèse est que des mutations au niveau des gènes de cette voie seraient trop délétères pour permettre le développement de l'embryon (*Conlon et al., 1994*).

3) La voie FGF :

A ce jour, seules des mutations sur *FGF8* et *FGFR1* ont été retrouvées dans l'HPE (*Arauz et al., 2010 ; Dubourg et al., 2018*). La voie des FGF est impliquée dans le maintien du signal SHH dans la plaque préchordale via une interaction entre *FGFR3* et proNODAL, le précurseur de NODAL (*Ellis et al., 2015*). Le complexe *FGFR3*-proNODAL inhiberait les BMPs qui à leur tour inhibent SHH. Cette voie est également impliquée dans la ventralisation du tube neural via *Zic2* (*Warr et al., 2008*) ainsi que dans la mise en place des préogéniteurs neuraux hypothalamiques (*Fu et al., 2019*).

4) Les autres gènes :

Plusieurs autres gènes ont été retrouvés mutés chez des patients HPE (*Mercier et al., 2011, Dubourg 2018*), dont *ZIC2* qui interagit avec les effecteurs GLI de la voie SHH (*Solomon et al., 2010*) et *SIX3*, un facteur de transcription qui régule l'expression de *SHH* dans la plaque neurale antérieure (*Lacbawan et al., 2009*).

D/ La voie de signalisation NOTCH et l'HPE

Une analyse chromosomique par puce à ADN sur une cohorte de 111 patients a permis de mettre en évidence une délétion redondante en 6qter chez 4 patients non apparentés. La plus petite région commune de 2,2 Mb contenait le gène Delta-like1 (*DLL1*), codant pour un ligand de la voie de signalisation NOTCH (Dupé et al., 2011). Cette voie définit de nombreux mécanismes d'interactions cellulaires se déroulant principalement entre cellules voisines. Elle contrôle ainsi des processus cruciaux du développement comme le destin cellulaire, la prolifération, la différenciation ou encore l'apoptose. La voie NOTCH a été très bien décrite dans les processus de somitogenèse et neurogenèse mais n'avait jamais été impliquée dans l'HPE. Depuis 2011, le gène *DLL1* est analysé par l'équipe chez les patients atteints d'HPE. Il est retrouvé muté dans 1,2% des cas (Dubourg et al., 2018), de façon isolée ou en association avec des variants sur d'autres gènes. Néanmoins, les modèles animaux inactivés pour un gène de la voie NOTCH ne présentent pas de phénotype d'HPE (e.g. *Dll1*^{-/-}, Hrabe de Angelis et al., 1997 ; *Notch1*^{-/-}, Conlon et al., 1995 ; *Notch2*^{-/-}, Hamada et al., 1999). Ceci est probablement dû à la redondance fonctionnelle existant entre les ligands et les récepteurs de cette voie de signalisation. Cependant, les embryons mutants nuls pour le gène *Rbpj*, l'unique effecteur de la voie NOTCH (Figure 17) présentent un fort retard de croissance et une létalité précoce (E9.5, Oka et al., 1995).

L'équipe a récemment décrit un rôle spécifique de NOTCH dans la neurogenèse cérébrale, et notamment dans la ligne médiane ventrale du cerveau antérieur (Ratié et al., 2014). Par ailleurs, il a été montré que la signalisation NOTCH régulait positivement la réponse au signal SHH dans les cellules ventrales du tube neural (Kong et al., 2015 ; Stasiulewicz et al., 2015). Au-delà, de son rôle au cours de la neurogenèse, la voie NOTCH pourrait exercer un rôle de potentialisateur de la voie SHH dans le cerveau antérieure.

E/ Les modes de transmission et le modèle multigénique :

Le mode de transmission de l'HPE a d'abord été considéré comme étant dominant. Mais certaines mutations étaient décrites chez des patients à phénotype sévère et étaient héritées de parents apparemment sains ; une transmission autosomique dominante à pénétrance incomplète et expressivité variable (Odent et al., 1998) ou avec « facteurs modificateurs » (Roessler et al., 2012) a donc été proposée. Il est aujourd'hui admis que cette pathologie implique de nombreux gènes et que plusieurs modes de transmission sont possibles : dominant, récessif, digénique ou oligogénique (Figure 22 ; Mercier et al., 2013 ; Dupé et al., 2017 ; Dubourg et al., 2018).

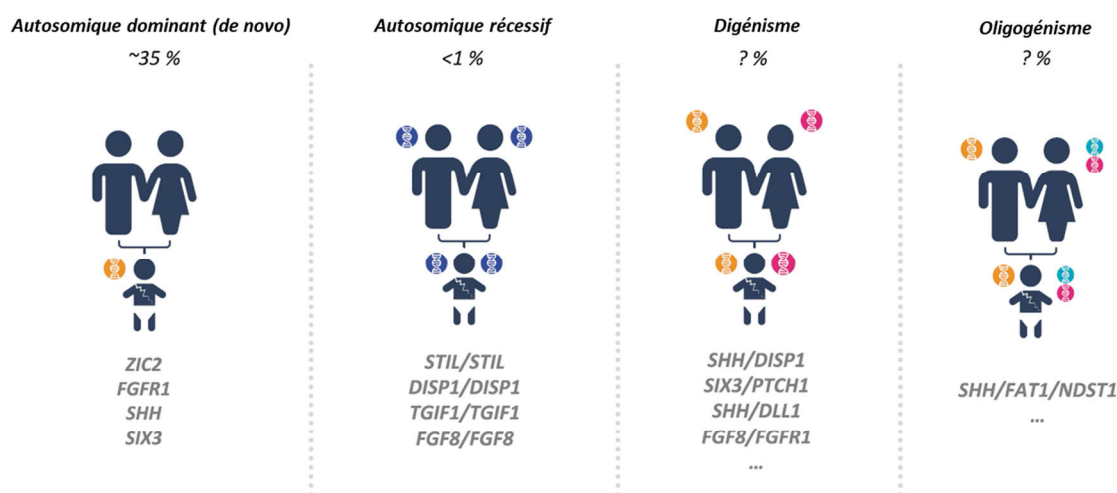


Figure 22 : Les différents modes de transmission dans l'HPE et leurs proportions parmi les cas documentés dans notre cohorte. (selon Dubourg et al., 2018)

Chez l'humain, une transmission par le mode dominant est retrouvée chez 35% des patients présentant une HPE isolée chez qui un diagnostic moléculaire a été posé (Dubourg et al., 2018). Ces mutations sont principalement retrouvées dans les gènes SHH, ZIC2, SIX3 et FGFR1 et apparaissent *de novo*.

Une transmission autosomique récessive a été décrite pour des mutations dans les gènes TGIF1 (El-Jaick et al., 2007), FGF8 (McCabe et al., 2011 ; Hong et al., 2018) et STIL (Kakar et al., 2014). Des analyses dans des familles consanguines permettent parfois d'observer ce type de transmission, qui ne représente cependant que quelques cas isolés (moins de 1% des patients de notre cohorte).

Malgré les nombreux gènes impliqués dans l'HPE et l'avènement des technologies de séquençage haut débit, des mutations délétères ne sont identifiées que dans 30% des cas. Par ailleurs, dans la majorité d'entre eux, les variants retrouvés chez les patients sont hérités d'un parent apparemment

sain. Il apparait donc que l'HPE est une pathologie complexe pouvant mettre en jeu, de façon concomitante, des mutations dans plusieurs gènes.

Ce modèle multigénique est conforté par de nombreuses études réalisées chez la souris. En effet, de nombreux modèles murins mutés pour deux gènes dans la même voie de signalisation ou dans deux voies de signalisation différentes présentent un phénotype de type HPE. On retrouve ainsi des doubles hétérozygotes pour des gènes de la voie SHH (*Disp1/Shh*, Tian et al., 2005), de la voie NODAL (*Smad2/Nodal*, Nomura & Li, 1998 ; *Gdf/Nodal*, Andersson et al., 2006), de la voie des BMP (*Nog/Chrd*, Bachiller et al., 2000) et de la voie des FGF (*Fgfr1/Fgfr2*, Gutin et al., 2006). Des souris doubles hétérozygotes, mutées sur des gènes de voies différentes ont également été rapportées (*Six3/Shh*, Geng et al., 2008 ; *Chrd/Nodal*, Yang et al., 2010 ; *Dkk1/Nog*, Del Barco Barrantès et al., 2003 ; *Foxa2/Nodal*, Varlet et al., 1997b).

Ce phénomène est aussi observé chez le modèle poulet, où une inhibition chimique à des concentrations inférieures au seuil délétère des voies Nodal et SHH induit des phénotypes du spectre de l'HPE, de sévérité variable en fonction du degré d'inhibition (Mercier et al., 2013).

Tous ces phénomènes, tant chez l'homme que chez l'animal, ont pour point commun de perturber le signal SHH. Il semblerait que plus le signal SHH est diminué dans des régions spécifiques et lors de certaines phases clés du développement du cerveau antérieur, plus le phénotype de l'HPE est sévère. L'objet de ma thèse porte donc sur l'étude du rôle de la voie NOTCH dans ces phénomènes, en étudiant d'une part son implication dans la mise en place des structures ventrales du télencéphale, et d'autre part son lien avec la voie SHH et son rôle dans l'apparition d'un phénotype HPE.

OBJECTIFS DE THÈSE

OBJECTIFS DE THESE

Mon travail de thèse s'est inscrit dans le projet de l'équipe de « Génétique des Pathologies Liées au Développement », qui travaille sur la caractérisation de la physiopathologie de l'HPE et sur la compréhension des mécanismes impliqués dans le développement précoce du cerveau antérieur.

Nous sommes, au CHU de Rennes, le centre de référence national pour l'holoprosencéphalie ; nous possédons donc une cohorte unique de patients atteints de cette pathologie.

Comme il a été exposé dans l'introduction, la voie de signalisation NOTCH a été impliquée en 2011 par l'équipe dans l'HPE grâce à des analyses en puce à ADN. Nous essayons, depuis, de comprendre les liens entre la voie NOTCH, le développement du cerveau antérieur, et l'HPE.

Des études précédemment menées sur modèle poulet ont montré que la voie NOTCH était impliquée dans le développement précoce de l'hypothalamus, et ont permis de mettre en évidence de nouvelles cibles moléculaires. Mes travaux de thèse se sont, par conséquent, articulés sur 3 axes :

- Etudier le rôle de la voie NOTCH au cours de la neurogenèse précoce du cerveau antérieur. J'ai utilisé les modèles poulet et souris pour inactiver la voie NOTCH et étudier sa fonction.
- Etudier la relation entre la voie NOTCH et la voie SHH dans le développement du cerveau antérieur. J'ai analysé l'expression de *Shh* chez des embryons de souris ou de poulet déficients en voie de signalisation NOTCH. J'ai généré des mutants souris hypomorphes à la fois pour la voie NOTCH et la voie SHH pour tester une potentielle synergie entre ces 2 voies
- Analyser l'ADN des patients de notre cohorte à l'aide des nouvelles technologies de séquençage pour identifier de nouveaux gènes ou variants impliqués dans l'HPE.

RÉSULTATS

RESULTATS

PARTIE 1 : ROLE DE LA VOIE NOTCH AU COURS DE LA NEUROGENESE PRECOCE DU CERVEAU

ANTERIEUR

La première moitié de ma thèse a été consacrée à l'étude du rôle de la voie NOTCH dans la neurogenèse du cerveau antérieur. J'ai travaillé en collaboration avec Michelle Ware (post-doctorante) sur l'implication de la voie NOTCH au cours de la régulation des gènes proneuraux et dans l'apparition des premières populations neuronales dans le cerveau antérieur.

❖ **Article 1 : Notch signaling and proneural genes work together to control the neural building blocks for the initial scaffold in the hypothalamus**

Frontiers in Neuroanatomy; 2014

Michelle Ware, Houda Hamdi-Rozé and Valérie Dupé

Le rôle de la voie NOTCH a été largement étudié dans les processus de somitogenèse et de différenciation des neurones, mais peu d'études décrivent son rôle précis au cours de la neurogenèse précoce de l'hypothalamus. Nous avons donc décidé d'écrire une revue afin de rassembler les informations sur le développement de ces premiers neurones. Nous y avons également inclus des résultats originaux issus de nos expérimentations sur modèle poulet et souris, qui sont des études d'expression d'acteurs de la voie NOTCH et de ses cibles.

Une revue de la littérature nous a tout d'abord permis de décrire les domaines d'expression de *Shh* et de facteurs de transcription spécifiques de l'hypothalamus (*Nkx2.1* et *Nkx2.2*) afin de superposer ces domaines d'expression à l'apparition des premiers neurones chez 3 modèles animaux : le poisson zèbre, le poulet et la souris. Ainsi, les premiers neurones à apparaître sont les nTPOC (noyaux du tractus de la commissure post-optique) dans l'hypothalamus antérieur, à 16 hpf (heures post-fertilisation) chez le poisson zèbre, HH13 chez le poulet et E9.5 chez la souris.

Des travaux antérieurs de l'équipe avaient montré que, chez le poulet, la voie NOTCH apparaissait dans l'hypothalamus au stade HH11 (*Ratié et al., 2013*), avec une expression de *Dll1*, *Hes5* et *Hey1* dans l'hypothalamus antérieur. Afin d'étudier la mise en place de la voie NOTCH dans l'hypothalamus chez la souris, j'ai effectué des hybridations *in situ* ciblant les différents acteurs de la voie NOTCH à plusieurs stades de développement. J'ai ainsi montré que *Dll1* et *Hes5* s'exprimaient dès E8.5 et *Hey1* dès E9.0 dans l'hypothalamus antérieur, juste avant l'apparition des premiers neurones (nTPOC). Puis

ces trois gènes s'expriment à E9.5 dans l'hypothalamus mamillaire, où se différencieront les nMTT (noyaux du tractus mamillotegmental). Ces éléments montrent que l'activité NOTCH est précoce dans l'hypothalamus et joue un rôle dans la différenciation de ces populations neuronales.

J'ai également étudié l'expression d'un gène proneural, *Ascl1*, normalement régulé par la voie NOTCH dans plusieurs autres tissus neuraux. Nous avons montré qu'*Ascl1* est le seul gène proneural exprimé dans l'hypothalamus antérieur puis dans l'hypothalamus mamillaire. Grâce à des expérimentations de double marquage en hybridation *in situ*, nous avons montré que l'expression d'*Ascl1* se superposait à celle de *Shh* dans l'hypothalamus antérieur, au niveau des nTPOC.

Nous avons ensuite étudié l'expression de deux nouveaux gènes cibles de la voie NOTCH, parmi lesquelles *Tagln3* (Transgeline 3) et *Chga* (Chromogranine a). Ces deux gènes cibles avaient précédemment été identifiés chez le poulet grâce à des expérimentations d'inhibition de la voie NOTCH (Ratié et al., 2013).

Les données de littératures complétées par nos propres observations ont permis de montrer que les premiers neurones se différenciant au niveau de l'hypothalamus sont issus d'une boucle de régulation qui est similaire à celle décrite dans d'autres sites de neurogénèse.

A un stade très précoce chez le poulet (HH10), une boucle de régulation entre Notch, Hes5, *Ascl1* et *Dll1* se met en place dans l'hypothalamus antérieur et permet l'induction de la neurogenèse grâce au phénomène d'inhibition latérale. Il s'ensuit une activation des gènes cibles dont des marqueurs neuronaux (*Nhlh1*) qui s'expriment dès HH11, bien avant que les cellules ne deviennent des neurones matures (HH13).



Notch signaling and proneural genes work together to control the neural building blocks for the initial scaffold in the hypothalamus

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The vertebrate embryonic prosencephalon gives rise to the hypothalamus, which plays essential roles in sensory information processing as well as control of physiological homeostasis and behavior. While patterning of the hypothalamus has received much attention, initial neurogenesis in the developing hypothalamus has mostly been neglected. The first differentiating progenitor cells of the hypothalamus will give rise to neurons that form the nucleus of the tract of the postoptic commissure (nTPOC) and the nucleus of the mammillotegmental tract (nMTT). The formation of these neuronal populations has to be highly controlled both spatially and temporally as these tracts will form part of the ventral longitudinal tract (VLT) and act as a scaffold for later, follower axons. This review will cumulate and summarize the existing data available describing initial neurogenesis in the vertebrate hypothalamus. It is well-known that the Notch signaling pathway through the inhibition of proneural genes is a key regulator of neurogenesis in the vertebrate central nervous system. It has only recently been proposed that loss of Notch signaling in the developing chick embryo causes an increase in the number of neurons in the hypothalamus, highlighting an early function of the Notch pathway during hypothalamus formation. Further analysis in the chick and mouse hypothalamus confirms the expression of Notch components and *Ascl1* before the appearance of the first differentiated neurons. Many newly identified proneural target genes were also found to be expressed during neuronal differentiation in the hypothalamus. Given the critical role that hypothalamic neural circuitry plays in maintaining homeostasis, it is particularly important to establish the targets downstream of this Notch/proneural network.

Keywords: early axon scaffold, forebrain, differentiation, tract of the postoptic commissure, mammillotegmental tract, hypothalamus patterning, ASCL1

INTRODUCTION

The hypothalamus is an evolutionary ancient structure in the rostral brain that plays a central role in the regulation of physiological processes such as hunger, thermoregulation, reproduction and behavior in adult vertebrates. The adult hypothalamus is subdivided into regions, each containing well documented clusters of neurons with defined functions (Simerly, 2004). Countless work involving physiological and genetic studies has focused on signaling molecules and transcription factors that control hypothalamus morphogenesis and the emergence of different neuronal subtypes (Shimogori et al., 2010). However, relatively little attention has been paid to the process through which the initial neurons are induced and specified in the primordium of the vertebrate hypothalamus, despite their key roles in pioneering

the major axon pathways in the forebrain (Wilson et al., 1990; Mastick and Easter, 1996; Ware and Schubert, 2011). The first differentiating cells of the hypothalamus will give rise to neurons that form the nucleus of the tract of the postoptic commissure (nTPOC) and the nucleus of the mammillotegmental tract (nMTT). Recent advances in the chick model has established that a Notch/proneural regulatory loop is implicated very early during the differentiation of these neurons (Ratié et al., 2013). The aim of this review is to highlight a role for Notch signaling during nTPOC and nMTT differentiation; including key findings from zebrafish, chick and mouse models, which has contributed to our understanding of this field. A potential cascade involving *Ascl1* and target genes will be discussed to determine the possible regulation of these initial hypothalamic neurons.

PATTERNING OF THE VERTEBRATE HYPOTHALAMIC PRIMORDIUM

During early embryogenesis the hypothalamus develops within the secondary prosencephalon (Puelles and Rubenstein, 2003;

Abbreviations: AH, anterior hypothalamus; bHLH, basic helix-loop-helix; MH, mammillary hypothalamus; MTT, mammillotegmental tract; TH, tuberal hypothalamus; NPC, neural progenitor cell; nTPOC, nucleus of the tract of the postoptic commissure; nMTT, nucleus of mamillo-tegmental tract; TPOC, tract of the postoptic commissure.

Martinez-Ferre and Martinez, 2012; Puelles et al., 2012). Developmental studies performed in zebrafish, chick and mouse indicate Sonic Hedgehog (SHH), secreted by the underlying prechordal plate mesendoderm, induces the formation of the hypothalamus (Dale et al., 1997; Mathieu et al., 2002; Aoto et al., 2009). Loss of *Shh* leads to missing ventral structures including the hypothalamus in zebrafish (Varga et al., 2001) and mouse (Chiang et al., 1996). In humans, mutations in the *Shh* gene results in holoprosencephaly, the most frequent human brain malformation that includes hypothalamic defects (Mercier et al., 2011). However, SHH alone is not sufficient to induce specific hypothalamus identity. The prechordal plate expresses numerous other secreted proteins that are involved in the development of the overlying hypothalamus primordium including Wnt antagonists, NODAL and Bone Morphogenic Proteins (BMP; Pera and Kessel, 1997; Kiecker and Niehrs, 2001; Mathieu et al., 2002; Manning et al., 2006; Cavodeassi and Houart, 2012).

Specific patterning of the hypothalamus begins when the hypothalamic primordium expresses the transcription factor *Nkx2.1* from Hamburger and Hamilton stage (HH)8 in chick and embryonic day (E)8 in mouse (Shimamura et al., 1995; Pera and Kessel, 1998; Sussel et al., 1999; Crossley et al., 2001). This expression of *Nkx2.1*, along with *Nkx2.2* is dependent on the presence of *Shh* in the prechordal plate (Barth and Wilson, 1995; Pera and Kessel, 1997; Rohr et al., 2001; Mathieu et al., 2002). SHH is then required to coordinate tissue growth and acquisition of anteroposterior (AP), dorsoventral (DV) and mediolateral patterning of the hypothalamus (Manning et al., 2006; Szabó et al., 2009).

At HH10, *Shh*, *Nkx2.1* and *Nkx2.2* expression expands in the basal plate of the chick prosencephalon, with the same rostral expression at the level of the presumptive anterior hypothalamus (AH) that corresponds to the prospective chiasmatic area (also called suboptical domain) (Crossley et al., 2001). A new *Nkx2.1* expression domain develops at HH12, just rostral to the hypothalamus in the basal telencephalon called the postoptic area (POA). In zebrafish and mouse, the same dynamic expression patterns of *Shh*, *Nkx2.1* and *Nkx2.2* is present within the hypothalamus (Figure 1). By HH13 in chick and E9.5 in the mouse, *Shh* and *Nkx2.1* expression has expanded further and the hypothalamic primordium is morphologically evident. Studies in chick show that once the hypothalamic primordium is established, SHH down-regulation mediated by local production of BMPs is necessary for establishing region-specific transcriptional profiles (Patten and Placzek, 2002; Manning et al., 2006; Ohyama et al., 2008). This leads to the subdivisions of the primordial hypothalamus into three regions, the AH, the tuberal hypothalamus (TH) and the mammillary hypothalamus (MH), with each region expressing specific markers (Figure 1; Alvarez-Bolado et al., 2012; Wolf and Ryu, 2013).

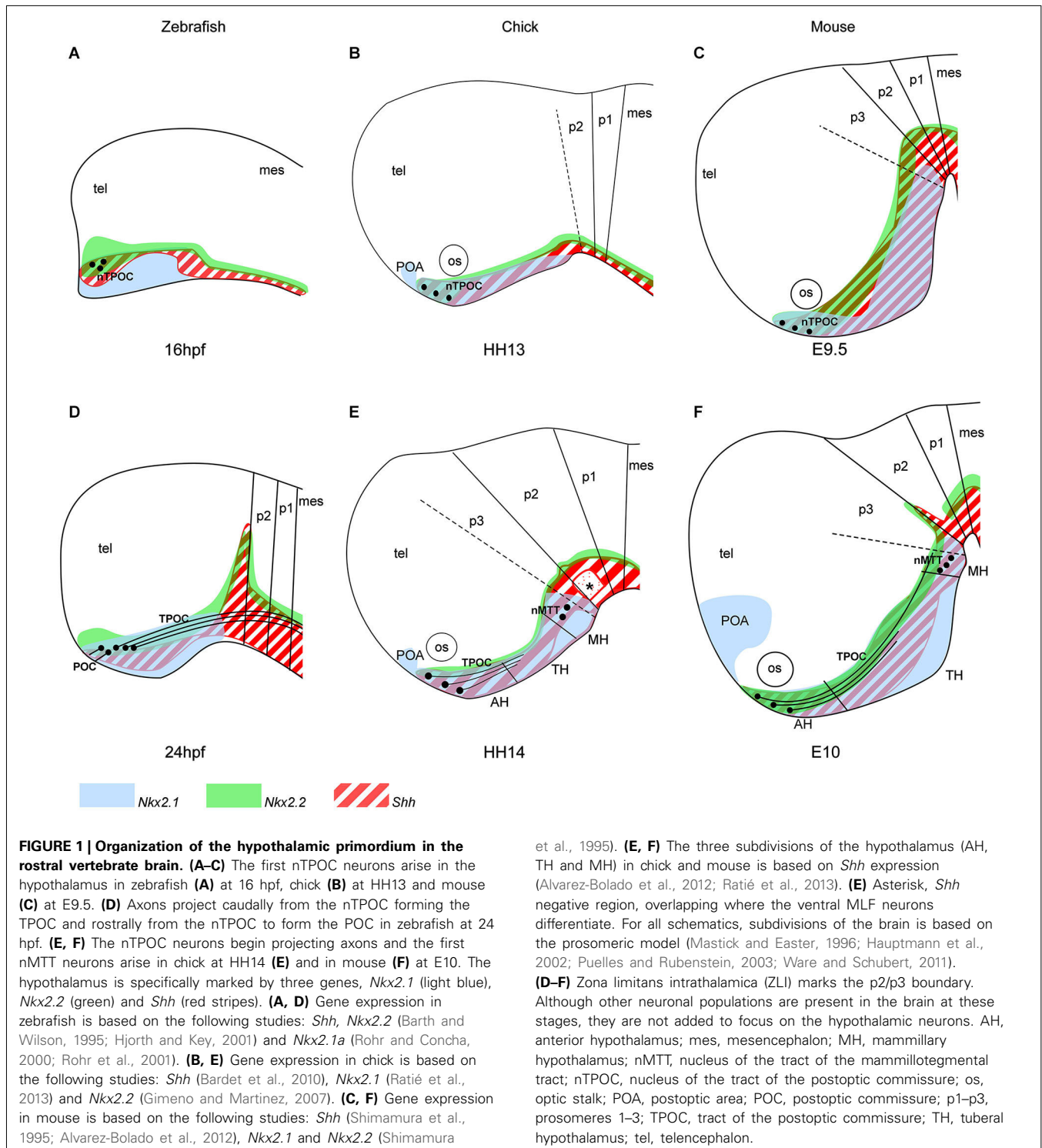
INITIAL NEUROGENESIS IN THE VERTEBRATE HYPOTHALAMUS

The first neurons that differentiate in the vertebrate brain give rise to the highly conserved early axon scaffold (Chitnis and Kuwada,

1990; Wilson et al., 1990; Easter et al., 1993; Mastick and Easter, 1996; Barreiro-Iglesias et al., 2008; Ware and Schubert, 2011; Ware et al., 2014). This is an important structure for the guidance of later, follower axons allowing more complex connections to form. Predating the mature hypothalamic neuronal clusters, two small GABAergic positive populations differentiate within the hypothalamus (Figure 1; Patel et al., 1994). The first neurons in the hypothalamic primordium differentiate to give rise to the nTPOC (also termed the ventro-rostral cluster (vrc) in anamniotes) at 16 hpf in zebrafish (Figure 1A; Chitnis and Kuwada, 1990; Ross et al., 1992) and HH13 in chick (Figure 1B; Ware and Schubert, 2011). An early birth-dating study has shown that hypothalamic neurogenesis in the mouse begins at E10 (Shimada and Nakamura, 1973). However, it is well-known that the initial nTPOC neurons arise at E9.5, suggesting neurogenesis begins earlier than previously thought (Figure 1C; Easter et al., 1993; Mastick and Easter, 1996; Ricaño-Cornejo et al., 2011). From the nTPOC neurons, axons extend and project caudally within the basal plate. The tract of the postoptic commissure (TPOC) axons project into the mesencephalon where these axons form part of the ventral longitudinal tract (VLT) along with the medial longitudinal fascicle (MLF) and later the mammillotegmental tract (MTT; Ware and Schubert, 2011). The MTT forms from a second set of neurons (nMTT) that differentiate later in the caudal hypothalamus of amniotes from HH14 in chick and E10 in mouse (Figures 1E,F; Puelles et al., 1987; Easter et al., 1993; Mastick and Easter, 1996). While the presence and location of the nTPOC is conserved in all vertebrates studied, the nMTT is not present in zebrafish, at least during early development (Barreiro-Iglesias et al., 2008). Neurons do however form later in the zebrafish MH, but it is not possible to comment on the homology with the nMTT (Wolf and Ryu, 2013). The postoptic commissure (POC) forms by 24 hpf, projecting axons from the nTPOC rostrally to form a commissure across the rostral midline connecting the left and right sides of the neural tube (Figure 1D; Ross et al., 1992; Bak and Fraser, 2003). The POC is likely to form in chick and mouse at later stages but has not been studied exhaustively (Croizier et al., 2011; Ware and Schubert, 2011).

The prosomeric model and hypothalamic markers such as *Shh*, *Nkx2.1* and *Nkx2.2* confirms the nTPOC and nMTT neurons form within the hypothalamus (Figure 1; Hjorth and Key, 2001; Puelles and Rubenstein, 2003). The nTPOC arises just below the optic stalk at the midline of the AH area and the nMTT in the lateral edge of the caudal hypothalamus in the MH (Figure 1; Easter et al., 1993). The nTPOC neurons differentiate along the boundaries of many gene expression areas in zebrafish (Macdonald et al., 1994), however the mechanism by which these neurons differentiate has been overlooked.

Some zebrafish and mouse mutants are available where the formation of these hypothalamic axon tracts is affected. Some genes are implicated in the differentiation of the neurons such as *Six3* (Ando et al., 2005), but many studies focus on the effect of gene inactivation on axon guidance, including *Fgf8* (Shanmugalingam et al., 2000), *Pax6* (Mastick et al., 1997; Nural and Mastick, 2004), *Slits* and *Robos* (Ricaño-Cornejo et al., 2011)



and *Sim1/Sim2* (Marion et al., 2005). Functionally, the TPOC is important for the guidance of other axon tracts. Ablation of the TPOC axons in the zebrafish embryo affects the patterning of the early axon scaffold (Chitnis and Kuwada, 1991). In zebrafish *Cyclops* mutants, the TPOC does not form, leading to the misguidance of the tract of the posterior commissure

(TPC) axons (Patel et al., 1994). More recently, a study in the embryonic mouse has shown later hypothalamic axons from the melanin-concentrating hormone (MCH) neurons use the TPOC for guidance (Croizier et al., 2011). While the potential function of the TPOC neurons is not known, lipophilic tracing shows that the TPOC axons project into the hindbrain, although the

target of these axons remains a mystery (Ware and Schubert, 2011). It is also unclear whether these neurons are still present postnatally, it could be that their sole purpose is to provide axons for guidance and then simply die after connections are made in the adult brain (Easter et al., 1993). The MTT may also function in the guidance of other tracts but this has not been studied exhaustively. In mouse, the MTT is likely to guide the mammillothalamic tract (MTH) that forms later in mouse contributing to the principle mammillary tract (Marion et al., 2005). The MTH is not known to form in zebrafish or chick. The MTT axons project to the tegmentum and are described as having a role in visceral function and processing special information in the adult human brain (Alpeeva and Makarenko, 2007; Kwon et al., 2011).

No attention has been brought to the mechanism by which the nTPOC and nMTT neurons differentiate, until 2013, when Notch components were first described as being present very early in the hypothalamus of the developing chick embryo (Ratié et al., 2013). A basic PubMed search of the key words Notch and hypothalamus generated very few publications and many of which are based in adult models or describe differentiation of late forming embryonic neurons (Chapouton et al., 2011; Aujla et al., 2013). This indicates a surprising lack of investigations surrounding neurogenesis of the initial hypothalamus neurons, when considering these early neurons have been described through-out the 1990s in different vertebrate species. As these neurons contribute to the early axon scaffold and are essential for the set-up of more complex connections, it is surely essential to understand how they differentiate and how they are specified. Finally, considering Notch along with the proneural network is a well-known signaling pathway, little is known about the implication of Notch signaling or neurogenic factors involved in the formation of the nTPOC and nMTT neurons.

NEUROGENESIS AND THE NOTCH/PRONEURAL NETWORK

Notch signaling is an evolutionary conserved signaling pathway involved in cell-cell communication regulating multiple processes throughout development. The Notch signaling pathway has previously been reviewed in detail, here a brief outline is described (Pierfelice et al., 2011). First identified in *Drosophila*, the Notch pathway has been confirmed to have similar roles in vertebrates (Coffman et al., 1990; Artavanis-Tsakonas and Simpson, 1991; Artavanis-Tsakonas et al., 1999). The core pathway consists of the interaction between a transmembrane Notch receptor anchored in one cell, with a transmembrane Notch ligand (Delta or Serrate/Jagged) in a neighboring cell. Upon receptor-ligand binding a series of proteolytic cleavages are triggered that releases the intracellular domain of Notch (NICD), which forms a nuclear complex with recombination signal binding protein for immunoglobulin kappa J region (RBPJ). This complex activates the transcription of target genes (Tamura et al., 1995; Fortini, 2009). The best characterized direct targets of the NICD/RBPJ complex are the Hes (Hairy-Enhancer of Split) and Hey (Hes related type) genes (Jarriault et al., 1995; Maier and Gessler, 2000). They are class-C basic helix-loop-helix (bHLH) proteins that function as transcriptional repressors and

can function together as homodimers or heterodimers (Iso et al., 2003).

One function of Notch relies on lateral induction, which is defined as the process by which a ligand-expressing cell stimulates those cells nearby to upregulate ligand expression, promoting ligand propagation and coordinated cell behavior (Eddison et al., 2000). The other function of Notch is lateral inhibition, whereby a ligand-expressing cell inhibits the expression of the ligand in the neighboring cells, therefore preventing those cells from adopting the same fate and generating a patched cellular pattern (Bray, 2006). It is associated with salt-and-pepper like patterns of gene expression (Fior and Henrique, 2009). For example, these two modes of Notch pathway operation coexist during inner ear development. Each mode relies on an associated gene regulatory network (Kiernan, 2013; Neves et al., 2013). Expression and functional studies suggest that lateral induction and lateral inhibition are associated with different Notch ligands that initiate signaling (Brooker et al., 2006; Saravanamuthu et al., 2009; Petrovic et al., 2014). The association of DLL1 with lateral inhibition is a general theme during neural development (Henrique et al., 1995; Adam et al., 1998; Kageyama et al., 2010).

Notch signaling has a very well-known role in neurogenesis, controlling the balance between proliferation of neural progenitor cells (NPCs) and differentiation of NPCs into neuronal and glial cells (Campos-Ortega, 1993; Chitnis et al., 1995; de La Pompa et al., 1997; reviewed by Paridaen and Huttner, 2014). In the neuroepithelium, neuron production is mostly controlled by lateral inhibition, where a regulatory loop is formed, with proneural genes controlling the expression of Notch ligands (Bertrand et al., 2002). The ligand, DLL1, can bind and activate NOTCH in neighboring cells. When the Notch signaling pathway is activated, transcriptional repressors (such as Hes or Hey genes) are expressed that prevent expression of proneural genes, inhibiting differentiation and therefore cells remain as progenitors. Cells expressing the ligand and therefore lacking Notch signaling can no longer express transcriptional repressors, leading to the upregulation of bHLH proneural transcription factors such as *Ascl1* or *Neurog1/2*. Under this Notch/proneural network the cell can exit the cell cycle and undergoes neural differentiation (Bertrand et al., 2002). This differentiation step is controlled by several classes of transcription factors that determine the identity of the neuron produced. Among them, a number of bHLH differentiation genes are switched on, such as *Nhlh1* or *NeuroD4*, followed by specific neuronal genes.

NOTCH SIGNALING IN THE VERTEBRATE HYPOTHALAMUS PRIMORDIUM

There are numerous studies investigating the expression and function of Notch components, proneural genes and downstream targets. However, as mentioned previously there is very little data describing the role of Notch signaling during the differentiation of the nTPOC and nMTT neurons. When Notch signaling is inhibited in the developing chick embryo, the number of nTPOC neurons increases, along with ectopic expression of many genes within the hypothalamus, confirming Notch has a role during hypothalamic neurogenesis at this early stage (Ratié et al., 2013).

This study describes a typical neurogenic phenotype expected for the loss of Notch function, working by lateral inhibition.

For the first time, the Notch components *Dll1*, *Hes5* and *Hey1* are shown to be expressed just before HH11 in the presumptive AH of the chick embryonic brain where the first nTPOC neurons will differentiate at HH13 (Ware and Schubert, 2011; Ratié et al., 2013). Expression of Notch components during initial neurogenesis in the zebrafish and mouse has been extensively studied, however for much of the data, it is difficult to interpret the expression in the hypothalamic primordium as no special attention was given to this area at early stages. The expression of Notch receptors in zebrafish are first described at 16 hpf in the prosencephalon and appear to overlap in the area where the nTPOC forms (Bierkamp and Campos-Ortega, 1993; Dyer et al., 2014). In mouse, while *Notch3* is ubiquitously expressed in the neuroectoderm from E8.0, *Notch2* and *Hes1* are expressed in the ventral prosencephalon from E8.5 and *Notch1* is expressed from E9.5 (Reaume et al., 1992; Williams et al., 1995; Koop et al., 1996). Remarkably, little information is present in the literature about when these genes are first expressed in the developing hypothalamus (Bettenhausen et al., 1995; de La Pompa et al., 1997; Leimeister et al., 1999; Barsi et al., 2005). Therefore, in this review, expression of Notch components are analyzed using *in situ* hybridization data to deal with this deficiency (Figure 2). *Dll1*, *Hes5* and *Hey1* mRNA probes are used to show the presence of Notch activity, focusing more specifically in the hypothalamus. At E8.0, *Dll1*, *Hes5* and *Hey1* are not expressed in the mouse presumptive hypothalamus (Figures 2A–C), it is only from E8.5, before the initial neurons differentiate that *Dll1* and *Hes5* expression is first observed (Figures 2E,E',F,F', arrowheads). Flat-mounted preparations of the ventral midline reveal a salt-and-pepper like pattern for these genes in the rostral hypothalamus (Figures 2E',F'). Expression continues in the AH at E9 for *Dll1* and *Hes5* (Figures 2I,J, arrowhead), while *Hey1* expression first starts to be expressed in the same region (Figure 2K, arrowhead). At E9.5, *Dll1*, *Hes5* and *Hey1* are first expressed in the MH where the nMTT neurons will differentiate at E10 (Figures 2M–O, unfilled arrowhead). It is important to note that the genes analyzed here are not specific for either the nTPOC or nMTT, but are also expressed by other early developing neurons such as those in the nucleus of the mesencephalic tract of the trigeminal nerve (nmesV; Figure 2). This is not surprising as Notch is a very general pathway involved in neuron progenitor expansion (Kageyama et al., 2009). Flat-mounted preparations performed at E9.5 confirm the localized expression of *Hes5* (Figure 2N) and *Hey1* (Figure 2O) in the AH.

Bringing together the data from the literature and *in situ* hybridization of mouse embryos presented here, this highlights that Notch signaling is active very early in the AH and MH where the nTPOC and nMTT neurons will develop respectively (Mastick and Easter, 1996; Ratié et al., 2013). The data also suggests that redundancy could be strong between the direct Notch target genes as multiple transcriptional repressors such as *Hes1*, *Hes5* and *Hey1* are expressed in the developing hypothalamus.

Like with the expression studies described in this section, no functional data about neurogenesis in the early hypothalamus

is available in zebrafish and mouse. There are several models lacking Notch signaling, which exhibit an increase in neurons throughout the embryo (de La Pompa et al., 1997; Itoh et al., 2003). For example, in the zebrafish and mouse mindbomb/Mib1 mutants, *Dll1* ubiquitination is affected and aberrant neurogenesis due to lower expression of *Hes1* and *Hes5* is observed throughout the embryo (Itoh et al., 2003; Barsi et al., 2005; Koo et al., 2005). A similar phenotype is also present in RBPJ mutant mice, where Notch activity is absent (Oka et al., 1995; de La Pompa et al., 1997). As all these mutant mice display early lethality, no description is available to indicate whether neurogenesis is disturbed in the hypothalamus. Conditional loss-of-function mice lacking RBPJ, using *Nkx2.1-Cre* to specifically knock-out Notch signaling in the hypothalamus, shows that Notch signaling is essential for the differentiation of late arcuate hypothalamic neurons in the mouse from E13.5 (Aujla et al., 2013). This study did not identify a role in the initial neurons, but we would assume there would be an increase in the number of nTPOC and nMTT neurons in these mutant mice.

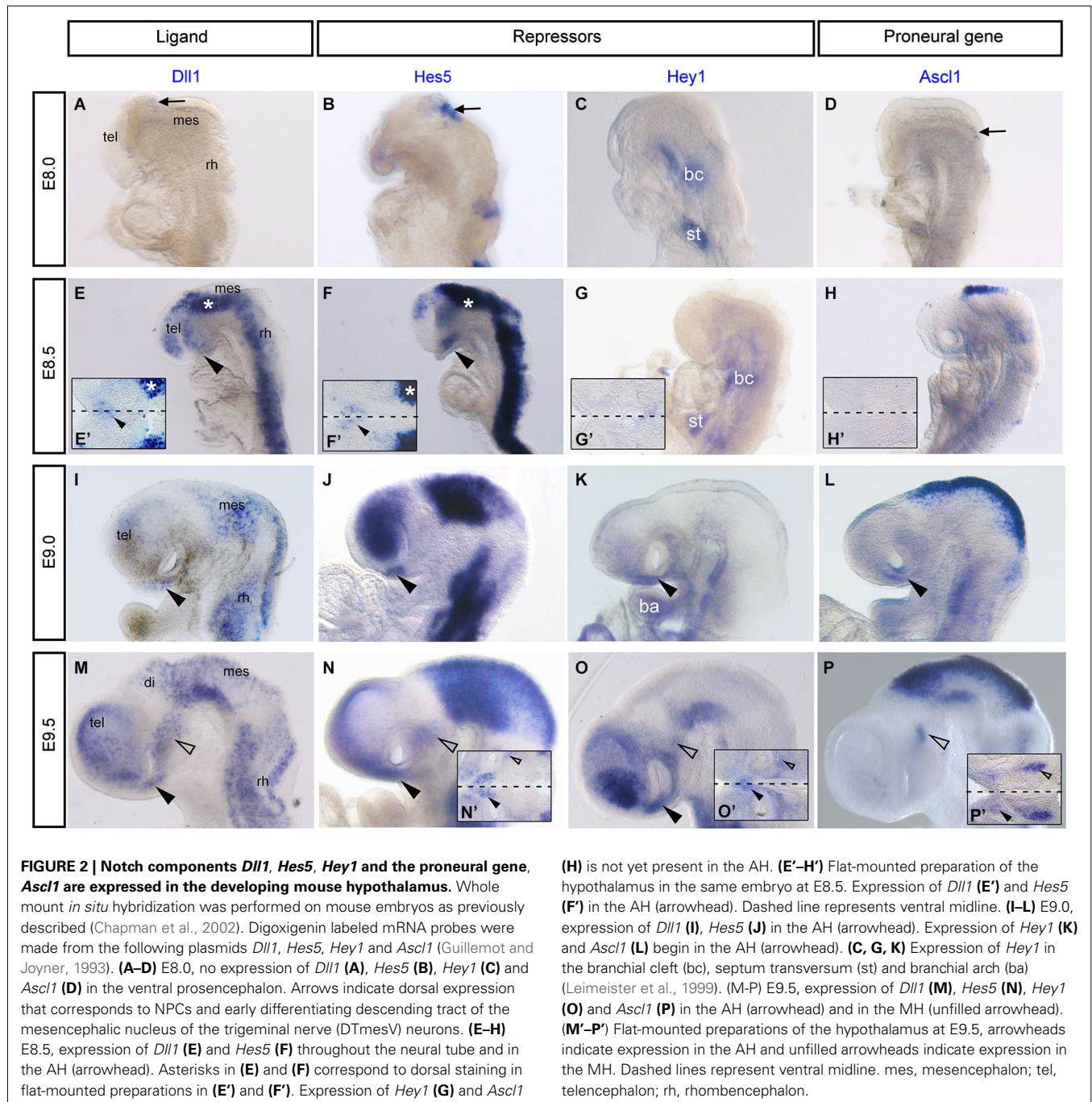
Many other knock-out or ectopic expression studies of Notch components describe an effect on neurogenesis throughout the embryo. The *Dll1* mutant mouse has not been well studied for a neurogenesis phenotype (Hrabe de Angelis et al., 1997; Przemeczek et al., 2003). However, *Dll1* does regulate primary neurogenesis in the *Xenopus* embryo (Chitnis et al., 1995).

There appears to be much redundancy between genes of the Notch pathway, which could explain why a function for Notch during nTPOC neuronal differentiation has not been described before in the mouse. For example, *Hes5* does not show any phenotype in single mutants (Cau et al., 2000). Double or triple knock-out mice produce more obvious phenotypes and prove redundancy occurs between these genes (Hatakeyama et al., 2004; Kageyama et al., 2008a). The absence of both *Hes1* and *Hes5* leads to aberrant neuronal localization. Interestingly, expression of *Dll1* and *Ascl1* is highly upregulated in the ventral diencephalon of E9.5 *Hes1/Hes5* double mutants as are the number of β III-tubulin (Tuj1) positive cells (Hatakeyama et al., 2004). The capacity of these bHLH proteins to do the same job, may also explain why there is discrepancy between their expressions in chick compared with mouse. For example, flat-mounted preparations of chick embryos at HH15 (Figure 3A, arrowhead) and HH14 (Ratié et al., 2013) confirm specific expression of *Hey1* in the AH, whereas expression is throughout the developing hypothalamus in the mouse (Figures 2O,O').

PRONEURAL GENE EXPRESSION IN THE VERTEBRATE HYPOTHALAMUS PRIMORDIUM

Induction of the Notch/proneural loop is essential in the developing hypothalamus as this will eventually lead to the correct number of cells differentiating into nTPOC and nMTT neurons as well as maintaining the progenitor population.

Ascl1 is a well-studied proneural bHLH transcription factor, its expression and function during early embryogenesis has been well described in many vertebrates (Johnson et al., 1990; Ferreira et al., 1993; Guillemot and Joyner, 1993; Jasoni et al., 1994; Mcnay et al., 2006). In zebrafish, *Ascl1* expression appears early



at 12 hpf in cells prior to the appearance of markers indicative of overt differentiation, by 16 hpf, *Ascl1* expression overlaps with the nTPOC (Allende and Weinberg, 1994; Ando et al., 2005). In the Notch inhibited chick model, embryos display an upregulation of *Ascl1* expression in the AH, overlapping the nTPOC (Ratié et al., 2013). Flat-mounted preparations of HH15 chick hypothalamus show that *Ascl1* expression overlaps with *Shh* expression in the AH (Figure 3B, arrowhead). The expression of *Ascl1* in the hypothalamus is examined further by *in situ* hybridization between E8 and E9.5 in the mouse embryo, like

with the Notch components, expression of *Ascl1* has been badly interpreted in this region (Figures 2D,H,L,P). *Ascl1* starts to be expressed in the developing hypothalamus at E9.0 (Figure 2L, arrowhead). At E9.5, flat-mounted preparations indicate that *Ascl1* is specifically expressed in a salt-and-pepper like pattern in the AH (Figures 2P,P', arrowhead) and in the MH (Figures 2P,P', unfilled arrowhead). *Ascl1* is important for the differentiation of late hypothalamic neurons because in *Ascl1* knock-out mice differentiation of neuroendocrine neurons is disturbed (McNay et al., 2006). Although the authors did not specifically look at the

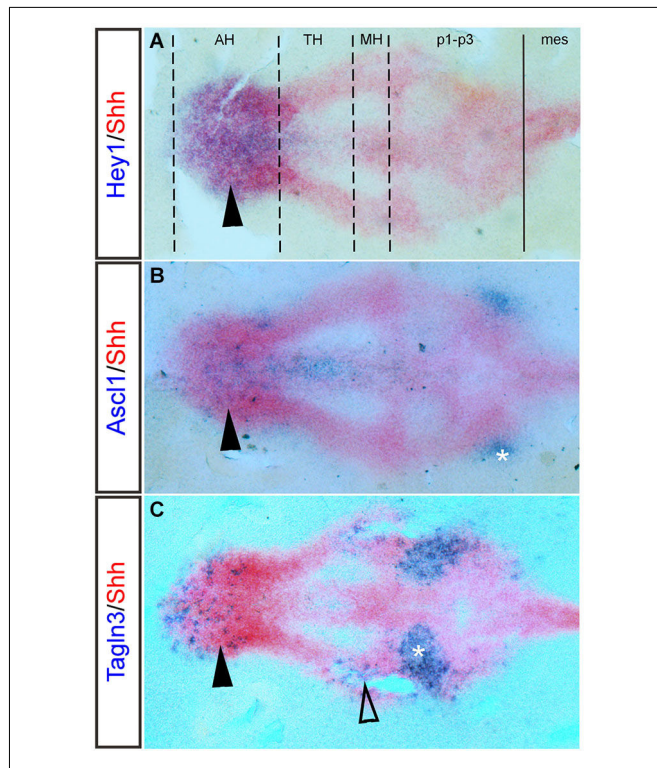


FIGURE 3 | *Hey1*, *Ascl1* and *Tagln3* expression overlaps with *Shh* in the chick hypothalamus primordium. Double labeling of *Hey1*, *Ascl1* and *Tagln3* (Purple, Digoxigenin labeled probes) at HH15 with the dynamic hypothalamic marker, *Shh* (Red, Fluorescein labeled mRNA probe) in chick confirms expression of these genes in the hypothalamic domains. **(A)** *Hey1* expression is specifically expressed in the anterior hypothalamus (AH) (arrowhead), overlapping the area where the nTPOC neurons will differentiate. Dashed lines indicate the boundaries of the hypothalamic domains, while the solid line marks the diencephalic-mesencephalic boundary (DMB). **(B)** *Ascl1* expression is located in the AH (arrowhead). Asterisk labels the nTPOC located in p1 (Ware and Schubert, 2011). **(C)** *Tagln3* expression is located in the AH (arrowhead) and in the mammillary hypothalamus (MH), overlapping where the nMTT neurons differentiate (unfilled arrowhead). Asterisk labels the ventral medial longitudinal fascicle (nMLF) located in p2 (Ware and Schubert, 2011). mes, mesencephalon; p1-p3, prosomeres 1–3; TH; tuberal hypothalamus.

nTPOC or nMTT neurons it can be assumed these neurons will be affected.

During initiation of neuronal differentiation various proneural genes are recruited, but the specific proneural genes involved could be different between species and neuronal populations. Here, the expression of other proneural genes has been researched in the developing hypothalamus. Remarkably, as *Neurog1/2* are not expressed in the hypothalamus (Ratié et al., 2013), *Ascl1* appears to be the only proneural gene expressed in the ventral chick AH, at least during early development. Lateral inhibition is the process controlling differentiation of these neurons but the precise mechanisms is different between chick and mouse. There are several lines of evidence to suggest this including restriction of *Ascl1* expression to the AH in chick (Figure 2B), where in mouse *Ascl1* is expressed in both the AH and MH (Figure 2P). To date, no other proneural gene has been described in the

developing chick MH. *Neurog1* and *Neurog2* are not found in the ventral hypothalamus of zebrafish and mouse (Ando et al., 2005; Mcnay et al., 2006; Osório et al., 2010), but a third member of the neurogenin family, *Neurog3* has been identified, specifically expressed in the AH (Wang et al., 2001; Villasenor et al., 2008; Pelling et al., 2011). *Neurog3* expression is regulated by *Ascl1* (Mcnay et al., 2006), but in *Neurog3* mutant mice there is no effect on early neurogenesis in the hypothalamus (Pelling et al., 2011; Anthwal et al., 2013). This lack of phenotype could be due to redundancy between the two proneural genes. It would be interesting to analyse *Ascl1/Neurog3* mutant mice to determine whether there is an additional defect in the formation of the nTPOC.

Additionally, in zebrafish and mouse, *Ascl1* and *Neurog3* may act together to control the processes of lateral inhibition leading to the differentiation of the nTPOC, whereas in chick differentiation is specifically regulated by *Ascl1*.

As the capacity to regulate differentiation steps during neurogenesis is shared by all the proneural genes (Guillemot, 2007), it may explain why neuronal differentiation in the vertebrate hypothalamus is not conserved.

DESCRIPTION OF PRONEURAL TARGET GENES WITHIN THE HYPOTHALAMIC PRIMORDIUM

In the absence of Notch activity during nTPOC differentiation in the chick hypothalamus, *Ascl1* is upregulated and induces expression of a wide spectrum of neuron specific genes (Castro et al., 2011; Ratié et al., 2013). While upregulation of some neuronal genes like *Nhlh1* or *Stmn2* is expected in tissue lacking Notch signaling, other genes identified are not associated with a role in hypothalamic development, such as Transgelin 3 (*Tagln3*) and Chromogranin A (*Chga*). *Nhlh1* and *Chga* mutant mice are available, but there is no phenotype or effect on neurogenesis, suggesting redundancy with other genes (Krüger and Braun, 2002; Hendy et al., 2006; Schmid et al., 2007). *Tagln3* appears to be a good marker because it is strongly expressed in the areas where both the nTPOC and nMTT form. In a flat-mounted preparation of HH15 chick hypothalamus, double labeling with *Shh* and *Tagln3* reveals expression of *Tagln3* in the AH and MH where the nTPOC and nMTT neurons are respectively located (Figure 3C, arrowhead and unfilled arrowhead). *Tagln3* is also expressed in the ventral MLF population, which are the first neurons to develop in the brain (Figure 3C, asterisk). While these target genes are all expressed in post-mitotic neurons (Theodorakis et al., 2002; Pape et al., 2008; Xie et al., 2008; Burzynski et al., 2009; Ratié et al., under review) no specific function can be attributed to these genes during nTPOC and nMTT development.

Another set of genes are specifically upregulated in the chick AH when Notch signaling is inhibited, *Slit1* and *Robo2*, which are well-known components involved in axon guidance (Chisholm and Tessier-Lavigne, 1999). They guide the TPOC axons through the hypothalamus (Devine and Key, 2008; Ricaño-Cornejo et al., 2011) and the regulation of these genes is Notch dependent (Ratié et al., 2013).

Analysis of the promoter regions in *Slit1*, *Robo2*, *Tagln3* and *Chga* reveal binding sites of *Hes5*, *Hey1*, *Ascl1* and *Nhlh1*

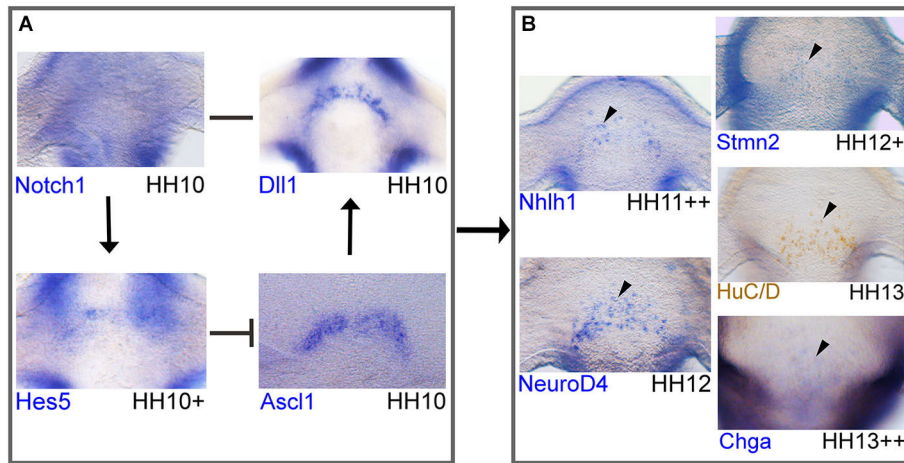


FIGURE 4 | Network of Notch/proneural genes and initial expression of downstream targets in the developing chick hypothalamus.

Whole-mount *in situ* hybridization or immunohistochemistry of markers in chick. Immunohistochemistry protocol has been described elsewhere (Lumsden and Keynes, 1989). Anti-HuC/D mouse (1:500; molecular probes; A21271) primary antibody was detected with a peroxidase-conjugated rabbit-anti-mouse secondary antibody (1:2000; Jackson ImmunoResearch; 315-035-045). Probes were obtained from cDNA and subcloned into pCRII-TOPO (Invitrogen) to make RNA probes or plasmids were obtained from other sources: *Dll1* and *Notch1* (kind gifts from Dr Frank Schubert).

(A, B) Frontal view of the AH in the developing hypothalamus where the nTPOC neurons will differentiate. This network of genes is based on *in silico* results and data from Ratié et al., 2013. Expression of all markers, except

Notch1 have a horseshoe shape. (A) Notch network loop in NPCs. *Notch1* expression is ubiquitous throughout the hypothalamus at HH10. *Dll1* and *Ascl1* expression in the hypothalamus at HH10. *Hes5* expression in the hypothalamus at HH10+. (B) Genes are upregulated in post-mitotic differentiating neurons. Expression of *Nhlh1* at HH11+ and *NeuroD4* at HH12. *Stmn2* expression at HH12+, *HuC/D* expression at HH13 and *Chga* expression, first appears at HH13++ in very few cells in the developing hypothalamus. Genes are expressed in a salt-and-pepper like pattern (arrowhead). Expression confirms Notch components and *Ascl1* are expressed first, followed by the expression of downstream targets. Arrows represent activation of downstream targets. Barred lines represent repression of downstream targets. A single line represents direct binding between ligand and receptor.

providing further evidence these target genes are part of the Notch/proneural regulatory network involved in neuronal differentiation in the hypothalamus (Ratié et al., 2013).

MOLECULAR CASCADE OF NEUROGENESIS ONSET IN THE CHICK HYPOTHALAMUS PRIMORDIUM

In order to corroborate this network of genes, the expression of Notch components and target genes is analyzed by *in situ* hybridization in the chick hypothalamus to provide further evidence for the existence of a molecular cascade that is Notch/proneural dependent.

The molecular cascade begins with the expression of Notch components and proneural genes followed by other bHLH transcription factors, target genes and well-known neuronal markers (Figure 4). *Notch1*, *Hes5*, *Dll1*, *Ascl1*, *Nhlh1*, *NeuroD4*, *Stmn2*, *HuC/D* and *Chga* are examples chosen to evaluate the stage of their first expression in the AH (Figure 4). At HH10, the first components to be expressed in the developing hypothalamus are *Notch1*, *Dll1* and *Ascl1*, followed by *Hes5* that form a regulatory loop (Figure 4A; Ratié et al., 2013). This mechanism has been well described in the literature for the induction of neurogenesis by lateral inhibition (Bertrand et al., 2002; Kageyama et al., 2008b).

Notch1 is present in the AH, ubiquitously expressed (Figure 4A) compared with *Dll1*, *Ascl1* and *Hes5* that are expressed in a salt-and-pepper like pattern with a horseshoe shape (Figure 4). This is in agreement with a lateral inhibition

model taking place in the AH. In this model, when *Ascl1* is active in a NPC, this can upregulate other bHLH genes such as *Nhlh1* (Ratié et al., 2013). *Nhlh1* and *NeuroD4* are analyzed as they are known markers of differentiation and expressed in the hypothalamus (Murdoch et al., 1999; Abu-Elmagd et al., 2001; Ratié et al., 2013). These genes are expressed from HH12, with *Nhlh1* expression appearing slightly earlier at HH11++ (Figure 4B). Other genes are upregulated from around HH13 such as, the well-known neuronal markers *Stmn2* and *HuC/D* but also new markers such as *Chga* (Figure 4B; Ratié et al., 2013).

BrdU labeling suggests *Dll1* expressing cells have exited the cell cycle (Henrique et al., 1995; Myat et al., 1996) therefore NPCs destined to become nTPOC neurons exit the cell cycle around HH10 as seen with *Dll1* expression (Figure 4A). It suggests that as early as HH10, the *Dll1* positive cells of the hypothalamus are destined to become neurons several stages before they become mature neurons expressing markers such as *Stmn2* or *HuC/D* at HH13. These results provide further evidence that the Notch/proneural loop is active in the hypothalamus from a very early stage before the first neurons appear.

CONCLUDING REMARKS

In this review, data has been discussed implicating the Notch/proneural network with a role during the differentiation of the first two groups of neurons that develop in the hypothalamus, the nTPOC and nMTT. There is still specific functional data

lacking in the hypothalamus to conclude the specific mechanisms in which these neurons differentiate, but a general picture using expression data and interpretation of other functional models has been achieved. The same Notch/proneural network is likely to regulate differentiation of nTPOC neurons in zebrafish, chick and mouse. Considering the conservation of the TPOC axon tract and the Notch signaling pathway, this is not surprising. Data regarding proneural gene expression in the chick MH is still too scarce to conclude, but some of the components of the Notch/proneural network are expressed in the mouse MH before the nMTT neurons differentiate. This expression suggests the same mechanisms occur between the nTPOC and nMTT, only the players for nMTT differentiation are yet to be found in chick.

It is still not known what triggers this Notch/proneural loop in these hypothalamic NPCs. Neuronal specification during spinal cord development is initially generated by activities of two competing signaling pathways: SHH and BMP/Wnt (Ericson et al., 1997; Jessell, 2000; Liem et al., 2000). Evidence is emerging to suggest that SHH and BMP may play a similar role in the differentiation of the early hypothalamic neurons (Manning et al., 2006; Ahsan et al., 2007; Szabó et al., 2009; Alvarez-Bolado et al., 2012). However, how these signaling pathways integrate the Notch/proneural network has to be investigated in the developing hypothalamus. Future work will require a study to identify transcription factors that are necessary for the patterning of the AH and MH very early during vertebrate development.

One thing is clear, this review highlights lots of open questions regarding initial neuronal differentiation in the hypothalamus as well as general patterning of the hypothalamic regions. We hope that this review will encourage the scientific communities to investigate the phenotype of their mutants during earlier stages when the nTPOC and nMTT neurons develop.

A final thought, distinct late hypothalamic cell types dysfunction can lead to metabolic or homeostatic disorders and there is evidence that this is the case in congenital obesity (Gibson et al., 2004; Bingham et al., 2008). Therefore, could a defect in the induction and specification of the initial neurons lead to such disorders as these neurons are essential to the axon tract formation of the late hypothalamic neurons (such as the MCH neurons) (Croizier et al., 2011).

AUTHOR CONTRIBUTIONS

Michelle Ware and Valérie Dupé set up and designed the experiments. Michelle Ware and Houda Hamdi-Rozé performed the experiments. Michelle Ware and Valérie Dupé wrote the manuscript. All authors read, discussed and edited the manuscript.

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❖ **Article 2 : Regulation of downstream neuronal genes by proneural transcription factors during initial neurogenesis in the vertebrate brain**

Neural Development; 2016

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Les travaux évoqués précédemment nous ont permis de mieux appréhender la mise en place de la voie NOTCH dans l'hypothalamus chez les modèles poulet et souris. Nous avons par la suite étudié la cascade moléculaire impliquant la voie NOTCH, les gènes proneuraux et plusieurs gènes cibles dans la mise en place des premiers neurones du cerveau antérieur.

Des travaux de l'équipe avaient permis d'identifier, chez l'embryon de poulet, de nouvelles cibles du réseau NOTCH/gènes proneuraux engagés dans la neurogenèse de l'hypothalamus *Tagln3*, *Chga* et *Cntn2* (Ratié et al., 2013, Ratié et al., 2014). Dans l'article ci-dessous, nous étudions les territoires d'expression de ces cibles, ainsi que de *Nhlh2* et *Stmn2* (marqueurs neuronaux déjà connus) chez les embryons de souris. Nous avons également étudié les domaines d'expression des gènes proneuraux *Ascl1*, *Ngn1* (Neurogénine 1) et *Ngn2* entre les stades E8.5 et E10.5 chez la souris et HH10 et HH18 chez le poulet. Nous avons notamment montré qu'au niveau de l'hypothalamus, les expressions de ces gènes sont restreintes et complémentaires. Ainsi, *Ascl1* est exprimé dans la partie antérieure de l'hypothalamus (au niveau des noyaux du tractus de la commissure post-optique ou nTPOC), alors que les neurogénines 1 et 2 sont exprimées dans les noyaux du tractus mamillotegmental ou nMTT.

Ce travail montre que, chez le poulet et la souris, ces marqueurs ont des domaines d'expression distincts, et les différentes populations neuronales ont chacune une « signature » avec une combinaison de marqueurs neurales bien spécifique et relativement conservée entre les deux espèces.

Pour étudier le lien spécifique entre la voie NOTCH et l'expression de *Ascl1*, *Nhlh1* et *Tagln3* dans le cerveau antérieur, nous avons utilisés des souris inactivées pour la voie NOTCH. Cependant, l'inactivation d'un des récepteurs ou d'un des ligands de la voie NOTCH n'entraîne qu'une faible diminution de l'activité NOTCH (Conlon et al., 1995 ; Przemack et al., 2003), en raison de la redondance de ces composants. Le moyen le plus efficace pour obtenir cette inactivation est donc d'invalider le gène *Rbpj*, l'effecteur principal de la voie NOTCH. Cependant, le knockout de ce gène entraîne la mort *in utero* des embryons à un stade trop précoce, avant la spécification des premiers neurones du cerveau antérieur (Oka et al., 1995). L'équipe a donc fait le choix de travailler sur des mutants conditionnels *Rbpj*, en utilisant un système de recombinaison Cre/loxP (Sauer, 1993). Dans

notre modèle, la Cre Recombinase est située en aval d'un promoteur ubiquitaire Rosa26 et est inductible au tamoxifène, ce qui nous permet de contrôler le stade exact à partir duquel la voie NOTCH est inhibée et donc de s'affranchir de la létalité embryonnaire précoce (souche **Rbpj^{L/L}; Rosa26-creER^{T2}**).

Chez les embryons de souris, lorsque la voie NOTCH est inactivée à partir de E8, nous avons constaté une surexpression d'*Ascl1*, de *Nhlh1* et *Tagln3* dans leurs domaines d'expression respectifs, avec une perte du motif poivre et sel normalement induit par le phénomène d'inhibition latérale. Ces résultats confirment le rôle de la voie NOTCH dans la régulation de ces gènes aux stades précoces du développement.

Afin d'étudier les conséquences de la surexpression d'*Ascl1*, nous avons effectué des expériences d'électroporation *in ovo* sur embryons de poulet où nous avons injecté, dans le tube neural en développement, de l'ARNm d'*Ascl1* murin. Nous avons ainsi montré qu'une surexpression d'*Ascl1* dans le mésencéphale induit une expression ectopique des marqueurs neuronaux *Nhlh1*, *Stmn2*, *Tagln3* et *Chga*. Ceci confirme la régulation de l'expression de ces gènes par *Ascl1*. Nous avons également induit, toujours par électroporation *in ovo*, la surexpression de *Ngn2*, qui donne des résultats similaires, ce qui montre une redondance fonctionnelle entre les gènes proneuraux.

RESEARCH ARTICLE

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Regulation of downstream neuronal genes by proneural transcription factors during initial neurogenesis in the vertebrate brain

Michelle Ware^{1,3}, Houda Hamdi-Rozé^{1,2}, Julien Le Fric¹, Véronique David^{1,2} and Valérie Dupé^{1*}

Abstract

Background: Neurons arise in very specific regions of the neural tube, controlled by components of the Notch signalling pathway, proneural genes, and other bHLH transcription factors. How these specific neuronal areas in the brain are generated during development is just beginning to be elucidated. Notably, the critical role of proneural genes during differentiation of the neuronal populations that give rise to the early axon scaffold in the developing brain is not understood. The regulation of their downstream effectors remains poorly defined.

Results: This study provides the first overview of the spatiotemporal expression of proneural genes in the neuronal populations of the early axon scaffold in both chick and mouse. Overexpression studies and mutant mice have identified a number of specific neuronal genes that are targets of proneural transcription factors in these neuronal populations.

Conclusion: Together, these results improve our understanding of the molecular mechanisms involved in differentiation of the first neuronal populations in the brain.

Keywords: Notch, Embryonic, Early axon scaffold, Neurogenin, Ascl1, Rbpj, Tagln3, Chga

Background

In the embryonic rostral brain, the first neurons differentiate in very specific domains and project axons to give rise to the early axon scaffold. This is an evolutionary conserved structure, formed from longitudinal, transversal and commissural axon tracts that act as a scaffold for the guidance of later axons [12, 55, 57, 59]. Each tract is formed from a small neuronal population, including the nucleus of the medial longitudinal fascicle (nMLF), the nucleus of the tract of the postoptic commissure (nTPOC), the nucleus of the mammillo-tegmental tract (nMTT), the nucleus of the tract of the posterior commissure (nTPC) and the nucleus of the descending tract of the mesencephalic nucleus of the trigeminal nerve (nmesV) (see Table 1 for abbreviations). Despite the importance of these tracts for ensuring the correct formation of later complex connections, the molecular mechanisms involved in differentiation and

specification of the neuronal populations that give rise to the early axon scaffold tracts has largely been ignored.

In all neuronal tissue, expression of specific neuronal transcription factors needs to be tightly controlled to ensure the correct patterning of neuronal populations both temporally and spatially [3]. This patterning is regulated in part by the Notch signalling pathway, which has remained highly conserved throughout vertebrate evolution. Lateral inhibition with feedback regulation allows Notch signalling to maintain the number of neural progenitor cells (NPCs) by controlling the number of neighbouring cells that can exit the cell cycle and subsequently undergo neural differentiation [14]. Cell cycle exit is controlled by a limited number of basic helix-loop-helix (bHLH) proneural genes that are both necessary and sufficient to activate neurogenesis [5, 28]. Loss of function studies indicate that proneural transcription factors direct not only general aspects of neuronal differentiation, but also specific aspects of neuronal identity within NPCs [23, 39, 60]. These proneural transcription factors include ASCL1 and members of the Neurogenin family. In many neuronal tissues these proneural genes are expressed in complementary domains

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Table 1 Abbreviations used throughout the paper

Cda	Circumferential descending axons
Di	diencephalon
dCortex	dorsal cortex
DMB	diencephalic-mesencephalic boundary
DTmesV	descending tract of the mesencephalic nucleus of the trigeminal nerve
Ep	epiphysis
LC	locus coeruleus
Mes	mesencephalon
MLF	medial longitudinal fascicle
MRB	mesencephalic-rhombencephalic boundary
MTT	mammilotegmental tract
nIII	nucleus of the oculomotor nerve
nIV	nucleus of the trochlear nerve
nmesV	nucleus of the descending tract of the mesencephalic nucleus of the trigeminal nerve
nMLF	nucleus of the medial longitudinal fascicle
nMTT	nucleus of the tract of the mammilotegmental tract
nTPC	nucleus of the tract of the posterior commissure
nTPOC	nucleus of the tract of the postoptic commissure
Os	optic stalk
p1, p2, p3	prosomere 1, prosomere 2, prosomere 3
pros	prosencephalon
Ptec	pretectum
Pth	prethalamus
Rh	rhombencephalon
Tel	telencephalon
TPC	tract of the posterior commissure
TPOC	tract of the postoptic commissure
vCortex	ventral cortex

[5, 13, 32, 37], suggesting that they contribute to the specificity of neuronal populations. In recent years, there has been emphasis on determining their downstream target genes, with proneural transcription factors playing a pivotal role in the transcriptional cascade that specifies neurons by activating general neuronal markers, either directly or indirectly [21]. Global profiling approaches are beginning to identify a large number of target genes that could be directly regulated by ASCL1 [2, 8, 16, 50, 58]. Recently, by inhibiting the Notch signalling pathway with the chemical inhibitor N-[3,5-difluorophenacetyl-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) during early chick development, new neuronal markers including Transgelin 3 (*Tagln3*), Chromogranin A (*Chga*) and Contactin 2 (*Cntn2*) were identified and introduced to a network of downstream proneural target genes [43]. Analysis of their expression, as well as the known neuronal markers, *Nhlh1* and Stathmin 2 (*Stmn2*), revealed interesting patterns

overlapping with the first neuronal populations of the early axon scaffold in the developing chick brain [44].

Identifying gene regulatory networks are essential for understanding the molecular cascades involved in subtype specification of neurons. Here, we describe the molecular cascade implicating Notch signalling, proneural genes and downstream targets at the level of the first neuronal populations that give rise to the early axon scaffold in both chick and mouse embryos. We identified several target genes that are known neuronal markers (*Nhlh1*, *Tagln3*, *Chga*, *Cntn2* and *Stmn2*), which are likely to play an essential role in the differentiation of these neuronal populations.

Methods

Chick embryos

Fertilised chicken (*Gallus gallus*) eggs were obtained from E.A.R.L. Les Bruyères (France). Eggs were incubated in a humidified incubator at 38 °C until the required developmental stages described according to Hamburger and Hamilton [19].

Generation and genotyping of mutant mouse embryos

To generate conditional RBPj knock-out mice, RBPj^{fl/fl} [20] mice were crossed with R26R^{creERT2} [3] mice. To activate cre recombinase, tamoxifen (Sigma) was dissolved in sunflower oil at a concentration of 10 mg/ml. 5 mg of tamoxifen was injected by intraperitoneal (IP) injection at embryonic day (E) 7.5 and embryos were harvested at E9.5. Heterozygous *Ascl1* delta null mutant mice were used in this study [18]. Genotyping of RBPj mutant embryos and *Ascl1* delta null mutant embryos was performed as previously described [7, 20]. Animal experimentation protocols were reviewed and approved by the Direction Départementale des Services Vétérinaires and are conformed to the European Union guidelines (RL2010/63/EU).

In ovo electroporation

The pCAGGS-IRES-nuclearGFP (pCIG) plasmid was used for control experiments. The overexpression constructs for rat *Ascl1* and mouse *Neurog2* were previously cloned into the pCIG plasmid [9]. The expression constructs were used at a concentration of 1 µg/µL⁻¹, with Fast Green (Sigma) added at 0.2% to facilitate visualisation of the DNA solution. The DNA solution was injected into the rostral neural tube of chick embryos at Hamburger and Hamilton stage (HH) 10-11, using a nanoinjector (Drummond Scientific). Electrodes were placed either side of the neural tube, targeting the mesencephalon. Five pulses of 15 V/50 ms were applied, using a square wave pulse electroporator (CUY21SC; Nepa Gene Co., Ltd). After electroporation, the eggs were sealed and incubated for a further 24 h.

In situ hybridisation and immunohistochemistry

All embryos were fixed in 4% PFA/PBS at 4 °C overnight, rinsed and processed for whole-mount RNA *in situ* hybridisation or immunohistochemistry. Anti-sense probes were generated either from plasmids cloned as previously described [43] or plasmids provided as a gift. The protocol for single and double *in situ* hybridisation has been previously described [43]. For double labelling, Digoxigenin and Fluorescein labelled probes were incubated together. The Digoxigenin antibody (Roche) was added first, followed by the NBT/BCIP reaction. After inactivation of the colour reaction, the embryos were fixed with 4% PFA overnight, then the Fluorescein antibody (Roche) was added, followed by fast red reaction (VectorRed). The immunohistochemistry protocol with anti-HuC/D (1:500; molecular probes; A21271) and anti-

neurofilament (1:1000; Invitrogen; 13–0700) has previously been described [30].

Results**Expression of neuronal markers during early development of the mouse brain**

Recently, a number of neuronal markers, described as part of the Notch/proneural network, were shown to be specifically expressed in the early neuronal populations of the chick brain [44]. To investigate the role of this network during formation of these neuronal populations in the developing mouse brain, the expression patterns of those markers, *Nhlh1*, *Tagln3*, *Chga*, *Cntn2* and *Stmn2* were analysed between E8.5 and E10.5 (Fig. 1). The conservation of gene expression was analysed by comparison with chick data (Table 2). Similar to the expression

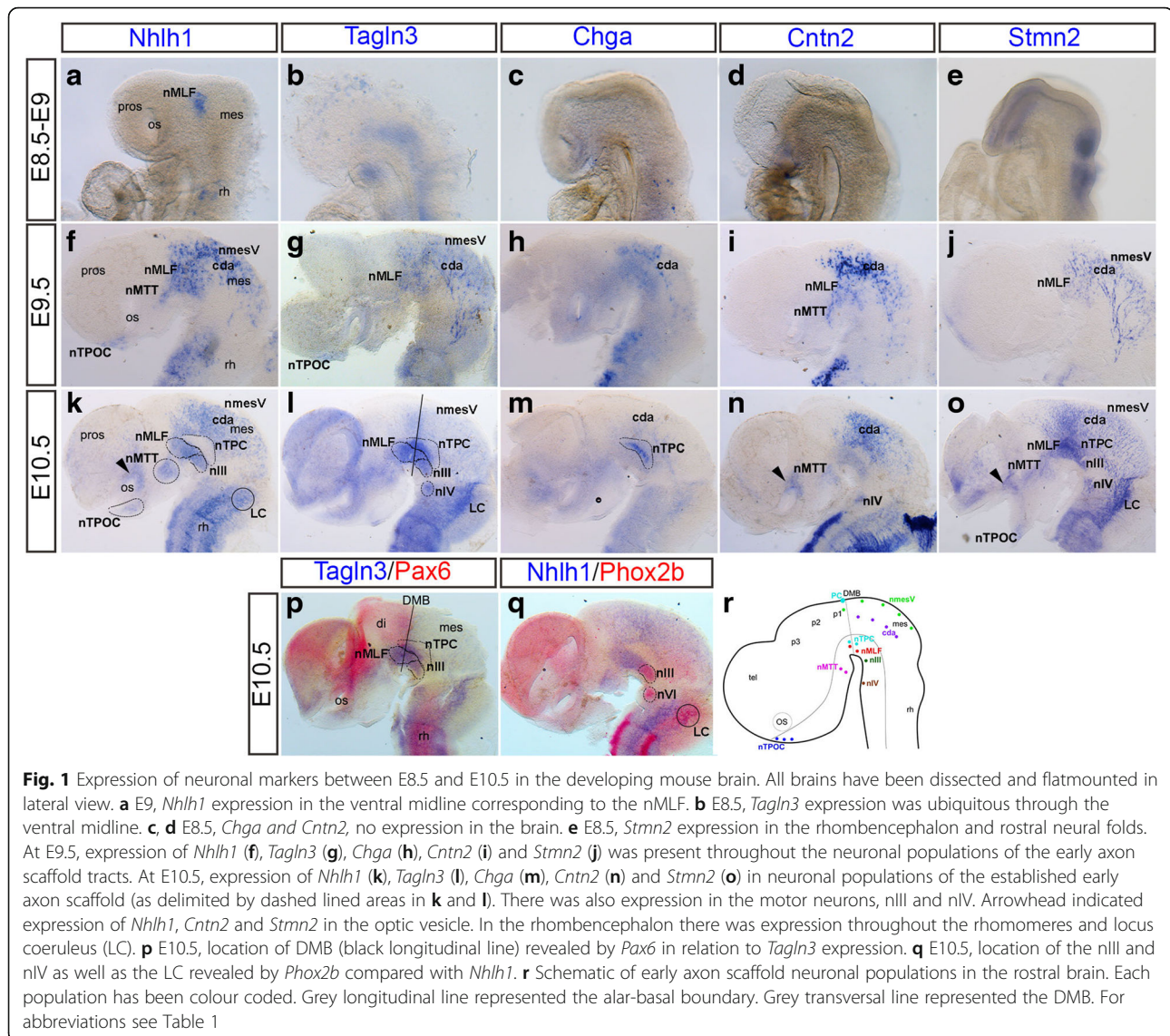


Table 2 Expression of *Nhlh1*, *Tagln3*, *Chga*, *Cntn2* and *Stmn2* in the developing chick and mouse brains

	Mouse E9.5-E10.5					Chick HH12-HH17				
	<i>Nhlh1</i>	<i>Tagln3</i>	<i>Chga</i>	<i>Cntn2</i>	<i>Stmn2</i>	<i>Nhlh1</i>	<i>Tagln3</i>	<i>Chga</i>	<i>Cntn2</i>	<i>Stmn2</i>
cda	✓	✓	✓	✓	✓	N/A	N/A	N/A	N/A	N/A
nmesV	✓	✓			✓	✓				✓
nMLF	✓	✓		✓	✓	✓	✓			✓
nMTT	✓	✓		✓	✓	✓	✓		✓	✓
nTPC		✓	✓		✓			✓		
nTPOC	✓	✓			✓	✓	✓	✓		✓
nIII	✓	✓			✓	✓	✓		✓	✓
nIV		✓		✓	✓	✓	✓		✓	✓

Ticks indicate where expression was present in the early axon scaffold populations and the motor neurons. Expression in the mouse brain between E9.5 and E10.5, compared in the chick brain between HH12 and HH17 (taken from [44] and Fig. 7)

patterns observed in the chick embryo [44], these neuronal markers were differentially expressed throughout the early neuronal populations in the brain (Fig. 1 and Table 2), cranial ganglia and spinal cord (data not shown) in the developing mouse embryo. We show that these genes were not pan-neuronal markers, but instead have characteristic expression domains at the level of these first neuronal populations developing in the brain.

At E8.5, there was no expression of these markers along the dorsal midline corresponding to the nmesV (Fig. 1a-e). This was surprising as the nmesV were the first neurons to arise in the rostral brain at E8.5 [12] and expression of *Nhlh1* and *Tagln3* predated the appearance of neurons in the chick brain [44]. *Nhlh1* expression was the first of these markers to be switched on in the ventral diencephalon corresponding to the nMLF (Fig. 1a). *Tagln3* was ubiquitously expressed throughout the ventral brain (Fig. 1b), while *Chga* and *Cntn2* were not yet expressed (Fig. 1c, d). *Stmn2* was expressed at E8.5 in the rostral prosencephalon and the rhombencephalon (Fig. 1e). At E9.5, expression of these markers were switched on in various neuronal populations (Fig. 1f-j and Table 2).

By E10.5, *Nhlh1*, *Tagln3* and *Stmn2* were expressed in almost all the neuronal populations of the brain (Fig. 1 k, l, o), while *Chga* and *Cntn2* were expressed more specifically (Fig. 1m, n). There was a clear gap between the circumferential descending axons (cda) and the nMLF where *Nhlh1* and *Cntn2* were not expressed (Fig. 1k, n), correlating to where the nTPC neurons were located. In contrast, *Tagln3*, *Chga* and *Stmn2* were expressed in the nTPC (Fig. 1l, m, o). Double labelling with *Pax6* (Fig. 1p) was used to mark the diencephalic-mesencephalic boundary (DMB) and confirmed the expression of *Tagln3* in the nMLF and nTPC within both the diencephalon and mesencephalon [33].

During development of the early axon scaffold, the oculomotor (III) and trochlear (IV) motor neurons also differentiated at the ventral midline. As the nucleus of

the oculomotor nerve (nIII) was not easily identifiable from the nMLF and nTPC at E10.5. Therefore, *Phox2b* was used as a specific marker of the motor neurons [40] to distinguish these populations (Fig. 1q). All the neuronal markers except *Chga* were expressed in the nIII (Fig. 1k-o). *Tagln3*, *Cntn2* and *Stmn2* were expressed in the nucleus of the trochlear nerve (nIV) (Fig. 1l, n, o).

While the expression of these markers in the mouse brain was largely conserved with chick, there were some subtle differences. For example, *Chga* was not expressed along the dorsal midline of the mesencephalon in the mouse (Fig. 1h, m and Table 2). Similar to chick, expression of *Cntn2* was not expressed in the nmesV along the mesencephalic roof, but in contrast *Cntn2* was expressed in the cda neurons in the mouse mesencephalon (Fig. 1i, n). Expression of the later markers, *Chga*, *Cntn2* and *Stmn2* in the mesencephalon at E9.5 suggested cda neurons were already present at this stage (Fig. 1h, i, j). The cda neurons were likely to be homologous to the tectobulbar neurons in the chick brain [27]. However, there was no expression of these neuronal markers in the same region of the chick mesencephalon suggesting differences in neuronal differentiation of these neurons (Table 2).

Having described the expression of these genes within the early neuronal populations in the mouse brain (Fig. 1r), the goal of this study was to determine what regulated the expression of these genes during initial neurogenesis in the rostral brain and during early axon scaffold formation. Having previously shown the involvement of the Notch signalling pathway in the expression of *Nhlh1*, *Tagln3*, *Chga*, *Cntn2* and *Stmn2* in chick, we first looked at the Notch/proneural network [43].

Expression of *Ascl1* and neuronal markers in the early neuronal populations in the brain was regulated by Notch signalling in mouse

So far, *Ascl1* has been the only proneural gene to have its expression described in detail during formation of the

early neuronal populations in the mouse brain. Expression was first detected in the brain at E8.0 in the nmesV before neuronal differentiation [34, 56]. We wanted to determine if the relationship between *Ascl1* and Notch signalling was similar to that already described in other central nervous system regions [47]. RBPj mutant mice have been commonly used to study the role of Notch inhibition [11, 36]. However, as the full RBPj knock-out mouse was embryonic lethal at E9, before the neuronal populations of the early axon scaffold tracts were fully established, we created a conditional mutant mouse by crossing RBPj^{fl/fl} [20] and R26R^{creERT2} mice [3]. Initially pregnant females were injected with 5 mg of tamoxifen at E6.5, before Notch signalling was active in the brain. However, the embryos displayed a typical Notch deficient phenotype with a strong developmental delay and it was not possible to compare brain development from this stage (results not shown). After injection of 5 mg tamoxifen, one day later at E7.5, we were able to rescue the early lethality and obtained RBPj^{fl/fl};R26R^{creERT2} embryos with an apparent similar morphology to the control embryos at E9.5. To confirm Notch signalling was knocked down in these embryos, *Hes5* expression was analysed (Fig. 2a, b; $n = 10$). *Hes5* was downregulated, but expression was not completely lost throughout the RBPj mutant brain (Fig. 2b). This result indicated a partial inhibition of Notch was established in these RBPj mutant embryos.

In the control embryos, *Ascl1* was normally expressed throughout the early neuronal populations, including the nTPOC, nmesV and nTPC (Fig. 2c, c'; $n = 10$). There was also expression along the dorsal and ventral rhombencephalon, the locus coeruleus (LC), the pretectum (Ptec) and the prethalamus (Pth) (Fig. 2c). Expression in the control brain was in a salt-and-pepper like pattern (Fig. 2c', arrowhead). When Notch signalling was knocked down, *Ascl1* expression was upregulated throughout the RBPj mutant brain and the salt-and-pepper like pattern was lost (Fig. 2d, d'; $n = 10$). Although *Ascl1* expression was upregulated, the neuronal populations remained identifiable. This showed that Notch signalling negatively regulates neurogenesis and that lateral inhibition involving *Ascl1* was implicated in the differentiation of the neuronal populations of the early axon scaffold tracts in mouse brain.

Compared to control embryos, there was no *Ascl1* expression in some regions of these RBPj mutant brains, such as, the Pth and nTPC. As *Ascl1* should be expressed in these populations already, this suggested there was already a developmental delay in these mutant embryos (Fig. 2d).

Using this RBPj mutant model, we also investigated the expression of the pan-neuronal markers, *Nhlh1* and *Tagln3* (Fig. 2e-h; $n = 5$). Both genes were upregulated

throughout the neuronal populations that give rise to the early axon scaffold tracts, which genetically confirmed expression of these genes was regulated by the Notch pathway (Fig. 2f, h).

Complementary and restricted expression of proneural genes in the developing mouse brain

As proneural genes are essential transcription factors for neurogenesis [5], we wanted to determine whether they played a role in regulating the expression of these neuronal markers. While the expression patterns of proneural genes have been widely described in populations throughout the peripheral and central nervous systems [18, 31, 32, 48], a detailed description during initial neurogenesis in the brain was lacking. Therefore, we first needed to confirm the expression patterns of proneural genes in these early neuronal populations. The expression patterns of *Neurog1* and *Neurog2* were analysed in the developing mouse brain in comparison to *Ascl1* (Fig. 3 and Table 3). Other proneural genes were not described here, such as *Atoh1*, which was not expressed in the ventral brain (data not shown) and *Neurog3* was only expressed in the developing hypothalamus [41, 52].

Ascl1 was first expressed in the brain from E8 along the dorsal midline of the mesencephalon [56]. *Neurog1* was also first expressed along the dorsal midline of the mesencephalon, slightly later at E8.5 (Fig. 3b). This expression of *Ascl1* (Fig. 3a) and *Neurog1* corresponded to the positioning of the nmesV. *Neurog2* was first expressed at E8.5 in the ventral brain, corresponding to the nMLF (Fig. 3c).

By E9.5, while *Ascl1* expression was mostly restricted to the dorsal midline of the mesencephalon (Fig. 3d), *Neurog1* expression expanded throughout the entire mesencephalon (Fig. 3e) and *Neurog2* was not expressed in the dorsal mesencephalon (Fig. 3f). At this stage, *Ascl1* was also expressed in the nTPOC, nTPC and Pth (Fig. 3d), *Neurog1* was expressed in the nMLF (Fig. 3e) and *Neurog2* was expressed in the nMTT, nMLF, the caudal thalamus (Fig. 3f; unfilled arrowhead) and in the dorsal optic vesicle (Fig. 3f; arrowhead).

At E10.5, *Ascl1*, *Neurog1* and *Neurog2* were differentially expressed throughout the early neuronal populations of the developing brain (Fig. 3g, h, i, j and Table 2). For example, both *Neurog1* and *Neurog2* were expressed in the caudal thalamus (Fig. 3h, i, unfilled arrowhead), the nMLF and the nIII (Fig. 3h, i), while *Ascl1* expression was restricted either side of the caudal thalamus in the Pth and in the Ptec (Fig. 3g). By E10.5, the mesencephalon contained both DTmesV neurons along the dorsal midline and cda neurons that were not clearly distinct from each other [33]. Expression of *Neurog1* overlapped with both the cda and nmesV (Fig. 3h), while *Ascl1* expression was more nmesV specific (Fig. 3g).

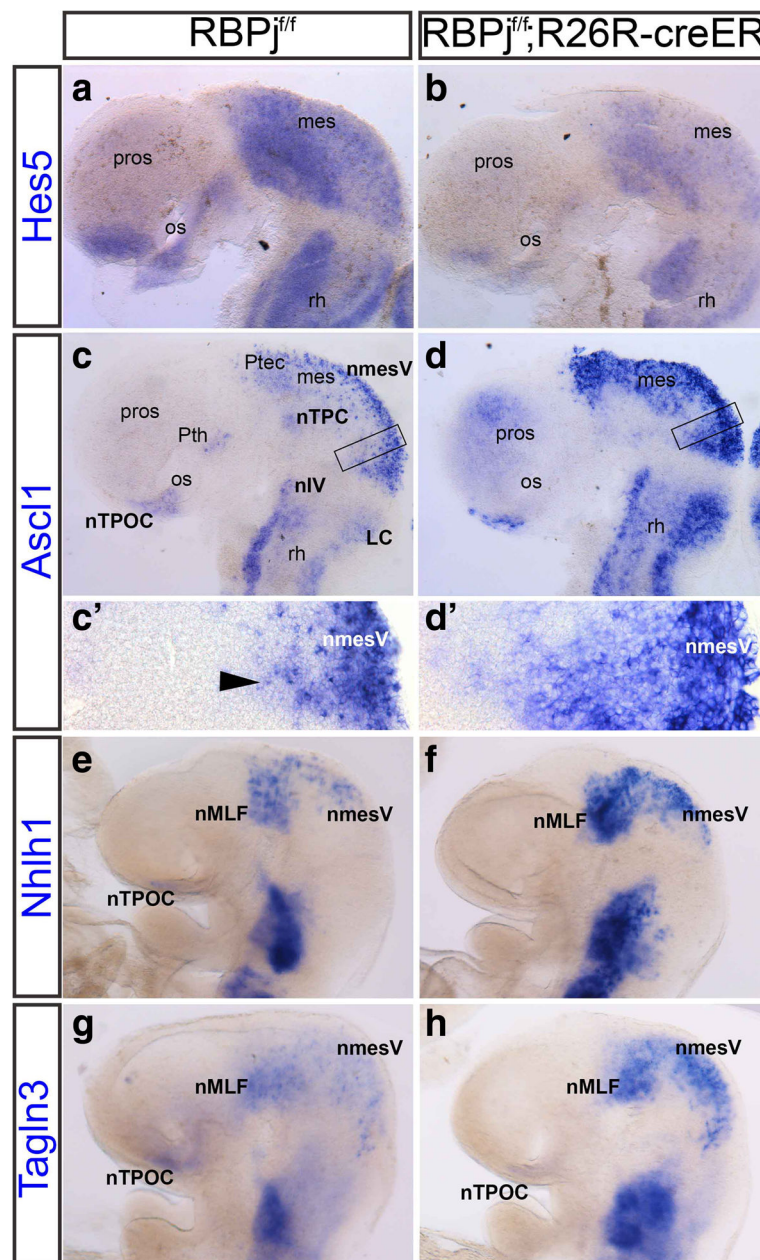


Fig. 2 Loss of Notch signalling affects expression of *Hes5*, *Ascl1*, *Nhlh1* and *Tagln3* in the mouse brain. **(a-d)** All brains have been dissected and flatmounted in lateral view. **e-h** Whole mount embryos. **a, b**, $n = 10$ Expression of *Hes5* at E9.5 within the embryonic mouse brain of the control **(a)** and RBPJ mutant **(b)**. **c, c'**, **d, d'**, $n = 10$ *Ascl1* expression in the neuronal populations, which give rise to the early axon scaffold tracts at E9.5 of the control **(c, c')** and RBPJ mutant brains **(d, d')**. Boxes in **c** and **d** indicate higher magnification in **c'** and **d'** respectively. Arrowhead indicates normal salt-and-pepper like expression of *Ascl1*. Control and mutant embryos were compared from the same littermates. **e, f**, $n = 5$ *Nhlh1* expression in control **(e)** and RBPJ mutant **(f)**. **g, h**, $n = 5$ *Tagln3* expression in control **(g)** and RBPJ mutant **(h)**. Expression of *Nhlh1* and *Tagln3* was upregulated throughout the brain. For abbreviations see Table 1

In the prosencephalon and mesencephalon, there was very little overlap between the expression of *Ascl1* and the two Neurogenin genes. The only exception was at the level of the nmesV (Fig. 3g, h, i; Table 3) where *Ascl1* and *Neurog1* expression overlapped. This mutual

exclusivity of proneural gene expression was especially obvious at the level of the nTPC and the cortex (Fig. 3g, h, i). With respect to the neuronal populations of the early axon scaffold tracts, the nTPC and nTPOC were the only populations to express a single proneural gene,

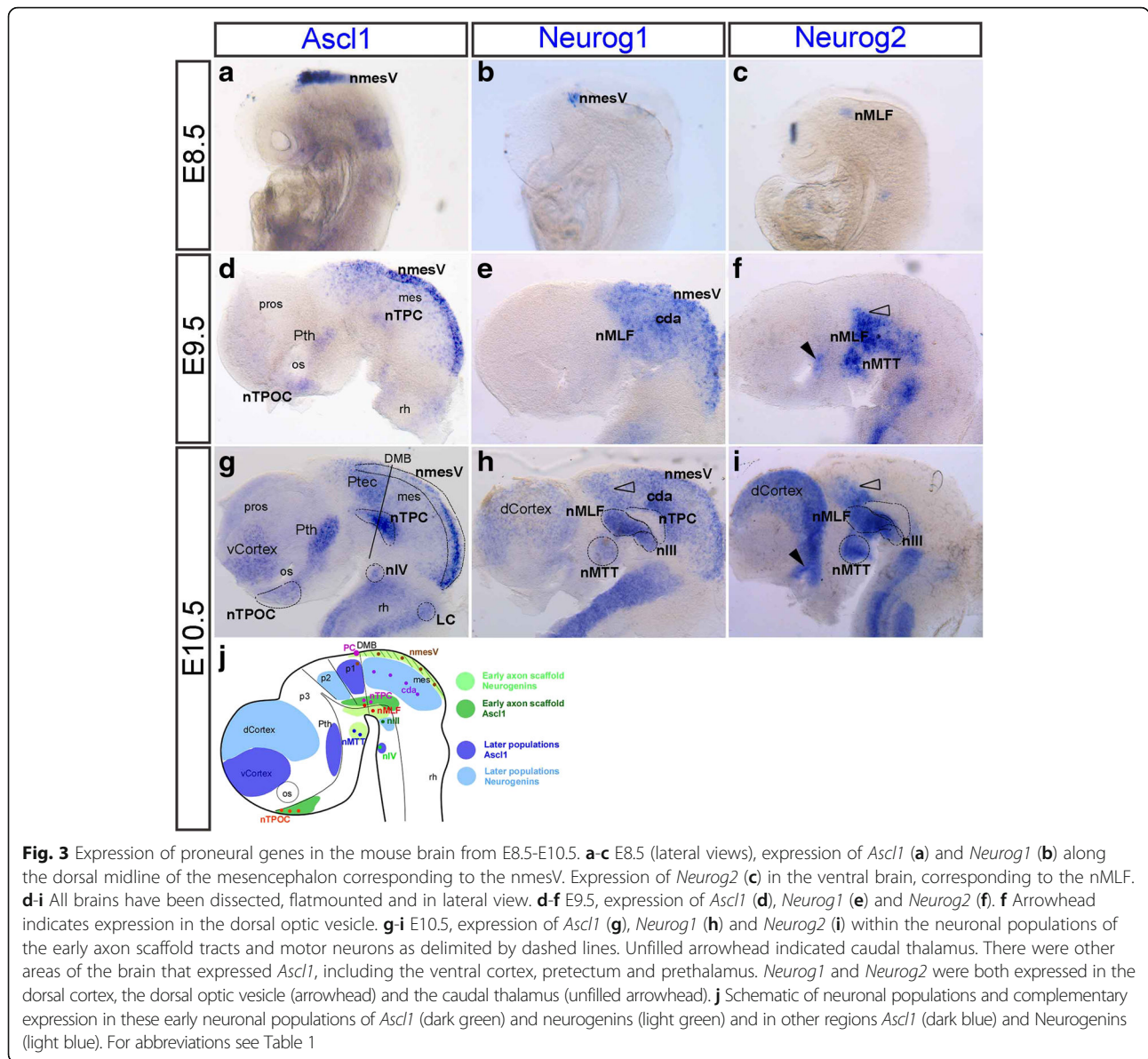


Table 3 Comparison of proneural gene expression in the chick and mouse brains

	<i>Ascl1</i>		<i>Neurog1</i>		<i>Neurog2</i>	
	Chick	Mouse	Chick	Mouse	Chick	Mouse
nmesV	✓	✓	✓	✓	✓	
nMLF			✓	✓	✓	✓
nMTT			✓	✓	✓	✓
nTPC	✓	✓				
nTPOC	✓	✓				
nIII			✓	✓	✓	✓
nIV	✓	✓	✓			

Ticks indicate where expression was located in early axon scaffold neuronal populations and motor neurons at HH18 in chick and E10.5 in mouse

Ascl1 (Fig. 3g). Although the nTPOC only expressed *Ascl1* here, *Neurog3* was also expressed in the hypothalamus, although not in this specific set of the early neurons [52, 53].

These expression studies have revealed a close relationship between proneural and neuronal markers in the developing mouse brain. In order to test whether the neuronal markers described in this study were specific targets of these proneural genes we decided to use the chick model. Therefore, we needed to determine whether expression of the proneural genes was conserved in the early neuronal populations by analysing and comparing the expression patterns of *Ascl1*, *Neurog1* and *Neurog2* in the developing chick brain.

Differential expression of proneural genes was highly conserved between the chick and mouse brains

In the developing chick brain, *Neurog2* was the first proneural gene to be expressed from HH8 in the progenitors that will give rise to the MLF neurons (Fig. 4c). *Ascl1* was first expressed in the brain at HH10 corresponding to the nTPOC (Fig. 4a). The expression of these proneural genes predated any of the downstream target genes and differentiated neuronal populations [44, 57]. *Neurog1* was first expressed in the brain from HH13 within the nmesV and nIII (Fig. 4b). Expression of *Ascl1* expanded to the nmesV from HH11 (data not shown), and then at HH14 the nTPC (Fig. 4d). By HH18, expression of *Ascl1* (Fig. 4g), *Neurog1* (Fig. 4h) and *Neurog2* (Fig. 4i) was in various neuronal populations of the early axon scaffold tracts and the motor neurons. *Neurog2* was expressed in the nMTT and dorsally above the MLF (Fig. 4h, arrowhead). Similar to mouse, the expression of these genes was mostly in complementary populations, expression of all three proneural genes only overlapped in the dorsal mesencephalon within the nmesV (Fig. 4g, h, i). *Neurog1* and *Neurog2* also overlapped in the nIII (Fig. 4h, i). From HH18, proneural genes were expressed in other neuronal populations of the brain. For example, expression of neurogenins dorsal to the MLF in both

chick and mouse corresponded to the caudal thalamus (Fig. 4g, h, i, unfilled arrowhead).

We showed that the expression of these proneural genes in the chick and mouse brains was highly conserved, however, there were some slight differences (Table 3). For example, *Neurog2* was expressed in the chick nmesV (Fig. 4i), but not in the mouse (Fig. 3i). Compared with mouse, there was less overlap of all the proneural genes in the chick as *Neurog2* was not as widely expressed throughout the populations in chick (Table 3). Interestingly, while the expression domains were conserved, the timing of expression was not always the same. For example, *Neurog2* expression was switched on first in chick (Fig. 4c), while *Ascl1* expression was switched on first in mouse. This was likely to be a reflection of the difference in timing of the first neuronal populations forming in the brain. The nmesV formed first in mouse [12] and the nMLF formed first in chick [57].

Expression of proneural genes overlapped with the expression of neuronal markers in the early neuronal populations of both the chick and mouse brains

Together, the proneural genes analysed here overlapped with the expression of all the neuronal markers in both the chick and mouse (Figs. 1, 3, 4). However, their

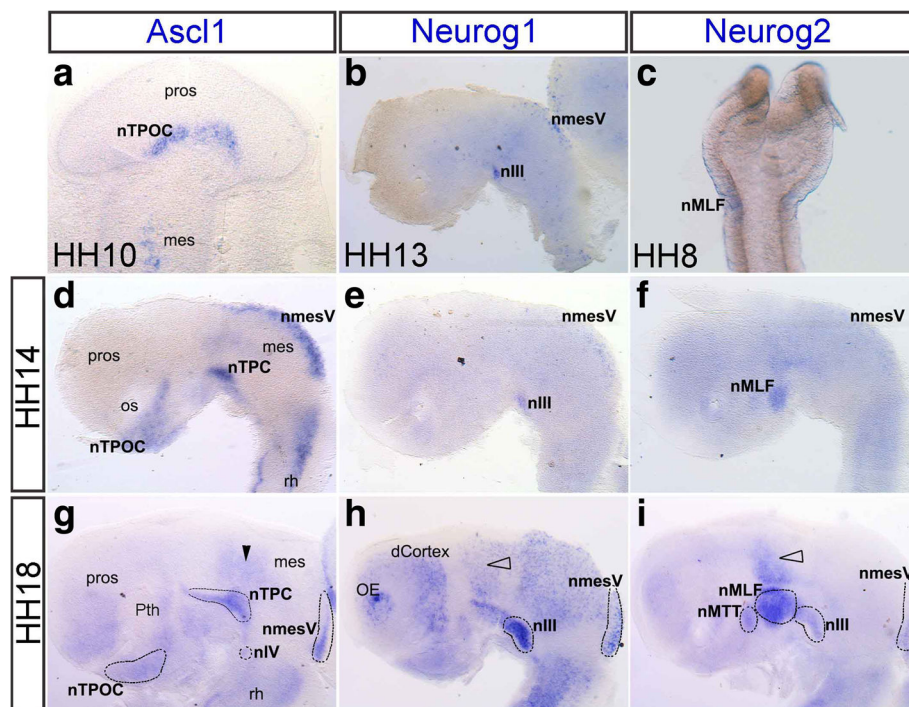


Fig. 4 *Ascl1*, *Neurog1* and *Neurog2* expression in complementary regions of the chick brain. **a-c** First expression of *Ascl1* (**a**, ventral view) at HH10 in the hypothalamus, *Neurog1* (**b**, dissected, lateral view) at HH13 in the mesencephalon and *Neurog2* (**c**, ventral view) at HH8 in the nMLF. **d-f** HH14 (dissected brain, lateral view). Expression of *Ascl1* (**d**), *Neurog1* (**e**) and *Neurog2* (**f**). **g-i** HH18 (dissected brain lateral view). Expression of *Ascl1* (**g**), *Neurog1* (**h**) and *Neurog2* (**i**). Expression in the pretectum (arrowhead). Expression in the caudal thalamus (unfilled arrowhead). For abbreviations see Table 1

expression did not correlate completely with either the domain of *Ascl1* or the neurogenins. In terms of neuronal marker expression, no single proneural gene completely overlapped with the complete expression of a target gene. *Tagln3* expression, for example, did not completely overlap with *Ascl1* (Figs. 11 and 3g). In chick, *Tagln3* expression was detected in the nMLF and *Neurog2* was the only proneural gene to be expressed in this region, while in mouse both *Neurog1* and *Neurog2* were expressed. This expression analysis suggested that different proneural genes were likely to regulate the same neuronal markers. In contrast to this observation, in both chick and mouse, *Chga* was specifically expressed in the nTPC with *Ascl1* being the only proneural gene in this population (Figs. 1m, 3g, 4g). To test this specificity, we overexpressed *Ascl1* and *Neurog2* in the chick brain.

***Ascl1* overexpression induced ectopic neuronal differentiation and misguided axon projection in the developing chick mesencephalon**

Previously, upregulation of *Ascl1* in other regions of the embryo led to increased number of neurons [4, 15, 24]. First, the identity of the cells that were electroporated and subsequently overexpressed *Ascl1* was investigated using HuC/D and Neurofilament pan-neuronal antibodies. Embryos were electroporated at HH10, just after neural tube closure, targeting the mesencephalic cells as the proneural and neuronal markers were not widely expressed in this region and there were few post-mitotic neurons (Fig. 5b, d). After 24 h, the number of HuC/D positive post-mitotic neurons increased when *Ascl1* was overexpressed in the chick brain (Fig. 5a, *a'* arrowhead; $n = 3$). These results confirmed that the *Ascl1* construct used here had the ability to induce neurogenesis in cells that were not yet destined to become neurons. Eventually neurons in this region will become tectobular forming the ventral commissure [57]. While HuC/D only showed an increase in the number of neurons, Neurofilament labelled both neurons and their projecting axons (Fig. 5c, d). Interestingly, some of these axons appeared to project along the same path as the DTmesV axons into the rhombencephalon (Fig. 5c, arrow). However, some axons were projecting rostrally back towards the diencephalic-mesencephalic boundary (DMB) (Fig. 5c', unfilled arrowhead), and some axons appeared to be curling back on themselves (Fig. 5c', arrowhead). These results confirmed neurons differentiated from cells that ectopically expressed *Ascl1*, however, their ability to follow the correct path was affected.

Overexpression of *Ascl1* and *Neurog2* caused ectopic expression of the same target genes in the chick brain

To establish a possible specificity of the proneural gene for one of the neuronal markers, we electroporated *Ascl1*

and *Neurog2* and analysed the effect on expression of the neuronal markers *Nhlh1*, *Tagln3*, *Chga* and *Stmn2*.

In embryos electroporated with the pCIG control plasmid ($n \geq 3$), no ectopic expression of *Nhlh1*, *Tagln3*, *Chga* and *Stmn2* was observed in cells expressing the control plasmid and each gene was normally expressed within the early neuronal populations (Fig. 6a, e, i, m). When either rat *Ascl1* (minimum $n = 3$ for each gene) or mouse *Neurog2* (minimum $n = 3$ for each gene) were overexpressed, cells that ectopically expressed the proneural gene, also expressed the markers *Nhlh1* (Fig. 6b, d), *Tagln3* (Fig. 6f, h), *Chga* (Fig. 6j, l) and *Stmn2* (Fig. 6n, p). As rat and mouse sequences were used, the ectopically expressing cells could be labelled specifically with a rat or mouse RNA riboprobe, therefore highlighting only the cells that were ectopically expressing the gene (Fig. 6; red). As only one half of the brain was electroporated, the other half acted as an internal control (Fig. 6c, g, k, o). The untransfected side of the embryo showed no ectopic expression of the gene and resembled the pCIG embryo. *Pax6* and *Sox10* were tested as negative controls to confirm the specificity of the electroporation, as they were not known to be downstream targets of proneural genes. When *Ascl1* was overexpressed, neither *Pax6* (Additional file 1: Figure S1A, B; $n = 3$) or *Sox10* (data not shown; $n = 3$) were upregulated. Together, these results suggested that both *ASCL1* and *NEUROG2* were able to regulate the same neuron specific genes tested here.

Loss of *Ascl1* led to discrete loss of *Tagln3* and *Chga* expression in the developing mouse brain

Ascl1 was specifically expressed in some neuronal populations where other proneural gene expression was missing, for example, in the nTPC (Fig. 3g). Therefore, to determine whether *Ascl1* had a specific role in the regulation of the neuronal genes within the early neuronal populations, *Ascl1* null mutant embryos were analysed to investigate the expression of the pan-neuronal gene *Tagln3* (Fig. 7; $n = 3$). Surprisingly, *Ascl1* null mutant embryos still expressed *Tagln3* in all of the neuronal populations at E10 (Fig. 7b), except the LC (Fig. 7b, unfilled arrowhead). The LC was already known to be affected in *Ascl1* mutant mice [22, 37]. We also investigated the expression of *Chga* in *Ascl1* null mutant embryos as its expression was more specific in the early neuronal populations (Fig. 1). Remarkably, in the *Ascl1* mutant embryos, *Chga* expression was specifically lost in the nTPC, while expression in the ganglia was not affected (Fig. 7d, d', filled arrowhead; $n = 2$). *Chga* expression was also downregulated in the cda and in the LC (Fig. 7d, unfilled arrowhead) compared with the control embryos.

Discussion

The organisation of the initial neuronal populations of the brain giving rise to the early axon scaffold has been

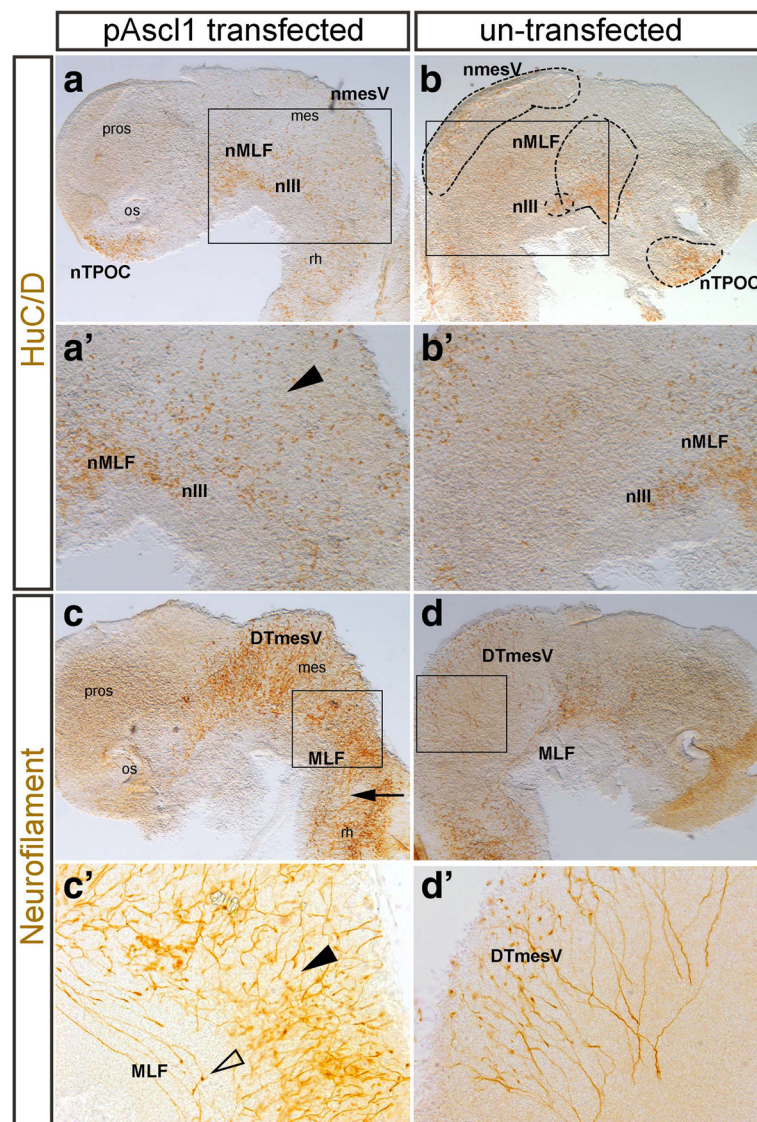


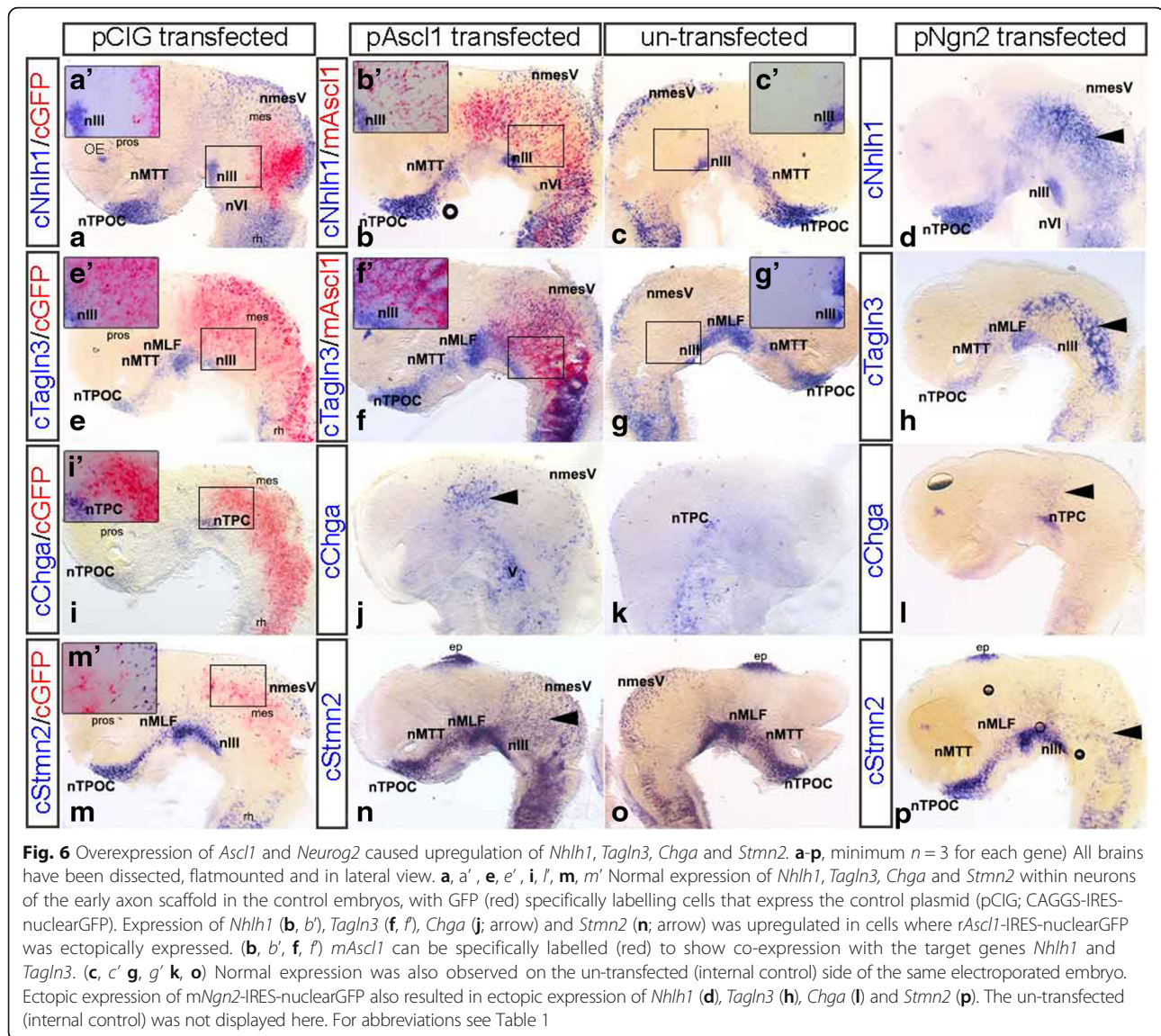
Fig. 5 *Ascl1* overexpression leads to ectopic neuronal differentiation. All brains have been dissected, flatmounted and in lateral view. **a, b, a', b'**; $n = 3$ The neuronal populations were labelled with HuC/D in the chick brain after electroporation with the p*Ascl1* plasmids. Box indicates higher magnification image. **a, a'** More HuC/D positive cells were visible in the mesencephalon (arrowhead). **b, b'** The un-transfected half of the brain showed normal distribution of neurons. **c, d, c', d'**; $n = 3$ The neuronal populations and their associated axon tracts were labelled with Neurofilament in the chick brain after electroporation with the p*Ascl1* plasmid. **c** There was an increase in the number of neurons and axons in the mesencephalon. Some of these neurons projected axons into the hindbrain (arrow), not seen in control side (**d**). Box indicates higher magnification image. (**c'**) Some axons did not project correctly. In the ventral brain axons projected rostrally towards the DMB (arrowhead) and other axons within the mesencephalon projected in a curved shape (arrowhead), not directly ventral like the axons in the control (**d**). **d, d'** Normal distribution of neurons and axons projected in the correct way. For abbreviations see Table 1

studied in great detail in zebrafish, chick and mouse [33, 57, 59]. However, the molecular mechanisms that underlie the specification of these early differentiating neurons remain undetermined. Our study shows that differentiation of these neurons is tightly regulated by the Notch/proneural network and reveals important new expression descriptions of proneural and neuronal markers in the early axon scaffold in both chick and mouse. This work adds further evidence to suggest evolutionary

conservation of the genetic mechanisms that control neuron differentiation between birds and mammals.

Expression of specific neuronal markers reveals genes that potentially play an essential role in the differentiation and specification of the populations that give rise to the early axon scaffold

Very few specific markers are described in the individual neuronal populations of the developing vertebrate brain



at early stages during the formation of the early axon scaffold tracts. This study describes 5 genes, *Nhlh1*, *Tagln3*, *Chga*, *Cntn2* and *Stmn2* that are expressed in specific neuronal populations and play a role in the Notch/proneural network. These are all known neuronal markers that mediate critical biological processes required to induce neuronal identity [35, 44]. *Nhlh1* and *Tagln3* are involved in fate determination, whereas *Chga*, *Cntn2* and *Stmn2* are expressed during terminal differentiation. There is some evidence that these neuronal genes play a specific role in determining the identity or function of these distinct neuronal clusters. For example, *Cntn2* has a role in the guidance of the MLF axons [61], and the specific expression of *Chga* in the nTPC in both the chick and mouse brains, suggests that nTPC may have a neuroendocrine function [49].

Despite the fact that *Nhlh1*, *Tagln3*, and *Stmn2* are considered pan-neuronal markers they have, to some extent, specific expression at the level of the first neurons establishing the early axon scaffold tracts in the amniote brain [55]. We show that each of these neuronal populations have a specific combination of these neurogenic markers during differentiation (Table 2). This means that very early during development these neurons acquire a specific identity. Most importantly, with a few exceptions, the expression pattern of these neuronal markers is highly conserved between chick and mouse (Table 2). Still, it is surprising to see that *Nhlh1* and *Tagln3* are not expressed in the mouse nmesV until after the first neurons differentiated at E8.5 [55], whereas *Nhlh1* and *Tagln3* are early markers for post-mitotic neurons in the chick [44]. Further analysis will be required to determine

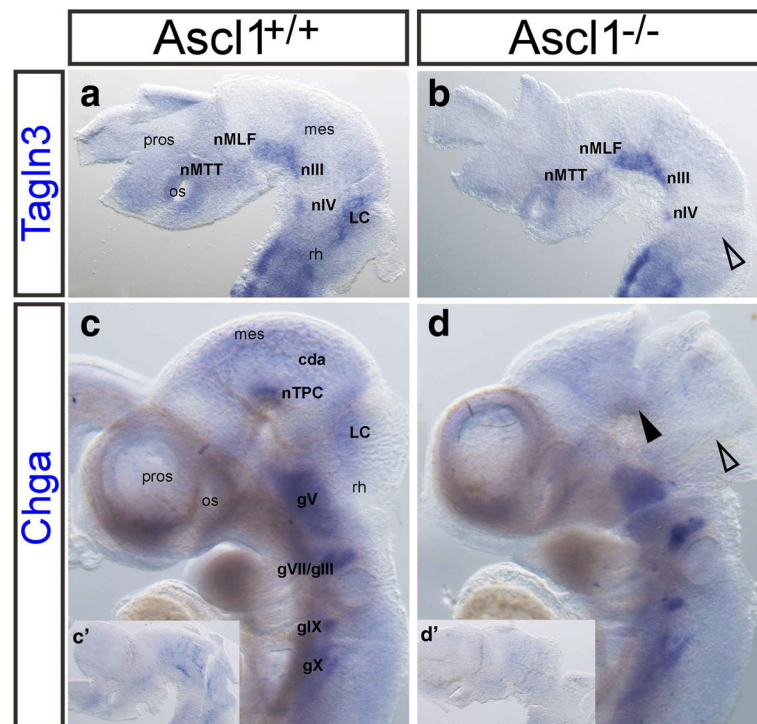


Fig. 7 Loss of *Ascl1* led to very specific downregulation of *Tagln3* and *Chga*. Expression of *Tagln3* in control (**a**, $n = 3$) and *Ascl1* null mutant embryos (**b**, $n = 3$). Expression was lost specifically in the locus coeruleus (LC; unfilled arrowhead). (**c**, **d**) Whole mount embryos. Expression of *Chga* in control (**c**, $n = 3$) and *Ascl1* null mutant embryos (**d**, $n = 2$). Expression was specifically lost in the nTPC (filled arrowhead), LC and cda. (**c'**, **d'**) Inserts indicate *Chga* expression in flatmounted brains in lateral view of the embryos in **c** and **d**. For abbreviations see Table 1. gV: trigeminal ganglion; gVII/VIII: facial and vestibulocochlear ganglia; gIX: petrosal ganglion; gX: nodose ganglion

the function of this discrepancy as ultimately these neuronal populations express the same genes in both the chick and mouse brains.

A relationship between Notch signalling, proneural genes and downstream targets is essential for the correct patterning of early neuronal populations in the developing vertebrate brain

Numerous studies support the idea that the Notch signalling pathway and proneural genes act together in a feedback loop to promote initial neurogenesis [5, 10, 29, 43]. However, in the developing brain, this has only been observed in the chick embryo via DAPT treatment [43]. By the inhibition of Notch signalling, this study confirms the role of Notch signalling in the Notch/proneural molecular circuitry that operates within the developing mouse brain similar to the other neural structures to control neurogenesis.

Compensation by proneural genes is not neuronal population specific

We show that a complex pattern of proneural gene expression exists during the generation of the initial neuronal populations in the brain. This seems to be the general situation in most regions of the central nervous

system [32]. Therefore, it is not surprising that *Ascl1* and *Neurog1/2* play a central role in the selection of neuronal progenitor subtypes by regulating downstream target genes [2, 5, 13, 37]. Genomic approaches (CHIP on chip, ChIP-seq and RNA-seq) are powerful tools that have led to the identification of hundreds of targets of ASCL1 [6, 8] and NEUROG2 [28]. However, the relationship between the proneural genes and these target genes, is yet to be functionally shown. In the present study, as the neuronal markers *Nhlh1*, *Tagln3*, *Chga*, *Cntn2* and *Stmn2*, are expressed in very similar expression patterns to the proneural genes, we propose that precise proneural genes regulate expression of specific neuronal genes, including, in specific neuronal populations of the early axon scaffold tracts.

Interestingly, we show that the nTPC has a very specific expression identity. These neurons do not express the pan-neuronal markers *Nhlh1* and *Tagln3*, they are the only neurons to have a strong expression of *Chga*, and *Ascl1* is the exclusively expressed proneural gene. Furthermore, in both the chick and mouse brains, expression of *Chga* is excluded from neuronal populations expressing *Neurog1* and *Neurog2*. This observation strengthens the argument for a specific function of ASCL1 in the development of specific neuroendocrine

neurons [34], and this is in accordance with the down-regulation of *Chga* in the *Ascl1* null mutant embryo.

This study shows that regulating expression of the target genes analysed here is not specific to either the over-expression of *Ascl1* or *Neurog2*, suggesting proneural genes are functionally equivalent (at least to induce neuronal identity). Indeed, while proneural genes are expressed in complementary regions, there are numerous studies that show they are able to compensate for each other [26, 37, 45]. It has been demonstrated that *Neurog2* has the capacity to rescue the development of *Ascl1*-dependent neurons [34, 37]. It is therefore not surprising that in the *Ascl1* null mutant embryos, the expression of *Tagln3* is not downregulated in neuronal populations expressing more than one proneural gene. This suggests there is compensation of other proneural genes in these populations. However, *Tagln3* expression is not downregulated in the nVI where *Ascl1* is the exclusively expressed proneural gene is unexpected. Other known proneural genes, *Neurog1*, *Neurog2*, *Neurog3* and *Atoh1* seem to be not expressed in the nVI. What is regulating *Tagln3* here is yet to be determined.

The highly conserved expression patterns of the proneural genes in the early ventral forebrain argue against a model of stochastic induction. An important selection pressure may exist to maintain this complementary proneural gene expression within the chick and mouse brains. We still have to determine why these neuronal target genes are expressed in some populations but not others, especially if these genes can be regulated by any proneural gene. It has been demonstrated, that these proneural genes are not always functionally equivalent and this capacity appears to vary in different regions of the nervous system [37]. How the divergent function of the proneural genes is established remain ambiguous. Further analysis of mice containing targeted mutations in both the *Ascl1* and *Neurog2* genes should be informative in answering this question.

The regional cues are likely to be involved in controlling the position of the various neuronal populations that give rise to the early axon scaffold tracts

Questions still remain, including what is controlling the specification of the individual neuronal populations that give rise to the early axon scaffold tracts and other early populations.

If proneural genes can regulate the same target genes, we still need to determine the specific genes or combination of genes (in a cascade) that regulate identity of each individual neuronal population of the early axon scaffold. Although a single proneural gene is sufficient to induce neuronal features, the additional expression of other factors is necessary to generate specific identity, for example, in fibroblasts [51, 54]. Thus, there is another layer of

complexity with other regional cues such as those produced by homeobox genes [17, 42]. Specification of neurons in the neural tube relies on combinations of bHLH and other transcription factors to activate or repress specific neurogenic programs. Homeobox genes, such as, *Sax1* could play a role in specifying the nMLF subtypes [46], as gain of function of *Sax1* results in an enlargement of the nMLF area [1]. However, other homeobox genes need to be found in order to explain the patterning of the neuronal populations of the early axon scaffold tracts.

Initially, a critical step is the establishment of morphogen gradients controlling the distinct sets of transcription factors resulting in the establishment of progenitor domains [25]. Such a mechanism has not yet been described during the establishment of the progenitor domains of the axon scaffold. It may be a different mechanism, as these populations of neurons are not distributed along specific axis. Sonic hedgehog (SHH), one of the main signalling molecules involved in neurogenesis patterning [38] is differentially expressed in the ventral forebrain [56] and mostly likely plays a critical role in the formation of the early axon scaffold tracts [1].

Conclusions

The organisation of the brain is more complex and harbours a greater diversity of neurons compared with the spinal cord. However, to our knowledge, no study investigating the specification of the neuronal populations that give rise to the early axon scaffold in any mutant mouse models has been done. Our present study gives essential tools to explore more accurately the formation of these neuronal populations in mutant models. This will provide a better understanding of how these early neurons differentiate in a specific territory with a specific identity.

Additional file

Additional file 1: Figure S1. *Ascl1* overexpression did not cause ectopic expression of *Pax6*. (a, b, n = 3) Brain was dissected, flatmounted and in lateral view. There was no upregulation of *Pax6* when the embryo was electroporated with the pAscl1 plasmid, which confirmed the specificity of the plasmid. The un-transfected side also showed normal expression of *Pax6*. For abbreviations see Table 1. (PDF 486 kb)

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Availability of data and material

All data generated or analysed during this study are included in this published article [and its supplementary information files].

Authors' contributions

M.W. and Va.D. set up and designed the experiments. M.W., H.R. and J.F. performed the experiments. M.W., Ve.D. and Va.D. wrote the manuscript. All authors discussed and edited the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Animal experimentation protocols were reviewed and approved by the Direction Départementale des Services Vétérinaires and are conformed to the European Union guidelines (RL2010/63/EU).

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PARTIE 2 : RELATIONS ENTRE LA VOIE NOTCH ET LA VOIE SHH DANS LE DEVELOPPEMENT DU CERVEAU ANTERIEUR

❖ Article 3 : The NOTCH pathway is a novel regulator of SHH signaling during early brain development.

En préparation

Houda Hamdi-Rozé, Michelle Ware, Hélène Guyodo, Aurélie Rizzo, Maïlys Rupin, Véronique David and Valérie Dupé.

Au cours du développement, *Shh* est exprimé dans tout le tube neural ventral y compris l'hypothalamus. Tôt au cours du développement, les expressions des effecteurs de la voie NOTCH et SHH colocalisent dans l'hypothalamus (cf article 1). Au cours de ce travail, je me suis attachée à décrire les interactions existant entre ces 2 voies qui pourraient expliquer l'implication de la voie NOTCH dans l'apparition de l'HPE.

Nous avons tout d'abord décrit la cinétique de l'expression de la voie NOTCH et SHH au cours du développement précoce du cerveau antérieur. J'ai donc effectué des hybridations *in situ* ciblant les gènes *Hes5*, *Nkx2.1* et *Ptch1* sur le modèle poulet. Elles montrent que la voie SHH est active dans l'hypothalamus avant la mise en place de la voie NOTCH dans cette région (à partir de HH10 chez le poulet), et que cette activité NOTCH est restreinte à la partie antérieure de l'hypothalamus.

Pour comprendre la relation entre les voies NOTCH et SHH, nous avons étudié l'expression de la voie SHH chez des embryons de poulets (inhibition au DAPT) ou de souris (*Rbpj*^{-/-}) inactivés pour la voie NOTCH. Nous avons observé, chez les deux modèles, une perte d'expression spécifique du gène *Shh* et de ses cibles *Nkx2.1* et *Ptch1* dans l'hypothalamus antérieur. L'expression de *Shh* est conservée dans le reste du tube neural. Ces résultats suggèrent une régulation spécifique de la voie SHH par la voie NOTCH au niveau de l'hypothalamus antérieur.

Afin d'étudier les conséquences de cette perte de l'activité SHH dans l'hypothalamus antérieur, nous avons étudié le phénotype cérébral chez notre modèle conditionnel *Rbpj*^{L/L}; Rosa26-creER^{T2} (cf. article 2). Nous avons constaté qu'une inactivation de la voie NOTCH juste avant sa mise en place dans l'hypothalamus induit une perte d'expression de *Shh* et de *Nkx2.1* ainsi qu'une hypoplasie du télencéphale.

Ces résultats montrent par ailleurs que NOTCH maintient l'activité SHH dans l'hypothalamus antérieur à un moment précis du développement, entre E8 et E9.

Pour approfondir l'étude de l'interaction génétique entre ces deux voies de signalisation, et étant donné que les modèles *Shh*^{-/-} et *Rbpj*^{-/-} présentent des malformations majeures, nous avons décidé d'étudier un modèle présentant une diminution partielle et simultanée des deux voies, le modèle double hétérozygote *Shh*^{+/-}, *Rbpj*^{+/-}.

Les individus doubles hétérozygotes étaient viables et d'apparence morphologique normale. Une étude histologique a néanmoins montré, chez des embryons âgés de E18.5, une glande hypophysaire dysmorphique chez les doubles hétérozygotes *Shh*^{+/-}; *Rbpj*^{+/-}. Chez ces individus, l'hypophyse s'insère dans un foramen présent au niveau du basisphénoïde. Ce foramen est dû à la persistance du canal pharyngo-hypophysaire, un vestige de la liaison entre la poche de Rathke et l'ectoderme oral lors du développement de l'hypophyse.

La persistance du canal pharyngo-hypophysaire est la conséquence de la malformation de l'hypophyse, dont le développement est sous le contrôle de l'activité SHH dans l'hypothalamus. Ces résultats rejoignent ceux observés dans la littérature chez des modèles où la voie SHH est inhibée (*Khonsari et al., 2013*). Par ailleurs, le phénotype observé chez ces doubles mutants hétérozygotes est similaire à celui décrit chez des formes mineures de patients avec une holoprosencéphalie (*Kjaer, 2015*). Ce travail nous a permis de générer le premier modèle souris pour cette forme d'HPE.

Dans cet article nous montrons que la voie NOTCH régule l'activité SHH dans la partie antérieure de l'hypothalamus, pendant une courte fenêtre temporelle au cours du développement cérébral. La voie NOTCH peut donc être ajoutée à la multitude de régulateurs impliqués dans le maintien de l'activité SHH. Cette observation ajoute un élément supplémentaire à l'hypothèse de travail de l'équipe selon laquelle l'HPE est une pathologie multigénique nécessitant l'accumulation de variants hypomorphes dans différents gènes afin de produire un phénotype. Ce phénotype serait plus ou moins sévère selon les gènes impliqués. Nous contribuons ainsi à la compréhension des mécanismes moléculaires engagés dans la grande variabilité phénotypique observée chez les patients HPE.

The NOTCH pathway is a novel regulator of SHH signalling during early brain development

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Running title: Notch regulates Shh signalling during brain development

Abstract

One important signalling pathway for development of the brain is Sonic Hedgehog (SHH). In humans, SHH haploinsufficiency is the predominant cause of holoprosencephaly, a structural brain malformation with phenotypical heterogeneity and incomplete penetrance. To fulfil its function during early development, *SHH* must be strictly expressed in a temporally and spatially defined manner. Genetic approaches in mice have revealed several signalling pathways implicated in the tight regulation of *Shh*. However, there are still some shadow areas regarding the regulation of *Shh* during forebrain development. In this study, we have used a genetic approach in mouse and a chemical inhibition approach in chick to demonstrate that activation of the NOTCH pathway is necessary to maintain *Shh* expression in a specific

area of the ventral forebrain, the anterior hypothalamus. Our data also shows that haploinsufficiency of the SHH and NOTCH signalling pathways synergise to produce midline defects reminiscent to holoprosencephaly, such as a malformed pituitary gland. These results provide new molecular mechanisms underlying the extreme phenotypic variability observed in human holoprosencephaly.

Introduction

The development of the vertebrate brain is complex and requires a tightly controlled spatial and temporal orchestration of numerous signalling pathways. Among these pathways is Sonic Hedgehog (SHH), an essential morphogenetic signal dictating cell fate decisions during early forebrain development (Lupo et al., 2006). Both embryological evidence in chick and genetic evidence in mouse have shown that SHH secreted by the axial mesoderm underlying the neural plate initiates development of the ventral midline in the brain (Chiang et al., 1996; Dale et al., 1997). SHH then induces expression of itself in the ventral midline of the developing forebrain, which will become the hypothalamus primordium. Subsequently, during hypothalamic patterning, SHH also acts as a local signal to subdivide the developing hypothalamus in subregions (Shimogori et al., 2010; Xie and Dorsky, 2017). From rostral to caudal regions, the presumptive hypothalamus can be subdivided into three regions, the anterior, tuberal and mammillary hypothalamus. The anterior hypothalamus is the first to produce differentiated neurones giving rise to the tract of the postoptic commissure (Ware et al., 2015). Each hypothalamic region has distinct patches of nuclei and SHH signalling is essential for the differentiation of these resident neurons (Alvarez-Bolado et al., 2012; Corman et al., 2018). In addition, recent advances have shown, in both the chick and mouse brains, that SHH produced in the hypothalamus is essential for development of the infundibulum and Rathke's pouch that will form the pituitary gland (Carreno et al., 2017; Fu et al., 2017; Zhao et al., 2012). Therefore, accurate regulation of *Shh* expression is crucial to pattern the ventral region of the brain, as well as, for cell proliferation and cell specification (Corman et al., 2018; Fu et al., 2017). Therefore, to ensure correct formation of the forebrain, *Shh* must be expressed in a temporally and spatially defined manner. The complexity of this regulation is such that numerous signalling pathways (i.e. NODAL, FGF, BMP) have been found to regulate *Shh* transcription during early brain patterning (Bertrand and Dahmane, 2006).

Consistent with its crucial role during development, *SHH* haploinsufficiency is the predominant cause of holoprosencephaly (HPE), a human structural brain malformation (Mercier et al., 2011; Nanni et al., 1999). This can result in graduated malformation of the forebrain characterised by the failure of the cerebral hemispheres and optic vesicles to separate into bilateral structures (Fallet-Bianco, 2018). SHH haploinsufficiency can also contribute to less severe phenotypes such as microcephaly, hypothalamic-dysfunction, pituitary insufficiency with moderate facial disorder like hypotelorism (abnormally close-set eyes) or a single median incisor (Mercier et al., 2011; Rosenfeld et al., 2010). Only these mild forms of HPE are compatible with life (Weiss et al., 2018). Genetic research on HPE patients and their families over the past two decades has implicated other signalling pathways in the disease such as FGF, NODAL and NOTCH (Dubourg et al., 2016; Dupé et al., 2011; Roessler et al., 2009). Whereas it has been shown that NODAL and FGF act in the pathology by regulation of SHH activity (Lupo et al., 2006), the exact mechanism by which NOTCH dysfunction could cause HPE-like phenotype is unexplained.

NOTCH signalling is an important evolutionary conserved mechanism known to control cell fates through local interaction. This pathway utilises a membrane bound ligand that when in contact with a receptor leads to the release of a cytoplasmic fragment of the receptor which then forms a complex with RBPJ capable of transcriptional regulation (Selkoe and Kopan, 2003). Early in development, NOTCH is implicated in the correct patterning of the first neuronal population of the developing hypothalamus (Ratié et al., 2013; Ware et al., 2016) and colocalisation of SHH activity and NOTCH components, such as *Dll1* and *Hes5*, has been described in both chick and mouse embryos, at the level of the developing ventral forebrain corresponding to the hypothalamus primordium (Aujla et al., 2015; Ratié et al., 2013; Ware et al., 2016).

This study focuses on a unique aspect of canonical NOTCH function in the developing anterior hypothalamus. Using chick and mouse embryonic models, we demonstrate that embryos lacking NOTCH activity during early forebrain development exhibit a specific reduction of SHH signalling in the anterior hypothalamus. This leads to a phenotype that is reminiscent to an inactivation of *Shh* in this tissue. This data demonstrates that the NOTCH pathway is implicated in HPE through the regulation of *Shh* expression and, thus may contribute to the phenotypic heterogeneity that is observed in HPE patients.

Materials and Methods

Generation of the mouse lines and genetic crosses

To generate conditional RBPj knock-out mice, *RBPj^{L/L}* (Han et al., 2002) mice were crossed with *R26RCreER^{T2}* (Badea et al., 2003) mice to generate *RBPj^{L/L};CreER^{T2}*. To activate Cre recombinase, Tamoxifen (Sigma) was dissolved in sunflower oil at a concentration of 10 mg/ml. Tamoxifen (5 mg) or vehicle was injected intraperitoneally (IP) at embryonic day (E) 7.75 and embryos were harvested at E9.5, E10.5 or E11.5. *Rbpj^{L/L};R26mTmG^{+/+}* mice (Muzumdar et al., 2007) were crossed with *Rbpj^{L/L};Cre^{ERT2+/-}* mice to evaluate Cre-mediated excision. *Rbpj^{+/-}* (gift from Dr. Tasuku Honjo) and *Shh^{+/-}* mice (Jackson Laboratories, Bar Harbor, ME, USA) have been described previously (Chiang et al., 1996; Oka et al., 1995). Embryos and mice were genotyped by PCR on DNA samples prepared from tail tips, yolk sacs or whole embryos. Primer sequences and PCR protocols are available on request. The mice were maintained in a room with controlled temperature (21-22°C) under a 12-12 light-dark cycle (light cycle from 7:00 to 19:00) with ad libitum access to the food and water.

Chick embryos and *ex ovo* roller culture

Fertilised chicken (*Gallus gallus*) eggs were obtained from E.A.R.L. Les Bruyères (France). Eggs were incubated in a humidified incubator at 38°C until the required developmental stages described according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). Embryos were collected at HH9 and cultured as described previously (Dupé and Lumsden, 2001). Loss of function experiments were performed with the γ -secretase inhibitor DAPT (Sigma, France) dissolved in DMSO. Embryos were treated with 40 μ M of DAPT in L15 culture medium, supplemented with chick serum. Control embryos were treated with DMSO.

In situ hybridisation

All embryos were fixed in 4% PFA/PBS at 4°C overnight, rinsed and processed for whole-mount RNA *in situ* hybridisation. Sense and anti-sense probes were generated from plasmids cloned as previously described (Ratié et al., 2013) or plasmids provided as a gift. The protocol for single and double *in situ* hybridisation has been previously described (Ratié et al., 2013). For double labelling, Digoxigenin and Fluorescein labelled probes were incubated together. The Digoxigenin antibody (Roche) was added first, followed by the NBT/BCIP reaction.

After inactivation of the colour reaction, the embryos were fixed with 4% PFA overnight, then the Fluorescein antibody (Roche) was added, followed by fast red reaction (VectorRed).

Histology and Skeletal preparation

For histology, E18.5 embryos were fixed in Bouin's fluid for 7 days, embedded in paraffin, serially sectioned and stained with Haematoxylin and Light Green. E18.5 embryos were dissected, skinned and eviscerated. Subsequently, embryos were fixed in acetic acid/ethanol overnight and stained for four days in an Alcian Blue solution (Sigma: A3157). Remaining tissue was digested in 1% potassium hydroxide. Bones were then stained with Alizarin (Sigma; A5533), and embryos were cleared by incubation with progressively increasing concentrations of glycerol.

Ethics statement

All experiments were carried out in accordance with the European Communities Council Directive of 24 November. VD, as the principal investigator in this study, was granted the accreditation 35-123 to perform the reported experiments and the experimental procedures were authorised by the French Ministry of Research committee C2EA-07 under the protocol N°2016010514029297.

Results

Sonic hedgehog signalling was active in the presumptive hypothalamus before NOTCH activity in the chick embryo

To analyse the potential interaction between NOTCH and SHH signalling at the level of the developing hypothalamus, the chick was used as a model since molecular features defining the antero-posterior domains have been extensively described (Fu et al., 2017; Manning et al., 2006). We first performed a detailed study of the expression of SHH effector target genes (*Nkx2.1* and *Ptch1*) and a NOTCH effector target gene (*Hes5*) (Fig. 1). It is well known that SHH signalling induces expression of *Nkx2.1* in the presumptive hypothalamus from HH8 (4-somites stages); making *Nkx2.1* the first marker that defines the hypothalamic progenitor state at HH8 (Pera and Kessel, 1997). *Hes5*, a direct transcriptional target of NOTCH and

expression of *Hes5* has been considered a reliable readout of NOTCH signalling (de la Pompa et al., 1997; Ratié et al., 2013).

To assess whether there is NOTCH activity when hypothalamus tissue becomes *Nkx2.1*-positive, we performed a double labelling of *Nkx2.1* and *Hes5* at HH9 (7 somite-stage) (Fig. 1A). This staining confirmed that *Hes5* was expressed in the midbrain at this stage, but not in the *Nkx2.1*-positive cells corresponding to the forming hypothalamus (Bracket in Fig. 1A). This result indicated that SHH signalling was active in the early developing hypothalamus to induce *Nkx2.1* before NOTCH signalling was detected.

Overlapping activity of NOTCH and SHH signalling was restricted to the anterior hypothalamus

PTCH1 is a SHH binding receptor upregulated at sites of active SHH signalling (Cohen et al., 2015). In the chick brain, *Hes5* and *Ptch1* were both described to have their expression restricted in the anterior hypothalamus (AH) at stage HH13 (Ratié et al., 2013; Fu et al., 2017). To clarify further the timing of *Ptch1* and *Hes5* expression in this area, we performed an analysis by staging the embryos by somite number. At HH10 (10-somite stage), the first *Hes5* (Fig. 1B) and *Ptch1* (Fig. 1C) transcripts were expressed, at the level of the AH domain between the optic vesicles (OV). In agreement with the expression being located in the AH, at HH12 (16-somite stage), double labelling of *Nkx2.1* and *Hes5* showed that the expression of *Hes5* was confined within the anterior limit of *Nkx2.1* expression corresponding to the AH (Fig. 1D). This expression analysis revealed that NOTCH activity started at HH10 in the hypothalamus and the location of expression was restricted in the AH which also corresponded to the anterior limit of *Shh* expression at this stage (Fig. 1E; (Manning et al., 2006).

NOTCH activity maintained *Shh* expression in the chick anterior hypothalamus

We next investigated the potential interaction of NOTCH and SHH signalling during early differentiation of the AH. To address this, we used DAPT, a pharmacological inhibitor of NOTCH signalling, and microarray analysis. After only 3 hours, this DAPT treatment caused a rapid downregulation of *Hes5* expression (Sup Fig. 1C and (Ratié et al., 2013).

As NOTCH activity was first detected in the hypothalamus at HH10, chick embryos were treated with DAPT from HH9 and harvested after 16 hours *ex ovo* roller culture, at approximately stage HH14 (Sup Fig. 1A). RNA-seq data was compared between DAPT-treated forebrain and DMSO-treated forebrain (Sup Fig. 1). DAPT treatment resulted in the differential expression of 1558 genes, with 769 genes upregulated and 789 genes downregulated (Ratié et al., 2013). Interestingly, both *Shh* and *Ptch1* were significantly downregulated in DAPT-treated embryos compared to DMSO-treated embryos (Sup Fig. 1B). This result suggested that NOTCH signalling could play a role in the regulation of SHH signalling.

To confirm this finding, we performed whole-mount *in situ* hybridisation on chick embryos treated with DAPT. All embryos (n=25) in which NOTCH signalling was eliminated by DAPT treatment from HH9 showed a consistent downregulation of *Shh* in the forebrain (Fig. 2A). Dissected neural tube highlighted specific *Shh* downregulation in the AH while *Shh* expression was essentially normal posteriorly, along the ventral neural tube (Fig. 2B). To examine the consequence of the loss of *Shh* in the AH domain of DAPT treated embryos, we examined the expression of *Nkx2.1* and *Ptch1*. In DAPT-treated embryos, *Ptch1* expression was absent in the AH (Fig. 2C). *Nkx2.1* expression was also lacking in the AH while it was still expressed in the tubero-mamillary hypothalamus (T-MH), although weaker compared to control embryos (Fig. 2D). From this expression analysis, we concluded that NOTCH signalling from HH10 was required to maintain *Shh* expression and its activity in the chick AH.

To show that the influence of NOTCH activity on hypothalamus development was temporally very specific, the same *ex ovo* roller culture experiments were done at HH10, HH11 and HH12 (Sup Fig 2). The embryos treated with DAPT at HH10 (10 somites) gave similar results to the embryos treated at HH9, with no *Shh* expression in the AH (Sup Fig. 2B, F). DAPT treatment at HH11 and HH12 gave a partial loss of *Shh* expression in the AH (Sup Fig. 2C, D, G, H). There was no more effect on *Shh* expression in the AH when DAPT treatment took place after HH12 (data not shown). This analysis of chick embryos with a loss of NOTCH function showed that NOTCH signalling maintained *Shh* expression and its activity in the AH was highly specific during a short time window between HH9 and HH12.

Inhibition of *Shh* expression and signalling in the ventral forebrain of *Rbpj*^{-/-} mice

While our analysis using the chick embryo suggested an interaction between the SHH and NOTCH signalling pathways in the AH, we wanted to confirm this genetic interaction using the mouse model. To perturb NOTCH signalling we utilised a knockout allele of *Rbpj* (Oka et al., 1995), a common effector required for the activity of all four mammalian NOTCH receptors (Jarriault et al., 1995). *Rbpj* mutant mice are commonly used to study the role of NOTCH signalling, because inhibition of this transcriptional activator abrogates NOTCH signalling during development (de la Pompa et al., 1997).

The expression of *Shh* and *Nkx2.1* was analysed by *in situ* hybridisation in these *Rbpj* null-mutant (*Rbpj*^{-/-} embryos). At E9.0, the anterior limit of *Shh* expression corresponded to the AH (Fig. 3A;(Blaess et al., 2014). At this stage, *Rbpj*^{-/-} embryos (n=5) showed an overall downregulation of *Shh* expression along the neural tube (Fig. 3C), and *Shh* expression was totally absent in the AH from E9.0 (Fig. 3C, arrowhead).

The NOTCH pathway has been shown to be crucial for early cardiac development, therefore, from E9.0, *Rbpj*^{-/-} embryos suffered from a severe developmental delay (Blaess et al., 2014; de la Pompa et al., 1997). Despite this delay, we managed to obtain *Rbpj*^{-/-} embryos at E9.5 (n=3). In these embryos, *Nkx2.1* expression was totally absent in the AH (Fig. 3D, arrowhead) and reduced in the T-MH. Normal expression of *Nkx2.1* in the thyroid primordium was conserved in the *Rbpj*^{-/-} embryos despite the developmental delay (Fig. 3D, asterisk). Notably, these *Rbpj*^{-/-} embryos displayed hypoplastic forebrain vesicles (Fig. 3D, arrow).

These data suggested that, like in chick embryos, NOTCH signalling was crucial to maintain the expression of *Shh* and *Nkx2.1* in the mouse AH. They showed that the specific regulation of *Shh* expression by NOTCH in the AH was conserved between the chick and mouse embryos.

Conditional inhibition of the NOTCH pathway leads to telencephalic hypoplasia

We hypothesised that *Rbpj*^{-/-} embryos that did not express *Shh* in the AH would subsequently exhibit a phenotype characteristic of a specific loss of function of *Shh* in this area. Mouse models with a specific loss of function of *Shh* in the hypothalamus primordium were previously generated (Carreno et al., 2017; Zhao et al., 2012). These mice displayed a particular phenotype with abnormal hypothalamus and hypoplastic telencephalon. Despite the hypoplastic forebrain we observed in E9.5 *Rbpj*^{-/-} embryos (arrow in Fig. 3D), further observation of the developing forebrain was compromised in these *Rbpj* knockout embryos as

they are dying at this stage from heart failure (Oka et al., 1995). Therefore, to attempt to rescue this phenotype, we used *Rbpj^{L/L};CreER^{T2}* mice where the activity of the Cre-recombinase could be induced at a specific time point during pregnancy using an injection of Tamoxifen (Ware et al., 2016). *Rbpj^{L/L}* excision was fully efficient 12 hours after tamoxifen injection (Sup Fig. 3). This allowed us to excise the *Rbpj* gene and thus inhibit the NOTCH signalling pathway at a chosen developmental stage, before the establishment of NOTCH in the AH. NOTCH activity was first detected in the mouse prospective hypothalamus at E8.5 (Ware et al., 2014). Therefore, injection of 4 mg of tamoxifen was first done at E7.5 to get an inhibition of NOTCH at E8.5. However, this treatment induced morphological anomalies reminiscent to the *Rbpj^{-/-}* embryo phenotypes. This included a shortening of the antero-posterior axis and lethality at E9.5. These results indicated that we were not able to obtain a rescue of the heart phenotype when the embryos were treated with Tamoxifen at E7.5 (Sup Table 1 and Sup Fig. 4).

Therefore, we performed injections at various stages (Sup Fig. 4). When the pregnant mouse was treated with Tamoxifen at E7.75, *Rbpj^{L/L};CreER^{T2}* embryos collected at E9.5 had an apparent similar morphology compared to the control *Rbpj^{L/L}* (Fig. 4A and Sup Fig. 4). Genotypic analysis of embryos between E9.5 and E11.5 revealed no statistical deviation from the expected Mendelian ratios (Sup Table 1). However, by E12.5, we were not able to harvest live *Rbpj^{L/L};CreER^{T2}* embryos (Sup Table 1). Therefore, by treating embryos at E7.75 with 4 mg tamoxifen, we have only partially rescued the early lethality caused by a complete loss of NOTCH signalling.

In order to evaluate the effect of this conditional inactivation on the developing hypothalamus, we analysed the expression of *Nkx2.1* at E9.5. We observed half (n=4/8) of the *Rbpj^{L/L};CreER^{T2}* embryos treated at E7.75 had a specific downregulation of *Nkx2.1* in the AH (Fig. 4A). Therefore, by inactivating NOTCH activity from E7.75, we were able to generate embryos with a specific downregulation of *Nkx2.1* in the AH, which was reminiscent to what we obtained with *Rbpj^{-/-}* embryos. Importantly, these rescued embryos did not show the developmental delay that was characteristic of *Rbpj^{-/-}* mutant. These embryos were thus partially rescued but still displayed a mispatterning in the AH. The delayed lethality in these conditional mutant mice gave us the opportunity to study the consequence of this mispatterning in older embryos. At E10.5, mRNA distribution of *Shh* in *Rbpj^{L/L};CreER^{T2}* embryos from the same littermates was extremely variable (Fig. 4B). *Shh* expression in the ventral forebrain ranged from slightly downregulated to completely absent (Fig. 4B).

The embryos with no *Shh* expression in the AH always had a reduction in size of the telencephalic vesicles (Fig. 4B, asterisk). The phenotypic and molecular variability we observed was probably due to the fact that when we injected tamoxifen into the pregnant mice, embryos were not all at exactly the same stage of development.

We have further taken advantage of these partially rescued embryos to test the expression of *Fgf10*, a marker specific for the T-MH. *Fgf10* expression extended into the AH in mouse embryos with a specific AH deletion of *Shh* (Carreno et al., 2017; Zhao et al., 2012). Similarly, in our conditional model *Rbpj^{L/L};CreER^{T2}*, half of the E9.5 (n=5) embryos, had the expression domain of *Fgf10* clearly extended anteriorly (Fig. 4C, bracket). This analysis indicated an anterior expansion of the expression domain of a posterior hypothalamic marker in *Rbpj^{L/L};CreER^{T2}* similar to what was previously described for *Shh* deficient mutants (Carreno et al., 2017; Zhao et al., 2012). At E10.5, *Rbpj^{L/L};CreER^{T2}* embryos (n=22) displayed various level of growth retardation of the developing brain, from apparent normal morphological brain to mild forebrain defects, corresponding to a reduction of the size of the telencephalic vesicles (Fig. 4B, asterisk). In order to assess the telencephalic hypoplasia of these embryos, we analysed *Bmp7* expression as expression was normally found in the dorsal telencephalic vesicles (Danesh et al., 2009). By E11.5, the *Bmp7* expression domain in the telencephalon was severely reduced in half of the *Rbpj^{L/L};CreER^{T2}* embryos (n=6/12) (Fig. 4D, bracket). This observation indicated that embryos with reduced NOTCH activity from E7.75 have telencephalic vesicles that were reduced in size, which was also reminiscent to the phenotype of the mouse models with a specific loss of function of *Shh* in the hypothalamus primordium (Carreno et al., 2017; Zhao et al., 2012).

Altogether, these findings suggested that the conditional loss of NOTCH signalling from E7.75 caused a downregulation of *Shh* in the AH and thus a failure for the forebrain to develop properly. Interestingly, tamoxifen treatment 6 hours later at E8.0 produced no telencephalic vesicle hypoplasia and no effect on the expression of *Shh* at the level of the ventral forebrain (Sup Fig. 4). Thus, timing of tamoxifen treatment was crucial. This suggested, that similar to DAPT-treated chick embryos, in mouse embryos, NOTCH signalling was implicated in the appropriate control of *Shh* expression in the AH during a narrow window of time, around E8.0.

NOTCH signalling cooperates with SHH for pituitary gland formation

To test the genetic interaction between the SHH and NOTCH signalling pathways in the mouse, we sought to explore the consequences of a partial downregulation of both pathways by genetically deleting one copy of *Shh* and one copy of *Rbpj* in mice.

Unlike, homozygous mutants, that were not viable, *Rbpj*^{+/-} and *Shh*^{+/-} heterozygous mutants have been described as phenotypically unremarkable relative to their control littermates (Chiang et al., 1996; Oka et al., 1995). In this study, double heterozygous *Shh*^{+/-};*Rbpj*^{+/-} mice were created by intercrossing *Shh*^{+/-} mice and *Rbpj*^{+/-} mice. These intercrosses produced offspring that were viable with no obvious phenotypes. Genotyping of E18.5 embryos and 3 weeks old mice revealed the expected mendelian mode of inheritance for all genotypes (Sup Table 2).

When histological head sections of *Shh*^{+/-};*Rbpj*^{+/-} mice (n=4) were analysed at E18.5 a number of phenotypes were observed (Fig. 5). Defects included a dysplastic pituitary gland as well as a remnant connection between the anterior part of the pituitary (the adenohipophyseal gland) and the oral ectoderm (Fig. 5C and Sup Fig. 5). We also analysed E18.5 *Shh*^{+/-};*Rbpj*^{+/-} skeletal preparations (n=20) and discovered a malformation of the sphenoid corresponding to an agenesis of the presphenoid bone and a fully penetrant opening at the level of the basisphenoid midline (Fig. 5F).

During normal development, the pituitary gland develops from the interaction of the infundibulum, a region of the hypothalamus, and the Rathke's pouch, a derivative of oral ectoderm (Takuma et al., 1998; Treier et al., 1998). Then the formation of the sphenoid bone establishes a definitive barrier between the pituitary gland and oral cavity (Sup Fig. 5).

It is presumed that a deviation in the development of the pituitary gland interferes with closure of the basisphenoid bone leading to an abnormal fenestration along the midline (Khonsari et al., 2013). The resulting hole is called a buccohypophyseal canal. In all mammals, this canal normally closes in the early stage of development when the link between the Rathke's pouch and oral ectoderm has disappeared. This canal is an ancestral vertebrate trait that has been lost in mouse and human by modulation of SHH signalling (Khonsari et al., 2013). In the brain of E18.5 *Shh*^{+/-};*Rbpj*^{+/-} embryos, the pituitary gland normally located between the hypothalamus and the basisphenoid bone was misplaced into the nasopharyngeal cavity through an abnormal opening in the midline of the basisphenoid bone (Fig. 5C and Sup Fig. 5). This indicated that the sphenoid bone cartilage was unable to develop properly in these *Shh*^{+/-};*Rbpj*^{+/-} mutant mice. A foramen was also observed at the level of the basisphenoid midline in E18.5 *Shh*^{+/-} (n=17) embryos, but never as large compared to *Shh*^{+/-}

; *Rbpj*^{+/-} mutants (Fig. 5E). Interestingly, these results suggested that the basisphenoid bone was the most sensitive tissue to *Shh* insufficiency.

These observations indicated that morphogenesis of the pituitary gland was impaired in *Shh*^{+/-}; *Rbpj*^{+/-} mice which led to basisphenoid abnormalities. This phenotype was probably due to a cumulative impact of NOTCH and SHH insufficiency during brain development.

Discussion

Dynamic domains of *Shh* expression pattern the ventral forebrain and a misbalance in SHH activity can cause a large spectrum of brain and craniofacial malformations (Lipinski et al., 2014; Marcucio et al., 2005). Here, we show for the first time that the NOTCH signalling pathway, is implicated in the control of *Shh* expression in the AH and is thus critical for early forebrain patterning and the occurrence of holoprosencephaly (HPE).

The use of diverse model systems to study forebrain development provides evidence that the molecular pathways regulating development of the ventral forebrain are conserved from fish to mammals (Grinblat and Lipinski, 2019; Xie and Dorsky, 2017). This conservation allows the opportunity to address questions regarding the implication of NOTCH signalling in human HPE by using the chick and mouse models. A potential implication of NOTCH signalling in early forebrain patterning has been recognised previously with alterations in one of the NOTCH ligands, *DLL1*, being described in patients with HPE (Dubourg et al., 2016; Dupé et al., 2011). In agreement with this, NOTCH signalling is present in the ventral forebrain of both chick and mouse embryos during a stage of development compatible with an implication in HPE pathology (Ratié et al., 2013; Ware et al., 2014; Zhu et al., 2006). However, when and by which molecular mechanism the NOTCH signalling pathway may regulate early development of the ventral forebrain remained unknown.

To begin understanding the relationship between the SHH and NOTCH signalling pathways, our expression studies show an overlap in the expression of NOTCH components (*Hes5*) and SHH components (*Nkx2.1* and *Ptch1*) in the ventral forebrain. This suggests that at least with expression in the same regions, the two pathways could regulate each other. It is well established in the chick that, initially, *Shh* is expressed throughout the presumptive hypothalamus from HH7 (1-somite stage) to HH14 (22-somite stage) and, subsequently, *Shh* expression becomes specifically restricted to the AH (Manning et al., 2006), where it

promotes anterior fate and neurogenesis (Fu et al., 2017). It is also established that during development the amount and duration of SHH exposure determine cellular responses (Dessaud et al., 2007; Mercier et al., 2013). The AH is a region in which high SHH activity is needed and therefore the consequent developmental outcomes are even more dosage sensitive (Carreno et al., 2017; Fu et al., 2017; Zhao et al., 2012).

Our expression studies show that NOTCH activity is strictly found in the AH from HH10, which is compatible with an implication in the establishment of the forebrain midline. Consistent with this, we demonstrate in both the chick and the mouse that NOTCH signalling is necessary to maintain *Shh* expression in the AH during a short time window, between HH10 and HH12 in chick and around E8.0 in mouse. This time window corresponds to developmental stages that are crucial for the specification of the forebrain midline. How NOTCH signalling contributes to maintaining *Shh* expression in the AH during this period is not well defined. However, we know that NOTCH signalling pathway plays a key role in cell fate choice during AH neurogenesis (Ratié et al., 2013; Ware et al., 2016) in balancing the number of progenitor cells with that of differentiating neurons (Pierfelice et al., 2011). Our previous studies show that transient inhibition of NOTCH signalling during early hypothalamic development enhances neurogenesis in the AH. This means that inhibition of NOTCH signalling from HH10 during hypothalamus differentiation causes a rapid decline in downstream components of the NOTCH signalling pathway (e.g. *Hes5*) leading to the upregulation of the proneural bHLH gene, *Ascl1*, followed by a precocious neurogenesis and excessive number of cells differentiated into neurons (Ratié et al., 2013; Ware et al., 2016). Thus, NOTCH signalling, through the repression of *Ascl1*, is necessary in the AH to maintain anterior progenitor cells and suppress neuronal differentiation. Once the anterior progenitor cells of the AH differentiate into neurones, *Shh* is downregulated in these cells (Fu et al., 2017). These data suggest that inactivation of NOTCH signalling causes precocious differentiation of the anterior progenitor cells into neurons promoting a premature downregulation of *Shh*, and therefore a downregulation of *Nkx2.1* and *Ptch1* in the AH.

Importantly, NKX2.1 and SHH are specific markers of the ventral cells of the forebrain (Pera and Kessel, 1997), as a consequence our study shows that the ventral fate of the forebrain is reduced when NOTCH activity is not appropriate.

We observe a specific downregulation of *Nkx2.1* in the AH, an anterior expansion of *Fgf10* and a reduction in the size of the telencephalic vesicles in the mouse embryo when NOTCH signalling is removed just before the onset of its activity in the AH. As this phenotype is also a common occurrence in the AH-specific-*Shh*-deficient model (Carreno et al., 2017; Zhao et al., 2012), we assume that it is the reduction in SHH signalling in the AH that might underlie the forebrain phenotype of *Rbpj^{L/L};CreER^{T2}* embryos.

Therefore, our observations incriminate NOTCH as a new signalling pathway, among multiple (e.g. FGF, NODAL, BMP), implicated in HPE occurrence through the control of *Shh* expression (Carreno et al., 2017; Davis and Camper, 2007; Fu et al., 2017; Manning et al., 2006; Zhao et al., 2012; Zhu et al., 2007).

This has been confirmed by generating double heterozygous (*Shh^{+/-};Rbpj^{+/-}*) mice; as the persistent buccohypophyseal canal we observed in these animals is a marker of variation in the dosage of SHH during midline morphogenesis (Khonsari et al., 2013). The same anomalies we observe in this study, although associated with other severe anomalies, are present in *Gas1^{-/-}* (Seppala et al., 2014) and *Cdo^{-/-}* (Cole and Krauss, 2003) mutant mice, two membrane proteins that act as agonists of SHH signalling during development. This indicates that the phenotype in our *Shh^{+/-};Rbpj^{+/-}* mouse is probably due to a supplementary deficiency of SHH signalling during pituitary formation compared to the single heterozygous mutant *Shh^{+/-}*. These findings support a lower threshold of NOTCH activity being sufficient to cause clinically significant abnormalities in mice with a genetic mutation in the *Shh* gene.

Persistence of the buccohypophyseal canal and pituitary gland aplasia represents an abnormality that occurs in human HPE (Kjaer, 2015; Kjaer and Fischer-Hansen, 1995). In these patients, Adenopituitary (aP) gland tissues are located subpharyngeally and the sella turcica, which provides room for the pituitary gland in the body of the sphenoid bone, is partly absent. In these cases, similar to *Shh^{+/-};Rbpj^{+/-}* mice, it is presumed that a deviation in the development of the pituitary gland results in an abnormality of the sella turcica (Kjaer, 2015). Therefore, a more detailed characterisation of this HPE-like animal model (*Shh^{+/-};Rbpj^{+/-}*) is important, as it is the first example of living mouse models with these unique microsigns of HPE. This further investigation on the *Shh^{+/-};Rbpj^{+/-}* adult mice will allow evaluating the impact of such anomaly on the cranial growth and physiology (such as growth hormone concentration). This will provide an excellent opportunity for understanding normal and abnormal development of the midline and may ultimately aid in the treatment of patient displaying mild form of HPE.

The severity of HPE defects ranges from the complete absence of midline structures to a single upper incisor. It is becoming increasingly clear that phenotypic heterogeneity of HPE is dependent of the level of diminution of SHH as well as the timing and location of these insufficiency (Cordero et al., 2004; Lipinski et al., 2014; Mercier et al., 2013; Zhao et al., 2012). The earlier and higher the decrease of SHH is, the more severe the HPE will be. Here, we show that *Shh* expression that is NOTCH-dependent takes place in a specific area of the ventral brain (AH) during a late phase of its development. At this stage, SHH secreted by the axial mesoderm underlying the neural plate has already initiate development of the ventral midline in the brain (Dale et al., 1997) and we know that even a total absence of SHH in the AH does not give rise to severe HPE (for example, an alobar form with cyclopia) (Cordero et al., 2004; Lipinski et al., 2014; Zhao et al., 2012). Thus the telencephalic hypoplasia we describe here for *Notch*-deficient mice represents a congenital brain anomaly with a later manifestation compared to severe HPE, due to differences in the timing and location of SHH attenuation (Cordero et al., 2004; Zhao et al., 2012). Thus, inhibition of NOTCH signalling in the AH on its own during forebrain development cannot produce severe HPE, but only microforms such as abnormal hypothalamic-pituitary axis and microcephaly. This is in agreement with the recent identification of neurodevelopmental disorders apparently not typical to HPE but associated to haploinsufficiency of *DLL1*. In these patients, intellectual disability and variable brain malformations such as microcephaly are described (Fischer-Zirnsak et al., 2019). This may indicate that variants in NOTCH component genes underlie some cases of HPE microform in humans and that such putative mutations might contribute to more severe forms of HPE when in combination with other genes involved in midline development. However, up to now, *DLL1* variants have been linked to only a small number of HPE patients (Dubourg et al., 2016). We propose to include the numerous components of NOTCH signalling to the panel of HPE genes already tested in HPE patients.. In particular, the NOTCH receptors that are expressed during early forebrain development (NOTCH1, NOTCH2 and NOTCH3) (Williams et al., 1995; Zhu et al., 2006); it may increase the contribution of variants in gene related to NOTCH signalling in HPE patients.

This functional study shows how haploinsufficiency of the *Shh* and *Rbpj* genes may contribute to the HPE phenotype although this is not sufficient for severe HPE. Especially since recent findings reveal that variants in SHH-related genes may represent risk factors and collectively contribute to the HPE phenotype (Dubourg et al., 2018; Hong et al., 2017; Kim et al., 2019; Mouden et al., 2016). This study has clinical implications, as many HPE cases are

not yet fully explained during genetic testing. These results illustrate the need for expansion of the current diagnosis criteria to better capture the full range of brain and facial dysmorphology in disorders related to SHH-deficiency.

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Figure legends

Figure 1: SHH and NOTCH activity in the chick anterior hypothalamus

Comparison of *Nkx2.1*, *Hes5* and *Ptch1* expression in the prospective hypothalamus (H), through either single labelling (B,C) or double labelling (A,D). The dotted lines delimit the anterior hypothalamus (AH) with the tubero-mamillary hypothalamus (T-MH). (E) Schematic of HH10 embryo showing expression domains of *Hes5*, *Ptch1*, *Nkx2.1* and *Shh* (Manning et al., 2006) in the developing hypothalamus. Mb, midbrain; OV, optic vesicles.

Figure 2: The identity of the anterior hypothalamic was lost in DAPT-treated chick embryos

A comparison of gene expression in embryos after 16 hours of roller culture with DMSO (control) and DAPT at HH9 (A, B) Expression of *Shh* transcripts were detected in the anterior (AH) and tubero-mamillary hypothalamus (T-MH) in the control embryos, but was completely undetectable in the AH in embryos treated with DAPT at HH9 (arrowhead). (B) A ventral view of the dissected neural tube. (C,D) *Ptch1* and *Nkx2.1* expression was also lost in the AH of DAPT-treated embryos (arrowhead in C,D). Fb, forebrain; E, endoderm; Ov, optic vesicle.

Figure 3: Defective expression of *Shh* and *Nkx2.1* in the ventral forebrain of *Rbpj*^{-/-} mouse mutants.

Whole-mount *in situ* hybridisation analysis of *Shh* and *Nkx2.1* expression in mouse embryos. (A,C) *Shh* expression at E9.0 and (B,D) *Nkx2.1* expression at E9.5; *Shh* and *Nkx2.1* expression in the ventral forebrain was absent in *Rbpj*^{-/-} (arrowheads). Brackets (A-B) designate the prospective hypothalamus (H) and its antero-posterior domains, anterior (AH) and tubero-mamillary (T-MH) domains. Asterisks (B,D) indicate the thyroid primordium. The arrow in D indicates forebrain hypoplasia in *Rbpj*^{-/-} mutants. Fb, forebrain; Ot, otic vesicle.

Figure 4: Forebrain hypoplasia in *RBPJ*^{L/L};*CreER*^{T2} embryos. *RBPJ*^{L/L} and *RBPJ*^{L/L};*CreER*^{T2} tamoxifen treated embryos at E7.75. Whole-mount *in situ* hybridisation analysis of *Nkx2.1*, *Shh*, *Fgf10* and *BMP7* in embryos of the indicated genotype and stage (A D). In A, arrowhead indicates the absence of *Nkx2.1* mRNA in the AH. In B, embryos are from the same littermate. Asterisk indicates hypoplastic telencephalic vesicle. In C, *Fgf10*

expression was restricted (Curly Bracket) in the tubero-mamillary hypothalamus (T-MH) in the control (*RBPj^{L/L}*) and larger curly bracket indicates the expansion of the *Fgf10* expression in a more anterior region of the hypothalamus. In D, Curly brackets indicate *Bmp7* expression in the telencephalic vesicles. AH, anterior hypothalamus; H, hypothalamus, Ot, otic vesicle; OV, optic vesicle; T, telencephalic vesicle.

Figure 5: Brain and cranial bone defects were observed in *Shh^{+/-};Rbpj^{+/-}* mutant embryos (A-C) Hematoxylin and eosin staining of frontal section through E18.5 heads. Note the remnant connection (yellow arrow) between the anterior part of the pituitary gland and the oral ectoderm (OE). (D-F) Ventral views of cranial preparations of E18.5 embryos stained with Alizarin red and Alcian blue for bone and cartilage, respectively. Mandibles have been removed for visualisation. (E) White arrow indicates the persistent buccohypophyseal canal at the level of the midline in *Shh^{+/-}* mutants. (F) Asterisk indicates the enlarged canal in *Shh^{+/-};Rbpj^{+/-}* basisphenoid. 3V, third ventricle; BS, basisphenoid; H, hypothalamus; OC, otic capsule; PT, pituitary gland; PS, presphenoid; PX, pharynx.

The Notch pathway is a novel regulator of SHH signalling during early brain development

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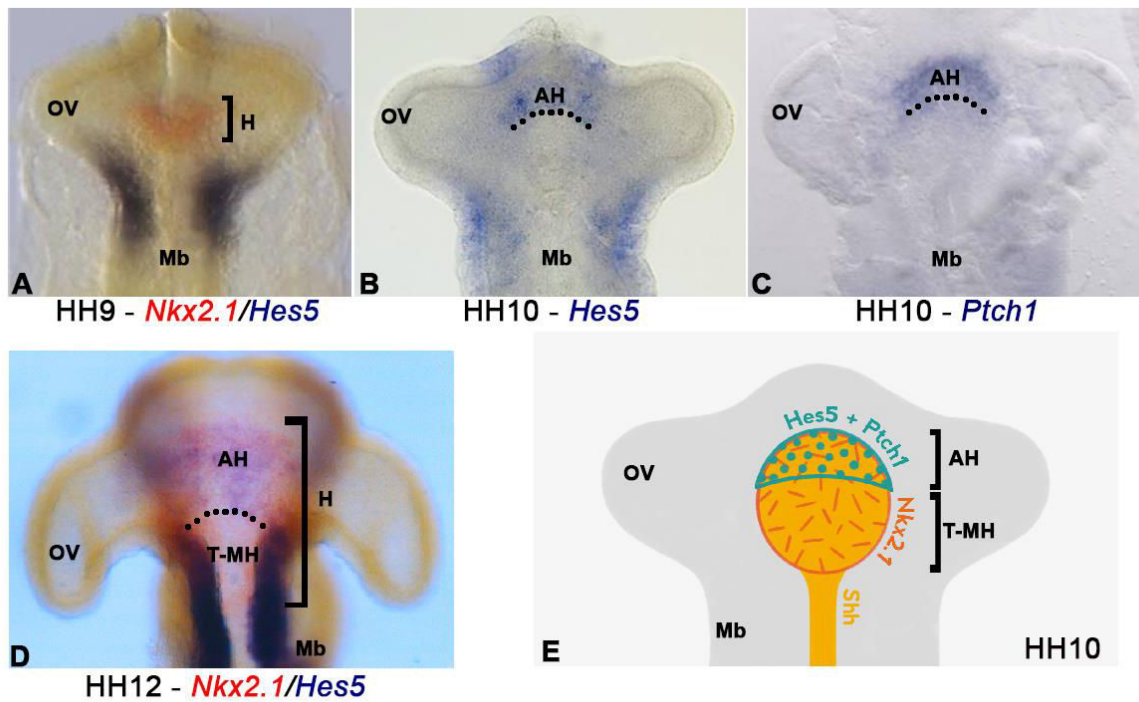


Figure 1: SHH and NOTCH activity in the chick anterior hypothalamus.

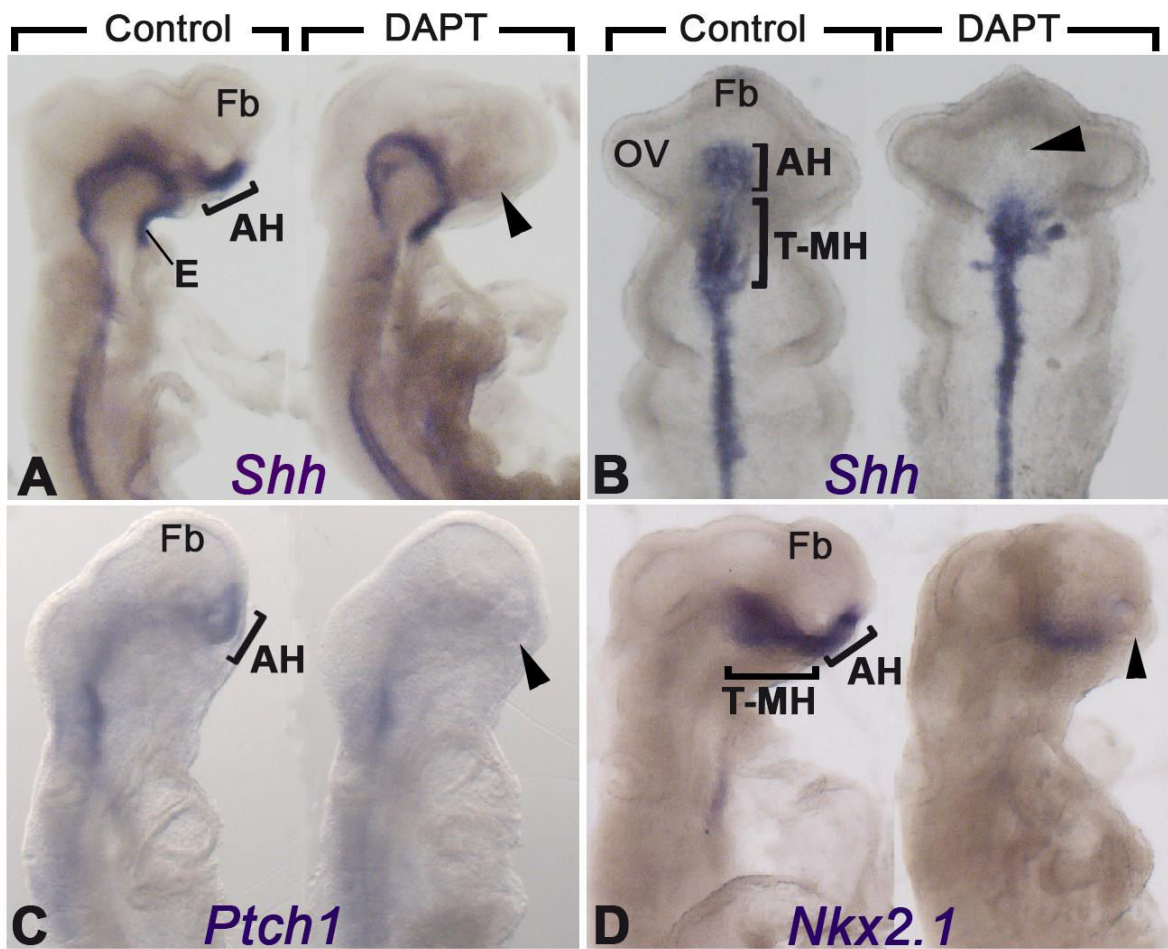


Figure 2: The identity of the anterior hypothalamic was lost in DAPT-treated chick embryos.

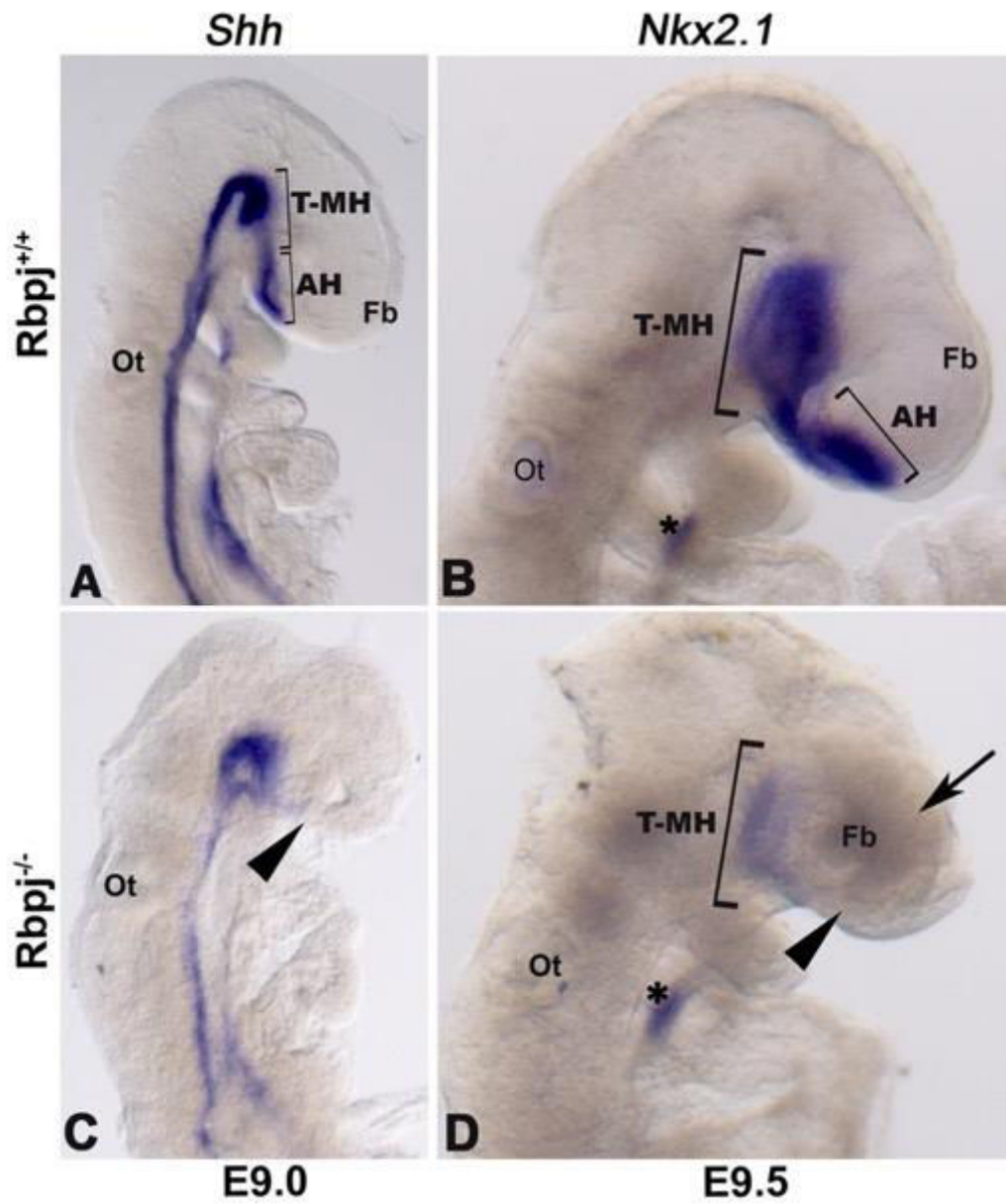


Figure 3: Defective expression of *Shh* and *Nkx2.1* in the ventral forebrain of *Rbpj*^{-/-} mouse mutants.

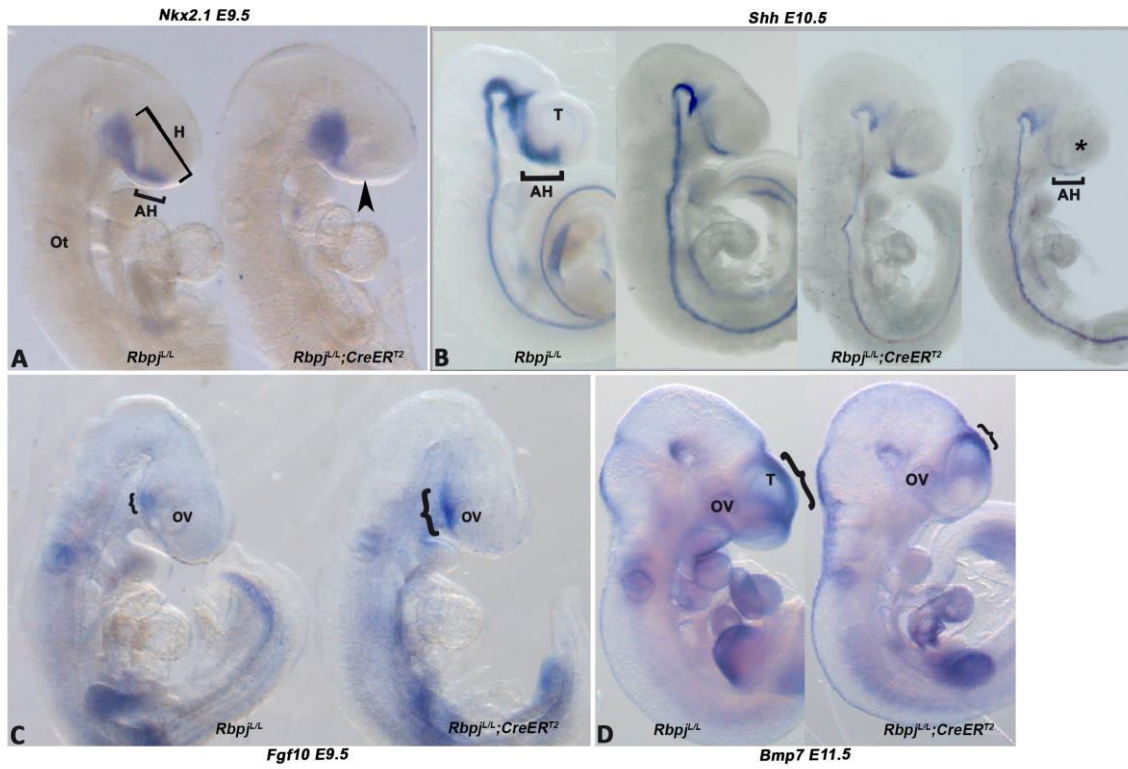


Figure 4: Forebrain hypoplasia in $RBPj^{L/L};CreER^{T2}$ embryos.

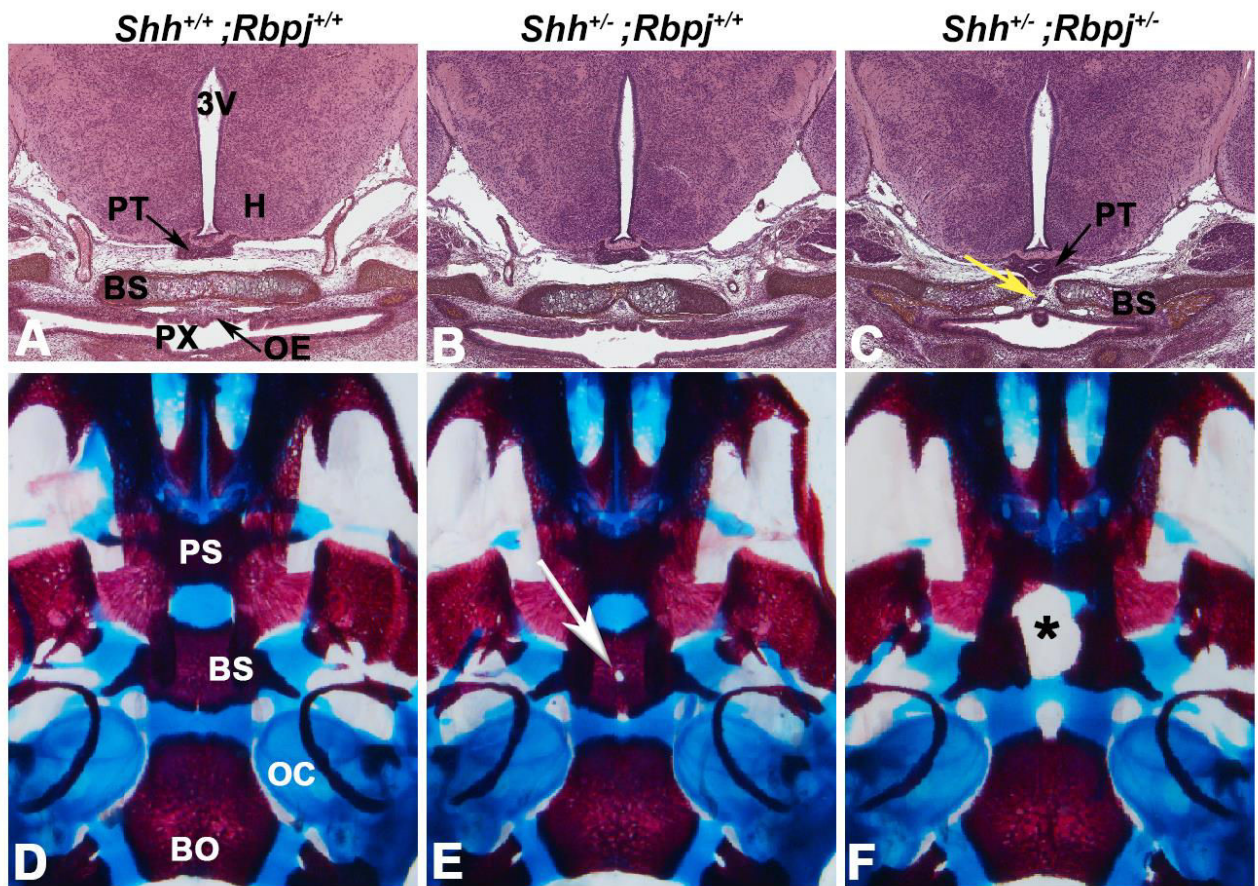


Figure 5: Brain and cranial bone defects were observed in *Shh*^{+/-}; *Rbpj*^{+/-} mutant embryos.

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Supplementary Table1. Number of mice obtained for each genotype of intercrossed *Rbpj*^{L/L} mice with *Rbpj*^{L/L};CreER^{T2} mice.

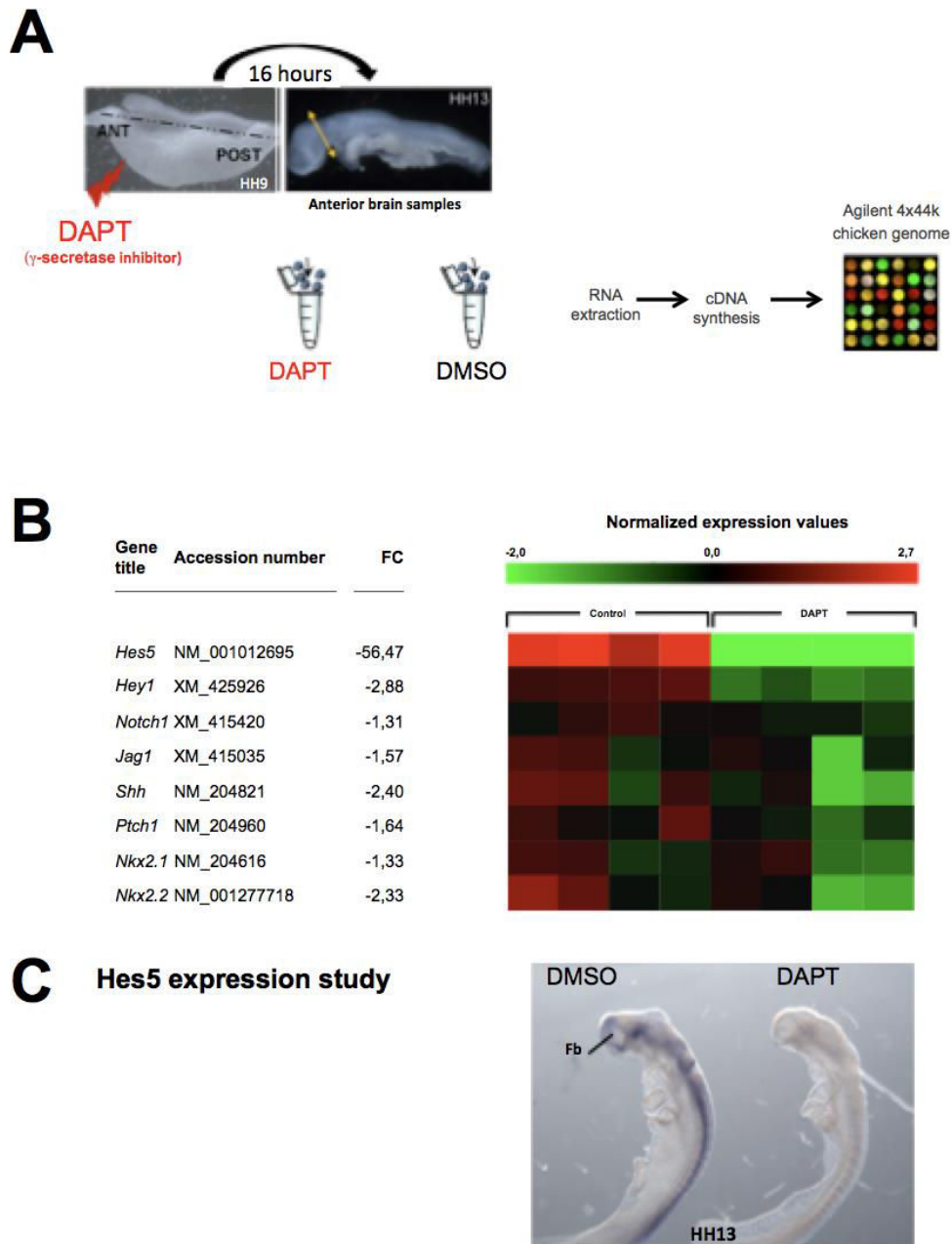
Tamoxifen treatment	Harvested	Genotypes (% expected)*	
		<i>Rbpj</i> ^{L/L} (50%)	<i>Rbpj</i> ^{L/L} ;R26Cre ^{ERT2} (50%)
Stage	Stages		
E7.5	E9.5	12 (57%)	9 (43%)
E7.5	E10.5	14 (93%)	1 (7%)
E7.5	E11.5	7 (100%)	0 (0%)
E7.75	E9.5	28 (55%)	23 (45%)
E7.75	E10.5	25 (53%)	22 (47%)
E7.75	E11.5	14 (58%)	10 (42%)
E7.75	E12.5	20 (91%)	2 (9%)
E8.0	E9.5	7 (44%)	9 (56%)
E8.0	E10.5	8 (44%)	10 (56%)
E8.0	E11.5	12 (54%)	10 (46%)
E8.0	E12.5	6 (55%)	5 (45%)

* derived from expected Mendelian ratios

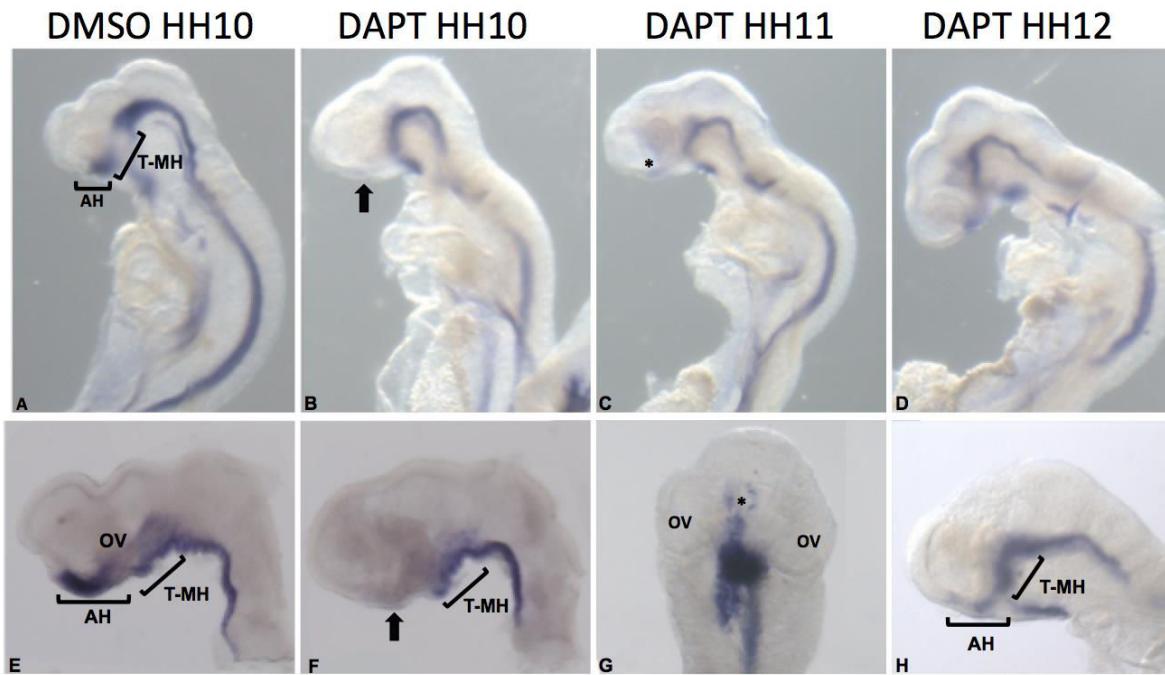
Supplementary Table2. Genotypes obtained from Shh^{+/-} x Rbpj^{+/-} intercrossed

	Genotypes (% expected)*				Total
	Shh ^{+/-} ;Rbpj ^{+/-} 25%	Shh ^{+/-} ;Rbpj ^{+/+} 25%	Shh ^{+/-} ;Rbpj ^{-/-} 25%	Shh ^{+/-} ;Rbpj ^{+/-} 25%	
Stage					
E18.5	22	26	24	21	93
3 weeks	15	12	13	17	57

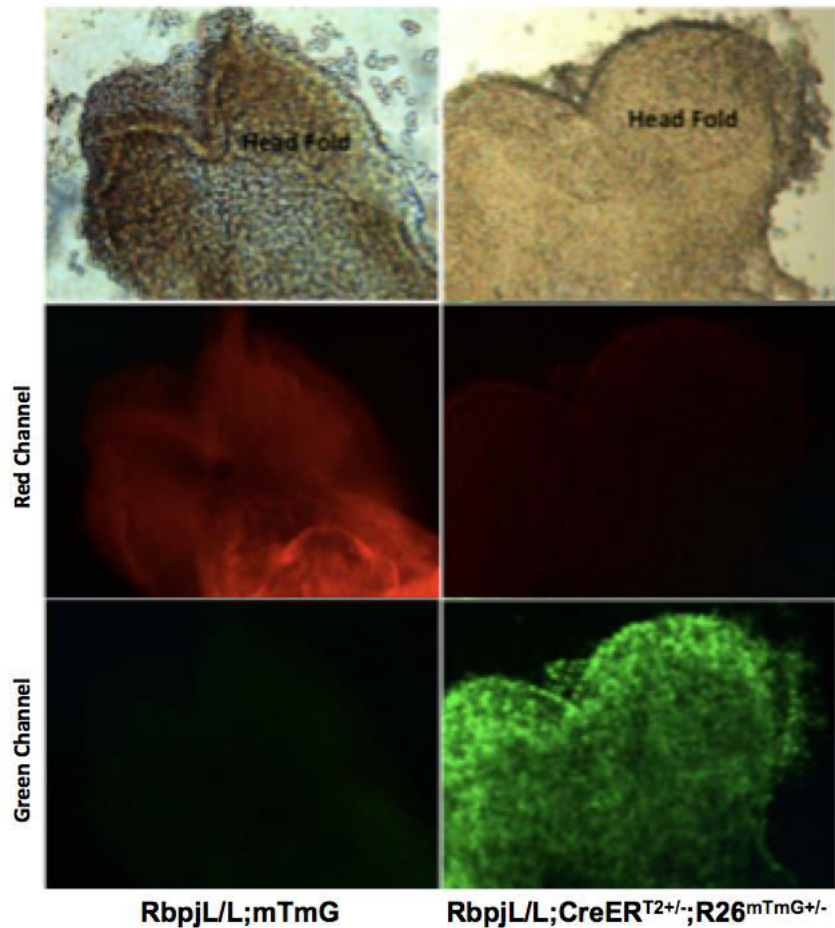
* derived from expected Mendelian ratios



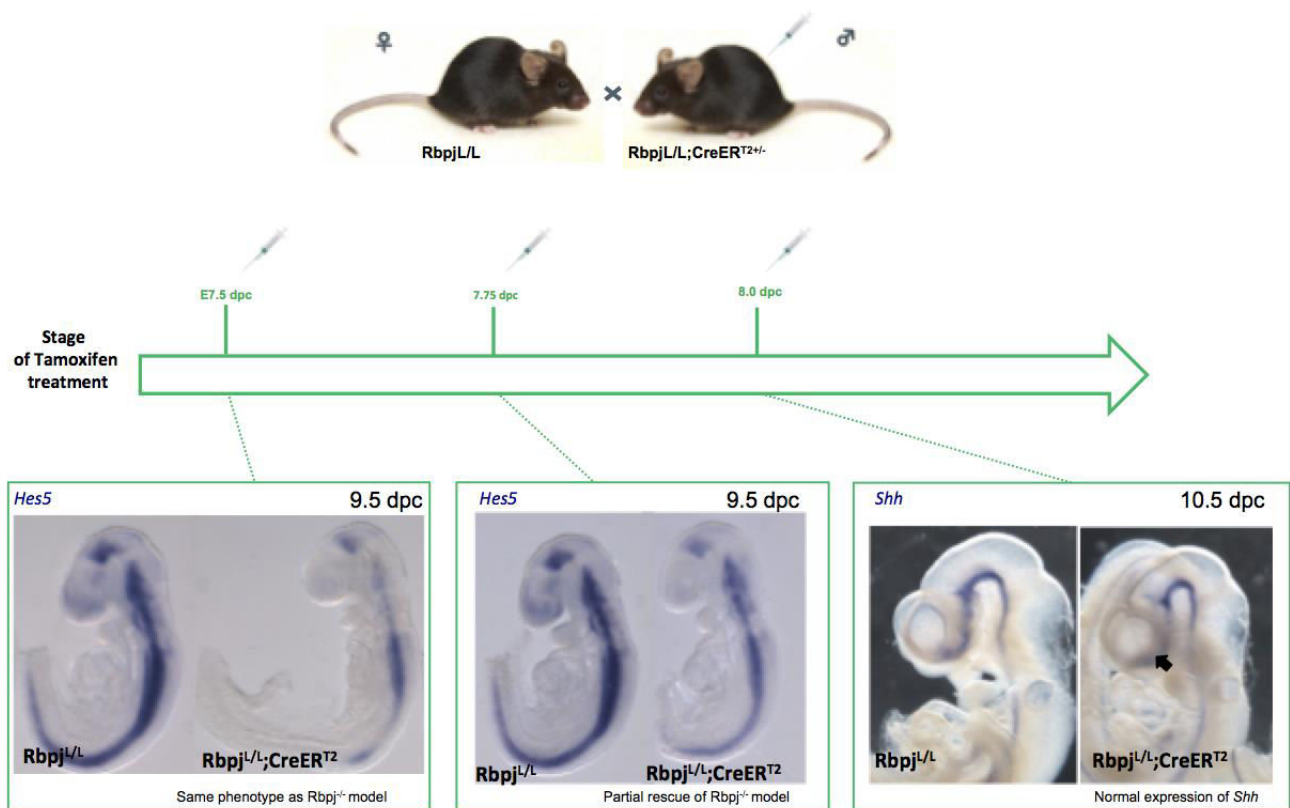
Supplementary figure 1: Transcriptome analysis of Notch inhibited chick roller culture embryos. A) Diagram of protocol. HH9 embryos were treated with N-[3.5-difluorophenacetyl-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) or Dimethyl sulfoxide (DMSO; control) for 16 hours. The global change in gene expression of the forebrain was compared by microarray analysis. RNA expression profiling on microarray method has been described elsewhere (Ratié et al., 2013). B) The Gene Ontology term contained 1558 enriched genes; 789 were downregulated and 769 upregulated. Results reveal *Shh* and *Ptch1* are downregulated. Here we have listed 8 genes. The accession number and the fold change are given. C) DAPT treatment caused a downregulation of the direct NOTCH target gene *Hes5*. Fb, forebrain.



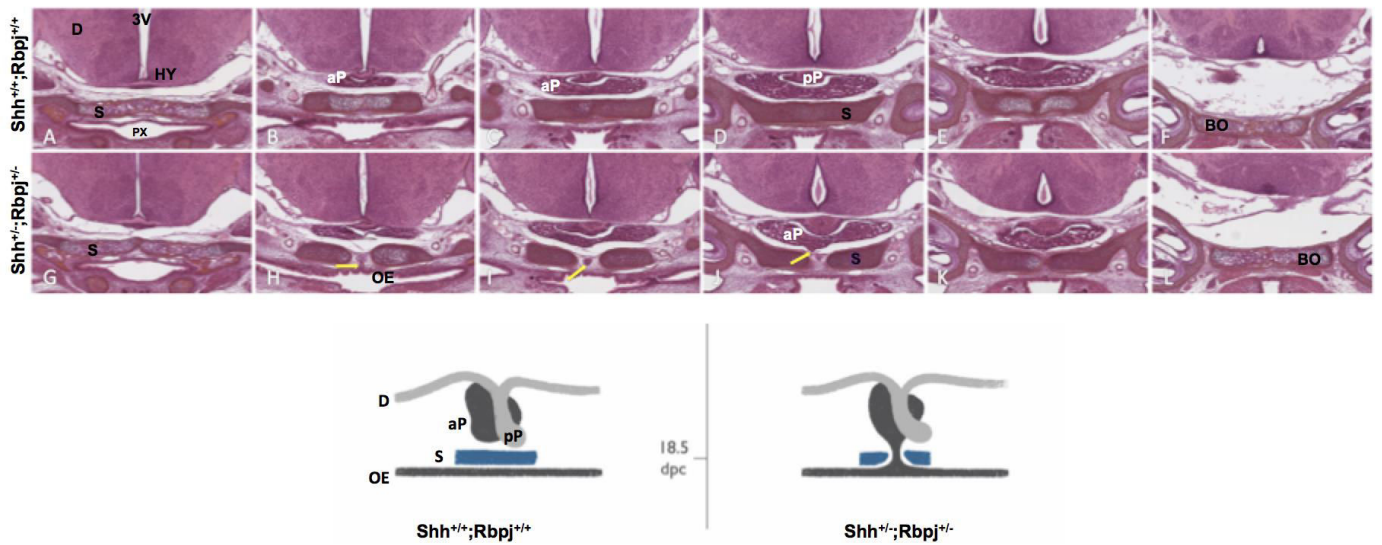
Supplementary figure 2: Expression of *Shh* at the level of the developing hypothalamus after inhibition of NOTCH signalling from stage HH10, HH11 or HH12. *Shh* expression in DMSO-treated chick embryos from HH10 (A,E) and DAPT-treated embryos from HH10 (B,F), HH11 (C,G) and HH12 (D,H). (E,F) dissected neural tube of A and B respectively. (G) Ventral view of C. Asterisk indicates scattered expression. AH: Anterior Hypothalamus; OV: optic vesicle; T-MH: Tubero-mammillary hypothalamus.



Supplementary figure 3: Conditional inactivation of *Rbpj* and Cre-inducible green fluorescent protein (GFP). *Rbpj*^{L/L};*R26mTmG*^{+/+} mice were crossed with *Rbpj*^{L/L};*Cre*^{ERT2+/-} mice. Tamoxifen (4mg) was injected to pregnant females at 7.75 dpc. *Rbpj*^{L/L} excision was efficient as 12 hours after Tamoxifen injection, red signal was barely detectable in the *Rbpj*^{L/L};*Cre*^{ERT2+/-};*R26mTmG*^{+/+} embryos and most of the cells expressed GFP (green).



Supplementary figure 4: Timing of Tamoxifen treatment is critical. *Rbpj^{L/L};R26mTmG^{+/+}* mice were crossed with *Rbpj^{L/L};CreER^{T2+/-}* mice and treated at E7.5, E7.75 and E8.0 after injection of 4 mg tamoxifen at E7.5, the *Rbpj^{L/L};CreER^{T2+/-}* embryos harvested at E9.5 had a severe developmental delay typical of a Notch deficient phenotype (n=20). After injection of 4 mg tamoxifen at E7.75, we were able to rescue early lethality and obtain *Rbpj^{L/L};CreER^{T2+/-}* embryos with an apparent similar morphology to the control embryos at E9.5 (n=30). To confirm NOTCH signalling was knocked down in these embryos, *Hes5* expression was analysed. *Hes5* was strongly down-regulated but not completely lost (n=8). After injection of 4 mg tamoxifen at E8.0, no morphological discrepancy between *Rbpj^{L/L};CreER^{T2+/-}* (n=40) and control embryos was observed. Notably, *Shh* expression at the level of the ventral hypothalamus was identical between control and *Rbpj^{L/L};CreER^{T2+/-}* embryos (black arrow).



Supplementary figure 5: Persistent buccohypophyseal canal in *Shh*^{+/-};*Rbpj*^{+/-}.

Haematoxylin and Eosin staining on frontal serial sections from the anterior to the posterior pituitary gland in control (A-F) and *Shh*^{+/-};*Rbpj*^{+/-} mutant (G-L) embryos at E18.5. Note the sphenoid bone (S) defect and the modification of anterior lobe (aP) of the pituitary gland in mutant. Yellow arrows indicate that the oral ectoderm (OE) is still connected to pituitary gland. Abbreviations: 3V, third ventricle; aP, anterior lobe of the pituitary gland; BO, basioccipital bone; D, diencephalon; HY, hypothalamus; pP, posterior lobe of the pituitary gland; PX, pharynx. S, sphenoid bone

PARTIE 3 : NOUVEAUX GENES IMPLIQUES DANS L'HPE ET REDEFINITION DES MODES DE TRANSMISSION

En parallèle de mon activité de recherche, je suis praticien hospitalier au laboratoire de génétique moléculaire et génomique au CHU de Rennes. Je travaille donc en étroite collaboration avec les praticiens responsables du diagnostic moléculaire des patients atteints d'HPE. Ma formation de Biologiste en Génétique Moléculaire m'a donc permis de participer activement aux discussions autour du diagnostic chez les patients et dans la recherche de nouveaux gènes candidats impliqués dans l'HPE. En effet, un diagnostic moléculaire est posé dans seulement 40% des HPE non chromosomique, non syndromiques (*Dubourg et al., 2018*). Notre équipe hospitalo-universitaire tente donc, par différentes stratégies d'analyse des ADN des patients (cartographie par homozygotie, exomes, génomes, etc...), de mettre en évidence l'implication de nouveaux gènes candidats dans la survenue de cette pathologie.

❖ **Article 4 : Homozygous STIL mutation causes holoprosencephaly and microcephaly in two siblings.**

PLOS ONE ; 2015

Charlotte Mouden, Marie de Tayrac, Christèle Dubourg, Sophie Rose, Wilfrid Carré, Houda Hamdi-Rozé, Marie-Claude Babron, Linda Akloul, Bénédicte Héron-Longe, Sylvie Odent, Valérie Dupé, Régis Giet, Véronique David

Afin d'identifier de nouveaux gènes candidats impliqués dans l'holoprosencéphalie, l'équipe a analysé l'ADN de patients issus de familles consanguines, en posant comme hypothèse l'existence d'un variant homozygote par descendance et d'un mode de transmission autosomique récessif. La cohorte d'échantillons d'ADN de patients HPE du CHU de Rennes comporte une quinzaine de familles consanguines avec un ou plusieurs cas d'HPE, et 10 individus isolés (ADN des parents non disponible) issus d'unions consanguines. Aucun remaniement, ni mutation pathogène dans un gène connu de l'HPE n'avait été retrouvé chez ces patients.

Pour 6 de ces familles consanguines, l'équipe a procédé à une cartographie des régions d'homozygotie par génotypage des SNP sur le génome entier, afin d'isoler dans chaque famille des régions candidates susceptibles de comporter des mutations homozygotes par descendance. Celles-ci ont ensuite été recherchées par séquençage de l'exome pour un enfant atteint par famille et un seul de ses parents.

Parmi ces 6 familles, l'une d'elles présentait un variant candidat intéressant, partagé par les deux enfants atteints et situé dans le gène *STIL* (appelé également *MCPH7*). Ce gène encode une protéine centrosomale, impliquée dans la formation du cil primaire. *Stil* a déjà été étudié sur modèle animal. Il existe notamment des souris *knock out* pour ce gène (*Stil*^{-/-}) qui présentent une absence de formation de la ligne médiane au niveau du prosencéphale, caractéristique de l'HPE (*Izraeli et al., 1999*). Des variants dans *STIL* ont par ailleurs été rapportés dans des cas de microcéphalies (*Kumar, 2009*). Or, les deux enfants atteints dans la famille étudiée présentent, en plus des phénotypes d'HPE lobaire et semi-lobaires, une sévère microcéphalie.

Le variant retrouvé chez ces patients touche un résidu conservé dans l'évolution. Il était prédit délétère par 8 outils de prédictions bio-informatiques sur les 10 employés. Afin de prouver fonctionnellement que la mutation avait un impact sur l'activité de la protéine STIL, nous avons mis en place un test cellulaire, en collaboration avec l'équipe de Régis Giet (Equipe Cytoskeleton and Cell Proliferation, IGDR).

Des études précédentes avaient montré qu'une déplétion de STIL par interférence à l'ARN bloquait la duplication des centrioles (*Vulprecht et al., 2012*). Nous avons donc étudié l'effet de la mutation sur le rôle de STIL dans la duplication des centrioles. Une expérience a été réalisée en éteignant la protéine STIL endogène par siRNA (small interfering RNA) puis en faisant un sauvetage avec la protéine GFP-STIL sauvage ou mutée. La duplication des centrioles a été évaluée en microscopie à fluorescence et a montré que la protéine STIL mutée ne restaurait pas totalement le phénotype centriolaire induit par la déplétion de la protéine STIL endogène. Ce résultat est conforme aux prédictions bio-informatiques et valide la présence d'une mutation délétère au niveau de ce gène.

RESEARCH ARTICLE

Homozygous *STIL* Mutation Causes Holoprosencephaly and Microcephaly in Two Siblings

Charlotte Mouden¹, Marie de Tayrac^{1,7}, Christèle Dubourg^{1,3}, Sophie Rose¹, Wilfrid Carré³, Houda Hamdi-Rozé^{1,3}, Marie-Claude Babron⁴, Linda Akloul⁵, Bénédicte Héron-Longe⁶, Sylvie Odent^{1,5}, Valérie Dupé¹, Régis Giet², Véronique David^{1,3*}

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Abstract

Holoprosencephaly (HPE) is a frequent congenital malformation of the brain characterized by impaired forebrain cleavage and midline facial anomalies. Heterozygous mutations in 14 genes have been identified in HPE patients that account for only 30% of HPE cases, suggesting the existence of other HPE genes. Data from homozygosity mapping and whole-exome sequencing in a consanguineous Turkish family were combined to identify a homozygous missense mutation (c.2150G>A; p.Gly717Glu) in *STIL*, common to the two affected children. *STIL* has a role in centriole formation and has previously been described in rare cases of microcephaly. Rescue experiments in U2OS cells showed that the *STIL* p.Gly717Glu mutation was not able to fully restore the centriole duplication failure following depletion of endogenous *STIL* protein indicating the deleterious role of the mutation. *In situ* hybridization experiments using chick embryos demonstrated that expression of *Stil* was in accordance with a function during early patterning of the forebrain. It is only the second time that a *STIL* homozygous mutation causing a recessive form of HPE was reported. This result also supports the genetic heterogeneity of HPE and increases the panel of genes to be tested for HPE diagnosis.

Introduction

Holoprosencephaly (HPE) (#236100) is the most frequent congenital malformation of the brain (1 in 10,000 live births; 1 in 250 conceptuses). HPE is characterized by impaired forebrain cleavage, midline facial anomalies and wide phenotypic spectrum. The clinical spectrum ranges

from alobar HPE to semilobar and lobar HPE generally associated with facial anomalies [1]. HPE is a severe pathology with mental retardation and developmental delay in all affected live newborns, with poor or symptomatic treatment. In addition, the midline malformation affects the development of the hypothalamus and the pituitary gland, leading to frequent endocrine disorders like temperature, heart rate and respiration instabilities, hypogonadism, thyroid hypoplasia or diabetes insipidus. The oromotor dysfunction is also affected, with feeding and swallowing difficulties [2]. Only 20% of children with alobar HPE survive after the first year of life, and 50% of infants with semi-lobar HPE are alive after 12 months [3]. Isolated HPE presents a high genetic heterogeneity and to date heterozygous mutations in 14 genes have been identified in HPE patients, 4 major genes (*Sonic hedgehog* or *SHH*, *ZIC2*, *SIX3*, *TGIF1*) and 10 genes considered as minor genes (*PTCH1*, *TDGF1*, *FAST1*, *GLI2*, *DISP1*, *FGF8*, *GAS1*, *CDON*, *NODAL* and *DLL1*) [4–7]. These genes encode proteins playing a role in early development, belonging mostly to signaling pathways like Sonic Hedgehog (SHH) or Nodal [8]. However, many patients remain without a molecularly confirmed diagnosis. In 70% of isolated cases, mutations in *SHH*, *SIX3* and *TGIF1* are inherited from a parent unaffected or harboring a microform of HPE [1], suggesting that other events are necessary to develop the disease. Thus, the mode of inheritance described as autosomal dominant with an incomplete penetrance and a variable expression has evolved to the assumption of a multi-hit pathology requiring two or more events involving several genes from the same or different signaling pathways.

The existence of rare consanguineous families suggests the possibility that autosomal recessive inheritance may account for a substantial part of this disorder. In the case of a rare recessively inherited disorder and known consanguinity, the initial assumptions are that the disease is caused by a homozygous variant inherited from both parents and that this variant resides within a large homozygous region.

To test this hypothesis and to improve the genetic basis of HPE, we have employed homozygosity mapping in two affected siblings and their mother issued from a Turkish consanguineous family (parents were first cousins), coupled with a next-generation sequencing approach on DNA from the mother and only one of the affected siblings (Fig. 1A). DNA of the father was not available.

Material and Methods

All patient samples in this study were obtained with informed consent according to the protocols approved by the local ethics committee (Rennes hospital).

Homozygosity Mapping

Genome-wide genotyping was undertaken using Illumina 300K single nucleotide polymorphism (SNP) mapping array beadchip (Illumina), on DNA of individuals I2 (mother), I13 (sibling—girl) and I15 (sibling—boy). The BlueFuse Multi software v3.3 (BlueGnome) was used to identify homozygous regions in all the family members. Only the regions longer than 1Mb and carrying at least 100 consecutive homozygous SNPs were selected. In parallel, homozygous regions and inbreeding coefficients were estimated/analyzed using FSuite pipeline [9].

Whole exome sequencing and variants filtering

Exome sequencing was performed on DNA of the boy I15 and of the mother I2 by Integragen using SureSelect V5 capture kit (Agilent) on HiSeq system (Illumina). The mean coverage was 80x, 95% of sequences had at least 10-times coverage and 91% of sequenced bases had a quality score greater than or equal to Q30. Bioinformatics analysis has been conducted with the Illumina pipeline analysis CASAVA v1.8. The sequenced reads were aligned to the hg19 human

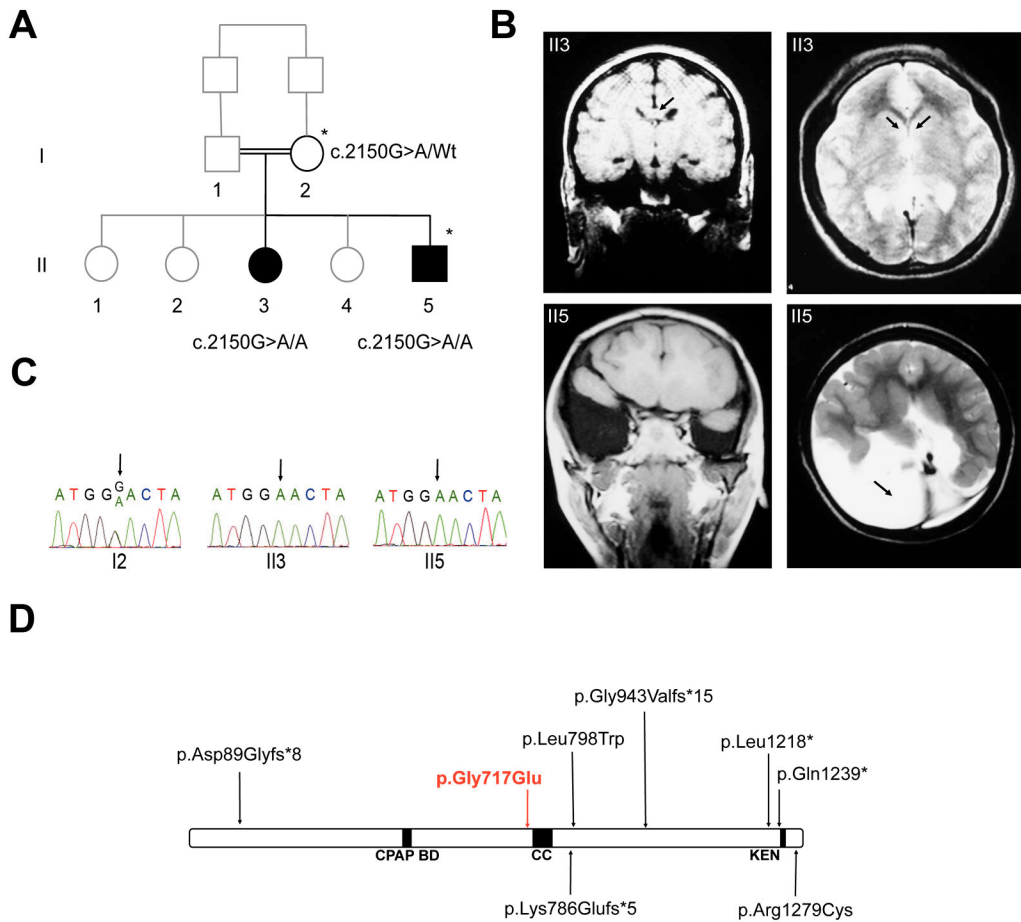


Fig 1. Pedigree of the consanguineous family, brain MRI of the affected siblings, Sanger validation of the c.2150G>A (p.Gly717Glu) *STIL* mutation, and schematic report of all *STIL* mutations reported so far. (A) Pedigree of the inbred family. Closed symbols indicate individuals affected with holoprosencephaly. Family members marked with an asterisk were analyzed by whole exome sequencing. (B) Coronal (on the left) and axial (on the right) brain MRI in individuals II3 and II5 at 12 and 5 years old respectively. II3: lobar HPE, the arrow in the coronal section shows the corpus callosum, and the arrows in the axial section show the absence of visualization of frontal horns, and a partial agenesis of the corpus callosum; II5: semi-lobar HPE, the arrow on axial MRI shows the absence of occipital lobe and a large unilateral temporal and occipital fluid cavity communicating. (C) Sanger validation was performed for the 3 available individuals II2, II3 and II5. The c.2150G>A mutation in *STIL* revealed a segregation with HPE in the two affected children. (D) Distribution of mutations previously reported in the literature on *STIL* protein. All mutations were present in a homozygous state [21,23,29] except those represented under the protein, which were two compound heterozygous mutations [28]. The p.Leu1218* mutation was found twice in two different families. The mutation reported in this study is p.Gly717Glu (in red) and is located in the central domain of the protein. Three important domains were represented here, the CPAP binding domain from amino acid 429 to 448, the coiled-coil domain (CC) from amino acid 720 to 750 and the KEN box, located between amino acids 1243–1245.

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reference genome with Eland v2 before variant calling (SNV and INDEL) with the CASAVA suite. The variants were then integrated into the Integragen proprietary online tool ERIS v2.0 (<http://eris.integragen.com/>) and filtered according to their genotype, allele frequency, and variant position within the gene. Based on the assumption that the mutation underlying the HPE was recessive in this inbred family, only mutations at a homozygous state in the boy (II5) and at a heterozygous state in the mother (II2) were retained. Resulting variants were annotated using a February 2014 build of ANNOVAR [10]. This software maps variants to RefSeq genes, known variations and frequencies from dbSNP137; it also annotates the predicted functional consequences of missense variants using six prediction algorithms (SIFT, Polyphen2, LRT, Mutation Taster, Mutation Assessor and FATHMM) and three conservation scores (PhyloP, GERP++ and SiPhy) from the dbNSFP v2.0 [11]. Predictions by two new ANNOVAR in-

house prediction scores were also performed. These algorithms, MetaSVM and MetaLR, use a radial Support Vector Machine (SVM) model that is based on multiple other scores, and according to the ANNOVAR website, the model outperforms other algorithms (developed by Coco Dong and Dr. Xiaoming Liu, University of Southern California, http://www.openbioinformatics.org/annovar/annovar_filter.html). Complementary annotations were performed using Condel v2.0 [12], Alamut v2.3 (Interactive Biosoftware) and Ingenuity Variant Analysis (www.ingenuity.com/variants) softwares.

Sanger sequencing

The *STIL* mutation c.2150G>A was confirmed in the mother I2 and the boy II5, and searched for in the girl II3 by classic Sanger sequencing. This was done using the BigDye terminator cycle sequencing kit (Applied Biosystems) on an ABI3130xl sequencer (Applied Biosystems) and analyzed using SeqScape software v2.6 (Life Technologies).

Centriole duplication assay

The plasmid expressing GFP-STIL p.Gly717Glu was obtained by site-directed mutagenesis from a pEGFP-C1 plasmid containing cDNA of GFP-STIL, kindly provided by Alwin Kramer (German Cancer Research Center, Heidelberg, Germany). These plasmids were resistant to the siRNA used for depletion of endogenous STIL. The sequence of the siRNA used was 5'-CAGU AACUCUAGCAAUAA-3'. U2OS cells (human osteosarcomas cells) were grown at 37°C in DMEM media containing 10% fetal calf serum and 100 U/ml of streptomycin and penicillin. Rescue experiments were performed as described in Franck *et al* [13]. Cells were transfected using JetPRIME transfection reagent (Polyplus Transfection), first with 100 pmol/ml of siRNA, and 24h later with 600 ng/ml of plasmids pEGFP-STIL WT or pEGFP-STIL p.Gly717-Glu. At the same time, cells were synchronized in G1/S phase with aphidicolin at 4 µl/ml. Cells were fixed 36h after plasmid transfection with methanol. For counting, centrioles were labeled using a mouse anti-centrin antibody (Millipore, clone 20H5) and GFP-STIL was labeled using a rabbit anti-GFP antibody (Abcam, ab-89314). The experiments were repeated at least three times and a minimum of 50 cells were counted for each experiment.

In Situ Hybridization

Fertilized chicken (*Gallus gallus*) eggs were obtained from EARL Les Bruyères (France). For the required developmental stages Hamburger and Hamilton (HH) 8, HH10 and HH16, eggs were incubated in a humidified incubator at 38°C [14]. After harvesting, chick embryos were fixed with 4% paraformaldehyde in phosphate buffered saline. The *Stil* probe was generated by PCR using the *Gallus gallus* NCBI *Stil* sequence, and subcloned in the pCRII-TOPO vector (Invitrogen, Cergy Pontoise, France). The recombinant plasmid was used to transcribe the sense and antisense RNA probes, labeled with digoxigenin. Whole-mount *in situ* hybridization was performed as previously described [15].

Results and Discussion

The two HPE affected children (born alive) and their three healthy siblings were seen in France, the girl (II3) was 12 years of age and the boy (II5) was 5 years of age (Fig. 1A, B).

For II3, her weight was 21.7 kg (-2.5 Standard Deviation, SD), height 1.24 m (-3SD), and occipitofrontal circumference (OFC) 41 cm (microcephaly <-7SD). Although the OFC was not recorded at birth, microcephaly was reported to be congenital. She had a severe intellectual disability, said only a few words, with a good understanding. Her walk was normal and she had

no behavioral or sleep defects. Brain MRI showed lobar HPE, absence of ventricular frontal horns, partial agenesis of the corpus callosum, both anterior and of the splenium (Fig. 1B).

For II5, his weight was 14 kg (-2SD), height 1 m (-2SD), and OFC 41.5 cm (microcephaly <-8SD). Microcephaly was also congenital. He had hypotelorism, major behavioral disorders, with episodes of self-aggression and sleep disorders. He had no language or acquired sphincter control. His walk was normal. He developed generalized tonic clonic seizures. Brain MRI revealed a semi-lobar HPE, atrophy of the vermis, partial agenesis of the corpus callosum, absence of occipital lobe and a large temporal and occipital fluid cavity communicating with the right ventricular junction (Fig. 1B). Both karyotype and Comparative Genomic Hybridization (CGH) Array detected no chromosomal abnormalities for both II3 and II5.

The homozygosity mapping identified 11 identical by descent regions larger than 1Mb, shared by the two affected siblings and heterozygous in the mother. Exome sequencing of the boy (II5) and the mother (I2) revealed homozygous mutations in 7 genes located in these regions, among which 6 were on the same largest region of 18Mb located on 1p23 between rs230280 and rs12402927 (*CTRC*, *SPEN*, *WDR65*, *STIL*, *ORC1*, *LCCR7*), and 1 was located on 12q24 (*PGAM5*).

Frequencies of these mutations in public databases, bioinformatics predictions, conservation properties and physico-chemical gap between the wild-type and mutated amino acid were analyzed (Table 1). Regarding these elements, the 3 mutations presenting the most deleterious criteria were those located in *CTRC*, *ORC1* and *STIL*. The *CTRC* gene encoded the serine protease chymotrypsin C, a protein produced in small quantities by pancreatic cells that degrades trypsin. Mutations in *CTRC* have been associated with hereditary pancreatitis [16]. *CTRC* was not expressed in the mouse adult brain (Expression Atlas, European Molecular Biology Laboratory), and the only known link with development is its association with enamel development, the hard mineralized surface of teeth [17]. The combination of these elements permitted the *CTRC* mutation to be discarded. The *ORC1* mutation was intriguing because mutations in this

Table 1. Characteristics of homozygous candidate mutations.

Gene	Mutation	Amino acid conservation	Physico-chemical gap	Minor allele frequency		Bioinformatics predictions				
				1000G	ESP6500	Condel	SIFT	PolyPhen2	MetaSVM	MetaLR
CTRC	p. Glu96Lys	very conserved	low	MAF = 0,0005	no	deleterious	deleterious	probably damaging	deleterious	deleterious
SPEN	p. Arg672Gln	no data	low	no	no	neutral	tolerated	probably damaging	tolerated	tolerated
WDR65	p. Arg891Gln	poorly conserved	low	no	no	neutral	tolerated	benign	tolerated	tolerated
STIL	p. Gly717Glu	very conserved	high	no	no	deleterious	deleterious	probably damaging	deleterious	tolerated
ORC1	p. Arg728His	very conserved	low	MAF = 0,0005	no	deleterious	deleterious	probably damaging	tolerated	tolerated
LRRC7	p. Pro755Ser	moderately conserved	high	no	no	neutral	tolerated	benign	tolerated	tolerated
PGAM5	p. Arg118His	very conserved	low	MAF = 0,0005	MAF = 0,000308	deleterious	tolerated	possibly damaging	tolerated	tolerated

The amino acid conservation and physico-chemical gap come from Alamut software. Minor Allele Frequency (MAF) in the 1000 Genomes Project (1000G, April 2012), in the Exome Sequencing Project (ESP6500, October 2012) and bioinformatics predictions by SIFT, PolyPhen2, MetaSVM and MetaLR were performed using ANNOVAR.

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gene were previously associated with Meier-Gorlin syndrome, a microcephalic primordial dwarfism [18]. *ORC1* encodes the subunit 1 of the origin recognition complex, a multi-subunit DNA binding complex, which is a key component of the DNA replication licensing machinery, and also plays a role in controlling centriole and centrosome copy number in human cells [19]. Except for microcephaly, the phenotype of Meier-Gorlin patients does not match with the phenotype of the affected siblings II3 and II5. In fact, the Meier-Gorlin syndrome has been characterized by a proportional short stature and microcephaly, with mean heights of approximately -5.5SD and -6SD at 5 and 12 years old [20]. Mean heights of II5 and II3 were -2SD and -2.5SD, which was significant but not major and did not correlate with their severe microcephaly. Moreover, the *ORC1* mutation found in the family was already described in the 1000 Genomes Project (1000G, April 2012), and predicted tolerated both by MetaSVM and MetaLR from ANNOVAR.

The mutation that presented the most deleterious criteria was the *STIL* homozygous mutation c.2150G>A (p.Gly717Glu). Indeed, almost all bioinformatic tools used predicted that this mutation was likely to affect the protein function. This mutation was located in exon 12 of the gene *STIL* (or *SIL*; *SCL/TAL1 interrupting locus*) (RefSeqNG_012126.1), encoding a pericentriolar and centrosomal protein. It was absent in 1000G and the Exome Sequencing Project (ESP6500, October 2012) databases, and in the Genome Management Application (University of Miami Health System) that contains genomic data of about 200 Turkish families.

This mutation concerned a highly conserved nucleotide and amino acid through 12 species including *Tetraodon* and *Xenopus*, and the physico-chemical gap between Glycine and Glutamic acid is high.

Segregation analysis by Sanger sequencing in the 3 available family members (II2, II3 and II5) confirmed the recessive inheritance of the c.2150G>A (p.Gly717Glu) mutation in the *STIL* gene, with consistent genotypes (Fig. 1C).

STIL mutations have been described in rare cases of autosomal recessive primary microcephaly (MCPH) [21–23]. MCPH is a genetically heterogeneous disease characterized by an intellectual deficit and a pronounced reduction in brain volume, with or without architectural anomalies, depending of the mutated gene. Eleven genes have been identified so far as being involved in the cause of this disease and most of these genes encode either centrosomal proteins or proteins associated with the poles of the mitotic spindle [24]. Several clinical and molecular genetic studies on microcephaly have been published. These studies have shown that in typical microcephaly, mutations in the *ASPM* gene were the most prevalent (14.1%) and mutations in *STIL* were less frequent (2.2%). They described several truncating mutations in the C terminus of the protein *STIL* (Fig. 1D) [21]. These truncating mutations deleted a motif involved in proteasomal degradation, called the KEN box, which would make *STIL* resistant to proteasomal degradation and cause centriole amplification. The mutation found in our HPE patients (p.Gly717Glu) was located in a conserved central part of the protein near a coiled-coil domain of the protein, but not in the regions interacting with the CPAP protein required for centriole assembly (Fig. 1D) [25].

Mouse embryos homozygous for the mutated *Stil* allele displayed multiple abnormalities, including forebrain midline defects and die by embryonic day (E) 10.5 [26]. These defects were reminiscent to a HPE phenotype and led Karkera *et al.* to look for an association with HPE and *STIL* mutations; however no causative mutations were noted in the 83 HPE patients studied [27]. We sequenced *STIL* in a series of 21 patients presenting HPE and microcephaly with 8 of them born from consanguineous parents, but we did not identify any mutations (data not shown). Among these patients, 12 have a European origin, 7 have a North-African origin, and 2 have a Middle-Eastern origin. Recently, two papers reported the implication of recessive *STIL* mutations, in a family with microcephaly associated with some midline defect [28], and

in a consanguineous family with microcephaly and HPE [29] (Fig. 1D). Altogether, these results suggest that *STIL* complements the already long list of genes involved in HPE.

The *STIL* gene comprises 18 exons, and the protein was identified as being required for cell-cycle mitotic entry as well as for centriole formation and duplication [30]. Depletion of *STIL* blocks centriole duplication, while overexpression results in the generation of extra centrioles, an event known as centrosome amplification. These results suggest that the expression levels of *STIL* needs to be precisely controlled and this was achieved by proteasomal degradation during mitotic exit [25,30,31].

In order to test the deleterious role of the *STIL* mutation p.Gly717Glu, we implemented transitory rescue experiments in U2OS cells (Fig. 2). Cells were first transfected with a siRNA targeting endogenous *STIL*, and 24h later with plasmids expressing RNAi resistant GFP-*STIL* WT or GFP-*STIL* p.Gly717Glu (Fig. 2A). Centrioles were counted 36h after transfection of GFP-*STIL* plasmids, allowing expression of the WT and the mutant GFP-tagged variants of *STIL*, and depletion of the endogenous *STIL* by RNAi. In order to have a homogeneous cell population, cells were synchronized in G1/S phase by aphidicolin treatment that blocked DNA synthesis but allowed centrioles to continue to replicate. In control U2OS cells, we observed approximately 93% of cells containing 4 centrioles and 7% contained less than 4 centrioles (Fig. 2B). As described in the literature [30], in absence of plasmid, the number of centrioles per cell decreased when cells were transfected with the *STIL* siRNA, with a majority of cells (80%) displaying less than 4 centrioles (Fig. 2B). When cells were transfected with GFP-*STIL* WT and siRNA, the GFP-*STIL* WT was able to rescue the duplication of centrioles, and after 36h, about 60% of cells had 4 or more centrioles. By contrast, when cells were transfected with siRNA and GFP-*STIL* p.Gly717Glu, the rescue was less efficient. Indeed, after 36h, 67% of the cells still had less than 4 centrioles (Fig. 2B) as illustrated in synchronized G1/S phase cells (Fig. 2C). However, we observed a partial rescue, suggesting a reduced but not null activity of *STIL* p.Gly717Glu on centriole duplication. The same result was observed in non-synchronized U2OS cells (S1 Fig.), suggesting a deleterious role of the mutation p.Gly717Glu for *STIL* in our patients.

The *Stil* gene, highly conserved among vertebrates, has been described to be widely expressed in the developing mouse embryo [26]. However no data is currently available about the early expression of *Stil* during vertebrate forebrain patterning. *In situ* hybridization (ISH) was used to determine whether the expression of *Stil* coincides with a regulatory function during ventral forebrain development when HPE arises.

The first detailed description of *Stil* expression in the developing vertebrate forebrain has been presented here (Fig. 3A, C, D and E). *Stil* was expressed during the first stages of chick brain development (HH8) consistent with *Stil* having a role in ventral forebrain patterning (Fig. 3A). Significantly, the ventral view of the corresponding flat-mounted neural tube revealed the ventral neuroectodermal surface specifically expressing *Stil* (Fig. 3C). *Stil* was expressed in the ventral forebrain in a domain that overlaps with the anterior expression domain of *Shh* (Fig. 3B), the main HPE gene [1]. Similarly, *Sufu*, a repressor of *Shh* pathway, was also expressed in this area [32]. Remarkably, *in vitro* experiments have shown that *STIL* interacts in the cytoplasm with *SUFU* to regulate *Shh* signaling [33–35]. This role may also be disturbed by the abnormal behavior of the mutated GFP-*STIL* (p.Gly717Glu) protein. Later between HH10 and HH16 (Fig. 3D, E), when patterning of the dorso-ventral forebrain has already initiated, *Stil* was expressed in all the neuroectoderm tissue. When using a *Stil* sense probe as control, no staining was observed (data not shown). This expression study suggested that *STIL* may have a function during early patterning of the forebrain that could be linked to *Shh* signaling. An abnormal *STIL* protein would lead to a disturbed *Shh* signaling which could explain the HPE phenotype. Subsequently, the ubiquitous expression of *STIL* during brain growth could be responsible for microcephaly appearance through its implication in centriole formation [24].

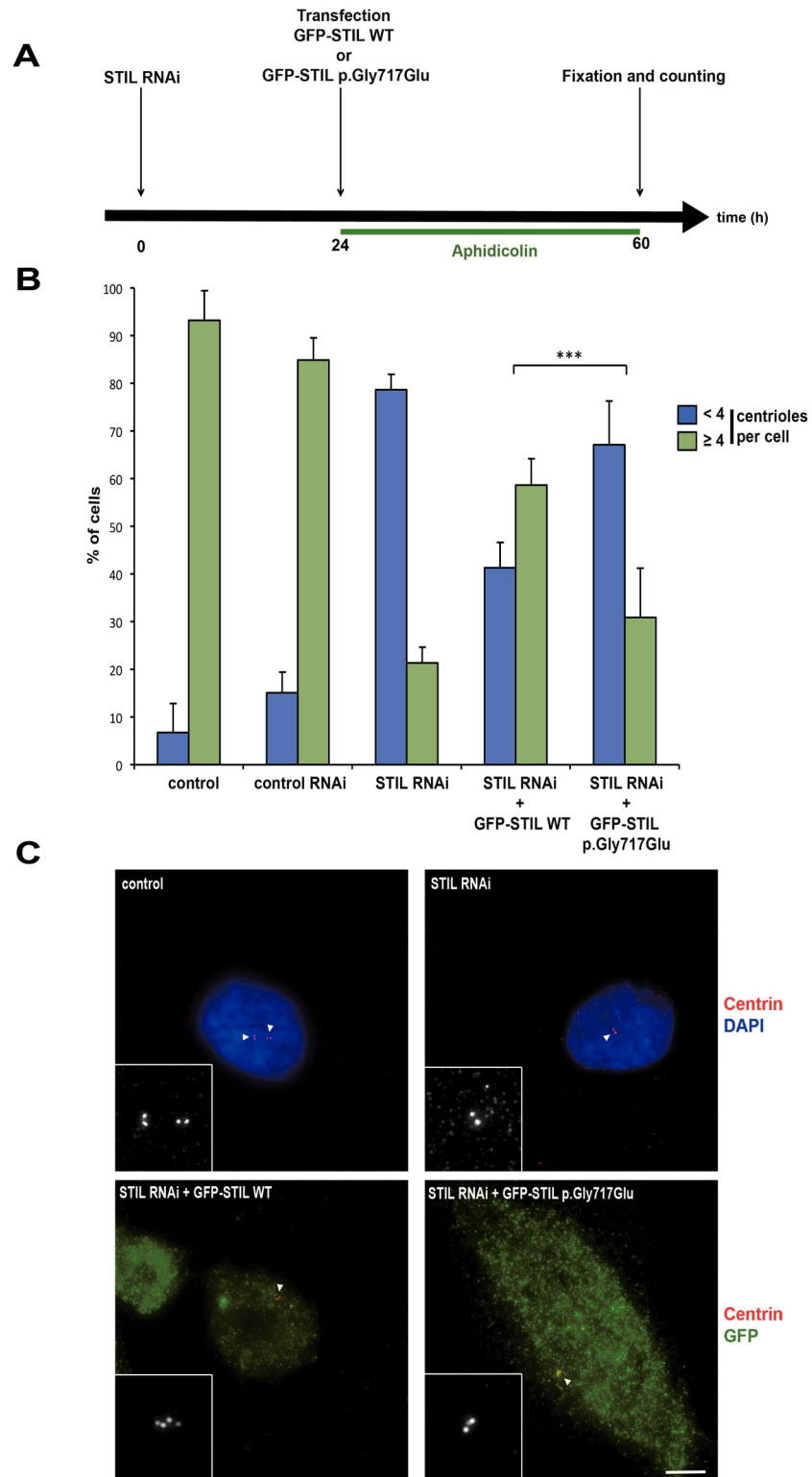


Fig 2. p.Gly717Glu cannot fully restore STIL depletion in synchronized U2OS cells. (A) Protocol used to assay the centriole duplication potential of WT and mutant STIL proteins. U2OS were treated with STIL RNAi, and transfected 24h later with GFP-STIL WT or GFP-STIL p.Gly717Glu constructs in aphidicolin containing medium (4 μ g/ml). Cells were fixed and counted 36h later to allow centriole duplication. (B) Percentages of S phase cells containing <4 or ≥ 4 centrioles following control RNAi (scrambled) and STIL RNAi, followed or not

by transfection with GFP-STIL WT or GFP-STIL p.Gly717Glu ($p < 0,001^{***}$). (C) Examples of S phase-arrested cells following different treatments. A control cell with 4 centrioles (top left panel), a STIL RNAi treated cell with 2 centrioles (top right panel), a STIL-depleted cell expressing GFP-STIL WT with 4 centrioles (bottom left), and a STIL-depleted cell expressing GFP-STIL p.Gly717Glu with 2 centrioles are displayed (bottom right). Centrin is shown in red (and in monochrome in the insets), DNA is blue (top panels) and GFP is green (bottom panels). The white arrowheads indicate the centriole region. The bar represents 10 μ m.

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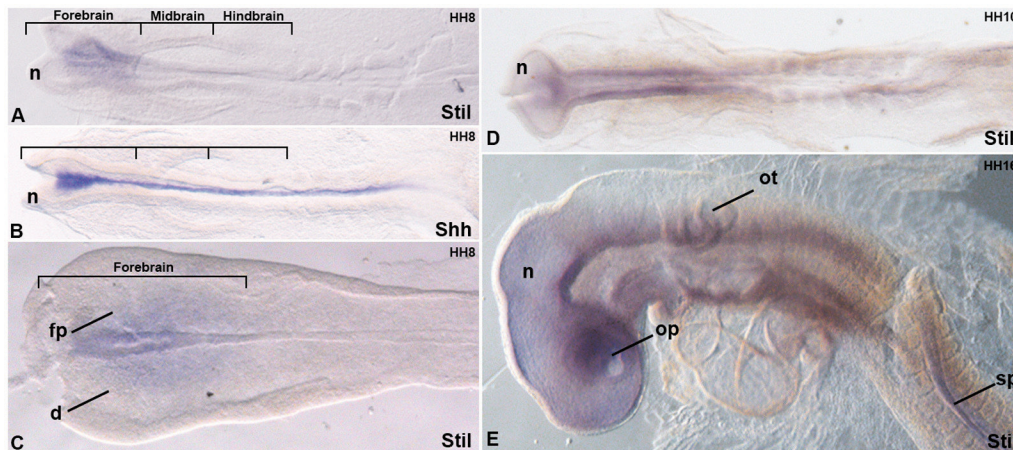


Fig 3. *Stil* was specifically expressed in the developing chick forebrain. *In situ* hybridization analysis of *Stil* (A, C-E) was performed on whole-mount chick embryos at developmental stages HH8, HH10 and HH16. Expression of *Shh* (B) was analyzed at HH8. A, B and D are dorsal views, E is a lateral view. (C) Flat-mounted preparation of the forebrain of the above embryo (A). Brackets indicate the compartments of the brain. d, dorsal border of the forebrain; fp, floor plate; n, neurectoderm; ot, otic vesicle; op, optic vesicle, sp, spinal cord.

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This study confirms that homozygosity mapping associated with next generation sequencing in consanguineous families can be of great interest to identify new genes in heterogeneous pathologies. In fact, the results presented here confirm that mutations in *STIL* can cause HPE associated with microcephaly, with a recessive mode of inheritance. These findings have clinical application since this new gene will increase the panel of genes already tested for holoprosencephaly diagnosis.

Supporting Information

S1 Fig. GFP-STIL p.Gly717Glu failed to complement STIL-dependent centriole duplication in U2OS cells. U2OS cells were subjected to control RNAi and STIL RNAi together with expression of siRNA resistant GFP-STIL or GFP-STIL p.Gly717Glu for 48h. The cells were then fixed and stained for GFP (green), tubulin (blue) and centrin (red, and monochrome in the lower panels). (A) Control cell with 4 centrioles (left), STIL-depleted cell expressing GFP-STIL WT with 4 centrioles (middle), STIL-depleted cell expressing GFP-STIL p.Gly717Glu with 3 centrioles (right) during mitosis. Centrioles were indicated by the triangles and the insets show the centriole regions. Bar represents 10 μ m. (B) Percentages of the interphase and mitotic cells containing 1, 2, 3, 4 or >4 centrioles following control RNAi (scrambled) and STIL RNAi with or without co-transfection with GFP-STIL WT or GFP-STIL p.Gly717Glu. The number of centriole was quantified in the GFP positive cells. Most STIL-depleted cells (70%) expressing GFP-STIL WT displayed 4 or more centrioles against only 30% for GFP-STIL p.Gly717Glu expressing cells ($p < 0,001^{***}$).

(TIF)

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Author Contributions

Conceived and designed the experiments: V. David MDT RG V. Dupé CD MCB. Performed the experiments: CM SR HHR. Analyzed the data: WC MDT CM SR. Contributed reagents/materials/analysis tools: BHL LA SO. Wrote the paper: V. David CM RG V. Dupé.

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❖ **Article 5 : Complex mode of inheritance in holoprosencephaly revealed by whole exome sequencing**

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Les mutations retrouvées dans les gènes de l'HPE sont majoritairement des mutations hétérozygotes héritées de parents asymptomatiques ou présentant une forme beaucoup plus atténuée que leur enfant. Pour expliquer cette hétérogénéité phénotypique, nous avons émis l'hypothèse que l'HPE avait une origine multigénique. Afin d'explorer cette hypothèse, l'équipe a réalisé le séquençage exomique en « trios » (deux parents et un enfant par famille) de 11 familles où l'enfant atteint présentait une mutation hétérozygote dans un des trois gènes de l'HPE *SHH*, *SIX3* et *TGIF1*, qu'il avait hérité de l'un de ses deux parents. L'analyse des séquençages a été adaptée de façon à rechercher une ou plusieurs autres mutations chez l'enfant, héritées de l'autre parent ou apparues *de novo*.

Grâce à cette approche, et après des étapes de filtres bio-informatiques pour éliminer les variants hérités du parent transmetteur de la première mutation et ne retenir que ceux dans des gènes en lien avec l'HPE, un variant a particulièrement retenu notre attention. Il a été retrouvé dans le gène *DISP1* dans une famille comportant 3 fœtus atteints de HPE et présentant tous les 3 le même variant dans *SHH*. Le variant dans *SHH* avait été hérité de leur père et n'était pas présent chez les deux sœurs saines. Le variant *DISP1* était lui hérité de la mère, présent chez deux des trois fœtus (le troisième n'ayant pu être testé) et chez l'une des deux sœurs saines.

Outre le mode de transmission compatible avec une hypothèse de multigénisme, ce variant était particulièrement intéressant puisque *DISP1* joue un rôle dans la sécrétion de *SHH*. Des embryons de souris présentant une mutation délétère dans *Disp1* présentent une diminution du signal *SHH* (Caspary et al., 2002). Ces arguments renforcent l'hypothèse d'une transmission digénique, pouvant expliquer la survenue de cette pathologie.



Original Article

Complex mode of inheritance in holoprosencephaly revealed by whole exome sequencing

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Holoprosencephaly (HPE) is the most common congenital cerebral malformation, characterized by impaired forebrain cleavage and midline facial anomalies. Heterozygous mutations in 14 genes have been associated with HPE and are often inherited from an unaffected parent, underlying complex genetic bases. It is now emerging that HPE may result from a combination of multiple genetic events, rather than from a single heterozygous mutation. To explore this hypothesis, we undertook whole exome sequencing and targeted high-throughput sequencing approaches to identify mutations in HPE subjects. Here, we report two HPE families in which two mutations are implicated in the disease. In the first family presenting two fetuses with alobar and semi-lobar HPE, we found mutations in two genes involved in HPE, *SHH* and *DISP1*, inherited respectively from the father and the mother. The second reported case is a family with a 9-year-old girl presenting lobar HPE, harbouring two compound heterozygous mutations in *DISP1*. Together, these cases of digenic inheritance and autosomal recessive HPE suggest that in some families, several genetic events are necessary to cause HPE. This study highlights the complexity of HPE inheritance and has to be taken into account by clinicians to improve HPE genetic counselling.

Conflict of interest

All authors declare that they have no conflicts of interest in the research.

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Key words: *DISP1* – holoprosencephaly – multigenic inheritance – *SHH* – whole exome sequencing

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Holoprosencephaly (HPE) is the most frequent cerebral malformation, with an occurrence of approximately 1 in 250 embryos and 1.3 in 10,000 births (1). HPE is characterized by a failure to define the midline of the forebrain and midface, with different degrees of severity from a lobar brain to alobar forms associated with cyclopia. Mild manifestations or microforms include ocular hypotelorism, microcephaly and a single central maxillary incisor (2). The mode of inheritance of HPE has been extensively discussed in the literature, and several genetic models have been proposed: autosomal dominant transmission, autosomal recessive transmission or association of mutations in multiple genes (2–5). All these studies point out a strong genetic heterogeneity, with several causative genes identified (Table 1). It mostly implicates heterozygous mutations in *SHH*, *ZIC2*, *SIX3* and *TGIF1*, which are considered as the four major genes involved in HPE. Heterozygous mutations in the minor genes, *GLI2*, *PTCH1*, *DISP1*, *FOXH1*, *NODAL*, *TDGF1*, *CDON*, *GAS1*, *DLL1* and *FGF8*, have been identified with a lower frequency (2, 7). Recently, two recessive inheritance cases of HPE have been described, implicating mutations in the gene *STIL* (Table 1) (8, 9). Importantly, these genes are all involved in signalling pathways implicated in brain development (4, 9–16) and alteration of SHH signalling appears to be the most common cause of HPE (17).

Although the major genes have been formally involved, their penetrance is usually incomplete with an intra-familial phenotypic variability. Actually, mutations located in these genes are inherited from a parent, asymptomatic or displaying a microform of HPE, in 70% of the cases (2). For example, the same *SHH*

mutation can be found in individuals harbouring either alobar HPE or minor forms (18). Consequently, the clinical variability could be because of the abnormalities in other genes that have a function in the same or interacting signalling pathways (19, 20).

This is strongly supported by the description of mouse models carrying mutations in two genes of the same or different signalling pathways. For example, although *Gas1*^{-/-} mutant mice exhibit partial fusion of the medial nasal processes and *Shh*^{+/-} mice appear normal, *Gas1*^{-/-}; *Shh*^{+/-} mice embryos display complete fusion of the medial nasal processes (21), reminiscent of a HPE phenotype. Such examples of animal models are numerous and all support the hypothesis that HPE could be because of a cumulative partial inhibition of signalling pathways implicated in forebrain development (6, 22).

However, there are only a few examples in the literature that suggest that HPE could be related to combined failures of several HPE genes in human (Table 2), including patients with co-occurring mutations or deletions in *SHH/TGIF1* and *SHH/ZIC2* (23). Nevertheless, sequencing of the four major genes (*SHH*, *ZIC2*, *TGIF1* and *SIX3*) in large HPE cohorts has not allowed to validate this hypothesis (2, 5, 14). Thus, the mode of inheritance of HPE is still unclear.

Identifying more genes in families in which polygenic inheritance is suspected would be very beneficial to understand the pathogenic mechanism of this developmental disorder. This is now facilitated by the recent development of next generation sequencing technologies (19). In this study, we performed whole exome sequencing (WES) in a family where the father carries a mutation in *SHH*, transmitted to three fetuses with

Table 1. Characteristics of genes and mutations implicated in HPE^a

Gene	Chromosomal locus	Mutation frequency in non-syndromic HPE	Percentage of inherited mutations from a parent	Zygosity state	References
<i>SHH</i>	7q36	High (12%)	70%	Heterozygous	(5, 23, 44, 55–57)
<i>ZIC2</i>	13q32	Medium (9%)	30%	Heterozygous	(5, 44, 58–61)
<i>SIX3</i>	2p21	Medium (5%)	70%	Heterozygous	(5, 44, 62–66)
<i>TGIF1</i>	18p11.3	Medium (1.7%)	Mainly inherited	Heterozygous	(5, 67, 68)
<i>PTCH1</i>	9q22.3	Low	Mainly inherited	Heterozygous	(4, 69, 70)
<i>TDGF1</i>	3p23-p21	Low	NA	Heterozygous	(10)
<i>GLI2</i>	2q14	Low	Mainly inherited	Heterozygous	(69, 71, 72)
<i>DISP1</i>	1q42	Low	Mainly inherited	Heterozygous	(38, 39)
<i>FGF8</i>	10q24	Low	Mainly inherited	Heterozygous–Homozygous	(11, 46)
<i>FOXH1</i>	8q24.3	Low	NA	Heterozygous	(73)
<i>NODAL</i>	10q22.1	Low	NA	Heterozygous	(74)
<i>GAS1</i>	9q21.33	Low	Mainly inherited	Heterozygous	(12)
<i>DLL1</i>	6q27	Low	Inherited	Heterozygous	(15)
<i>CDON</i>	11q24.2	Low	NA	Heterozygous	(13)
<i>STIL</i>	1p33	Low	Inherited	Heterozygous–Homozygous	(8, 9, 75)

HPE, holoprosencephaly; NA, not available.

^aThe mutation frequencies are given in qualitative terms: ‘high’ is for genes mutated in more than 10% of HPE cases, ‘medium’ for genes mutated between 10 and 1%, and ‘low’ for genes mutated in less than 1% of HPE cases. Mutation frequencies were calculated based on our local HPE cohort (>1000 cases). Mainly inherited: inherited mutations from a parent are predominant among the reported cases; inherited: All reported mutations are inherited from a parent. CNV or large indels encompassing whole genes are not included in this table.

Complex mode of inheritance in holoprosencephaly

Table 2. Digenic inheritance in human HPE cases^a

Gene	Mutation	References
<i>SHH</i>	p.Gly290Asp	(23)
<i>ZIC2</i>	p.Ala461_Ala470dup	
<i>SHH</i>	p.Pro424Ala	(23)
<i>TGIF1</i>	del18p11	
<i>SHH</i>	p.del378_380	(23)
<i>TGIF1</i>	p.Thr151Ala	
<i>GLI2</i>	p.Arg151Gly	(70)
<i>PTCH1</i>	p.Thr328Ala	
<i>SIX3</i>	p.Ala93Asp	(4)
<i>PTCH1</i>	p.Ala393Thr	
<i>SIX3</i>	p.Ala284Pro	(65)
<i>ZIC2</i>	p.Trp304Arg	
<i>SHH</i>	p.Leu218Pro	(12)
<i>GAS1</i>	p.Asp270Tyr	
<i>SHH</i>	p.Cys363Tyr	(12)
<i>GAS1</i>	p.Asp288Gly	
<i>SHH</i>	p.Pro347Gln	In this report
<i>DISP1</i>	p.Met1096Thr	

^aMutations are given in proteic nomenclature, except the del18p11 which carries the *TGIF1* gene off.

semi-lobar and alobar HPE. We hypothesized that the foetuses have all inherited a second mutation in another gene from the mother. This original strategy was powerful as it revealed a second mutation in *DISP1* shared by the mother and the HPE foetuses. This family is the first one in which mutations in the two HPE-associated genes, *SHH* and *DISP1*, have been identified. Furthermore using a targeted next-generation sequencing (NGS) method, the involvement of *DISP1* in HPE was reinforced by the observation of two *DISP1* compound heterozygous mutations in another HPE family. These results support the complexity of HPE inheritance and raise important questions about how clinicians should consider the inheritance mode of HPE.

Materials and methods

Patients and samples

Patients presenting midline abnormalities and suspicion of HPE were referred from the Centre Hospitalier Universitaire of Rennes (France) and the Hôpital Cochin (Paris, France) through the network of reference centres for developmental anomalies and malformation syndromes (CLAD centres). Patients and parents blood and tissue samples were obtained from the processing of biological samples through the Centre de Ressources Biologiques (CRB) Santé of Rennes BB-0033-00056 (<http://www.crbsante-rennes.com>). The research protocol was conducted under French legal guidelines and fulfilled the requirements of the local institutional ethics committee. The parents of the photographed patient assented to include photographs in a scientific publication.

F1 family

The mother II2 had three terminations of pregnancy with foetuses (III1, III2 and III3) harbouring semi-lobar or

alobar HPE (Fig. 1a and Table 3a). The F1 family also includes two other healthy girls III4 and III5, being 12- and 11-years-old, respectively. The father II1, the grandfather II and the uncle II3 all present microcephaly. This latter had four children among which three (III6, III7 and III9) harbour microcephaly and minor facial midline malformations (hypotelorism). The father II1 also had two healthy siblings, II5 and II6 (Table 3a). DNA was available for all family members except II4.

F2 family

The F2 family includes three members, the two healthy parents I1 and I2 and their 9-year-old girl III1 who displayed a lobar HPE (Fig. 2 and Table 3b). She has two healthy siblings but their DNA was not available. Pregnancy was normal. A wide cleft palate was observed at birth, which was surgically treated. III1 was then referred to genetic counselling at 5-and-half-years-old for psychomotor retardation (started walking at 21 months and had a language delay) and learning difficulties. On clinical examination, she weighed 16.5 kg (−1 SD), was 109.5 cm tall, and had microcephaly (−2 SD). She presented facial dysmorphism with flat face, short nose, small mouth and hypotelorism (Fig. 2c,d). At the sight of these clinical observations, we recommended a molecular diagnosis of HPE. Molecular testing of 18 HPE genes was performed on the DNA of daughter III1, revealing the presence of two compound heterozygous mutations in the minor HPE gene *DISP1*. Subsequently, to confirm the presence of HPE, MRI was performed and showed a mild form of lobar HPE with a very localized fusion of hemispheres in the forebrain (Fig. 2b).

Whole exome sequencing

WES was performed by the Genoscope on the DNA of the two parents, II1 and II2, and one of the foetuses III2 in F1 family, using ‘SeqCap EZ Exome v3.0’ capture (Roche, Basel, Switzerland) on HiSeq™2000 platform (Illumina, San Diego, California, United States). Exomes were homogeneously sequenced with a mean coverage of 93% of the targeted bases with read depth greater than 20X, and an overall mean depth of coverage of 112X. Bioinformatic analyses were conducted with the Illumina pipeline analysis CASAVA 1.8. The sequenced reads were aligned on the reference human genome 19 (hg19) with Eland v2.0 before variant calling (SNV and INDEL) with the CASAVA suite. Resulting variants were annotated using ANNOVAR v2.0 (http://www.openbioinformatics.org/annovar/annovar_filter.html). Variants population frequencies were extracted from three different public databases [the Exome Sequencing Project (ESP6500), the 1000 Genomes Project (1000g, 2014) and the Exome Aggregation Consortium (ExAC02)]. Several bioinformatics predictions tools were used to predict conservation (GERP++, PHYLOP, SiPHY, and PHASTCONS ELEMENTS 46-WAY) (24–27) and deleterious effect of SNVs and INDELS (SIFT, POLYPHEN-2 HDIV and HVAR, LRT, MUTATIONTASTER,

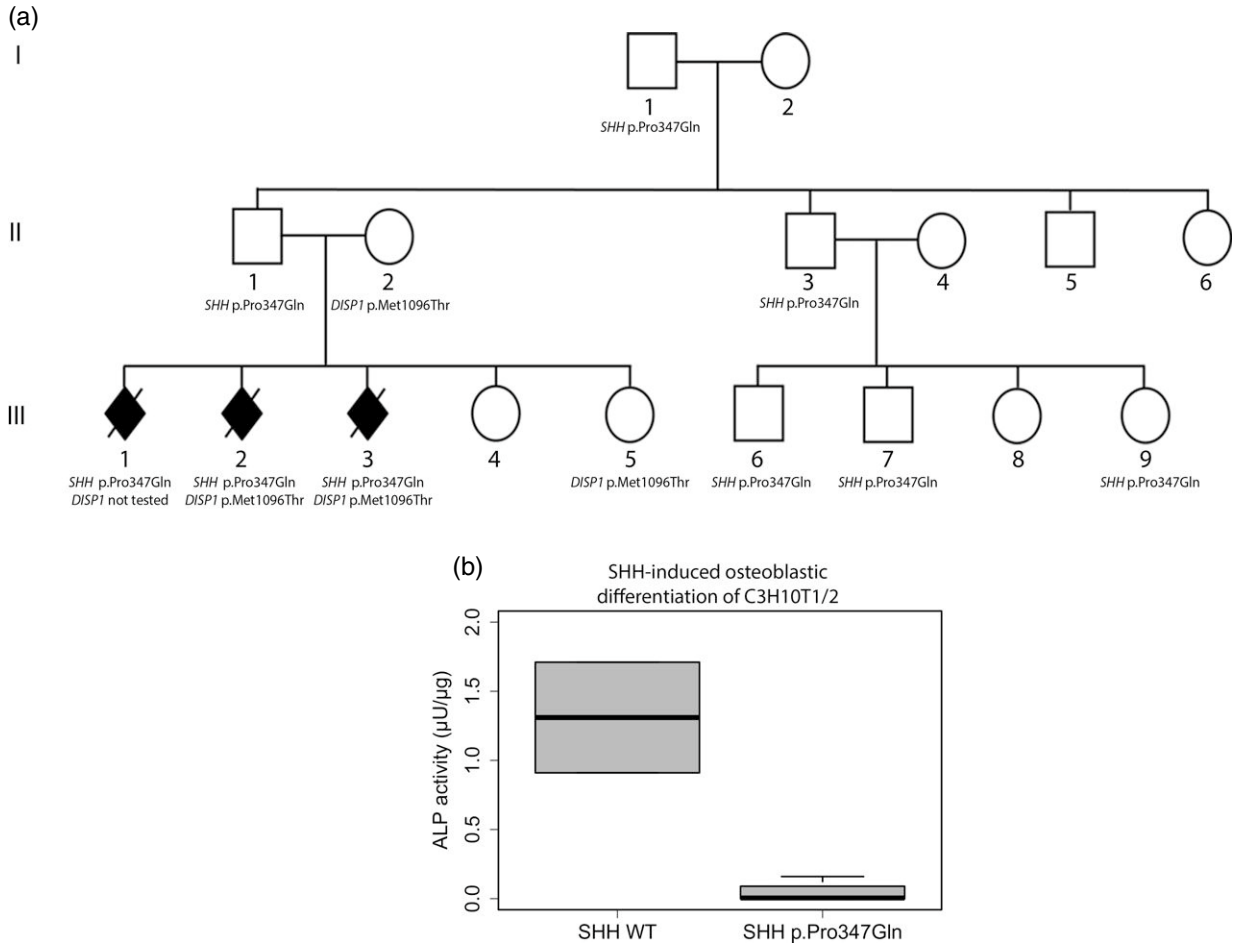


Fig. 1. Pedigree of F1 family and functional test of the *SHH* p.Pro347Gln mutation. (a) Black symbols refer to HPE subjects. Mutations were represented in proteic nomenclature. The F1 family consisted of 17 members. Nine of them harbour the *SHH* p.Pro347Gln mutation, inherited from the paternal grandfather II1. In addition to the *SHH* mutation, foetuses III2 and III3 have a *DISP1* p.Met1096Thr mutation, transmitted by their mother II2. The foetus III1 was not tested for *DISP1*, as DNA quantity was insufficient. (b) Alkaline Phosphatase (ALP) activity measured in total protein extracts from C3H10T1/2 cells transfected with plasmids containing *SHH* WT or *SHH* p.Pro347Gln.

MUTATIONASSESSOR, FATHMM, RADIAL SVM, LR and CADD) (28–34). Visual inspection of candidate variants was performed with Integrative Genome Viewer (IGV, Broad Institute, Cambridge, Massachusetts, United States).

Targeted high-throughput sequencing

Targeted NGS was performed using Ion Torrent technology (Life Technologies, Carlsbad, California, United States) on DNA from the girl III1 in F2 family. Two pools of 711 primer pairs were designed (Ion Ampliseq technology, Life Technologies) to sequence all the exons of a panel of 18 genes involved in HPE or candidates (*SHH*, *ZIC2*, *TGIF1*, *SIX3*, *DISP1*, *CDON*, *GAS1*, *SUFU*, *FGF8*, *FGFR1*, *NODAL*, *HHAT*, *SUFU*, *TDGF1*, *PTCH1*, *FOXH1*, *SOX2* and *DLL1*) and 2 *SHH* expression regulatory regions, spanning 111 kb. Libraries were sequenced with Ion PGM™ System (Life Technologies). A PGM-specific pipeline incorporated in the Ion Torrent server (Torrent Suite version 4.0.2; Life Technologies) was used to perform the following steps:

reads alignment on hg19, targeted regions coverage analysis, filtering and removal of poor signal reads. Variant calling was performed with the Ion Torrent Variant Caller version 4.0. Mutations were annotated using ANNOVAR v2.0 as described for WES analysis and with ALAMUT software (Interactive Biosoftware, Rouen, France).

Sanger sequencing

Sanger sequencing in F1 and F2 families assessed the intra-familial segregation of the candidate mutations found by NGS. This was performed using the BigDye terminator cycle sequencing kit (Applied Biosystems, Waltham, Massachusetts, United States) on an ABI3130xl sequencer (Applied Biosystems) and analysed using SEQSCAPE software v2.6 (Life Technologies).

Functional validation of the *SHH* p.Pro347Gln mutation

The human *SHH* cDNA (RefSeq NM_000193) was cloned in a pMSCVneo (Clontech, Mountain View, California, United States) vector. A mutated plasmid

Table 3. Phenotypic description of families F1 and F2 members^a

	Sex	Age	Brain MRI	Face
(a) Phenotypic description of family F1 members ^a				
I1	M	81	Microcephaly (−5 SD)	–
I2	F	79	–	–
II1	M	48	Microcephaly (−5 SD)	–
II2	F	48	–	–
II3	M	46	Microcephaly (−4.5 SD)	Hypotelorism
II5	M	42	–	–
II6	F	35	–	–
III1	M (foetus)	–	Semi-lobar HPE	Proboscis, macroglossy
III2	F (foetus)	–	Alobar HPE	Flat face hypotelorism, premaxillary agenesis, cleft lip/palate
III3	F (foetus)	–	Severe HPE	NA
III4	F	12	–	–
III5	F	11	–	–
III6	M	12	Microcephaly (−4 SD)	Hypotelorism, cleft lip/palate
III7	M	11	Microcephaly (−3 SD)	–
III8	F	9	–	–
III9	F	6	Microcephaly (−4 SD)	–
(b) Phenotypic description of family F2 members				
I1	M	34	–	–
I2	F	33	–	–
II1	F	9	Lobar HPE microcephaly (−2 SD)	Flat face, short nose, hypotelorism, arched palate, cleft palate, microstomia, narrow palpebral fissures

F, Female; M, Male; MRI, magnetic resonance imaging; NA, not available; SD, standard deviation.

^aThe head circumferences of individuals harbouring microcephaly are given in SD. The dash means that the phenotype is normal.

containing the *SHH* p.Pro347Gln mutation was obtained by site-directed mutagenesis using the QuikChange XL Site-directed Mutagenesis Kit (Stratagene, La Jolla, California, United States). Plasmids containing cDNA of *SHH* WT or *SHH* p.Pro347Gln were transfected in C3H10T1/2 cells using Transfast (Promega, Fitchburg, Wisconsin, United States). Six days later, the alkaline phosphatase (ALP) activity was measured as previously described (35), reliable to the differentiation of C3H10T1/2 into osteoblasts under SHH action.

Results

F1 family

In the F1 family, we showed by Sanger sequencing that a p.Pro347Gln mutation in *SHH* (c.1040C>A of RefSeq NM_000193) was present in fetuses III1, III2 and III3 inherited from the father II1 (Fig. 1a). It was inherited from the grandfather II and was also transmitted to the uncle II3, displaying microcephaly and hypotelorism. This uncle transmitted the *SHH* mutation to three children (III6, III7 and III9), also harbouring microcephaly and hypotelorism. WES analysis validated the known heterozygous *SHH* mutation in the father II1 presenting microcephaly and hypotelorism, and in the HPE foetus III2.

The deleterious effect of the *SHH* p.Pro347Gln mutation was evaluated using an adaptation of a cell-based assay previously described (35). The efficiency of SHH signalling in the presence of mutation was evaluated by quantifying the SHH-dependent differentiation

of mesenchymal cells (C3H10T1/2) into osteoblasts. This was assessed by measuring the activity of the ALP in C3H10T1/2 cells, 6 days after transfection with the pMSCVneo plasmids containing either cDNA of *SHH* WT or cDNA of *SHH* p.Pro347Gln. The ALP activity of the cells expressing the mutated *SHH* was 0.04 μ U by μ g of protein extract, whereas it was 1.31 μ U/ μ g in the protein extract from cells expressing *SHH* WT, meaning that C3H10T1/2 cells failed to undergo osteoblastic differentiation under action of mutated SHH. This strongly reflects the deleteriousness of the p.Pro347Gln mutation (Fig. 1b).

Based on the assumption that HPE observed in the foetus III2 resulted from the association of the *SHH* mutation with another mutation, variants were filtered according to this inheritance pattern. Only mutations inherited from the mother II2 were selected in the child III2. Intronic variants were filtered out, as well as synonymous variants and those that had a population allele frequency over 1% in any of the three public databases 1000g, ESP6500, and ExAC02. Thirty-four variants were selected, and a prioritization was performed using the cumulative predictions of 10 bioinformatics tools. The 10 first-ranked candidate mutations are presented in Table S1. Most of these genes were already associated with genetic syndromes without any obvious link with forebrain development. We thus focused on a mutation in *DISP1*, known to be involved in HPE. This mutation is a substitution of a thymine in cytosine at location c.3287 of *DISP1* (NM_032890), leading to the change of a methionine in threonine at location p. 1096. This mutation

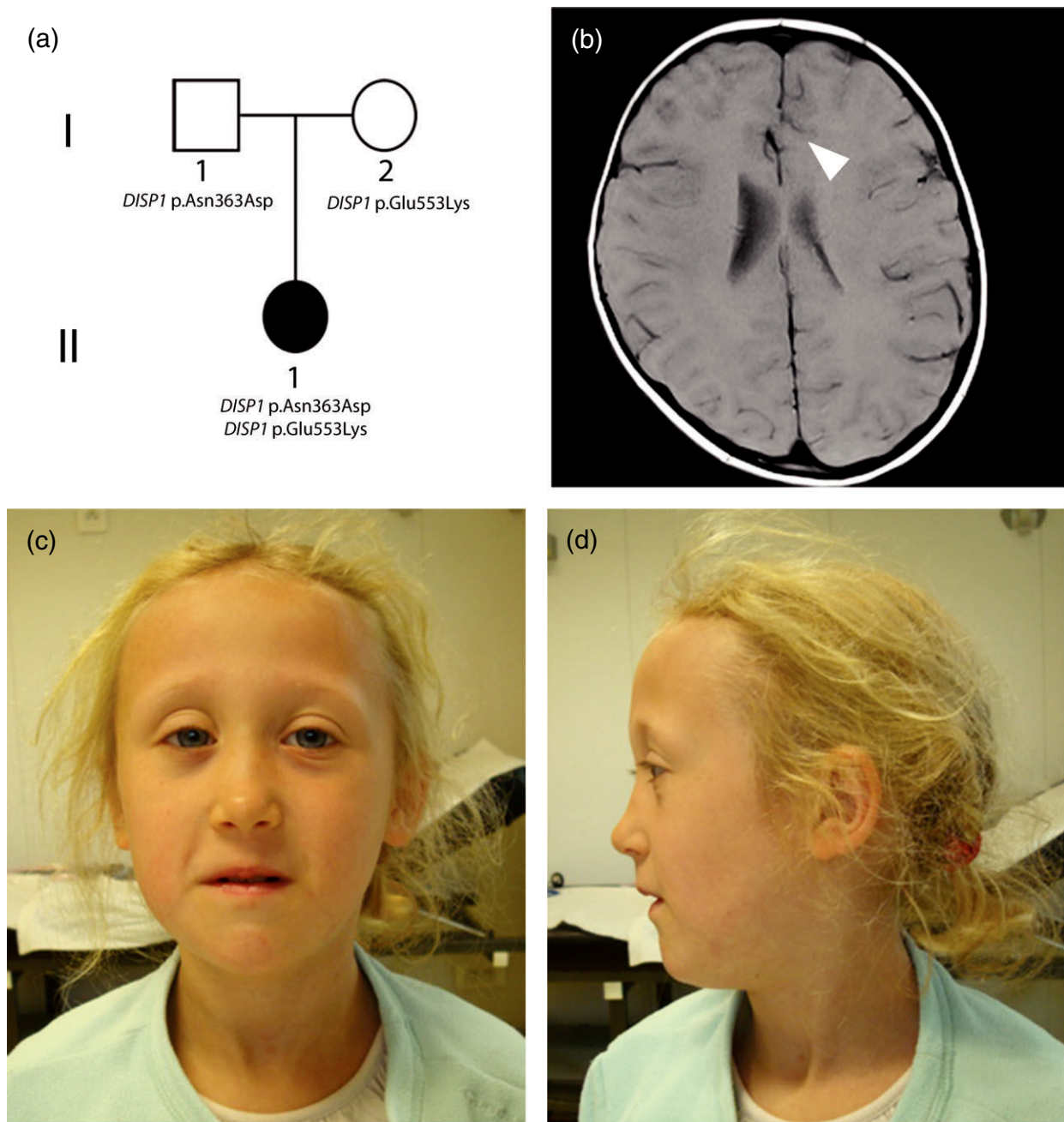


Fig. 2. Pedigree of F2 family and MRI of individual III1 of F2 family. (a) The F2 family consisted of three members. The daughter III1 has two *DISP1* compound heterozygous mutations, *DISP1* p.Asn363Asp, inherited from the father I1, and *DISP1* p.Glu553Lys, inherited from the mother I2. (b) Axial brain MRI of individual III1 of F2 family, harbouring a minor form of HPE, showing a very localized fusion of the hemispheres in the forebrain (white arrow). Facial (c) and lateral (d) photographs of the daughter III1 showing mild facial malformations.

was listed as rs144673025 in dbSNP database and has a minor allele frequency of 0.55% in ESP6500, 0.1398% in 1000 g, and 0.6189% in ExAC02. Three bioinformatics prediction tools classified this mutation as deleterious (LRT, MUTATIONTASTER, and FATHMM), whereas other tools predicted it as tolerated (POLYPHEN-2, SIFT, RADIAL SVM, LR, and MUTATIONASSESSOR) (Table 4).

We used Sanger sequencing to search for the *DISP1* c.3287T>C mutation in individuals III3, III4 and III5 (Fig. 1). DNA was no longer available to look for this

DISP1 mutation in foetus III1. A perfect co-segregation of the two mutations, *SHH* p.Pro347Gln and *DISP1* p.Met1096Thr, was observed in HPE fetuses III2 and III3. Among the two healthy sisters, III4 has no mutation whereas III5 carries the *DISP1* mutation only.

F2 family

The F2 family was screened for 18 HPE candidate genes using targeted NGS for molecular diagnosis on the DNA

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Table 4. Characteristics of *DISP1* and *SHH* mutations found in F1 and F2 families^a

Gene	Nucleic acid change	Amino acid change	Minor allele frequencies				Bioinformatic predictions
			dbSNP	ESP6500	1000G	ExAC	
<i>DISP1</i>	c.T3287C	p.Met1096Thr	rs144673025	0.005	0.001	0.006	D:3 P:1 T:6
<i>SHH</i>	c.C1040A	p.Pro347Gln	–	0	0	0	D:9 P:1 T:0
<i>DISP1</i>	c.A1087G	p.Asn363Asp	–	0	0	0	D:7 P:2 T:1
<i>DISP1</i>	c.G1657A	p.Glu553Lys	–	0	0	0	D:5 P:1 T:4

D, deleterious; P, possibly deleterious; T, tolerated.

^aMutations reported in F1 family (*DISP1* p.Met1096Thr; *SHH* p.Pro347Gln) and in F2 family (*DISP1* p.Asn363Asp; *DISP1* p.Glu553Lys) were annotated using ANNOVAR. Minor alleles frequencies were extracted from dbSNP build 138, in the Exome Sequencing Project containing sequencing data from 6500 exomes (ESP6500), from the 1000 Genome Project release of 2014 (1000G), and from the Exome Annotation Consortium (ExAC) containing sequencing data from 60,700 exomes. Bioinformatic predictions were given by 10 predictions tools (SIFT, POLYPHEN-2 HDIV and HVAR, LRT, MUTATIONTASTER, MUTATIONASSESSOR, FATHMM, RADIAL SVM, LR and CADD).

of the daughter III. Two heterozygous mutations were identified in the exon 10 of *DISP1*: the c.1087A>G transition leading to a missense mutation p.Asn363Asp and the c.1657G>A transition leading to a missense mutation p.Glu553Lys. Sanger sequencing on DNA of the parents showed that the p.Asn363Asp mutation was inherited from the father I1, and the p.Glu553Lys mutation was inherited from the mother I2 (Fig. 2a).

The *DISP1* p.Asn363Asp mutation was absent from public databases and was predicted deleterious by 7 out of 10 of the prediction tools used. The second mutation, p.Glu553Lys, was also predicted deleterious by the majority of the prediction tools and was also absent from public sequencing databases (Table 4). According to the ALAMUT software and the other conservation scores used, these two mutations were in highly conserved regions at both the nucleotide and the amino acid level.

Discussion

The genetic heterogeneity of HPE is supported by reduced penetrance and the absence of obvious genotype–phenotype correlation. This is especially true for patients carrying heterozygous *SHH* mutations, as 45% harbour microforms, 45% present severe HPE and 10% are apparently asymptomatic (18). Using WES, we report here the first co-segregation of mutations in *SHH* and *DISP1* with severe HPE. Our results suggest that these mutations are combining to give a severe phenotype and provide strong evidence that digenic inheritance is a significant genetic model for HPE.

Relationships between SHH and DISP1 in HPE

In this study, we have investigated one family carrying a deleterious mutation in *SHH* and displaying variable expressivity of the disease. This mutation causes the change of proline in glutamine, which results in a severe reduction of SHH activity. Although microcephaly is not a typical sign of HPE, we can consider that the *SHH* mutation is responsible for this mild form of HPE in the present family (2). *SHH* mutation shows full penetrance

with microcephaly, whereas a second mutation in *DISP1* appears to be necessary to obtain a more severe HPE in three fetuses. This is also supported by the finding that the mother and a clinically normal sister carried only the mutation in *DISP1*.

In this study, we also describe the first HPE case with compound heterozygous mutations in *DISP1*. Altogether, we find three different mutations (Fig. 3) in conserved regions of *DISP1*, including one in the sterol-sensing domain (SSD). The exact role of the SSD remains unclear, although most SSD containing proteins have been implicated in intracellular trafficking (36, 37). These three missense mutations in *DISP1* provide new arguments for the implication of this gene in HPE. Few other mutations in *DISP1* have been previously described in patients only harbouring microforms of HPE (38, 39). Noteworthy, all these *DISP1* mutations were inherited from clinically unaffected parents. This gives evidence that additional factors are necessary to potentiate these mutations of *DISP1* and to lead to HPE. As *DISP1* mediates the secretion of SHH from producing cells and allows consequent paracrine signalling (40), we hypothesized that these mutations have an impact on SHH signalling. Mice data also strongly support the implication of *Disp1* in HPE (40–42). Heterozygous knockout for *Disp1* are undistinguishable from the wild-type whereas *Disp1*^{-/-} embryos do not survive beyond E9.5 because of heart development defects. They also display cyclopia reminiscent to severe HPE (41). Further analysis of these mutants permitted to show that SHH signalling was disrupted in *Disp1*^{-/-} embryos, indicating that *Disp1* is essential for proper SHH signalling. Thus, *Disp1* is most probably critical for ventral forebrain induction through its interaction with Shh pathway. Consequently, the co-segregation of a mutation in *SHH* and a mutation in *DISP1* with severe HPE strongly suggests that cumulative effects lead to severe impairment of forebrain development.

Inheritance in HPE

Some authors have proposed autosomal recessive inheritance in HPE (3, 43). However, despite systematic

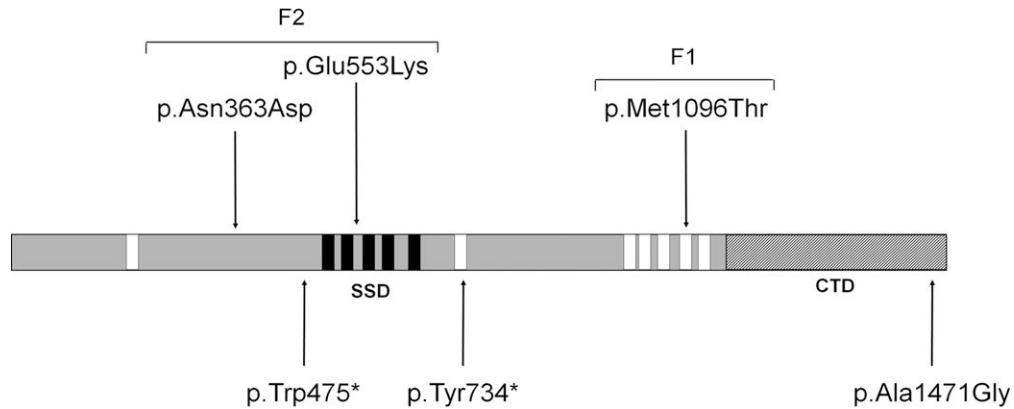


Fig. 3. Distribution of all *DISP1* point mutations reported so far in HPE subjects. Mutations are shown on a schematic representation of *DISP1* protein. The white and black rectangles represent transmembrane helical domains, including the sterol-sensing domain (SSD) from amino acids 486–658 (black). The hatched area is the C-terminal domain (CTD, 360 last amino acids of the protein). Mutations presented by families F1 and F2 are represented at the top, whereas mutations previously reported in the literature are represented at the bottom. The p.Trp475* and p.Tyr734* mutations were described by Roessler et al. (38). The first mutation was present in a girl with seizures, developmental delay, midline cleft lip/palate and mild decortication, inherited from her mother. The second mutation was transmitted from a mother to her daughter who presents facial malformations: bilateral cleft lip/palate, hypotelorism, upslanting palpebral fissures and solitary maxillary central incisor. The p.Alc1471Gly mutation was reported by in a boy, born with heart abnormalities (ventricular septal defect and abnormal aorta), Bochdalek congenital diaphragmatic hernia and left-sided cleft lip with bilateral cleft palate (39).

sequencing, homozygous mutations in the major HPE genes *SHH*, *ZIC2* and *SIX3*, were never described in HPE cohorts (2, 5, 44). Nonetheless, some autosomal recessive cases implicating minor HPE genes (Table 1) have been reported. In 2007, a first case of recessive inheritance of *TGIF1* mutations was described, with the finding of two compound heterozygous mutations (45). A loss of function homozygous mutation in *FGF8* has also been identified in one consanguineous HPE family (46). More recently, hypomorphic alleles of *STIL* were implicated in two cases of autosomal recessive inheritance in HPE patients (8, 9). It was proposed that *STIL* had a function during early brain development linked to SHH signalling (9, 47). Here, we describe a first HPE patient displaying two mutated *DISP1* alleles whereas the two clinically normal parents carry only one mutated allele. This strongly supports that the presence of two mutations in a minor HPE gene exacerbates the risk of developing a HPE phenotype. Furthermore, the mild phenotype of the HPE patient described in this manuscript suggests that *DISP1* activity is decreased such that it leads to a significant impairing of SHH pathway responsible for mild HPE, but above the threshold that would result in severe HPE and early lethality, as indicated by the mice model (41).

Currently, single heterozygous mutations are mainly reported in HPE cases (Table 1) and believed to account for the HPE phenotype (5). But still, it cannot be excluded that a mutation in another gene may underlie the observed forebrain defects. However, double heterozygous mutations for two HPE genes were rarely reported (Table 2). This is not really surprising because HPE genes are key developmental genes, and strong deleterious mutations in two of these genes are probably not viable (43, 48). Thus, it is important to design the pipeline of WES analysis in order to avoid discarding mutations predicted to have a mildly deleterious effect

in development genes. This strategy has enabled us to identify a new *DISP1* mutation in a family presenting two fetuses with alobar and semi-lobar HPE associated to a deleterious mutation in *SHH*. The identification of these two altered genes that have functional relationships in multiple affected individuals in one family strongly supports a digenic inheritance (19).

Polygenic inheritance has now been established for more and more other complex inheritance diseases among which digenic inheritance is the simplest form (19, 20). This mode of inheritance was reported in Kallmann syndrome (KS) (49), characterized by a defective hormonal reproductive axis and sense of smell. This developmental pathology was firstly described as autosomal dominant or X-chromosome linked. Further studies permitted to refine the genetics of KS by describing several patients harbouring two mutations in different genes (50, 51), combining major and minor KS genes, and giving evidence of a digenic inheritance of KS. This was also reported in patients with Alport syndrome, presenting mutations in two different collagen IV genes (*COL4A3* and *COL4A4*) (52). In some nephropathies, mutations in two genes encoding glomerular proteins nephrin and podocin (*NPHS1* and *NPHS2*) were identified in several patients (53). Such a multigenic inheritance is also well described in ciliopathies like Bardet–Biedl syndrome (BBS) with many patients harbouring mutations in two or more *BBS* genes (54). These cases illustrate how, in complex syndromes with variable severity, polygenic inheritance plays a role in the clinical expression of the disease. This is particularly relevant when there are functional relationships between mutated genes, as this is the case for HPE genes (43). A first online database dedicated to digenic diseases (DIDA, <http://dida.ibsquare.be/>) is now available (20). The cases described in our manuscript fully meet the required criteria to be included in DIDA database (20).

By describing new HPE families with no classical autosomal dominant inheritance, our work refines the genetic bases of HPE. This discovery has significant implications for genetic counselling especially for risk assessment of patient relatives. Clinical geneticists have to be aware of such different patterns of heritability, and WES or at least sequencing of a large panel of HPE genes, should be performed to establish a molecular diagnosis.

Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web-site.

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❖ **Article 6 : Mutational spectrum in Holoprosencephaly shows that FGF is a new major signaling pathway.**

Human Mutation; 2016

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Dans le laboratoire de diagnostic moléculaire pour l'HPE, au CHU de Rennes, la recherche de variants chez les patients atteints était historiquement faite par séquençage de Sanger et se limitait aux 4 gènes dits « majeurs » (*SHH*, *ZIC2*, *SIX3* et *TGIF1*). Depuis l'avènement du séquençage haut débit (NGS, New Generation Sequencing), nous avons mis en place un panel ciblant 20 gènes impliqués dans l'HPE ou considérés comme de très bon candidats : *SHH*, *ZIC2*, *SIX3*, *TGIF1*, *GLI2*, *PTCH1*, *GAS1*, *TDGF1*, *CDON*, *DISP1*, *FOXH1*, *NODAL*, *FGF8*, *HHAT*, *DLL1*, *SUFU*, *SOX2*, *RBM33*, *LMBR1* et *FGFR1*. L'analyse a été menée, en deux ans, sur 257 échantillons (131 fœtus et 126 enfants vivants). Un variant délétère a été retrouvé chez 24% des patients, et un variant de signification inconnue chez 10% d'entre eux.

Ce travail a permis d'actualiser la répartition des fréquences de variants dans les différents gènes et de les classer comme suit : *SHH* (variant délétère chez 5.8% des patients testés), *ZIC2* (4.7%), *GLI2* (3.1%), *SIX3* (2.7%), *FGF8* (2.3%), *FGFR1* (2.3%), *DISP1* (1.2%), *DLL1* (1.2%), and *SUFU* (0.4%). *SHH*, *ZIC2* et *SIX3* restent considérés comme des gènes majeurs.

Ce travail a également permis de renforcer le rôle de la voie FGF dans l'apparition de l'HPE. *FGF8*, précédemment décrit dans l'HPE, semble avoir une fréquence de mutations plus importante que ce qui avait été rapporté (*Arauz et al., 2010; McCabe et al., 2011*). Des mutations dans le gène *FGFR1*, qui était jusqu'alors généralement associé au syndrome de Hartsfield ou au syndrome de Kallmann, peuvent aussi donner des formes d'HPE typiques non syndromiques. Nous décrivons notamment le cas d'un patient présentant une HPE lobaire avec fente labio-palatine bilatérale, diabète insipide et dysplasie septo-optique. Ce variant a été retrouvé chez son père à l'état de mosaïque dans le sang circulant. Son père, de façon cohérente avec le mosaïcisme, présentait une forme mineure d'HPE avec une hypoplasie unilatérale droite de l'orbiculaire de la lèvre supérieure, une fente nasale bilatérale, et une agénésie du corps calleux.

Enfin, ce travail a montré que dans 16% des cas, des variants dans les gènes dits « mineurs » sont associés à un second évènement sur un autre gène, ce qui renforce l'hypothèse d'une transmission multigénique.

Mutational Spectrum in Holoprosencephaly Shows That FGF is a New Major Signaling Pathway

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ABSTRACT: Holoprosencephaly (HPE) is the most common congenital cerebral malformation in humans, characterized by impaired forebrain cleavage and midline facial anomalies. It presents a high heterogeneity, both in clinics and genetics. We have developed a novel targeted next-generation sequencing (NGS) assay and screened a cohort of 257 HPE patients. Mutations with high confidence in their deleterious effect were identified in approximately 24% of the cases and were held for diagnosis, whereas variants of uncertain significance were identified in 10% of cases. This study provides a new classification of genes that are involved in HPE. *SHH*, *ZIC2*, and *SIX3* remain the top genes in term of frequency with *GLI2*, and are followed by *FGF8* and *FGFR1*. The three minor HPE genes identified by our study are *DLL1*, *DISP1*, and *SUFU*. Here, we demonstrate that fibroblast growth factor signaling must now be considered a major pathway involved in HPE. Interestingly, several cases of double mutations were found and argue for a polygenic inheritance of HPE. Altogether, it supports that the implementation of NGS in HPE diagnosis is required to improve genetic counseling. Hum Mutat 37:1329–1339, 2016. © 2016 Wiley Periodicals, Inc.

KEY WORDS: holoprosencephaly; FGF signaling pathway; multigenic inheritance; targeted NGS; brain malformation

Introduction

Holoprosencephaly (HPE; MIM# 236100) is the most frequent congenital brain malformation (one in 10,000 live births, one in 250 conceptuses). It results from incomplete midline division of the prosencephalon between 18th and 28th day of gestation, affecting both the forebrain and the face [Dubourg et al., 2007; Marcotelles and Laquerriere, 2010]. The clinical spectrum is very wide, ranging from severe HPE with a single cerebral ventricle and cyclopia to clinically unaffected carriers in familial HPE. Three classic anatomical classes have been described, in decreasing order of severity: lobar, semilobar, and lobar HPE. The full spectrum of HPE also includes middle interhemispheric variants (MIH) or syntelencephaly, septo-optic HPE, and microforms characterized by midline defects (e.g., single maxillary median incisor [SMMI] or hypotelorism) without the brain malformations typical of HPE [Barkovich et al., 2002; Simon et al., 2002; Lazaro et al., 2004; Hahn et al., 2010].

Not only is HPE highly variable phenotypically, but also very heterogeneous etiologically [Bendavid et al., 2010; Pineda-Alvarez et al., 2010; Roessler and Muenke, 2010]. HPE may be due to chromosome abnormalities, such as trisomy 13, 18, and triploidy, or may be one of the components of a multiple malformation syndrome, such as Smith–Lemli–Opitz or CHARGE syndrome. The Hartsfield syndrome associates HPE with ectrodactyly, with and without cleft lip and palate. HPE may also result from exposure to maternal diabetes during gestation [Johnson and Rasmussen, 2010; Miller et al., 2010]. Isolated HPE presents a high genetic heterogeneity. To date, heterozygous mutations in 15 genes have been identified in HPE patients with four major genes (*Sonic hedgehog* or *SHH*: MIM# 600725; *ZIC2*: MIM#603073; *SIX3*: MIM# 603714; *TGIF1*: MIM# 602630), and 11 genes that are considered as minor genes (*PTCH1*: MIM#601309; *TDGF1*: MIM# 187395; *FOXH1*: MIM# 603621; *GLI2*: MIM# 165230; *DISP1*: MIM# 607502; *FGF8*: MIM# 600483; *GAS1*: MIM# 139185; *CDON*: MIM# 608707; *NODAL*: MIM# 601265; *DLL1*: MIM# 606582; and very recently *STIL*: MIM# 181590) [Arauz et al., 2010; Bendavid et al., 2010; Bae et al., 2011; Dupe et al., 2011; Pineda-Alvarez et al., 2012; Mouden et al., 2015].

Additional Supporting Information may be found in the online version of this article.

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Table 1. Distribution of Holoprosencephaly Types and Mutations in the Cohort of 257 Patients

Type	All (male, female)	SHH	ZIC2	GLI2	SIX3	FGF8	FGFR1	DISP1	DLL1	SUFU
Alobar	62 (24, 38)	9.7%	8.1%	–	6.5%	1.6%	–	–	1.6%	–
Semilobar	54 (26, 28)	3.7%	5.6%	–	1.9%	3.7%	3.7%	–	1.9%	–
Lobar	43 (27, 16)	2.3%	9.3%	2.3%	2.3%	2.3%	2.3%	4.7%	–	–
Syntelencephaly	12 (7, 5)	8.3%	–	–	8.3%	8.3%	–	–	–	–
Microform	80 (42, 38)	6.3%	–	8.8%	–	1.3%	1.3%	1.3%	1.3%	1.3%
Hartsfield	3 (3, 0)	–	–	–	–	–	66.7%	–	–	–
Kallmann	3 (1, 2)	–	–	–	–	–	–	–	–	–
Total	257	5.8%	4.7%	3.1%	2.7%	2.3%	2.3%	1.2%	1.2%	0.4%

These genes encode proteins playing a role in early brain development, which mostly belong to the signaling pathway Shh, and to a lesser extent Nodal and Fgf pathways [Arauz et al., 2010; Mercier et al., 2013]. Mutations in *SHH*, *SIX3*, and *TGIF1* are inherited from an unaffected parent or parent harboring only a microform of HPE in 70% of the cases [Mercier et al., 2011]. It suggests that other events are necessary to develop the disease. Consequently, the mode of inheritance initially described as autosomal dominant with an incomplete penetrance and a variable expression has been redefined [Odent et al., 1998; Mouden et al., 2016]. HPE is now listed as a polygenic disease having multiple inheritance modes. Among them, polygenic inheritance would require two or more events involving genes from the same or different signaling pathways with functional relationship. This polygenic inheritance plays a role in the variability of the phenotype, especially when there is a functional relationship between mutated genes, as this is the case for HPE genes [Mercier et al., 2013]. This has significant implications for genetic counseling and for risk assessment of patient relatives.

Until recently, HPE molecular diagnosis had relied on the detection of point mutations in the four main HPE genes (*SHH*, *ZIC2*, *SIX3*, and *TGIF1*) by Sanger sequencing and on the search for deletions in either known HPE genes or in the entire genome (using CGH array).

Targeted next-generation sequencing (NGS) has been proven in the recent years to be very beneficial clinically, especially for the molecular diagnosis of genetically heterogeneous diseases, such as intellectual disability, hearing loss [Shearer et al., 2010], and ciliopathies-like Bardet–Biedl syndrome [M'Hamdi et al., 2014]. Targeted NGS appears to be more suitable for routine clinical practice than whole-exome sequencing as it provides better coverage of particular genes for a lower cost and easier and quicker data interpretation [Rehm, 2013]. Therefore, we have developed a targeted NGS panel for the molecular diagnosis of HPE by screening 20 genes positively involved in HPE or defined as candidates for this disorder using the Ion Torrent AmpliSeq and Ion Personal Genome Machine (PGM) strategy.

In a cohort of 271 HPE probands tested since the beginning of 2014, we were able to provide a diagnosis in approximately 24% of patients. We also show that components of the FGF signaling pathway are clearly involved in HPE.

Materials and Methods

Sample Collection

A total of 257 patients (131 fetuses and 126 living children) with normal conventional karyotype were referred by the French geneticists from eight different CLAD (Centres Labellisés pour les Anomalies du Développement) of the country, French centers of prenatal diagnosis (CPDPN), fetopathologists from the French Fetopathology Society (SOFFOET), as well as several European centers. The

257 patients are described in Table 1. This cohort includes 130 males and 127 females, who have been diagnosed with alobar ($n = 62$), semilobar ($n = 54$), lobar ($n = 43$), syntelencephaly ($n = 12$), HPE microform ($n = 80$), Hartsfield syndrome ($n = 3$), or Kallmann syndrome ($n = 3$). All samples were obtained with informed consent according to the protocols approved by the local ethics committee (Rennes hospital).

Gene Selection and Panel Design

Gene selection was based on their proved or suspected involvement in HPE, or in syndromes including HPE, membership in signaling pathways implicated in HPE, and expression in the developing forebrain compatible with HPE. Known regulatory regions of *SHH* (*LMBR1*: MIM# 605522; *RBM33*) have also been included.

The panel was designed with Ion AmpliSeq™ Designer (Life Technologies, Carlsbad, CA, USA). It includes coding and flanking intronic sequences (50 base pairs) of the following 20 genes: *SHH* (NM_000193.2), *ZIC2* (NM_007129.3), *SIX3* (NM_005413.3), *TGIF1* (NM_170695.2), *GLI2* (NM_005270.4), *PTCH1* (NM_000264.3), *GAS1* (NM_002048.2), *TDGF1* (NM_003212.3), *CDON* (NM_016952.4), *DISP1* (NM_032890.3), *FOXH1* (NM_003923.2), *NODAL* (NM_018055.4), *FGF8* (NM_033163.3), *HHAT* (NM_018194.4) (MIM# 605743), *DLL1* (NM_005618.3), *SUFU* (NM_016169.3) (MIM# 607035), *SOX2* (NM_003106.3) (MIM# 184429), *RBM33* (NM_053043.2), *LMBR1* (NM_022458.3), and *FGFR1* (NM_023110.2) (MIM# 136350). It covers 111 kb.

Library Preparation and DNA Sequencing

An adapter-ligated library was constructed with the Ion AmpliSeq Library Kit 2.0 (Life Technologies) following the manufacturer's protocol. Briefly, 10 ng of DNA was amplified in two pooled reactions and then gathered together. Amplicons were partially digested at primer sequences before ligation with Ion Torrent adapters P1 and A, and the adapter-ligated products were then purified with AMPure beads (Beckman Coulter Genomics, Brea, CA), and PCR-amplified for seven cycles. The resulting libraries of 11 patients were equalized using the Ion Library Equalizer Kit (Life Technologies) and then pooled.

Sample emulsion PCR, emulsion breaking, and enrichment were performed with the Ion PGM Template OT2 200 Kit (Life Technologies), according to the manufacturer's instructions. Briefly, an input concentration of one DNA template copy per ion sphere particle (ISP) was added to emulsion PCR master mix, and the emulsion was generated with an Ion OneTouch system (Life Technologies). Next, ISPs were recovered, and template-positive ISPs were enriched with Dynabeads MyOne Streptavidin C1 beads (Life Technologies). The Qubit 2.0 fluorometer (Life Technologies) was used to confirm ISP enrichment. An Ion PGM 200 Sequencing Kit was used for

sequencing reactions, as recommended in the protocol, and chips 316 were used to sequence-barcoded samples on the Ion Torrent PGM for 500 dNTP-flows.

In order to achieve a complete coverage of at least the four main genes for each patient, six fragments, respectively, one in *SHH*, four in *ZIC2*, and one in *SIX3*, were systematically studied by Sanger method. Depending on the coverage, analysis of other genes was completed according to the patient phenotype by Sanger sequencing.

Bioinformatical Analysis

The sequencing data produced by the PGM were first processed with the Torrent Suite 4.2.1, Ion Torrent platform-specific pipeline including signal processing, adapter trimming, filtering of poor signal-profile reads, and alignment to the hg19 human reference genome with TMAP (Torrent Mapping Alignment Program). Four independent variant calling algorithms from the Torrent suite were used.

The four VCF (variant calling format) files were combined and annotated with ANNOVAR (February 2014 build) [Wang et al., 2010]. A gene-based annotation identified whether SNPs cause protein-coding changes and the amino acids that were affected based on RefSeq. A filter-based annotation identified variants and their associated frequency that were reported in the following databases: dbSNP138, 1000-Genome (1000G), NHLBI-ESP, ExAC (Exome Aggregation Consortium), and ClinVar [Landrum et al., 2014]. ANNOVAR was also used to annotate the predicted functional consequences of missense variants using dbNSFP (database for synonymous SNP's functional predictions) v2.6 (<http://sites.google.com/site/jpopgen/dbNSFP>) [Liu et al., 2011, 2013]. This database compiles prediction scores and interpretation from 10 different algorithms: SIFT [Kumar et al., 2009], Polyphen2.HDIV [Adzhubei et al., 2010], Polyphen2.HVAR [Adzhubei et al., 2010], LRT [Chun and Fay, 2009], MutationTaster [Schwarz et al., 2010], MutationAssessor [Reva et al., 2011], FATHMM [Shihab et al., 2013], CADD [Kircher et al., 2014], MetaSVM [Dong et al., 2015], and MetaLR [Dong et al., 2015] (Suppl. Tables S1 and S2). Three conservation scores (GERP++ [Davydov et al., 2010], PhyloP [Siepel et al., 2006], and SiPhy [Garber et al., 2009]) are also included in dbNSFP v2.6 (Suppl. Tables S1 and S2).

The variant annotation was completed with “in-house” data regarding variants frequency within each run, across runs, and during previous annotation helping to identify recurring false positives and polymorphisms. Furthermore, only variants with a frequency less than 1/1,000 in 1000G, EVS (Exome Variant Server), ExAC held our interest.

After variants validation by visualization with IGV (Integrative Genomics Viewer), complementary annotations were performed using Condell v2.0 [Gonzalez-Perez and Lopez-Bigas, 2011] and Alamut Visual v2.4.5 (Interactive Biosoftware, Rouen, France) to estimate variant pathogenicity. The information given by different tools were re-examined with caution to provide accurate results: PolyPhen [Adzhubei et al., 2013], SIFT [Kumar et al., 2009], Mutation Taster [Schwarz et al., 2014], and Align-GVGD [Tavtigian et al., 2006] were tested for exonic variants. In order to study the effect of potential splice variations, Alamut Visual integrates various splice-site prediction methods: SpliceSiteFinder-like [Zhang, 1998], MaxEntScan [Yeo and Burge, 2004], NNSPLICE [Reese et al., 1997], GeneSplicer [Perteau et al., 2001], Human Splicing Finder [Desmet et al., 2009], ESEFinder [Cartegni et al., 2003], RESCUE-ESE [Fairbrother et al., 2002], and EX-SKIP [Raponi et al., 2011] were inter-

rogated. The first five gave scores increased with the importance of the predicted impact on the splice.

Finally, a variant was retained for diagnosis when a majority of tools predicted it as potentially deleterious and/or when family pedigree segregation was consistent. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1. We use the tool ProteinPaint (<http://pecan.stjude.org>) for visualizing amino acid changes corresponding to the retained variants [Zhou et al., 2015].

Mutation Validation

All variants with a potential deleterious effect were confirmed by Sanger sequencing. They were submitted to ClinVar (ClinVar accessions SCV000268717 – SCV000268738 on <http://www.ncbi.nlm.nih.gov/clinvar/>). Segregation analyses were performed whenever DNA was available for additional family members.

Results

Targeted NGS analysis of the 257 patients identified candidate and diagnosis variants in 23.7% of the cases: mutations with high confidence in their deleterious effect in three of the main genes: *SHH*, *ZIC2*, and *SIX3* were identified in 13.2% of the cases (34/257), and in other tested genes in 10.5% (27/257). For these cases, we were able to give a diagnosis. We also found variants classified as variants of uncertain significance (VUS) in 10% (26/257) of the cases.

From these data, the 10 first-ranked genes involved in HPE are *SHH* (5.8%), *ZIC2* (4.7%), *GLI2* (3.1%), *SIX3* (2.7%), *FGF8* (2.3%), *FGFR1* (2.3%), *DISP1* (1.2%), *DLL1* (1.2%), and *SUFU* (0.4%) (Table 1; Fig. 1). All variants were found in a heterozygous state and were held for diagnosis.

SHH, *ZIC2*, and *SIX3* Retain Their Position of Major Genes

Description of the *SHH*, *ZIC2*, and *SIX3* mutations is provided in Figures 1 and 2. As previously described by Mercier et al. [2011], our results confirmed that *SHH* is the major gene implicated in HPE. *SHH* mutations are mostly missense (Fig. 1) and are inherited in 80% of cases of this study. The spectrum of clinical manifestations associated with *SHH* mutations is very large and includes severe forms as well as microforms. *ZIC2* is the second major gene, which is affected by all types of mutations: missense (42%), frameshift, and nonsense (42%), and also splice mutations (16%). *ZIC2* alterations are generally associated with severe HPE forms and few facial features and are de novo in 92% of cases in our study. Proband with *SIX3* mutation mostly had severe HPE correlated with severe facial features. Like *SHH* mutations, *SIX3* variants are mostly inherited.

Altogether, these results support that mutations in *SHH* and *SIX3* are highly inherited, whereas most of the *ZIC2* mutations are de novo.

GLI2 Is Mostly Involved in Midline Abnormalities

Six *GLI2* heterozygous variants were held for diagnosis (Figs. 1 and 3; Table 2; Suppl. Table S2).

The c.596dupG/p.Ala200Argfs*151 (A200Rfs*151) mutation was identified in a boy with nasal pyriform aperture atresia and was inherited from his asymptomatic mother.

The c.790C>T/p.Arg264* (R264*) mutation was identified in a 2-year-old girl with isolated solitary median maxillary central incisor and was inherited from her asymptomatic mother.

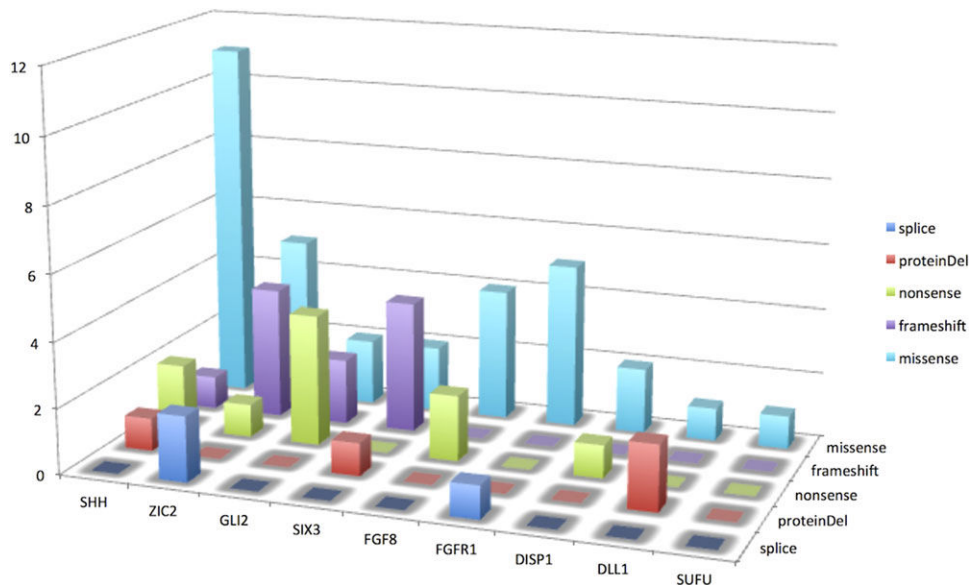


Figure 1. Distribution of mutations held for diagnosis in the top 10 holoprosencephaly genes tested by targeted NGS.

The c.2064delC/p.Ser690Alafs*5 (S690Afs*5) mutation was identified in a 20-year-old girl with hexadactyly, choanal atresia, hypopituitarism, and cerebellar atrophy. This mutation occurred de novo.

The c.2237G>A/p.Trp746* (W746*) mutation was identified in a male fetus aborted because of lobar HPE, premaxillary agenesis, hexadactyly, pituitary hamartoma, and short femur. Moreover, his karyotype revealed a mosaic fragility on chromosome 3 (3p24.1, so very far from *TDGF1*). This mutation was not inherited from his mother, and DNA from the father was unavailable.

The c.4761G>C/p.*1587Tyrext*46 (*1587Y) mutation was found in a 16-year-old boy with hypopituitarism, solitary median maxillary central incisor, and choanal atresia. It was inherited from his asymptomatic mother.

The c.349G>A/p.Ala117Thr (A117T) variant was found in two brothers, one with hypopituitarism and optic atrophy, the other with bilateral cleft lip and palate. This variant was inherited from the father presenting only subtle hypotelorism. The effect of this variant is uncertain as it involves a moderately conserved amino acid and the physicochemical gap between alanine and threonine is low (Grantham distance = 58).

Except the A117T, which is of uncertain clinical significance, all the other variations modify the stop codon. They are inherited in the majority of cases, implicating that these variants in *GLI2* clearly show incomplete penetrance.

Altogether, the mutations in *GLI2* are mostly associated with spectrum linked to midline and characterized by solitary median maxillary central incisor and pituitary insufficiency. Only one is associated with classic HPE.

FGF8 Reaches the Top Genes

Six patients of our cohort presented heterozygous variations in *FGF8* gene (Figs. 1 and 3; Table 2; Suppl. Table S2).

A fetus with semilobar HPE presented the c.356C>T/p.Thr119Met (T119M) variant in *FGF8* in association with a splice mutation in *FGFR1*. The couple had already had a termination of pregnancy due to semilobar HPE and the paternal

grandmother presents a right cleft lip. DNA samples were not available, preventing further Sanger validation.

The c.317C>A/p.Ala106Glu (A106E) was identified in a 4-year-old boy with semilobar HPE. This variant implicates a highly conserved amino acid (through 13 species until *Fugu*) located in the interleukin-1/heparin-binding growth factor domain. It is predicted as possibly damaging by SIFT, PolyPhen, and Mutation taster. This mutation occurred de novo. This is the first time that a *FGF8* mutation is described in association with syntelencephaly.

The c.385C>T/p.Arg129* (R129*) was identified twice in two unrelated families. The first patient is a boy with alobar HPE and the second one is a boy with syntelencephaly. In both cases, the mutation was inherited from the asymptomatic father.

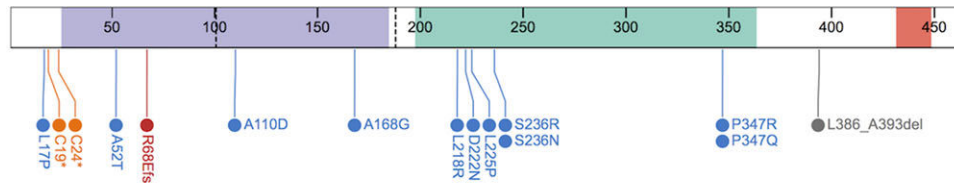
The c.617G>A/p.Arg206Gln (R206Q) was also identified twice in two unrelated families. The first case is a 3-year-old girl with microform (pyriform aperture stenosis, solitary median maxillary central incisor, hypotelorism) presenting an additional variant in *DLL1* (p.Asp601_Ile602delinsVal). These two variants are also present in her older sister who was operated on for bilateral cleft lip and palate and are inherited from the mother presenting hypotelorism and microretrognathism. So there is an apparent cosegregation of these mutations with minor signs of HPE spectrum in this family. The second case is a female fetus with lobar HPE.

Overall, the mutation frequency (2.2%) in *FGF8* demonstrates that this gene can be classified as a major gene.

FGFR1 Is a New Major Gene in HPE

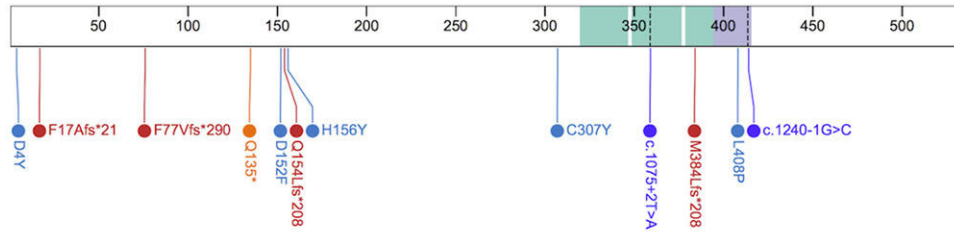
Six heterozygous variants in *FGFR1* (NM_023110.2) were identified in our cohort: five in the intracellular tyrosine kinase domain (TKD, amino acids 478–767): p.Gly485Val, p.Gly490Arg, p.Gly643Asp, c.1977+1G>A, p.Glu692Lys, and one in the extracellular ligand-binding domain (p.Arg250Pro) (Figs. 1 and 3; Table 2; Suppl. Table S2).

The c.1454G>T/p.Gly485Val (G485V) and the c.1468G>C/p.Gly490Arg (G490R) were identified in patients with Harstfield



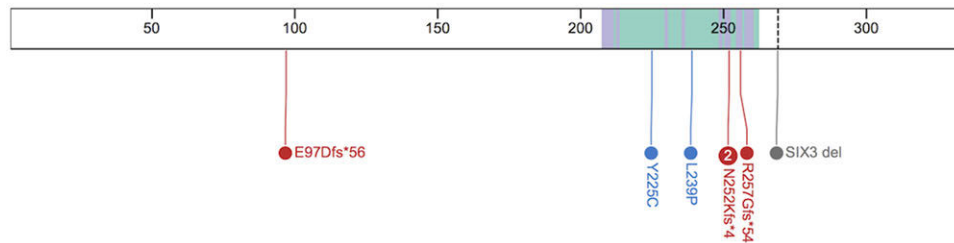
SHH NM_000193

- **HH_signal** Hedgehog amino-terminal signalling domain; pfam01085
- **Hint** Hedgehog/Intein domain; cd00081
- **other** heparin binding site (glycine box) [chemical binding]



ZIC2 NM_007129

- **zf-H2C2_2** Zinc-finger double domain; pfam13465
- **zf-C2H2** Zinc finger, C2H2 type; cl15478



SIX3 NM_005413

- **homeodomain** Homeodomain; cd00086
- **DNA binding** DNA-binding site [nucleotide binding]

- MISSENSE
- NONSENSE
- PROTEINDEL
- FRAMESHIFT
- SPLICE

Figure 2. Mutational landscape of *SHH*, *ZIC2*, and *SIX3* genes. This comprehensive visualization of sequence mutations was performed with ProteinPaint (<http://pecan.stjude.org>). Mutations are presented as filled circles with colors corresponding to mutation type: blue for missense, orange for nonsense, brown for deletion, red for frameshift, and purple for splice mutations. The GenBank references used were NM_000193.2 for *SHH*, NM_007129.3 for *ZIC2* and NM_005413.3 for *SIX3*.

syndrome and occurred de novo. The latter has already been reported by Simonis et al. [2013].

The c.1928G>A/p.Gly643Asp (G643D) mutation occurred de novo in a patient with nasal pyriform aperture hypoplasia, single central incisor, and intellectual deficiency. It involves a highly conserved residue (through 16 species from *Caenorhabditis elegans* to *Homo sapiens*) located in the serine-threonine/tyrosine-protein kinase catalytic domain and the physicochemical gap between glycine and aspartate is important (Grantham distance = 94). AlignGVGD, SIFT and MutationTaster predict a deleterious effect.

The c.1977+1G>A variant was identified in a patient with semilobar HPE in association with a variant in *FGF8*, p.Thr119Met, as described above. The c.1977+1G>A variant is predicted to induce a skipping of exon 17 by all five splice prediction tools.

The c.2074G>A/p.Glu692Lys (E692K) mutation was identified in a fetus with HPE and cleft lip and palate, and was inherited from his mother with hypogonadotropic hypogonadism.

The c.749G>C/p.Arg250Pro (R250P) mutation was identified in a boy with lobar HPE and bilateral cleft lip and palate. Sanger sequencing suggested a very low proportion of the mutated base (cytosine) to the normal base (guanine) in the father leucocyte DNA (Fig. 4).

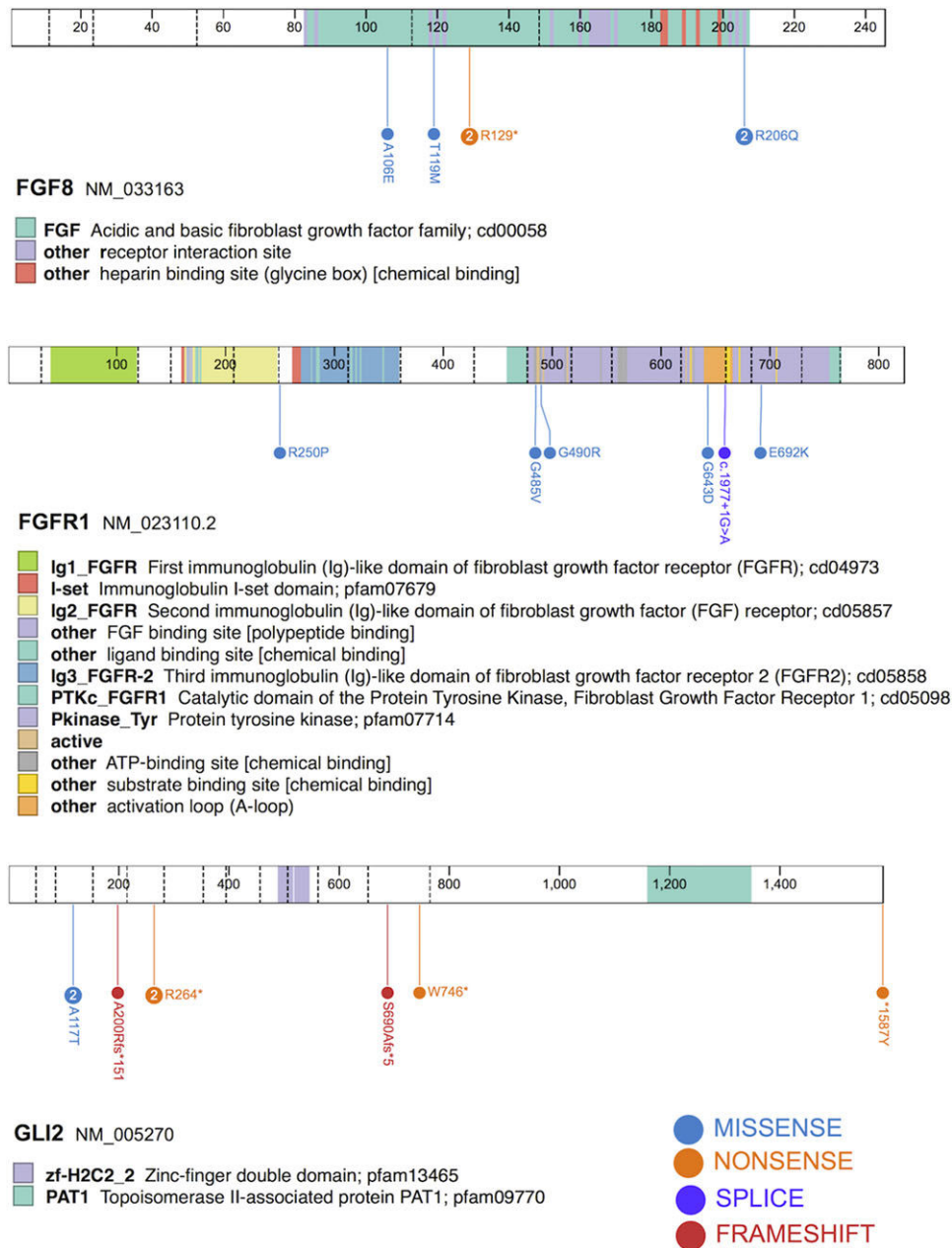


Figure 3. Mutational landscape of *FGF8*, *FGFR1*, and *GLI2* genes, performed with ProteinPaint. The GenBank references used were NM_033163.3 for *FGF8*, NM_023110.2 for *FGFR1*, and NM_005413.3 for *GLI2*.

This was confirmed by NGS sequencing showing mosaicism for the presence of the mutation (GRCh37 genome build: g.38282214C>G) with a frequency of 6% in the peripheral blood, and was perfectly correlated with the phenotype of the father presenting a microform with a right unilateral hypoplasia of the orbicularis of the upper lip and bilateral nasal slot, and MRI showed agenesis of the corpus callosum. The 15-month-old boy now presents diabetes insipidus and septo-optic dysplasia.

Mutations in *FGFR1* were recently described in Hartsfield syndrome (OMIM 300571), which is a rare and unique association of HPE and ectrodactyly, with or without cleft lip and palate, and variable additional features [Simonis et al., 2013; Hong et al., 2016]. Here, we identified four *FGFR1* mutations in patients presenting HPE without extremities abnormalities.

Minor HPE Genes Present Mutations That Are Associated with a Second One in Most of the Cases

The three HPE minor genes identified by our study are *DLL1*, *DISP1*, and *SUFU* (Fig. 1; Table 2; Suppl. Table S2).

In the *DLL1* gene, we identified twice the same mutation c.1802_1804del/p.Asp601_Ile602delinsVal (or 601_602del) in two unrelated patients. First, this mutation was found in a patient with semilobar HPE and has already been reported by our group [Dupé et al., 2011]. Second, it was identified in a 3-year-old girl with microform (pyriform aperture stenosis, solitary median maxillary central incisor, hypotelorism). It was found in association with a VUS in *FGF8* (R206Q); the two variants perfectly cosegregate with the phenotype in the family and may be implicated in the phenotype as we

Table 2. Characteristics of Variants Identified in *GLI2*, *FGF8*, *FGFR1*, *DLL1*, *DISP1*, and *SUFU*, and Associated Phenotypes

Gene	Variant (gDNA)	Variant (cDNA)	Variant (protein)	Deleterious score or effect	Patient's phenotype	Inheritance	Paired mutation
<i>GLI2</i>	g.121708913G>A	c.349G>A	p.Ala117Thr	D:2 P:1 T:7	Hypopituitarism, optic atrophial/bilateral cleft	Father (hypotelorism)	
	g.121712959dupG	c.596dupG	p.Ala200Argfs*151	Frameshift	NPAS	Mother	
	g.121726436C>T	c.790C>T	p.Arg264*	Stop gain	SMMCI	Mother	
	g.121743961delC	c.2064delC	p.Ser690Alafs*5	Frameshift	Hexadactyly, choanal atresia, hypopituitarism, cerebellar atrophial	De novo	
	g.121744134G>A	c.2237G>A	p.Trp746*	Stop gain	Lobar HPE, premaxillary agenesis, pituitary hamarthoma, hexadactyly	Not inherited from the mother	
<i>FGF8</i>	g.121748251G>C	c.4761G>C	p.*1587Tyrext*46	Stop loss	Hypopituitarism, SMMCI, choanal atresia	Mother	
	g.103534509G>T	c.317C>A	p.Ala106Glu	D:9 P:0 T:1	Semilobar HPE	De novo	
	g.103531308G>A	c.356C>T	p.Thr119Met	D:10 P:0 T:0	Semilobar HPE	ND	<i>FGFR1</i> : c.1977+1G>A
	g.103531279G>A	c.385C>T	p.Arg129*	Stop gain	Alobar HPE	Father	
	g.103530204C>T	c.617G>A	p.Arg206Gln	D:9 P:0 T:1	Syntelencephaly NPAS, SMMCI, hypotelorism	Mother (hypotelorism, microretrognathism)	<i>DLL1</i> : p.Asp601_Ile602 delinsVal
<i>FGFR1</i>	g.38282214C>G	c.749G>C	p.Arg250Pro	D:8 P:0 T:2	Lobar HPE, cleft lip palate	Father (microform): mosaicism 6%	
	g.38275486C>A	c.1454G>T	p.Gly485Val	D:10 P:0 T:0	Hartsfield syndrome	De novo	
	g.38275472C>G	c.1468G>C	p.Gly490Arg	D:10 P:0 T:0	Hartsfield syndrome	De novo	
	g.38272346C>T	c.1928G>A	p.Gly643Asp	D:9 P:0 T:1	NPAS, SMMCI, DI	De novo	
	g.38272296C>T	c.1977+1G>A	p.?	Splicing	Semilobar HPE	ND	<i>FGF8</i> : p.Thr119Met
	g.38271782C>T	c.2074G>A	p.Glu692Lys	D:10 P:0 T:0	HPE, cleft	Mother (hypogonadotropic hypogonadism)	
<i>DLL1</i>	g.170592563_170592565del	c.1802_1804del ACA	p.Asp601_Ile602del insVal	Deletion/insertion	Semilobar HPE NPAS, SMMCI, hypotelorism	Father Mother (hypotelorism, microretrognathism)	<i>FGF8</i> : p.Arg206Gln
	g.170592125G>A	c.2117C>T	p.Ser706Leu	D:10 P:0 T:0	Alobar HPE	Father	<i>SHH</i> : p.Leu386_Ala393del
<i>DISP1</i>	g.223175826A>G	c.1087A>G	p.Asn363Asp	D:10 P:0 T:0	Lobar HPE, hypotelorism	Father	<i>DISP1</i> : p.Glu553Lys
	g.223176396G>A	c.1657G>A	p.Glu553Lys	D:5 P:1 T:4		Mother	<i>DISP1</i> : p.Asn363Asp
	g.223177637G>A	c.2898G>A	p.Trp966*	Stop gain	HPE microform	ND	<i>SUFU</i> : p.Pro341Leu
<i>SUFU</i>	g.104359301C>T	c.1022C>T	p.Pro341Leu	D:3 P:2 T:5	HPE microform	ND	<i>DISP1</i> : p.Trp966*

The GenBank references used for nucleotide numbering were NM_005270.4 for *GLI2*, NM_033163.3 for *FGF8*, NM_023110.2 for *FGFR1*, NM_005618.3 for *DLL1*, NM_032890.3 for *DISP1*, NM_016169.3 for *SUFU*, and NM_000193.2 for *SHH*. Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1. The deleterious score was given by 10 predictions tools (SIFT, Polyphen2_HDIV, Polyphen2_HVAR, LRT, MutationTaster, MutationAssessor, FATHMM, CADD, MetaSVM, and MetaLR).

* For detailed prediction data, see Suppl. Table S2.

D, deleterious; P, possibly deleterious; T, tolerated; NPAS, nasal pyriform aperture stenosis; SMMCI, solitary median maxillary central incisor; ND: not determined.

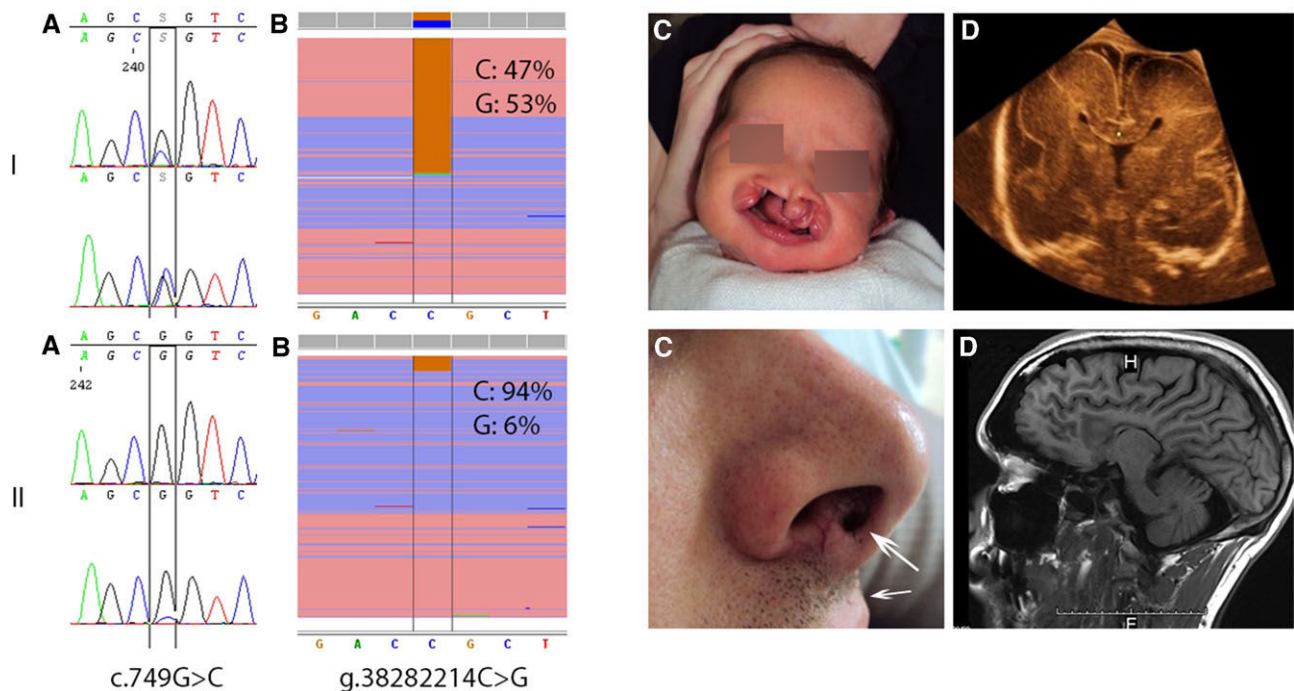


Figure 4. p.Arg250Pro (R250P) mutation in *FGFR1*. (I) Proband. (II) Father. **A:** Partial *FGFR1* electrophoregrams (upper: forward strand; lower: reverse strand) and c.749G >C (NM_023110.2) mutation identified in proband in a heterozygous state, and in father in trace on the reverse strand. **B:** Visualization of the g.38282214C >G (GRCh37 genome build) variation in IGV obtained by targeted NGS. **C:** Facial photograph of the proband with bilateral cleft lip palate; lateral photograph of the father with right unilateral hypoplasia of the orbicularis of the upper lip and bilateral nasal slot. **D:** Prenatal MRI showing lobar HPE in the proband, and MRI showing agenesis of the corpus callosum in the father.

have shown that Fgf pathway might regulate expression of *DLL1* in the chick developing brain [Dupé et al., 2011].

We also found the c.2117C>T/p.Ser706Leu (S706L) mutation in the *DLL1* gene in a fetus with alobar HPE in association with an in-frame deletion in *SHH* (c.1157_1180del/p.Leu386_Ala393del). The two mutations were however inherited from her asymptomatic father.

Regarding the *DISP1* gene, we identified two compound heterozygous mutations in a 9-year-old girl with a mild form of lobar HPE, facial dysmorphism, and hypotelorism: the c.1087A>G transition leading to a missense mutation p.Asn363Asp (N363D) and the c.1657G>A transition leading to a missense mutation p.Glu553Lys (E553K). The p.Asn363Asp mutation was inherited from the father and the p.Glu553Lys mutation was inherited from the mother [Mouden et al., 2016].

In one polymalformative fetus with bilateral cleft lip and facial dysmorphism suggesting HPE microform, we found a nonsense heterozygous mutation c.2898G>A or p.Trp966* (W966*) in *DISP1*, associated with a mutation in *SUFU* (c.1022C>T/p.Pro341Leu) that substitutes the last base of exon 8 and that is predicted deleterious by most bioinformatics prediction tools mutation. Family study unfortunately could not be performed because DNA samples were not available.

These results suggest that mutations in minor genes would be found more often in HPE patients with polygenic inheritance.

Discussion

HPE is a very complex disorder, both in clinical and genetic terms involving two or more genetic events. We present here the first large HPE series studied by targeted NGS, and we provide a new

classification of genes involved in HPE. *SHH*, *ZIC2*, and *SIX3* remain the top genes in terms of importance with *GLI2*, and are followed by *FGF8* and *FGFR1*. The fraction of mutations in the major genes (*SHH*, *ZIC2*, *SIX3*) is reduced in the present study compared with previous studies [Mercier et al., 2011]; it is probably due to the present cohort that included more patients with microforms and syntelencephaly. *TGIF1* was previously classified as a major HPE gene [Mercier et al., 2011] but did not present any mutation in our study. Similarly, *PTCH1*, *GAS1*, *TDGF1*, *CDON*, *FOXH1*, *NODAL*, and *SHH*-regulating sequences *LMBR1* and *RBM33* showed no mutations held for diagnosis in the 257 cases sequenced. New case control studies need to be performed in larger cohorts to better evaluate their role and diagnosis potential in HPE. Such studies may be much more capable to evaluate the implication of rare variants. The candidate *HHAT* and *SOX2* genes did not present any mutation either.

Significantly, the identification of numerous mutations in *FGF8* and *FGFR1* in our cohort strengthens FGF signaling involvement in HPE.

FGF8 is a ligand of the large fibroblast growth factor (FGF) family and is important for gonadotropin-releasing hormone neuronal development with human mutations resulting in hypogonadotropic hypogonadism and Kallmann syndrome [Falardeau et al., 2008; Hardelin and Dode, 2008]. Our targeted NGS approach demonstrates that mutation in *FGF8* occurs more commonly than previously thought [Arauz et al., 2010; McCabe et al., 2011]. The phenotype associated with *FGF8* alterations is variable and mutation can be de novo or inherited. Interestingly, the same inherited nonsense mutation (p.Arg129*) was identified in two unrelated patients, one with a severe HPE and the other with a mild form. It supports that another event could be necessary to lead to severe HPE.

We also describe here convincing examples of *FGFR1* mutations in patients with isolated HPE. *FGFR1* belongs to the tyrosine kinase receptor superfamily and contains an extracellular ligand-binding domain with three immunoglobulin (Ig)-like domains (D1–D3) and a cytoplasmic domain responsible for tyrosine kinase activity (Fig. 3). The clinical manifestations of *FGFR1* alterations are very heterogeneous since loss-of-function mutations in *FGFR1* have been linked to Kallman syndrome [Dode et al., 2003; Albuissou et al., 2005; Villanueva and de Roux, 2010], hypogonadotropic hypogonadism with or without anosmia [Costa-Barbosa et al., 2013; Vizeneuve et al., 2013; Villanueva et al., 2015], and Hartsfield syndrome [Simonis et al., 2013; Hong et al., 2016]. Gain-of-function mutations in *FGFR1* have also been identified in about 5% of Pfeiffer syndrome with or without craniosynostosis [Chokdeemboon et al., 2013]. We describe here one case of *FGFR1* mutation (p.Glu692Lys) associated both with Kallmann syndrome and HPE. The location of this mutation is consistent with Kallmann syndrome as mutations of neighboring residues (p.Leu590Pro, p.Ile693Phe) were already described in patients with this syndrome [Dodé et al., 2007; Bailleul-Forestier et al., 2010].

Out of the six *FGFR1* mutations described in our study, two were found in patients with Hartsfield syndrome. Previous reports of Hartsfield syndrome implicate *FGFR1* mutations in the ATP-binding site and the protein TKD [Simonis et al., 2013; Dhamija et al., 2014; Hong et al., 2016]. These mutations would have a dominant-negative activity that would account for the most severe phenotype of Hartsfield syndrome [Hong et al., 2016]. Concordantly, the two *FGFR1* mutations (p.Gly485Val, p.Gly490Arg) that are associated with Hartsfield syndrome in our cohort are localized in the region coding for ATP-binding site (Fig. 3). However, two of the mutations identified in HPE patients without abnormalities of the extremities are also found in the region coding for activation loop of the protein TKD (p.Gly643Asp; c.1977+1G>A). We hypothesized that these *FGFR1* mutations rather lead to a classic loss of function [Hong et al., 2016]. *FGF8* and *FGFR1* are not the only members of the FGF family to be expressed in the early forebrain. Other members should be considered as strong potential candidates for HPE.

FGF signaling pathway plays a dominant role in embryonic development and is essential for ventral telencephalon development and digits formation [Li et al., 2005; Gutin et al., 2006; Ellis et al., 2015]. FGF signaling is involved in maintaining *Shh* expression in the prechordal tissue, where it plays a crucial role in the induction of the ventral forebrain [Ellis et al., 2015]. *FGFR1* also maintains expression of *Shh* in the developing limb [Li et al., 2005]. According to our hypothesis, dominant-negative *FGFR1* mutations would lead to a more severe downregulation of *Shh* activity as compared with a classic loss of function. It would explain the presence of limb defect in Hartsfield syndrome similar to those observed in the *Shh*^{-/-} knockout mice [Chiang et al., 1996].

The knowledge of the mode of inheritance in HPE has evolved since the description of an autosomal-dominant model with an incomplete penetrance and a variable expression [Odent et al., 1998] through an autosomal-dominant model with modifier genes [Roessler et al., 2012]. Thanks to our NGS strategy targeting 20 genes, we have shown that 16% of mutations kept for diagnosis was found in association with a second one (*FGF8/FGFR1*, *FGF8/DLL1*, *DLL1/SHH*, *DISP1/DISP1*, *DISP1/SUFU*). These cases of double mutations in two different genes—and even in the same one—strengthen the polygenic inheritance previously illustrated by Mouden et al. (2016). Here, a second event in *FGF8* was identified in one patient with *FGFR1* mutation. In the same way, a gene synergistic interaction between a deleterious *FGFR1* allele transmitted

from one parent, and a loss-of-function allele in *FGF8* from the other parent was recently described in two sisters with semilobar and lobar HPE, respectively [Hong et al., 2016]. Altogether, these observations strongly suggest that a cumulative effect on the FGF signaling pathway leads to HPE. We showed that most of the mutations were inherited mainly from an asymptomatic parent, which suggests that another event could be necessary to cause HPE. The important and wide variability of expression from an asymptomatic to severe form for a same mutation, the incomplete penetrance and the identification of several mutations in the same patient argue for this oligogenic inheritance. Furthermore, the description of numerous mouse models carrying mutations in two genes of the same or different signaling pathways involved in forebrain development strongly support this mode of inheritance by showing that a cumulative partial inhibition of signaling pathways is necessary to develop HPE [Allen et al., 2007; Krauss, 2007; Mercier et al., 2013]. However, only a few examples of digenic inheritance in human were reported in the literature until now [Nanni et al., 1999; Ming and Muenke, 2002; Lacbawan et al., 2009; Hong et al., 2016; Mouden et al., 2016]. The present study demonstrates that digenism would not be so rare in human HPE. Systematic implementation of NGS in HPE diagnosis will be necessary to account for this multigenic inheritance and to improve genetic counselling.

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❖ **Article 7 : Integrated Clinical and Omics Approach to Rare Diseases: Novel Genes and Oligogenic Inheritance in Holoprosencephaly.**

Brain; 2019

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L'holoprosencéphalie a d'abord été définie comme une pathologie à transmission autosomique récessive (*Cohen et Gorlin, 1969*) puis autosomique dominante avec expressivité variable (*Odent et al., 1998*) et enfin autosomique dominante avec « gènes modificateurs » (*Roessler et al., 2012*). En réalité, il semble que plusieurs modes de transmission sont possibles : autosomique dominant (e.g. *ZIC2*), autosomique récessif (e.g. *STIL* et *DISP1*) et oligogénique (e.g. *SHH*) (*Dubourg et al., 2018*). Cette hypothèse d'oligogénisme peut expliquer les notions d'expressivité variable et de gènes modificateurs, mais elle restait controversée. Récemment, des études ont montré que des pathologies à transmission non-Mendélienne (ce qui semble être le cas de l'HPE) pourraient être dues à l'accumulation de variants hypomorphes dans différents gènes (*Li et al., 2017*). Ces hypothèses de transmission ne sont pas prises en compte dans les algorithmes bio-informatiques utilisées classiquement dans les analyses de séquençage haut débit (exome et génome). Nous avons donc proposé une nouvelle stratégie d'analyse intégrant les données cliniques à l'étude génétique afin de mieux spécifier l'impact des variants identifiés.

Dans cette étude, l'équipe a analysé 26 familles avec HPE par exome (analyse en trio). Les patients atteints avaient déjà eu un dépistage classique (analyse des gènes de l'HPE par NGS ciblé - cf articles 4 et 5-, CGH-array et MLPA) sans pour autant qu'aucune altération évidente n'ait été retrouvée.

Dans le but d'orienter l'analyse de ces exomes, nous avons établi, à partir de bases de données humaines et animales (souris) une liste des phénotypes cliniques retrouvés dans l'HPE ainsi que les gènes qui y sont associés.

Enfin, à l'aide d'une base de donnée RNA-seq, défini des réseaux de gènes co-exprimés avec *SHH*, *ZIC2*, *SIX3* et *TGIF* dans le cerveau entre la 4^{ème} et la 10^{ème} semaine de développement embryonnaire.

En combinant ces 3 approches (analyse classique sélectionnant des variants rares dans la population générale et prédits délétères, analyse phénotypique, données d'expression), nous avons pu isoler 232 variants d'intérêt dans 9 familles. La suite de l'étude s'est faite en étudiant les variants présents famille par famille et en les interprétant en fonction des spécificités phénotypiques retrouvées.

Ce travail a permis d'établir une liste de 180 gènes tous impliqués dans la régulation de la voie SHH, le cil primaire ou la voie Wnt et la polarité planaire. L'analyse des exomes a retrouvés des variants dans 19 gènes, dont 15 n'avaient jusqu'à alors jamais été associés à l'HPE chez l'homme. Nous avons notamment retrouvé des récurrences dans les gènes *FAT1* et *SCUBE2*, toujours associées à des variants dans d'autres gènes, impliqués dans l'HPE (*SHH*, *PTCH1*) ou en lien avec la voie SHH (*NDST1*, *COL2A1*, *HIC1*,...). Nous avons également identifié des variants dans des gènes impliqués dans la mise en place du cil primaire, ce qui, en perturbant la transduction du signal SHH, peut induire un phénotype HPE.

Enfin, en comparant l'incidence de combinaisons de variants dans plusieurs gènes à des bases de données contrôle nationales et européennes, on a montré une surreprésentation significative de ces combinaisons dans notre cohorte, ce qui renforce l'hypothèse de transmission oligogénique dans l'holoprosencéphalie.

Integrated clinical and omics approach to rare diseases: novel genes and oligogenic inheritance in holoprosencephaly

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Holoprosencephaly is a pathology of forebrain development characterized by high phenotypic heterogeneity. The disease presents with various clinical manifestations at the cerebral or facial levels. Several genes have been implicated in holoprosencephaly but its genetic basis remains unclear: different transmission patterns have been described including autosomal dominant, recessive and digenic inheritance. Conventional molecular testing approaches result in a very low diagnostic yield and most cases remain unsolved. In our study, we address the possibility that genetically unsolved cases of holoprosencephaly present an oligogenic origin and result from combined inherited mutations in several genes. Twenty-six unrelated families, for whom no genetic cause of holoprosencephaly could be identified in clinical settings [whole exome sequencing and comparative genomic hybridization (CGH)-array analyses], were reanalysed under the hypothesis of oligogenic inheritance. Standard variant analysis was improved with a gene prioritization strategy based on clinical ontologies and gene co-expression networks. Clinical phenotyping and exploration of cross-species similarities were further performed on a family-by-family basis. Statistical validation was performed on 248 ancestrally similar control trios provided by the Genome of the Netherlands project and on 574 ancestrally matched controls provided by the French Exome Project. Variants of clinical interest were identified in 180 genes significantly associated with key pathways of forebrain development including sonic hedgehog (*SHH*) and primary cilia. Oligogenic events were observed in 10 families and involved both known and novel holoprosencephaly genes including recurrently mutated *FAT1*, *NDST1*, *COL2A1* and *SCUBE2*. The incidence of oligogenic combinations was significantly higher in holoprosencephaly patients compared to two control populations ($P < 10^{-9}$). We also show that depending on the affected genes, patients present with particular clinical features. This study reports novel disease genes and supports oligogenicity as clinically relevant model in holoprosencephaly. It also highlights key roles of SHH signalling and primary cilia in forebrain development. We hypothesize that distinction between different clinical manifestations of holoprosencephaly lies in the degree of overall functional impact on SHH signalling. Finally, we underline that integrating clinical phenotyping in genetic studies is a powerful tool to specify the clinical relevance of certain mutations.

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Abbreviations: GoNL = Genome of the Netherlands; HPE = holoprosencephaly; WES = whole exome sequencing

Introduction

Holoprosencephaly (HPE1, OMIM #236100) is a severe developmental defect resulting from incomplete forebrain cleavage. The disease is characterized by incomplete separation of cerebral hemispheres with several anatomical classes ranging from microforms to alobar HPE. Affected individuals present with typical craniofacial midline defects of varying severity including proboscis, cleft lip and palate, ocular hypotelorism and solitary median incisor. HPE occurs in about 1 in 10 000 to 20 000 live births worldwide (Mercier *et al.*, 2011).

The genetic basis of HPE remains unclear and different transmission patterns have been described including autosomal dominant, recessive and digenic inheritance (Dubourg *et al.*, 2018). Most mutations associated with HPE display incomplete penetrance and variable expressivity, i.e. close relatives carrying the same pathogenic variant can be asymptomatic or present distinct HPE-spectrum anomalies (Mercier *et al.*, 2011). Sonic hedgehog (*SHH*) was the first discovered gene implicated in HPE (Roessler *et al.*, 1996) and its variants remain the most common cause of non-chromosomal HPE (Dubourg *et al.*, 2018). In 2011, molecular screening of 645 HPE probands revealed that mutations in the *SHH*, *ZIC2*, *SIX3* and *TGIF1* genes were the most frequent ones and collectively accounted for 25% of cases (Mercier *et al.*, 2011). The following studies reported that *GLI2* might also be considered as a major HPE gene in terms of frequency (Dubourg *et al.*, 2016), although variants in *GLI2* rarely result in classic HPE but instead cause a distinct phenotype that includes pituitary insufficiency and subtle facial features (Bear *et al.*, 2014). Pathogenic variants in *FGF8*, *FGFR1*, *DISP1*, and *DLL1* were also found in ~7% of HPE cases (Dupé *et al.*, 2011; Dubourg *et al.*, 2016). The other HPE genes reported so far are *TDGF1*, *FOXH1*, *TGIF1*, *CDON*, *NODAL*, *GAS1*, *STIL* and *SUFU* whose frequency is not established due to the small number of reported cases (Mouden *et al.*, 2015, 2016; Dubourg *et al.*, 2018; Kruszka *et al.*, 2018).

Clinical genetic testing of HPE has improved, but ~70% of familial cases remain without a clear molecular diagnosis. Most of known HPE genes belong to the *SHH* pathway, which represents the primary pathway implicated in the disease (Mercier *et al.*, 2013; Dubourg

et al., 2016; Kruszka *et al.*, 2018). Therefore, defective *SHH*-related processes are likely to be substantially involved in HPE.

Whole-exome sequencing (WES) has been successful for Mendelian disease-gene discovery and differential diagnosis (Bamshad *et al.*, 2011). WES analysis uses filtering approaches for candidate variant prioritization combined with comprehensive clinical evaluation. A variety of additional strategies has been developed to further improve the performance of WES in clinical settings. Collaborative platforms such as Matchmaker Exchange (Philippakis *et al.*, 2015) are used to search for recurrence in patients affected by similar phenotypes. Integrative variant-prioritization algorithms such as the Exomiser suite (Smedley *et al.*, 2015) combine WES with different phenotype-driven approaches (based on clinical data and cross-species phenotype comparisons) and analysis of protein interactome data. As useful as they are, these strategies are limited: collaborative platforms are not efficient in case of very rare genetic diseases while pipelines such as Exomiser are not designed to study non-Mendelian disorders. Studying HPE faces these two challenges: (i) HPE live-born infants are excessively rare; and (ii) although HPE is considered a Mendelian disorder, the wide range of severity must necessitate strong modifying factors such that a single pathogenic variant may be neither necessary nor sufficient for pathogenesis.

Recent studies have highlighted that non-Mendelian disease phenotypes could present an oligogenic aetiology and result from accumulation of inherited low-penetrance variants in multiple genes (Li *et al.*, 2017). However, such events are likely overlooked in clinical genetic studies if variants are inherited from a clinically unaffected parent.

In this study, we address the additional yield that can be obtained for HPE patients who underwent medical WES evaluation in clinical settings that failed to establish a molecular diagnosis. Given the wide clinical spectrum of the disease, as well as incomplete penetrance and variable expressivity of HPE mutations, we raised the possibility that the low diagnostic yield is partly due to the complex aetiology of HPE and hypothesized that a part of unsolved HPE cases results from oligogenic events, i.e. accumulation of several rare hypomorphic variants in distinct, functionally connected genes.

Our study involved patients for whom no disease aetiology could be determined by conventional diagnostic approaches. Similarly to previous WES studies (Lee *et al.*, 2014; Stark *et al.*, 2017), we used clinically-driven prioritization approach to identify genes associated with specific clinical features as reported in gene-phenotype reference databases and mouse models. Complementarily, we developed and used a prioritization strategy based on gene co-expression networks of the developing human brain to select genes with spatio-temporal expression patterns compatible with those of known HPE genes. Finally, we used in-depth clinical phenotyping together with cross-species similarities to further strengthen the evidence of causality.

This study highlights novel HPE genes and identifies new disease-related pathways including the primary cilia pathway. Our findings also illustrate the high degree of oligogenicity of HPE and suggest that the disease requires a joint effect of multiple hypomorphic mutations.

Materials and methods

Patient selection and preliminary genetic analyses

Study protocol was approved by the Ethics Committee of Rennes Hospital. Patients diagnosed with HPE and relatives were recruited using the clinical database of Holoprosencephaly Reference Center of Rennes Hospital. Study participation involved informed written consent, availability of clinical data, and either DNA or peripheral blood sample.

The main selection criterion for this study was the absence of clear genetic cause of HPE after conventional diagnostic procedures. As part of routine diagnosis, all patients were scanned for rare damaging mutations by targeted HPE gene-panel sequencing (Dubourg *et al.*, 2016) and for copy number variants (CNVs) using comparative genomic hybridization (CGH)-array and multiplex ligation-dependent probe amplification (MLPA). Patients for whom no genetic cause of HPE (i.e. a fully-penetrant causal mutation in known HPE gene or a chromosomal aberration/copy number variant explaining the pathology) could be established, underwent trio-based WES for further analysis. WES was performed using standard procedures as previously described (Mouden *et al.*, 2015, 2016). The scheme for variant classification followed the American College of Medical Genetics and Genomics association (ACMG) guidelines (Richards *et al.*, 2015) and included a hypothesis-free analysis of all *de novo* and homozygous variants on a family-by-family basis. Patients for whom no such variants of clinical interest had been detected were considered eligible for the hypothesis of oligogenic inheritance and included in this study.

Variant selection under oligogenic hypothesis

As discussed in previous studies, ACMG guidelines are useful in identifying variants with strong effect on phenotype but are unhelpful in case of modifier variants (Hong *et al.*, 2017).

Therefore, the ACMG classification was not taken into account for variant selection dedicated to the analysis of oligogenic events. WES trio data were reanalysed using more permissive settings (filtering protocols used in this study are described in the Supplementary material). The exome analysis was complemented with two gene prioritization strategies based on available clinical knowledge and co-expression networks.

Clinically-driven approach

We established two clinician-generated lists of relevant phenotypes reminiscent of HPE in human and mouse models, respectively (Supplementary Table 3). Genes associated with the phenotypes of interest were identified with publicly available clinical resources and associated ontologies. Human gene-phenotype associations were extracted from relevant databases (Supplementary Fig. 1) using R package *VarFromPDB* (<https://github.com/cran/VarfromPDB>). The Mouse Genome Informatics (MGI) (Smith *et al.*, 2018) database and a homemade workflow were used to retrieve genes associated with any of the corresponding phenotypes in mouse mutants. Human and mouse results were combined and redundancy was removed to establish a list of clinically-driven candidate genes associated with HPE-related anomalies (Supplementary Table 4).

Identification of HPE-related genes by weighted gene co-expression network analysis

We used weighted gene co-expression network analysis (WGCNA) (Langfelder and Horvath, 2008) on the RNA-Seq data from the Human Development Biology Resource (HDBR) (Lindsay *et al.*, 2016) to identify genes sharing highly similar expression patterns with four classical genes associated with HPE (*SHH*, *SIX3*, *ZIC2* and *TGIF1*) during cerebral development. Data from samples corresponding to forebrain, cerebral cortex, diencephalon, telencephalon and temporal lobe structures taken between the fourth and 10th post-conception weeks were selected (Supplementary Fig. 9). RNA-seq data were analysed with the iRAP pipeline (<https://github.com/nunofonseca/irap>). We used R package WGCNA to construct co-expression networks and identify modules of co-expressed genes. The detailed protocols for WGCNA analysis are described in the Supplementary material. The Topological Overlap Matrix (TOM) matrix was used to establish a list of transcriptome-driven candidate genes sharing highly similar expression profiles with *SHH*, *ZIC2*, *SIX3* and *TGIF1* (Supplementary Table 5).

Integration and identification of oligogenic events

The two gene prioritization schemes were combined with the WES results to identify a restricted list of rare variations located in genes identified by either the transcriptomic or the clinical prioritization approach (Fig. 1). Further analyses of the candidate variants were performed on a family-by-family basis. Oligogenic events were defined as combinations of candidate variants in ≥ 2 genes co-segregating with disease, i.e. unique to

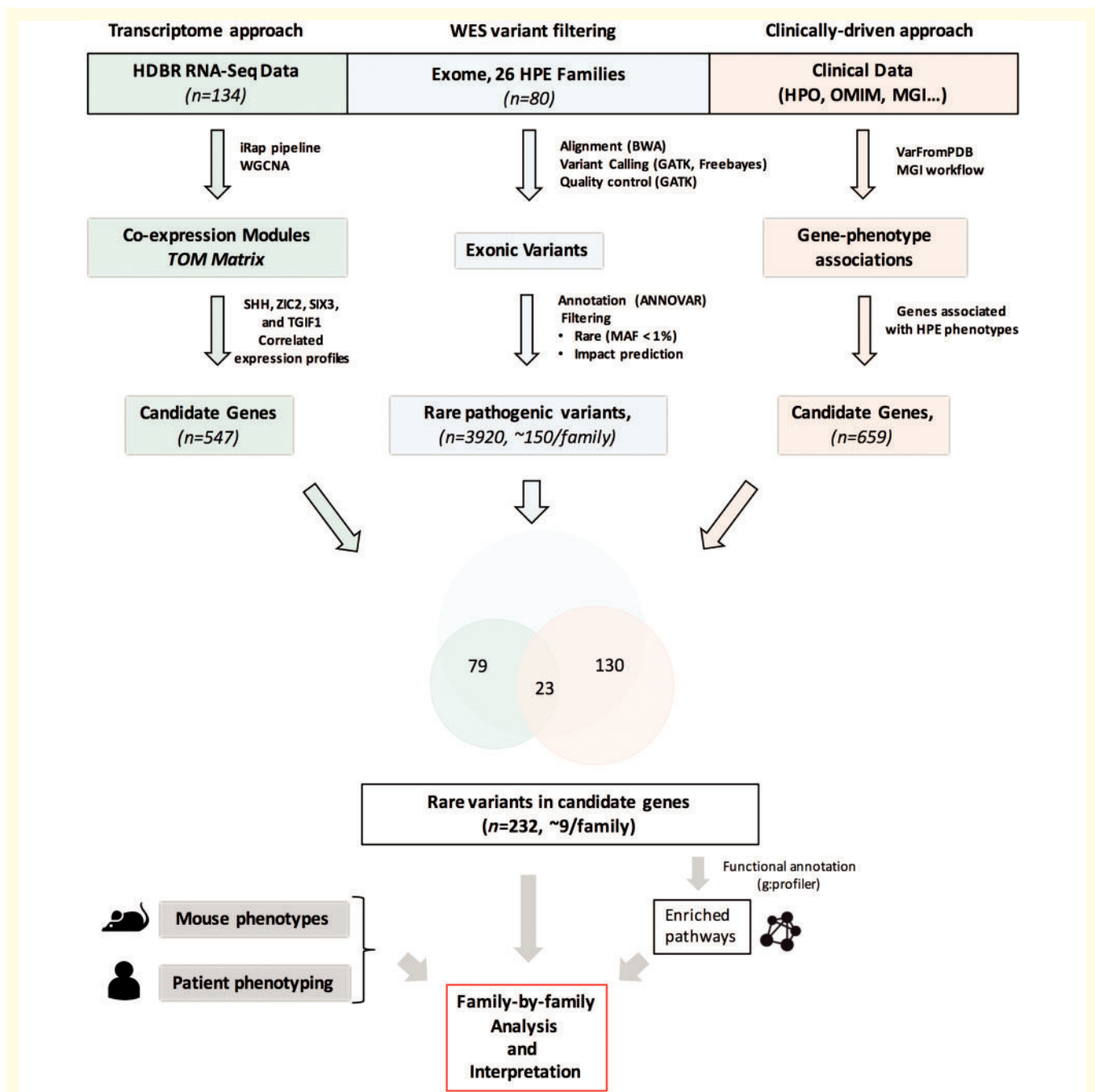


Figure 1 Flow chart illustrating the prioritization strategy. Classical WES analysis was performed (blue) and combined with two prioritization approaches: (i) based on gene co-expression networks (green); and (ii) based on clinical knowledge (salmon). Details of the pipeline are also provided in the Supplementary material. Variant overlaps were selected and further analysed by functional annotation analysis and on a family-by-family basis, by integrating a comprehensive clinical phenotyping of patients and exploration of cross-species similarities.

the affected individuals of each family. Variants could be either inherited from the parents—at least one each from the mother and the father—or occur *de novo* in the affected child.

To evaluate the impact of candidate genes further, we performed deep clinical phenotyping to characterize similarities between unrelated patients and/or published knockout mice. Special attention was given to genes harbouring distinct rare variants in at least two affected patients with striking phenotypic overlap. Phenotypic overlaps between patients and mouse

mutants deficient for the corresponding candidate genes were also examined. The most interesting oligogenic combinations of rare deleterious variants in the affected children were finally discussed during multidisciplinary meetings.

To determine significantly enriched biological processes and pathways, functional annotation was performed by *g:profiler* (<http://biit.cs.ut.ee/gprofiler>) and Bonferroni adjusted *P*-value were considered significant below a value of 0.05 (KEGG, REACTOME and Gene Ontology Biological Processes).

Control cohorts and validation

To test whether the identified oligogenic combinations were specific to the HPE cohort, we used SNV and INDELS data from 248 healthy trios (744 individuals) provided by Genome of the Netherlands (GoNL) sequencing project as a control cohort (Genome of the Netherlands Consortium, 2014). Additional control cohort consisting of 574 unrelated French individuals was provided by the French Exome Project (FREX).

We applied the same variant filtering approach and the same strategy for selection of oligogenic events. Proportion of families and/or individuals presenting oligogenic events were then compared between HPE cohort and the control cohorts. *P*-values were calculated using two-sided Fisher's exact test (*fisher.test* function in R, version 3.4.2).

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request.

Results

Clinical findings

We assembled a cohort of 26 families representing a total of 80 individuals including 29 affected children diagnosed with lobar ($n=3$), semilobar ($n=11$), alobar ($n=13$) or microform HPE ($n=2$) (Table 1). Common HPE clinical manifestations were observed among the probands and included cleft lip and palate (38%), hypotelorism (34%), microcephaly (31%) and arhinencephaly (31%). Ancestry analysis identified that 24 families were of European descent and two of South East Asia and African descent (Supplementary Fig. 10). Eight parents presented minor signs of midline facial anomalies and three parents were diagnosed with HPE microforms.

The initial targeted sequencing had identified point mutations in known HPE genes in 13 families and a full heterozygous deletion of *SIX3* gene had been detected by CGH-array in one family (Fig. 2 and Supplementary Fig. 8). All anomalies were later confirmed by WES analysis. They were inherited from asymptomatic or mildly affected parents and were considered as insufficient to fully explain the pathogenesis of HPE, suggesting that the presence of additional risk factors was required for the disease to occur.

HPE variants overview and identification of disease-related pathways

Combined clinically- and transcriptome-driven analysis of the exome data identified a total of 232 rare candidate variants in 180 genes (Fig. 1 and Supplementary Table 6). All variants presented a minor allele frequency below 1% and were predicted to be highly deleterious to protein function (Supplementary material). One hundred and fifty-three variants concerned genes associated with HPE phenotypes

Table 1 Clinical description of 26 HPE families

Category and feature	<i>n</i>	%
Proband sex		
Male	6	21
Female	20	69
Unknown	3	10
Total	29	100
Clinical phenotype of the parents		
Unaffected	40	78
Minor sign	8	16
Hypotelorism	4	8
Incomplete iris	1	2
Epicanthus	1	2
Narrow palate	1	2
Nasal anomaly	1	2
HPE microform	3	6
Total	51	100
Clinical characteristics of the probands		
HPE	29	100
Lobar	3	10
Semilobar	11	38
Alobar	13	45
Microform	2	7
Cleft lip/palate	11	38
Hypotelorism	10	34
Microcephaly	9	31
Arhinencephaly	9	31
Agenesis of corpus callosum	7	24
Flat head (plagiocephaly)	6	21
Thalami Fusion	6	21
Ventricles Fusion	6	21
Premaxillary agenesis	5	17
Fusion frontal lobes	4	14
Flat nose	4	14
Proboscis	3	10
Cyclopia	2	7
Total	29	100
Families with mutations in HPE genes		
SHH	4	15.4
ZIC2	1	3.8
SIX3	5*	19.2
TGIF1	2	7.7
PTCH1	1	3.3
ZIC2/GLI2	1	3.8
No mutation	12	46.2
Total	26	100.0
Family ethnicity		
European	21	81
African	1	4
South Asian	1	4
Admix	3	12
Total	26	100.0

*For *SIX3*, point mutations were found in four families (targeted sequencing) and a heterozygous deletion was detected by CGH-array in one family.

among which 32 were located in genes reported to induce HPE-like phenotypes in mutant mice (Supplementary Table 8). One hundred and two variants were located in genes sharing expression profiles highly similar to those of

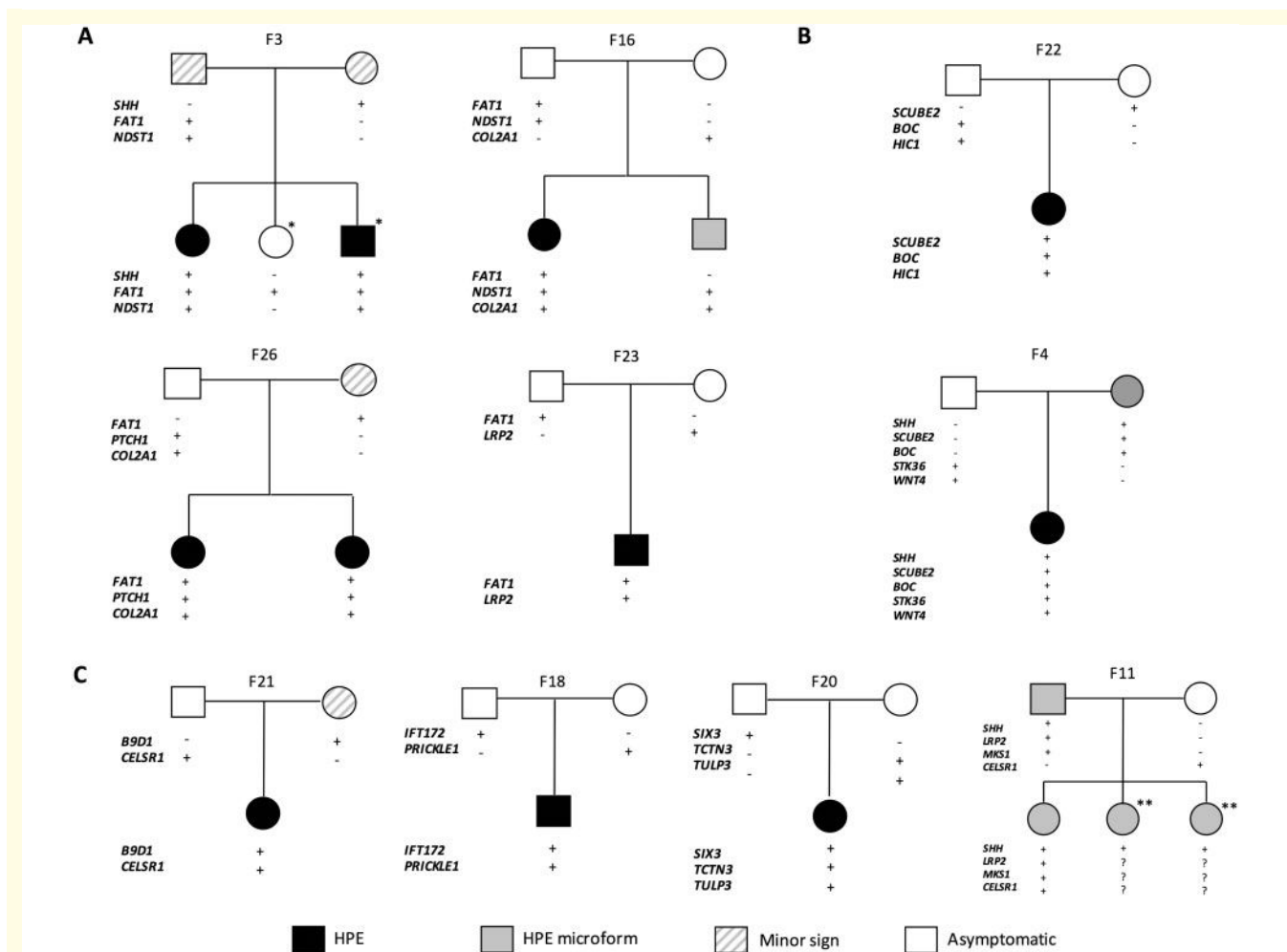


Figure 2 Oligogenic events reported in this study. Candidate genes are listed for each family. Individuals carrying or not carrying the variants are identified by the plus or minus sign symbols, respectively. Variant information is available in Tables 2, 3 and Supplementary Table 6. **(A)** Oligogenic events involving *FAT1*. **(B)** Oligogenic events involving variants in *SCUBE2* and *BOC*. **(C)** Oligogenic events involving mutations in genes related to the primary cilium. *Not available for WES, clinical phenotyping and Sanger sequencing of *SHH*, *FAT1* and *NDST1* were performed. **Samples not available, Sanger sequencing of *SHH* was performed in the referring laboratory.

HPE genes. Overlap between phenotype and gene co-expression network analysis contains 23 variants including 14 previously described mutations in known HPE genes (*SHH*, *ZIC2*, *SIX3*, *GLI2*, *TGIF1* and *PTCH1*).

Consistent with known disease aetiology, functional profiling of the 180 genes revealed a significant enrichment for biological processes implicated in forebrain development (Supplementary Table 7) including Sonic Hedgehog signalling pathway (REAC:5358351, P -value = 2.79×10^{-3} ; KEGG:04340, P -value = 10^{-4}), Primary Cilia (REAC:5617833, P -value = 10^{-6} ; GO:0060271, P -value = 2×10^{-6}) and Wnt/Planar Cell Polarity (PCP) signalling pathway (GO:0016055, P -value = 2×10^{-5}). The SHH pathway is the primary pathway implicated in HPE and the primary cilium is required for the transduction of SHH signalling (Gorivodsky *et al.*, 2009; Murdoch and Copp, 2010) while components of Wnt/PCP

pathway regulate both SHH signalling and primary cilia (Goetz *et al.*, 2009; Murdoch and Copp, 2010).

In-depth analyses highlighted 10 families with oligogenic events (Fig. 2) clustered among 19 genes (Tables 2 and 3) that functionally relate to disease-relevant pathways (Fig. 3). These combinations of variants were unique to the affected probands. The main findings are presented below and full reports are available in the Supplementary material.

Recurrent oligogenic events involving *FAT1*

Four different families, i.e. 15% of the 26 families studied here, presented oligogenic events involving *FAT1* in combination with rare variants in known HPE genes (*SHH*, *PTCH1*), as well as in *NDST1*, *COL2A1* and *LRP2*

genes (Fig. 2A). *FAT1* is a protocadherin and its knock-down in mice causes severe midline defects including HPE (Ciani *et al.*, 2003); in *Drosophila* it has been shown to regulate the PCP pathway (Rock *et al.*, 2005). *LRP2*, *NDST1* and *COL2A1* are all functionally relevant to the SHH pathway (Fig. 3): *NDST1* and *COL2A1* mice mutants exhibit HPE phenotype and reduced SHH signalling in the forebrain (Grobe *et al.*, 2005; Leung *et al.*, 2010), while *LRP2* acts as an auxiliary receptor of SHH during forebrain development and its inactivation in mouse similarly leads to HPE phenotype (Christ *et al.*, 2012).

Oligogenic events involved the following combinations: *SHH/FAT1/NDST1* (Family F3), *FAT1/NDST1/COL2A1* (Family F16), *FAT1/COL2A1/PTCH1* (Family F26) and *FAT1/LRP2* (Family F23) (Fig. 2A, Tables 2 and 3). Details are provided in the Supplementary material, Case report 1.

In Family F3, Sanger sequencing of additional family members revealed that the *SHH/FAT1/NDST1*

combination was unique to the affected individuals (Fig. 2A). For Family F16, only the foetus carrying the *FAT1/NDST1/COL2A1* combination was affected by semi-lobar HPE, while the sibling carrying *NDST1/COL2A1* variants presented only a microform (Fig. 2A). These observations are fully consistent with the oligogenic inheritance model where accumulation of multiple variants in genes associated to HPE phenotypes and/or HPE-related molecular pathways is required.

Recurrent oligogenic events involving *SCUBE2/BOC* implicated in SHH signalling

Two families presented oligogenic events implicating combined variants in the *BOC* and *SCUBE2* genes (Fig. 2B, Tables 2 and 3). *BOC* is an auxiliary receptor of SHH and was recently reported as an HPE modifier in humans (Hong

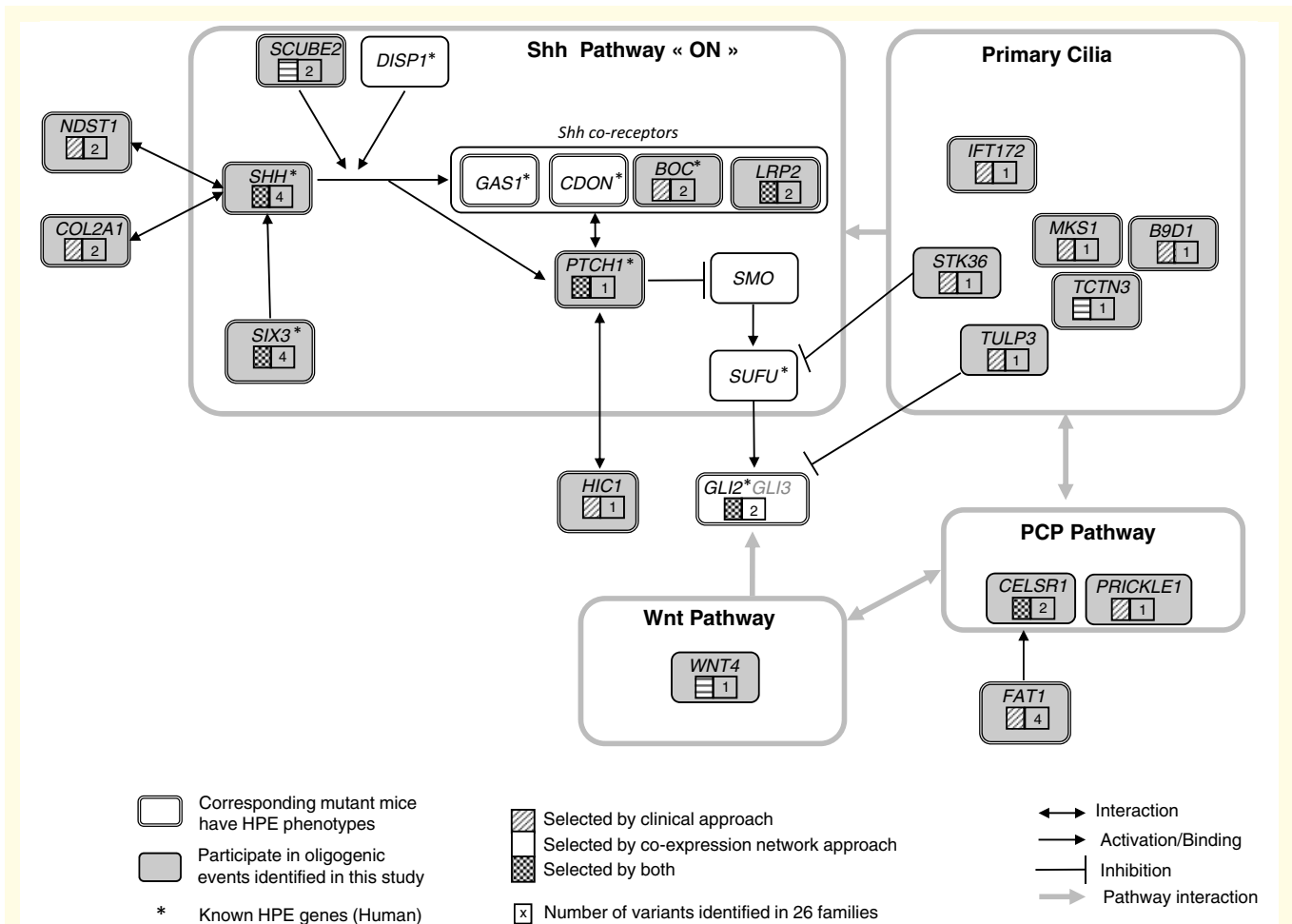


Figure 3 Implication of the candidate genes in the signalling pathways involved in HPE. Key affected pathways and genes are presented. Under each gene name, the selection methods (clinical or co-expression networks approach or both) is shown on the left and the number of variants for each gene is shown on the right. Genes known in HPE are marked with an asterisk, and genes for which corresponding mutant mice have HPE phenotypes are surrounded by a double line. The genes implicated in oligogenic events in the study are indicated with a grey background.

et al., 2017). *SCUBE2* shares a highly similar expression pattern with *SHH* and *SIX3* and is implicated in the release of *SHH* from the secreting cell (Jakobs *et al.*, 2014). In Family F4, a combination of *SCUBE2/BOC* variants was associated with additional variants in *SHH*, *STK36* (see below) and *WNT4*, a member of the Wnt pathway, implicated in regulation of SHH signalling (Murdoch and Copp, 2010). In Family F22, the *SCUBE2* variant results in a premature stop codon at position 525 (Supplementary Fig. 7), which results in truncation of its CUB domain and is predicted to directly affect its SHH-related activity (Jakobs *et al.*, 2014). This family presented an additional candidate variant in *HIC1*, which genetically interacts with *PTCH1* (Briggs *et al.*, 2008). Mice deficient for *HIC1* exhibit craniofacial defects including HPE (Carter, 2000).

The reported variant combinations were observed exclusively in the affected probands and were absent in asymptomatic individuals. Altogether, these results reveal recurrent mutations in *SCUBE2/BOC* and further strengthen the oligogenic inheritance model of HPE.

Implication of primary cilium in HPE

Remarkably, five families presented candidate variants in genes related to the primary cilium: *STK36*, *IFT172*, *B9D1*, *MKS1*, *TCTN3* and *TULP3* (Fig. 2C). Ciliary proteins are known to play essential roles in the transduction of SHH signalling downstream of *PTCH1* during forebrain development (Goetz *et al.*, 2009; Murdoch and Copp, 2010).

STK36, also known as ‘fused’, is a ciliary protein implicated in SHH signalling and associated to craniofacial phenotypes (Goetz *et al.*, 2009; Murdoch and Copp, 2010). *IFT172* codes for a core component of intraflagellar transport complex IFT-B required for ciliogenesis and regulation of SHH signal transduction. Moreover, *Ift172*^{-/-} mice exhibit reduced expression of *Shh* in the ventral forebrain and severe craniofacial malformations including HPE (Gorivodsky *et al.*, 2009). *B9D1*, *MKS1* and *TCTN3* are all members of the transition zone protein complex implicated in regulation of ciliogenesis (Garcia-Gonzalo *et al.*, 2011). The disruption of *B9d1* and *Mks1* in mouse models causes craniofacial defects that include HPE (Dowdle *et al.*, 2011; Wheway *et al.*, 2013). Although no mouse model is available for *TCTN3*, its expression profile is highly similar to that of *SHH* and disruption of its protein complex partners (*TCTN1*, *TCTN2*, *CC2D2A*, *MKS1*, *B9D1*) leads to HPE in mouse (Dowdle *et al.*, 2011; Garcia-Gonzalo *et al.*, 2011; Wheway *et al.*, 2013). Moreover, *TCTN3* was shown to be necessary for the transduction of SHH signal and *TCTN3* mutations were found in patients affected by ciliopathies (Thomas *et al.*, 2012). Finally, *TULP3* is a critical repressor of *Shh* signalling in mice and is associated with various craniofacial defects (Murdoch and Copp, 2010).

Additional variants observed in these families include a heterozygous deletion of *SIX3*, missense mutations in *SHH*, *SCUBE2*, *BOC* and *LRP2* (described above) as well as two genes implicated in PCP pathway (Fig. 3): *CELSR1* (two

families) and *PRICKLE1*, both associated with craniofacial defects in mouse mutants (Fig. 2C) (Goetz *et al.*, 2009; Murdoch and Copp, 2010; Yang *et al.*, 2014). Similar to previously described cases, the oligogenic events were present exclusively in the affected children.

Given the essential role of the primary cilium in SHH signal transduction, these observations strongly suggest that rare variants in ciliary genes contribute to the disease onset in these families.

Correspondence between affected genes and secondary clinical features

To provide additional evidence, we performed an in-depth analysis of secondary clinical features associated with HPE in our patients. Deep clinical phenotyping identified clinical similarities between unrelated patients (Tables 2 and 3) as well as overlaps of secondary clinical features between patients and the corresponding mouse mutants.

Interestingly, the two patients with variants in ciliary genes (*IFT172/PRICKLE1* and *SIX3/TCTN3/TULP3*) both presented with polydactyly, a clinical feature commonly associated with ciliopathies (Goetz *et al.*, 2009). Importantly, the patient with the oligogenic combination *IFT172/PRICKLE1* presented with a large set of overlapping clinical features with the corresponding mouse mutants including polydactyly, cleft palate and eye defects (Gorivodsky *et al.*, 2009; Yang *et al.*, 2014).

Of note, the two unrelated patients having variants in *FAT1* and *NDST1* shared a large set of specific secondary clinical features, including mandibular and ear abnormalities. Intrauterine growth restriction was found exclusively in the two patients with *COL2A1* variants. The most severely affected child in Family F16 (*FAT1/NDST1/COL2A1*) presented a strong overlap with *NDST1*-null and *COL2A1*-null mutant mice (HPE, mandibular anomalies, absent olfactory bulb, abnormal nose morphology) (Grobe *et al.*, 2005; Leung *et al.*, 2010). Similarly, proboscis and eye defects were observed in both *FAT1/NDST1/SHH* patient and *FAT1*^{-/-} mice (Ciani *et al.*, 2003).

Finally, the two unrelated *SCUBE2/BOC* cases in Families F4 and F22 presented with cebocephaly, a midline facial anomaly characterized by ocular hypotelorism and a single nostril, which was absent in all other patients. Consistently, *SCUBE2* is highly expressed in the nasal septum in mouse (Xavier and Cobourne, 2011), and cebocephaly was previously associated with *CDON*—another known HPE gene sharing highly similar functions and structure with *BOC* (Zhang *et al.*, 2006).

While these clinical features are not specific to HPE, the described overlaps provide additional support for disease implication of the presented candidate variants.

Statistical validations

The identified oligogenic events were clustered among 19 genes (Fig. 2, Tables 2 and 3). To assess the frequency of

Table 4 Statistical validations: Fisher's exact test analysis for oligogenic events

Comparison	HPE	GoNL	FREX	P-value		
				HPE versus GoNL	HPE versus FREX	GoNL versus FREX
Families with oligogenic events	10/26 (38%)	3/248 (1.2%)	NA	2.301×10^{-9}	NA	NA
Children harbouring rare deleterious variants in two or more candidate genes	13/29 (45%)	6/248 (2.4%)	NA	1.902×10^{-10}	NA	NA
All individuals harbouring rare deleterious variants in two or more candidate genes	21/80 (26%)	14/744 (1.8%)	16/574 (2.7%)	3.237×10^{-14}	1.521×10^{-11}	0.35

Oligogenic inheritance is defined as presence of combined rare deleterious variants in two or more genes, described in Table 3 and Fig. 2. The proportion of individuals harbouring combined rare deleterious variants in the identified genes is significantly higher in HPE cohort as compared to two control populations GoNL and FREX (Fisher's exact test).

healthy individuals presenting similar variant combinations in these genes, we applied the same family-by-family variant analysis to the 248 control trios provided by GoNL. This control cohort was chosen as 24/26 (92%) of the HPE families included in the study were of European descent (Supplementary Fig. 10).

The approach identified three families among controls presenting variant combinations satisfying the criteria that we established for the oligogenic events (gene, variant and parental inheritance). The three oligogenic events found in the control cohort were *FAT1/B9D1*, *SCUBE2/PTCH1* and *SCUBE2/LRP2/PTCH1/CELSR1* (Supplementary Table 9). Although one *SCUBE2* variant (p.Thr285Met) was found in both the HPE and the control cohort, none of the combinations found among controls corresponded to oligogenic events identified in the HPE cohort. The incidence of oligogenic events was significantly lower in the GoNL families (3/248, 1.2%) as compared to the HPE cohort (10/26, 38%) with a Fisher's exact test *P*-value of 2.301×10^{-9} (Table 4).

Three additional children of the GoNL cohort harboured combinations of rare deleterious variants in two or more candidate genes. However, in these cases, all variants were inherited from the same parent. Therefore, these combinations were not considered as oligogenic events similar to those of HPE patients. Nevertheless, even when taking into account these three additional cases, the proportion of children having variants in two or more candidate genes was significantly different between the HPE cohort (13/29, 45%) and the GoNL cohort (6/248, 2.4%) with a Fisher's exact test *P*-value of 1.902×10^{-10} .

Finally, 14 individuals of the GoNL cohort (parents and children combined) harboured rare deleterious variants in two or more genes. Without taking into account the relatedness between the GoNL individuals, the proportion of individuals having variants in two or more candidate genes remained significantly different between the HPE cohort (21/80, i.e. 26%) and the GoNL control cohort (14/744, 1.8%), as confirmed by Fisher's exact test (*P*-value = 3.237×10^{-14}).

To assess the frequency of control individuals presenting rare variant combinations in the identified candidate genes

further (Fig. 2, Tables 2 and 3), we analysed a second control cohort. The FREX data were chosen as they consist of 574 unrelated French individuals ancestrally matching the HPE cohort.

Screening of the FREX cohort revealed that 16/574 individuals (i.e. 2.7%) harboured rare deleterious variants in two or more candidate genes. This proportion was statistically different from that observed in the HPE cohort (21/80, 26% versus 16/574, 2.7%; *P*-value = 1.521×10^{-11} , Fisher's exact test).

Additionally, the two control cohorts (GoNL and FREX) did not present statistically significant differences in terms of proportions of individuals having rare deleterious variants in two or more candidate genes: 14/744 (1.8%) for the GoNL cohort versus 16/574 (2.7%) for the FREX (*P*-value = 0.35, Fisher's exact test).

The analysis of the GoNL and FREX cohorts illustrates that the incidence of combined rare deleterious variants in the identified candidate genes is significantly higher in HPE patients as compared to a control population. All performed comparisons showed a statistically significant *P*-value between the cases and the controls (Table 4), thus providing evidence for oligogenicity as clinically relevant model in HPE.

Discussion

In this study, we addressed the relevance of oligogenic model for unsolved HPE cases. We provide evidence that the onset of HPE arises from the combined effects of hypomorphic variants in several genes belonging to critical biological pathways of brain development. To circumvent the limitations of classical WES analysis in complex rare disorders, we combined clinically-driven and co-expression network analyses with classical WES variant prioritization. This strategy was applied to 26 HPE families and allowed prioritization of 180 genes directly linked to the SHH signalling, cilium and Wnt/PCP pathways (Fig. 3). The analysis of oligogenic events in patients with HPE anomalies revealed 19 genes including 15 genes previously unreported in human HPE patients (Tables 2 and 3). All these genes

are either associated with HPE phenotypes in corresponding mouse models (such as *FAT1*, *NDST1*), present highly similar expression patterns with already known HPE genes in the developing brain (such as *SCUBE2*, *TCTN3*), or both. We observed co-occurrence of mutations in several gene pairs such as *FAT1/NDST1* and *SCUBE2/BOC*, which provides additional arguments towards their implication in HPE. The incidence of oligogenic combinations was significantly higher in HPE patients compared to the GoNL and FREX control populations. We additionally show that in-depth evaluation of secondary clinical features in patients with HPE anomalies and comparison to published mouse knockout models may provide additional arguments for the causality of candidate genes.

The main challenge in disease-gene discovery by WES is to identify disease-related variants among a large background of non-pathogenic polymorphisms (Bamshad *et al.*, 2011; MacArthur *et al.*, 2014). For example, the presented *FAT1* encodes a large protocadherin gene spanning over 139 kb in the human genome and presenting over 2000 missense variants with a minor allele frequency below 1% in the gnomAD database. Despite this high number of variations found in the general population, rare variants in *FAT1* were recently implicated in several genetic disorders including facioscapulohumeral dystrophy-like disease (Puppo *et al.*, 2015). Hence, correct interpretations and conclusions require extremely careful assessment of available biological and clinical knowledge.

To improve the pertinence of our study, we developed a strategy to restrict the potential candidates by targeting genes with biological and clinical arguments for their implication in the disease. Implication of a given gene in a disease is often supported by the similarity between the human pathology and the phenotype obtained in relevant animal models (MacArthur *et al.*, 2014). Accordingly, in this study, the main evidence of causality for candidate genes was that their disruption leads to clinically-defined HPE-related phenotypes in corresponding published mutant mouse models. Unlike other phenotypes, such as reduced body weight (Reed *et al.*, 2008), holoprosencephaly is a rare effect of gene knockout in mice as it is associated with <1% of knockout mice (as reported in the MGI database). Recent exome sequencing studies have applied similar phenotype-driven approaches to identify causal variants in monogenic disorders. Dedicated tools have been developed to that aim (Exomiser, Phive) (Smedley *et al.*, 2015) but none are designed for non-Mendelian traits involving hypomorphic variants with mild effects. We provide a method to specifically address such cases and show that further developments are necessary to improve the diagnosis of genetic disorders, especially by taking into account oligogenic inheritance. Inclusion of carefully defined mouse mutant phenotypes is of powerful value as certain phenotypes like HPE are very informative due to their rarity.

Prioritization tools can also include protein–protein interaction (PPI) network information, which improves

performance in cases where candidate genes do not have an associated knockout mouse model. However, PPI-based prioritization is limited when disease investigation requires incorporation of tissue-specific data. The key process affected by HPE is the elaboration of the forebrain and its dorso-ventral patterning (Fernandes and Hébert, 2008). Deciphering the biological mechanisms involved in the early brain development is therefore necessary to provide relevant information to select disease-related genes. To incorporate tissue-specificity, we performed analysis using the RNA-Seq data of embryonic human brain at the earliest available developmental stages (from 4 to 17 post-conception weeks) as provided by the Human Development Biology Resource (Lindsay *et al.*, 2016). We defined relevant co-expression modules and selected candidate genes of which expression patterns follow those of known HPE genes. Further analysis showed that the resulting candidate genes, such as *SCUBE2* and *TCTN3*, are pertinent as they are equally implicated in the SHH pathway that is the primary HPE pathway (Thomas *et al.*, 2012; Jakobs *et al.*, 2014). Co-expression analysis provides additional insight into disease pathogenesis by establishing the first link between previously unrelated genes. A future challenge will be to generalize this approach, but such a task will face the necessity to incorporate disease relevant co-expression modules that need to be pre-computed.

Patients exhibiting HPE anomalies present enrichment of rare variants in genes related to the SHH pathway, as well as to the Wnt/PCP and primary cilia pathways, which were both shown to functionally interact with and regulate SHH pathway (Goetz *et al.*, 2009; Gorivodsky *et al.*, 2009; Murdoch and Copp, 2010; Wheway *et al.*, 2013). Accumulation of multiple rare variants in genes related to these pathways will likely disrupt the dorso-ventral gradient of the SHH morphogen (Fernandes and Hébert, 2008), leading to an incomplete cleavage of the forebrain and, ultimately, to HPE. In this model, distinction between different manifestations of HPE lies in the degree of overall functional impact on SHH signalling (Mercier *et al.*, 2013). Moreover, depending on the affected genes and pathways, HPE patients would present different secondary clinical features.

The observed overlapping secondary clinical features further support the causality of the reported variants for HPE. As hypomorphic mutations do not have the same impact as the complete inactivation of a gene in most cases, phenotypic overlaps may be challenging to detect and require expert assessment of clinical and biological data. For example, mice deficient in *NDST1* exhibit agnathia (Grobe *et al.*, 2005) (absence of the lower jaw) while unrelated patients presenting candidate variants in *NDST1* exhibit prognathia and retrognathia (abnormal positioning of the lower jaw), respectively. All three phenotypes are part of the same spectrum of mandibular anomalies. From a clinical perspective, overlap of secondary clinical features between the patient and the animal models provides additional critical evidence of a causal relationship between

candidate gene and disease. A key issue here remains the semantic representation of patient's phenotype and the use of a well-established phenotypic ontology during the examination processes. Explorations of secondary clinical features should be performed in future studies of genetic diseases.

Additional molecular screenings in larger populations of HPE patients are necessary to definitely assess the implication of our candidate genes in the disease. Therefore, we propose to include these novel genes into future genetic screenings of HPE patients.

In conclusion, this paper presents novel genes implicated in HPE and illustrates that HPE presents an oligogenic inheritance pattern requiring the joint effect of multiple genetic variants acting as hypomorphic mutations. The proposed inheritance pattern accounts for a wide clinical spectrum of HPE and explains the significant part of cases in which no molecular diagnosis could be established by conventional approaches. It also explains the incomplete penetrance and variable expressivity of inherited causal mutations observed in the reported cases of HPE (Mercier *et al.*, 2011). We propose that in cases of non-Mendelian diseases with variable phenotypes, the possibility of oligogenic inheritance needs to be evaluated. Exploration of such events will improve the diagnostic yield of complex developmental disorders and will contribute to better understanding of the mechanisms that coordinate normal and pathological embryonic development.

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Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online.

Appendix I

Full collaborator details are available in the online Supplementary material.

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***DISCUSSION GÉNÉRALE
ET PERSPECTIVES***

DISCUSSION GENERALE ET PERSPECTIVES

L'objectif de ma thèse était d'étudier la voie NOTCH au cours du développement précoce du cerveau antérieur, son rôle dans la neurogenèse et dans la régulation du signal SHH. Ce travail s'inscrivait dans le projet de l'équipe de mieux comprendre les mécanismes moléculaires mis en jeu dans une pathologie du développement précoce du cerveau (l'holoprosencéphalie). Mes travaux ont permis non seulement de décrire des processus de développement normaux du cerveau antérieur mais aussi de contribuer à comprendre les mécanismes physiopathologiques à l'origine d'une maladie rare.

LA VOIE NOTCH CONTROLE LA NEUROGENESE PRECOCE DANS L'HYPOTHALAMUS

Précédemment, des travaux de l'équipe avaient montré chez le poulet que le ligand *Dll1* et les cibles de la voie NOTCH, *Hes5* et *Hey1*, étaient exprimés dans l'hypothalamus antérieur à partir de HH11 (soit 13 somites, *Ratié et al., 2013*). Durant ma thèse, j'ai montré chez la souris que la voie NOTCH s'exprimait dans le cerveau à partir de E8.0 au niveau du mésencéphale mais qu'elle n'était active dans l'hypothalamus antérieur qu'à partir de E8.5 (8 à 12 somites), soit juste avant l'apparition des premiers neurones (nTPOC) dans cette zone (*Mastick & Easter, 1996*). Son expression est ensuite également retrouvée, à E9.5, au niveau de la zone mamillaire de l'hypothalamus dans les nMTT. Nous avons montré que la voie NOTCH se mettait initialement en place via une boucle de régulation, avec une expression ubiquitaire de *Notch1* dans le cerveau et une expression spécifique dans l'hypothalamus antérieur de *Dll1*, *Hes5* et *Ascl1* selon un motif « poivre et sel ». Cette boucle de régulation est donc très conservée entre le poulet et la souris (*Ratié et al., 2013*). Elle est caractéristique du mécanisme d'inhibition latérale lié à la voie NOTCH déjà décrite dans de nombreux autres tissus embryonnaires (*Bertrand et al., 2002 ; Kageyama et al., 2008*). Par ailleurs, dans ces tissus, l'expression des acteurs de la voie NOCTH précède celle des marqueurs neuronaux tels que *Nhlh1* et *NeuroD4* puis de *Stmn2* et *HuC/D* (*Murdoch et al., 1999; Abu-Elmagd et al., 2001*); ce que nous observons également au niveau de l'hypothalamus. Ces observations montrent que la voie NOTCH est impliquée dans la neurogenèse précoce hypothalamique via des mécanismes moléculaires classiquement utilisés par les autres sites de neurogenèse de l'embryon.

La voie NOTCH a été impliquée depuis longtemps dans la neurogenèse (Mizutani et al., 2007 ; Imayoshi et al., 2010), mais son rôle spécifique au niveau de l'hypothalamus avait été seulement décrit à des stades tardifs dans l'apparition, par exemple, les neurones à fonction endocrine (Aujla et al., 2011; Aujla et al., 2013). Des études menées sur des mutants conditionnels $Rbpj^{fl/fl}; Nkx2.1^{Cre}$ où la voie NOTCH est inhibée spécifiquement dans l'hypothalamus, montrent son rôle important dans l'induction de la différenciation des neurones endocrines de l'hypothalamus (e.g. les neurones GHRH) (Aujla et al., 2013). Par ailleurs, ces mêmes neurones sont absents chez les mutants $Ascl1^{-/-}$ (McNay et al., 2006). Ces résultats suggèrent que le mécanisme de détermination neurale nécessaire à la neurogenèse des neurones endocrines de l'hypothalamus dépende d'une boucle de régulation entre la protéine proneurale ASCL1 et la voie NOTCH. Cependant, les auteurs de ces travaux n'ont pas étudié le développement des premiers neurones se différenciant dans l'hypothalamus comme les nTPOC.

Nos travaux montrent que la voie NOTCH est également impliquée dans la spécification des premiers neurones hypothalamiques (nTPOC et nMTT). Le rôle exact de ces populations n'est pas encore établi mais il semble que leurs axones formeraient des faisceaux qui serviraient de guide pour les projections axonales du prosencéphale (Easter et al., 1993 ; Ware et al., 2015)

Les souris conditionnelles utilisées ($Rbpj^{L/L}; Rosa26-creER^{T2}$), nous ont permis de montrer qu'une perte de fonction de RBPJ entraîne une surexpression de *Ascl1* et une perte du motif poivre et sel dans le prosencéphale, ce qui avait déjà été décrit chez le KO pour $Rbpj^{-/-}$ et $Notch1^{-/-}$ au niveau du tube neural (De la Pompa et al., 1997). Cette surexpression correspond à une différenciation prématurée des progéniteurs neuraux en neurones. De façon similaire, nous montrons, qu'au niveau de l'hypothalamus, via le processus d'inhibition latérale, la voie NOTCH permet de maintenir des cellules à l'état de progéniteurs. De la même manière, chez notre modèle conditionnel, l'inactivation de la voie NOTCH déséquilibre ce processus; *Ascl1* est surexprimé, les progéniteurs neuronaux se différencient prématurément et l'organisation en poivre et sel est perdue.

Les souris mutées pour les gènes proneuraux (*Ascl1*, *Neurog1* et *Neurog2*) présentent une diminution du nombre de neurones dans de nombreux territoires neurogéniques tels que les ganglions crâniens, l'épithélium olfactif, la rétine ou la moelle épinière (Fode et al., 2000 ; Guillemot & Joyner, 1993 ; Guillemot et al., 1993 ; Ma et al., 1998). Il est établi que ces gènes proneuraux (*Ascl1*, *Neurog1* et *Neurog2*) peuvent avoir des territoires d'expression et des rôles différents dans la différenciation des sous-types de neurones. En effet, au niveau de la moelle épinière et du télencéphale dorsal, *Ascl1* et *Neurog2* possèdent des fonctions divergentes pour la spécification de

sous-types neuronaux (*Nieto et al., 2001 ; Parras et al., 2002 ; Sun et al., 2001*). Les gènes proneuraux sont donc non seulement impliqués dans le choix du destin neural mais également dans le choix des sous-types neuronaux. Nos travaux montrent qu'il en est de même au niveau de l'hypothalamus, où, chez le poulet comme chez la souris, on retrouve *Ascl1* spécifiquement dans les nTPOC et les *Neurog1* et *2* dans les nMTT. Ainsi, les gènes proneuraux semblent exclusifs dans les territoires neurogéniques et *Ascl1* est le gène proneural exprimé dans l'hypothalamus antérieur.

Nos expériences d'électroporation réalisées dans le mésencéphale confirment la capacité d'ASCL1 à induire la neurogenèse dans des cellules neurectodermiques, mais elles montrent également que *Tagln3* et *Chga* sont des cibles de ce gène. Ces gènes avaient précédemment été identifiés car ils étaient fortement surexprimés dans le l'hypothalamus antérieure lorsque la voie NOTCH était inhibée (*Ratié et al., 2013*). L'expression de ces deux gènes est donc dépendante de la boucle de régulation NOTCH/ASCL1.

Les transgélines (TAGLN) sont des protéines associées aux microtubules dont le rôle principal est se lier à l'actine et de stabiliser le cytosquelette (*Assinder et al., 2009*). Nous avons montré que l'expression de *Tagln3* est pan-neurale (*Ratié et al., 2013 ; Ware et al., 2016*). CHGA (Chromogranine A) est une protéine neuroendocrine localisée dans les vésicules sécrétoires des neurones et des cellules endocrines (*Helle et al., 2007*). Lors du développement embryonnaire précoce, CHGA est exprimée spécifiquement dans le noyau du tractus de la commissure postérieure (nTPC- chez la souris et le poulet) et dans les nTPOC (chez le poulet), suggérant que ces neurones peuvent avoir une activité neuroendocrine (*Ratié et al., 2013 ; Ware et al., 2016*), mais son rôle dans ces populations neuronales reste à définir.

L'étude du domaine d'expression de *Chga* suggère que ce marqueur neuronal est activé spécifiquement par ASCL1 et pas par NEUROG1/2. Cependant, nos études de surexpression chez le poulet, montrent que ASCL1 comme NEUROG2 sont suffisants pour activer *Chga* dans le neurectoderme mésencéphalique.

Ces résultats suggèrent que même si ASCL1 et les NEUROG ont des territoires d'expressions bien distincts, qui sont très conservés au cours de l'évolution, ils ont la capacité d'activer l'expression des mêmes marqueurs neuronaux. Des études réalisées à l'aide de souris transgéniques ont effectivement montré que *Ascl1* pouvait remplacer *Neurog2* au cours de la différenciation neuronale au niveau du télencéphale dorsal et la moelle épinière ventrale (*Fode et al., 2000*) et inversement *Neurog2* est capable d'induire les neurones dont la différenciation est normalement dépendante de *Ascl1* (*Parras et al., 2002*).

Les domaines d'expression mutuellement exclusifs des gènes proneuraux et leur grande conservation ne peuvent donc pas expliquer à eux seuls l'expression spécifique des marqueurs neuraux, *Tagln3* et *Chga*. Cependant, une étude récente a montré que des protéines proneurales, ASCL1 et NEUROG2 modifierait, chacune à sa manière, le paysage chromatinien et donc l'accessibilité aux promoteurs des gènes cibles pour d'autres facteurs de transcription (Aydin et al., 2019). Les auteurs proposent l'hypothèse selon laquelle, dans des progéniteurs où la conformation de la chromatine est permissive, *Ascl1* et *Neurog2* induiraient facilement des modifications importantes de la chromatine et permettraient de recruter des facteurs de transcriptions différents et d'orienter le sous-type neuronal. A l'inverse, pour des progéniteurs où la chromatine a une conformation relativement fixe qui oriente déjà fortement le destin neuronal, l'intervention d'*Ascl1* ou *Ngn2* peut indifféremment conduire au recrutement de facteurs de transcriptions qui auront un rôle équivalent dans la spécification neuronale. Ainsi, les neurones exprimant *Tagln3* et *Chga* seraient donc déjà engagés vers un sous-type neuronal spécifique, et la différenciation définitive serait induite par *Ascl1* ou *Ngn2*.

Cette première partie de travail de thèse m'a donc permis de poser les bases de la neurogenèse dépendant de la voie NOTCH au niveau de l'hypothalamus présomptif. Nous avons également mis en évidence de nouvelles cibles de la boucle de régulation NOTCH/ASCL1, qui sont des marqueurs neuraux, *Tagln3* et *Chga*.

LA VOIE NOTCH REGULE SHH DANS L'HYPOTHALAMUS

Etant donné l'implication de la voie NOTCH dans la neurogenèse précoce de l'hypothalamus, et le rôle primordial de la voie SHH dans la mise en place de ce tissu, nous avons étudié une possible interaction entre ces 2 voies. Chez le poulet comme chez la souris, la voie SHH est active dans l'hypothalamus antérieur avant l'apparition de la voie NOTCH (Manning et al., 2006 ; Hamdi-Rozé et al., en préparation). Cependant, chez les embryons inactivés pour la voie NOTCH, l'expression de *Shh* est globalement diminuée et même absente au niveau de l'hypothalamus antérieur. Dans la littérature, il existe plusieurs modèles souris avec une inhibition ciblée de la voie SHH dans l'hypothalamus. C'est le cas des souris *Hesx1^{Cre/+}; Shh^{fl/-}*, où l'inactivation de *Shh* est spécifique au cerveau antérieur (Carreno et al., 2017 ; Andoniadou et al., 2007), et des souris *SBE2^{Cre/+}; Shh^{fl/-}* où *Shh* est inactivé dans les cellules exprimant SBE2, un régulateur transcriptionnel de *Shh* spécifique à l'hypothalamus (Zhao et al., 2012 ; Jeong et al., 2006). Les phénotypes observés dans nos embryons conditionnels *Rbpj^{+/L}* ; *Rosa26-creER^{T2}* sont très similaires à ceux décrits pour ces modèles, tant au

niveau des expressions géniques (perte d'expression de *Nkx2.1*, expansion du territoire d'expression de *Fgf10*) que de la morphologie (hypoplasie du télencéphale). Ceci implique que le phénotype au niveau du prosencéphale présenté par les embryons déficients en activité NOTCH pourrait être dû à une diminution spécifique de l'activité SHH dans l'hypothalamus antérieur.

Il a été montré chez le poulet que lorsque les progéniteurs neuronaux hypothalamiques se différencient en neurones, elles perdent leur capacité à exprimer *Shh* (Fu et al., 2017). Or, il est établi que l'activité de la voie NOTCH dans les cellules hypothalamiques permet leur maintien à l'état de progéniteurs neuronaux (Ratié et al., 2013). Il est donc possible que la perte d'expression de *Shh* dans l'hypothalamus antérieure soit secondaire à la différenciation précoce des progéniteurs neuronaux due à une perte d'activité de NOTCH.

Par ailleurs, des études menées sur les progéniteurs neuronaux de la moelle épinière chez le poulet et la souris ont montré que la voie NOTCH agissait sur ces progéniteurs pour augmenter leur sensibilité à SHH, et que cette action se faisait via la diminution de PTCH1 au niveau du cil primaire, facilitant ainsi l'entrée de SMO et l'activation des GLI (Kong et al., 2015 ; Stasiulewicz et al., 2015). Récemment, il a été montré, dans la moelle épinière du poisson zèbre, que la voie NOTCH agissait également sur la voie SHH de manière indépendante du cil primaire, en contrôlant directement la transcription des gènes de la famille des GLI (Jacobs & Huang, 2019). Ceci rejoint d'autres études qui ont également montré un rôle du complexe NICD/RBPJ dans la régulation de l'expression de *Gli2* et *Gli3* chez la souris (Li et al., 2012). L'ensemble de ces mécanismes qui implique la voie NOTCH pourrait collectivement contribuer à réguler la disponibilité de la voie SHH au cours du développement de l'hypothalamus.

Par ailleurs, l'implication des cils dans la pathologie a été montrée par notre équipe, en caractérisant une mutation homozygote dans le gène *STIL* chez des patients HPE (Mouden et al., 2015). *STIL* est une protéine permettant la duplication des centrioles (Vulprecht et al., 2012), structures qui se trouvent à la base des cils primaires et qui permettent leur développement (Bettencourt-Dias & Glover, 2005). Une mutation dans ce gène entraîne une diminution du nombre de centrioles et une perturbation de la ciliogenèse (Mouden et al., 2015) et donc du signal SHH. *STIL* agit également directement sur la transduction du signal SHH dans les neurones des mammifères (Sun et al., 2014).

UNE DEREGLATION DE LA VOIE NOTCH INDUIT UN PHENOTYPE HPE

Même si les mécanismes moléculaires réellement impliqués dans la régulation de *Shh* par la voie NOTCH restent à définir, nos observations montrent qu'une perturbation de l'activité de NOTCH dans l'hypothalamus provoque une diminution de l'expression de *Shh*. Or, la concentration de SHH au niveau du cerveau antérieur ventral doit être finement régulée pour un développement normal du cerveau (Cordero et al., 2004). La perturbation du signal SHH due à un dysfonctionnement de la voie NOTCH pourrait donc contribuer au développement d'anomalies de type HPE.

Les modèles animaux utilisés habituellement et inhibés pour les voies NOTCH et SHH (respectivement *Rbpj*^{-/-} et *Shh*^{-/-}) présentent des malformations multiples et une létalité précoce ne nous permettant pas d'analyser spécifiquement la fonction de ces voies au cours du développement hypothalamique (Oka et al., 1995; Chiang et al., 1996). Cependant, en générant un modèle conditionnel où la voie NOTCH est inhibée à partir de E7.75, nous montrons que l'inactivation de NOTCH entraîne une hypoplasie du télencéphale, ou microcéphalie, qui est également retrouvée chez les patients HPE (Cohen, 2006).

Par ailleurs, le phénotype observé chez les double-hétérozygotes *Rbpj*^{+/-};*Shh*^{+/-} valide l'existence d'une synergie entre ces 2 voies de signalisation. Ces mutants présentent une glande hypophysaire dysmorphique et une malformation des os sphénoïdes ; ce qui est caractéristique d'anomalies de la ligne médiane. Cette malformation est nommée « persistance du canal pharyngo-hypophysaire ». Elle a pour origine une anomalie du développement de la poche de Rathke qui induit une persistance du pédicule pharyngo-hypophysaire et oblige l'os sphénoïde à se développer autour de ce pédicule résiduel.

Des anomalies comparables ont été retrouvées chez des souris mutées dans les corécepteurs de la voie SHH que sont *Cdon* et *Gas1* (Cole & Krauss, 2003, Khonsari et al., 2013, Seppala et al., 2014). Par ailleurs, en inhibant *Shh* seulement dans l'hypothalamus antérieur (Carreno et al., 2017), il en résulte une malformation de l'adénohypophyse avec non disjonction totale de la poche de Rathke de l'ectoderme oral et anomalie du basisphénoïde. C'est donc une diminution du signal SHH dans l'hypothalamus qui entraîne une malformation de l'hypophyse, elle-même à l'origine d'un défaut secondaire au niveau de l'os basisphénoïde (Khonsari et al., 2013).

Des malformations de l'hypophyse associées à un défaut de formation de l'os sphénoïde ont été décrites à plusieurs reprises chez des patients HPE (Kjaer & Fischer-Hansen, 1995; Lo et al., 1998; Kjaer, 2015). Elles peuvent être isolées ou associées à des malformations plus typiques de l'HPE. Nos

souris *Shh*^{+/-} ; *Rbpj*^{+/-}, où l'anomalie hypophysaire est isolée, constituent un modèle de microforme d'HPE.

Des travaux d'inhibition de la voie SHH par la cyclopamine chez l'embryon de poulet ont montré que le phénotype HPE dépend de la concentration de cyclopamine (*Mercier et al., 2013*) mais ils montrent également que plus la voie SHH est inhibée tôt, plus le phénotype HPE induit est sévère (*Cordero et al., 2004*). En effet, SHH exerce son action sur le développement morphologique du prosencéphale du stade préchordal (E7.75 chez la souris) jusqu'à la différenciation de l'hypothalamus (E10 chez la souris). Il est donc cohérent que plus le dysfonctionnement se produit tôt et plus le phénotype HPE est sévère. Nous montrons que la voie NOTCH n'intervient que tardivement pour maintenir l'expression de *Shh* dans l'hypothalamus antérieur ; une perte de fonction de l'activité NOTCH à elle seule ne peut donc pas induire une anomalie sévère du développement du prosencéphale liée à une diminution de SHH.

Nos travaux nous ont donc permis de générer un modèle animal avec une microforme d'HPE correspondant à une dysmorphie hypophysaire. Nos individus doubles hétérozygotes ne semblaient pas présenter de trouble de la fertilité ou de croissance, mais il serait intéressant d'étudier les fonctions de leur axe hypothalamo-hypophysaire via des prises de température, des dosages d'hormones, des études du nyctémère et de la prise alimentaire, afin de tester une potentielle dérégulation de l'hypophyse.

La malformation hypophysaire explique les troubles endocriniens souvent observés (diabète, troubles thyroïdiens, hypernatrémies, ...) chez les patients HPE (*Levey et al., 2010 ; Vergier et al., 2019*). Néanmoins, le diagnostic d'HPE est rarement évoqué chez un enfant présentant des troubles de l'axe hypothalamo-hypophysaire, associé ou non à une déficience intellectuelle. Une analyse des gènes de l'HPE devrait être effectuée chez ces patients, avec une attention particulière portée aux gènes de la voie NOTCH s'ils n'ont pas de signes *a priori* typiques de l'HPE.

Dans notre démarche diagnostique, l'équipe a, depuis plusieurs années, impliqué la voie NOTCH dans la survenue de l'HPE via le gène *DLL1* : une analyse par puce à ADN (CGH-array) sur une cohorte de 111 patients a montré une délétion récurrente en 6qter chez 4 patients dont la plus petite région commune comportait le gène *DLL1* (*Dupé et al., 2011*). Depuis, plusieurs patients avec des mutations ponctuelles dans ce gène ont été identifiés. Ainsi, *DLL1* est retrouvé muté chez 1,2% des patients de notre cohorte locale et fait partie des 10 gènes les plus fréquemment impliqués dans l'HPE (*Dubourg et al., 2016*). Une équipe allemande a récemment recensé 15 cas internationaux (via la plateforme coopérative Gene Matcher) de patients présentant un variant délétère dans *DLL1* (*Fischer-Zirnsak et al., 2019*). Les variants étaient hérités ou *de novo*. Ces patients présentaient tous un trouble du

neurodéveloppement, sans toutefois décrire de cas typique d'HPE. Ceci montrerait qu'une mutation dans la voie NOTCH ne suffit pas, à elle seule, pour causer une HPE sévère.

La voie NOTCH ayant été impliquée en pathologie humaine seulement au travers de variants dans *DLL1*, il était jusqu'alors le seul gène de cette voie inclus dans les panels diagnostics (Dubourg et al., 2016 ; Roessler et al., 2018). L'accessibilité aux technologies d'exome et de génome étant facilitée, les pipelines d'analyse utilisés chez les patients atteints devraient désormais inclure tous les acteurs de cette voie de signalisation (Kim et al., 2019). Néanmoins, les variants dans les gènes des récepteurs NOTCH ou dans les ligands DLL et JAG sont souvent associés à des pathologies cardiaques (Garg et al. 2005), hépatiques (syndrome Alagille, McDaniel et al., 2006), squelettiques (Bulman et al., 2000), artérielles (Joutel et al., 2004) sans description de malformations du système nerveux. La voie NOTCH ayant de multiples rôles lors du développement embryonnaire, les variants induisant une HPE pourraient être différents de ceux impliqués dans les autres atteintes organiques.

L'HPE EST UNE PATHOLOGIE MULTIGENIQUE

La clé de voute du développement du cerveau antérieur est le maintien d'une concentration stable de SHH (Dessaud et al., 2007). La régulation de cette concentration doit se faire de manière très fine en fonction du stade de développement et du tissu embryonnaire (Figure 23). Cela implique que, potentiellement, tout variant retrouvé au niveau de gènes impliqués, de près ou de loin, dans le contrôle de l'activité de SHH peut contribuer à l'apparition d'un phénotype HPE. En effet, un dysfonctionnement dans l'un des acteurs intervenant dans cette régulation perturbera le signal SHH de manière plus ou moins accentuée, sur une période plus ou moins longue et dans un tissu spécifique (Geng et al., 2008 ; Zhao et al., 2012 ; Ellis et al., 2015). Ainsi, plus SHH sera diminué précocement et de façon ubiquitaire (par exemple le modèle souris *Shh*^{-/-}), plus le phénotype sera sévère (HPE lobaire avec cyclopie) (Chiang et al., 1996). A contrario, si SHH est diminué de façon plus tardive et plus modérément, dans un territoire précis, le phénotype HPE sera moins sévère et les atteintes plutôt isolées. Par exemple, nous n'observons jamais de phénotype sévère, comme une cyclopie, chez nos mutants conditionnels *Rbpj*^{+/-}; Rosa26-creER^{T2} traités au tamoxifène à E7.75; ce qui suggère que la perte de *Shh* que nous observons dans l'hypothalamus est trop tardive pour entraîner un phénotype HPE sévère. De même, une diminution partielle à la fois de l'activité NOTCH et de l'activité SHH (mutant *Rbpj*^{+/-}; *Shh*^{+/-}) n'entraîne qu'une anomalie localisée de l'hypophyse. Chez ces animaux, le signal SHH est donc assez diminuée pour entraîner une anomalie de la ligne médiane

mais pas suffisamment pour permettre une anomalie sévère de type HPE. Cependant, il serait essentiel de tester l'expression de marqueurs dorso-ventraux du cerveau antérieur, tels que *Pax6* et *Dlx2* pour le montrer.

Le phénotype observé chez les patients HPE peut donc être la résultante de l'accumulation de variants dans plusieurs gènes, ce qui constitue la définition du mode de transmission multigénique.

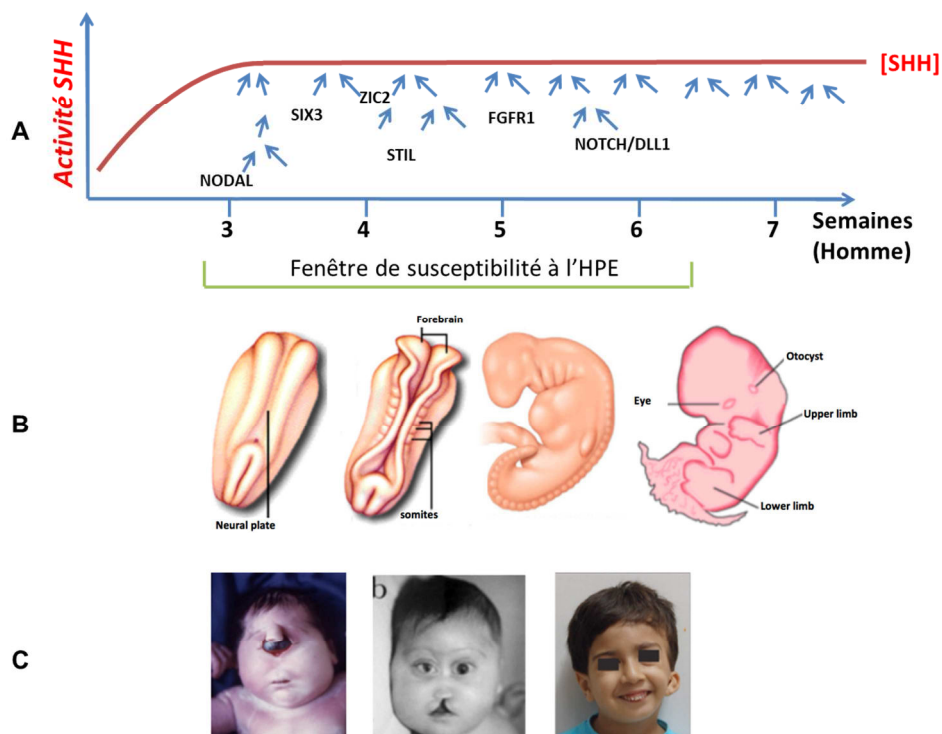


Figure 23 : Maintien de la concentration de SHH et apparition de l'holoprosencéphalie. (A) Représentation de la concentration de SHH au cours du développement de l'embryon humain et les voies de signalisation et facteurs de transcription impliqués dans son maintien. (B) Stades de développement de l'embryon humain sensibles à une diminution du signal SHH. (C) phénotypes HPE de sévérité décroissante, allant de la cyclopie avec proboscis à l'hypotélorisme avec fente labio-palatine puis à une incisive médiane unique. L'accumulation de variants hypomorphes dans les gènes impliqués dans la voie SHH ou dans sa régulation provoquera une HPE ou des anomalies neurodéveloppementales, dont le phénotype sera d'autant plus sévère que la concentration sera diminuée tôt et/ou de façon importante.

Dans notre cohorte, les mutations ponctuelles dans *DLL1* sont souvent héritées et retrouvées isolées ou associées à des mutations dans d'autres gènes. Il a été décrit par exemple que la mutation c.1802_1804del/p.Asp601_Ile602delinsVal dans *DLL1* est associée à un VSI (variant de signification inconnue) dans *FGF8* (c.617G>A/p.Arg206Gln) chez une patiente de 3 ans présentant une incisive médiane unique, un hypotélorisme et une sténose de l'orifice piriforme (*Dubourg et al., 2016*). Elle avait hérité le variant *DLL1* de son père asymptotique et le variant *FGF8* de sa mère qui ne présentait qu'un hypotélorisme. Cette situation est caractéristique d'une pathologie multigénique, avec des variants hypomorphes présents chez chacun des parents sains ou présentant une forme moins sévère que l'enfant. Notre hypothèse est que les effets des variants se cumulent pour impacter la concentration de SHH ; suffisamment pour engendrer une anomalie de la ligne médian chez l'enfant.

Les analyses sur les patients et leurs familles ont grandement évolué ces dernières années. Elles sont passées du séquençage en Sanger de 4 gènes majeurs (*SHH, ZIC2, SIX3, TGIF*) au séquençage de l'exome entier via la technologie haut débit. Ces nouvelles approches nous permettent d'identifier de nombreux variants qui sont, grâce à des pipelines bio-informatiques spécifiques, triés et priorisés. Néanmoins, étant donné la variabilité phénotypique et la complexité des mécanismes physiopathologiques mis en jeu, il est souvent difficile d'affirmer avec certitude qu'un variant est délétère ou bénin.

Par conséquent, nous avons besoin de réaliser des études fonctionnelles pour valider l'impact de ces variants. Nous avons par exemple utilisé le modèle cellulaire pour tester le potentiel effet délétère de nouveaux variants (e.g. gène *STIL*, *Mouden et al., 2015*). Cependant, ces expériences dépendent de la fonction du gène incriminé et nécessitent de longues mises au point à chaque nouveau gène candidat. Par ailleurs, grâce aux données de séquençage produites aujourd'hui, nous sommes passés d'une stratégie d'implication de gène à une stratégie d'implication de variant. En effet, l'impact des variants peut être bénin ou délétère, selon un mode perte de fonction, gain de fonction ou dominant négatif. Ainsi, pour le gène *FGFR1*, il a été montré que des variants hétérozygotes type perte de fonction induisaient une HPE, alors que des variants hétérozygotes à effet dominant négatif (dont l'action va donc bloquer l'activité de la protéine issue de l'allèle sauvage restant) entraînaient un syndrome de Hartsfield, avec un phénotype plus sévère associant HPE et atteinte des membres (*Dubourg et al., 2016 ; Hong et al., 2016 ; Hong et al., 2018*).

Nous utilisons déjà des modèles cellulaires pour tester les variants d'épissage (technologie « Minigen ») et les variants synonymes (*Kim et al., en soumission*). Les variants faux-sens - les plus fréquents - sont toutefois plus difficiles à étudier puisque leurs impacts dépendent du gène et de sa

fonction. Par ailleurs, le modèle cellulaire ne nous permet pas d'étudier les conséquences de l'accumulation de plusieurs variants dans différents gènes.

Pour continuer à progresser sur la physiopathologie de cette maladie précoce du développement, il faudrait pouvoir accéder aux tissus incriminés aux stades où se met en place l'anomalie afin de réaliser des approches transcriptomiques. Cependant, comme l'anomalie se met en place très tôt au cours du développement (*Dubourg et al., 2007*), il n'est pas possible d'avoir accès à ces tissus.

Aujourd'hui, il existe une nouvelle technologie très innovante qui permet de réaliser des cultures 3D de cellules à partir de cellules iPS (Induced Pluripotent Stem cells, ou cellules souches pluripotente induites ; *Scudellari, 2016*). Cette technologie permet de produire des organoïdes cérébraux qui peuvent récapituler en partie les premiers stades de développement du cerveau (*Lee et al., 2017*). Grâce à notre cohorte, nous pouvons produire des cellules iPS à partir de patients HPE pour générer des organoïdes cérébraux. Une telle approche permettra à l'avenir de disposer de tissus possédant une signature ARN similaire à un cerveau embryonnaire. Cet outil couplé à des approches d'édition de gène (Crisper/Cas9) permettrait d'étudier de façon plus systématique l'impact des variants identifiés chez les patients sur le développement cérébral précoce. Et, ainsi, de non seulement étudier l'effet délétère potentiel d'un variant mais également sa fonction physiopathologique.

En conclusion, nos travaux, renforcent les études menées par d'autres équipes (*Gupta & Sen, 2016 ; Xie & Dorsky, 2017*) sur les voies de signalisation impliquées dans le développement précoce du cerveau antérieur, et tendent à soutenir la même hypothèse : l'holoprosencéphalie est une pathologie complexe multigénique, causée par une modification de la concentration de SHH au cours du développement du le cerveau antérieur et craniofacial (*Roessler et al., 2009a ; Kim et al., 2019*). La variabilité phénotypique dépend du moment auquel a lieu cette modification de concentration et de son importance. Cette complexité couplée au nombre important de patients sans diagnostic moléculaire laisse à penser qu'il existe probablement de nombreux autres gènes et variants impliqués dans la pathologie. La régulation de la concentration de SHH devant être très précises, cela suppose que les variants impliqués peuvent avoir un impact faible (variants hypomorphes) et que c'est leur accumulation qui induit un phénotype. Cette hypothèse modifie les pratiques en matière d'analyse diagnostic et donc le conseil génétique pour les patients et leurs familles. Cependant, le diagnostic moléculaire est plus difficile à établir avec certitude et le risque de récurrence (et les décisions prénatales en découlant) est à évaluer avec prudence.

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ANNEXES

ANNEXES

Annexe 1 : Poster présenté au congrès de la British Society for Developmental Biology (BSDB), du 10 au 13 Avril 2016, à Warwick (Royaume-Uni).



Notch maintains Shh expression during the patterning of the ventral forebrain

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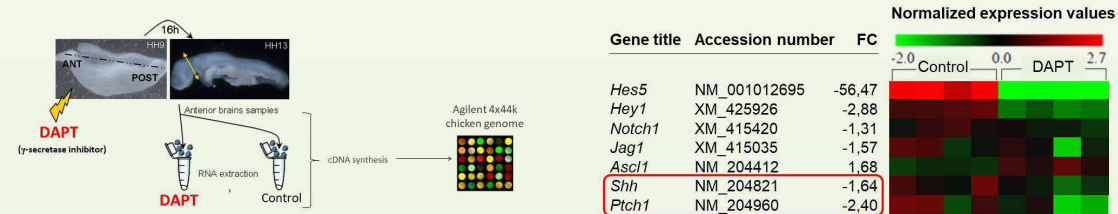
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Introduction

Holoprosencephaly (HPE) is a malformation of the brain characterized by impaired forebrain cleavage in which Sonic Hedgehog (Shh) mutations account for the majority of the human cases. Recently a potential role of the Notch pathway in the onset of this disease has been shown (Dupé et al., 2011). Whereas a connection between the Notch and Shh pathways has been shown recently in the spinal cord, nothing is known about the potential crosstalk between these pathways during the establishment of the ventral forebrain. Our transcriptome analysis of embryos treated with DAPT showed a decrease of *Shh* expression in the developing forebrain. Further studies have shown that the control of *Shh* expression by Notch takes place between HH7 and HH10. Finally, inhibition of the Shh and Notch pathways act synergistically to promote severe HPE-like phenotypes.

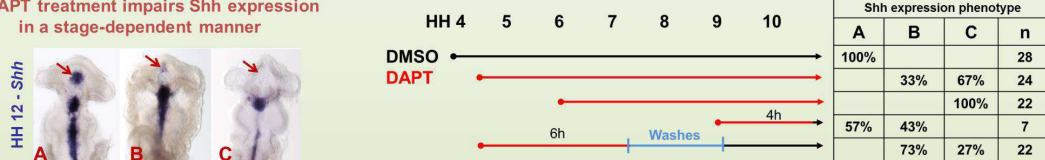
Dupé V, Rochard L, Mercier S, et al., NOTCH, a new signaling pathway implicated in holoprosencephaly. *Hum Mol Genet.* 2011.

Transcriptome Analysis of the anterior brain after Notch inhibition



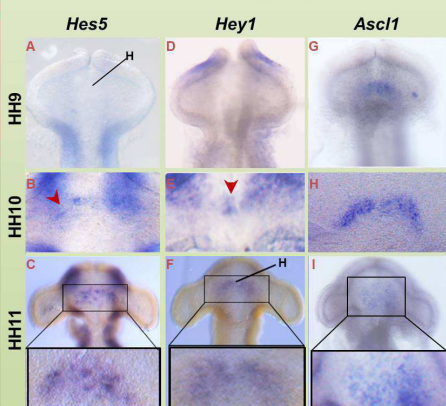
Notch inhibition was confirmed by a downregulation of Notch effectors (*Hes5*, *Hey1*, *Notch1* and *Jag1*) and upregulation of the proneural gene *Ascl1*. We obtained a significant downregulation of *Shh* and *Ptch1* expression (red box), suggesting that the Notch pathway could regulate *Shh* during early forebrain development.

DAPT treatment impairs Shh expression in a stage-dependent manner



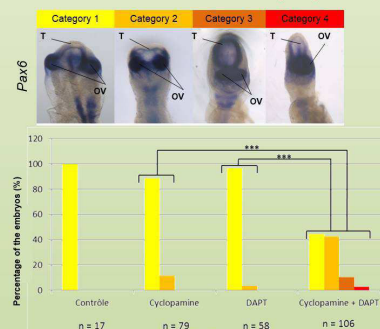
In control embryos, *Shh* is expressed in the rostral forebrain (A, red arrow). In DAPT-treated embryos from HH4 or HH6, *Shh* expression was decreased (B) or disappears (C) specifically in this region. However, DAPT treatment from HH9 impacts *Shh* signaling only in about half the embryos. Interestingly, early DAPT treatment followed by washes between HH7 and HH9 allows a rescue of the *Shh* expression. These results converge toward a regulation of *Shh* by the Notch pathway in the ventral forebrain between HH7 and HH10.

Notch/proneural gene expression in chick embryos during early development



To establish the timing of Notch pathway, *in situ* hybridizations were performed with two of its effectors (*Hes5* and *Hey1*) and one target gene (*Ascl1*). *Hes5* and *Hey1* seem to appear in the prymordium hypothalamus (H) between HH9 and HH10 (fig. B and E, arrowheads). *Ascl1* is present at HH9 (fig. G). These results indicate that the activation of Notch pathway in the early stages of forebrain development may take place around HH10.

A combined inhibition of Notch and Shh leads to cyclopia



Pax6 mRNA was used as a marker for optic vesicles (OV). Cultured embryos treated with DAPT from HH4 or subthreshold concentrations of cyclopamine (*Shh* inhibitor) present two well separated optic vesicles in category 1 and category 2. Interestingly, a combined inhibition of both pathways produced a significant increase in the number of embryos with major hypotelorism (category 2 and 3) or a complete cyclopia, the main features of the HPE spectrum.

Conclusion: We demonstrate a functional relationship between Notch and Shh pathways during early development of the ventral forebrain. These data suggest that Notch does not promote *Shh* expression in the ventral brain but rather maintains its expression between embryonic stages HH7 and HH9. Furthermore, the double inhibition of these two signalling pathways can lead to a HPE-like phenotype. These findings support the hypothesis that genetic heterogeneity plays a key role in HPE etiology and may contribute to its phenotypic variability.

Annexe 2 : Poster présenté aux Assises de Génétique Humaine, du 24 au 26 Janvier 2018, à Nantes (France).



La régulation de *SHH* par la voie NOTCH pour expliquer l'hétérogénéité phénotypique de l'holoprosencéphalie.



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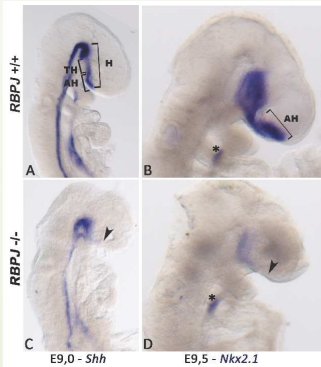


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Introduction

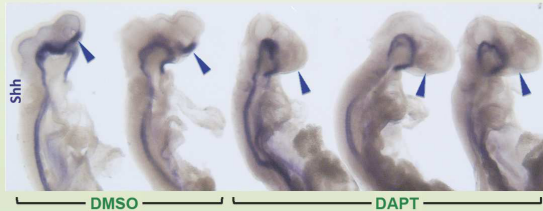
L'holoprosencéphalie (HPE) est une anomalie cérébrale accompagnée d'anomalies faciales. Elle est causée par un défaut de développement de la ligne médiane au niveau du prosencéphale et se caractérise par un large spectre phénotypique en fonction du degré de séparation des hémisphères cérébraux (formes lobaire, semilobaire, alobaire ou microforme). Cette hétérogénéité phénotypique s'accompagne d'une hétérogénéité génétique. A ce jour, 16 gènes ont été impliqués dans l'apparition de l'HPE. Ces gènes sont tous engagés dans la régulation de l'activité de Sonic Hedgehog (SHH) au cours du développement précoce du cerveau antérieur ventral. Les études menées sur les modèles animaux montrent que la perturbation temporelle de la signalisation SHH est en corrélation avec la gravité des phénotypes holoprosencéphaliques : plus l'altération est forte et se produit tôt au cours du développement céphalique, plus le phénotype est sévère. La voie de signalisation NOTCH a été associée à l'HPE, mais les mécanismes moléculaires impliquant cette voie au cours du développement précoce du cerveau restent inconnus. Notre étude a été menée sur des modèles animaux inhibés pour la voie NOTCH : des embryons de souris knock-out (KO) et des embryons de poulet traités au DAPT (un inhibiteur chimique de la voie NOTCH).

La suppression de la voie NOTCH induit l'inhibition de la signalisation SHH dans l'hypothalamus antérieur



L'expression de *Shh* et de sa cible *Nkx2.1* ont été analysées par hybridation *in situ* chez des embryons de souris KO pour un effecteur de la voie NOTCH, *Rbpj*. Chez les embryons contrôles (A,B), *Shh* et *Nkx2.1* sont détectés le long de l'axe antéro-postérieur de l'hypothalamus (H). Chez les mutants *Rbpj*^{-/-}, aucune expression n'est détectable dans la région antérieure de l'hypothalamus (AH) (flèches : C, D).

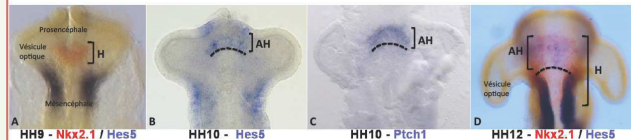
Chez l'embryon de poulet, l'inhibition de la voie NOTCH à partir du stade HH9 induit une disparition spécifique de *shh* dans l'hypothalamus antérieur (AH).



Inhibition de la voie NOTCH à l'aide d'un inhibiteur chimique (DAPT) sur des embryons de poulet mise en culture à partir du stade HH9 et Hybridation *in situ* du gène *Shh* au stade HH12. *Shh* est spécifiquement absent au niveau de l'AH (flèches bleues).

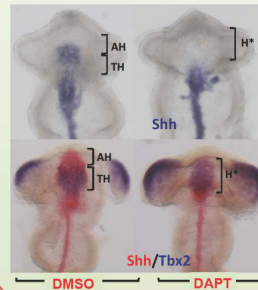
La voie NOTCH est impliquée dans le maintien de l'expression de *Shh* tardivement au cours du développement du cerveau antérieur.

La voie SHH est active dans l'hypothalamus avant l'apparition de la voie NOTCH



Chez l'embryon de poulet, SHH active son gène cible *Nkx2.1* au niveau de l'ébauche de l'hypothalamus (H) dès le stade HH8 (en rouge au stade HH9 fig. A). Le gène cible de la voie NOTCH, *Hes5*, ne commence à s'exprimer dans la région antérieure de l'hypothalamus (AH) qu'à partir du stade HH10 (B). *Ptch1*, le récepteur de la voie SHH, est également un marqueur spécifique de l'hypothalamus antérieur (C). A HH12, *Nkx2.1* et *Hes5* sont coexprimés dans la région antérieure de l'hypothalamus (D).

Le contrôle de SHH par la voie NOTCH fait intervenir TBX2

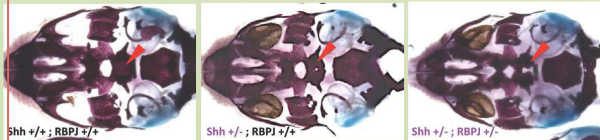


Au cours du développement normal du cerveau antérieur, TBX2 inhibe l'expression de *Shh* au niveau de l'hypothalamus tubéral (TH). Chez les embryons traités au DAPT, la perte d'expression de *Shh* dans l'hypothalamus antérieur (AH) est corrélée avec une expression ectopique de *Tbx2* dans cette région. Notre étude montre qu'en l'absence de la voie NOTCH, *Tbx2* n'est plus réprimé dans l'hypothalamus antérieur et va réprimer alors réprimer *Shh*.

Notch → Tbx2 → Shh

Conclusion : La voie NOTCH régule l'expression de *Shh*, le gène majeur de l'HPE. Cette interaction se fait via l'activité inhibitrice du facteur de transcription TBX2. La régulation de *Shh* par NOTCH intervient tardivement au cours du développement du cerveau antérieur. Un défaut de la signalisation de NOTCH seul ne peut donc pas conduire à un phénotype HPE sévère. Ces résultats montrent que l'hétérogénéité génétique contribue à la variabilité phénotypique de la pathologie.

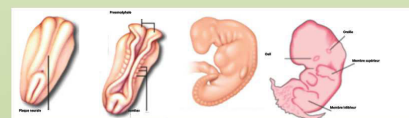
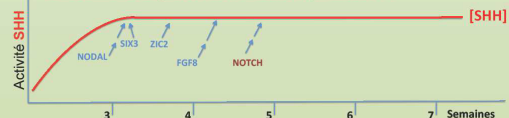
Forme mineure d'holoprosencéphalie chez les mutants *Shh*^{+/-} ; *Rbpj*^{+/-}



Préparation squelettique de fœtus de E18,5, issus du croisement entre des parents *Shh*^{+/-} et *Rbpj*^{+/-}.

Nous avons produit des doubles mutants hétérozygotes *Shh*^{+/-} ; *Rbpj*^{+/-} et observé chez 100% des animaux (n=6) une anomalie du basisphénoïde plus importante que chez les mutants *Shh*^{+/-} (n=5). Cette anomalie traduit la présence d'une malformation de l'hypothalamus.

Le maintien de la concentration de SHH est cruciale pendant une longue période de développement du cerveau ; ce qui nécessite le recrutement de plusieurs voies de signalisation :



Période de susceptibilité HPE

Titre : Etude du rôle de la voie NOTCH dans la régulation de *Shh* au cours du développement du cerveau antérieur chez les vertébrés

Mots clés : *Shh*, *Notch*, holoprosencéphalie, hypothalamus, neurone, multigénisme

Résumé : L'holoprosencéphalie (HPE) est une pathologie du développement définie par un défaut de clivage du cerveau antérieur le long de la ligne médiane. Elle est caractérisée par une hétérogénéité phénotypique et une pénétrance incomplète.

Le morphogène SHH est la principale molécule impliquée dans l'apparition de l'HPE. La voie NOTCH a été également impliquée dans l'apparition d'HPE chez les patients. Dans cette étude, j'ai montré que c'est en maintenant l'expression de *Shh* dans le cerveau ventral que NOTCH contribue à l'apparition de l'HPE. J'ai également montré que la voie NOTCH était impliquée dans la neurogenèse précoce au niveau de l'hypothalamus.

Enfin, des mutants souris hypomorphes pour la voie SHH et la voie NOTCH présentent des anomalies discrètes de la ligne médiane correspondant à une dysplasie de la glande hypophysaire et une malformation de l'os basisphénoïde. Les mêmes anomalies sont observées chez les patients HPE. Ce travail nous a donc permis de générer un modèle animal reproduisant une forme d'HPE qui repose sur la double inactivation partielle de deux voies de signalisation moléculaire. Ce résultat renforce l'hypothèse d'une transmission multigénique pour cette pathologie complexe du développement de la ligne médiane.

Title: Study of the role of NOTCH pathway in *Shh* regulation during vertebrate brain development

Keywords: *Shh*, *Notch*, holoprosencephaly, hypothalamus, neuron, multigenism

Abstract: Holoprosencephaly (HPE) is a developmental pathology defined by a lack of forebrain cleavage along the midline and characterized by phenotypic heterogeneity and incomplete penetrance.

The morphogen SHH is the main molecule involved in the onset of HPE. The NOTCH pathway has also been implicated in the occurrence of HPE in patients. In this study, I showed that NOTCH pathway activation was necessary to maintain *Shh* expression in the ventral forebrain. Furthermore, I demonstrated the implication of NOTCH pathway in early neurogenesis in the hypothalamus.

Finally, a hypomorphic mouse model for both SHH and NOTCH pathways has shown the appearance of pituitary gland dysplasia associated with basisphenoid bone malformations reminiscent of abnormalities observed in HPE patients. This work has allowed us to generate an animal model reproducing an HPE phenotype based on the partial inactivation of two molecular signaling pathways. This result reinforces the hypothesis of a multigenic transmission for this complex pathology that affects midline development.