UNIVERSIDADE DE LISBOA

Faculdade de Medicina



The role of multiple DSL ligands

- Dll1, Dll4 and Jag1 –

in vertebrate neurogenesis

Susana F. Rocha

Degree of Doctor of Philosophy in Biomedical Sciences

Specialization in Basic Neurosciences

Lisboa 2009

UNIVERSIDADE DE LISBOA

Faculdade de Medicina



The role of multiple DSL ligands

- Dll1, Dll4 and Jag1 –

in vertebrate neurogenesis

Susana F. Rocha

Degree of Doctor of Philosophy in Biomedical Sciences

Specialization in Basic Neurosciences

Supervisor / *Orientador* - Professor Domingos Henrique

Lisboa 2009

A impressão desta dissertação foi aprovada pela Comissão Coordenadora do Conselho Científico da Faculdade de Medicina de Lisboa em reunião de 22 de Setembro de 2009.

As opiniões expressas são da exclusiva responsabilidade do seu autor.

The current thesis presents data during my PhD research project, developed at Instituto de Medicina Molecular in the period of October 2004 to September 2008, under the supervision of Professor Doutor Domingos Henrique (Faculdade de Medicina, Universidade de Lisboa). Part of this project was developed in collaboration with Doctor Nicoletta Kessaris (Wolfson Institute for Biomedical Research, UCL), who received me in her laboratory from June 2007 till June 2008. The research described in the present thesis was supported by a fellowship from the Fundação para a Ciência e Tecnologia, BD/18745/2004 – POCI 2010.

The data presented in this dissertation was purely the result of work carried out by me and it is clearly acknowledged in the text whenever data or reagents produced by others were utilized. The opinions expressed in this publication are the exclusive responsibility of the author and this work has not been submitted for any degree at this or any other university.

The scientific board of the Medical School of the University of Lisbon approved the printing of this thesis on the 22nd of September 2009.

The results presented in this Thesis are published in the following article included in the appendix:

Rocha, SF. *et al* (2009). *Dll1* and *Dll4* function sequentially in the retina and pV2 domain of the spinal cord to regulate neurogenesis and create cell diversity. *Developmental Biology* **328**, 54-65

Ciência.Inovação 2010
Programa Operacional Ciência e Inovação 2010
MINISTÉRIO DA CIÊNCIA, TECNOLOGIA E ENSINO SUPERIOR



“I have tried not to falter; I have made missteps along the way. But I have discovered the secret that after climbing a great hill, one only finds that there are many more hills to climb. I have taken a moment here to rest, to steal a view of the glorious vista that surrounds me, to look back on the distance I have come. But I can rest only for a moment, for with freedom come responsibilities, and I dare not linger, for my long walk is not yet ended.”

Nelson Mandela *in* Long Walk to Freedom

TABLE OF CONTENTS

Acknowledgements		i
Summary		iii
Sumário		v
Abbreviations		ix
Index of Figures		xiii
Index of Tables		xv
Chapter I: General Introduction		1
I.1	THE NOTCH PATHWAY	4
I.1.1	The discovery of *Notch* and the concept of lateral inhibition	4
I.1.1.1	Lateral inhibition, equivalence groups and Notch	5
I.1.1.2	Notch and the neurogenic genes	9
I.1.2	The core components of the Notch pathway	10
I.1.2.1	The DSL family of Notch ligands	11
I.1.2.2.	The Notch family of receptors	14
I.1.2.3	Activation of the Notch pathway	15
I.1.2.4	The CSL transcription factor	17
I.1.2.5	Hes proteins, the downstream targets of Notch	18
I.1.3	Regulation of development by Notch signalling	19
I.1.3.1	Lateral inhibition versus Lateral induction	19
I.1.3.2	Modulation of the Notch pathway	24
I.2	NOTCH AND VERTEBRATE NEUROGENESIS	30
I.2.1	Vertebrate neurogenesis	30
I.2.2	Functions of Notch during CNS development	33
I.2.2.1	Neural progenitor maintenance	33
I.2.2.2	Cell fate decisions	34
I.2.2.3	Other less explored functions: cell migration, neurite outgrowth and cell survival	36
I.3	FUNCTION(S) OF THE DSL LIGANDS	38
I.3.1	DSL ligands in the nervous system	38
I.3.1.1	*Delta-like 1*	38
I.3.1.2	*Delta-like 3*	39
I.3.1.3	*Delta-like 4*	40
I.3.1.4	*Jag1*	40
I.3.1.5	*Jag2*	41
I.3.2	Multilpe DSL ligands for a single cause	42
I.3.2.1	The inner ear	42

I.3.2.2 The skin epidermis 44
I.3.2.3 T-cell hematopoietic lineage 45
I.4 NOTCH AND DISEASE 47
I.5 AIMS AND SCOPES OF THIS THESIS 49

Chapter II: Establishment of the RNAi gene silencing technique in chick embryos 51
II.1 INTRODUCTION 53
II.2 CHOOSING A STRATEGY TO ESTABLISH RNAI IN THE CHICK EMBRYO 54
II.2.1 The discovery of RNAi and its molecular mechanism 54
II.2.1.1 The RNA interference mechanism 55
II.2.1.2 miRNA processing 56
II.2.2 Technical advances and limitations in gene silencing by RNAi 57
II.2.2.1 What to use? Long dsRNA, chemically synthesized siRNAs or vector-based expression of siRNAs/shRNAs? 57
II.2.2.2 siRNA- versus shRNA-mediated gene silencing 59
II.2.2.3 Influence of the backbone structure of shRNAs on gene silencing efficiency 60
II.2.2.4 Promoters driving expression of shRNAs 63
II.2.2.5 Criteria for selecting the target sequence for the shRNAs 65
II.3 DESIGNING SHRNAS FOR THE NOTCH PATHWAY 65
II.4 LOSS-OF-FUNCTION OF THE NOTCH PATHWAY BY RNAI 69
II.4.1 Neurogenesis is not affected by *cNotch1* targeting shRNAs 69
II.4.2 cU6 promoter shows high levels of expression 71
II.4.3 RNAi-mediated gene silencing in the chick embryo is not reproducible 72
II.5 DISCUSSION 74

Chapter III: *Dll1* and *Dll4* function sequentially, in the retina and pV2 domain of the spinal cord, to regulate the rate of neurogenesis and create neural cell diversity 77
III.1 INTRODUCTION 79
III.2 THE DLL1 AND DLL4 LIGANDS IN THE DEVELOPING MOUSE RETINA 81
III.2.1 *Dll1* and *Dll4* are expressed sequentially in the developing mouse retina 81
III.2.2. *Dll1*- and *Dll4*-expressing cells contribute to various cell fates in the retina 85
III.2.3 *Dll1* loss-of-function in the retina leads to a mild neurogenic phenotype 86
III.2.4 Disruption of retinal morphology in $Dll1^{f/f}$,$Chx10^{Cre/+}$ mutants 90
III.3 THE *DLL1* AND *DLL4* LIGANDS IN THE DEVELOPING MOUSE NEURAL TUBE 94
III.3.1 *Dll1* and *Dll4* are expressed sequentially in the pV2 domain of the mouse neural tube 94
III.3.2 *Dll1* as the sole Notch ligand regulating neurogenesis 97
III.3.2.1 *Dll1* is the main responsible for progenitor maintenance in the spinal cord 97

III.3.2.2 Dll1 does not affect neuronal type specification 100
III.3.3 The *Dll1-Dll4* circuitry regulating neurogenesis in the pV2 domain 109
III.3.3.1 Both *Dll1* and *Dll4* contribute to progenitor maintenance in the neuroepithelium 109
III.3.3.2 *Dll4* functions to generate neuronal diversity in the pV2 domain 110
III.4 DLL4 SIGNALS TO CELLS ALREADY COMMITTED TO DIFFERENTIATION 111
III.5 DISCUSSION 114
III.5.1 The sequential expression of *Dll1* and *Dll4* in the developing retina and pV2 domain of the neural tube 116
III.5.2 A single Notch ligand is necessary and sufficient to regulate the rate of neurogenesis 117
III.5.3 Dll1 and the generation of cell diversity 118
III.5.3.1 Neuron-neuron specification 119
III.5.3.2 Neuron-glial specification 121
III.5.4 A unique circuitry involving two Dll ligand to regulate neurogenesis 122
III.5.5 Two ligands, two outcomes of Notch signalling: proliferation and diversisfication 123
III.5.5.1 Regulation of the rate of neurogenesis: maintenance of a pool of progenitors 124
III.5.5.2 Cell fate diversification 124
III.5.6 Dll1 and Dll4 mediate temporally overlapping yet distinct functions 126
III.5.6.1 *Dll1*- and *Dll4*-expressing cells have different signalling abilities 126
III.5.6.2 Dll1/Dll4 and progenitor maintenance in physiological conditions 127
III.5.7 Possible mechanisms of Dll4-mediated cell fate diversification 128
III.5.7.1 Clues from the pV2 domain of the neural tube 129
III.5.7.2 The Dll1/Dll4 circuitry in the developing retina 132

Chapter IV: The role of *Jag1* in regulating neurogenesis in the embryonic neural tube and forebrain 135

IV.1 INTRODUCTION 137
IV.2 THE JAG1 LIGAND IN THE DEVELOPING MOUSE NEURAL TUBE 138
IV.2.1 *Jag1* is expressed in discrete domains, dI6 and pV1, of the neural tube 138
IV.2.2 Jag1 loss-of-function does not result in a neurogenic phenotype, in the developing neural tube 140
IV.2.3 Interneurons derived from the *Jag1*-expressing domains do not present migratory defects 143
IV.3 THE JAG1 LIGAND IN THE DEVELOPING MOUSE FOREBRAIN 144
IV.3.1 *Jag1* is expressed in discrete domains of the forebrain that overlap with *Dll1* expression 144
IV.3.2 Jag1 is able to activate Notch in the developing mouse forebrain 146
IV.3.3 Is Jag1 involved in Martinotti cell production? 150
IV.4 DISCUSSION 152
IV.4.1 Redundant functions of Jag1 and Dll1 in the neural tube 152

IV.4.2 Different Dll1- and Jag1-mediated Notch outputs 153

Chapter V: Concluding remarks 157

Chapter VI: Materials and Methods 163
VI.1 MOLECULAR BIOLOGY TECHNIQUES 165
VI.1.1 Preparation and transformation of competent *E.coli* bacteria 165
VI.1.1.1 Preparation and transformation of chemically competent *E.coli* bacteria 165
VI.1.1.2 Preparation and transformation of electrocompetent *E.coli* bacteria 166
VI.1.2 Plasmid DNA purification 167
VI.1.3 DNA and RNA quantification 167
VI.1.4 Restriction digestions and ligation reactions 167
VI.1.5 Analysis and isolation of DNA by agarose gel electrophoresis 168
VI.2 GENERATION OF SHRNA-EXPRESSING VECTORS 168
VI.2.1 Selection of genetargeting sequences by the nearest-neighbour model 168
VI.2.2 Target sequences selected and oligonucleotides used for cloning 170
VI.2.3 Cloning strategies 171
VI.2.3.1 DNA vectors used 171
VI.2.3.2 Generation of plasmids expressing shRNAs with 21nt stem 171
VI.2.3.3 shRNAs with 29nt stem and leader sequence 172
VI.3 CHICK EMBRYO MANIPULATION 173
VI.3.1 In ovo chick embryo electroporation 173
VI.3.2 Tissue embedding and preparation of cryostat sections 173
VI.4 MOUSE MANIPULATION 174
VI.4.1 Mouse breeding 174
VI.4.2 Dissection of embryos 175
VI.4.3 Perfusion of adult mice 175
VI.4.4 Genotyping 175
VI.4.5 Administration of BrdU 176
VI.4.6 Tissue embedding and preparation of cryostat sections 177
VI.5 IMMUNOHISTOCHEMISTRY 177
VI.5.1 Immunoflourescence on cryostat sections of embryonic tissue 177
VI.5.2 Immunofluorescence on cryostat section of adult tissue 178
VI.5.3 BrdU labelling 179
VI.5.4 Detection of β-Galactosidase by X-gal staining 180
VI.6 *IN SITU* HYBRIDIZATION 180
VI.6.1 Antisense RNA probe synthesis 180
VI.6.1.1 DNA template preparation 180
VI.6.1.2 Probe synthesis 181
VI.6.2 *In situ* hybridization on cryostat sections 182
VI.7 IMAGING 182

VI.8 CONTROLS AND STATISTICAL ANALYSIS 183
VI.9 CONSTRUCTION OF A DLL1-ICRE TARGETING VECTOR 183
VI.9.1 DNA plasmids 183
VI.9.2 PCR amplification of homology arms for homologous recombination 184

Chapter VII: References 187

Appendix 213

ACKNOWLEDGEMENTS

My first and greatest THANK YOU goes to my boss, Professor Domingos Henrique, whose motivation, knowledge, pleasure in learning and teaching has inspired me during these last years. Thank you for all that you have taught me, for widening my horizons and for always sharing your honest opinion (even when harsh). It was, and still is much appreciated. Thank you for accepting me in your lab and for being not only a demanding boss but a good friend. Sorry for the headaches and the occasional stubbornness.

I thank also my second PhD supervisor, Dr. Nicoletta Kessaris, for having me in her lab at the UCL during my last year. It was a very enriching experience, where I learnt a lot. Thank you for all the support.

A big thank you to Rita Fior, one of the senior PhD students in the lab when I started. Thanks for being a model and for all the scientific and personal discussions.

A very big thank you to all members of the UBD: Geni, Cristina, Gonçalo, Alina, Sara, Filipe, Lopes, Elsa and Claudia. Thanks for the lab environment, all the fun, *frases do dia* and coffee drinks. And of course, for the help in the lab and scientific input. Thank you also to all members of our lab co-inhabitants, the Morphogenesis Unit.

I would like to thank all my lab colleagues in London. Don't be jealous but I have two special thanks. First, to Joana who arrived in London at the same time as I did. Thanks for listening and for sharing many good (and less good) times with me. Second, to 'my favourite English guy', Mat Grist. Thank you for being so much fun in the lab and for singing to the sound of Mika, lol.

Thank you to Professor Ralf Adams for making it possible for me to write this thesis at the MPI, in Muenster.

A very big thank you to my family, specially my mother. Without you I would not be who I am today. Thank you for everything. Last, but definitely not least, thank you to my husband, Rui, for making me smile everyday and for all your love.

SUMMARY

Notch signalling is an evolutionarily conserved pathway in metazoans that relies on direct cell-to-cell communication to generate cell diversity, playing an essential role during formation of multicellular organisms. Notch signalling is mediated by interactions betweeen Notch receptors and DSL ligands that are localized at the membrane surface of neighbouring cells. This receptor-ligand interaction leads to various proteolytic events of the receptor that ultimately results in the cleavage of the intracellular domain of Notch (NICD). The NICD fragment then translocates to the nucleus, where it associates with the CSL transcription factor and activates transcription of downstream targets. Some of these encode transcriptional repressors that inhibit the expression of genes encoding DSL ligands. In this way, negative feedback loops are created that enhance the signal-sending character of one cell, while enhancing the signal-receiving character of the other cell. This molecular mechanism allows the amplification of small existing differences between two interacting cells with similiar developmental potential.

In the nervous system, Notch activity has been described to be essential for various processes, among which neurogenesis, cell fate, cell survival and migration. Notch signalling is able to mediate such a wide variety of functions within the same tissue, despite the pathway converging systematically into the activation of the CSL transcription factor. However, it has been demonstrated that the existence of multiple DSL ligands and Notch modulators may influence the output of Notch signalling and, in this way, exert different functions.

The aim of the work described in this thesis was to understand the function and contribution of various DSL ligands to the proper development of the vertebrate nervous system. For this, my studies focused on three genes that encode DSL ligands, namely *Dll1*, *Dll4* and *Jag1*. Within the developing vertebrate nervous system, these are found either in complementary or overlapping expression patterns, which *per se* is indicative of possibly different roles. However, many questions remain unanswered and require further study.

In this thesis, I describe in detail the expression pattern of *Dll1*, *Dll4* and *Jag1* in the developing spinal cord, retina and forebrain of mouse embryos. Although it was already known that *Dll1* and *Jag1* have a complementary expression pattern in the

neural tube, I show that the 'striped' expression of *Jag1* is restricted to the pV1 and dI6 domains. Through the analysis of conditional knockout mice for *Dll1* and *Jag1*, I provide the first evidence that *Jag1* is able to activate Notch signalling within these domains of the neural tube and, in this way, is sufficient to maintain a pool of neural progenitors. In contrast, in the developing forebrain where *Jag1* expression overlaps with that of *Dll1*, it is not able to activate Notch signalling or, at least, not sufficiently. Although it remains to be clarified why Jag1 presents different signalling abilies in these two tissues, its function remains elusive even in the neural tube, where I show that although sufficient, Jag1-mediated Notch signalling is not necessary for this process and that the expression of *Dll1* in the neighbouring domains is able to fully compensate for the absence of *Jag1*. In fact, my work demonstrates that a single DSL ligand, Dll1, is necessary and sufficient to fullfill the function of progenitor maintenance in all three neural tissues analysed. Consequently, Dll1-mediated Notch signalling allows the the generation of different cell fates when these arise over time, as is the case for neuron-glial cell production.

In specific regions of the developing vertebrate nervous system, for instance in the pV2 domain of the neural tube and retina, multiple cell types arise from the same progenitor population during the same time-window. In these regions, a genetic circuitry employs two distinct DSL ligands, Dll1 and Dll4, which are sequentially expressed in cells committing to neuronal differentiation. In addition, in these regions where both *Dll1* and *Dll4* are co-expressed, I show by double in situ hybridization that *Dll1*-expressing nascent neurons also exhibit *hes5* expression, i.e., Notch activation. Moreover, activation of the Notch pathway is never observed in *Dll4*-expressing that are also nascent neurons, given their lack of progenitor cell markers. Analysis of *Dll1* mutants revealed an expected neurogenic phenotype. However, and unlike mutants where Notch signalling is completely compromised, this premature differentiation is not accompanied by the loss cell fate diversity. Altogether, this work has led to the proposal of a model in which the sequential expression of *Dll1* and *Dll4* are part of a molecular mechanism that permits a given cell to undergo successive rounds of Notch signalling, which correlate with two cell fate decisions. Dll1-induced Notch signalling is required in an initial phase to mediate the progenitor-neuron decision. Once a cell has committed to neuronal differentiation, it again uses Notch signalling, mediated by Dll4, to prevent surrounding cells that are embarking on neuronal differentiation from adopting the same fate.

SUMÁRIO

A sinalização Notch é uma via de sinalização que é conservada no reino animal e que depende da comunição directa entre células para permitir a geração de diversidade celular. Desta forma, a via Notch é fundamental para o desenvolvimento de organismos multicelulares. A sinalização Notch é mediada por interacções estabelecidas entre receptores Notch e ligandos DSL, que se encontram na superfície membranar de células vizinhas. Esta interacção entre receptor-ligando desencadeia várias reacções proteolíticas do receptor, que resultam por fim na clivagem do domínio intracelular de Notch (NICD). Após a translocação do fragmento NICD para o núcleo, este associa-se com o factor de transcrição CSL e activa a expressão de genes alvos. Alguns destes genes alvos codificam para repressores transcricionais que inibem a expressão de ligandos DSL. Desta forma, é criado um mecanismo de retroacção negativa que amplifica o capacidade sinalizadora de uma célula, ao mesmo tempo que re-enforça o carácter receptor da célula vizinha. Este mecanismo molecular permite a amplificação de pequenas diferenças que possam existir entre duas células que interactuem e que têm potenciais de desenvolvimento semelhantes.

No sistema nervoso, a actividade Notch é essencial para vários processos, entre os quais a neurogénese, a especificação de tipos celulares, a viabilidade e a migração celular. Apesar desta via de sinalização convergir sistematicamente na activação do factor de transcrição CSL, a sinalização Notch é capaz de mediar variadas funções no mesmo tecido. Contudo, é sabido que a existência de múltiplos ligandos DSL e moduladores de Notch podem influenciar o resultado final da sinalização Notch e, desta forma, exercer diferentes funções.

O objectivo do trabalho descrito nesta tese era o de compreender a função e a contribuição de vários ligandos DSL para o desenvolvimento correcto do sistema nervoso em vertebrados. Para tal, os meus estudos focaram-se em três genes que codificam para ligandos DSL, nomeadamente *Dll1, Dll4* e *Jag1*. Estes genes apresentam, no sistema nervoso embrionário, padrões de expressão que tanto podem ser complementares, como podem ocorrer de uma forma coincidente. Esta diversidade nos padrões de expressão dos vários ligands, sugere por si só, a possibilidade de exercerem diferentes funções. No entanto, muitas questões permanecem por ser respondidas e requerem mais estudo.

Nesta tese, descrevo em detalhe o padrão de expressão de *Dll1, Dll4* e *Jag1* no tubo neural, retina e telencéfalo. Embora já fosse sabido que *Dll1* e *Jag1* são expressos de forma complementar no tubo neural, eu mostro que as 'riscas' de expressão de *Jag1* correspondem aos domínios restritos de pV1 e dl6. Através da análise de ratinhos com perda de função condicional para *Dll1* e *Jag1*, pude mostrar pela primeira vez que Jag1 é capaz de activar a sinalização Notch nestes mesmos domínios neurais, e que, desta forma, é suficiente para a manutenção de uma populaçao de progenitores. No telencéfalo onde a expressão de *Jag1* se sobrepõe com a de *Dll1*, *Jag1* não é capaz de activar a via Notch ou pelo menos, não de forma suficiente. Embora permaneça por esclarecer porque é que Jag1 apresenta diferentes capacidades de activação da via Notch no tubo neural e no telencéfalo, a sua função continua por esclarecer até no tubo neural, onde eu mostro que apesar de ser suficiente para mediar inibição lateral, sinalização Notch mediadada por Jag1 não é necessária para este processo. A expressão de *Dll1* nos domínios adjacentes é capaz de compensar completamente pela perda de função de *Jag1*. Aliás, eu mostro que um único ligando DSL, *Dll1*, é necesário e suficiente para levar a cabo a função de manutenção de progenitores nos três tecidos neuronais analisados. Consequentemente, sinalização Notch mediada por Dll1 permite a geração de diferentes tipos celulares quando estes são criados ao longo do tempo, tal como é o caso da produção de neurónios e de glia.

Em regiões específicas do sistema nervoso de vertebrados, como por exemplo, no domínio pV2 do tubo neural e na retina, vários tipos celulares ao produzidos simultaneamente. Nestas regiões, um circuito genético faz uso de dois ligandos DSL, Dll1 e Dll4, que são expressos de forma sequencial em células que se começam a diferenciar em neurónios. Em adição, eu mostro que nestas regiões onde Dll1 e Dll4 são co-expressos, é possível observarmos a expressão de *hes5*, isto é, a activação de Notch em neurónios nascentes que expressam *Dll1*. Curiosamente, esta activação de Notch não é observada em células que expressam *Dll4*, apesar destas também serem neurónios nascentes uma vez que não apresentam marcadores de progenitores. A análise de mutantes para *Dll1* revelou um fenótipo neurogénico esperado. No entanto, e em contraste com mutantes em que a sinalização Notch se encontra completamente comprometida, a diferenciação neuronal prematura observada não se fez acompanhar por uma perda na diversidade de tipos celulares. Este trabalho levou à proposta de um modelo em que a expressão sequencial de *Dll1 e Dll4* cria um mecanismo molecular que permite uma dada célula receber sucessivos sinais Notch, que correlacionam com duas

decisões de destino celular. Sinalização Notch mediada por Dll1 é necessária numa primeira fase para mediar a decisão progenitor versus neurónio. Quando uma célula inicia a sua diferenciação neuronal, recorre mais uma vez à sinalização Notch, desta vez mediada por Dll4, para impedir células adjacentes, e que também se encontram em diferenciação, de adoptarem a mesma identidade.

ABBREVIATIONS

β-Gal	β-Galactosidase
AC	Anchor cell
AD	Alzheimer's disease
Amp	Ampicillin
ANK	Ankyrin repeats
AP	Alkaline phosphatase
APP	β-amyloid precursor protein
AS-C	*Achaete-scute* complex
bHLH	Basic helix-loop-helix
BMPs	Bone Morphogenetic Proteins
bps	Base pairs
BrdU	Bromodeoxyuridine
C. elegans	*Caenorhabditis elegans*
CADASIL	Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy
CKO	Conditional knockout
CMZ	Ciliary margin zone
CNS	Central nervous system
CR	Cysteine-rich region
CR	Calretinin
CSL	Human CBf1, fly Suppressor of hairless, worm Lag1
DIG	Digoxigenin
Dlk-1	*Delta-like1*
Dll1	*Delta-like1*
Dll3	*Delta-like3*
Dll4	*Delta-like4*
dMGE	Dorsal medial ganglionic eminence
DNA	Deoxyribonucleic acid
DNER	Delta/Notch EGF-related receptor
Dpp	Decapentaplegic
DRG	Dorsal root ganglion
DSE	Distal sequence element
DSL	Delta, Serrate and Lag2
dsRNA	Double-stranded RNA
DV	Dorsoventral
Dx	deltex
E(spl)	*Enhancer-of-split*
E. coli	*Escherichia coli*
EGFRs	Epidermal Growth Factor-like Repeats

EGL	External germinal layer
EMS	Ethyl methanesulfonate
FCD	Focal cortical dysplasia
FLUO	Fluorescein
GFP	Green fluorescent protein
HA	Homolgy arm
HATs	Histone acetylases
HCs	Hair cells
HDAC1	Histone deacetylase 1
hes	*Hairy* and *enhancer of split*
HFs	Hair follicles
HH	Hamilton and Hamburger
hk	*hook*
hpe	Hours post-electrporation
hrs	Hours
IFE	Interfollicular epidermis
IGL	Internal granular layer
IMM	Institute for Molecular Medicine
INM	Interkinetic nuclear migration
INs	Interneurons
Jag1	*Jagged1*
Jag2	*Jagged2*
LFng	Lunatic Fringe
LGE	Lateral ganglionic eminence
LMC	Lateral motoneuron column
LNR	LIN-12/Notch-related region
MAM	Mastermind
MFng	Manic Fringe
MGE	Medial ganglionic eminence
Mib-1	Mindbomb-1
Mib-2	Mindbomb-2
min	Minutes
miRNA	Micro RNA
MMC	Medial motoneuron column
MNs	Motoneurons
mRNA	Messenger RNA
MZ	Mantle zone
NB	Neuroblast
Neur	Neuralized
NEXT	Notch Extracellular Truncation
NICD	Notch Intracellular Domain

NLS	Nuclear localizing signal
NSCs	Neural stem cells
nts	Nucleotides
o/n	overnight
OPCs	Oligodendrocyte precursor cells
PCR	Polymerase chain reaction
PDZ	PSD-95/Dlg/ZO-1
PEST	Proline, glutamate, serine and threonine rich region
PNS	Peripheral nervous system
POA	Pre-optic area
PSB	Pallial-subpallial boundary
PSE	Proximal sequence element
RAM	RBPJκ-Associated Molecule
RFng	Radical Fringe
RGC	Retinal ganglion cells
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RPE	Retinal pigmented epithelium
RTK	Receptor Tyrosine Kinase
SC	Spinal cord
SCs	Supporting cells
sec	Seconds
SGs	Sebaceous glands
Shh	Sonic Hedgehog
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
snRNA	Small nuclear RNA
SOP	Sensory organ precursor
Spdo	Sanpodo
Sst	Somatostatin
Su(dx)	Suppressor of deltex
Su(H)	Suppressor of hairless
SVZ	Subventricular zone
TM	Transmembrane domain
UTR	Untranslated region
vSMCs	Vascular smooth muscle cells
VU	Ventral uterine precursor cell
VZ	Ventricular zone
Wg	Wingless
WIBR	Wolfson Institute for Biomedical Research

YFP	Yellow fluorescent protein

INDEX OF FIGURES

Figure I.1	Wigglesworth's experiments on sensory bristle patterning in the *Rhodnius prolixus*	5
Figure I.2	Equivalence groups and lateral inhibition	7
Figure I.3	Conserved structure of the DSL family of Notch ligands between different species	12
Figure I.4	Conserved structure of the Notch family of receptor proteins between different species	15
Figure I.5	Activation of the Notch pathway and signal transduction	17
Figure I.6	Neural versus epidermal cell fate decision in *Drosophila*	21
Figure I.7	Lateral inhibition versus lateral induction	22
Figure I.8	Modulation of Notch signalling by Fringe in the *Drosophila* wing	23
Figure I.9	Models for the role of Notch ligand endocytosis	25
Figure I.10	Sensory organ specification in the *Drosophila* adult	28
Figure I.11	Regionalization of the neural tissue	32
Figure I.12	Lateral inhibition mediated through Notch-Delta interactions regulates neuronal differetiation	33
Figure I.13	Notch signalling and cell fate decisions in the vertebrate nervous system	35
Figure I.14	Stages of inner ear development and expression patterns of *Jag1, Jag2* and *Dll1*	43
Figure I.15	Expression of DSL ligands in the interfollicular epidermis and hair follicle	45
Figure I.16	Notch signalling in lymphogenesis	46
Figure II.1	The discovery and molecular mechanism of RNAi	56
Figure II.2	Strategies for construction of DNA vector-based expression of siRNAs or shRNAs	60
Figure II.3	Structural modifications in the backbone of miRNA precursors and their impact on gene silencing effectiveness	62
Figure II.4	Alignment of the nucleotide sequences of the mouse, human and chick U6 promoters	64
Figure II.5	Thermodynamic profiles, target sequences and hairpin structures of designed *cNotch1* shRNAs	68
Figure II.6	Effect of shRNAs, targeting the *cNotch1* gene, on the Notch pathway and neuronal differentiation	70
Figure II.7	Expression levels of cU6 transcripts are high and stable	72
Figure II.8	Thermodynamic profiles, target sequences and hairpin structures of designed *cPresenilin1/2* and *cSuH* shRNAs	73
Figure III.1	*Dll* ligands are expressed transiently and sequentially during neuronal differentiation in the retina	83
Figure III.2	Cells expressing *Dll1*- and *Dll4* give rise to various retinal cell types	86
Figure III.3	Neurogenesis in E11.5 *Dll1* mutant retinas	87
Figure III.4	Enhanced neurogenesis in the retinas of $Dll1^{f/f};Chx10^{Cre/+}$ mutants	89
Figure III.5	$Dll1^{f/f},Chx10^{Cre/+}$ retinas show disruption of the neuroepithelial organization, with the appearance of rosette-like structures	92
Figure III.6	Apical-basal organization preserved within the rosette-like structure of $Dll1^{f/f},Chx10^{Cre/+}$ retinas	93

Figure III.7	Maintenance of Notch activity in retinas of *Dll1* mutants	94
Figure III.8	Sequential expression of *Dll1* and *Dll4* in the pV2 domain of the mouse neural tube, at E11.5	96
Figure III.9	Neurogenic phenotype in the neural tube of *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos	97
Figure III.10	Maintenance of neural progenitors is restricted mainly to *Jag1*-expressing domains, in the neural tube of E11.5 *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos	98
Figure III.11	*Dll1* mutant embryos lose glial cell fates due to excessive neuronal differentiation	99
Figure III.12	Quantification of neuronal cell types produced in the neural tube of wild-type and *Dll1*-mutant embryos , at E11.5	100
Figure III.13	Recombination efficiency of the *Nestin-Cre* line in the pMN domain	101
Figure III.14	Neurogenesis in the pMN domain of *Dll1$^{LacZ/Ki}$* embryos	104
Figure III.15	Neurogenesis in the pV0 domain of the neural tube at E11.5	107
Figure III.16	Neurogenesis in the *Dll1*- and *Dll4*-expressing pV2 domain	110
Figure III.17	Activation of the Notch pathway in cells committed to neuronal differentiation, in *Dll4*-expressing regions of the CNS	113
Figure III.18	Cell diversity created in time: progression through distinct competence states	122
Figure III.19	Cell diversity created during the same time window	125
Figure III.20	Generation of cell diversity through Dll4-mediated lateral inhibition between equivalent and bipotent sibling cells	130
Figure IV.1	Restriction of the two Jag1 stripes in the neural tube to the dI6 and pV1 domains	139
Figure IV.2	Absence of a neurogenic phenotype in *Jag1$^{f/f}$,Nestin$^{Cre/+}$* E11.5 neural tubes	142
Figure IV.3	Migration of V1 interneurons derived from Jag1-expressing domain do nt present migratory defects upon *Jag1* inactivation	144
Figure IV.4	Expression of *Jag1* in the embryonic forebrain, at E11.5-E13.5	145
Figure IV.5	Both Dll1 and Jag1 are capable of activating Notch signalling, although to different extents	147
Figure IV.6	Altered forebrain morphology at the LGE/MGE boundary level in *Dll1$^{f/f}$,Nkx6.2^{iCre}* but not *Jag1$^{f/f}$,Nkx6.2^{iCre}* embryos	149
Figure IV.7	Production and final localization of Martinotti cells are unaffected in the absence of Jag1 function	151
Figure VI.1	Calculation of the thermodynamic stability of each base pair	169
Figure VI.2	pSouper vector map	171
Figure VI.3	Cloning of vectors expressing shRNAs with 21bp	172
Figure VI.4	PCR-based strategy for quick generation of plasmids expressing 29bp stem hairpins with a 27nt leader sequence	173
Figure VI.5	Cloning strategy of a targetingvector for the generation of a transgenic *Dll1^{iCre}* mouse line	185

INDEX OF TABLES

Table I.1	Neurogenci loci identified in a screen for embryonic lethal mutants	9
Table I.2	Core components of the Notch pathway	11
Table II.1	Effect of different shRNAs targeting distinct components of the Notch pathway	73
Table VI.1	Genes targeted for silencing by RNAi and oligonucleotides used to clone the respective shRNA-expressing vectors	170
Table VI.2	Primers used for genotyping	176
Table VI.3	Specific fixations and/or antigen retrieval treatments used for several antibodies	179
Table VI.4	Constructs used as templates for *in vivo* transcription reactions	181
Table VI.5	Oligonucleotides used for amplification of *Dll1* homology arms	184

CHAPTER I

GENERAL INTRODUCTION

I. GENERAL INTRODUCTION

The formation of a multicellular organism is a complex process that requires the spatio-temporal coordination of proliferation, establishment of different cell fates and the organisation of these cells into tissues and organs. This process has inspired and puzzled philosophers and biologists over many centuries. How can a single cell be able to generate an organism composed of multiple organs and specialized tissues that, in turn, exhibit cellular variety?

Elegant experiments in the field of experimental embryology, namely experiments performed by Hans Driesch, Thomas Morgan and Hans Spemann (Gilbert, 2006), have shown that cells from early developmental stages, such as blastomeres, have the potential to contribute to the formation of any organ or tissue, or even to generate a complete organism when isolated from the remaining cells of the blastula. However, as development proceeds, the potency of these cells becomes restricted to specific paths of differentiation. This process is generally named cell fate specification and is promoted by the diversification of gene expression patterns in individual cells, as a result of the activity of both intrinsic and extrinsic factors.

Despite the numerous cell types present in a multicellular organism, particularly in vertebrates, it is quite amazing and spectacular that five major and universal signalling pathways are at the basis of regulating multiple processes in development, accounting for the specification of correct cell fates. These are the sonic hedgehog (Shh), bone morphogenetic proteins (BMPs), Wnt, Notch and receptor tyrosine kinase (RTK) signalling pathways. Each pathway regulates transcription of its target genes by using a restricted set of transcriptions factors and it is quite remarkable that each of these signalling pathways is capable of executing such a wide variety of functions and decisions during development. For example, the activation of the Notch pathway converges systematically into the activation of the SuH/CBF1 transcription factor, which mediates the subsequent regulation of all Notch targets. What enables each of these signalling pathway to act iteratively throughout embryonic and adult development, even within the same tissue and yet produce a multiplicity of developmental outputs? Cell context is a frequent and apparently simple answer for this complicated question and can be understood as the proteome of a cell or tissue. Which components of the pathway of interest are present in that environment? At

what levels? What other interacting proteins which regulate or may be regulated by other pathways are present?

The Notch pathway is one of the five main signalling pathways that is used time and time again to regulate the development of almost very, if not every cell type in metazoans (reviewed in (Canalis, 2008; Crosnier et al., 2006; Dotto, 2008; Laky and Fowlkes, 2008; Louvi and Artavanis-Tsakonas, 2006; Niessen and Karsan, 2008; Siekmann et al., 2008)). This PhD thesis has been focused on this particular pathway and, specifically, on the role that the various Notch ligands may have in promoting the myriad of Notch functions. Therefore, in this first chapter a general overview on the Notch pathway will be presented. After a brief historic perspective, I will proceed to describe the components of the pathway, as well as the molecular mechanisms that regulate the pathway. Next, I will describe the role of the Notch pathway in vertebrate neurogenesis and emphasize the known roles of each Notch ligand within the nervous system. Finally, I will focus on some studies that have approached the function of distinct ligands in systems where various ligands are expressed and their role in inducing different Notch outputs.

I.1 THE NOTCH PATHWAY

I.1.1 The discovery of *Notch* and the concept of lateral inhibition

In the early 20th century, Thomas H. Morgan started using *Drosophila melanogaster* (fruit fly) to study the mechanisms governing inheritance. In order to do so, Morgan screened for and used flies with easily detectable mutations (Morgan, 1917). Among the many mutations observed by T.H. Morgan, there was one that resulted in an abnormal aspect of the fly's wings: the presence of "notches" on the margin of the wing (Mohr, 1919). As a consequence, the gene responsible for this phenotype was named *Notch*. This phenotype was due to a hypomorphic allele and it was only in the 30s, with the analysis of homozygous loss-of-function alleles of the *Notch* locus, that Donald F. Poulson revealed the importance of *Notch* (Poulson, 1937; Poulson, 1940). Besides the embryonic lethality, *Notch* mutant *Drosophila* embryos displayed a neurogenic phenotype, i.e, a severe hyperplasia of the nervous tissue, and the loss of epidermal tissues. These results suggested a role for Notch in cell fate choices, a

decision between the epidermal and the neural cell fate (reviewed in (Louvi and Artavanis-Tsakonas, 2006)).

I.1.1.1 Lateral inhibition, equivalence groups and Notch

Although Notch signalling is widely known for its role in the lateral inhibition mechanism, the very concept of lateral inhibition was born independently of Notch. The first experiments hinting at such a mechanism were described in 1940 by Vincent B. Wigglesworth. He spent his research career intrigued with 'pattern formation' and used *Rhodnius prolixus*, a blood-sucking bug, to approach systems with a well defined and repetitive patterning, such as the formation of sensory bristles (which compose the peripheral nervous system in insects), pigmentation cells and muscle insertions (Wigglesworth, 1940).

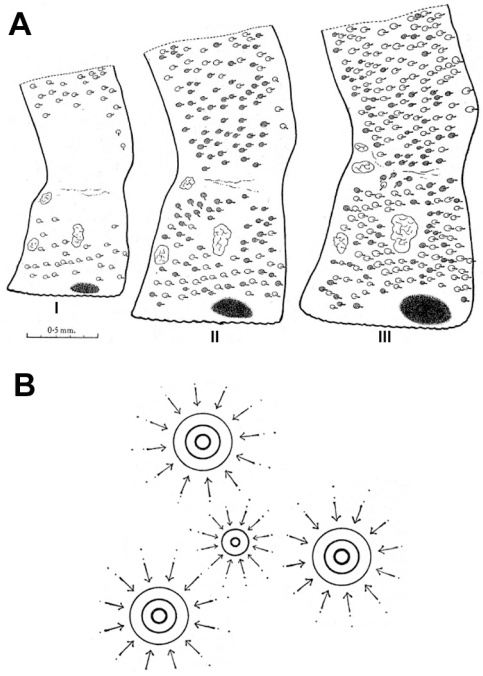


Figure I.1: Wigglesworth's experiments on sensory bristle patterning in the *Rhodnius prolixus*.
Burns on the epidermis of a 3rd instar *R. prolixus* nymph (I) resulted in the loss of sensory bristles, that were generated *de novo* at later instar nymphs (II and III). These newly formed bristles maintained their even spacing between them. **(B)** Wigglesworth's model of inhibition: pre-existing bristles (concentric circles) inhibited neighbouring epidermal cells from acquiring a sensory bristle fate by absorbing (arrows), and consequently depleting a certain factor, necessary for bristle formation and activity, form the surrounding epidermal cells. Adapted from (Wigglesworth, 1940).

In the specific case of sensory bristle formation, Wigglesworth was able to deduce that these were derived from epidermal cells, reappearing *de novo* after a lesion that damaged the bristles. These newly formed bristles were, like the remaining ones, evenly spaced and this precision in bristle spacing indicated that prosensory epidermal cells were sensitive to their position relatively to the already existing

sensory bristles (Figure I.1). This led Wigglesworth to ask, "*What is the nature of the inhibitory influence exerted by the plaques* [and bristles] *upon the surrounding cells?"* (Wigglesworth, 1940). At the time, Wigglesworth proposed a model in which the pre-existing bristles would deplete a 'factor', produced by the surrounding epidermal cells, that was essential for their activity. Consequently, new bristles would be inhibited from forming within the proximity of already pre-existing bristles, due to the absence or low levels of this 'factor' (Figure I.1).

Whereas Wigglesworth's experiments described for the first time the existence of an inhibitory influence of one cell on neighbouring cells, i.e, lateral inhibition, as a mechanism of creating cell diversity, it was only much later that an equally important concept was defined, the concept of equivalence group. An equivalence group can be defined as a group of cells that share a similar developmental potential, independently of ultimately acquiring the same cell fate or not. Evidence for the existence of equivalence groups was first shown through classical studies of cell lineage tracing and cell ablation in *Caenorhabditis elegans* (*C. elegans*) (Kimble, 1981; Kimble and Hirsh, 1979; Sulston and White, 1980). The invariant character of most cell lineages during *C. elegans* development led initially to the idea that the fate of each individual cell was determined exclusively by its parent. Hence, each cell behaved in cell-autonomous fashion (reviewed in (Davidson, 1976)). However, there are some exceptions, such as the ancestry of the anchor cell (AC) and the ventral uterine precursor cell (VU), which are required for vulval induction and production of uterus cells, respectively. The AC and VU cells derive from two cells, Z1 and Z4. Lineage tracing of these cells showed that they are equivalent and display an identical potential to acquire an AC or VU fate: the probability of Z1 acquiring an AC or VU fate is 50% and the same is true for Z4 (Kimble and Hirsh, 1979). However, if the AC fate is assumed by Z1, then Z4 will always assume the VU fate and vice-versa. Further studies demonstrated that the generation of different cell fates from initially equivalent cells was dependent on cell-cell interactions and lateral inhibition (Kimble, 1981; Kimble and Hirsh, 1979; Sulston and White, 1980). Laser-ablation of either Z1 or Z4 resulted invariably in the specification of the remaining cell as AC. Furthermore, if the Z1 and Z4 precursor cells were separated and therefore unable to interact, both would become ACs. Although the AC/UV cell fate decision is the simplest example of an equivalence group, these results showed the existence of a predominant cell fate (AC) that exerts a negative inhibition on its neighbouring cell, thereby preventing it from also acquiring the AC fate (Figure I.2, A).

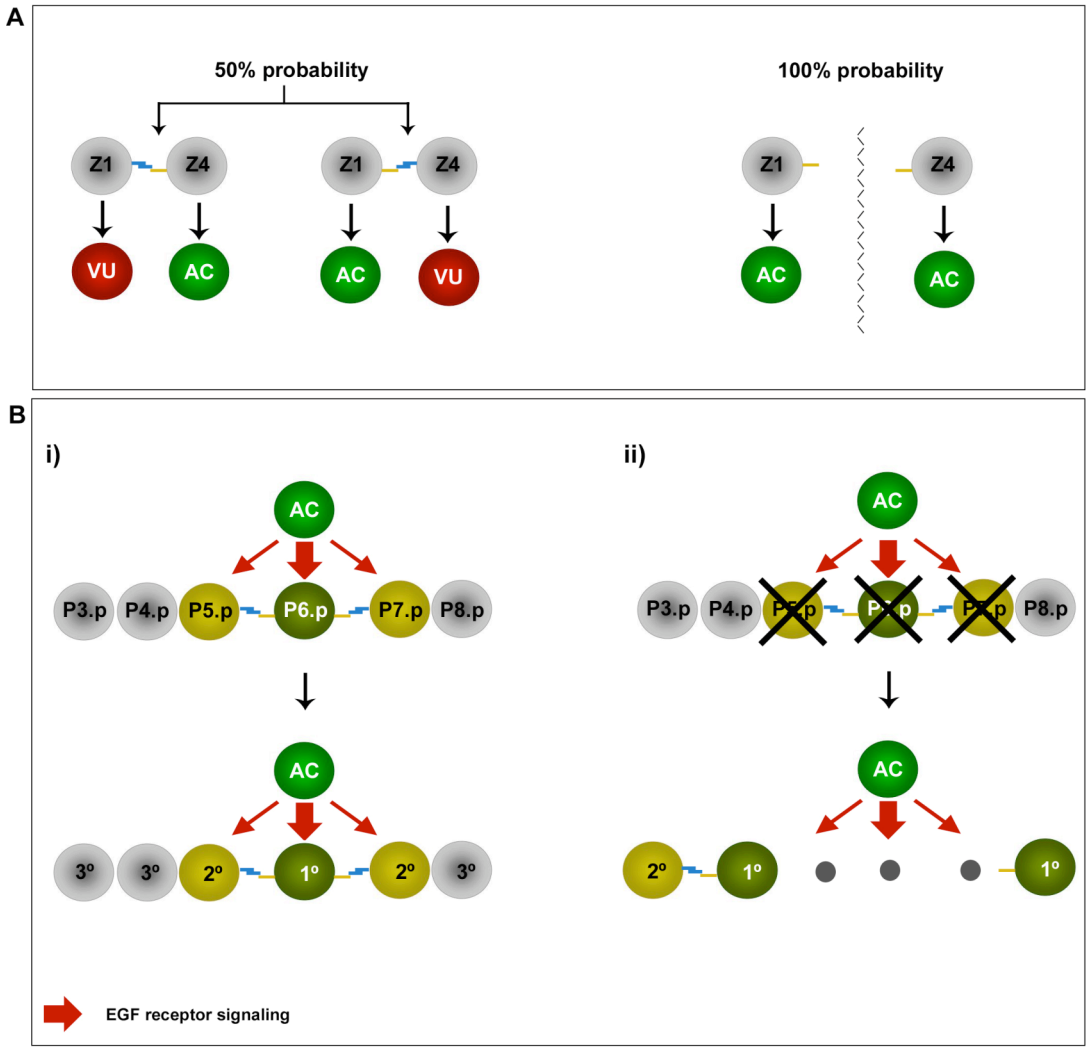


Figure I.2: Equivalence groups and lateral inhibition.

(A) The AC/VU decision, the simplest example of an equivalence group. The two precursor cells, Z1 and Z4, have an equal potential to acquire an AC or UV fate **(i)**. Despite having the same potential, the same fate is never adopted by the two cells. One of the precursor cells gains, stochastically, higher levels of Notch ligand (represented in yellow) and signals to its neighbouring cell, thereby inhibiting it from acquiring the AC fate. When the Z1 and Z4 precursors are separated and therefore unable to 'communicate', Notch is not activated and both cells assume the default fate, the AC fate **(ii)**. **(B)** The six vulval precursors (VPCs) are another example of equivalence group. Hence, all 6 precursors are able to acquire one of the three possible fates (1º, 2º and 3º), however the potential to do so, is not identical among them: the VPCs are biased towards a specific fate **(i)**. Secretion of EGF by the AC induces EGF signalling in the VPCs. Maximal activation of EGF signaling occurs in the P6.p cell, which is the cell closer to the source of EGF. As a consequence, expression of downstream targets such as, the DSL ligands, are up-regulated. The closer the VPC to the EGF source, the stronger the activation of the pathway. The P6.p cell signals to neighbouring cells and activation of the Notch pathway inhibits these cells from acquiring the 1º fate **(i)**. Despite the bias of each VPC to acquire a certain fate, laser ablation studies **(ii)**, revealed that cells are able to acquire any of the three alternative fates Adapted from (Sternberg, 2005).

Although the AC/VU cell fate decision occurs between a pair of precursor cells with equivalent potential, cells within an equivalence group do not need to be identical in potential. For instance, vulval development in *C. elegans* is dependent on a group of six vulval precursors (VPCs – P3.p to P8.p), each distributed in a specific manner along the anterior-posterior axis (Figure I.2, B). These six VPCs acquire three distinct fates (1º, 2º and 3º) and lineage tracing of each VPC revealed that VPC P6.p invariantly acquires the 1º fate, while VPC P5.p and P7.p undertake the 2º fate and the remaining VPCs acquire the 3º fate (reviewed in (Sternberg, 2005)). Nevertheless, experiments involving ablation of single and/or combinations of different VPCs demonstrated that all VPCs can replace the ablated cell (Figure I.2, B), despite the normal bias towards a particular fate (Sulston and White, 1980).

Similar laser ablation experiments were performed in other animal models, such as leeches (Weisblat and Blair, 1984), grasshoppers (Doe and Goodman, 1985a; Doe and Goodman, 1985b) and ascidians (Nishida and Satoh, 1989). Together, these independent studies confirmed the existence of equivalence groups and lateral inhibition as a mechanism to control binary cell fates, i.e, a mechanism that ensures that a group of equivalent cells acquire one of two cell fates (neuroblast versus epidermis, AC versus UV). Of these studies, I would like to refer to some of the concluding remarks of Doe and Goodman, where the concepts of equivalence group and lateral inhibition are nicely linked. Firstly, both *Drosophila* bristles and grasshopper neuroblast (NBs) arise at specific positions of the ectoderm, thus "*positional information in the ectoderm gives groups of cells* [equivalence group]*… a high probability of forming a NB or chaeta* [bristle]… *cell interactions* [within] *the group results in one cell differentiating into a NB or chaeta* [that] *inhibits its neighbors from undertaking an identical developmental pathway…"* (Doe and Goodman, 1985a).

In *Drosophila*, such equivalence groups with neural potential are referred to as proneural clusters and consequently, the genes involved in their specification are known as proneural genes, which belong to the *achaete-scute* complex (AS-C). Mutations that abolish the activity of AS-C genes lead to the loss of neural cells (bristles in insects) (Cabrera et al., 1987; Garcia-Bellido and Santamaria, 1978; Jimenez and Campos-Ortega, 1990; Martin-Bermudo et al., 1991). Conversely, the inactivation of genes involved in the lateral inhibition step, leads to an increased production of neural cells as observed for example in *Notch* mutants (Poulson, 1937; Poulson, 1940). This second group of genes is known as neurogenic genes. Thus, proneural and neurogenic genes function at opposing ends: proneural genes are

required to confer neural competence to ectodermal cells, whereas neurogenic genes are necessary to restrict this potential to a single cell.

I.1.1.2 Notch and neurogenic genes

In order to identify the genetic basis governing the establishment of developmental potential in a spatiotemporal manner, large-scale genetic screens were performed in *Drosophila* in the early 80's. *Drosophila* were treated with ethyl methanesulfonate (EMS), a mutagenic chemical that produces random mutations in DNA, and embryos were screened for EMS-induced lethal mutations (Jurgens et al., 1984; Nusslein-Volhard et al., 1984; Wieschaus et al., 1984). Among the many loci identified, several resulted in phenotypes very similar to the already known loss-of-function of *Notch*, i.e, a neurogenic phenotype (Table I.1). Hence, these loci contained the so called neurogenic genes.

Table I.1: Neurogenic loci identified in screen for embryonic lethal mutants. Adapted from (Jurgens et al., 1984; Nusslein-Volhard et al., 1984; Wieschaus et al., 1984).

Locus	Phenotype	Number of alleles			Map position[a)]
		total	weak	ts	
big brain (bib)	ventral cuticle absent, hypertrophy of central nervous system	5	–	–	32
Delta (Dl)	no ventral cuticle; hypertrophy of central nervous system; dominant "Dl" phenotype in adults	10	3	2	66.2
Enhancer of split (E(spl))	no ventral cuticle; hypertrophy of central nervous system	1	–	–	89
master mind (mam)	ventral cuticle absent, hypertrophy of central nervous system	9	7	–	71
neuralised (neu)	no ventral cuticle; hypertrophy of central nervous system	3	1	1	50.0
Notch (N)	hypertrophy of ventral nervous system, ventral cuticle absent	12	2	3	3

Although the precise cellular and molecular mechanisms underlying the epidermal-versus-neural cell fate decision were not understood, it was clear that this event was genetically controlled. Thus, following the identification of these neurogenic loci, extensive studies were performed with the aim of cloning and characterizing the

genes within each of the identified locus (Artavanis-Tsakonas et al., 1983; Hartley et al., 1988; Price et al., 1993; Rao et al., 1990; Smoller et al., 1990; Vassin and Campos-Ortega, 1987). The characterization of the proteins encoded by the neurogenic genes and very elegant work based on genetic epitasis (de-la-Concha et al., 1988; Dietrich and Campos-Ortega, 1984; Vassin et al., 1985) revealed the existence of a regulatory circuitry between these genes. Moreover, the molecular analysis of the putative proteins encoded by the *Notch* (Wharton et al., 1985) and *Delta* locus (Vassin et al., 1987) predicted that these two molecules were cell surface proteins. This led to the idea that functional interactions between neurogenic genes could occur at the cell membrane level, which was in accordance with the already known dependence of cell fate decisions on cell-cell interactions. Given the restricted spatial expression of *Delta* to regions of active neurogenesis, Campos-Ortega proposed a model in which Delta would be the source of the inhibitory effect of differentiating cells on neighbouring ectodermal cells (de-la-Concha et al., 1988). Reception of this inhibitory signal by ectodermal cells would lead to repression of the neural fate, repression dependent on the activity of the *Enhancer-of-split* (*E(spl))* genes, which were shown to be epistatically downstream of both *Delta* and *Notch*.

The hypothesis proposed by Campos-Ortega was confirmed by subsequent studies that unveiled the molecular mechanisms of the Notch pathway, the mediator of lateral inhibition. The following sections will therefore be dedicated to the description of the molecular components and modulators of the Notch signaling pathway.

I.1.2 The core components of the Notch pathway

The Notch pathway is well known for its role in many developmental processes mediated through cell-cell interactions. This cell-cell communication system is evolutionarily conserved and occurs through direct interaction between cell surface proteins, the Notch receptor and its ligand, which belongs to either the Delta or Serrate protein families. The interaction between receptor and ligand at the cell membrane surface leads to the activation of the Notch pathway that involves also the function of a transcription factor of the CSL (human CBF1, fly Suppressor of hairless, worm Lag1) family to transduce the signal to the nucleus and ultimately regulate the transcription of downstream targets. Although the above description is an over-simplification of Notch signalling, it points out the core components of the pathway: receptor, ligand and transcription factor (Table I.2). Each of these components is described in more detail below.

Table I.2: Core components of the Notch pathway
Su(H) – Suppressor of hairless; Dll – Delta-like

	C.elegans	*Drosophila*	*Chick*	*Mammals*
Receptor	LIN-12	Notch	Notch1	Notch1
	GLP-1		Notch2	Notch2
				Notch3
				Notch4
Ligand	Lag-2	Delta	Delta1	Dll1
	APX-1		Delta4	Dll3
	ARG-2			Dll4
	DSL-1	Serrate	Serrate1	Jag1
			Serrate2	Jag2
CSL transcription factor	LAG-1	Su(H)	Su(H)	CBF1/RBPJκ

I.1.2.1 The DSL family of Notch ligands

The Notch ligands belong to two protein families, the Delta and Serrate (the latter also known as Jagged in mammals). A related ligand was also identified in *C. elegans* and named Lag-2, hence the acronym DSL ligands (Delta, Serrate and Lag2). Whereas *Drosophila* presents only one *Delta* and one *Serrate* gene, five genes encoding DSL ligands have been identified and cloned in vertebrates: *Delta-like 1* (*Dll1*), *Delta-like 3* (*Dll3*), *Delta-like 4* (*Dll4*), *Jagged1* (*Jag1*) and *Jagged2* (*Jag2*) (Dunwoodie et al., 1997; Henrique et al., 1995; Lindsell et al., 1995; Myat et al., 1996; Shawber et al., 1996).

Although Delta and Serrate ligands are both transmembrane proteins and share similar sequence motifs, they are structurally distinct (Figure I.3). The DSL ligands present a conserved extracellular domain that contains a unique DSL domain at the N-terminal portion of the protein and several Epidermal Growth Factor-like Repeats (EGFRs). Previous studies using truncated forms of DSL ligands have shown that their binding and activation of Notch receptors is dependent on the DSL domain, together with the first two EFGRs (Parks et al., 2006; Shimizu et al., 1999). Additionally, the identification and characterization of Dlk-1 (Delta-like 1), a related transmembrane protein that is unable to activate Notch receptors and lacks the DSL

domain has confirmed the requirement of this domain for Notch activation (Baladron et al., 2005; Bray et al., 2008; Wang and Sul, 2006). However, over-expression studies have revealed a role for Dlk-1 in *cis*-inhibition of Notch activity, both in mammalian cells and *Drosophila* (Baladron et al., 2005; Bray et al., 2008), although the molecular mechanisms responsible for *cis*-inhibition have not been elucidated. Another related protein, Delta/Notch-like EGF-related receptor (DNER) that also lacks the DSL domain has been identified (Eiraku et al., 2002), being expressed in post-mitotic neurons of the mouse central nervous system. Contrarily to Dlk-1, DNER seems to be able to activate Notch in adjacent glial cells (Eiraku et al., 2005). Therefore, whether there are exceptions to the requirement of the DSL domain for the activation of the Notch pathway is still an open question.

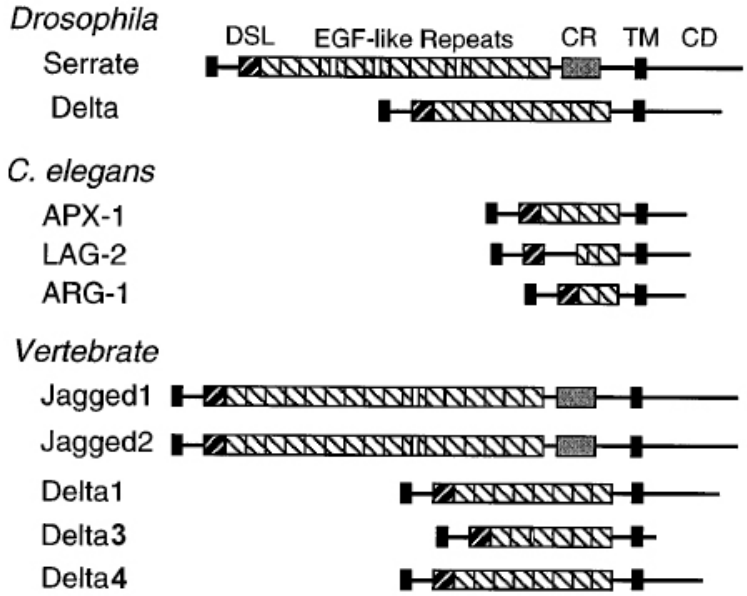


Figure I.3: Conserved structure of the DSL family of Notch ligands between different species.
CR – cysteine-rich region; TM – transmembrane domain; CD – cytoplasmic domain. Adapted from (Weinmaster, 1997).

As already mentioned, the DSL ligands display mutiple EGFRs in tandem and the number of EGFRs on the extracellular domain of the Delta and Serrate ligands constitute one of the main differences between these two protein families. Serrate ligands display up to twice as many EGFRs than Delta ligands (Figure I.3). Nevertheless, the functional importance of these EGFRs is not completely understood. For example, it has been shown that a LAG-2 mutant protein lacking

EGFRs is still able to rescue *lag2* deficient *C. elegans* (Henderson et al., 1997). On the other hand, mutations in specific EGFRs of the *Drosophila* Delta protein result in reduced binding affinity with Notch and, consequently, in reduced Notch activation (Lieber et al., 1992).

Serrate proteins present also a cysteine-rich region (CR) that is absent from the Delta proteins. It is thought that this CR region may negatively modulate ligand-receptor interactions, since Delta-Notch interactions have been reported to be more stable than Serrate-Notch (Fehon et al., 1990; Rebay et al., 1991).

Concerning the intracellular domain, little conservation is observed between the various DSL ligands. However, several experiments have shown that both the transmembrane and intracellular domains of DSL ligands are fundamental for Notch activation as ligands lacking the membrane anchor and/or the intracellular tail fail to activate the Notch receptor. In fact, such DSL constructs have been widely used as dominant-negative forms of the DSL ligands (Chitnis et al., 1995; Hukriede and Fleming, 1997; Hukriede et al., 1997; Sun and Artavanis-Tsakonas, 1996). Only recently has new light been shed on the molecular mechanisms that regulate the signalling activity of DSL ligands through the identification of specific proteins that interact with their intracellular domain (for review see (Pintar et al., 2007; Schweisguth, 2004)). Although DSL ligands do not share significant sequence homology in their intracellular domains, most ligands (all except mammalian Jag2 and Dll3) present several conserved lysine residues and a C-terminal PDZ (PSD-95/Dlg/ZO-1) ligand-binding domain, which are involved in endocytosis-dependent ligand activity and interactions with the cytoskeleton, respectively. In addition, recent reports have described proteolytic events of the intracellular domain of DSL ligands and even its localization in the nucleus, suggesting the possibility that these ligands mediate bi-directional signalling (Ascano et al., 2003; Bland et al., 2003; Klueg et al., 1998). This topic will be further explored in Section I.1.3.2.

Although special attention has been given in recent years to the functional importance of the membrane and intracellular domains of DSL ligands, the situation in *C. elegans* seems to be an exception. The DSL ligands of *C. elegans* differ from those of vertebrates and *Drosophila*, since over-expression of the secreted, extracellular forms of LAG-2 and APX-1 are able to rescue *lag-2* deficient worms and activate the GLP-1/Notch receptor (Fitzgerald and Greenwald, 1995). In fact, seven DSL ligands lacking the transmembrane and intracellular domains have been computationally predicted in the *C. elegans.* Of these secreted DSL ligands, DSL-1

has been shown to activate GLP1 signalling and is thus able to rescue *lag-2* deficient worms (Chen and Greenwald, 2004).

The structural differences observed among the several DSL ligands has naturally prompted up the question of whether they are all able to activate Notch and with equal efficiency. Studies approaching the signalling ability of DSL ligands have shown that all are capable of activating Notch, with the exception of mammalian Dll3 (Geffers et al., 2007; Ladi et al., 2005). However, differences have been observed in the binding affinities between different DSL ligands and Notch receptors and/or in the ability of certain ligand-receptor combinations to activate the pathway (Besseyrias et al., 2007; Fleming et al., 1997; Hicks et al., 2000; Panin et al., 1997). Many of these differences in binding or activating the Notch receptor are dependent on cell context and the presence of specific Notch modulators. An example of such modulators are the glycosyltransferases of the Fringe protein family, which enhances Delta-mediated Notch signalling while significantly reducing that mediated by Serrate (see Section I.1.3.2).

I.1.2.2 The Notch family of receptors

The Notch receptor is a large transmembrane protein that was first characterized in *Drosophila* (Wharton et al., 1985). Whereas only one *Notch* gene is present in the *Drosophila* genome, four vertebrate *Notch* genes have been identified and cloned (Table I.2) (Artavanis-Tsakonas et al., 1995; Greenwald, 1994; Lardelli et al., 1995; Uyttendaele et al., 1996). All Notch receptor proteins exhibit a strikingly similar structure and are characterized by the presence of specific domains within their extracellular and intracellular regions (Figure I.4).

The extracellular portion of Notch receptors has multiple EGFRs, which vary in number according to the Notch receptor and species. Although the sequence of the *Drosophila* Notch receptor has been known for two decades, little is known about the function and relevance of these EGFRs. In fact, only two of these repeats, the EGFRs 11 and 12 in the *Drosophila* Notch protein, have been shown to be necessary for receptor-ligand interactions (Rebay et al., 1991). C-terminal to the EGFRs, there is a cysteine-rich region, the LNR (LIN-12/Notch-related region) that negatively regulates the activation of the pathway (Greenwald and Seydoux, 1990; Lieber et al., 1993; Lyman and Young, 1993).

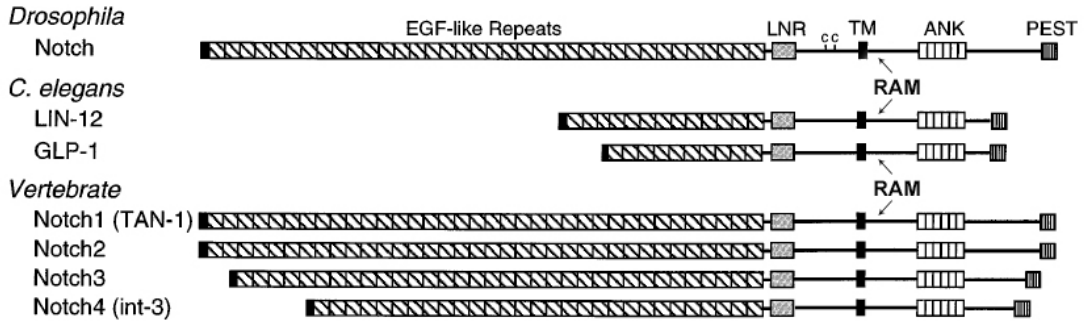


Figure I.4: Conserved structure of the Notch family of receptor proteins between different species.

LNR – LIN-12/Notch-related region; TM – transmembrane domain; RAM – RBPJκ-Associated Molecule; ANK – ankyrin repeats; PEST – proline, glutamate, serine and threonine rich region. Adapted from (Weinmaster, 1997).

The intracellular region of Notch receptor contains several known domains that are required for Notch signalling. The RAM domain (RBPJκ-Associated Molecule) is localized immediately downstream of the transmembrane domain and was shown to interact with the CSL transcription factor (Honjo, 1996). The ANK domain is localized C-terminal to the RAM domain and consists of six ankyrin repeats that are both necessary and sufficient for Notch activity (Lieber et al., 1993; Lyman and Young, 1993; Rebay et al., 1991). Finally, at the C-end of the Notch receptors there is a conserved PEST domain (proline-glutamate-serine-threonine), which is involved in protein turnover (Greenwald, 1994; Rogers et al., 1986).

The intracellular domain of the various Notch receptors differ from each other in the number of amino acid residues located between the ANK and PEST domains. This region seems to bind negative regulators of Notch signalling, such as Numb and Dishevelled (Axelrod et al., 1996; Guo et al., 1996; Zhong et al., 1996), thereby raising the possibility that these interactions can modulate the activity of distinct Notch receptors.

I.1.2.3 Activation of the Notch pathway

Until 1998, there were essentially two lines of thought to explain how the Notch signal is transduced within cells. The first one, proposed by Artavanis-Tsakonas and

colleagues, hypothesized that a CSL transcription factor was associated with the cytoplasmic RAM domain of the Notch receptor, and that upon ligand-receptor interaction it would be released and translocated to the nucleus (Fortini and Artavanis-Tsakonas, 1994). However, accumulating evidence suggested a second hypothesis in which the intracellular domain of the Notch receptor could have a more active role in signal transduction, by acting in the nucleus itself. Favouring this second hypothesis was the finding that the cytoplasmic region of Notch contained a nuclear localizing signal (NLS) that is essential for Notch activity (Kopan et al., 1994; Stifani et al., 1992), and also that the truncated form of the Notch receptor consisting only of its intracellular domain is sufficient to rescue *Notch* mutant phenotypes (Lieber et al., 1993; Roehl and Kimble, 1993; Struhl et al., 1993). However, the fact that the Notch receptor was never detected within the nucleus was a drawback for the acceptance of this second hypothesis. Until elegant work from M. Lecourtois and F. Schweisguth showed, although indirectly, that the Notch receptor is proteolytically cleaved upon its interaction with Delta and translocated to the nucleus (Lecourtois and Schweisguth, 1998). Subsequent experiments confirmed this proteolytic processing of the Notch receptor, which involves not a single step but various (described below in Figure I.5).

De novo synthesis of Notch receptors is followed by a Furin-dependent cleavage of the proteins at the S1 site, in the *trans*-Golgi network (Logeat et al., 1998). The cleavage products accumulate at the cell surface and are held together as a heterodimer through a Ca^{2+}-dependent non-covalent bond (Rand et al., 2000). Note that the existence of the Notch receptor as a heterodimer only occurs in vertebrates and not in *Drosophila* (Kidd and Lieber, 2002). Upon interaction with a DSL ligand, a conformational change of the receptor occurs that exposes a specific site (S2) to a second proteolytic event, mediated by metalloproteases of the ADAM/TACE/Kuzbanian family (Brou et al., 2000; Lieber et al., 2002; Mumm et al., 2000). Cleavage of Notch at the S2 site releases the extracellular domain that is *trans*-endocytosed into the ligand-expressing cell (Parks et al., 2000). The activated membrane-tethered form of Notch is known as NEXT (Notch Extracellular Truncation) and is further processed at the S3 and S4 sites, located within the transmembrane domain of the receptor (Okochi et al., 2002; Schroeter et al., 1998), by the γ-secretase activity of the Presenilin-Nicastrin-Aph1-Pen2 protein complex (De Strooper et al., 1999; Ray et al., 1999). The processing of NEXT results in the release of the intracellular domain of Notch (NICD – Notch IntraCelullar Domain), which translocates to the nucleus and associates with the CSL transcription factor

(reviewed in (Bray and Furriols, 2001)) and the Mastermind coactivator (MAM) (Petcherski and Kimble, 2000), forming a ternary complex.

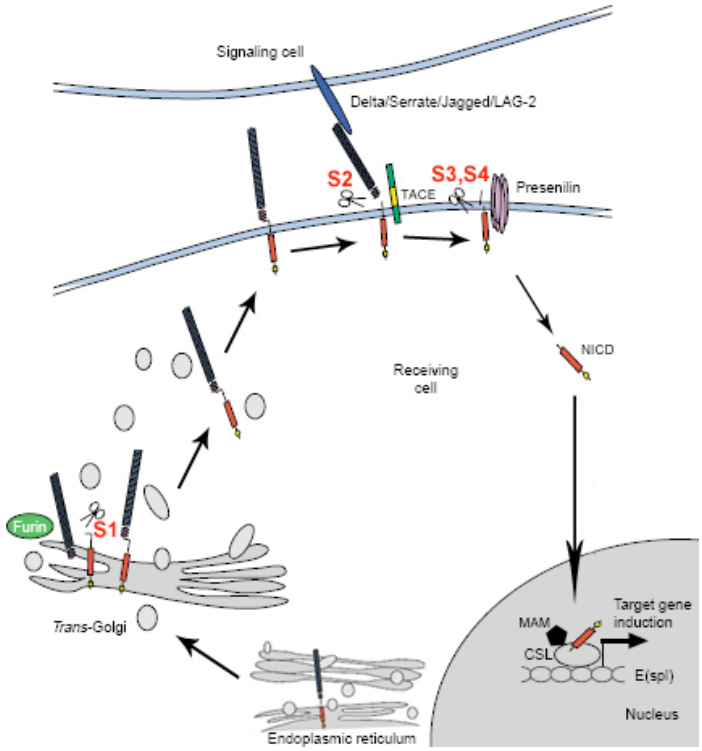


Figure I.5: Activation of the Notch pathway and signal transduction. Several proteolytic events within the Notch protein (S1-S4), its interaction with ligands and the nuclear translocation of NICD is required for Notch activity. In the nucleus, NICD associates with the CSL transcription factor and MAM coactivator, forming a ternary complex. This transcription complex binds to the promoters of downstream targets of the Notch pathway (*E(spl), hes, her, hey* genes) and activates their transcription.

I.1.2.4 The CSL transcription factor

The activity of the ternary complex formed by Notch to CSL and MAM, leads to the transcriptional activation of genes containing promoters with Su(H)/CBF-1-binding sites (Jarriault et al., 1995). In fact, the activation of the Notch pathway causes a switch in the transcriptional activity of the CSL transcription factor, which in the absence of NICD acts as a transcriptional repressor of the same target genes (reviewed in (Bray and Furriols, 2001; Mumm and Kopan, 2000)).

At the basis of this repressor to activator switch of the CSL transcription factor is the recruitment of histone acetylases (HATs) by NICD to the Notch/CSL/MAM complex (Kurooka and Honjo, 2000). The recruitment of HATs to the transcriptional complex is thought to displace and counteract the co-repressor complex containing SMRT and the histone deacetylase 1 (HDAC1) (Kao et al., 1998), previously assembled at the Su(H)/CBF1 binding sites (reviewed in (Bray and Furriols, 2001; Mumm and Kopan, 2000)).

There are many Su(H)/CBF-1-binding sites throughout the genome (Rebeiz et al., 2002) but it is still not known which correspond to actual downstream targets of Notch signalling. The best known targets are the genes from the *hes* (*hairy* and *enhancer of split*) family.

I.1.2.5 Hes proteins, the downstream targets of Notch

The Hes proteins, which are basic helix-loop-helix-Orange (bHLH-O) transcriptional repressors, are direct downstream effectors of Notch signalling. These proteins present four characteristic structural domains: the basic, HLH, Orange and WRPW domains (reviewed in (Davis and Turner, 2001)).

The HLH domain allows the homo- and heterodimerization of Hes proteins, which then bind to specific DNA sequences (E- and N-boxes) through their basic domain. Upon binding of Hes proteins to DNA, its C-terminal WRPW motif recruits co-repressor factors like Groucho, which lead to the transcriptional repression of the target genes, such as the proneural genes. Additionally, Hes proteins may also repress transcription by competing with bHLH transcriptional activators for the same DNA binding boxes (Grbavec and Stifani, 1996; Jennings et al., 1999; Paroush et al., 1994).

In addition to a mechanism dependent on DNA-binding, bHLH-O proteins can inhibit transcription by directly interacting and forming heterodimers with bHLH activator proteins, through their HLH and Orange domains (Dawson et al., 1995). For example, it is known that although Hes5 does not bind to E-boxes, it inhibits the binding of the bHLH transcriptional activator E47 to E-boxes by forming a heterodimer. This results in the repression of gene expression dependent on E47 activation (Akazawa et al., 1992), through its inactivation or deviation from E47-responsive promoters.

I.1.3 Regulation of development by Notch signalling

The Notch signalling pathway is involved in many developmental processes such as proliferation, cell fate decisions, cell survival and migration (reviewed in (Lai, 2004)). How does Notch signalling act to exert such panoply of functions? In particular, when the pleiotropy of the Notch pathway is not due to a pleiotropy of downstream transcriptional targets?

To discuss these questions, I will first approach and describe two processes mediated by Notch signalling, lateral inhibition and lateral induction, which control binary cell fate decisions and the establishment of morphological boundaries, respectively. Additionally, I will enumerate several mechanisms that regulate and modulate Notch signalling, enabling the creation of very specific outputs.

I.1.3.1 Lateral Inhibition versus Lateral Induction

Lateral inhibition

The concept of lateral inhibition has already been described in the context of vulval development in *C. elegans* and neuroblast and sensory bristle development in *Drosophila*. Lateral inhibition serves the purpose of mediating binary cell fate decisions and, in the original model, it involves the interaction between two (or more) cells sharing the same developmental potential, i.e, 'equivalent' cells. Both cells would send inhibitory signals that prevent acquisition of a primary fate but, at the end, only one of the two signalling cells acquires this fate, while the second acquires an alternative fate. In this model, the two equivalent cells are initially both signal-sending and signal-receiving. How they become different and acquire alternative fates has been the focus of intense investigation. The genetic and molecular dissection of the mechanism underlying the singling out of neural precursors in the embryonic CNS (neuroblasts) and PNS (sensory organ precursors – SOPs) of *Drosophila* has provided some answers to this question.

Genetic studies in *Drosophila* have shown that proneural genes of the AS-C are essential for the formation of neural precursors in the CNS and PNS. The AS-C genes are expressed in specific regions of the *Drosophila* neurectoderm, in groups of cells from which neural precursors will arise. These groups of cells are known as 'proneural clusters' and it is predicted that all proneural cells have a similar potential to become a neuroblast (Cabrera et al., 1987; Romani et al., 1987). The *AS-C* genes

encode transcription factors and their activity is essential to confer neural potential to these cells, as shown by the absence of neural precursors and subsequently of neuronal structures in *AS-C* mutants (Garcia-Bellido, 1979; Garcia-Bellido and Santamaria, 1978; Ghysen and Dambly-Chaudiere, 1988). Conversely, ectopic expression of *AS-C* results in the induction of supranumerary neural precursors (Brand and Cabrera, 1988; Hinz et al., 1994; Rodriguez et al., 1990). The singling out of a neural precursor within each proneural cluster is accompanied by a restriction in the expression pattern of the *AS-C* genes, which occurs first in all cells of the proneural cluster and ends in a single cell, the presumptive neural precursor (NB or SOP). How is this singling out of a neural precursor achieved?

As already mentioned, the loss of activity of neurogenic genes leads to an impairment in the sorting out of a single neural precursor. Thus, upon inactivation of a neurogenic gene, all cells of the proneural cluster acquire the neural fate (Lehmann et al., 1983). In order to understand the mode of action of the neurogenic genes in the singling out of a neural precursor, Pat Simpson and collaborators performed elegant experiments in *Drosophila*, involving mosaics with different doses of the neurogenic genes *Notch* and *Delta* (Heitzler and Simpson, 1991; Heitzler and Simpson, 1993). This work demonstrated that cells were able to 'sense' the relative levels of Delta and Notch (Figure I.6) and, as a result, cells exhibiting higher levels of Delta (or lower levels of Notch) always acquired the neural fate while the neighbouring wild-type cells were repressed from doing so, acquiring instead an epidermal fate. Conversely, cells exhibiting higher levels of Notch (or lower levels of Delta) were always inhibited from acquiring the neural fate. Hence, Notch was shown to be required for the reception of the inhibitory signal emitted from the presumptive neuroblast, with Delta being the source of this signal. Together with the membrane localization of the Notch and Delta proteins, and the ability of Notch-expressing cells to aggregate to Delta-expressing cells (Fehon et al., 1990), these results were consistent with the idea that Notch functioned as a receptor and Delta as its ligand. From these early results, Pat Simpson suggested the existence of a "*feedback mechanism whereby cells producing less receptor relative to their neighbours* [would] *be more efficient at signalling*" (Heitzler and Simpson, 1991).

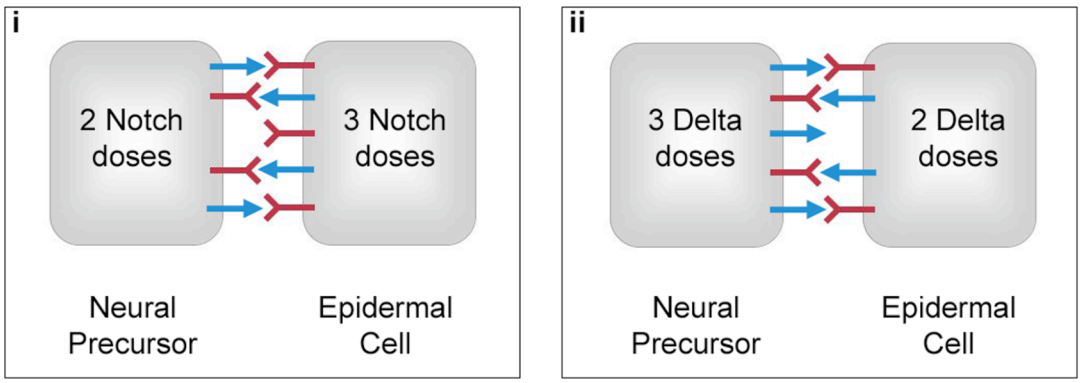


Figure I.6: Neural versus epidermal cell fate decision in *Drosophila*.
The generation of *Drosophila* mosaics with different doses of the neurogenc genes, *Notch* **(i)** and *Delta* **(ii)**, demonstrated that cells are able to 'sense' the relative levels of Notch and Delta. As a result, cells with higher levels of Delta (or lower of Notch) acquire the neural precursor fate, while cells lower levels of Delta (or higher of Notch) acquire the epidermal fate. Adapted from (Heitzler and Simpson, 1991).

Indeed, Notch-mediated lateral inhibition with such a feedback loop was later demonstrated in the *Drosophila*. The downstream targets of Notch activation, the *E(spl)* complex, were shown to be transcriptional repressors that function to silence the activity of the proneural genes (Bailey and Posakony, 1995; Knust et al., 1992; Lecourtois and Schweisguth, 1995). In addition to this, it was shown that the proneural transcription factors activate the transcription of *Delta* (de Celis et al., 1991; Hinz et al., 1994; Kunisch et al., 1994). Thus, the repression of proneural gene expression in cells with higher levels of Notch leads not only to a predictable decline in its neural potential, but also to decreased expression of the Delta ligand. This renders the *Notch*-expressing cell less competitive when compared to neighbouring *Delta*-expressing cells, which in turn become more efficient at signalling. As a consequence, *Delta*-expressing cells are singled out from the remaining cells of the proneural cluster. Furthermore, since a strongly ligand-expressing cell inhibits all surrounding cells from expressing the ligand, a natural consequence of this 'halo' of inhibition is that a second strongly ligand-expressing cell can only emerge from a region far from it, thereby generating a salt-and-pepper pattern (Figure I.7, A).

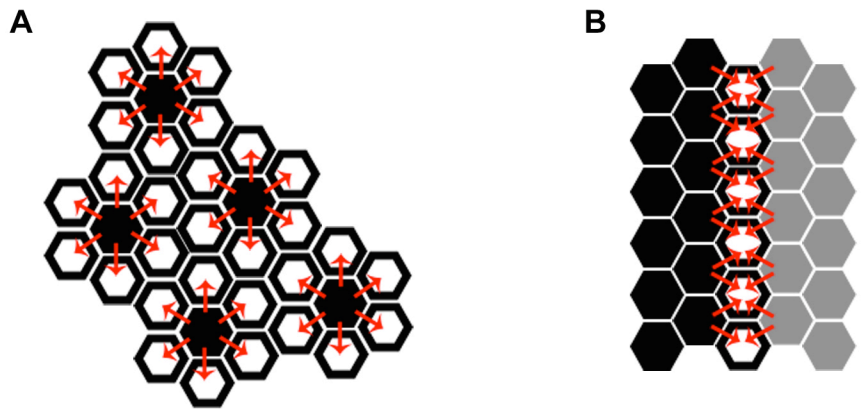


Figure I.7: Lateral Inhibition versus Lateral Induction.
Lateral inhibition leads to singling out of individual cell fates. The signal-sending cell (filled cells) signals to all surrounding cells (red arrows) and inhibits these from acquiring the same fate. A salt-and-pepper pattern is created originating for example, the evenly spaced bristles of the *Drosophila.* **(B)** Lateral induction leads to the formation of sharp boundaries. Signal-sending cells (filled black and grey cells) flanking the boundary cells, cooperate in specifying/maintaining the boundary cells type. This cooperation is accomplished by modulating the direction of Notch signalling to a specific group of cells (open cells), through the differential expression of Fringe proteins between the two compartments flanking the boundary cells. Adapted from (Bray, 1998).

Lateral induction

Notch signalling can also function to promote boundary formation, through the induction of a new cell type (Figure I.7, B). There are various examples of Notch involvement in boundary formation, namely during vertebrate somitogenesis, zebrafish hindbrain segmentation and formation of *Drosophila* wing margin (reviewed in (Blair, 2004; Irvine and Vogt, 1997; Takahashi, 2005)).

The formation of the *Drosophila* wing margin is the best known example of lateral induction. The wing margin is formed by a specialized group of cells that regulates the production of morphogens, such as Wg (Wingless) and Dpp (Decapentaplegic), which are essential for the growth of the wing blade. These specialized cells with organizer character are established at the interface between the dorsal and ventral compartments of the imaginal disc, where Notch activation is concentrated.

Both *Delta* and *Serrate* are expressed in the *Drosophila* imaginal wing disc, however their expression patterns are complementary. *Delta* is expressed in the ventral compartment, while *Serrate* is present only in the dorsal cell population. How then is Notch activation restricted to the interface between these two cell populations?

Fringe (Fng), a glycosyltransferase that modifies the Notch receptor post-translationally, modulates the sensitivity of receptor activation to specific ligands. Notch glycosylation by Fringe enhances Delta-mediated Notch activity and inhibits signalling mediated by the Serrate ligand (Fleming et al., 1997). *Fringe* and *Serrate* are co-expressed in the dorsal compartment of the imaginal wing disc and this inhibits Serrate–induced activation of Notch in this compartment. Thus, *Serrate*-expressing cells are only able to signal to cells on the ventral compartment, which do not express Fringe. On the other hand, Delta-mediated Notch activation is enhanced when Notch is glycosylated by Fringe, which leads *Delta*-expressing cells in the ventral compartment to signal to cells on the dorsal field of the wing (Figure I.8). The outcome of this opposing directionality of the signal is the appearance of high levels of Notch activation along a very restricted region, the interface between the dorsal-ventral compartments and the induction of a new cell type (reviewed in (Herranz and Milan, 2006)).

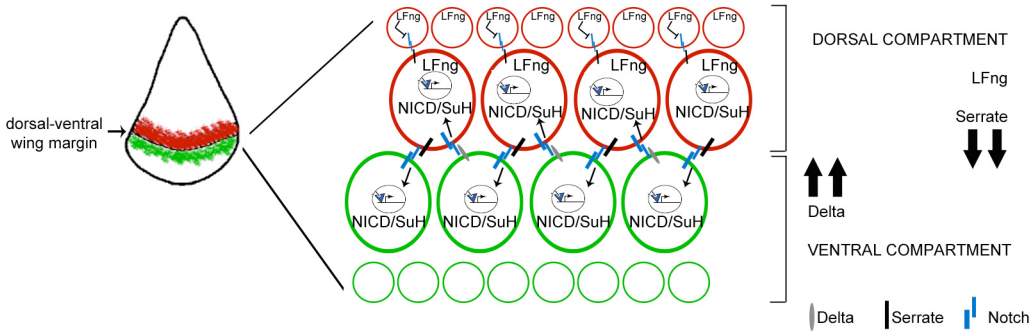


Figure I.8: Modulation of Notch signalling by Fringe in the *Drosophila* wing.
Serrate and Fringe are co-expressed in the dorsal compartment of the *Drosophila* wing (red cells) while Delta is restricted to the ventral compartment (green cells). Fringe-mediated Notch glycosylation inhibits Serrate-induced activation of Notch, thus Serrate-expressing cells are only able to signal to cells on the ventral compartment. On the other hand, Delta-mediated Notch activation is enhanced when Notch is glycosylated by Fringe, which leads to Delta-expressing cells, in the ventral compartment to signal to the cells on the dorsal field of the wing. By modulating the signalling activity of each ligand expressed in the *Drosophila* wing, the activation of Notch signalling is concentrated at the interface of dorsal and ventral compartments. Adapted from (Irvine and Vogt, 1997).

In addition to the generation of a new cell type at the boundary between two distinct cell compartments, there is another hallmark of lateral induction that is of fundamental importance. It was demonstrated in *Drosophila* that Notch activation positively regulates the expression of Delta and Serrate at the dorsoventral (DV)

boundary of the wing (de Celis and Bray, 1997; Panin et al., 1997). This positive feedback loop is necessary to maintain high levels of ligand expression and hence, maintain high levels of Notch activation in this region of the wing. This positive regulation of DSL ligand expression contrasts with the mechanism of lateral inhibition, where activated Notch inhibits ligand expression through a negative feedback loop.

I.1.3.2 Modulation of the Notch pathway

As mentioned in previous sections, Notch signalling occurs through the reiterative usage of the same core components and without secondary messengers, yet is able to execute a wide range of functions, inclusively within the same tissue. Since the early work performed by Pat Simpson and collaborators (Heitzler and Simpson, 1991), it has been known that the relative levels of Notch signalling influences the outcome of the signalling pathway. Thus several regulatory/modulatory mechanisms, explored below, act upon the Notch pathway in order to finely adjust the signaling output in terms of directionality, intensity and/or duration.

Endocytosis and ubiquitination – in the early 70's, the loss-of-function of *shibire* (*shi*), which encodes for a *Drosophila* homologue of Dynamin (Chen et al., 1991; van der Bliek and Meyerowitz, 1991), was described to have phenotypes very similar to those of various neurogenic mutants (Poodry et al., 1973; Shellenbarger and Mohler, 1978). Dynamin is required for the pinching off of clathrin-coated endocytic vesicles and this suggests that endocytosis plays an important role in the Notch pathway. Indeed, analysis of Delta localization revealed that this protein is present not only at the cell surface but also in intracellular vesicles, which are of endocytic nature. In endocytic mutants, such as *shi* and *hook* (*hk*), these Delta-containing intracellular vesicles are absent (Kramer and Phistry, 1996; Kramer and Phistry, 1999; Parks et al., 1995) and the importance of DSL ligand internalization was nicely demonstrated by the work performed by Muskavitch and collaborators. The analysis of a *Delta* allele encoding a protein that is persistently on the cell surface revealed a reduction in its signaling capacity and consequently, a phenotype identical to that of a *Delta* loss-of-function mutation (Parks et al., 2000; Parks et al., 1995).

Currently, several models are considered to explain the regulation of Notch activity through endocytosis of the Delta and Serrate ligands (reviewed in (Schweisguth, 2004)). One model proposes that endocytosis of the ligand upon its interaction with the receptor provides the physical force required for shedding of the extracellular domain of the Notch receptor, facilitating the exposure of the S2 cleavage site to the activity of γ-secretase (Figure I.9, A). This is supported by the observation that the extracellular domain of Notch is indeed *trans*-endocytosed into the ligand-expressing cells (Parks et al., 2000).

A second model suggests that DSL ligands are initially produced in an inactive form and that only upon internalization and recycling back to the cell membrane do they become active (Figure I.9, B). It is thought that recycling of the ligands might be either necessary for a post-translational activating modification or for its re-insertion into specific membrane domains. In support of this model, phenotypic analysis of *Drosophila rab11* and *sec15* mutants, which encode components of the recycling endosome and exocyst, respectively, show that these genes are needed for Delta recycling, Notch activation and consequently, specification of the SOP lineage (Emery et al., 2005; Jafar-Nejad et al., 2005).

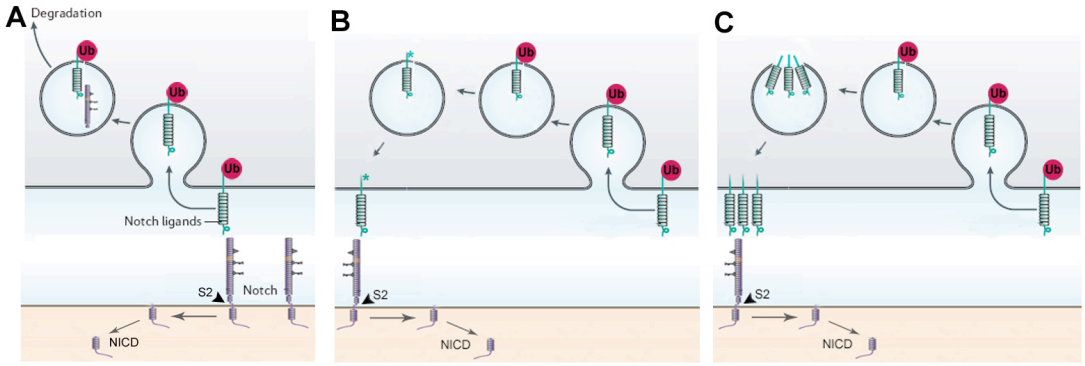


Figure I.9: Models for the role of Notch ligand endocytosis.

(A) Notch ligand endocytosis facilitates shedding of the extracellular domain of Notch (S2 cleavage). Recycling of Notch ligand is required to activate the ligand, possibly through post-transcriptional modifications (asterisk, **B**) or through re-insertion of clustered ligands into specific microdomains of the cell membrane **(C)**. Adapted from (Bray, 2006).

A third and final hypothesis has been set forward to explain the need of DSL ligand recycling for Notch activity: the ubiquitin-dependent promotion of ligand clustering. Previous studies had shown that the Delta ligand activates the Notch pathway more

efficiently when pre-clustered at the cell membrane surface (Hicks et al., 2002). Therefore, it was suggested that the ubiquitination and internalization of the ligands could be involved in their clustering and consequently higher activity (Figure I.9, C).

Recently, three E3 ubiquitin-ligases have been identified and shown to regulate the endocytosis of DSL ligands in *Drosophila* and vertebrates. These are Mindbomb-1 (Mib-1), Mindbomb-2 (Mib-2) and Neuralized (Neur). In fact, *Neur* had been already identified as a neurogenic gene in the screen performed by Kludig and colleagues (Jurgens et al., 1984). Interestingly, Neur-dependent ubiquitination of the Delta ligand has been shown to be involved not only in targeting the ligand for endosomal recycling but also in its degradation (Deblandre et al., 2001). This implies that ubiquitination of DSL ligands may be involved in enhancing their signalling ability, as well as the degradation of the activated ligand, thereby constituting a mechanism of fine temporal regulation of Notch activation.

Ligand processing – Like the Notch receptors, it has been shown that the DSL ligands are also prone to proteolytic events mediated by the metalloprotease Kuzbanian (Klueg et al., 1998; Qi and *al*, 1999). Although the role of ligand processing remains to be understood, *Kuzbanian* loss-of-function results in ectopic activation of Notch, suggesting that proteolysis of DSL ligands might be necessary for their downregulation (Mishra-Gorur et al., 2002; Sapir et al., 2005). Another plausible function of ligand processing might be to generate soluble ligand fragments capable of antagonizing Notch activity (Hicks et al., 2002; Mishra-Gorur et al., 2002; Sun and Artavanis-Tsakonas, 1997).

Regulation of Notch receptor activation

Numb and Sanpodo – Numb is a conserved membrane-associated protein that inhibits Notch signalling. Two mechanisms, based on studies in *Drosophila*, have been described for the negative regulation of Notch signalling by Numb. Firstly, it has been shown that Numb interacts directly both with the intracellular domain of Notch and α-adaptin, which is one of the subunits of the AP2 complex that links cargos targeted for endocytosis to the clathrin coat of transport vesicles (Berdnik et al., 2002; Guo et al., 1996; Santolini et al., 2000). Thus, Numb might regulate Notch signaling by promoting endocytosis of Notch receptors and their degradation. In the second proposed mechanism, Numb functions by inducing the endocytosis of a

positive regulator of Notch signaling, Sanpodo (Spdo) (Dye et al., 1998; Skeath and Doe, 1998) preventing in this way the interaction of this membrane protein with Notch and the activation of the pathway (Hutterer and Knoblich, 2005; O'Connor-Giles and Skeath, 2003).

Cell fate specification within the SOP lineage is an illustrative example of the combination of several of the regulatory mechanisms mentioned above (Figure I.9). As previously described, Notch-mediated lateral inhibition singles out SOPs from proneural clusters present in the *Drosophila* neuroectoderm. These SOPs then undergo successive rounds of asymmetric cell division to generate the *Drosophila* adult sensory organ, which is composed by four distinct cell types: the socket, the shaft, the sheath and the neuron (Figure I.10, A). The SOP (or pI cell) divides into an anterior (pIIb) and a posterior (pIIa) cell. The pIIb cell will then divide and give rise to a pIIIb cell and a glial cell, which is does not become part of the *Drosophila* sensory organ. The pIIIb cell goes on to originate the neuron and the sheath cell through another asymmetric division and the pIIa cell gives rise to the shaft (externally visible hair) and the socket cell.

In each of these cell divisions, Notch signaling is activated in one of the daughter cells in a unidirectional manner and, thereby, assures the acquisition of different cell fates between sibling cells (Figure I.10, B). The unidirectionality of the signal from pIIb to pIIa, for example, involves the asymmetric inheritance of several factors (Neur, Rab11 and Neur) to the pIIB cell (Figure I.10, C).

The asymmetric localization of the Neur E3 ubiquitin ligase to the pIIb cell promotes monoubiquitination and endocytosis of Delta, which is required for Notch activation on the neighbouring cell (in this case, its sibling pIIa cell) (Le Borgne and Schweisguth, 2003). In addition to Neur, it was recently found that the small GTPase Rab11 also accumulates in the pIIb cell, but not in the pIIa cell, and positively regulates the activity of the DSL ligand. It was shown that upon division of the SOP cell, Delta is endocytosed and targeted to recycling endosomes where Rab11 is localized. These results suggest a role for Rab11 in recycling and activating the Delta ligand exclusively in the pIIb cell. In support of this, Knoblich and collaborators observed that in the presence of a dominant-negative form of Rab11, Notch activation is indeed compromised (Emery et al., 2005). While Neur and Rab11 function in the pIIb cell to enhance the signaling abilities of the DSL ligand, the asymmetric inheritance of Numb by the pIIb cell (Rhyu et al., 1994) reduces the ability of this cell to activate Notch by promoting endocytosis and/or degradation of

Notch and of its positive regulator Spdo (Hutterer and Knoblich, 2005). The simultaneous presence of these three distinct regulatory mechanisms assures the directionality of the Notch-Delta signalling and the correct cell fate specification.

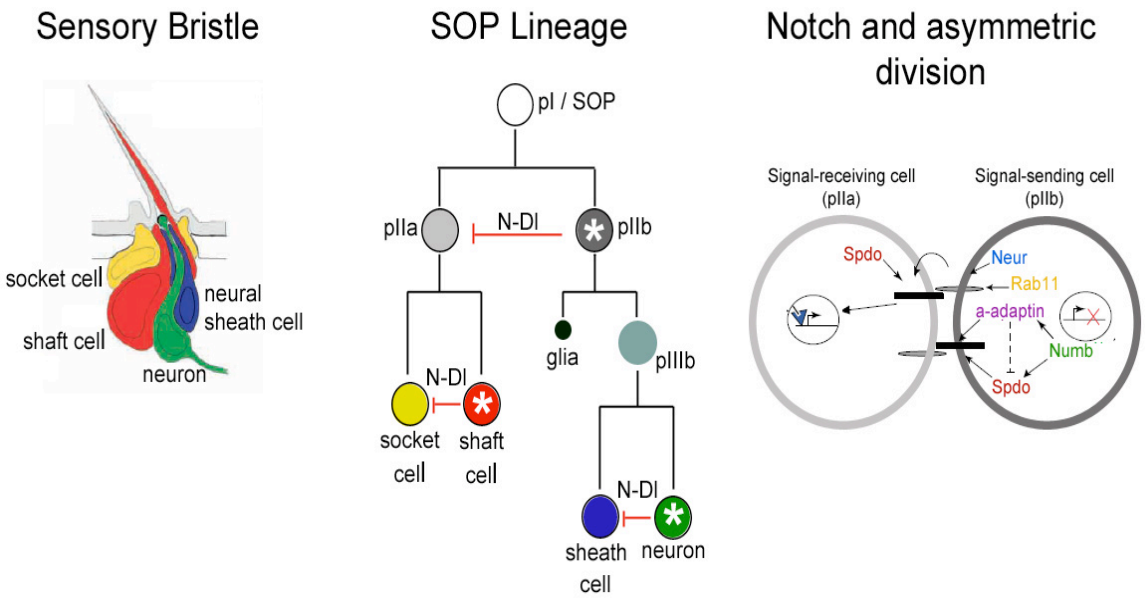


Figure I.10: Sensory organ specification in the *Drosophila* adult

The sensory organ of the adult *Drosophila* is composed by four distinct cell types that derive from the SOP through rounds of successive cell divisions. In each of these cell divisions, one of the sibling cells (marked with an asterisk) inherits several modulators of Notch signalling (Numb, Neur and Rab11) which pre-determine the signal-sending cell. Notch-Delta interaction then sort out the two possible fates at each division (pIIa/pIIb, socket/shaft and sheath/neuron). Adapted from (Adam et al., 1998; Bardin et al., 2004).

Glycosylation – The Notch receptor is a target of several glycosylation events, the first of which mediated by an *O*-fucosyl transferase (O-Fut) whose activity is required for the generation of a functional receptor (Okajima and Irvine, 2002; Sasamura et al., 2003; Shi and Stanley, 2003). Other glycosylation processes, mediated by glycosyltransferases of the Fringe family, determine the sensitivity of Notch receptor activation to specific ligands. As described above, Notch glycosylation by Fringe enhances Delta-mediated Notch activity and inhibits signalling mediated by the Serrate ligand in *Drosophila* (Fleming et al., 1997). This mechanism of Notch activity modulation is conserved in vertebrates where three Fringe proteins, Lunatic Fringe (LFng), Manic Fringe (MFng) and Radical Fringe (RFng) have been described. In mammalian cells, it has been shown that Notch glycosylation by LFng and MFng

enhances Dll1-mediated Notch activity, while inhibiting that mediated by the Jag1 ligand (Bruckner et al., 2000; Fleming et al., 1997; Hicks et al., 2000; Moloney et al., 2000; Panin et al., 1997; Yang et al., 2005). However, in a different context where signaling is mediated by Notch2, LFng is able to potentiate the signaling activity of both Dll1 and Jag1 (Hicks et al., 2000). Additionally, it has been reported that RFng (but not LFng nor MFng) enhances signalling induced either by Dll1 or Jag1 (Yang et al., 2005). Thus, the signalling output from different pairs of ligand-receptors can be modulated by Fringe-mediated glycosylation.

It still remains to be understood how this modulation of Notch 'sensitivity' to signalling through specific ligands is accomplished. It has been suggested that glycosylation may influence the binding of specific ligands to the glycosylated EGFRs of the Notch receptors (Bruckner et al., 2000; Moloney et al., 2000). However, this hypothesis has been contradicted by other studies where ligand-receptor binding was shown not to be affected by Fringe activity (Hicks et al., 2000; Yang et al., 2005).

Receptor ubiquitination – The regulation of receptor turnover is a critical regulatory process in most signaling pathways, since it determines the amount of receptor available to interact with ligands. Accordingly, several E3 ubiquitin ligases have been implicated in regulating Notch turnover either positively or negatively, through the regulation of its degradation. For instance, the Itch/NEDD4/Suppressor of Deltex (Su(dx)) family of E3 ubiquitin ligases have been shown to negatively Notch signalling by targeting the receptor for degradation (Lai, 2002; Qiu et al., 2000; Sakata et al., 2004).

Nevertheless, ubiquitination and endocytosis of Notch is not always related to protein degradation and consequently, to downregulation of signaling. For instance, it has been shown in *Drosophila* that Notch is ubiquitination by another E3 ubiquitin ligase, Deltex (Dx), prevents Notch from entering the lysosomal degrading pathway (Hori et al., 2004; Matsuno et al., 1995). Thus, it has been suggested that Dx and Su(dx) function antagonistically to regulate endosomal sorting instead of endocytosis *per se.* Nevertheless, the precise mechanisms by which Deltex operates remain to be understood.

I.2 NOTCH AND VERTEBRATE NEUROGENESIS

The nervous system is a highly complex system that is responsible for regulating all aspects of body function. It allows the perception of both the internal and external environment, the distinction of a wide variety of stimuli and the establishment of specific responses. This process of perception and subsequent response is dependent on a sophisticated network of neurons and glial cells – on the order of 10^{11} neurons and ten times more glia in the brain – that are reproducibly organized in circuitries. Remarkably, these neurons comprise several types that are generated in the correct proportions, migrate to correct positions and establish the correct connections and circuitries. Thus, the formation of the nervous system requires a tight regulation of several developmental steps, namely neural induction, cell proliferation, cell fate determination, migration, neurite growth and synapse formation.

The Notch signalling pathway was first observed to be fundamental for the appropriate development of the *Drosophila* nervous system over 70 years ago, and is also involved in many of these processes in vertebrates (reviewed in (Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005)). In the developing vertebrate nervous system, all five DSL ligands are expressed in different spatiotemporal patterns suggesting that they play several distinct roles and, in this way, account for the pleiotropy of Notch functions during CNS development.

In this section, I will first describe the process of neurogenesis in vertebrates and approach the general roles of Notch signalling in the vertebrate CNS. Then, I will focus on each of the DSL ligands, namely on their individual expression patterns and phenotypes observed in loss-of-function assays.

I.2.1 Vertebrate neurogenesis

The CNS is composed by thousands of millions of cells that are mostly generated during embryonic development. The generation of this enormous amount of cells naturally requires that a pool of undifferentiated neural progenitor cells has to be maintained throughout embryonic development. Moreover, only regulated maintenance of neural progenitors can assure that these are exposed to the different cues available at different time points of development, instructing or pre-determining neural progenitors to acquire a certain neuronal subtype or glial identity. This process

ensures the production of both early and late cell fates, as well as the proportions in which they are produced. Thus, in order to understand neurogenesis it is necessary to comprehend the processes that underlie the production of neural progenitors (proliferation) and how they exit cell cycle to enter differentiation.

The vertebrate CNS is composed by several structures, namely the forebrain, midbrain, hindbrain, spinal cord and retina. Within each of these structures, neural progenitors are organized in a polarized neuroepithelium: each cell spans the entire width of the neuroepithelium and are anchored to each other at the apical surface of the neuroepithelium by several specialized junctions (Figure I.11, B) (Aaku-Saraste et al., 1996; Bancroft and Bellairs, 1975; Schoenwolf and Kelley, 1980).

When a neural progenitor withdraws from the cell cycle, it loses its apical attachment, migrates out of the ventricular zone (VZ) of the neuroepithelium and accumulates in the mantle zone (MZ; Figure I.11, A). This neural tissue regionalization is not only restricted to distinct localizations of neural progenitors and differentiated neurons, but also to the different phases of the cell cycle at which the progenitors may be found (reviewed in (Hollyday, 2001)). Although neural progenitor cells are bound to both the apical and basal surfaces of the neuroepithelium, the nucleus migrates along the axis of the cell. This cell movement is known as interkinetic nuclear migration (INM) and the position of the nucleus in relation to the apicobasal surfaces is indicative of the cell cycle phase of the neural progenitor at a given timepoint. For instance, the nucleus of cells undergoing mitosis are always localized at the most apical side of the neuroepithelium. After cell division, cells enter the G1 phase and their nucleus migrate basally, where cells enter S-phase and duplicate their DNA content, to then enter the G2 phase, where the nuclei start to migrate back to the luminal surface for a new mitotic event (Figure I.11, B).

Although INM was first described in the 30's (Sauer, 1935), the functional importance of this process still remains unclear. INM has been intensely studied in recent years, yet the conclusions taken from these studies remain controversial and unclear. In some reports, it has been suggested that this nuclear migration of neural progenitors promotes cell fate diversity (Baye and Link, 2007; Cappello et al., 2006; Del Bene et al., 2008; Xie et al., 2007), while others have observed no alterations in neurogenesis upon disruption of INM (Imai et al., 2006). Those in favour of a possible role of INM in cell fate diversification have suggested that the polarized character of neural progenitors, i.e., the presence of distinct complexes at the apical and basal surfaces, may lead to differential responses between neuroepithelial cells. Thus, the presence

of adherens- and tight junction-associated signaling proteins on the apical surface (Cappello et al., 2006; Cui et al., 2007) and basally localized focal adhesion-associated signaling molecules (Li and Sakaguchi, 2002; Wozniak et al., 2004) leads to the activation of specific pathways in neighbouring progenitor cells when these present their cell body closer to the apical or basal surfaces, respectively.

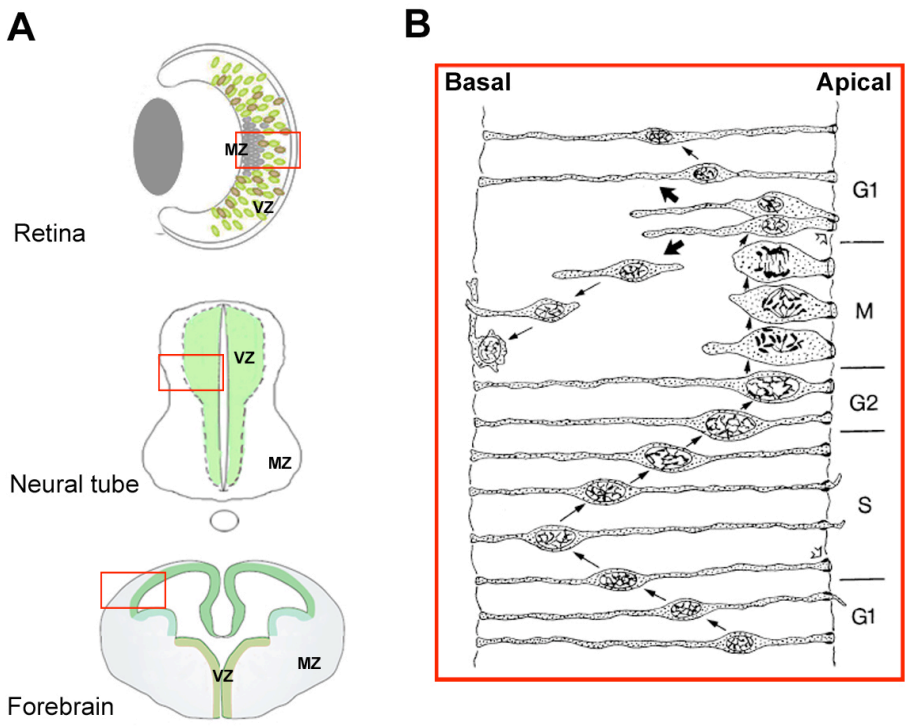


Figure I.11: Regionalization of the neural tissue

(A) Neural progenitor cells are confined to the ventricular zone (VZ), which is always localized at the most apical side of the tissue, while cells that have exited the cell cycle and embarked on neuronal differentiation migrate and accumulate in the mantle zone (MZ). **(B)** Within the ventricular zone, the neural progenitors are further regionalized according to their cell cycle phase: mitotic nuclei are only localized at the most apical surface of the neuroepithelium, while cells in S-phase have their nucleus closer to the basal side and cells in G1 and G2 are cycling between these two opposing positions. Adapted from (Hollyday, 2001).

I.2.2 Functions of Notch during CNS development

I.2.2.1 Neural progenitor maintenance

The Notch pathway is of major importance in many events determining the correct development of the nervous system and its role in progenitor maintenance is without any doubt the most well understood and studied (reviewed in (Louvi and Artavanis-Tsakonas, 2006)). However, there are some differences in the function of Notch in controlling neural development between flies and vertebrates. In *Drosophila*, Notch is first involved in an epidermal versus neural cell fate decision. In fact, *Notch* loss-of-function leads to hyperplasia of the neural tissue, which is over-produced at the expense of epidermis. In vertebrates, the situation is different since there is no evidence that Notch plays a role in specification of ectodermal cells to the neural or epidermal lineage (reviewed in (Stern, 2002)). Instead, the loss of Notch activity leads to neural tissue hypoplasia. This contrasting effect is due to excessive and premature neuronal differentiation at the expense of neural progenitors (de la Pompa et al., 1997; Hatakeyama et al., 2004; Lutolf et al., 2002; Yun et al., 2002). A direct consequence of this early progenitor depletion is the loss of late neuronal cell fates and of glial cells.

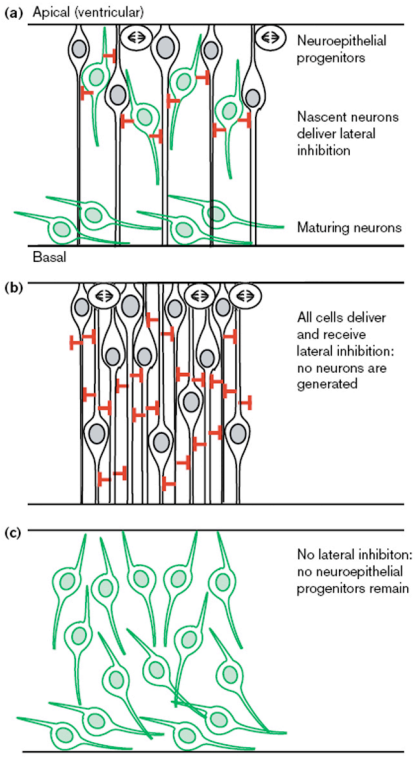


Figure I.12: Lateral inhibition mediated through Notch-Delta interactions regulates neuronal differentiation.
Differentiating neurons (green cells) are *Delta*-expressing cells that interact with neighbouring neural progenitor cells (black cells). By activating the Notch pathway in the neural progenitors, these are inhibited from embarking on neuronal differentiation. **(b)** In a situation of excessive Notch signalling, for instance when the number of signalling cells is increased by over-expression of Delta, Notch activity is induced in all cells, thus neuronal differentiation does not occur. **(c)** Conversely, blockage of Notch signalling (by over-expression of a dominant-negative form of Delta) leads to the depletion of progenitor cells due to excessive and premature neuronal differentiation. Adapted from (Henrique et al., 1997).

Like in the epidermal-versus-neural cell fate decision that occurs in *Drosophila* proneural clusters, the decision to remain or not as a progenitor cell in the vertebrate nervous system is also mediated by the balance of two sets of genes, the proneural and the neurogenic genes. Interestingly, not all proneural genes provide the same competence and they may also bias the neural competent cells to specific neuronal fates (reviewed in (Ross et al., 2003; Vetter and Brown, 2001). The balance between these two groups of bHLH proteins is maintained by Notch signalling. Within the neuroepithelium, individual cells that are exiting cell cycle and embarking on neuronal differentiation display higher levels of proneural gene expression and activate the transcription of the Delta ligand (Chitnis et al., 1995; Henrique et al., 1995; Ma et al., 1996; Murciano et al., 2002), which signals to neighbouring cells. As a result of Notch activation in these cells, proneural gene expression is repressed, leading to the inhibition of neuronal differentiation. The importance of this cross-talk between differentiating neuronal cells and progenitor cells (Figure I.12) has been demonstrated in many studies involving the over-expression and/or loss-of-function of several components of the Notch pathway, including Notch receptors, ligands, downstream targets and proteases (reviewed in (Yoon and Gaiano, 2005)).

The recent discovery of ongoing neurogenesis in adulthood (reviewed in (Riquelme et al., 2008)) and the expression of Notch receptors and ligands in the subventricular zone (SVZ) and hippocampus, the two sites where adult neurogenesis is observed (Givogri et al., 2006; Irvin et al., 2004; Irvin et al., 2001; Stump et al., 2002), has led to the analysis of Notch function in adult stages. The analysis of hemizygous adult mice, carrying only one allele for the Notch1 receptor and the Jag1 ligand, has suggested that the role of Notch signalling in the maintenance of neural progenitors/neural stem cells is extended into adulthood (Nyfeler et al., 2005).

I.2.2.2 Cell fate decisions

The role of Notch in cell fate specification has been explored in great detail in systems such as the developing nervous system of *Drosophila* (reviewed in (Gaiano and Fishell, 2002)) and the *C.elegans* vulva (Sternberg, 2004). In the vertebrate nervous system, this role has been focused mostly on the selection of neural versus glial cell fates (Figure I.13).

Many reports have shown that Notch signalling is required for glial cell specification. The absence of Notch signalling leads to a lack of glial cell production, while over-

activation of the pathway leads to the acquisition of the glial fate at the expense of the neuronal fate (Dorsky et al., 1995; Furukawa et al., 2000; Jadhav et al., 2006a; Scheer et al., 2001). Nevertheless, it has been controversial whether Notch plays a direct or indirect role in this process of neuron-versus-glia decision, i.e., whether it has an instructive or permissive character in glial fate specification. Although both neurons and glial cells appear to derive from a common precursor, neurons are generated earlier during the development of the nervous system, while glial cell are generated later (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). This implies that if the primary function of Notch in maintaining uncommitted progenitor cells is compromised, later cell fates will not be produced. In this case, the function of Notch signaling would be merely permissive.

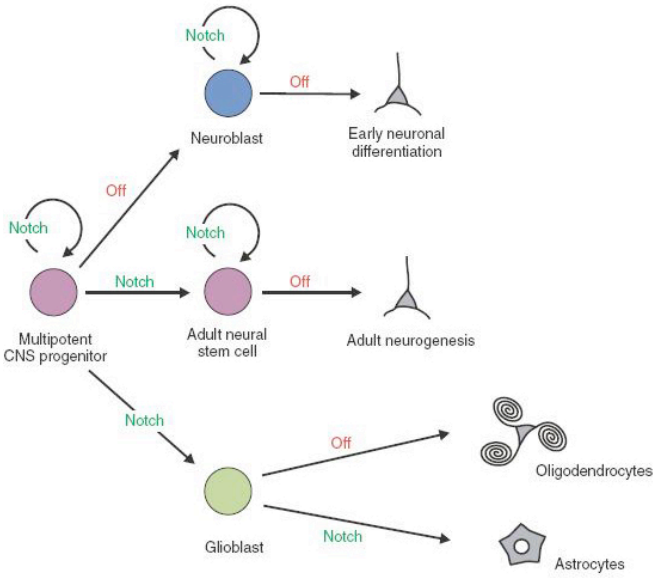


Figure I.13: Notch signalling and cell fate decisions in the vertebrate nervous system.
The role of Notch signalling in cell fate decisions in the vertebrate nervous system has remained until recently limited to the neural versus glial cell fates. Adapted from (Yoon and Gaiano, 2005)

Early studies in the developing neural retina favoured the hypothesis where Notch signalling acts instructively to induce the glial fate, in particular the astrocytic or Muller glial fate. These conclusions arose mainly from studies in which retinal progenitors of the nervous system were forced to express constitutively activated Notch, where it was shown that prolonged activation of the Notch pathway leads cells into acquiring a glial cell fate (Bao and Cepko, 1997; Furukawa et al., 2000; Scheer et al., 2001). Nevertheless, work performed by Bill Harris (Dorsky et al., 1995) and more recently by Cepko's lab (Jadhav et al., 2006a) argues against an instructive role of Notch in inducing the glial fate. By also over-activating the Notch pathway in retinal progenitors, these authors have shown that, although constitutive Notch

signalling causes retinal cells to acquire a glial fate, glial features are only present at the correct developmental stage, despite the constitutive Notch signaling from early embryonic stages.

Until recently, neurogenesis in the neural tube was viewed in a way that may be too simplistic. The neural tube is subdivided into distinct progenitor domains and it has been largely accepted that a distinct neuronal subtype would be generated from each progenitor domain, followed by the production of glial cells (reviewed in (Wilson and Maden, 2005)). Nevertheless, there has been increasing evidence that several neuronal subtypes can be generated simultaneously within the same progenitor domain (Alvarez et al., 2005; Ericson et al., 1997; Karunaratne et al., 2002; Shin et al., 2007; Yeo and Chitnis, 2007). The exact role, if any, of Notch signaling in determining different neuronal cell fate within a single progenitor domain of the neural tube is still not fully understood.

I.2.2.3 Other less explored functions: cell migration, neurite outgrowth and cell survival

In addition to the role in progenitor maintenance and cell fate decisions, other functions have been suggested for the Notch pathway in the nervous system, namely during cell migration, neurite outgrowth and cell survival. In this section, I refer to the evidence indicating a possible role for Notch in these developmental processes and the underlying mechanisms.

Notch was first associated with cell migration upon the analysis of *Dll1*$^{-/-}$ mice, which display a disruption of neural crest migration (De Bellard et al., 2002). Neural crest cells emerge from the dorsal neural tube and migrate segmentally through the rostral half of the somites to their final destinations. In the absence of Dll1-mediated Notch signaling, the migration of these cells is no longer confined to the rostral half of the somites. It was suggested that Notch intervenes in neural crest cell migration by regulating the expression of the EphrinB2 ligand and of the Eph receptors, which are well known for their role in cell migration and axon guidance (reviewed in (Klein, 2004)).

In addition, recent work on the mouse cerebellum, has shown that Notch signaling is required for the proper migration of cerebellar granule cells (Weller et al., 2006). However, the authors of this report suggest that the role of Notch may be indirect. The absence of Notch signaling may lead to defects in the production of glial cells

(late cell fate), which have been shown to be required from neuronal migration (Anthony et al., 2005).
Also, several studies in *Drosophila* have implicated Notch signaling in glial cell migration (Edenfeld et al., 2007) and morphogenetic movements that are involved, for instance, in the formation of the foregut proventiculus (Fuss et al., 2004). Moreover, it has been shown that Notch signaling regulates the expression of cytoskeletal proteins such as Short-stop and RhoA (Fuss et al., 2004; Zecchini et al., 1999), which are essential for cell movement and influence directly or indirectly the dynamics of the cytoskeleton.

Notch function is usually associated to progenitor cells of the nervous system. However, the Notch receptor has been detected in differentiated cortical neurons, where it may have a role in neurite outgrowth and ramification (Franklin et al., 1999; Redmond et al., 2000; Sestan et al., 1999). These studies have shown that Notch signaling determines the length and number of neurites through cell-cell contact. Interestingly, it was also shown that the regulation of neurite outgrowth determined the number of interneuronal contacts, which is increased with Notch activation (Sestan et al., 1999). These results, together with the finding that mice carrying one mutated allele of the Notch receptor display spatial learning and memory deficits (Costa et al., 2003), hint towards a role of Notch in neuronal plasticity. Work done by Furukawa and collaborators, which made use of a transgenic antisense approach, also showed deficits in hippocampal long-term potentiation and memory, thus providing more evidence for a role of Notch in neuronal plasticity (Wang et al., 2004). Nevertheless, the role of Notch in differentiated neurons remains elusive.

The analysis of *Notch1* mouse mutants reveals an increase in the number of apoptotic neural progenitor cells and differentiating neurons in the forebrain (Mason et al., 2006) and cerebellum (Lutolf et al., 2002). These *in vivo* results are in accordance with *in vitro* experiments where Notch was shown to promote cell survival through the up-regulation of anti-apoptotic genes (*Bcl-2* and *Mcl-1*). However it was shown that the expression of the downstream targets (the Hes proteins) is not sufficient to promote cell survival (Oishi et al., 2004). Moreover, the over-expression of a dominant-negative form of RBP-Jκ did not interfere with the ability of Notch to promote cell survival, suggesting that the role of Notch in cell survival is mediated by a RBPJκ-independent mechanism. Another study has shown quite the opposite, that Notch promotes cell death (Yang et al., 2004). Nevertheless, in this study, transgenic

mice carrying 10-20 copies of activated Notch were used, raising the possibility that these high levels of Notch might in some way be toxic for the cell.

I.3 FUNCTION(S) OF THE DSL LIGANDS

I.3.1 DSL ligands in the nervous system

In the vertebrate nervous system, all five Notch ligands are expressed in different spatial and temporal patterns, suggesting that they may play distinct roles. Nevertheless, few studies have been aimed at understanding the function of specific and individual DSL ligands, mainly due to early embryonic lethality, between E9.5 and E11.5, of homozygous mutants for *Dll1* (Hrabe de Angelis et al., 1997), *Dll4* (Duarte et al., 2004) and *Jag1* (Xue et al., 1999), or peri-natally in the case of *Jag2* (Jiang et al., 1998).

Therefore, I will first describe the known expression patterns of each of ligand in the nervous system and the neural phenotypes observed in loss-of-function assays. In a second part, I will focus on various systems in which more than one DSL ligand function together during normal development.

I.3.1.1 *Delta-like 1*

Dll1 is by far the most widely expressed Notch ligand in the developing vertebrate nervous system. It is expressed throughout the anterior-posterior axis of the neural tube (spinal cord and brain), as well as in the developing ear and retina. *Dll1*$^{-/-}$ mice present severe hemorrhage as early as E10 and die at E11.5 (Hrabe de Angelis et al., 1997). *Dll1* is expressed mostly in isolated cells of the CNS that are embarking on differentiation (Bao and Cepko, 1997; Henrique et al., 1995; Lindsell et al., 1996). *Dll1* mutant mice embryos display defects in the CNS with excessive neuronal differentiation at the expense of neural progenitor cells (Hrabe de Angelis et al., 1997; Yun et al., 2002). These results are in accordance with the observed neurogenic phenotype in experiments using a dominant-negative form of the Dll1 ligand in *Xenopus* (Chitnis et al., 1995), zebrafish (Haddon et al., 1998b) and chick embryos (Dorsky et al., 1997; Henrique et al., 1997). The dominant negative form of the Delta protein (and Jagged) corresponds to a truncated form of the ligand lacking

its intracellular domain. This truncated form of Delta has been shown to reduce Notch signalling significantly (Appel and Eisen, 1998; Chitnis et al., 1995; Haddon et al., 1998b; Henrique et al., 1997; le Roux et al., 2003). However, the use of dominant negative forms of DSL ligands result in inhibition of all Notch signaling and does not allow to pinpoint the specific functions of each ligand, individually.

Although the exact mechanisms underlying the dominant-negative effects are not completely understood, it has been proposed that the truncated forms bind directly to endogenous ligands and create an inactive complex, thereby interfering with signal presentation (Chitnis et al., 1995). This mechanism would imply that cells expressing the dominant-negative protein would be unable to signal to neighbouring cells. Instead, this cell would be prone to receiving signals from its neighbours and, therefore, be inhibited from embarking on neuronal differentiation. However, work done by Lewis and collaborators argues against this model, since they clearly showed in the chick retina that isolated cells expressing dominant-negative Delta1 always entered neuronal differentiation (Henrique et al., 1997), hinting that these cells became 'deaf' to signaling form neighbouring cells. A possible explanation is that dominant-negative DSL ligands bind in *cis* to Notch receptors, inhibiting the activation of Notch. Thus, dominant-negative DSL ligands inhibit overall Notch signaling and do not allow the functional analysis of each specific DSL ligand.

I.3.1.2 *Delta-like 3*

The Dll3 ligand is an atypical Notch ligand in the sense that it is the only one unable to activate the Notch pathway and whose complete loss-of-function does not result in lethality (Kusumi et al., 1998). The *Dll3* gene is expressed mainly in the presomitic mesoderm, somites, brain and spinal cord (Campos et al., 2001; Dunwoodie et al., 1997). In the neural tube, *Dll3* is expressed in post-mitotic neurons that are localized at the boundary between the ventricular and mantle zone, along the whole dorsoventral axis (Dunwoodie et al., 1997). Although loss-of-function of *Dll3* leads to a severe disruption in the formation of morphological boundaries between somites (Dunwoodie et al., 2002; Kusumi et al., 1998), mutants show no obvious phenotype in the neural tube. Work performed by Gerry Weinmaster and collaborators suggested that Dll3 in not only unable to activate Notch signaling in cultured cells but also that it is able to antagonize Notch signaling, possibly by competing with the Dll1 ligand (Ladi et al., 2005). Accordingly, neuronal differentiation was promoted when Dll3 was overexpressed in zebrafish (Ladi et al., 2005), contrasting with the opposite

phenotype obtained with overexpression of the Dll1 ligand (Haddon et al., 1998b). However, a role for Dll3 in antagonizing Notch signaling is still controversial, since a recent study that analysed increasing dosages of Dll3 in mice did not observe any evidence pointing towards this, despite confirming the inability of Dll3 to activate Notch (Geffers et al., 2007). Based on domain swapping between the Dll3 and Dll1 ligands, it was suggested that the inability of Dll3 to mediate Notch activation might be due to the almost absent cytoplasmic domain, which does not allow internalization of the ligand and its subsequent activation by post-translational modifications or clustering. Furthermore, Dll3 lacks the two most N-terminal EGFRs when compared with other Dll ligands. These domains were shown to be fundamental for the Notch activating activity of Dll ligands (Geffers et al., 2007).

I.3.1.3 *Delta-like 4*

The Dll4 ligand has received the attention of many research groups due to its restricted expression to the arterial vessels during angiogenesis (Shutter et al., 2000) and its haploinsufficiency: *Dll4*$^{+/-}$ and *Dll4*$^{-/-}$ embryos are embryonic lethal as early as E9.5-E10.5, due to severe vascular defects (Duarte et al., 2004). The early embryonic lethality of these embryos and the lack of conditional knockout mice up until very recently (Koch et al., 2008), had made impossible the analysis of the function of this gene is other tissues where it is expressed (Benedito and Duarte, 2005). In the nervous system, *Dll4* has been shown to be specifically expressed in a single progenitor domain of the neural tube, the p2 domain, and also in the developing retina.

I.3.1.4 *Jag1*

The *Jag1* gene expression pattern was first described in the neural tube, where it is expressed in a complementary pattern to *Dll1* (Lindsell et al., 1996; Lindsell et al., 1995; Myat et al., 1996). This complementary expression of *Dll1* and *Jag1* extends along the whole anterior-posterior axis of the neural tube, with the exception of the developing forebrain, where the expression of the two genes overlaps (Lindsell et al., 1996). A complementary expression pattern between *Jag1* and *Dll1* is also observed in the developing retina (Bao and Cepko, 1997; Lindsell et al., 1996), where *Dll1* transcription is restricted to the neurogenic region where active neurogenesis occurs

while *Jag1* is only expressed in the ciliary margin zone (CMZ), which is considered to be a neural stem region where neither *Notch1*, nor its downstream targets are expressed (Bao and Cepko, 1997; Kubo et al., 2003). *Jag1* is also expressed in the developing inner ear: its expression occurs initially throughout all cells of the presumptive prosensory patch, from where hair and supporting cells will be generated, and later becomes restricted only to the supporting cells (Morrison et al., 1999).

Jag1$^{-/-}$ mouse embryos present severe vascular defects and die at E10.5-E11.5 (Xue et al., 1999). Due to the embryonic lethality of *Jag1*$^{-/-}$ mice, few studies have approached the function of this gene in the CNS and the only reported studies employed misexpression experiments, using the full-length and dominant-negative forms of the Jag1 protein (le Roux et al., 2003). However, these experiments did not uncover any detectable role for the Jag1 ligand in neural tube neurogenesis. The recent generation of conditional knockout mice for the *Jag1* allele, have allowed the approach of the function of the gene in the developing inner ear (Brooker et al., 2006; Kiernan et al., 2006). The function of *Jag1* in the inner ear will be further explored in the next section (Section I.3.2), since this system involves other DSL ligands, namely *Dll1* and *Jag2*, whose functions have also been accessed.

In addition to the developing inner ear, a role for Jag1 in cell migration has also been described in the cerebellum (Weller et al., 2006). Nevertheless, the authors have suggested that the role of Jag1 in migration is an indirect one, since mice lacking *Jag1* expression in the cerebellum display a reduction in Bergmann glia, which act as a scaffold for migrating neuron. Another function that has been associated with Jag1-mediated Notch signaling is the regulation of oligodendrocyte maturation. For instance, it has been shown that Jag1, present in axons of retinal ganglion cells, signals to neighbouring oligodendrocytes in order to inhibit their full differentiation and subsequent myelinization of the axons before they reach their targets (Wang et al., 1998).

I.3.1.5 *Jag2*

Unlike other DSL ligands that are expressed in the neural tube, *Jag2* is not expressed within the ventricular region, being instead expressed in postmitotic neurons located at the ventral horn (Shawber et al., 1996). *Jag2* is transcribed also in the inner nuclear layer of the retina (Valsecchi et al., 1997) and in nascent hair cells

of the developing inner ear (Lanford et al., 2000). *Jag2*$^{-/-}$ mice die immediately after birth due to respiratory problems (Jiang et al., 1998).

I.3.2 Multiple DSL ligands for a single cause

The Notch pathway is a signaling pathway that plays many roles in developmental signalling. How is this signaling achieved? An interesting hypothesis is the differential action of distinct DSL ligands, which are frequently expressed within the same spatial and temporal space. In recent years, the generation of conditional knockout mice has allowed the analysis of the loss-of-function of individual DSL ligands in various systems, where more than one ligand acts to regulate development. Therefore, in this section I will focus on the role of various ligands in three such systems: the inner ear, the skin epidermis and the T-cell hematopoietic lineage.

I.3.2.1 The Inner Ear

The inner ear is responsible both for the sense of hearing and balance. The inner ear derives from an initially simple structure, the otic placode, which undergoes complex morphological changes and gives rise to a membraneous labyrinth with semicircular canal and utricle dorsally, and saccule and cochlea ventrally (Figure I.14, A). In parallel with these morphological changes, two Notch-dependent events take place: first the production of neuroblasts (neurogenesis), followed by hair cell production and differentiation.

Neurogenesis in the vertebrate inner ear occurs at an early developmental stage (E9-E10 in the mouse and HH13 in the chick) (Adam et al., 1998; Morrison et al., 1999), with neuronal progenitors being specified in a restricted domain of the otic placode (Adam et al., 1998). *Dll1* expression is restricted to single cells in this domain, located at the anterior region of the otic vesicle and referred to as the proneural domain, due to the expression of proneural genes. *Dll1*-expressing cells are neuronal precursors that will delaminate into the surrounding mesenchymal space to form the VIII cranial ganglion (Carney and Silver, 1983). Treatment of chick embryos will an inhibitor Notch signaling results in an excessive production of neuronal progenitors (Abello et al., 2007), revealing that Notch signaling regulates the production of neuronal precursors in the inner ear through lateral inhibition.

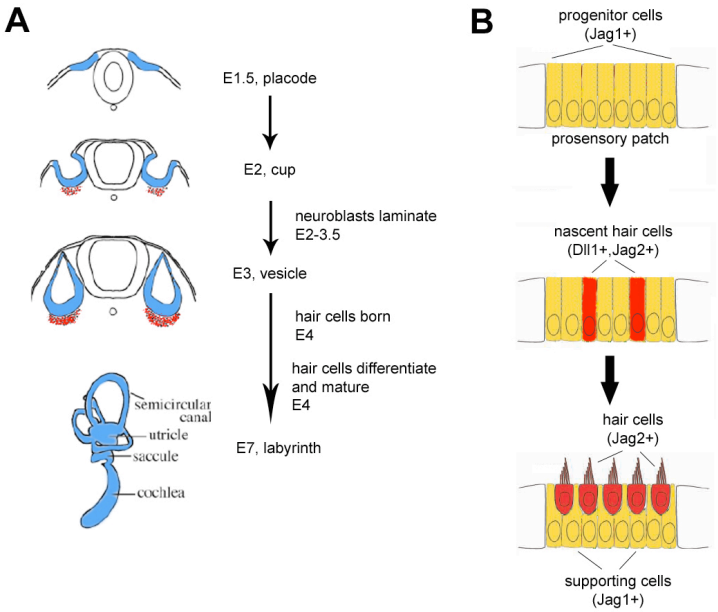


Figure I.14: Stages of inner ear development and expression pattern of *Jag1*, *Jag2* and *Dll1*.
(A) Stages of inner ear development in the chick embryos. **(B)** *Jag1* expression is initiated in progenitor cells (yellow cells) of the prosensory patches of the inner ear, before hair cell (in red) differentiation and later becomes restricted to supporting cells. Nascent hair cells, which arise from the prosensory patches, express *Dll1* and *Jag2*. Adapted from (Adam et al., 1998; Daudet and Lewis, 2005).

After neuroblast delamination has terminated, the first hair cells (HCs) are born in specific domains known as sensory patches. These HCs occur in a salt-and-pepper pattern and express both *Dll1* and *Jag2* (Figure I.14, B) (Adam et al., 1998; Lanford et al., 1999; Morrison et al., 1999), suggesting that HCs production is regulated by Notch-mediated lateral inhibition: differentiating HCs would inhibit neighbouring cells from acquiring the same cell fate by activating Notch through the action of the Dll1 and Jag2 ligands. The analysis of the zebrafish *mindbomb* mutant, where Notch signaling is impaired, provided evidence for this hypothesis, since an excessive production of HCs was observed at the expense of supporting cells (SCs), the alternative cell fate within the sensory patches. In fact, the whole of the sensory patches differentiated into HCs and no SCs were detected (Haddon et al., 1998a). Also, over-expression of the activated form of Notch (NICD) in sensory patches inhibits cells from differentiating as HCs (Daudet and Lewis, 2005). Nevertheless, the picture seems slightly more complex given that the loss-of-function of *Jag2* and/or *Dll1* does not fully phenocopy the results observed in the zebrafish *mindbomb* mutant: although HCs are produced in excess, a depletion in SCs is not observed

(Brooker et al., 2006; Kiernan et al., 2005; Kiernan et al., 2006; Lanford et al., 1999). In addition, NICD is also able to promote HC production in non-sensory regions (Daudet and Lewis, 2005). This is preceded by induction of *Jag1* expression, a marker of presumptive sensory patches (or prosensory patches), quite some time before the onset of hair cell production (Adam et al., 1998; Morrison et al., 1999). Indeed, analysis of conditional knockout mice for *Jag1* has shown that this gene is required at early stages of inner ear development for the establishment of the prosensory regions. In the absence of *Jag1* function, prosensory patch formation is impaired and causes a significant reduction of both the hair and supporting cell population (Brooker et al., 2006; Kiernan et al., 2006).

Thus, the results obtained for the loss-of-function of Jag1 and Dll1/Jag2 ligands point towards a dual role of Notch signaling in the development of the inner ear. First, Jag1-mediated Notch signalling acts to establish groups of cells competent to generate hair and supporting cells in the prosensory patches. In a second phase, Notch signaling acts to limit the number of cells within the prosensory region that acquire the hair cell fate, through lateral inhibition mediated primarily by Dll1 and Jag2.

I.3.2.2 The Skin Epidermis

Distinct populations of stem cells within the mammalian epidermis undergo self-renewal and produce progeny that differentiate into hair follicles (HFs), interfollicular epidermis (IFE) and sebaceous glands (SGs). Several Notch receptors (Notch1-4) and DSL ligands (Dll1, Jag1 and Jag2) are expressed at specific sites in the IFE and HFs (Figure I.15). Notch1 activity alone has been shown to be necessary for SG maturation in the adult epidermis and for proper HF morphology (Pan et al., 2004; Vauclair et al., 2005). Combined loss-of-function of *Notch1* and *Notch2* causes HF defects, also observed in *Presenilin* mutants, but only the triple *Notch1*, *Notch2* and *Notch3* mutant mimics the complete γ-secretase phenotype (Pan et al., 2004).

Loss-of-function studies involving each of the DSL ligands expressed in the epidermis have revealed distinct functions for each ligand. These studies have shown that Jag1 is the primary ligand involved in regulating HF differentiation (Estrach et al., 2006), while Dll1 regulates IFE proliferation and differentiation and Jag2 seems to be dispensable in these decisions. Additionally, a role in cell adhesion has also been attributed to Dll1. It has been shown *in vitro* that IFE cells over-

expressing Dll1 cluster together (Lowell et al., 2000). The cohesiveness between Dll1-expressing cells was shown to be dependent on the cytoplasmic domain of Dll1, more specifically on its PDZ domain and independent of RBP-Jκ (Lowell and Watt, 2001).

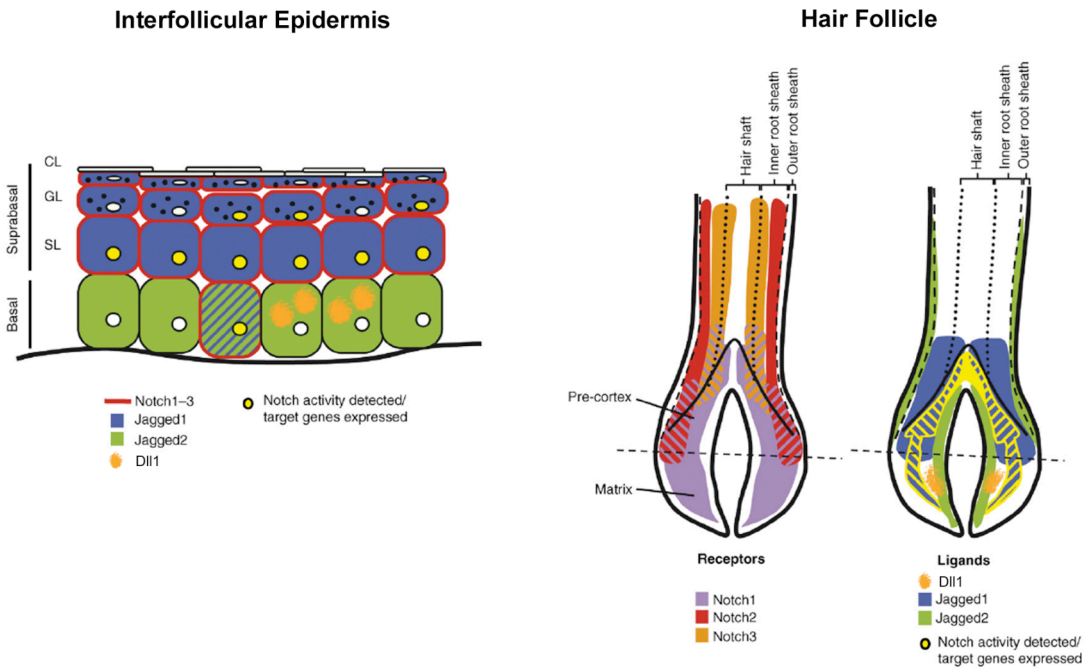


Figure I.15: Expression DSL ligands in the interfollicular epidermis and hair follicle.
In the interfollicular epidermis, *Jag1* is expressed in the suprabasal layers and in some basal cells. *Jag2* expression is restricted to cells of the basal layer, while *Dll1* transcripts are present in clusters of basally localized cells, which possibly correspond to stem cell clusters. In the hair follicle, *Jag1* is expressed in the bulb pre-cortex and suprabasal layers of the outer root sheath, while *Jag2* expression occurs in the follicle bulb and in the basal layer of the outer root sheath. Once again, *Dll1* transcripts are found in clusters of cells at the base of the hair follicle. Adapted from (Watt et al., 2008).

I.3.2.3 T-cell Hematopoietic Lineage

Receptors and ligands of the Notch pathway are widely expressed in the hematopoietic system. Notch activity has been shown to be required at various steps of hematopoiesis (Figure I.16), including specification of the T-cell fate as opposed to the B-cell fate, and possibly also in the specification of cell subtypes within the T- and B-cell lineage subtype (reviewed in (Radtke et al., 2004)). In addition, it is thought that Notch signaling may also play a role in the formation and maintenance of the stem cell niche for hematopoietic stem cells (HSCs) in the bone marrow (Calvi et al.,

2003; Stier et al., 2002; Varnum-Finney et al., 2003; Varnum-Finney et al., 2000). However, studies performed with conditional loss-of-function mouse models fail to confirm a role for Notch signalling in HSC maintenance (Han et al., 2002; Radtke et al., 1999; Saito et al., 2003) and hence, this issue remains controversial and requires further studies. Of the processes mentioned above, the best-known role for Notch in the hematopoietic system is the specification of the T-cell fate. Therefore, in this section I will only focus on recent findings on the role of DSL ligands in promoting T-cell lineage commitment and differentiation.

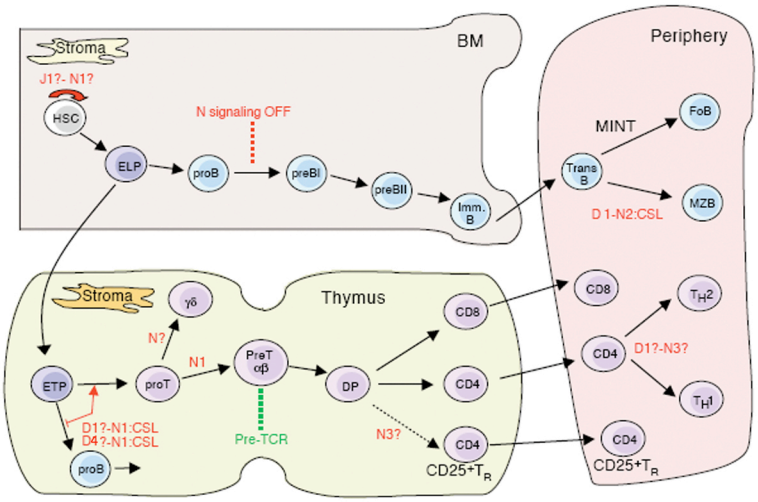


Figure I.16: Notch signalling in lymphogenesis.

Maintenance of hematopoietc stem cells (HSC) within the bone marrow occurs in stem cell niches, which are in close contact to Jag1-expressing osteoblasts. Notch signalling is required to inhibit B-cell commitment and in this way, allow the acquisition of the T-cell fate. Within the T- and B-cell lineages, Notch also participates in subtype cell fate decisions: αβ T-cells versus γδ T-cells and marginal zone B cells (MZB) versus follicular B cells (FoB). Adapted from (Radtke et al., 2004).

It is already known for almost a decade that the conditional inactivation of *Notch1*, (Radtke et al., 1999; Wilson et al., 2001) or *RBP*-Jκ (Han et al., 2002) in bone marrow progenitors causes these cells to differentiate into B-cells in the thymus, instead of T-cells. Multiple Notch receptors (*Notch1-3*) and DSL ligands (*Dll1, Dll4, Jag1, Jag2*) are expressed within the thymus and analysis of mutant mice for each of the expressed receptors has shown that T-cell lineage commitment is dependent solely on Notch1 activity, since neither Notch2 nor Notch3 are able to compensate for the absence of Notch1 function. In fact, *Notch3* mutant mice do not exhibit any hematopoietic phenotype (Krebs et al., 2003) and *Notch2*-deficient mice present

defects at the levels of B-cell subtype specification (Saito et al., 2003). These findings raised the question of which DSL ligand is then responsible for activating the Notch1-dependent T-cell fate commitment.

Given that conditional inactivation of *Jag1* or *Jag2* does not disturb hematopoiesis (Jiang et al., 1998; Mancini et al., 2005), these DSL ligands can be excluded as being the mediators of Notch signalling during the process of T-cell commitment. Thus, Dll1 and/or Dll4 must account for the Notch-dependent commitment to the T-cell fate. The Dll1 ligand was favoured for a long time as the DSL ligand responsible for Notch1 receptor activation during T lymphopoiesis, due to in vitro work showing that *Dll1*-expressing stromal cells were sufficient to drive complete development of mature and functional T-cells (Jaleco et al., 2001; Schmitt and Zuniga-Pflucker, 2002). However, recent reports have demonstrated that *Dll4*-expressing stromal cells are also capable of supporting full T-cell development (Besseyrias et al., 2007; Hozumi et al., 2004). Furthermore, the analysis of mice lacking Dll1 activity in hematopoietic progenitors revealed no defects in T-cell commitment. Instead, *Dll1* mutant mice displayed defects in B-cell subtype specification, a process that is dependent on Notch2 signalling (Hozumi et al., 2004). These results indicate that Dll1 is either not required for T-cell commitment and maturation or that its loss-of-function is compensated by Dll4. Recent analysis of conditionally deleted *Dll4* in the thymus has confirmed that this ligand is indeed essential and sufficient to regulate T-cell lymphopoiesis. Loss-of-function of *Dll4* in the thymus completely blocks T-cell development and leads to ectopic B-cell development within the thymus (Hozumi et al., 2008; Koch et al., 2008). This correlates with the finding that *Dll1* is not expressed in the thymic tissue (with exception of blood vessels) (Koch et al., 2008).

I.4 NOTCH AND DISEASE

The Notch pathway is involved in cell-cell communication between many cell types and in a vast range of tissues, regulating the acquisition of the proper cell fates during embryonic development and also in the adult. Thus, disruption of this conserved signalling pathway has consequences for several cell types in many tissues. Dysregulation of the Notch pathway has been implicated in tumour growth (reviewed in (Dotto, 2008)), in proliferation versus differentiation and also in cell survival. Recently, more attention has been given to brain tumorigenesis, due to the abnormal expression of several Notch ligands observed in tumors of glial origin

(Ignatova et al., 2002). These brain tumours display a decrease in the expression of *Dll1* and *Jag2* but not of *Jag1,* and an increase in the 'stemness' of cells within the tumour. In fact, several studies have observed the existence of neural stem-like cells from glial tumours (Ignatova et al., 2002; Toda et al., 2001). The dysregulation of only some Notch ligands in these brain tumours might be reflective of different Notch roles mediated by each ligand or combination of ligands. Thus a deeper understanding of how ligand-specific Notch signalling determines the outcome is important.

Mutations of Presenilin and the Notch3 receptor are the best characterized in terms of neurological pathologies. For instance, Presenilin, which is required for both Notch and β-amyloid precursor protein (APP) proteolysis, is known to be involved in neuronal diseases such as focal cortical dysplasia (FCD) and Alzheimer's Disease (AD) (reviewed in (Cotter et al., 1999; Selkoe and Kopan, 2003)). FCD is characterized by impaired cortical lamination and by the abnormal presence of neural cells expressing both neuronal and glial markers. These neuronal migration and differentiation defects lead to the disruption of neuronal circuitries and ultimately predispose to a variety of clinical pathologies, the most common being epilepsy (Cotter et al., 1999; Morrison and Nakhutina, 2007). As for AD, which is well known for its progression to dementia, this pathology occurs due to the aggregation of β-amyloid peptides (Aβ-42) into amyloid fibrils. Amyloid peptides (either Aβ-40 or Aβ-42) are generated from sequential proteolysis of APP by β- and γ-secretase activity. Mutations in Presenilin, abrogates the γ-secretase-dependent proteolysis and leads to an increased production of the Aβ-42 peptide, which is more hydrophobic and thus more susceptible to aggregation (Selkoe and Kopan, 2003).

Mutations of the Notch3 receptor has been identified as the cause of CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy), a disease that is predominantly characterized by neurologic pathology (Abe et al., 2002). CADASIL patients show pyschiatric disturbances, migraines, pseudobulbar palsy and progressive vascular dementia (reviewed in (Louvi et al., 2006)). However, Notch3 expression in the adult is virtually restricted to vascular smooth muscle cells (vSMCs) and not present in neural tissue (Prakash et al., 2002; Villa et al., 2001). It is known that the absence of Notch3 in vSMCs results in the death of these cells and although the arteriopathy is widespread, it is the occurance of strokes in brain vessels that accounts for most of the clinical manifestations. Nevertheless, the molecular mechanisms by which Notch3 in vSMCs leads to these neurological pathologies is still not fully understood.

Although Notch signalling is involved in many human diseases, including neurological diseases, further study is necessary to define the precise mechanisms and the developmental context through which the distinct ligands and receptors of this pathway regulate development and affect disease.

I.5 AIMS AND SCOPE OF THIS THESIS

The work described in this thesis aims to understand the role of DSL ligands during vertebrate neurogenesis. I focused my studies on three distinct regions of the CNS, the retina, neural tube and forebrain, where multiple DSL ligands are known to be expressed.

As previously mentioned, null mutants in the various genes encoding DSL ligands are lethal at early stages of embryonic development, with the exception of *Jag2*. This precluded studies on the specific functions of individual ligands during nervous system development. As an alternative, some approaches using gain-of-function experiments and mis-expression of dominant-negative forms of DSL ligands were employed. However, these studies were not very informative. In particular, because dominant-negative forms cause overall inhibition of Notch signalling and does not allow one to access the function of each specific ligand, especially when multiple ligands are expressed in the same tissue. Therefore, in order to gain insight into the function of each ligand, conditional loss-of-function assays are of fundamental importance.

When I started my PhD, gene targeting of mouse embryonic stem cells was the only method to access the loss-of-function of a gene in vertebrates. Given the embryonic lethality of null mouse mutants for all genes encoding DSL ligands, the unavailability of conditional knockout (CKO) mice for the ligands and the time-consuming process of generating CKO lines by gene targeting, I decided to adopt initially a different strategy. In order to understand the role and requirement of multiple DSL ligands during neurogenesis, my first aim was to establish the recently discovered phenomenon of RNA interference (RNAi) as a gene-silencing technique in the chick embryo. This would allow me to approach the function of any gene of interest in an easy and rapid manner. However, the results obtained from several independent experiments targeting distinct components of the Notch pathway (presented in Chapter II) revealed that the RNAi technique is poorly reproducible and that the levels of gene knock-down are insufficient to generate clear and reproducible

phenotypes. I thus decided to continue my studies on the role of several DSL ligands expressed in the nervous system, using knockout and conditional knockout mouse models, which had been generated in the meantime by others.

During this second part of my PhD, I focused on the role of three DSL ligands during neurogenesis: Dll1, Dll4 and Jag1. These three ligands present interesting expression patterns, which are suggestive of distinct roles. *Dll1* and *Dll4* are expressed in overlapping domains both in the retina and in a single domain of the developing neural tube, the pV2 domain. On the other hand, *Jag1* is expressed in a complementary pattern to *Dll1* throughout the whole of the neural tube, with the exception of the forebrain, where the Jag1-expressing domains co-express *Dll1*.

In Chapter III, I provide a detailed description of the expression pattern of the *Dll1* and *Dll4* ligands in the developing retina and pV2 domain of the neural tube. Additionally, I fate mapped these cells, in order to understand if the ligands were expressed in different subpopulations of cells that would acquire distinct neuronal cell fates. These initial observations allowed me to propose a model in which Dll1 and Dll4 are expressed in a sequential manner within the same cell, between commitment to neuronal differentiation and full neuronal differentiation. Functional studies were then performed for the Dll1 ligand through the use of *Dll1* CKO mice. The inactivation of *Dll1* in neural tissue resulted in the expected neurogenic phenotype, however the defects did not phenocopy those obtained with overall inactivation of Notch signalling: cell fate diversity was not compromised.
Most of the results presented in this Chapter have been published in the paper Rocha et al (2009).

Finally, in Chapter IV, I describe the work performed to access the function of *Jag1* in the developing neural tube and embryonic forebrain. I started by analysing the expression pattern of *Jag1* in relation to *Dll1*, which is also expressed in both of these systems. Despite the different spatial relationship between these two ligands (complementary in the neural tube and overlapping in the forebrain), the analysis of the *Jag1* CKO mutant revealed no neural defect, suggesting that Dll1 is able to compensate for the absence of *Jag1* activity. Thus, no evident role was observed for *Jag1* at the levels of cell fate decisions, neural progenitor maintenance, nor cell migration.

CHAPTER II

ESTABLISHMENT OF THE RNAi GENE SILENCING TECHNIQUE IN CHICK EMBRYOS

II.1 INTRODUCTION

The chick embryo has been widely used as a vertebrate model to study embryonic development since the early days of Aristotle, and has contributed significantly to the understanding of embryonic developmental processes (reviewed in (Stern, 2004)). The chick embryo provides an attractive model in embryology due to its accessibility, easy manipulation, efficient *in ovo* electroporation and imaging (Bronner-Fraser, 1996; Muramatsu et al., 1997; O'Donovan et al., 2008; Saunders and Gasseling, 1962). Nevertheless, loss-of-function approaches in the developing chick embryo were limited to the administration of drugs, whose specificity can sometimes be doubtful, or to the misexpression of dominant-negative forms of specific proteins, which act by suppressing the function of the endogenous proteins.

As previously mentioned, the main aim of my PhD project was to understand the function and/or necessity of multiple DSL ligands in the developing vertebrate nervous system. Therefore, it was of fundamental importance to be able to manipulate the function of each of these ligands in a specific manner. Previous studies have made use of dominant-negative forms of DSL ligands, which lack most of their intracellular domains and were shown to block Notch signalling quite efficiently (Appel and Eisen, 1998; Chitnis et al., 1995; Haddon et al., 1998b; Henrique et al., 1997). Although, the mechanism by which these dominant-negative proteins inhibit Notch signaling is not completely understood, it is well established that these truncated DSL ligands block all Notch signaling, irrespectively of the DSL ligand expressed in the tissue of interest. Clear examples of this are several studies that have used a *Xenopus* dominant-negative Dll1 protein to inhibit Notch signalling in zebrafish neurogenesis (Appel and Eisen, 1998; Haddon et al., 1998). The extracellular domain of xDll1 presents a 55%-75% homology with the multiple ligands expressed in the neural tube of the zebrafish and, nevertheless, is able to block Notch signalling very efficiently. Since the dominant-negative form of a single DSL ligand is sufficient to block all Notch signalling, the usage of dominant-negative forms did not seem a valid assay to approach the functional importance of each of the ligands expressed in the chick developing nervous system. Therefore, the establishment of new loss-of-function techniques became fundamental in order to be able to perform functional studies with the chick embryo.

Fortunately or not, the beginning of my PhD coincided with the start of a very “sexy” and promising technology based on the RNA interference (RNAi) gene-silencing phenomenon. RNAi is an evolutionarily conserved process in which double-stranded

RNA (dsRNA) leads to specific degradation of homologous mRNA. Since its discovery in 1998 (Fire et al., 1998), RNAi proved to be capable of silencing almost any gene in organisms such as *C. elegans* and *Drosophila* in a rapid, easy and inexpensive manner, making it one of the most promising and powerful genetic tools. Therefore, due to its great potential in gene manipulation, this technology became a very ambitioned technique in cells and vertebrate models, namely in the chick embryo. Hence, my first aim was the establishment of the RNAi gene silencing technology in the developing chick embryo.

II.2 CHOOSING A STRATEGY TO ESTABLISH RNAi IN THE CHICK EMBRYO

RNAi is now a well-established technique that is widely used in large-scale studies of gene function and therapeutic applications (reviewed in (Boutros and Ahringer, 2008; Kim and Rossi, 2008)). However, when I started my PhD in 2003, the first RNAi-based experiments in mammalian cells had only recently been reported (Caplen et al., 2001; Elbashir et al., 2001a; Xia et al., 2002) and a boom of theoretical and technical information on the mechanisms through which dsRNA-dependent gene silencing occurred, as well as methods of delivering this powerful tool into vertebrate cells, was constantly being published. Therefore, the first aim of my PhD was to develop a strategy that could efficiently knockdown by RNAi the expression of any gene of interest in the chicken embryo. To better explain the options I made, I will start by making a brief introduction on the mechanism governing RNAi-mediated gene silencing and then describe the state of the art, from a technical point of view.

II.2.1 The discovery of RNAi and its molecular mechanism

There is evidence since the early 80's that small RNA molecules are able to negatively regulate gene expression (Izant and Weintraub, 1984; Nellen and Lichtenstein, 1993) but the underlying mechanisms remained largely unclear for more than a decade. In the early 90's, Victor Ambros and collaborators identified a small non-coding gene in *C. elegans*, *lin-4*, that negatively regulates the expression of *lin-14* (Lee et al., 1993). Interestingly, the RNA transcript of *lin-4* displayed antisense complementarity to multiple sites in the 3' UTR region of *lin-14.* Thus, it was hypothesized that small antisense RNA molecules, such as *lin-4* RNA, are able to regulate gene expression by pairing with complementary sequences in the 3' UTR

of target mRNA and thereby repress their translation. However, elegant work performed in *C. elegans* by Craig Mello and co-workers (Figure II.1, a) demonstrated that injection of single-stranded antisense RNA does not lead to gene silencing. Instead, it was shown that it is double-stranded RNA (dsRNA) that has the potential to efficiently silence gene expression (Fire et al., 1998). Accordingly, subsequent studies revealed that naturally occurring small non-coding genes, such as *lin-4*, also known as microRNAs (miRNAs), were predicted to fold into a stem-loop structure. Furthermore, the 'silencing properties' of miRNAs are located within the dsRNA stem-like structure.

II.2.1.1 The RNA interference mechanism

RNA-mediated gene silencing or RNA interference is an evolutionarily conserved pathway in eukaryotic cells, through which dsRNA is used to manipulate gene expression post-transcriptionally (reviewed in (Bartel, 2004)). This process integrates two distinct steps, the initiation and effector steps (Figure II.1, b).

The initiation step involves the cleavage of long dsRNA into smaller fragments known as small interfering RNAs (siRNAs). The cleavage of dsRNA into siRNAs is accomplished by Dicer, a ribonuclease of the RNase III class that yields dsRNA molecules of ~21 base pairs (bps), with characteristic 3' overhangs of 2 nts and 5'-phosphate and 3'-hydroxyl termini (Bernstein et al., 2001; Zamore et al., 2000). These siRNAs are necessary and sufficient to induce RNA-mediated gene silencing. Once generated, siRNAs lead to the activation of the effector step and consequently to the silencing of the target gene.

The effector step starts with the incorporation of the siRNA into a multienzymatic complex, known as the RNA-Induced Silencing Complex (RISC). Once this complex is assembled, it is activated by the unwinding of the two siRNA strands, staying only one of its strands to guide RISC to its homologous target mRNA while the other is degraded. The pairing of the remaining siRNA strand and the target mRNA, leads to the cleavage of the latter at a single site, yielding two products: one without a cap and the other without the poly(A) tail, which confers instability and facilitates its degradation by RNases.

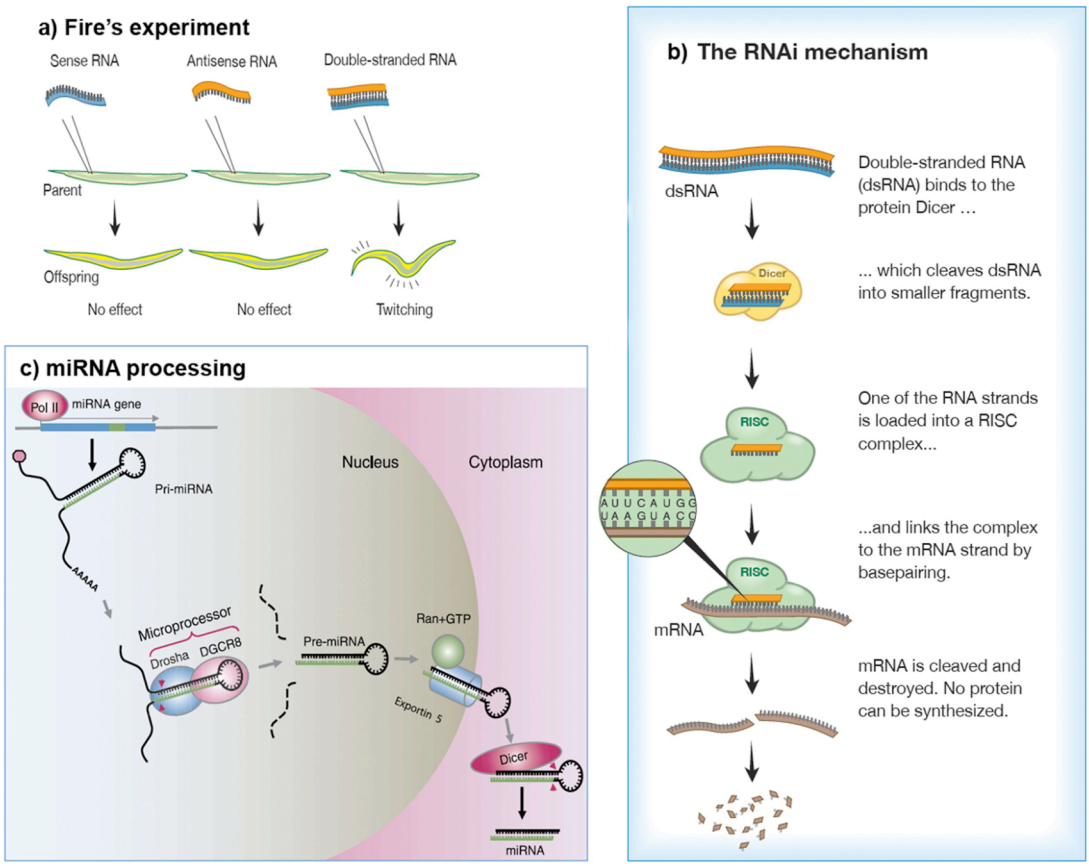


Figure II.1: The discovery and molecular mechanism of RNAi

(a) *Unc-22* is a gene that is abundant in the *C. elegans* muscle and a decrease in its expression levels was already known to produce a severe 'twitching' phenotype. in 1998, Andrew Fire and Craig Mello injected RNA molecules (sense, anti-sense and double-stranded) coding for *Unc-22*, and tested each of these molecules for their ability to silence *Unc-22* expression. The expected phenotype for the loss-of-function of *Unc-22* was only observed upon injection of dsRNA. **(b)** Schematic representation of the RNAi mechanism. **(c)** Processing steps involved in the maturation of endogenously transcribed miRNAs. Adapted from (Gregory and Shiekhattar, 2005).

II.2.1.2 miRNA processing

miRNA are endogenously produced RNA sequences that fold into a stem-loop structure and that are able to mediate gene silencing. miRNAs are initially transcribed as primary transcripts that undergo several maturation steps before becoming 'mature' miRNAs (Figure I.1, c). The primary transcripts, also known as pri-miRNAs, contain a cap and a poly(A) tail, which are cleaved in the nucleus by Drosha, a RNase III endonuclease. Upon cleavage by Drosha, the pri-miRNA yields a shorter stem-loop structure of ~70 nucleotides, termed pre-miRNA., which are subsequently processed to mature miRNAs in the cytoplasm by Dicer. As with long

dsRNA, cleavage of pre-miRNAs by Dicer results in small dsRNAs of ~21bp that are derived from the stem region of the hairpin structure (Lee et al., 2003; Lee et al., 2002b; Zeng and Cullen, 2003). Once the mature miRNA is produced, it makes use of the same molecular machinery as siRNAs to interfere with protein-coding mRNA. Despite siRNAs and miRNAs sharing the same molecular machinery, distinct effector molecules interfere differently with the target mRNA. While siRNAs mediate sequence-specific mRNA degradation, miRNAs lead to translational repression of the target mRNA. Furthermore, and unlike siRNAs, complete complementarity between the miRNA and the target mRNA is not observed (reviewed in (Bartel, 2004)).

II.2.2 Technical advances and limitations in gene silencing by RNAi

II.2.2.1 What to use? Long dsRNA, chemically synthesized siRNAs or vector-based expression of siRNAs/shRNAs?

By 2003, RNAi was already a well established biological tool for gene silencing in organisms such as plants (Chuang and Meyerowitz, 2000; Waterhouse et al., 1998), *C. elegans* (Fraser et al., 2000; Montgomery et al., 1998; Timmons and Fire, 1998) and *Drosophila* (Caplen et al., 2000; Clemens et al., 2000; Hammond et al., 2000). In each of these models, RNAi could be induced by mere injection of dsRNA. Although RNAi proved to be extremely simple and efficient in invertebrates, the application of this promising technique in vertebrate models was still very limited, mainly due to the non-specific effects of long dsRNA. Vertebrate cells respond naturally to the presence of dsRNA by activating an immune response through the induction of interferon synthesis, which leads to non-specific inhibition of protein translation and mRNA degradation (Manche et al., 1992; Minks et al., 1979; Stark et al., 1998). Thus, the endogenous mechanism of gene silencing by RNAi seemed to be inadequate for vertebrates.

Nevertheless studies performed by Tuschl and colleagues (Elbashir et al., 2001a; Harborth et al., 2001) revealed that RNAi-mediated gene silencing involves the cleavage of long dsRNA into ~21 nucleotide RNA fragments (siRNAs). These siRNAs were shown to be sequence-specific effector molecules that are necessary and sufficient for gene silencing. More importantly, dsRNA smaller than 30bp are unable to activate the interferon response (Manche et al., 1992; Minks et al., 1979),

and can by-pass non-specific effects of the interferon response. The first successive attempts to silence vertebrate genes with such small dsRNAs were performed by transfecting chemically synthesized siRNAs into cells (Caplen et al., 2001; Elbashir et al., 2001a; Xia et al., 2002). The transfection of vertebrate cells with siRNAs presented several limitations such as the low efficiency of downregulation of targeted genes, the high cost of chemically synthesized siRNAs, the transient nature of the response due to the rate of cell growth, with the consequent dilution of the siRNAs and the transfection process *per se* (by electroporation or by using lipid-based reagents).

To circumvent the limitations of direct delivery of siRNAs, several groups established vector-based expression of gene silencing effector molecules, i.e., siRNAs or short hairpin RNA (shRNA) structures that resemble miRNA intermediate precursors (Brummelkamp et al., 2002; Lee et al., 2002a; McManus et al., 2002; Miyagishi and Taira, 2002a; Miyagishi and Taira, 2002b; Paddison et al., 2002a; Paddison et al., 2002b; Paul et al., 2002; Sui et al., 2002; Yu et al., 2002). These studies showed that vector-based expression of siRNAs/shRNAs can mediate gene silencing as efficiently, if not more, than that achieved with direct delivery of siRNAs, without triggering the interferon response. Furthermore, expression of these RNAi effector molecules was stable, allowing the analysis of loss-of-function phenotypes over longer time periods. Based on these advantages, I opted to follow a strategy based on siRNA/shRNA-expressing vectors in the chick embryo, instead of using chemically or *in vitro* synthesized siRNAs. Furthermore, electroporation of DNA vectors into the chick embryo is rapid and easy, while previous attempts in the lab to electroporate chemically synthesized siRNAs were unsuccessful.

Whereas injection of long dsRNA in invertebrates yields hundreds of different siRNAs, of which several are bound to target the gene of interest efficiently, the same does not happen when the silencing strategy chosen is reduced to a plasmid expressing a single siRNA/shRNA. Thus, it became necessary to understand the mechanisms through which RNAi occurs and the factors determining the gene silencing efficiency. Which is more efficient, siRNAs or shRNAs? What backbone structure is the best? What promoters to use? And ultimately, which targeting sequence to use?

II.2.2.2 siRNA- versus shRNA-mediated gene silencing

In the first experiments using DNA vectors to drive the expression of siRNAs, these systems were designed in such a way that the antisense and sense strands were synthesized independently and driven from tandem promoters (Miyagishi and Taira, 2002a; Miyagishi and Taira, 2002b). Given that gene silencing by RNAi requires siRNA duplexes (Boutla et al., 2001; Elbashir et al., 2001b; Nykanen et al., 2001), this strategy presents a rate limiting step: these independently transcribed sense and antisense strands still have to pair *in vivo* to form functional siRNAs (Figure II.2, a).

Almost in parallel, a second strategy for generating DNA vector-based expression of siRNAs was developed. The main difference between this strategy and the one described above is that both strands of the siRNA duplex are transcribed in a single RNA molecule, a shRNA (McManus et al., 2002; Paddison et al., 2002a; Paddison et al., 2002b). The main advantage of this strategy is that duplex formation only requires folding back of the hairpin (Figure II.2, b), which should be easier and more efficient than the pairing of two strands synthesized separately within the cell. However, shRNAs require Dicer processing whereas siRNAs do not. Nevertheless, several groups compared the gene silencing efficiency mediated by vector-expressed siRNAs and shRNAs and concluded that shRNAs were equally, if not more, efficient than siRNAs (Brummelkamp et al., 2002; McManus et al., 2002; Miyagishi and Taira, 2002a; Yu et al., 2002). Moreover, this dependency on Dicer activity may actually yield an advantage to shRNAs comparatively to siRNAs, since a second role has been associated with this RNase III. It has been shown that Dicer plays direct role in transporting its cleavage products to the RNA-induced silencing complex, RISC (Liu et al., 2003; Ma et al., 2004; Pham et al., 2004), where targeting of the antisense strand to its complementary mRNA occurs, leading to its subsequent degradation. Taking into account studies comparing the gene silencing activity of vector-expressed siRNAs and shRNAs and the possible structural advantages of shRNAs, I decided to generate vectors expressing shRNAs targeting genes of interest.

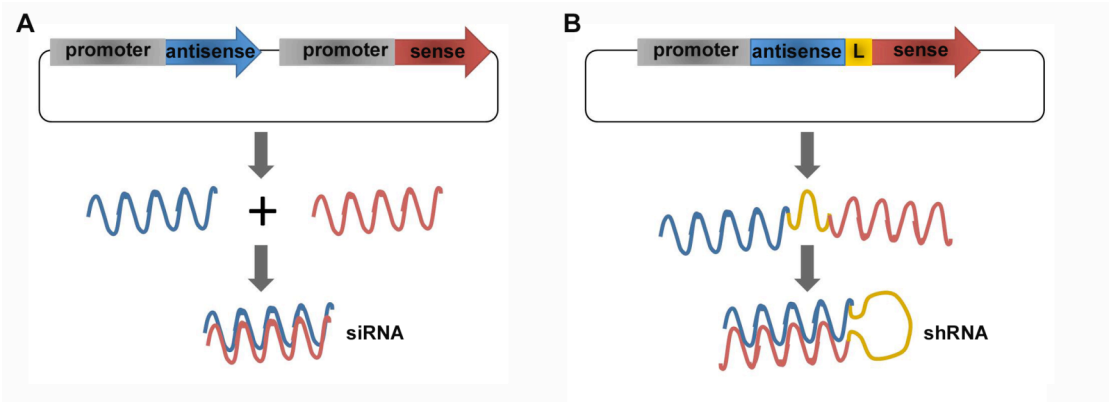


Figure II.2: Strategies for construction of DNA vector-based expression of siRNAs or shRNAs. **(A)** Two tandem promoters are responsible for the independent expression the antisense and sense strands of the siRNA duplex, which thereafter have to pair in the cell. **(B)** One promoter drives the expression of a single molecule containing both sense and antisense strands, linked by a loop (L), forming a shRNA that is subsequently processed by Dicer into a siRNA. Figure adapted from (Miyagishi and Taira, 2002b).

II.2.2.3 Influence of the backbone structure of shRNAs on gene silencing efficiency

Although the siRNA and miRNA pathways function *in vivo* and are conserved among species, siRNAs occur as a response to viral infection: they are the Dicer-mediated cleavage products of long dsRNA fragments produced by viruses. On the other hand, miRNA precursors are encoded within the genome and have been shown to negatively regulate the expression of endogenous genes that present target sequences complementary to miRNAs. These naturally encoded miRNAs are conserved among species, therefore various studies were conducted with the aim of creating hairpins that resemble the naturally occurring precursor molecules of miRNAs and to define sequence/structural requirements necessary for efficient miRNA processing and function in mammalian cells (Zeng et al., 2002).

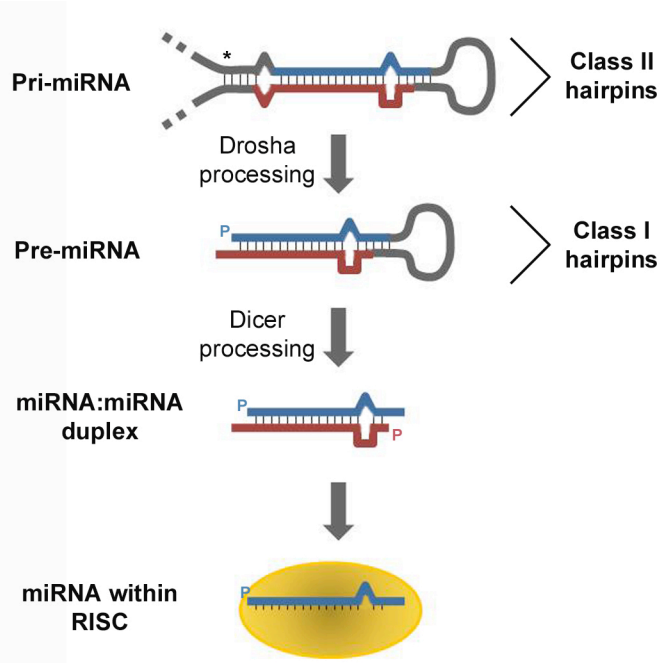
Two main backbone structures were generated that resemble distinct miRNA precursors: pre-miRNAs that are intermediate precursors (class I hairpins) and pri-miRNAs, which are the most immature form of miRNA precursors (class II hairpins; Figure II.3, a). miRNA precursors do not have a perfect hairpin structure, in fact, they present several nucleotide mismatches (bulges) within their stem-like structures. It is the presence of these mismatches that account for gene silencing through inhibition of mRNA translation instead of mRNA degradation. Work performed by various groups that generated several modified forms of class I and II hairpins (summarized in Figure II.3, b), contributed with further understanding to the structural aspects that

are critical for the silencing activity of hairpins (Brummelkamp et al., 2002; McManus et al., 2002; Paddison et al., 2002a; Zeng and Cullen, 2003; Zeng et al., 2002). Modifications altering the position and/or character of the internal bulges of miRNA precursors, or impairing the full complementarity between both strands at the base of the stem (in class II hairpins; asterisk in Figure II.3, a), revealed that the efficiency of miRNA-mediated gene silencing is dependent on the integrity of the stem-like structure of the miRNA precursor and that bulges within the stem add an extra level of complexity (McManus et al., 2002; Paddison et al., 2002a; Zeng and Cullen, 2003). Therefore, it was concluded from these studies that the hairpin structures with highest gene silencing potential were those that exhibited simple hairpins with complete homology. By using the simple class I hairpins, with full complementarity between both strands of the stem and, of course, between the antisense strand and the target mRNA, the production of bonafide siRNAs is assured and the gene silencing efficiency remains solely dependent on the target sequence. In this way, the number of variants influencing the effectiveness of the functional experiments is decreased.

Within the class I hairpins, several structural aspects were assayed, such as the length of the stem, the size and composition of the loop and whether the loop linked to the 5' or 3´end of the antisense strand (Brummelkamp et al., 2002; McManus et al., 2002; Paddison et al., 2002; Zeng and Cullen, 2003). Of these, the linkage of the loop with the antisense strand appeared to affect profoundly the gene silencing activity. Linkage of the loop with the 5' end of the antisense strand resulted in low silencing efficiency, while if linked to the 3' end the hairpin was effective. Although this result was not interpreted mechanistically, work done by Jayasena´s and Zamore´s labs showed that the 5' end of the antisense strand plays a decisive role in assuring the incorporation of the correct strand of the siRNA (the antisense strand) in the silencing complex (see section II.2.5; (Khvorova et al., 2003; Schwarz et al., 2003)). It is possible that the presence of the loop linkage at the 5' end of the antisense strand may hinder this function.

In my work, I decided to use a hairpin structure with an already characterized loop (Brummelkamp et al., 2002), linking the 3' antisense to the 5' antisense strand. Also, since hairpins with full complementarity in the stem region were found to be more effective, nucleotide mismatches were also minimized.

A



B

Modifications	Effect on miRNA expression/processing	Effect on miRNA-mediated gene silencing ability
Vary stem size between 21-29bp (Class I hairpin)	---	↓ moderately for 21bp stem
Decrease size of terminal loop	↓ moderately	↓ moderately
Terminal loop at 5' antisense strand	---	↓ significantly
Terminal loop at 3' antisense strand	---	no effect
Composition of antisense strand	no effect	↓ totally
Alteration of position/character of internal bulges	↓ moderately	↓ moderately
Composition of base of the stem * (Class II hairpins)	no effect	no effect
Pairing of base of the stem * (Class II hairpins)	↓ totally	↓ totally

Figure II.3: Structural modifications in the backbone of miRNA precursors and their impact on gene silencing effectiveness.

(A) miRNA biogenesis pathway depicting the immature and intermediate forms of miRNA precursors.

(B) Effect of modifications introduced at different levels of the precursor backbone structure, on miRNA expression/processing and/or on gene silencing activity.

II.2.2.4 Promoters driving expression of shRNAs

RNA Polymerase III promoters are known to drive the expression of small nuclear RNAs (reviewed in (Paule and White, 2000)) and have been widely used in the generation of siRNA/shRNA-expressing vectors. Most reported assays of RNAi have used the well characterized mouse or human U6 or H1 promoters (Baer et al., 1990; Kunkel et al., 1986; Reddy, 1988), which have been shown to efficiently drive expression of shRNAs. Mammalian U6 promoters have been undoubtedly more frequently used (Katahira and Nakamura, 2003; Lee et al., 2002; McManus et al., 2002; Miyagishi and Taira, 2002a; Miyagishi and Taira, 2002b; Paddison et al., 2002; Paul et al., 2002; Sui et al., 2002; Xia et al., 2002; Yu et al., 2002), than H1 promoters (Brummelkamp et al., 2002; Miyagishi and Taira, 2002a). It is worth noting, that unlike the majority of the transcripts driven by RNA Pol III, U6 transcripts lack any requirement for intragenic promoter elements since all the regulatory sequences of U6 transcripts are localized upstream of the transcript (Kunkel et al., 1986; Reddy, 1988). This characteristic allows one to substitute the endogenous U6 gene with a shRNA, while assuring the maintenance of efficient and high levels of expression. Furthermore, U6 transcripts are maintained in the nucleus (Bertrand et al., 1997; Good et al., 1997), which might contribute to minimize the activation of non-specific immune responses that occur in the cytoplasm. Furthermore, at least one report that directly compared the transcriptional efficiencies of H1 and U6-promoters concluded that U6-driven shRNA expression results in higher gene silencing levels than those of H1-driven expression (Miyagishi and Taira, 2002a).

Work carried out by Engelke and co-workers on the effective expression of small RNA molecules driven by the U6 promoter showed that the presence of a 27nt leader sequence, which comprises the first 27nt of the U6 RNA gene, increases the stability of the uncapped 5'end of the U6 transcript (Good et al., 1997). In fact, the addition of this leader sequence 5´to the shRNA can increase the level of transcripts in the cell up to 12-fold. In addition, the two variants of the U6 promoter, U6(+1) and U6(+27), were compared in their ability to mediate gene silencing (Paul et al., 2002). Although both U6(+1) and U6(+27) promoters led to similar reductions in gene expression, the results obtained with the U6(+27) promoter were more consistent and exhibited greater gene silencing efficiencies. However, it has been suggested that accumulation of shRNA transcripts can induce off-target silencing and activate non-specific effects, such as cellular toxicity and the interferon response (Bridge et al., 2003; Jackson et al., 2003). Therefore, a direct correlation between shRNA levels in the cell and gene silencing efficiency and cannot always be drawn. For this reason

and because the transcriptional activity of the cU6 promoter had not been assayed previously, I decided to generate shRNA-expressing vectors with different transcriptional activities, i.e, vectors with both the U6(+1) and U6(+27) promoters.

Although the vast majority of studies using vector-mediated RNAi in vertebrates made use of the mammalian U6 promoter, these experiments were performed almost exclusively in mammalian cells lines. Until late 2003, only two attempts had been made to introduce this novel technique in the chick embryo using vector-based shRNA (Katahira and Nakamura, 2003; Pekarik et al., 2003). Although these studies reported efficient gene silencing with mammalian U6 promoters, bioinformatic analysis of the human, mouse and chick U6 promoters (hU6, mU6 and cU6 promoters, respectively) reveals a significant divergence in the regulatory regions (Figure II.4), in particular at the proximal sequence element (PSE), an element essential for the formation of a stable transcription complex. Furthermore, slight sequence differences at the PSE have been shown to affect transcription levels (Domitrovich and Kunkel, 2003). I therefore used a chick specific promoter of the encoded U6 snRNA, in order to maximize transcription levels.

```
mU6  cggccccctcgcacagacttgtgggagaagctcggctactcccctgccccggtta--------------------
hU6  cgcgcgccgagcccaaggtcgggcaggaagagggcctatttcccatgattccttcat------------------
cU6  TGAAGAAACGAAGCCAGACCCGGCACTCAGCGGGCAGCCCGCGCCTCCCGCCGCCCCACAGTGCCGCGCGCGTG

mU6  -atttgcatataatatttcctagtaactatagaggcttaatgtgcgataaaagacagataatctgttctttttta
hU6  -atttgcatatacgatacaaggctgttagagagataattagaattaatttgactgtaaacacaaagatattagt
cU6  CATTTGCATGGCGCGGTGCTCGCAGG------------GGGAAACTCACCCCCTCAAGTCCGCCCCCCGCTTCC

mU6  atactagctacattttacatgataggcttggatttctataagagatacaaatactaaattattattttaaaaaa
hU6  acaaaatacgtgacgtagaaagtaataatttcttgggtagtttgcagtttt----aaaattatgttttaaaa--
cU6  CGCCCGCTGTCCCG---CACCTCATCAGTGCTGTGCGCTGTCTGTGTCCCCCAGCACGCACTCTTTG-----CT

mU6  cagcacaaaaggaaactcaccctaactgtaaaagtaattgtgtgttttgagac-tataaatatcccttgga
hU6  -tggactatcatatgcttaccgtaacttgaaagtatttcgatttcttggctttt-tata-------------
cU6  GTTCTTACCCGGAGGCTTGCCCTATCCTTAAGGTTTCTATTTTTTAGG-----TATAAATACTGCT----
```

Figure II.4: Alignment of the nucleotide sequences of the mouse, human and chick U6 promoters.

The distal sequence element (DSE), highlighted in green, is an octamer and 100% conserved among the three species; the proximal sequence element (PSE), composed by 19nts, is highlighted in blue, showing a 79% homology between mammals and only 58% homology between mammals and chick; the TATA box is highlighted in yellow.

II.2.2.5 Criteria for selecting the target sequence for the shRNAs

The selection of the target sequence of the shRNA is the most critical step when using RNAi to silence gene expression. The target sequence is important at several steps of the RNAi pathway and it is insufficient for the antisense strand to be 100% complementary to the target mRNA. Elegant studies have shown that the targeting sequence determines which of the two strands composing the siRNA (sense or antisense) gets loaded into the RISC complex where it mediates gene silencing. Briefly, statistical and *in vitro* studies revealed that the selection of the strand to be incorporated into the RISC depends on a property of the siRNA known as asymmetry: the siRNA strand selected to enter RISC is the one with the most unstable 5' end (Figure II.4, A). Therefore, as silencing will only occur if the antisense strand is incorporated into the silencing complex, it should present a more unstable 5' end comparatively to that of the sense strand. Furthermore, the efficient degradation of target mRNA is dependent on two additional factors. Firstly, the antisense strand should not present a strong secondary structure that might hinder full siRNA:mRNA pairing and secondly, cleavage and subsequent degradation of target mRNA is dependent on a certain instability in the positions 8-12nts (Figure II.4, A). This localized instability confers flexibility to the siRNA:mRNA region, facilitating the access of endonucleases and its cleavage (Khvorova et al., 2003; Reynolds et al., 2004; Schwarz et al., 2003). Thus, the thermodynamic properties of the siRNA duplex determine several sub-steps in the effector step - duplex unwinding, strand selection and mRNA degradation efficiency – and were critical in the design and selection of shRNAs in this work.

II.3 DESIGNING SHRNAS FOR THE NOTCH PATHWAY

Although my particular interest was the functional analysis of the distinct Notch ligands, I decided to establish and optimize the RNAi technique by first designing a principle-of-proof experiment. For this, I chose to target a gene that would inhibit overall Notch signalling in the neural tube. Loss of Notch activity in the embryonic nervous system has been very well characterized and is known to lead to an easily detectable neurogenic phenotype, i.e, excessive neuronal differentiation at the expense of neural progenitors (Baker and Yu, 1997; de la Pompa et al., 1997; Dorsky et al., 1995; Handler et al., 2000; Henrique et al., 1997; Jiang et al., 1996; Lutolf et al., 2002; Ye et al., 1999; Yun et al., 2002). *cNotch1* is expressed

throughout the entire ventricular region of the developing nervous system, while the expression of *cNotch2* is restricted to the floorplate (le Roux et al., 2003; Lindsell et al., 1995). Thus, *cNotch1* is the main Notch receptor expressed in the neural tube and its knock-down should undoubtedly result in a marked neurogenic phenotype as that described for the *Notch1* knockout mouse (Lutolf et al., 2002), irrespectively of the signalling DSL ligand.

The *cNotch1* gene was analysed with several online softwares that select candidate target sequences: siRNA Target Finder provided by Ambion (www.ambion.com/techlib/misc/siRNA_finder.html), RNAi Target Sequence Prediction supplied by BBSRC ChickEST Database (www.chick.umist.ac.uk) and siRNA Target Designer from Promega (www.promega.com/siRNADesigner/program/). Only the coding DNA sequence was used in this analysis, since it is known that the 3' UTR of multiple mRNAs is frequently targeted by a single miRNA. The first studies showing this involved the negative regulation of the *C. elegans lin-14* gene by RNA duplex formation with the *lin-4* gene, which encodes a 22nt RNA (Ha et al., 1996; Lee et al., 1993; Wightman et al., 1993). Furthermore, *lin-4* was shown to bind the 3' UTR of a second gene, *lin-28* leading to its down-regulation (Moss et al., 1997). These experiments showed that the 3´UTR of many mRNAs contain conserved elements that are complementary to certain miRNAs and moreover, that several genes make use of the same sequences and consequently of the same miRNA (reviewed in (Lai, 2002)). Therefore, by not including the 3' UTR in this analysis for siRNA design, sequences that could target several other genes besides my gene of interest were avoided. All the putative target sequences retrieved from the online services referred to above were next subjected to several selection criteria.

First, sequences that that contained four or more consecutive thymidines were eliminated, since the presence of at least four thymidines in tandem defines the transcription termination sequence of any RNA Polymerase III. Second, the thermodynamic characteristics of the remaining sequences were analysed. As previously mentioned, the antisense strand of the siRNA is selected for incorporation into the silencing complex, RISC, when its 5' end is more unstable than the 5' end of the sense strand. Also, unstable pairing between siRNA and mRNA at the site of endonuclease cleavage facilitates mRNA cleavage, which occurs approximately at the tenth nucleotide of the antisense strand and is then followed by mRNA degradation (Khvorova et al., 2003; Reynolds et al., 2004; Schwarz et al., 2003; Silva et al., 2004; Silva et al., 2003). Therefore, target sequences presenting an "A" or a

"T" at its 3' end and/or a "G" or a "C" at nucleotide 10, were not considered. For each of the remaining putative siRNAs, a thermodynamic profile was determined and analysed. The method proposed by Jayasena's lab for determining the thermodynamic profile or stability of RNA duplexes (Khvorova et al., 2003), is based on the Oligo 5.0 Software (MBI Cascade, Co, USA). This program determines the free energy of each base pair in a given sequence by using the nearest-neighbour model (see Section VI.2.1 in Materials and Methods; (Freier et al., 1986; Sugimoto et al., 1994; Xia et al., 1998)). From the various profiles, I selected two *cNotch1*-specific targeting sequences (*A5* and *L2*; Figure II.5, B,C). These *cNotch1* target sequences show all the required hallmarks for efficient incorporation into the silencing complex (exemplified in Figure II.5, A) and subsequent gene silencing: low stability of antisense 5' end compared to antisense 3' end ($\Delta G_{5'end} - \Delta G_{3'end} < -0.35$ kcal.mol^{-1}; (Khvorova et al., 2003)) and low stability in the central region of the antisense strand, where cleavage of the mRNA will occur. Nevertheless, since previous reports have shown that the internal stability of a RNA:RNA duplex can be significantly reduced with the introduction of mismatches (Khvorova et al., 2003; Schwarz et al., 2003; Silva et al., 2003), individual nucleotides in the 3' end of the sense strand were altered (red nucleotides in Figure II.5, B,C), in order to create mismatches and further reduce the stability of the antisense 5' end, without altering its full complementarity with the target mRNA.

shRNA-expressing vectors using both the U6(+1) and the U6(+27) promoters were generated, due to their different transcriptional activities. Given that the U6(+27) promoter transcribes an initial portion of its endogenous snRNA (first 27nts), the target sequence to be cloned downstream of it no longer needs to be initiated with a "G". By no longer restricting putative targeting sequences to only those starting with a "G", I was able to select a sequence with a more favourable energetic profile (higher $|\Delta G_{5'end} - \Delta G_{3'end}|$ value). Such is the case of the *cNotch1 L2* target sequence. Thus the *cNotch1 L2* target sequence was used to create a U6(+27) promoter-driven expression of 29nt shRNA and the *cNotch1 A5* target sequence was used to generate U6(+1) promoter-driven expression of 21nt shRNAs. As negative controls, I either electroporated the empty vector containing only the cU6 promoter or a vector expressing a shRNA targeting a different gene, *cPar3* (see sequence in Section VI.2.2 in Materials and Methods), which is involved in maintaining the integrity of the neuroepithelium and that in principle should not interfere with the Notch pathway.

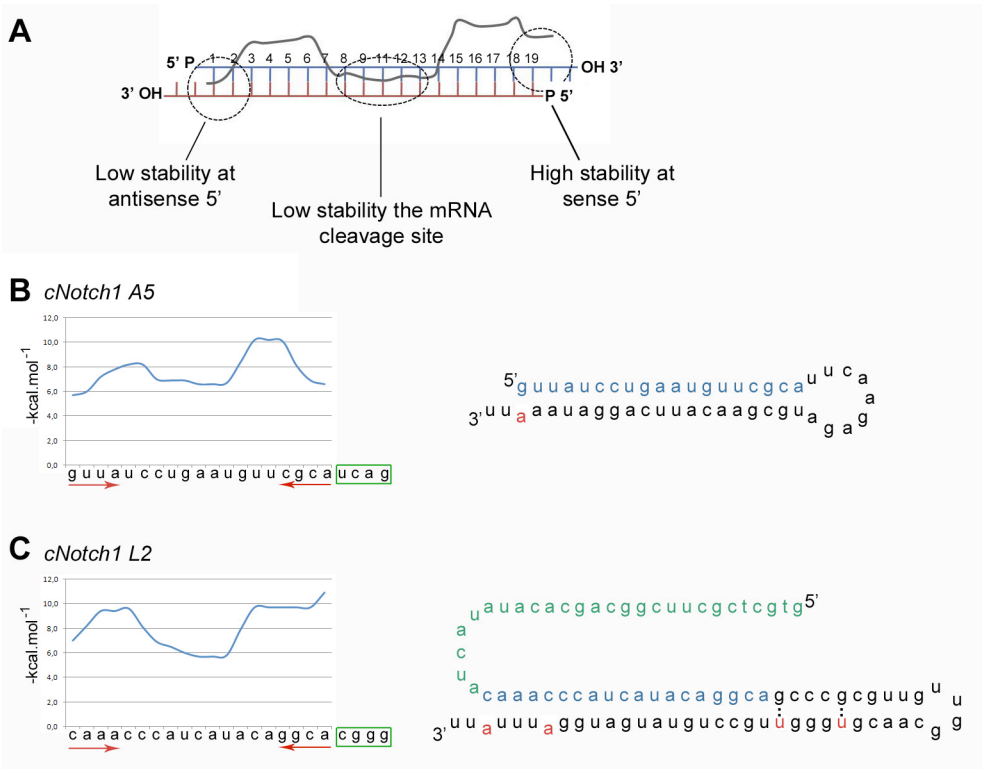


Figure II.5: Thermodynamic profiles, target sequences and hairpin structures of designed *cNotch1* shRNAs.

(A) Scheme depicting the thermodynamic characteristics of a siRNA that are required for efficient gene silencing. The graph overlaid with the siRNA represents the absolute free energy associated with the corresponding base pairs. An efficient siRNA should have low stability (low free energy) in the 5' region of the antisense strand when compared to its 3' region, which facilitates the unwinding and incorporation of the antisense strand into RISC. In addition, and low stability in the central region of the antisense strand, where cleavage of the mRNA will occur. **(B,C)** Thermodynamic profiles of targeting sequences, A5 and L2 respectively, directed against the *cNotch1* gene and hairpin structure adopted. Antisense sequence is indicated below corresponding energetic profile. Hairpin structures were designed so that the loop links to the 3' end of the antisense strand (in blue) and the 5´end of the sense strand. Mismatches created in the sense strand are depicted in red and the leader sequence of the *cNotch1 L2* shRNA is in green.

II.4 LOSS-OF-FUNCTION OF THE NOTCH PATHWAY BY RNAI

The well characterized neurogenic phenotype of mutants of the Notch pathway (Baker and Yu, 1997; de la Pompa et al., 1997; Dorsky et al., 1995; Handler et al., 2000; Hatakeyama et al., 2004; Henrique et al., 1997; Jiang et al., 1996; Lutolf et al., 2002; Ye et al., 1999; Yun et al., 2002), allowed me to easily screen for effective gene silencing in chick embryos electroporated with the vector-expressed shRNAs targeting the *cNotch1* gene. Embryos were analysed at two distinct timepoints, 4-8 hours post-electroporation (hpe) and at 24hpe. The first timepoint allowed me to access the immediate effects on Notch signaling, i.e, the effect on its downstream targets (Figure II.6, A), while possible consequences in neuronal differentiation would be detectable in the second timepoint.

II.4.1 Neurogenesis is not affected by *cNotch1* targeting shRNAs

The neural tube of ~HH11 chick embryos (Hamburger and Hamilton, 1951) was electroporated in parallel with each of the constructs targeting the *cNotch1* gene and control expression vectors. To determine if *cNotch1* had been efficiently silenced, I performed *in situ* hybridization to detect transcriptional activity of this gene (Figure II.6, B,F,J,N). When the shRNA vectors were electroporated into one side of the neural tube (identified as GFP-positive by co-electroporation of a GFP-expressing vector), no difference was observed in the expression levels of *cNotch1* mRNA when compared to the non-electroporated side, both with control vectors (Figure II.6, B,N) and shRNAs vectors designed to knock-down *cNotch1* (Figure II.6, F,J). The absence of a visible reduction in the expression levels of *cNotch1* suggested that the gene silencing technique was ineffective, or that the shRNAs designed to target this gene were not the best. Nevertheless, taking into account that only a fraction of the neural tube cells are electroporated (~1/3), and that RNAi-mediated gene silencing does not results in a knockout situation (complete loss of gene expression) but instead in a partial knockdown of gene expression, I decided analyze the expression of direct downstream targets and neuronal differentiation (Figure II.6, A).

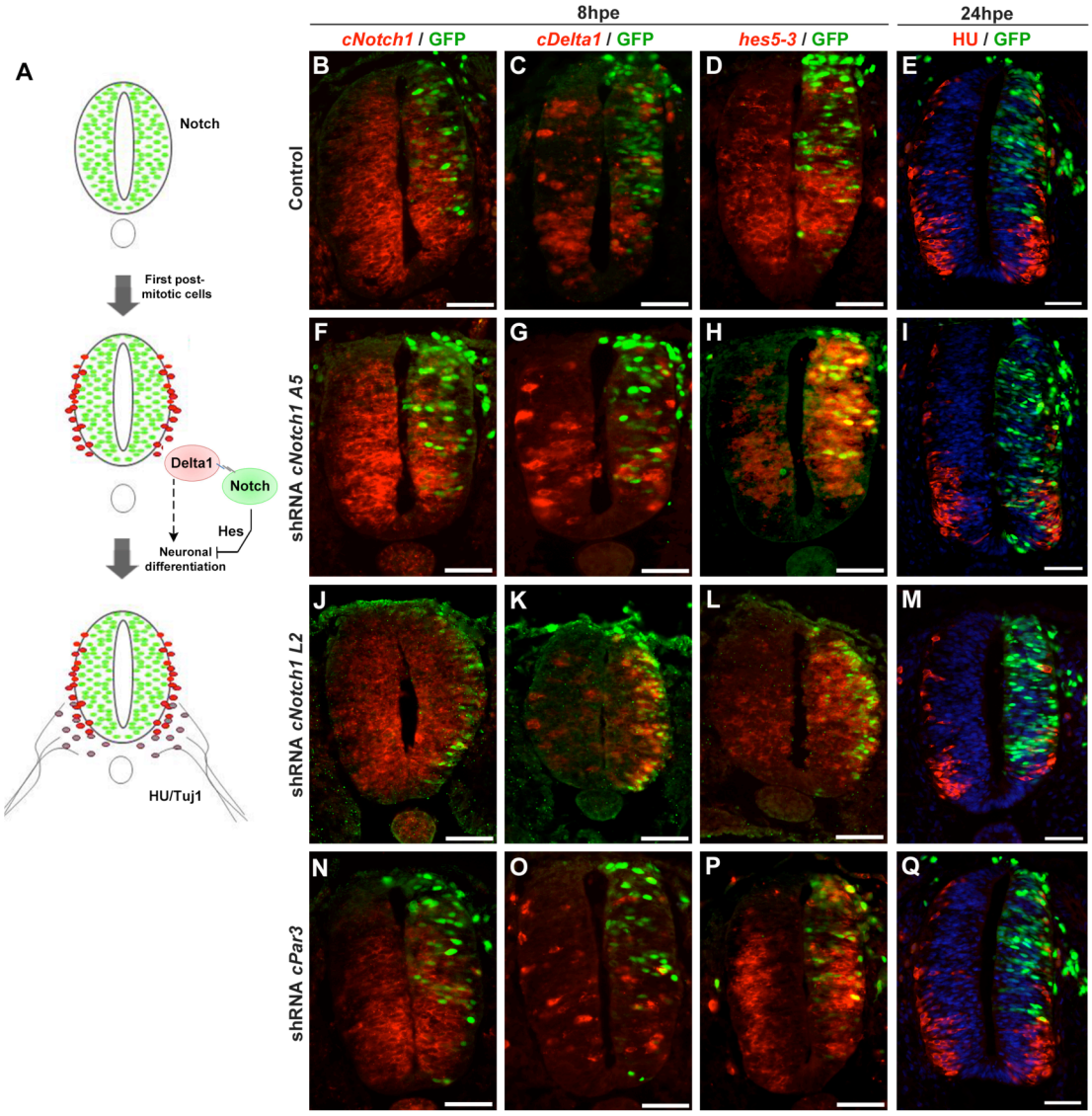


Figure II.6: Effect of shRNAs, targeting the *cNotch1* gene, on the Notch pathway and neuronal differentiation.

The regulation of the rate of neurogenesis is mediated by Delta-Notch signalling, which inhibits neuronal differentiation through lateral inhibition. Sections of embryos electroporated with mock plasmid **(B-E)**, shRNA *cNotch1 A5* **(F-I)**, shRNA *cNotch1 L2* **(J-M)** and shRNA *cPar3* **(N-Q)** were immunostained with HU (24hpe), a marker of differentiated neurons **(E,I,M,Q)** and the expression of distinct components of the Notch pathway were analysed (8hpe): *cNotch1* **(B,F,J,N)**, *cDelta1* **(C,G,K,O)** and *hes5-3* **(D,H,L,P)**. All embryos were co-electroporated with a plasmid expressing eGFP, in order to allow identification of electroporated cells. Scale bars=50μm

Upon reduction of Notch receptor levels, a decrease in the expression of its direct downstream targets (*hes5-1, hes5-2, hes5-3*) is expected, together with an increase in the number of cells embarking on neuronal differentiation that express *cDelta1*. Analysis of embryos collected 8hpe, revealed that the majority did not present

differences in the expression levels of the downstream targets, nor in the number of cells expressing *cDelta1* (Table II.1). Furthermore, when embryos collected 24hpe were immunostained for HU, a marker for differentiated neurons, no difference could be observed in the number of differentiated neurons between the electroporated and non-electroporated side of the neural tube (Figure II.6, E,I,M,Q; n=12 and n=10 for shRNA *cNotch1 A5* and *L2*, respectively). However, a reduced number of embryos exhibited an increased not only in *cDelta1* expression (Figure II.5, C,G,K,O and Table II.1) but also of the downstream targets (results shown only for *hes5-3*; Figure II.5, D,H,L,P and Table II.1). Assuming that these observations are a consequence of the down-regulation of the *cNotch1* gene, a simple interpretation of these apparently conflicting results might be that the ectopic *cDelta1*-expressing cells activate the Notch pathway in neighbouring wild-type cells, causing up-regulation of *hes5-3.* This would be a cell non-autonomous effect. Actually, the results show that the majority of the *hes5-3*-expressing cells were not electroporated with the *cNotch1* shRNA expressing vectors, confirming that the upregulation of this gene is cell non-autonomous (Figure II.6, H,L). Only a very restricted number of the embryos electroporated with *cNotch1* targeting shRNA-expressing vectors displayed the expected phenotype, with an increased number of cells embarking on differentiation (*cDelta1*-expressing cells). This could be due to either a weak or unstable expression of cU6-driven transcripts, an unspecific off target effect or simply due to poor reproducibility of the RNAi technique.

II.4.2 cU6 promoter shows high levels of expression

In order to approach the hypothesis involving the stability and expression levels of cU6 transcripts, a ~250bp fragment from the ampicillin resistence gene was cloned downstream of the cU6 promoter (cU6:Amp-snRNA). Embryos were electroporated with the cU6:Amp-snRNA vector and harvested at 6hpe and 24hpe. I performed whole mount *in situs* for the 'Amp snRNA' fragment and observed that the cU6 promoter drives high levels of expression at least until 24hpe (Figure II.7). Therefore, it seems unlikely that low gene silencing efficiency is due to low levels of shRNA expression.

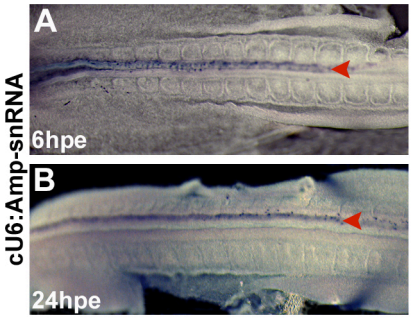


Figure II.7: Expression levels of cU6 transcripts are high and stable

II.4.3 RNAi-mediated gene silencing in the chick embryo is not reproducible

Embryos electroporated with the control vector expressing shRNAs targeting the *cPar3* gene never presented unspecific up-regulation of *hes5.3* or *cDelta1* expression (Figure II.6, O,P and Table II.1). These results suggested that it would be rather unlikely for the neurogenic phenotype, observed in only a small percentage of the embryos electroporated with *cNotch1* targeting shRNA-expressing vectors, to be non-specific. Nevertheless, in order to understand if the transcriptional changes observed were specific or mere off-target effects of RNAi, vectors expressing shRNAs targeting another two components of the Notch pathway, *Presenilin1/2* (Figure II.8, A) and *Suppressor of Hairless* (*SuH*; Figure II.8, B), were generated. Both genes encode for proteins that are essential for activation of the Notch pathway. Note that in the chick embryo there are two isoforms of the *Presenilin* gene (*Presenilin1/2*), therefore a shRNA was designed to target a region of homology between both isoforms. In this way, redundancy should not be able to mask a possible silencing effect of the RNAi.

As described previously for the RNAi-mediated gene silencing of *cNotch1*, the majority of the embryos electroporated with vectors expressing shRNAs targeting *Presenilin1/2* or *SuH* did not present any phenotype (Table II.1). In fact, none of the shRNA *SuH* embryos were any different from wild-type embryos, whereas 2/17 embryos electroporated with shRNAs targeting *Presenilin1/2* exhibited upregulation of *cDelta1* and *hes5-3*.

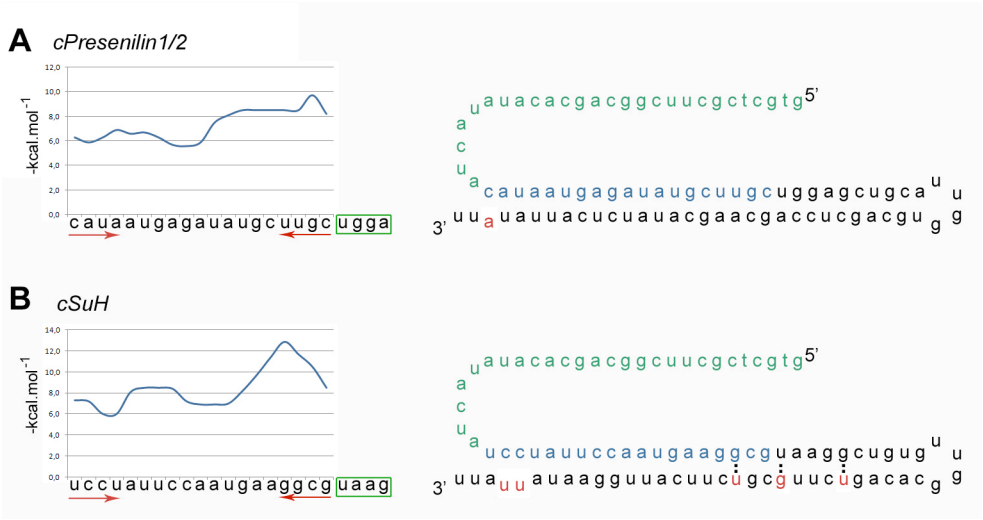


Figure II.8: Thermodynamic profiles, target sequences and hairpin structures of designed *cPresenilin1/2* and *cSuH* shRNAs.

Thermodynamic profiles of targeting sequences directed against the *cPresenilin1/2* **(A)** and *cSuH* **(B)** genes and hairpin structure adopted. Antisense sequence is indicated below corresponding energetic profile and nucleotides in green boxes are the immediate downstream nucleotides taken into consideration when determining the stability of the 3' end of the antisense strand (see Section VI.2.1 in Materials and Methods). Hairpin structures were designed so that the loop links to the 3' end of the antisense strand (in blue) and the 5´end of the sense strand. Mismatches created in the sense strand are depicted in red and the leader sequence of the shRNAs is in green.

These results indicate that the increase in *cDelta1* and *hes5-3* expression levels, are most probably due to RNAi-mediated gene silencing of genes involved in the Notch pathway. Nevertheless, only a very reduced number of electroporated embryos reveal this phenotype, despite high and stable transcription of the cU6-driven expression of shRNAs. It thus seems that shRNA-mediated gene silencing in the chick embryo is not very reproducible.

Table II.1: Effect of different shRNAs targeting distinct components of the Notch pathway.
cN1=cNotch1; cPsen1/2=cPresenilin1/2; cSuH=cSuppressor of hairless

No. Embryos	**Electroporated shRNA construct**					
	GFP	***cPar3* + GFP**	***cN1-A5* + GFP**	***cN1* L2 + GFP**	***cPsen1/2* + GFP**	***cSuH* + GFP**
Total	36	28	33	123	17	15
↑ cDll1 / hes5-3	0	0	3	8	2	0

II.5 DISCUSSION

RNAi is an eukaryotic regulatory mechanism that uses dsRNA molecules to modulate gene activity. This is accomplished by degrading mRNA that exhibits full complementarity to the target sequence of the dsRNA. Thus, this evolutionarily conserved gene silencing mechanism acts in a sequence specific manner. The discovery of the RNAi gene silencing mechanism has undoubtedly opened many doors in several fields of research. Not only did reverse genetic approaches became available for animal models that until recently presented limited genetic manipulation possibilities, such as the chick embryo, but also introduced a novel and rapid way of performing large-scale screening for novel genes involved in tissue- or pathway-specific functions. Furthermore, RNAi has been extensively used in experiments aimed at developing therapeutic approaches to control disease-associated genes. It is thus easy to understand the impact that the discovery of the RNAi gene silencing mechanism has brought to the biological world.

It was with the perspective of generating the loss-of-function phenotypes for any gene of interest in a simple, robust and reproducible manner that my first year and half of my PhD was dedicated to the establishment of the RNAi technology in the chick embryo, the animal model used in the lab at the time. However, I was unable to set this technique to function in a consistent and reproducible manner. Although three distinct components of the Notch pathway, *cNotch1, cPresenilin1/2* and *cSuH*, were targeted, a neurogenic phenotype was only observed in 6% - 9% of all electroporated embryos. Thus, I concluded that gene silencing by RNAi was not sufficiently reproducible in order to be used as a standard technique. Furthermore, besides the low reproducibility, the neurogenic phenotype observed was also questionable, since the increase in *cDelta1*-expressing was not followed by an increase in the number of differentiated cells, as it is known to occur in the neural tube of *Notch1* knockout mice (Yang et al., 2006) and triple *Hes1,Hes3,Hes5* mutant mice (Hatakeyama et al., 2004), where complete impairment of Notch signaling leads to massive neuronal differentiation.

Currently, there are few reports on RNAi-based loss-of-function assays performed in chick embryos. In fact, some of these reports reached the same conclusion and describe the RNAi technique as an ineffective method for routine gene silencing in chick embryos (Alvarez et al., 2006; Hernandez and Bueno, 2005). Work performed by Hernandez and Bueno is in agreement with the results reported in this thesis: the authors observe no decrease in the expression levels of the targeted genes, despite

the employment of several strategies and targeting sequences (Hernandez and Bueno, 2005). Furthermore, off-target effects have been reported in RNAi-mediated gene silencing in the chick nervous system (Alvarez et al., 2006). Importantly, it has been shown that the off-target effects of RNAi are not restricted to the induction of the interferon response but can also affect expression of other non-related genes. Studies concerning miRNA-mediated gene silencing have revealed that as little as 6-7nt of the miRNA sequence, the seed, is sufficient to trigger the silencing of genes (Anderson et al., 2008; Lim et al., 2005). This adds the additional complication that siRNAs designed to target regions within the 3'UTR of several mature transcripts may also target other genes than the gene of interest.

Nevertheless, several groups have reported successful gene knockdown using RNAi in the chick (Chesnutt and Niswander, 2004; Dai et al., 2005; Das et al., 2006; Katahira and Nakamura, 2003; Kawakami et al., 2003; Pekarik et al., 2003). In fact, some of these groups have used RNAi as a standard technique. For instance, Stoeckli and co-workers have systematically used long dsRNA, ranging from 400bp to ~2kb, to silence genes of interest (Baeriswyl and Stoeckli, 2008; Bourikas et al., 2005; Mauti et al., 2007; Pekarik et al., 2003). Although it is known that long dsRNA activates the interferon response, none of these studies did controls to verify the absence of apoptosis due to the activation of the immune response (Bridge et al., 2003; Sledz et al., 2003). Although off-targets effects have been widely reported, many of the studies performed have not done sufficient controls to exclude off-target phenotypes. An example of this, is work done by Belmonte and co-workers where they approach the function of the *Mkp3* gene (Kawakami et al., 2003). Since the expression of this gene is regulated by the PI(3)K/Akt pathway, which is known to be anti-apoptotic, the authors reasoned that Mkp3 could have a role in preventing apoptosis during limb formation. To test this hypothesis, vectors expressing siRNAs targeting the *Mkp3* gene were used and an increase in apoptosis was observed. However, whereas 40% of the electroporated embryos presented an increase in apoptosis, a mere 18% exhibited downregulation of the target gene, Mkp3. Furthermore, the control assay was limited to electroporation with a vector expressing GFP, which does not allow one to eliminate the possibility of siRNA-induced apoptosis.

Furthermore, although some of the studies using RNAi in chick embryos have reported down-regulation of the targeted genes, the degree of gene silencing has been quite modest (Chesnutt and Niswander, 2004; Dai et al., 2005; Katahira and Nakamura, 2003) and in some cases, not sufficient for a detectable functional

phenotype. Such is the case for the down-regulation achieved for the *En1/2* genes by expression of a shRNA complementary to both genes (Katahira and Nakamura, 2003), which fail to show any morphological different of the mid-hindbrain boundary, despite previous experiments having shown that *En1*$^{-/-}$,*En2*$^{-/-}$ mouse embryos lack the mid-hindbrain region (Liu and Joyner, 2001).

Recently, Stuart and colleagues designed a novel strategy for optimal gene silencing in the chick embryo, based on retroviral RNAi vectors expressing miRNAs (Das et al., 2006). In order to maximize miRNA processing by Drosha and Dicer, a conserved chicken operon was adapted. Interestingly, the *cNotch1* gene is also targeted in this study. Cells electroporated with the *cNotch1* miRNA and therefore, cells in which Notch signalling should be reduced/abolished, are all localized to the mantle zone of the neural tube, whereas embryos electroporated with a mock miRNA present evenly distributed electroporated cells between the ventricular and mantle zones. This result is in agreement with the predicted: cells devoid of Notch signalling escape lateral inhibition and differentiate. Nevertheless, although the results indicate that the Notch pathway was inhibited in the electroporated cells, the phenotype observed is far from presenting the severe phenotypes obtained from complete knockout models (Hatakeyama et al., 2004; Lutolf et al., 2002; Yang et al., 2006).

Overall, RNAi-mediated gene silencing in the chick embryo does not seem reliable, nor efficient. The work here presented, and the work published by others (Alvarez et al., 2006; Hernandez and Bueno, 2005) has shown that the phenotypes obtained by RNAi have very low reproducibility and that the levels of gene knock-down are insufficient for one to obtain clear and obvious phenotypes, contrasting largely with complete knockout models. For all these reasons, I continued my studies on the role of DSL ligands expressed in the nervous system using knockout and conditional knockout mouse models.

CHAPTER III

DLL1 AND *DLL4* FUNCTION SEQUENTIALLY, IN THE RETINA AND pV2 DOMAIN OF THE SPINAL CORD, TO REGULATE THE RATE OF NEUROGENESIS AND CREATE NEURAL CELL DIVERSITY

Most of the results presented in this chapter have been published in the paper:

Rocha, SF. *et al* (2009), *Dll1* and *Dll4* Function Sequentially, in the Retina and pV2 Domain of the Spinal Cord, to Regulate the Rate of Neurogenesis and Create Neural Cell Diversity, *Developmental Biology*, **328**, 54-65

III.1 INTRODUCTION

The vertebrate central nervous system (CNS) is composed by a wide variety of neuronal and glial cell types that are reproducibly generated in the correct proportion among individuals of the same specie. Therefore, development of the CNS requires a fine regulation of neuronal production in time, space, number and identity. In the embryonic neural tube, cell diversity is first established through a morphogenic gradient of Sonic Hedgehog (Shh) that specifies distinct progenitor domains along the dorsoventral (DV) axis. Within each domain, progenitors have a well-defined identity and are competent to produce only a restricted set of neural cell types during development (reviewed in (Briscoe and Novitch, 2008; Jessell, 2000)). However, little is known about how the final balance of the various cell types in each domain is achieved, whether it involves interactions with other cells or is mainly determined by an intrinsic program. In other regions of the developing CNS, like the neural retina, neurogenesis is regulated in a different way: progenitors are not organized into specific domains and seem competent to generate all the cell types present in the adult tissue. Thus, various mechanisms must be in place at different regions of the developing nervous system to regulate the fine-grained patterns of neural differentiation in the vertebrate CNS.

The Notch pathway has been implicated in several aspects of neural development, from the generation and maintenance of neural progenitors to the control of dendritic morphology (reviewed in (Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005)). A role for Notch signalling in controlling cell type specification is also well established in *Drosophila*, in particular during the generation of the various cell types that form sensory organs in the fly peripheral nervous system. During the process, five different cells are formed by three consecutive asymmetric cell divisions, starting from a single progenitor, and the final fate of each cell is determined by successive signalling events mediated by the Notch receptor (Bardin et al., 2004). A role for Notch signaling in controlling cell fate diversity during vertebrate neurogenesis has been also reported in the chick and mouse spinal cord pV2 domain (Del Barrio et al., 2007; Peng et al., 2007) and was recently extended to the epiphysis and spinal cord of zebrafish embryos (Cau et al., 2008; Shin et al., 2007; Yeo and Chitnis, 2007). In the vertebrate inner ear and retina, Notch activity is also needed to diversify cell fates, as shown by various experiments where absence of Notch signaling results in an unbalanced production of specific cell types in these organs (Brooker et al., 2006; Daudet and Lewis, 2005; Kiernan et al., 2005; Kiernan et al., 2006). For instance,

treatment of the developing inner ear with inhibitors of the Notch pathway caused the formation of sensory patches devoid of supporting cells and composed solely of hair cells (Daudet and Lewis, 2005; Takebayashi et al., 2007), showing that Notch signalling regulates a binary decision between these two types of cells in the otic epithelium. In the retina, chemical inhibition of Notch (Kubo et al., 2005) or genetic inactivation of *Notch1* signaling (Jadhav et al., 2006b; Yaron et al., 2006) also disturbs cell fate specification, causing the formation of an increased number of cone photoreceptors, at the expense of other retinal cell types. Thus, in addition to its well-known function in controlling progenitor availability during vertebrate neurogenesis, Notch signalling also plays an important role in neuronal type diversification.

Whether these multiple functions of the Notch pathway rely on the reiterated usage of the same molecular components at different steps of neuronal production, or involve distinct circuitries to control independently each step is not yet known. Vertebrates contain various genes encoding DSL ligands, which are all expressed in the developing vertebrate nervous system with distinct spatiotemporal patterns. An attractive possibility is that different ligands mediate distinct Notch functions at each step of the neurogenesis process. These ligands belong to two groups of structurally distinct proteins, those with a structure related to the *Drosophila* Delta protein and those related to the *Drosophila* Serrate protein (also named Jagged in vertebrates. Mice, for instance, have two genes encoding Jagged ligands, *Jagged1* (*Jag1*) and *Jagged2* (*Jag2*), and three genes encoding Delta ligands, *Delta1*, *Delta3* and *Delta4* (also known as *Dll1*, *Dll3* and *Dll4*). Two of these genes, *Jag1* and *Dll1*, are expressed with striking complementary patterns in the embryonic neural tube, in cells committed to neuronal differentiation (Lindsell et al., 1996; Myat et al., 1996), while *Dll3* and *Jag2* are expressed later in differentiating neurons across all dorso-ventral (DV) domains of the neural tube (Dunwoodie et al., 1997; Kusumi et al., 1998; Shawber et al., 1996; Valsecchi et al., 1997). Recently, *Dll4* was reported to be expressed in a restricted domain of the neural tube, the pV2 domain and in the developing mouse retina (Benedito and Duarte, 2005), two regions where *Dll1* is also expressed. The *Jagged* genes are also expressed in the developing neural retina, *Jag1* in cells of the ciliary margin and lens (Bao and Cepko, 1997; Lindsell et al., 1996), and *Jag2* in differentiated retinal ganglion cells (RGCs), after they reach the ganglion cell layer (Valsecchi et al., 1997).

These various expression patterns of Notch ligands in the developing mammalian CNS reveal that different regions of the embryo employ distinct genetic networks to regulate neuronal production, probably reflecting the existence of region-specific strategies to assemble functional circuits in the nervous system. However, little is known about the specific role of each ligand in these networks and how they contribute to the multiple functions of Notch signaling during vertebrate neurogenesis. Lack of detailed loss-of-function studies for each of the Notch ligands in the developing nervous system has prevented the elucidation of these questions, mostly due to the early embryonic lethality of mice carrying mutations in the various *Dll* and *Jag* genes (Louvi and Artavanis-Tsakonas, 2006).

In this Chapter, I describe the work done to obtain a detailed analysis of the expression of *Dll1* and *Dll4* during neurogenesis in the mouse neural retina and spinal cord. To address the functions of *Dll1* and *Dll4*, I analysed mice where *Dll1* is conditionally inactivated in the developing CNS. My results support a model where the two Dll ligands regulate subsequent steps of neuronal production in the retina and pV2 domain: while Dll1 serves primarily to activate Notch signalling in neighbouring progenitors and prevent their untimely differentiation, Dll4's main function is to generate diversity within the population of differentiating neurons.

III.2 THE DLL1 AND DLL4 LIGANDS IN THE DEVELOPING MOUSE RETINA

III.2.1 *Dll1* and *Dll4* are expressed sequentially in the developing mouse retina

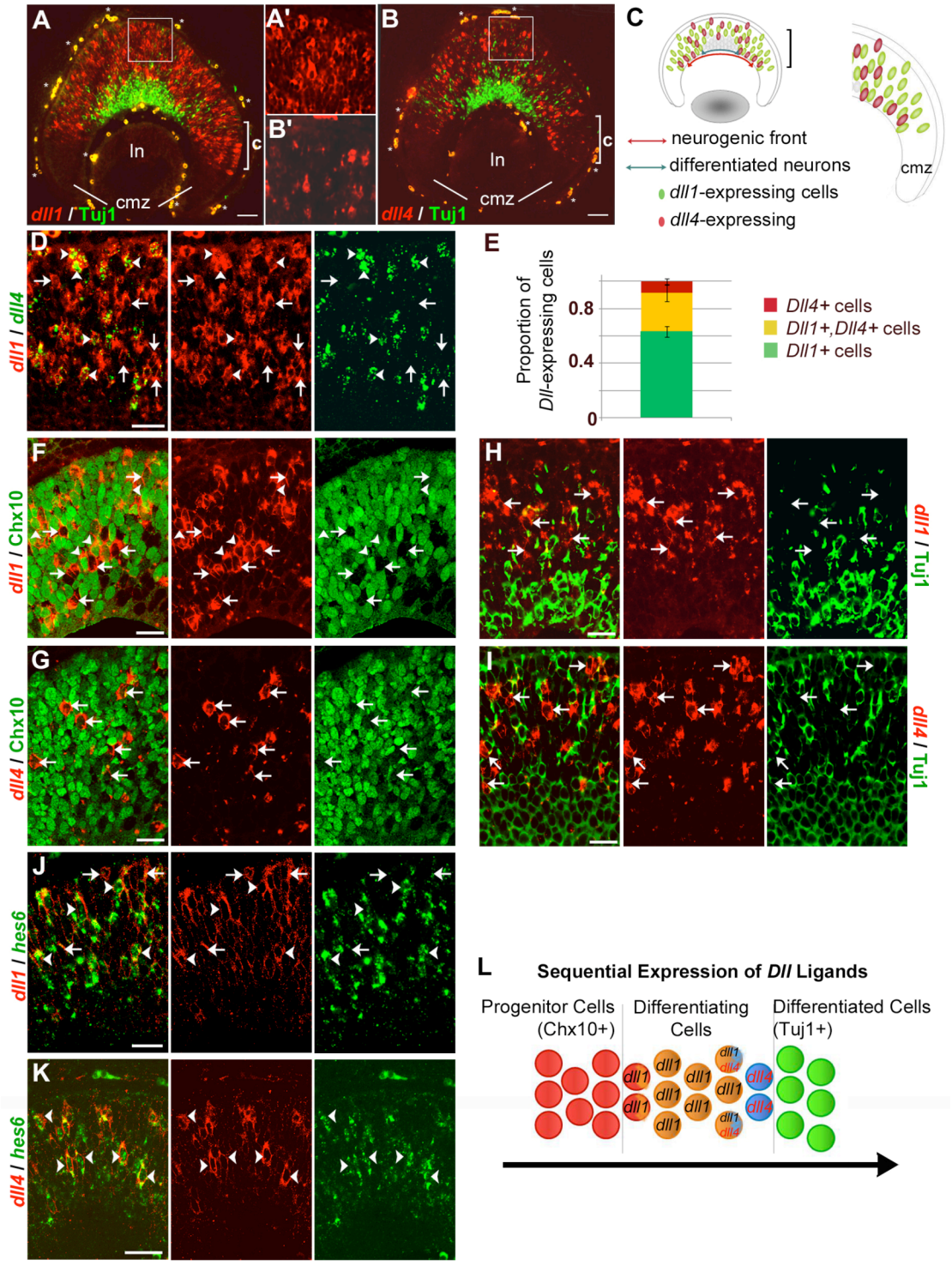
It has previously been shown that expression of *Dll1* and *Dll4* in the mouse retina starts as early as E10.5 (Hrabe de Angelis et al., 1997) and E11.5 (Benedito and Duarte, 2005), respectively. A more detailed study of the expression of these two genes during mouse retinal development revealed that *Dll1* and *Dll4* are expressed in cells located in the ventricular region of the retinal neuroepithelium, with more cells expressing *Dll1* than *Dll4* (Figure III.1, A,B). At E14.5, for instance, the number of cells expressing *Dll1* is 2.5 times higher than the number of *Dll4*-expressing cells (n=1355 cells). Also, cells expressing *Dll1* are often found in clusters and exhibit variable levels of expression, while *Dll4*-expressing cells appear usually isolated and show higher levels of expression (Figure III.1, A',B'). Expression of the two genes occurs first in the central retina and spreads peripherally along the expanding

neurogenic region, being always absent from the ciliary margin zone (CMZ). However, *Dll1*-expressing cells are located at more peripheral positions than *Dll4*-expressing cells, suggesting that *Dll1* is expressed before *Dll4* as progenitors enter neuronal differentiation, following the central-to-periphery wave of neurogenesis (Figure III.1, C; (Prada et al., 1991)). This temporal difference in expression might indicate that the two genes are expressed in the same cells but at subsequent phases of the differentiation program. Alternatively, each gene might be expressed in distinct populations of differentiating neurons, with *Dll4* being active in a population that starts to differentiate later than the *Dll1*-expressing population.

Figure III.1: *Dll* ligands are expressed transiently and sequentially during neuronal differentiation in the retina.

Dll1 **(A,A')** and *Dll4* **(B,B')** are expressed within the neurogenic region and are excluded from the CMZ (**A** and **B** are adjacent sections). **(A',B')** Cells with *Dll1* expression are more frequent than *Dll4*-expressing cells and show more variable levels of mRNA. At the front of the neurogenic wave that spreads from the central retina to the periphery (bracketed region), expression of both *Dll1* and *Dll4* precedes the appearance of differentiated Tuj1+ neurons. However, the domain of *Dll1*-expressing cells extends more to the periphery, suggesting that *Dll1* is expressed before *Dll4* during retinal neurogenesis. A cartoon depicting these findings is shown in **(C)**. Double *in situ* hybridization for *Dll1* and *Dll4* **(D)** shows the existence of a subpopulation co-expressing both genes. Most *Dll4*-expressing cells co-express *Dll1* (arrowheads in **D**), although the inverse is not true (arrows in **D**). Cell countings indicating the contribution of each of the *Dll*-expressing populations **(E)**. Confocal images of Chx10 immunostaining (a marker for RPCs, in green) and *Dll1* **(F)** or *Dll4* **(G)** expression shows that *Dll1* transcription is initiated in $Chx10^{+}$ progenitors (arrowheads in **F**), while no *Dll4*-expressing cells co-express Chx10 (arrows in **G**). Simultaneous detection of differentiated $Tuj1^{+}$ neurons and expression of *Dll1* **(H)** or *Dll4* **(I)**, shows that both genes are expressed before neuronal differentiation. Double *in situ* hybridization for *hes6* and *Dll1* **(J)** or *Dll4* **(K)** reveals that practically all cells expressing *Dll4* also express *hes6*. In contrast, only a fraction of the *Dll1*-expressing cells co-express *hes6* (arrowheads). **(L)** Summary of our findings supporting a model in which the two *Dll* ligands are expressed at subsequent steps of the neurogenic programme, during retinal differentiation, with *Dll1* expression preceding that of *Dll4*.

Asterisks in **(A,B)** mark autofluorescence of red blood cells surrounding the RPE and in the vitreal space. ln – lens, CMZ – ciliary margin zone, RPE – retinal pigmented epithelium. Scale bars **(A,B)** = 50μm; **(D,F-K)** = 25μm


A
A'
B
B'
C
ln
cmz
dll1 / Tuj1
dll4 / Tuj1
neurogenic front
differentiated neurons
dll1-expressing cells
dll4-expressing
D
dll1 / dll4
E
Proportion of Dll-expressing cells
0.8
0.4
0
Dll4+ cells
Dll1+,Dll4+ cells
Dll1+ cells
F
dll1 / Chx10
G
dll4 / Chx10
H
dll1 / Tuj1
I
dll4 / Tuj1
J
dll1 / hes6
K
dll4 / hes6
L
Sequential Expression of Dll Ligands
Progenitor Cells (Chx10+)
Differentiating Cells
Differentiated Cells (Tuj1+)

To address these hypotheses, I started by asking whether *Dll1* and *Dll4* expression occurs in unique or overlapping populations of retinal cells, using double *in situ* hybridization at E12.5 and E14.5. As expected, given the higher number of *Dll1*-expressing cells, I found that two thirds of these cells do not show *Dll4* expression. On the other side, I found that the majority (77 ± 0.1%) of the *Dll4*-expressing cells do co-express *Dll1* (Figure III.1, D), with only a minor population of retinal cells expressing exclusively *Dll4* (less than 10% of the total number of retinal cells expressing a *Dll* gene; Figure III.1, E). Thus, although these results do not completely exclude the alternative hypothesis, the existence of a significant population co-expressing the two *Dll* genes is more consistent with the view that *Dll1* and *Dll4* are expressed sequentially in the same retinal cells.

To further characterize the cells expressing each *Dll* gene, *Dll1/Dll4* in situ hybridization was combined with immunostainings for Chx10 (a marker of retinal progenitor cells; (Chen and Cepko, 2000)) and Tuj1 (a marker of early differentiating neurons; (Menezes and Luskin, 1994)), at E12.5 (Figure III.1, F-I). Although *Dll1* was previously described to be mainly expressed in nascent neurons (Henrique et al., 1997; Murciano et al., 2002), we found that 20.2 ± 4.2% of the *Dll1*-expressing cells are still positive for the retinal progenitor marker Chx10 (n=1624 cells, Fig. 1F). On the contrary, *Dll4* is never expressed in $Chx10^+$ cells (Figure III.1, G), indicating that *Dll1* expression occurs at an earlier phase of neuronal differentiation than *Dll4*. In addition, no expression of *Dll1* and *Dll4* could be detected in differentiated $Tuj1^+$ neurons (Figure III.1, H,I), revealing that the two genes are only active within a restricted time window, between neuronal commitment and differentiation. Furthermore, double *in situ* hybridization for *Dll1*/*Dll4* and *hes6*, a gene expressed in differentiating neurons (Koyano-Nakagawa et al., 2000), reveals that all *Dll4*-expressing cells express also *hes6* (Figure III.1, J,K), while only a fraction of $Dll1^+$ show co-expression with *hes6*. Together, these findings strongly favor the initial hypothesis that the two *Dll* genes are expressed sequentially in differentiating retinal neurons, at subsequent steps of the neurogenic programme, with *Dll1* expression preceding that of *Dll4* (Figure III.1, 1L).

III.2.2 *Dll1*- and *Dll4*-expressing cells contribute to various cell fates in the retina

If, indeed, *Dll1* and *Dll4* are sequentially expressed in differentiating retinal neurons, the two genes must be part of the same neurogenic programme in retinal cells, independently of their final fate. To test this prediction, I investigated whether retinal cells expressing *Dll1* or *Dll4* have preferential fates or, instead, can differentiate into any of the various retinal cell types. With this purpose, the fate of retinal cells expressing each *Dll* gene was followed by analysing the expression of beta-galactosidase (β-Gal) in the retinas of heterozygous $Dll1^{LacZ/+}$ and $Dll4^{LacZ/+}$ mice (Duarte et al., 2004; Hrabe de Angelis et al., 1997), taking advantage of β-Gal's perdurance in expressing cells, long after *Dll1* or *Dll4* transcription was switched-off. This allows the identification of cells expressing each Dll ligand by double immunostaining for β-Gal and specific markers for each retinal cell type, at different stages of retinal development. The results show co-localization of the β-Gal reporter from either $Dll1^{LacZ/+}$ or $Dll4^{LacZ/+}$ retinas with Brn3b (a marker for RGCs; Figure III.2, A,B), CRABP (a marker for amacrine cells; Figure III.2, C,D), NF165 (a marker for horizontal cells; Figure III.2, E,F) and recoverin (a marker for photoreceptors; Figure III.2, G,H). Due to weak β-Gal levels at postnatal stages, when the bipolar cell marker PKC is expressed, it was not possible to check for colocalization of these two markers. Nonetheless, the results show that the expression of *Dll1* and *Dll4* is not confined to specific retinal cell types, thereby excluding the hypothesis that the two *Dll* genes participate in distinct differentiation programmes, active in separate but co-existing populations of retinal progenitors. Instead, the finding that *Dll1*- and *Dll4*-expressing cells contribute to the various retinal cell types provides further evidence that the two genes act in the same neurogenic program to regulate neuronal differentiation in the retina.

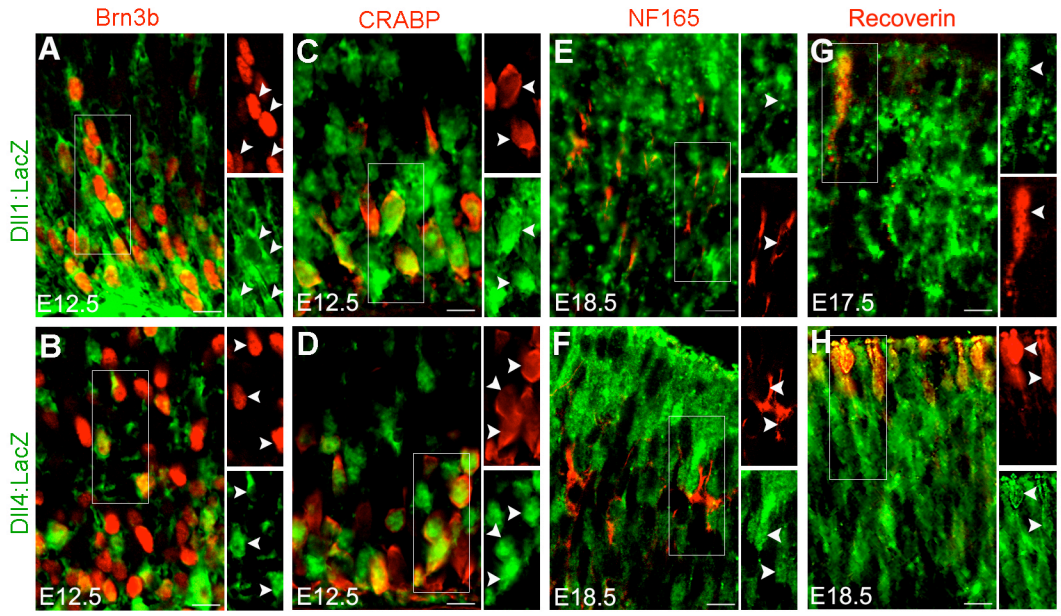


Figure III.2: Cells expressing *Dll1*- and *Dll4* give rise to various retinal cell types

Dll1$^{LacZ/+}$ and *Dll4*$^{Lacz/+}$ mice were used to follow the fate of cells expressing each of the Dll ligands by immunostaining for the β-Gal reporter and specific markers for each retinal cell type. Both Dll1::LacZ **(A,C,E,G)** and Dll4::LacZ **(B,D,F,H)** cells show co-labelling with markers for retinal ganglion cells at E12.5 **(A,B)**, amacrine at E12.5 **(C,D)**, horizontal at E17.5 **(E,F)** and photoreceptor cells at E17.5-E18.5 **(G,H)**, indicating that cells expressing either Notch ligand contribute to all retinal cell fates. Scale bars = 10 μm.

III.2.3 *Dll1* loss-of-function in the retina leads to a mild neurogenic phenotype

To further understand the role of the two Dll ligands in the neural retina, the phenotype of mutant mice where *Dll1* is inactive was analyzed. Since *Dll1*$^{-/-}$ null mice are embryonic lethal as early as E10.5 (Hrabe de Angelis et al., 1997), the function of the Dll1 ligand in retinal development was approached by analyzing *Dll1*-floxed mice (Hozumi et al., 2004) expressing Cre recombinase under the control of the *Chx10* promoter (referred as *Dll1*$^{f/f}$*;Chx10*$^{Cre/+}$), which drives Cre expression exclusively in RPCs (Rowan and Cepko, 2004). The analysis was done in embryos from E11.5 onwards, which corresponds to the onset of retinal neurogenesis (Young, 1985). Since retinal ganglion cells (RGCs) are the only differentiated neuronal type present in the mouse retina at E11.5 (Carter-Dawson and LaVail, 1979), I started the analysis by looking for the presence of Brn3b (a specific marker of RGCs) and Tuj1 (a pan-neuronal marker in the retina, with the expection of photoreceptors) in control and mutant *Dll1*$^{f/f}$*;Chx10*$^{Cre/+}$ retinas. No significant difference in the number of RGCs could be observed between normal and mutant retinas (Figure III.3, A,B). In addition,

all Tuj1^{+} cells are also Brn3b^{+}, in both control and mutant retinas, indicating that all differentiated neurons are RGCs and that no other neuronal type started to differentiate prematurely in *Dll1* mutant retinas.

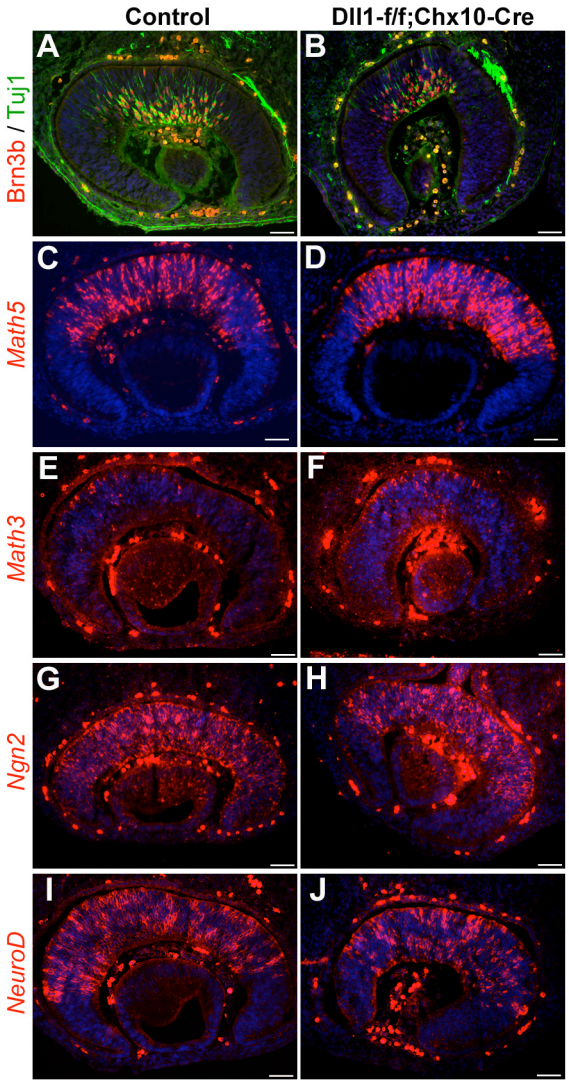


Figure III.3: Neurogenesis in E11.5 *Dll1* mutant retinas.
(A,B) At E11.5, wild-type and mutant retinas (*Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$) present no difference in the number of Brn3b+ RGCs, nor in the number of total neurons (Tuj1+ cells). **(C-J)** In situ hybridization for bHLH-encoding genes *Math5* **(C,D)**, *Math3* **(E,F)**, *Ngn2* **(G,H)** and *NeuroD* **(I,J)**, show no significant difference between control and mutant embryos, with the exception of *Math5* that is increased in *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas **(C)** compared to those of control littermates **(D)**. Scale bars = 50µm.

I then assessed the expression of several bHLH-encoding genes that have been shown to control differentiation of various retinal cell types, namely *Math5* (Brown et al., 1998; Brown et al., 2001; Liu et al., 2001; Wang et al., 2001), *Math3* (Hatakeyama et al., 2001; Inoue et al., 2002), *Ngn2* (Ma and Wang, 2006) and *NeuroD* (Ahmad et al., 1998; Inoue et al., 2002; Morrow et al., 1999). I found that only *Math5*, a gene implicated in RGC production, is upregulated in the retinas of *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ mice at E11.5, when compared to control retinas (Figure III.3, C-J).

Thus, although the absence of *Dll1* does not lead to an increase in the number of differentiated retinal neurons at E11.5, the genetic circuitry promoting RGC differentiation seems to be already active in a higher number of RPCs. I therefore analyzed RGCs production one day later (E12.5) and found that the number of these cells generated in the absence of *Dll1* increases 34,2% when compared to control retinas (31.4 ± 0.2% of cells are Brn3b$^+$ in *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas, while 23.4 ± 1.3% are Brn3b$^+$ in control retinas. Figure III.4, A-C). This increase in neurogenesis correlates with a decrease of *hes5* expression (Figure III.4, D,E) and an increase of *hes6* expression in E12.5 *Dll1* mutant retinas (Figure III.4, F,G), confirming that absence of *Dll1* causes a reduction in Notch signalling and excessive RGC differentiation.

While the retina at E12.5 produces almost exclusively RGCs, from E12.5 onwards several neuronal types are generated in overlapping periods (Carter-Dawson and LaVail, 1979; Young, 1985). To measure the overall rate of retinal neurogenesis at later stages, a single dose of BrdU was administered at E13.5 and retinas were harvested for analysis one day later. Double immunostaining for BrdU and Chx10 allowed me to identify cells (BrdU$^+$/Chx10$^-$) that had exited the cell cycle and initiated differentiation during this 24h period (Figure III.4, H). Although the epithelial architecture of *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas is disturbed (see Section III.1.4), counting the number of BrdU$^+$/Chx10$^-$ cells in relation to the total number of BrdU$^+$ cells reveals an enhanced rate of neurogenesis in *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas when compared to control retinas (Figure III.4, I-K), with an overall increase of 38.6% (control = 28.4 ± 2.7%; Dll1$^{f/f}$,Chx10$^{Cre/+}$= 39.4 ± 4.6%; $p<0.05$). In parallel, a significant reduction (~45%) in the number of Chx10$^+$ progenitors was also observed in *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas, when compared with control retinas (control = 7.2 ± 0.16 cells per mm^2; *Dll1$^{f/f}$,Chx10$^{Cre/+}$* = 3.9 ± 0.09 Chx10$^+$ cells per mm^2; Figure III.4, L).

The increased rate of neurogenesis is also shown by the faster accumulation of newborn neurons in mutant *Dll1$^{LacZ/flox}$,Chx10$^{Cre/+}$* retinas, when compared with phenotypically normal retinas from *Dll1$^{LacZ/+}$* embryos. In both cases, newborn neurons can be identified by β-Gal expression, driven by the *Dll1* promoter. However, there is a higher number of Dll1:LacZ neurons in E14.5 retinas of *Dll1$^{LacZ/f}$,Chx10$^{Cre/+}$* embryos (Figure III.4, M,N), indicating that more cells have committed to neuronal differentiation in the absence of Dll1 signalling. Thus, although the neural retina expresses two distinct Notch ligands, *Dll1* and *Dll4*, the results indicate that the absence of *Dll1* is sufficient to increase the rate of neurogenesis throughout several

timepoints of retinal development and cause a significant depletion of progenitors, as a result of the reduction in Notch signalling activity.

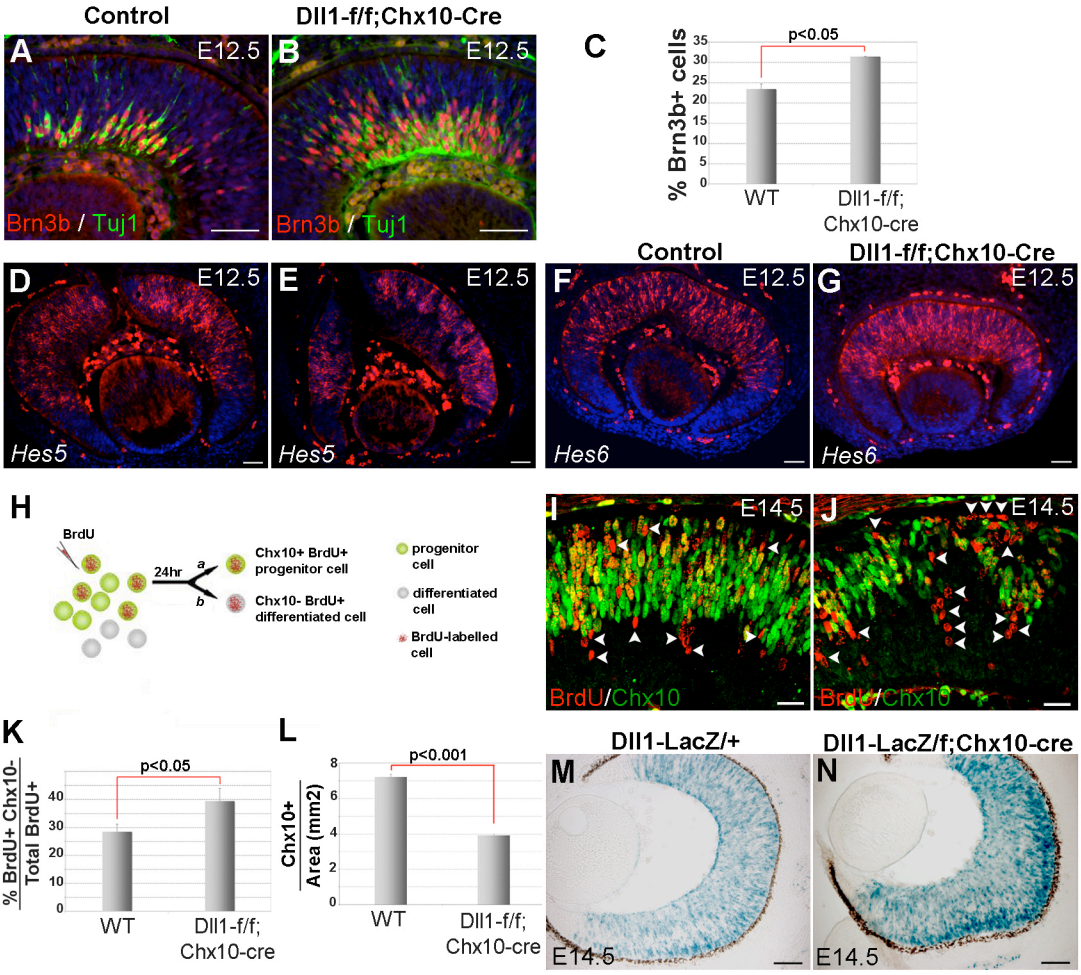


Figure III.4: Enhanced neurogenesis in the retinas of *Dll1$^{f/f}$;Chx10$^{Cre/+}$* mutants.

(A,B,C). Enhanced neurogenesis is accompanied by a decrease in *hes5* expression **(D,E)** and an increase in *hes6* expression **(F,G)**, correlating with a reduction in Notch signalling. **(H)** Scheme depicting the strategy to measure the rate of retinal neurogenesis, through the administration of a single dose of BrdU 24hrs prior to dissection, followed by detection of Chx10, a retinal progenitor marker. Double immunostaining for BrdU and Chx10 allows the identification of retinal progenitors that exited the cell cycle and entered differentiation over the 24hr period (BrdU^{+},Chx10^{-} cells). Counting these cells in relation to the total population of BrdU^{+} cells is indicative of the rate of neurogenesis. **(I,J)** Sections of wild-type and *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas showing cells that remained as progenitors (BrdU^{+},Chx10^{+}, yellow nuclei) and cells that committed to differentiation (BrdU^{+},Chx10^{-}, white arrowheads). **(K,L)** *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas show a ≅38% increase in the rate of neurogenesis when compared to control retinas and a matching decrease in the Chx10^{+} progenitor population **(L)**. An higher number of Dll1::LacZ neurons is observed in *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas **(N)** when compared with retinas from normal *Dll1$^{Lacz/+}$* mice **(M)**. Scale bars **(A-B,D-G,M-N)** = 50µm ; **(M,N)** = 25µm.

III.2.4 Disruption of retinal morphology in *Dll1$^{f/f}$,Chx10$^{Cre/+}$* mutants

A likely consequence of this depletion in neuroepithelial progenitors is the observed disruption of the retinal neuroepithelium in E14.5 *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas (Figure III.4), with the appearance of rosette-like structures similar to those described in *Notch1* and *Hes1* mutant retinas (Jadhav et al., 2006; Tomita et al., 1996; Yaron et al., 2006). These rosettes in *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas are formed mainly by Chx10^{+} retinal progenitors, organized around a central lumen, and are interspersed with regions of the neuroepithelium where differentiated neurons accumulate (Figure III.5B). Immunostaining for N-Cadherin, ZO-1 and PAR3 reveals that these progenitors are polarized, with the apical side forming the luminal surface where they enter mitosis like in a normal retinal neuroepithelium (Figure III.5, C-F). Within these rosettes, progenitors are engaged in neurogenesis and competent to produce all retinal cell types, which maintain their spatial relationships in the neuroepithelium, as shown by the appearance of recoverin-positive photoreceptors close to the apical lumen in E17.5 rosettes or the presence of horizontal cells at intermediate positions of the epithelial rosettes (Figure III.6).

The formation of these “progenitor cell rosettes” indicates that *Dll1* inactivation does not lead to a complete exhaustion of RPCs, most likely due to some remaining Notch activity, as revealed by the persistent expression of *hes5* in rosettes from E14.5 mutant retinas (Figure III.7, A,B). This might be due to mosaic expression of the Cre recombinase driven by the *Chx10* promoter, allowing the emergence of cells where *Dll1* has not been inactivated and is still able to signal. However, this hypothesis is unlikely, as a similar rosette phenotype can be observed in *Dll1* mutant retinas when gene inactivation is driven by two other Cre-expressing lines, FoxG1-Cre (Hebert and McConnell, 2000) and Nestin-Cre (Tronche et al., 1999) (data not shown). Moreover, I could also detect a rosette phenotype in retinas of embryos heteroallelic for a null and a hypomorphic allele of *Dll1* (*Dll1$^{LacZ/Dll1ki}$*; Figure III.7, C,D) (Schuster-Gossler et al., 2007), which show as well an increased rate of neurogenesis. Thus, the remaining Notch activity and the persistence of retinal progenitors in *Dll1* mutant retinas cannot be exlained by incomplete gene inactivation. Instead, these findings might easily be accounted for by the activity of Dll4, which continues to be expressed in *Dll1*-mutant retinas (Figure III.7, E,F) and could signal to maintain a pool of RPCs.

What is then the role of each Dll ligand in the genetic circuitry regulating retinal neurogenesis? Do they have a redundant function? Or, given the sequentiality in their expression, do they also play unique roles at different steps of the neurogenesis process? To address these questions, I turned my attention to the developing spinal cord, where these ligands are also expressed within the neurogenic zone, in both unique and overlapping patterns.

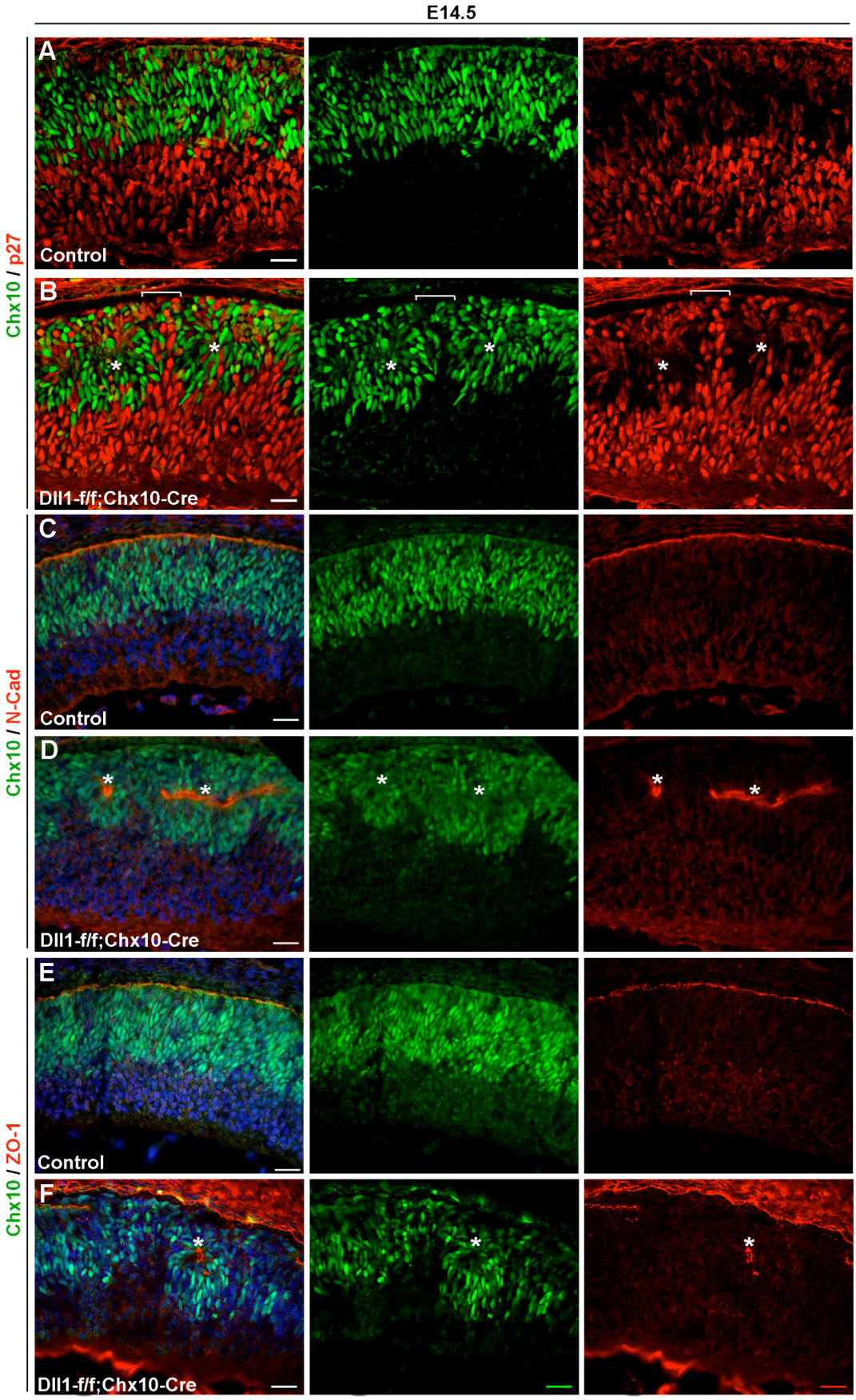

E14.5
A
Control
Chx10 / p27
B
Dll1-f/f;Chx10-Cre
C
Control
Chx10 / N-Cad
D
Dll1-f/f;Chx10-Cre
E
Control
Chx10 / ZO-1
F
Dll1-f/f;Chx10-Cre

Figure III.5: *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas show disruption of the neuroepithelial organization, with the appearance of rosette-like structures.

(A) In the wild-type E14.5 retinal epithelium, Chx10^{+} progenitors are located in the ventricular regions while p27+ neurons accumulate outside this region. **(B)** In E14.5 *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas, Chx10^{+} progenitors are arranged in rosettes (asterisks) and surrounded by post-mitotic neurons (p27^{+}) that form apical-to-basal "corridors" of ectopically differentiated neurons (bracket). **(C-F)** The rosette's neuroepithelium maintains its apico-basal polarity, with N-Cadherin **(C,D)** and ZO1 **(E,F)** being present at the center of rosettes in *Dll1*-mutant retinas. Scale bars **(A,B)** = 25µm ; **(C-F)** = 50µm

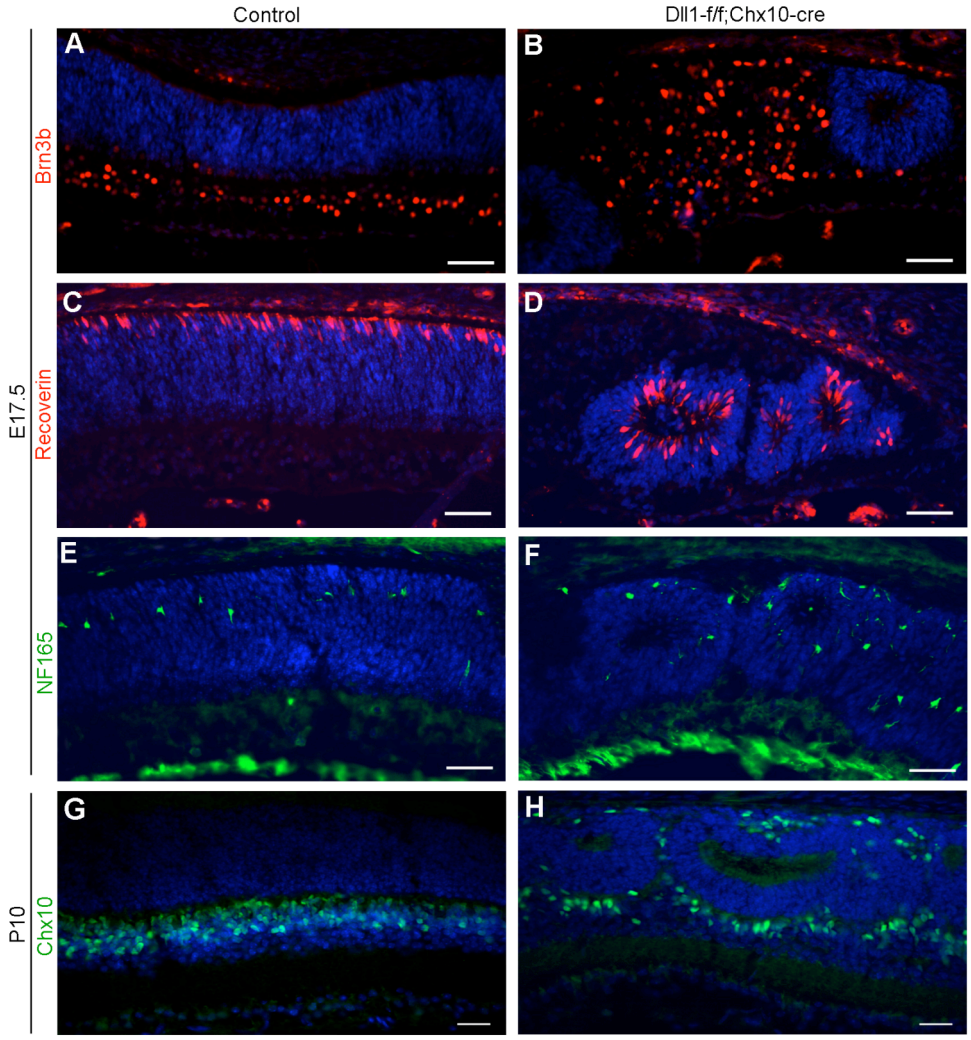


Figure III.6: Apical-basal organization preserved within the rosette-like structure of *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas

Neurogenesis continues in *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas and laminar organization of rosettes follows the normal arrangement like in normal retinas, with RGCs being excluded from the progenitor-containing neuroepithelium **(A,B)**, photoreceptor cells accumulating at the most apical region of the neuroepithelium **(C,D)**, horizontal cells located at intermediate positions **(E,F)** and bipolar cells located further away from the apical surface **(G,H)**. Scale bars **(A-H)** = 50µm

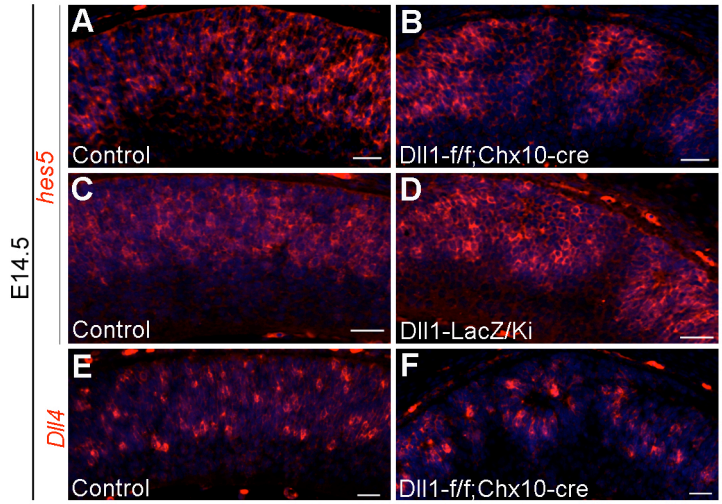


Figure III.7: Maintenance of Notch activity in retinas of *Dll1* mutants.
In situ hybridization for *hes5* indicates that Notch activity is not completely lost in *Dll1*$^{f/f}$*,Chx10*$^{Cre/+}$ retinas **(A,B)**. The persistence of Notch signalling is also observed in retinas of *Dll1*$^{LacZ/Dll1ki}$ embryos that are heteroallelic for a null and a hypomorphic allele of *Dll1* **(C,D)**, and which are not dependent on recombination events. The continued expression of the *Dll4* in the neuroepithelium of *Dll1* mutants retinas **(E,F)**, accounts for the continued activation of the Notch pathway. Scale bars **(A,F)** = 50µm

III.3 THE *DLL1* AND *DLL4* LIGANDS IN THE DEVELOPING MOUSE NEURAL TUBE

III.3.1 *Dll1* and *Dll4* are expressed sequentially in the pV2 domain of the mouse neural tube

The neural tube, like the retina, expresses various Notch ligands within the ventricular region, namely *Dll1* (Chitnis et al., 1995; Henrique et al., 1995), *Dll4* (Benedito and Duarte, 2005) and *Jag1* (Lindsell et al., 1996; Myat et al., 1996). These are expressed in specific progenitor domains of the developing neural tube, from which distinct neuronal cell types arise (reviewed in (Wilson and Maden, 2005)). *Dll1* is expressed throughout most of the dorsal-ventral axis of the ventricular zone, being excluded from the *Jag1*-expressing domains (Lindsell et al., 1996; Myat et al., 1996), while *Dll4* expression is restricted to the pV2 domain, where *Dll1* is also expressed (Benedito and Duarte, 2005; Del Barrio et al., 2007; Peng et al., 2007).

To investigate in more detail the expression pattern of the two *Dll* genes in the pV2 domain and compare it to the retina, I once again took advantage of β-Gal's perdurance to fate map *Dll1*- and *Dll4*-expressing cells, using *Dll1*$^{LacZ/+}$ and *Dll4*$^{LacZ/+}$

mice. In addition, I also performed double in situ hybridization for *Dll1* and *Dll4* on sections of E11.5 mouse embryos. The pV2 domain gives rise to two interneuron subtypes, V2a and V2b, which are characterized by Chx10 and GATA3 expression, respectively (Ericson et al., 1997). To see if the two Dll ligands are expressed in cells that contribute to both pV2 fates, as described for the retina (Figure III.2), double immunostaining for β-Gal and Chx10 was performed. I observed that *Dll4*-expressing cells contribute exclusively to the V2a interneuron subtype of the pV2 domain of the developing neural tube (Figure III.8, A)., while *Dll1*-expressing cells contribute to both the V2a (arrows in Figure III.8, B) and V2b lineages (arrowheads in Figure III.8, B). The expression of Dll1 in V2b interneurons was inferred indirectly as double immunostaining for β-Gal and GATA3, a V2b marker, was not possible. Nonetheless, the presence of Dll1:LacZ cells which do not express Chx10 but are closely neighbouring Dll1:LacZ-positive V2a interneurons suggests that *Dll1*-expressing cells contribute to both V2 interneuron subtypes. Furthermore, all V2a interneurons (Chx10-expressing cells) are Dll4:LacZ positive (Figure III.8, A), indicating that not only is *Dll4* expression confined to a specific cell fate in the pV2 domain, but also occurs in all V2a interneurons during their progression into differentiation. Despite this difference with the retina, analysis by double *in situ* hybridization show pV2 cells expressing only one of the ligands, as well as coexpression of the two *Dll* genes in several pV2 cells (Figure III.8, C). I next compared the localization of Dll1:LacZ and Dll4:LacZ cells in spinal cords of $Dll1^{LacZ/+}$ and $Dll4^{LacZ/+}$ embryos, respectively. While Dll1:LacZ cells can still be detected in the ventricular region, Dll4:LacZ cells are only located in the mantle zone (Figure III.8, D,E), indicating that *Dll1* starts to be expressed earlier than *Dll4* in differentiating V2 interneurons. Together with the in situ data, these results indicate that the expression of *Dll1* and *Dll4* during neurogenesis in the pV2 domain closely resembles what we have described above for the neural retina.

Since $Dll4^{-/-}$ null mice are embryonic lethal between E9.5-E10.5 (Duarte et al., 2004) and no conditional KO mice were available, I was not able to approach directly the function of *Dll4* during neurogenesis. Nevertheless, the analysis of *Dll1* deficient neural tubes allows one to study separately the functions of the *Dll1* and *Dll4* genes during neurogenesis. First, and unlike in the retina, the analysis of the domains where *Dll1* is the sole DSL ligand expressed enables one to by-pass any situation of redundancy or compensation by other ligands and thereby access the function of *Dll1*. Second, in *Dll1* mutants, the pV2 domain offers a situation where only *Dll4* is expressed. This allows one to infer indirectly the role of *Dll4* in regulating

neurogenesis in this particular domain of the neural tube and extrapolate to the developing retina. Therefore, I will next analyse the effect of the loss-of-function of *Dll1* during neurogenesis in the domains where it is the only Notch ligand to be expressed, and then the effect of the absence of *Dll1* in the pV2 domain, where *Dll4* will be the only ligand available to regulate neurogenesis.

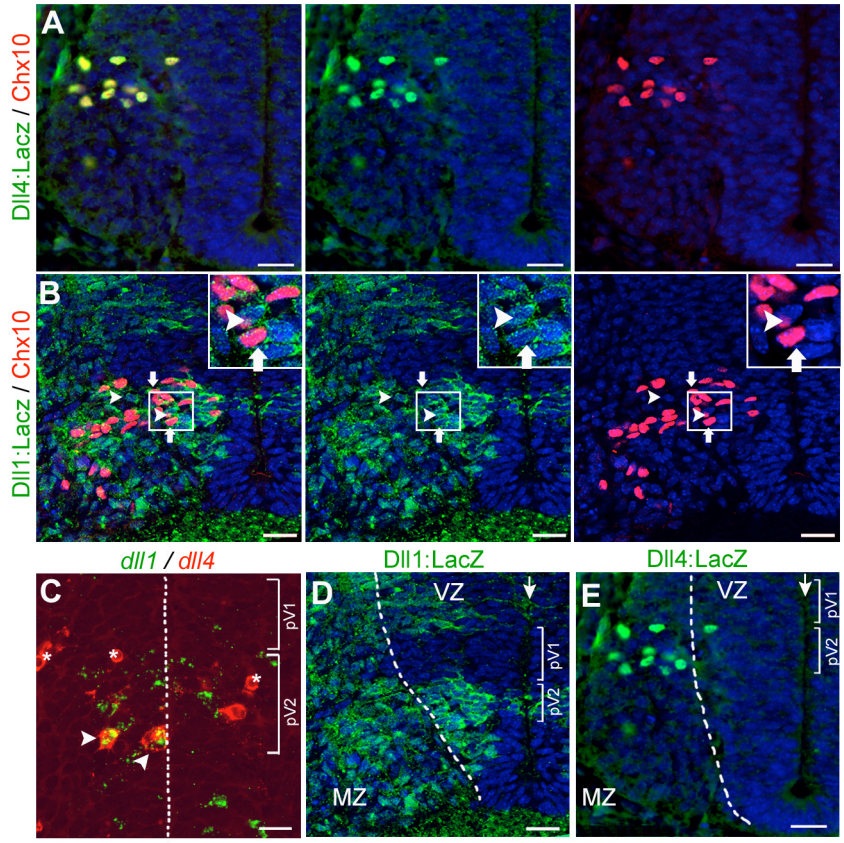


Figure III.8: Sequential expression of *Dll1* and *Dll4* in the pV2 domain of the mouse neural tube, at E11.5.

The pV2 domain is the only domain in the neural tube where *Dll1* and *Dll4* are both expressed.**(A)** *Dll4*$^{LacZ/+}$ mice show that *Dll4* is only expressed in the Chx10+ V2a lineage, while *Dll1*$^{LacZ/+}$ mice show that *Dll1* is expressed both in V2a (arrows in **B**) and V2b interneurons (arrowheads in **B**). **(C)** Close view of the expression of *Dll1* and *Dll4* in the pV2 domain, showing co-expression of the two genes in some cells (arrowheads; dashed line is indicating the midline). Like in the retina, more cells express *Dll1* than *Dll4*. Immunostaining for the β-Gal reporter of *Dll1*$^{LacZ/+}$ and *Dll4*$^{Lacz/+}$ mice shows Dll1:LacZ expression already in the VZ **(D)**, while Dll4:LacZ is only detected in more differentiated cells that have moved in the MZ **(E)**. MZ – mantle zone, VZ – ventricular zone. Scale bar **(C)** = 20µm; **(A,B,D,E)** = 25µm

III.3.2 *Dll1* as the sole Notch ligand regulating neurogenesis

The function of the Dll1 ligand in the neural tube was approached using *Dll1*-floxed mice expressing cre recombinase under the control of the rat *Nestin* promoter (referred as *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$), which expresses Cre in all neural progenitor cells (Tronche et al., 1999). Embryos were collected from E10.5-E12.5 and neuronal production was evaluated at the forelimb level, both quantitatively and qualitatively.

III.3.2.1 *Dll1* is the main responsible for progenitor maintenance in the spinal cord

Spinal cords of embryos ranging from E10.5 to E12.5 were analyzed by immunostaining with Tuj1 or NF165 antibodies, which detect differentiated neurons (Fig. 9A-F). No significant difference could be detected in the number of neurons in wild type and Dll1$^{f/f}$,Nestin$^{Cre/+}$ embryos at E10.5 (Figure III.9, A,B). At E11.5, however, the mutant neural tubes are severely affected (Figure III.9, C,D) and massive neuronal differentiation is observed in the absence of Dll1 signalling. Concomitantly, the progenitor population is severely depleted (Figure III.9, C,D) and a striking reduction in neural tube size is observed at E12.5, when compared with wild type littermates (Figure III.9, E,F).

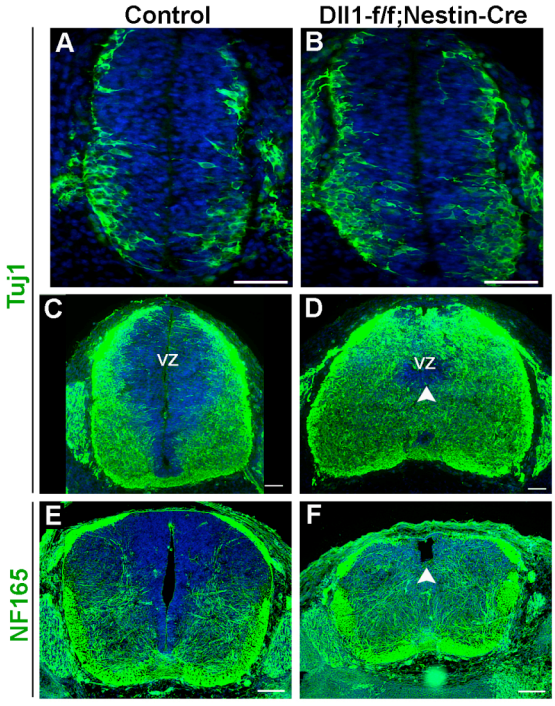


Figure III.9: Neurogenic phenotype in the neural tube of *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ embryos.
At E10.5, no significant differences in neuronal differentiation are observed between control **(A)** and *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ neural tubes **(B)**. At E11.5, massive neuronal differentiation can be observed in *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ neural tubes **(D)**, in comparison with wild type neural tube **(C)**. The *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ neural tube is almost devoid of ventricular zone (vz, arrowhead) where neural progenitors are located. **(E,F)** At E12.5, progenitor depletion leads to an atrophy of the neural tube. Neurons are visualized with the anti-Tuj1 or anti-NF165 antibody. Scale bars **(A-D)** = 50μm; **(E-F)** = 100μm.

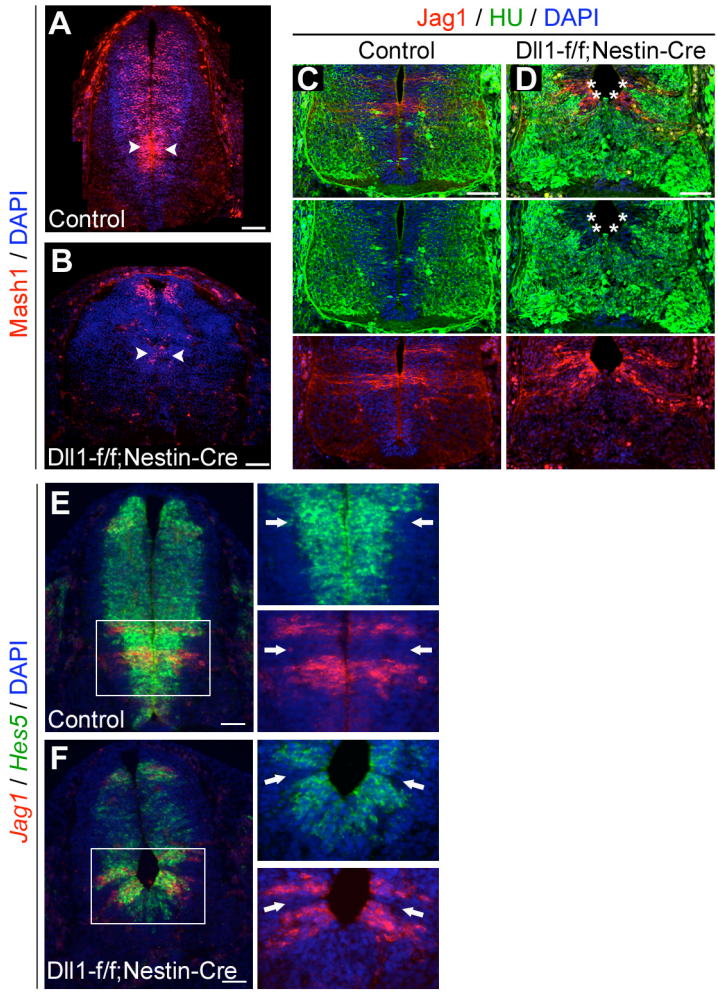


Figure III.10: Maintenance of neural progenitors is restricted mainly to *Jag1*-expressing domains, in the neural tube of E11.5 *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos.

Immunostaining for Mash1 **(A,B)** shows the presence of very few pV2 progenitor cells (arrowheads) in *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos **(B)** compared to control littermates **(A)**. **(C,D)** Double immunostaining for Jag1 and HU, a marker of differentiated neurons. In control neural tubes **(C)**, Jag1 expression is observed in discrete progenitor domains (HU-negative). However, in neural tubes of *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos **(D)**, Jag1 and HU reveal a striking complementary pattern in *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos, indicating that most of the progenitor cells in *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos belong to *Jag1*-expressing domains (asterisks). In situ hybridization for *hes5* shows that Notch signalling is active throughout the whole dorsoventral axis of normal neural tubes **(E)**, while *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos only present Notch signalling in the *Jag1*-expressing domains **(F)**. White arrows in **(E,F)** indicate the *Dll1*-expressing pV0 domain that is localized between both *Jag1*-expressing domains: no Notch signalling is observed in the pV0 domain of *Dll1$^{f/f}$,Nestin$^{Cre/+}$* neural tubes **(H)**. Scale bars **(A-F)** = 50µm

Nonetheless, a cluster of progenitors remain at the center of the deformed neural tube in Dll1$^{f/f}$,Nestin$^{Cre/+}$ embryos at E11.5 and E12.5, surrounding a small lumen that is still present (arrowhead in Figure III.9, D,F). By in situ hybridization and immunostaining, I found that these cells include the remaining pV2 progenitors

(arrowhead in Figure III.10, A;B), as well as *Jag1*-expressing progenitors (Figure III.10, C,D) which originate from the neural tube domains where this gene is active, namely the pV1 and pD6 domains ((Matise and Joyner, 1997); see also Section IV.2.1 Chapter IV). Moreover, *hes5* expression could also be detected in these domains, showing that Notch signaling is active and preventing progenitor differentiation, in contrast to the domains where Dll1 is inactive and no other ligand is expressed (Figure III.10, E,F).

Another consequence of the progenitor depletion in *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ neural tubes is the reduced generation of glial cells, which are born later from the same progenitors that first give rise to neurons (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). Immunodetection of S100, a marker of glial cells, shows a clear decrease in the production of this late cell type (Figure III.11). The few remaining glial cells in *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ neural tubes are possibly derived from the *Jag1*-expressing and/or pMN domains that remain unaffected in *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ neural tubes. Together, these results show that, in the spinal cord domains where Dll1 is the only expressed Notch ligand, it is necessary and sufficient to maintain progenitors and ensure their availability throughout neurogenesis.

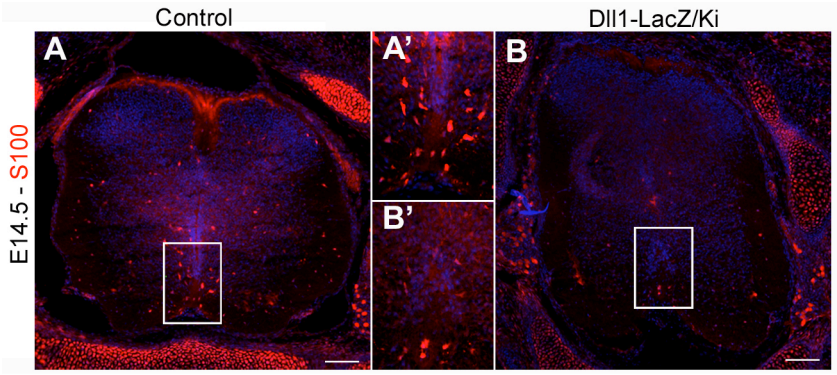


Figure III.11: *Dll1* mutant embryos lose glial cell fates due to excessive neuronal differentiation. Immunostaining for the S100, a marker of astrocytes, shows many astrocytes in the E14.5 wild type neural tube **(A)**, while almost none are observed in *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ embryos **(B)**. Scale bars **(A,B)** = 50µm.

III.3.2.2 Dll1 does not affect neuronal type specification

The Notch pathway has been implicated not only in the maintenance of a neural progenitor pool but, in some cases, also in mediating neuronal type specification (Brooker et al., 2006; Cau et al., 2008; Kiernan et al., 2005; Kiernan et al., 2006; Shin et al., 2007). Therefore, I asked whether absence of *Dll1* results not only in premature differentiation but also in unbalanced production of specific neuronal types.

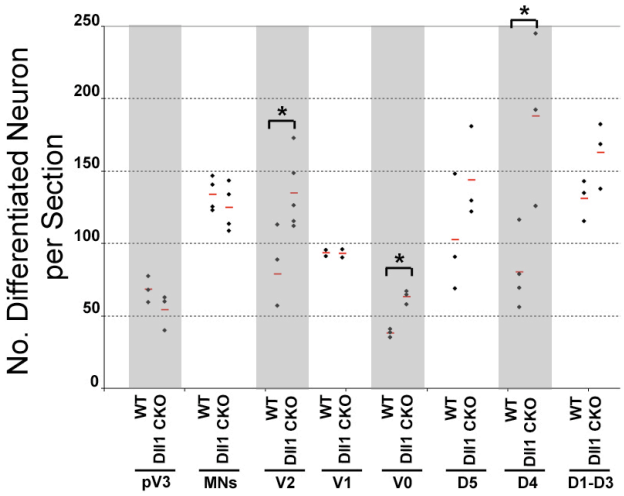


Figure 12: Quantification of neuronal cell types produced in the neural tube of wild-type and *Dll1*-mutant embryos, at E11.5.

In order to approach this question, I quantified the different neuronal types produced from the various dorsoventral domains of the spinal cord by using antibodies or combinations of antibodies specific for each of the different neuronal cell types produced in the neural tube. The results confirms excessive neuronal differentiation in all *Dll1*-expressing domains from *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ embryos (Figure III.12), when compared with control embryos. The only neural domains that are not affected are those that do not express *Dll1* (pV1 domain) or the most ventral domains (pV3 and pMN) where recombination is poor and *Dll1* loss-of function is not complete (Figure III.13). Thus, these results suggest that *Dll1* is not involved in creating cell fate diversity in the spinal cord.

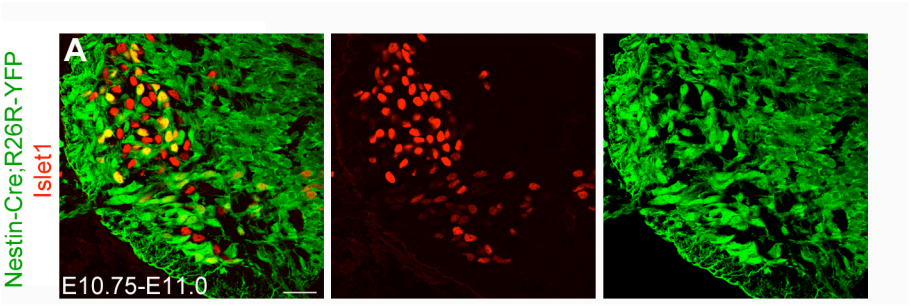


Figure III.13: Recombination efficiency of the Nestin-Cre line in the pMN domain
At E11.75-E11.0, all motoneurons have exited cell cycle and differentiated. Recombination is poor in this domain, as shown by crossing the Nestin-Cre line with the R26R-YFP reporter line and immunolabelling with Islet1, a marker of MNs. Scale bars = 50µm

The process of neuronal specification in the spinal cord has been exhaustively studied and it has been shown to depend on the dorsoventral patterning system, which subdivides the spinal cord into various cellular domains along the dorsoventral axis. Within each of these domains, progenitors have a well-defined identity and are restricted in their differentiation potential (reviewed in (Briscoe and Ericson, 2001)). Nevertheless, recent reports have shown that some domains of the neural tube give rise to various cell subtypes (Alvarez et al., 2005; Ericson et al., 1997; Karunaratne et al., 2002; Pierani et al., 2001; Shin et al., 2007; Yeo and Chitnis, 2007; Zhou et al., 2000). The pV2 domain is an example of such a domain that produces different neuronal subtypes, V2a and V2b interneurons. Importantly, it is thought that these two types of interneurons arise from a common progenitor, a progenitor that has the potential to acquire either fate. Therefore, I decided to reapproach the possibility of Dll1-mediated Notch signalling being involved in neuronal subtype specification. For this, I focused on single domains of the developing spinal cord, which are known to produce various neuronal subtypes that might arise from equivalent progenitors. This approach contrasts from the previous, in which I compared the production of neurons derived from different domains of the spinal cord, where neural progenitors are distinct in their differentiation potential.

The pMN, pV2 and pV0 domains are known to produced various neuronal cell types, therefore I turned my attention first to the the pMN and the pV0 domains, which express only *Dll1* and then to the pV2 domain that expresses both *Dll1* and *Dll4*.

Dll1 and motoneuron subtype specification

Like other domains of the developing neural tube, the pMN domain also presents a first phase of neurogenesis followed by gliogenesis. This ventral domain of the neural tube first produces motoneurons (MNs), which are among the first neurons to differentiate in the developing neural tube (Nornes and Carry, 1978) and then oligodendrocytes. Several studies have shown that Notch signalling is both required for the production oligodendrocyte precursor cells (OPCs; (Grandbarbe et al., 2003; Park and Appel, 2003)) and in a subsequent step, necessary to inhibit premature differentiation of the OPCs into mature oligodentrocytes (Appel et al., 2001; Genoud et al., 2002; Wang et al., 1998). These experiments have reflected the role of Notch signalling in maintaining a pool of progenitor cells, by inhibiting their premature differentiation into either MNs or oligodendrocytes. However, few studies have been conducted to look at a possible involvement of Notch in MN subtype specification. The pMN domain generates several MN subtypes that occupy esterotyped positions in the neural tube and target distinct and specific muscles (reviewed in (Landmesser, 2001; Lumsden, 1995)). Although several studies have enabled us to understand how MNs are diversified, most of this work has described mechanisms acting at postmitotic stages of MN differentiation. Thus it is plausible that transcriptional networks further upstream might already play a role in MN subtype specification. For instance, the mouse forebrain generates ventral and dorsal MNs (vMNs and dMNs, respectively) and it has been shown that the co-expression of *Ngn2* and *Olig2* specifies the ventral subtype (Mizuguchi et al., 2001). Therefore, I questioned if Dll1-mediated Notch signalling could mediate MN subtype specification.

As mentioned above, the analysis of $Dll1^{f/f}$;$Nestin^{Cre/+}$ embryos was not suitable to approach the function of the Dll1 ligand in MN differentiation, due to the weak recombination levels observed in the pMN domain (Figure III.13). Therefore, I analysed embryos heteroallelic for a null and a hypomorphic allele of *Dll1* ($Dll1^{LacZ/Ki}$). Embryos were collected at E13.5, a timepoint at which the different motoneuron subtypes express specific molecular markers and occupy esterotyped positions in the ventral horns of the spinal cord (Lumsden, 1995). Production of MNs was evaluated at the forelimb level by immunostaining for MNR, a marker of all MN subtypes (Figure III.14, B,C) and for Islet1, Lim1 and Lim3 (Figure III.14, D-E) that are markers of specific MNs subtypes (Figure III.14, A). Visualization of all MNs with MNR2 reveals a clear decrease in the number of MNs in the spinal cord of $Dll1^{LacZ/Ki}$

embryos when compared to that of control littermates (Figure III.14, B,C). This decrease in the number of MNs was also reflected on the number of Islet1-positive MNs, as well as on the number of Lim3+/Islet1+ positive motoneurons, which are absent in the spinal cord of *Dll1*$^{LacZ/Ki}$ embryos when compared to that of wild-type littermates (asterisk in Figure III.14, D,E). In addition to the decrease in the number of MNs in *Dll1* mutant embryos, an abnormal morphology of the ventral horn was observed. The cell bodies of the neurons contained within the ventral horn of *Dll1*$^{LacZ/Ki}$ embryos are no longer separated from the extraspinal tissue by white matter (arrow in Figure III.14, C,E). Thus, although Lim3+/Islet1+ positive motoneurons constitute a MN subtype, which appears to not be present in the *Dll1* mutant, I was not able to conclude whether this is due to a misspecification of this MN subtype or, for instance, to an impairment in overall MN production given the visible decrease in total MNs or even due to a possible leakage of MNs into the extraspinal tissue.

In order to approach the possibilities mentioned above, I decided to analyse MN production at early stages. For this, I analysed embryos at E10.0-E11.0 at the forelimb level. Overall motoneuron production was analysed at the peak/late period of MN genesis (E10.0-E10.25) and after completion of MN production (E10.75-E11.0) (Arber et al., 1999). Counting Islet1+ cells revealed a 1,95 ± 0,1 fold increase in the number of differentiated MNs at E10.0-E10.25 (control = 63,8 ± 12,7 cells per section; *Dll1*$^{LacZ/Ki}$ = 127,5 ± 9,2 cells per section; p≤0.001; Figure III.14, F,G). At E10.75-E11.0, however, this increase in MN production is no longer detectable. In fact, the number of Islet1+ MNs is equal, if not decreased in the *Dll1* mutant neural tube (control = 220 ± 29,2 cells per section; *Dll1*$^{LacZ/Ki}$ = 193 ± 20,3 cells per section; Figure III.14, H,I).

This raises the question of what happens to the excessive MNs observed at earlier stages? Did the prematurely differentiated MNs enter apoptosis? Previous reports have shown that the absence of Notch signalling in the nervous system can enhance apoptosis (Genoud et al., 2002; Lutolf et al., 2002). Nevertheless, this hypothesis is unlikely since immunostaining for cleaved Caspase3, a marker of apoptosis, reveals no significant differences between control and *Dll1*$^{LacZ/Ki}$ embryos (Figure III.14, P,Q).

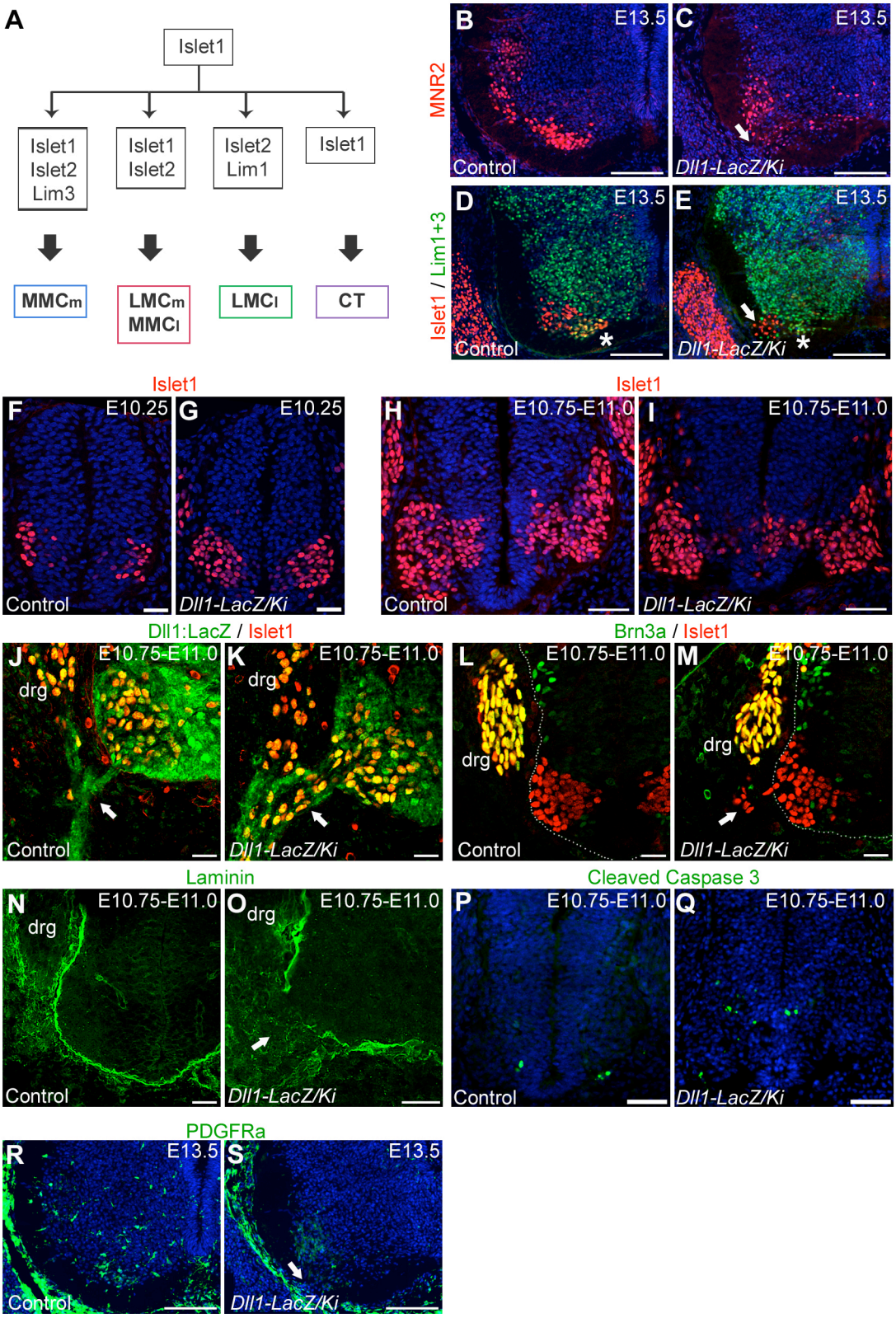

A
Islet1
Islet1 Islet2 Lim3
Islet1 Islet2
Islet2 Lim1
Islet1
MMCm
LMCm MMCl
LMCl
CT
B E13.5
C E13.5
MNR2
Control
Dll1-LacZ/Ki
D E13.5
E E13.5
Islet1 / Lim1+3
Control
Dll1-LacZ/Ki
Islet1
F E10.25
G E10.25
Control
Dll1-LacZ/Ki
Islet1
H E10.75-E11.0
I E10.75-E11.0
Control
Dll1-LacZ/Ki
Dll1:LacZ / Islet1
J E10.75-E11.0
drg
K E10.75-E11.0
drg
Control
Dll1-LacZ/Ki
Brn3a / Islet1
L E10.75-E11.0
drg
M E10.75-E11.0
drg
Control
Dll1-LacZ/Ki
Laminin
N E10.75-E11.0
drg
O E10.75-E11.0
drg
Control
Dll1-LacZ/Ki
Cleaved Caspase 3
P E10.75-E11.0
Q E10.75-E11.0
Control
Dll1-LacZ/Ki
PDGFRa
R E13.5
S E13.5
Control
Dll1-LacZ/Ki

Figure III.14: Neurogenesis in the pMN domain of *Dll1*$^{LacZ/Ki}$ embryos.
(A) Scheme showing the different MN subtypes produced in the mammalian neural tube. Each of the differentiated subtypes expresses a different combination of LIM genes. Overall MN production was assessed at E13.5 by immunostaining for MNR2 **(B,C)**. *Dll1*$^{LacZ/Ki}$ embryos **(C)** show a decrease in the number of MNs when compared to littermates **(B)**. This decrease in the number of MNs was also reflected on the number of Islet1-positive MNs, as well as on the number of Lim1+/Lim3+/Islet1+ positive MNs **(D,E)**, which are absent in the spinal cord of *Dll1*$^{LacZ/Ki}$ embryos **(E,** asterisk**)** when compared to that of wild-type littermates **(D,** asterisk**).** At early stages of MN production, (E10.25), a clear increase in MN production (Islet1+ cells) is observed in the absence of Dll1 **(G)** when compared to control littermates **(F)**. The excessively produced MNs in *Dll1*$^{LacZ/Ki}$ embryos are no longer detectable from E10.75 onwards **(H,I)**, due to migratory defects of the MNs that escape into the extraspinal region **(**arrows in **K,M)**, whereas MNs in control embryos accumulate in the ventral horn of the spinal cord **(J,L)**. E10.75 wild-type embryos display an intact basal membrane **(N)**, while the ventral horn of *Dll1*$^{LacZ/Ki}$ embryos show loss of Laminin, a marker of the basal membrane that delimits de neural tube structure **(O)**. Immunostaining for activated Caspase 3 **(P,Q)**, reveals no difference in cell death between *Dll1*$^{LacZ/Ki}$ embryos **(Q)** and control littermates **(P)**. Immunostaining for the PDGFRα, shows many OPCs in the E13.5 wild type neural tube **(R)**, while almost none are observed in *Dll1*$^{LacZ/Ki}$ embryos **(S).** drg – dorsal root ganglion. Scale bars **(B-G, J-O, R-S)** = 25μm; **(H,I,P,Q)** = 50μm

A more detailed analysis reveals that *Dll1*$^{LacZ/Ki}$ embryos present migration defects of the MN population. Immunostaining for β-Gal and Islet1 shows that all MNs are derived from *Dll1*-expressing cells and that, in control embryos, these accumulate in the ventral horn of the developing neural tube (Figure III.14, J). However, in *Dll1*$^{LacZ/Ki}$ embryos, Islet1+ cells are observed in the axonal projections that exit from the ventral horn of the spinal cord (arrow in Figure III.14, K). Since Islet1 is a marker not only of MNs, but also of sensory neurons in the dorsal root ganglion (DRG) and of a subset of dorsal interneurons of the spinal cord (Ericson et al., 1992; Thor et al., 1991), the identity of these extraspinal Islet1+ cells was questioned. While both DRG neurons and MNs express Islet1, only the former cell type expresses Brn3a. Thus, by performing double immunostaining for Islet1 and Brn3a (Figure III.14, L,M), I could conclude that the extraspinal Islet1+,Brn3a- cells along the ventral axonal projections are indeed MNs (arrow in Figure III.14,M). Furthermore, the basal lamina that envolves and delimits the spinal cord is disrupted in the ventral horn of *Dll1*$^{LacZ/Ki}$ embryos (Figure III.14, N,O), coincident with the observed escaping MNs.

Like in the remaining domains of the developing neural tube, the inactivation of *Dll1* leads to a neurogenic phenotype where MNs are produced in excess at the expense of glial cell types, more specifically OPCs (Figure III.14, R,S). This is in accordance with previous reports (Grandbarbe et al., 2003; Park and Appel, 2003) that show that

Notch signalling is necessary for the maintenance of a progenitor pool in the pMN domain that subsequently generates OPCs.

Dll1 and V0 interneuron subtype specification

The pV0 domain generates ventral commissural interneurons that establish local intersegmental connections with neurons of the ventral horn (Moran-Rivard et al., 2001). Work performed by Jessell and colleagues have shown that the expression of *Dbx1*, a homeobox gene, is restricted to the pV0 domain (Pierani et al., 1999; Pierani et al., 2001). Furthermore, it was shown that this *Dbx1*-expressing domain is subdivided into dorsal and ventral subregions: the ventral region, $pV0_V$, coexpresses *Dbx2* and *Pax6* (a marker of ventral neural progenitors), while the dorsal $pV0_D$ region coexpresses *Dbx2* and *Pax7* (a marker of dorsal neural progenitors). Although fate mapping of the pV0 domain shows that both $pV0_V$- and $pV0_D$–derived interneurons share the same medial migratory route, these two interneuron subtypes are molecularly distinct: interneurons from the $pV0_V$ domain express Evx1, whereas differentiated $pV0_D$-derived interneurons do not (Pierani et al., 2001). Even though little is known about the individual and/or unique functions of the $V0_V$ and $V0_D$ interneurons, I questioned if Dll1-mediated Notch signalling could participate in cell fate decisions involving these two V0 interneuron subtypes. Although no marker is known to be specific for the $V0_D$ interneuron subtype, one is able to distinguish these from the $V0_V$ subtype by marking all pV0-derived interneurons and simultaneously immunolabeling for Evx1: pV0-derived interneurons expressing Evx1 are $V0_V$, whereas pV0-derived interneurons that do not express Evx1 are $V0_D$.

The expression of the *Dbx1* gene has been reported to be restricted to the pV0 domain of the developing neural tube (Pierani et al., 1999), therefore I used a transgenic mouse line expressing iCre under the control of the *Dbx1* promoter (unpublished data from N. Kessaris, UCL, UK) in order to visualize all pV0-derived interneurons. Since the pV0 domain is flanked dorsally and ventrally by the *Jag1*-expressing domains, pV1 and pD6, respectively (Matise and Joyner, 1997) and our unpublished data – see Section IV.2.1), I checked the restricted recombination pattern of the $Dbx1^{iCre}$ mouse line, by crossing it to reporter mice, *R26R-YFP* (Srinivas et al., 2001) and performing immunostaining for Jag1. The majority of the recombined cells were localized between the *Jag1*-expressing domains (arrowheads in Figure III.15, A), indicating that this *Dbx1*-iCre transgenic mouse line is specific and restricted to the pV0 domain of the developing neural tube.

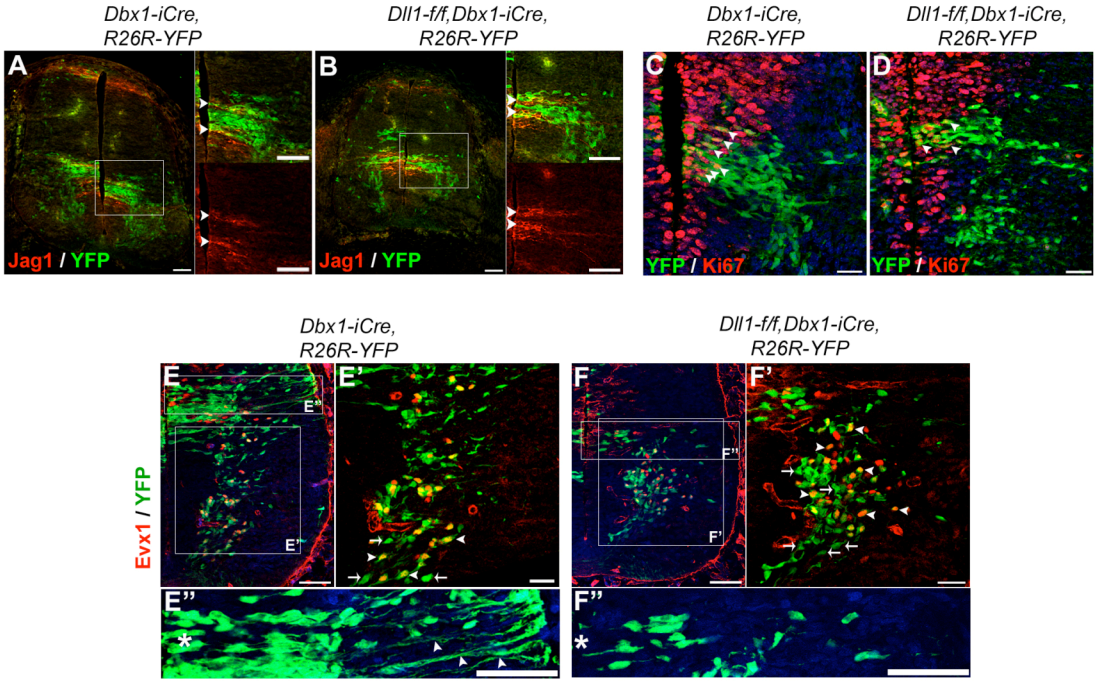


Figure III.15: Neurogenesis in the pV0 domain of the neural tube at E11.5.

(A) Recombination mediated by the *Dbx1-iCre* line is restricted to the pV0 domain, which is flanked ventrally and dorsally by Jag1-expressing cells (arrowheads in magnification). **(B)** Embryos lacking Dll1 activity in the pV0 domain (*Dll1*$^{f/f}$,*Dbx1*$^{iCre/+}$,*R26R-YFP*) show a decrease in the number of YFP+ progenitor cells in the ventricular zone (compare arrowhead in **A** and **B**), with an almost collapse of the region between the two Jag1 domains. (**C**,**D**) Double immunostaining for YFP and Ki67, a marker of proliferating cells, confirms the decrease in the number of pV0 progenitor cells (YFP+,Ki67+) in *Dll1*$^{f/f}$,*Dbx1*$^{iCre/+}$,*R26R-YFP* embryos (**D**) when compared to control littermates (**C**), which leads to the almost complete disappearance of the gap separating the two Jag1-expressing domains. Control embryos present YFP-expressing $V0_D$ (YFP+/Evx1+) and $V0_V$ (YFP+/Evx1-) interneurons (**E'**, arrows and arrowheads, respectively) that migrate along a medial-ventral route and also YFP+ cells with radial glial morphology (arrowhead in **E''**). Despite the increased production of V0 interneurons observed in *Dll1*$^{f/f}$,*Dbx1*$^{iCre/+}$,*R26R-YFP* mutant embryos, $V0_D$ and $V0_V$ interneurons (**F'**, arrows and arrowheads, respectively) appear to be present in equal proportions and follow the same migratory route as in control embryos. However YFP+ cells resembling radial glial are no longer detected. Scale bars **(A,B,E,F)** = 50µm; **(E',F',C,D)** = 25µm.

The function of the Dll1 ligand in determining cell decisions among the pV0-derived interneuron subtypes was therefore approached using *Dll1*-floxed mice expressing iCre recombinase under the control of the *Dbx1* promoter and a reporter allele (referred as *Dll1*$^{f/f}$,*Dbx1*$^{iCre/+}$,*R26R-YFP*). At E11.5, a clear reduction in the number of ventricular YFP+ cells was observed in the *Dll1* mutant mice (arrowheads in Figure III.15, A,B and asterisk in Figure III.15, E'',F''), revealing a decrease of pV0 progenitors. As a consequence, one can observe an almost complete "fusion" of the

adjacent Jag1-expressing domains (arrowheads in Figure III.15, A,B). This decrease in *Dbx1*-expressing progenitor cells is also confirmed by double immunostaining for YFP and Ki67, a marker of proliferating cells, as few YFP+,Ki67+ cells are observed in *Dll1*$^{f/f}$*,Dbx1*$^{iCre/+}$ neural tubes when compared to control littermates (arrowheads in Figure III15, C,D). The premature loss of pV0 progenitors is accompanied by a ~32% increase in neuronal differentiation, as revealed by the higher number of Evx1+ interneurons (control = 76,4 ± 4,8 cells/section; *Dll1*$^{f/f}$*,Dbx1*$^{iCre/+}$ = 101,2 ± 1,5 cells/section; p<0,05). Despite the increased number of differentiated V0 interneurons in the *Dll1* mutant, these interneurons display a medial migration towards the ventral neural tube as in control embryos (Figure III.15, A,B and E,F). Although neuronal differentiation in the pV0 domain was determined by counting only the Evx1+ $V0_V$ subtype, double immunostaining for YFP and Evx1+ shows that over-production of $V0_V$ interneurons (YFP+,Evx1+; arrowheads in Figure III.15, E',F') did not occur at the expense of $V0_D$ interneurons (YFP+,Evx1-; arrows in Figure III.15, E',F'). Thus, my results indicate that Dll1-mediated Notch activity plays no role in the specification of $V0_V$ and $V0_D$ interneurons in the V0 domain of the spinal cord.

I have already described above that Dll1-mediated Notch signalling is required for the production of glial cells, astrocytes and oligodendrocytes. This observation is once more confirmed in *Dll1*$^{f/f}$*,Dbx1*$^{iCre/+}$*,R26R-YFP* embryos. Previous work performed by Kessaris and colleagues, using another transgenic Dbx1-iCre line (Fogarty et al., 2005), showed that the *Dbx1*-expressing domain generates radial glia, which later differentiates into oligodendrocytes. At E11.5, YFP+ cells with a typical radial glial morphology are also observed in control embryos: these cells extend a single thin process from the luminal to the pial surface (arrowheads in Figure III.15,E''; reviewed in (Bentivoglio and Mazzarello, 1999)). However, in *Dll1* mutant embryos (*Dll1*$^{f/f}$*,Dbx1*$^{iCre/+}$*,R26R-YFP*), I do not observe any of these cells with radial glial morphology (compare E'' and F'' in Figure III.15).

Altogether, these results confirm previous observations that *Dll1* activity is necessary to maintain a pool of progenitor cells and thereby assure the production of late cell fates, such as astrocytes and oligodendrocytes.

III.3.3 The *Dll1-Dll4* circuitry regulating neurogenesis in the pV2 domain

Dll1 is expressed in almost all domains of the developing mouse neural tube and, with the exception of the pV2 domain, it is the only DSL ligand there expressed. This suggests that not only is Dll1 necessary for the maintenance of the neural progenitors but also that it is sufficient. Why then is a Dll1/Dll4 circuitry specifically necessary in the pV2 domain of the neural tube (and in the retina as well), if Dll1-mediated signalling is necessary and sufficient to regulate neurogenesis?

III.3.3.1 Both *Dll1* and *Dll4* contribute to progenitor maintenance in the neuroepithelium

The results above described indicate that the expression of *Dll1* and *Dll4* during neurogenesis is very similar, both in the developing retina and the pV2 domain of the neural tube (Figure III.8). Given the fact that only two neuronal subtypes, V2a and V2b, are produced in the V2 domain, when compared to seven major cell types produced in the retina, I chose the pV2 domain as a simpler system to approach the function of the *Dll1*/*Dll4* circuitry. In a first step, I analysed the effects of *Dll1* inactivation in the pV2 domain. Embryos were collected at E11.5 and neuronal production in the pV2 domain was evaluated in the pV2 domain at the forelimb level, in control and $Dll1^{f/f}$;$Nestin^{Cre/+}$ neural tubes. Immunostaining of control and *Dll1* mutant neural tubes for Chx10, a marker of V2a INs (Figure III.16, A,B), and GATA3, a marker of V2b INs (Figure III.16, C,D) revealed an increase in the total number of V2 interneurons in *Dll1* mutant embryos, when compared to control embryos, indicating that *Dll1* is necessary to regulate neuronal differentiation in the pV2 domain (Figure III.16, E). This is in agreement with previous work on *Presenilin1* and *Notch1* mutants (Del Barrio et al., 2007; Peng et al., 2007; Yang et al., 2006), where pV2 progenitors have also been shown to differentiate in excess. However, by performing double immunostaining for Jag1 and Sox2, a marker of progenitor cells, I could still detect $Sox2^+$ progenitors ventral of the Jag1-expressing domains. These $Sox2^+$ progenitors are most likely in the pV2 domain of *Dll1* mutant spinal cords (Figure III.16, F-G´ - arrowhead), suggesting that, like in the *Dll1* mutant retina, Dll4 is activating Notch and maintaining progenitors in the pV2 domain. This was confirmed by comparing the expression of *hes5* in $Dll1^{f/f}$;$Nestin^{Cre/+}$ and wild-type embryos: while *hes5* expression is maintained in the *Dll4*-expressing pV2 domain of

Dll1 mutant embryos (Figure III.16, H,I - arrowhead), there is a severe reduction on the levels of *hes5* in other spinal cord domains where *Dll1* is the only expressed Notch ligand (Figure III.16, H,I - bracket). Together, these results show that both *Dll1* and *Dll4* contribute to activate Notch signalling in neighbouring progenitors to prevent their differentiation, in the pV2 domain as well as in the retina.

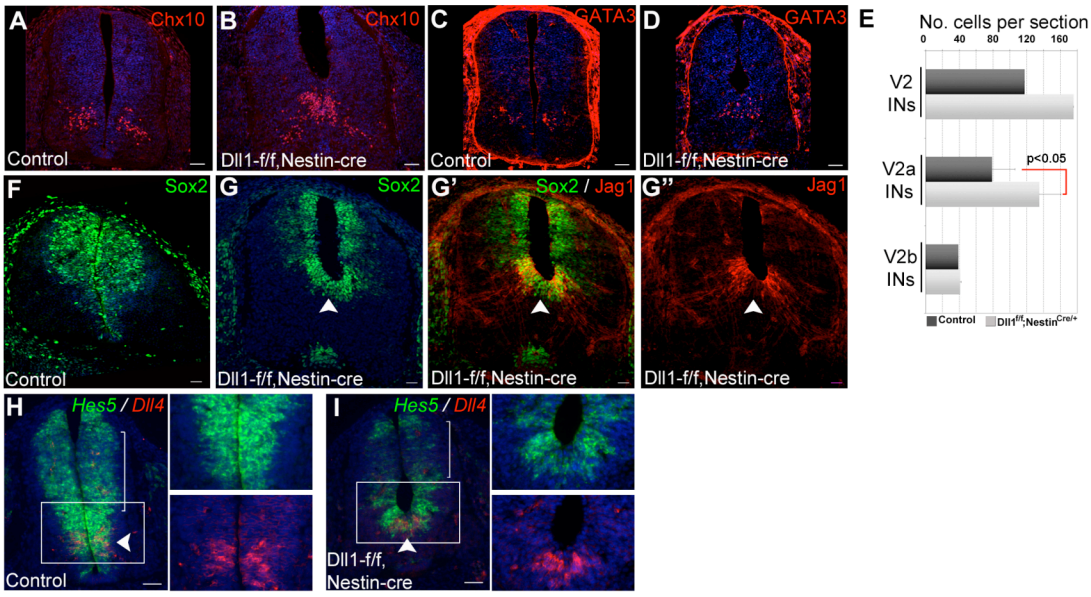


Figure III.16: Neurogenesis in the *Dll1*- and *Dll4*-expressing pV2 domain.
Analysis of neurogenesis in the pV2 domain of *Dll1* mutants shows that the overall increase in V2 interneuron production is due to an increase of the Chx10^{+} V2a subtype **(A,B,E)**, without alterations in the generation of the GATA3^{+} V2b subtype **(C,D,E)**. **(F)** E11.5 wild-type embryos have a progenitor population (Sox2+ cells) distributed throughout the neural tube, while *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ embryos display a reduction in the number of progenitors in domains expressing only the *Dll1* **(G)**. In the pV2 domain (arrowhead in G,G') and in the adjacent *Jag1*-expressing V1 domain (in red), Sox2+ progenitors are maintained **(G,G',G'')**. **(H,I)** Analysis of *hes5* expression shows that Notch signalling is still active in pV2 progenitors (arrowhead), despite the absence of Dll1. Note the strong reduction in *hes5* expression in domains expressing only *Dll1* (bracket). Also, in *Dll1* mutants, *Dll4* continues to be expressed in V2 interneurons **(I)**. INs – interneurons; Scale bars **(B-I)** = 50 µm

III.3.3.2 *Dll4* functions to generate neuronal diversity in the pV2 domain

The results shown in previous sections reveal that *Dll4* can also contribute to progenitor maintenance in the retina and in a restricted domain of the spinal cord, where it shares this function with *Dll1*. However, this is unlikely to be the major role of *Dll4* during neurogenesis as it, alone, could not provide enough Notch activity to maintain a normal pool of progenitors in the absence of *Dll1*. So, what other functions

does *Dll4* have in the retina and pV2 domain? To answer this question, I analysed neuronal differentiation in the pV2 domain when *Dll1* is mutated and only *Dll4* is active. In this situation (E11.5 $Dll1^{f/f}$;$Nestin^{Cre/+}$ embryos), I found that the production of V2a interneurons is increased in comparison to control embryos, with no significant alteration in the population of V2b interneurons (Figure III.16, A-E).

These results contrast with what has been previously reported in *Presenilin1* and *Notch1* mutants, where Notch signaling is abolished and all pV2 progenitors differentiate as V2a interneurons, at the expense of the V2b fate (Del Barrio et al., 2007; Peng et al., 2007; Yang et al., 2006). The presence of both neuronal subtypes in the V2 region of *Dll1* mutant spinal cords, therefore implies that the generation of distinct neuronal fates from a common progenitor is mainly controlled by Dll4-mediated Notch signalling, with Dll1 being dispensable in this decision. Analysis of neuronal differentiation in *Dll1* mutant retinas shows a similar phenotype, with the various retinal subtypes being produced when Dll4 is the only active Notch ligand (Figure III.6). Thus, the results described above reveal that *Dll1* and *Dll4* have overlapping but different functions during neurogenesis in the retina and pV2 domain: while *Dll1* is the main player controlling the entry of progenitors into differentiation, *Dll4*'s main role is to regulate the acquisition of distinct fates by differentiating neurons.

III.4 DLL4 SIGNALS TO CELLS ALREADY COMMITTED TO DIFFERENTIATION

I have so far shown that *Dll1* and *Dll4* are part of a genetic circuitry in which the two genes are expressed sequentially, and suggested that the two Dll ligands act in concert in the developing retina and pV2 domain of the neural tube. Whereas Dll1 acts upon neighbouring progenitor cells and, through Notch activation, inhibits these from embarking on differentiation, the subsequent activity of Dll4 would lead to a second phase of Notch signalling to diversify cell fates. Which cels are involved in Dll4-mediated Notch interaction to regulate the pV2 cell fate decision?

To address this question, I performed double in situ hybridization to detect simultaneously the *Dll4*-expressing cells and the cells where Notch is activated, identified by their expression of *hes5*. As a control, I did double in situ hybridization with *Dll1* and *hes5*, which are expected to be active in adjacent cells as result of Dll1-mediated Notch activity, *Dll1* in newborn neurons and *hes5* in neural progenitors (Chitnis et al., 1995; Henrique et al., 1995; Henrique et al., 1997). This pattern could

be confirmed in regions of the neural tube where Dll1 is the only expressed ligand (Figure III.17, Aii). However, in both the pV2 domain and neural retina, I could detect cells co-expressing *Dll1* and *hes5* (arrowheads in Figures III.17, Ai and III.17, C), as well as cells expressing *Dll1* and no *hes5*. No cells co-expressing both *Dll4* and *hes5* were found in the pV2 domain or retina (Figure III.17, B and Figure III.17, D). These results show to a further extent the similarity between the pV2 domain and developing retina and also that in these regions of the CNS, the presence of *Dll4* seems to correlate with the activation of the Notch pathway in cells that are already committed to differentiation (*Dll1*-expressing cells).

Altogether, the results described above and in previous sections indicate that the consecutive expression of *Dll1* and *Dll4* in the same cells reflect a sequential role for each of the genes. In this Dll1-Dll4 circuitry, I propose that *Dll1* acts as the main player in maintaining a pool of progenitors and *Dll4* regulates the acquisition of distinct fates by differentiating neurons, possibly by signalling to cells newly committed to neuronal differentiation. This hypothesis is very attractive, since previous work done by Cepko and collaborators has suggested that retinal cell type determination may occur at or after the final cell division.

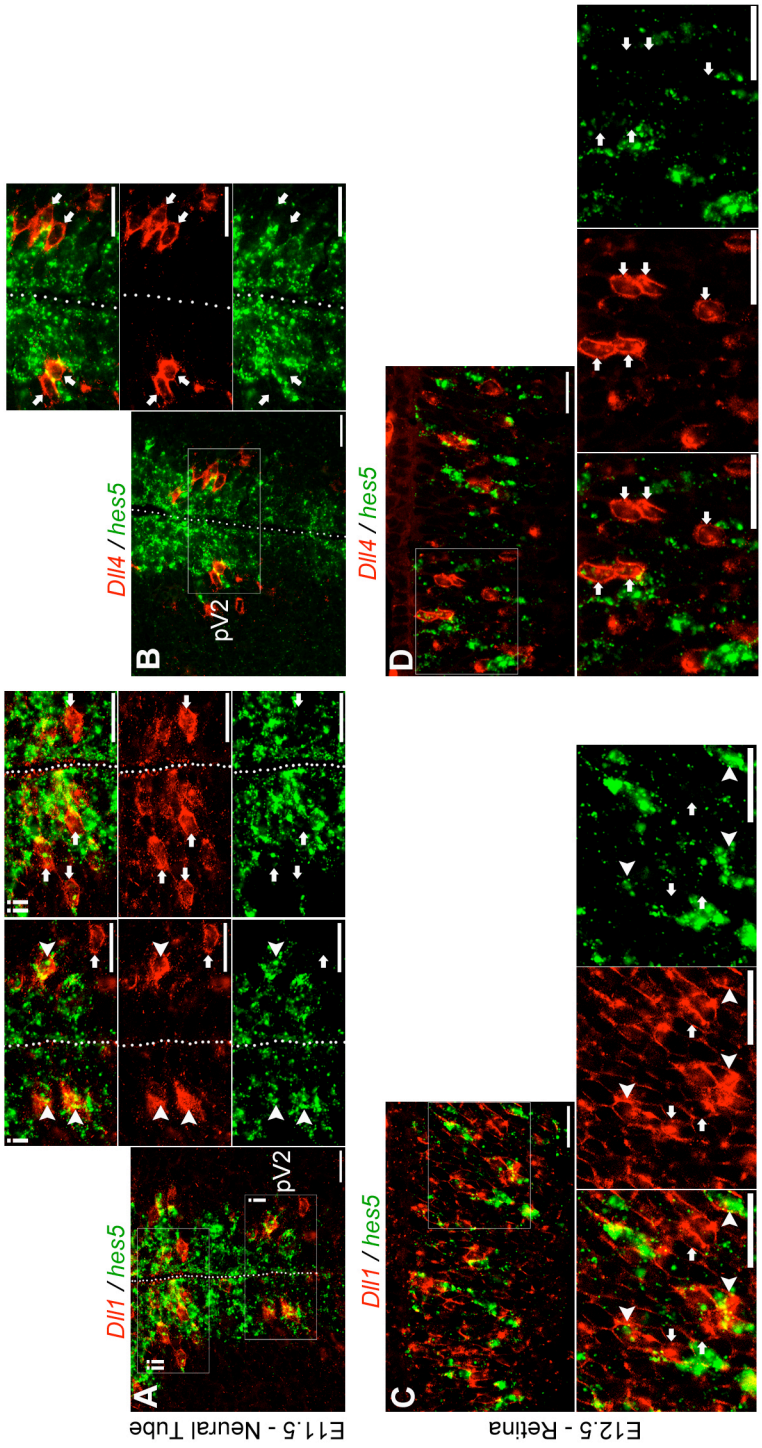


Figure III.17: Activation of the Notch pathway in cells committed to neuronal differentiation, in *Dll4*-expressing regions of the CNS.
(A-D) Double in situ hybridization for *hes5* and *Dll1* **(A,C)** or *Dll4* **(B,D)**, in sections of E11.5 neural tubes **(A,B)** and E12.5 developing retinas **(C,D)**. In domains where *Dll1* is the only ligand to be expressed, Notch activity, monitored by the expression of the downstream target *hes5*, is excluded from newborn neurons (*Dll1*-expressing cells; arrows in **A,ii**). However, in regions where the *Dll4* ligand is also expressed, such as the pV2 domain **(A,i)** and the retina **(C)**, cells embarking on differentiation can still show Notch activity (*Dll1+*; arrowheads in **C**). These *Dll1+,hes5+* cells compose only a fraction of the *Dll1*-expressing cells (arrows in **C**). As for *Dll4*-expressing cells, these never present coexpression of *hes5* (arrows in **B,D**). Dashed lines in **(A,B)** indicate midline of neural tube. Apical side of retinas **(C,D)** is up. Scale bars = 25µm.

III.5 DISCUSSION

Someone in the library is typing away crazily and ruffling paper sheets. Through the corner of my eye I see the hedges swaying with the wind. I sigh and rub my eye. Flip-flop, flip-flop, there he comes again, with jingling keys. Humm, with fresh coffee too… I can smell it. He sips it noisily. Irritating! I sigh and fidget my feet. And during all this information reception, I am continuously breathing, pumping blood, trying to cope with the heat and processing some self-satisfying chocolate. My fingers continue gliding over the keyboard, searching for the right characters, while searching for the right words in my mind to write this thesis. Ahh.. for a moment, I had silence. Managed to ignore what surrounds me and focus on this.

All this, that I am consciously aware of, in less than a minute and all regulated by the nervous system. There is no doubt that the vertebrate nervous system is remarkably complex! It is responsible for regulating all aspects of body function: it perceives both the internal and external environment, distinguishes a wide variety of stimuli and establishes specific responses to each of these. It is therefore not surprising that not only millions of neurons (10^{11}) are required to regulate all body function, but also a wide range of specific cell types is needed to receive specific stimuli, deliver the information and execute a specific response. In order for such a vast variety of neurons to be reproducibly generated among individuals within a given species, neurogenesis has to occur over an extended time period. Therefore, regulatory mechanisms must exist to finely regulate proliferation and differentiation, assuring in this way that the developing nervous system can continuously generate new neural progenitor cells, while generating the correct number and types of neuronal and glial cells.

In addition to the numerous cell types present in the CNS, particularly in vertebrates, it is equally remarkable that five major and universal signalling pathways (Shh, BMP, Wnt, Notch and RTK signalling pathways) are at the basis of regulating multiple processes in development and account for instance, for the specification of the correct cell fates, proliferation and migration. The Notch pathway, for instance, on which the work presented in this thesis is focused on, has been implicated in the generation and maintenance of neural progenitors, cell viability, cell fate determination, boundary formation, neuronal migration and control of dendritic

morphology (reviewed in (Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005)). Whether these multiple functions of the Notch pathway rely on the reiterated usage of the same molecular components at different steps of neuronal production, or involve distinct circuitries to control independently each step is not yet known. Vertebrates contain various genes encoding Notch ligands, which are expressed in the developing vertebrate nervous system with distinct spatiotemporal patterns, suggesting the existence of distinct genetic networks to regulate neuronal production. Thus, an attractive possibility is that different ligands mediate distinct Notch functions at each step of the neurogenesis process. In an attempt to elucidate the biological role of combinatorial expression patterns of the Notch ligands in eliciting different Notch responses, this thesis focuses on several ligands that are expressed in the CNS.

More specifically, in this particular chapter, I have focused on the Dll1 and Dll4 ligands. I first describe a comprehensive study of the expression of these two genes and show that they are co-expressed in a population of differentiating neurons in the retina and pV2 domain of the spinal cord, with *Dll1* starting to be expressed earlier than *Dll4* in these cells. The results disclose novel aspects of the dynamic expression of *Dll* genes during neurogenesis, and give a new perspective on how Notch signalling operates to control consecutive steps of neuronal production in vertebrate embryos. More importantly, my observations from detailed analysis of *Dll1* mutant embryos fit well with our findings on the co-expression of *Dll1* and *Dll4*, revealing that both genes have overlapping but distinct functions in the retina and pV2 domain: while *Dll1* is necessary and sufficient to maintain neural progenitors, it is dispensable for neural cell type diversification, a process also mediated by Notch signalling and where *Dll4* plays the major role. In conclusion, the results here presented uncover a novel genetic circuitry involving the sequential activity of two *Dll* genes to coordinate consecutive steps of neuronal production in two regions of the developing CNS: first, to control the rate of neurogenesis and, second, to create cell diversity in the population of differentiating neurons.

Here I will discuss the results described in this chapter and explore possible models that have been developed during the course of this work.

III.5.1 The sequential expression of *Dll1* and *Dll4* in the developing retina and pV2 domain of the neural tube

In this work, I performed a detailed analysis of the expression of *Dll1* and *Dll4* during neurogenesis in the mouse retina and spinal cord and show that the two genes are sequentially expressed in cells entering neuronal differentiation, with *Dll1* starting to be expressed before *Dll4*. This conclusion is supported by the following evidence:

a) In the marginal regions of the retina, *Dll1* expression is detected in cells at more peripheral locations than *Dll4*-expressing cells. Given the central-to periphery wave of neurogenesis, whereby peripheral cells are less advanced in differentiation than more central cells (Prada et al., 1991), this implies that *Dll1* starts to be expressed at an earlier phase of the differentiation process than *Dll4*.

b) *Dll1* expression can be detected in retinal cells still expressing the progenitor marker Chx10 (Rowan and Cepko, 2004), while no *Dll4*-expressing cell show expression of this marker. On the contrary, all *Dll4*-expressing cells express *hes6*, a gene which is active in differentiating neurons, while only a fraction of the cells coexpress *Dll1* and *hes6*.

c) Double in situ hybridization reveals that the majority (~80%) of *Dll4*-expressing cells also express *Dll1*, thereby showing that expression of the two *Dll* ligands occur in the same population of differentiating neurons and not in separate populations. Since *Dll1* starts to be expressed before *Dll4*, we can therefore conclude that the two genes are sequentially expressed in this population.

In addition, analysis of retinas from $Dll1^{LacZ/+}$ and $Dll4^{LacZ/+}$ mice at E12.5 reveals that *Dll1* is expressed in all RGCs during their progression into differentiation (Figure III.2, A). This implies that, at least for RGCs, all *Dll4*-expressing cells must have gone through a phase of *Dll1* expression and suggests that the minor population of retinal cells expressing only *Dll4* are cells that have already switched-off *Dll1* but still express *Dll4*, before advancing into the next stages of differentiation. Taken together, these findings support a model (Figure III.1, L) where the two *Dll* ligands are expressed sequentially in differentiating retinal neurons, starting with *Dll1* expression when cells commit to differentiation. It follows a period where *Dll1* and *Dll4* are both transcribed in newborn neurons, after which the expression of *Dll1* fades away faster than that of *Dll4*, with both genes being finally switched off, before overt neuronal

differentiation. The same model applies to the differentiation of V2 interneurons in the developing spinal cord, where I also show that *Dll1* starts to be transcribed before *Dll4* in pV2 progenitors, followed by a period where the two *Dll* genes are coexpressed and then turned off before full differentiation.

In a recent work by Reh and collaborators, a similar sequential expression pattern for the *Dll1* and *Dll4* genes was found in the chick retina (Nelson and Reh, 2008), with *Dll1* being already expressed in mitotically active progenitors, while *Dll4* was only transcribed in differentiating neurons. However, the authors were not able to discern whether *Dll1* and *Dll4* are expressed in the same or in separate populations of retinal cells. In the work here described, I now show that the two *Dll* genes are sequentially expressed in the same population of differentiating neurons in the mouse retina and pV2 domain of the spinal cord, implying that *Dll1* and *Dll4* are part of the same neurogenic program active during neurogenesis in these regions of developing CNS. However, can we establish that this Dll1→Dll4 neurogenic programme is common to all retinal and pV2 domain-derived progenitors? In particular, given the marked disproportion of *Dll1*- and *Dll4*-expressing cells and the transient character of this expression (between commitment to and overt neuronal differentiation)? How then will this Dll1-Dll4 circuitry regulate neurogenesis? I will attempt to answer this question in the following sections.

III.5.2 A single Notch ligand is necessary and sufficient to regulate the rate of neurogenesis

This neurogenic program involving the coexpression of two *Dll* ligands in differentiating neurons is unique to the retina and the pV2 domain. In the remaining regions of the neural tube, cells entering differentiation express just one Notch ligand, either *Jag1* or *Dll1*, which are expressed in separate populations of the spinal cord, while *Dll3* is transcribed later in differentiating neurons that no longer express *Dll1* or *Jag1* (Campos et al., 2001; Dunwoodie et al., 1997). In the developing retina, other ligands are also expressed but in different patterns relatively to *Dll1* and *Dll4*: *Jag1* is only transcribed in cells of the ciliary margin and lens (Bao and Cepko, 1997; Lindsell et al., 1996), while *Jag2* in expressed in differentiated RGCs after they reach the ganglion cell layer (Valsecchi et al., 1997). Altogether, the various expression patterns of Notch ligands in the developing mammalian CNS reveal the existence of distinct genetic circuitries controlling neurogenesis in different regions of the nervous

system. However, little is known on how these circuitries contribute to the final diversity of neural cell types and their global organization in the developing nervous system. This is mainly due to the early embryonic lethality of mice carrying mutations in the various *Dll* and *Jag* genes, which have precluded loss-of-function studies on their role during neurogenesis.

In this work, I analyse and compare the function of two of these Notch circuitries, one active in the spinal cord and involving only *Dll1*, and another involving the coexpression of *Dll1* and *Dll4* in the retina and pV2 domain. In the first case, I show that inactivation of *Dll1* in the spinal cord domains where it is the sole expressed ligand leads to a severe neurogenic phenotype with excessive neuronal differentiation and depletion of neural progenitors. This phenotype is very similar to that described for *Hes1*;*Hes3*;*Hes5* triple mutant embryos (Hatakeyama et al., 2004), in which complete impairment of Notch signalling results also in total depletion of neural progenitors. Thus, one can conclude that the activity of a single ligand is sufficient (and necessary) for the Notch pathway to function and maintain neural progenitors throughout neurogenesis. The analysis of neurogenesis in *Jag1*-expressing domains, in a *Dll1* mutant background, further confirms the sufficiency of one Notch ligand in preserving progenitors and controlling the rate of neurogenesis. In addition, these results indicate that Jag1 performs the same function as Dll1 in the domains of the developing spinal cord where it is expressed, activating Notch signalling in progenitors to control their differentiation. Whether Jag1 might play other functions in these domains is not yet clear, but the analysis of conditional *Jag1* mutants and *Dll1,Jag1* double mutants might provide some answers to this question (see Chapter IV).

III.5.3 Dll1 and the generation of cell diversity

Although the Notch pathway has been mainly associated with a function in inhibiting neuronal differentiation, Notch also participates in other processes such as neuronal migration, dendrite arborization, boundary formation and cell fate determination (reviewed in (Louvi and Artavanis-Tsakonas, 2006)).

The role of Notch in cell fate specification has been explored in great detail in systems such as the developing vulva of *C. elegans* (Sternberg, 2004) and the nervous system of *Drosophila melanogaster* (Gaiano and Fishell, 2002). Recently, a role for cell fate determination has also been reported in vertebrate models. In the

inner ear, for instance, Notch mediates the cell fate decision between hair cell and supporting cell (Brooker et al., 2006; Daudet and Lewis, 2005; Kiernan et al., 2005; Kiernan et al., 2006) and in the retina, loss of Notch activity also disturbs cell fate specification, causing the formation of an increased number of cone photoreceptors, at the expense of other retinal cell types (Jadhav et al., 2006b; Kubo et al., 2005; Yaron et al., 2006). Therefore I questioned whether Dll1-mediated Notch signaling could also participate in cell fate specification.

III.5.3.1 Neuron-neuron specification

Despite the excessive neuronal differentiation observed in *Dll1* mutants, no alterations in the identity of the neurons being produced could be detected, showing that Dll1-mediated Notch signalling controls the rate of neurogenesis but not the type of neurons generated in each domain.

It is known that DV patterning of the neural tube subdivides the spinal cord into various cellular domains along the DV axis, each domain containing progenitors with a well-defined identity and restricted in their differentiation potential (Briscoe and Ericson, 2001). Thus, one can argue that a possible role for Dll1-mediated Notch signalling in cell fate diversification will not be detectable by looking at neuronal cell types derived from distinct progenitor domains, since progenitors from each domain might already be restricted in their differentiation potential. Nevertheless, recent reports have shown that single domains, which are comprised by progenitor cells with similar differentiation potential, can generate various cell subtypes (Alvarez et al., 2005; Ericson et al., 1997; Karunaratne et al., 2002; Pierani et al., 2001; Shin et al., 2007; Yeo and Chitnis, 2007; Zhou et al., 2000). Therefore, I focused on two domains that are known to each give rise to multiple neuronal subtypes, namely the pMN and pV0 domains, and approached the possibility of Dll1-mediated signalling participating in cell fate decisions.

The analysis of motoneuron subtype specification in embryos lacking Dll1 activity did not enable us to establish any conclusions concerning a possible role for Dll1 determining cell fates. These embryos displayed migration defects in the motoneuron population. Motoneurons were found to migrate into the extraspinal region and migrate along the axons exiting the ventral horns of the neural tube. Although I did not look further into this phenotype, similar phenotypes have been reported elsewhere and associated to the absence of neural crest-derived boundary cap cells

(Bron et al., 2007; Chauvet and Rougon, 2008; Mauti et al., 2007; Vermeren et al., 2003), which cluster at the ventral horn of the neural tube and confine motor neurons within the spinal cord. The activity of the Dll1 ligand has already been shown to be important for neural crest cell specification and migration (Cornell and Eisen, 2000; De Bellard et al., 2002; Wakamatsu et al., 2000). Since we made use of *Dll1*$^{LacZ/Ki}$ mice (null and hypomorphic alleles) to approach the function of Dll1-mediated Notch signalling in the pMN domain, one possibility is that the MN migration defects are a consequence of neural crest defects caused by Dll1 deletion in the dorsal neural tube. An alternative explanation arises from the reported finding that inactivation of the *HB9* gene, which is necessary cell autonomously for the consolidation of MN identity, also displays an identical phenotype, with leakage of MNs into the extraspinal region (Arber et al., 1999). Indeed, several studies have shown that both MNs and neural crest-derived boundary cap cells participate in MN migration/confinement within the neural tube structure, through forward and reverse semaphorin-plexin signalling between these two cell types (Bron et al., 2007; Chauvet and Rougon, 2008; Mauti et al., 2007).

Experiments deleting Dll1 activity specifically in the pMN domain, for example through the use of a Cre recombinase line driven by a *Olig2* promoter, would be necessary in order to be able to draw any conclusions in relation to the function of Dll1 in MN specification, other than progenitor maintenance. However, the analysis of V1 interneuron subtype production indicates that Dll1 does not mediate cell fate specification in this domain, since both V1 interneuron subtypes are excessively produced. Furthermore, analysis of the neuronal subtypes produced both in the pV2 domain of the neural tube and retina of *Dll1* deficient embryos, also suggests that Dll1 does not participate in neuronal cell fate decisions (discussed further below).

Nevertheless, recent work performed by Goulding and colleagues describes a possible role for Dll1-mediated Notch signalling in promoting a dIL_B fate over a dIL_A fate in the dorsal part of the spinal cord (Mizuguchi et al., 2006). dIL_A and dIL_B neurons were described to arise from a single dorsal domain, dIL, during a second phase of neurogenesis that occurs between E12-E13.5 (Gross et al., 2002; Muller et al., 2002). Within the dIL domain, progenitors with distinct potentials are present: Ptf1a-positive cells give rise to dIL_A neurons, while Ptf1a-negative originate dIL_B neurons. Although the dIL domain has been described to result from an expansion of the dI4 domain that is present during the early phase of dorsal neural tube neurogenesis (E10.5-E11.5), the similarities among dIL_A/dIL_B and dI4/dI5 are striking at the molecular level, both in the progenitor and differentiated states. Thus, it is

tempting to raise the possibility that the dIL domain derives from the intermingling of dI4- and dI5-derived progenitor cells. In such scenario, an early progenitor depletion due to Dll1 inactivation could account for a disproportion in the number of available dIL_A and dIL_B progenitor cells. Although evidence is provided that the production of the dIL_B neuronal fate is decreased in the absence of Notch signalling, with no alteration in the dIL_A fate (Mizuguchi et al., 2006), this decrease in the number of dIL_B neurons might be an indirect consequence of the early progenitor depletion due to Dll1 inactivation and not necessarily due to an active role of Dll1-mediated Notch signalling in promoting a dIL_B fate over a dIL_A fate.

III.5.3.2 Neuron-glial specification

Work done in the developing nervous system of *Drosophila*, shows that Notch signalling acts at several steps: in the selection of neural precursors (neuroblasts) and in the selection of neural versus glial fates (Gaiano and Fishell, 2002). In this chapter of my thesis, as in previous reports (Furukawa et al., 2000; Hojo et al., 2000; Jadhav et al., 2006a), it is shown that Notch signalling, in this case mediated by Dll1, is required for glial cell specification. The inactivation of *Dll1* in the developing neural tube leads to the almost complete loss of both the astrocyte and oligodendrocyte lineages due to an over-production of neuronal cell fates. Although it is true that *Dll1* is necessary for the generation of glial cell fates, it remains unknown to what extent this specification is a direct or indirect consequence of Dll1-mediated Notch signalling. Many reports have argued both in favour and against an active role of the Notch pathway in establishing the glial lineage. Experiments using Notch loss-of-function mutants have favoured a model in which the absence of glial cell fate specification is primarily due to premature depletion of progenitor cells due to excessive neuronal differentiation. In this case, no instructive role for Notch signaling is ascribed (Dorsky et al., 1995; Jadhav et al., 2006a). On the other hand, experiments based on the over-activation of the Notch pathway have supported the view that Notch plays an active and instructive role in the neurogenesis → gliogenesis switch (Chambers et al., 2001; Furukawa et al., 2000; Gaiano et al., 2000; Scheer et al., 2001; Tanigaki et al., 2001). For instance, recent studies performed by Cepko and collaborators have shown that retinal progenitor cells where Notch signalling is constitutively active acquire a glial fate (Jadhav et al., 2006a). Although these observations appear to support an instructive role for Notch in promoting the glial fate at the expense of neurons, this might not be the case.

Cepko's results show that despite the constitutive activation of the Notch pathway as early as E12, these retinal progenitor cells only acquire glial features postnatally, at the same time as wild-type cells. Therefore, it is unlikely that Notch activation acts in an instructive manner, promoting the ectopic formation of glial cells. Instead, by allowing retinal progenitors to remain proliferative and undifferentiated, these cells are permissive to extrinsic and intrinsic signals that direct or bias them towards a particular fate at specific developmental time points and therefore, allow progenitor cells to progress through multiple competence states (Figure III.18).

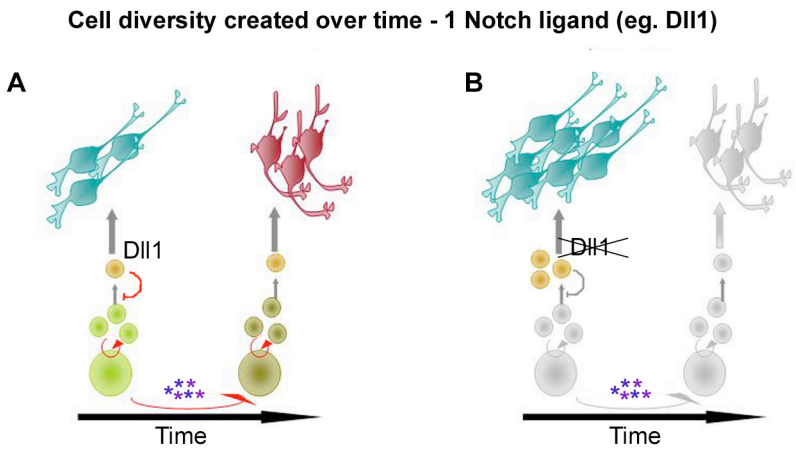


Figure III.18: Cell diversity created in time: progression through distinct competence states.
(A) Cells exiting the cell cycle and embarking on differentiation (yellow cell) express a Notch ligand, for example *Dll1*, which inhibits neighbouring cells from also embarking on differentiation. Therefore, by mediating lateral inhibition, the Notch pathway maintains a pool of progenitor cells (light green cells). By maintaining a pool of uncommitted progenitors, these are able to respond to the different signals (intrinsic and/or extrinsic - asterisks) that are available at different developmental times, and change their competence (dark green cells). This time-dependent change in the cell´s competence state allows it to differentiate into a distinct cell type from the ones produced at an earlier developmental stage. I have shown that a single DSL ligand is sufficient to assure the production of sequentially generated cell types neural cell types, such as neurons and glia, through the maintenance of a pool of progenitor cells.
(B) A failure to maintain the activity of this single DSL ligand (or of the Notch receptor) yields identical phenotypes: an excessive production of neurons at the expense of the progenitor population. This precocious depletion of progenitor cells results in the loss of the later cell fate.

III.5.4 A unique circuitry involving two Dll ligands to regulate neurogenesis

Although the general rule in the developing spinal cord seems to be that a single Notch ligand is enough to regulate neuronal production, the work here presented shows that in the pV2 domain, and also in the neural retina, two ligands – Dll1 and

Dll4, are active during neurogenesis in an overlapping population of differentiating neurons. The restriction of the Dll1/Dll4 circuitry to the retina and pV2 domain raises the interesting question of why do these regions of the developing CNS employ a different circuitry to regulate neuronal production, when compared with the other regions of the neural tube. One explanation might be related to the fact that the generation of various neuronal cell types in the retina and pV2 domain is not separated in time nor in space. This contrasts with the other domains of the SC where pre-patterning along the dorsoventral axis leads to the delimitation of several sub-domains, each containing progenitors committed to produce a single type of neuron, or various types but at consecutive periods, as is the case for the pMN domain (reviewed in (Kessaris et al., 2001; Rowitch, 2004)). Here, Notch signalling is mainly required to maintain a pool of progenitors able to generate all the successive cell types and my work indicates that the activity of a single ligand is necessary and sufficient to accomplish this function.

In the retina and pV2 domain, the final cell fate is not dependent on the time and place of their generation, instead it is decided in situ through cell-cell interactions mediated by Notch signalling. Indeed, loss of Notch function in the retina results in the overproduction of cone photoreceptors at the expense of other cell types like RGCs and amacrine cells (Jadhav et al., 2006b; Yaron et al., 2006) and in a V2b-to-V2a cell fate switch in the pV2 domain of the NT (Del Barrio et al., 2007; Peng et al., 2007). Hence, Notch activity in these regions of the CNS not only provides a signal to sustain progenitors during neurogenesis but it is also essential to generate cell diversity in the population of retinal neurons.

III.5.5 Two ligands, two outcomes of Notch signalling: proliferation and diversification

A role for Notch signalling in controlling cell fate diversity during vertebrate neurogenesis was recently extended to the zebrafish epiphysis and spinal cord (Cau et al., 2008; Shin et al., 2007; Yeo and Chitnis, 2007), involving in all cases the participation of more than one Notch ligand. In this work, I describe some findings that might provide additional clues to understand how a circuitry, involving two Dll ligands, operates to mediate two sequential functions of Notch signalling: the maintenance of a pool of progenitors and cell fate diversification.

III.5.5.1 Regulation of the rate of neurogenesis: maintenance of a pool of progenitors

As mentioned previously, the rate of neurogenesis is increased both in neural tube and in the developing retina when *Dll1* is inactivated. This is in accordance with previous work where the Notch pathway has been inactivated by different means (Del Barrio et al., 2007; Jadhav et al., 2006b; Peng et al., 2007; Yang et al., 2006; Yaron et al., 2006). Furthermore, while the analysis of domains expressing exclusively the *Dll1* ligand have revealed that the action of Dll1 alone is sufficient to regulate the rate of neurogenesis, the analysis of the pV2 domain of the spinal cord and of the retina (where *Dll1* and *Dll4* are both expressed), have shown that not only is Dll1 sufficient to do so but also that it is necessary. Dll4-mediated Notch signalling is not able to fully replace Dll1 in regulating the rate of neurogenesis. These results suggest that in these regions of the CNS where *Dll1* and *Dll4* are both expressed, *Dll1* continues to be the main player in the maintenance of uncommitted progenitors cells (Figure III.19).

III.5.5.2 Cell fate diversification

Despite the excessive neuronal production and in contrast with the *Notch1* and *Presenilin1* loss-of-function mutants where cell fate switches were observed (Del Barrio et al., 2007; Jadhav et al., 2006b; Peng et al., 2007; Yaron et al., 2006), the remaining progenitor cells in *Dll1* mutants give rise to all neuronal types, both in the retina and pV2 domain of the neural tube. These results, together with Dll1 over-expression experiments performed by the Richardson and Sharma labs (Del Barrio et al., 2007; Peng et al., 2007), indicate that it is not Dll1-mediated signalling that participates in this Notch-dependent cell fate decision. On the other hand, the Richardson and Sharma labs have shown through over-expression experiments that Dll4-mediated Notch signalling is capable of inhibiting, in a cell non-autonomous manner, the V2a interneuron fate and in this way provide the possibility of the cell acquiring a V2b cell fate. I now show that Dll4, the only remaining Notch ligand in the pV2 domain of *Dll1* mutants (and neurogenic region of the retina), is not only capable of inhibiting the V2a cell fate but also that it is sufficient to maintain the production of both interneuron cell fates. My results support a model in which the two functions of Notch signaling in the retina and pV2 domain are carried out by a novel circuitry comprising the sequential activity of Dll1 and Dll4, with the first ligand providing the major contribution to progenitor maintenance and the second ligand regulating the acquisition of diverse cell fates by differentiating neurons (Figure III.19).

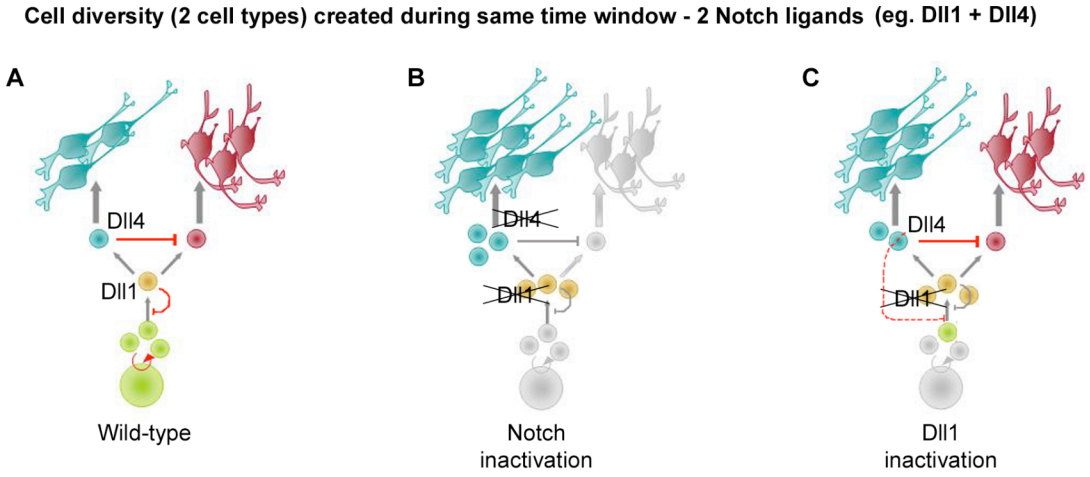


Figure III.19: Cell diversity created during the same time window.

(A) The creation of different cell types during the same time window and from common progenitors, such is the case in the pV2 domain and the retina, can be explained by the action of Notch signalling in two subsequent steps. A first phase of Notch signalling would be elicited when cells exiting the cell cycle and embarking on differentiation (yellow cell) express *Dll1,* thereby inhibiting neighbouring progenitor cells (green cells) from adopting the same neuronal fate. Thus, a pool of progenitor cells is maintained through lateral inhibition mediated by the interaction between progenitor cells and *Dll1*-expressing newborn neurons. Following *Dll1* expression, a second ligand is expressed. This second ligand, *Dll4*, initiates a second phase of Notch activity, which leads cells that have already started differentiation to acquire different cell fates. **(B)** The full inactivation of the Notch pathway, in the pV2 domain for example, leads both to the depletion of neural progenitors, as well as to the loss of cell diversification. Excessive neuronal differentiation is observed, with the overproduction of the V2a fate (blue neurons) at the expense of the V2b fate (red/grey neurons). **(C)** On the other hand, the loss-of-function of Dll1-mediated Notch signalling leads only to excessive neuronal differentiation but without the loss of any cell type. This is explained by the presence and action of the Dll4 ligand on cells that are embarking on differentiation and that are inhibited from differentiating into V2a cells.

The model I propose above contrasts with that from Reh and colleagues (Nelson and Reh, 2008), where *Dll1* is proposed to be expressed in clusters of progenitor cells and mediate mutual inhibition between them, leading to the segregation of individual cells as *Dll4*-expressing newborn neurons. Such a mechanism implies that all differentiating neurons would express both *Dll1* and *Dll4* during their path to differentiation. Although this hypothesis is attractive and consistent with my finding that *Dll1* is normally expressed in clusters of retinal cells, other results from my work do not support this model. First, while *Dll1* is expressed in all RGCs during their progression into differentiation (Figure III.2A), only a fraction of the RGC population is derived from *Dll4*-expressing cells (Figure III.2, B). Moreover, whereas *Dll1* expression is frequently observed in clusters of retinal cells that could signal among

themselves, the same is not true in the pV2 domain of the neural tube (Figure III.17, Ai). Here, as in the rest of the *Dll1*-expressing domains of the developing neural tube, *Dll1*-expressing cells occur as isolated cells. Conceptually, this salt-and-pepper expression pattern does not allow for cross-activation of the Notch pathway among *Dll1*-expressing cells. This is confirmed by the lack of cells co-expressing *Dll1* and *hes5* in all domains of the neural tube, with the exception of the pV2 domain where, as in the retina, *Dll4* is expressed and therefore, a good candidate for activating Notch signalling in *Dll1*-expressing cells (Figure III.17).

III.5.6 Dll1 and Dll4 mediate temporally overlapping yet distinct functions

One obvious questions raised by the model presented in this thesis is how two ligands that are co-expressed and both capable of activating the Notch pathway carry out different functions? The detailed expression patterns described in this thesis for the *Dll1* and *Dll4* genes, together with the *Dll1* loss-of-function data, might provide some explanations.

III.5.6.1 *Dll1*- and *Dll4*-expressing cells have different signaling abilities

As in the other regions of the spinal cord where *Dll1* is the only ligand to be expressed, all differentiating neurons in the pV2 domain and retina express *Dll1* (see for example Figure III.2, A, where all RGCs are Dll1:LacZ positive). By performing double in situ hybridization for *Dll1* and *hes5* in domains of the neural tube where *Dll1* is the only expressed ligand, I observed that the Notch pathway is only activated in cells within the ventricular zone of the neural tube. Furthermore, none of the *hes5*-expressing cells co-express *Dll1*, indicating that Dll1 is only capable of activating Notch in uncommitted progenitor cells (Figure III.17, Aii). This observation is in agreement with the well established model that *Dll1*-expressing cells activate Notch in neighbouring progenitor cells and excludes the possibility of Dll1 mediating any Notch-dependent function in cells already embarking on differentiation. However, while Dll1-expressing cells only activates Notch in progenitor cells, Dll4 seems to activate this pathway also in cells that are already embarking on differentiation and that express *Dll1* (Figure III.17, Ai). The activation of the Notch pathway in *Dll1*-expressing differentiating cells is readily observed both in the retina and the pV2 domain of the developing NT, where *Dll4* is

also expressed. Nevertheless, the analysis of the loss-of-function of *Dll4* in the retina and pV2 domains of the neural tube is required to confirm this hypothesis.

The different signalling abilities of the Dll1 and Dll4 ligands can be due to a stronger interaction of Dll4 with Notch1, comparatively to the Dll1/Notch1 interaction. In fact, Radkte and colleagues have recently shown that Dll4 has a higher affinity for Notch1 than Dll1 (Besseyrias et al., 2007). This observation raises the possibility of Dll4 being able to bind to and activate Notch even when this receptor exists in lower levels, as in the case of cells committed to differentiation and that have already or are undergoing their last cell division. This is in agreement with the idea that Dll1 would only signal to progenitor cells, which have higher levels of Notch1, being able to promote their maintenance as progenitors. However, Dll1 would be incapable of signalling among committed cells to finely regulate the acquisition of the different cell fates characteristic of a given competence state. This last function could be carried out by Dll4, which has higher affinity for the Notch1 receptor.

The existence of different ligand-dependent efficiencies of Notch activation can account for the preference of Dll4 as the ligand able to function in creating cell diversity. Also it supports the hypothesis of Dll1 being dispensable and possibly not required at all in this process. Nevertheless, the analysis of *Dll4* mutants is required to test this hypothesis.

III.5.6.2 Dll1/Dll4 and progenitor maintenance in physiological conditions

In this chapter, I have shown that in the absence of Dll1 function, the Dll4 ligand is capable of signalling to progenitor cells, activate Notch in these and thereby contribute to their maintenance, even if only partially. But does Dll4 play a significant role in progenitor maintenance in physiological conditions? Once again, only the analysis of *Dll4* mutants will provide a clear answer to this question. Nevertheless, several observations allow me to suggest that Dll4 does not have a relevant role in progenitor maintenance and that it may actually be totally dispensable for this process.

Detailed analysis of the *Dll1* and *Dll4* expression patterns shows that these two genes are not only expressed sequentially in the same cells, with *Dll1* transcription preceding that of *Dll4,* but also that their expression overlaps temporally in such a way that most *Dll4*-expressing cells still express *Dll1*. This implies that *Dll1*-

expressing cells are already inhibiting neuronal differentiation of neighbouring progenitor cells before the onset of *Dll4* expression. Therefore, one can speculate that, in physiological conditions, Dll4 is not required for maintenance of a neural progenitor pool since Dll1 initiates this function first and is sufficient to do so, as already discussed above.

In a situation where *Dll1* has been inactivated, *Dll4* is able to compensate partially for the loss-of-function of this gene and thereby attenuate to a certain degree the neurogenic phenotype. However, unlike *Dll1*, *Dll4* is not expressed in all differentiating cells. For instance, in the pV2 domain, *Dll4* is expressed exclusively in cells destined to acquire the V2a interneuron fate, but not in cells of the V2b lineage (see Figure III.8). Also, in the retina, it is only transcribed in a subset of each retinal cell type (Figure III.2, B). In fact, this restricted expression of *Dll4* to only a subset of differentiating neurons might be fundamental for its role in creating cell fate diversity among differentiating neurons. Nevertheless, in a *Dll1*-mutant background, this reduced number of *Dll4*-expressing cells in relation to the number of differentiating cells leads to a decreased number of signal-sending cells and consequently to a decrease in the number of cells maintained as progenitors.

III.5.7 Possible mechanisms of Dll4-mediated cell fate diversification

How is the expression of *Dll4* restricted only to a subset of differentiating cells? Are cells with high levels of *Dll4* expression sorted out by classic lateral inhibition? Does *Dll4* expression occur stochastically in a subset of cells and inhibit neighbouring cells from doing so? Or is a subset of cells predetermined genetically to express the *Dll4* gene? How can Dll4-mediated Notch signalling regulate the diversification of neuronal cell types, especially in a more complex system such as the retina, where *Dll4*-expressing cell contribute to multiple neuronal cell types? These are some of the questions which arise from the work presented throughout this chapter and that remain to be completely understood. In this section of the discussion, I will attempt to answer some of these questions, based on studies published by others and/or results of my own. Nevertheless, further work is required to test and/or validate any proposed mechanism and unravel the mode of action by which Dll4 and consequently the Dll1/Dll4 circuitry functions. Although both the pV2 domain of the spinal cord and the retina make use of the Dll1/Dll4 circuitry to regulate neurogenesis, the generation of seven cell types in the retina increases the level of complexity when compared to the pV2 domain, which only generates two interneuron

subtypes. Therefore, I will first explore the pV2 domain in more detail and then adjust/extrapolate to the retinal system.

III.5.7.1 Clues from the pV2 domain of the neural tube

How does Notch signalling and more specifically Dll4-mediated Notch signalling act upon V2 interneuron development? Previous studies have shown that the V2a and V2b interneuron fates are generated during the same time period (Del Barrio et al., 2007; Peng et al., 2007), arguing against time-dependent changes in the intrinsic competence of the pV2 progenitor cells, through a mechanism such as the one depicted in Figure III.17. Instead, the authors propose that the V2a and V2b interneuron fates arise from bipotent progenitors cells, through a mechanism where both daughter cells resulting from a terminal mitosis express the Dll4 ligand (Del Barrio et al., 2007). According to their model, these *Dll4*-expressing sibling cells would undergo the classical process of lateral inhibition and the cross-talk between these two daughter cells would ultimately result in one of them becoming the signal-sending cell and the other the signal-receiving cell (Lewis, 1998). The cell with higher levels of Dll4 (signal-sending) would acquire a V2a fate, while the signal-receiving cell would differentiate as a V2b interneuron (Figure III.20). This model ths proposes the existence of Dll4-mediated Notch signalling between equivalent and bipotential pV2 cells and is in primarily based on two observations. First, the identification of intermediate progenitors cells expressing simultaneously markers of pV2 progenitor cells (FoxN4 and Mash1), as well as markers of differentiated V2a and V2b interneurons (Lim3 and GATA2, respectively), suggest the existence of equivalent and bipotential progenitor cells (Peng et al., 2007). Second, *Dll4* expression was frequently observed in pairs of cells in the ventricular region, where mitosis occurs and therefore raising the hypothesis of sibling cells exchanging signals in order to determine their final interneuron fate (Del Barrio et al., 2007).

Very recently, another study concerning the production the V2a and V2b interneuron cell types in the zebrafish spinal cord (Kimura et al., 2008), has proposed a similar model. Higashijima and collaborators have shown that indeed daughter cells resulting from the final cell division of pV2 progenitors produce both the V2a and V2b fate. This is in accordance with the models proposed by the Richardson and Sharma labs, where each of the bipotential sibling cells will acquire one of the two possible cell fates, i.e, a pV2 progenitor would divide asymmetrically in terms of cell fate. Nevertheless, fate mapping of mouse V2 interneurons is necessary to confirm

whether the V2a and V2b cell fates also arise from sibling cells in mammals, as is the case in the zebrafish neural tube.

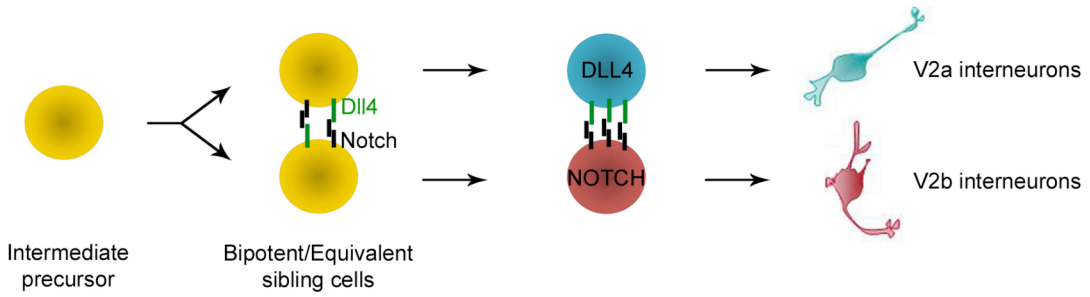


Figure III.20: Generation of cell fate diversity through Dll4-mediated lateral inhibition between equivalent and bipotent sibling cells.

pV2 progenitor cells acquire an intermediate state (yellow cell) and express simultaneously markers of uncommitted progenitor cells (FoxN4/Mash1) and of the two possible final interneuron fates (Lim3/GATA2). These intermediate precursor cells undergo final mitosis to yield two equivalent sibling cells that express *Dll4*. Cell-cell interactions are established between the sibling cells, through the Dll4 ligand and the Notch1 receptor. Lateral inhibition between these two cells establishes feedback loops that ultimately lead to one of the cells acquiring increased levels of Dll4 ligand (blue cell) and the other cell exhibiting higher levels of activated Notch (red cell). This simple mechanism of lateral inhibition between two initially equivalent cells creates different transcriptional profiles within each of these cells, enabling them to acquire distinct cell fate: a V2a or a V2b interneuron fate.

Although the zebrafish *Dll4* ortholog is not expressed in the V2 lineage (Kimura et al., 2008), Higashijima and coworkers show that another Notch ligand, *DeltaC*, is expressed at higher levels in the daughter cell that will ultimately adopt the V2a fate. This observation is in agreement with the already described model, where Dll4 is enriched in cells committed to the V2a lineage. Nevertheless, another hypothesis is also suggested to explain the high levels of *DeltaC* in only one of the sibling cells: the unequal distribution of *DeltaC* mRNA between the daughter cells of an intermediate progenitor which already expresses *DeltaC* before its final division. This model is quite attractive, since it strongly resembles the mechanisms used during the formation of SOPs, where cell divisions involving Numb-dependent asymmetric segregation of Notch signaling account for the acquisition of different cell fates between sibling cells (Bardin et al., 2004). Nevertheless, the random orientation of division axes of pV2 intermediate progenitors and the even distribution pattern of

Numb during cell division does not support a asymmetric cell fate decision according to the same mechanisms used in SOP formation.

The results presented in this thesis are conflicting with the two models described above. Firstly, fate mapping analysis in *Dll4*$^{LacZ/+}$ mouse embryos, which allows the identification of cells that have expressed *Dll4* through the detection of β-Gal, reveals that *Dll4* is only detected in cells that commit to the V2a lineage and is never active in the V2b lineage. This finding seems to rule out a model in which the V2a/V2b decision involves interactions between equivalent newborn neurons expressing *Dll4* to sort out their fates. Nevertheless, one can always argue that the equivalent sibling cells express *Dll4* at initially very low levels and that these levels would not increase in cells committing to the V2b fate, raising the possibility that the reporter β-Gal protein does not accumulate to detectable levels in the V2b lineage. The generation of a stable *Dll4* reporter mouse line would allow one to discriminate if *Dll4* expression is indeed restricted only to the V2a lineage, as suggested by the fate mapping results here presented or, alternatively, if *Dll4* is initially expressed in equivalent and bipotent newborn neurons. For this purpose a targeting vector has been produced in the lab (by Carlos Rodrigues), which shall allow the generation of a Dll4^{iCre} transgenic mouse line.

If we assume the possibility that Dll4 is expressed exclusively in cells of the V2a lineage, then it is plausible to consider a situation similar to that proposed by Higashijima and colleges, where *Dll4* could be asymmetrically expressed or inherited by pV2 sibling cells. Nevertheless, this hypothesis seems less likely, not only due to the absence of a mechanism to explain the asymmetric expression of *Dll4* in sibling V2 cells but also because of previous studies that have shown the presence of *Dll4* mRNA at apparently equal levels, in both sibling cells during terminal mitosis (Del Barrio et al., 2007). Although highly speculative, if we bear in mind the conclusions withdrawn from the fate mapping analysis in *Dll4*$^{LacZ/+}$ mouse embryos and consider the possibility of *Dll4* being expressed only in the V2a lineage, then the presence of *Dll4* mRNA in both V2 sibling cells suggests that the production of the V2a and V2b cell fates might not occur in a pair-like manner, as in the zebrafish (Kimura et al., 2008). As mentioned above, only fate mapping of mouse V2 interneurons will confirm if in mammals the V2a and V2b cell fates are also produced in a pair-like manner. In order to pursue this question, one would ideally like to label isolated cells embarking on differentiation. Therefore, I have constructed a targeting vector that will allow the production of a transgenic mouse line expressing iCre under the influence of the *Dll1* promoter (*Dll1*^{iCre}; see Materials and Methods, Section VI.9), which is active in all

differentiating neurons. By crossing this mouse line with a stable but weakly recombining reporter line, for example the R26R-GFP mouse line (Mao et al., 2001), one should be able to label isolated *Dll1*-expressing cells and achieve single cell resolution to determine the fate of the progeny of these cells.

Nevertheless, in a situation where *Dll4* expression is restricted to a subset of differentiating neurons (the V2a lineage in the case of the pV2 domain of the neural tube) and where the alternative cell fates do not arise from sibling cells, then one must assume that Dll4-mediated Notch signalling must occur between differentiating *Dll4*-expressing V2a cells and bipotent pV2 cells. This implies that Dll4 exerts its role in cell fate decision by signalling either to intermediate progenitors that are yet to accomplish their last cell division and/or to newborn post-mitotic V2 interneurons that are still bipotent, resulting in the inhibition of their V2a potential and acquisition of the alternative V2b fate. Thus, the choice between V2a and V2b fates can thus be viewed as a simple binary decision based on asymmetric Notch signalling, where V2a neurons use Dll4 to prevent neighbouring cells from acquiring the same fate.

III.5.7.2 The Dll1/Dll4 circuitry in the developing retina

Since the same Dll1/Dll4 circuitry is active in the developing neural retina, could a similar binary logic apply to cell fate diversification in this tissue and regulate how the various retinal cell types are generated? This hypothesis is compatible with the finding that *Dll4* expression is not restricted to any particular retinal cell type, hence this ligand can be used iteratively to sort out the successive cell fate decisions that occur throughout retinal development. However, it is unlikely that the same Dll4 signal could act differently at each binary decision to induce a specific and well defined fate in interacting cells. In any case, even in the pV2 domain, a direct role for Dll4 in inducing a V2b fate in target cells is not supported by my results and that of others, as the excess of *Dll4*-expressing V2a neurons in *Dll1* mutants did not cause the predictable increase in the number of V2b neurons and over-expression assays of Dll4 are controversial (Del Barrio et al., 2007; Peng et al., 2007). Instead, I favor a model in which Dll4-mediated Notch activation produces an “unspecific” inhibition/freezing in differentiation in all target cells, which might include multipotent progenitors with different competences (which are also targeted by Dll1) or uncommitted and bipotent post-mitotic cells. The outcome of this inhibition would be that multipotent progenitors will progress in their intrinsic competence and become able to generate a different set of retinal cell types when released from inhibition,

while bipotential cells will acquire the alternative fate available in each binary decision. So, as retinal cells enter differentiation and start to express Dll4, they create a “halo of inhibition” around them that affects neighbouring cells in distinct ways, thereby contributing to diversify the panoply of cell fates generated at each given time in the retina. For this model to work, one requires that not all differentiating cells create such a “halo of Notch activity” as, otherwise, a continuous field of inhibition would be generated in the retinal neuroepithelium and block all differentiation. This agrees with the findings that only about one third of the cells entering differentiation as RGCs express *Dll4* and that these cells are dispersed throughout the retinal neuroepithelium in a “salt and pepper” pattern, creating an uniform but not continuous distribution of inhibitory signals.

In this way, the sequential expression of the two *Dll* ligands results in the two independent functions of Notch signalling in the retina and pV2 domain: maintaining progenitors and generating cell diversity.

CHAPTER IV

THE ROLE OF *JAG1* IN REGULATING NEUROGENESIS IN THE EMBRYONIC NEURAL TUBE AND FOREBRAIN

IV.1 INTRODUCTION

Notch signalling is crucial for many processes during the development of the nervous system. Vertebrates express several Notch ligands, which can present distinct or overlapping spatiotemporal expression patterns within the same tissue. The existence of several Notch receptors and ligands, together with their different expression patterns (Irvin et al., 2004; Lindsell et al., 1996; Stump et al., 2002), provide the possibility of multiple receptor-ligand combinations and of distinct functional outputs. For instance, in the neural tube various Notch ligands are expressed (*Dll1, Dll4* and *Jag1*) within the ventricular region. However, little is known about the function of the Jag1 ligand. Like previous studies that attempted to understand the role of *Dll1* and *Dll4* in spinal cord neurogenesis, studies concerning *Jag1* have also been limited to misexpression studies performed in the chick embryo (le Roux et al., 2003; Sakamoto et al., 1998). Although the *Jag1* gene is expressed in two stripes throughout the entire anterior-to-posterior axis of the forebrain and spinal cord (Lindsell et al., 1996; Myat et al., 1996), these studies did not observe any obvious effects on neural proliferation or differentiation. It was therefore suggested that the weak interaction established between Jagged1 and the Notch1 receptor (Shimizu et al., 1999) could be insufficient to activate Notch signalling to minimum threshold levels to play a role in progenitor maintenance, like the Dll1 ligand. Recent work on *Jag1* in the cerebellum has not detected any neurogenic phenotype upon inactivation of *Jag1*. Instead, *Jag1*-expressing cells that differentiate into granule cells present a migration defect (Weller et al., 2006). In the inner ear, *Jag1* has been described to be necessary for the establishment of prosensory regions and generation of hair cells (Brooker et al., 2006; Kiernan et al., 2006). Also in the adult, a role for *Jag1* in the maintenance of neural stem cells in the subventricular zone (SVZ) has been proposed (Nyfeler et al., 2005). Within the CNS and besides the neural tube, *Jag1* is also expressed in discrete domains of the forebrain and in the ciliary margin zone (CMZ) of the retina (Bao and Cepko, 1997; Lindsell et al., 1996), nevertheless its role has never been approached in these systems.

In this chapter, I describe my work addressing the role of *Jag1* during neurogenesis in the developing mouse spinal cord and forebrain. I identify the two distinct *Jag1*-expressing domains of the intermediate region of the neural tube, one dorsal (dI6) and one ventral (pV1), which are the only spinal cord domains where *Dll1* is not expressed. This striking complementary expression between *Jag1* and *Dll1* is not observed in the forebrain, where *Jag1* expression overlaps with that of *Dll1* in the

dorsal medial ganglionic eminence (dMGE) and at the pallial-subpallial boundary (PSB). The analysis of *Dll1* mutant embryos revealed that, both in the spinal cord and forebrain, Jag1 is able to activate the Notch pathway and, consequently, the transcription of downstream targets. Nevertheless, a role for *Jag1* in the regulation of the rate of neurogenesis, in cell type specification and/or in cell migration could not be observed upon the analysis of *Jag1* mutant embryos.

IV.2 THE JAG1 LIGAND IN THE DEVELOPING MOUSE NEURAL TUBE

IV.2.1 *Jag1* is expressed in discrete domains, dI6 and pV1, of the neural tube

Previous studies have shown that the expression of *Jag1* in the mouse neural tube starts as early as E10.5-E11.5 (Lindsell et al., 1996; Lindsell et al., 1995; Myat et al., 1996). In accordance with published in situ hybridization data, I show here by immunostaining that Jag1 appears initially as two bilateral and symmetrical stripes in the IR of the neural tube and that by E11.5, a third and more dorsal stripe is detected immediately ventral to the roof plate (yellow arrows in Figure IV.1, B-E). Immunostaining shows the presence of Jag1 along most of the cell membrane, spanning from the luminal surface to the basal end in the mantle zone of the developing spinal cord (arrowheads in Figure IV.1, B').

In order to determine the exact domains of the neural tube expressing the *Jag1* ligand, double immunostainings were performed for *Jag1* and specific markers for distinct dorsal and ventral domains, in E11.5 sections of the mouse neural tube (Figure IV.1, A). pV0-derived Evx1+ interneurons (Moran-Rivard et al., 2001; Pierani et al., 1999), which have not yet migrated ventrally and that are still within the ventricular zone, are localized immediately dorsal to the ventral most stripe of Jag1 (Figure IV.1, C). On the other hand, this domain of Jag1 is immediately dorsal to newly produced Chx10+ cells (Figure IV.1, D), which label V2 interneurons (Ericson et al., 1997). Thus, and in accordance with previous work from the Joyner lab (Matise and Joyner, 1997), it is possible to conclude that the ventral most stripe of Jag1 in the spinal cord corresponds to the ventral pV1 domain. The same type of analysis revealed that the dorsal stripe of Jag1 is immediately ventral of the dI5 progenitor domain that gives rise to interneurons expressing Brn3a. Although Brn3a also labels sensory neurons derived from the three most dorsal domains of the neural tube (dI1-

dI3), newly generated dI5 interneurons (brackets in Figure IV.1, E) are easily distinguishable from the ventrally migrating sensory neurons. These results suggest that this dorsal mid stripe of Jag1 corresponds to the dI6 domain, the most ventral domain of the dorsal neural tube.

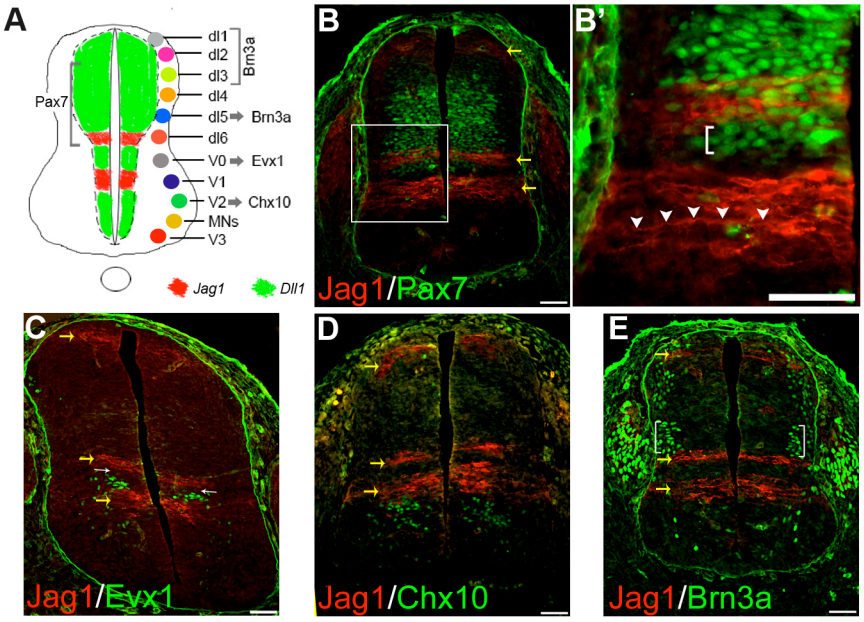


Figure IV.1: Restriction of the two Jag1 stripes in the neural tube to the dI6 and pV1 domains.
(A) Diagram depicting the progenitor domains along the DV axis of the neural tube. Markers used to determine the domains expressing the Jag1 ligand are also indicated. Jag1 expression is restricted to the dI6 and pV1 domains and *Dll1* is expressed in the remaining progenitor domains. **(B-E)** Localization of Jag1 stripes (yellow arrows), in E11.5 mouse neural tube, relatively to neuronal subtypes that derive from specific DV domains. **(B,B')** In the mid-region of the neural tube, the dorsal and ventral stripes of Jag1 are located in the ventral region of the dorsal spinal cord (Pax7+) and in the ventral neural tube (Pax7-), respectively. **(B')** High magnification of the inset in (B), shows that the Pax7+ domain expands slightly more ventrally than the dorsal stripe of Jag1 (bracket). The Jag1 ligand localizes along the cell membrane, labeling the processes (arrowheads) that attach the cells to the luminal and basal surface. **(C)** Evx1+ cells, derived from the ventral pV0 domain, are localized immediately dorsal to the ventral stripe of Jag1. Although these Evx1+ cells are ventral in relation to the dorsal Jag1 stripe, there is a gap between these two cell populations (white arrows). This gap corresponds to the dorsal region of the V0 domain that expresses Pax7 (bracket in B'). **(D)** Chx10+ INs derived from the pV2 domain are immediately ventral to the ventral most stripe of Jag1. **(E)** Brn3a+ cells derived from the dI5 domain of the neural tube (brackets) are immediately dorsal to the dorsal stripe of Jag1. Scale bars **(B-E)** = 50μm.

Double immunostaining for Jag1 and Pax7 further confirm these results (Figure IV.1, B). Pax7 is a marker for most progenitors of the dorsal neural tube and higher levels of this protein are found in the three most ventral domains of the dorsal neural tube (dI4-dI6), while lower levels of Pax7 are found in the dorsal dI3 progenitor domain. Although the dorsal stripe of Jag1 overlaps with the ventral most region of the Pax7+ domain, high magnification shows that the Pax7+ domain extends slightly more ventrally (~2-3 cells width) than this Jag1 stripe (Figure IV.1, B'). This restricted ventral domain of Pax7+ has already been described to correspond to the $pV0_D$ domain (white arrows in Figure IV.1, C) (Pierani et al., 2001). Altogether, these results show that the two stripes of *Jag1* expression correspond to specific and discrete progenitor domains of the developing mouse spinal cord, the dI6 and the pV1 domains.

IV.2.2 Jag1 loss-of-function does not result in a neurogenic phenotype, in the developing neural tube

To understand the role of the Jag1 ligand in the developing neural tube, the phenotype of mutant mice where *Jag1* is inactive was analyzed. Since *Jag1*$^{-/-}$ null mice are embryonic lethal as early as E10.5 (Xue et al., 1999), the function of the Jag1 ligand in neural tube was approached by analyzing *Jag1*-floxed mice (Brooker et al., 2006) expressing Cre recombinase under the control of the rat *Nestin* promoter (referred as *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$). The Nestin-cre mouse line drives the expression of Cre recombinase in all neural progenitor cells (Tronche et al., 1999).

Embryos were collected at E11.5 and analysed at the forelimb level. To evaluate the recombination efficiency of the *Jag1* conditional allele, immunostaining for Jag1 was performed (Figure IV.2, A,B). Given that *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$ embryos presented a complete loss of the Jag1 protein in the neural tube, when compared to control embryos, I next analysed the production of interneurons derived from the *Jag1*-expressing pV1 domain and also from the surrounding *Dll1*-expressing domains. The production of interneurons derived from the second *Jag1*-expressing domain, the dI6 progenitor domain, was not evaluated due to the lack of specific markers for these cells. Immunostaining for En1, a specific marker of V1 interneurons, revealed that the number of pV1-derived interneurons was not significantly altered in *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$ neural tubes, when compared to control littermates (control = 105.8 ± 26.3 cells per section; *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$ = 128.8 ± 38.4 cell per section; $p>0.1$; n=3 embryos; Figure IV.2, C-E). Also, in the adjacent *Dll1*-expressing domains no significant alterations in

the number of neurons was observed, neither in Evx1-expressing V0 INs (control = 80.9 ± 11.2 cells per section; *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$ = 73.6 ± 11.0; Figure IV.2, E), nor Chx10-expressing V2 INs (control = 102.8 ± 6.7 cells per section; *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$ = 108.2 ± 17.5; Figure IV.2, E).

The absence of a neurogenic phenotype in the *Jag1*-expressing pV1 domain of *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$ embryos was quite surprising. More so, because results presented in the previous chapter of this thesis have shown that the Jag1 ligand is both able to activate Notch and to prevent pV1 neural progenitors from differentiating through lateral inhibition (Figure III.9, G,H). These contradictory observations led me to question whether the Notch pathway is affected in *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$ neural tubes or if signals from the neighbouring *Dll1*-expressing domains could compensate for the loss of Jag1 activity. In situ hybridization for *hes5*, a direct downstream target of Notch signalling, shows that Notch activity in *Jag1*-deficient neural tubes remained intact (Figure IV.2, F,G), suggesting that indeed Dll1 can compensate for the loss of Jag1.

Since it is well established that the Dll1 and Jag1 ligands are expressed in a striking complementary pattern (Lindsell et al., 1996; Myat et al., 1996), I wondered if Dll1 compensated for the absence of Jag1 by expanding its domain of expression into the pV1 domain. In situ hybridization for *Dll1* revealed that the expression of *Dll1* remained unaltered in *Jag1*-deficient neural tubes. *Dll1* transcription continues to be excluded from the pV1 domain, as indicated by the persistence of a gap in *Dll1* expression in the ventral neural tube (arrowheads in Figure IV.2, H,I). These results imply that *Dll1*-expressing cells present at the boundary between the pV0/pV1 and pV1/pV2 domains are sufficient to signal to neural progenitors in the adjacent pV1 domain and inhibit their untimely differentiation.

The Dll1-mediated maintenance of pV1 neural progenitor cells in *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$ neural tubes was further confirmed by analysing *Dbx1* and *Dbx2* expression. The *Dbx1*-expressing domain corresponds to the pV0 domain and the dorsal and ventral *Dbx2*$^{+}$*,Dbx1*$^{-}$ expressing domains correspond to the dI6 and pV1 domains, respectively (Pierani et al., 2001). Double in situ hybridization for *Dbx1* and *Dbx2* reveals an identical pattern between control and *Jag1*$^{f/f}$*,Nestin*$^{Cre/+}$ neural tubes, at E11.5 (Figure IV.2, J,K), with the *Dbx2*-expressing domain still expanding ventrally to the *Dbx1* domain. This is another indication that neural progenitors from the *Jag1*-expressing domain had indeed been preserved, despite the inactivation of the *Jag1* gene.

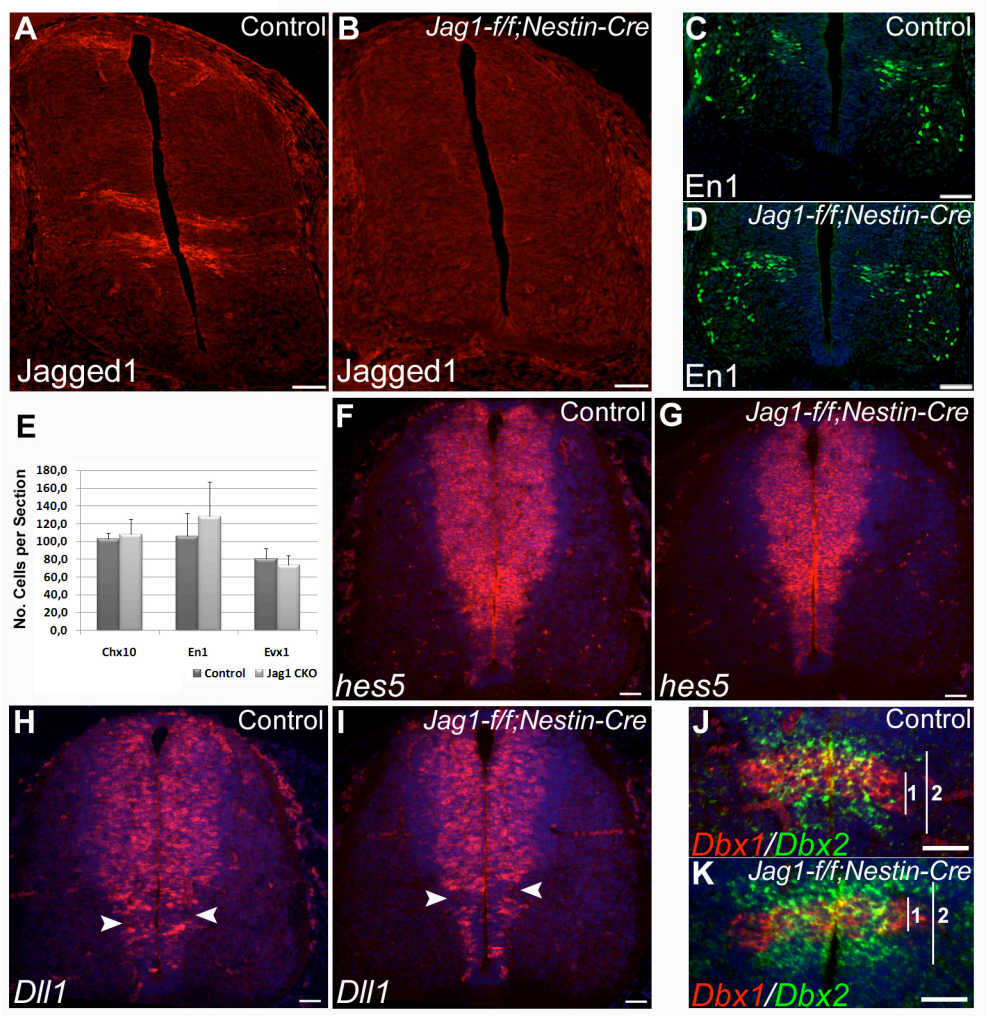


Figure IV.2: Absence of a neurogenic phenotype in *Jag1$^{f/f}$;Nestin^{Cre}* E11.5 neural tubes.

(**A,B**) Efficient recombination of the *Jag1* conditional knockout allele in *Jag1$^{f/f}$,Nestin^{Cre}* mice, demonstrated by Jag1 immunostaining. Jag1 protein is no longer observed in the *Jag1* mutant neural tube (**B**), comparatively to control embryos (**A**). (**C,D,E**) V1 interneurons, immunolabelled for En1, produced in control (**C**) and *Jag1$^{f/f}$,Nestin^{Cre}* (**D**) neural tubes present identical migratory pathways and exist in similar numbers. (**E**) As a negative control, the production of interneurons from neighbouring *Dll1*-expressing domains (Chx10+ and Evx1+) was evaluated and observed to be unaltered in *Jag1$^{f/f}$,Nestin^{Cre}* mice, comparatively to control embryos. (**F,G**) Expression of *hes5*, a direct target of the Notch pathway. Consistent with the absence of a neurogenic phenotype, the expression of *hes5* in *Jag1$^{f/f}$,Nestin^{Cre}* neural tube (**G**) is indistinguishable from that of control embryos (**F**). Like in wild-type embryos (**H**), the expression pattern of the *Dll1* Notch ligand in *Jag1$^{f/f}$,Nestin^{Cre}* neural tubes (**I**) continues to exhibit a gap (arrowheads) where no *Dll1* transcripts are detected and that corresponds to the pV1 domain. (**J,K**) Double in situ hybridization for the *Dbx1* and *Dbx2* genes in sections of control (**J**) and *Jag1* mutant (**K**) neural tubes shows that maintenance of pV1 progenitors. *Dbx2*-expressing domain (bar indicated with 2) extends more dorsally and ventrally than that of *Dbx1* (bar indicated with1). The ventral *Dbx2*-positive and *Dbx1*-negative domain corresponds to the pV1 progenitors. Scale bars (**A-D, F-K**) = 50µm

IV.2.3 Interneurons derived from the *Jag1*-expressing domains do not present migratory defects

The expression of *Dll1* and *Jag1* in specific and mutually exclusive DV domains of the neural tube (Lindsell et al., 1996; Lindsell et al., 1995; Myat et al., 1996), together with the finding that Dll1 and Jag1 activate Notch within their domains of expression, suggests that both ligands exert the same function (progenitor maintenance through lateral inhibition) but in different progenitor domains. Nevertheless, the analysis of *Jag1* conditional knockout mutants has shown that Jag1 is apparently dispensable for this function, since Dll1 is able to fully compensate for the absence of Jag1. Therefore, I asked what other role, if any, could Jag1 have in the developing neural tube.

Jag1 is extensively expressed in the cerebellum, including in cerebellar granule cells (Stump et al., 2002). Recently, Weller *et al* have shown that like in the neural tube, Jag1 function does not seem to be required to regulate the production of cerebellar neurons. Instead they have described a role for Jag1 in cell migration (Weller et al., 2006). Granule cells, which in wild-type embryos derive from *Jag1*-expressing cells, exhibit an impaired migration in *Jag1*-deficient mice. These cells do not complete their migration from the external germinal layer (EGL) to the internal granular layer (IGL). Could it be that in the neural tube, Jag1 also has a function in cellular migration?

In order to approach this question, the localization of En1-immunolabelled interneurons was examined in neural tube sections of E11.5 control and $Jag1^{f/f}$;$Nestin^{Cre/+}$ embryos. Double immunostaining for En1 and Islet1 (a marker of motoneurons) revealed a similar distribution pattern of the V1 interneurons both in control and $Jag1^{f/f}$;$Nestin^{Cre/+}$ embryos (Figure IV.3), in accordance with the described migratory pathway of $En1^+$ interneurons at the forelimb level (Matise and Joyner, 1997). Although many newly differentiated V1 interneurons are still localized within the lateral ventricular region (arrowhead in Figure IV.3, A,B), $En1^+$ cells that have already migrated ventrally are observed in the ventral horn, in close association with $Islet1^+$ motoneurons. Most of these ventral cells are positioned in such a way that they surround the forming lateral motoneuron column (LMC; asterisk in Figure IV.3, A,B) both on the medial side, as well as on the most marginal surface of the neural tube, adjacent to the LMC. Therefore, these results do not support a role for Jag1 in V1 IN migration.

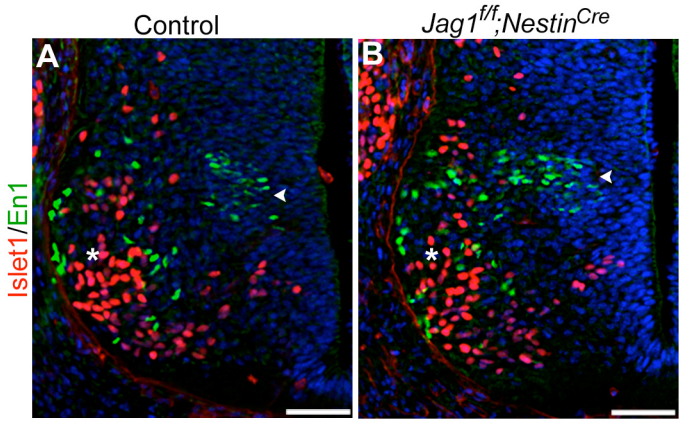


Figure IV.3: Migration of V1 interneurons derived from *Jag1*-expressing domain do not present migratory defects upon *Jag1* inactivation.
(**A,B**) Immunostaining for motoneurons (Islet1) and V1 interneurons (En1) in control (**A**) and *Jag1*$^{f/f}$*;Nestin*^{Cre} (**B**) neural tubes, of E11.5 embryos. Both in wild-type and *Jag1*-mutants, differentiated V1 interneurons are found still exiting the ventricular region (arrowhead) and also in the ventral horn surrounding the forming LMC (asterisk) medially and laterally. Scale bar (A,B) = 50μm

Altogether, the results obtained from the analysis of *Jag1*-mutant embryos have not shed much light on the possible function(s) of *Jag1* in neural tube neurogenesis. Given that Jag1 is able to activate Notch in the dI6 and pV1 domains to prevent neural progenitors from differentiating, it is plausible that this might, in fact, be Jag1's only function. To gain more insight on the function of Jag1 during mammalian neurogenesis, I turned my attention to the developing forebrain, where *Jag1* is also expressed in discrete domains.

IV.3 THE JAG1 LIGAND IN THE DEVELOPING MOUSE FOREBRAIN

IV.3.1 *Jag1* is expressed in discrete domains of the forebrain that overlap with *Dll1* expression

The *Jag1* gene has previously been described to be expressed in the mouse forebrain from E13.5 onwards and in a single stripe in the sulcus limitans (Lindsell et al., 1996). I now show that *Jag1* is strongly expressed in the developing forebrain of the mouse embryo, as early as E11.5, at the level of the sulcus between the lateral and medial ganglionic eminence (LGE and MGE), extending to the whole of the

dMGE (arrowhead in Figure IV.4, B). At E11.5, *Jag1* expression also starts to be detectable, although at very low levels, at the pallial-subpallial boundary (PSB; arrows in Figure IV.4, B). At E12.5, this second domain of *Jag1* expression is more evident (arrows in Figure IV.4, D), as well as the appearance of scattered *Jag1*-expressing cells in the pre-optic area (POA) of the ventral telencephalon (asterisk in Figure IV.4, D). Unlike in the hindbrain and neural tube, the expression of *Jag1* does not occur in a complementary pattern relatively to that of *Dll1*, instead the expression of these two genes overlap at the *Jag1*-expressing domains, since *Dll1* is expressed throughout the entire neuroepithelium of the developing forebrain (Figure IV.4, C; (Lindsell et al., 1996)).

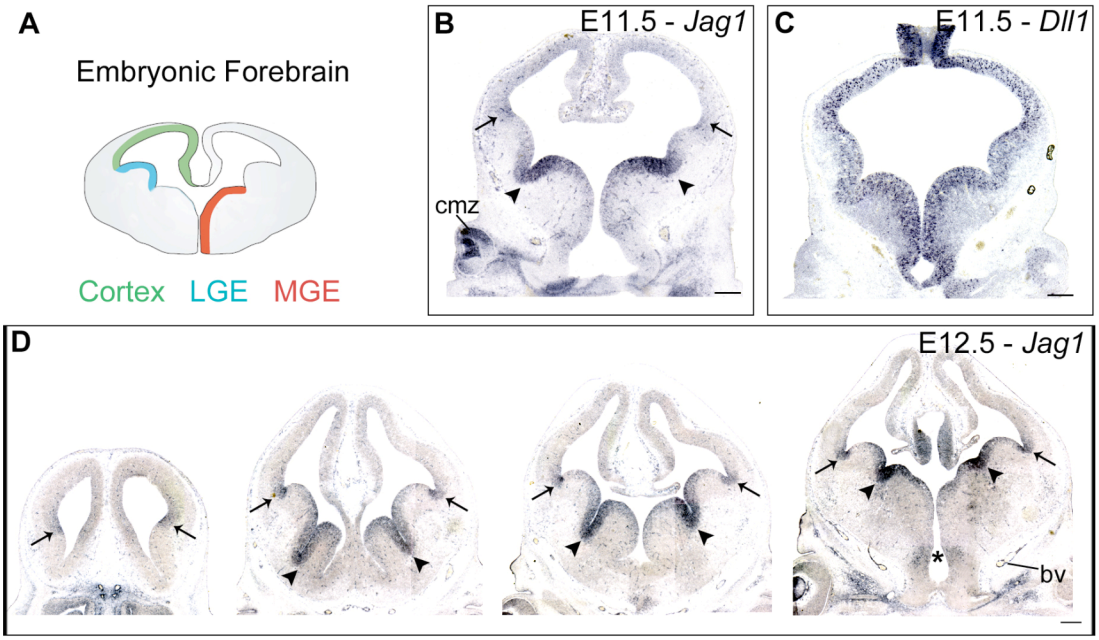


Figure IV.4: Expression of *Jag1* in the embryonic forebrain, at E11.5-E12.5.

(A) Cartoon depicting morphologically identifiable structures of the early embryonic telencephalon, in a coronal section. The dorsal telencephalon gives rise to the cortex or pallium, while the ventral telencephalon or subpallium give rise to the lateral ganglionic emninence (LGE) and to the medial ganglionic eminence (MGE). **(B,C)** Expression of *Jag1* **(B)** and *Dll1* **(C)** at E11.5. While, *Dll1* is expressed throughout the entire neuroepithelium, the expression of *Jag1* is restricted to specific domains. *Jag1*-expression is detected, at E11.5 **(C)** and E12.5 **(D)**, at the pallial-subpallial boundary (PSB; arrows) and at the dorsal MGE (arrowheads). At E12.5, *Jag1* expression is also weakly observed in scattered cells in the neuroepithelium of the pre-optic area (POA, asterisk in **D**). cmz – ciliary margin zone; bv – blood vessel. Scale bars **(B-D)** = 40µm

IV.3.2 Jag1 is able to activate Notch in the developing mouse forebrain

The overlapping expression of *Dll1* and *Jag1* in the developing mouse forebrain raises the question if both ligands are able to activate Notch signalling. This question gains more revelance when considering the fact that *LFng* is expressed throughout the neuroepithelium of the forebrain (Ishii et al., 2000) and that previous studies have shown that LFng interferes with Jag1-mediated Notch activity (Bruckner et al., 2000; Hicks et al., 2000; Yang et al., 2005).

In order to approach this question, Notch activity was evaluated in mouse embryos lacking the activity of either the Jag1 or Dll1 ligands. Since *Jag1*$^{-/-}$ and *Dll1*$^{-/-}$ null mice are embryonic lethal as early as E10.5-E11.5 (Hrabe de Angelis et al., 1997; Xue et al., 1999), the ability of each of these ligands to activate Notch was approached by analysing *Jag1*- (Brooker et al., 2006) and *Dll1*-floxed (Hozumi et al., 2004) mice, expressing iCre recombinase (codon-improved Cre recombinase; (Shimshek et al., 2002)) under the control of the *Nkx6.2* promoter (referred as *Jag1*$^{f/f}$*,Nkx6.2*^{iCre} and *Dll1*$^{f/f}$*,Nkx6.2*^{iCre}). The Nkx6.2-iCre mouse line drives the expression of iCre at high levels in neural progenitors of the dorsal MGE, as early as E10.5 (Fogarty et al., 2007). By conditionally inactivating the *Dll1* allele in the dMGE, one can extrapolate whether *Jag1*, which is the only remaining ligand to be expressed, can activate the Notch pathway and eventually compensate for the loss-of-function of *Dll1*. The same rationale is valid for the opposite situation (inactivation of *Jag1* to access Dll1-mediated Notch signalling). Therefore, *Jag1*$^{f/f}$*,Nkx6.2*$^{iCre/+}$, *Dll1*$^{f/f}$*,Nkx6.2*$^{iCre/+}$ and respective control embryos were dissected at E12.5, and in situ hybridization for *hes5* was performed on coronal sections of the forebrain (Figure IV.5, A-B,E-F).

Jag1$^{f/f}$*,Nkx6.2*$^{iCre/+}$ embryos exhibited no difference in *hes5* expression (Figure IV.5, A,B). Both the pattern and levels of mRNA expression from the Notch downstream target, *hes5,* were undistinguishable from control littermates. Immunostaining for Jag1 show that the levels of Jag1 protein in the dMGE are significantly reduced (arrowheads in Figure IV.5, C,D) in *Jag1*$^{f/f}$*,Nkx6.2*$^{iCre/+}$ embryos comparatively to control embryos. This result excludes the possibility that the sustained *hes5* expression is due to inefficient recombination of the *Jag1* allele. Most likely, the maintenance of *hes5* expression in the dMGE of *Jag1*$^{f/f}$*,Nkx6.2*$^{iCre/+}$ embryos is due to Dll1-mediated Notch activity. Moreover, the comparable levels of *hes5* transcription, together with the viability of adult *Jag1*$^{f/f}$*,Nkx6.2*$^{iCre/+}$ mice, suggest that Dll1 is able to fully compensate for the absence of *Jag1*.

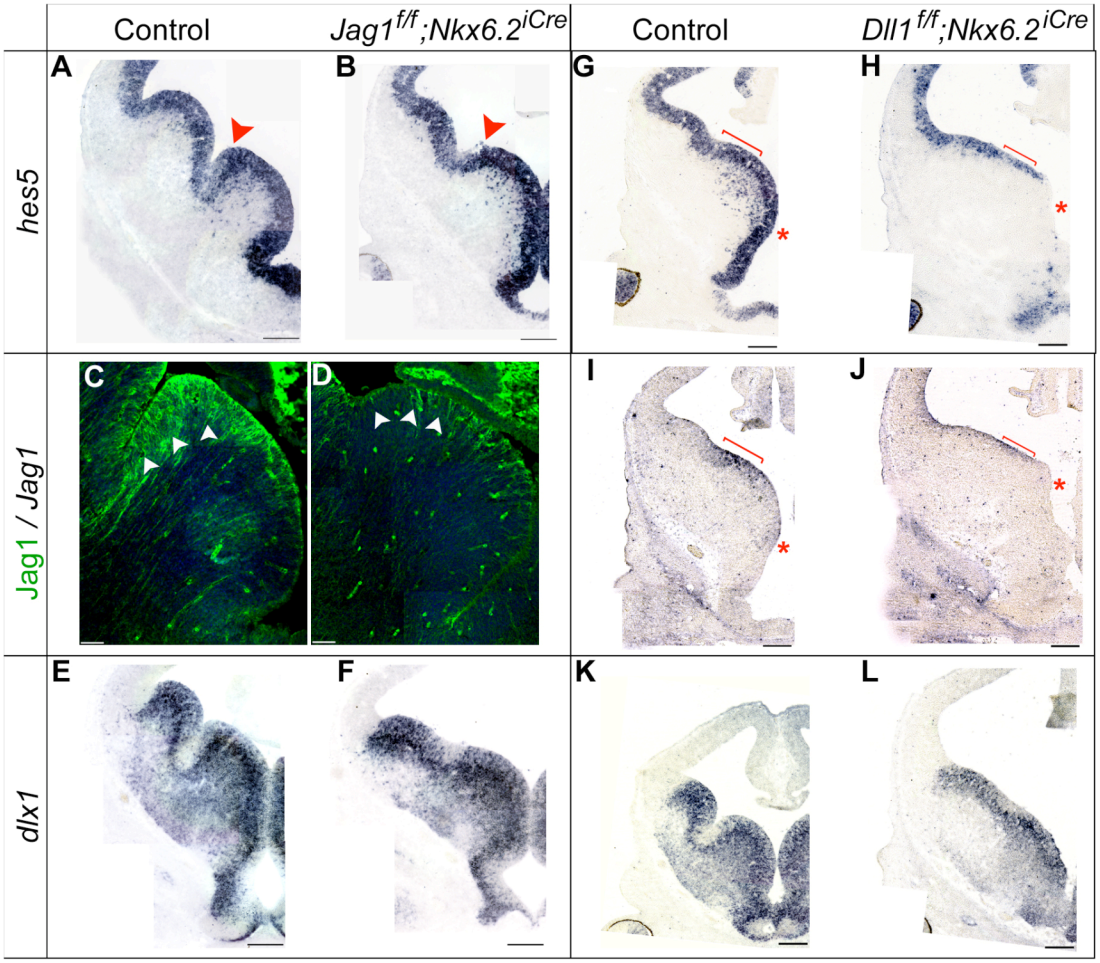


Figure IV.5. Both Dll1 and Jag1 are capable of activating Notch signalling, although to different extents.

(A-B,G-H) In situ hybridization for *hes5* in sections of E12.5 *Jag1*$^{f/f}$*;Nkx6.2*^{iCre} **(A,B)**, *Dll1*$^{f/f}$*;Nkx6.2*^{iCre} embryos **(G,H)** and littermate controls. Expression of *hes5* is unaffected in the dMGE of *Jag1* mutants (red arrowheads in **A,B**) when compared to control embryos. Unaffected Notch signalling in the absence of Jag1 function is not due to inefficient deletion of *Jag1*, since Jag1 protein levels are significantly decreased in the dMGE of *Jag1*$^{f/f}$*;Nkx6.2*^{iCre} embryos **(D)**, when compared to control embryos **(C)**. *Jag1* mutants (**F**) do not exhibit any difference in the expression of *Dlx1*, a marker for progenitor cells in both the VZ and SVZ, when compared to control embryos (**E**). In *Dll1*-mutants **(H)**, expression of *hes5* is severely affected when compared to wild-type littermates **(G)**. In *Dll1*$^{f/f}$*;Nkx6.2*^{iCre} embryos, *hes5* expression is significantly decreased in the dMGE (red bracket) and completely lost in the ventral region of the MGE (red asterisk). These regions correspond to *Jag1*-expressing and non-*Jag1*-expressing domains **(I,J)**, respectively. (**K,L**) In situ hybridization for *dlx1*, confirms the loss of progenitor cells in the forebrain of *Dll1*$^{f/f}$*;Nkx6.2*^{iCre} embryos (**L**), comparatively to control littermates (**K**). Scale bar **(A-B, E-K)** = 40μm and **(C,D)** = 10μm

While no apparent differences in the expression of *hes5* were detectable in *Jag1$^{f/f}$,Nkx6.2$^{iCre/+}$* embryos, *Dll1$^{f/f}$,Nkx6.2$^{iCre/+}$* embryos displayed a severe reduction in *hes5* expression, when compared to control embryos (Figure IV, G,H). At more ventral levels of the MGE (asterisk in Figure IV.5, G,H), where *Jag1* is not expressed (asterisk in Figure IV.5, I,J), *hes5* expression is completely lost. In the dMGE, where *Jag1* is expressed (bracket in Figure IV.5, I,J), *hes5* expression is greatly reduced. Comparison of the dMGE region of *Dll11$^{f/f}$,Nkx6.2$^{iCre/+}$* and control embryos reveals also a significant thinning of the neuroepithelium, which is suggestive of a loss of neural progenitors. In situ hybridization for *dlx1*, a gene that is expressed in progenitor cells of both the ventricular zone (VZ) and subventricular zone (SVZ) (Bulfone et al., 1993; Yun et al., 2002), is greatly reduced in *Dll1* mutants (Figure IV.5, K,L). Interestingly, and in agreement with the unaltered expression of *hes5* in *Jag1* mutant embryos, the expression pattern of *Dlx1* also remains unaffected in *Jag1$^{f/f}$,Nkx6.2^{iCre}* embryos (Figure IV.5, E,F). These observations, which are in accordance with the previously described neurogenic phenotype in *Dll1* null embryos (Yun et al., 2002), confirm the requirement of Dll1 in progenitor cell maintenance and indicate that Jag1 is either not involved in this function or is, at least, dispensable. The loss of neural progenitors in *Dll1$^{f/f}$,Nkx6.2^{iCre}* embryos and the consequent thinning of the VZ may explain the reduced expression of *Jag1* in *Dll1*-mutants (bracket in Figure IV.5, J). This weaker Jag1 expression might, nevertheless, be sufficient to maintain some Notch activity within the dMGE (bracket in Figure IV.5, H). One may also argue that the maintenance of Notch activity may be explained by incomplete *Dll1* inactivation. However, the results suggest that this is unlikely given that *hes5* expression, hence Notch activation, is completely lost in the region ventral to the MGE (asterisk in Figure IV.5, H), where Nkx6.2^{iCre} has been shown to recombine poorly, when compared to the dMGE (Fogarty et al., 2007).

Morphological differences are also clear in the forebrain of *Dll1$^{f/f}$,Nkx6.2^{iCre}* embryos, when compared with control littermates, namely that complete absence of the LGE/MGE sulcus. The development of the mouse olfactory bulb is dependent an invagination of the neuroepithelium that originates a sulcus very similar to that of the LGE/MGE (Hebert et al., 2003). Hebert *et al* proposed that a decrease in proliferation is required for the initial bulb invagination and consequent sulcus formation. If one extrapolates this hypothesis to the development and morphology of the forebrain, one would expect less proliferation in the LGE/MGE sulcus compared to the proliferating and expanding 'bulges' of the ganglionic eminences (arrows in Figure IV.6, A). Indeed, this is observed upon BrdU administration 1hr before embryo

dissection and subsequent detection by immunostaining (Figure IV.6, B). Wild-type E11.5 embryos show a higher density of BrdU-positive cells in the MGE (arrowhead in Figure IV.6, B), when compared to the LGE/MGE sulcus (marked by *Nkx6.2* expression and asterisk in Figure IV.6, B). This distribution of proliferating cells is no longer observed in *Dll1*$^{f/f}$*,Nkx6.2*^{iCre} embryos where the MGE shows a significant decrease in the number of proliferating cells (arrowhead in Figure IV.6, C) relatively to the LGE/MGE boundary, where the sulcus is no longer present (asterisk in Figure IV.6, C). These results suggest that the rate of neurogenesis is an important factor for the correct morphogenesis of various neural tissues. Furthermore, in *Jag1*$^{f/f}$*,Nkx6.2*^{iCre} embryos, where I have shown that neurogenesis remains unaltered, no morphological defects are observed in the developing forebrain (Figure IV.6, D).

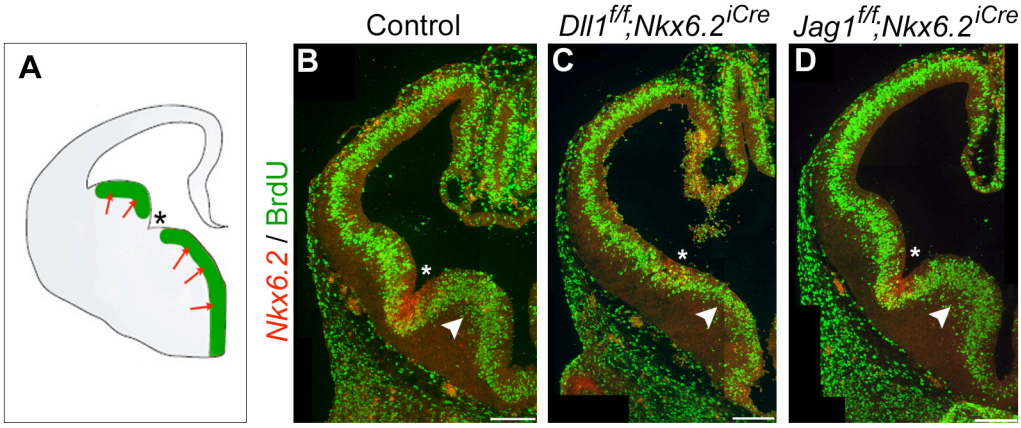


Figure IV.6: Altered forebrain morphology at the LGE/MGE boundary level in *Dll1*$^{f/f}$*,Nkx6.2*^{iCre} but not *Jag1*$^{f/f}$*,Nkx6.2*^{iCre} embryos.

(A) Diagram representing the influence of unequal cell proliferation in tissue morphology. This hypothesis, first proposed for appropriate olfactory bulb morphogenesis (Hebert et al., 2003), suggests that decreased cell proliferation within a certain area of the neuroepithelium (asterisk) leads to the invagination of this region and subsequent formation of a sulcus, while neighbouring neuroepithlium, which have a higher proliferation rate expand and form bulges (red arrows). **(B-C)** Immunostaining for proliferating BrdU-positive cells (administered 1hr before dissection) was combined with *Nkx6.2* in situ hybridization, which clearly labels the LGE/MGE boundary (asterisk). Both control littermates **(B)** and *Jag1*$^{f/f}$*,Nkx6.2*^{iCre} embryos **(D)** show a higher density of BrdU-positive cells in the MGE relatively to the MGE/LGE boundary. This relation is not maintained in Dll*1*$^{f/f}$*,Nkx6.2*^{iCre} embryos **(C)**, where increased neurogenesis has significantly reduced neural progenitors in the MGE and compromised the formation of the LGE/MGE sulcus. Scale bar **(B-D)** = 40μm.

Altogether, these results reveal that, like in the neural tube, both Dll1 and Jag1 are able to activate the Notch pathway in the developing mouse forebrain. However, there is a major difference between the forebrain and the spinal cord. In the latter, the Jag1 ligand is sufficient to regulate neurogenesis in the domains where it is expressed, while in the forebrain it is insufficient. Although residual Notch signalling is maintained in the dMGE when *Jag1* is the sole ligand to be expressed (in a *Dll1*-mutant background), progenitor depletion is observed. These results suggest that Dll1 and Jag1 have different signaling abilities, with Dll1 being able to activate Notch signaling more efficiently than Jag1.

Given that Dll1-mediated signaling is sufficient to maintain neural progenitors and regulate the rate of neurogenesis in the developing mouse forebrain, I next questioned if Jag1 could alternatively play a role in cell fate specification and/or migration.

IV.3.3 Is Jag1 involved in Martinotti cell production?

In the previous sections, I showed that *Jag1* expression occurs in restricted domains of the developing forebrain, in the PSB and in the dMGE. The restricted expression of *Jag1* in the dMGE is of particular interest, since a specific cortical interneuron subtype, known as Martinotti cells, was recently shown to derive exclusively from this domain (Fogarty et al., 2007). Therefore, I questioned if Jag1 could have a role in specifying or regulating the production of this particular cell fate. To approach this question, I analysed the production of Martinotti cells in control and $Jag1^{f/f}$,$Nkx6.2^{iCre}$ mice. Mice were sacrificed and analysed at adult stages (6 weeks post-natally), when interneurons have completed their tangential migration from the subpallial to the cortex and acquired the expression of specific calcium-binding proteins which allow their subtype identification (reviewed in (Wonders and Anderson, 2006)). Martinotti cells can be identified as a subpopulation of the calretinin-positive (CR^+) cells in the mouse cortex that coexpress somatostatin (Sst) (Xu et al., 2006). I therefore did double immunostaining for CR and Sst on coronal sections of the mouse brain, at the level of the subventricular zone (SVZ), in control and *Jag1* mutant littermates (Figure IV.7).

Countings of CR^+,Sst^+ cells in relation to the total number of CR+ cells, revealed no difference between mice lacking *Jag1* function in the dMGE (from early embryonic stages) and control littermates ($Control_{Litter1}$ = 23.7 ± 3.3% versus

Jag1$^{f/f}$*,Nkx6.2*^{iCre}$_{Litter1}$ = 24.2 ± 4.7% with p>0.9 and Control$_{Litter2}$ = 15.5 ± 2.6% versus *Jag1*$^{f/f}$*,Nkx6.2*^{iCre}$_{Litter2}$ = 16.4 ± 1.2% with p>0.6; Figure IV.6, A-C). The differences observed between the two litters that were analysed were due to a decrease in the overall number of Sst^{+} cells, which may be possibly due to differences in tissue fixation between the different litters. Nevertheless, these results show that Jag1 is not required for specification of this interneuron population that is derived exclusively from the *Jag1*-expressing region of the dMGE.

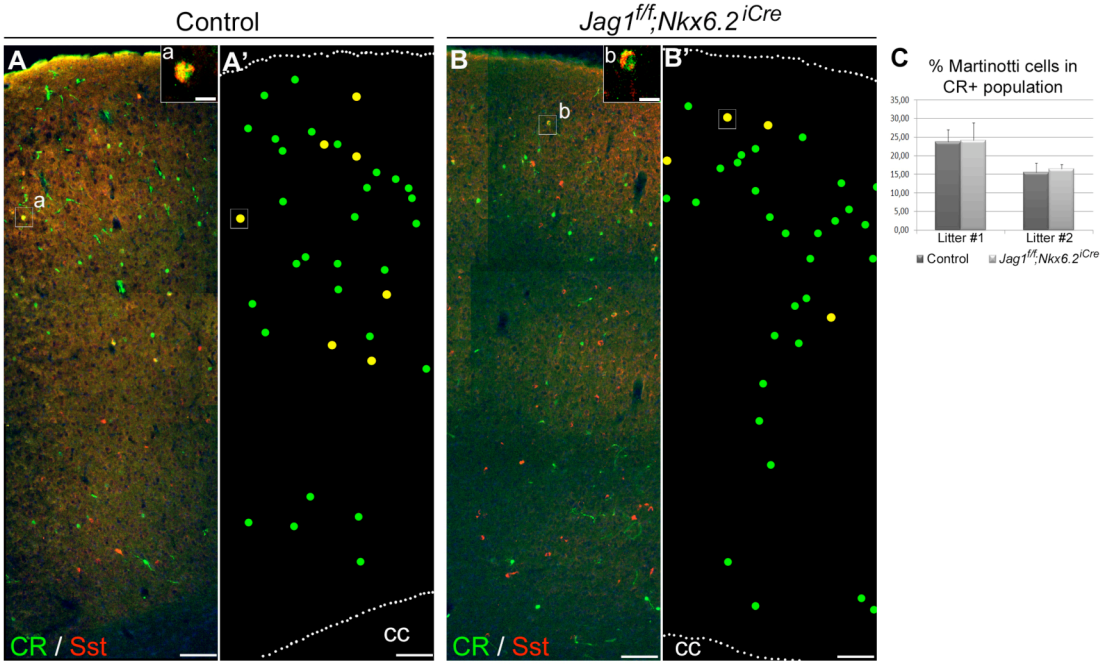


Figure IV.7: Production and final localization of Martinotti cells are unaffected in the absence of Jag1 function.

(**A,B**) Double immunostaining for calretinin (CR) and somatostatin (Sst) in control (**A**) and *Jag1*$^{f/f}$*;Nkx6.2*^{iCre} (**B**) adult cortex. Double labeled cells represent a specific cortical interneuron subtype, the Martinotti cells (**a,b** - zoomed in inset). (**A'B'**) Schematic representation of CR^{+} (green dots) and CR^{+},Sst^{+} (yellow dots) cells in (**A**) and (**B**), respectively. Martinotti cells show an identical distribution pattern in the cortex of *Jag1*$^{f/f}$*;Nkx6.2*^{iCre} (**B'**) mice when compared to control mice (**A'**): they are localized in the upper layers of the cortex. (**C**) Countings of the percentage of Martinotti cells in relation to the total number of CR^{+} cells was not affected in the absence of Jag1 function. cc – corpus callosum. Scale bars (**A,A',B,B**) = 20µm, (**a,b**) = 2,5µm

Interneurons within the dorsoventral axis of the cortex organize themselves in cortical layers in an "inside-out" manner, so that the distribution of Ins reflects their time of birth (Fairen et al., 1986; Miller, 1985; Peduzzi, 1988). The localization of the CR^{+},Sst^{+} Martinotti cells was identical in both control and *Jag1*-mutant mice (see distribution of CR^{+} - green dots – and CR^{+},Sst^{+} - orange dots – in Figure IV.7, A',B'):

all CR^+,Sst^+ cells were observed in the upper layers of the cortex. This indicates that Jag1 is not involved in regulating the rate or timing of neurogenesis (at least of Martinotti cells), since their appropriate localization within the cortex indicates that they were generated during the correct developmental time point.

IV.4 DISCUSSION

In this study, I have attempted to understand the role of the Jag1 ligand in mammalian neurogenesis, namely in the developing neural tube and forebrain. I have made use of mice with a conditional deletion of the *Jag1* allele to study both of these systems. A role for Jag1 in the regulation of the rate of neurogenesis, in cell type specification and/or in cell migration could not be observed. Nevertheless, I have shown that Jag1 is able to activate the Notch pathway both in the spinal cord and in the forebrain, albeit to different extents. *Dll1* is able to compensate for the loss-of-function of *Jag1* both in the neural tube and forebrain of the developing mouse embryo and is thus sufficient to maintain neural progenitors. *Jag1*, on the contrary, is unable to fully compensate for the loss of *Dll1* function, even in regions in the developing forebrain, where expression of *Dll1* and *Jag1* overlap. And importantly, I have provided the first evidence that Jag1 is active in lateral inhibition in the spinal cord and consequently in maintenance of progenitor cells.

IV.4.1 Redundant functions of Jag1 and Dll1 in the neural tube

The analysis of *Dll1* mutants in the previous chapter of this thesis allowed me to approach and confirm the ability of Jag1 to activate the Notch pathway and inhibit neural progenitor differentiation in the spinal cord. These results provide the first in vivo evidence in vertebrates that Jag1 is able to regulate neurogenesis through lateral inhibition like the Dll1 ligand. The functional equivalence of the Serrate and Delta ligands during several developmental processes in the *Drosophila* embryo, has already been established (Gu et al., 1995; Zeng et al., 1998). For instance, although Serrate is not expressed during embryonic neuroblast segregation in *Drosophila*, ectopic expression of Serrate in embryos lacking the Delta ligand is able to partially recover the severe neuronal hyperplasia observed in Delta-deficient embryos (Gu et al., 1995). Moreover, although the role of Notch signalling in SOP lineage cell fate decisions (Hartenstein and Posakony, 1989; Heitzler and Simpson, 1991; Heitzler

and Simpson, 1993; Posakony, 1994) and wing vein patterning (de Celis et al., 1997; Huppert et al., 1997; Sturtevant and Bier, 1995) is well established, the deletion of either one of the DSL ligands does not fully reproduce the Notch phenotype. Only the deletion of both Serrate and Delta mimics the loss-of-function of *Notch*, suggesting again that the two ligands are redundant (Zeng et al., 1998).

These observations in *Drosophila* are interesting and are extendable to the work presented in this thesis (Chapter III and IV), where neurogenesis within specific domains of the neural tube (dI6 and pV1) is not disturbed upon the inactivation of either *Dll1* or *Jag1*. However, preliminary data (C. Ramos and D. Henrique) indicates that the simultaneous loss-of-function of both genes causes premature neurogenesis in these domains of the developing spinal cord, yielding a phenotype that is indistinguishable from that of *Notch1* mutants (Yang et al., 2006).

IV.4.2 Different Dll1- and Jag1-mediated Notch outputs

Besides the functional redundancy/equivalence of Serrate and Delta ligands, i.e that Delta and Serrate are able to carry out the same functions through the activation of the Notch pathway, studies performed in *Drosophila* also suggest that the signalling output derived from each ligand is different, as Delta was able to fully compensate for the absence of Serrate but the converse was not true (Gu et al., 1995; Zeng et al., 1998).

Delta and Serrate/Jagged ligands are structurally distinct and the structural differences between the two protein families have been suggested to play a role in determining the signalling efficiency mediated by specific ligand-receptor Notch ligands, the Delta and Serrate protein families differ mainly in the number of EGF repeats (EGFRs) and the presence of a cysteine-rich (CR) region in the extracellular domain (reviewed in (Fleming, 1998; Weinmaster, 1997)). In vertebrates, Jagged ligands present twice as many EGFRs comparatively to Delta ligands, which tend to have only eight (six in the case of Dll3). Furthermore, Jagged ligands also present a CR, region while Delta ligands do not. Although it is thought that certain EGFRs may stabilize or modify ligand-receptor interactions and that the CR region might have a role in selecting specific Notch receptors, little is understood as to how these extracellular domains can determine/influence ligand-receptor interactions.

Another factor that is known to modulate ligand-receptor interactions are the Fringe proteins. The Fringe proteins (Lunatic, Manic and Radical Fringe in vertebrates) are glycosyltransferases that modify the extracellular domain of the Notch receptor. By doing so, the ability of Jagged- and Delta-mediated signaling is altered (Bruckner et al., 2000; Fleming et al., 1997; Hicks et al., 2000; Moloney et al., 2000; Yang et al., 2005). The activity of Fringe potentiates Dll1-mediated activation of the Notch1 receptor, while inhibiting Notch1 activation through the Jag1 ligand. However, when the Notch pathway relies on the activation of the Notch2 receptor, the presence of Fringe potentiates both Dll1- and Jag1-mediated Notch (Hicks et al., 2000). Thus, the expression of Fringe can, *per se*, modify the output of Notch signalling and create differential levels of Notch activation within the same system. Therefore, mechanisms that modulate Notch signalling are of outstanding importance (reviewed in (Bray, 2006; Schweisguth, 2004)).

For instance, in the work presented in this thesis, I show that Jag1 is able to activate Notch and regulate neurogenesis within its domains of expression of the spinal cord. However, the same is not true in the developing forebrain. This possibily results from different signaling abilities of Jag1 in the neural tube and forebrain, which can easily be a consequence of Notch modulation by *LFng*. It is well established that LFng activity inhibits Jag1-mediated Notch activation, therefore the complementary expression between *Jag1* and *LFng* in the neural tube (Sakamoto et al., 1997) inhibits Jag1 from signalling to cells in neighbouring domains where Notch is glycosylated by LFng. The activity of LFng restricts Jag1-mediated signalling exclusively to *Jag1*-expressing domains and this accounts for the inability of Jag1 to compensate for the absence of Dll1 function in neighbouring domains. On the other hand, *Jag1* expression in the developing forebrain overlaps both with *Dll1* and *LFng*. The simultaneous expression of these genes probably creates a situation in which Dll1-signalling is enhanced, while Jag1-mediated Notch activation is reduced. Progenitor cells may therefore experience high or low levels of Notch activation depending on the signalling ligand and respond differently to the surrounding cues.

In fact, recent work developed in the mouse by Adams and colleagues has shown that Jag1 can antagonize *in vivo* Dll-mediated Notch signaling when Notch is modified by Fringe glycosyltransferases (Benedito et al., 2009). This inhibitory function of Jag1 on Notch activity was shown to contribute to the heterogeneity in the levels of Notch activation within a population of cells, which then creates differences in gene expression between adjacent cells (tip versus stalk cell in the particular case of the vasculature). This antagonistic role of Jag1 has also been observed in other

tissues, such as the pancreas (Golson et al., 2009), indicating that it might be a molecular mechanism that is conserved in various systems. In addition, work performed by Gaiano and co-workers has suggested that differential Notch signalling levels can distinguish neural stem cells (NSCs) from proliferating intermediate progenitors (Mizutani et al., 2007) and a role for Jag1 has been proposed for the maintenance of NSCs in the adult mouse SVZ (Nyfeler et al., 2005). Could it be that an identical mechanism is used in the embryo to maintain a pool of NSCs through to adulthood? This seems unlikely, given that *Jag1* is only expressed in the dMGE and pallial-subpallial boundary (PSB) and that these embryonic regions in the mouse forebrain to not originate the entire extent of the adult SVZ, where NSCs reside (Young et al., 2007).

In this chapter I have studied the role of Jag1 in the developing mouse neural tube and forebrain. I have done so by assessing various aspects of neural development, including neurogenesis rate, cell fate specification and neural migration. However, I was unable to attribute a role for Jag1 in any of these processes. In order to better understand the function of Jag1 in neurogenesis, a more cellular approach would be convenient. It would be of interest to characterize with more detail the *Jag1*-expressing cells with respect to the cell cycle, to cells expressing *Dll1* and *LFng*, and also to cells with activated Notch. For instance, it has been assumed that *Jag1*, like *Dll1*, is expressed in nascent neurons, although this has never been shown. In fact, a characteristic of *Dll1* expression in nascent neurons and its role in mediating lateral inhibition is the resulting salt and pepper pattern. Such expression pattern is not observed for Jag1. Instead, *Jag1*-expressing cells are usually densely distributed within their domains of expression. Moreover, it has been shown by others that *Jag1* is expressed in the CMZ of the developing retina (Bao and Cepko, 1997; Lindsell et al., 1996), a region where neurogenesis is not active and thus, at least here, *Jag1* is not expressed in nascent neurons. It would be equally important to trace *Jag1*-expressing cells and fate-map them. Indeed, more detailed studies are required to unravel the function of *Jag1* in vertebrate neurogenesis.

CHAPTER V

CONCLUDING REMARKS

V. CONCLUDING REMARKS

The Notch pathway has been one of the most studied signalling pathways in the last two decades. This highly conserved pathway was first discovered in invertebrates and shown to be important for embryonic development and for adulthood homeostasis. Notch acts at developmental forkroads where, without instructing which path a given cell should take, it restricts the number of cells following the same fate. In this way, Notch signaling 'allows' a group of potentially similar cells to acquire distinct cell fates and, in this way, contributes to the generation of different cell types that are required for the development of a functional organ/tissue. In addition to its role in generating cell diversity, Notch has also been implicated in other cellular functions that are essential for homeostasis, namely proliferation and cell survival. The mechanisms used by the Notch pathway to act, time and time again, in such a broad spectrum of functions are still not fully understood.

The tendency to simplify biological processes may turn these more intuitive but less representative of the reality. The same is true for developmental processes where Notch signalling is important. In fact, scientific evidence obtained in different model organisms and developmental systems have shown that various molecular components and genetic cascades are involved in Notch signalling. This is well demonstrated by the existence of multiple DSL ligands and Notch modulators that, depending on the tissue and cell context, may exert different functions. During my PhD, I attempted to elucidate the role and contribution of different DSL ligands (Dll1, Dll4 and Jag1) to the development of the mouse nervous system.

In this thesis, I have analysed the function and the expression pattern of three mouse genes encoding DSL ligands (*Dll1, Dll4* and *Jag1*) in the neural tube, retina and forebrain. In some of the regions studied, only one of these genes is expressed, whereas in others two of the genes are co-expressed within the same neural domain. *Jag1* expression overlaps with that of *Dll1* in the forebrain and is complementary to *Dll1* in the neural tube. I was able to provide the first clear evidence that Jag1 is able to mediate Notch signalling in the developing spinal cord, being sufficient (although dispensable) to maintain neural progenitors within its domains of expression (the pV1 and dl6 domains of the neural tube). Analysis of *Jag1* loss-of-function mutants did not yield any detectable phenotype, suggesting that *Jag1* function may be completely compensated by *Dll1* and thus dispensable for proper neural development.

Nevertheless, one cannot exclude the possibility of Jag1 having a function apart from neural progenitor maintenance that would only be translated into a detectable phenotype at later stages of development. For instance, one may question if *Jag1*-expressing neurons acquire the correct identity and whether neuronal connections are properly established in these mutants. Therefore, a careful analysis of the *Jag1* mutants at late embryonic timepoints, or even postnatally, may yield clues into other possible functions of this ligand during vertebrate neurogenesis. For instance, it is known that *Jag1* is the only DSL-encoding gene that is expressed in the SVZ of the adult mouse brain (Irvin et al., 2004) and this system may be more interesting to approach the role of Jag1 because compensation by other DSL ligands might not occur.

By analysing *Dll1* loss-of-function mouse embryos, I have confirmed a role for this ligand in neural progenitor maintenance in all analysed tissues of the developing mouse CNS. I have shown that a single DSL ligand, Dll1, is necessary and sufficient to maintain a pool of neural progenitors and, in this way, allow the generation of different cell fates when these arise over time (for example, neuron-glial cell production). However, when more than one cell type is generated from the same neural domain and during the same temporal interval, such as the developing retina and pV2 domain of the spinal cord, two DSL ligands are employed, Dll1 and Dll4. I have provided evidence that these two genes are expressed sequentially and that although their function may partially overlap, this sequential expression may underlie two rounds of Notch signalling to allow a given cell to undergo successive fate decisions. First, Dll1-induced Notch signalling is used to mediate the progenitor-neuron decision; in a second phase, cells that are already committed to neural differentiation require a second Notch signalling event, possibly mediated by Dll4, to prevent all from acquiring the same fate, thereby establishing cell diversity. Although this model requires further investigation, namely the analysis of *Dll4* mutants, it provides a mechanism that is conceptually similar to that present in the SOP lineage of *D. melanogaster*, where consecutive events of Notch signalling also account for successsive cell fate decisions (reviewed in (Bardin et al., 2004)). However, many questions remain unanswered and require further study.

First, it would be of interest to understand what determines the asymmetry in Notch signalling among differentiating neurons, i.e., what determines which differentiating neurons are signal-sending or signal–receiving cells. In the pV2 domain of the developing spinal cord, only two neuronal types, V2a and V2b, are produced and this

question may seem 'simple' given the directionality of Dll4-mediated Notch signalling, from V2a precursor to surrounding cells that acquire the alternative V2b fate. The asymmetric expression of *Dll4* has been proposed to result from lateral inhibition, where one of two initially equivalent cells would 'win the competition' and become the signalling cell (Del Barrio et al., 2007; Peng et al., 2007). However, this model implies that in an initial phase, both equivalent cells express *Dll4*. I have in this thesis shown that this may not be the case. Instead, fate mapping of *Dll4*-expressing cells through the detection of β-Gal activity in *Dll4*$^{LacZ/+}$ mice has indicated that this gene is only expressed in cells that will acquire the V2a fate. These results suggest that *Dll4* expression is pre-determined by a specific genetic circuitry present in only some differentiating V2 cells.

A question that arises as a consequence of this hypothesis is which are the players in this genetic circuitry? Genes encoding DSL ligands are known to be transcriptionally regulated by bHLH proteins. Mash1, NeuroD and Neurogenin1/2 are among some of these proteins which are expressed in the pV2 domain of the neural tube and are therefore possible candidates for regulating *Dll4*-expression. In fact, over-expression of *Mash1* in the chick neural tube has been shown to upregulate *Dll4* transcription (Del Barrio et al., 2007). However, studies perfomed by Thomas Reh's group in the chick retina have suggested that *Mash1* is a potential candidate for regulating *Dll1* expression and not that of *Dll4* (Nelson and Reh, 2008). Further studies are thus required to unravel the components of this genetic circuitry and how it operates to establish the asymmetric *Dll4* expression among differentiating V2 neurons in the developing vertebrate spinal cord.

Should the existence of such a specific genetic circuitry be unveiled in the pV2 domain of the neural tube, an immediate question would be if the same genetic cascade is employed in the retina? Despite the conserved character of the *Dll1/Dll4* circuitry both in the retina and pV2 domain of the neural tube, the retina presents two major differences which complicate the feasibilty of a 'fixed' genetic circuitry tightly regulating *Dll4* expression. First and unlike the neural tube, the retinal neuroepithelium generates a wide variety of neuronal cell types. Second, and most importantly, *Dll4* expression is not restricted to a single neuronal type, nor only to a fraction of the neuronal types produced. Instead, this gene seems to be expressed in some differentiating neurons from all neuronal types. The extent of this 'randomness' in *Dll4* expression is not yet firmly established and requires the development of a stable reporter line for *Dll4*-expressing cells, in order for one to fate map these cells and determine what proportion of cells, within each retinal cell type, express (or have

expressed) *Dll4*? This fate mapping might answer whether all neuronal cell types have an equal contribution of *Dll4*-expressing cells or whether there is a cell type with a significantly higher contribution. For instance, if a given retinal cell 'type A' is derived exclusively or mostly from *Dll4*-expressing cells, while others fates have little contribution, Dll4-mediated Notch signalling in the retina could also be directional, from 'type A' cells to neighbouring cells that would acquire the alternative fates B, C, etc. In such scenario, Dll4 would function mainly to control the production of 'type A' cells and consequently, ensure the generation of the remaining cell types. If we assume that this 'type A' cell are photoreceptors, this hypothesis would support the models proposed by Cepko's and Ashery-Padan's labs (Jadhav et al., 2006; Yaron et al. 2006), where Notch signalling is required to specifically inhibit photoreceptor production.

However, if no significant difference is observed in the contribution of *Dll4*-expressing cells among the several retinal cell types, then it remains to be answered how signalling by Dll4 may be differently perceived by the different cells undergoing neuronal committment. One possible molecular mechanism that allows Notch signalling to be perceived in distinct manners by cells exposed to the same signal is the differential expression of modulators in the signal receiving-cells. An example of such modulators are the Fringe proteins that have recently been proposed to contribute to different outputs in Notch signalling in processes as diverse as myelopoiesis, lymphopoiesis, angiogenesis and neurogenesis and, in this way, promote cell heterogenity. Whether this is the case in retinal cells undergoing neuronal committment is not known. The expression pattern of the three genes encoding for Fringe proteins (*LFng, MFng* and *RFng*) has not been yet analysed in detail in the retina. In fact, it is not known whether these genes are expressed in differentiating retinal cells or only restricted to neural progenitors. In the neural tube, it has been shown by Wilkinson and collaborators that expression of *LFng* is negatively regulated by proneural genes and in this way, restricted to progenitor cells, being negatively regulated by proneural genes when cell commit to differentiation.

In summary, I have proposed in this thesis a novel genetic circuitry comprising the sequential expression and function of two DSL ligands, *Dll1* and *Dll4*, which functions to create cell diveristy in specific regions of the developing mouse nervous system. Nonetheless, further studies are required to fully understand this mechanism.

CHAPTER VI

MATERIALS AND METHODS

VI.1 MOLECULAR BIOLOGY TECHNIQUES

VI.1.1 Preparation and transformation of competent *E. coli* bacteria

Throughout this work competent *Escherichia coli* bacteria were used for transformation with plasmid DNA. According to the lab, Unit of Developmental Biology at the Institute for Molecular Medicine (IMM) or Developmental Biology Group at the Wolfson Institute for Biomedical Research (WIBR), different protocols were used to prepare competent cells. The resulting *E. col*i bacteria were either chemically or electro- competent. The bacterial strains of *E. coli* used were DH5α, DH5T and XL-1 Blue.

VI.1.1.1 Preparation and transformation of chemically competent *E.coli* bacteria

Preparation of chemically competent bacteria was based on the protocol described by Inoue *et al* (Inoue et al., 1990). The bacterial strains of *Escherichia coli* used were DH5α and DH5T. Cultures of these bacterial strains were made competent for transformation with plasmid DNA by treatment with calcium chloride ($CaCl_2$). A single colony was placed in 25mL of LB medium and cultured for 6-8 hours (hrs) at 37ºC, with vigorous shaking. This starter culture was used to inoculate three 1L flasks (each containing 250mL of SOB medium), of which the first received 10mL of the starter culture, the second received 4mL and the third received 2mL. All three flasks were incubated overnight (o/n) at 18-22ºC with shaking, until one of the flasks reached an OD_{600nm} of 0.6. Further growth of the bacteria was interrupted by cooling the culture to 4ºC. The culture was then centrifuged at 2500g for 10 minutes (min) at 4ºC. The pellet was gently resuspended in 80mL of ice-cold Inoue buffer (55mM $MnCl_2$, 15mM $CaCl_2$, 250mM KCl, 10mM PIPES pH6.7), centrifuged at 2500g for 10min at 4ºC and resuspended once more in 20mL of ice-cold Inoue buffer. After complete resuspension, 1.5mL of DMSO was added to the solution and cells were incubated an additional 10min in ice before freezing 0.25mL aliquots in liquid nitrogen and stored at -80ºC.

For plasmid DNA transformation, frozen aliquots of competent cells were thawed on ice. Plasmid DNA (0.01-0.8 µg) was incubated with 100µL of cells on ice for 30min. The cells/DNA mix was heat-shocked for 90 seconds (sec) at 42ºC and then incubated on ice for 2min. 600µL of SOB medium was added to the mix, which was

then incubated with shaking at 37ºC, for 45min. The mix was centrifuged for 30sec and 600µL of the supernatant solution were discarded. The cells were resuspended in the remaining volume and plated on appropriate selective LB agar media and incubated at 37ºC o/n.

VI.1.1.2 Preparation and transformation of electrocompetent *E. coli* bacteria

A single bacterial colony was placed in 10mL of SOC medium and allowed to grow in a shaking incubator o/n at 37ºC. This starter culture was used to inoculate two 2L flasks (each containing 400mL of SOC medium), each of which received 4mL of the starter culture. The flasks were incubated o/n at 37ºC with shaking, until the cultures reached an OD_{600nm} of at least 0.6-0.8. Further growth of the bacteria was interrupted by aliquoting the cultures into pre-chilled 50mL Falcon (25mL of culture per Falcon) and incubating these for 15min, in ice. The cells were then centrifuged at 1500g for 15min at 4ºC, the pellets were washed twice by gently resuspending each pellet in 25mL of ice-cold ultra-clean H_2O and centrifuging at 1500g for 15min at 4ºC. After these two washes, each pellet was resuspended in 5mL of ice-cold 10% glycerol (v/v). Resuspensions were pooled into two falcons (40mL resuspension in each) and centrifuged at 1500g for 15min at 4ºC. Finally, each cell pellet was resuspended in 250µL of ice-cold 10% glycerol, aliquoted (42µL aliquots), frozen in liquid nitrogen and stored at -80ºC.

For plasmid DNA transformation, frozen aliquots of competent cells were thawed on ice. Plasmid DNA (0.01-0.8 µg) was added to 42µL of cells. The cells/DNA mix was placed in 0.2cm cuvettes (Bio-Rad) on ice and electroporated at 2.5kV (25µFD capacitance and 200Ω resistance) using a Bio-Rad MicroPulser. 500µL of LB medium was added to the mix, which was then incubated with shaking at 37ºC, for 45min. The mix was centrifuged for 30sec and 400µL of the supernatant solution were discarded. The cells were resuspended in the remaining volume and plated on appropriate selective LB agar media and incubated at 37ºC o/n.

VI.1.2 Plasmid DNA purification

For small scale preparation of plasmid DNA (~5-10µg), 2mL of a 3mL o/n bacterial culture of transformed competent cells, in the appropriate selective LB medium, was processed using the *Wizard Plus SV Minipreps DNA Purification System* (Promega), according to the manufacturer's instructions.

For large scale preparations of plasmid DNA (200-400 µg), 50mL of the selective LB medium was inoculated with 0.5mL of plasmid bacterial culture and shaken at 37ºC o/n and processed using the *Genopure Plasmid Midi Kit* (Roche), according to the manufacturer's instructions.

VI.1.3 DNA and RNA quantification

The concentration of DNA and RNA was determined by spectrophotometry using GeneQuant*pro* or Nanodrop spectrophotometer. One A_{260} unit corresponds to 50 µg/mL of double stranded DNA and to 35 µg/mL of single stranded RNA (Sambrook and Russell, 2001). The purity of the nucleic acid preparation was estimated by the ration between the readings obtained at 260nm and 280nm (pure preparations of DNA and RNA show ratio values of 1.8-2.0).

VI.1.4 Restriction digestions and ligation reactions

Enzymatic restriction of DNA was performed for approximately 1hr using 5-10U of commercially available restriction enzymes and respective buffers (Promega, Roche, Fermentas, New England Biolabs). The volume of enzyme used in each reaction, never exceeded 10% of the total reaction volume.

Ligation reactions were carried out in a final volume of 10µL, for a total DNA amount of 0.5µg. The ligation reactions were performed o/n, at 4ºC for blunt-end reactions and at 15ºC for cohesive-end ligation, using 1U of T4 DNA Ligase (Promega) and the respective ligation buffer.

VI.1.5 Analysis and isolation of DNA by agarose gel electrophoresis

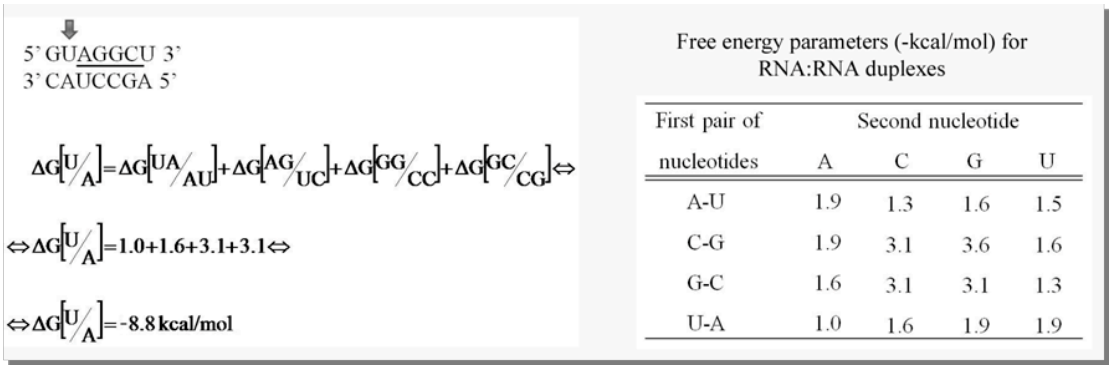
To separate and estimate the size of DNA fragments, agarose gel electrophoresis was carried out. Gels were prepared by heating agarose (Invitrogen) until complete dissolution in 1x TAE buffer (40mM Tris, 1mM EDTA, 0.35% glacial acetic acid). The final agarose concentration depended on the size of DNA to be resolved: 2.0% (w/v) for <0.5kb and 1.0% (w/v) for 1-10kb. DNA was visualized by the addition of ethidium bromide (GibcoBRL) to the gel, to a final concentration of 0.2µg/mL. DNA samples were mixed with 1x Loading buffer (60% Glycerol (v/v) : 10mM EDTA with traces of OrangeG – Sigma) and electrophoresis was carried out in 1x TAE buffer at 5-10 V/cm of gel length, until the desired resolution was achieved. DNA was visualized under an ultraviolet light at 340nm and the size of the fragments was estimated by comparison with linear DNA strands of known molecular weight (*1kb Plus DNA Ladder* – Invitrogen or *Hyperladder I* - Bioline). The region of the gel containing the DNA fragment of interest was excised under ultraviolet light at 365nm and purified using *Wizard Plus SV Gel and PCR Clean-up System* (Promega), following to the manufacture's instructions.

VI.2 GENERATION OF SHRNA-EXPRESSING VECTORS

VI.2.1 Selection of gene targeting sequences by the nearest-neighbour model

The nearest-neighbour model takes into account the neighbouring nucleotides of each base pair. This is of extreme importance since two sequences composed by the same nucleotides but with a different sequence can exhibit different thermodynamic stabilities, while sequences with different nucleotide compositions can present the same stability (Le Novere, 2001). According to the Oligo 5.0 Software, the neighbouring context for each base pair corresponds to the four bases pairs immediately downstream of it. Therefore, the free energy associated to each base pair is equal to the sum of the free energy of each of the individual base pairs composing the pentamer (Figure VI.1). We made use of published thermodynamic parameters (Khvorova et al., 2003) to create thermodynamic profiles of any siRNA duplex. Upon introduction of the nucleotide sequence of the antisense strand into a programmed Excel worksheet, these thermodynamic profiles were generated automatically.

The calculation of the stability of a certain RNA:RNA duplex through the nearest-neighbour model presents some limitations. How does one determine the free energy associated to each of the last four nucleotides of the 19nt antisense sequence, since these no longer present a pentamer context? Jayasena and colleagues have by-passed this situation by considering a 23nt sequence: 19nt targeting sequence + 4 downstream nucleotides (personal communication). Although these additional 4nts are an extension of the fully complementary siRNA to the target mRNA, they are not present in the processed 19nt siRNA and can influence significantly the calculated stability of the 3' end of the antisense strand. Therefore, besides determining the thermodynamic profiles based on the antisense sequence together with this 4nt extension (for example see green boxes in Figure II.4), we also inferred the relative stability between the 3' and 5' ends of the antisense strand through their G/C content (red arrows in Figure II.4).



Free energy parameters (-kcal/mol) for RNA:RNA duplexes

First pair of nucleotides	Second nucleotide A	C	G	U
A-U	1.9	1.3	1.6	1.5
C-G	1.9	3.1	3.6	1.6
G-C	1.6	3.1	3.1	1.3
U-A	1.0	1.6	1.9	1.9

Figure VI.1: Calculation of the thermodynamic stability of each base pair.

The free energy (ΔG) of a certain base pair (for example, the pair U-A depicted with the arrow) is determined by the neighbouring nucleotide context (the 4 base pairs immediately downsteam – underlined): it is equal to the sum of the free energy of each of the individual base pairs composing the pentamer.

VI.2.2 Target sequences selected and oligonucleotides used for cloning

In order to establish the RNAi gene silencing technique in chick embryos, target sequences were selected for several genes. These target sequences are listed in the table below (Table VI.1), as well as oligonucleotides (Sigma Genosys) that were used to clone shRNAs targeting these selected sequences.

Table VI.1: Genes targeted for silencing by RNAi and oligonucleotides used to clone the respective shRNA-expressing vectors.

Psen1/2 – Presenilin1/2, SuH – Suppressor of Hairless

Gene	mRNA target sequence (5'→3')	Oligonucleotides (5'→3')
cNotch1 (A5)	UGCGAACAUUCAGGAUAAC	AATTCGTTATCCTGAATGTTCGCATTCAAGAGATGC GAACATTCAGGATAAATTTTTGGGAT
		ATCCCAAAAATTTATCCTGAATGTTCGCATCTCTTG AATGCGAACATTCAGGATAACG
cNotch1 (L2)	CAACGCGGGCUGCCUGUAUG AUGGGUUUG	GCGCGGGCGGCCGCTCCTGCTGCGCGCAGCTCTC C
		TATGAATTCAAAAATAAATCCATCATACAGGCAACC CACGTTGCCAACAACGCGGGCTGCCTGTATGATG GGTTTGTAGTATATGTGCTGCCGAA
cPsen1/2	UGCAGCUCCAGCAAGCAUAUC UCAUUAUG	GCGCGGGCGGCCGCTCCTGCTGCGCGCAGCTCTC C
		TATGAATTCAAAAATATAATGAGATATGCTTGCTGG AGCTGCACCAATGCAGCTCCAGCAAGCATATCTCA TTATGTAGTATATGTGCTGCCGAA
cSuH	CACAGCCUUACGCCUUCAUUG GAAUAGGA	GCGCGGGCGGCCGCTCCTGCTGCGCGCAGCTCTC C
		TATGAATTCAAAAATAATATTCCAATGAAGACGCAA GACTGTGCCAACACAGCCTTACGCCTTCATTGGAA TAGGATAGTATATGTGCTGCCGAA
cPar3	AGGCUUUCUCAAGCAGAAC	AATTCGTTCTGCTTGAGAAAGCCTTTCAAGAGAAG GCTTTCTCAAGAAGAACTTTTTGGGAT
		ATCCCAAAAAGTTCTTCTTGAGAAAGCCTTCTCTTG AAAGGCTTTCTCAAGCAGAACG

VI.2.3 Cloning strategies

VI.2.3.1 DNA vectors used

Several plasmid construct were generated to do RNAi based studies in the chick embryo. Plasmid constructs to express several shRNAs targeting genes of the Notch pathway were cloned into the pSouper vector (kindly provided by Steve Wilson, Sheffield University). The pSouper plasmid consists of a chick U6 promoter (see Figure II.3 for promoter sequence and Figure VI.2 for vector restriction map) cloned into a pBluescript II KS backbone (Stratagene). The commercially available bacterial cloning vector pGEM T-Easy (Promega) were used for sub-cloning strategies.

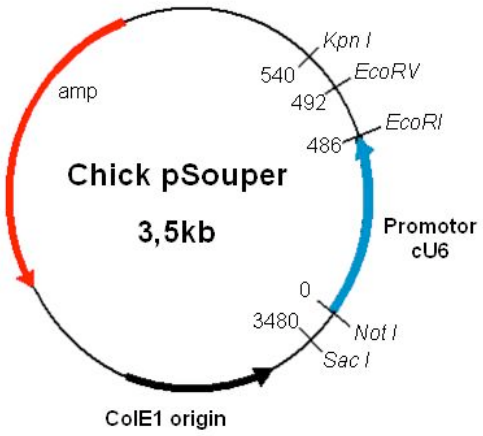


Figure VI.2: pSouper vector map. The cU6 promoter is cloned into the multiple cloning site of the pBluescript II KS vector at *NotI* and *EcoRI*.

VI.2.3.2 Generation of plasmids expressing shRNAs with 21nt stem

Oligonucleotides encoding shRNAs targeting the *cNotch1* (*cNotch1 A5* shRNA) and *cPar3* genes were paired to form a 62bp double-stranded DNA fragment. This DNA fragment contained *EcoRI* and *EcoRV* polylinkers that allowed its directional cloning into the pSouper vector and also the termination sequence of the RNA Polymerase III at the 3' end of the shRNA (Figure VI.3).

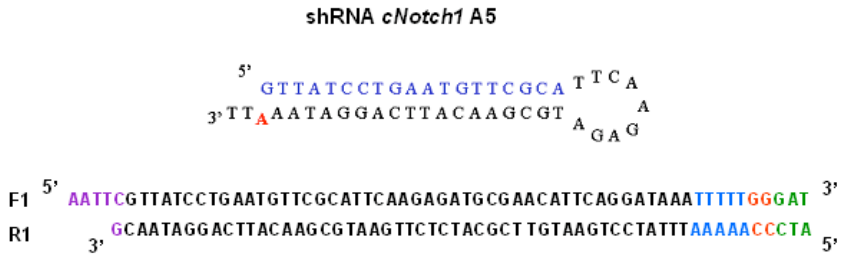


Figure VI.3: Cloning of vectors expressing shRNAs with 21bp.
Oligonucleotides for the cloning of the 21nt stem shRNAs, were designed to have the entire coding for the shRNA (in black), a *EcoRI* restriction site at 5' (in purple), a *EcoRV* restriction site at 3' (in green), a RNA Polymerase III termination sequence (in blue) and two G's after the termination sequence to confer stability (in red).

VI.2.3.3 ShRNAs with 29nt stem and leader sequence

To generate plasmids expressing 29bp stem hairpins with, a 27nt leader sequence, I followed a PCR-based strategy, proposed by Hannon's lab (Paddison et al., 2004). This PCR strategy makes use of a DNA template, composed by the U6 promoter sequence followed by the snRNA sequence (the pSouper vector), and two specific primers: a U6 forward primer that targets the 5' region of the promoter and a hairpin primer that corresponds to the whole shRNA coding sequence (72nt) + 21nt of homology to the U6 snRNA downstream of the U6 promoter (Figure VI.4,). By using these two primers, it was possible to produce a PCR product with the full cU6 promoter, followed by the leader sequence (first 27nt of the snRNA) and the shRNA. This PCR product was then purified and cloned into the pGEM T-Easy vector.

All vectors generated for RNAi studies were sequence verified. Samples were sequenced by Genomed at the IMM or by Stabvida.

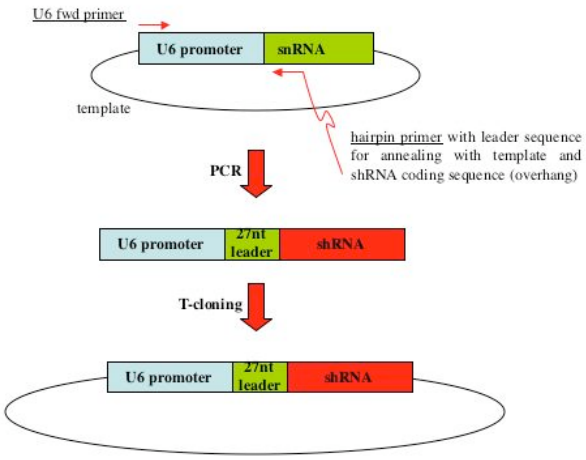


Figure VI.4: PCR-based strategy for quick generation of plasmids expressing 29bp stem hairpins with a 27nt leader sequence.

VI.3 CHICK EMBRYO MANIPULATION

VI.3.1 In ovo chick embryo electroporation

Super-coiled plasmid DNAs encoding for shRNAs were injected into the neural tube of HH11-12 chick embryos, at a concentration of 2 µg/µL in PBS. As a positive control of electroporation and in order to visualize electroporated cells, I co-electroprated the pCIG vector, a kind gift of Andy McMahon (Megason and McMahon, 2002), at 0,4 µg/µL. Platinum electrodes (Nepagene CUY613G), distanced 4mm between the anode and cathode, were placed parallel to the neural tube and embryos were pulsed 4 time (30V/50ms), using an Electro Square Poartor ECM830 (BTX). Embryos were incubated for 8hr or 24hr at 38ºC, harvested and fixed in 4% paraformaldehyde:PBS (PFA), at 4ºC, o/n.

VI.3.2 Tissue embedding and preparation of cryostat sections

After fixation, the tissue was washed in 1x PBS, passed first through a 15% sucrose:PBS solution, followed by a 30% sucrose:PBS solution for cryoprotection and then embedded in 7.5% gelatin : 15% sucrose : PBS and frozen in cold isopenthane (-70ºC). Frozen tissue was stored at -80ºC indefinitely and

cryosectioned (Leica CM 3050 cryostat) whenever needed. Embryonic tissue was sectioned at 12µm and collected on Superfrost slides.

VI.4 MOUSE MANIPULATION

VI.4.1 Mouse breeding

The *Chx10-Cre* (Rowan and Cepko, 2004) and *FoxG1-Cre* strains (Hebert and McConnell, 2000) were obtained from Jackson Lab. The *Nestin-Cre* (Tronche et al., 1999) and R26RYFP (Srinivas et al., 2001) strains were a kind gift from Rüdiger Klein and Nicoletta Kessaris, respectively. *Dll4*^{LacZ} embryos (Duarte et al., 2004) were a kind gift from António Duarte and the conditional mutants, *Dll1-flox* (Hozumi et al., 2004) and *Jag1-flox* (Brooker et al., 2006) were kindly provided by Julian Lewis. The *Dll1*^{LacZ} (Hrabe de Angelis et al., 1997), *Dll1*^{Ki} (Schuster-Gossler et al., 2007) and the *Nkx6.2-iCre* (Fogarty et al., 2007) strains were previously described. Finally, the *Dbx1-iCre* was generated in Nicoletta Kessaris' Lab and not yet published.

Mice carrying the conditional *Dll1-flox* allele were crossed with each Cre strain and double heterozygous progeny were identified by PCR. These were then crossed with mice homozygous for the conditional allele, to produce litters containing conditional knockout mice (*Dll1-flox/flox;Chx10-Cre, Dll1-flox/flox;Nestin-Cre, Dll1-flox/flox;FoxG1-Cre, Dll1-flox/flox;Dbx1-iCre* or *Dll1-flox/flox;Nkx6.2-iCre*) and sibling controls.

Mice carrying the conditional *Dll1-flox* allele were crossed with *Dll1*$^{LacZ/+}$ mice to generate *Dll1*$^{LacZ/flox}$ mice. These were then crossed to *Dll1-flox/+,Chx10-Cre* double heterozygous mice, to produce litters containing mice with one null and one conditional knockout allele (*Dll1-LacZ/flox,Chx10-cre*) and sibling controls.

The *Dll1*$^{LacZ/+}$ and *Dll1*$^{Ki/+}$ were intercrossed to obtain *Dll1*$^{LacZ/Ki}$ mutants and sibling controls.

Mice carrying the conditional *Jag1-flox* allele were crossed with each Cre strain and double heterozygous progeny were identified by PCR. These were then crossed with mice homozygous for the conditional allele, to produce litters containing conditional knockout mice (*Jag1-flox/flox;Nestin-Cre* or *Jag1-flox/flox;Nkx6.2-iCre*) and sibling controls.

VI.4.2 Dissection of embryos

Crosses were set and vaginal plugs were checked every morning to obtain embryos at the stages of interest. Embryos were staged as E0.5 on the morning of the vaginal plug. The dams were culled with CO_2 and the embryos were dissected in RNAse-free PBS. A portion of the yolk sac or tail of each embryo was extracted during dissection and later used for genotyping (see section IV.4.4). Once dissected, embryos were collected in fixative medium: 4% paraformaldehyde:PBS (PFA) for *in situ* hybridization and immunofluorescence or 0.2% glutaraldehyde for X-Gal staining. Fixation with PFA was in general performed at 4ºC, o/n, except when using particular antibodies (see Table VI.3) and fixation with glutaraldehyde was done at r/t, ~1hr.

VI.4.3 Perfusion of adult mice

Whenever the tissue of interest was the adult brain, mice were anaesthetised with a mixture of Hypnorm (0.315mg/mL of fentanyl citrate and 10mg/mL of fluanisone - VetaPharma) and Hypnovel (5mg/mL of midazolam - Roche). Each mouse was injected with 160-200μL of the anaesthesic (1:2:1, Hypnorm:H_2O:Hypnovel). Once unconscious, mice were perfused with ~40mL of cold 4% PFA:PBS via the left ventricle. Once perfusion was complete, the brain was dissected out, sectioned into 2mm slices and left in 4% PFA:PBS o/n, at 4ºC, embedded and frozen (see section VI.4.6).

VI.4.4 Genotyping

Genomic DNA was extracted from tissue biopsy of post-natal mouse tails, or from embryonic tissue. Each sample of tissue was placed in a 1.5 mL eppendorf and mixed with 50μL of Lysis Buffer (50mM KCl, 10mM Tris-Hcl pH8.0, 2mM $MgCl_2$, 0.1mg/mL gelatin, 0.45% Nonidet-P40, 0.45% Tween-20, 0.5mg/mL Proteinase K) and incubated at 55ºC during 6hr to o/n. After complete lysis, samples were incubated at 90ºC for 15min, in order to inactivate Proteinase K activity.

Each genotyping PCR reaction was done in a final volume of 25μL and contained 1x PCR Buffer (Promega), 1.5mM $MgCl_2$ (Promega), 0.2mM of each primer (from Sigma-Aldrich or MWG; Table VI.2), 1U of Taq DNA Polymerase and 2μL of genomic DNA.

Table VI.2: Primers used for genotyping

Mouse Line	Primers (5' → 3' sequence)
Dll1$^{LacZ/+}$	GTTGCACCACAGATGAAACGC TGCAGCTTCAGCTCAAATACGC AAGCCAGACGAAGAGGAAACCG
Dll1^{Flox}	CACACCTCCTACTTACCTGA GAGAGTACTGGATGGAGCAAG
Dll1^{Ki}	AGACTGCCTTGGGAAAAGCG AAGGGGAGAAGATGCTTGATAACC
Dll4$^{LacZ/+}$	GGGGAATCAGCTTTTCAGGAA CGAACTCCTGCAGCCGCAGCT ACGACGTTGTAATACGAC
Jag1^{Flox}	TGAACTCAGGACAGTGCTCT GTTTCAGTGTCTGCCATTGC
Cre lines	GGACATGTTCAGGATCGCCAGGCG GCATAACCAGTGAAACAGCATTGCTG
iCre lines	GAGGGACTACCTCCTGTACC TGCCCAGAGTCATCCTGGC
Rosa26R^{YFP}	AAAGTCGCTCTGAGTTGTTAT GGAGCGGGAGAAATGGATATG GCGAAGAGTTTGTCCTCAACC

VI.4.5 Administration of BrdU

BrdU (bromodeoxyuridine - Sigma) administration was used to measure the rate of retinal neurogenesis at E14.5. BrdU was administered to pregnant dams (E13.5) as an intraperitoneal (IP) injection, at a final concentration of 100mg/kg. Embryos were dissected 24hr after BrdU administration and the tissue was processed for BrdU detection (see section VI.5.3).

VI.4.6 Tissue embedding and preparation of cryostat sections

After fixation, the tissue was washed in 1x PBS, passed through a 20% sucrose:PBS solution for cryoprotection and then embedded in 7.5% gelatin : 15% sucrose : PBS or in OCT and frozen in cold isopenthane (-70ºC). Frozen tissue was stored at -80ºC indefinitely and cryosectioned (Leica CM 3050 cryostat) whenever needed. Embryonic tissue and post-natal retinas were sectioned at 12µm and collected on Superfrost slides, while adult brain tissue was sectioned at 30µm and collected into wells containing 1x PBS (see section VI.5.2).

VI.5 IMMUNOHISTOCHEMISTRY

VI.5.1 Immunofluorescence on cryostat sections of embryonic tissue

Immunofluorescence was performed as follows: sections were washed 10min, in PBS at 37°C to remove the embedding gelatine and then rinsed 2 x 5min in PBS. Sections were then treated with 0.1M Glycine in PBS (10min), permeabilized with 0.5% Triton-100X in PBS (10min) and blocked with 10% Fetal Bovine Serum (FBS) in TBST (20mM Tris-HCl pH8, 150mM NaCl, 0.05% Tween-20) for 1hr at r/t. Primary antibodies were diluted in blocking solution and incubated o/n, at 4°C. After primary antibody binding, sections were washed 3 x 10min in TBST. Appropriate secondary antibodies were diluted in blocking solution and incubated for 1hr, at r/t. All sections were counterstained with DAPI (0.15%, w/v) for 5min and mounted with Dako Fluorescence Mounting Medium.

Primary antibodies used were: mouse anti-β-galactosidase (Promega, 1/500), rabbit anti-β-galactosidase (Molecular Probes, 1/500), mouse anti-BrdU (Sigma, 1/1000), mouse anti-Brn3a (Chemicon, 1/100), rabbit anti-Brn3b (Babco, 1/600), rabbit anti-activated-Caspase 3 (Cell Sig. Technol., 1/200), sheep anti-Chx10 (Exalpha, 1/100), mouse anti-CRABP (Affinity Bioreagents, 1/1000), rabbit anti-En-1 (kind gift from A. Joyner, 1/100), mouse anti-Evx1 (DSHB, 1/100), mouse anti-GATA3 (Santa Cruz, 1/50), rabbit anti-GFP (Abcam, 1/1000), mouse anti-HU (Molecular Probes, 1/500), mouse anti-Islet1 (DSHB, 1/1000), rabbit anti-Jag1 (Santa Cruz, 1/50), rabbit anti-Laminin (BRL, 1/100), mouse anti-Lim3 (DSHB, 1/100), , mouse anti-MNR2 (DSHB,

1/100), mouse anti-neurofilament NF165 (DSHB, 1/1000), mouse anti-N-Cadherin (BD Transduction Lab., 1/100), mouse anti-Nkx2.2 (DSHB, 1/50), mouse anti-p27-Kip1 (Transduction Lab, 1/50), rabbit anti-Pax2 (Covance, 1/200), rat anti-PDGFRα (BD Biosciences, 1/500), rabbit anti-Recoverin (Chemicon, 1/1000), rabbit anti-S100β (Sigma, 1/100), rabbit anti-Sox2 (Chemicon, 1/200), mouse anti-Tuj1 (Covance, 1/500) and mouse anti-ZO-1 (Zymed, 1/50). Secondary antibodies used were: HRP-conjugated goat anti- mouse and goat anti-rabbit (Molecular Probes, 1/1000) to do FITC-Tyramide amplification (Perkin Elmer) and Alexa 488/594-conjugated goat anti-mouse, goat anti-rabbit and donkey anti-sheep (Molecular Probes, 1/400).

Note that several antibodies require specific fixations and/or antigen retrieval treatments which are referred to in Table VI.3.

VI.5.2 Immunofluorescence on cryostat sections of adult tissue

Immunofluorescence on sections of adult tissue (30µm) was performed on free floating sections. Sections were placed in wells with PBS and blocked with freshly made blocking buffer (10% sheep serum, 0.1% Triton-X100 in PBS) for 1hr at r/t, with agitation. Primary antibodies were diluted in blocking buffer and incubated o/n, at 4°C with agitation. After primary antibody binding, sections were washed 3 x 15min in PBS. Appropriate secondary antibodies and DAPI were diluted in blocking buffer and incubated for 2hr at r/t, with agitation. Sections were washed 3 times in PBS before transferring sections to slides and mounting with Dako Fluorescence Mounting Medium. Primary antibodies used were: mouse anti-Calretinin (Swant, 1/500) and rabbit anti-Somatostatin (Peninsular, 1/200) were used as primary antibodies, and Alexa 488/568-conjugated goat anti-mouse and goat anti-rabbit (Molecular Probes, 1/400) were used as secondary antibodies.

Table VI.3: Specific fixations and/or antigen retrieval treatments used for several antibodies.

Antigen		Fixation/Pretreatment Requirements	Observations
β-galactosidase Chx10		Methanol pretreatment	Sections were incubated in 3%H_2O_2:Methanol for 30min, at r/t prior to blocking
BrdU		Formamide and HCl pretreatment	See section VI.5.3
Brn3a	N-Cadherin	Weak tissue fixation	Tissue was fixed with 4%PFA:PBS for a short period of time (stage dependent) at 4°C: E10.5 – 20min E11.5 – 45min E12.5 - 60min E13.5 – 90min
En-1	Nkx2.2		
Evx1	p27-Kip1		
GATA3	Pax2		
Jag1	PDGFRα		
Lim3	S100β		
MNR2			

VI.5.3 BrdU labelling

Detection of BrdU incorporation was performed as follows: cryosections were incubated in a 50% formamide : 1x SSC solution, at 65ºC for 1hr (when not subjected previously to *in situ* hybridization) and then incubated in 2N HCl, at 37ºC for 30min. After acidic treatment, the sections were neutralized with 2 x 10min 0.1M Tris-HCl (pH8.0) and rinsed twice with 1x PBS. From here onward, sections were blocked as in Section VI.5.1 and BrdU incorporation was detected using a mouse anti-BrdU antibody (1:1000, Sigma).

Whenever, BrdU labelling was necessary with another antibody labelling, which was sensitive to the HCL treatment, a different protocol was followed. Briefly, after performing the immunostaining for the “sensitive” antibody, sections were fixed 10min in an acidic alcohol solution (70% ethanol, 10% glacial acetic acid) and 20min in 70% ethanol at -20ºC, followed by a 15min wash with 1% Triton-X100:PBS and finally by 15min HCl treatment (6N HCl, 1% Triton-X100, PBS). After acidic

treatment, the sections were neutralized with 3 x 5min PBS. From here onward, sections were blocked as in Section VI.5.1 and BrdU incorporation was detected using the anti-BrdU antibody referred above.

VI.5.4 Detection of β-Galactosidase by X-Gal staining

β-Galactosidase (β-Gal) detection requires a glutaraldehyde-based fixation (0.2% glutaraldehyde : 5mM EGTA (pH8.0) : 2mM $MgCl_2$: PBS – LacZ Fixative). Embryos were fixed at r/t for 30min-1hr (depending on the stage), embedded, frozen and cryosectioned (see section VI.4.2).

Briefly, sections were degelatinated in 1x PBS for 15min, at 37ºC, rinsed twice in 1x PBS, refixed with LacZ Fixative and washed 3 x 10min in LacZ Washing Buffer (2mM $MgCl_2$: 0.02% Nonidet-P40 : PBS). Finally sections were stained in 5mM potassium ferrocyanide : 5mM potassium ferricyanide : 0.1% X-Gal : LacZ Washing Buffer. Sections were left to stain in the dark, at 37ºC for 4hrs to o/n. Sections were then dehydrated and mounted with DPX Mountant for Histology (Fluka).

VI.6 *IN SITU* HYBRIDIZATION

VI.6.1 Antisense RNA probe synthesis

During the course of this work, several RNA anti-sense probes were used for *in situ* hybridization on cryosections of mouse embryos. Digoxigenin- (DIG) and Fluorescein- (FLUO) labelled RNA anti-sense probes were synthesized *in vitro* by T3, T7 or SP6 RNA polymerase, from plasmid templates containing cDNAs of several genes (Table VI.4).

VI.6.1.1 DNA template preparation

DNA template preparation was performed as follows: 10µg of plasmid DNA was linearized in a final volume of 100µL, using 50U of the appropriate restriction enzyme, for 2 hours (hrs) at 37ºC. After confirmation of complete digestion, the reaction was finalized by adding 5µL of 10% SDS (w/v) and 1µL of Proteinase K

(20mg/mL - Sigma) and allowing the sample to incubate at 55ºC, for 15min. To exclude proteins, the DNA template was subjected to column purification using *Wizard Plus SV Gel and PCR Clean-up System* (Promega).

VI.6.1.2 Probe synthesis

Anti-sense transcripts were produced by using 1µg of linearized plasmid DNA and 20U of the appropriate RNA polymerase in the presence of 30mM DTT, 1x DIG/FLUO-NTP mix (1mM ATP, CTG, GTP, 0.65mM UTP and 0.35mM DIG/FLUO-UTP), 40U RNasin (Promega) and 1x Transcription Buffer (Stratagene), in a final volume of 25µL. After incubating at 37ºC for 3hrs, the sample was precipitated by adding 20.5µL RNAse-free H_2O, 2µL of 0.5M EDTA (pH 8.0), 2.5µL of 8M LiCl and 150µL of ethanol, incubating at -20ºC for at least 1hr. After centrifugation, the RNA precipitate was washed with ethanol, resuspended in 100µL of 10mM EDTA and, after brief denaturation at 70ºC for 15min, checked by agarose gel electrophoresis.

Table VI.4: Constructs used as templates for *in vitro* transcription reactions

	Gene	Restriction enzyme	RNA polymerase	References
Chick Probes	*Delta1*	*NotI*	T3	(Henrique et al., 1995)
	hes5-3	*NotI*	T3	RIKEN EST B7
	Notch1	*BamHI*	T3	Clone12
Mouse Probes	*Dll1*	*NotI*	T7	
	Dll4	*BamHI*	T3	
	Hes5	*HindIII*	T3	
	Hes6	*EcoRI*	T3	
	Jagged1	*EcoRI*	T3	
	Math3	*PvuII*	T7	
	Math5	*AscI*	T7	Gift from N. Brown (Brown et al., 1998)
	NeuroD	*EcoRI*	T3	
	Ngn2	*NotI*	T7	

VI.6.2 *In situ* hybridization on cryostat sections

For *in situ* hybridization, embryos were fixed in 4% PFA/PBS at 4ºC, o/n and embedded as described above (see VI.4.6). Hybridization on cryostat sections was done as previously described (Myat et al., 1996) with modifications.

Double *in situ* hybridization on cryostat sections was done by combining DIG- and FLUO-labelled anti-sense RNA probes. The protocol was optimized during the course of this thesis. Briefly, sections were hybridized with DIG- and FLUO-labelled RNA probes, after which post-hybridization washes and blocking were carried out as described in (Myat et al., 1996). Because weaker probes yield better results with Fast Red development comparatively to Tyramide staining, sections were then incubated with the antibody that binds specifically to the weaker probe (anti-DIG-AP or anti-FLUO-AP, 1:2000 - Roche). After an o/n incubation at 4ºC, sections were washed with TBST and the staining reaction for AP (alkaline phosphatase) was performed using Fast Red (Roche). After the development of the first probe, sections were washed in PBS and incubated with the antibody that specifically recognizes the second probe (anti-FLUO-POD or anti-DIG-POD, 1:500 - Roche), for 1hr at r/t. After incubation with the POD-conjugated antibody, sections were washed 3 x 10min in TNT (0.1M Tris-HCl pH7.5, 0.15M NaCl, 0.05% Tween-20) and the second probe was developed by incubating the sections with Tyramide-FITC:amplification buffer (1:50, TSA^{TM}-Direct NEN^{TM} Kit - Perkin Elmer) for 10min, at r/t. Sections were washed 3 x 5min in TNT, counterstained with DAPI and mounted with Dako Fluorescence Mounting Medium.

VI.7 IMAGING

Bright field images were acquired using a Zeiss Axioplan microscope, equipped with a Hamamatsu C4742-95 camera. Immunostained sections were visualized using the fluorescence microscope Leica DMR, equipped with a Leica DC 350F digital camera. For confocal imaging, sections were analysed using the laser confocal microscope Zeiss LSM510. All images acquired were treated for noise reduction and colour adjustments with Adobe Photoshop.

VI.8 CONTROLS AND STATISTICAL ANALYSIS

Countings of the number of retinal progenitor cells (Chx10+) in E14.5 retinas were done from confocal images of the central region of the retina. Images were cropped to contain the entire width of the retina and Chx10 immunostained cells were counted with ImageJ software and normalized to the area analysed (cells/mm^2).

Differentiation of RGCs at E12.5, was determined by sectioning and immunostaining whole retinas for Brn3b. Total Brn3b+ cells were counted for each section, using ImageJ software, and normalized to the total number of DAPI+ cells.

Quantification of neuron subtypes in sections of neural tube was done by counting cells immunopositive for the described antibodies in images taken with a 40x objective on a Leica Leica DM5000B fluorescence microscope. In order to quantify the different neuronal types produced from the various spinal cord DV domains, at E11.5, cell type specific antibodies were used: pV3 (Nkx2.2), MNs (Islet1 localized in the ventral horn), V2 interneurons (Chx10), V1 interneurons (En1), V0 interneurons (Evx1), dI5 neurons (Brn3a+ cells ventral to dI4 Pax2+ cells), dI4 (dorsal Pax2+ cells), dI1-dI3 (Brn3a+ cells dorsal to dI4 Pax2+ cells).

A minimum of 3 embryos were counted (8 sections each minimum) for each phenotype (wild-type or mutants). Images from double labelling experiments in retina sections were taken with a confocal microscope (Zeiss LSM510 META). Statistical analysis of presented results was done using the Student's t test (paired).

VI.9 CONSTRUCTION OF A DLL1-ICRE TARGETING VECTOR

During the course of my work, I produced a targeting vector to be used in the generation of a *Dll1^{iCre}* transgenic mouse line. I describe below the cloning vectors used and the amplified PCR fragments and finally, present in Figure VI.5 the cloning strategy used for the production of this target vector.

VI.9.1 DNA plasmids

Several plasmids were used for the construction of the *Dll1^{iCre}* targeting vector. These plasmids either contained cDNA sequence encoding for iCre (iCre@TOPO) or a kanamycin selection cassette (Poly-frt-Kan-frt@pBlue-AscPac) and were kindly provided by Nicoletta Kessaris. The commercially available bacterial cloning vector pGEM T-Easy (Promega) were used for sub-cloning strategies.

VI.9.2 PCR amplification of homology arms for homologous recombination

The *Dll1*^{iCre} targeting vector was designed to recombine with the first exon of the *Dll1* gene, i.e, the gene of interest (*iCre*) will replaced the first exon of the *Dll1* gene, leaving the remaining introns and exons intact. This strategy is thought to minimize the deletion of possible enhancers and regulatory elements within the *Dll1* gene. Therefore, a 5' and 3' homology arm (HA) were amplified using a BAC clone (RP23-64I11) as a template (see oligonucleotides used in Table VI.5). *AscI* and *NcoI* were added as polylinkers to the 5' HA and *SpeI* and *PacI* to the 3' HA. The *NcoI* restriction site on the 3' end of the 5' HA contains the ATG of the gene.

Table VI.5: Oligonucleotides used for amplification of *Dll1* homology arms

	Oligonucleotides (5'→3')
5' homology arm	TAGGCGCGCCGCAGTCCTTGCCA GACCATGGTACCGCTGGACGCCC
3' homology arm	CATACTAGTGTGGAGGGAGGGGAGTC GCTTAATTAACGATGCCTGCGCCAT

PCR reactions were prepared in a final volume of 50µL (400µg template BAC plasmid, 1x PCR Buffer with 1.5mM $MgSO_4$, 0.2mM dNTPs, 5U PFU DNA polymerase – Stratagene - and 2mM of each primer). Amplification was performed with an initial heating at 94ºC for 2min, followed by 25 cycles at 94ºC,45sec for denaturation, 67ºC, 30sec for annealing and 72ºC, 1min for extension.

PCR products were subcloning into the pGEM T-Easy vector and sequence verified. Subsequent cloning steps were performed as indicated in Figure VI.5, B.

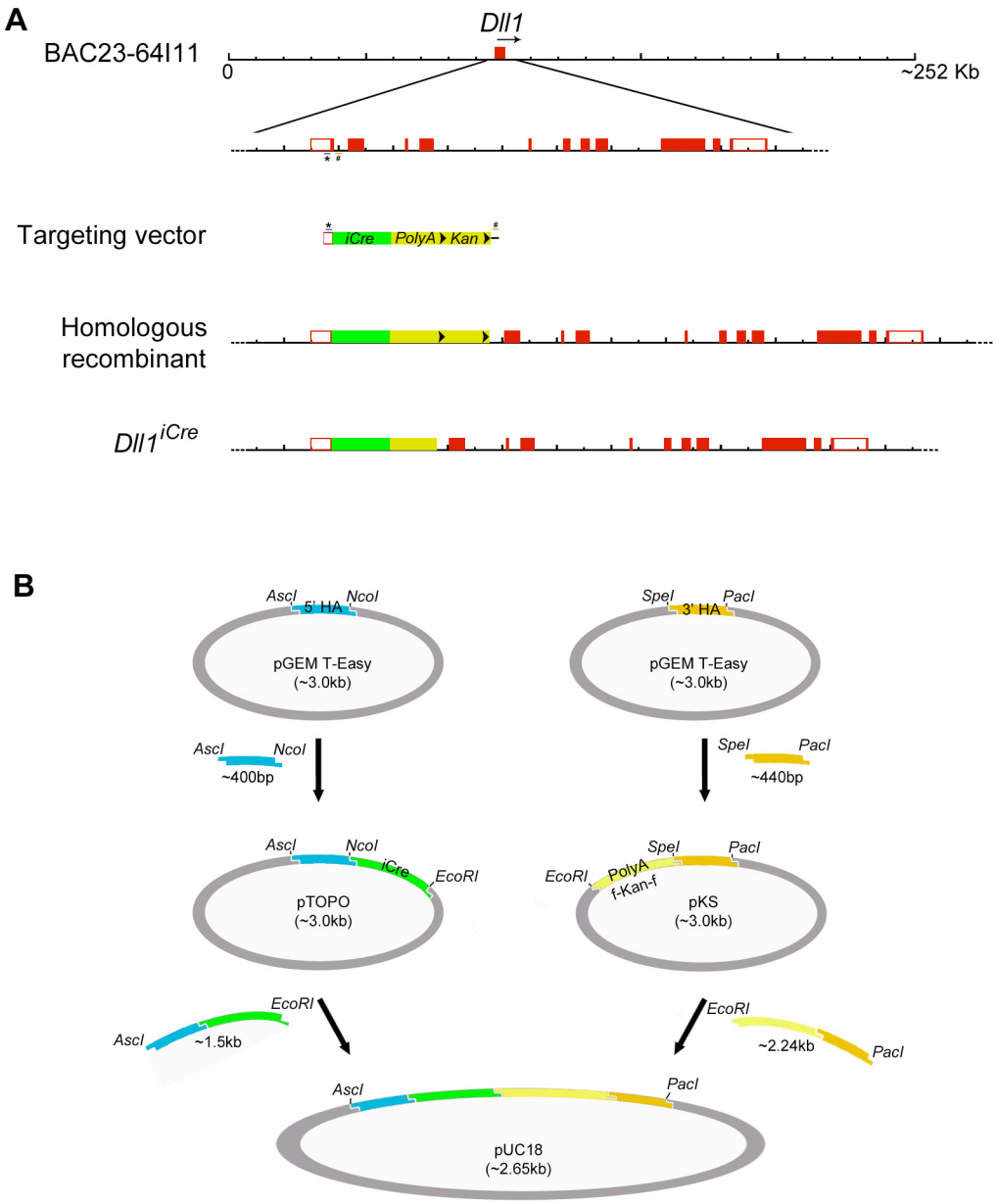


Figure VI.5: Cloning strategy of a targeting vector for the generation of a transgenic *Dll1*^{iCre} mouse line.

(A) Representation of the *Dll1*^{iCre} mouse line to be generated using BAC recombineering.

(B) Scheme of sub-cloning steps performed to generate the final targeting vector.

CHAPTER VII

REFERENCES

CHAPTER VII: REFERENCES

Aaku-Saraste, E., Hellwig, A. and Huttner, W. B. (1996). Loss of occludin and functional tight junctions, but not ZO-1, during neural tube closure--remodeling of the neuroepithelium prior to neurogenesis. *Dev Biol* **180**, 664-79.

Abe, K., Murakami, T., Matsubara, E., Manabe, Y., Nagano, I. and Shoji, M. (2002). Clinical Features of CADASIL. *Ann N Y Acad Sci* **977**, 266-72.

Abello, G., Khatri, S., Giraldez, F. and Alsina, B. (2007). Early regionalization of the otic placode and its regulation by the Notch signaling pathway. *Mech Dev* **124**, 631-45.

Adam, J., Myat, A., Le Roux, I., Eddison, M., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1998). Cell fate choices and the expression of Notch, Delta and Serrate homologues in the chick inner ear: parallels with Drosophila sense-organ development. *Development* **125**, 4645-54.

Ahmad, I., Acharya, H. R., Rogers, J. A., Shibata, A., Smithgall, T. E. and Dooley, C. M. (1998). The role of NeuroD as a differentiation factor in the mammalian retina. *J Mol Neurosci* **11**, 165-78.

Akazawa, C., Sasai, Y., Nakanishi, S. and Kageyama, R. (1992). Molecular characterization of a rat negative regulator with a basic helix-loop-helix structure predominantly expressed in the developing nervous system. *J Biol Chem* **267**, 21879-85.

Alvarez, F. J., Jonas, P. C., Sapir, T., Hartley, R., Berrocal, M. C., Geiman, E. J., Todd, A. J. and Goulding, M. (2005). Postnatal phenotype and localization of spinal cord V1 derived interneurons. *J Comp Neurol* **493**, 177-92.

Alvarez, V. A., Ridenour, D. A. and Sabatini, B. L. (2006). Retraction of synapses and dendritic spines induced by off-target effects of RNA interference. *J Neurosci* **26**, 7820-5.

Anderson, E. M., Birmingham, A., Baskerville, S., Reynolds, A., Maksimova, E., Leake, D., Fedorov, Y., Karpilow, J. and Khvorova, A. (2008). Experimental validation of the importance of seed complement frequency to siRNA specificity. *Rna* **14**, 853-61.

Anthony, T. E., Mason, H. A., Gridley, T., Fishell, G. and Heintz, N. (2005). Brain lipid-binding protein is a direct target of Notch signaling in radial glial cells. *Genes Dev* **19**, 1028-33.

Appel, B. and Eisen, J. S. (1998). Regulation of neuronal specification in the zebrafish spinal cord by Delta function. *Development* **125**, 371-80.

Appel, B., Givan, L. A. and Eisen, J. S. (2001). Delta-Notch signaling and lateral inhibition in zebrafish spinal cord development. *BMC Dev Biol* **1**, 13.

Arber, S., Han, B., Mendelsohn, M., Smith, M., Jessell, T. M. and Sockanathan, S. (1999). Requirement for the homeobox gene Hb9 in the consolidation of motor neuron identity. *Neuron* **23**, 659-74.

Artavanis-Tsakonas, S., Matsuno, K. and Fortini, M. E. (1995). Notch signaling. *Science* **268**, 225-32.

Artavanis-Tsakonas, S., Muskavitch, M. A. and Yedvobnick, B. (1983). Molecular cloning of Notch, a locus affecting neurogenesis in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **80**, 1977-81.

Ascano, J. M., Beverly, L. J. and Capobianco, A. J. (2003). The C-terminal PDZ-ligand of JAGGED1 is essential for cellular transformation. *J Biol Chem* **278**, 8771-9.

Axelrod, J. D., Matsuno, K., Artavanis-Tsakonas, S. and Perrimon, N. (1996). Interaction between Wingless and Notch signaling pathways mediated by dishevelled. *Science* **271**, 1826-32.

Baer, M., Nilsen, T. W., Costigan, C. and Altman, S. (1990). Structure and transcription of a human gene for H1 RNA, the RNA component of human RNase P. *Nucleic Acids Res* **18**, 97-103.

Baeriswyl, T. and Stoeckli, E. T. (2008). Axonin-1/TAG-1 is required for pathfinding of granule cell axons in the developing cerebellum. *Neural Develop* **3**, 7.

Bailey, A. M. and Posakony, J. W. (1995). Suppressor of hairless directly activates transcription of enhancer of split complex genes in response to Notch receptor activity. *Genes Dev* **9**, 2609-22.

Baker, N. E. and Yu, S. Y. (1997). Proneural function of neurogenic genes in the developing Drosophila eye. *Curr Biol* **7**, 122-32.

Baladron, V., Ruiz-Hidalgo, M. J., Nueda, M. L., Diaz-Guerra, M. J., Garcia-Ramirez, J. J., Bonvini, E., Gubina, E. and Laborda, J. (2005). dlk acts as a negative regulator of Notch1 activation through interactions with specific EGF-like repeats. *Exp Cell Res* **303**, 343-59.

Bancroft, M. and Bellairs, R. (1975). Differentiation of the neural plate and neural tube in the young chick embryo. A study by scanning and transmission electron microscopy. *Anat Embryol (Berl)* **147**, 309-35.

Bao, Z. Z. and Cepko, C. L. (1997). The expression and function of Notch pathway genes in the developing rat eye. *J Neurosci* **17**, 1425-34.

Bardin, A. J., Le Borgne, R. and Schweisguth, F. (2004). Asymmetric localization and function of cell-fate determinants: a fly's view. *Curr Opin Neurobiol* **14**, 6-14.

Bartel, D. P. (2004). MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* **116**, 281-97.

Baye, L. M. and Link, B. A. (2007). Interkinetic nuclear migration and the selection of neurogenic cell divisions during vertebrate retinogenesis. *J Neurosci* **27**, 10143-52.

Benedito, R. and Duarte, A. (2005). Expression of Dll4 during mouse embryogenesis suggests multiple developmental roles. *Gene Expr Patterns* **5**, 750-5.

Benedito, R., Roca, C., Sorensen, I., Adams, S., Gossler, A., Fruttiger, M. and Adams, R. H. (2009). The notch ligands Dll4 and Jagged1 have opposing effects on angiogenesis. *Cell* **137**, 1124-35.

Bentivoglio, M. and Mazzarello, P. (1999). The history of radial glia. *Brain Res Bull* **49**, 305-15.

Berdnik, D., Torok, T., Gonzalez-Gaitan, M. and Knoblich, J. A. (2002). The endocytic protein alpha-Adaptin is required for numb-mediated asymmetric cell division in Drosophila. *Dev Cell* **3**, 221-31.

Bernstein, E., Caudy, A. A., Hammond, S. M. and Hannon, G. J. (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**, 363-6.

Bertrand, E., Castanotto, D., Zhou, C., Carbonnelle, C., Lee, N. S., Good, P., Chatterjee, S., Grange, T., Pictet, R., Kohn, D. et al. (1997). The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *Rna* **3**, 75-88.

Besseyrias, V., Fiorini, E., Strobl, L. J., Zimber-Strobl, U., Dumortier, A., Koch, U., Arcangeli, M. L., Ezine, S., Macdonald, H. R. and Radtke, F. (2007). Hierarchy of Notch-Delta interactions promoting T cell lineage commitment and maturation. *J Exp Med* **204**, 331-43.

Blair, S. S. (2004). Developmental biology: Notching the hindbrain. *Curr Biol* **14**, R570-2.

Bland, C. E., Kimberly, P. and Rand, M. D. (2003). Notch-induced proteolysis and nuclear localization of the Delta ligand. *J Biol Chem* **278**, 13607-10.

Bourikas, D., Pekarik, V., Baeriswyl, T., Grunditz, A., Sadhu, R., Nardo, M. and Stoeckli, E. T. (2005). Sonic hedgehog guides commissural axons along the longitudinal axis of the spinal cord. *Nat Neurosci* **8**, 297-304.

Boutla, A., Delidakis, C., Livadaras, I., Tsagris, M. and Tabler, M. (2001). Short 5'-phosphorylated double-stranded RNAs induce RNA interference in Drosophila. *Curr Biol* **11**, 1776-80.

Brand, M. and Cabrera, C. V. (1988). Two groups of interrelated genes regulate early neurogenesis in *Drosophila melangaster*. *Roux's Arch. Dev. Biol.* **197**, 457-470.

Bray, S. (1998). Notch signalling in Drosophila: three ways to use a pathway. *Semin Cell Dev Biol* **9**, 591-7.

Bray, S. and Furriols, M. (2001). Notch pathway: making sense of suppressor of hairless. *Curr Biol* **11**, R217-21.

Bray, S. J. (2006). Notch signalling: a simple pathway becomes complex. *Nat Rev Mol Cell Biol* **7**, 678-89.

Bray, S. J., Takada, S., Harrison, E., Shen, S. C. and Ferguson-Smith, A. C. (2008). The atypical mammalian ligand Delta-like homologue 1 (Dlk1) can regulate Notch signalling in Drosophila. *BMC Dev Biol* **8**, 11.

Bridge, A. J., Pebernard, S., Ducraux, A., Nicoulaz, A. L. and Iggo, R. (2003). Induction of an interferon response by RNAi vectors in mammalian cells. *Nat Genet* **34**, 263-4.

Briscoe, J. and Ericson, J. (2001). Specification of neuronal fates in the ventral neural tube. *Curr Opin Neurobiol* **11**, 43-9.

Briscoe, J. and Novitch, B. G. (2008). Regulatory pathways linking progenitor patterning, cell fates and neurogenesis in the ventral neural tube. *Philos Trans R Soc Lond B Biol Sci* **363**, 57-70.

Bron, R., Vermeren, M., Kokot, N., Andrews, W., Little, G. E., Mitchell, K. J. and Cohen, J. (2007). Boundary cap cells constrain spinal motor neuron somal migration at motor exit points by a semaphorin-plexin mechanism. *Neural Develop* **2**, 21.

Bronner-Fraser, M. (1996). Manipulations of neural crest cells or their migratory pathways. *Methods Cell Biol* **51**, 61-79.

Brooker, R., Hozumi, K. and Lewis, J. (2006). Notch Ligands with Contrasting Functions: Jagged1 and Delta1 in the Mouse Inner Ear. *Development* **133**, 1277-1286.

Brou, C., Logeat, F., Gupta, N., Bessia, C., LeBail, O., Doedens, J. R., Cumano, A., Roux, P., Black, R. A. and Israel, A. (2000). A novel proteolytic cleavage involved in Notch signaling: the role of the disintegrin-metalloprotease TACE. *Mol Cell* **5**, 207-16.

Brown, N. L., Kanekar, S., Vetter, M. L., Tucker, P. K., Gemza, D. L. and Glaser, T. (1998). Math5 encodes a murine basic helix-loop-helix transcription factor expressed during early stages of retinal neurogenesis. *Development* **125**, 4821-33.

Brown, N. L., Patel, S., Brzezinski, J. and Glaser, T. (2001). Math5 is required for retinal ganglion cell and optic nerve formation. *Development* **128**, 2497-508.

Bruckner, K., Perez, L., Clausen, H. and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* **406**, 411-5.

Brummelkamp, T. R., Bernards, R. and Agami, R. (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**, 550-3.

Bulfone, A., Puelles, L., Porteus, M. H., Frohman, M. A., Martin, G. R. and Rubenstein, J. L. (1993). Spatially restricted expression of Dlx-1, Dlx-2 (Tes-1), Gbx-2, and Wnt-3 in the embryonic day 12.5 mouse forebrain defines potential transverse and longitudinal segmental boundaries. *J Neurosci* **13**, 3155-72.

Cabrera, C. V., Martinez-Arias, A. and Bate, M. (1987). The expression of three members of the achaete-scute gene complex correlates with neuroblast segregation in Drosophila. *Cell* **50**, 425-33.

Calvi, L. M., Adams, G. B., Weibrecht, K. W., Weber, J. M., Olson, D. P., Knight, M. C., Martin, R. P., Schipani, E., Divieti, P., Bringhurst, F. R. et al. (2003). Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature* **425**, 841-6.

Campos, L. S., Duarte, A. J., Branco, T. and Henrique, D. (2001). mDll1 and mDll3 expression in the developing mouse brain: role in the establishment of the early cortex. *J Neurosci Res* **64**, 590-8.

Canalis, E. (2008). Notch signaling in osteoblasts. *Sci Signal* **1**, pe17.

Caplen, N. J., Fleenor, J., Fire, A. and Morgan, R. A. (2000). dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. *Gene* **252**, 95-105.

Caplen, N. J., Parrish, S., Imani, F., Fire, A. and Morgan, R. A. (2001). Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci U S A* **98**, 9742-7.

Cappello, S., Attardo, A., Wu, X., Iwasato, T., Itohara, S., Wilsch-Brauninger, M., Eilken, H. M., Rieger, M. A., Schroeder, T. T., Huttner, W. B. et al. (2006). The Rho-GTPase cdc42 regulates neural progenitor fate at the apical surface. *Nat Neurosci* **9**, 1099-107.

Carney, R. S., Alfonso, T. B., Cohen, D., Dai, H., Nery, S., Stoica, B., Slotkin, J., Bregman, B. S., Fishell, G. and Corbin, J. G. (2006). Cell migration along the lateral cortical stream to the developing basal telencephalic limbic system. *J Neurosci* **26**, 11562-74.

Carter-Dawson, L. D. and LaVail, M. M. (1979). Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. *J Comp Neurol* **188**, 263-72.

Cau, E., Quillien, A. and Blader, P. (2008). Notch resolves mixed neural identities in the zebrafish epiphysis. *Development* **135**, 2391-401.

Chambers, C. B., Peng, Y., Nguyen, H., Gaiano, N., Fishell, G. and Nye, J. S. (2001). Spatiotemporal selectivity of response to Notch1 signals in mammalian forebrain precursors. *Development* **128**, 689-702.

Chauvet, S. and Rougon, G. (2008). Semaphorins deployed to repel cell migrants at spinal cord borders. *J Biol* **7**, 4.

Chen, C. M. and Cepko, C. L. (2000). Expression of Chx10 and Chx10-1 in the developing chicken retina. *Mech Dev* **90**, 293-7.

Chen, M. S., Obar, R. A., Schroeder, C. C., Austin, T. W., Poodry, C. A., Wadsworth, S. C. and Vallee, R. B. (1991). Multiple forms of dynamin are encoded by shibire, a Drosophila gene involved in endocytosis. *Nature* **351**, 583-6.

Chesnutt, C. and Niswander, L. (2004). Plasmid-based short-hairpin RNA interference in the chicken embryo. *Genesis* **39**, 73-8.

Chitnis, A., Henrique, D., Lewis, J., Ish-Horowicz, D. and Kintner, C. (1995). Primary neurogenesis in Xenopus embryos regulated by a homologue of the Drosophila neurogenic gene Delta. *Nature* **375**, 761-6.

Chuang, C. F. and Meyerowitz, E. M. (2000). Specific and heritable genetic interference by double-stranded RNA in Arabidopsis thaliana. *Proc Natl Acad Sci U S A* **97**, 4985-90.

Clemens, J. C., Worby, C. A., Simonson-Leff, N., Muda, M., Maehama, T., Hemmings, B. A. and Dixon, J. E. (2000). Use of double-stranded RNA interference in Drosophila cell lines to dissect signal transduction pathways. *Proc Natl Acad Sci U S A* **97**, 6499-503.

Cornell, R. A. and Eisen, J. S. (2000). Delta signaling mediates segregation of neural crest and spinal sensory neurons from zebrafish lateral neural plate. *Development* **127**, 2873-82.

Costa, R. M., Honjo, T. and Silva, A. J. (2003). Learning and memory deficits in Notch mutant mice. *Curr Biol* **13**, 1348-54.

Cotter, D. R., Honavar, M. and Everall, I. (1999). Focal cortical dysplasia: a neuropathological and developmental perspective. *Epilepsy Res* **36**, 155-64.

Crosnier, C., Stamataki, D. and Lewis, J. (2006). Organizing cell renewal in the intestine: stem cells, signals and combinatorial control. *Nat Rev Genet* **7**, 349-59.

Cui, S., Otten, C., Rohr, S., Abdelilah-Seyfried, S. and Link, B. A. (2007). Analysis of aPKClambda and aPKCzeta reveals multiple and redundant functions during vertebrate retinogenesis. *Mol Cell Neurosci* **34**, 431-44.

Dai, F., Yusuf, F., Farjah, G. H. and Brand-Saberi, B. (2005). RNAi-induced targeted silencing of developmental control genes during chicken embryogenesis. *Dev Biol* **285**, 80-90.

Das, R. M., Van Hateren, N. J., Howell, G. R., Farrell, E. R., Bangs, F. K., Porteous, V. C., Manning, E. M., McGrew, M. J., Ohyama, K., Sacco, M. A. et al. (2006). A robust system for RNA interference in the chicken using a modified microRNA operon. *Dev Biol* **294**, 554-63.

Daudet, N. and Lewis, J. (2005). Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. *Development* **132**, 541-51.

Davidson, E. H. (1976). Gene activity in early development. New York: Academic Press.

Davis, R. L. and Turner, D. L. (2001). Vertebrate hairy and Enhancer of split related proteins: transcriptional repressors regulating cellular differentiation and embryonic patterning. *Oncogene* **20**, 8342-57.

Dawson, S. R., Turner, D. L., Weintraub, H. and Parkhurst, S. M. (1995). Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. *Mol Cell Biol* **15**, 6923-31.

De Bellard, M. E., Ching, W., Gossler, A. and Bronner-Fraser, M. (2002). Disruption of segmental neural crest migration and ephrin expression in delta-1 null mice. *Dev Biol* **249**, 121-30.

de Celis, J. F. and Bray, S. (1997). Feed-back mechanisms affecting Notch activation at the dorsoventral boundary in the Drosophila wing. *Development* **124**, 3241-51.

de Celis, J. F., Bray, S. and Garcia-Bellido, A. (1997). Notch signalling regulates veinlet expression and establishes boundaries between veins and interveins in the Drosophila wing. *Development* **124**, 1919-28.

de Celis, J. F., Marí-Beffa, M. and Garcia-Bellido, A. (1991). Function of *trans*-acting genes of the *achaete-scute* complex in sensory organ patterning in the mesonotum of *Drosophila*. *Roux's Arch. Dev. Biol.* **200**, 64-76.

de la Pompa, J. L., Wakeham, A., Correia, K. M., Samper, E., Brown, S., Aguilera, R. J., Nakano, T., Honjo, T., Mak, T. W., Rossant, J. et al. (1997). Conservation of the Notch signalling pathway in mammalian neurogenesis. *Development* **124**, 1139-48.

De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J. et al. (1999). A presenilin-1-dependent gamma-secretase-like protease mediates release of Notch intracellular domain. *Nature* **398**, 518-22.

de-la-Concha, A., Dietrich, U., Weigel, D. and Campos-Ortega, J. A. (1988). Functional Interactions of Neurogenic Genes of Drosophila Melanogaster. *Genetics* **118**, 499-508.

Deblandre, G. A., Lai, E. C. and Kintner, C. (2001). Xenopus neuralized is a ubiquitin ligase that interacts with XDelta1 and regulates Notch signaling. *Dev Cell* **1**, 795-806.

Del Barrio, M. G., Taveira-Marques, R., Muroyama, Y., Yuk, D. I., Li, S., Wines-Samuelson, M., Shen, J., Smith, H. K., Xiang, M., Rowitch, D. et al. (2007). A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool. *Development* **134**, 3427-36.

Del Bene, F., Wehman, A. M., Link, B. A. and Baier, H. (2008). Regulation of neurogenesis by interkinetic nuclear migration through an apical-basal notch gradient. *Cell* **134**, 1055-65.

Dietrich, U. and Campos-Ortega, J. A. (1984). The expression of neurogenic loci in imaginal epidermal cells of Drosophila melanogaster. *J Neurogenet* **1**, 315-32.

Doe, C. Q. and Goodman, C. S. (1985a). Early events in insect neurogenesis. I. Development and segmental differences in the pattern of neuronal precursor cells. *Dev Biol* **111**, 193-205.

Doe, C. Q. and Goodman, C. S. (1985b). Early events in insect neurogenesis. II. The role of cell interactions and cell lineage in the determination of neuronal precursor cells. *Dev Biol* **111**, 206-19.

Domitrovich, A. M. and Kunkel, G. R. (2003). Multiple, dispersed human U6 small nuclear RNA genes with varied transcriptional efficiencies. *Nucleic Acids Res* **31**, 2344-52.

Dorsky, R. I., Chang, W. S., Rapaport, D. H. and Harris, W. A. (1997). Regulation of neuronal diversity in the Xenopus retina by Delta signalling. *Nature* **385**, 67-70.

Dorsky, R. I., Rapaport, D. H. and Harris, W. A. (1995). Xotch inhibits cell differentiation in the Xenopus retina. *Neuron* **14**, 487-96.

Dotto, G. P. (2008). Notch tumor suppressor function. *Oncogene* **27**, 5115-23.

Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D. and Rossant, J. (2004). Dosage-sensitive requirement for mouse Dll4 in artery development. *Genes Dev* **18**, 2474-8.

Dunwoodie, S. L., Clements, M., Sparrow, D. B., Sa, X., Conlon, R. A. and Beddington, R. S. (2002). Axial skeletal defects caused by mutation in the spondylocostal dysplasia/pudgy gene Dll3 are associated with disruption of the segmentation clock within the presomitic mesoderm. *Development* **129**, 1795-806.

Dunwoodie, S. L., Henrique, D., Harrison, S. M. and Beddington, R. S. (1997). Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. *Development* **124**, 3065-76.

Dye, C. A., Lee, J. K., Atkinson, R. C., Brewster, R., Han, P. L. and Bellen, H. J. (1998). The Drosophila sanpodo gene controls sibling cell fate and encodes a tropomodulin homolog, an actin/tropomyosin-associated protein. *Development* **125**, 1845-1856.

Edenfeld, G., Altenhein, B., Zierau, A., Cleppien, D., Krukkert, K., Technau, G. and Klambt, C. (2007). Notch and Numb are required for normal migration of peripheral glia in Drosophila. *Dev Biol* **301**, 27-37.

Eiraku, M., Hirata, Y., Takeshima, H., Hirano, T. and Kengaku, M. (2002). Delta/notch-like epidermal growth factor (EGF)-related receptor, a novel EGF-like repeat-containing protein targeted to dendrites of developing and adult central nervous system neurons. *J Biol Chem* **277**, 25400-7.

Eiraku, M., Tohgo, A., Ono, K., Kaneko, M., Fujishima, K., Hirano, T. and Kengaku, M. (2005). DNER acts as a neuron-specific Notch ligand during Bergmann glial development. *Nat Neurosci* **8**, 873-80.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**, 494-8.

Elbashir, S. M., Lendeckel, W. and Tuschl, T. (2001b). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15**, 188-200.

Emery, G., Hutterer, A., Berdnik, D., Mayer, B., Wirtz-Peitz, F., Gaitan, M. G. and Knoblich, J. A. (2005). Asymmetric Rab 11 endosomes regulate delta recycling and specify cell fate in the Drosophila nervous system. *Cell* **122**, 763-73.

Ericson, J., Rashbass, P., Schedl, A., Brenner-Morton, S., Kawakami, A., van Heyningen, V., Jessell, T. M. and Briscoe, J. (1997). Pax6 controls progenitor cell identity and neuronal fate in response to graded Shh signaling. *Cell* **90**, 169-80.

Ericson, J., Thor, S., Edlund, T., Jessell, T. M. and Yamada, T. (1992). Early stages of motor neuron differentiation revealed by expression of homeobox gene Islet-1. *Science* **256**, 1555-60.

Estrach, S., Ambler, C. A., Lo Celso, C., Hozumi, K. and Watt, F. M. (2006). Jagged 1 is a beta-catenin target gene required for ectopic hair follicle formation in adult epidermis. *Development* **133**, 4427-38.

Fairen, A., Cobas, A. and Fonseca, M. (1986). Times of generation of glutamic acid decarboxylase immunoreactive neurons in mouse somatosensory cortex. *J Comp Neurol* **251**, 67-83.

Fehon, R. G., Kooh, P. J., Rebay, I., Regan, C. L., Xu, T., Muskavitch, M. A. and Artavanis-Tsakonas, S. (1990). Molecular interactions between the protein products of the neurogenic loci Notch and Delta, two EGF-homologous genes in Drosophila. *Cell* **61**, 523-34.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-11.

Fitzgerald, K. and Greenwald, I. (1995). Interchangeability of Caenorhabditis elegans DSL proteins and intrinsic signalling activity of their extracellular domains in vivo. *Development* **121**, 4275-82.

Fleming, R. J. (1998). Structural conservation of Notch receptors and ligands. *Semin Cell Dev Biol* **9**, 599-607.

Fleming, R. J., Gu, Y. and Hukriede, N. A. (1997). Serrate-mediated activation of Notch is specifically blocked by the product of the gene fringe in the dorsal compartment of the Drosophila wing imaginal disc. *Development* **124**, 2973-81.

Fogarty, M., Grist, M., Gelman, D., Marin, O., Pachnis, V. and Kessaris, N. (2007). Spatial genetic patterning of the embryonic neuroepithelium generates GABAergic interneuron diversity in the adult cortex. *J Neurosci* **27**, 10935-46.

Fogarty, M., Richardson, W. D. and Kessaris, N. (2005). A subset of oligodendrocytes generated from radial glia in the dorsal spinal cord. *Development* **132**, 1951-9.

Fortini, M. E. and Artavanis-Tsakonas, S. (1994). The suppressor of hairless protein participates in notch receptor signaling. *Cell* **79**, 273-82.

Franklin, J. L., Berechid, B. E., Cutting, F. B., Presente, A., Chambers, C. B., Foltz, D. R., Ferreira, A. and Nye, J. S. (1999). Autonomous and non-autonomous regulation of mammalian neurite development by Notch1 and Delta1. *Curr Biol* **9**, 1448-57.

Fraser, A. G., Kamath, R. S., Zipperlen, P., Martinez-Campos, M., Sohrmann, M. and Ahringer, J. (2000). Functional genomic analysis of C. elegans chromosome I by systematic RNA interference. *Nature* **408**, 325-30.

Freier, S. M., Kierzek, R., Jaeger, J. A., Sugimoto, N., Caruthers, M. H., Neilson, T. and Turner, D. H. (1986). Improved free-energy parameters for predictions of RNA duplex stability. *Proc Natl Acad Sci U S A* **83**, 9373-7.

Furukawa, T., Mukherjee, S., Bao, Z. Z., Morrow, E. M. and Cepko, C. L. (2000). rax, Hes1, and notch1 promote the formation of Muller glia by postnatal retinal progenitor cells. *Neuron* **26**, 383-94.

Fuss, B., Josten, F., Feix, M. and Hoch, M. (2004). Cell movements controlled by the Notch signalling cascade during foregut development in Drosophila. *Development* **131**, 1587-95.

Gaiano, N. and Fishell, G. (2002). The role of notch in promoting glial and neural stem cell fates. *Annu Rev Neurosci* **25**, 471-90.

Gaiano, N., Nye, J. S. and Fishell, G. (2000). Radial glial identity is promoted by Notch1 signaling in the murine forebrain. *Neuron* **26**, 395-404.

Garcia-Bellido, A. (1979). Genetic Analysis of the Achaete-Scute System of DROSOPHILA MELANOGASTER. *Genetics* **91**, 491-520.

Garcia-Bellido, A. and Santamaria, P. (1978). Developmental Analysis of the Achaete-Scute System of DROSOPHILA MELANOGASTER. *Genetics* **88**, 469-486.

Geffers, I., Serth, K., Chapman, G., Jaekel, R., Schuster-Gossler, K., Cordes, R., Sparrow, D. B., Kremmer, E., Dunwoodie, S. L., Klein, T. et al. (2007). Divergent functions and distinct localization of the Notch ligands DLL1 and DLL3 in vivo. *J Cell Biol* **178**, 465-76.

Genoud, S., Lappe-Siefke, C., Goebbels, S., Radtke, F., Aguet, M., Scherer, S. S., Suter, U., Nave, K. A. and Mantei, N. (2002). Notch1 control of oligodendrocyte differentiation in the spinal cord. *J Cell Biol* **158**, 709-18.

Ghysen, A. and Dambly-Chaudiere, C. (1988). From DNA to form: the achaete-scute complex. *Genes Dev* **2**, 495-501.

Gilbert, S. F. (2006). Developmental Biology: Palgrave Macmillan, Sinauer Associates.

Givogri, M. I., de Planell, M., Galbiati, F., Superchi, D., Gritti, A., Vescovi, A., de Vellis, J. and Bongarzone, E. R. (2006). Notch signaling in astrocytes and neuroblasts of the adult subventricular zone in health and after cortical injury. *Dev Neurosci* **28**, 81-91.

Golson, M. L., Le Lay, J., Gao, N., Bramswig, N., Loomes, K. M., Oakey, R., May, C. L., White, P. and Kaestner, K. H. (2009). Jagged1 is a acompetitive inhibitor of Notch signaling in the embryonic pancreas. *Mech Dev*.

Good, P. D., Krikos, A. J., Li, S. X., Bertrand, E., Lee, N. S., Giver, L., Ellington, A., Zaia, J. A., Rossi, J. J. and Engelke, D. R. (1997). Expression of small, therapeutic RNAs in human cell nuclei. *Gene Ther* **4**, 45-54.

Grandbarbe, L., Bouissac, J., Rand, M., Hrabe de Angelis, M., Artavanis-Tsakonas, S. and Mohier, E. (2003). Delta-Notch signaling controls the generation of neurons/glia from neural stem cells in a stepwise process. *Development* **130**, 1391-402.

Grbavec, D. and Stifani, S. (1996). Molecular interaction between TLE1 and the carboxyl-terminal domain of HES-1 containing the WRPW motif. *Biochem Biophys Res Commun* **223**, 701-5.

Greenwald, I. (1994). Structure/function studies of lin-12/Notch proteins. *Curr Opin Genet Dev* **4**, 556-62.

Greenwald, I. and Seydoux, G. (1990). Analysis of gain-of-function mutations of the lin-12 gene of Caenorhabditis elegans. *Nature* **346**, 197-9.

Gregory, R. I. and Shiekhattar, R. (2005). MicroRNA biogenesis and cancer. *Cancer Res* **65**, 3509-12.

Gross, M. K., Dottori, M. and Goulding, M. (2002). Lbx1 specifies somatosensory association interneurons in the dorsal spinal cord. *Neuron* **34**, 535-49.

Gu, Y., Hukriede, N. A. and Fleming, R. J. (1995). Serrate expression can functionally replace Delta activity during neuroblast segregation in the Drosophila embryo. *Development* **121**, 855-65.

Guo, M., Jan, L. Y. and Jan, Y. N. (1996). Control of daughter cell fates during asymmetric division: interaction of Numb and Notch. *Neuron* **17**, 27-41.

Ha, I., Wightman, B. and Ruvkun, G. (1996). A bulged lin-4/lin-14 RNA duplex is sufficient for Caenorhabditis elegans lin-14 temporal gradient formation. *Genes Dev* **10**, 3041-50.

Haddon, C., Jiang, Y. J., Smithers, L. and Lewis, J. (1998a). Delta-Notch signalling and the patterning of sensory cell differentiation in the zebrafish ear: evidence from the mind bomb mutant. *Development* **125**, 4637-44.

Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D. and Lewis, J. (1998b). Multiple delta genes and lateral inhibition in zebrafish primary neurogenesis. *Development* **125**, 359-70.

Hamburger, V. and Hamilton, H. L. (1951). A series of normal stages in the development of the chick embryo. 1951. *Journal of Morphology* **88**, 49-92.

Hammond, S. M., Bernstein, E., Beach, D. and Hannon, G. J. (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404**, 293-6.

Han, H., Tanigaki, K., Yamamoto, N., Kuroda, K., Yoshimoto, M., Nakahata, T., Ikuta, K. and Honjo, T. (2002). Inducible gene knockout of transcription factor recombination signal binding protein-J reveals its essential role in T versus B lineage decision. *Int Immunol* **14**, 637-45.

Handler, M., Yang, X. and Shen, J. (2000). Presenilin-1 regulates neuronal differentiation during neurogenesis. *Development* **127**, 2593-606.

Harborth, J., Elbashir, S. M., Bechert, K., Tuschl, T. and Weber, K. (2001). Identification of essential genes in cultured mammalian cells using small interfering RNAs. *J Cell Sci* **114**, 4557-65.

Hartenstein, V. and Posakony, J. W. (1989). Development of adult sensilla on the wing and notum of Drosophila melanogaster. *Development* **107**, 389-405.

Hartley, D. A., Preiss, A. and Artavanis-Tsakonas, S. (1988). A deduced gene product from the Drosophila neurogenic locus, enhancer of split, shows homology to mammalian G-protein beta subunit. *Cell* **55**, 785-95.

Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F. and Kageyama, R. (2004). Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. *Development* **131**, 5539-50.

Hatakeyama, J., Tomita, K., Inoue, T. and Kageyama, R. (2001). Roles of homeobox and bHLH genes in specification of a retinal cell type. *Development* **128**, 1313-22.

Hebert, J. M., Lin, M., Partanen, J., Rossant, J. and McConnell, S. K. (2003). FGF signaling through FGFR1 is required for olfactory bulb morphogenesis. *Development* **130**, 1101-11.

Hebert, J. M. and McConnell, S. K. (2000). Targeting of cre to the Foxg1 (BF-1) locus mediates loxP recombination in the telencephalon and other developing head structures. *Dev Biol* **222**, 296-306.

Heitzler, P. and Simpson, P. (1991). The choice of cell fate in the epidermis of Drosophila. *Cell* **64**, 1083-92.

Heitzler, P. and Simpson, P. (1993). Altered epidermal growth factor-like sequences provide evidence for a role of Notch as a receptor in cell fate decisions. *Development* **117**, 1113-23.

Henderson, S. T., Gao, D., Christensen, S. and Kimble, J. (1997). Functional domains of LAG-2, a putative signaling ligand for LIN-12 and GLP-1 receptors in Caenorhabditis elegans. *Mol Biol Cell* **8**, 1751-62.

Henrique, D., Adam, J., Myat, A., Chitnis, A., Lewis, J. and Ish-Horowicz, D. (1995). Expression of a Delta homologue in prospective neurons in the chick. *Nature* **375**, 787-90.

Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D. and Lewis, J. (1997). Maintenance of neuroepithelial progenitor cells by Delta-Notch signalling in the embryonic chick retina. *Curr Biol* **7**, 661-70.

Hernandez, V. H. and Bueno, D. (2005). RNA interference is ineffective as a routine method for gene silencing in chick embryos as monitored by fgf8 silencing. *Int J Biol Sci* **1**, 1-12.

Herranz, H. and Milan, M. (2006). Notch and affinity boundaries in Drosophila. *Bioessays* **28**, 113-6.

Hicks, C., Johnston, S. H., diSibio, G., Collazo, A., Vogt, T. F. and Weinmaster, G. (2000). Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2. *Nat Cell Biol* **2**, 515-20.

Hicks, C., Ladi, E., Lindsell, C., Hsieh, J. J., Hayward, S. D., Collazo, A. and Weinmaster, G. (2002). A secreted Delta1-Fc fusion protein functions both as an activator and inhibitor of Notch1 signaling. *J Neurosci Res* **68**, 655-67.

Hinz, U., Giebel, B. and Campos-Ortega, J. A. (1994). The basic-helix-loop-helix domain of Drosophila lethal of scute protein is sufficient for proneural function and activates neurogenic genes. *Cell* **76**, 77-87.

Hojo, M., Ohtsuka, T., Hashimoto, N., Gradwohl, G., Guillemot, F. and Kageyama, R. (2000). Glial cell fate specification modulated by the bHLH gene Hes5 in mouse retina. *Development* **127**, 2515-22.

Hollyday, M. (2001). Neurogenesis in the vertebrate neural tube. *Int J Dev Neurosci* **19**, 161-73.

Honjo, T. (1996). The shortest path from the surface to the nucleus: RBP-J kappa/Su(H) transcription factor. *Genes Cells* **1**, 1-9.

Hori, K., Fostier, M., Ito, M., Fuwa, T. J., Go, M. J., Okano, H., Baron, M. and Matsuno, K. (2004). Drosophila deltex mediates suppressor of Hairless-independent and late-endosomal activation of Notch signaling. *Development* **131**, 5527-37.

Hozumi, K., Mailhos, C., Negishi, N., Hirano, K., Yahata, T., Ando, K., Zuklys, S., Hollander, G. A., Shima, D. T. and Habu, S. (2008). Delta-like 4 is indispensable in thymic environment specific for T cell development. *J Exp Med* **205**, 2507-13.

Hozumi, K., Negishi, N., Suzuki, D., Abe, N., Sotomaru, Y., Tamaoki, N., Mailhos, C., Ish-Horowicz, D., Habu, S. and Owen, M. J. (2004). Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. *Nat Immunol* **5**, 638-44.

Hrabe de Angelis, M., McIntyre, J., 2nd and Gossler, A. (1997). Maintenance of somite borders in mice requires the Delta homologue DII1. *Nature* **386**, 717-21.

Hukriede, N. A. and Fleming, R. J. (1997). Beaded of Goldschmidt, an antimorphic allele of Serrate, encodes a protein lacking transmembrane and intracellular domains. *Genetics* **145**, 359-74.

Huppert, S. S., Jacobsen, T. L. and Muskavitch, M. A. (1997). Feedback regulation is central to Delta-Notch signalling required for Drosophila wing vein morphogenesis. *Development* **124**, 3283-91.

Hutterer, A. and Knoblich, J. A. (2005). Numb and alpha-Adaptin regulate Sanpodo endocytosis to specify cell fate in Drosophila external sensory organs. *EMBO Rep* **6**, 836-42.

Ignatova, T. N., Kukekov, V. G., Laywell, E. D., Suslov, O. N., Vrionis, F. D. and Steindler, D. A. (2002). Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* **39**, 193-206.

Imai, F., Hirai, S., Akimoto, K., Koyama, H., Miyata, T., Ogawa, M., Noguchi, S., Sasaoka, T., Noda, T. and Ohno, S. (2006). Inactivation of aPKClambda results in the loss of adherens junctions in neuroepithelial cells without affecting neurogenesis in mouse neocortex. *Development* **133**, 1735-44.

Inoue, H., Nojima, H. and Okayama, H. (1990). High efficiency transformation of Escherichia coli with plasmids. *Gene* **96**, 23-8.

Inoue, T., Hojo, M., Bessho, Y., Tano, Y., Lee, J. E. and Kageyama, R. (2002). Math3 and NeuroD regulate amacrine cell fate specification in the retina. *Development* **129**, 831-42.

Irvin, D. K., Nakano, I., Paucar, A. and Kornblum, H. I. (2004). Patterns of Jagged1, Jagged2, Delta-like 1 and Delta-like 3 expression during late embryonic and postnatal brain development suggest multiple functional roles in progenitors and differentiated cells. *J Neurosci Res* **75**, 330-43.

Irvin, D. K., Zurcher, S. D., Nguyen, T., Weinmaster, G. and Kornblum, H. I. (2001). Expression patterns of Notch1, Notch2, and Notch3 suggest multiple functional roles for the Notch-DSL signaling system during brain development. *J Comp Neurol* **436**, 167-81.

Irvine, K. D. and Vogt, T. F. (1997). Dorsal-ventral signaling in limb development. *Curr Opin Cell Biol* **9**, 867-76.

Ishii, Y., Nakamura, S. and Osumi, N. (2000). Demarcation of early mammalian cortical development by differential expression of fringe genes. *Brain Res Dev Brain Res* **119**, 307-20.

Izant, J. G. and Weintraub, H. (1984). Inhibition of thymidine kinase gene expression by anti-sense RNA: a molecular approach to genetic analysis. *Cell* **36**, 1007-15.

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G. and Linsley, P. S. (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* **21**, 635-7.

Jadhav, A. P., Cho, S. H. and Cepko, C. L. (2006a). Notch activity permits retinal cells to progress through multiple progenitor states and acquire a stem cell property. *Proc Natl Acad Sci U S A* **103**, 18998-9003.

Jadhav, A. P., Mason, H. A. and Cepko, C. L. (2006b). Notch 1 inhibits photoreceptor production in the developing mammalian retina. *Development* **133**, 913-23.

Jafar-Nejad, H., Andrews, H. K., Acar, M., Bayat, V., Wirtz-Peitz, F., Mehta, S. Q., Knoblich, J. A. and Bellen, H. J. (2005). Sec15, a component of the exocyst, promotes notch signaling during the asymmetric division of Drosophila sensory organ precursors. *Dev Cell* **9**, 351-63.

Jaleco, A. C., Neves, H., Hooijberg, E., Gameiro, P., Clode, N., Haury, M., Henrique, D. and Parreira, L. (2001). Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. *J Exp Med* **194**, 991-1002.

Jarriault, S., Brou, C., Logeat, F., Schroeter, E. H., Kopan, R. and Israel, A. (1995). Signalling downstream of activated mammalian Notch. *Nature* **377**, 355-8.

Jennings, B. H., Tyler, D. M. and Bray, S. J. (1999). Target specificities of Drosophila enhancer of split basic helix-loop-helix proteins. *Mol Cell Biol* **19**, 4600-10.

Jessell, T. M. (2000). Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat Rev Genet* **1**, 20-9.

Jiang, R., Lan, Y., Chapman, H. D., Shawber, C., Norton, C. R., Serreze, D. V., Weinmaster, G. and Gridley, T. (1998). Defects in limb, craniofacial, and thymic development in Jagged2 mutant mice. *Genes Dev* **12**, 1046-57.

Jiang, Y. J., Brand, M., Heisenberg, C. P., Beuchle, D., Furutani-Seiki, M., Kelsh, R. N., Warga, R. M., Granato, M., Haffter, P., Hammerschmidt, M. et al. (1996). Mutations affecting neurogenesis and brain morphology in the zebrafish, Danio rerio. *Development* **123**, 205-16.

Jimenez, F. and Campos-Ortega, J. A. (1990). Defective neuroblast commitment in mutants of the achaete-scute complex and adjacent genes of D. melanogaster. *Neuron* **5**, 81-9.

Jurgens, G., Wieschaus, E., Nusslein-Volhard, C. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. II. Zygotic loci on the third chromosome. *Roux's Arch. Dev. Biol.* **193**, 283-295.

Kao, H. Y., Ordentlich, P., Koyano-Nakagawa, N., Tang, Z., Downes, M., Kintner, C. R., Evans, R. M. and Kadesch, T. (1998). A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev* **12**, 2269-77.

Karunaratne, A., Hargrave, M., Poh, A. and Yamada, T. (2002). GATA proteins identify a novel ventral interneuron subclass in the developing chick spinal cord. *Dev Biol* **249**, 30-43.

Katahira, T. and Nakamura, H. (2003). Gene silencing in chick embryos with a vector-based small interfering RNA system. *Dev Growth Differ* **45**, 361-7.

Kawakami, Y., Rodriguez-Leon, J., Koth, C. M., Buscher, D., Itoh, T., Raya, A., Ng, J. K., Esteban, C. R., Takahashi, S., Henrique, D. et al. (2003). MKP3 mediates the cellular response to FGF8 signalling in the vertebrate limb. *Nat Cell Biol* **5**, 513-9.

Kessaris, N., Pringle, N. and Richardson, W. D. (2001). Ventral neurogenesis and the neuron-glial switch. *Neuron* **31**, 677-80.

Khvorova, A., Reynolds, A. and Jayasena, S. D. (2003). Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**, 209-16.

Kidd, S. and Lieber, T. (2002). Furin cleavage is not a requirement for Drosophila Notch function. *Mech Dev* **115**, 41-51.

Kiernan, A. E., Cordes, R., Kopan, R., Gossler, A. and Gridley, T. (2005). The Notch ligands DLL1 and JAG2 act synergistically to regulate hair cell development in the mammalian inner ear. *Development* **132**, 4353-62.

Kiernan, A. E., Xu, J. and Gridley, T. (2006). The Notch Ligand JAG1 Is Required for Sensory Progenitor Development in the Mammalian Inner Ear. *PLoS Genet* **2**, e4.

Kimble, J. (1981). Alterations in cell lineage following laser ablation of cells in the somatic gonad of Caenorhabditis elegans. *Dev Biol* **87**, 286-300.

Kimble, J. and Hirsh, D. (1979). The postembryonic cell lineages of the hermaphrodite and male gonads in Caenorhabditis elegans. *Dev Biol* **70**, 396-417.

Kimura, Y., Satou, C. and Higashijima, S. I. (2008). V2a and V2b neurons are generated by the final divisions of pair-producing progenitors in the zebrafish spinal cord. *Development*.

Klein, R. (2004). Eph/ephrin signaling in morphogenesis, neural development and plasticity. *Curr Opin Cell Biol* **16**, 580-9.

Klueg, K. M., Parody, T. R. and Muskavitch, M. A. (1998). Complex proteolytic processing acts on Delta, a transmembrane ligand for Notch, during Drosophila development. *Mol Biol Cell* **9**, 1709-23.

Knust, E., Schrons, H., Grawe, F. and Campos-Ortega, J. A. (1992). Seven genes of the Enhancer of split complex of Drosophila melanogaster encode helix-loop-helix proteins. *Genetics* **132**, 505-18.

Koch, U., Fiorini, E., Benedito, R., Besseyrias, V., Schuster-Gossler, K., Pierres, M., Manley, N. R., Duarte, A., Macdonald, H. R. and Radtke, F. (2008). Delta-like 4 is the essential, nonredundant ligand for Notch1 during thymic T cell lineage commitment. *J Exp Med*.

Kopan, R., Nye, J. S. and Weintraub, H. (1994). The intracellular domain of mouse Notch: a constitutively activated repressor of myogenesis directed at the basic helix-loop-helix region of MyoD. *Development* **120**, 2385-96.

Koyano-Nakagawa, N., Kim, J., Anderson, D. and Kintner, C. (2000). Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. *Development* **127**, 4203-16.

Kramer, H. and Phistry, M. (1996). Mutations in the Drosophila hook gene inhibit endocytosis of the boss transmembrane ligand into multivesicular bodies. *J Cell Biol* **133**, 1205-15.

Kramer, H. and Phistry, M. (1999). Genetic analysis of hook, a gene required for endocytic trafficking in drosophila. *Genetics* **151**, 675-84.

Krebs, L. T., Xue, Y., Norton, C. R., Sundberg, J. P., Beatus, P., Lendahl, U., Joutel, A. and Gridley, T. (2003). Characterization of Notch3-deficient mice: normal embryonic development and absence of genetic interactions with a Notch1 mutation. *Genesis* **37**, 139-43.

Kubo, F., Takeichi, M. and Nakagawa, S. (2003). Wnt2b controls retinal cell differentiation at the ciliary marginal zone. *Development* **130**, 587-98.

Kubo, F., Takeichi, M. and Nakagawa, S. (2005). Wnt2b inhibits differentiation of retinal progenitor cells in the absence of Notch activity by downregulating the expression of proneural genes. *Development* **132**, 2759-70.

Kunisch, M., Haenlin, M. and Campos-Ortega, J. A. (1994). Lateral inhibition mediated by the Drosophila neurogenic gene delta is enhanced by proneural proteins. *Proc Natl Acad Sci U S A* **91**, 10139-43.

Kunkel, G. R., Maser, R. L., Calvet, J. P. and Pederson, T. (1986). U6 small nuclear RNA is transcribed by RNA polymerase III. *Proc Natl Acad Sci U S A* **83**, 8575-9.

Kurooka, H. and Honjo, T. (2000). Functional interaction between the mouse notch1 intracellular region and histone acetyltransferases PCAF and GCN5. *J Biol Chem* **275**, 17211-20.

Kusumi, K., Sun, E. S., Kerrebrock, A. W., Bronson, R. T., Chi, D. C., Bulotsky, M. S., Spencer, J. B., Birren, B. W., Frankel, W. N. and Lander, E. S. (1998). The mouse pudgy mutation disrupts Delta homologue Dll3 and initiation of early somite boundaries. *Nat Genet* **19**, 274-8.

Ladi, E., Nichols, J. T., Ge, W., Miyamoto, A., Yao, C., Yang, L. T., Boulter, J., Sun, Y. E., Kintner, C. and Weinmaster, G. (2005). The divergent DSL ligand Dll3 does not activate Notch signaling but cell autonomously attenuates signaling induced by other DSL ligands. *J Cell Biol* **170**, 983-92.

Lai, E. C. (2002a). Micro RNAs are complementary to 3' UTR sequence motifs that mediate negative post-transcriptional regulation. *Nat Genet* **30**, 363-4.

Lai, E. C. (2002b). Protein degradation: four E3s for the notch pathway. *Curr Biol* **12**, R74-8.

Lai, E. C. (2004). Notch signaling: control of cell communication and cell fate. *Development* **131**, 965-73.

Laky, K. and Fowlkes, B. J. (2008). Notch signaling in CD4 and CD8 T cell development. *Curr Opin Immunol* **20**, 197-202.

Landmesser, L. T. (2001). The acquisition of motoneuron subtype identity and motor circuit formation. *Int J Dev Neurosci* **19**, 175-82.

Lanford, P. J., Lan, Y., Jiang, R., Lindsell, C., Weinmaster, G., Gridley, T. and Kelley, M. W. (1999). Notch signalling pathway mediates hair cell development in mammalian cochlea. *Nat Genet* **21**, 289-92.

Lanford, P. J., Shailam, R., Norton, C. R., Gridley, T. and Kelley, M. W. (2000). Expression of Math1 and HES5 in the cochleae of wildtype and Jag2 mutant mice. *J Assoc Res Otolaryngol* **1**, 161-71.

Lardelli, M., Williams, R. and Lendahl, U. (1995). Notch-related genes in animal development. *Int J Dev Biol* **39**, 769-80.

Le Borgne, R. and Schweisguth, F. (2003). Notch signaling: endocytosis makes delta signal better. *Curr Biol* **13**, R273-5.

Le Novere, N. (2001). MELTING, computing the melting temperature of nucleic acid duplex. *Bioinformatics* **17**, 1226-7.

le Roux, I., Lewis, J. and Ish-Horowicz, D. (2003). Notch activity is required to maintain floorplate identity and to control neurogenesis in the chick hindbrain and spinal cord. *Int J Dev Biol* **47**, 263-72.

Lecourtois, M. and Schweisguth, F. (1995). The neurogenic suppressor of hairless DNA-binding protein mediates the transcriptional activation of the enhancer of split complex genes triggered by Notch signaling. *Genes Dev* **9**, 2598-608.

Lecourtois, M. and Schweisguth, F. (1998). Indirect evidence for Delta-dependent intracellular processing of notch in Drosophila embryos. *Curr Biol* **8**, 771-4.

Lee, N. S., Dohjima, T., Bauer, G., Li, H., Li, M. J., Ehsani, A., Salvaterra, P. and Rossi, J. (2002a). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* **20**, 500-5.

Lee, R. C., Feinbaum, R. L. and Ambros, V. (1993). The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* **75**, 843-54.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. et al. (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**, 415-9.

Lee, Y., Jeon, K., Lee, J. T., Kim, S. and Kim, V. N. (2002b). MicroRNA maturation: stepwise processing and subcellular localization. *Embo J* **21**, 4663-70.

Lehmann, R., Jimenez, F., Dietrich, U. and Campos-Ortega, J. A. (1983). On the phenotype and development of mutants of early neurogenesis in *Drosophila melanogaster*. *Roux's Arch. Dev. Biol.* **192**, 62-74.

Li, M. and Sakaguchi, D. S. (2002). Expression patterns of focal adhesion associated proteins in the developing retina. *Dev Dyn* **225**, 544-53.

Lieber, T., Kidd, S., Alcamo, E., Corbin, V. and Young, M. W. (1993). Antineurogenic phenotypes induced by truncated Notch proteins indicate a role in signal transduction and may point to a novel function for Notch in nuclei. *Genes Dev* **7**, 1949-65.

Lieber, T., Kidd, S. and Young, M. W. (2002). kuzbanian-mediated cleavage of Drosophila Notch. *Genes Dev* **16**, 209-21.

Lieber, T., Wesley, C. S., Alcamo, E., Hassel, B., Krane, J. F., Campos-Ortega, J. A. and Young, M. W. (1992). Single amino acid substitutions in EGF-like elements of Notch and Delta modify Drosophila development and affect cell adhesion in vitro. *Neuron* **9**, 847-59.

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P., Linsley, P. S. and Johnson, J. M. (2005). Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. *Nature* **433**, 769-73.

Lindsell, C. E., Boulter, J., diSibio, G., Gossler, A. and Weinmaster, G. (1996). Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. *Mol Cell Neurosci* **8**, 14-27.

Lindsell, C. E., Shawber, C. J., Boulter, J. and Weinmaster, G. (1995). Jagged: a mammalian ligand that activates Notch1. *Cell* **80**, 909-17.

Liu, A. and Joyner, A. L. (2001). EN and GBX2 play essential roles downstream of FGF8 in patterning the mouse mid/hindbrain region. *Development* **128**, 181-91.

Liu, Q., Rand, T. A., Kalidas, S., Du, F., Kim, H. E., Smith, D. P. and Wang, X. (2003). R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway. *Science* **301**, 1921-5.

Liu, W., Mo, Z. and Xiang, M. (2001). The Ath5 proneural genes function upstream of Brn3 POU domain transcription factor genes to promote retinal ganglion cell development. *Proc Natl Acad Sci U S A* **98**, 1649-54.

Logeat, F., Bessia, C., Brou, C., LeBail, O., Jarriault, S., Seidah, N. G. and Israel, A. (1998). The Notch1 receptor is cleaved constitutively by a furin-like convertase. *Proc Natl Acad Sci U S A* **95**, 8108-12.

Louvi, A., Arboleda-Velasquez, J. F. and Artavanis-Tsakonas, S. (2006). CADASIL: a critical look at a Notch disease. *Dev Neurosci* **28**, 5-12.

Louvi, A. and Artavanis-Tsakonas, S. (2006). Notch signalling in vertebrate neural development. *Nat Rev Neurosci* **7**, 93-102.

Lowell, S., Jones, P., Le Roux, I., Dunne, J. and Watt, F. M. (2000). Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem-cell clusters. *Curr Biol* **10**, 491-500.

Lowell, S. and Watt, F. M. (2001). Delta regulates keratinocyte spreading and motility independently of differentiation. *Mech Dev* **107**, 133-40.

Lumsden, A. (1995). Neural development. A 'LIM code' for motor neurons? *Curr Biol* **5**, 491-5.

Lutolf, S., Radtke, F., Aguet, M., Suter, U. and Taylor, V. (2002). Notch1 is required for neuronal and glial differentiation in the cerebellum. *Development* **129**, 373-85.

Lyman, D. and Young, M. W. (1993). Further evidence for function of the Drosophila Notch protein as a transmembrane receptor. *Proc Natl Acad Sci U S A* **90**, 10395-9.

Ma, Q., Kintner, C. and Anderson, D. J. (1996). Identification of neurogenin, a vertebrate neuronal determination gene. *Cell* **87**, 43-52.

Ma, W. and Wang, S. Z. (2006). The final fates of neurogenin2-expressing cells include all major neuron types in the mouse retina. *Mol Cell Neurosci* **31**, 463-9.

Manche, L., Green, S. R., Schmedt, C. and Mathews, M. B. (1992). Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell Biol* **12**, 5238-48.

Mancini, S. J., Mantei, N., Dumortier, A., Suter, U., MacDonald, H. R. and Radtke, F. (2005). Jagged1-dependent Notch signaling is dispensable for hematopoietic stem cell self-renewal and differentiation. *Blood* **105**, 2340-2.

Mao, X., Fujiwara, Y., Chapdelaine, A., Yang, H. and Orkin, S. H. (2001). Activation of EGFP expression by Cre-mediated excision in a new ROSA26 reporter mouse strain. *Blood* **97**, 324-6.

Martin-Bermudo, M. D., Martinez, C., Rodriguez, A. and Jimenez, F. (1991). Distribution and function of the lethal of scute gene product during early neurogenesis in Drosophila. *Development* **113**, 445-54.

Mason, H. A., Rakowiecki, S. M., Gridley, T. and Fishell, G. (2006). Loss of notch activity in the developing central nervous system leads to increased cell death. *Dev Neurosci* **28**, 49-57.

Matise, M. P. and Joyner, A. L. (1997). Expression patterns of developmental control genes in normal and Engrailed-1 mutant mouse spinal cord reveal early diversity in developing interneurons. *J Neurosci* **17**, 7805-16.

Matsuno, K., Diederich, R. J., Go, M. J., Blaumueller, C. M. and Artavanis-Tsakonas, S. (1995). Deltex acts as a positive regulator of Notch signaling through interactions with the Notch ankyrin repeats. *Development* **121**, 2633-44.

Mauti, O., Domanitskaya, E., Andermatt, I., Sadhu, R. and Stoeckli, E. T. (2007). Semaphorin6A acts as a gate keeper between the central and the peripheral nervous system. *Neural Develop* **2**, 28.

McManus, M. T., Petersen, C. P., Haines, B. B., Chen, J. and Sharp, P. A. (2002). Gene silencing using micro-RNA designed hairpins. *Rna* **8**, 842-50.

Megason, S. G. and McMahon, A. P. (2002). A mitogen gradient of dorsal midline Wnts organizes growth in the CNS. *Development* **129**, 2087-98.

Menezes, J. R. and Luskin, M. B. (1994). Expression of neuron-specific tubulin defines a novel population in the proliferative layers of the developing telencephalon. *J Neurosci* **14**, 5399-416.

Miller, M. W. (1985). Cogeneration of retrogradely labeled corticocortical projection and GABA-immunoreactive local circuit neurons in cerebral cortex. *Brain Res* **355**, 187-92.

Minks, M. A., West, D. K., Benvin, S. and Baglioni, C. (1979). Structural requirements of double-stranded RNA for the activation of 2',5'-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. *J Biol Chem* **254**, 10180-3.

Mishra-Gorur, K., Rand, M. D., Perez-Villamil, B. and Artavanis-Tsakonas, S. (2002). Down-regulation of Delta by proteolytic processing. *J. Cell Biol.* **159**, 313-324.

Miyagishi, M. and Taira, K. (2002a). Development and application of siRNA expression vector. *Nucleic Acids Res Suppl*, 113-4.

Miyagishi, M. and Taira, K. (2002b). U6 promoter-driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. *Nat Biotechnol* **20**, 497-500.

Mizuguchi, R., Kriks, S., Cordes, R., Gossler, A., Ma, Q. and Goulding, M. (2006). Ascl1 and Gsh1/2 control inhibitory and excitatory cell fate in spinal sensory interneurons. *Nat Neurosci* **9**, 770-8.

Mizuguchi, R., Sugimori, M., Takebayashi, H., Kosako, H., Nagao, M., Yoshida, S., Nabeshima, Y., Shimamura, K. and Nakafuku, M. (2001). Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. *Neuron* **31**, 757-71.

Mizutani, K., Yoon, K., Dang, L., Tokunaga, A. and Gaiano, N. (2007). Differential Notch signalling distinguishes neural stem cells from intermediate progenitors. *Nature* **449**, 351-5.

Mohr, O. L. (1919). Character changes caused by mutation of an entire region of a chromosome in *Drosophila*. *Genetics* **4**, 275-282.

Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. et al. (2000). Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-75.

Montgomery, M. K., Xu, S. and Fire, A. (1998). RNA as a target of double-stranded RNA-mediated genetic interference in Caenorhabditis elegans. *Proc Natl Acad Sci U S A* **95**, 15502-7.

Moran-Rivard, L., Kagawa, T., Saueressig, H., Gross, M. K., Burrill, J. and Goulding, M. (2001). Evx1 is a postmitotic determinant of v0 interneuron identity in the spinal cord. *Neuron* **29**, 385-99.

Morgan, T. (1917). The theory of the gene. *Am Nat* **51**, 513-544.

Morrison, A., Hodgetts, C., Gossler, A., Hrabe de Angelis, M. and Lewis, J. (1999). Expression of Delta1 and Serrate1 (Jagged1) in the mouse inner ear. *Mech Dev* **84**, 169-72.

Morrison, C. E. and Nakhutina, L. (2007). Neuropsychological features of lesion-related epilepsy in adults: an overview. *Neuropsychol Rev* **17**, 385-403.

Morrow, E. M., Furukawa, T., Lee, J. E. and Cepko, C. L. (1999). NeuroD regulates multiple functions in the developing neural retina in rodent. *Development* **126**, 23-36.

Moss, E. G., Lee, R. C. and Ambros, V. (1997). The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. *Cell* **88**, 637-46.

Muller, T., Brohmann, H., Pierani, A., Heppenstall, P. A., Lewin, G. R., Jessell, T. M. and Birchmeier, C. (2002). The homeodomain factor lbx1 distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. *Neuron* **34**, 551-62.

Mumm, J. S. and Kopan, R. (2000). Notch signaling: from the outside in. *Dev Biol* **228**, 151-65.

Mumm, J. S., Schroeter, E. H., Saxena, M. T., Griesemer, A., Tian, X., Pan, D. J., Ray, W. J. and Kopan, R. (2000). A ligand-induced extracellular cleavage regulates gamma-secretase-like proteolytic activation of Notch1. *Mol Cell* **5**, 197-206.

Muramatsu, T., Mizutani, Y., Ohmori, Y. and Okumura, J. (1997). Comparison of three nonviral transfection methods for foreign gene expression in early chicken embryos in ovo. *Biochem Biophys Res Commun* **230**, 376-80.

Murciano, A., Zamora, J., Lopez-Sanchez, J. and Frade, J. M. (2002). Interkinetic nuclear movement may provide spatial clues to the regulation of neurogenesis. *Mol Cell Neurosci* **21**, 285-300.

Myat, A., Henrique, D., Ish-Horowicz, D. and Lewis, J. (1996). A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. *Dev Biol* **174**, 233-47.

Nellen, W. and Lichtenstein, C. (1993). What makes an mRNA anti-sense-itive? *Trends Biochem Sci* **18**, 419-23.

Nelson, B. R. and Reh, T. A. (2008). Relationship between Delta-like and proneural bHLH genes during chick retinal development. *Dev Dyn* **237**, 1565-80.

Niessen, K. and Karsan, A. (2008). Notch signaling in cardiac development. *Circ Res* **102**, 1169-81.

Nishida, H. and Satoh, N. (1989). Determination and regulation in the pigment cell lineage of the ascidian embryo. *Dev Biol* **132**, 355-67.

Nornes, H. O. and Carry, M. (1978). Neurogenesis in spinal cord of mouse: an autoradiographic analysis. *Brain Res* **159**, 1-6.

Novitch, B. G., Chen, A. I. and Jessell, T. M. (2001). Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. *Neuron* **31**, 773-89.

Nusslein-Volhard, C., Wieschaus, E. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. *Roux's Arch. Dev. Biol.* **193**, 267-282.

Nyfeler, Y., Kirch, R. D., Mantei, N., Leone, D. P., Radtke, F., Suter, U. and Taylor, V. (2005). Jagged1 signals in the postnatal subventricular zone are required for neural stem cell self-renewal. *Embo J* **24**, 3504-15.

Nykanen, A., Haley, B. and Zamore, P. D. (2001). ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* **107**, 309-21.

O'Connor-Giles, K. M. and Skeath, J. B. (2003). Numb inhibits membrane localization of Sanpodo, a four-pass transmembrane protein, to promote asymmetric divisions in Drosophila. *Dev Cell* **5**, 231-43.

O'Donovan, M. J., Bonnot, A., Mentis, G. Z., Arai, Y., Chub, N., Shneider, N. A. and Wenner, P. (2008). Imaging the spatiotemporal organization of neural activity in the developing spinal cord. *Dev Neurobiol* **68**, 788-803.

Oishi, K., Kamakura, S., Isazawa, Y., Yoshimatsu, T., Kuida, K., Nakafuku, M., Masuyama, N. and Gotoh, Y. (2004). Notch promotes survival of neural precursor cells via mechanisms distinct from those regulating neurogenesis. *Dev Biol* **276**, 172-84.

Okajima, T. and Irvine, K. D. (2002). Regulation of notch signaling by o-linked fucose. *Cell* **111**, 893-904.

Okochi, M., Steiner, H., Fukumori, A., Tanii, H., Tomita, T., Tanaka, T., Iwatsubo, T., Kudo, T., Takeda, M. and Haass, C. (2002). Presenilins mediate a dual intramembranous gamma-secretase cleavage of Notch-1. *Embo J* **21**, 5408-16.

Paddison, P. J., Caudy, A. A., Bernstein, E., Hannon, G. J. and Conklin, D. S. (2002a). Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev* **16**, 948-58.

Paddison, P. J., Caudy, A. A. and Hannon, G. J. (2002b). Stable suppression of gene expression by RNAi in mammalian cells. *Proc Natl Acad Sci U S A* **99**, 1443-8.

Paddison, P. J., Silva, J. M., Conklin, D. S., Schlabach, M., Li, M., Aruleba, S., Balija, V., O'Shaughnessy, A., Gnoj, L., Scobie, K. et al. (2004). A resource for large-scale RNA-interference-based screens in mammals. *Nature* **428**, 427-31.

Pan, Y., Lin, M. H., Tian, X., Cheng, H. T., Gridley, T., Shen, J. and Kopan, R. (2004). gamma-secretase functions through Notch signaling to maintain skin appendages but is not required for their patterning or initial morphogenesis. *Dev Cell* **7**, 731-43.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-12.

Park, H. C. and Appel, B. (2003). Delta-Notch signaling regulates oligodendrocyte specification. *Development* **130**, 3747-55.

Parks, A. L., Klueg, K. M., Stout, J. R. and Muskavitch, M. A. (2000). Ligand endocytosis drives receptor dissociation and activation in the Notch pathway. *Development* **127**, 1373-85.

Parks, A. L., Stout, J. R., Shepard, S. B., Klueg, K. M., Dos Santos, A. A., Parody, T. R., Vaskova, M. and Muskavitch, M. A. (2006). Structure-function analysis of delta trafficking, receptor binding and signaling in Drosophila. *Genetics* **174**, 1947-61.

Parks, A. L., Turner, F. R. and Muskavitch, M. A. (1995). Relationships between complex Delta expression and the specification of retinal cell fates during Drosophila eye development. *Mech Dev* **50**, 201-16.

Paroush, Z., Finley, R. L., Jr., Kidd, T., Wainwright, S. M., Ingham, P. W., Brent, R. and Ish-Horowicz, D. (1994). Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. *Cell* **79**, 805-15.

Paul, C. P., Good, P. D., Winer, I. and Engelke, D. R. (2002). Effective expression of small interfering RNA in human cells. *Nat Biotechnol* **20**, 505-8.

Paule, M. R. and White, R. J. (2000). Survey and summary: transcription by RNA polymerases I and III. *Nucleic Acids Res* **28**, 1283-98.

Peduzzi, J. D. (1988). Genesis of GABA-immunoreactive neurons in the ferret visual cortex. *J Neurosci* **8**, 920-31.

Pekarik, V., Bourikas, D., Miglino, N., Joset, P., Preiswerk, S. and Stoeckli, E. T. (2003). Screening for gene function in chicken embryo using RNAi and electroporation. *Nat Biotechnol* **21**, 93-6.

Peng, C. Y., Yajima, H., Burns, C. E., Zon, L. I., Sisodia, S. S., Pfaff, S. L. and Sharma, K. (2007). Notch and MAML Signaling Drives Scl-Dependent Interneuron Diversity in the Spinal Cord. *Neuron* **53**, 813-27.

Petcherski, A. G. and Kimble, J. (2000). LAG-3 is a putative transcriptional activator in the C. elegans Notch pathway. *Nature* **405**, 364-8.

Pham, J. W., Pellino, J. L., Lee, Y. S., Carthew, R. W. and Sontheimer, E. J. (2004). A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila. *Cell* **117**, 83-94.

Pierani, A., Brenner-Morton, S., Chiang, C. and Jessell, T. M. (1999). A sonic hedgehog-independent, retinoid-activated pathway of neurogenesis in the ventral spinal cord. *Cell* **97**, 903-15.

Pierani, A., Moran-Rivard, L., Sunshine, M. J., Littman, D. R., Goulding, M. and Jessell, T. M. (2001). Control of interneuron fate in the developing spinal cord by the progenitor homeodomain protein Dbx1. *Neuron* **29**, 367-84.

Pintar, A., De Biasio, A., Popovic, M., Ivanova, N. and Pongor, S. (2007). The intracellular region of Notch ligands: does the tail make the difference? *Biol Direct* **2**, 19.

Poodry, C. A., Hall, L. and Suzuki, D. T. (1973). Developmental properties of Shibire: a pleiotropic mutation affecting larval and adult locomotion and development. *Dev Biol* **32**, 373-86.

Posakony, J. W. (1994). Nature versus nurture: asymmetric cell divisions in Drosophila bristle development. *Cell* **76**, 415-8.

Poulson, D. (1937). Chromosomal deficiencies and the embryonic development of *Drosophila melanogaster*. *Proc Natl Acad Sci U S A* **23**, 133-137.

Poulson, D. (1940). The effects of certain X-chromosome deficiencies on the embryonic development of *Drosophila melanogaster*. *J. Exp. Zool.* **83**, 271-325.

Prada, C., Puga, J., Perez-Mendez, L., Lopez And, R. and Ramirez, G. (1991). Spatial and Temporal Patterns of Neurogenesis in the Chick Retina. *Eur J Neurosci* **3**, 1187.

Prakash, N., Hansson, E., Betsholtz, C., Mitsiadis, T. and Lendahl, U. (2002). Mouse Notch 3 expression in the pre- and postnatal brain: relationship to the stroke and dementia syndrome CADASIL. *Exp Cell Res* **278**, 31-44.

Price, B. D., Chang, Z., Smith, R., Bockheim, S. and Laughon, A. (1993). The Drosophila neuralized gene encodes a C3HC4 zinc finger. *Embo J* **12**, 2411-8.

Qi, H. and *al, e.* (1999). Processing of the notch ligand Delta by the metalloprotease Kuzbanian. *Science* **283**, 91-94.

Qiu, L., Joazeiro, C., Fang, N., Wang, H. Y., Elly, C., Altman, Y., Fang, D., Hunter, T. and Liu, Y. C. (2000). Recognition and ubiquitination of Notch by Itch, a hect-type E3 ubiquitin ligase. *J Biol Chem* **275**, 35734-7.

Radtke, F., Wilson, A., Mancini, S. J. and MacDonald, H. R. (2004). Notch regulation of lymphocyte development and function. *Nat Immunol* **5**, 247-53.

Radtke, F., Wilson, A., Stark, G., Bauer, M., van Meerwijk, J., MacDonald, H. R. and Aguet, M. (1999). Deficient T cell fate specification in mice with an induced inactivation of Notch1. *Immunity* **10**, 547-58.

Rand, M. D., Grimm, L. M., Artavanis-Tsakonas, S., Patriub, V., Blacklow, S. C., Sklar, J. and Aster, J. C. (2000). Calcium depletion dissociates and activates heterodimeric notch receptors. *Mol Cell Biol* **20**, 1825-35.

Rao, Y., Jan, L. Y. and Jan, Y. N. (1990). Similarity of the product of the Drosophila neurogenic gene big brain to transmembrane channel proteins. *Nature* **345**, 163-7.

Ray, W. J., Yao, M., Mumm, J., Schroeter, E. H., Saftig, P., Wolfe, M., Selkoe, D. J., Kopan, R. and Goate, A. M. (1999). Cell surface presenilin-1 participates in the gamma-secretase-like proteolysis of Notch. *J Biol Chem* **274**, 36801-7.

Rebay, I., Fleming, R. J., Fehon, R. G., Cherbas, L., Cherbas, P. and Artavanis-Tsakonas, S. (1991). Specific EGF repeats of Notch mediate interactions with Delta and Serrate: implications for Notch as a multifunctional receptor. *Cell* **67**, 687-99.

Rebeiz, M., Reeves, N. L. and Posakony, J. W. (2002). SCORE: a computational approach to the identification of cis-regulatory modules and target genes in whole-genome sequence data. Site clustering over random expectation. *Proc Natl Acad Sci U S A* **99**, 9888-93.

Reddy, R. (1988). Transcription of a U6 small nuclear RNA gene in vitro. Transcription of a mouse U6 small nuclear RNA gene in vitro by RNA polymerase III is dependent on transcription factor(s) different from transcription factors IIIA, IIIB, and IIIC. *J Biol Chem* **263**, 15980-4.

Redmond, L., Oh, S. R., Hicks, C., Weinmaster, G. and Ghosh, A. (2000). Nuclear Notch1 signaling and the regulation of dendritic development. *Nat Neurosci* **3**, 30-40.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S. and Khvorova, A. (2004). Rational siRNA design for RNA interference. *Nat Biotechnol* **22**, 326-30.

Rhyu, M. S., Jan, L. Y. and Jan, Y. N. (1994). Asymmetric distribution of numb protein during division of the sensory organ precursor cell confers distinct fates to daughter cells. *Cell* **76**, 477-91.

Riquelme, P. A., Drapeau, E. and Doetsch, F. (2008). Brain micro-ecologies: neural stem cell niches in the adult mammalian brain. *Philos Trans R Soc Lond B Biol Sci* **363**, 123-37.

Rodriguez, I., Hernandez, R., Modolell, J. and Ruiz-Gomez, M. (1990). Competence to develop sensory organs is temporally and spatially regulated in Drosophila epidermal primordia. *Embo J* **9**, 3583-92.

Roehl, H. and Kimble, J. (1993). Control of cell fate in C. elegans by a GLP-1 peptide consisting primarily of ankyrin repeats. *Nature* **364**, 632-5.

Rogers, S., Wells, R. and Rechsteiner, M. (1986). Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis. *Science* **234**, 364-8.

Romani, S., Campuzano, S. and Modolell, J. (1987). The achaete-scute complex is expressed in neurogenic regions of Drosophila embryos. *Embo J* **6**, 2085-2092.

Ross, S. E., Greenberg, M. E. and Stiles, C. D. (2003). Basic helix-loop-helix factors in cortical development. *Neuron* **39**, 13-25.

Rowan, S. and Cepko, C. L. (2004). Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. *Dev Biol* **271**, 388-402.

Rowitch, D. H. (2004). Glial specification in the vertebrate neural tube. *Nat Rev Neurosci* **5**, 409-19.

Saito, T., Chiba, S., Ichikawa, M., Kunisato, A., Asai, T., Shimizu, K., Yamaguchi, T., Yamamoto, G., Seo, S., Kumano, K. et al. (2003). Notch2 is preferentially expressed in mature B cells and indispensable for marginal zone B lineage development. *Immunity* **18**, 675-85.

Sakamoto, K., Nakamura, H., Takagi, M., Takeda, S. and Katsube, K. (1998). Ectopic expression of lunatic Fringe leads to downregulation of Serrate-1 in the developing chick neural tube; analysis using in ovo electroporation transfection technique. *FEBS Lett* **426**, 337-41.

Sakamoto, K., Yan, L., Imai, H., Takagi, M., Nabeshima, Y., Takeda, S. and Katsube, K. (1997). Identification of a chick homologue of Fringe and C-Fringe 1: involvement in the neurogenesis and the somitogenesis. *Biochem Biophys Res Commun* **234**, 754-9.

Sakata, T., Sakaguchi, H., Tsuda, L., Higashitani, A., Aigaki, T., Matsuno, K. and Hayashi, S. (2004). Drosophila Nedd4 regulates endocytosis of notch and suppresses its ligand-independent activation. *Curr Biol* **14**, 2228-36.

Santolini, E., Puri, C., Salcini, A. E., Gagliani, M. C., Pelicci, P. G., Tacchetti, C. and Di Fiore, P. P. (2000). Numb is an endocytic protein. *J. Cell Biol.* **151**, 1345-1352.

Sapir, A., Assa-kunik, E., Tsruya, R., Schejter, E. and Shilo, B. Z. (2005). Unidirectional Notch signslling depends on continuous cleavage of Delta. *Development* **132**, 123-132.

Sasamura, T., Sasaki, N., Miyashita, F., Nakao, S., Ishikawa, H. O., Ito, M., Kitagawa, M., Harigaya, K., Spana, E., Bilder, D. et al. (2003). neurotic, a novel maternal neurogenic gene, encodes an O-fucosyltransferase that is essential for Notch-Delta interactions. *Development* **130**, 4785-95.

Sauer, F. C. (1935). *J. Comp. Neurol.* **62**, 377-405.

Saunders, J. W., Jr. and Gasseling, M. T. (1962). Cellular death in morphogenesis of the avian wing. *Dev Biol* **5**, 147-78.

Scheer, N., Groth, A., Hans, S. and Campos-Ortega, J. A. (2001). An instructive function for Notch in promoting gliogenesis in the zebrafish retina. *Development* **128**, 1099-107.

Schmitt, T. M. and Zuniga-Pflucker, J. C. (2002). Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. *Immunity* **17**, 749-56.

Schoenwolf, G. C. and Kelley, R. O. (1980). Characterization of intercellular junctions in the caudal portion of the developing neural tube of the chick embryo. *Am J Anat* **158**, 29-41.

Schroeter, E. H., Kisslinger, J. A. and Kopan, R. (1998). Notch-1 signalling requires ligand-induced proteolytic release of intracellular domain. *Nature* **393**, 382-6.

Schuster-Gossler, K., Cordes, R. and Gossler, A. (2007). Premature myogenic differentiation and depletion of progenitor cells cause severe muscle hypotrophy in Delta1 mutants. *Proc Natl Acad Sci U S A* **104**, 537-42.

Schwarz, D. S., Hutvagner, G., Du, T., Xu, Z., Aronin, N. and Zamore, P. D. (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**, 199-208.

Schweisguth, F. (2004). Notch signaling activity. *Curr Biol* **14**, R129-38.

Selkoe, D. and Kopan, R. (2003). Notch and Presenilin: regulated intramembrane proteolysis links development and degeneration. *Annu Rev Neurosci* **26**, 565-97.

Sestan, N., Artavanis-Tsakonas, S. and Rakic, P. (1999). Contact-dependent inhibition of cortical neurite growth mediated by notch signaling. *Science* **286**, 741-6.

Shawber, C., Boulter, J., Lindsell, C. E. and Weinmaster, G. (1996). Jagged2: a serrate-like gene expressed during rat embryogenesis. *Dev Biol* **180**, 370-6.

Shellenbarger, D. L. and Mohler, J. D. (1978). Temperature-sensitive periods and autonomy of pleiotropic effects of l(1)Nts1, a conditional notch lethal in Drosophila. *Dev Biol* **62**, 432-46.

Shi, S. and Stanley, P. (2003). Protein O-fucosyltransferase 1 is an essential component of Notch signaling pathways. *Proc Natl Acad Sci U S A* **100**, 5234-9.

Shimizu, K., Chiba, S., Kumano, K., Hosoya, N., Takahashi, T., Kanda, Y., Hamada, Y., Yazaki, Y. and Hirai, H. (1999). Mouse jagged1 physically interacts with notch2 and other notch receptors. Assessment by quantitative methods. *J Biol Chem* **274**, 32961-9.

Shimshek, D. R., Kim, J., Hubner, M. R., Spergel, D. J., Buchholz, F., Casanova, E., Stewart, A. F., Seeburg, P. H. and Sprengel, R. (2002). Codon-improved Cre recombinase (iCre) expression in the mouse. *Genesis* **32**, 19-26.

Shin, J., Poling, J., Park, H. C. and Appel, B. (2007). Notch signaling regulates neural precursor allocation and binary neuronal fate decisions in zebrafish. *Development*.

Shutter, J. R., Scully, S., Fan, W., Richards, W. G., Kitajewski, J., Deblandre, G. A., Kintner, C. R. and Stark, K. L. (2000). Dll4, a novel Notch ligand expressed in arterial endothelium. *Genes Dev* **14**, 1313-8.

Siekmann, A. F., Covassin, L. and Lawson, N. D. (2008). Modulation of VEGF signalling output by the Notch pathway. *Bioessays* **30**, 303-13.

Silva, J., Chang, K., Hannon, G. J. and Rivas, F. V. (2004). RNA-interference-based functional genomics in mammalian cells: reverse genetics coming of age. *Oncogene* **23**, 8401-9.

Silva, J. M., Sachidanandam, R. and Hannon, G. J. (2003). Free energy lights the path toward more effective RNAi. *Nat Genet* **35**, 303-5.

Skeath, J. B. and Doe, C. Q. (1998). Sanpodo and Notch act in apposition to Numb to distinguish sibling neuron fates in the Drosophila CNS. *Development* **125**, 1857-1865.

Sledz, C. A., Holko, M., de Veer, M. J., Silverman, R. H. and Williams, B. R. (2003). Activation of the interferon system by short-interfering RNAs. *Nat Cell Biol* **5**, 834-9.

Smoller, D., Friedel, C., Schmid, A., Bettler, D., Lam, L. and Yedvobnick, B. (1990). The Drosophila neurogenic locus mastermind encodes a nuclear protein unusually rich in amino acid homopolymers. *Genes Dev* **4**, 1688-700.

Srinivas, S., Watanabe, T., Lin, C. S., William, C. M., Tanabe, Y., Jessell, T. M. and Costantini, F. (2001). Cre reporter strains produced by targeted insertion of EYFP and ECFP into the ROSA26 locus. *BMC Dev Biol* **1**, 4.

Stark, G. R., Kerr, I. M., Williams, B. R., Silverman, R. H. and Schreiber, R. D. (1998). How cells respond to interferons. *Annu Rev Biochem* **67**, 227-64.

Stern, C. D. (2002). Induction and initial patterning of the nervous system - the chick embryo enters the scene. *Curr Opin Genet Dev* **12**, 447-51.

Stern, C. D. (2004). The chick embryo--past, present and future as a model system in developmental biology. *Mech Dev* **121**, 1011-3.

Sternberg, P. W. (2004). Developmental biology. A pattern of precision. *Science* **303**, 637-8.

Sternberg, P. W. (2005). Vulval development. *WormBook*, 1-28.

Stier, S., Cheng, T., Dombkowski, D., Carlesso, N. and Scadden, D. T. (2002). Notch1 activation increases hematopoietic stem cell self-renewal in vivo and favors lymphoid over myeloid lineage outcome. *Blood* **99**, 2369-78.

Stifani, S., Blaumueller, C. M., Redhead, N. J., Hill, R. E. and Artavanis-Tsakonas, S. (1992). Human homologs of a Drosophila Enhancer of split gene product define a novel family of nuclear proteins. *Nat Genet* **2**, 119-27.

Struhl, G., Fitzgerald, K. and Greenwald, I. (1993). Intrinsic activity of the Lin-12 and Notch intracellular domains in vivo. *Cell* **74**, 331-45.

Stump, G., Durrer, A., Klein, A. L., Lutolf, S., Suter, U. and Taylor, V. (2002). Notch1 and its ligands Delta-like and Jagged are expressed and active in distinct cell populations in the postnatal mouse brain. *Mech Dev* **114**, 153-9.

Sturtevant, M. A. and Bier, E. (1995). Analysis of the genetic hierarchy guiding wing vein development in Drosophila. *Development* **121**, 785-801.

Sugimoto, N., Katoh, M., Nakano, S., Ohmichi, T. and Sasaki, M. (1994). RNA/DNA hybrid duplexes with identical nearest-neighbor base-pairs have identical stability. *FEBS Lett* **354**, 74-8.

Sui, G., Soohoo, C., Affar el, B., Gay, F., Shi, Y., Forrester, W. C. and Shi, Y. (2002). A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci U S A* **99**, 5515-20.

Sulston, J. E. and White, J. G. (1980). Regulation and cell autonomy during postembryonic development of Caenorhabditis elegans. *Dev Biol* **78**, 577-97.

Sun, X. and Artavanis-Tsakonas, S. (1996). The intracellular deletions of Delta and Serrate define dominant negative forms of the Drosophila Notch ligands. *Development* **122**, 2465-74.

Sun, X. and Artavanis-Tsakonas, S. (1997). Secreted forms of DELTA and SERRATE define antagonists of Notch signaling in Drosophila. *Development* **124**, 3439-48.

Takahashi, Y. (2005). Common mechanisms for boundary formation in somitogenesis and brain development: shaping the 'chic' chick. *Int J Dev Biol* **49**, 221-30.

Takebayashi, S., Yamamoto, N., Yabe, D., Fukuda, H., Kojima, K., Ito, J. and Honjo, T. (2007). Multiple roles of Notch signaling in cochlear development. *Dev Biol* **307**, 165-78.

Tanigaki, K., Nogaki, F., Takahashi, J., Tashiro, K., Kurooka, H. and Honjo, T. (2001). Notch1 and Notch3 instructively restrict bFGF-responsive multipotent neural progenitor cells to an astroglial fate. *Neuron* **29**, 45-55.

Thor, S., Ericson, J., Brannstrom, T. and Edlund, T. (1991). The homeodomain LIM protein Isl-1 is expressed in subsets of neurons and endocrine cells in the adult rat. *Neuron* **7**, 881-9.

Timmons, L. and Fire, A. (1998). Specific interference by ingested dsRNA. *Nature* **395**, 854.

Toda, M., Iizuka, Y., Yu, W., Imai, T., Ikeda, E., Yoshida, K., Kawase, T., Kawakami, Y., Okano, H. and Uyemura, K. (2001). Expression of the neural RNA-binding protein Musashi1 in human gliomas. *Glia* **34**, 1-7.

Tomita, K., Ishibashi, M., Nakahara, K., Ang, S. L., Nakanishi, S., Guillemot, F. and Kageyama, R. (1996). Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. *Neuron* **16**, 723-34.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P. C., Bock, R., Klein, R. and Schutz, G. (1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* **23**, 99-103.

Uyttendaele, H., Marazzi, G., Wu, G., Yan, Q., Sassoon, D. and Kitajewski, J. (1996). Notch4/int-3, a mammary proto-oncogene, is an endothelial cell-specific mammalian Notch gene. *Development* **122**, 2251-9.

Valsecchi, C., Ghezzi, C., Ballabio, A. and Rugarli, E. I. (1997). JAGGED2: a putative Notch ligand expressed in the apical ectodermal ridge and in sites of epithelial-mesenchymal interactions. *Mech Dev* **69**, 203-7.

van der Bliek, A. M. and Meyerowitz, E. M. (1991). Dynamin-like protein encoded by the Drosophila shibire gene associated with vesicular traffic. *Nature* **351**, 411-4.

Varnum-Finney, B., Brashem-Stein, C. and Bernstein, I. D. (2003). Combined effects of Notch signaling and cytokines induce a multiple log increase in precursors with lymphoid and myeloid reconstituting ability. *Blood* **101**, 1784-9.

Varnum-Finney, B., Xu, L., Brashem-Stein, C., Nourigat, C., Flowers, D., Bakkour, S., Pear, W. S. and Bernstein, I. D. (2000). Pluripotent, cytokine-dependent, hematopoietic stem cells are immortalized by constitutive Notch1 signaling. *Nat Med* **6**, 1278-81.

Vassin, H., Bremer, K. A., Knust, E. and Campos-Ortega, J. A. (1987). The neurogenic gene Delta of Drosophila melanogaster is expressed in neurogenic territories and encodes a putative transmembrane protein with EGF-like repeats. *Embo J* **6**, 3431-3440.

Vassin, H. and Campos-Ortega, J. A. (1987). Genetic Analysis of Delta, a Neurogenic Gene of Drosophila melanogaster. *Genetics* **116**, 433-445.

Vassin, H., Vielmetter, J. and Campos-Ortega, J. A. (1985). Genetic interactions in early neurogenesis of Drosophila melanogaster. *J Neurogenet* **2**, 291-308.

Vauclair, S., Nicolas, M., Barrandon, Y. and Radtke, F. (2005). Notch1 is essential for postnatal hair follicle development and homeostasis. *Dev Biol* **284**, 184-93.

Vermeren, M., Maro, G. S., Bron, R., McGonnell, I. M., Charnay, P., Topilko, P. and Cohen, J. (2003). Integrity of developing spinal motor columns is regulated by neural crest derivatives at motor exit points. *Neuron* **37**, 403-15.

Vetter, M. L. and Brown, N. L. (2001). The role of basic helix-loop-helix genes in vertebrate retinogenesis. *Semin Cell Dev Biol* **12**, 491-8.

Villa, N., Walker, L., Lindsell, C. E., Gasson, J., Iruela-Arispe, M. L. and Weinmaster, G. (2001). Vascular expression of Notch pathway receptors and ligands is restricted to arterial vessels. *Mech Dev* **108**, 161-4.

Wakamatsu, Y., Maynard, T. M. and Weston, J. A. (2000). Fate determination of neural crest cells by NOTCH-mediated lateral inhibition and asymmetrical cell division during gangliogenesis. *Development* **127**, 2811-21.

Wang, S., Sdrulla, A. D., diSibio, G., Bush, G., Nofziger, D., Hicks, C., Weinmaster, G. and Barres, B. A. (1998). Notch receptor activation inhibits oligodendrocyte differentiation. *Neuron* **21**, 63-75.

Wang, S. W., Kim, B. S., Ding, K., Wang, H., Sun, D., Johnson, R. L., Klein, W. H. and Gan, L. (2001). Requirement for math5 in the development of retinal ganglion cells. *Genes Dev* **15**, 24-9.

Wang, Y., Chan, S. L., Miele, L., Yao, P. J., Mackes, J., Ingram, D. K., Mattson, M. P. and Furukawa, K. (2004). Involvement of Notch signaling in hippocampal synaptic plasticity. *Proc Natl Acad Sci U S A* **101**, 9458-62.

Wang, Y. and Sul, H. S. (2006). Ectodomain shedding of preadipocyte factor 1 (Pref-1) by tumor necrosis factor alpha converting enzyme (TACE) and inhibition of adipocyte differentiation. *Mol Cell Biol* **26**, 5421-35.

Waterhouse, P. M., Graham, M. W. and Wang, M. B. (1998). Virus resistance and gene silencing in plants can be induced by simultaneous expression of sense and antisense RNA. *Proc Natl Acad Sci U S A* **95**, 13959-64.

Watt, F. M., Estrach, S. and Ambler, C. A. (2008). Epidermal Notch signalling: differentiation, cancer and adhesion. *Curr Opin Cell Biol* **20**, 171-9.

Weinmaster, G. (1997). The ins and outs of notch signaling. *Mol Cell Neurosci* **9**, 91-102.

Weisblat, D. A. and Blair, S. S. (1984). Developmental interdeterminacy in embryos of the leech Helobdella triserialis. *Dev Biol* **101**, 326-35.

Weller, M., Krautler, N., Mantei, N., Suter, U. and Taylor, V. (2006). Jagged1 ablation results in cerebellar granule cell migration defects and depletion of Bergmann glia. *Dev Neurosci* **28**, 70-80.

Wharton, K. A., Johansen, K. M., Xu, T. and Artavanis-Tsakonas, S. (1985). Nucleotide sequence from the neurogenic locus notch implies a gene product that shares homology with proteins containing EGF-like repeats. *Cell* **43**, 567-81.

Wieschaus, E., Nusslein-Volhard, C. and Jurgens, G. (1984). Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. III. Zygotic loci on the X-chromosome and forth chromosome. *Roux's Arch. Dev. Biol.* **193**, 296-307.

Wigglesworth, V. (1940). Local and general factors in the development of 'pattern' in *Rhodnius prolixus*. *J. Exp. Zool.* **17**, 180-200.

Wightman, B., Ha, I. and Ruvkun, G. (1993). Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. *Cell* **75**, 855-62.

Wilson, A., MacDonald, H. R. and Radtke, F. (2001). Notch 1-deficient common lymphoid precursors adopt a B cell fate in the thymus. *J Exp Med* **194**, 1003-12.

Wilson, L. and Maden, M. (2005). The mechanisms of dorsoventral patterning in the vertebrate neural tube. *Dev Biol* **282**, 1-13.

Wonders, C. P. and Anderson, S. A. (2006). The origin and specification of cortical interneurons. *Nat Rev Neurosci* **7**, 687-96.

Wozniak, M. A., Modzelewska, K., Kwong, L. and Keely, P. J. (2004). Focal adhesion regulation of cell behavior. *Biochim Biophys Acta* **1692**, 103-19.

Xia, H., Mao, Q., Paulson, H. L. and Davidson, B. L. (2002). siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol* **20**, 1006-10.

Xia, T., SantaLucia, J., Jr., Burkard, M. E., Kierzek, R., Schroeder, S. J., Jiao, X., Cox, C. and Turner, D. H. (1998). Thermodynamic parameters for an expanded nearest-neighbor model for formation of RNA duplexes with Watson-Crick base pairs. *Biochemistry* **37**, 14719-35.

Xie, Z., Moy, L. Y., Sanada, K., Zhou, Y., Buchman, J. J. and Tsai, L. H. (2007). Cep120 and TACCs control interkinetic nuclear migration and the neural progenitor pool. *Neuron* **56**, 79-93.

Xu, X., Roby, K. D. and Callaway, E. M. (2006). Mouse cortical inhibitory neuron type that coexpresses somatostatin and calretinin. *J Comp Neurol* **499**, 144-60.

Xue, Y., Gao, X., Lindsell, C. E., Norton, C. R., Chang, B., Hicks, C., Gendron-Maguire, M., Rand, E. B., Weinmaster, G. and Gridley, T. (1999). Embryonic lethality and vascular defects in mice lacking the Notch ligand Jagged1. *Hum Mol Genet* **8**, 723-30.

Yang, L. T., Nichols, J. T., Yao, C., Manilay, J. O., Robey, E. A. and Weinmaster, G. (2005). Fringe glycosyltransferases differentially modulate Notch1 proteolysis induced by Delta1 and Jagged1. *Mol Biol Cell* **16**, 927-42.

Yang, X., Klein, R., Tian, X., Cheng, H. T., Kopan, R. and Shen, J. (2004). Notch activation induces apoptosis in neural progenitor cells through a p53-dependent pathway. *Dev Biol* **269**, 81-94.

Yang, X., Tomita, T., Wines-Samuelson, M., Beglopoulos, V., Tansey, M. G., Kopan, R. and Shen, J. (2006). Notch1 signaling influences v2 interneuron and motor neuron development in the spinal cord. *Dev Neurosci* **28**, 102-17.

Yaron, O., Farhy, C., Marquardt, T., Applebury, M. and Ashery-Padan, R. (2006). Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. *Development* **133**, 1367-78.

Ye, Y., Lukinova, N. and Fortini, M. E. (1999). Neurogenic phenotypes and altered Notch processing in Drosophila Presenilin mutants. *Nature* **398**, 525-9.

Yeo, S. Y. and Chitnis, A. B. (2007). Jagged-mediated Notch signaling maintains proliferating neural progenitors and regulates cell diversity in the ventral spinal cord. *Proc Natl Acad Sci U S A* **104**, 5913-8.

Yoon, K. and Gaiano, N. (2005). Notch signaling in the mammalian central nervous system: insights from mouse mutants. *Nat Neurosci* **8**, 709-15.

Young, K. M., Fogarty, M., Kessaris, N. and Richardson, W. D. (2007). Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. *J Neurosci* **27**, 8286-96.

Young, R. W. (1985). Cell differentiation in the retina of the mouse. *Anat Rec* **212**, 199-205.

Yu, J. Y., DeRuiter, S. L. and Turner, D. L. (2002). RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci U S A* **99**, 6047-52.

Yun, K., Fischman, S., Johnson, J., Hrabe de Angelis, M., Weinmaster, G. and Rubenstein, J. L. (2002). Modulation of the notch signaling by Mash1 and Dlx1/2 regulates sequential specification and differentiation of progenitor cell types in the subcortical telencephalon. *Development* **129**, 5029-40.

Zamore, P. D., Tuschl, T., Sharp, P. A. and Bartel, D. P. (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**, 25-33.

Zecchini, V., Brennan, K. and Martinez-Arias, A. (1999). An activity of Notch regulates JNK signalling and affects dorsal closure in Drosophila. *Curr Biol* **9**, 460-9.

Zeng, C., Younger-Shepherd, S., Jan, L. Y. and Jan, Y. N. (1998). Delta and Serrate are redundant Notch ligands required for asymmetric cell divisions within the Drosophila sensory organ lineage. *Genes Dev* **12**, 1086-91.

Zeng, Y. and Cullen, B. R. (2003). Sequence requirements for micro RNA processing and function in human cells. *Rna* **9**, 112-23.

Zeng, Y., Wagner, E. J. and Cullen, B. R. (2002). Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol Cell* **9**, 1327-33.

Zhong, W., Feder, J. N., Jiang, M. M., Jan, L. Y. and Jan, Y. N. (1996). Asymmetric localization of a mammalian numb homolog during mouse cortical neurogenesis. *Neuron* **17**, 43-53.

Zhou, Q., Choi, G. and Anderson, D. J. (2001). The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. *Neuron* **31**, 791-807.

Zhou, Y., Yamamoto, M. and Engel, J. D. (2000). GATA2 is required for the generation of V2 interneurons. *Development* **127**, 3829-38.

APPENDIX

Developmental Biology 328 (2009) 54–65

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/developmentalbiology

Dll1 and *Dll4* function sequentially in the retina and pV2 domain of the spinal cord to regulate neurogenesis and create cell diversity

Susana Ferreira Rocha [a], Susana Santos Lopes [b], Achim Gossler [c], Domingos Henrique [a,b,*]

[a] Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal
[b] Instituto Gulbenkian de Ciência, Ap. 14, Oeiras, Portugal
[c] Institute for Molecular Biology, Medizinische Hochschule Hannover, Carl-Neuberg-Strasse 1, D-30625 Hannover, Germany

ARTICLE INFO

Article history:
Received for publication 10 November 2008
Revised 26 December 2008
Accepted 5 January 2009
Available online 14 January 2009

Keywords:
Notch signaling
Delta ligands
Neurogenesis
Retina
Spinal cord
Cell fate diversity

ABSTRACT

Signalling mediated by Notch receptors is known to have multiple functions during vertebrate neural development, regulating processes like progenitor differentiation and cell type diversification. Various Notch ligands are expressed in the developing nervous system and their activities might contribute to this multiplicity of functions. Here, we show that two *Delta-like* genes, *Dll1* and *Dll4*, are sequentially expressed in differentiating neurons of the embryonic mouse retina and spinal cord's pV2 domain, with *Dll1* starting to be expressed before *Dll4*. Analysis of *Dll1* mutants reveals this gene is necessary and sufficient to maintain a pool of progenitors in the embryonic neuroepithelium. Accordingly, in the spinal cord domains where Dll1 is the only expressed Notch ligand, its inactivation leads to an increased rate of neurogenesis and premature differentiation of neural progenitors. In contrast, in the pV2 domain and retina where *Dll1* is co-expressed with *Dll4*, progenitors are not exhausted and cell diversity is maintained. Together, our results support a model where *Dll1* and *Dll4* are part of a unique genetic circuitry that regulates subsequent steps of neurogenesis in the retina and pV2 domain: while *Dll1* serves to prevent the untimely differentiation of neural progenitors, *Dll4* might function to generate diversity within the population of differentiating neurons.
© 2009 Elsevier Inc. All rights reserved.

Introduction

The Notch pathway has been implicated in several aspects of neural development, from the generation and maintenance of neural progenitors to the control of dendritic morphology (reviewed in Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005). A role for Notch signalling in controlling cell type specification is also well established in *Drosophila*, in particular during the generation of the various cell types that form sensory organs in the fly peripheral nervous system (reviewed in Bardin et al., 2004). During vertebrate neurogenesis, Notch signaling have been also implicated in cell fate diversity, for instance in the chick and mouse spinal cord pV2 domain (Del Barrio et al., 2007; Peng et al., 2007), as well as in the epiphysis and spinal cord of zebrafish embryos (Cau et al., 2008; Shin et al., 2007; Yeo and Chitnis, 2007). In the vertebrate inner ear and retina, Notch activity is also needed to diversify cell fates, as shown by various experiments where absence of Notch signaling results in an unbalanced production of specific cell types in these organs (Brooker et al., 2006; Kiernan et al., 2005, 2006, Jadhav et al., 2006; Yaron et al., 2006). For instance, treatment of the developing inner ear with inhibitors of the Notch pathway caused the formation of sensory patches devoid of supporting cells and composed solely of hair cells (Daudet and Lewis, 2005; Takebayashi et al., 2007), showing that Notch signalling regulates a binary decision between these two cell types in the otic epithelium. In the retina, chemical inhibition of Notch signalling (Kubo et al., 2005) or genetic inactivation of *Notch1* (Jadhav et al., 2006; Yaron et al., 2006) also disturbs cell fate specification, causing the formation of an increased number of cone photoreceptors, at the expense of other retinal cell types. Thus, in addition to its well-known function in controlling progenitor availability during vertebrate neurogenesis, Notch signalling plays also an important role in neuronal type diversification.

Vertebrates contain various genes encoding Notch ligands, which are expressed in the developing nervous system with distinct spatio-temporal patterns, and an attractive possibility is that different ligands mediate distinct Notch functions at each step of the neurogenesis process. These ligands belong to two groups of structurally distinct proteins, those related to the *Drosophila* Delta protein and those related to the *Drosophila* Serrate protein (also named Jagged in vertebrates). Mice, for instance, have two genes encoding Jagged ligands, *Jagged1* (*Jag1*) and *Jagged2* (*Jag2*), and three genes encoding Delta ligands, *Delta1*, *Delta3* and *Delta4* (also known as *Dll1*, *Dll3* and *Dll4*). Two of these genes, *Jag1* and *Dll1*, are expressed with striking complementary patterns in the embryonic neural tube (NT), in cells

* Corresponding author. Instituto de Medicina Molecular, Faculdade de Medicina de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal. Fax: +351 21 7999504.
E-mail address: henrique@fm.ul.pt (D. Henrique).

0012-1606/$ – see front matter © 2009 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2009.01.011

committed to neuronal differentiation (Lindsell et al., 1996; Myat et al., 1996), while *Dll3* and *Jag2* are expressed later in differentiating neurons (Dunwoodie et al., 1997; Kusumi et al., 1998; Shawber et al., 1996; Valsecchi et al., 1997). Recently, *Dll4* was reported to be expressed in a restricted domain of the NT, the pV2 domain, and in the developing mouse retina (Benedito and Duarte, 2005), two regions where *Dll1* is also expressed. The *Jagged* genes are also expressed in the developing neural retina, *Jag1* in cells of the ciliary margin and lens (Bao and Cepko, 1997; Lindsell et al., 1996), and *Jag2* in differentiated retina ganglion cells (RGCs), after they reach the ganglion cell layer (Valsecchi et al., 1997).

These various expression patterns of Notch ligands in the developing mammalian CNS reveal that different regions of the embryo employ distinct genetic networks to regulate neuronal production, probably reflecting the existence of region-specific strategies to assemble functional circuits in the nervous system. However, little is known about the specific role of each ligand in these networks and how they contribute to the multiple functions of Notch signaling during vertebrate neurogenesis. Lack of detailed loss-of-function studies for each of the Notch ligands in the developing nervous system has prevented the elucidation of these questions, mostly due to the embryonic lethality of mice carrying mutations in the various *Dll* and *Jag* genes (Louvi and Artavanis-Tsakonas, 2006).

In the work here described, we first performed a detailed analysis of the expression of *Dll1* and *Dll4* during neurogenesis in the mouse neural retina and spinal cord, and show that these two genes are transiently expressed during differentiation of V2 and retinal neurons. Our results indicate that *Dll1* starts to be expressed before *Dll4*, in cells still showing progenitor markers. As these cells advance in their differentiation, *Dll4* starts to be transcribed and the two *Dll* genes are coexpressed during a restricted period, being then switched off before overt neuronal differentiation. To address the functions of this *Dll1*/*Dll4* circuitry, we studied mice where *Dll1* is conditionally inactivated in the developing CNS. Our results support a model where the two Dll ligands regulate subsequent steps of neuronal production in the retina and pV2 domain: while Dll1 serves primarily to activate Notch signalling in neighbouring progenitors and prevent their untimely differentiation, Dll4's main function is to generate diversity within the population of differentiating neurons.

Materials and methods

Mouse breeding

The *Chx10-Cre* strain (Rowan and Cepko, 2004) was obtained from Jackson Lab. The $Dll4^{LacZ}$ (Duarte et al., 2004), *Nestin-Cre* (Tronche et al., 1999) and *Dll1-flox* (Hozumi et al., 2004) strains were a kind gift from António Duarte, Rüdiger Klein and Julian Lewis, respectively. The $Dll1^{LacZ}$ and $Dll1^{Ki}$ strains were previously described (Hrabe de Angelis et al., 1997; Schuster-Gossler et al., 2007).

Mice carrying the conditional *Dll1-flox* allele were crossed with each Cre strain and double heterozygous progeny were identified by PCR. These were then crossed with mice homozygous for the conditional allele, to produce litters containing conditional knockout mice (*Chx10-Cre/+;Dll1-flox/flox or Nestin-Cre/+;Dll1-flox/flox*) and sibling controls. The $Dll1^{LacZ/+}$ and $Dll1^{Ki/+}$ were intercrossed to obtain $Dll1^{LacZ/Ki}$ mutants and sibling controls.

Immunohistochemistry and in situ hybridization

Embryos were fixed in 4% paraformaldehyde at 4 °C, then cryoprotected in 30% sucrose and embedded in 7.5% gelatin:15% sucrose. Cryostat sections (12 μm) were used for immunofluorescence and in situ hybridization, as described (Fior and Henrique, 2005). Antibodies used in this work are described in Table 1 (Supplementary material).

Cell counts and microscopy

Countings of the number of retinal progenitor cells in E14.5 retinas were done in confocal images of the central region of the retina. Images were cropped to contain the entire width of the retina and $Chx10^+$ cells were counted with ImageJ software and normalized to the area analysed (cells/mm^2). Differentiation of RGCs at E12.5 was determined by sectioning and immunostaining whole retinas for Brn3b. $Brn3b^+$ cells were counted in each section and normalized to the total number of $DAPI^+$ cells. Quantification of neuronal subtypes in NT sections was done by counting cells immunopositive for the described antibodies in images taken with a 40× objective on a Leica DM5000B fluorescence microscope. The following cell type specific antibodies were used: Nkx2.2 (pV3), Islet1 and MNR-2 (motoneurons), Chx10 (V2 interneurons), En1 (V1 interneurons), Evx1 (V0 interneurons), Brn3a (dl5 neurons identified as positive cells localized ventrally to dl4 Pax2+ cells), Pax2 (dl4, identified as dorsally localized and Brn3a-negative), Brn3a (dl1–dl3, identified as positive cells localized dorsally to dl4 Pax2+ cells). Antibodies are described in Supplementary Table S1. A minimum of 3 embryos were counted (minimum of 8 sections per embryo) for each phenotype. Images from double labelling experiments in retina sections were taken with a confocal microscope (Zeiss LSM510 META). In all pictures, apical is up. Statistical analysis of results was done using the Student's *t* test (paired).

Results

Dll1 and Dll4 are expressed sequentially in the developing mouse retina

Previous studies have shown that expression of *Dll1* and *Dll4* in the mouse retina starts as early as E10.5 (Hrabe de Angelis et al., 1997) and E11.5 (Benedito and Duarte, 2005), respectively. We have studied in more detail the expression of these two genes during mouse retinal development and found that both *Dll1* and *Dll4* are expressed in cells located in the ventricular zone (VZ) of the retinal neuroepithelium, with more cells expressing *Dll1* than *Dll4* (Figs. 1A, B). At E14.5, for instance, the number of cells expressing *Dll1* is 2.5 times higher than the number of *Dll4*-expressing cells (n=1355 cells). Also, cells expressing *Dll1* are often found in clusters and exhibit variable levels of expression, while *Dll4*-expressing cells appear usually isolated and show higher levels of expression (Figs. 1A′, B′). Expression of the two genes is first detected in cells located in the central retina. As the retina grows, expression spreads peripherally along the expanding neurogenic region, being always absent from the ciliary margin zone (CMZ). However, *Dll1*-expressing cells are located at more peripheral positions than *Dll4*-expressing cells (Figs. 1A–C), implying that *Dll1* is expressed before *Dll4* as progenitors enter neuronal differentiation, following the central-to-periphery wave of neurogenesis (Prada et al., 1991). This temporal difference in expression might indicate that the two genes are expressed in the same cells but at subsequent phases of the differentiation program. Alternatively, each gene might be expressed in distinct populations of retinal cells, with *Dll1* being active in a population that starts to differentiate earlier than the *Dll4*-expressing population.

To address these hypotheses, we performed double in situ hybridization in sections of E12.5 and E14.5 retinas. We found that the majority (78%) of the *Dll4*-expressing cells do co-express *Dll1* (Figs. 1D, E), while only one third of the *Dll1*-expressing cells show co-expression of *Dll4.* To further characterize the cells expressing each *Dll* gene, we combined *Dll1*/*Dll4* in situ hybridization with immunostainings for Chx10, present in retinal progenitor cells (RPCs) (Liu et al., 1994), and Tuj1, which marks differentiating neurons (Lee et al., 1990), at E12.5 and E14.5 (Figs. 1F–I and data not shown). Although *Dll1* was previously described to be mainly expressed in nascent neurons (Henrique et al., 1997), we found that 20.2±4.2% of the *Dll1*-expressing

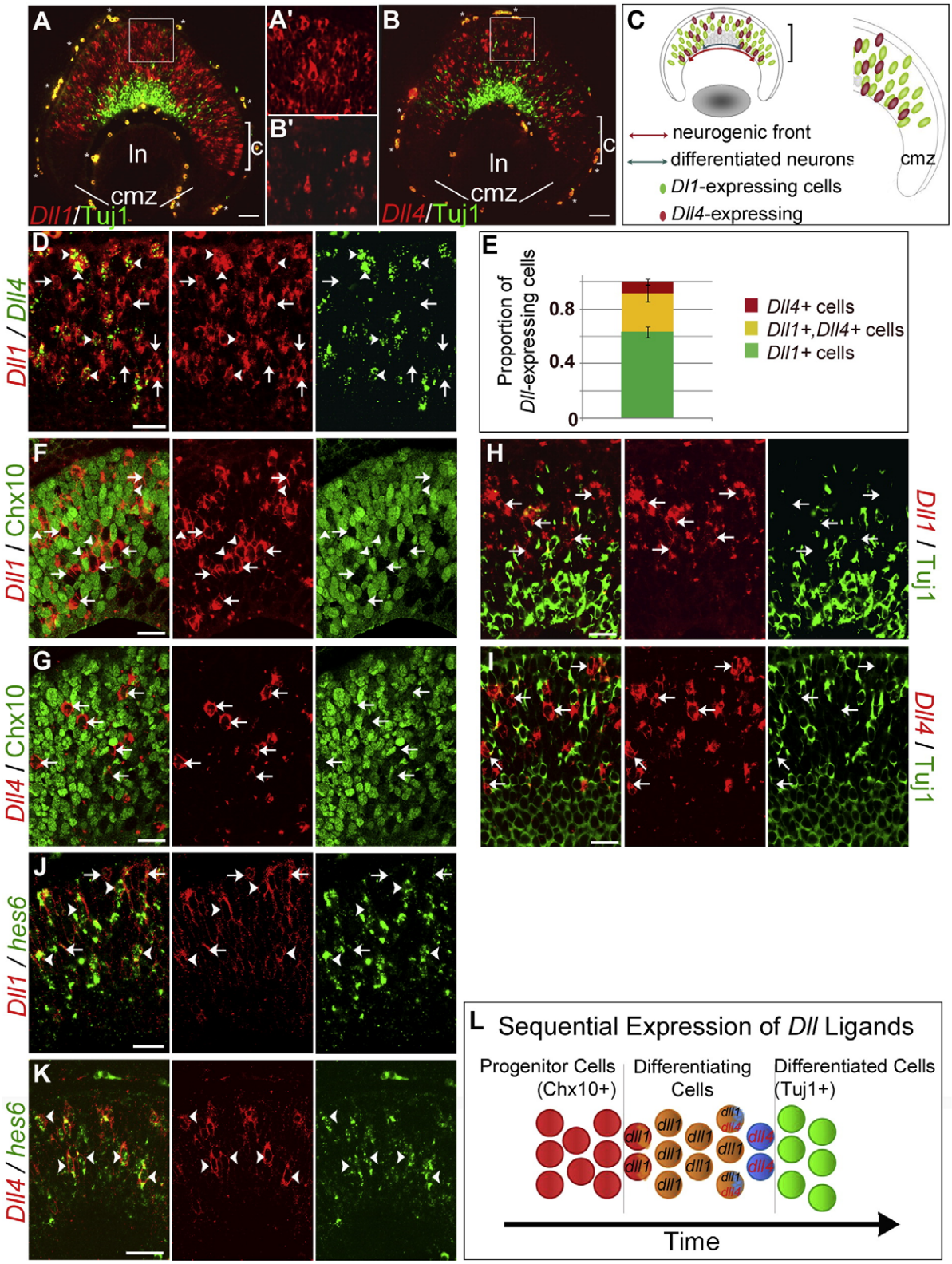


Fig. 1. *Dll* ligands are expressed transiently and sequentially in the developing retina. (A, B) Cells with *Dll1* expression are more frequent than *Dll4*-expressing cells and show more variable levels of mRNA (A′, B′). At the front of the neurogenic wave that spreads from the central retina to the periphery (bracketed region), expression of both *Dll1* and *Dll4* precedes the appearance of differentiated Tuj1^{+} neurons (A and B are adjacent sections). However, the domain of *Dll1*-expressing cells extends more to the periphery. A cartoon depicting these findings is shown in (C). (D) Confocal images of double in situ hybridization for *Dll1* and *Dll4* shows the existence of a subpopulation of retinal cells co-expressing both genes. Most *Dll4*-expressing cells co-express *Dll1* (arrowheads in D), although the inverse is not true (arrows in D). (E) Cell countings indicating the contribution of each of the *Dll*-expressing populations. Confocal images of Chx10 immunostaining (a RPC marker) and *Dll1* (F) or *Dll4* (G) expression shows that *Dll1* transcription is initiated in RPCs (arrowheads in F), while no *Dll4*-expressing cells co-express Chx10 (arrows in G). (H, I) Absence of simultaneous detection of Tuj1^{+} neurons and expression of *Dll1* (H) or *Dll4* (I), shows that both genes are expressed before overt neuronal differentiation. (J, K) Confocal images of double in situ hybridization for *hes6* and *Dll1* (J) or *Dll4* (K) reveals that practically all cells expressing *Dll4* co-express *hes6*. In contrast, only a fraction of the *Dll1*-expressing cells co-express *hes6* (arrowheads). (L) Summary of our findings supporting a model in which the two *Dll* ligands are expressed at subsequent steps of the neurogenic programme, with *Dll1* expression preceding that of *Dll4*. Asterisks mark red blood cells surrounding the RPE and in the vitreal space. ln — lens, CMZ — ciliary margin zone, RPE — retinal pigmented epithelium. Scale bars (A, B)=50 μm; (D, F–K)=25 μm.

cells are still positive for the RPC marker Chx10 ($n=1624$ cells, Fig. 1F). On the contrary, *Dll4* is never expressed in Chx10$^+$ cells (Fig. 1G), indicating that *Dll1* expression occurs at an earlier phase of neuronal differentiation than *Dll4*. In addition, no expression of *Dll1* and *Dll4* could be detected in differentiated Tuj1$^+$ neurons (Figs. 1H, I), revealing that the two genes are only active within a restricted time window, between neuronal commitment and differentiation. Furthermore, double in situ hybridization for *Dll1*/*Dll4* and *hes6*, a gene expressed in differentiating neurons (Koyano-Nakagawa et al., 2000), reveals that all *Dll4*-expressing cells co-express *hes6* (Fig. 1K), while only a fraction of *Dll1*$^+$ cells do so (Fig. 1J). Together, these findings indicate that the two *Dll* genes are expressed sequentially in differentiating retinal cells, at subsequent steps of the neurogenic program, with *Dll1* expression preceding that of *Dll4* (Fig. 1L).

Dll1- and Dll4-expressing cells contribute to various cell fates in the retina

If, indeed, *Dll1* and *Dll4* are sequentially expressed in differentiating retinal neurons, the two genes must be part of the same neurogenic program in retinal cells, independently of their final fate. To test this prediction, we investigated whether cells expressing *Dll1* or *Dll4* have preferential fates or, instead, can differentiate into any of the various retinal cell types. With this purpose, the fate of retinal cells expressing each *Dll* gene was followed by analysing the expression of beta-galactosidase (β-Gal) in the retinas of heterozygous *Dll1*$^{LacZ/+}$ and *Dll4*$^{LacZ/+}$ mice (Duarte et al., 2004; Hrabe de Angelis et al., 1997), taking advantage of β-Gal perdurance in expressing cells, after *Dll1* or *Dll4* transcription had been switched-off. This allows the identification of cells that have expressed each *Dll* ligand by double immunostaining for β-Gal and specific markers for each retinal cell type, at different stages of retinal development. The results show co-localization of the β-Gal reporter from either *Dll1*$^{LacZ/+}$ or *Dll4*$^{LacZ/+}$ retinas with markers for RGCs (Brn3b, Figs. 2A, B), amacrine cells (CRABP, Figs. 2C, D), horizontal cells (NF165, Figs. 2E, F) and photoreceptors (recoverin, Figs. 2G, H). Thus, our results show that the expression of *Dll1* and *Dll4* is not confined to specific retinal cell types, thereby excluding the hypothesis that the two *Dll* genes participate in distinct differentiation programs, active in separate but co-existing populations of retinal progenitors.

Dll1 loss-of-function in the retina leads to a mild neurogenic phenotype

To further understand the role of Dll/Notch signaling in the neural retina, we analyzed the phenotype of mutant mice where *Dll1* is inactive. To circumvent the early embryonic lethality of *Dll1*$^{-/-}$ null mice (Hrabe de Angelis et al., 1997), the function of the Dll1 ligand was approached by analyzing *Dll1*-floxed mice (Hozumi et al., 2004) expressing Cre recombinase under the control of the *Chx10* promoter (referred to as *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$), which drives Cre expression exclusively in RPCs (Rowan and Cepko, 2004). Our analysis was done from E11 onwards, which corresponds to the onset of retinal neurogenesis (Young, 1985). Since RGCs are the only differentiated neuronal type present in the mouse retina at E11.5 (Carter-Dawson and LaVail, 1979), we started our analysis by looking for the presence of Brn3b (a specific marker for RGCs) and Tuj1 (a pan-neuronal marker in the retina, with the exception of photoreceptors) in control and *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas. No significant difference in the number of RGCs could be observed between normal and mutant retinas at this stage (data not shown). In addition, all Tuj1$^+$ cells are also Brn3b$^+$, in both control and mutant retinas, indicating that no other neuronal type started to differentiate prematurely in *Dll1* mutant retinas.

We then assessed the expression of several bHLH-encoding genes that have been shown to control differentiation of various retinal cell types, namely *Math5*, *Math3*, *Ngn2* and *NeuroD* (reviewed in Hatakeyama and Kageyama, 2004). We found that only *Math5*, a gene implicated in RGC production (Brown et al., 2001), is upregulated in the retinas of *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ mice at E11.5, when compared to control retinas (Figs. 3A, B and data not shown). Thus, although the absence of *Dll1* does not lead to an increase in the number of differentiated retinal neurons at E11.5, the genetic circuitry promoting RGC differentiation seems to be already active in a higher number of RPCs. We therefore analyzed RGC production one day later (E12.5) and found that their number increases 34.2% in the absence of *Dll1* (31.4±0.2% of cells are Brn3b$^+$ in *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas, versus 23.4±1.3% in control retinas, $p<0.05$; Figs. 3C–E). This increase in neurogenesis correlates with a decrease of *hes5* expression (Figs. 3F, G) and an increase of *hes6* expression in E12.5 *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas (Figs. 3H, I), confirming that absence of *Dll1* causes a reduction in Notch signalling and excessive RGC differentiation.

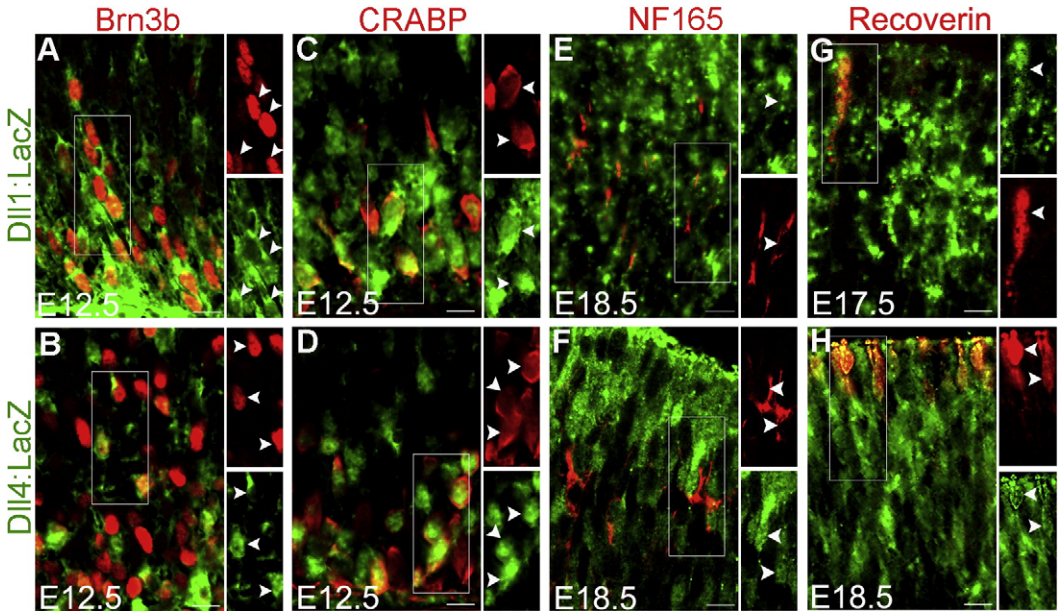


Fig. 2. Cells expressing *Dll1* and *Dll4* give rise to various retinal cell types. Immunostaining for the β-Gal reporter and specific markers for each retinal cell type, in *Dll1*$^{LacZ/+}$ and *Dll4*$^{Lacz/+}$ retinas. Both Dll1::LacZ (A, C, E, G) and Dll4::LacZ (B, D, F, H) cells show co-labelling with markers for retinal ganglion cells (A, B), amacrine (C, D), horizontal (E, F) and photoreceptor cells (G, H). Scale bars = 10 μm.

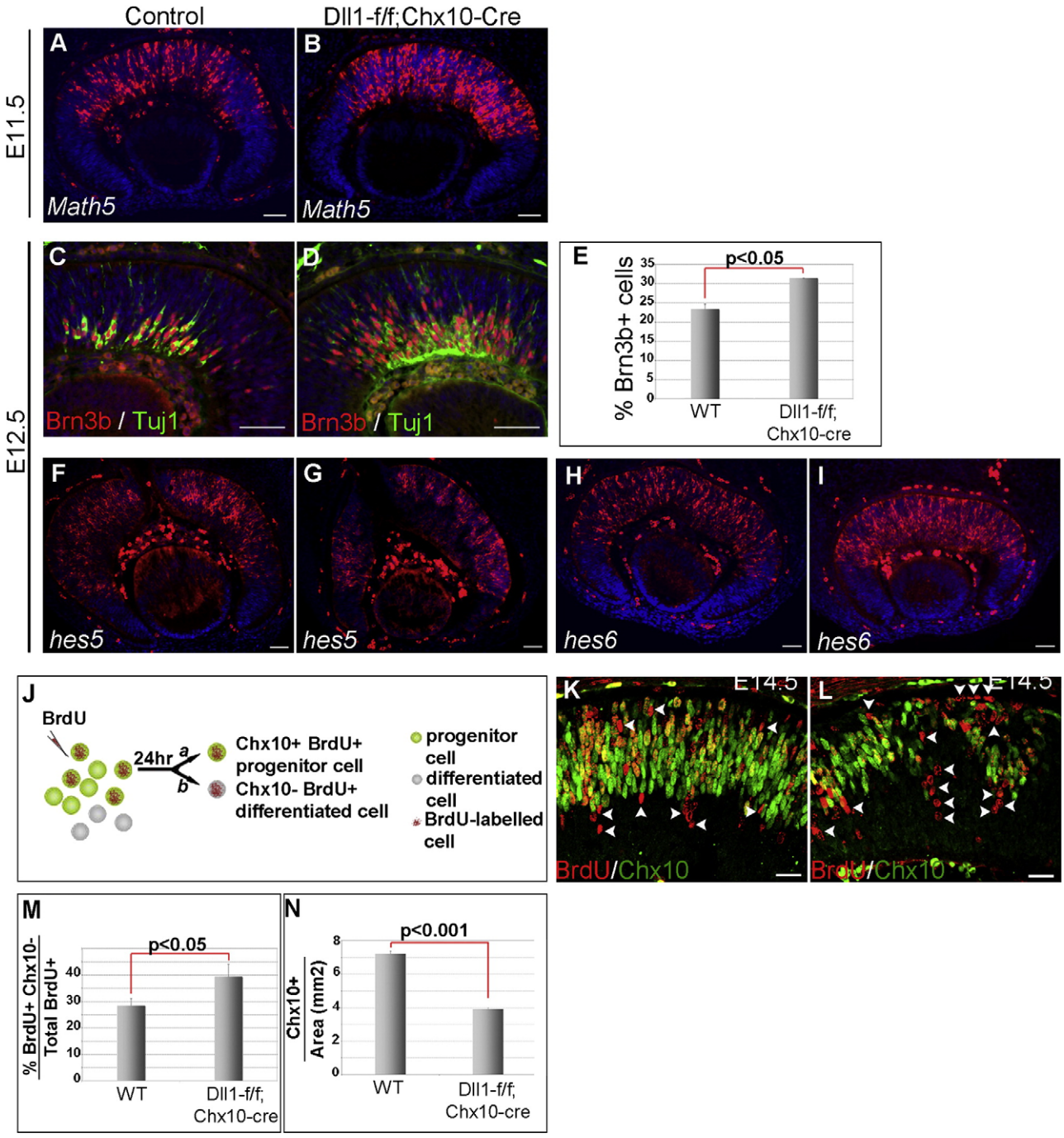


Fig. 3. Enhanced neurogenesis in *Dll1*-mutant retinas. (A, B) At E11.5, mutant retinas (*Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$) show increased *Math5* expression when compared to control littermates. This precedes the increase in RGC differentiation, as shown by the higher number of Brn3b^{+} RGCs detected in mutant E12.5 retinas (C–E). Enhanced neurogenesis is accompanied by a decrease in *hes5* expression (F, G) and an increase in *hes6* expression (H, I), correlating with a reduction in Notch signalling. (J) Scheme depicting the strategy to measure the rate of retinal neurogenesis, through the administration of a single dose of BrdU 24 h prior to dissection. (K, L) Confocal images of sections of wild-type and *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas showing cells that remained as progenitors (BrdU^{+},Chx10^{+}, yellow nuclei) and cells that committed to differentiation (BrdU^{+},Chx10^{-},arrowheads). (M,N) *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas show a ≅38% increase in the rate of neurogenesis when compared to control retinas and a matching decrease in the RPC population (L). Scale bars (A–D, F–I)=50 μm; (M, N)=25 μm.

While the retina at E12.5 produces almost exclusively RGCs, from E12.5 onwards several neuronal types are generated in overlapping periods (Carter-Dawson and LaVail, 1979; Young, 1985). To measure the overall rate of retinal neurogenesis at later stages, we administered a single dose of BrdU at E13.5 and harvested retinas for analysis one day later. Double immunostaining for BrdU and Chx10 allowed us to identify cells (BrdU^{+}/Chx10^{-}) that had exited the cell cycle and initiated differentiation during this 24 h period (Fig. 3J). Although the epithelial architecture of *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas is disturbed (see below), the number of BrdU^{+}/Chx10^{-} cells in relation to the total number of BrdU^{+} cells reveals an enhanced rate of neurogenesis in *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas when compared to control retinas (Figs. 3K–M), with an overall increase of 38.6% (control=28.4±2.7%; *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$=39.4±4.6%; $p<0.05$). In parallel, we also observed a significant reduction (~45%) in the number of Chx10^{+} RPCs in *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$ retinas, when compared with control retinas (control=7.2±0.16 cells/mm^{2}; *Dll1*$^{f/f}$,*Chx10*$^{Cre/+}$=3.9±0.09 Chx10^{+} cells per mm^{2}; $p<0.001$; Fig. 3N). Thus, although the neural retina expresses two distinct Notch ligands, *Dll1* and *Dll4*, our results indicate that the absence of *Dll1* is sufficient to increase the rate of neurogenesis throughout several timepoints of retinal development and cause a

significant depletion of progenitors, as a result of the reduction in Notch signalling activity.

Disruption of retinal morphology in the Dll1 mutants

A likely consequence of the RPC depletion is the observed disruption of the retinal neuroepithelium in *Dll1*-mutant retinas (Fig. 4) at E14.5, with the appearance of rosette-like structures similar to those described in *Notch1* and *Hes1* mutant retinas (Jadhav et al., 2006; Tomita et al., 1996; Yaron et al., 2006). These rosettes are formed mainly by Chx10^{+} RPCs, organized around a central lumen, and are interspersed with regions of the neuroepithelium where differentiated neurons accumulate (Fig. 4B). Immunostaining for N-Cadherin, ZO-1 and PAR3 reveals that these progenitors are polarized, with the apical side forming the luminal surface where they enter mitosis like in a normal retinal neuroepithelium (Figs. 4C–E and data not shown). Within these rosettes, progenitors are engaged in neurogenesis and competent to produce all retinal cell types, which maintain their spatial relationships in the rosette neuroepithelium, as shown by the appearance of recoverin-positive photoreceptors close to the apical lumen in E17.5 rosettes or the presence of horizontal cells at intermediate positions of the neuroepithelial rosettes (Fig. S1 in supplementary material).

The formation of "progenitor cell rosettes" indicates that *Dll1* inactivation does not lead to a complete exhaustion of RPCs, most likely due to some remaining Notch activity, as revealed by the persistent expression of *hes5* in rosettes from E14.5 mutant retinas (Figs. 4F, G). This might be due to mosaic expression of the Cre recombinase driven by the *Chx10* promoter, allowing the emergence of cells where *Dll1* has not been inactivated and is still able to signal. However, this hypothesis is unlikely, as a similar rosette phenotype can be observed in recombination-independent retinas of embryos

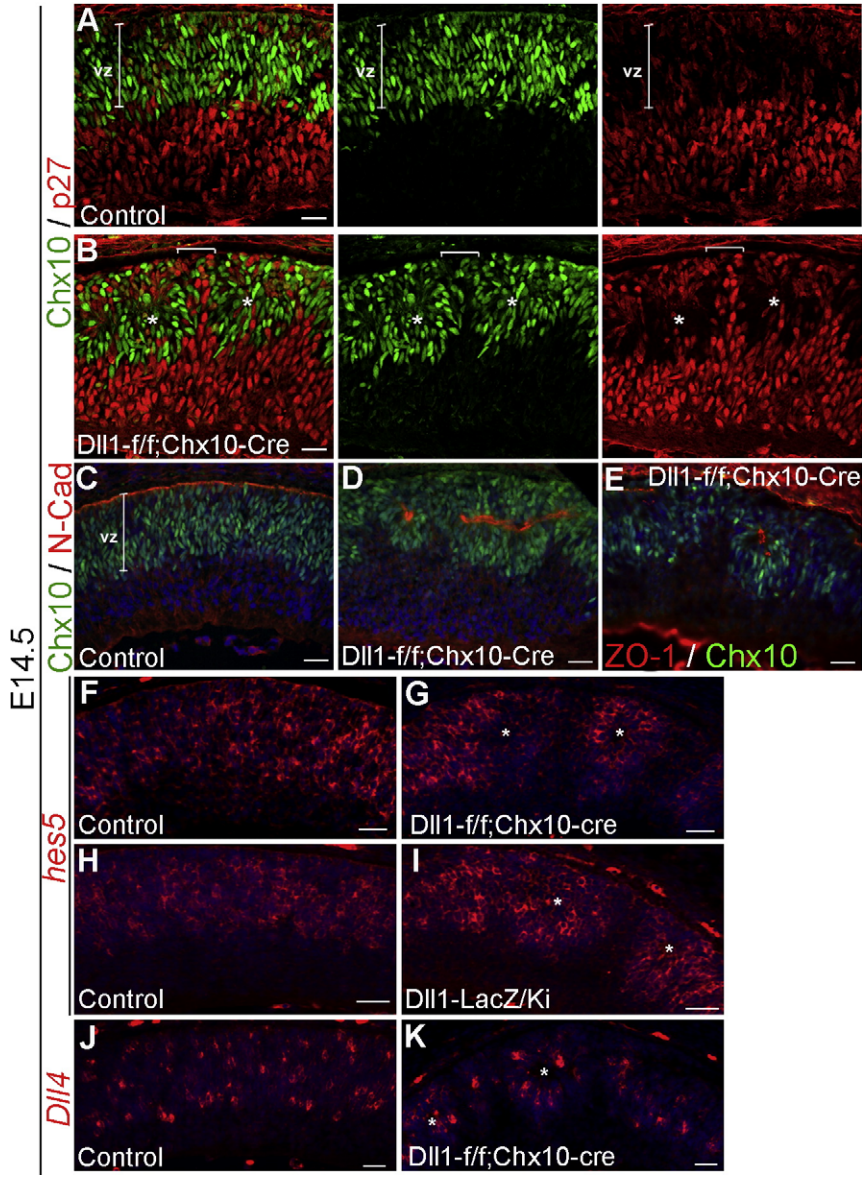


Fig. 4. *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas show disruption of neuroepithelial organization, with the appearance of rosette-like structures and maintenance of Notch activity. (A) In the wild-type E14.5 retinal epithelium, Chx10^{+} progenitors are located in the VZ while p27+ neurons accumulate outside this region. (B) In E14.5 *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas, Chx10^{+} progenitors are arranged in rosettes (asterisks) and surrounded by post-mitotic neurons (p27^{+}) that form apical-to-basal "corridors" of ectopically differentiated neurons (bracket). (C–E) Retinal cells within rosettes maintain their apico-basal polarity, with N-Cadherin and ZO1 being present at the center of rosettes in *Dll1*-mutant retinas. (F, G) Expression of the Notch target, *hes5*, in wild-type and *Dll1$^{f/f}$,Chx10$^{Cre/+}$* retinas show that, despite the absence of *Dll1*, *hes5* is still expressed in progenitors within rosettes (asterisks). (H, I) A different combination of mutant alleles, *Dll1$^{LacZ/Ki}$* (one null and one hypomorphic allele), displays a similar rosette phenotype and maintenance of Notch activity. (J, K) *Dll4* expression is maintained in *Dll1*-mutant retinas. Scale bars (A, B)=25 μm; (C–K)=50 μm.

heteroallelic for a null and a hypomorphic allele of *Dll1* (*Dll1*$^{LacZ/ki}$) (Schuster-Gossler et al., 2007), which also show increased neurogenesis (Figs. 4H, I and data not shown). Thus, the remaining Notch activity and the persistence of RPCs in *Dll1*-mutant retinas are unlikely to be due to incomplete gene inactivation. Instead, our findings might easily be accounted for by the activity of *Dll4*, which continues to be expressed in *Dll1*-mutant retinas (Figs. 4J, K) and could signal to maintain a pool of RPCs.

Both Dll1 and Dll4 contribute to progenitor maintenance in the neuroepithelium

To address the role of each Dll ligand in the genetic circuitry regulating neurogenesis, we turned our attention to the developing spinal cord, where *Dll1* and *Dll4* are also expressed within the neurogenic zone, in both unique and overlapping patterns. *Dll1* is expressed throughout most of the dorsal–ventral axis in the VZ, being excluded from the *Jag1*-expressing domains (Lindsell et al., 1995; Myat et al., 1996), while *Dll4* expression is restricted to the pV2 domain, where *Dll1* is also expressed (Benedito and Duarte, 2005; Del Barrio et al., 2007; Peng et al., 2007).

To investigate in more detail the expression pattern of *Dll1* and *Dll4* in the pV2 domain and compare it to the retina, we first performed double in situ hybridization in sections of E11.5 mouse NT. We could detect coexpression of the two *Dll* genes in cells of the pV2 domain, as well as some cells expressing only one of the ligands (Fig. 5A). We next compared the localization of Dll1:LacZ and Dll4:LacZ cells in spinal cords of *Dll1*$^{LacZ/+}$ and *Dll4*$^{LacZ/+}$ embryos, respectively. While Dll1:LacZ cells can still be detected in the VZ, Dll4:LacZ cells are only located in the mantle zone (Figs. 5B, C), revealing that *Dll1* starts to be expressed earlier than *Dll4* in differentiating V2 interneurons. Together with the in situ data, these results indicate that the expression of *Dll1* and *Dll4* during neurogenesis in the pV2 domain closely resembles what we have described above for the neural retina.

The pV2 domain, however, provides a simpler system to study the function of these two genes during neurogenesis, as it gives rise to only two neuronal subtypes, V2a and V2b (Karunaratne et al., 2002), in contrast with the retina where seven major cell types are generated (reviewed in Cepko, 1996). To analyse the effects of *Dll1* inactivation in the pV2 domain, *Dll1*-floxed mice were crossed with Nestin-Cre mice that express Cre recombinase in neural tube progenitors (Tronche et al., 1999). Embryos were collected at E11.5 and neuronal production in the spinal cord was evaluated at the forelimb level, in control and *Dll1*$^{f/f}$,*Nestin*$^{Cre/+}$ NTs. Our results reveal an increase in the number of V2 interneurons in *Dll1* mutant embryos, when compared to control embryos, indicating that *Dll1* is necessary to regulate neuronal differentiation in the pV2 domain (Fig. 5D). This is in agreement with previous work on *Presenilin1* and *Notch1* mutants (Del Barrio et al., 2007; Peng et al., 2007; Yang et al., 2006), where pV2 progenitors have also been shown to differentiate in excess. However, in the NT of *Dll1*-mutant embryos, Notch signaling is still active and progenitors are still present in the pV2 domain, albeit in reduced numbers, as

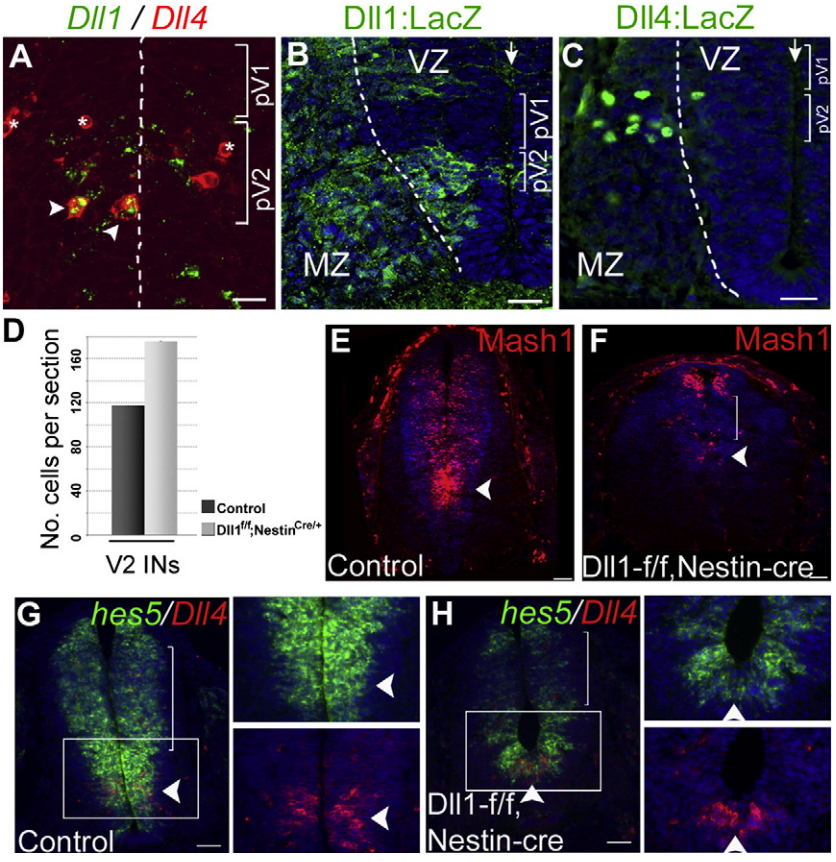


Fig. 5. Neurogenesis in the pV2 domain of E11.5 *Dll1*-mutant embryos. (A) Close view of *Dll1* and *Dll4* expression in the pV2 domain, showing co-expression of the two genes in some V2 cells (arrowheads; asterisks mark red blood cells and dashed-line indicates the midline). (B, C) Immunostaining for the β-Gal reporter in *Dll1*$^{LacZ/+}$ and *Dll4*$^{LacZ/+}$ mice shows Dll1:LacZ expression in the VZ, while Dll4:LacZ is only detected in cells that reside already in the MZ (arrows indicate lumen of NT). (D) The production of V2 interneurons is increased in *Dll1* mutants, relatively to control neural tubes. (E, F) A small population of Mash1^{+} progenitors are maintained in the pV2 domain of *Dll1*-mutant embryos (arrowhead in E, F). Note that more dorsal progenitors in the domains expressing only Dll1 (bracket) are severely reduced in the *Dll1*-mutant embryos (F) comparatively to control embryos (E). (G, H) Notch signalling is still active in pV2 progenitors of *Dll1*-mutant NTs, as shown by the strong *hes5* expression close to the most ventral aspect of the remaining lumen (arrowhead in H), in contrast with the severe reduction of *hes5* transcripts in other spinal cord domains where only *Dll1* is expressed (bracket in H). *Dll4* expression is also maintained in the remaining pV2 domain of *Dll1*-mutant embryos (arrowhead in H). In B, C and D–H, nuclei are visualized in blue by DAPI staining. VZ — ventricular zone; MZ — mantle zone; INs — interneurons; Scale bars (A)=20 μm; (B, C)=25 μm; (E–H)=50 μm.

shown by the presence of Mash1^{+} pV2 progenitors close to the remaining lumen in the most ventral domain of mutant spinal cords (Figs. 5E, F). In addition, *Dll4* is still expressed in the remaining pV2 domain of *Dll1*-mutant embryos, together with the Notch target *hes5* (Figs. 5G, H), confirming that, like in the retina, Dll4 is activating Notch and maintaining progenitors in the pV2 domain.

Together, these results show that both Dll1 and Dll4 contribute to activate Notch signalling in neighbouring progenitors to prevent their differentiation, in the pV2 domain as well as in the retina.

Dll1 is the main ligand responsible for progenitor maintenance in the spinal cord

Even though both Dll ligands seem to contribute to progenitor maintenance, the earlier and broader expression of *Dll1* in the neuroepithelium makes it a better candidate to be the main player in maintaining a pool of progenitors throughout neurogenesis. To test this, we monitored neurogenesis in control and *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos, focusing first in the NT domains where *Dll1* is the sole ligand to be expressed. At E10.5, we could not detect any significant difference in the number of neurons in wild-type and *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos (data not shown). At E11.5, however, mutant NTs are severely affected (Figs. 6A, B) and massive neuronal differentiation is observed. Concomitantly, the progenitor population is severely depleted and a striking reduction in NT size is observed at E12.5, when compared with wild-type littermates (Figs. 6C, D). Nonetheless, a cluster of progenitors remains at the center of the deformed NT in *Dll1$^{f/f}$, Nestin$^{Cre/+}$* embryos at E11.5 and E12.5 (Fig. 6B). These include the remaining pV2 progenitors (Figs. 5F, H), as well as progenitors from the *Jag1*-expressing domains (Figs. 6E, F), namely the pV1 and pD6 domains (Matise and Joyner, 1997, and our unpublished results). We could also detect *hes5* expression in these domains, showing that Notch signaling is active and preventing progenitor differentiation, in contrast to the domains where *Dll1* is inactive and no other ligand is expressed (Figs. 6E, F).

Quantification of the different neuronal types produced from the various spinal cord DV domains confirms the excessive neuronal differentiation from all *Dll1*-expressing domains in *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos (Fig. 6G), when compared with control embryos. However, the number of V1 interneurons derived from a *Jag1*-expressing domain is not altered. Also, the most ventral neuronal types (V3 interneurons and motoneurons) seem unaffected, most likely because

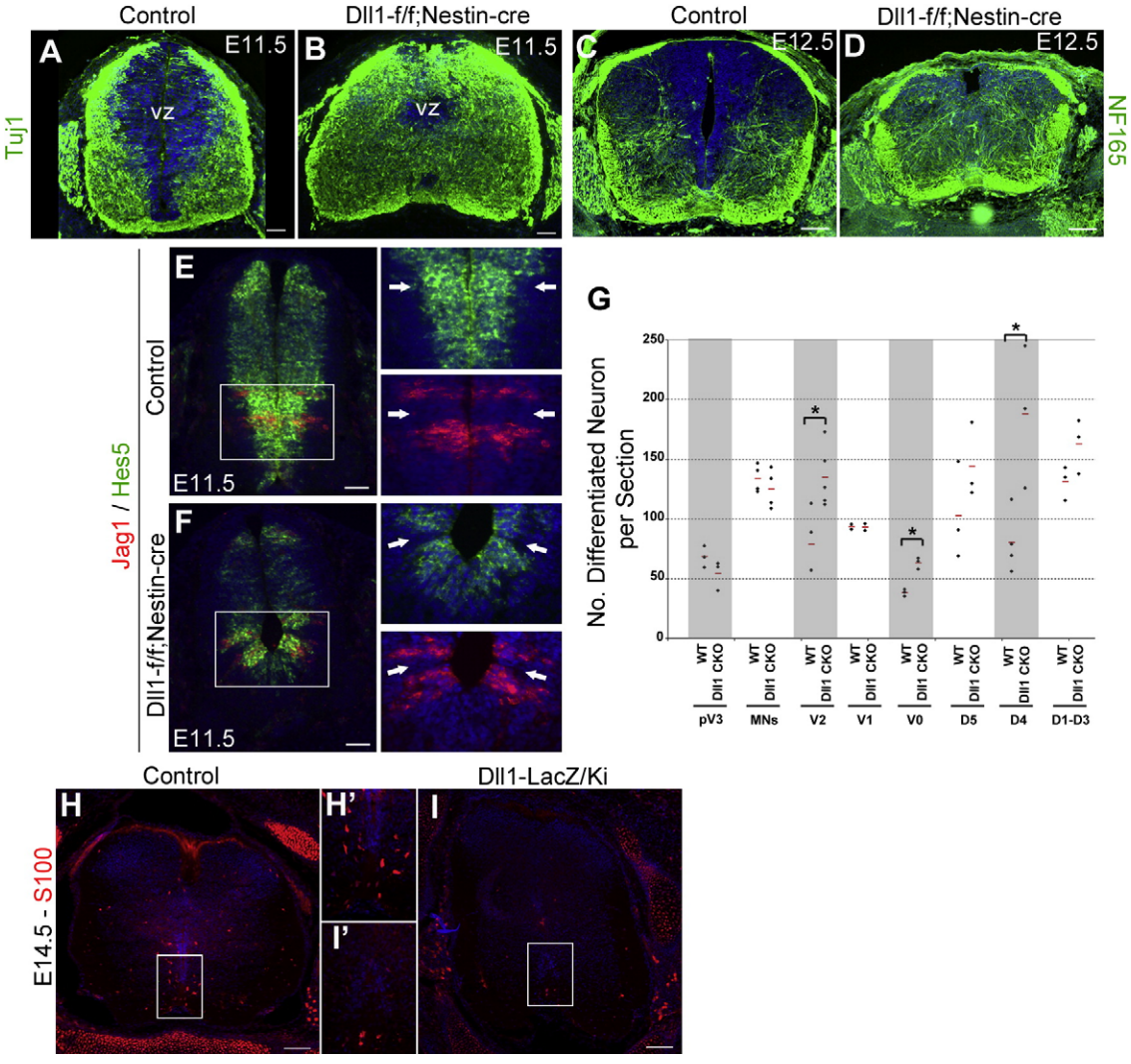


Fig. 6. Neurogenic phenotype in the NT of *Dll1$^{f/f}$,Nestin$^{Cre/+}$* embryos. (A, B) Massive neuronal differentiation can be observed in *Dll1$^{f/f}$,Nestin$^{Cre/+}$* NTs at E11.5, which are almost devoid of ventricular zone (VZ). (C, D) At E12.5, progenitor depletion leads to an atrophy of the NT. Excess of neurons are visualized with the anti-NF165 antibody. (E, F) Expression of *hes5* shows that Notch signalling is still active in the *Jag1*-expressing domains of *Dll1$^{f/f}$,Nestin$^{Cre/+}$* NTs, contrasting with the adjacent *Dll1*-expressing pV0 domain (arrows in E, F) where *hes5* expression is abolished (F). (G) Quantification of neuronal cell types produced in the various NT domains of wild-type and *Dll1*-mutant embryos, at E11.5. (H,I) Immunostaining with S100 antibody shows many astrocytes in the E14.5 wild-type neural tube (H, H′), while almost none are observed in *Dll1* mutant embryos (I, I′). Scale bars (A, B, E, F, H, I)=50 μm; (C, D)=100 μm.

of the weak Nestin-Cre activity at the time when these ventral neurons are produced (see Fig. S2 in supplementary material).

Another consequence of the progenitor depletion in *Dll1* mutant NTs is the reduced generation of glial cells, which are born later from the same progenitors that first give rise to neurons (Mizuguchi et al., 2001; Novitch et al., 2001; Zhou et al., 2001). Immunodetection of S100, a marker of glial cells, shows a clear decrease in the production of this late cell type in the absence of *Dll1* activity (Figs. 6H, I). The few remaining glial cells are possibly derived from the *Jag1*-expressing and/or pMN domains that remain unaffected in *Dll1* mutant NTs.

Together, these results show that, in the spinal cord domains where *Dll1* is the only expressed Notch ligand, its activity is necessary and sufficient to maintain progenitors and ensure their availability throughout neurogenesis.

Dll4 functions to generate neuronal diversity in the pV2 domain

The results shown in previous sections reveal that Dll4 can also contribute to progenitor maintenance in the retina and in a restricted domain of the spinal cord, where it shares this function with Dll1. However, this is unlikely to be the major role of Dll4 during neurogenesis as it, alone, could not provide enough Notch activity to maintain a normal pool of progenitors in the absence of Dll1. Then, what other functions does Dll4 have in the retina and pV2 domain? A possible answer arises from the analysis of neuronal differentiation in the pV2 domain of *Dll1*-mutant embryos, a condition where Dll4 is now the only active Notch ligand. In these embryos (E11.5, $Dll1^{f/f}$; $Nestin^{Cre/+}$), both V2a and V2b interneurons are produced, with no significant alteration in the population of V2b interneurons and an increase in the V2a population, when compared to control embryos (Figs. 7A–E). These results contrast with what has been previously reported in *Presenilin1* and *Notch1* mutants, where abolition of Notch signaling causes all pV2 progenitors to differentiate as V2a interneurons, at the expense of the V2b fate (Del Barrio et al., 2007; Peng et al., 2007). Our finding that both neuronal subtypes are still produced in the V2 region of *Dll1*-mutant spinal cords therefore implies that Dll1 is dispensable for the V2a–V2b binary decision, which is most likely controlled by Dll4-mediated Notch signalling. Analysis of neuronal differentiation in *Dll1* mutant retinas shows also that the various retinal cell types keep being produced when Dll4 is the only active Notch ligand (see Fig. S1 in supplementary material). Together, these results suggest that *Dll1* and *Dll4* have overlapping but also different functions during neurogenesis in the retina and pV2 domain: while *Dll1* is the main player controlling the entry of progenitors into differentiation, *Dll4* regulates the acquisition of distinct fates by differentiating neurons.

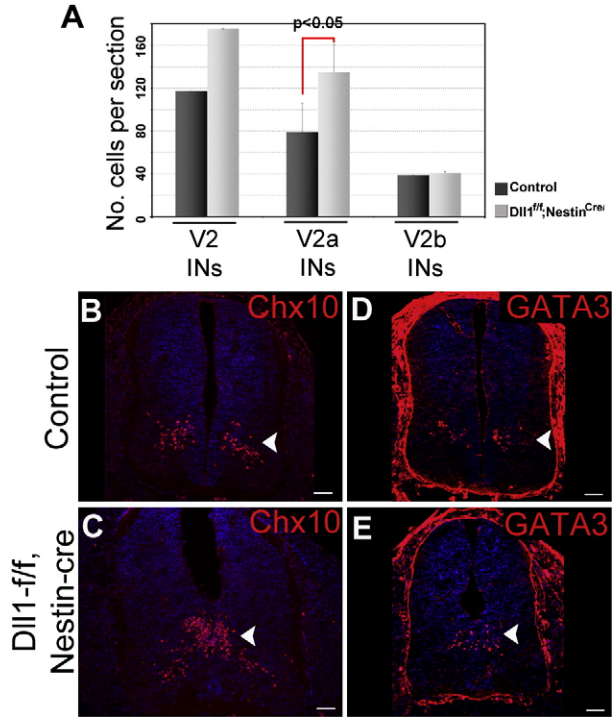


Fig. 7. Both V2a and V2b are produced in the V2 domain of *Dll1*-mutant spinal cords. The overall increase in V2 interneuron production in *Dll1*-mutant pV2 domain (A) is due to an increase of the $Chx10^{+}$ V2a subtype (B, C), without alterations in the generation of the $GATA3^{+}$ V2b subtype (D, E). Scale bars (B–E)=50 μm.

Discussion

In this work, we have shown that neurogenesis in the mouse retina and in the V2 domain of the spinal cord is regulated by the activity of two distinct Dll ligands, Dll1 and Dll4. We report that the two *Dll* genes are transiently co-expressed in differentiating neurons, although *Dll1* starts to be expressed before *Dll4*, when cells commit to differentiation. This led us to ask whether the two Dll ligands regulate similar or different phases of the neurogenesis process. Our results show that both Dll1 and Dll4 can activate Notch signaling in neural progenitors of the retina and pV2 domain, contributing in this way to regulate the rate of neurogenesis in these regions of the developing CNS. In addition, analysis of *Dll1* mutants reveals that the Dll1 ligand is the major player in this process, while Dll4 might play a key function during the establishment of cell diversity within the population of retinal and V2 neurons.

The sequential expression of Dll1 and Dll4 in the developing retina

In the developing nervous system of vertebrates, Notch signaling is generally viewed as a "universal" regulator of neuronal differentiation, being essential to maintain a population of neural progenitors throughout the extended period of neurogenesis. Signaling in these progenitors is activated by contact of Notch receptors with active ligands in adjacent cells and results in the inhibition of their differentiation (reviewed in Louvi and Artavanis-Tsakonas, 2006; Yoon and Gaiano, 2005). In this paper, we focused on the role of two Dll ligands, Dll1 and Dll4, and performed a detailed analysis of their expression during neurogenesis in the mouse retina and spinal cord. We show that the two *Dll* genes are sequentially expressed in cells entering neuronal differentiation, with *Dll1* starting to be expressed before *Dll4*. This conclusion is supported by the following evidence:

a) In the marginal regions of the retina, *Dll1* expression is detected in cells at more peripheral locations than *Dll4*-expressing cells. Given the central-to periphery wave of neurogenesis, whereby peripheral cells are less advanced in differentiation than more central cells (Prada et al., 1991), this implies that *Dll1* starts to be expressed at an earlier phase of the differentiation process than *Dll4*.
b) *Dll1* expression can be detected in retinal cells still expressing the progenitor marker Chx10 (Rowan and Cepko, 2004), while no *Dll4*-expressing cell show expression of this marker. On the contrary, all *Dll4*-expressing cells express *hes6*, a gene which is active in differentiating neurons (Koyano-Nakagawa et al., 2000), while only a fraction of the cells coexpress *Dll1* and *hes6*.
c) Double in situ hybridization reveals that the majority (~80%) of *Dll4*-expressing cells also express *Dll1*, thereby showing that expression of the two *Dll* ligands occur in the same population of differentiating neurons and not in separate populations. Since *Dll1* starts to be expressed before *Dll4*, we can therefore conclude that the two genes are sequentially expressed in this population.

In addition, analysis of retinas from $Dll1^{LacZ/+}$ and $Dll4^{LacZ/+}$ mice at E12.5 reveals that *Dll1* is expressed in all RGCs during their progression into differentiation (Fig. 2A), whereas *Dll4* is expressed in only a subset

of RGCs. This implies that, at least for RGCs, all *Dll4*-expressing cells must have gone through a phase of *Dll1* expression. Moreover, it suggests that the minor population of retinal cells expressing only *Dll4*, are cells that have already switched-off *Dll1* but still express *Dll4*, before advancing into the next stages of differentiation.

Taken together, our findings support a model (Fig. 1L) where the two *Dll* ligands are expressed sequentially in differentiating retinal neurons, starting with *Dll1* expression when cells commit to differentiation. It follows a period where *Dll1* and *Dll4* are both transcribed in newborn neurons, after which the expression of *Dll1* fades away faster than that of *Dll4*, with both genes being finally switched off, before overt neuronal differentiation. The same model applies to the differentiation of V2 interneurons in the developing spinal cord, where we also show that *Dll1* starts to be transcribed before *Dll4* in pV2 progenitors (Figs. 5B, C).

In a recent work by Reh and collaborators, a similar sequential expression pattern for the *Dll1* and *Dll4* genes was found in the chick retina (Nelson and Reh, 2008), with *Dll1* being already expressed in mitotically active progenitors, while *Dll4* was only transcribed in differentiating neurons. However, the authors were not able to discern whether *Dll1* and *Dll4* are expressed in the same or in separate populations of retinal cells. We now show, in the mouse retina and pV2 domain of the spinal cord, that the two *Dll* genes are sequentially expressed in the same population of differentiating neurons, implying that *Dll1* and *Dll4* are part of the same neurogenic program active during neurogenesis in these regions of developing CNS.

One question that remains to be addressed in future work is why only a subset of differentiating retinal neurons expresses *Dll4*, in contrast with the broader expression of *Dll1*. One possible mechanism is based on previous findings in zebrafish embryos, where the singling-out of a primary neuron expressing *DeltaB* from a cluster of cells expressing two other *Delta* genes (*DeltaA* and *DeltaD*) is mediated by Notch activity (Haddon et al., 1998). In the mouse retina, a similar process might be involved and a *Dll4*-expressing cell could emerge from a cluster of cells expressing *Dll1* through a process involving Notch-mediated lateral inhibition. However, this singling-out is unlikely to be mediated by *Dll1*, as we could not observe a predictable increase in the number or density of *Dll4*-expressing cells in the neuroepithelium of *Dll1* mutant embryos (Figs. 4J, K and 5G, H).

A single Notch ligand is necessary and sufficient to regulate neurogenesis

In contrast to the retina and pV2 domain, cells entering differentiation in other regions of the NT express just one Notch ligand, either *Jag1* or *Dll1*, which are transcribed in separate cell populations. Another *Dll* gene, *Dll3*, is transcribed later in differentiating neurons but its expression does not overlap with that of *Dll1* or *Jag1* (Campos et al., 2001; Dunwoodie et al., 1997). In the developing retina, other ligands are also expressed but in different patterns relatively to *Dll1* and *Dll4*: *Jag1* is only transcribed in cells of the ciliary margin and lens (Bao and Cepko, 1997; Lindsell et al., 1996), while *Jag2* is expressed in differentiated RGCs after they reach the ganglion cell layer (Valsecchi et al., 1997). Altogether, these different patterns of Notch ligands in the developing mammalian CNS highlight the operation of distinct genetic circuitries controlling neurogenesis in the nervous system.

In this paper, we analyse and compare the function of two of these Notch circuitries, one active in the spinal cord and involving only *Dll1*, and another involving the coexpression of *Dll1* and *Dll4* in the retina and pV2 domain. We show that inactivation of *Dll1* in the spinal cord domains where it is the sole expressed ligand leads to a severe neurogenic phenotype, with excessive neuronal differentiation and depletion of neural progenitors. This phenotype is very similar to that described for *Hes1*;*Hes3*;*Hes5* triple mutant embryos (Hatakeyama et al., 2004), in which complete impairment of Notch signalling results also in total depletion of neural progenitors. However, inactivation of *Dll1* does not disturb Notch signalling in adjacent domains of the spinal cord expressing *Jag1*, where neural progenitors are preserved and neuronal production is normal. We can thus conclude that the activity of a single ligand is sufficient (and necessary) to maintain neural progenitors throughout neurogenesis. In addition, our results imply that Jag1 performs the same function as Dll1 in the domains of the developing spinal cord where it is expressed, activating Notch signalling in progenitors to control their differentiation. Whether Jag1 might play other functions in these domains is not yet clear, but the analysis of conditional *Jag1* mutants and *Dll1*/*Jag1* double mutants might provide some answers to this question.

A unique circuitry involving two Dll ligands to regulate neurogenesis

Although the general rule in the developing spinal cord seems to be that a single Notch ligand is enough to regulate neuronal production, our work shows that, in the pV2 domain and in the neural retina, two ligands — Dll1 and Dll4, are active during neurogenesis in an overlapping population of differentiating neurons. This raises the question of why these particular regions of the developing CNS employ a different Notch circuitry to regulate neuronal production. One explanation might be related to the fact that the generation of various neuronal cell types in the retina and pV2 domain is not separated in time nor in space, in contrast with the other regions of the spinal cord where different cell types arise from compartmentalized domains of the neuroepithelium and are born at different times. In these domains, Notch signalling is mainly required to maintain a pool of progenitors capable of generating all the successive cell types. To accomplish this function, our experiments show that the activity of a single ligand is necessary and sufficient. On the contrary, in the retina and pV2 domain, progenitors are multipotent and not constrained by their location, with the various cell types being produced simultaneously throughout the neurogenic period (Carter-Dawson and LaVail, 1979; Young, 1985; Peng et al., 2007). The final fate of cells in these regions is therefore decided in situ through cell–cell interactions and our work implies that a novel circuitry involving the sequential action of two Dll ligands is at work to coordinate two consecutive steps of neuronal production: first, to control the rate of neurogenesis and, second, to create cell diversity in the population of differentiating neurons.

As a result, inactivation of *Dll1* in these regions does not cause a neurogenic phenotype as severe as the one observed in other regions expressing only *Dll1*. In the retina, for instance, absence of Dll1 causes an initial acceleration of neurogenesis and a reduction in the progenitor population, leading to disruption of the neuroepithelium and formation of neural rosettes. However, *Dll4* continues to be expressed and Notch signaling is still active in *Dll1* mutant retinas, preventing a complete depletion of retinal progenitors. Moreover, we observed that the remaining progenitors in *Dll1* mutant retinas give rise to the normal set of retinal cell types. This is different from what has been described in *Notch1* mutant retinas (Jadhav et al., 2006; Yaron et al., 2006) which, in addition to the excessive differentiation of progenitors and formation of neural rosettes, show also alterations in cell type diversity, with cone photoreceptors being overproduced at the expense of other cell types like RGCs and amacrine cells. Taken together, these findings reveal that Notch activity in the developing retina not only provides a signal to sustain progenitors during neurogenesis but it is also essential to generate cell diversity in the population of retinal neurons. In addition, our results support a model in which these two functions of Notch signaling are carried out by a novel circuitry comprising the sequential activity of two Dll ligands, with Dll1 providing the major contribution to progenitor maintenance and Dll4 regulating the acquisition of diverse cell fates by differentiating neurons.

In the pV2 domain of the spinal cord, where the Dll1/Dll4 circuitry is also active, previous work points towards a similar role for Dll4 in generating cell diversity (Del Barrio et al., 2007; Peng et al., 2007). In

this domain, bipotential pV2 progenitors are known to give rise to two distinct cell fates (V2a and V2b) in a process that is mediated by Notch signalling, as shown by the excess of V2a neurons in mutants without Notch activity (Del Barrio et al., 2007; Peng et al., 2007), at the expense of V2b neurons. Moreover, gain-of-function experiments in the chick embryo indicate that only Dll4, and not Dll1, is able to activate this function of Notch signalling during V2 neurogenesis. We now show that *Dll1* is indeed dispensable during the choice of V2a versus V2b fate, as both cell types are produced in *Dll1*-mutant embryos, implying that *Dll4* is the major player in the V2a/V2b decision. Nonetheless, we have found that production of V2a neurons is increased in *Dll1* mutant embryos, whereas the number of V2b neurons is unaltered. This is likely due to a higher number of pV2 progenitors entering differentiation in the absence of *Dll1* and acquiring the primary V2a fate. Then, as *Dll4* starts to be expressed, it manages to maintain the remaining pV2 progenitors and, simultaneously, ensures that the two V2 fates are generated, balancing their production. The end result is the production of a normal number of V2b neurons and an excess of V2a neurons, due to the initial burst in their generation.

Two ligands and two outcomes of Notch signalling

The next challenge will be to understand how the *Dll1*/*Dll4* circuitry operates to mediate the two sequential functions of Notch signalling. Our results support a model where *Dll1* acts first to regulate the timing of progenitor differentiation while *Dll4* functions in a second step to control cell fate diversity. However, our results show that there is a period in the life of pV2 and retinal cells when the two *Dll* genes are simultaneously expressed, making it difficult to understand how the two proposed functions of Notch signaling can each be ascribed to one of the Dll ligands. A possible mechanism to explain the different activities of each ligand is based on the finding that Dll4's interaction with Notch1 is stronger than the Dll1–Notch1 interaction (Besseyrias et al., 2007), raising the hypothesis that Dll4 produces a longer lasting Notch1 activation in target cells. One can thus speculate that the weaker Dll1 signal is just enough to prevent retinal progenitors from committing to differentiation, while Dll4-mediated inhibition might last enough time for the progenitors' intrinsic competence program to advance and acquire the capacity to generate a different array of retinal cell types, when released from Notch inhibition. We therefore propose that the two functions of Notch signalling in the retina and pV2 domain, namely maintaining progenitors and generating cell diversity, are based on the sequential activity of the two Dll ligands, coupled with the evolving competence of retinal progenitors. Future experiments shall address the mechanisms underlying these Notch functions in the retina and pV2 domain.

Acknowledgments

We thank Sara Ferreira and Alina Costa for excellent technical help and all lab members for help and critical reading of the manuscript. This work was supported by Fundação para a Ciência e Tecnologia — FCT (PTDC/SAU-OBD/75106/2006). S.F.R. and S.L. were recipients of FCT fellowships.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.01.011.

References

Bao, Z.Z., Cepko, C.L., 1997. The expression and function of Notch pathway genes in the developing rat eye. J. Neurosci. 17, 1425–1434.
Bardin, A.J., Le Borgne, R., Schweisguth, F., 2004. Asymmetric localization and function of cell-fate determinants: a fly's view. Curr. Opin. Neurobiol. 14, 6–14.
Benedito, R., Duarte, A., 2005. Expression of Dll4 during mouse embryogenesis suggests multiple developmental roles. Gene Expr. Patterns 5, 750–755.
Besseyrias, V., Fiorini, E., Strobl, L.J., Zimber-Strobl, U., Dumortier, A., Koch, U., Arcangeli, M.L., Ezine, S., Macdonald, H.R., Radtke, F., 2007. Hierarchy of Notch–Delta interactions promoting T cell lineage commitment and maturation. J. Exp. Med. 204, 331–343.
Brooker, R., Hozumi, K., Lewis, J., 2006. Notch ligands with contrasting functions: Jagged1 and Delta1 in the mouse inner ear. Development 133, 1277–1286.
Brown, N.L., Patel, S., Brzezinski, J., Glaser, T., 2001. Math5 is required for retinal ganglion cell and optic nerve formation. Development 128, 2497–2508.
Campos, L.S., Duarte, A.J., Branco, T., Henrique, D., 2001. mDll1 and mDll3 expression in the developing mouse brain: role in the establishment of the early cortex. J. Neurosci. Res. 64, 590–598.
Carter-Dawson, L.D., LaVail, M.M., 1979. Rods and cones in the mouse retina. II. Autoradiographic analysis of cell generation using tritiated thymidine. J. Comp. Neurol. 188, 263–272.
Cau, E., Quillien, A., Blader, P., 2008. Notch resolves mixed neural identities in the zebrafish epiphysis. Development 135, 2391–2401.
Cepko, C.L., 1996. The patterning and onset of opsin expression in vertebrate retinae. Curr. Opin. Neurobiol. 6, 542–546.
Daudet, N., Lewis, J., 2005. Two contrasting roles for Notch activity in chick inner ear development: specification of prosensory patches and lateral inhibition of hair-cell differentiation. Development 132, 541–551.
Del Barrio, M.G., Taveira-Marques, R., Muroyama, Y., Yuk, D.I., Li, S., Wines-Samuelson, M., Shen, J., Smith, H.K., Xiang, M., Rowitch, D., et al., 2007. A regulatory network involving Foxn4, Mash1 and delta-like 4/Notch1 generates V2a and V2b spinal interneurons from a common progenitor pool. Development 134, 3427–3436.
Duarte, A., Hirashima, M., Benedito, R., Trindade, A., Diniz, P., Bekman, E., Costa, L., Henrique, D., Rossant, J., 2004. Dosage-sensitive requirement for mouse Dll4 in artery development. Genes Dev. 18, 2474–2478.
Dunwoodie, S.L., Henrique, D., Harrison, S.M., Beddington, R.S., 1997. Mouse Dll3: a novel divergent Delta gene which may complement the function of other Delta homologues during early pattern formation in the mouse embryo. Development 124, 3065–3076.
Fior, R., Henrique, D., 2005. A novel hes5/hes6 circuitry of negative regulation controls Notch activity during neurogenesis. Dev. Biol. 281, 318–333.
Haddon, C., Smithers, L., Schneider-Maunoury, S., Coche, T., Henrique, D., Lewis, J., 1998. Multiple Delta genes and lateral inhibition in zebrafish primary neurogenesis. Development 125, 359–370.
Hatakeyama, J., Kageyama, R., 2004. Retinal cell fate determination and bHLH factors. Semin. Cell Dev. Biol. 15, 83–89.
Hatakeyama, J., Bessho, Y., Katoh, K., Ookawara, S., Fujioka, M., Guillemot, F., Kageyama, R., 2004. Hes genes regulate size, shape and histogenesis of the nervous system by control of the timing of neural stem cell differentiation. Development 131, 5539–5550.
Henrique, D., Hirsinger, E., Adam, J., Le Roux, I., Pourquie, O., Ish-Horowicz, D., Lewis, J., 1997. Maintenance of neuroepithelial progenitor cells by Delta–Notch signalling in the embryonic chick retina. Curr. Biol. 7, 661–670.
Hozumi, K., Negishi, N., Suzuki, D., Abe, N., Sotomaru, Y., Tamaoki, N., Mailhos, C., Ish-Horowicz, D., Habu, S., Owen, M.J., 2004. Delta-like 1 is necessary for the generation of marginal zone B cells but not T cells in vivo. Nat. Immunol. 5, 638–644.
Hrabe de Angelis, M., McIntyre II, J., Gossler, A., 1997. Maintenance of somite borders in mice requires the Delta homologue DII1. Nature 386, 717–721.
Jadhav, A.P., Mason, H.A., Cepko, C.L., 2006. Notch 1 inhibits photoreceptor production in the developing mammalian retina. Development 133, 913–923.
Karunaratne, A., Hargrave, M., Poh, A., Yamada, T., 2002. GATA proteins identify a novel ventral interneuron subclass in the developing chick spinal cord. Dev. Biol. 249, 30–43.
Kiernan, A.E., Cordes, R., Kopan, R., Gossler, A., Gridley, T., 2005. The Notch ligands DLL1 and JAG2 act synergistically to regulate hair cell development in the mammalian inner ear. Development 132, 4353–4362.
Kiernan, A.E., Xu, J., Gridley, T., 2006. The notch ligand JAG1 is required for sensory progenitor development in the mammalian inner ear. PLoS Genet. 2, e4.
Koyano-Nakagawa, N., Kim, J., Anderson, D., Kintner, C., 2000. Hes6 acts in a positive feedback loop with the neurogenins to promote neuronal differentiation. Development 127, 4203–4216.
Kubo, F., Takeichi, M., Nakagawa, S., 2005. Wnt2b inhibits differentiation of retinal progenitor cells in the absence of Notch activity by downregulating the expression of proneural genes. Development 132, 2759–2770.
Kusumi, K., Sun, E.S., Kerrebrock, A.W., Bronson, R.T., Chi, D.C., Bulotsky, M.S., Spencer, J.B., Birren, B.W., Frankel, W.N., Lander, E.S., 1998. The mouse pudgy mutation disrupts Delta homologue Dll3 and initiation of early somite boundaries. Nat. Genet. 19, 274–278.
Lee, M.K., Tuttle, J.B., Rebhun, L.I., Cleveland, D.W., Frankfurter, A., 1990. The expression and posttranscriptional modification of a neuron-specific beta-tubulin isotype during chick embryogenesis. Cell. Motil. Cytoskeleton 17, 118–132.
Lindsell, C.E., Shawber, C.J., Boulter, J., Weinmaster, G., 1995. Jagged: a mammalian ligand that activates Notch1. Cell 80, 909–917.
Lindsell, C.E., Boulter, J., diSibio, G., Gossler, A., Weinmaster, G., 1996. Expression patterns of Jagged, Delta1, Notch1, Notch2, and Notch3 genes identify ligand-receptor pairs that may function in neural development. Mol. Cell. Neurosci. 8, 14–27.
Liu, I.S., Chen, J.D., Ploder, L., Vidgen, D., van der Kooy, D., Kalnins, V.I., McInnes, R.R., 1994. Developmental expression of a novel murine homeobox gene (Chx10): evidence for roles in determination of the neuroretina and inner nuclear layer. Neuron 13, 377–393.

Louvi, A., Artavanis-Tsakonas, S., 2006. Notch signalling in vertebrate neural development. Nat. Rev. Neurosci. 7, 93–102.
Matise, M.P., Joyner, A.L., 1997. Expression patterns of developmental control genes in normal and Engrailed-1 mutant mouse spinal cord reveal early diversity in developing interneurons. J. Neurosci. 17, 7805–7816.
Mizuguchi, R., Sugimori, M., Takebayashi, H., Kosako, H., Nagao, M., Yoshida, S., Nabeshima, Y., Shimamura, K., Nakafuku, M., 2001. Combinatorial roles of olig2 and neurogenin2 in the coordinated induction of pan-neuronal and subtype-specific properties of motoneurons. Neuron 31, 757–771.
Myat, A., Henrique, D., Ish-Horowicz, D., Lewis, J., 1996. A chick homologue of Serrate and its relationship with Notch and Delta homologues during central neurogenesis. Dev. Biol. 174, 233–247.
Nelson, B.R., Reh, T.A., 2008. Relationship between Delta-like and proneural bHLH genes during chick retinal development. Dev. Dyn. 237, 1565–1580.
Novitch, B.G., Chen, A.I., Jessell, T.M., 2001. Coordinate regulation of motor neuron subtype identity and pan-neuronal properties by the bHLH repressor Olig2. Neuron 31, 773–789.
Peng, C.Y., Yajima, H., Burns, C.E., Zon, L.I., Sisodia, S.S., Pfaff, S.L., Sharma, K., 2007. Notch and MAML signaling drives Scl-dependent interneuron diversity in the spinal cord. Neuron 53, 813–827.
Prada, C., Puga, J., Perez-Mendez, L., Lopez And, R., Ramirez, G., 1991. Spatial and temporal patterns of neurogenesis in the chick retina. Eur. J. Neurosci. 3, 1187.
Rowan, S., Cepko, C.L., 2004. Genetic analysis of the homeodomain transcription factor Chx10 in the retina using a novel multifunctional BAC transgenic mouse reporter. Dev. Biol. 271, 388–402.
Schuster-Gossler, K., Cordes, R., Gossler, A., 2007. Premature myogenic differentiation and depletion of progenitor cells cause severe muscle hypotrophy in Delta1 mutants. Proc. Natl. Acad. Sci. U. S. A. 104, 537–542.
Shawber, C., Boulter, J., Lindsell, C.E., Weinmaster, G., 1996. Jagged2: a serrate-like gene expressed during rat embryogenesis. Dev. Biol. 180, 370–376.
Shin, J., Poling, J., Park, H.C., Appel, B., 2007. Notch signaling regulates neural precursor allocation and binary neuronal fate decisions in zebrafish. Development 134, 1911–1920.
Takebayashi, S., Yamamoto, N., Yabe, D., Fukuda, H., Kojima, K., Ito, J., Honjo, T., 2007. Multiple roles of Notch signaling in cochlear development. Dev. Biol. 307, 165–178.
Tomita, K., Ishibashi, M., Nakahara, K., Ang, S.L., Nakanishi, S., Guillemot, F., Kageyama, R., 1996. Mammalian hairy and Enhancer of split homolog 1 regulates differentiation of retinal neurons and is essential for eye morphogenesis. Neuron 16, 723–734.
Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R., Schutz, G., 1999. Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nat. Genet. 23, 99–103.
Valsecchi, C., Ghezzi, C., Ballabio, A., Rugarli, E.I., 1997. JAGGED2: a putative Notch ligand expressed in the apical ectodermal ridge and in sites of epithelial–mesenchymal interactions. Mech. Dev. 69, 203–207.
Yang, X., Tomita, T., Wines-Samuelson, M., Beglopoulos, V., Tansey, M.G., Kopan, R., Shen, J., 2006. Notch1 signaling influences v2 interneuron and motor neuron development in the spinal cord. Dev. Neurosci. 28, 102–117.
Yaron, O., Farhy, C., Marquardt, T., Applebury, M., Ashery-Padan, R., 2006. Notch1 functions to suppress cone-photoreceptor fate specification in the developing mouse retina. Development 133, 1367–1378.
Yeo, S.Y., Chitnis, A.B., 2007. Jagged-mediated Notch signaling maintains proliferating neural progenitors and regulates cell diversity in the ventral spinal cord. Proc. Natl. Acad. Sci. U. S. A. 104, 5913–5918.
Yoon, K., Gaiano, N., 2005. Notch signaling in the mammalian central nervous system: insights from mouse mutants. Nat. Neurosci. 8, 709–715.
Young, R.W., 1985. Cell differentiation in the retina of the mouse. Anat. Rec. 212, 199–205.
Zhou, Q., Choi, G., Anderson, D.J., 2001. The bHLH transcription factor Olig2 promotes oligodendrocyte differentiation in collaboration with Nkx2.2. Neuron 31, 791–807.