Analysis of the Expression Pattern of Transmembrane proteins with extracellular leucine rich repeats (eLRRs) in the developing nervous system

Disha Chauhan

Dipòsit Legal: L.858-2014
http://hdl.handle.net/10803/145645

Analysis of the Expression Pattern of Transmembrane Proteins with Extracellular Leucine Rich Repeats (eLRRs) in the Developing Nervous System

Doctoral Thesis by
Disha Chauhan
Molecular and Developmental Neurobiology
IRBLLEIDA, Universitat de Lleida

Supervisor
Dr. Joaquim Egea
Molecular and Developmental Neurobiology
IRBLLEIDA

Lleida, 2014

Analysis of the Expression Pattern of Transmembrane Proteins with Extracellular Leucine Rich Repeats (eLRRs) in the Developing Nervous System

Memòria de tesi doctoral presentada per Disha Chauhan per optar al grau de Doctor per la Universitat de Lleida.

Treball realitzat a l'Institut de Recerca Biomèdica de Lleida (IRBLLEIDA) a la Unitat *Molecular and Developmental Neurobiology* del grup consolidad Cicle Cel·lular del Departament de Ciències Mèdiques Bàsiques de la Universitat de Lleida, sota la direcció del Doctor Joaquim Egea Navarro.

El treball realitzat en aquesta tesi doctoral ha estat finançat pels projectes de recerca del Miniterio de Economía y Competitividad *Function of the FLRT Family of LRR Proteins in the Nervous System Development and Connectivity* (BFU2010-1805) i pel *Career Integration Grant* de la UE (programa Marie Curie) *Novel Molecular Mechanisms of Neuron Migration in the Developing Cortex and their Contribution to Related Diseases* (Neuromigration, 293980) La doctoranda Disha Chauhan ha gaudit durant aquest temps d'una beca predoctoral de l'AGAUR, FI-DGR 2011.

Lleida, 28 de Maig de 2014,

Doctorand — Director de tesi

Disha Chauhan — Dr. Joaquim Egea Navarro

Brain, the only organ which uses itself to understand itself

INDEX

INDEX

ACKNOWLEDGEMENTS

Acknowledgements

There are no proper words to convey my deep gratitude and respect for my Supervisor, Dr. Joaquim Egea Navarro. “Happiness is the Road”, I understand his words better now when I’m at first checkpoint of my Journey. He has inspired me to become an independent researcher and helped me realize the power of critical reasoning. He inspired to ‘be’ by ‘being’ one. And anytime in life if ever I’ll be lost I will remember his ‘Bon Courage’. I hope someday I’ll make him Proud.

My sincere thanks must also go to Dr. Carme Espinet, She generously gave her time to offer her expertise of years at all levels and constant encouragement.

I am very grateful to collaborators Dr. David Ginty, Professor of Neurobiology, Harvard Medical School, United States for providing ISLR2 antibody making this work possible and Dr. Isabel Novoa Garcia, Director, Biobanc, HUVH, Barcelona, Spain for providing human fetal brain sections and for their suggestions and generosity.

I am thankful to many people around me for making this work possible by helping in all possible ways, Dr. Loreta Medina, Dr. Matthieu Vermeren, Dr. Antonio Abellan Rodenas, Dr. Mario Encinas, Dr. Xavi Dolcet, Dr. Carles Canti, Dr. Anaïs Panosa Borràs and Dr. Judit Ribas Fortuny.

There is no way to express how much it meant to me to have been a member of Molecular and Developmental Neurobiology Laboratory. These brilliant friends and colleagues inspired me over the three years, Dr. Serafi Cambray for teaching me lots of lab lessons, Maria Jose Menal Castellote & Catherine Fleitas Perez for being most amazing lab companion and amigas, Ma Inmaculada Montoliu Sanchis for being a constant help understanding my broken Spanish and taking care of all my needs in lab, And as said we learn most while teaching, I have learned so much from Pau Marfull and I am grateful for it to him , I’m also thankful to Julia Sanchez, Blanca Peguera Carre, Montse Ortega Fernandez for making working even more fun with their presence and transferring their never ending energy to everyone in lab. Each of them in their unique way has their contribution in making this Thesis possible.

I have deep gratitude for my friend Alba Naudi Farre for being such a wonderful friend and giving me so much love. I'm especially thankful to Neus, Conce and entire Farre family for never letting me miss my family in India.

I cannot forget friends in the IRBLLEIDA who helped me learning technical skills and motivated me with their joyous presence all the time. I'm thankful to Milica, MariaVittoria, Mireia, Esther, Alexandra, Isabel, Estella, Montse, Paulo, Marta. I'm also thankful to the whole Animal house facility and cell culture staff for their kind and timely help.

Many friends have helped me to stay in high spirits through these three years, cheered me on, and celebrated each accomplishment I'm grateful to have friends and partners of all crimes Upasana Sengupta, Ana Munana, Charumathi Pushparaj and Arindam Das. I'm blessed to have friend who were with me not just emotionally but made sure that I stay fit physically, José Maria and Liliana Santos, I'll never forgive them for killing me with their training. PhD itself was adventurous enough but I'm so glad I met Ariadna Brugues to make my stay in Spain more memorable with all the adventures I did with her and for all the love I received from her parents. I'm also thankful to Dr. Hiren Karathia, Falguni Seth, Melodia and Sandra.

A sincere thanks to my all friends and seniors who inspite of being far were constant source of encouragement and support to me. I'm deeply thankful to Dr. Niroj Kumar Sethy, Dr. Yasmin Ahmad, Dr. Kalpana Shrivastava Bhardwaj, Dr. Saurabh Saxena, Swarna Sharad Rao, Shiladitya Mitra.

I'm thankful to IRBLLEIDA, AGAUR, Generalitat de Catalunya and the University of Lleida for their prompt support, this research would not have been possible without their support. I'm also thankful to my Thesis Committee. I'm thankful to SPAIN for last memorable three years of my life.

Finally, I must express my very profound gratitude to My Parents, My Sister and Saurabh Gayali, my best friend and partner, for their unconditional trust and endless patience. I am thankful to them for encouraging me in all pursuits and inspiring me to follow my dreams, they believed in me. It's their love that I could be what I am.

Disha Chauhan

ABBREVIATIONS

aa	Amino acid
AEP	Anterior entopeduncular area
AMIGO	Amphoterin induced gene and ORF
AP	Apical Progenitors
ApoER2	Apolipoprotein E receptor 2
ATP	Adenosine triphosphate
BCIP	5-bromo-4-chloro-3-indolylphosphate
BDNF	Brain derived neurotrophic factor
BP	Basal Progenitors
CAM	Cell adhesion molecule
Cdk	Cyclin dependent kinase
CDS	Coding sequence
CGE	Caudal ganglionic eminence
CNS	Central nervous system
CP	Cortical plate
CR	CajalRetzius
DAPI	4',6-diamidino-2-phenylindole
DCC	deleted in colorectal cancer
DCx	Doublecortin
DEGA	Differentially expressed in human gastric adenocarcinoma
Dlx	Drosophila distal less
DMEM	Dulbecco's Modified eagle medium
DNA	Deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DRGs	Dorsal root ganglion
E	Embryonic Day
ECD	Extracellular Domain
ECL	Enhanced chemiluminescence
ECM	Extracellular Matrix
EDTA	Ethylenediamine-tetra acetic acid

EGF	Epidermal growth factor
EtOH	Ethanol
FGF	Fibroblast growth factor
FGFR	Fibroblast growth factor receptor
FLRT	Fibronectin and leucine rich transmembrane protein
FN	Fibronectin
GABA	Gamma-aminobutyric acid
GDNF	Glial cell line derived neurotrophic factor
GE	Ganglionic eminences
HEK293T	Human Embryonic Kidney cells
HGF	Hepatocyte Growth Factor
HRP	horseradish peroxidase
ICD	Intracellular domain
Ig	Immunoglobulin
IPC's	Intermediate progenitor cells
ISH	*in situ* hybridization
ISLR	Immunoglobulin superfamily containing Leucine rich repeat
IZ	Intermediate zone
kDa	Kilo Dalton
LCS	Lateral cortical stream
LGE	Lateral Ganglionic Eminences
LINGO	Leucine rich repeat and Ig Domain containing -1
Linx	Leucine rich repeat domain and Immunoglobulin domain containing Axon extension
Lis	Lissencephaly
LRIG	Leucine rich repeats and immunoglobulin like domain protein
LRRCT	cysteine rich carboxyl terminal domain flanking leucine rich repeat
LRRIG	Leucine rich repeat containing Immunoglobulin domain
LRRNT	cysteine rich amino terminal domain flanikingleucine rich repeat
MGE	Medial Ganglionic Eminences
MZ	Marginal zone
NBT	Nitrobluetetrazolium
NLRR	Neuronal leucine rich repeat protein

Nrg	Neuregulin
OCD	Obsessive compulsive disorder
oRG	nonventricular radial glia
OSVZ	Outer subventricular zone
PAGE	Polyacrylamide gel electrophoresis
Pax	Paired box
PCR	Polymerase chain reaction
PDZ	PSD95/discs large/zona occludens-1
POA	Pre Optic Area
PP	Preplate
RGC's	Radial Glial cells
RMS	Rostral Migratory stream
RMS	Rostral Migratory stream
RNA	Ribonucleic acid
Robo	Roundabout
RT	Room temperature
SALM	Synaptic adhesion like molecule
SBH	Subcortical band heterotopia
SDF	Stromal derived factor
Sema	Semaphorin
SP	Sub Plate
SVZ	SubVentricular Zone
Tbr	T-brain gene
TCAs	Thalamocortical Axons
TM-eLRR	transmembrane protein containing extracellular leucine rich repeats
Trk	Tyrosine kinases
vRG	ventricular radial glia
VZ	Ventricular Zone

ABSTRACT

ABSTRACT

Tim Berners Lee quoted ‘There are billions of neurons in our brains, but what are neurons? Just Cells. The Brain has no knowledge until connections are made between neurons. All that we know, all that we are, comes from the way our neurons are connected’ (from Weaving the Web, 1999). The cerebral cortex, the substrate of our cognitive capabilities, is probably the most complex vertebrate structure and summarizes most of the features of the nervous system development and function. The mature human cortex comprises approximately 21-26 billion neurons and 30-40 billion glial cells (Pelvig et al., 2008) that interconnect in a highly specific pattern which emerges from sequential developmental events including cell migration, axon guidance, target selection, and synapse formation. Failing in any step of these processes has a profound impact in nervous system development and function. Thus, neuron migration defects in the developing cortex lead to neurological disorders, such as Lissencephaly, periventricular heterotopias, polymicrogyria, schizophrenia, and depression (Valiente and Marin, 2010). Neuron migration and axon guidance occurs through a playground of cellular environment where they integrate a plethora of diffusible and contact dependent cues to reach their proper targets and synaptic partners. These are the "axon guidance cues" and interact with specific receptors at the surface of the neuron (Dickson, 2002; Lichtman and Smith, 2008; Akins and Biederer 2006). Their name is somehow confusing because they have been also involved in synapse formation and function in the developing of non-neuronal systems like the blood vessels (Bouvree et al., 2008; Larrivee et al., 2009). Surprisingly, only few families of the axon guidance cues has been described suggesting that other molecules must be involved to explain the huge complex architecture and function of the nervous system. The identification of these new players and their role in neural connectivity is one of main challenges in molecular neurobiology in solving the puzzle of brain development.

Transmembrane proteins with extracellular leucine-rich repeats (eLRR-TMs) have recently emerged as important players in neuron connectivity. Indeed, comparative analysis shows that, during evolution, eLRR-TMs expression has expanded in mammals, mostly enriched in the nervous system. The LRR motif is a stretch of 20-30

amino acids rich in leucine residues. In the innate immune system, the LRR acts as a protein-protein binding motif able to interact with a wide variety of antigens. This diversity, together with their enriched expression in the nervous system, makes the eLRR-TMs to be good candidates to add complexity to the neuronal connectivity. This is further emphasized by the fact that several eLRR-TM proteins have been linked to human neurological and psychiatric disorders including epilepsy, Tourette's syndrome, night blindness, congenital insensitivity to pain (with mental retardation) and Alzheimer's disease (Matsushima et al. 2005). Indeed, some well knew eLRR-TMs have been already involved in cell migration, axon guidance and synapse formation. However for the majority of eLRR-TMs, their role in nervous system development and function is basically unknown. With the aim to assess the neuronal function of these uncharacterized eLRR-TMs, in the present thesis work we performed a small scale gene expression study, mainly by in situ hybridization, in the mouse and human brain at different developmental stages. The information that is currently available from original publications or from public resources about the expression of eLRR-TMs in the brain is fragmented; often lacks spatial localization or the studies were conducted only on adult tissue. Thus there is great need for such systematic gene expression mapping in developing stages giving closer insight on role of individual protein in nervous system development and function. Also it is interesting to compare the expression patterns of these genes between mouse and human, especially in the cerebral cortex, where important anatomical differences exist between species (presence of sulcus and circumvolutions in the human cortex). Thus, in the present thesis work, we have analyzed the mRNA expression pattern for several families of genes encoding eLRR-TM proteins at different developmental stages in mouse brain (from E13.5 to E17.5) and in human brain (at prenatal stages of 15 and 22 weeks). The results obtained are going to give important clues about the role of eLRR-TM proteins in nervous system development and function. In particular, the gene expression profiling of neocortex at various developmental time and regions will reveal candidate genes associated with different mechanisms of corticogenesis, especially when comparing mouse and human brain.

Resum

Tim Berners Lee va dir "existeixen bilions de neurones en el nostre cervell; però que són les neurones? només cèl·lules. El cervell no és funcional fins que les conneccions entre neurones s'han establert. Tot el que som i coneixem prové de com les nostres neurones estan interconnectades (de *Weaving the Web*, 1999). L'escorça cerebral, el substrat de les nostres capacitats cognitives, és possiblement l'estructura més complexa del vertebrats i resumeix la majoria de les característiques del desenvolupament i funció del sistema nerviós. L'escorça madura humana té aproximadament 21-26 bilions de neurones i 30-40 bilions de cèl·lules (Pelvig et al.,. 2008) que s'interconnecten seguint un patró molt específic que es forma seguint una seqüència molt precisa d'esdeveniments que tenen lloc principalment durant el desenvolupament i que inclouen migració, guia axonal, selecció del teixit diana i la formació de sinapsis. Defectes en qualsevol d'aquests processos te un impacte profund en el desenvolupament i funció del sistema nerviós. Així, defectes en la migració neuronal en l'escorça cerebral donen lloc a desordres neurològics i psiquiàtrics com la lisencefàlia, heterotòpies ventriculars, polimicrogíria, esquizofrènia i depressió (Valiente and Marin, 2010). Per arribar a contactar amb la cèl·lula diana, la migració neuronal i la guia axonal esta regulada pel context cel·lular, on les neurones han d'integrar la informació d'una amplia gama de factors, tant solubles com ancorats a la membrana (i que, per tant, requereixen de contacte cel·lular). Aquestes molècules son les "molècules de guia axonal" que interaccionen amb receptors específics en la superfície de la neurona (Dickson, 2002; Lichtman and Smith, 2008; Akins and Biederer, 2006). El seu nom confon una mica perquè aquestes molècules s'han implicat en moltes altres funcions com per exemple també en la formació de sinapsis així com en el desenvolupament de teixits no neuronals com el sistema vascular (Bouvree et al., 2008; Larrivee et al., 2009). Sorprenentment, només unes poques famílies de molècules de guia axonal has estat identificades lo qual suggereix que altres molècules queden per descobrir-se per tal d'explicar l'enorme complexitat de l'arquitectura neuronal i la seva funció. La identificació d'aquestes noves molecules i la caracterització de la seva funció és uns dels reptes més importants de la neurobiologia molecular.

Les proteïnes transmembrana amb repeticions de leucina en la seva part extracel·lular (eLRR-TMs) han esdevingut recentment com a important reguladores de la

connectivitat neuronal. De fet, anàlisis comparatius han mostrat que durant l'evolució, l'expressió de proteïnes eLRR-TMs, ha incrementat en mamífers, especialment al sistema nerviós. La seqüència LRR te uns 20-30 aminoàcids i està enriquida amb l'aminoàcid leucina. En el sistema immune innat, el LRR actua com a domini d'unió proteïna-proteïna que és capaç d'interaccionar amb una varietat molt amplia d'antígens. Aquesta diversitat, juntament amb la seva expressió enriquida al sistema nerviós fa que les proteïnes eLRR-TM siguin uns bons candidats per afegir complexitat a la connectivitat neuronal. Aquest idea ve reforçada per l'observació que molts gens que codifiquen per proteïnes eLRR-TM s'han associat a desordres neurològics i psiquiàtrics en humans com per exemple epilèpsia, el síndrome de Tourette, ceguera nocturna, insensibilitat congènita al dolor (amb retard mental) i la malaltia d'Alzheimer (Matsushima et al., 2005). De fet, algunes proteïnes eLRR-TM s'han relacionat recentment amb migració neuronal, guia axonal i la formació de sinapsis. No obstant això, per a la gran majoria de proteïnes eLRR-TM, el seu paper en el desenvolupament i la funció del sistema nerviós és desconeguda. Amb l'objectiu d'avaluar la funció neuronal d'aquestes proteïnes eLRR-TM poc o gens caracteritzades, en aquest treball de tesi doctoral hem fet un *screening* a petita escala d'expressió gènica, principalment per hibridació in situ, tant en teixit de cervell de ratolí com en cervell humà a diferents estadis de desenvolupament. La informació que es pot obtenir d'expressió d'aquests gens en el cervell a partir dels treballs publicats o de les bases de dades públiques és moltes vegades inexistent o està fragmentada, sovint manca localització espaial (l'estudi d'expressió se va fer en teixit homogeneïtzat sense poder distingir entre l'heterogeneïtat cel·lular i la localització espaial) o be els estudis s'han fet en teixit adult. Així que hi ha una necessitat d'una aproximació sistemàtica per mapar l'expressió d'aquests gens per tal de tenir una informació més precisa del seu paper durant el desenvolupament del sistema nerviós. També es molt interessant comparar el patró d'expressió d'aquests gens entre ratolí i humà, especialment en l'escorça, on existeixen diferencies anatòmiques importants entre espècies (per exemple la presencia de solcs en l'escorça humana). Així, en aquest treball hem analitzat l'expressió d'mRNA de membres de diferents famílies gèniques que codifiquen per eLRR-TMs en varis estadis de desenvolupament en ratolí (entre E13.5 i E17.5) i a l'escorça humana (a 15 i 22 setmanes). Els resultats obtinguts seran molt valuosos a l'hora d'entendre la funció dels gens que codifiquen per proteines eLRR-TM en el desenvolupament del sistema nerviós. En particular, el patró d'expressió a l'escorça cerebral a diferents estadis i diferents regions revelarà gens

candidats implicats en els mecanismes moleculars de la corticogènesis, especialment quan es compara el cervell de ratolí amb l'humà.

Resumen

Tim Berners Lee dijo "existen billones de neuronas en nuestro cerebro; pero que son las neuronas? sólo células. El cerebro no es funcional hasta que las conexiones entre neuronas se han establecido. Todo lo que somos y conocemos proviene de cómo nuestras neuronas están interconectadas (de *Weaving the Web*, 1999). La corteza cerebral, el substrato de nuestras capacidades cognitivas, es posiblemente la estructura más compleja de los vertebrados y resume la mayoría de las características del desarrollo y función del sistema nervioso. La corteza madura humana tiene aproximadamente 21-26 billones de neuronas y 30-40 billones de células (Pelvig et al.,. 2008) que se interconectan siguiendo un patrón muy específico que se forma siguiendo una secuencia muy precisa de acontecimientos que tienen lugar principalmente durante el desarrollo y que incluyen migración, guía axonal, selección del tejido diana y la formación de sinapsis. Defectos en cualquiera de estos procesos tiene un impacto profundo en el desarrollo y la función del sistema nervióso. Así, defectos en la migración neuronal en la corteza cerebral dan lugar a problemas neurológicos y psiquiátricos como la lisencefalia, heterotopias ventriculares, polimicrogiria, esquizofrenia y depresión (Valiente and Marin, 2010). Para llegar a conectar con la célula diana, la migración neuronal y la guía axonal está regulada por el contexto celular, donde les neuronas deben integrar la información de una amplia gama de factores, tanto solubles como anclados a la membrana (y que, por lo tanto, requieren de contacte celular). Estas moléculas son las "moléculas de guía axonal" que interaccionan con receptores específicos en la superficie de la neurona (Dickson, 2002; Lichtman and Smith, 2008; Akins and Biederer, 2006). Su nombre confunde un poco porque estas moléculas se han implicado en muchas otras funciones como por ejemplo en la formación de sinapsis así como en el desarrollo de tejidos no neuronales como el sistema vascular (Bouvree et al., 2008; Larrivee et al., 2009). Sorprendentemente, sólo unas pocas familias de moléculas de guía axonal han sido identificadas lo cual sugiere que otras moléculas quedan por descubrirse para explicar la enorme complejidad de la arquitectura neuronal y su función. La identificación de estas nuevas moléculas y su caracterització funcional es uno de los retos más importantes de la neurobiología molecular.

Las proteínas transmembrana con repeticiones de leucina en su parte extracelular (eLRR-TMs) se han convertido recientemente como importantes reguladores de la conectividad neuronal. De hecho, análisis comparativos han demostrado que durante la evolución, la expresión de proteínas eLRR-TMs, ha incrementado en mamíferos, especialmente en el sistema nervioso. La secuencia LRR tiene unos 20-30 aminoácidos y está enriquecida con el aminoácido leucina. En el sistema inmune innato, el LRR actúa como dominio de unión proteína-proteína que es capaz de interaccionar con una variedad muy amplia de antígenos. Esta diversidad, juntamente con su expressión enriquecida en el sistema nervioso hace que las proteínas eLRR-TM sean unos buenos candidatos para añadir complejidad a la conectividad neuronal. Esta idea se refuerza con la observación que muchos genes que codifican para proteínas eLRR-TM se han asociado a problemas neurológicos y psiquiátricos en humanos como por ejemplo epilepsia, el síndrome de Tourette, ceguera nocturna, insensibilidad congénita al dolor (con retraso mental) y la enfermedad de Alzheimer (Matsushima et al., 2005). De hecho, algunas proteínas eLRR-TM se han relacionado recientemente con la migración neuronal, guía axonal y la formación de sinapsis. A pesar de ello, para la gran mayoría de proteínas eLRR-TM, su papel en el desarrollo y la función del sistema nervioso es desconocida. Con el objetivo de evaluar la función neuronal de estas proteínas eLRR-TM poco o nada caracterizadas, en este trabajo de tesis doctoral hemos realizado un *screening* a pequeña escala de expresión génica, principalmente por hibridación in situ, tanto en tejido de cerebro de ratón como en cerebro humano en diferentes estadios de desarrollo. La información que se puede obtener de expresión de estos genes en el cerebro a partir de los trabajos publicados o de las bases de datos públicas es muchas veces inexistente o está fragmentada, a menudo falta localización espacial (el estudi de expresión se realiza en tejido homogeneizado sin poder distinguir entre la heterogeneidad celular y la localización espacial) o bien los estudios se han realizado en tejido adulto. Así que existe una necesidad de una aproximación sistemática para mapar la expresión de estos genes para tener una información mucho más precisa de su papel durante el desarrollo del sistema nervioso. También es muy interesante comparar el patrón de expresión de estos genes entre ratón y humano, especialmente en corteza, donde existen diferencias anatómicas importantes entre especies (por ejemplo la presencia de surcos en la corteza humana). Así hemos analizado la expresión de mRNA de diferentes miembros de varias familias de genes que codifican para eLRR-TMs en varios estadios de desarrollo en cerebro de ratón (entre E13.5 i E17.5) y en corteza

humana (a 15 i 22 semanas). Los resultados obtenidos serán muy valiosos a la hora de entender la función de estos genes en el desarrollo del sistema nervioso. En particular, el patrón de expresión en la corteza cerebral en diferentes estadios y diferentes regiones revelará genes candidatos implicados en los mecanismos moleculares de la corticogénesis, especialmente cuando se compara el cerebro de ratón con el humano.

1. INTRODUCTION

1. INTRODUCTION

1.1Mouse Brain Development

> *David Hunter Hubel, Canadian neurophysiologist famous for his work on the structure and function of the Visual cortex and co-recipient of 1981 Nobel Prize in Physiology stated "The Brain is a tissue. It is a complicated, intricately woven tissue, like nothing else we know of in the universe, but it is composed of cells, as any tissue is. They are, to be sure, highly specialized cells, but they function according to the laws that govern any other cells. Their electrical and chemical signals can be detected, recorded and interpreted and their chemicals can be identified; the connections that constitute the brain's woven feltwork can be mapped. In short, the brain can be studied, just as the kidney can".*

Enormous amount of work has been done to understand one of the greatest mysteries in biomedical research: the organization and function of vital organ of existence,The Brain. Different researchers use different model organisms but the ultimate goal is to understand how our brain works, from basic tasks to more complex and abstract functions like learning and memory. Dr. Henry Markram's Human Brain Project is one such landmark endeavor in modern neuroscience through which we can gain profound insights into what makes us human, develop new treatments for brain disease and plan our future approach to understand brain functioning. Also the federal Brain Mapping Project aims at conquering challenges such as epilepsy, autism and Alzheimer's disease. The mouse turned out to be a relevant model to understand the organization and function of the brain having an advantage of being a mammal and share striking similarity to humans in anatomy, physiology, and genetics. Furthermore, it was the only animal model commonly used in research that had all the major divisions of the human brain, including the cerebral cortex. Owing to these advantages, organization and development of mouse brain has been studied more extensively than the human one and still providing important information about new molecular players involved in neuronal connectivity and function.

The nervous system in mammals originates from a part of the dorsal ectoderm that is specified during early developmental stages to become neural ectoderm. This region is called the neural plate and the cells have a distinct columnar appearance compared to the rest of the ectoderm. At 6-7 somite stage (E8.5) neural plate folds inwards to form the neural tube which will finally give rise to the brain and the spinal cord (Ybot Gonzalez et al., 2002). The anterior part of the the neural tube enlarges into three primary vesicles at different levels: prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain) (Figure 1A). In mammals, the forebrain becomes much larger than other vesicles.

Mammalian forebrain is functionally the most complex of biological structures and controls most of higher brain functions like homeostasis, learning, memory, cognition and behaviour since all parts of brain involved in this functions develop from it. Following neural induction, the primary prosencephalon subdivides into anterior telencephalon and caudal diencephalon. The dorsal telencephalon develops into pallium (roof); including hippocampus and cerebral cortex and the ventral part called subpallium (base) gives rise to the striatum and globus pallidus (Lateral, Medial and Caudal Ganglionic Eminences) (Figure 1B-E). The pallium is probably the region of the brain development of which has been studied more extensively and which has provided with important information about basic principles and mechanisms of nervous system formation. The pallium gives rise to different parts of cerebral cortex. There has been contradiction among various researchers in defining cerebral cortex, which was classified into two major parts the neocortex and allocortex. The neocortex (also called isocortex or neopallium by some researchers) is the newest part of the cerebral cortex to evolve and “neo” was used referring to its relatively recent appearance in evolution. Neocortex is the part of the mature cerebral cortex which develops into a six layered structure while the part which comprises less than six layers like for instance the one forming the hippocampus is referred as “allocortex”. The present thesis work is mainly focused on the neocortex formation and some parts of allocortex (hippocampus) so to avoid any ambiguity we will refer to it in the whole text as “neocortex” and as “pallium” when referring in broader sense.

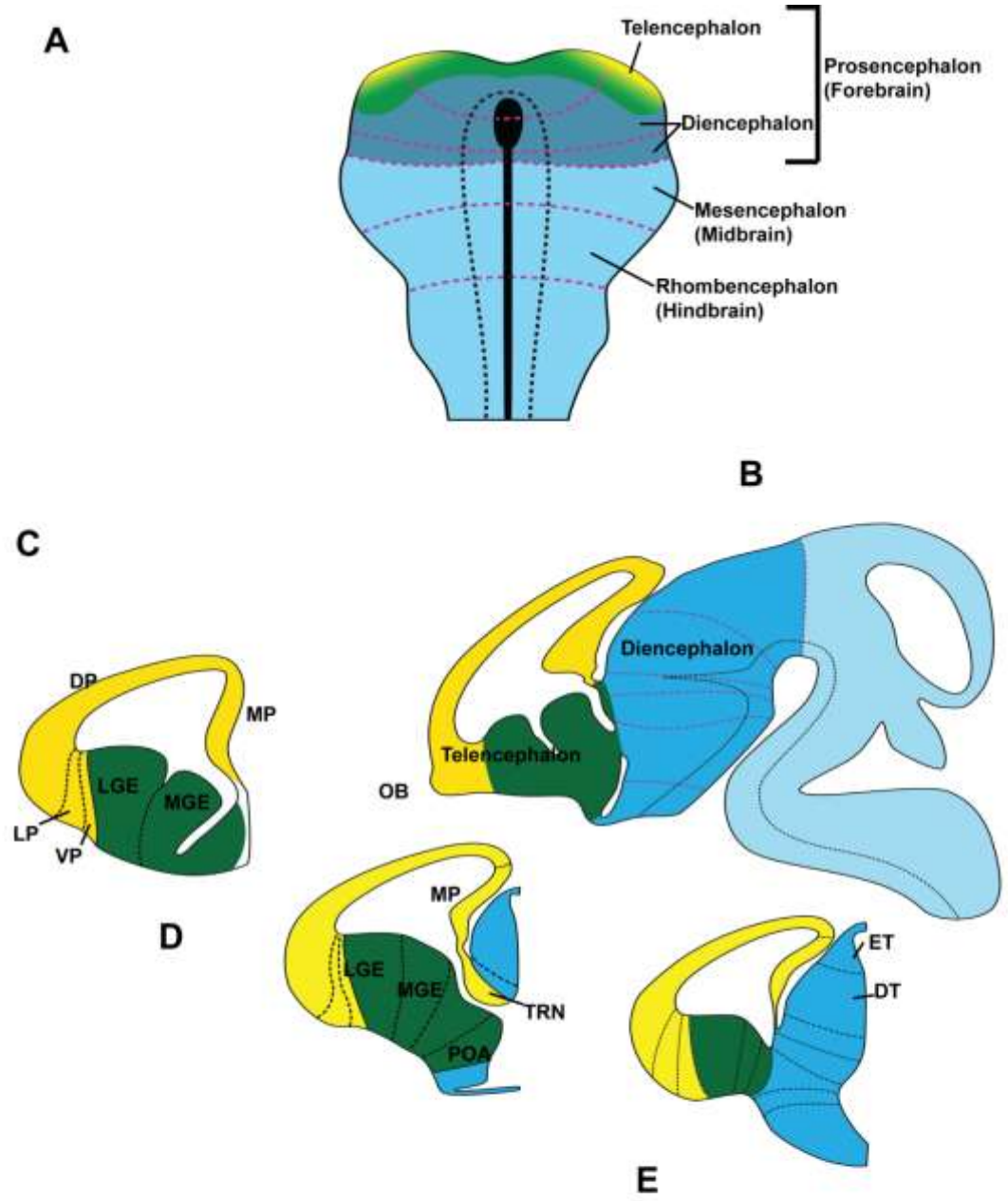


Figure1. Organization of Prosencephalon. (A) Schema of neural plate. (B) Schema of saggital section through the brain of an E13.5 mouse showing the prosencephalic subdivisions. (C-E) Transversal sections through the telencephalon and diencephalon. Abbreviations: DP,Dorsal Pallium; DT, Dorsal Thalamus; ET, Epithalamus; LGE, Lateral Ganglionic eminences; LP, Lateral Pallium; MGE, Medial ganglionic eminence; MP, Medial Pallium; POA, Preoptic Area; VP, Ventral pallium; TRN, Reticular Thalamic Nucelus. Yellow and green represents Telencephalon, Dark blue represents Diencephalon and Light blue represents Mesencephalon. Figure adapted fromPuelles et al., 2000.

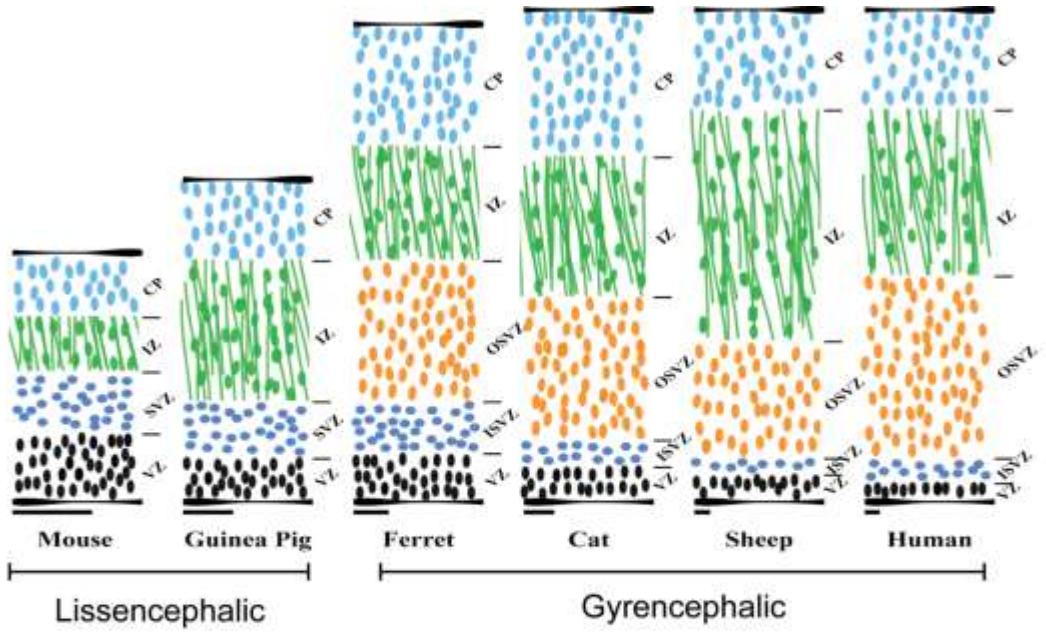


Figure 2. Schematic representation of neocortical layers in neocortex of lissencephalic and gyrencephalic mammals at equivalent developmental stages (Mouse, E15.5; Guinea Pig, E30; Ferret, P0; Cat, E46; Sheep, E65, Human, 16gw)on coronal sections at equivalent levels. Figure adapted from Reillo et al., 2010.

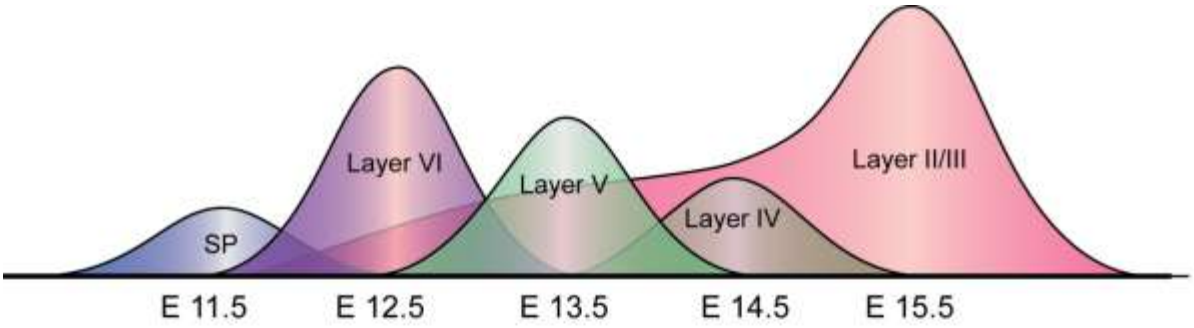
1.2 Mouse Neocortex Development

A folded sheet of brain tissue in mammals made of over 10 billion neurons called "neocortex", is the seat of our highest sensory, motor and cognitive abilities and has undergone pronounced expansion and development during evolution (Figure 2). In the mouse brain, like other animals such as lizards, the neocortical surface is smooth (it is also called "lissencephalic") and covers only the top part of the brain. In contrast, in the monkey and human brains, neocortex has sulci (and for this reason is also known as"gyrencephalic") and covers almost the entire brain. This anatomical difference is due to the expansion of neocortex during evolution and the acquisition of complex tasks and cognitive capabilities. The basic principles of neocortical development have been studied and can be divided into three major processes: neurogenesis, neural migration,

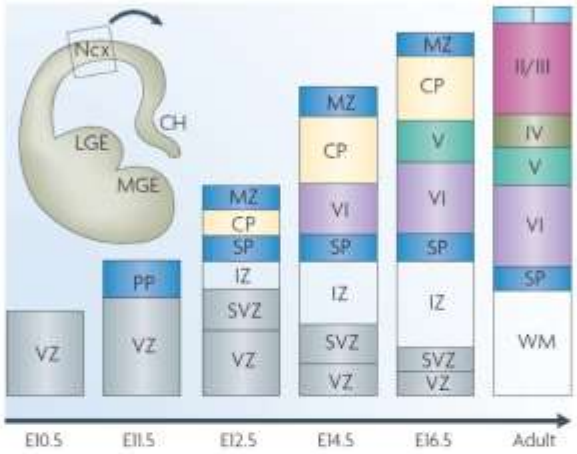
and functional connectivity. The mammalian neocortex has a basic six layered structure (layers I-VI). This laminated cortical architecture is built during embryonic development in a sequential manner (see below and Figure 2).

Corticogenesis happens between E10 to E19 (Dehay and Kennedy, 2007; Molyneaux et al., 2007). Cortical neurons arise from the proliferative regions at the margin of the embryonic cerebral ventricles (Rakic, 1982), the ventricular zone (VZ) covering the ventricle and the subventricular zone (SVZ) adjacent to the VZ. Prior to the onset of neurogenesis, cellular proliferation results in two daughter progenitor cells through symmetric cell division. The progenitors in the neuroepithelium are epithelial-like cells but soon during development change morphology and acquire a polarized shape with their cell body (soma) located in the VZ and a long process, which spans over the wall of neocortex, reaching the pial surface (Figure 3). These progenitors are then known as "radial glia cells" (RGCs) (Kriegstein and Noctor, 2004). Concurrently, RGCs start undergoing asymmetric cell division and one of the daughter cells called intermediate progenitor cells (IPCs) exits cell cycle and migrate out of the VZ to begin to differentiate into a neuron (Figure3; Bystron et. al., 2008, Bystron et. al., 2006).

The neocortex is formed by two principal neuronal subtypes, glutamatergic projection neurons (also known as pyramidal cells), which account for majority of neurons (approximately 80%) and local circuit GABAergic interneurons. However, the two types of neurons are generated in different regions of developing brain. Pyramidal cells are born in the adjacent SVZ/VZ while interneurons are generated in the proliferative regions of the subpallium (Corbin et al., 2001; Marin and Rubenstein, 2001; Rakic 2007). This different origin makes the neurons to use different strategies to migrate and disperse in the brain. Thus, pyramidal cells adopt a radial migration, in which they migrate from the progenitor zone toward the surface of the brain following the radial layout of the neural tube. In contrast, interneurons adopt a tangential migration, in which cells migrate orthogonally to the direction of radial migration (Marin and Rubenstein, 2003).



A. Sequential generation of cortical projection neuron subtypes



B. "Inside-out" development of the cerebral cortex

Figure 3. **Development of the cerebral cortex: sequential neurogenesis of projection neurons and formation of the cortical layers**.
(A) As indicated, projection neurons of distinct cortical layers are generated in overlapping temporal waves by progenitors of the adjacent neuroepithelium, the ventricular zone (VZ) and the subventricular zone such that deep layer neurons (layer V-VI) are generated before upper layer neurons (layer II-IV) (approximate timepoints indicated). (B) The cerebral cortex develops in an "inside-out" fashion: newborn neurons migrate radially from the ventricular regions towards the pial surface, and occupy progressively upper layers in the developing cortex. As represented, at E11.5 preplate (PP) is formed by the earliest born neurons. Around E12.5, a second cohort of neurons migrates into the PP forming the cortical plate (CP), which splits the PP into superficial marginal zone (MZ) and the deeply located subplate (SP). With the arrival of more and more newborn neurons, the CP starts to expand between the MZ and the SP and later born neurons migrate past earlier born neurons and settle underneath the MZ, thereby forming the multilayered structure of the neocortex. The formation of this layered array of neurons starts at E13.5-E14.5 and lasts untill postnatal stages. Note how the amount of progenitors in the SVZ and VZ decreases during development. Abbreviations: CP, Cortical Plate; E, embryonic day; IZ, intermediate zone; LGE, Lateral ganglionic eminences; MGE, Medial ganglionic eminences; MZ, MArginal zone; NCx, Neocortex; PP, Preplate; SP, Subplate; VZ, ventricular zone; WM, white matter. Figure adapted from Molyneaux et al., 2007.

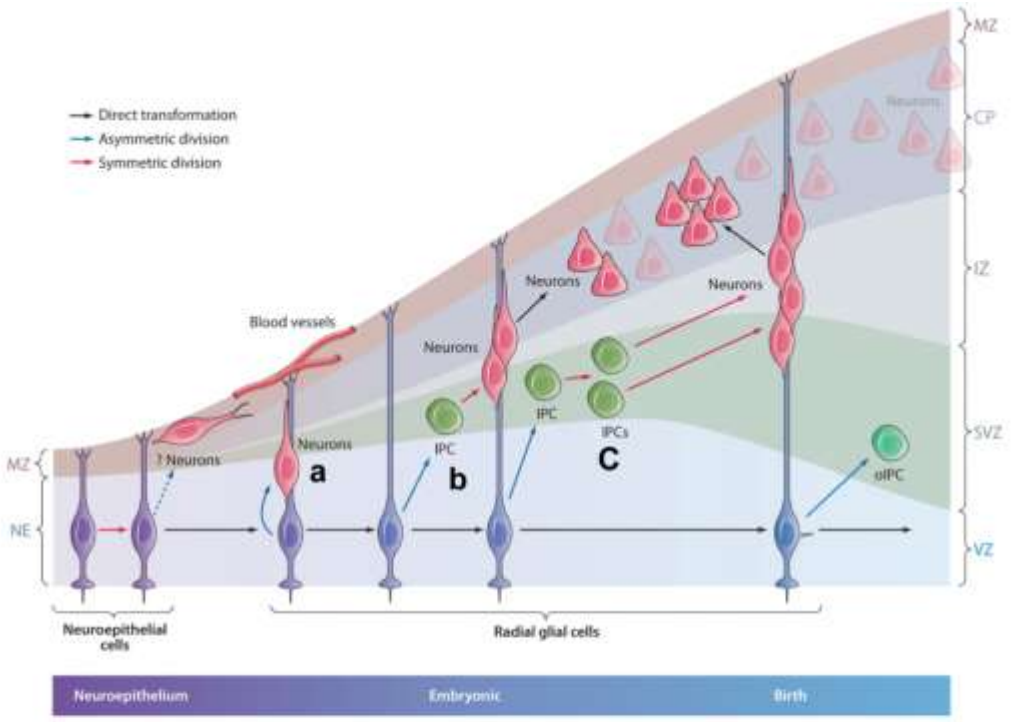


Figure 4. **Three modes of neurogenesis during cortical development.** RG in cortex generate neurons (a) directly through asymmetric division; (b) indirectly by generation of IPCs and one-round of amplification; or (c) indirectly again through IPCs, but with two rounds of division and further amplification. This additional amplification stage may be fundamental to increase cortical size during evolution . Abbreviations: CP, cortical plate; IPC, intermediate progenitor cell; IZ, intermediate zone; MZ, marginal zone; NE, Neuroepithelium; oIPC, oligodendrocytic progenitor cell; RG, radial glia; SVZ, subventricular zone; VZ, ventricular zone. Figure adapted from Kriegstein and Alvarez-Buylla, 2009.

Pyramidal neuron differentiation and migration takes place in a synchronous manner for successive cohorts of cells (Figure3). The first cohort of neural progenitors which migrates out of VZ constitutes the transient preplate at around the 11th Embryonic Day (E11) of mouse development. Simultaneously Cajal Retzius (CR) neurons appear at the outermost aspect of the preplate, arising from pallial sources (Takiguchi-Hayashi et al., 2004; Bielle et al., 2005; Yoshida et al., 2006). The next wave (~E13) of post mitotic neural progenitors forms cortical plate by entering the preplate and splitting it into a superficial layer the Marginal zone (MZ or layer I) and the deep subplate (SP). The

successive waves of migration positions neurons within the different layers of cortical plate giving rise to the characteristic six layers of neurons (Hatten, 1999; Gupta et al., 2002; Marin and Rubenstein, 2003). Birth dating using tritiated thymidine showed layers II-VI are generated in an "inside-out" sequence where earliest born neurons occupy the innermost layers and subsequent waves trespass them to form the more superficial layers (Angevine and Sidman, 1961; Rakic, 1974). A very remarkable phenomenon happens that radially migrating neurons (excitatory) and tangentially migrating neurons (inhibitory) born at same time, share the same layer inspite of the fact that GABAergic interneurons migrate much longer distance guided by a separate set of signals and cues (Marin and Rubenstein, 2003).

Unlike in radial migration, cells migrating tangentially tend to migrate with an orientation that is close to parallel to the VZ. These cells include mainly GABAergic interneurons and some cortical oligodendrocytes, originating in subpallium. Earlier experiments suggested Lateral Ganglionic eminence as the source of cells migrating tangentially (De Carlos et al., 1996; Tamamaki et al., 1997). But more recent studies based on DiI-Labelling experiments in slice cultures and several transplantation paradigms showed that these cells originated in various proliferative regions of subpallial telencephalon, including the LGE, medial ganglionic eminence, and POA/AEP (Anterior Preoptic area) (Anderson, 1997; Lavdas et al., 1999; Wichterle et al., 1999; Anderson et al., 2001; Marin et al., 2001; Wichterle et al., 2001). Two major tangential routes have been proposed: 1. From the medial ganglionic eminence (MGE) to the neocortex and hippocampus, and 2. From the lateral ganglionic eminence (LGE) to the olfactory bulb (Marin and Rubenstein, 2001; Kriegstein and Noctor, 2004), which in adulthood is called the rostral migratory stream (Figure 5). At Embryonic stage of 11.5 day in mouse the primary source of tangentially migrating neurons is MGE and AEP. Inteneurons migrate from MGE circumventing the striatum area and invade the subventricular zone (SVZ), the lower intermediate zone (IZ), and the subplate. LGE derived cells, meanwhile migrate to populate Olfactory bulb. The rostral migrations of cells from LGE proliferation region to the olfactory bulb persist untill adulthood. At late stages of telencephalic development, cells derived from LGE and MGE both robustly populate cortex, LGE neurons are directed toward the SVZ where they appear to become mitotically active (Marin and Rubenstein, 2001).

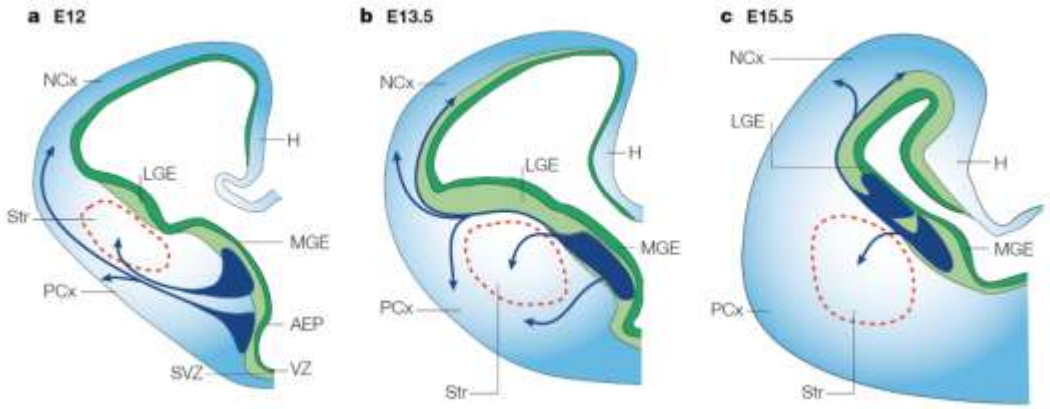


Figure 5: **Routes of tangential migration of immature interneurons from the subpallial telencephalon to the cortex**. Three spatially and temporally distinct routes can be distinguished. (a) Interneurons that migrate to the cortex and originating from the MGE and AEP in E12 embryonic stage, (b) At E13.5 stage interneurons originate from MGE but follow mainly a deeper route though some interneurons also follow superficial migration route like in earlier stage, (c) At E15.5 interneurons migrating to cortex also originate from LGE and goes through deep route. Abbreviations: AEP, Anterior entopeduncular area; H, hippocampus; LGE, Lateral ganglionic eminences, MGE, Medial ganglionic eminences, NCx, neocortex; PCx, piriform cortex; Str, Striatum; SVZ, Subventricular zone; VZ, ventricular zone. Marin and Rubenstein, 2001.

1.3 Migration Dynamics and Cortical Integration

Neuronal migration is a fundamental process in the development of the CNS as neurons are not born in their final adult location. So most neurons must migrate substantial distances from the ventricle to integrate into their final destination within the cortex. Migration of neurons is tightly regulated by various extracellular cues that ultimately trigger rearrangement of cytoskeletal components. The two main types of neurons that form the cortex and that take place from different origins needed two different modes of migration which became evident when lineage analysis proved that radially and tangentially migrating cells in the developing cortex arise from different progenitor cells (Mione et al., 1997 and Tan et al., 1998).

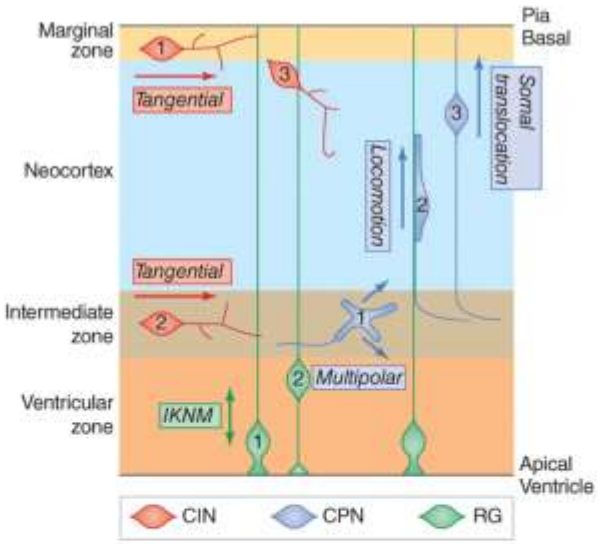


Figure 6: **Major Neuron Migrations in the developing mouse brain**: Cortical Interneurons (CINs, red) migrate tangentially along the marginal zone (1) and a intermediate zone (2) from their origins in the basal forebrain. Later they migrate into the cortical plate (3). Radial glia (RG, green) undergoes interkinetic nuclear movement (IKNM) with mitosis apical (1) and SPhase basal (2). Cortical Projection neurons (CPN,blue) migrate through three phases: multi-polar (1) and locomotion (2), and somal translocation (3). Figure adapted from Cooper, 2013.

Neuron migration happens mainly in two ways: locomotion (the main mechanism) and somal translocation (Figure 6). The highly polarized nature of migrating neurons, in the direction of migration, is achieved through generation of a single leading process acting as compass of migrating neurons which select direction by responding to chemotactic cues (Rakic, 1990; Yee et al., 1999). The morphology of the leading process varies in different neuronal types: in radially migrating cortical neurons they have single leading process (Rakic, 1972) while in cortical interneurons, the leading process branches as part of migratory cycles (Bellion et al., 2005; Martini et al., 2009; Marin et al., 2010). During locomotion cells migrate through repeated saltatory motions achieved by three steps. First, the cell extends a leading process approximately 50-100um from the soma and then the nucleus translocates into the leading process, a step referred as nucleokinesis (Figure 7). Nucleokinesis occurs in two steps as exhibited by time lapse imaging. A cytoplasmic swelling is formed in the same direction than the leading process containing the centrosome and a cage-like structure formed by microtubules. Centrosome forward movement induces the perinuclear microtubule network that extends from the nucleus to the centrosome to stretch, causing forward movement of the nucleus and other organelles into the swelling (Bellion et al., 2005; Schaar and

McConnell, 2005; Tsai and Gleeson, 2005). The perinuclear microtubule network regains its original form as nucleus moves forward (Solecki et al., 2004). Repetition of these two steps produces the typical saltatory movement of migrating neurons. In final step of locomotion, the trailing process is eliminated in the migrating neurons while pyramidal cells do not form a trailing process, extend their axon as they move (Schwartz and Meinecke, 1992; Horwitz and Parsons, 1999; Noctor et al., 2004). Locomotion is dependent on glia, radial glia therefore is needed in absence of pial attachment (Figure 7). The end is noted by leading process nearing the pia and the cell body reaches dense layers of immature neurons called primitive cortical zone (Nadarajah et al., 2001; Sekine et al, 2011).

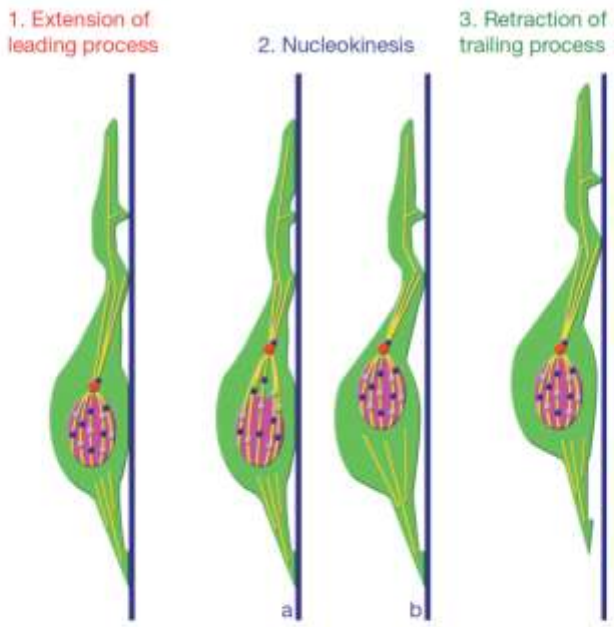


Figure 7. **Gliophilic radial migration**: 1. Migrating neurons first extend a leading process in the direction of migration, 2. this is followed by nucleokinesis beginning with the forward movement of the centrosome into the leading process. Forward movement of centrosome induces the perinuclear microtubule cage that extends from the nucleus to the centrosome to stretch, thereby mediating forward movement of the nucleus; 3. In the final step, retraction of the leading process enables the cell to move forward. Figure adapted from Solecki et al., 2006.

The second mode of migration is somal translocation which has been called so, as the entire cell soma moves towards the leading edge, not just the cell nucleus. It involves

nucleokinesis and trailing process but no extension of leading processes (Miyata et al.,2001; Nadarajah and Parnavelas, 2002; Miyata and Ogawa, 2007). This mode occurs as continuous, smooth movement; the leading edge is attached to the pial surface and is structured as fork, with processes that branch off a common branch point. This mode presumably doesn't require glial cells; pial attachment suffices for translocation of the neuron. This is the main mechanism of projection neurons early in cortical development or in the last cycle of locomotion (Figure 6; Miyata et al., 2001).

Radial migration can be easily called as major route of migration involved in formation of highly laminated structure in the CNS such as cerebral and cerebellar cortices. As mention above, glutamatergic cortical neurons in developing neocortex are born in an inside-out pattern where neurons for deepest layers are born and begin migration before neurons for superficial layers (Angevine and Sidman, 1961). Thus with development the distance to be migrated is increased for neurons. The radial migration thus is not a straight travelling as expected and later experiments using fluorescently labelled cells and time lapse imaging in slice cultures showed that projection neurons do not simply migrate along a straight radial trajectory from the VZ to CP but do display a more complex behavior characetrized by changes in morphology, orientation and speed of migration. (Tabata and Nakajima, 2003; Noctor et al., 2004). The first neurons that are born move directly to the CP adopting a characteristic bipolar morphology while they migrate. However, some of them move radially just a short distance. Then they pause for as long as 24 hours adopting a multipolar morphology and going through another round of cell division. These cells form another layer of progenitor cells adjacent to the VZ, which is called the SVZ. It is suggested that the arise of the SVZ has been essential for the expansion of the neocortex in mammals (Tabata et al., 2012). Progenitors in the VZ and SVZ are also known as Apical and Basal progenitors (APs and BPs), respectively. APs give rise to deep layer neurons, while BPs, give rise to upper layer neurons within the CP. Originally this phenomenon of migratory pause of cortical neurons in SVZ was termed 'Sojourning' (Bayer et al., 1991). Multipolar SVZ neurons are highly dynamic, extending and retracting processes though they don't migrate appreciable distance. Suggested by many research this behaviour allows the SVZ progenitors to search for environmental cues (Noctor et al., 2001; Tabata and Nakajima, 2003; Noctor et al., 2004; Tabata et al., 2009). For exiting the SVZ, BPs adopt again a bipolar morphology extending a leading process directed towards the pial surface in

order to reach the CP. However, some BPs extends a process towards the ventricle and often exhibiting a retrograde somal movement into the VZ (Noctor et al., 2004). Here again neurons might spend 24 hours near the margin of ventricle before proceeding for radial migration towards the CP (Noctor et al., 2004). Stage four migrations seems to be gliophilic and transition from one phase to another involves environmental signals and cue to guide some neurons to show retrograde motion while some proceed from second stage to fourth directly.

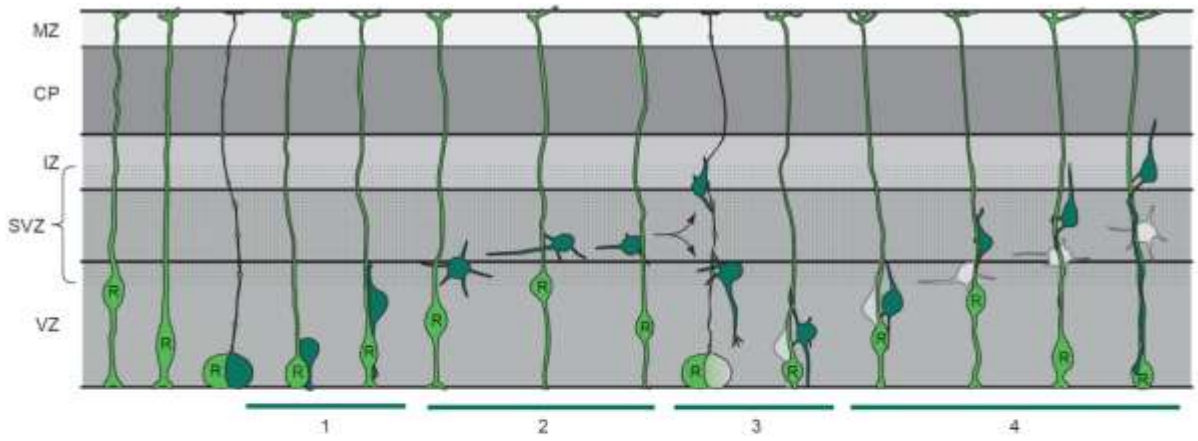


Figure 8. **Schematic Diagram showing cortical pyramidal neurons undergo distinct phases of locomotion migration.** Phase one involves radial movement of pyramidal neurons (dark green) from the site of origin at the ventricular surface to the subventricular zone. In Phase two, cells become multipolar and pause their migration in the lower intermediate zone and SVZ. Some neurons undergo phase three, which is characterized by retrograde motion toward the ventricle. Phase four is the final radial migration to the cortical plate (CP), guided by radial glial fibres. Radial glia (light green) remains mitotic, undergo interkinetic nuclear migration, and generate additional daughter cells (grey). Figure adapted from Kriegstein and Noctor, 2004.

Though Interneurons migrate tangentially through saltatory progression, some differences exist compared to pyramidal neurons. In both cases, neurons undergo extension and retraction of leading process which is sensitive to guidance cues followed by nucleokinesis but in the case of pyramidal neurons this leading process is unique while in interneurons can be branched (Moya and Valdeomillos, 2004; Bellion et al., 2005; Nasrallah et al., 2006; Britto et al., 2009: Martini et al., 2009). Tangential migration of interneurons relies on other types of cell interactions different from the

RGC process. Permissive homotypic interactions, where adjacent migrating cells are used as a substrate, are used when neurons move through hostile environment, for instance neuroblasts migrating from the lateral ventricles of the telencephalon toward the olfactory bulb in adult brain, also known as "chain migration" (Wichterle et al., 1997; Corbin et al., 2001; Marin and Rubenstein, 2001). Inhibitory homotypic interaction is also a commonly used strategy and is also known as "contact inhibition". Here neurons achieve directional migration in absence of chemotactic gradients as it favours cell movement towards area with less cell density. Cajal retzius cells are suggested to use this strategy to disperse throughout the surface of cerebral cortex during early corticogenesis (Borrell and Marin, 2006).

The trajectories of tangential migrating interneurons are more complex than pyramidal neurons. Normally the tangential migration occurs in ventrolateral to dorsomedial trajectory but evidences also suggests that interneurons can also follow multidirectional migration patterns within the MZ and IZ/SVZ streams (Ang et al., 2003; Tanaka et al., 2003, 2006, 2009). And similar to radial migration, migrating interneurons in the IZ/SVZ dive back to VZ before ascending back to its final position in cortical plate termed ventricle directed migration (Nadarajah et al., 2002; Ang et al., 2003). An important aspect of neuron migration is the heterotypic interactions. Pyramidal neurons use for instance the RGC processes as a scaffold to guide their final destination (gliophilic interaction). Interneurons do not use RGCs processes and instead use heterotypic interactions with either corticofugal or thalamocortical axons (neurophilic interaction) making projections into GE as substrates during tangential migration (Parnavelas, 2000; Denaxa et al., 2001Morante-Oria et al.,2003; Tanaka et al., 2003; Lopez Bendito et al., 2006).

1.4 Molecular Mechanisms Regulating Neuronal Migration

The molecular mechanisms controlling the migration of neurons in vivo are complex, as they involve coordination of many factors including extrinsic factors such as secreted molecules (axon guidance cues for instance), cell-cell and cell-extracellular matrix

adhesion and even neurotransmitters and intrinsic factors such as regulators of cytoskeleton dynamics and transcription factors.

Extrinsic Factors

Many secreted extracellular molecules like Slits, netrins, Semaphorins, and Reelin showed roles in regulating neuron migration but probably Reelin is the best characterized one in radial migration in the developing cortex. Reelin is a large extracellular glycoprotein secreted by cajal retzius cells in the marginal zone (D' Arcangelo et al., 1995; Ogawa et al., 1995; Hirotsune et al., 1995; Sheppard and Pearlman, 1997). The *Reeler* mouse was initially described in 1951 by Falconer (Falconer, 1951). These mice displayed abnormal behaviours such as ataxia, tremor and reeling gaits, reeler is an autosomal recessive mutation in the mouse that disorganizes cortical development (Caviness and Sidman, 1973; Caviness, 1976; Goffinet, 1979; Caviness, 1982, 1992).At the anatomical level, their brains displayed defective laminar organization of the cerebral cortex (Hamburgh, 1963; Rice and Curran, 1999; Lambert de Rouvroit and Goffinet, 2001). In detail, the preplate formation in *reeler* mice happens normally but the second cohort of cortical neurons fails to split the PP into the marginal zone and the subplate and a superplate structure is formed instead called a reeler cortical phenotype. Following cohorts of differentiating neurons do not trespass older ones and therefore the mice exhibit an inverted laminar pattern (Figure 9). Reelin plays multiple roles in regulating the proper laminar position of neurons in the neocortex by acting as a stop signal to terminate the radial migration of cortical neurons at the top of the cortical plate (Magdaleno et al., 2002; Frotscher, 2010). Reelin binds to two members of the lipoprotein family receptors, VLDLR and ApoER2 expressed by migrating neurons (D'Arcangelo et al., 1999; Heisberger et al., 1999). Recently it has been reported that Reelin signalling can be linked to the dynamics of actin filaments in migrating cells. Formation of new actin filaments is promoted by N-cofilin which is phosphorylated in the leading process of migrating neurons by Reelin signalling through ApoER2. Phosphorylated N-cofilin loses its capacity to depolymerise F-actin leading to stabilization of neuronal cytoskeleton, providing evidence to possibility that Reelin acts as a stop signal. In the postnatal mouse brain, Reelin acts as a detachment signal for chain-migrating interneuron precursors in the olfactory bulb according to recent study (Hack et al., 2002).

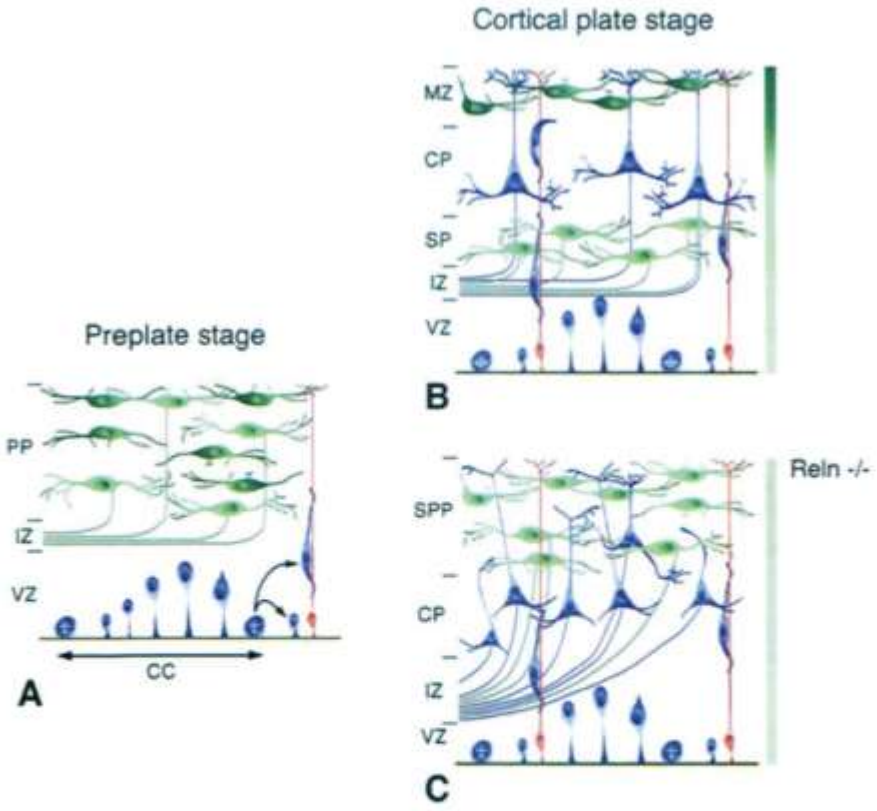


Figure 9. **Radial migration generates the laminar organization of the cortex**. (a) The preplate is constituted by two main types of early generated neurons: Cajal Retzius cells, which express Reelin (Dark green cells) and subplate neurons (light green cells). The Radial migration of cortical neurons (Blue cells) requires interaction with radial glia (Red Cells). (B) Migration of cortical neurons forms the cortical plate, which splits the preplate in two layers called the Marginal zone and subplate. (C) In reelin mutants, cortical neuron fail to invade the preplate and the cortical plate develops deep to Cajal Retzius and preplate cells (superplate). Figure adapted from Marin and Rubenstein, Chapter 5 book Mouse Development: patterning, morphogenesis and organogenesis.

Another family acting as guidance molecules in neuronal migration is Semaphorins. Semaphorin (Sema) 3A and 3F, have been shown to act as chemorepellents for migrating interneurons and regulate the sorting of interneurons towards the striatum and the cortex (Marin et al., 2001; Nobrega-Pereira et al., 2008). In contrast, Sema 3A has a role to act as a chemoattractant for apical dendrites of differentiating cortical neurons providing directionality to cell movement (Chen et al., 2008). Sema3A is present in superficial cortical layers and is able to attract new born neurons in transwell chemotaxis assays in vitro. In vivo, radial migration of cortical neurons mainly towards layer II/III in the CP was impaired by knockdown of neuropilin 1, the receptor of Sema3A, by in utero electroporation experiments (Chen et al., 2008). Thus Semaphorin-

3A serves to guide cortical projection neurons toward upper cortical layers. Other semaphorin has also displayed possible roles in radial migration for example Sema-6A which might initiate radial migration of cerebellar granule cells through modulation of nuclear translocation (Kerjan et al., 2005).

Netrin/Dcc (deleted in colorectal carcinoma) and Slit/Robo are two other major ligands/receptors families playing role as guidance cues. Chemoattractant netrin-1 is expressed mainly in MZ in cortex where it can interact with the active alpha3beta-1 integrin expressed by migrating interneurons. Inhibition of this interaction leads to disruption in interneuron migration and abnormal positioning in upper cortical plate (Stanco et al., 2009). Slits are diffusible repulsive cues present at LGE and striatum. Although mice lacking Slit1 and Slit2 interneuron migration stay unaffected, knock-out mice for Robo1 display an aberrant population of calbindin-expressing interneurons in the Slit3 expressing striatum, a normally repellent environment for interneurons (Zhu et al., 1999; Marillat et al., 2002; Marin et al., 2003; Andrews et al., 2006). Neuregulins (Nrgs) belong to the epidermal growth factor family of growth factors. Neuregulin-1(Nrg1) is expressed in the subpallium and in cortical IZ/SVZ/VZ working as both long range and short range attractant through its receptor ErB4 (Yau et al., 2003; Flames et al., 2004). Nrg1 is expressed as two isoforms in developing cortex (Flames et al., 2004).

The CRD-Nrg1 (Type III) is a membrane bound form that is expressed in the route followed by interneurons from LGE to cortex acting as permissive factor (Flames et al., 2004). In contrast the Ig-Nrg1 (type I/II) is a secreted form that is expressed mainly in developing pallium forming a long-range gradient which helps directing interneurons from MGE to cortex (Flames et al., 2004). However, the attractive role of Neuregulin has been questioned by recent publications (Li et al., 2012). Other soluble molecules have been also involved in neuron migration including the hepatocyte growth factor/ scatter factor (HGF/SF), glial cell line-derived neurotrophic factor (GDNF), brain derived neurotrophic factor (BDNF), and NT4. For instance, they are all expressed in interneuron migration trajectory and guide its migration properly (Powell et al., 2001; Polleux et al., 2002; Pozas and Ibanez, 2005). Special mention to chemokines, which have been shown to be essential in maintaining migration of interneurons at later stages within the cortex, in the MZ and SVZ (Tiveron et al. 2006; Li et al., 2008; Lopez-Bendito et al., 2008). Thus the stromal derived factor-1 (SDF-1 also known as Cxrcl2) is a potent chemoattractant for cells derived from MGE via its receptor CXC chemokine

receptor 4 (CXCR4) expressed by interneurons. Loss of either SDF-1 or CXCR4 expression does not prevent interneurons from reaching the cortex but migration is less organized and leads to disrupted distribution (Stumm et al., 2003; Tiveron et al., 2006; Li et al., 2008; Lopez-Bendito et al., 2008). Cajal Retzius cells also express CXCR4 and their attraction to meningeal SDF-1 is pivotal for tangential migration (Borrell and Marin, 2006; Paredes et al., 2006).

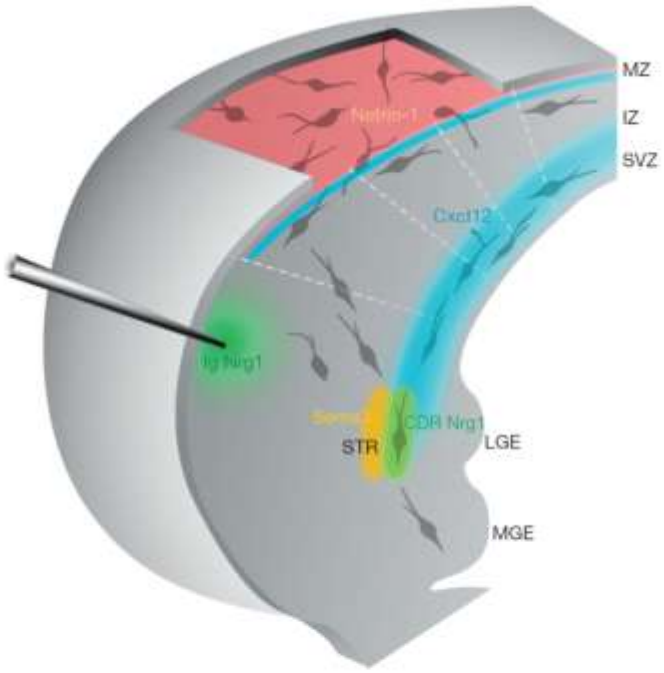


Figure 10. **Guidance Cues and Tangential Migration**. MGE-derived interneurons cross the LGE aided by membrane-bound neuregulin-1 (Type III Nrg1) acting as a permissive substrate. Interneurons avoid the developing striatum in response to class III semaphorins. Robo receptors are also involved in regulating the migration of cortical interneurons around the striatum. Type I/II Nrg1 is expressed in the developing pallium. Ig-Nrg1 is cleaved from the membrane; therefore it is likely that its gradient contributes to driving MGE-derived interneurons toward the cortex. In the pallium, the chemokine signaling CXCL12/CXCR4 promotes the tangential dispersion of interneurons through the SVZ and MZ. Netrin-1 interacting with the alpha3beta1 integrin expressed by interneurons also promotes their migration in the MZ. Cell dispersion in the MZ proceeds by multidirectional motion with frequent changes in cell polarity. Cell directionality depends on the angle of branch formation. Cells migrating in the SVZ generate small branching angles with high frequency. Neurons in the CP show a higher frequency of branches with large angles. Also interneurons form branches with wider angles when subjected to experimental chemoattractant gradients created in ectopic places, as indicated by experiments with the micropipette perfusion of Ig Nrg 1. Figure adapted from Marin et al., 2010.

Many cell adhesion molecules like integrins and astrotactin and structures (Gap junctions) plays vital role in neuron migration, for instance in mediating the interaction of migrating pyramidal neurons with the RGC processes during locomotion and radial migration (Stitt and Hatten, 1990; Anton et al.1997; Adams et al.2002). Integrins are glycoprotein transmembrane receptors mediating cell-cell and cell-extracellular matrix (ECM) interactions. Functional integrin is composed of alpha and beta subunits forming heterodimers in many combinations that gives more than 20 different integrin receptor subtypes (Juliano, 2002). All alpha subunits expressed in developing cortex like alpha3, alphaV and alpha-6 dimerize with beta integrin. Alpha subunits determine responses of integrin receptors by determining ligand specificity (Anton et al., 1999). Mutations in mice for these integrins genes exhibited distinct cortical lamination phenotypes demonstrating different role played by different subunits in neuronal migration (Marin and Rubenstein, 2003).Retarded neuronal migration and disrupted cortical lamination was observed in alpha3 mutants (Schmid et al., 2004). In vitro inhibition of apha3 integrin also reduces the speed of migration and cause decreased gliophilic interactions of neuron-RGC leading to detachment (Anton et al., 1999; Dulabon et al., 2000). Conditional knockdown of the β1 subunit leads to disrupted cortical lamination by perturbance of anchoring of RGCs to the pial surface (Cousin et al., 1997; Graus-Porta et al., 2001). While deletion of β1 integrin from RGCs resulted in cortical lamination disruption, selective β1 integrin deletion from cortical neurons doesn't produce any defect in migration or cortical lamination (Belvindrah et al., 2007). Integrins interact with ECM components such as fibronectin, laminin, and reelin (Sheppard et al., 1991; Dulabon et al., 2000). While integrins mediate cell-matrix interactions and mediate gliophilic to neurophilic switch. A gliophilic-to-neurophilic switch in the adhesive preference of developing cortical neurons occurs following the loss of alpha3beta1 integrin function (Anton et al, 1999), Gap junction channels can link together adjacent neurons to produce a cluster of electrically coupled cells firing synchronous action potentials, which is suggested to presage development of synaptic coupling (Yuste et al., 1992). This Gap Junctions are channels connecting opposing membranes through hydrophobic interactions and form an aqueous pore joining the cytoplasm of adjacent cells (Elias and Kriegstein, 2008). This connection allows exchange of ions and molecules up to approximately 1200 Da in size between coupled cells (Simon and

Goodenough, 1998). These proteins are composed of six Cx subunits, and at least 20 Cx genes have been identified. Recent work has defined more roles of gap junction channels in embryonic cortex development including regulation of proliferation, cytoskeletal elements and neuronal migration (Weissman et al., 2004; Elias et al., 2007; Olk et al., 2009). Furthermore, Gap junction adhesion sites promote stabilization of the neuronal leading processes and somal translocation (Elias et al., 2007).

Another cell surface glycoprotein expressed by migrating cortical and cerebellar neurons Astrotactin regulates adhesive interaction between migrating neurons and radial glial cells (Edmondson et al., 1988; Adams et al., 2002). NJPA1 or Neuron-glial junctional protein 1 which is radial glial membrane protein also play role in neuron-glial adhesion and migration (Anton et al., 1996).

Most of these factors have a direct or indirect effect on cytoskeleton dynamics affecting migration forming a complex decision making system. Neurotransmitters also play a vital role in modulating migration of cortical neurons. Neurotransmitters like GABA, glutamate and glycine have different functions in promotion of radial and tangential migration. The primary neurotransmitter present in the adult neocortex is GABA which is also found in the neocortical proliferative zones in neurogenic stages during development (Van Eden et al., 1989; Del Rio et al., 1992; Schwartz and Meinecke, 1992; Behar et al., 1996; Tsai and Leung, 2006). Migrating neurons express the two main types of receptors $GABA_A$ and $GABA_B$ receptors and also $GABA_C$ which is considered a variant of $GABA_A$. GABA receptor subtypes have different roles in migrating neurons and acts dose dependently, Low concentrations of GABA (femtomolar) exert a chemotaxic effect on neurons while higher concentration (micromolar) exert less specific chemokinetic effect increasing motility of cortical neurons. Neuronal migration induced by GABA *in vitro* is age and dose dependent (Behar et al., 1996). Like $GABA_B$ act as chemoattractant for cortical neurons while Luhmann and colleagues showed that GABA acts through $GABA_C$ to promote radial migration (Luhmann et al., 2006) $GABA_A$ receptor acted differently than other receptor by playing role in stopping the cortical migration (Behar et al., 2001; Denter et al., 2010). GABA is upregulated by migrating interneurons, but its role in migration is controversial. Pharmacological blocking of $GABA_AR$ impeded interneuron migration while other experiments suggested contradictory results (Behar et al., 1996; Lopez-Bendito et al., 2003; Cuzon et al., 2006). Other neurotransmitters have been also

involved in interneuron migration. For instance the subpallium acts as a source of dopamine which modulates interneuron migration (Crandall et al., 2007) while AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxasole-propionic acid) has been shown to initiate retraction of leading process and halt migration (Anton et al., 2013).

Apart from GABA other neurotransmitters which had important role in guiding radial migration was Glutamate, ATP and NMDA. Glutamate is the main excitatory neurotransmitter in the adult brain. Cajal retzius cells in the marginal zone releases glutamate while post mitotic neurons migrating to the cortical plate express glutamate receptors (del Rio et al., 1995). Glutamate has multiple roles in developing neocortex like stimulating neurite outgrowths, proliferation control, and chemoattractant stimulating migration from proliferative zones to the cortical plate (Pearce et al., 1987; Behar et al., 1999; Haydar et al., 2000). Similar roles have been exhibited by Glycine and NMDA also in radial migration of cerebellar granule neurons (Komuro and Rakic, 1993; Behar et al., 1999).

Intrinsic Factors

Lis1 is a microtubule associated protein that bind tubulin and other microtubule regulatory proteins (Sapir et al., 1997) including dynein/dynactin (Faulkner et al., 2000; Smith et al., 2000), Ndel1/NUDEL (Sasaki et al., 2000), and Nde1/ mNudE (Feng et al., 2000). It is suggested that Lis1 acts through promoting dynein ATPase activity (Mesngon et al., 2006) and knockdown of NdeI, which facilitates the interaction of Lis1 and Dynein, prevents many cells from migrating away from the proliferative zones(Sasaki et al., 2005). Mutations in Lis1 leads to a malformation called smooth brain (lisencephally) lacking sulci and gyri with disrupted cortical layers (Pilz et al., 1998). Lis1 also interacts with reelin signalling pathway to modulate neuronal migration (Assadi et al., 2003). Dcx is another microtubule associated protein interacting and stabilizing polymerized microtubules (des Portes et al., 1998; Gleeson et al., 1998; Francis et al., 1999; Horesh et al., 1999). It is suggested that Lis1 and Dcx works in concert to maintain microtubule polymerization and possibly in similar pathways. Dcx promotes neuronal migration and is found in leading processes of migrating and

differentiating neurons (Francis et al., 1999). Dcx is a major genetic cause of subcortical band heterotopia (Dobyns and Truwit, 1995).There are many more important regulators of cytoskeleton having decisive role in migration including Filamin A and Cdk5. Filamin A is an actin crosslinking protein produced by FLNA gene expressed by migrating neurons in prenatal cortex. Filamin A is thought to enhance cell motility by crosslinking F-actin, which in turn increases the viscosity of F-actin network (Stossel et al., 2001). Cdk5 is a kinase phosphorylating cytoskeletal proteins including microtubule and actin associated proteins. Focal adhesion kinase is phosphorylated by Cdk5 regulating organization of microtubules in migrating neurons (Xie et al., 2003). Cdk5 mutants showed similar defects in lamination of cortex as in reeler mice (Chae et al., 1997).

Transcription factors role in radial migration has been found very recently. COUP-TFII for instance has been found to be necessary and sufficient for tangential migration of CGE derived interneurons (Kanatani et al., 2008); COUP-TFII overexpression drives intereneurons into the IZ/SVZ migratory stream over the MZ stream (Kanatani et al. 2008). Pax6, expressed by radial glia in VZ has been proposed to have role in neuronal migration (Gotz et al., 1998). Pax6 knockout embryos show defects in migration of late born cortical precursors (Caric et al., 1997). Chimeric analysis showed that Pax6 is important for the radial migration of cortical cells from the SVZ to the overlying cortical plate (Talamillo et al., 2003). Another transcription factor expressed by intermediate progenitor cells located in the SVZ of the dorsal but not ventral forebrain Tbr2 has also been proposed to have role in proper layer formation of the dorsal neocortex (Englund et al., 2005; Sessa et al., 2008). Tbr2 expression is needed to loosen the intermediate progenitor cells from the ventricle surface and trigger migration towards the overlying SVZ, as Tbr2 downregulate the expression of cell adhesion molecules (Arnold et al., 2008). Neurogenins 1 and 2 (Ngn1/2) are proposed to enhance migration of projection neurons produced in the dorsal cortex (Nobrega-Pereira and Marin, 2009). The signalling mechanisms by which transcriptions factors exert their effects on migration are not clear. Previous work showed that Ngn2 induces expression of the Rho GTPase Rnd2 causing migration of cortical neurons (Heng et al., 2008). Another proneuronal factor, Ascl1, directly regulates Rnd3 expression and also promotes neuronal migration in the developing cortex (Pacary et al., 2011). Both Rnd2

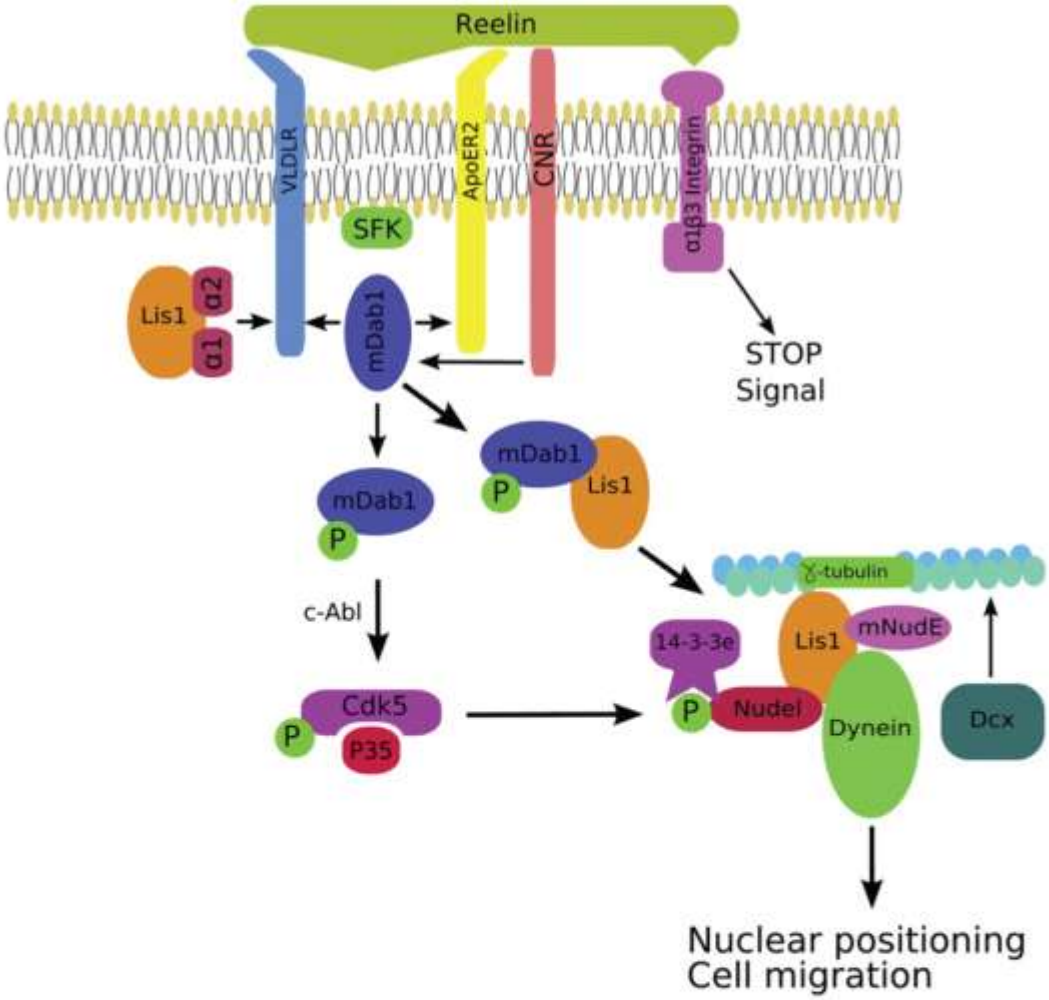


Figure11. **Lis1 and Reln signalling pathways for neuronal migration**. Extracellular Reln binds to one of the three receptors complexes: cadherin-related neuronal receptor (Cnr), VldlR/ApoER2 or 3β integrin. This binding activates mDab1, which can activate Cdk5/p35.Cdk5 can phosphorylate many intracellular targets including Nude1. Phosphorylated Nude1 forms a complex with Lis1, mNudE, Dyneins and microtubules. This complex is necessary for cell migration and nuclear positioning. Dcx is also believed to interact with Lis1 and modulates microtubule function. Figure adapted from Guerrini and Parrini, 2009.

and Rnd3 exert their effects on migration inhibiting RhoA signaling but at different levels: while Rnd2 control multipolar to bipolar transition in the SVZ/ IZ, Rnd3 controls locomotion in cortical plate (Heng et al., 2008; Pacary et al., 2011). As for interneurons a cohort of transcription factors have been shown to regulate tangential migration. Neuropilin-2 for instance can be repressed by both Nkx2.1 and Dlx1/2, controlling the migration trajectory of cortical and striatal interneurons (Le et al., 2007; Nobrega-

Pereira et al., 2008). While inhibition of Dlx1 and Dlx2 lead to no detectable interneuron migration (Anderson et al., 1997).

1.5 Axon Guidance

Differentiating neurons during brain development send out an axon tipped at its leading edge by a very motile structure named "growth cone" that explores the environment in order to find the appropriate synaptic target. This is achieved in a highly specific and directed manner leaving no space for any error; this error proof system is formed by molecular guidance cues presented by cells in the environment (Tessier-Lavigne and Goodman, 1996). The correct connectivity of nervous system relies on this ability of axons to locate and recognize their appropriate targets. Axon guidance is regulated by the expression of guidance cues that can act as chemo-attractants/repellants and contact-attractants/repellants either locally or over long distances (Tessier-Lavigne & Goodman, 1996) (Figure 12).

Several conserved families of axon guidance molecules have been discovered untill now, prominent among these are Netrins, Slits, Semaphorins and Ephrins as their mechanism of action is most understood by neurobiologists (Dickson B et al., 2002). Netrins form a conserved family of secreted proteins which have been shown to act as bifunctional neural guidance cues, exerting either attractive or repulsive effects (Dickson and Keleman, 2002). The repulsive effects of netrin involve the UNC5 family of receptors, and the attractive effects of netrin are mediated through the DCC family of receptors, although the DCC family of receptors has also been implicated in repulsion (Round and Stein, 2007). Semaphorins is another conserved family of axon guidance cues that include secreted and membrane bound proteins (Pasterkamp and Kolodkin, 2003). They are known for neural repulsion and, classified into eight groups on the basis of sequence similarity and distinctive structural features (Figure 13; Raper, 2000). Two receptor families have been implicated in mediating semaphorin function, the plexins and neuropilins **(**Figure 13; Winberg et al., 1998; Raper, 2000**).** Slits are a family comprised of large, secreted proteins containing four leucine-rich repeats

surrounded by conserved N- and C-terminal domains, nine EGF-like repeats, an ALPS domain and a cysteine-rich carboxyl terminal domain (Wong et al., 2002). Slits have a role mainly in neural repulsion mediated via a family of receptors called roundabout (Robo) (Battye et. al., 1999; Kidd et al., 1999). Similarly Ephrins are a family of membrane bound proteins (Himanen and Nikolov, 2003), with role in repulsion mainly along with attractive effects that are triggered by the receptor tyrosine kinase Ephs. The name of "axon guidance cues" is currently considered as misleading since these factors have been involved in many

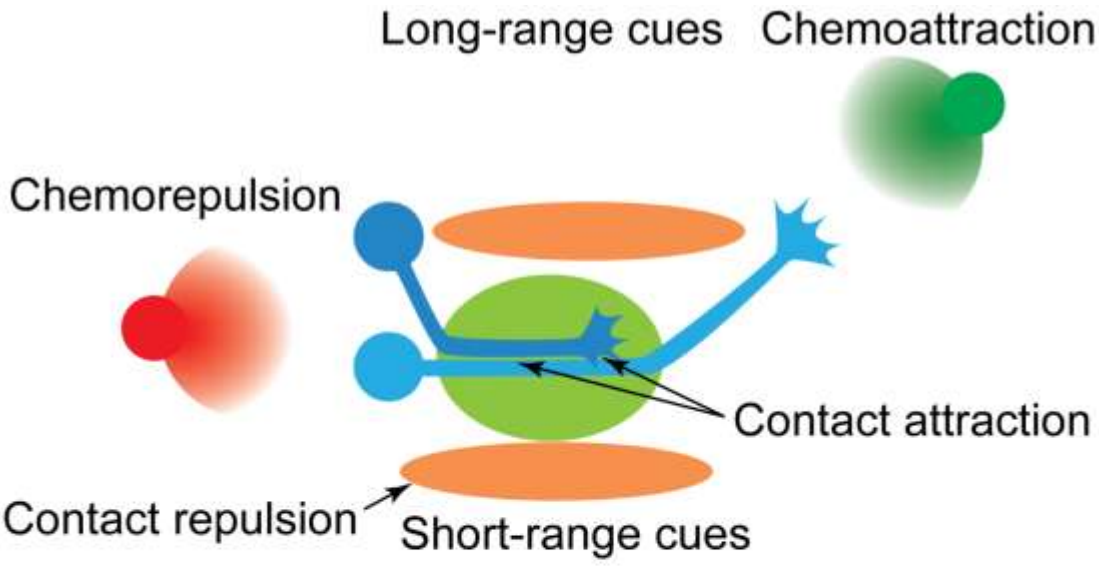


Figure 12. **Guidance mechanisms of axons and migrating neurons.**
Schematic representation of the four basic guidance mechanisms based on the nature of the molecule (soluble or membrane bound) and the elicited response (attractive or repulsive): chemoattraction, chemorepulsion, contact attraction and contact repulsion. Migrating neurons and extending axons possess the specialized structures to perceive directional signals-the leading process and growth cone, respectively. In this scenario, extending axons and migrating neurons are coarsely guided by attractive (+) and repulsive (-) diffusible cues towards the target area. On their way, they encounter guidepost cells providing short-range directional information by presenting membrane bound attractive or repulsive signals.

other functions, including non-neuronal aspects. As described above, many of these cues were shown to regulate neuron migration. In the other hand, Slit/Robo system has been implicated in the modulation of proliferation of the central nervous system

progenitors and EphRs. Robo and UNC5 have been shown to be involved in vascular system development (Segura et al., 2009; Borrell et al., 2012). The amazing complexity of nervous system contrasts with the characterization of relative small families of axon guidance cues. The function of these cues have been expanded by the observation that their temporal and spatial expression is tightly regulated, by finding that their activity can be finely tuned by co-receptors and by the fact that their signalling often crosstalk leading to a readout not predicted previously by the function of the individual cues (Dudanova and Klein, 2013; Levya et al., 2014). These data emphasizes the need of finding more molecular mechanisms involved in the construction of neural framework (see below Aims and Objectives).

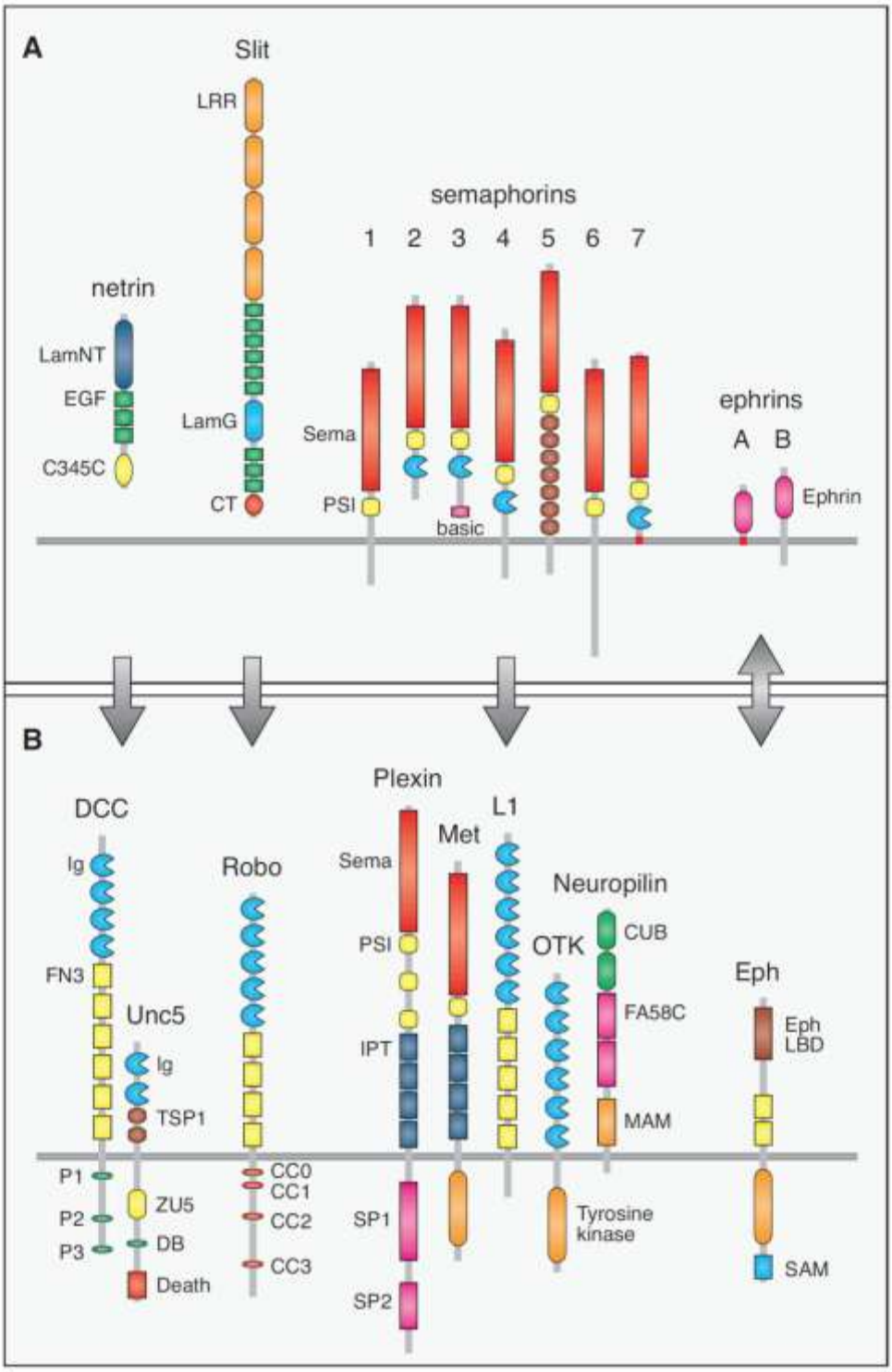


Figure 13. **Conserved families of guidance molecules** (A) and their receptors (B) Domain names are from SMART (http://smart.embl-heidelberg.de). P1 to P3, DB (DCC binding), CC0 to CC3, and SP1 and SP2 indicate conserved regions in the cytoplasmic domains of DCC, UNC-5, Robo, and Plexin receptors, respectively. Figure Adapted from Dickson J , 2002

1.6 Coordination of Neuronal migration and Axon guidance: development of Thalamocortical Projections

It's surprising that these two events are normally analyzed as independent processes even when it is evident that they must have been linked through evolution to ensure the precise formation of neural circuits.

After generation of distinct neuronal populations, immature neurons migrate from origin to more superficial positions of the neural tube, where axonal connections eventually occur (Hatten, 2002). Neurons then extend axons, which navigate through the developing brain following highly stereotyped routes guided by guidance molecules positioned in the extracellular environment to find specific targets (Tessier-Lavigne and Goodman, 1996; Dickson, 2002). Precise distribution of guidance molecules in time and space is an integral part of axon pathfinding. However, the information about the mechanisms controlling the precise timing and arrangement of guidance cues is very limited. Apart from neuroepithelial cells, postmitotic neurons also contribute in display of patterning information since expression of guidance cues is transferred from neural progenitors to postmitotic cells through the process of radial migration (Rakic, 1988).

Tangential migration evolved as a mechanism to increase the complexity of neuronal circuits because it allows neurons born from distant progenitor zones to intermingle in final common destination and applies to axons as well (Corbin et al., 2001; Marin and Rubenstein, 2001). Tangential migration is most prominent in ventral telencephalon where various major axonal tract, such as thalamocortical connection traverse. Thalamocortical projections constitute one of the most prominent higher level processing connections in the mammalian brain. Thalamocortical axons (TCAs) convey sensory and motor inputs to the cerebral cortex, where integration of this information leads to perception and the organization of appropriate responses (Lopez-Bendito et al., 2006). The functional complexity of the thalamocortical projection is the consequence of an extremely elaborate process of axon guidance, orderly linking the various thalamic nuclei with specific cortical regions. Thalamocortical axons originate in dorsal thalamus, continuing ventrally passing through ventral thalamus and turn dorsolaterally to extend through ventral telencephalon to neocortex. Concomitantly, projection neurons in the cortex send axons to the thalamus (corticothalamic axons) that meet

TCAs and it is thought that one set of axons serve as a substrate for the other to reach their final targets (hand-shake hypothesis) (Molnar et al., 2012). The mechanisms controlling the pathfinding of thalamocortical axons are not completely understood but it has been shown that neuron migration is required.

1.7 Human Neocortex Development

The description of the different aspects and mechanisms of brain development explained so far is based mainly on observations made in the mouse. Most of this information has been very valuable to understand our own brain development and connectivity. However, some specific traits of human brain, such as the increased neocortical volume and surface area and the emergence of convolutions of cortical sheet forming gyri and sulci, which overall is thought to be the basis of intellectual capacity, highlights the limitations of the mouse model (Reillo et al., 2011; Welker 1990; Rakic 1995). Therefore it is necessary to understand how molecular regulators of developing mouse brain can control human neocortical development and if specific molecular programs have evolved. However first we discuss cytoarchitectural differences and then various hypotheses about human neocortical development.

Summary of Human-Rodent Cytoarchitectural differences during neocortical development

The basic sequence in formation of neocortex is conserved across human and rodents, the neuroepithelial cells of the neocortical primordium proliferate and differentiate to become a complex multi-layered structure; however some transient layers in humans develop more complex as development progressesto acquire larger surface area with formation of gyri and sulci and thus thicker neocortex. The human PP, prior to segregation into MZ and SP, develops transiently into multiple layers namely the superficial Cajal-Retzius cell layer, the intermediate monolayer and deep pioneer plate (O'Leary and Nakagawa, 2002; Meyer, 2001; Meyer et al., 2000). Also the human SVZ is subdivided into ISVZ and OSVZ by a cell poor/fibre rich IFL (intermediate fibre layer) at around 12 pcw (Bayatti et al., 2008; Smart et al., 2002; Zecevic et al., 2005).

This layer is comprised of more heterogeneous populations of progenitor cells unlike rodent SVZ which contain only few layers of cells (Fietzz et al., 2010; Hansen et al., 2010; Martinez-Cerdeno et al., 2006). Human SP is another example of difference between rodent and human neocortex where human SP is expanded drastically by accumulation of vast amount of fibre tracts (Kostovic and Rakic, 1990) and is correlated with formation of gyri by bulging of CP caused by this fibre tract accumulation (Kostovic and Rakic, 1990). This elaborate and complex SVZ and SP underlie the thicker neocortex and surface area expansion in gyrencephalic humans. Another difference between two is appearance of transient layer of undifferentiated granular cells above the human MZ termed SG layer (Meyer, 2001) which is absent in rodents.

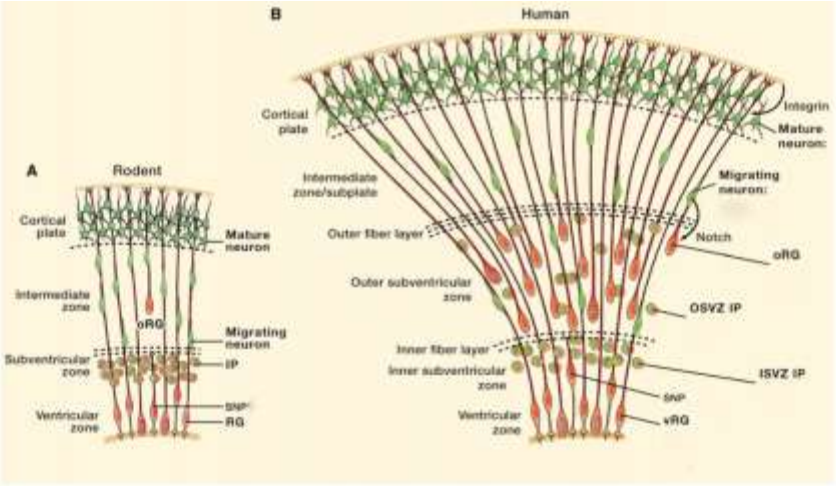


Figure 14 . **Contrasting Rodent and Human Neocortical Development**
(A) Current views of rodent corticogenesis are illustrated. Radial glial (RG) cells most often generate intermediate progenitor (IP) cells that divide to produce pairs of neurons. These neurons use RG fibers to migrate to the cortical plate. The historical view of neocortical development was that RG and neuronal progenitor cells were lineally distinct and that RG did not have a role in neurogenesis. Our current appreciation of the lineage relationship between RG cells, IP cells, and neurons has revised this view. The recent observation that small numbers of outer subventricular zone radial glia-like (oRG) cells exist in the mouse is also illustrated. (B) We highlight the lineage of oRG cells, IP cells, and migrating neurons (red to green) present in the human outer subventricular zone (OSVZ) and the increased number of radial fibers that neurons can use to migrate to the cortical plate. The number of ontogenetic "units" is significantly increased with the addition of oRG cells over ventricular RG (vRG) cells. Maintenance of oRG cells by Notch and integrin signaling is shown. Short neural precursors (SNP), a transitional cell form between RG and IP cells, are also depicted in (A) and (B). Lui et al., 2011.

In Mouse majority of RG are epithelial cells residing in VZ making full apical-basal contacts (Noctor et al., 2002). However in humans RG are organized differently in developing neocortex and are spanning ventricle to pia. Thymidine labelling studies suggested that proliferation of cells within OSVZ coincides with major wave of cortical neurogenesis (Rakic, 1974; Lukaszewicz et al., 2005) and thus it's possible that OSVZ contributes to neuron production. Also recent studies revealed the cellular heterogeneity of OSVZ, which includes both RG and IP cell types. The RG cells in OSVZ were called oRG and unlike vRG cells which are bipolar, the oRG cells are unipolar, with basal fiber towards the pia but without any apical fiber. oRG cells then divide asymmetrically to daughter cells that are bipolar, which remain undifferentiated over additional transit-amplifying cell cycles, and do not express neuronal markers such as TBR2. Each oRG

cell functions as a founder cell for an extended lineage during the cell cycles. This transit amplification helps in understanding the gyrencephalic brain development (Reillo et al., 2011). oRG cells continue to translocate towards pia while daughter cells undergo transit amplification and thus various theories hypothesize that OSVZ development leads to remodelling of migration scaffold and increase in neuron number in human neocortex were achieved through three steps; population of founder cells increased as oRG, oRG cells undergo multiple rounds of asymmetric divisions to generate IP cells which in turn amplify neuron production. It's just beginning of understanding molecular basis behind the suggested hypothesis for human neocortex development and evolution.Analysis of gene expression study will provide us with various candidates to prove suggested hypothesis.

1.8 Cerebral cortex anomalies resulting from defects in neuronal migration

Abnormal migrations lead to abnormal corticogenesis and abnormal cortical function in turn. Many Human Cerebral cortex anomalies were found linked to neuronal migration defects or mutation of factors involved in migration mechanisms. Cognitive and motor impairment and epilepsy are its most frequent consequences (Guerrini et al., 2008). Examples for such disorders are lissencephaly, pachygyria, polymicrogyria, heterotropia and focal cortical dysplasia but among them only in lissencephaly and periventricular heteroptopia role of defect in neuronal migration has been elucidated and thus providing us more information on development of cortex and neuronal migration mechanism. Lissencephaly (LIS) is characterized by absent (agyria) or decreased (pachygyria) convolutions, leading to cortical thickening and a smooth cerebral surface (Friede, 1989). Lissencephaly has been classified into several types, the most common, classical LIS or type 1 LIS, features a very thick cortex (10–20 mm vs. the normal 4 mm) and no other major brain malformations. The cytoarchitecture consists of four primitive layers, including an outer marginal layer which contains Cajal–Retzius neurons (layer 1), a superficial cellular layer which contains numerous large and disorganized pyramidal neurons (layer 2) corresponding to the true cortex, a variable cell sparse layer 3), and a deep cellular layer (composed of medium and small neurons) which extends more than half the width of the mantle (layer 4) (Figure 15) (Golden and Harding, 2004).

Subcortical band heterotopia (SBH) is a related disorder in which bands of gray matter are interposed in the white matter between the cortex and the lateral ventricles (Barkovich, 2000). Histopathology demonstrates that heterotopic neurons settle close to the 'true' cortex in a pattern suggestive of laminar organization (Harding, 1996). Pyramidal cells in the heterotopic band may be smaller than normal compared to those in the overlying cortex, which is usually structurally normal with the exception of shallow sulci (Golden and Harding, 2004).

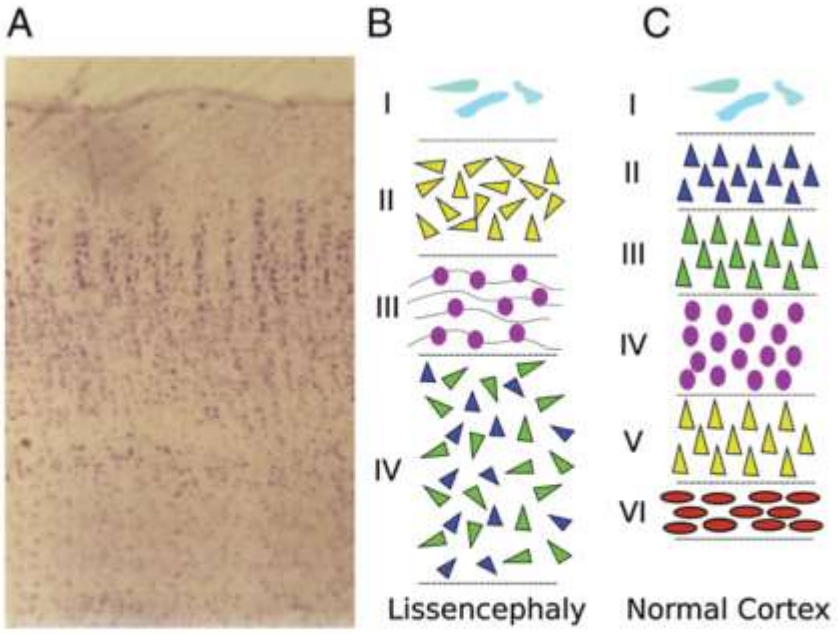


Figure15. (A) Histopathological section from a patient with lissencephaly. Scheme of (B) cortical layering in classical lissencephaly (4 non-organized layers) and (C) normal cortical laminar organization (6 layers). Figure adapted from Guerrini and Parrini, 2009.

1.9 The superfamily of transmembrane proteins with extracellular Leucine Rich Repeats (LRRs)

The LRR is a widespread structural domain that has been identified in over 2000 protein sequences in all life forms, from viruses to eukaryotes. It is involved in protein-protein interactions regulating signal transduction, cell adhesion, DNA repair, recombination, transcription, RNA processing, apoptosis and the mammalian innate immune response (Enkhbayer et al. 2004). This multifunctionality has been suggested to play important

roles in nervous system connectivity. Indeed, recent works suggest the involvement of proteins with LRRs in various aspects of nervous system development like neurite outgrowth (Tsuji L et al., 2004), fasciculation (Kuja-Panula Jet al., 2003) or synapse formation (Ko J et al., 2006; Kim S et al., 2006; O'Sullivan et al., 2012).

1.9.1 Structural features of Leucine Rich Repeats

Each leucine-rich motif is typically 20-30 amino acids long and usually rich in the hydrophobic amino acid leucine. The defining feature of the LRR motif is an 11 residue hallmark sequence LxxLxLxxNxL (x being any amino acid) in the N-terminal part (Kajava, 1998), although other hydrophobic residues can substitute the leucine and asparagines residues at the consensus positions. This sequence forms a β-strand and a loop region that connects with the C terminal part of the repeat, which is more variable in sequence and structure (Kobe and Deisenhofer 1994, 1995; Kajava 1998). Seven classes of the leucine-rich motifs had been identified based on lengths and consensus sequences of the variable segments of repeats. They are 'typical', 'RI-like', 'CC', 'PS', 'SDS22-like', 'bacterial' and 'TpLRR' and referred as subfamilies (Kajava, 2001). Individual leucine-rich motifs are arrayed in tandems of two or more repeats that together constitute the LRR domain. The first crystal structure of a protein consisting entirely of LRRs, the ribonuclease inhibitor, revealed that this arrangement in multiple repeats results in a curved, horseshoeshaped structure (Kobe & Deisenhofer, 1993). Protein domains with LRR architecture form curved solenoid structures where each repeat is a turn of the solenoid. The LRR superfamily thus belongs to a larger structural clan that includes many different types of solenoid folds (Kobe and Kajava, 2000). The concave side of the LRR domains is defined by a parallel β-sheet to which each LRR contributes with one strand. The strands are interwoven with a variety of structural elements in the convex side. The curved LRR architecture appears to be well suited for building protein-protein interaction modules and it is generally thought that the concave surfaces of the LRR structures contain their ligand binding sites. Such mode of binding has been demonstrated in several crystal structures of LRR protein domains in complex with their ligands, but some LRR proteins use alternative surfaces for ligand binding (Lovell et al.,, 2008). Typically the amino- and carboxyl- terminal LRRs are flanked by cysteine-rich flanking domains (LRRNT and LRRCT, respectively). The typically four cysteines in flanking domains form two disulphide bond pairs. By shielding the

otherwise exposed hydrophobic core at the ends of the LRRs, these capping domains serve essential functions in the folding of the LRR domain (Kobe and Kajava, 2001).

1.9.2 Structural features of neuronal transmembrane proteins containing LRRs

In previous works the alignment of amino acid sequence in the extracellular region of the LRRIG proteins disclosed that majority of identified LRRIG proteins were putative Type Ia transmembrane proteins and regulated various aspects of nervous system development like guidance of axons and dendrites to their target areas, mediating selection of appropriate targets, and in synaptic plasticity in adult nervous system (Chen et al., 2006; Homma, 2009). The extracellular LRR (eLRR)-containing proteins include a family of transmembrane proteins characterized by an LRR region and a transmembrane domain (TM). In addition, some of these TM proteins contain immunoglobulin domains (Ig) and/or Fibronectin type-III (FNIII) domains located at the carboxyl side of the LRR domain and contribute to the protein-protein (ligand-receptor) interactions. According to the repeats in the LRR domain, which ranges from 5-15, and the rest of domain structures, the eLRR-TMs cluster into different subfamilies (Chen, 2006; Dolan, 2007).

1.9.3 eLRR-TM proteins in Neural Development and Disease

Most of the genes encoding for eLRR-TM proteins are expressed in the nervous system (Beaubien & Cloutier 2009, Homma et al. 2009, Hong et al. 2009, Lauren et al. 2003). The functional role of these proteins for nervous system development and connectivity has been recently highlighted by different studies. For instance loss of LGI-1 (Leucine rich glioma inactivated -1) in mice causes lethal epilepsy. Extracellularly secreted LGI-1 links two epilepsy related receptors, ADAM22 and ADAM23 by forming a transynaptic protein complex, in absence of LGI-1 this complex is disrupted. LGI-1 gene targeted mouse provides a good model for human epilepsy (Fukata et al., 2010). Another example is LRRTM2, a key regulator of excitatory synapse development and function (de Wit et al., 2009). Also TrkA, TrkB, and TrkC, the receptors for neurotrophins, are a family of LRR domain containing proteins. Neurotrophin and Trk

receptor signaling play central roles in axon targeting, neuronal survival, and synaptogenesis in the vertebrate peripheral nervous system (PNS) (Segal, 2003).

The role of eLRR-TMs in nervous system function has been emphasized by the observation that many neurological or psychiatric disorders associated with the impared function of eLRR-TMs. For instance, Slitrks were found to be involved in Tourette's syndrome and OCD (Obsessive Compulsive Disorder) (Abelson et al., 2005; Katayama et al., 2010; Shmelkov et al., 2010). Another LRR candidate LGI1 has been associated to autosomal dominant lateral temporal lobe epilepsy (Kalachikov et al., 2002; Morante-Redolat et al., 2002). LRRN3 or NLRR3 has been associated with autism spectrum disorder (Sousa et al., 2010). However, detailed information concerning binding partners and functions of many of these eLRR-TM proteins are not yet characterized.

ISLR Family

This protein family of eLRR-TM proteins is comprised of two members, ISLR1 and ISLR2. Originally isolated from a subtracted retina cDNA library, the gene was named immunoglobulin superfamily containing LRR (ISLR). ISLR2 also called Linx (Leucine Rich Repeat Domain and Immunoglobulin Domain containing Axon extension), which was one of 18 members of a newly identified LIG gene family (Mac Laren et al., 2004). The deduced 745 aa protein has an N-terminal signal sequence, followed by 5 tandem LRRs flanked by cysteine rich LRR N terminal and C terminal domains, an immunoglobulin domain, a transmembrane domain, and a short C terminal cytoplasmic tail (Figure 16). ISLR was suggested to be a type Ia transmembrane protein (Nakai and Kanehisa, 1992). ISLR1 expression pattern was very specific and was found only in mesenchymal tissues surrounding nasal placode, the second branchial arch and trunk regions while no expression was detected in the developing nervous system. In contrast, ISLR2 expression was observed mainly in the nervous system suggesting that it could play important functions. Indeed, co-immunoprecipitation analysis showed that mouse Linx interacted with TrkA, TrkC, Ret and p75 and that also could form homomultimers (Mandai et al., 2010). Linx-/- mice could not survive after birth showing deficits in sensory and motor neuron axonal projections similar to (but less in extent) those described in NGF, TrkA and Ret mutant mice.

.

FLRT family

Fibronectin and leucine-rich-transmembrane proteins (FLRTs) are cell surface proteins that comprise three members, encoded by three different genes, *FLRT1*, *FLRT2* and *FLRT3* and are found exclusively in vertebrates (Lacy et al., 1999; Zipursky et al., 2006). The protein structure is similar and they consist of type I transmembrane proteins of 650-675 aa long with amino acid 41-55% identical to humans (Lacy et al., 1999). The extracellular part contains ten eLRR domains flanked by N-terminal and C-terminal cysteine-rich regions and a FNIII domain while the intracellular region consists of around 100 aminoacids without any conserved domain (Figure14) (Lacy et al., 1999). Potential N-linked glycosylation sites appear four times in the extracellular region and several putative phosphorylation sites in the intracellular domain were predicted (Lacy et al., 1999). Indeed, FLRT1 was recently shown to be phosphorylated at three tyrosine sites (Wheldon et al., 2010). FLRTs are expressed in many different tissues but all three isoforms are prevalent in the brain where they play multiple functions with multiple molecular mechanisms involved. FLRT2 was reported by our group to modulate axon guidance in vitro and cell migration in the developing cortex in vivo. This is achieved by the shedding of the extracellular domain of FLRT2 that then acts as a repulsive ligand for Unc5D receptors modulating the migration of the Tbr2+ IPCs from the SVZ to the CP during cortex development (Yamagishi et al., 2011). In the other hand, FLRT3 was identified as a postsynaptic binding partner for LPHN3 (Latrophilins), the target of the Vidow Spider venom (O'Sullivan et al., 2012). But FLRTs in the nervous system display also cell-autonomous functions. Our group has recently demonstrated that FLRT3 is a novel co-receptor for Robo1, controlling the attractive response of rostral TCAs to the guidance cues Netrin-1 and Slit1 and therefore, the correct topographic distribution of the TCAs. In particular, FLRT3 changes the repultive activity of Robo1 triggered by Slit1 into a signaling that induces DCC upregulation and Netrin-1-dependent attraction (Leyva et al., 2014).

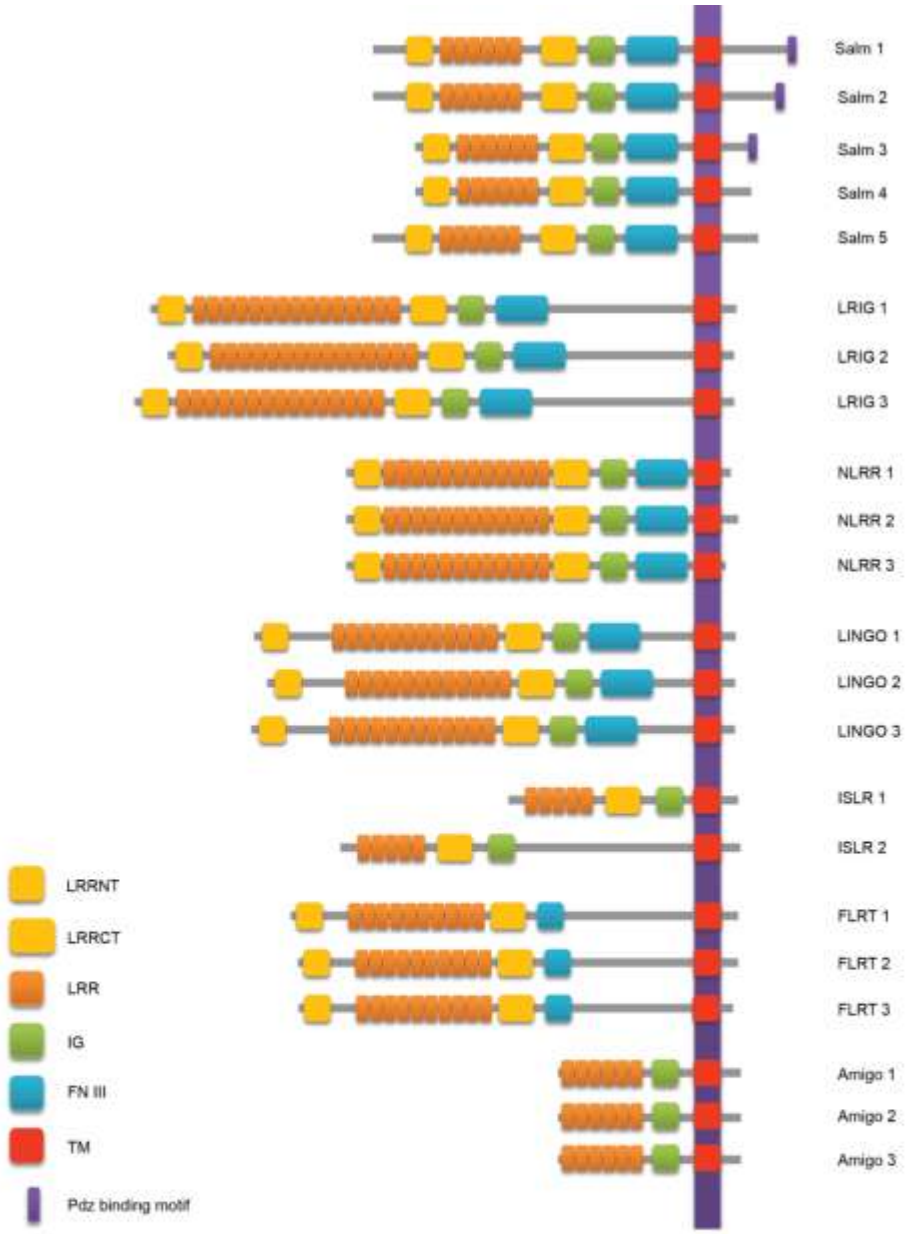


Figure 16. **Domain architecture of the eLRR containing transmembrane protein families.** The small yellow box represents LRRNTwhile bigger yellow box represents LRRCT, i.e . Leucine rich repeat flanking domain at N terminal and C terminal. ORange box represents Leucine Rich repeat domain and the number of this repeats varies from one family to naother. Green box represents Ig , Immunoglobuling Domain. Blue box represents fibronectin III domain and dark orange represents Transmembrane while smaller purple blocks represents PDZ binding motiff.

AMIGO family

AMIGO/Alivin family is comprised of three members identified by differential display analysis of neurons namely AMIGO1, AMIGO2 and AMIGO3. Three research groups were exploring these genes independently (Kuja-Panula et al., 2003; Ono et al., 2003; Rabenau et al., 2004) and found that a new gene was induced by the neurite-outgrowth promoting factor amphoterin hence named Amphoterin-induced gene and ORF (AMIGO). The three AMIGOs form a novel family of type I transmembrane proteins with six LRRs and a single C2-like Ig domain located next to transmembrane segment. AMIGO2 is also known as DEGA (differentially expressed in gastric adenocarcinomas) (Rabenau et al., 2004). Transcript level analyses suggested presence of AMIGO1 exclusively in nervous system while AMIGO-2 and AMIGO-3 are enriched in the brain as well as in other tissues (Hossain et al., 2012). The alignment for AMIGO, AMIGO2 and AMIGO3 shows that the most conserved regions between the three proteins are the LRRs, the transmembrane region and some parts of the cytoplasmic tail. AMIGO family has wide range of roles from nervous system to vascular system, AMIGO1 is an N-linked glycosylated transmembrane protein implicated in axon tract development with homophilic binding mechanisms (Kuja-Panula et al., 2003; Chen et al., 2012). Also it was shown that the members of the AMIGO family similar to LINGO1 are hypothesised to be able to bind each other in a heterophilic fashion in addition to homophilic binding detected in all family members suggesting cooperative role between them (Kuja Panula et al., 2003; Chen et al., 2006; Kajander et al., 2011). AMIGO2/Alivin 1 has been found to have role in inhibition of apoptosis and promoting survival of cerebellar granule neurons (Ono et al., 2003) and AMIGO3 is an NgR1/p75 co-receptor signalling axon growth inhibition in the acute phase of adult central nervous system injury (Ahmed et al., 2013) able to substitute for LINGO1 and interact with NgR and p75 to signal growth cone collapse. Apart from nervous system some studies suggest AMIGO-2 might play important role in the vascular system as a cell survival promoting factor for vascular ECs, probably as being involved in vascular development, angiogenesis and/or vascular remodelling (Hossain et al., 2011).

SALM family

SALM (Synaptic adhesion like molecules) family also known as Lrfn (Leucine rich repeat and fibronectin III domain containing) belongs to the superfamily of (LRR)-containing adhesion molecules. The family contains five members: SALM1, SALM2, SALM3, SALM4 and SALM5. The domain structure of these proteins is very similar with the extracellular region containing six LRRs flanked by cysteine rich capping domains (LRRNT and LRRCT), a C2-Ig domain, and a FNIII domain. The cytoplasmic region varies in length and ends with PDZ domain binding motif in SALMs1-3 but not found in SALM4 and SALM5 suggesting that individual SALMs have distinct signalling capabilities and functions (Morimura et al., 2006; Wang et al., 2006; Ko et al., 2009; Woo et al., 2009). These synaptic adhesion molecules play important roles in various stages of neuronal development mainly synapse formation, maturation and neurite outgrowth. SALM4 have unique role of increasing the number of primary processes extending from the cell body and SALM5 have been suggested to have role in progressive autism and familial schizophrenia also (Nam et al., 2011) It is also reported that SALMs affect both axon and dendrite outgrowth, although the effect varies with individual SALMs (Wang et al., 2008).

NLRR Family

Neuronal leucine rich repeat protein (NLRR) genes were first isolated from a mouse brain cDNA library and three members were since then been identified in mouse, rat and human; NLRR1, NLRR2 and NLRR3 (Fukamachi et al., 2002). NLRRs have been proposed to function as neuronal adhesion molecules (Fukamachi et al., 2002). These three genes encode for type I transmembrane proteins highly glycosylated, with 11 external LRRs, one FNIII domain and a short intracellular tails capable of mediating protein-protein interaction (Taniguchi et al., 1996). It has been reported that NLRR1 are highly expressed in unfavourable neuroblastoma and high level of NLRR1 correlate with poor clinical outcome of patients with neuroblastoma (Hamano et al., 2004), while NLRR3 and NLRR5 were expressed at higher levels in favourable neuroblastoma. NLRR2 expression didn't show any significant differences between favourable and unfavourable neuroblastomas (Hamano et al., 2004). Another study suggests NLRR-3 may be an important component of the pathophysiological response to brain injury

(Ishii et al., 1996). Also it is proposed that NLRR-3 potentiates Ras-MAPK signalling by facilitating internalization of EGF in clathrin-coated vesicles (Fukamachi et al., 2002).

LRIG Family

Leucine rich repeats and immunoglobulins (LRIG) like domains family comprises three members: LRIG1/Lig1, LRIG2 and LRIG3. These are single pass type I transmembrane protein whose extracellular domain contains 15 LRRs and three (Ig) like domains (Suzuki et al., 1996). The extracellular domains are highly conserved within the family but cytoplasmic domains diverge probably for divergence in function (Rio et al., 2013). LRIG1 is known to bea negative regulator of several oncogenic receptor tyrosine kinases, including members of ErbB family, the Met and Ret receptors (Laederich et al., 2004; Gur et al., 2004; Miller et al., 2008; Shattuck et al., 2007; Ledda et al., 2008). LRIG1 reduced expression has been linked to poor prognosis in breast, head and neck, lung and bladder cancers also including glioma (Liuslinder et al., 2007; Rouam et al., 2010; Thomasson et al., 2011; Krig et al., 2011; Sheu et al., 2013) suggesting that LRIG1 may function as a tumor suppressor (Hedman et al., 2002). LRIG1 is also proposed to be marker of quiescent stem cells population in mammalian epidermis (Jensen et al, 2009).

High LRIG2 expression is also poor prognostic marker in early stages of cervical cancers by immunohistochemistry (Hedman et al., 2010). Another work on pituitary adenomas suggests LRIG2 may be differentially regulated during tumour progression (Zhang et al., 2011). On other hand LRIG3 expression was restricted to the lateral canal during embryogenesis and its loss leads to defective inner ear morphogenesis since it's shown that LRIG3 acts to repress Netrin transcription during inner ear development (Abraira et al., 2008).

LINGO1

Leucine rich repeat and Ig domain containing 1 (LINGO-1), also known as LERN, belongs to new protein family comprising three human paralogs: LINGO-2, LINGO-3,

LINGO-4. LINGO-1 contains 12 LRR motifs flanked by N- and C- terminal capping domains, one C2- Ig domain, a TM domain and a short cytoplasmic tail (Mi et al., 2008). A canonical epidermal growth factor receptor like tyrosine phosphorylation site is present in cytoplasmic tail (Mi et al., 2008). LINGO-1 is highly conserved evolutionarily, with human and mouse orthologue sharing 99.5% identity. LINGO-1 was found expressed in brain and spinal cord, and was not detectable in non-neural tissues (Carim-Todd et al., 2003; Mi et al., 2004; Okafuji and Tanaka, 2005; Barrette et al., 2007; Lee et al., 2007; Llorens et al., 2008).

LINGO-1 is a key negative regulator of myelination, expressed in both neurons and oligodendrocytes. In neurons, LINGO-1 is an integral component of the Nogo receptor complex, which inhibits axonal growth via RhoA. In neurons, it functions as an essential coreceptor of the Nogo receptor complex that mediates the inhibition of axonal growth because of regulatory factors present in myelin (Mi et al., 2004).

2. AIM

2. AIMS AND OBJECTIVES

The development of neocortex and mechanisms involved in it such as neuronal migration and thalamocortical/corticofugal projections is of particular interest to our group since the molecules involved in this process are yet to be identified in mouse and humans. Development of neocortex is a process of high specificty spatio-temporal regulation of gene expression. Neocortex is seat for higher cognitive abilities such as learning and memory. These functions depend on precise connectivity of neuronal networks during cortex development which in turn depends on receptor proteins displayed on the surface of neurons. These receptors relay extracellular recognition events leading to cellular processes like axon guidance, neuron migration and synapse formation. Number of such receptors identified yet is comparatively very less to large cellular network regulated by this process suggesting involvement of many unknown candidates.

Our group have recently published role of *FLRT3* (eLRR-TM protein) as a Robo-1 interacting protein which is deciding factor for Netrin-1 attraction in developing axons (Levya-Diaz et al., 2014). This finding and knowing LRR is versatile protein binding motif makes it promising candidate in neural circuit formation since it allows interaction with a wide diversity of partners (Kobe and Deisenhofer, 1994; Kobe and Kajava, 2001) and also play vital roles in neuronal process outgrowth and synapse formation (Chen et al, 2006).This study proposes that expression profiling of eLRR-TM candidates at different developmental stages and regions will reveal new gene candidate and associated events involved in corticogenesis. Also dysfunction of eLRR lead to many neurological disorders in humans (Matsushima et al., 2005; Majercak et al., 2006; Abelson et al., 2005; Katayama et al., 2010; Budel et al., 2008). Different studies based on gene expression pattern have revealed the novel putative role of many families of gene but unfortunately most of the study has been done on Adult stage while most of fate determining neuronal connections occur in developmental stage, thus a comprehensive study at developmental stage of mouse embryonic brain and human fetal brain was need of time to understand better the involvement of such small but important families of protein.

The specific objectives are:

Objective 1: To perform an *in situ* hybridization screening of various families of genes encoding for eLRR-TMs at different developmental stages during mouse brain development and analyze the data in terms of temporal and spatial patterns.

Objective 2: From the screening in objective 1 and on the basis of the analysis of the expression patterns, select those candidates with potential functions in neuron migration and axon guidance and perform in vitro and in vivo functional assays.

1. MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals, reagents, commercial kits and enzymes

All chemicals and reagents were purchased from Fluka, Invitrogen, Merck, Sigma, Roche, Millipore, unless described otherwise in the methods section. Water used for buffers, solutions and reactions mixes was filtered using a Milli-Q-Water System (Millipore). Restriction endonucleases, polymerases and other DNA modifying enzymes were purchased from New England Biolabs, Roche and Takara. Plasmid preparations were done using the SIGMA Plasmid Miniprep or the SIGMA Midiprep kits. SIGMA PCR purification and QIAquick gel extraction kits were used for molecular cloning procedures.

3.2 Methods

3.2.1 Molecular Biology

3.2.1.1 Design and generation of shRNA Constructs

The RNAi targeting sequences used were designed using two strategies.We designed shRNA using http://www.broadinstitute.org/rnai/public/ webpage. From there we only selected the sequences that were in the coding sequence and not in the 3’ or 5’ UTR, since none of these two regions were present in the constructs we used to test shRNA. We designed 5 different shRNA for each gene, which we named from I1 to I5 for ISLR2 and from N1 to N5 for NLRR3.

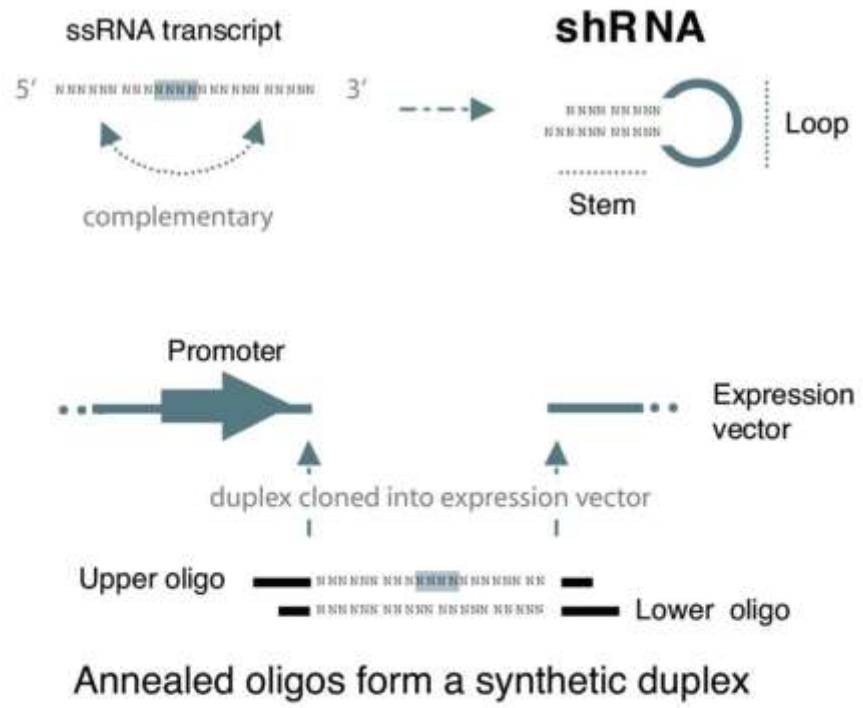


Figure 17. Schematic representation of cloning shRNA into suitable vector

3.2.1.2 Plasmids for *in situ* probes

For Mouse riboprobe preparation plasmid was obtained from S. Homma's Lab. For Human Riboprobe sequence were amplified from clone obtained from IMAGE Biosource via PCR and cloned into pCR-II-TOPO vector using TOPO cloning. The correct sequence and direction of insertion was determined by sequencing using T7- and SP6- promoter specific primers.

3.2.1.3 Generation of labelled riboprobes for *in situ* hybridization

Digoxigenin labelled riboprobes for mouse and human were generated using the plasmids described above. For sense and antisense probe synthesis, plasmids were linearized using the respective restriction enzymes, indicated in the materials section. After linearization of 10µg plasmid DNA, the vector was purified by phenol/chloroform extraction. The efficiency of the linearization and purification was examined using agarose gel electrophoresis, the quantity of purified DNA was determined using agarose

gel and spectrophotometer (Nanodrop 1000, Thermo Scientific) and the linearized plasmid was stored at -20^{o}C. For in vitro transcription of RNA, 200 ng of linearized plasmid was used in 20µl transcription reactions together with 2µl dig-RNA labelling mix (Roche), 2µ transcription buffer, 2 µl DTT 0.1 M, 1 µl RNAse inhibitor, 1 µl RNA Polymerase (T3, T7 or SP6) and RNAse-free H_2O. After 3 hours incubation at 37^{o}C, the transcription efficiency was assessed using agarose gel electrophoresis and the RNA was precipitated by addition of 100µl TE buffer, 10 µl LiCl 4M and 300 µl EtOH 100% with subsequent centrifugation at 13000 rpm for 15 min at 4^{o}C. The pellet was washed twice with 70% EtOH, dried on ice and resuspended in 100 µl TE. The riboprobe were aliquoted and stored at -80^{o}C. For in situ hybridization 10 µl/ml prehybridization solution was used.

3.2.1.4 Cloning

New England Biolab (NEB) or Roche enzymes and buffers were used for all the cloning steps. For subcellular localization, we generated N-terminal Myc tagged versions of ISLR2 and NLRR3. The general strategy was to amplify by PCR the cDNA of these two genes without their endogenous signal peptide (SP) and where a HA tag sequence was incorporated into the forward primer. This PCR product was then subcloned into the vector JEN148 that already contains the SP sequence of the transmembrane protein FLRT3 in pcDNA3. ISLR2 was amplified from the pYX-Asc (IMAGE BioSources) vector with the following primers MDNO244,F:5’ccg**ggatcc**gga<u>tatccttacgacgtgcctgactacgcc</u>gggcccgagccttgcgcctgtgttgaca agtacgcc3’ (BamHI restriction site in bold, HA-tag underlined);MDN-O-245 R: 5’ccg**ctcgag**<u>tca</u>gcccgctgtctgcctgtagttgccg3’ (XhoI in bold, Stop codon underlined). Primers structure is shown in Results section. This PCR product was then digested with XhoI and BamHI enzymes and subcloned into JEN148 digested with BamH1 and Xho1 (BamHI-XhoI digestion of JEN148 removes FLRT3 coding sequence leaving its SP in the vector) and dephosphorylated. For NLRR3 the primers we used are the following: MDN-O-242 F: 5’cc**ggatcc**gga<u>tatccttacgacgtccctgactacgcc</u>ggggataaaaaagtggattgcccccaattatgtacc3’(Bam HI restriction site in bold, HA-tag underlined); MDN-O-243 R: 5’ccg**cccggg**<u>tta</u>ggacatatttgtcggcactcctatagcc3’ (SmaI in bold, Stop codon underlined). This PCR product was then digested with XhoI and SmaI enzymes and subcloned into

JEN148 digested with XhoI -> Klenow (to turn the site a blunt-end) -> BamHI and dephosphorylated.

For overexpression in organotypic culture of brain slices, we neededto generate a pCAGIG plasmid with NLRR3-Myc. NLRR3myc was removed from pcDNA3 with BamHI and PmeI, then treated with Klenow and cloned into EcoRV-opened and dephosphorylated pCAGIG.

For knockdown study by lentiviral infection, we ordered the designed shRNA as primers, which were resuspended with water at 100 μM concentrations, and annealed by mixing 10 μL of forward and reversed primers and 5 μL of NEB Buffer 4. Annealing was performed in the ThermalCycler, which was set from 95°C gradially decreasing to 20°C in 175 min. These shRNA were diluted 1:1000 and ligated with FSVsi under the U6 promoter and between the LTR regions. Vector was digested with BamHI and AgeI, but was not dephosphorylated. We transformed the ligation into Stbl3 chemicocompetent cells instead of the regular DH5alpha competent bacteria, since this bacterial strain reduces the frequency of recombination of LTR-containing vectors. Correct cloning was checked by colony PCR using commercial Taq polymerase and the following primers: MDN-O-271 F: 5'ggctttatatatcttgtggaaagg3'; MDN-O-272 R: 5'acacacattccacagccggtaccc3'.

3.2.2 Tissue Culture

3.2.2.1 Cell Culture

HEK 293T cells were grown in DMEM (Invitrogen) +10% FBS + antibiotics (Penicillin + streptomycin) + glutamine and divided every 48-36 hours. For experiments with transfection, cells were cultivated in collagen coated either p60 plates or 24 well plates. This coating was performed by adding 1 ml or 0.5 ml respectively, of 0.1 mg/mL of collagen in 0.02N acetic acid

3.2.2.2 *Ex-vivo* Slice culture

Pregnant female mice were sacrificed by fast cervical dislocation at embryonic (E) day 13.5, E14.5, E15.5 or E17.5 (day of visualization of vaginal plug is considered 0.5).

After spraying them with ethanol they were opened with scissors in order to obtain the embryos which were collected on ice cold L15++ (L15, 0.25% HEPES and 35mM D-Glucose). On a dissection plate with cold L15++ we removed the embryos from the placenta and their heads were cut. After *ex vivo* electroporation (see later in Materials and methods), in clean plate with L15++, and under a lens microscope, brains were carefully removed from the head with a pair of forceps. Then they were embedded in 4% low melting agarose in L15++. When the block got solidified they were cut in the vibratome at 300 μm. Finally, slices were cultured in a Millipore Organotypic Cell Culture Insert pre-coated with PolyD-Lys and in SCM (slice culture medium).

3.2.2.3 Transfection

For transfection of HEK 293T cells in a collagen-treated p60 plates, 4 μg of DNA were mixed with 220 μL of OptiMEM. In another tube, 220 μL of OptiMEM were mixed with PEI (10 μl of PEI per 1 μg DNA). Content of both tubes is mixed in one, vortex and kept 10 min at RT. Before adding the DNA-PEI mix plates where washed two times with optiMEM. When the incubation time has finished, the DNA-PEI mix was further diluted with 560 μl of OptiMEM (1 ml in total) then added to the cells and incubated 1 hr at 37°C in the incubator. Then, the media was replaced by fresh media and kept in the incubator.

3.2.2.4 Lentivirus Production

Lentiviruses were produced by co-transfection in a p100 non-confluent of HEK 293T cells with 3 vectors: vector of interest (FSVsi with the shRNA or FSVsi empty as a control), pMD2G (containing the envelope genes of the virus) and psPAX2 (the packaging vector).Cells were transfected with 10 μg of each vector, as it is explained in the previously described transfection protocol. Next day media was changed, and transfection efficiency was checked in the fluorescent microscope, since FSVsi vector is containing GFP. By next morning, media was full of viruses, so was collected, centrifuged to discard cells, filtered in 0.45 μm filters and stored at -20°C ready to be used for infection.

This protocol, and all the work with lentiviruses, was done in a Biosafety Level II hood, and the materials for lentivirus kept in separated boxes from common materials

3.2.3 Biochemistry

3.2.3.1 Cell lysates

Lysis was done using SDS buffer.

3.2.3.2 Biotinylation Assay

For biotinylation10^6 HEK 293 cells were seeded in a collagen-coated p60 plates and transfected the next day with NLRR3 or ISLR2 both with intracellular Myc or GFP (as negative control and transfection control). All the biotinylation protocol was performed on ice and with ice cold buffers to maintain cell stability. The buffers were prepared each time just before starting the assay.First, cells were washed 5 min 3 times with 2 mL of PBS-CM (PBS with 0.9 mM CaCl2, 0.33mM MgCl2).Cells were incubated 30 min with 250 µL 0.5ng/µL sulfo-NHS-Biotin (SNB) in PBS-CM with rocking. One plate of each transfection was not biotinylated and instead was incubated only with PBS-CM, which was used as control for the streptavidin pull-down. After that cells were washed 3 times with 50mM NH4Cl in PBS-CM for 5 min to quench free biotin and washed again 3 times with PBS-CM only. Cells were lysed 15 min with 0.5 lysis buffer (1.25% Triton X-100, 0.25% SDS, 50 mM Tris ph8, 150mM NaCl, 0.4M iodoacetamide, 5mM EDTA and inhibitors of proteases (Roche)), scratched and collected to be sonicated. Cell debris was pelleted by centrifugation at 13000 rpm at 4°C for 15 min and supernatant was collected. Before proceeding to next step 50 µL of each sample was taken in order to have a total cell lysate control (input control). Twenty µL of pre-washed streptavidin beads were added to the remaining sample and were put 2 hr in incubation at 4 °C with rotating.Beads were collected by centrifugation at 1000 g at 4 °C for 3 min and washed 3 times with 1mL Wash Buffer (Triton X-100 0.5%, 0.1% SDS, 50 mM Tris ph8, 150mM NaCl, 5mM EDTA). Finally, the sample was eluted with SDS PAGE sample Buffer at 100°C for 10 min. Samples were analyzed by Western Blot.

3.2.3.3 Immunofluorescence Assay

For immunofluorescence assay HEK293T cells were grown on collagen-coated coverslips in 24-well plates and transfected either ISLR2-HA (tag in extracellular side) or FLRT3-Myc (as positive control with Myc at intracellular side) or left untransfected (negative control), two wells per transfection condition. With one of these wells we waited for 24 hours after transfection and with the other for 48 hours for fixation. We performed 3 different protocols for each transfection. For protocol 1,cells were washed in well with PBS and fixed 20 min with 500 µL 4% PFA. Then they were washed 10 min with NH4Cl 50 mM in PBS. After that, permeabilization was performed during15 min with 500 µL of 0.1% Triton in PBS on ice. Then,coverslip was processed directly for immunofluorescence to detect antigens located both outside and inside of the cell. The CVS was taken out the well and treated 20 min with 50 µL of blocking solution (BS: 4% donkey serum in PBS), followed by 1.5 hr of incubation at room temperature of 50 µL primary antibody (AntiMyc mouse 1:250 in BS, AntiHA rat 1:500). In next step cells were washed 5 min twice with PBS and then incubated for minimum 30 min with secondary antibody at room temperature (Donkey anti-mouse Alexafluor488 1:500 in BS, donkey anti-rat Alexafluor488 1:500 in BS) and DAPI 1:1000 in BS. Cells were finally washed with PBS and the CVS quickly rinsed with H2O before mounting with slowfade (Invitrogen). This preparation was sealed with polish nail and stored at 4°C under the dark until the analysis by immunofluorescence.

In Protocol 2 we performed first the immunodetection protocol 1 followed by immunodetection protocol 2. In both cases the same primary antibody was used but with different secondary antibodies, so we were able to assess how much of my protein was inside and outside of the cell. In the first part we used a secondary antibody Alexafluor488 as above, and after last wash cells were permeabilizied 15 min with 500 µL of 0.1% Triton in PBS, washed 20 min with 50 µL of BS, followed by 1.5 hr of incubation at room temperature of 50 µL primary antibody and secondary antibodies Alexafluor568 (Donkey anti-mouse Alexafluor568 1:500 in BS or donkey anti-rat Alexafluor568 1:500 in BS) and DAPI.

Protocol 3 is performed on live cells, in a way that they are fixed after incubation with primary antibody. To start 1 µL of primary antibody is incubated 1 hr at RT with 0.2 µL of secondary Alexafluor488 antibody in 50 µL of DMEM. The coverslip with cells is kept in DMEM until we take it off the plate. Cells are incubated 30 min with 50 µL of

the mix. After a wash, cells are fixed in 4% PFA, washed with PBS, permeabilizied with 0.1% Triton, incubated with primary antibody, washed in PBS and finally incubated with secondary antibody Alexaflour568.

3.2.3.4 Western Blotting

Samples were mixed with SDS PAGE sample Buffer and boiled at 95°C for 10 min before loading them into the 8% polyacrylamide gel. Electrophoresis was performed in the following parameters: 300V and 20mA/gel. For transfer the PVDF membrane was first activated 1 min in methanol, washed 1 min in water and 1 min more in Transfer Buffer. This step was performed by semi-dry method, applying 90 min a 300mV and 60mA/membrane current. After that, membrane was washed with TBST (Tris Buffer Saline Tween: 50 mM Tris, 150 mM NaCl, 0.05% Tween 20) and incubated 1h with 5% skimmed milk. After that membrane was washed 3 times in TBST and then incubated with 1.5mL of primary antibody (AntiMyc 1:1000 in TBST in all cases) ON at 4°. Next day, excess of primary antibody was washed 3 times with TBST and then the membrane was incubated 1h with 15 mL of secondary antibody (antimouse HRP 1:5000 in TBST). Membrane was then washed with TBST 6 times in 30 min approx. and revealed in Chemidoc. Quantification of band intensity was performed with ImageJ.

3.2.4 Animal handling and experiments

Pregnant wild type mice maintained on a CD1 background were used for gene expression analysis, Ex-vivo and In vivo electroporation experiments. All mice were housed in the animal house facility of the UdL under standard conditions and were treated according to the laws and regulations of the European Union and Spanish government. In all cases, the day of vaginal plug was considered to be E0.5. Mouse embryos from embryonic day 12.5 (E12.5) to 18.5 (E18.5) were used in the present study. Pregnant females were deeply anesthetized with isoflurane and then euthanatized by decapitation in order to collect their embryos.

3.2.4.1 *in utero* electroporation

All animal protocols were done according to ethical committee guidelines. Timed pregnant mice were anesthetized with isoflurane, their abdominal cavity cut open, and the uterine horns exposed. Approximately 2-3 µl of DNA solution was injected into the lateral ventricle of embryo aged 12.5-13.5 post coitum (E12.5-E13.5) using a pulled glass micropipette. Each embryo within its uterus was placed between tweezerstype electrodes (CUY650-P5; NEPA Gene, Chiba, Japan). The angle of inclination of the electrode varies with respect to the horizontal plane of the brain was zero for targeting the cortical ventricular zone (VZ). For GE-targeted electroporation the angle of the paddles were adjusted to 30 degree. Only a few embryos at a time were exposed, and care was taken to quickly place them back into the abdominal cavity to avoid excessive temperature loss. The wall and skin of the abdominal cavity were sutured closed, and embryos were allowed to develop normally until sacrificed for further study.

3.2.4.2 *ex-vivo* Electroporation

DNA-Fast green 0.4% was injected in the ventricle within isolated heads with a very thin injector. After that, 5 pulses of 0.5ms with a 150V voltage were applied in the appropriate direction so that DNA went into the cortical neurons in the VZ. To that purpose, positive electrode was placed in the frontal superior cortex.

3.2.5 Dissections, Tissue preparation and cryosectioning

3.2.5.1 Tissue Preparations

Mouse brain was fixed in 4%PFA Overnight at 4^{o}C and then washed subsequently with PBS to remove excess PFA. Brain was cryoprotected in 30% sucrose to avoid any dessication while storage in -80^{o}C till it sinks to bottom and then embedded in cryoprotective Tissue Tek.

3.2.5.2 Sectioning of Brains

Section of 16µm thickness was made from frozen section in cryostat at -24^{o}C. Air dry for one hour at RT and then stored at -80^{o}C.

3.2.6 Histology

3.2.6.1 *in situ* hybridization

For *in situ* hybridization, the embryo brains were fixed overnight in 4% paraformaldehyde (PFA) in PBS, and then allowed to sink to bottom in 30% sucrose at 4^{o}C followed by embedding in Cryoprotectant (Tissue Tek), stored at -80^{o}C. Serial brain cryosections were collected 16µm (Cryostat Leica CM 3000) on superfrost plus slides from Thermo Fisher, air dried and stored at -80^{o}C. *in situ* hybridization was performed using protocol adapted and modified from the protocols of Paul Gray, Inma Cobos, N.P. Pringle and W.D. Richardson, and others. Cryosections of brain tissue (20µm thick) stored at -80^{o}C were defrosted for 30 min at RT. Hybridization was carried out over night in hybridization buffer containing respective digoxigenin labelled riboprobes at 70^{o}C and slide was covered with cover slip. Sections were then rinsed and washed four times for 20 minutes each with washing buffer at 65^{o}C. Rinsed and washed with MABT three times for 20 minutes each at RT. Sections were incubated in blocking solutions for 60 min at RT. Subsequently, sections were incubated over night at 4^{o}C with an alkaline phosphatise-conjugated anti-digoxigenin antibody (Roche) diluted 1:2000 in blocking solution. Sections were then rinsed and washed six times for 20 minutes with MABT at RT, followed by rinse and wash of 10 minutes, three times with NTMT Buffer at RT. Signal was visualized with NBT/BCIP (Roche) diluted in NTMT, developing was carried out in dark at RT until signal appeared completely. Developing was stopped by three 5 min washes steps in PBT at RT. Sections were postfixed in 4% PFA in PBS for 15 min, rinsed and washed with PBS twice for 5 minutes. Rinsed and washed with water twice for 5 minutes. Air dried under hood. Mounted with glycerol-gelatin and stored at RT.

3.2.6.2 Immunofluorescence

Embryonic brains sectioned at 16µm on a cryosection were fixed in 4% PFA and further washed with PBS followed by PBS +NH_4CL wash and Permeabilization with PBST. Sections were then incubated with blocking solution and subsequently incubated overnight with Primary antibodies prepared in blocking solution. After washing, incubation was done with secondary antibody for 60-90 min at RT. Sections washed and mounted and stored at 4^{o}C.

3.2.7 Acquisition of Human Samples

All the Human fetal brain sections from spontaneous abortions were provided by Dr. Isabel Novoa Garcia, Director, Biobanc, HUVH Barcelona, Spain in cryoprotected conditions and I processed it further.

3.2.8 Dual Protocol of in situ hybridization and Immunofluorescence

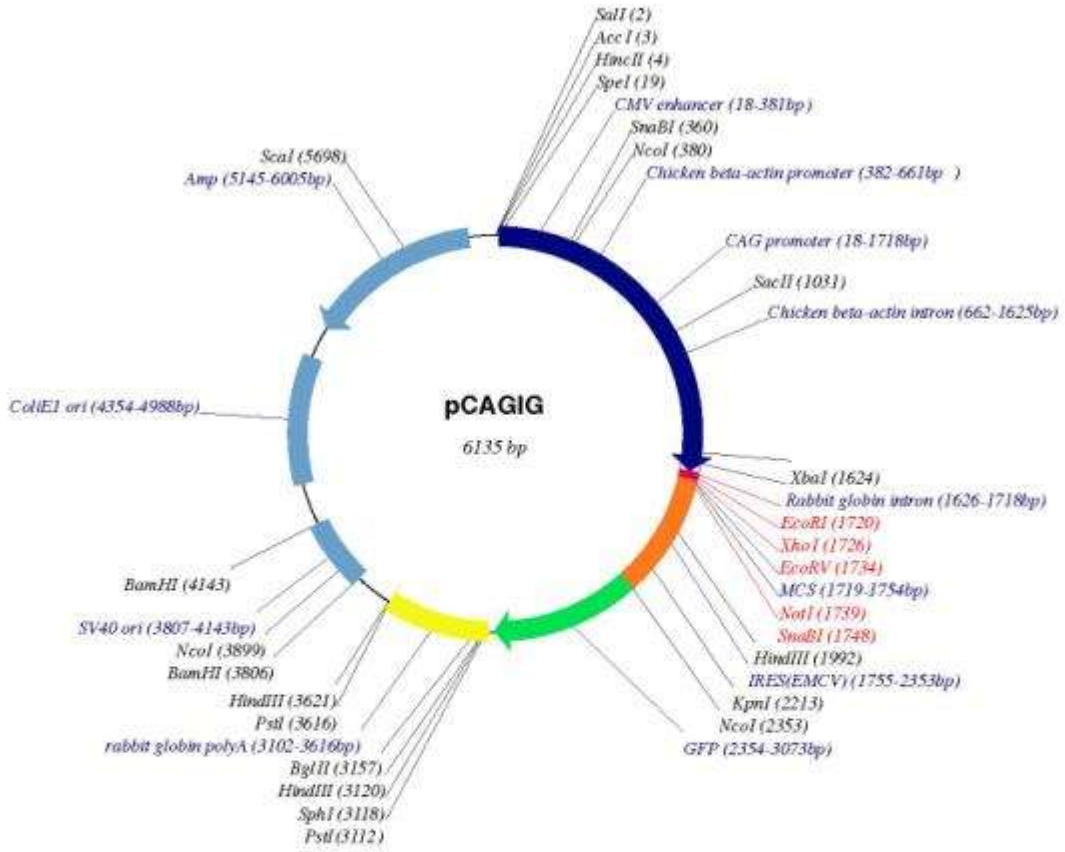
The whole procedure was similar to procedure I used individually for ISH and IF except reduced hybridization temperature for ISH to 55^{o}C from 70^{o}C.

3.2.9 Vectors Used

I used different vectors for different experiments in this thesis but explaining here three main vectors used for in vivo study of ISLR2 and NLRR3

3.2.9.1 pCAGIG

This vector was used to clone ISLR2 and NLRR3 for overexpression in ex vivo study since it also encodes IRES-GFP.



1711 1760

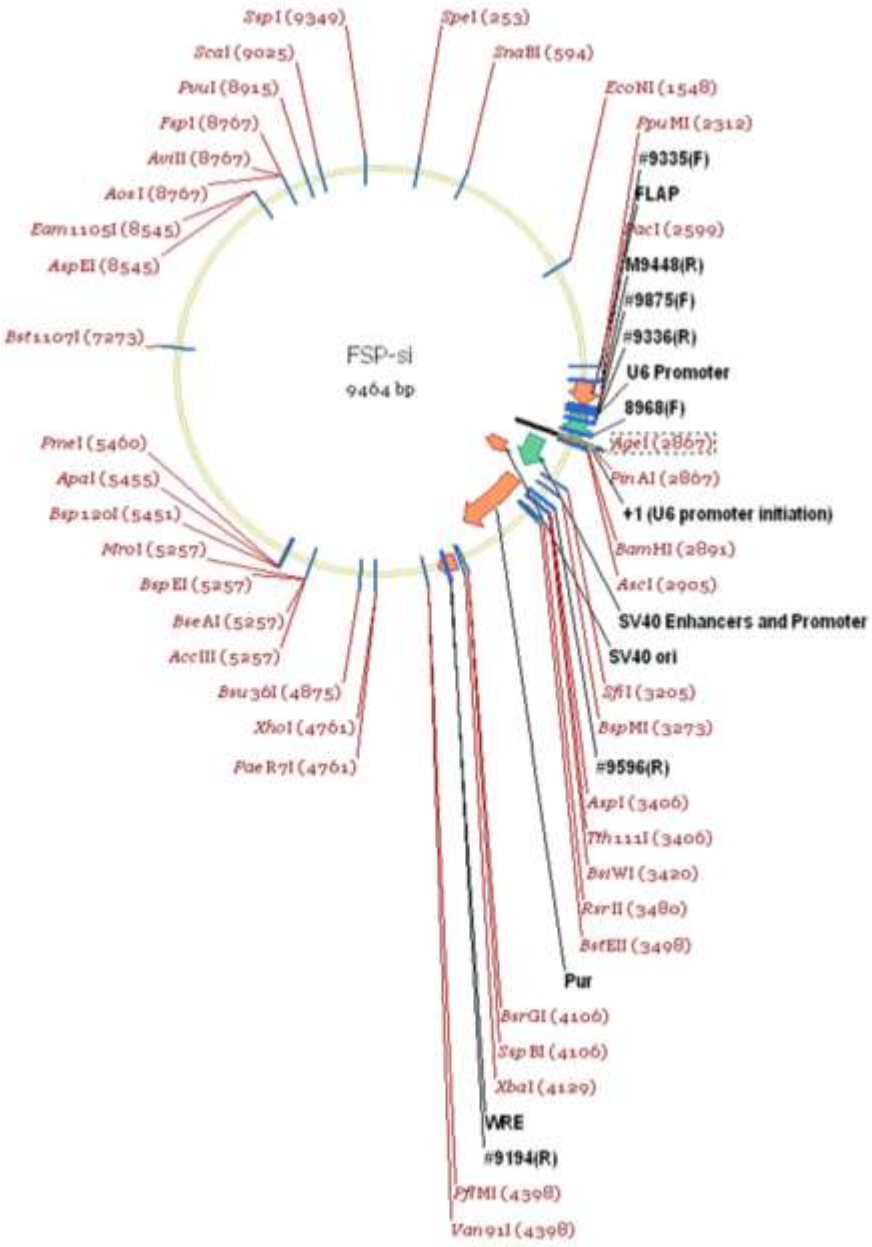
TTGGCAAAGAATTCCTCGAGGATATCGCGGCCGCACTCCTCAGGTGCAGG

EcoRI XhoI EcoRV NotI

IRES-GFP was from pMX-IRES-GFP (Nosaka et al., EMBO J. **18**, 4754-4765 (1999)).
Other parts were from pCAGGS (Niwa et al., Gene **108**, 193-199 (1991)) with modified multiple cloning sites (pCAGEN).
This vector was constructed by Takahiko Matsuda.

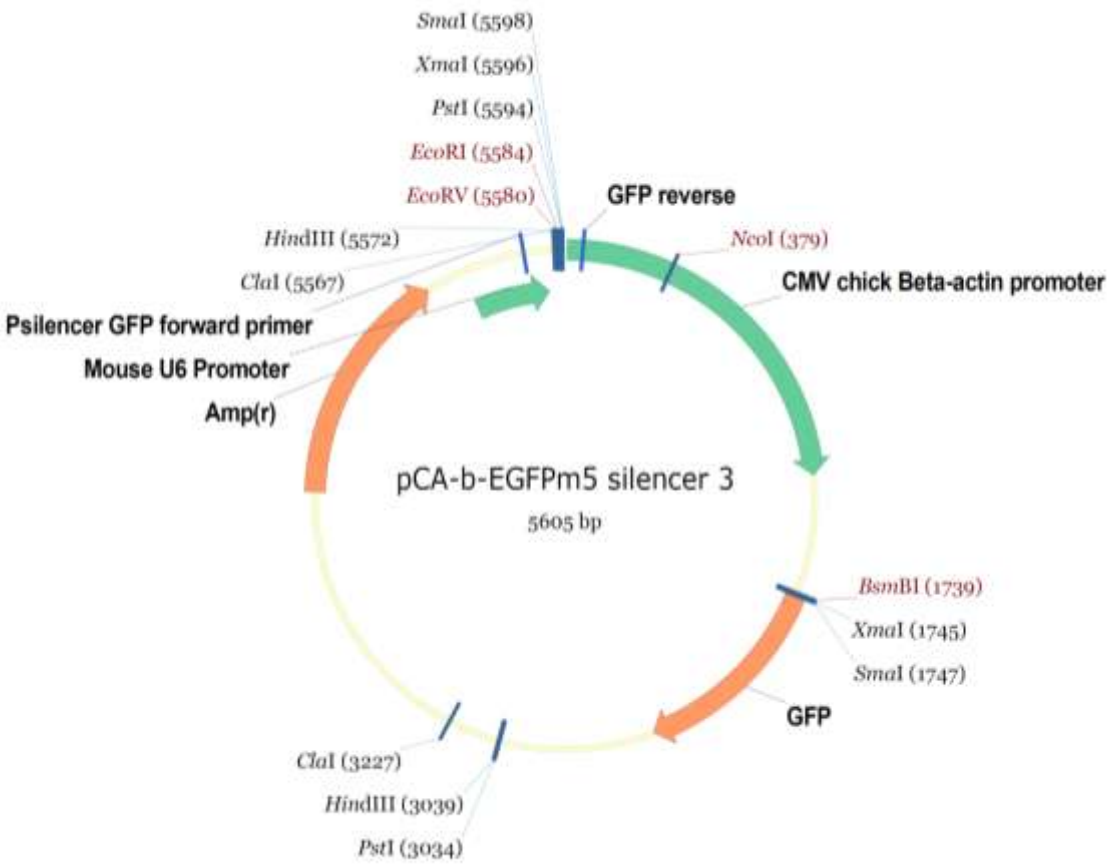
3.2.9.2 FSV-si

This vector was kind gift from Dr. Mario Encinas and I used it for cloning shRNAs for ISLR2 and NLRR3 and Lentivirus Production. Here I used BamHI and Age I as cloning sites. Also the Pur marker was replaced with venus YFP.



3.2.9.3 pCA-β-EGFPm5 silencer3

This vector was kind gift from Dr. Matthieu Vermeren, This vector has been used in many previous research works to understand neuronal migration by ex-vivo and in utero electroporation so I used this vector to replace lentiviruses, this vector also has GFP expression. I used ApaI and EcoRV as cloning sites for cloning shRNAs.



4. RESULTS

4. RESULTS

Analysis of Expression Pattern of transmembrane proteins with extracellular leucine rich repeats (eLRRs) in the developing nervous system.

In this study, I have used *in situ* hybridization to create an expression map for some families of eLRR-TMs distinguishing the areas expressing the transcripts in coronal sections of brain and analysis of this expression pattern in different developmental stages. For this I used serial coronal sections of mouse brain at E13.5, E15.5 and E17.5 developmental stages and coronal sections of Human foetal brain at post conceptual weeks (pcw) 15 and 22 of a small region from occipital lobe. Individual antisense digioxigenin-labelled probes were designed and synthesized for each of the genes mentioned separately for human and mouse. After this cataloguing and analysis of expression pattern I selected few genes and further analysed them using dual combination of *in situ* hybridization and immunofluorescence simultaneously in human fetal brain and mouse embryonic brain using specific neuronal marker antibodies.

Below, I explain the result for each gene for all the three types of analysis I did comparing the expression patterns in mouse and human fetal brain together. I referred nomenclature from Prenatal Mouse Brain Atlas: Color images and annotated diagrams of: Gestational Days 12, 14, 16 and 18 Sagittal, coronal and horizontal section by Uta Schambra.

4.1.1 Expression of *ISLR2* in the developing mouse and human brain

The mRNA expression pattern of *ISLR2* in mouse embryonic brain is very interesting (Figure 18). At E13.5, *ISLR2* signal was observed in the hippocampus, the neocortex (CP), the lateral ganglionic eminence (SVZ and caudate putamen) and in epithalamus (mainly in medial habenula) and the reticular thalamic nucleus in more posterior sections. Strong signal was also seen in septum (Figure 18 A-C). In posterior section prominent signal was also visible in different regions of hypothalamus (Figure 18C, H). *ISLR2* expression in neocortex was more restricted to cortical plate and subplate (Figure 18 and 19). Interestingly, like it is observed at later stages, *ISLR2* is not expressed in the VZ of cortex and expression was restricted to CP and no separate stream was observed in SP (Figure 19A).

At E15.5, expression of *ISLR2* was consistent in the developing hippocampus and neocortex (Figure 18 F-H). In the neocortex an additional expression region appears as fine line below the CP which probably are neurons in the subplate (Figure 18I and Figure 19B). It appears as if this line has extends into the amygdaloid area (Figure 20C). Expression in basal ganglia was detected even more prominent at E15.5, including the SVZ of the LGE, the caudate putamen (Figure 18E, H). *ISLR2* expression also appeared in piriform cortex, lateral olfactory tract and preoptic area expanded in hypothalamus (Figure 18E, F). Expression in Reticular thalamic nucleus and anterior amygdaloid area became stronger (Figure 18H).

At E17.5, expression of *ISLR2* was found in the anterior amygdaloid area and the lateral migratory stream reservoir (Figure 18I, J, K). In the neocortex, *ISLR2* expression becomes more diffuse in the lateral/ventral part labelling cells mainly in the deep layers of the differentitating CP (Figure 18J). In contrast, in the dorsal/medial part, including the cingulated cortex, *ISLR2* expression is maintained in the entire CP and stream in SP. At this stage, a stream of *ISLR2* labelled cells was detected in the SVZ of the neocortex (Figure 18J and Figure 19C). This stream merges with labelled cells from lateral ganglionic eminences and extends towards the hippocampus (Figure 20C) and was denoted as lateral migratory stream with its reservoir in anterior amygdaloid area and ganglionic eminence. In the thalamus *ISLR2* expression as evident is still maintained in

the dorsal part, including the medial habenula part of Epithalamus but, interestingly, is not expressed in the ventral thalamus at any of the stages analyzed (Figure 18C,H,K and Figure 20B). Expression in reticular thalamic nucleus became faint than in earlier stages. In hippocampus all the stages showed strong expression and by E17.5 *ISLR2* is found in CA1, CA2, CA3 and dentate gyrus (Figure 20A).

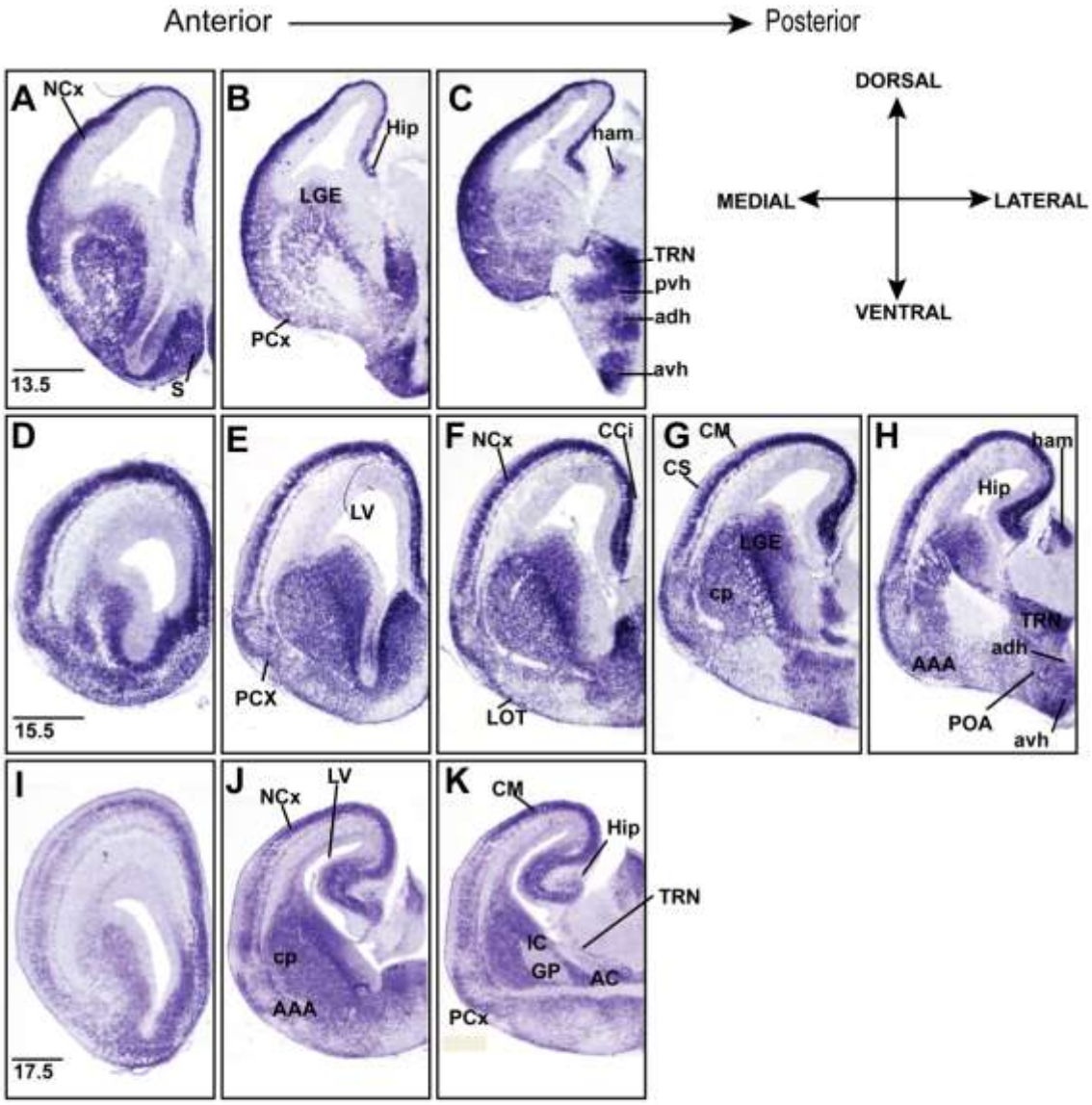


Figure 18 . **Expression of ISLR2 in the developing mouse brain**
(A-K) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-C), E15.5 (D-H), E17.5 (I-K) using a digoxigenin-labelled ISLR2 antisense probe on coronal brain sections of mouse embryos. A, D, E, I panels show anterior; B,F,G,J shows medial; C, H,K shows posterior sections of the brain, respectively. Abbreviations: AAA, Anterior amygdaloid area; AC, Anterior commissure; adh,Anterior dorsal hypothalamic nucleus; avh,Anterior ventral hypothalamic nucleus; CCi, Cingulate cortex; CM, Motor cortex;cp, Caudate putamen; CS, Sensory cortex; LGE, Lateral Ganglionic eminence; GP, Globus Pallidus; ham, medial habenula; Hip, Hippocampus; IC, Internal Capsule; LOT, Lateral Olfactory Tract; LV, Lateral Ventricle; NCx, Neocortex; PCx, Piriform Cortex; POA, Pre optic area; pvh, periventricular thalamic nucleus; S, Septum; TRN, Reticular Thalamic nucleus .Scale Bars: A-K, 200 μm.

An interesting downregulation of *ISLR2* expression was observed from E15.5 to E17.5 in anterior cortex (motor cortex); expression in this area was very prominent in E15.5 (Figure 18D) while in E17.5 (Figure 18I) expression was significantly reduced.

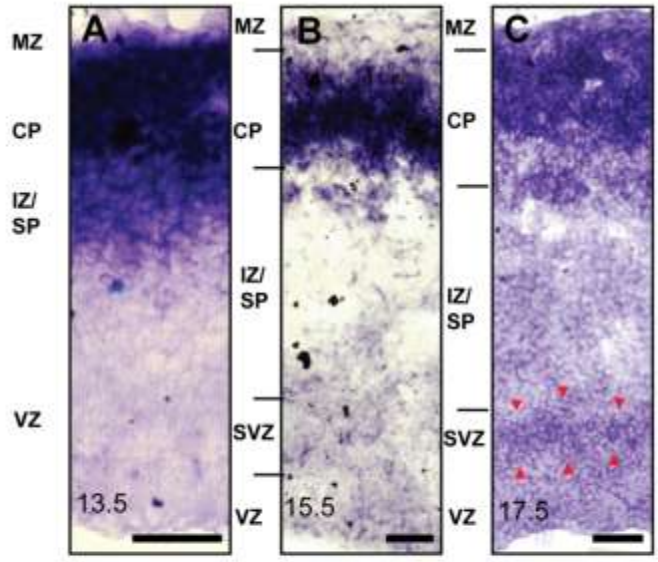


Figure 19. **Expression of ISLR2 in the developing cerebral cortex**. In situ hybridization analyses of coronal brain sections at E13.5 (A), E15.5 (B), E17.5 (C) using a digioxigenin labelled ISLR2 antisense probe. (A) High expression of ISLR2 in the E13.5 cortex restricted to cortical plate and intermediate zone.(B) At E15.5 ISLR2 is strongly expresed in cells located at cortical plate, a separate line in subplate and faintly in SVZ and VZ.. (C) At E17.5 the expression extends from cortical plate to Intermedaite zone and another stream at SVZ (indicated by red arrowhead). Abbreviations: CP, cortical plate; IZ, intermediate zone; MZ, Marginal zone; SVZ, subventricular zone; VZ, ventricular zone. Scale bars: A, 100 μm; B,C 50 μm.

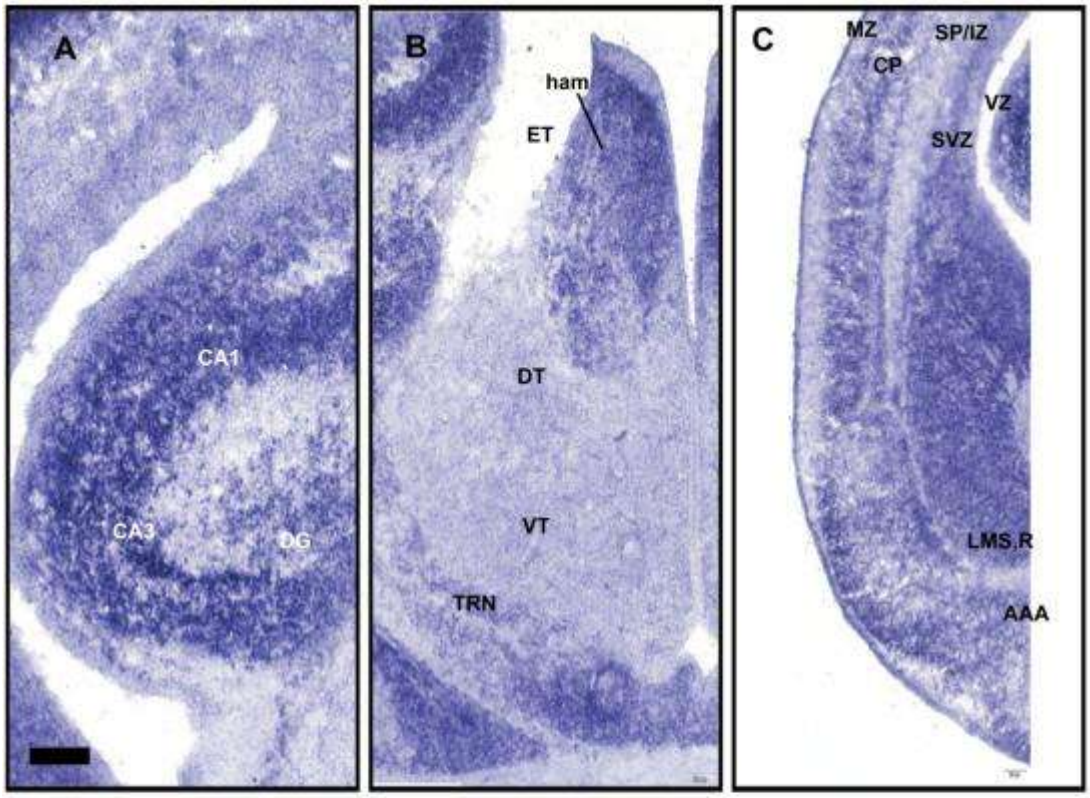


Figure 20 . **Higher magnified view of specific regions showing expression of ISLR2 in coronal section of mouse embryonic brain at E17.5** (Figure 18-K). (A) Hippocampus showing DG, CA1 and CA3 region,(B) Thalamus showing three main regions of Thalamus ET, DT and VT, (C) Lateral migratory stream showing reservoir. Abbreviations: AAA, Anterior amygdaloid area; CA, Cornu Ammonis; CP, Cortical plate; DG, Dentate Gyrus; DT, Dorsal Thalamus; ET, Epithalamus; ham, medial habenula; IZ, Intermediate zone; LMS,R, Lateral Migratory stream,Reservoir; MZ, Marginal zone; SP, Subplate; SVZ, Subventricular zone; TRN, Reticular Thalamic nucleus; VZ, Ventricular zone .Scale Bars: A-C, 100 µm.

The mRNA expression pattern of *ISLR2* in coronal section of human fetal brain from gestational week 15 gave specific staining in cortical area when compared with H&E stained similar section (Figure 21).

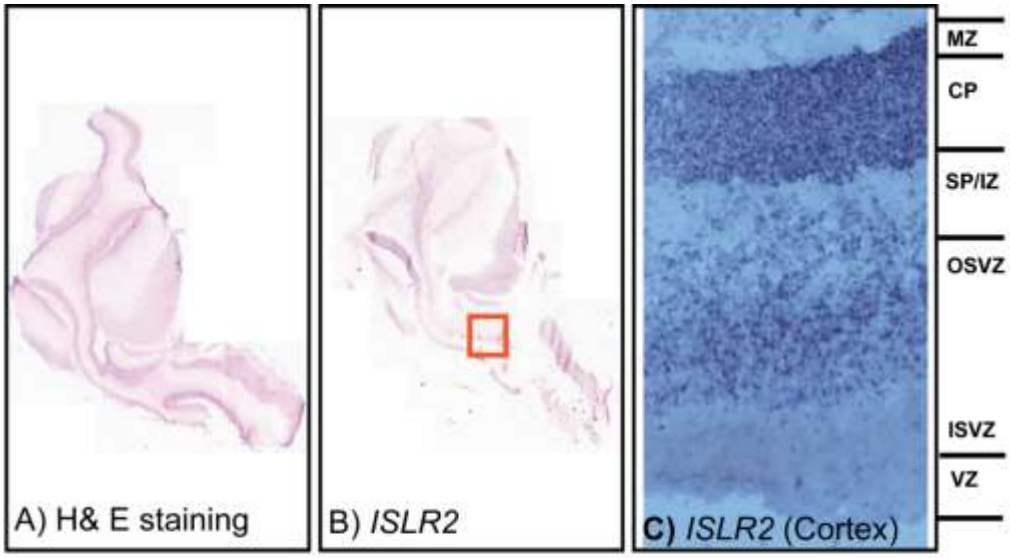


Figure 21. **Expression of *ISLR2* in the developing Human fetal brain.**
(A) H& E staining of coronal brain section of Occipital cortex area of human fetal brain at gestational week 15. (B) In situ hybridization (ISH) analyses of coronal brain section of occipital cortex area of human fetal brain at gestational week 15 using a digoxigenin labeled *ISLR2* antisense probe. (C) Higher Magnified view of Boxed area of neocortex from section B.

4.1.2 Characterization of the neurons expressing *ISLR2* in the developing mouse brain and human fetal brain.

From our ISH screening we selected *ISLR2* in order to analyze better its putative function in brain development, especially its possible role in neuronal migration and axon guidance. In particular, I tried to understand the phenotype of the *ISLR2*-expressing neurons with association with different markers for different neuronal subtypes and layer specific. To investigate neuronal subtype and layer specificity of ISLR2-expressing neurons in mouse and human developing neocortices, I performed IF of ISLR2 with CTIP2, SATB2, SOX2, DCx, TuJ-1 using serial coronal sections

(cryosections) in mouse at E13.5, E16.5 and E18.5 and occipital neocortices of dorsolateral sections of human fetal brain aged 15pcw and 22pcw.

Analysis of co-localization of transcription factor CTIP2 and SATB2 with ISLR2 in developing mouse and human brain

CTIP2 is a well established marker mainly for layer V cortical neurons. In order to investigate the presence of ISLR2 in this cortical layer, I performed double immune-fluorescent staining of ISLR2 with CTIP2. In mouse CTIP2 also labels the differentiating fields and SVZ of both lateral and medial ganglionic eminences while ISLR2 is expressed mainly in LGE but not, or very little, in MGE in both the stages (E13.5 and E16.5; Figure 22FGJK). In the cortex, ISLR2 expressing neurons express CTIP2 as well at this stage, indicating that ISLR2 labels differentiating neurons (see other markers below that suggest the same conclusion) (Figure 23A-E; Figure 24E-H). Subcellular localization (Figure 23F and 24I) shows that most of the CTIP2+ neurons (nuclear staining) display co-expression of ISLR2 in the cytoplasm and/or membrane. In the most lateral/ventral part of the cortex ISLR2 expression shows a fibre-like pattern within the IZ which probably are axons, either thalamocortical or corticufugal projections (see discussion) (Figure 23C). The upper cortical layers at this stage can be detected by SATB2 staining and as illustrated in Figure 28D-E, ISLR2 and SATB2 expression domains overlap at E18.5. Superficial layer neurons labelled with SATB2 are also faintly expressing ISLR2 at E18.5 (Figure 28CDE). However, a consistent staining of ISLR2 can be detected in the deepest layers of the cortex/upper part of the IZ (Figure 28C). Accordingly, ISLR2 expression is clearly visible in some parts of the thalamus as well as in vertical fibers within this structure (projecting axons) (Figure 23E). At older stages E16.5 and E18.5, ISLR2 expression in the cortex labels tangential structures in the IZ region as well as radial fibers that probably are incoming thalamocortical axons (Figure 24G; Figure 28C). Indeed, like at E13.5, the thalamus at this stage also shows ISLR2 staining in different nuclei (medial habenula of Epithalamus and reticular thalamic nuclei, as well projecting axons (Figure 22E).

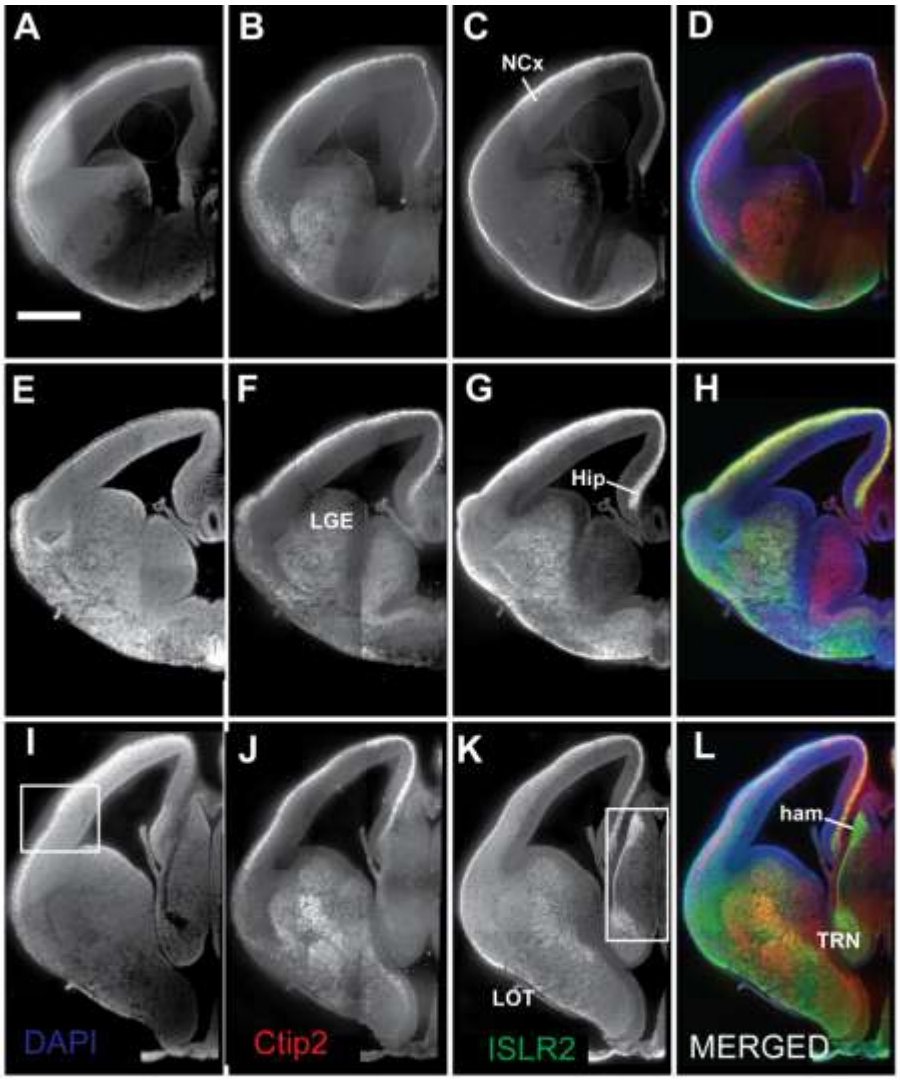


Figure 22. **ISLR2 (green) and Ctip2 (Red) double immunostaining was performed on E13.5 mouse brain sections from anterior (A-D) to posterior (I-L)**. Abbreviations: ham, medial habenula; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; LOT, Lateral Olfactory Tract ; NCx, Neocortex; TRN, Thalamic reticular nucleus. Scale Bars: A-L, 100 µm.White Boxed area has been shown in higher magnification in next figure.

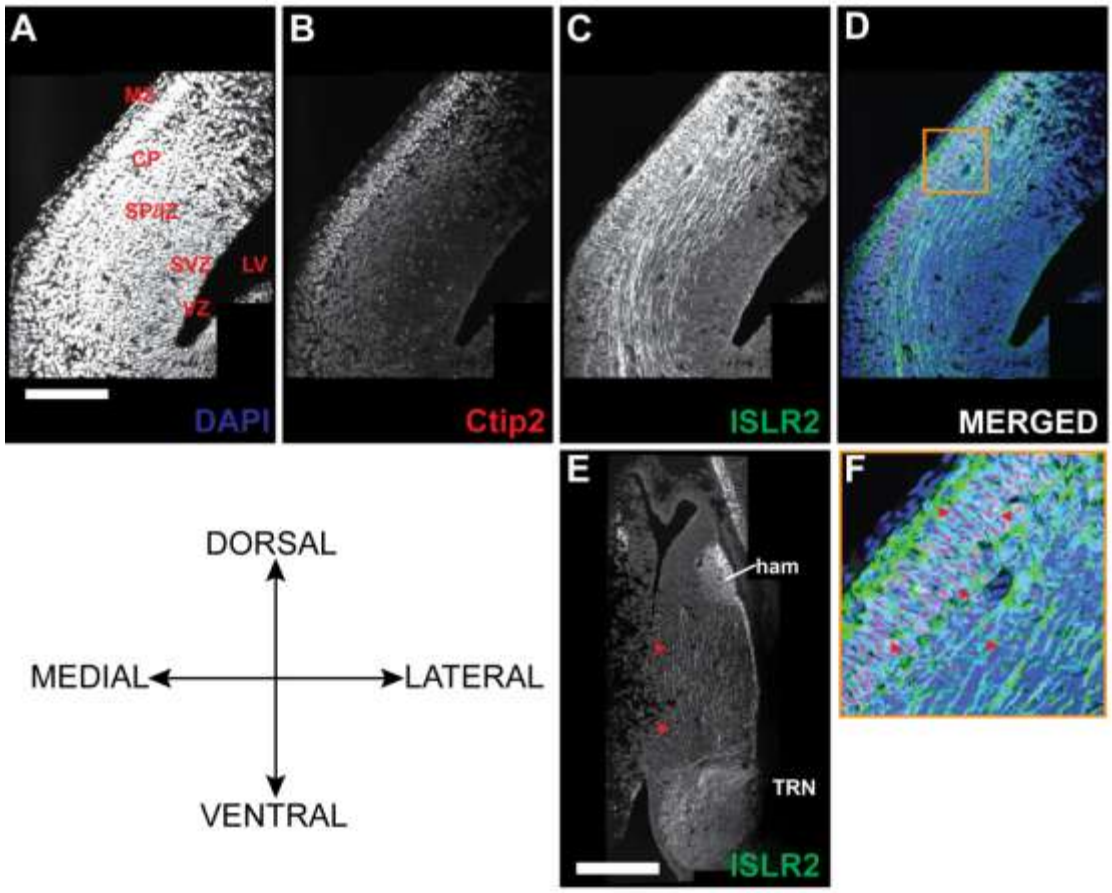


Figure 23. **Higher Magnification image of the boxed areas in Figure22** . A-D represents part of neocortex from a posterior coronal brain section double immunostained with ISLR2 and Ctip2 ; E represents part of Thalamus (red arrowheads showing axonal fibres stained with ISLR2 antibody) . Abbreviations: CP, Cortical plate; ham, medial habenula; Hip, Hippocampus; IZ, Intermediate zone; LGE, Lateral Ganglionic Eminence; LOT, Lateral Olfactory Tract; LV, Lateral Ventricle; MZ, Marginal zone; NCx, Neocortex; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular Zone; TRN, Thalamic reticular nucleus. Scale Bars A-E: 100μm.F represents part of Cortex magnified to show colocalization of ISLR2 with CTIP2 (pointed by red arrowheads).

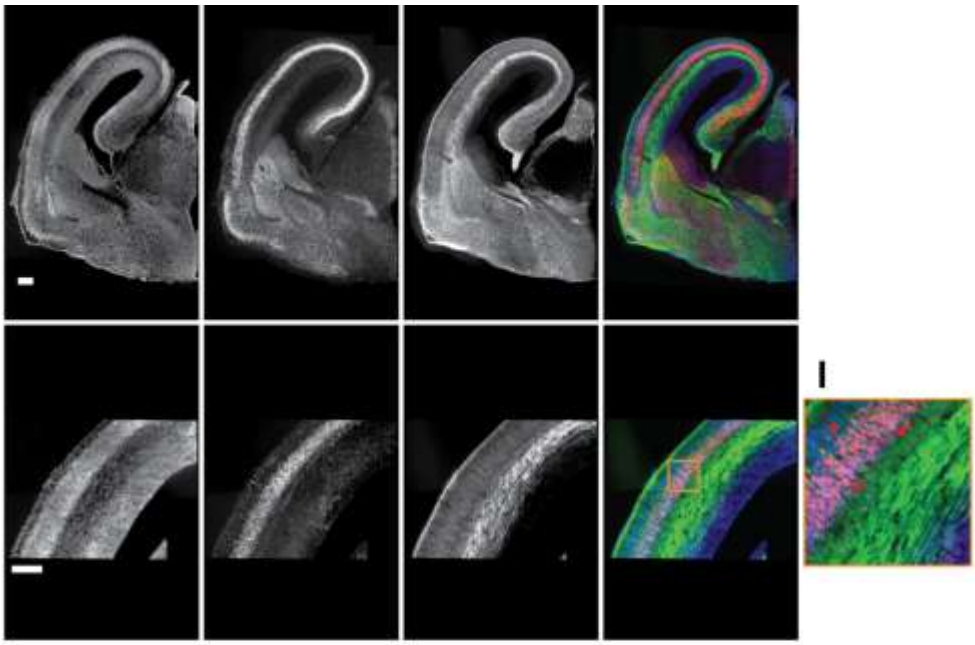


Figure 24. **ISLR2 and CTIP2 double immunostaining was performed on E16.5 mouse brain coronal posterior section (A-D)**. Higher Magnified View (20X) of neocortex region showing different layers (E-H). I represents part of Neocortex to show colocalization of ISLR2 and CTIP2 (pointed by red arrowheads). Abbreviations: CP, Cortical plate; ham, medial habenula; Hip, Hippocampus; IZ, Intermediate zone; LGE, Lateral Ganglionic Eminence; LOT, Lateral Olfactory Tract; LV, Lateral Ventricle; MZ, Marginal zone; NCx, Neocortex; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular Zone; TRN, Thalamic reticular nucleus. Scale Bars A-H: 100µm.

In human fetal brain sample the double immunostaining was analyzed in neocortical area (Figure 25) of coronal sections obtained from occipital lobe. Similar to mouse, ISLR2 is also expressed in CTIP2+ neurons in the CP in stage 15pcw sections. Interestingly few CTIP2+ neurons were detected also in the SP/IZ and OSVZ that were positive for ISLR2 as well (Figure 25A-H). As in the mouse, subcellular localization (Figure 25I) shows that most of the CTIP2+ neurons (nuclear staining) display co-expression of ISLR2 in the cytoplasm and/or membrane (Figure 25I). In contrast to mice, in the developing human brain ISLR2 expression was broader and extended into deeper regions like the ISVZ in 15pcw sections. When I analysed the CTIP2 and ISLR2 expression in expanded neocortex at later stages (22pcw), CTIP2 staining was strongly reduced gradually demonstrated in the deep region of cortical plate and very less number of neurons expressed CTIP2 (Figure 26 & 27). Few CTIP2 expressing neuron

were present in deeper layers (SP/IZ and OSVZ). Similar to 15pcw stage many neurons were double positive for CTIP2 and ISLR2 both.

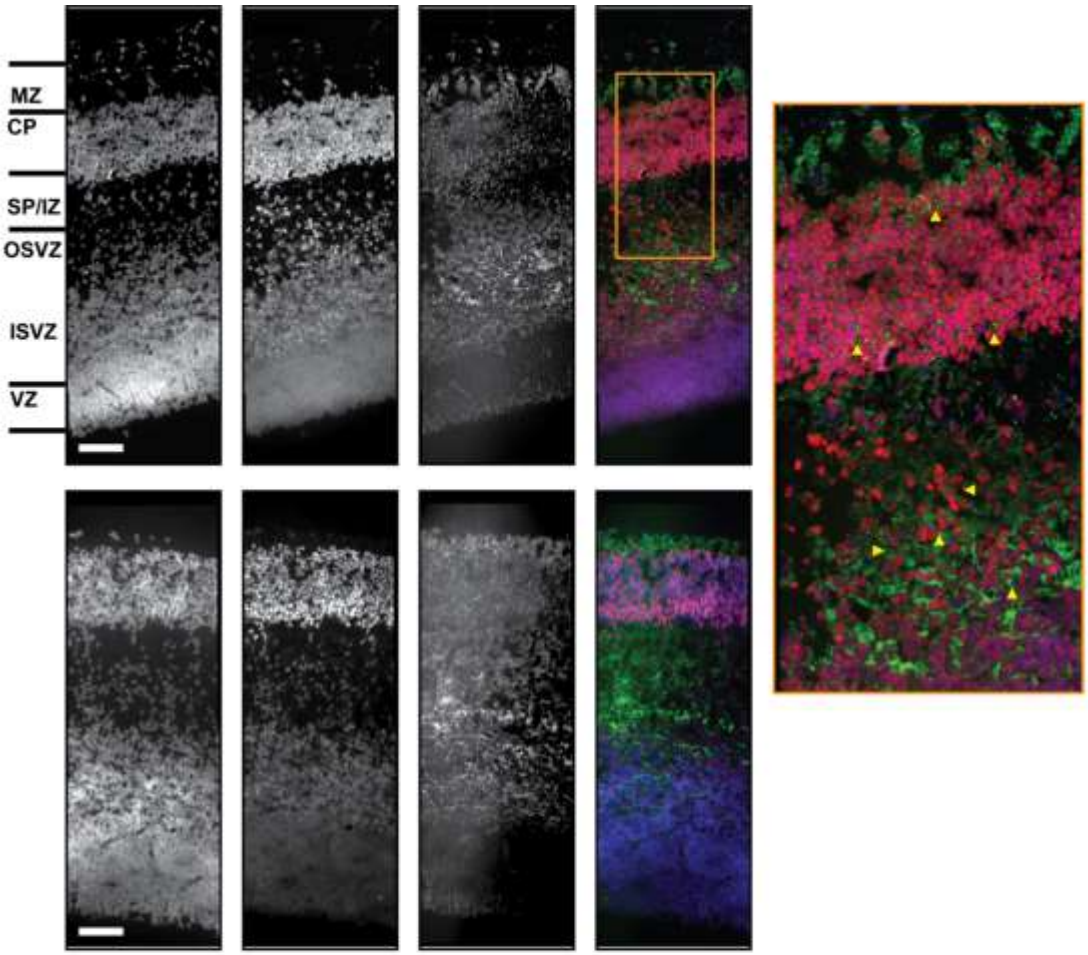


Figure 25. **Double immunofluorescense of ISLR2 (green) and CTIP2 (red) performed on 15 pcw human fetal brain coronal section, showing two neocortex region (A-D & E-H).** Higher Magnified View (20X) of neocortex region showing colocalization of ISLR2 and CTIP2 (pointed by yellow arrowheads) (I). ISLR2-immunopositive cells partially have CTIP2 in CP and many merged cells with CTIP2 and ISLR2 are diffusely demonstrated in deeper layers (SP/IZ & OSVZ). Abbreviations: CP, Cortical plate; ISVZ, Inner Subventricular zone; IZ, Intermediate zone; MZ, Marginal zone; OSVZ, Outer Subventricular zone; SP, Subplate; VZ, Ventricular Zone. Scale Bars A-H: 100µm

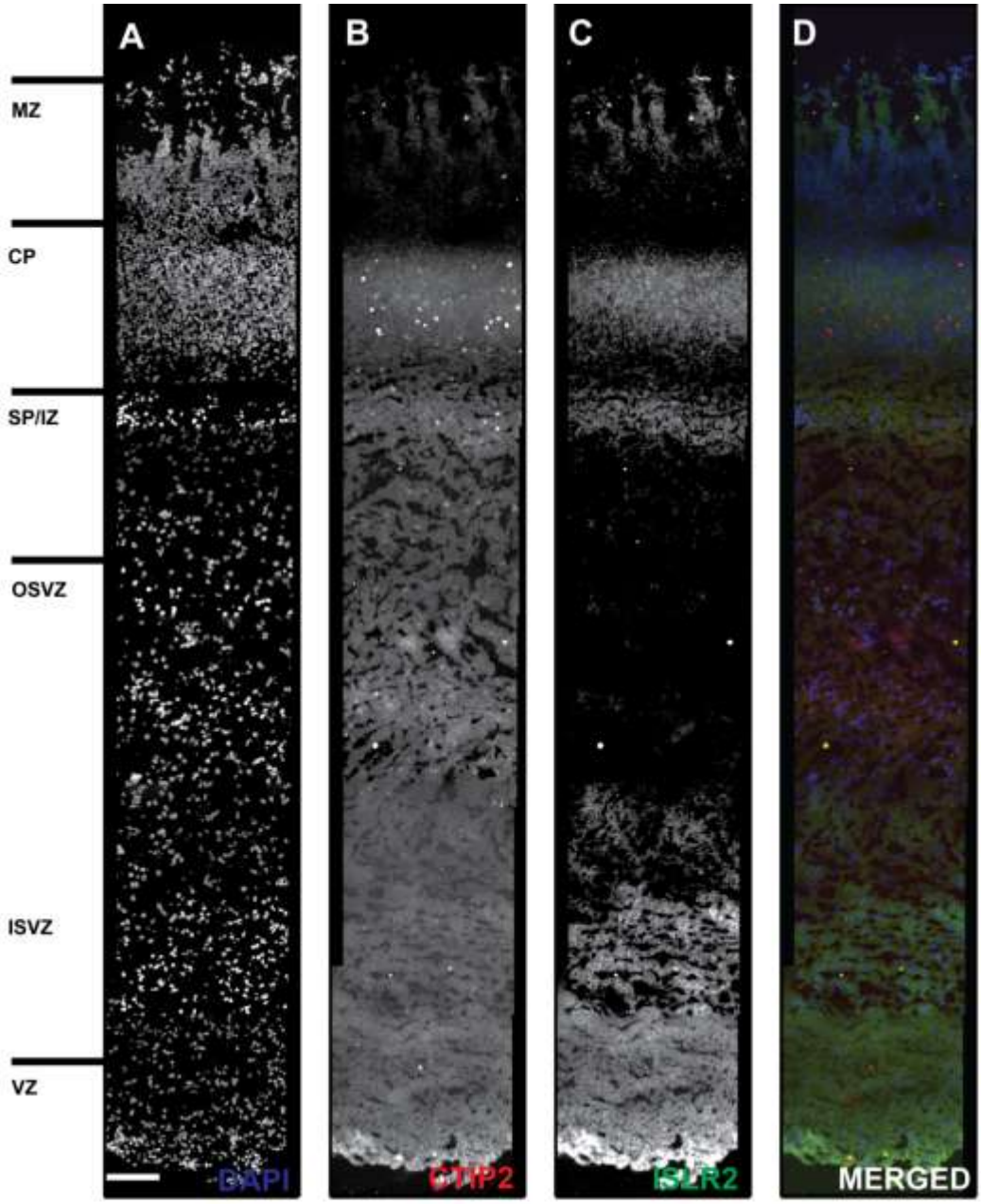


Figure 26. **Double immunofluorescense of ISLR2 (green) and CTIP2 (red) performed on 22 pcw human fetal brain coronal section, showing neocortex region (A-D)**. ISLR2-immunopositive cells partially have CTIP2 in CP and many merged cells with CTIP2 and ISLR2 are diffusely demonstrated in deeper layers (SP/IZ & OSVZ). Abbreviations: CP, Cortical plate; ISVZ, Inner Subventricular zone; IZ, Intermediate zone; MZ, Marginal zone; OSVZ, Outer Subventricular zone; SP, Subplate; VZ, Ventricular Zone. Scale Bars A-D: 100µm.

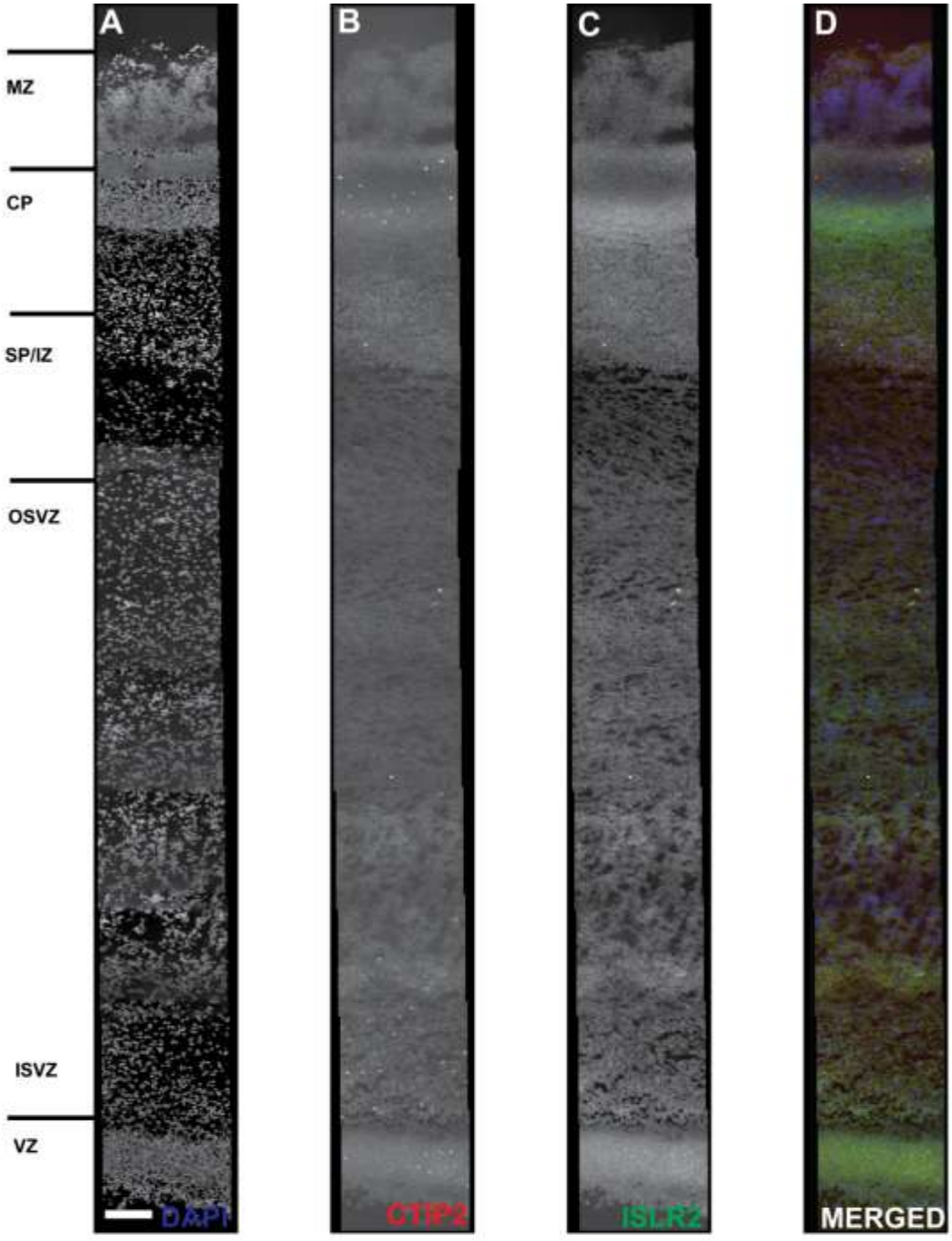


Figure 27. **Double immunofluorescense of ISLR2 (green) and CTIP2 (red) performed on 22pcw human fetal brain coronal section, showing neocortex region (A-D)**. ISLR2-immunopositive cells partially have CTIP2 in CP and many merged cells with CTIP2 and ISLR2 are diffusely demonstrated in deeper layers (SP/IZ & OSVZ). Abbreviations: CP, Cortical plate; ISVZ, Inner Subventricular zone; IZ, Intermediate zone; MZ, Marginal zone; OSVZ, Outer Subventricular zone; SP, Subplate; VZ, Ventricular Zone. Scale Bars A-D: 100μm.

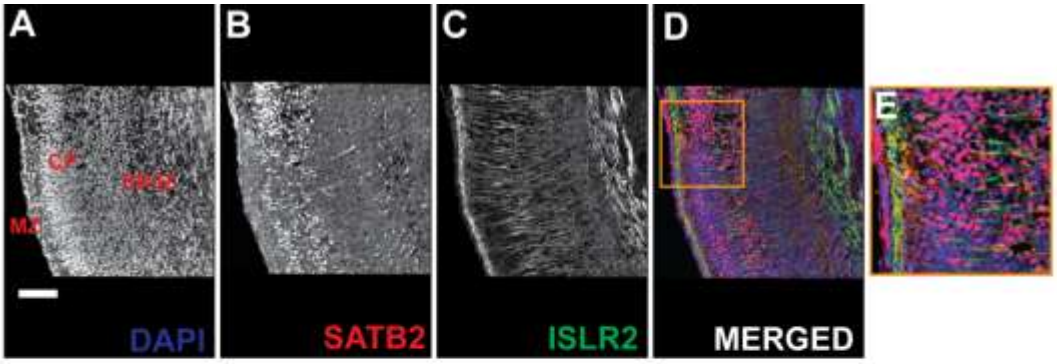


Figure 28. **Higher Magnified view of neocortex region of double immunostained with ISLR2 (Green) and SATB2 (Red) posterior coronal brain sections of E18.5 Mouse embryo (A-D) the boxed areas in Figure 28-E shows neurons doublepositive for both ISLR2 and SATB2.** SATB2 + cells were observed in superficial layers. Abbreviations: CP, Cortical plate; IZ, Intermediate zone; MZ, Marginal zone; SP, Subplate; Scale Bars A-D: 100µm.E represents part of Cortex magnified to show some neurons showing colocalization of ISLR2 with SATB2.

Analysis of Immunofluorescence of ISLR2 with progenitor cell marker SOX2

To investigate any possibility of ISLR2 in neuron proliferation I performed double immunofluorescence of ISLR2 with SOX2 on coronal sections of E16.5 mouse brain and coronal sections of 15pcw and 22pcw human fetal brain. In E16.5 mouse brain coronal sections SOX2 was clearly expressed by neurons in VZ of neocortex (Figure 29B,F) , no double positive cells for SOX2 and ISLR2 are observed in other layers and VZ layer was devoid of ISLR2 (Figure 29C,G). This complementary staining indicates that ISLR2 does not regulate neuron proliferation nor the early steps of neuronal fate (Figure 29D & H).

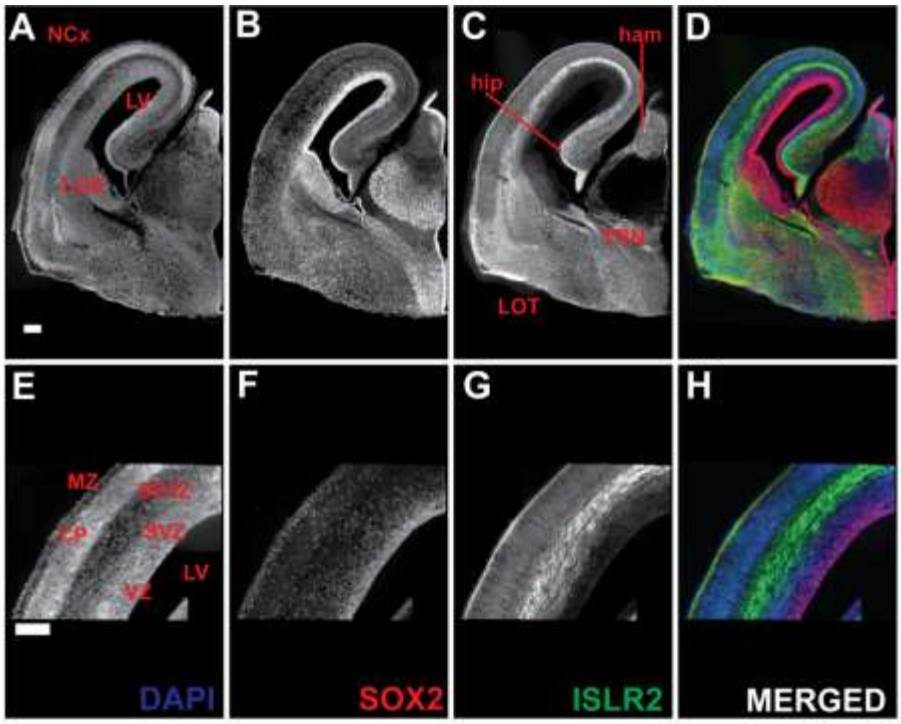


Figure 29. **ISLR2 (green) and Sox2 (red) double immunofluorescence was performed on E16.5 mouse brain coronal sections (A-D).** Higher magnified view (20X) of neocortex region showing different layers (E-H). Abbreviations: CP, Cortical plate; ham, medial habenula; Hip, Hippocampus; IZ, Intermediate zone; LGE, Lateral Ganglionic Eminence; LOT, Lateral Olfactory Tract; LV, Lateral Ventricle; MZ, Marginal zone; NCx, Neocortex; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular Zone; TRN, Thalamic reticular nucleus. Scale Bars A-H: 100µm.

In human fetal brain coronal sections SOX2 was clearly expressed by neurons in VZ of neocortex like in mouse though some SOX2 positive cells were found in ISVZ and OSVZ also (Figure 30B, F). However, no double positive cells for SOX2 and ISLR2 were observed, neither in VZ (where ISLR2 is absent) nor in the scattered SOX2+ neurons in the ISVZ and OSVZ (Figure 30C, G). So, as in the mouse, complementary expression patterns between ISLR2 and SOX2 suggest that ISLR2 does not regulate progenitor proliferation and/or early neuron fate (Figure 30D, H). While in later stage of human development; 22pcw the cells expressing SOX2 was extended across ISVZ, OSVZ and some to IZ also apart from VZ (Figure 31aB; Figure 31bB) and ISLR2 was expressed more in upper layers, in CP and SP/IZ not colocalizing with SOX2 (Figure31aCD; Figure31bCD).

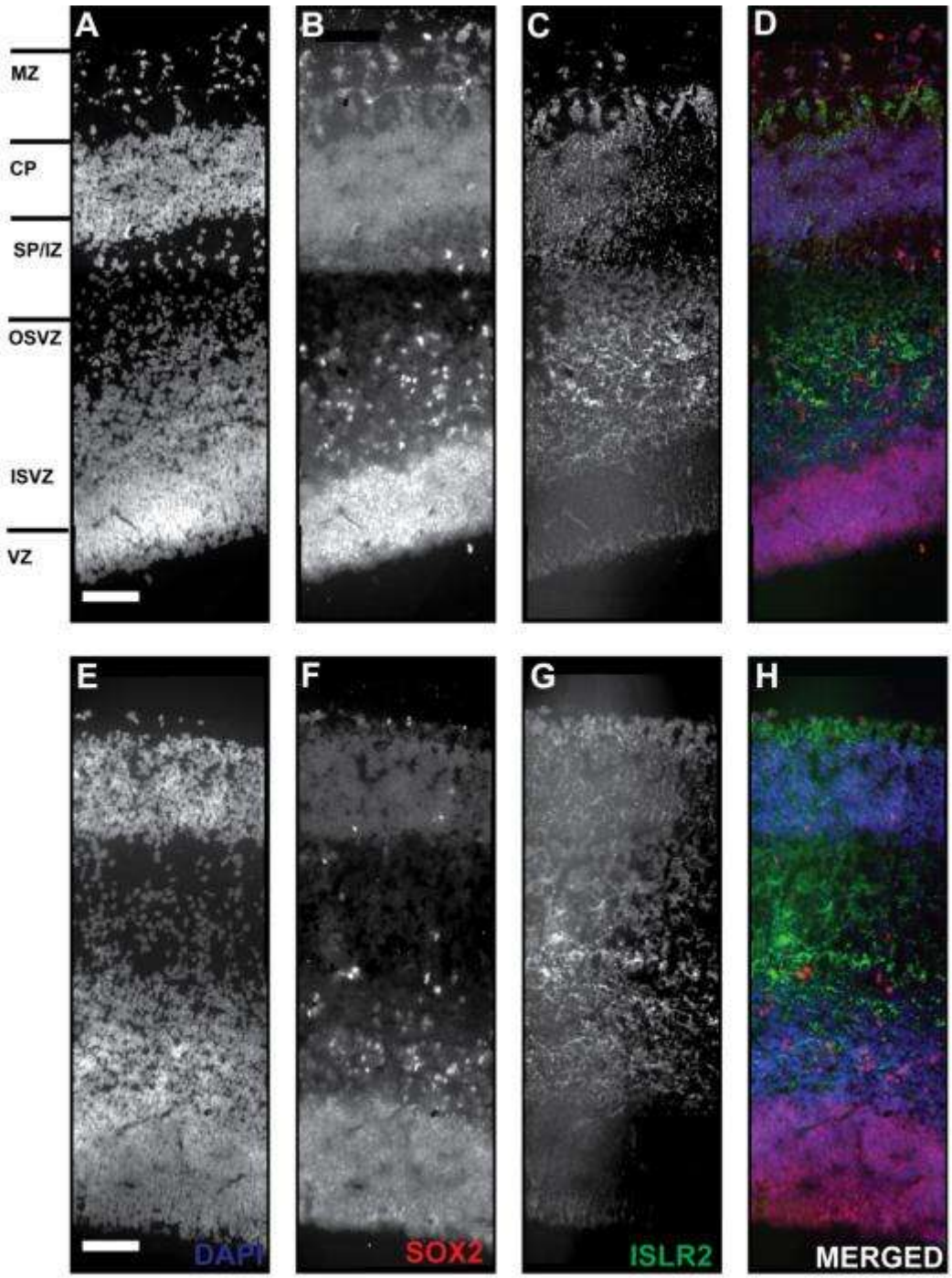


Figure 30. **Double immunofluorescence of ISLR2 (green) and SOX2 (red) performed on 15 pcw human fetal brain coronal section, showing two neocortex region (A-D & E-H).** No double immunopositive cells having both ISLR2 and SOX2 were observed-. Abbreviations: CP, Cortical plate; ISVZ, Inner Subventricular zone; IZ, Intermediate zone; MZ, Marginal zone; OSVZ, Outer Subventricular zone; SP, Subplate; VZ, Ventricular Zone. Scale Bars A-H: 100μm.

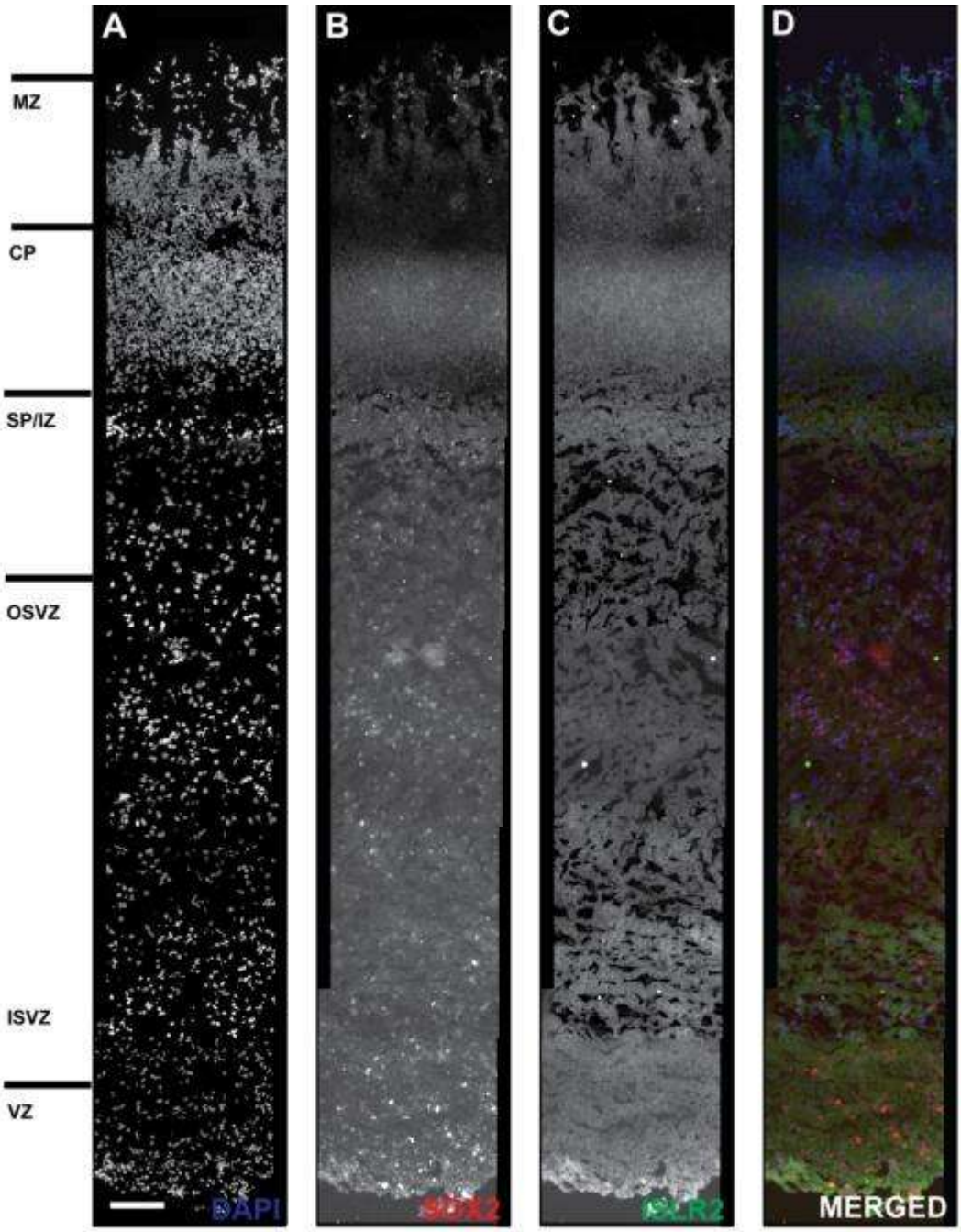


Figure 31a. **Double immunofluorescence of ISLR2 (green) and SOX2 (red) performed on 22 pcw human fetal brain coronal section, showing two neocortex region (A-D).** No double immunopositive cells having both ISLR2 and SOX2 were observed-. Abbreviations: CP, Cortical plate; ISVZ, Inner Subventricular zone; IZ, Intermediate zone; MZ, Marginal zone; OSVZ, Outer Subventricular zone; SP, Subplate; VZ, Ventricular Zone. Scale Bars A-D: 100µm.

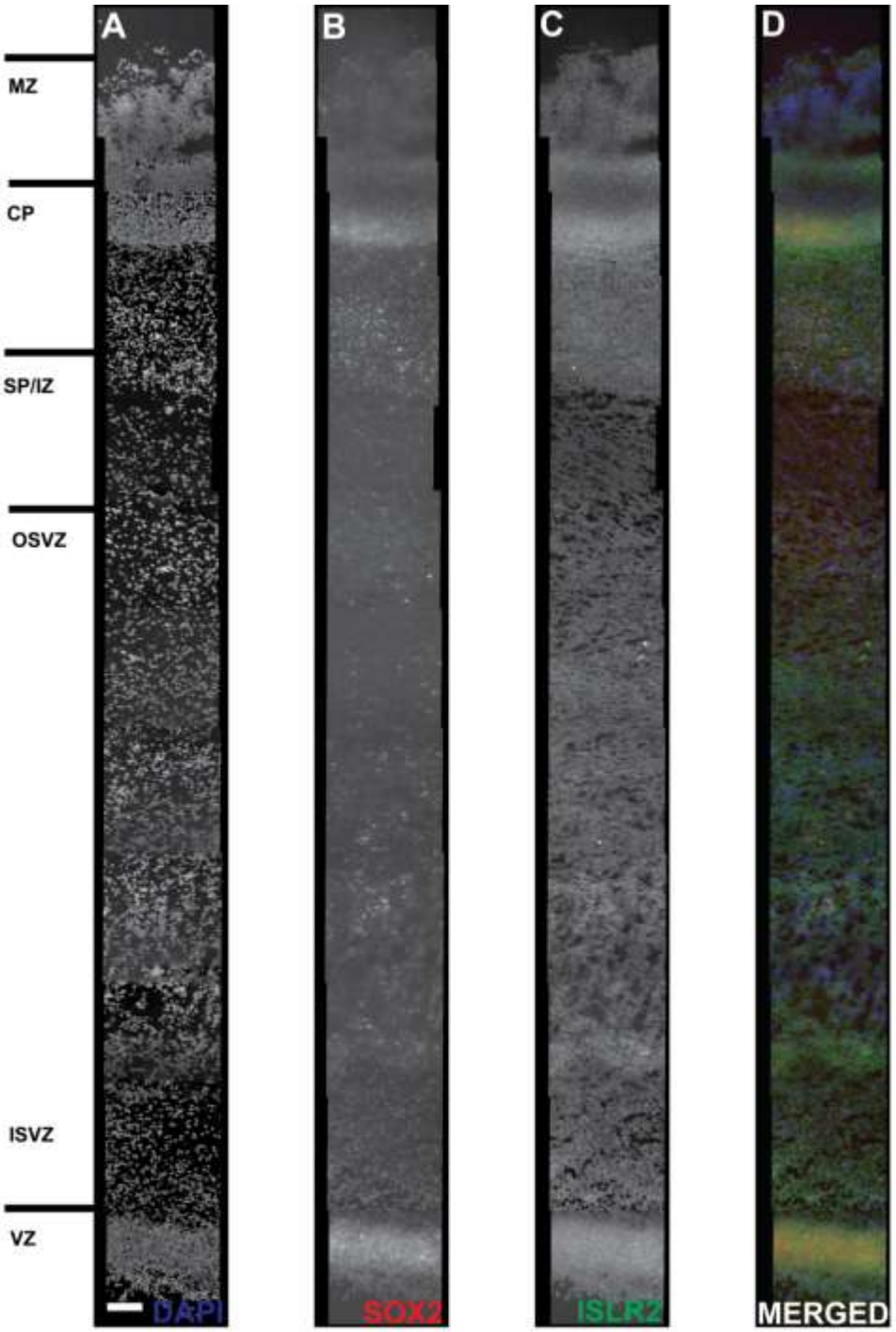


Figure 31b. **Double immunofluorescence of ISLR2 (green) and SOX2 (red) performed on 22 pcw human fetal brain coronal section, showing two neocortex region (A-D).** No double immunopositive cells having both ISLR2 and SOX2 were observed. Abbreviations: CP, Cortical plate; ISVZ, Inner Subventricular zone; IZ, Intermediate zone; MZ, Marginal zone; OSVZ, Outer Subventricular zone; SP, Subplate; VZ, Ventricular Zone. Scale Bars A-D: 100µm.

Analysis of double Immunofluorescence of ISLR2 and DCx

The above analysis has shown that ISLR2 is present in maturing neurons and, at the subcellular level, that it is localized both in the soma and in the axons in a cytoplasmic/membrane pattern. To confirm this expression we used another marker, doublecortin (DCx), which labels differentiating neurons. Expression of ISLR2 and DCx displayed striking overlapping expression in different regions of the brain, including neocortex, hippocampus and thalamus although DCx staining is more diffused when compared to ISLR2 (Figure 32A-L).

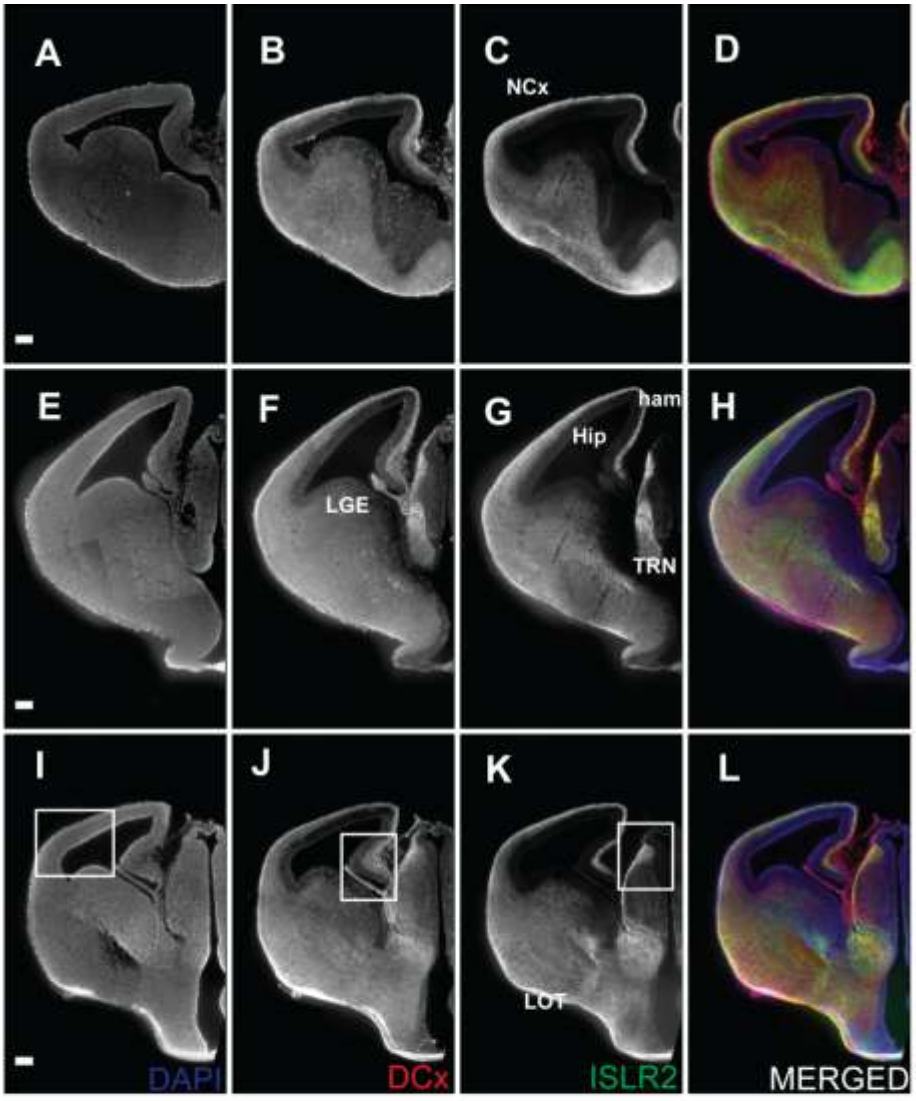


Figure 32. **ISLR2 (green) and DCx (red) double immunostaining was performed on E13.5 mouse brain sections from anterior (A-D) to posterior (I-L).** Abbreviations: ham, medial habenula; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; LOT, Lateral Olfactory Tract ; NCx, Neocortex; TRN, Thalamic reticular nucleus. Scale Bars: A-L, 100µm.White Boxed area has been shown in higher magnification in next figure.

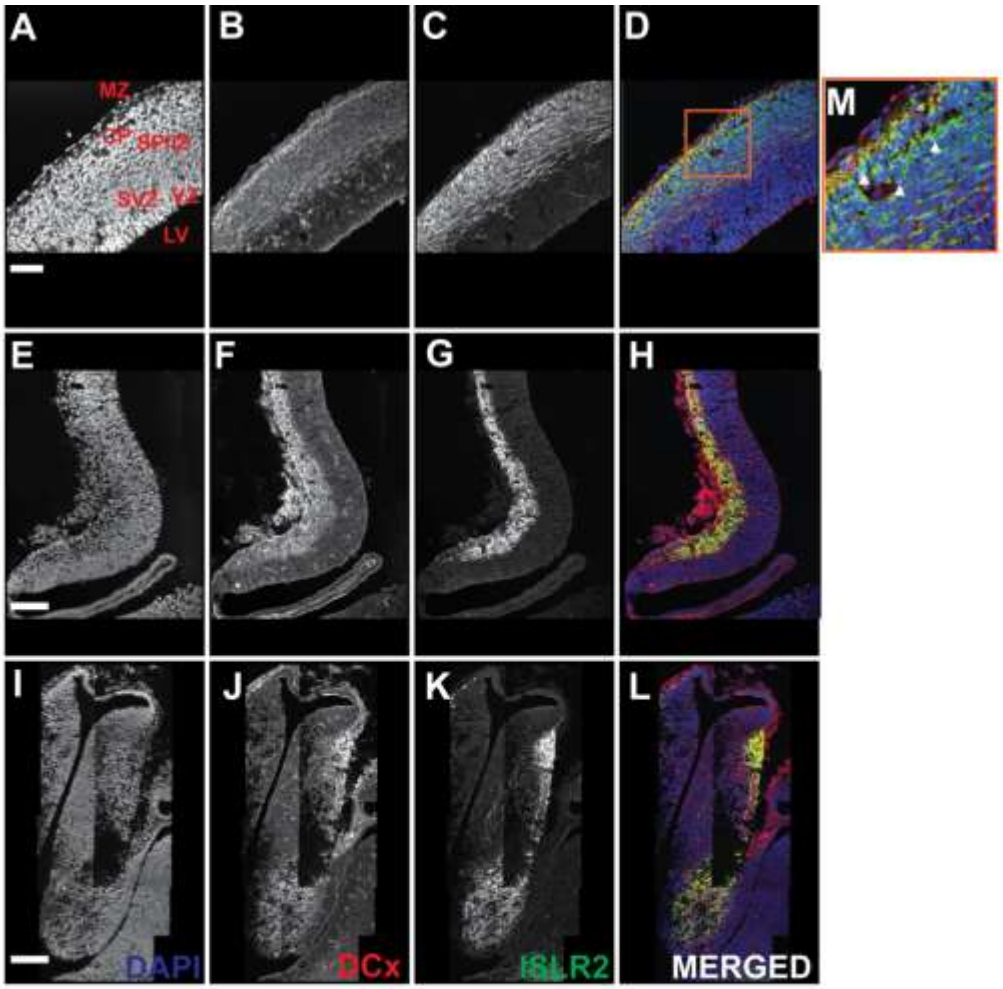


Figure 33. **Higher Magnification image of the boxed areas in Figure32 . A-D represents part of neocortex from a posterior coronal brain section double immunostained with ISLR2 and DCx; E-H represents part of Hippocampus; I-L represents part of Thalamus**. Abbreviations: CP, Cortical plate; IZ, Intermediate zone; LV, Lateral Ventricle; MZ, Marginal zone; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular Zone. Scale Bars A-L: 100µm. M represents part of Cortex magnified to show overlap of ISLR2 with DCx (pointed by white arrowheads).

In neocortex both DCx and ISLR2 are overlapping in upper layers and IZ layers also (Figure 33D, M). In hippocampus most of the cells positive for DCx were also positive for ISLR2 while in thalamus some of the DCx + cells were positive for ISLR2 (Figure 33G, H, K, L). Analysis of double Immunofluorescence of DCx and ISLR2 at E18.5 also suggests that ISLR2 and DCx overlap (Figure 34D-F) and both are expressed mainly in upper layers (I-IV).

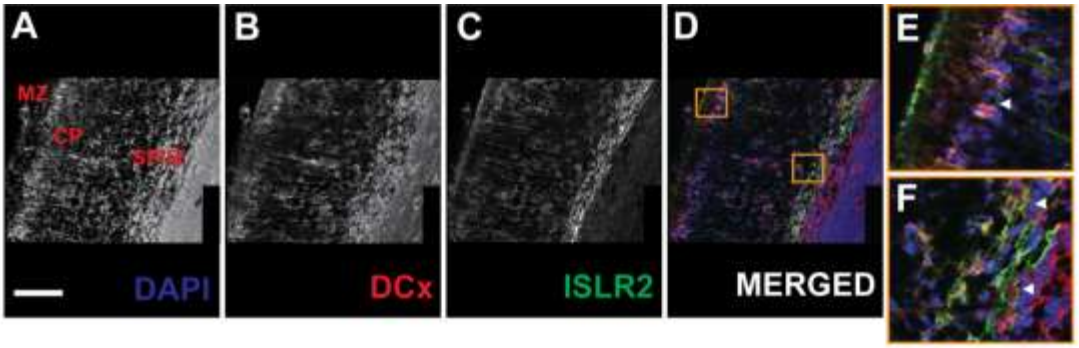


Figure 34. **Higher Magnified view of neocortex region of double immunostained with ISLR2 (Green) and DCx (Red) posterior coronal brain sections of E18.5 Mouse embryo (A-D).** Abbreviations: CP, Cortical plate; IZ, Intermediate zone; MZ, Marginal zone; SP, Subplate; Scale Bars A-D: 100µm.E represents part of Cortex magnified to show some neurons showing colocalization of ISLR2 with DCx (white arrowheads).

Analysis of Double immunostaining of ISLR2 and TuJ-1

On double immunostaining ISLR2 and TuJ-1 showed strong colocalization in all the regions of brain at E13.5, including neocortex, hippocampus and thalamus (Figure 35). In ganglionic eminences the area of TuJ-1 is more extended towards SVZ where ISLR2 expression is clearly reduced (Figure 35J-K). When observed at higher magnification also the colocalization was very evident in neocortex. In the neocortex ISLR2 and TuJ-1 colocalize perfectly in the axonal fibres (Figure 36 B-C).

At E18.5 co localization of ISLR2 and TuJ-1 was consistent to earlier developmental stages (Figure 37D). TuJ-1 is expressed in cortical plate very strongly expressed but in deeper CP expression gradually decreases to some extent gaining increment in IZ, while expression of ISLR2 was consistent from CP to IZ (Figure 37B-C).

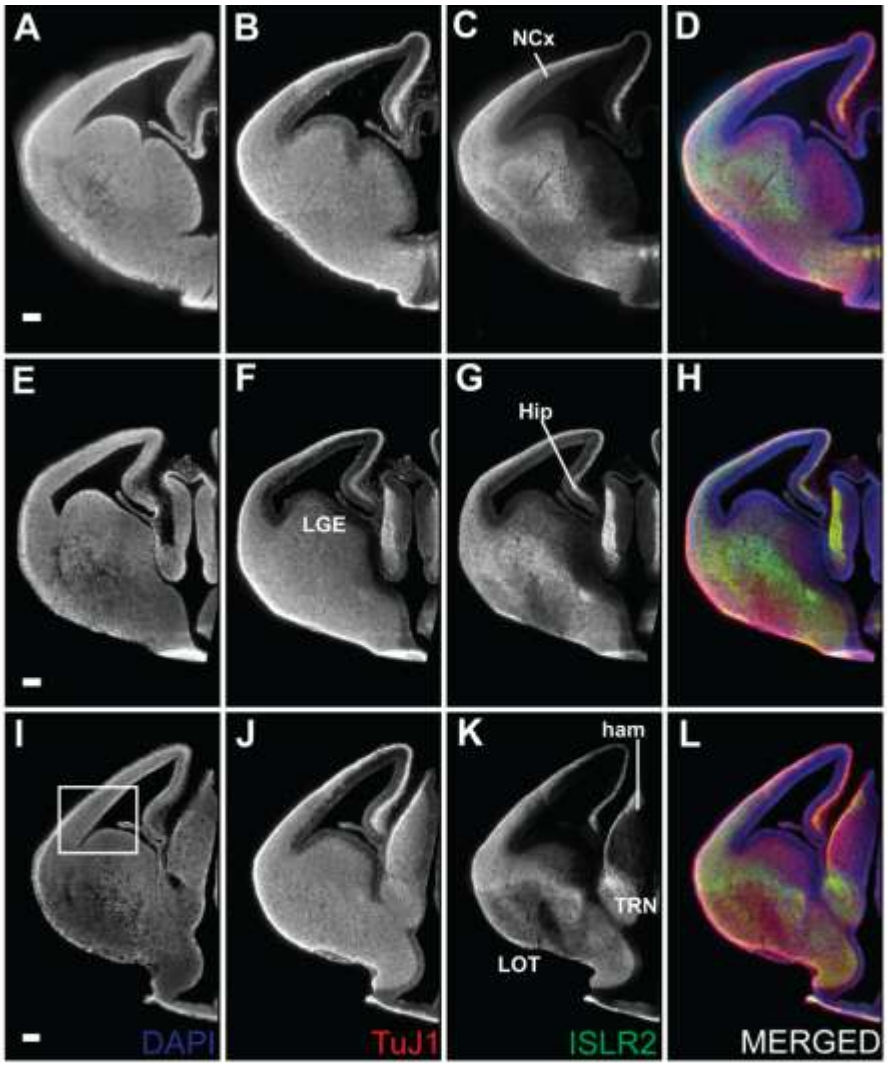


Figure 35. I**SLR2 and TuJ-1 double immunostaining was performed on E13.5 mouse brain sections from anterior (A-D) to posterior (I-L).** Abbreviations: ham, medial habenula; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; LOT, Lateral Olfactory Tract ; NCx, Neocortex; TRN, Thalamic reticular nucleus. Scale Bars: A-L, 100 μm.White Boxed area has been shown in higher magnification in next figure.

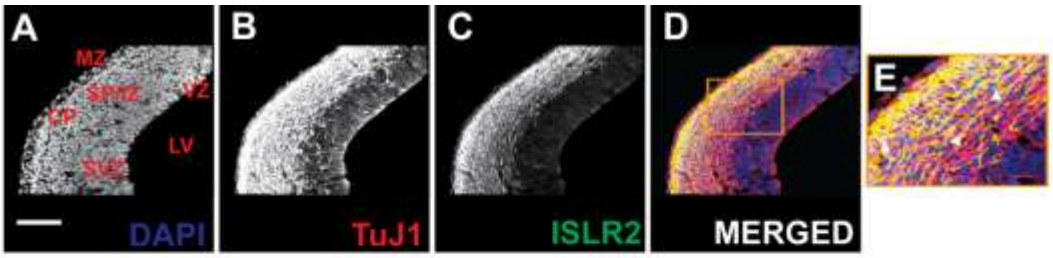


Figure 36. **Higher Magnification image of the boxed areas in Figure 35. A-D represents part of neocortex from a posterior coronal brain section double immunostained with ISLR2 and TuJ-1.** Abbreviations: CP, Cortical plate; IZ, Intermediate zone; LV, Lateral Ventricle; MZ, Marginal zone; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular Zone. Scale Bars A-D: 100µm. E represents part of Cortex magnified to show colocalization of ISLR2 with TuJ-1 (pointed by white arrowheads).

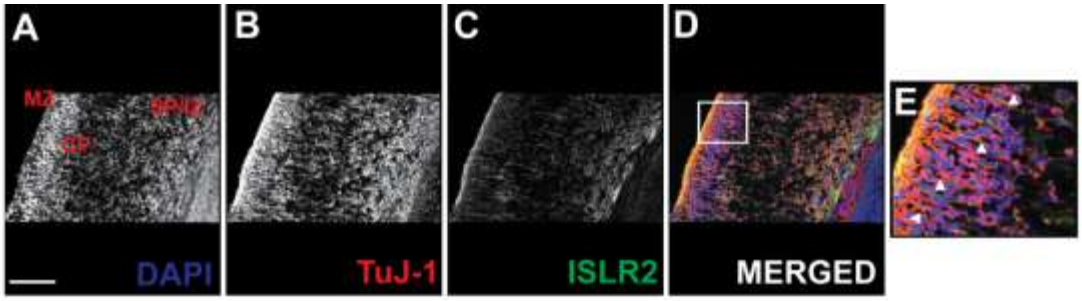


Figure 37. **Higher Magnified view of neocortex region of double immunostained with ISLR2 (green) and TuJ-1 (red) posterior coronal brain sections of E18.5 Mouse embryo (A-D)**. Abbreviations: CP, Cortical plate; IZ, Intermediate zone; MZ, Marginal zone; SP, Subplate; Scale Bars A-D: 100µm.E represents part of Cortex magnified to show some neurons showing colocalization of ISLR2 with TuJ-1 (white arrowheads).

Comparing expression of ISLR2 with FLRT3 in the developing mouse and human brain.

There is a special interest of our group in another eLRR-TM protein, FLRT3, for which much more is known concerning expression and function in the nervous system development (see also next section). I therefore wished to compare the expression pattern of both FLRT3 and ISLR2 in the same sections using specific antibodies at E13.5 stage. The FLRT3 and ISLR2 proteins were detected in somehow non-overlapping regions (Figure 38). As mentioned above, in the neocortex expression of ISLR2 was abundant in CP and in the axons in the IZ while FLRT3 was restricted to lower CP and upper IZ (Figure 39B, C). Expression of FLRT3 in ganglionic eminences decreases from anterior to posterior sections but is consistent for ISLR2 (Figure 38FG). Most interesting was thalamus where FLRT3 is restricted only to tip of ET while ISLR2 is expressed in medial habenula part of ET and extended to DT. ISLR2 is not expressed in lower DT and VT but shows strong expression in TRN.

Looking for any colocalization at cellular level we took closer look of neocortex, thalamus and hippocampus (Figure 39). Expression of FLRT3 was found mainly in membrane while ISLR2 was present more in cytoplasm than membrane. In hippocampus the expression for FLRT3 was very weak except in fimbria when compared to abundantly present ISLR2 (Figure 39F, G). While in Thalamus expression of FLRT3 was restricted more towards ventricular zone and epithalamus, ISLR2 avoided this area and was observed more towards medial habenula avoiding tip of ET where FLRT3 was expressed, while axonal fibres expressing ISLR2 was very clearly observed in lower DT and VT(Figure 39) as shown in earlier figure also.

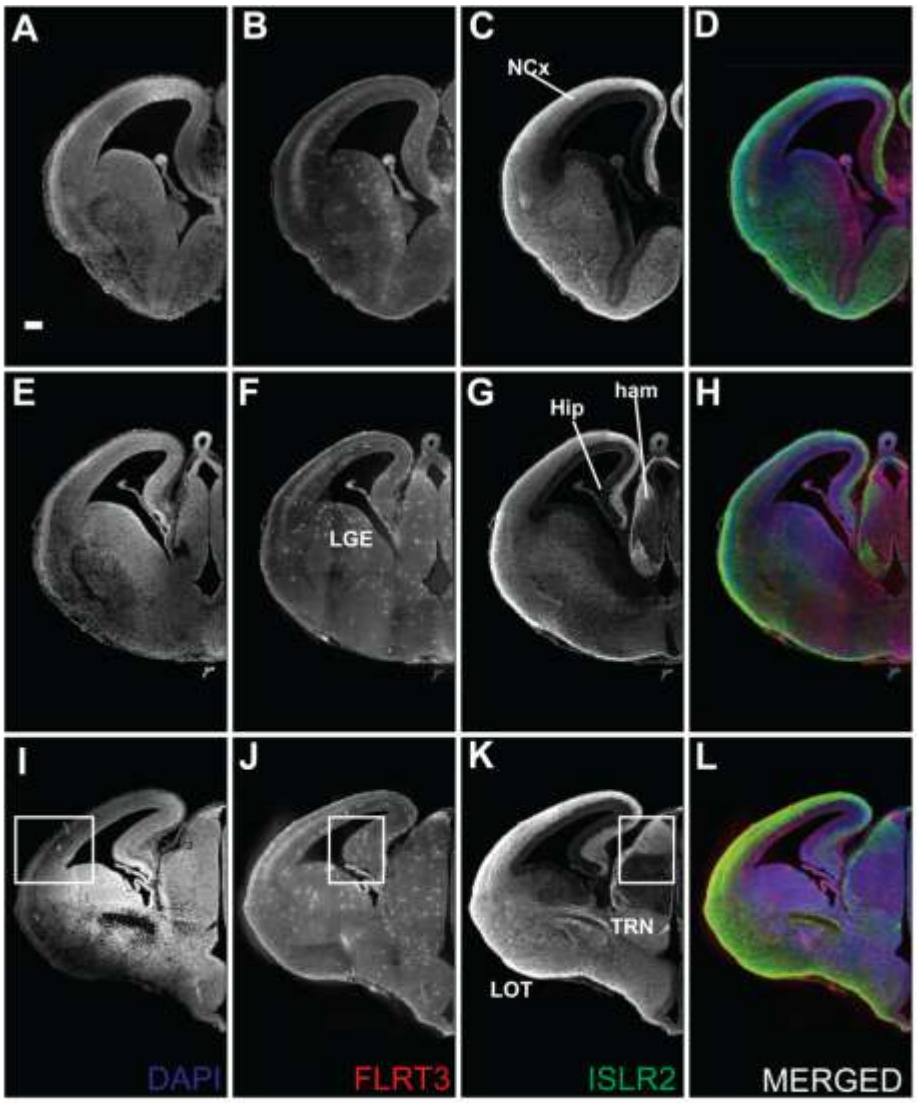


Figure 38. **ISLR2 and FLRT3 double immunostaining was performed on E13.5 mouse brain sections from anterior (A-D) to posterior (I-L)**. Abbreviations: ham, medial habenula; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; LOT, Lateral Olfactory Tract ; NCx, Neocortex; TRN, Thalamic reticular nucleus. Scale Bars: A-L, 100 µm.White Boxed area has been shown in higher magnification in next figure.

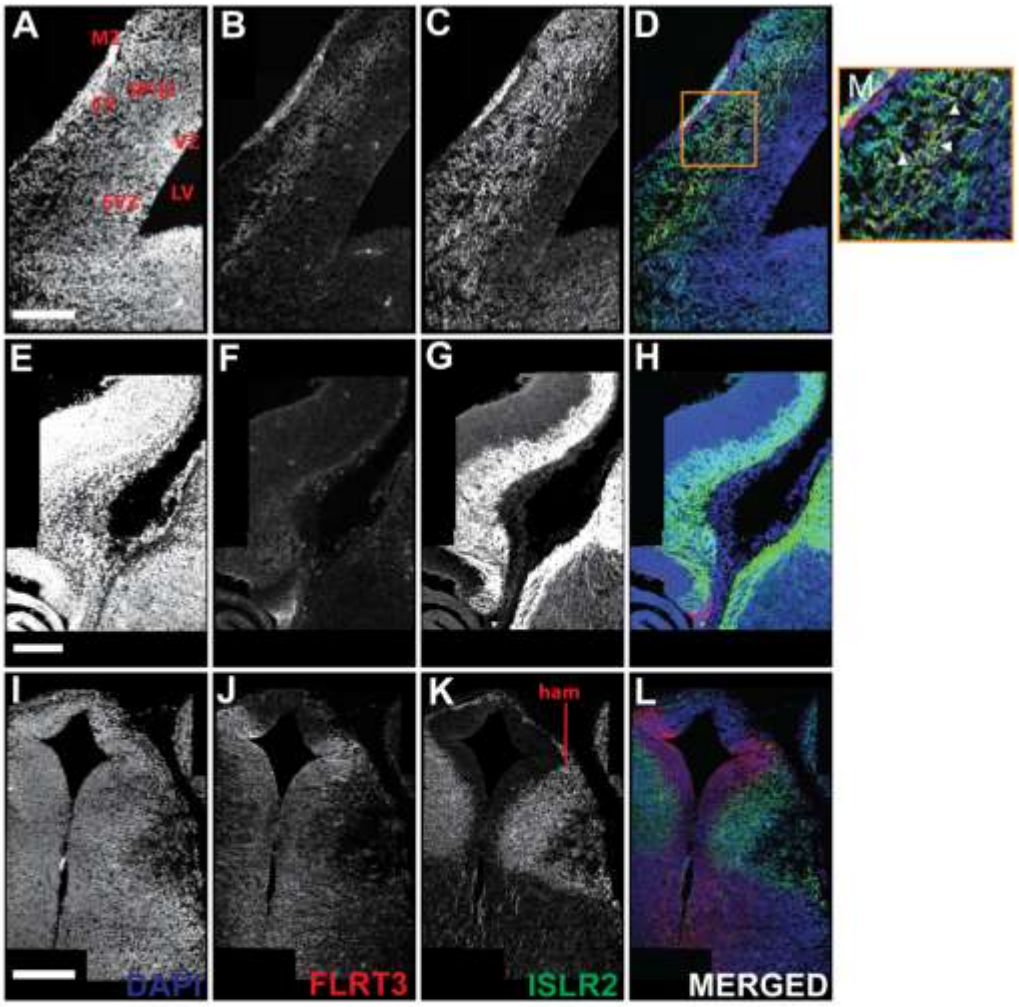


Figure 39. **Higher Magnification image of the boxed areas in Figure 38 . A-D represents part of neocortex from a posterior coronal brain section double immunostained with ISLR2 and FLRT3 ; E-H represents part of Hippocampus; I-L represents part of Thalamus.** Abbreviations: CP, Cortical plate; ham, medial habenula; IZ, Intermediate zone; LV, Lateral Ventricle; MZ, Marginal zone; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular Zone. Scale Bars A-L: 100µm. M represents part of Cortex magnified to show colocalization of ISLR2 with FLRT3 (pointed by white arrowheads).

4.2.1 Expression of *FLRT* family in the developing mouse and human brain

FLRT family of genes is having three members *FLRT1*, *FLRT2* and *FLRT3* and they display distinct expression patterns during brain development.

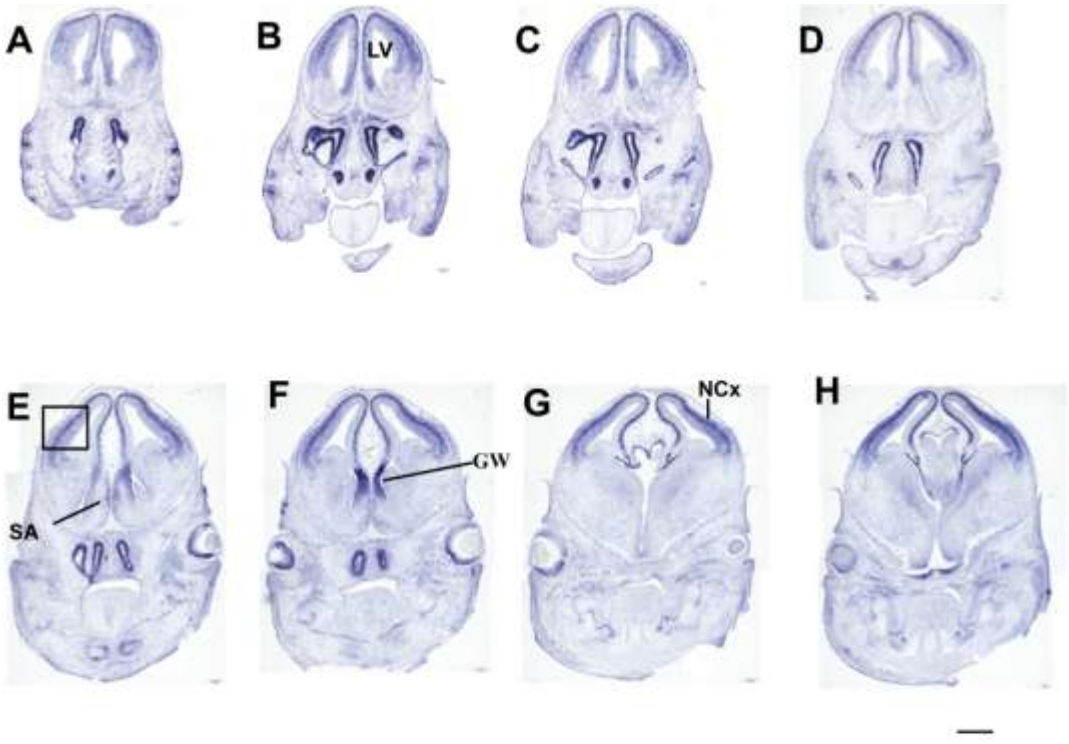


Figure 40. **Expression of *FLRT1* in the developing mouse brain**.
(A-H) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 using a digoxigenin-labelled Flrt1 antisense probe on coronal brain sections of mouse embryos. Abbreviations: GW, Glial Wedge; LV, Lateral Ventricle; NCx, Neocortex; OB, Olfactory bulb; SA, Septal area.Scale Bars: A-H, 200 µm. Boxed area in E has been shown in high magnification in Figure 41.

FLRT1 is expressed majorly in the pallium (mainly in the CP and a bit in the SVZ and little in subpallial regions during development (Figure 40; 42). Other areas of expression include the septal area (Figure 40E) and Glial wedge also shows strong expression of *FLRT1* at E13.5 (Figure 40F).

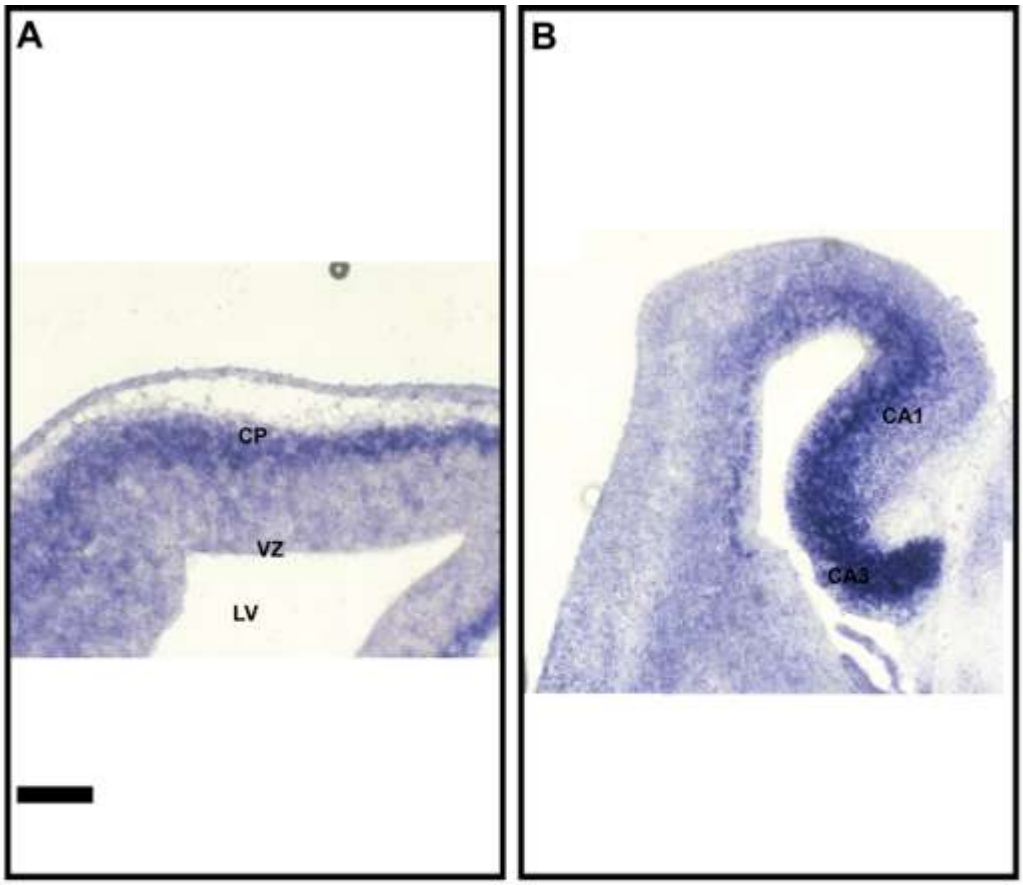


Figure 41. **Higher magnified view of specific regions showing expression of FLRT1 in coronal section of mouse embryonic brain**. (A) Cortex showing different layers at E13.5 stage (Figure 40E), (B) Hippocampus showing CA1 and CA3 regions (Figure 42E). Abbreviations: CA, Cornu Ammonis; CP, Cortical plate; LV, Lateral Ventricle; VZ, Ventricular zone .Scale Bars: A-B, 100 μm.

Hippocampal area also shows expression of *FLRT1*, which increases during development and mainly labels CA1 and CA3 regions (Figure42 C-F; Figure 41B). At E15.5 and E17.5 *FLRT1* expression in cortex became broader including expression in the CP and also in cells located in the IZ-SVZ, extending toward the hippocampus (Figure 42). However, when compared to E13.5, it seems as if overall *FLRT1* expression in the cortex gets reduced (compare Figure 42 and Figure 40).

In contrast to *FLRT1*, *FLRT2* had a broader expression pattern. Like *FLRT1*, *FLRT2* is expressed in the CP of the neocortex and in the hippocampal area at E13.5 (Figure 43). In the basal ganglia at this stage, *FLRT2* expression is found mainly in the SVZ of the lateral ganglionic eminence and little expression is detected in the MGE. Later in the development (E15.5 and E17.5) expression of *FLRT2* in the neocortex is maintained in

the CP and in the CA1 and CA3 regions of the hippocampus (Figure 44 F-H; Figure 45A). At E17.5, the expression of *FLRT2* in neocortex decreases in width restricting expression only to the more superficial layers of the cortical plate (Figure 44E-H).

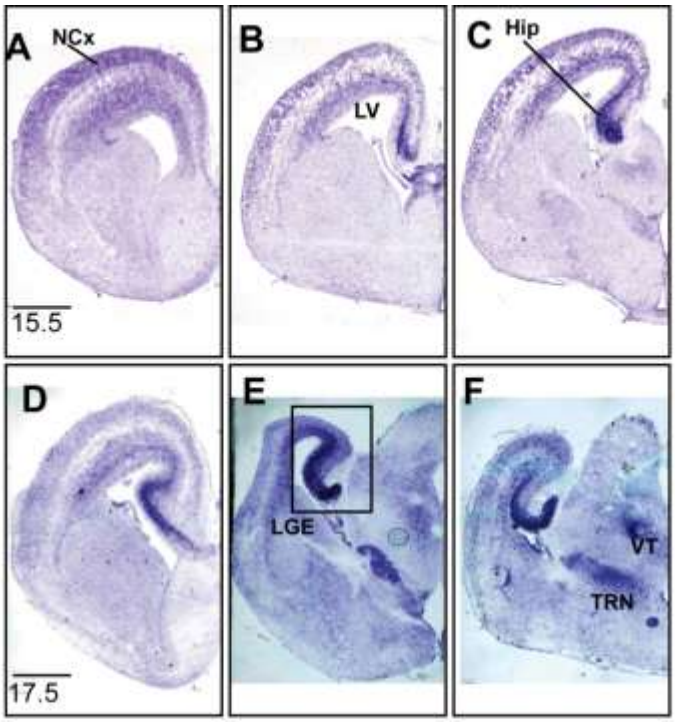


Figure 42. **Expression of *FLRT1* in the developing mouse brain**.
(A-F) In situ hybridization (ISH) analyses of coronal brain sections at E15.5 (A-C), E17.5 (D-F) using a digoxigenin-labelled *FLRT1* antisense probe on coronal brain sections of mouse embryos. A & D panels show anterior; B,shows medial; C, E,F shows posterior sections of the brain, respectively. Abbreviations: ET, Epithalamus; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; LV, Lateral Ventricle; NCx, Neocortex; TRN, Reticular thalamic nuclei; VT, Ventral thalamus. Scale Bars: A-F, 200 µm.

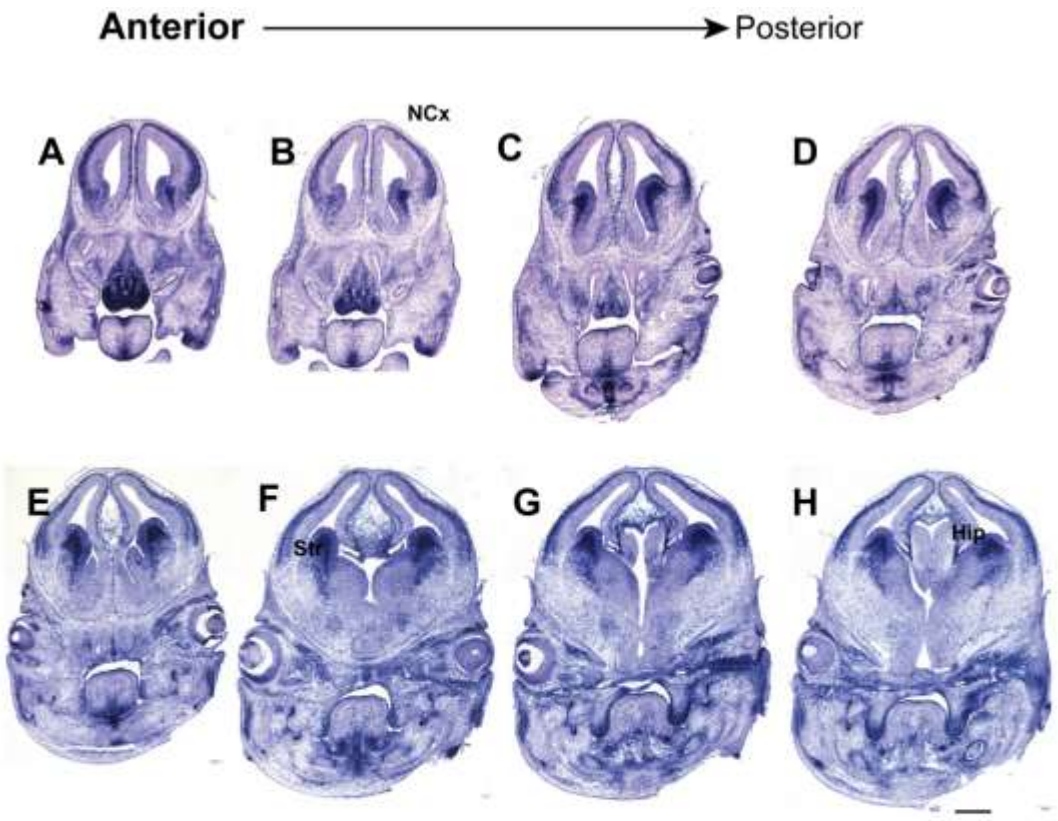


Figure 43. **Expression of *FLRT2* in the developing mouse brain.**
(A-H) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 using a digoxigenin-labelled *FLRT2* antisense probe on coronal brain sections of mouse embryos. Abbreviations: Hip, Hippocampus; NCx, Neocortex; Str, Striatum.Scale Bar: A-H, 200 μm.

FLRT2 expression in the basal ganglia at E15.5 and E17.5 is maintained in the SVZ of the LGE but is also detectable at this stage in the caudate putamen (high/dorsal to low ventral) (Figure 44BCEF). Interestingly, in both places *FLRT2* expression displays a gradient from dorsal/high to ventral/low revealing a region of *FLRT2* low expression between the two structures like a "corridor" (Figure 44B, C; Figure 45C). A strong signal appears at the reticular thalamic nuclei at E15.5 which faints at E17.5, also expression at medial habenula region of epithalamus was persistent in both E15.5 and

E17.5 stage (Figure 44DG; Figure 45C). A faint stream of *FLRT2* expression is visible in SP/IZ of neocortex (Figure 44B) which I didn't find in E17.5 (Figure 44E-H).

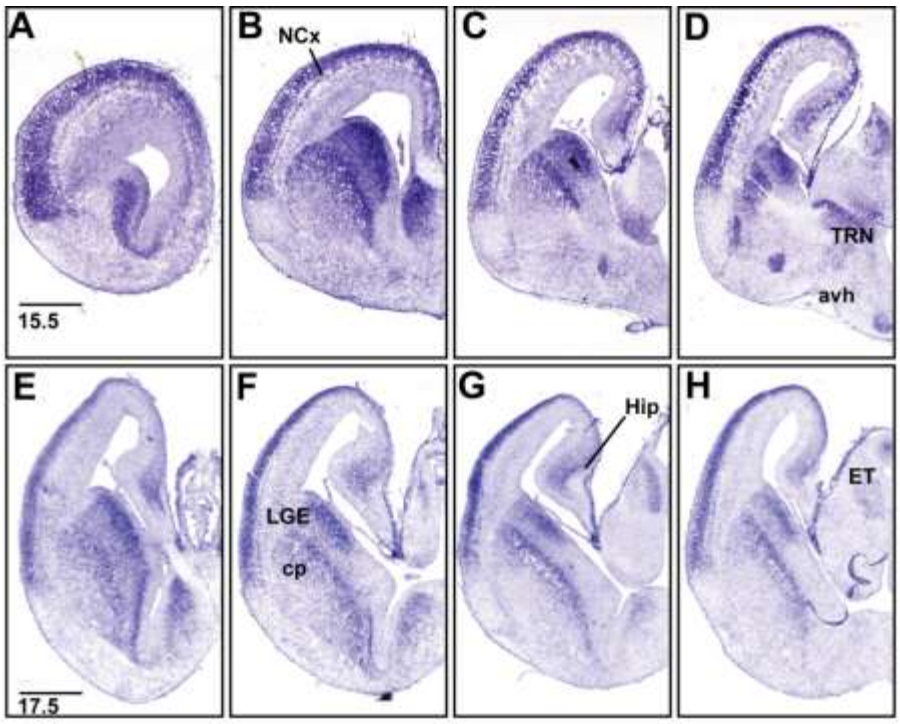


Figure 44. **Expression of *FLRT2* in the developing mouse brain.**
(A-H) In situ hybridization (ISH) analyses of coronal brain sections at E15.5 (A-D), E17.5 (E-H) using a digoxigenin-labelled *FLRT2* antisense probe on coronal brain sections of mouse embryos. A & B panels show anterior; C & E shows medial; D,F,G,H shows posterior sections of the brain, respectively. Abbreviations: avh,anterior ventricular hypothalamic nucleus; cp, Caudate putamen; ET, Epithalamus; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; NCx, Neocortex; TRN, Reticular thalamic nuclei. Scale Bars: A-H, 200 μm.

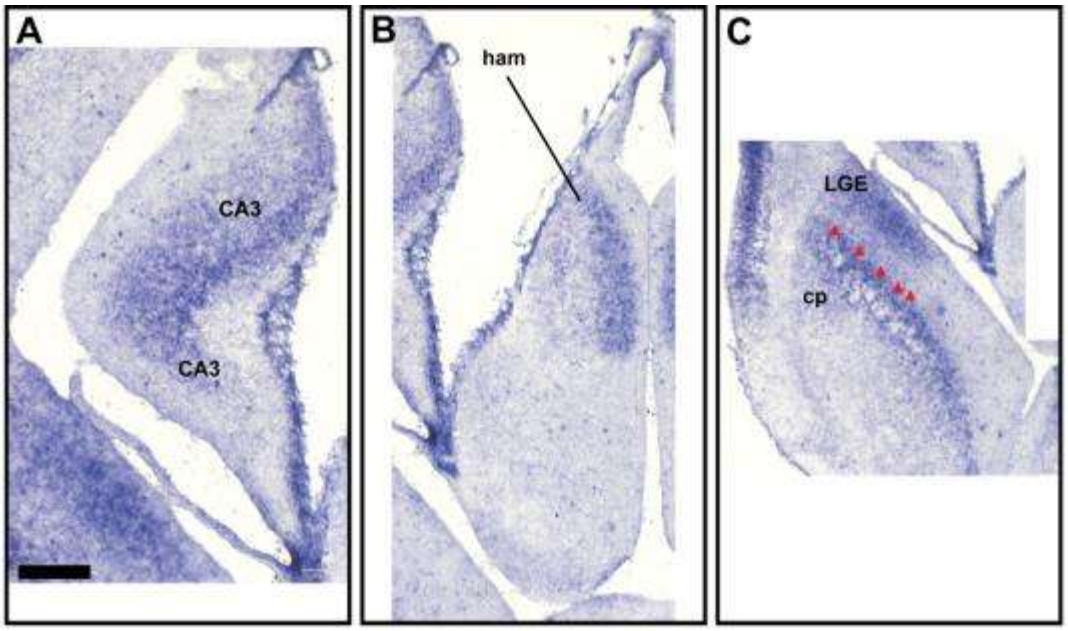


Figure 45. **Higher magnified view of specific regions showing expression of *FLRT2* in coronal section of mouse embryonic brain at E17.5**. (A)Hippocampus showing CA1 and CA3 regions (Figure 44F); (B) Thalamus showing specific expression of *FLRT2*, Figure 44G; (C) Corridor formation by *FLRT2* expression between LGE and cp, Figure 44G (indicated by red arrowheads). Abbreviations: CA, Cornu Ammonis; cp, Caudate putamen; ham, medial habenula; LGE, Lateral ganglionic eminence; LV, Lateral Ventricle; .Scale Bars: A-C, 100 µm.

FLRT3 was detected in areas of the brain which look like complementing *FLRT2* expression. At E13.5, *FLRT3* was observed faintly in neocortex though signal became strong in cingulate cortex region and hippocampal regions (Figure 46A-I). In the basal ganglia at this stage *FLRT3* was labelling very specifically the boundary between the LGE and MGE, especially in the progenitor area (Figure 46F-H; Figure 48D). Expression was also detectable strongly in the thalamus region (Figure 46I).

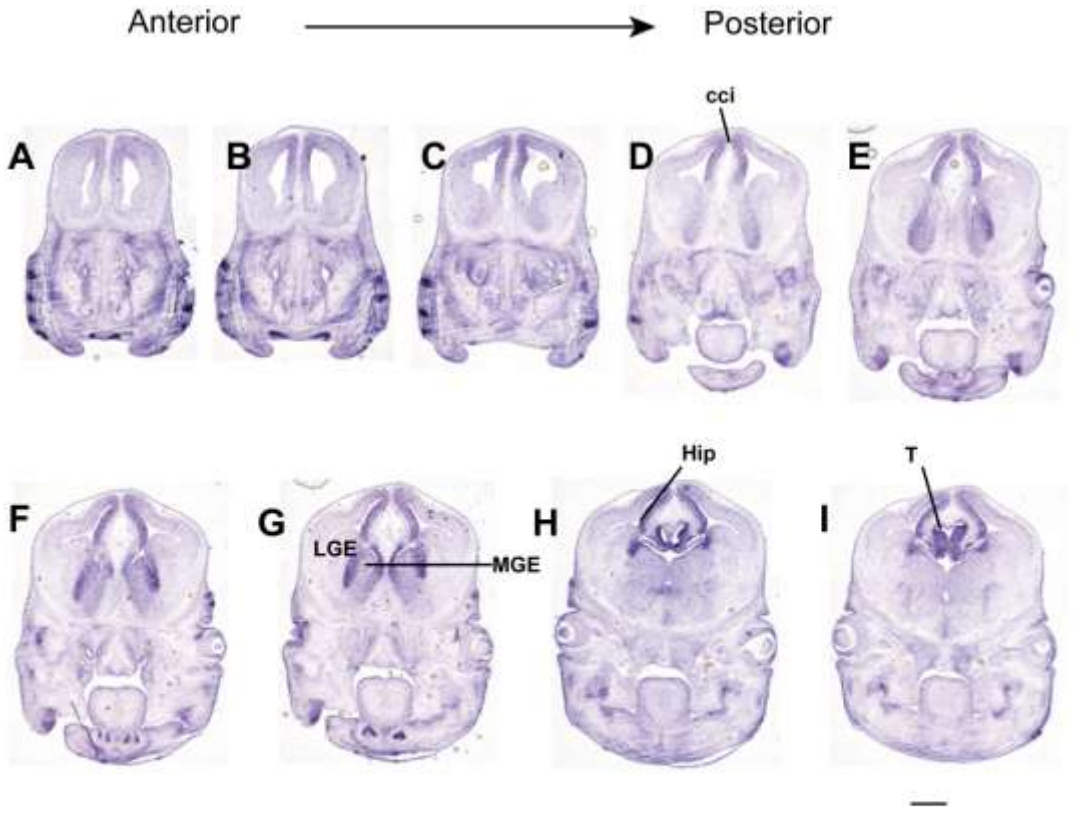


Figure 46. **Expression of *FLRT3* in the developing mouse brain.**
(A-I) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 using a digoxigenin-labelled *FLRT3* antisense probe on coronal brain sections of mouse embryos. Abbreviations: cci, cingulate cortex; T, Thalamus; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; MGE, Medial ganglionic eminence. Scale Bar: A-I, 200 µm.

FLRT3 expression in E15.5 and E17.5 becomes broader (Figure 47 A-F). At these stages *FLRT3* is clearly detected in the neocortex, in the upper layers of the CP and in the IZ, extending towards hippocampus (Figure 47; Figure 48A). In the hippocampus, *FLRT3* expression is especially high in DG, less in CA3 and very little is detected in CA1 (Figure 47E,F; Figure 48B). A strong signal appeared as well in anterior ventricular hypothalamic nucleus, in the differentiating striatum and piriform cortex (Figure 47E). Interestingly, *FLRT3* is also expressed in the VZ of the ET (Figure 47E, F; Figure 48C).

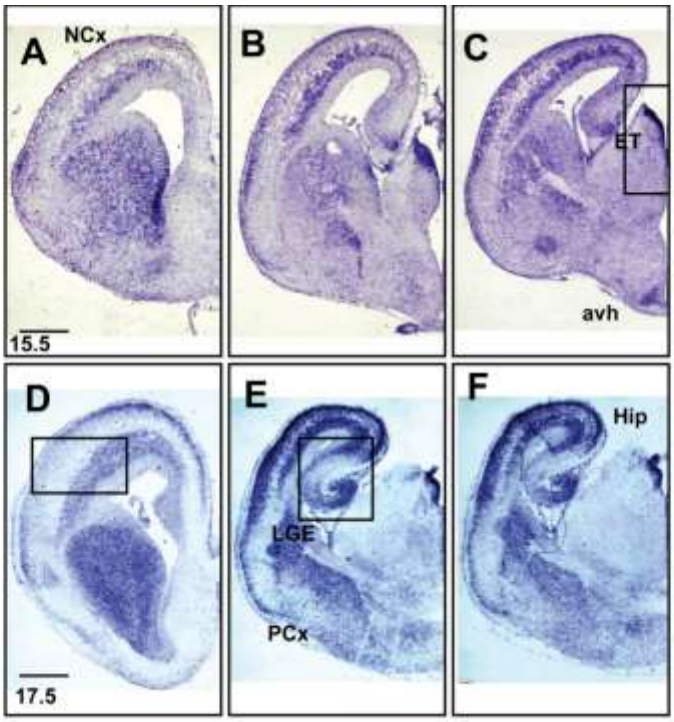


Figure 47. **Expression of *FLRT3* in the developing mouse brain**.
(A-F) In situ hybridization (ISH) analyses of coronal brain sections at E15.5 (A-C), E17.5 (D-F) using a digoxigenin-labelled *FLRT3* antisense probe on coronal brain sections of mouse embryos. A & D, panels show anterior; B shows medial; C, E,F shows posterior sections of the brain, respectively. Abbreviations: avh, anterior ventricular hypothalamic nucleus; ET, Epithalamus; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; NCx, Neocortex; PCx, Piriform Cortex. Scale Bars: A-F 200 μm. Boxed area are shown in higher magnification in Figure 48.

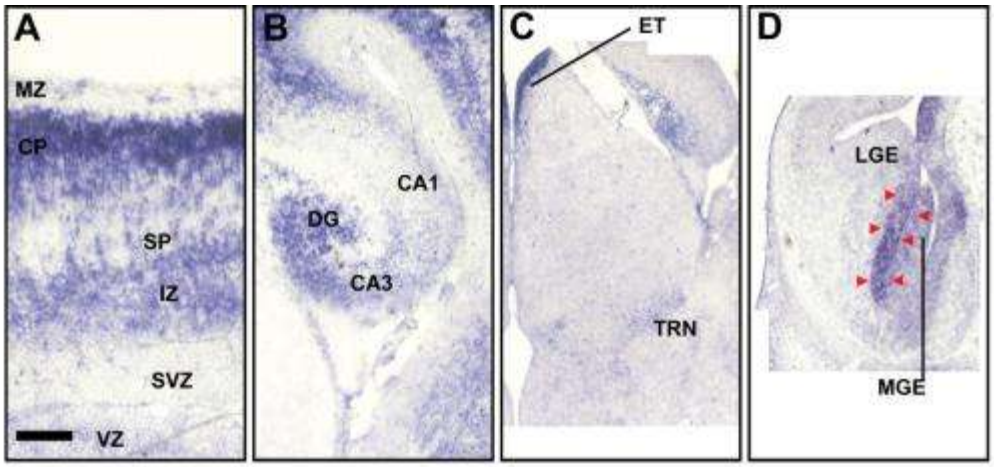


Figure 48. **Higher magnified view of specific regions showing expression of *FLRT3* in coronal section of mouse embryonic brain**. (A) Neocortex area showing expression of *FLRT3* in CP and IZ at E17.5 (Figure 47D);(B) Hippocampus showing DG, CA1 and CA3 region at E17.5 (Figure 47E);(C) Thalamus showing expression of *FLRT3* in VZ of ET and TRN at E15.5 (Figure 47C); (D) Expression of *FLRT3* forming a boundary between LGE and MGE at E13.5, indicated by red arrowheads (Figure 46G). CA, Cornu Ammonis; CP, Cortical plate; DG, Dentate Gyrus; ET, Epithalamus; IZ, Intermediate zone; LGE, Lateral Ganglionic eminence; MGE, Medial ganglionic eminence; MZ, Marginal zone; SP, Subplate; SVZ, Subventricular zone; TRN, Reticular Thalamic nucleus; VZ, Ventricular zone. Scale Bars: A-D, 100 μm.

4.2.2 Characterization of the neurons expressing *FLRT1* and *FLRT3* in the developing mouse brain and human fetal brain.

To analyse further the role of FLRTs in brain development, I studied FLRT members individually with different markers as I did before for ISLR2. I performed double immunofluorescence for FLRT2 and FLRT3 with different markers on mouse (E16.5) and human (15pcw) fetal brain sections. FLRT2 antibodies didn't work with our protocol in human samples and therefore I performed a dual protocol of ISH followed by an IHC on coronal sections comparing mouse and human fetal brain. Though for this dual protocol we used three markers CTIP2, PAX6, SOX2 and TBR2, only CTIP2 showed a consistent staining.While PAX6 worked only with human tissue, SOX2 didn't worked for dual protocol.

Analysis of dual ISH and IHC of transcription factor CTIP2 with FLRT1 in developing mouse and human brain.

I performed dual ISH and IHC on E16.5 mouse brain coronal section. I did ISH for *FLRT1* and then on same sections performed Immunofluorescence with CTIP2, SOX2 and TBR2 but only CTIP2 worked with this protocol. *FLRT1* is expressed in CP and thus colocalizes with CTIP2 (Figure 49B, C, E, F). *FLRT1* is also labelling SVZ and VZ, where CTIP2 is not expressed (Figure 49B, C, E, F). Besides this expression in the cortex, FLRT1 is also In LGE also the expression of CTIP2 and FLRT1 overlap and same regions are labelled by CTIP2 and FLRT1 in thalamus suggesting these neurons to be differentiating neuron (Figure C).

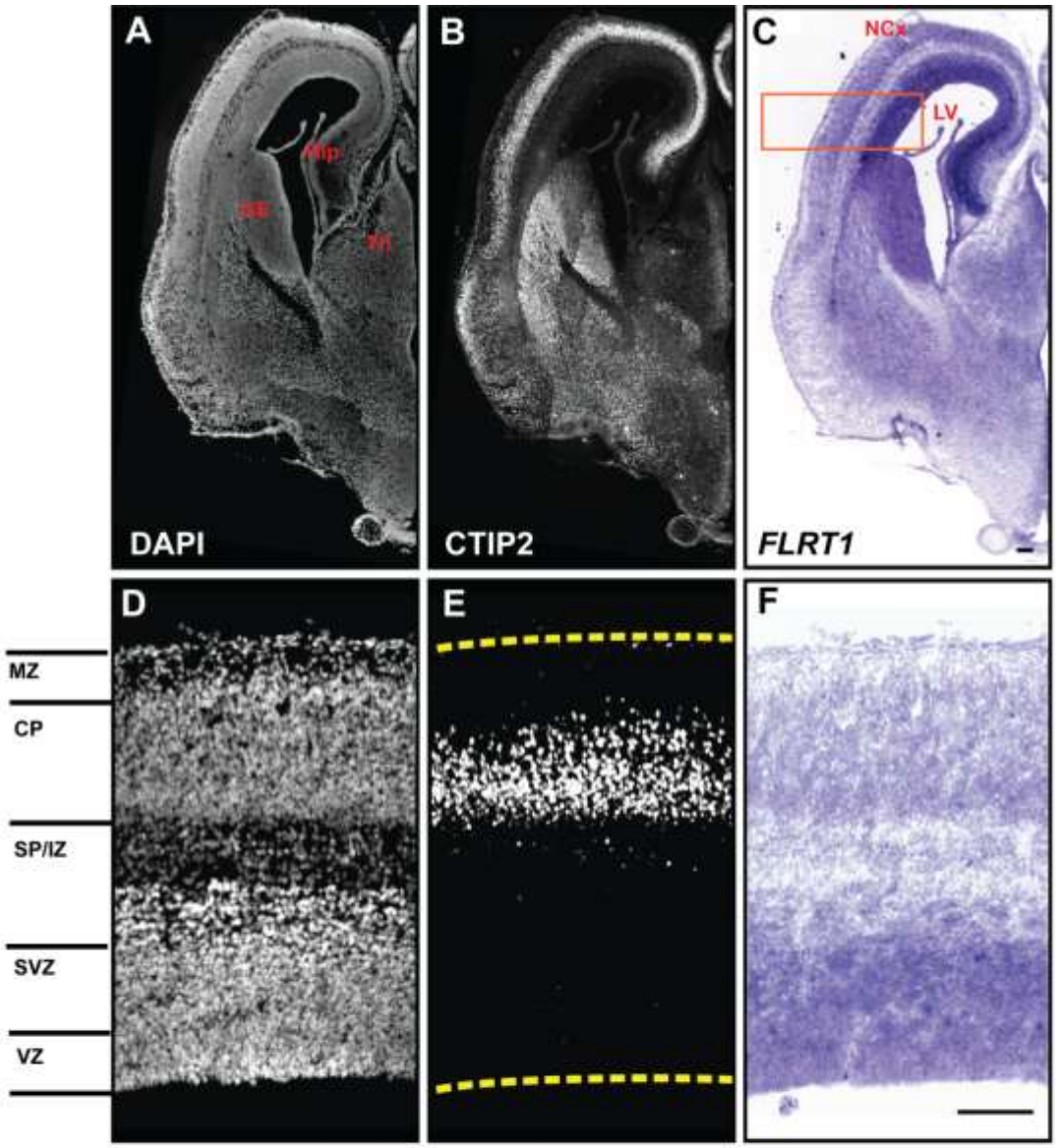


Figure 49. ***FLRT1* and CTIP2 dual ISH & IHC, performed on E16.5 mouse brain sections (A-C)**; Higher magnified view (20X) of neocortex region of C indicated by an orange box (D-F). Abbreviations: CP, Cortical plate; GE, Ganglionic Eminence; Hip, Hippocampus; IZ, Intermediate zone; LV, Lateral ventricle; MZ, Marginal zone; NCx, Neocortex; SP, Sub plate; SVZ, Subventricular zone; Th, Thalamus; VZ, Ventricular zone. Scale Bars: A-F: 100µm.

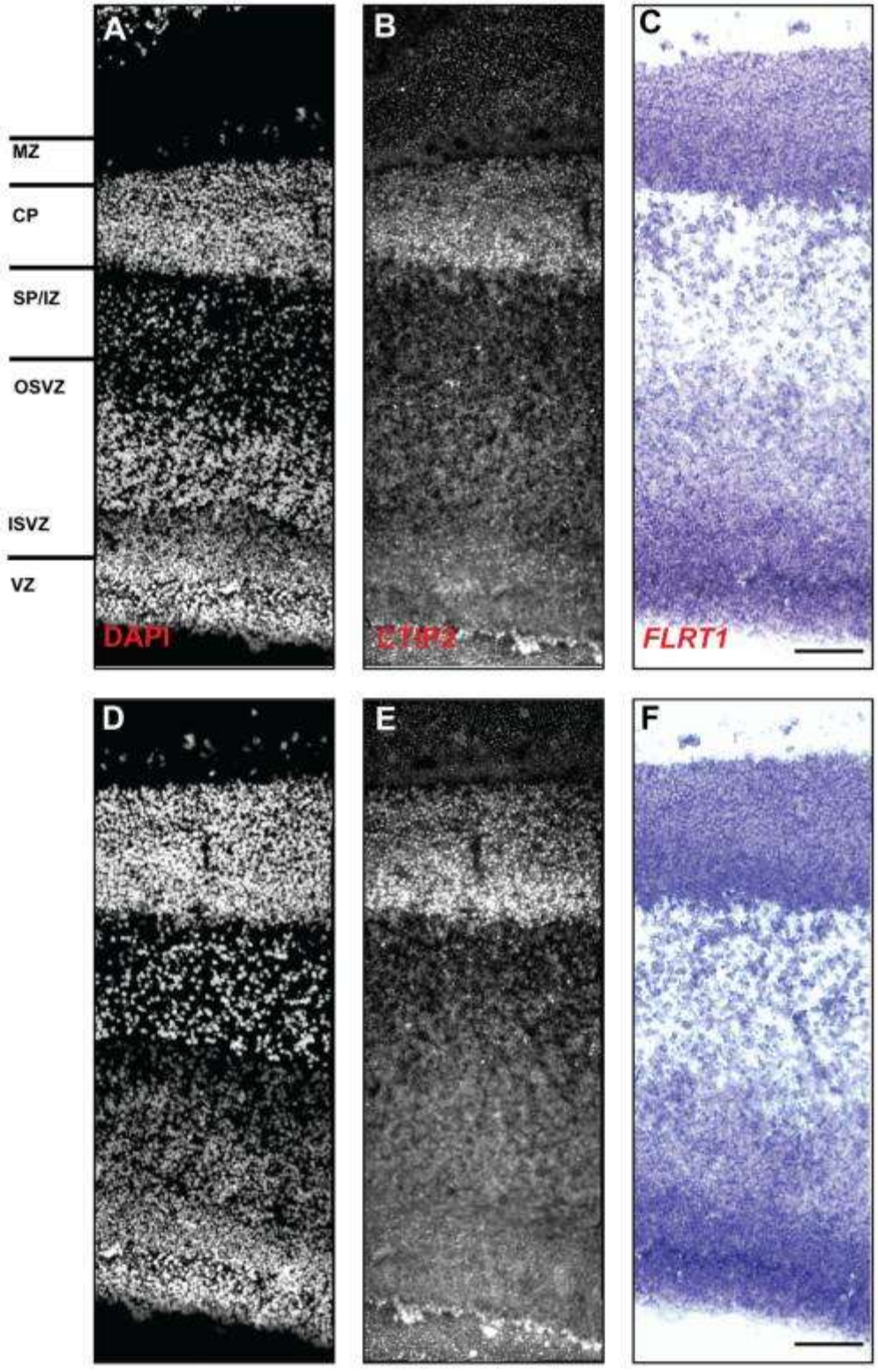


Figure 50 . **FLRT1 and CTIP2 dual ISH & IHC, performed on 15pcw human fetal brain coronal sections of occipital lobe;** Higher magnified view (20X) of two neocortex region (A-C & D-F) . Abbreviations: CP, Cortical plate; IZ, Intermediate zone; ISVZ, Inner subventricular zone; MZ, Marginal zone; OSVZ, Outer subventricular zone; SP, Sub plate; VZ, Ventricular zone. Scale Bars: A-F: 100µm.

In human sections from 15pcw, *FLRT1* is expressed in the CP as well and thus colocalizes with CTIP2 expression similar to mouse (Figure 50). Interestingly, *FLRT1* expression is also found in the VZ, ISVZ and cells in the OSVZ in a kind of a gradient, from ventral-high to dorsal-low (Figure 50). Expression in VZ for FLRT1 is strong which reduces gradually in ISVZ (Figure 50).

Analysis of double Immunofluorescence of FLRT3 with PAX6 and TBR2 in developing mouse and human brain

Next I performed double Immunofluorescence of FLRT3 with TBR2 in developing mouse and human brain sections. TBR2 is a known marker for neurogenic progenitors in the cortical SVZ although some staining can be detected in the basal VZ, in the lower IZ as well as in extracortical regions such as the TRN in the ventral thalamus and in the anterior ventricular hypothalamic nucleus (references and Figure 51B, F). In the mouse, neurons positive for FLRT3 that also express TBR2 were observed in very discrete places, in lower IZ and in hippocampal area (Figure 51D, H, marked with white arrowheads).

In human neocortex expression of TBR2 was observed in ISVZ mainly with some labelling in VZ and OSVZ extended to lower IZ. FLRT3 staining in human samples gave a lot of background (indicate in the figure these dotty background with asterisks for instance can make a comment in figure legend) but one could detect a specific, membrane-like, pattern in cells of the CP and VZ but not in IZ unlike in mouse. No doublepositive cells were visible in IZ of human neocortex.

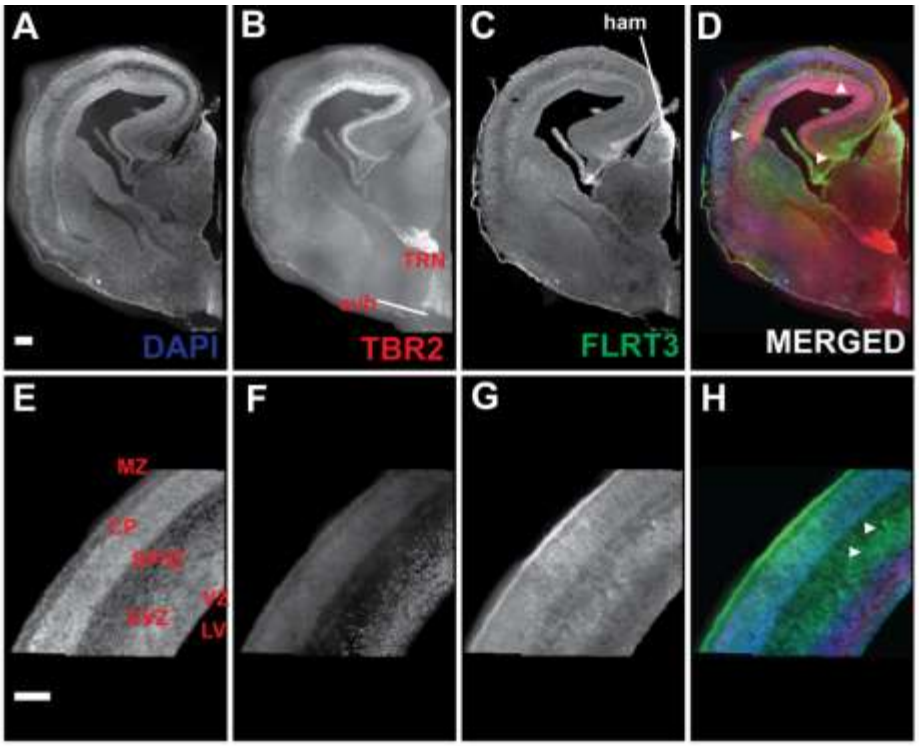


Figure 51. **FLRT3 (green) and TBR2 (red) double immunostaining was performed on E16.5 mouse brain coronal posterior section (A-D)**. Higher Magnified View (20X) of neocortex region showing different layers (E-H). . Abbreviations: avh, anterior ventricular hypothalamic nucleus; CP, Cortical plate; ham, medial habenula; IZ, Intermediate zone; LV, Lateral Ventricle; MZ, Marginal zone; NCx, Neocortex; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular Zone; TRN, Thalamic reticular nucleus. Scale Bars A-H: 100µm. White arrowheads showing very few doublepositive cells for FLRT3 and TBR2.

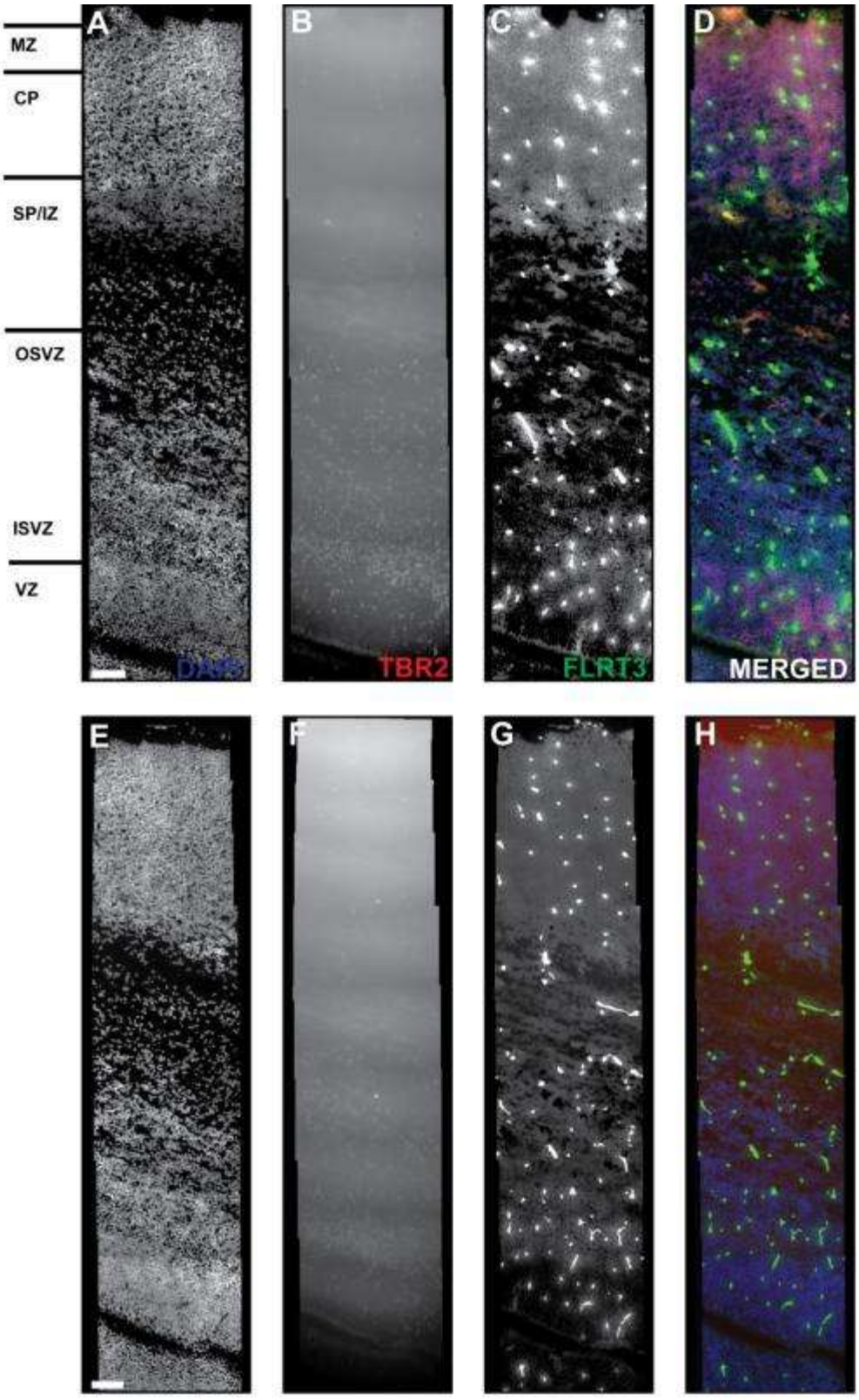


Figure 52. **Double immunofluorescense of FLRT3 (green) and TBR2 (red) performed on 15 pcw human fetal brain coronal section, showing two neocortex region (A-D & E-H).** Higher Magnified View (20X) of neocortex region. No Double-positive neurons have been observed. Abbreviations: CP, Cortical plate; ISVZ, Inner Subventricular zone; IZ, Intermediate zone; MZ, Marginal zone; OSVZ, Outer Subventricular zone; SP, Subplate; VZ, Ventricular Zone. Scale Bars A-H: 100µm.

4.3.1 Expression of AMIGO family in the developing brain

AMIGO family has three members *AMIGO1*, *AMIGO2* and *AMIGO3*. *AMIGO1* expression was difficult to be detected as a concrete staining in the brain throughout development (Figure 53 and 54). In contrast, a strong signal was observed in other structures of the head like in retina and in the palate mucosa at E13.5 with the same probe (Figure 53C, D), suggesting therefore that *AMIGO1* probe is fine and that the gene is very low expressed in the developing brain. Nevertheless it seems as if *AMIGO1* is somehow enriched in the proliferative regions of the pallium and subpallium at E13.5 (Figure 53 C,G) and in the dorsal thalamus at E15.5 (Figure 54B), the globus pallidus and the piriform cortex at E17.5 (Figure 54C).

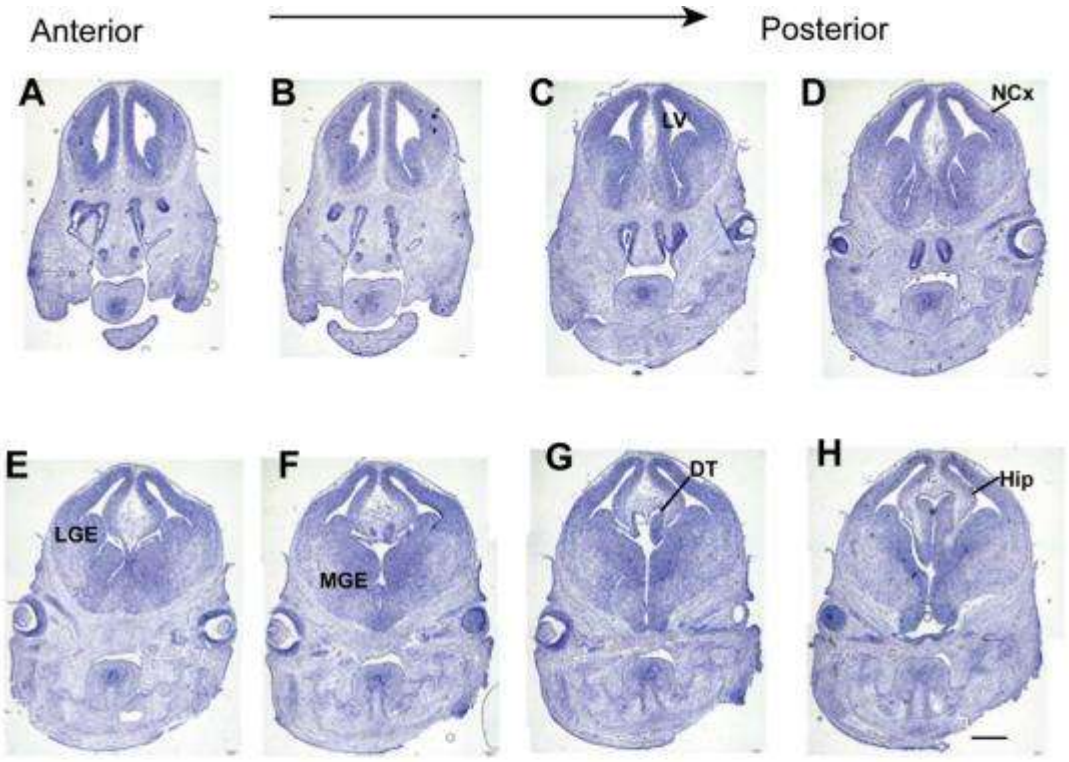


Figure 53. **Expression of *AMIGO1* in the Embryonic stage 13.5 of mouse brain**.(A-H) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 using a digoxigenin-labelled *AMIGO1* antisense probe on coronal head sections of mouse embryos. Abbreviations: DT, Dorsal Thalamus; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; LV, Lateral Ventricle; MGE, Medial Ganglionic eminence; NCx, Neocortex. Scale Bars: A-H 200 µm.

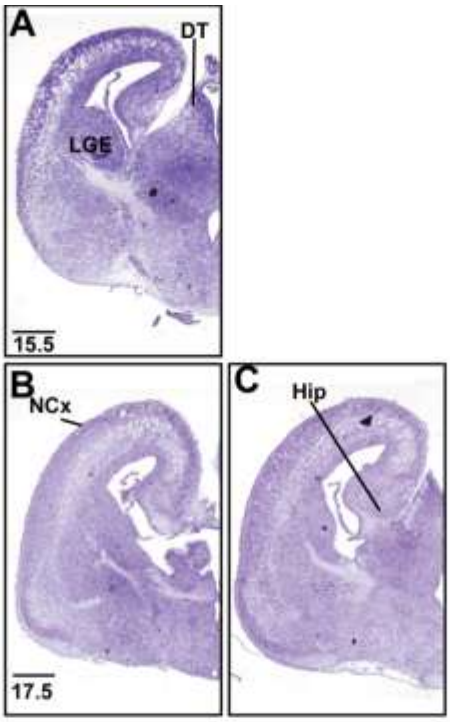


Figure 54 . **Expression of *AMIGO1* in the developing mouse brain.**
(A-C) In situ hybridization (ISH) analyses of coronal brain sections at E15.5 (A), E17.5 (B,C), using a digoxigenin-labelled *AMIGO1* antisense probe on coronal brain sections of mouse embryos. Panel A show posterior section E15.5 stage while B&C shows medial and posterior sections from E17.5. Abbreviations: DT,Dorsal Thalamus; Hip, Hippocampus; NCx, Neocortex.Scale Bars: A-C 200 µm.

AMIGO2 shows a more discrete expression pattern than *AMIGO1*. At E13.5 it shows expression in neocortex but only in the most superficial part, probably the marginal zone (Figure 55A-D). A strong signal was observed in anterior dorsal hypothalamic nucleus which is maintained at least at E15.5 (Figure 55D, H) Later in development, *AMIGO2* was found in the CP in a gradient from ventrolateral-high to dorsomedial-low (Figure 55E,F and I) and in the hippocampus, particularly in the CA1 region. In the basal ganglia a faint but consistent expression was detected in globus palidus (already in E13.5) and caudate putamen (Figure 55C, E, F, I). Finally, a very discrete and strong staining was observed in the dorsal part of the thalamus, in the habenula region (Figure 55F, G). This expression is specific for medial sections of the thalamus and is not

observed in more extreme anterior and posterior sections (complete panels Figure 55E-H).

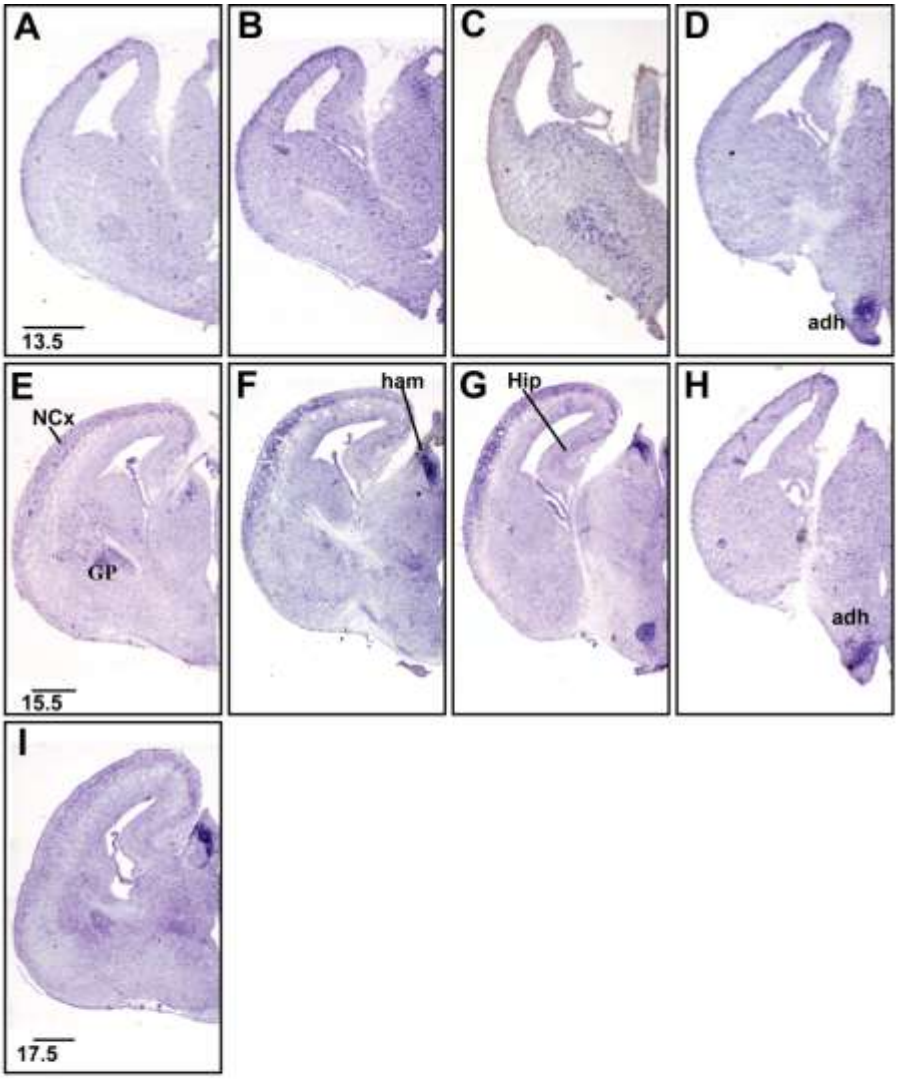


Figure 55 . **Expression of *AMIGO2* in the developing mouse brain.**
(A-I) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-D), E15.5 (E-H), E17.5 (I) using a digoxigenin-labelled *AMIGO2* antisense probe on coronal brain sections of mouse embryos. All the sections here are mostly posterior. Abbreviations: adh, anterior dorsal hypothalamic nucleus; GP, globus pallidus; ham, medial habenula; Hip, Hippocampus; ; NCx, Neocortex. Scale Bars: A-I, 200 μm.

As if for *AMIGO1*, *AMIGO3* also did not show a very clear staining during brain development (Figure 56). Only at E15.5 it was observed a gradient of *AMIGO3* expression in the CP from ventrolateral to dorsomedial (Figure 56E-H). Other areas,

although less clear, seem to express also *AMIGO3* although very faint: LGE (VZ and SVZ) at E15.5 and globus pallidus at E17.5 (Figures 56E, G, K).

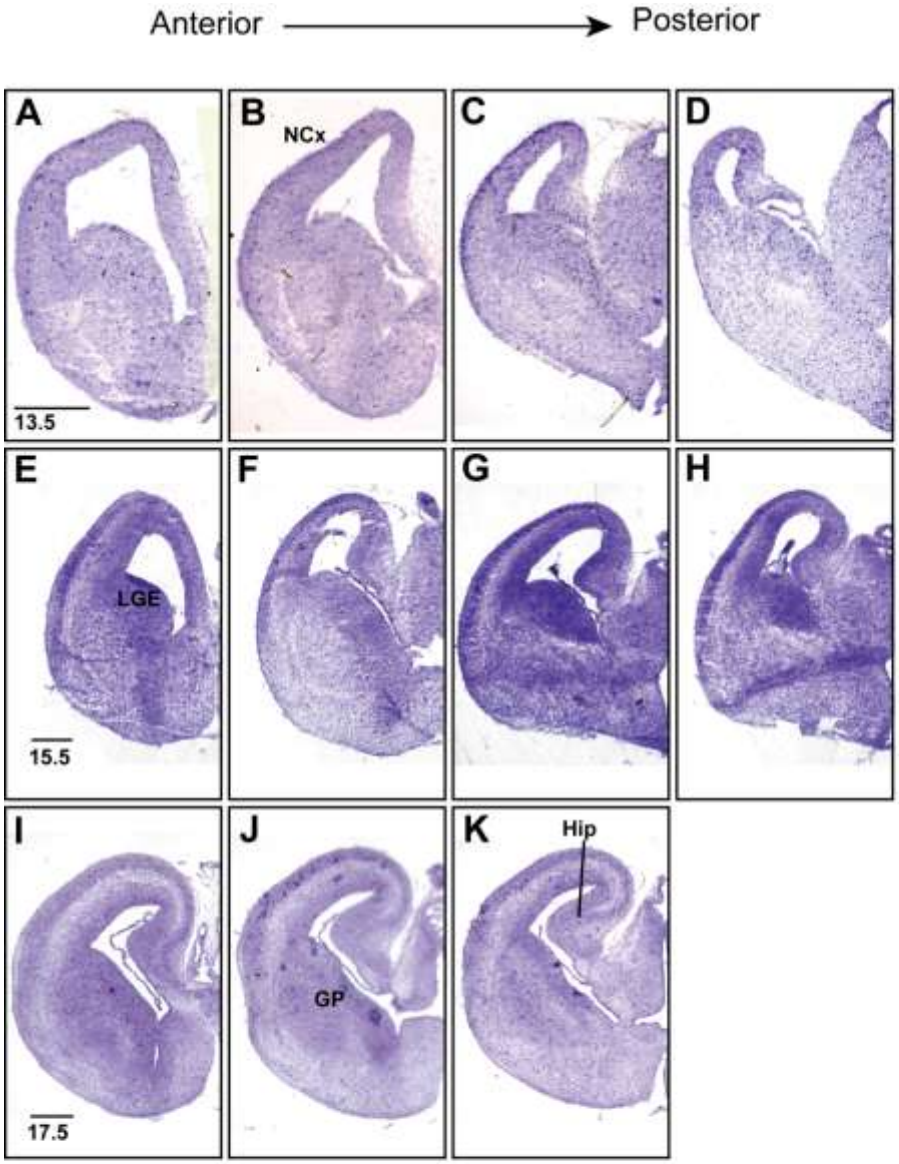


Figure 56 . **Expression of *AMIGO3* in the developing mouse brain.**
(A-K) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-D), E15.5 (E-H), E17.5 (I-K) using a digoxigenin-labelled *AMIGO3* antisense probe on coronal brain sections of mouse embryos. A, E, I panels show anterior; B,F,J shows medial; C,D, G,H,K shows posterior sections of the brain, respectively. Abbreviations: GP, Globus Pallidus; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; NCx, Neocortex. Scale Bars: A-K, 200 μm.

4.4.1 Expression pattern of *SALM* family in developing mouse brain

SALM family of genes is comprised of five members, *SALM1*, *SALM2*, *SALM3*, *SALM4* and *SALM5*. Except for *SALM3*, we detected specific expression patterns for all the *SALM* genes, especially for *SALM5*.

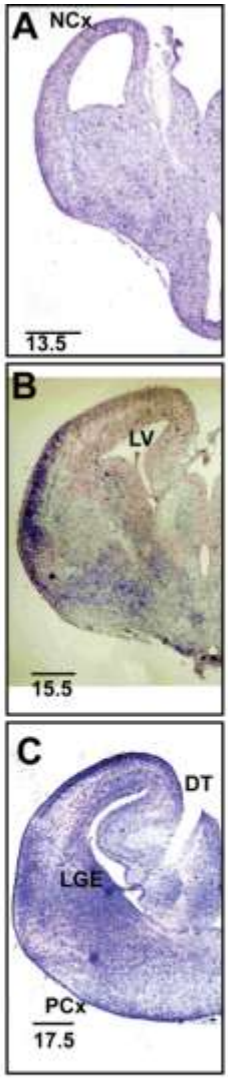


Figure 57. **Expression of *SALM1* in the developing mouse brain.**
(A-C) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A), E15.5 (B), E17.5 (C) using a digoxigenin-labelled *SALM1* antisense probe on coronal brain posterior sections of mouse embryos. Abbreviations: DT, Dorsal Thalamus; LGE, Lateral Ganglionic Eminence; LV, Lateral Ventricle; NCx, Neocortex; PCx, Piriform Cortex. Scale Bars: A-C, 200 μm.

For *SALM1*, expression was not very strong at stage E13.5 nor was very specific (Figure 57 A). However, it increased as development proceeds. At E15.5 for instance, a strong

signal was observed in neocortex (lateral/ventral part) and in piriform cortex (Figure 57B). *SALM1* expressing cells were also observed in the ventral part of the basal ganglia, including the region of the globus pallidus.

At E17.5, the expression was mainly observed in neocortex but more towards the dorsal end unlike in E15.5 (Figure 57C). The expression was restricted to the superficial layers in the CP and a faint line in the SVZ that extends from the LGE in the basal ganglia (Figure 58). Signal also appeared stronger than E15.5 in hippocampus and piriform cortex (Figure 57 C).

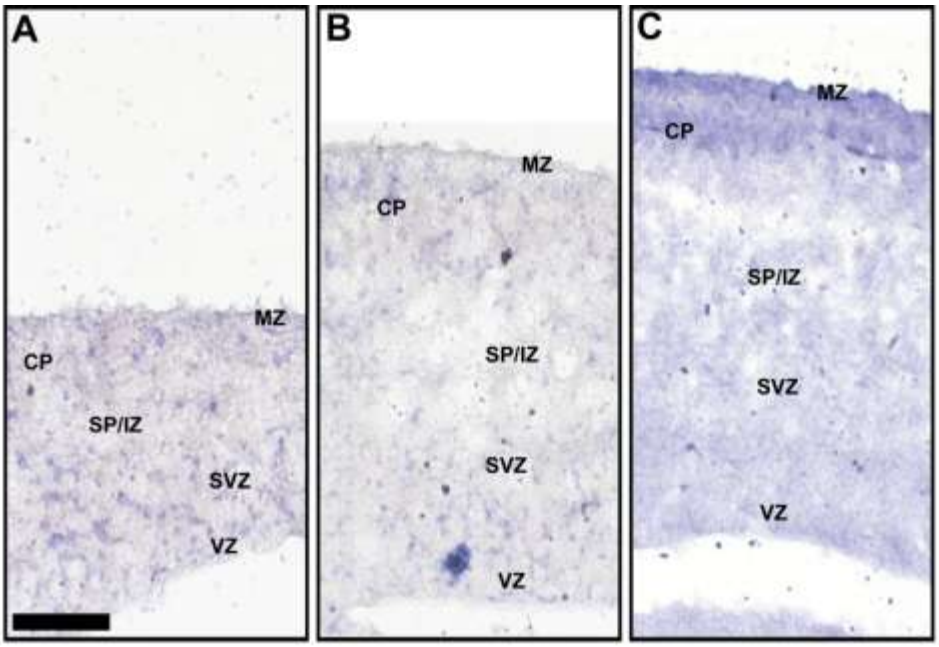


Figure 58. **Higher magnified view of neocortex showing expression of *SALM1* in coronal section of mouse embryonic brain at E13.5** (A), E15.5 (B), E17.5 (C). Abbreviations: CP, Cortical plate; IZ, Intermediate zone; MZ, Marginal zone; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular zone. Scale Bars: A-C, 100 µm.

SALM2 was expressed widely in neocortex at E13.5 stage and expression extended dorsally into the hippocampus area and ventrally into the piriform cortex. Also the thalamus showed a faint but consistent expression at this stage in the proliferative region (Figure 59 A). No drastic expression changes were observed from anterior to posterior at E13.5. Expression at later stages became broader (Figure 59B, C; Figure 60) and *SALM2* was detected in the CP, piriform cortex, hippocampus (CA1 and CA3

region specifically), in the globus pallidus of the basal ganglia and very strongly in thalamus, especially in the ventral part (Figure 59C, D; Figure 60).

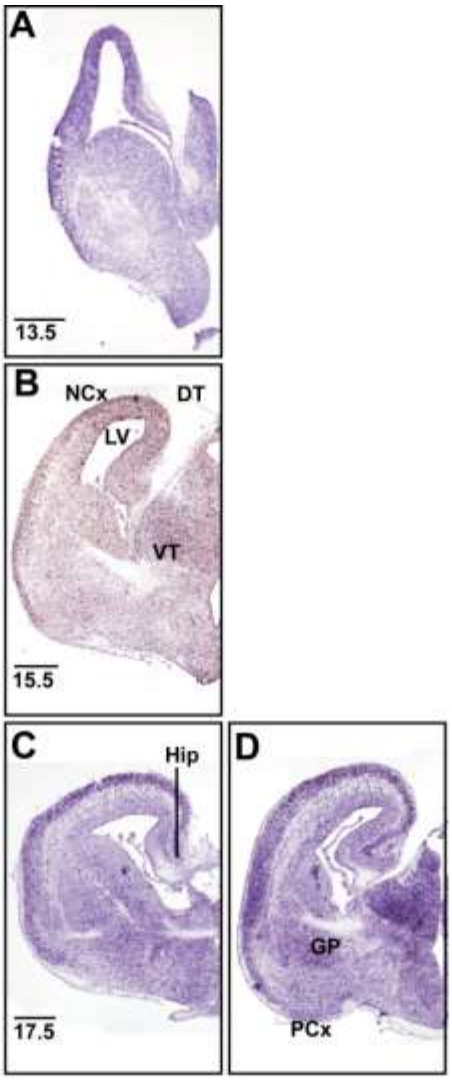


Figure 59. **Expression of *SALM2* in the developing mouse brain.**
(A-D) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A), E15.5 (B), E17.5 (C &D) using a digoxigenin-labelled *SALM2* antisense probe on coronal brain posterior sections of mouse embryos. Abbreviations: DT,Dorsal Thalamus; GP, Globus Pallidus; Hip, Hippocampus; LV, Lateral Ventricle; NCx, Neocortex; PCx, Piriform Cortex; VT, Ventral Thalamus.Scale Bars: A-D, 200 μm.

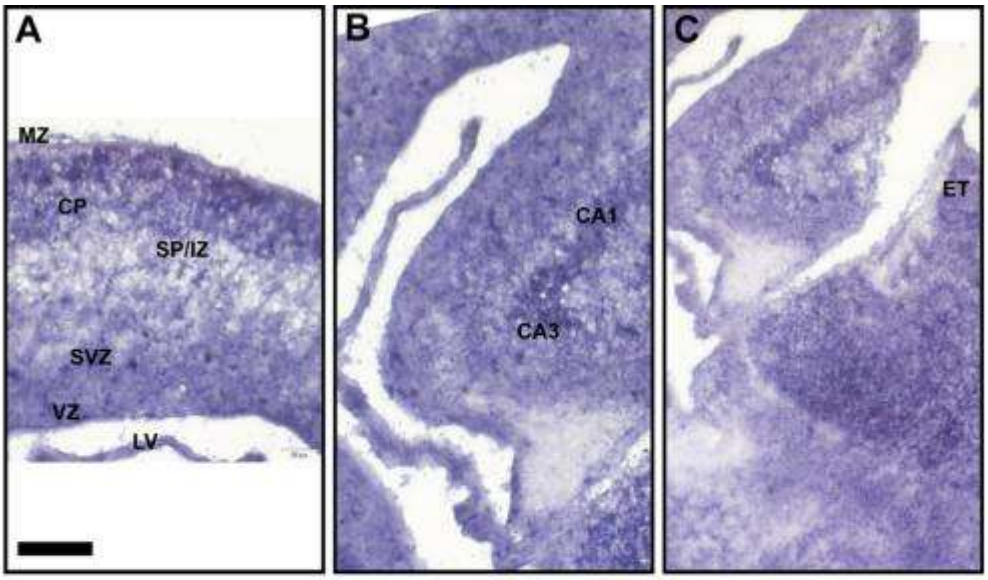


Figure 60. **Higher magnified view of specific regions showing expression of *SALM2* in coronal section of mouse embryonic brain .** (A) Neocortex area showing expression of *SALM2* in CP at E17.5 (Figure 60D); (B) Hippocampus showing CA1 and CA3 region at E17.5; (C) Thalamus showing expression of *SALM2*. Abbreviations: CA, Cornu Ammonis; CP, Cortical plate; ET, Epithalamus; IZ, Intermediate zone; MZ, Marginal zone; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular zone .Scale Bars: A-C, 100 µm.

SALM4 expression was also quiet similar to *SALM2* and also increase during development. At E13.5 expression was observed in cortical plate region which faintly extended towards hippocampus and thalamus (Figure 61 A, B). At E17.5 stage (Figure 61 C-F), expression was observed in the superficial layers of the CP but only in the medial part of the neocortex (Figure 61 C-F; Figure 62A). A faint but specific staining was detected as well in the ventricular zones of the neocortex and the LGE, in the hippocampus, PCx, VT, GP and caudate putamen (Figure 61; Figure 62).

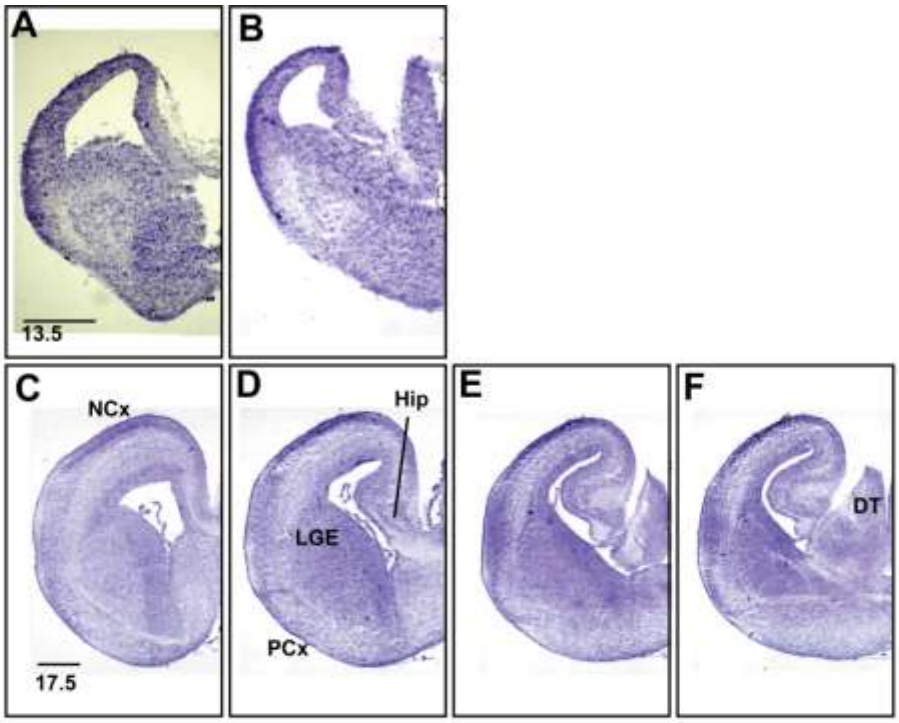


Figure 61. **Expression of *SALM4* in the developing mouse brain**.
(A-F) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-B), E17.5 (C-F) using a digoxigenin-labelled *SALM4* antisense probe on coronal brain sections of mouse embryos. A, C, D panels show anterior; B, E, F shows posterior sections of the brain, respectively. Abbreviations: DT,Dorsal Thalamus; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; NCx, Neocortex; PCx, Piriform Cortex. Scale Bars: A-F 200 μm.

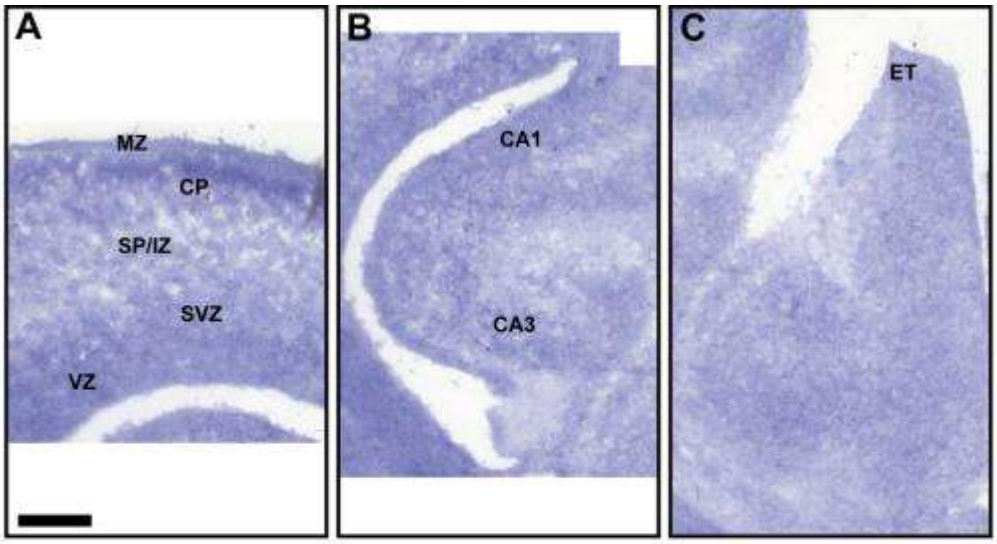


Figure 62. **Higher magnified view of specific regions showing expression of *SALM4* in coronal section of mouse embryonic brain.** (A) Neocortex area showing expression of *SALM4* in CP at E17.5 ; (B) Hippocampus showing CA1 and CA3 region at E17.5; (C) Thalamus showing expression of *SALM4*. Abbreviations: CA, Cornu Ammonis; CP, Cortical plate; ET, Epithalamus; IZ, Intermediate zone; MZ, Marginal zone; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular zone .Scale Bars: A-C, 100 μm.

SALM5 expression was very strong and specific in brain unlike other members of same family. Its expression increased and became wider during development (Figure 63). At E13.5 *SALM5* expression was observed in CP only extending into the hippocampal region and the piriform cortex (Figure 63A, B; Figure 64A).

At E15.5 stage the overall expression of SALM5 increased in the brain (Figure 63C-E). *SALM5* was still observed in the CP, the hippocampus and the piriform cortex but was also detected in the basal ganglia (GP), in the anterior amygdaloid area and in the ventral and dorsal thalamus. In the thalamus, the expression of SALM5 is not homogeneous and highlights some specific nuclei (Figure 63E).

At E17.5 (Figure 63F-I), the expression of SALM5 extended throughout the CP, in a gradient from superficial-high to deep-low (Figure 63 F-I; Figure 63C). Unlike other *SALM* family members SALM5 was not expressed in ventricular zone. Piriform cortex and anterior amygdaloid area showed strong signals for *SALM5* at this stage as in hippocampus (CA1 and CA3) and thalamus (Figure 63F-I; Figure 64D, E).

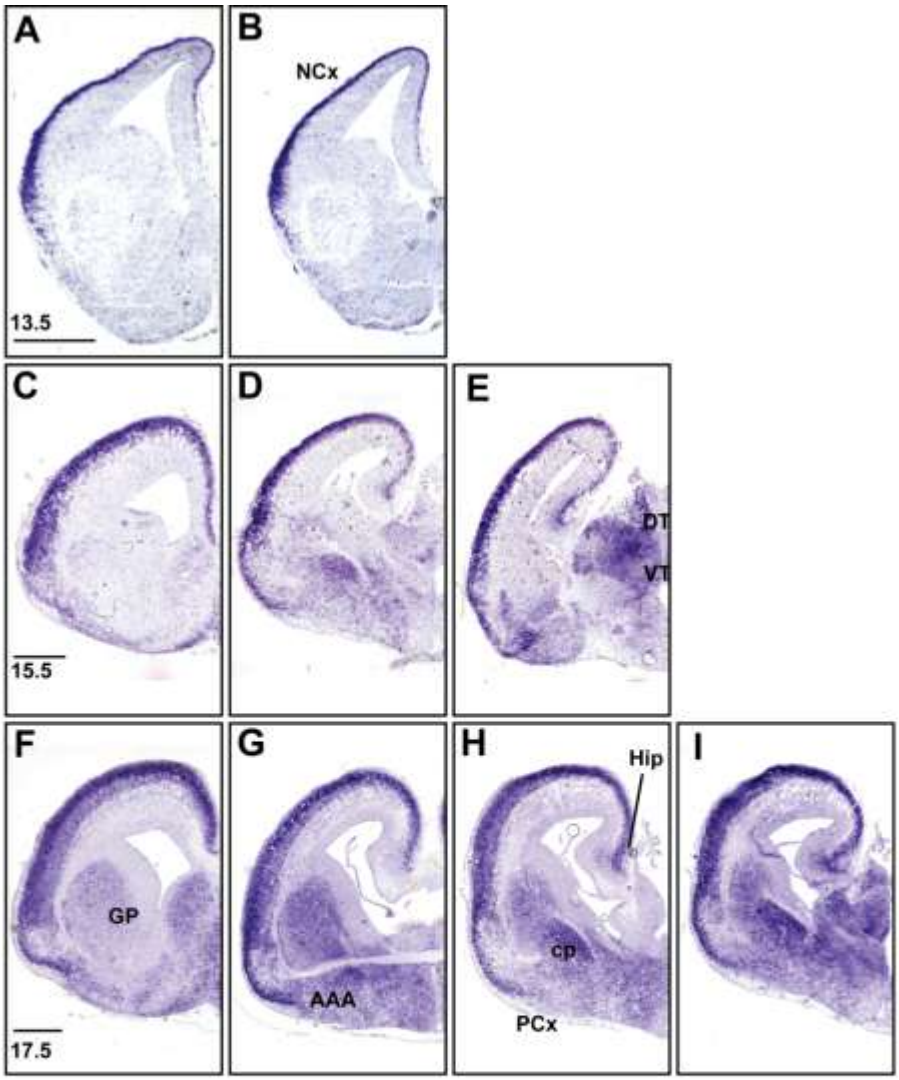


Figure 63. **Expression of *SALM5* in the developing mouse brain.**
(A-I) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-B), E15.5 (C-E), E17.5 (F-I) using a digoxigenin-labelled *SALM5* antisense probe on coronal brain sections of mouse embryos. A, C,F panels show anterior; B, D, G shows medial; E, H, I shows posterior sections of the brain, respectively. Abbreviations: AAA, Anterior amygdaloid area; cp, Caudate putamen; DT, Dorsal Thalamus; GP, Globus Pallidus; Hip, Hippocampus; NCx, Neocortex; PCx, Piriform Cortex; VT, Ventral Thalamus. Scale Bars: A-I, 200 μm.

.

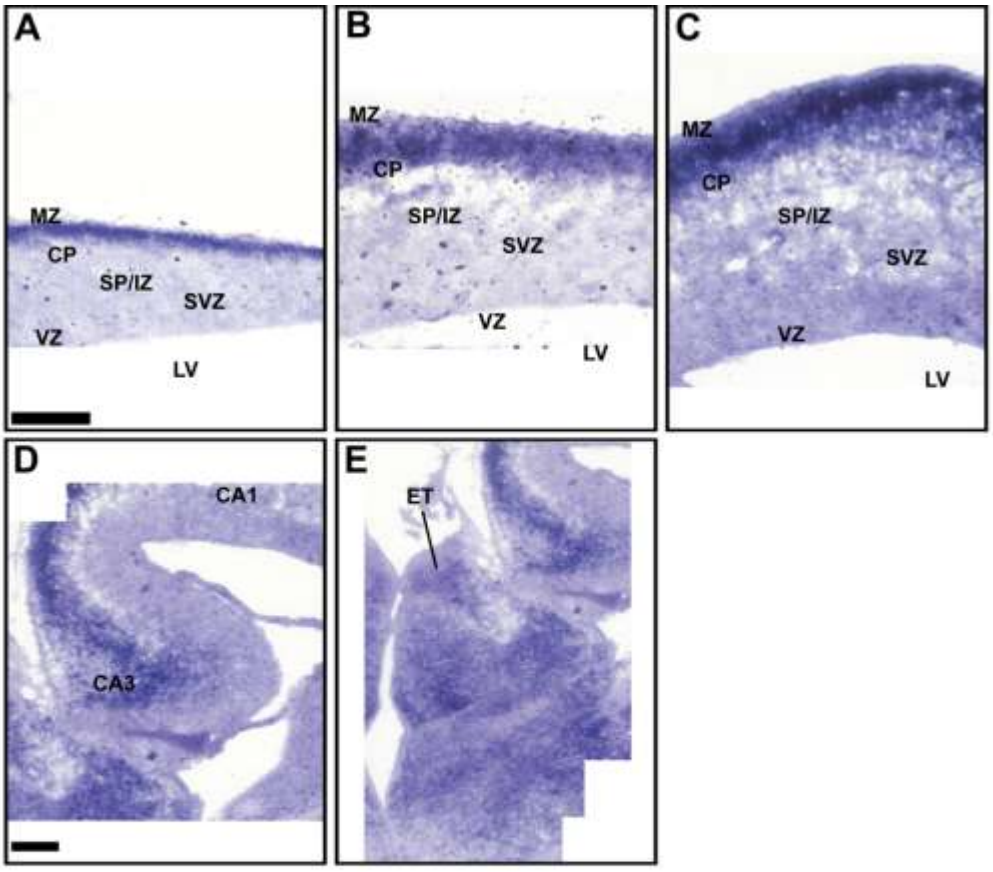


Figure 64. **Higher magnified view of specific regions showing expression of *SALM5* in coronal section of mouse embryonic brain**. (A,B,C) Neocortex area showing expression of *SALM5* in CP at E13.5, E15.5 and E17.5 respectively ; (D) Hippocampus showing CA1 and CA3 region at E17.5; (E) Thalamus showing expression of *SALM5*. Abbreviations: CA, Cornu Ammonis; CP, Cortical plate; ET, Epithalamus; IZ, Intermediate zone; MZ, Marginal zone; SP, Subplate; SVZ, Subventricular zone; VZ, Ventricular zone .Scale Bars: A-E, 100 µm.

4.5.1 Expression of NLRR family in the developing mouse brain

NLRR family consists of three members, *NLRR1*, *NLRR2* and *NLRR3*. *NLRR1* at E13.5 gave a particularly interesting expression pattern with the higher expression specifically in the hippocampal region (Figure 65A-C). Other areas of a fainter staining at this stage included the SVZ/VZ of the basal ganglia and the thalamus and the lateral/ventral CP extending into the piriform cortex (Figure 65 A-C).

At E15.5 expression in cortical plate became stronger, extending into the piriform cortex but not into the cingulated cortex. A faint staining is maintained in the VZ/SVZ of the basal ganglia and thalamus which is now observed also in the SV/SVZ of the

neocortex (Figure 65D-H). Interestingly, the strong expression of *NLRR1* in the hippocampal region has dissapeared at this stage (and later at E17.5) (Figure 65 D-H).

A gradient from E13.5 to E17.5 was observed with a decrease in expression of *NLRR1* with development. Expression in piriform cortex disappeared at E17.5 completely and also the intensity of *NLRR1* expressing cells decreased drastically. The expression around lateral ventricle in the VZ/SVZ also dissapeared at E17.5 stage of development. At E17.5, expression of *NLRR1* was reliably observed only in the upper layers of the cortical plate, specifically in dorsal part of the neocortex (Figure 65I-L). In posterior sections a faint signal was observed in hippocampus (Figure 65L).

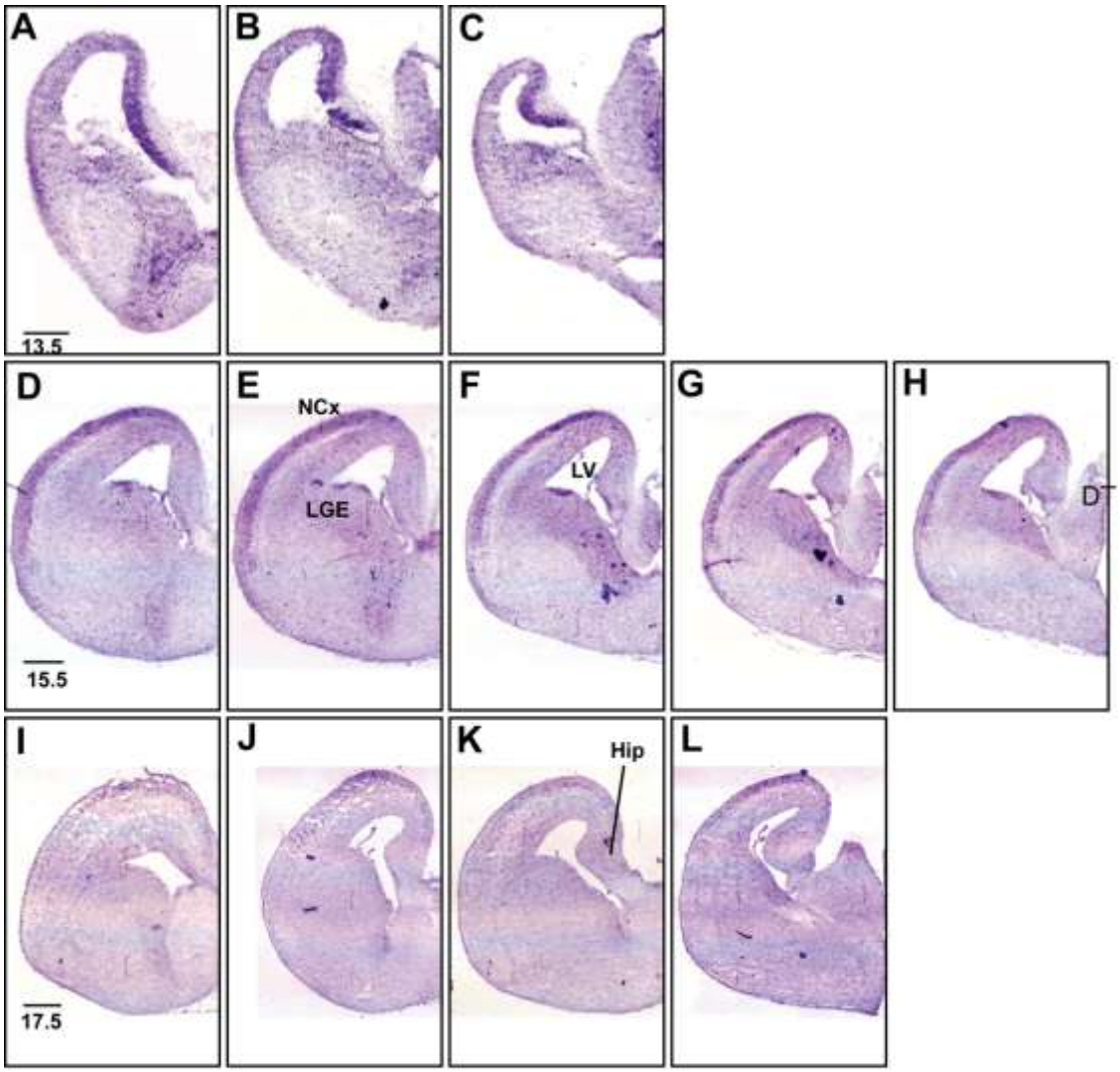


Figure 65. **Expression of *NLRR1* in the developing mouse brain.**
(A-L) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-C), E15.5 (D-H), E17.5 (I-L) using a digoxigenin-labelled *NLRR1* antisense probe on coronal brain sections of mouse embryos. A, D, E, I, J panels show anterior; B,F, K shows medial; C, G, H, L shows posterior sections of the brain, respectively. Abbreviations: DT, Dorsal Thalamus; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; LV, Lateral Ventricle; NCx, Neocortex. Scale Bars: A-L, 200 μm.

NLRR2 at E13.5 stage was mainly expressed in cortex extending into the hippocampal region and the piriform cortex (Figure 66A-E) and in the thalamus. NLRR2 expressing cells were also observed in anterior dorsal hypothalamic nucleus and in anterior ventral hypothalamic nucleus (Figure 66A-E).

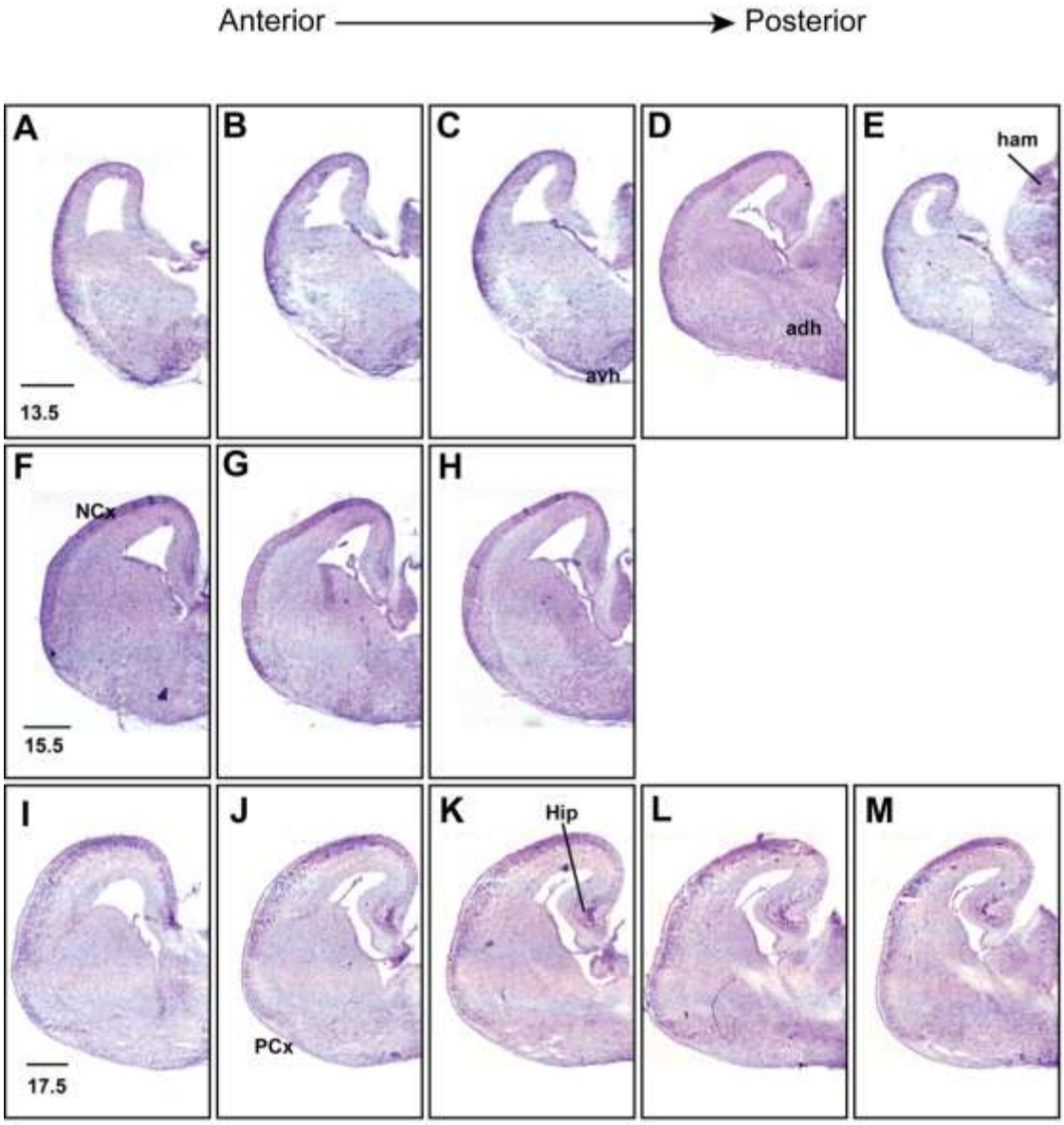


Figure 66. **Expression of *NLRR2* in the developing mouse brain.**
(A-M) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-E), E15.5 (F-H), E17.5 (I-M) using a digoxigenin-labelled *NLRR2* antisense probe on coronal brain sections of mouse embryos. A, F, I panels show anterior; B, G, J shows medial C,D,E,H,K,L,M shows posterior sections of the brain, respectively. Abbreviations: adh, anterior dorsal hypothalamic nucleus; avh, anterior ventral hypothalamic nucleus; ham, medial habenula; Hip, Hippocampus; NCx, Neocortex; PCx, Piriform Cortex. Scale Bars: A-M, 200 μm.

At E15.5 *NLRR2* expression was observed in cortical plate and also weak signal in piriform cortex; expression was also observed in ventricular zone of the thalamus (Figure 66F-H). At E17.5 the NLRR2 expression was stronger and was observed clearly in cortical plate (with higher expression in the dorsal part of the cortex) and piriform

cortex (Figure 66I-M). A fainter staining was observed in the thalamus, in medial habenula and some regions of the basal ganglia and hypothalamus mainly in anterior dorsal hypothalamic nuclei and anterior ventricular hypothalamic nuclei (Figure 66L-M). An interesting strong expression was observed in hippocampus, in particular in the CA3 region (Figure 66 L).

NLRR3 at E13.5 is expressed faintly in cortex and in thalamus but displayed a strong signal in ventral lateral geniculate nucleus (Figure 67A, B). At later stages *NLRR3* expression increases. At E15.5 the expression of *NLRR3* was observed in the cortex, in a gradient from ventral-high (including the piriform cortex) to dorsal-low without labelling neither the cingulated cortex nor the hippocampal region (Figure 67C). A faint stream of *NLRR3* expression was observed as well in the ventricular zone of the cortex (Figure 67C). *NLRR3* expression was also detected very strongly and in the reticular thalamic nuclei and in medial habenula and ventral thalamus. At E17.5, NLRR3 expression was still observed in CP (especially in the upper layers) exteding into the cingulated cortex and piriform cortex and including the septum and the anterior most part of the caudate putamen (Figure 67D-G). The neocortical-ventricular expression of *NLRR3* was more evident at this stage (Figure 67D-G). Detectable expression was found as well in the hippocampus (CA1 and CA3 regions) and strong staining remained in the thalamus (ventral part specially) at this stage.

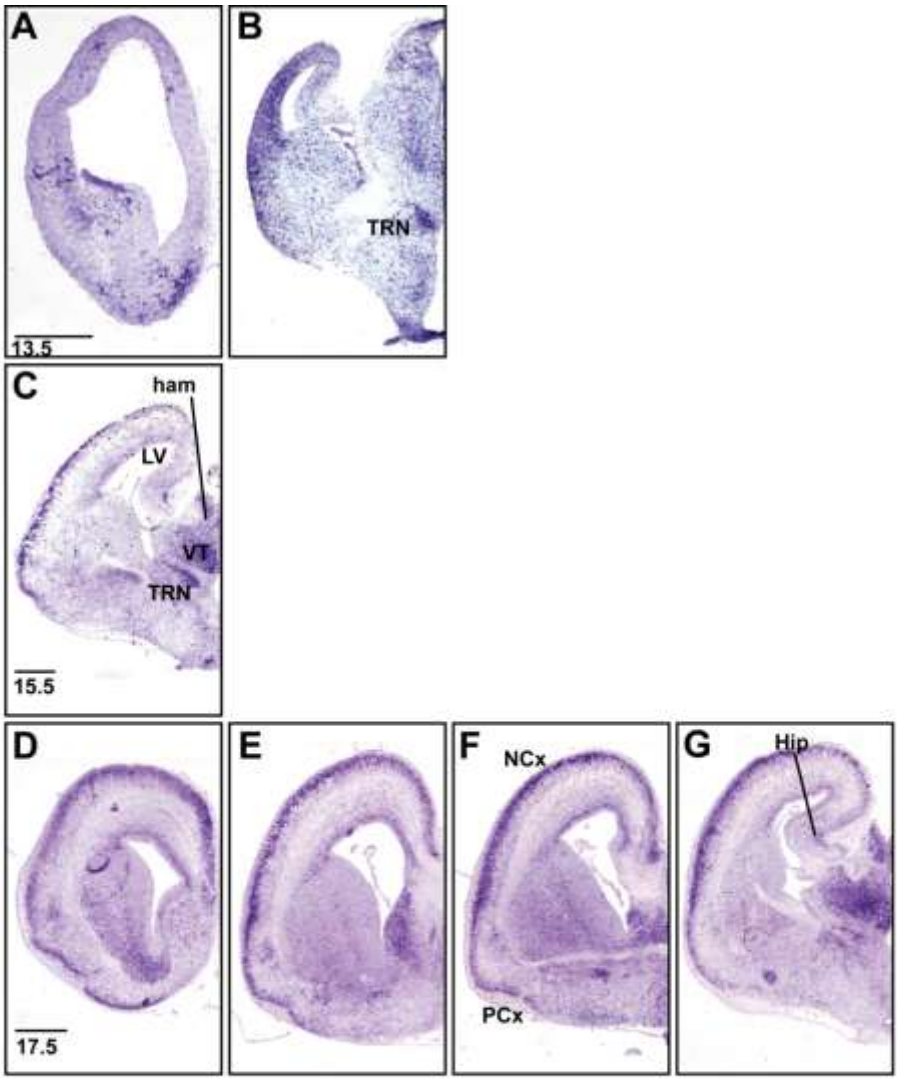


Figure 67. **Expression of *NLRR3* in the developing mouse brain.**
(A-G) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-B), E15.5 (C), E17.5 (D-G) using a digoxigenin-labelled *NLRR3* antisense probe on coronal brain sections of mouse embryos. A & D, panels show anterior; E, F shows medial; B, C, G shows posterior sections of the brain, respectively. Abbreviations: ham, medial habenula; Hip, Hippocampus; NCx, Neocortex; PCx, Piriform Cortex; TRN, Reticular thalamic nuclei; VT, Ventral Thalamus. Scale Bars: A-G, 200 μm.

NLRR3 at E13.5 is expressed faintly in cortex and in thalamus but displayed a strong signal in reticular thalamic nuclei nucleus (Figure 67 A, B). At later stages, *NLRR3* expression increases. At E15.5 the expression of *NLRR3* was observed in the cortex, in a gradient from ventral-high (including the piriform cortex) to dorsal-low without labelling the cingulated cortex and the hippocampal region (Figure 67C). A faint stream

of *NLRR3* expression was observed as well in the ventricular zone of the cortex (Figure 67C). *NLRR3* expression was also detected very strongly and in the reticular thalamic nuclei and VT at E17.5. At E17.5, NLRR3 expression was still observed in CP (especially in the upper layers) exteding into the cingulated cortex, in piriform cortex, in septum and in the anteror most part of the caudate putamen (Figure 67D-G). The neocortical-ventricular expression of *NLRR3* was more evident at this stage (Figure 67D-G). Detectable expression was found as well in the hippocampus and strong staining remained in the thalamus (ventral part specially) at this stage.

4.6.1 Expression of LRIG family in the developing mouse and human brain

LRIG family have three members; *LRIG1*, *LRIG2* and *LRIG3*. *LRIG1* had a very distinct expression pattern highlighting mainly proliferative regions. At E13.5 for instance it was heavily expressed in the ventricular zone of the cortex, hippocampus and third ventricle (Figure 68 A, B), while no expression (at least, undetectable under our ISH approach) was observed in any other region of brain. Expression was also observed in ventricular zone of septal area in anterior sections.

At E15.5 stage of development the ventricular expression pattern of *LRIG1* was still persistent in the cortex, septal area and specially the hippocampus (Figure 68C-F).Staining include as well the dorsal thalamus (Figure 68F). At E15.5 also the signal around third ventricle was maintained. Interestingly, a faint but consistent expression of *LRIG1* was also detected at this developmental stage in the cortical plate (Figure 68C-F).

At E17.5 the expression for *LRIG1* decreased drastically but remained restricted to thin layers around lateral ventricular zone of cortex, hippocampus and third ventricle, thalamus and hypothalamus (Figure 68G). Expression in cortical plate was not longer detectable at this stage and completely disappeared.

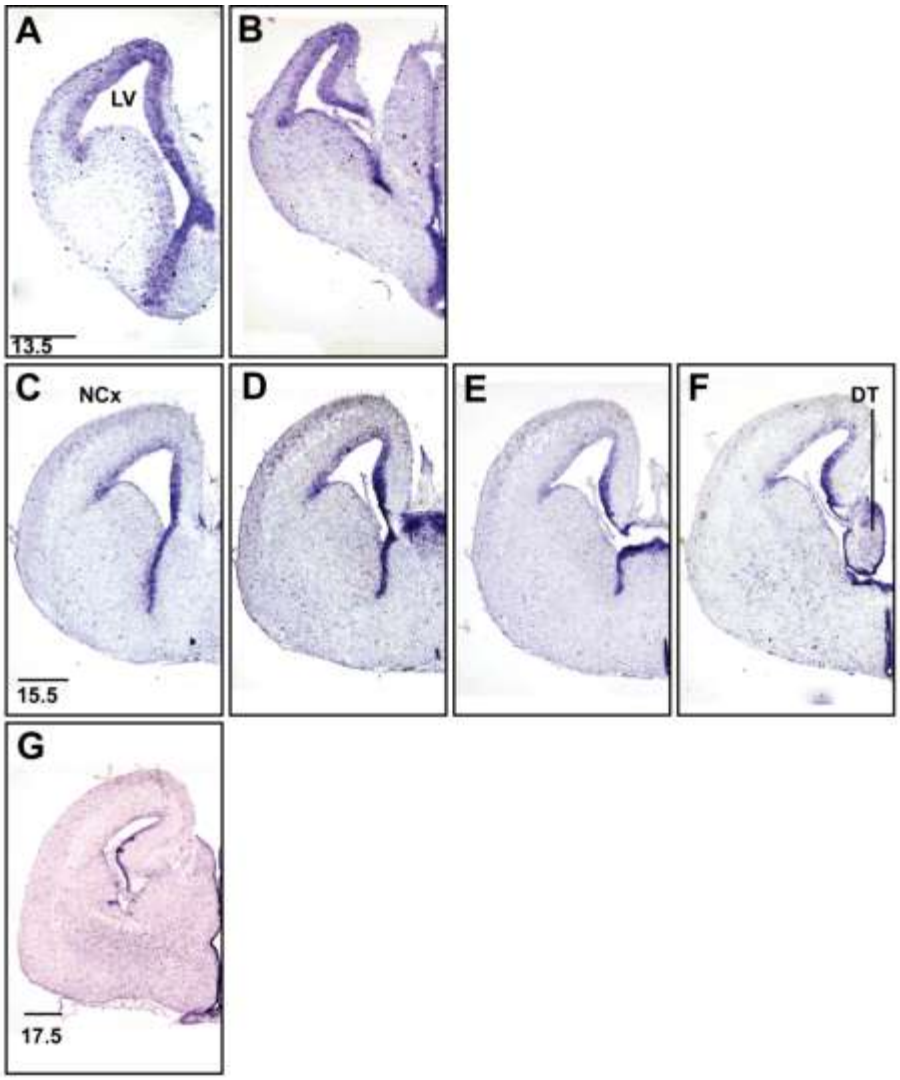


Figure 68. **Expression of *LRIG1* in the developing mouse brain**.
(A-G) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-B), E15.5 (C-F), E17.5 (G) using a digoxigenin-labelled *LRIG1* antisense probe on coronal brain sections of mouse embryos. A & C panels show anterior; D & E shows medial; B, F, G shows posterior sections of the brain, respectively. Abbreviations: DT, Dorsal Thalamus; LV, Lateral Ventricle; NCx, Neocortex. Scale Bars: A-G, 200 μm.

LRIG2 expression was mainly detected in cortical plate at E13.5 and E15.5 stages (Figure 69 A-I). At E17.5 stage the expression level of *LRIG2* became a bit broader and was detected in cortical plate, the hippocampus (stronger in CA1 region) and ventral and dorsal thalamus (Figure 69 J-N). At this stage *LRIG2* expression was also observed in ventricular zone surrounding lateral ventricle in cortex and ganglionic eminences and in the basal ganglia, in caudate putamen and globus pallidus (Figure 69J-N).

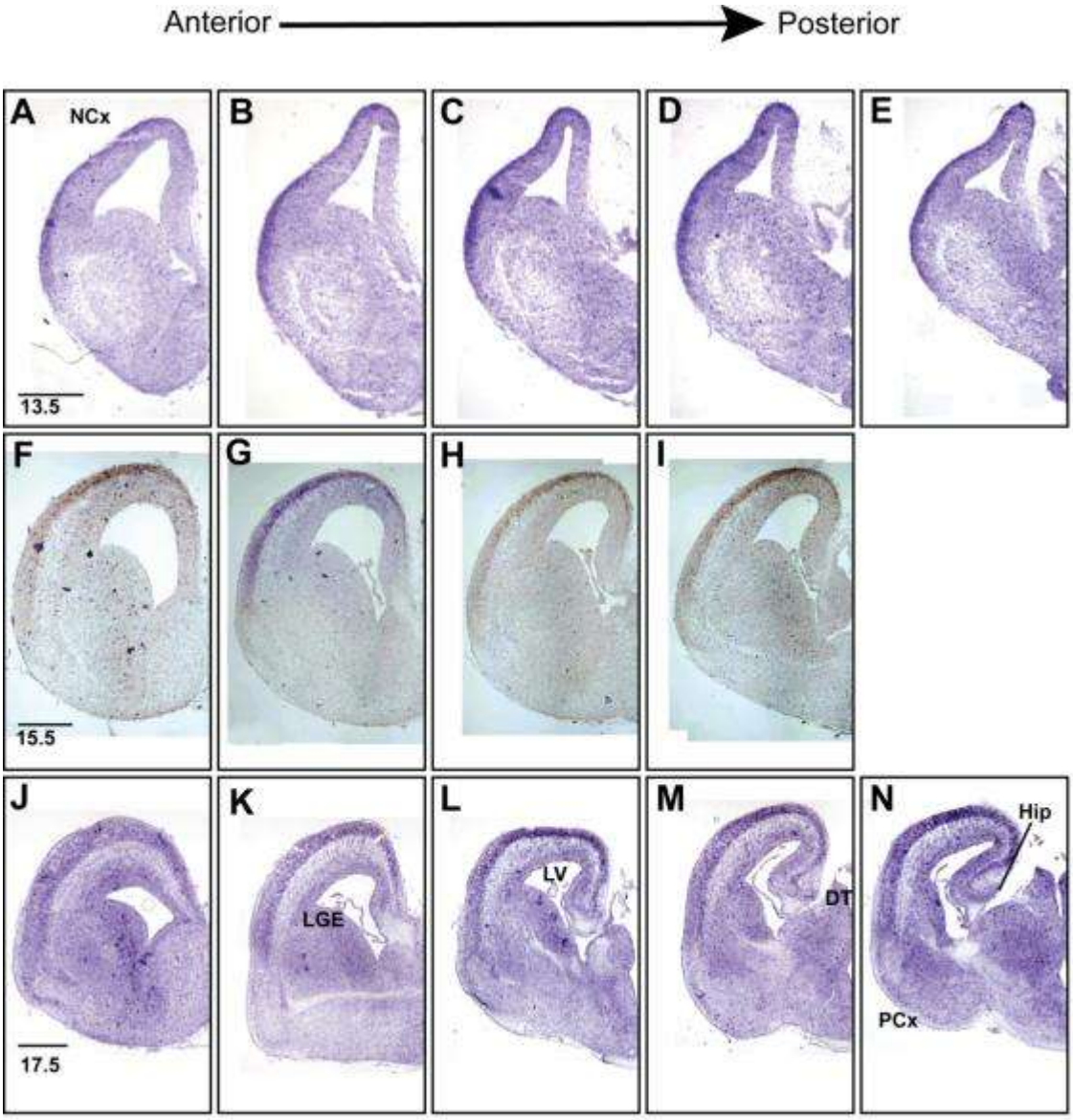


Figure 69. **Expression of *LRIG2* in the developing mouse brain.**
(A-N) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-E), E15.5 (F-I), E17.5 (J-N) using a digoxigenin-labelled *LRIG2* antisense probe on coronal brain sections of mouse embryos. A, B, F, G, J panels show anterior; C, D, H, K, L shows medial; E, I, L, M, N shows posterior sections of the brain, respectively. Abbreviations: DT, Dorsal Thalamus; Hip, Hippocampus; LGE, Lateral Ganglionic Eminence; LV, Lateral Ventricle; NCx, Neocortex; PCx, Piriform Cortex. Scale Bars: A-N, 200 µm.

LRIG3 expression was similar to *LRIG2* but somehow a bit weaker. At E13.5 and E15.5 expression of *LRIG3* was observed in the upper cortical plate only (Figure 70 A-G).

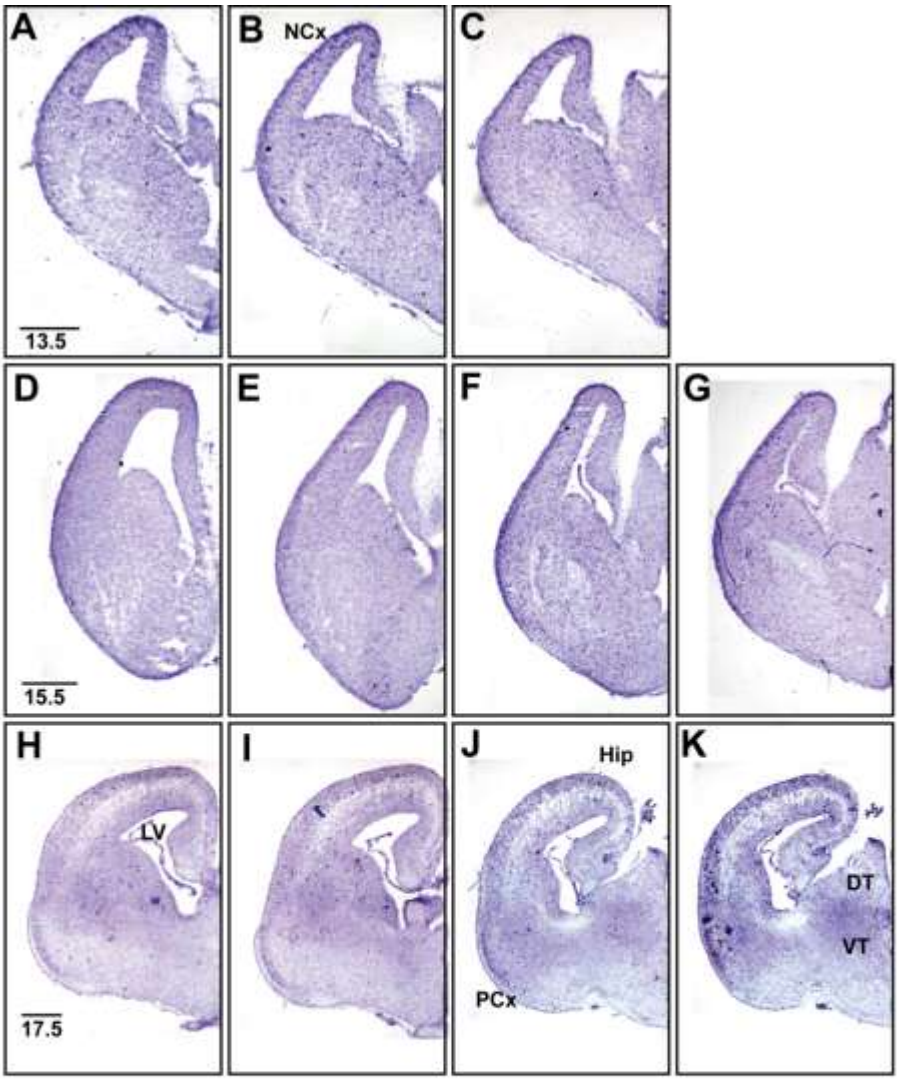


Figure 70. **Expression of *LRIG3* in the developing mouse brain**.
(A-K) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-c), E15.5 (D-G), E17.5 (H-K) using a digoxigenin-labelled *LRIG3* antisense probe on coronal brain sections of mouse embryos. D & I panels show anterior; A, E, I shows medial; ; B, C, F, G, J, K shows posterior sections of the brain, respectively. Abbreviations: DT,Dorsal Thalamus; Hip, Hippocampus; LV, Lateral Ventricle; NCx, Neocortex; PCx, Piriform Cortex; VT, Ventral Thalamus.Scale Bars: A-K, 500 μm.

LRIG3 expression at E17.5 was broader in the cortical plate and extended into the piriform cortex which was clearly labelled (Figure 70H-K). Faint expression in dorsal ventral thalamus was also detected (Figure 70K).

4.6.2 Characterization of the neurons expressing LRIG-1 in the developing mouse and human fetal brain

Analysis of expression pattern of *LRIG-1* gave interesting conclusions therefore I performed dual protocol of ISH with *LRIG-1* probes and IF with CTIP2, SOX2 and TBR2 in mouse and human fetal brain sections. As mentioned above only CTIP2 staining worked by using this double protocol.

Analysis of CTIP2 with LRIG-1 by dual ISH and IHC in developing mouse brain

LRIG1 as seen above also was expressed mainly in VZ/SVZ around neocortex, GE and hypothalamus. In cortex *LRIG-1* labelled cells of VZ and extended to SVZ but with decreased expression (Figure 71CF). CTIP2 was restricted to CP and upper IZ mainly in cortex and SVZ of GE (Figure 71BE). No co localization or overlap was observed between *LRIG-1* and CTIP2.

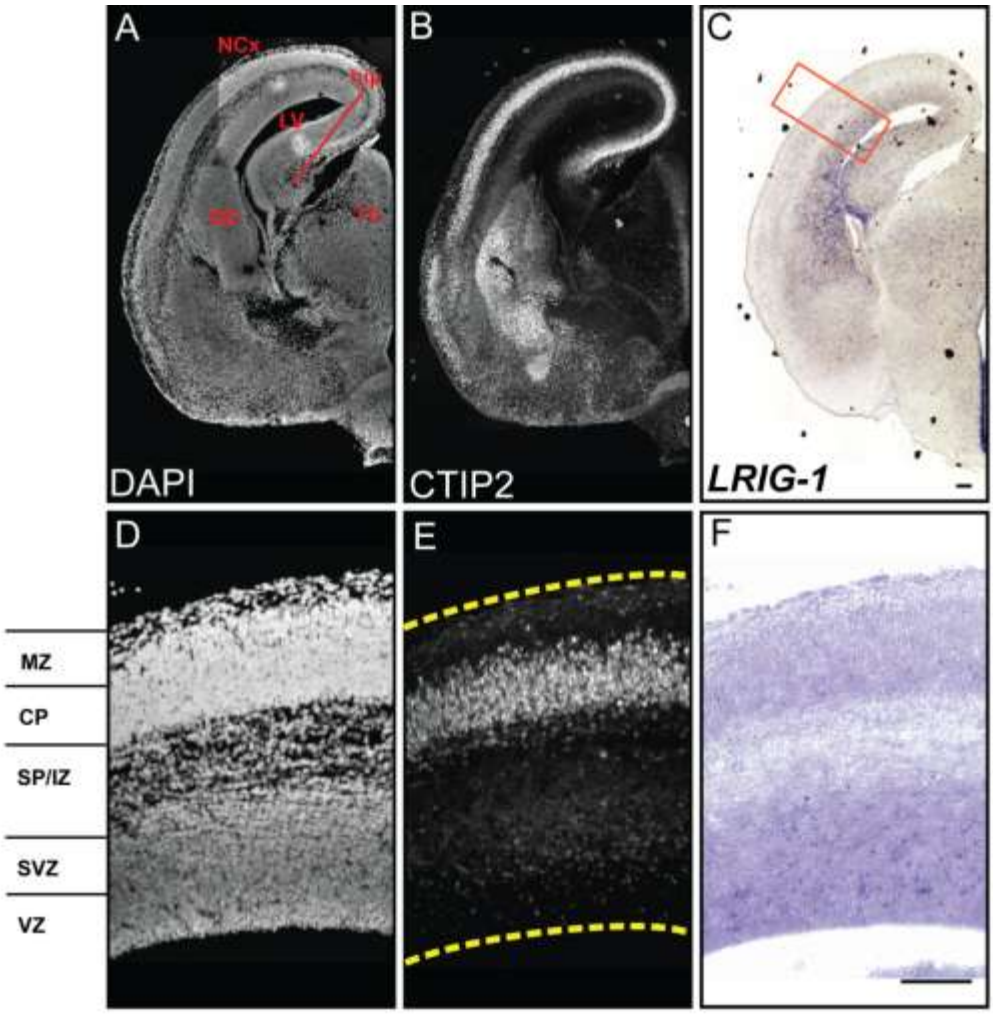


Figure 71. ***LRIG1*** **and CTIP2 dual ISH & IHC, performed on E16.5 mouse brain sections (A-C); Higher magnified view (20X) of neocortex region of C indicated by an orange box (D-F).** Abbreviations: CP, Cortical plate; GE, Ganglionic Eminence; Hip, Hippocampus; IZ, Intermediate zone; LV, Lateral ventricle; MZ, Marginal zone; NCx, Neocortex; SP, Sub plate; SVZ, Subventricular zone; Th, Thalamus; VZ, Ventricular zone. Scale Bars: A-F: 100µm.

Analysis of CTIP2 with LRIG-1 by dual ISH and IF in developing human brain

In human fetal brain sections *LRIG-1* expression was clearly detected in VZ but not in other layers of neocortex which is clearly similar to the pattern observed in mouse cortex (Figure 72C, G). As in the mouse, CTIP2 expression, which is also restricted to cortical plate, do not overlap with *LRIG-1* in humans (Figure 72 B, C, F, and G).

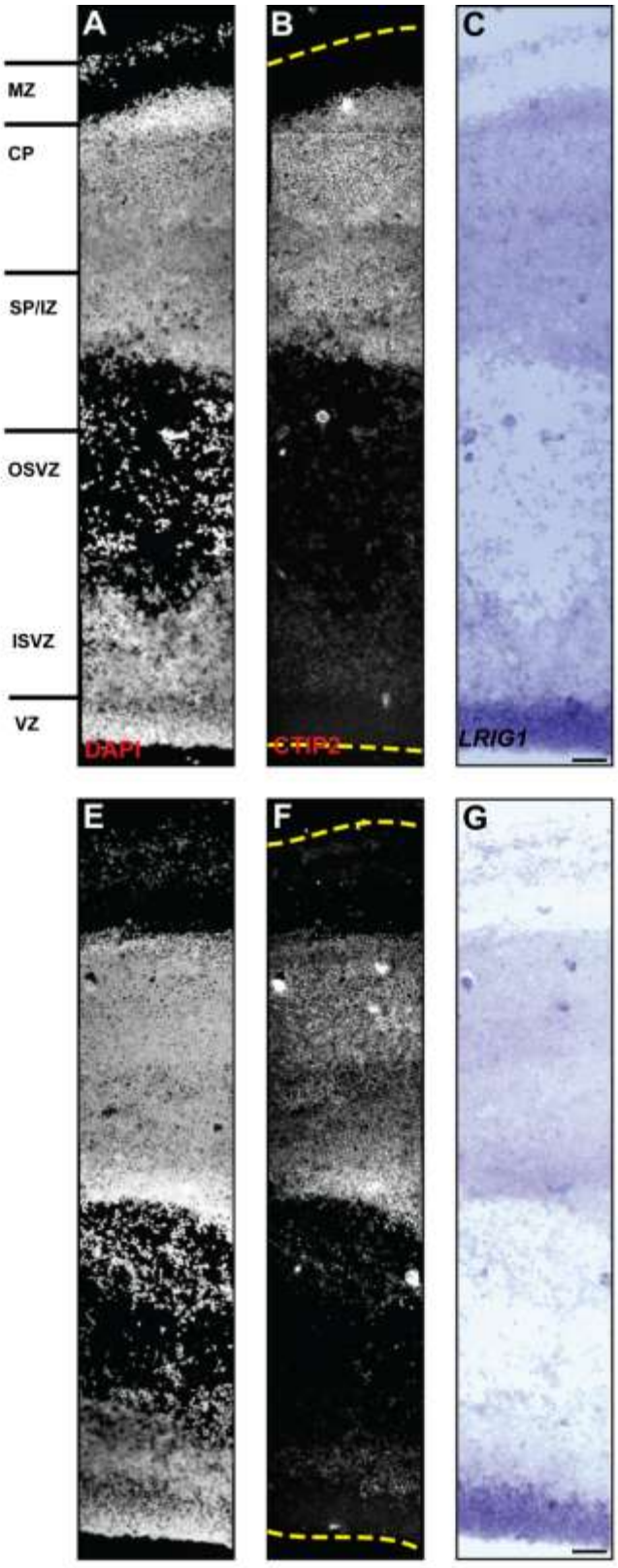


Figure 72. ***LRIG1*** **and CTIP2 dual ISH & IHC, performed on 15pcw human brain sections from occipital lobe (A-F); Higher magnified view (20X) of two neocortex region A-C & D-F.** Abbreviations: CP, Cortical plate; ISVZ, Inner subventricular zone; IZ, Intermediate zone; MZ, Marginal zone; OSVZ, Outer subventricular zone; SP, Sub plate; VZ, Ventricular zone. Scale Bars: A-F: 100µm.

4.7.1 Expression of Lingo1 in developing mouse brain

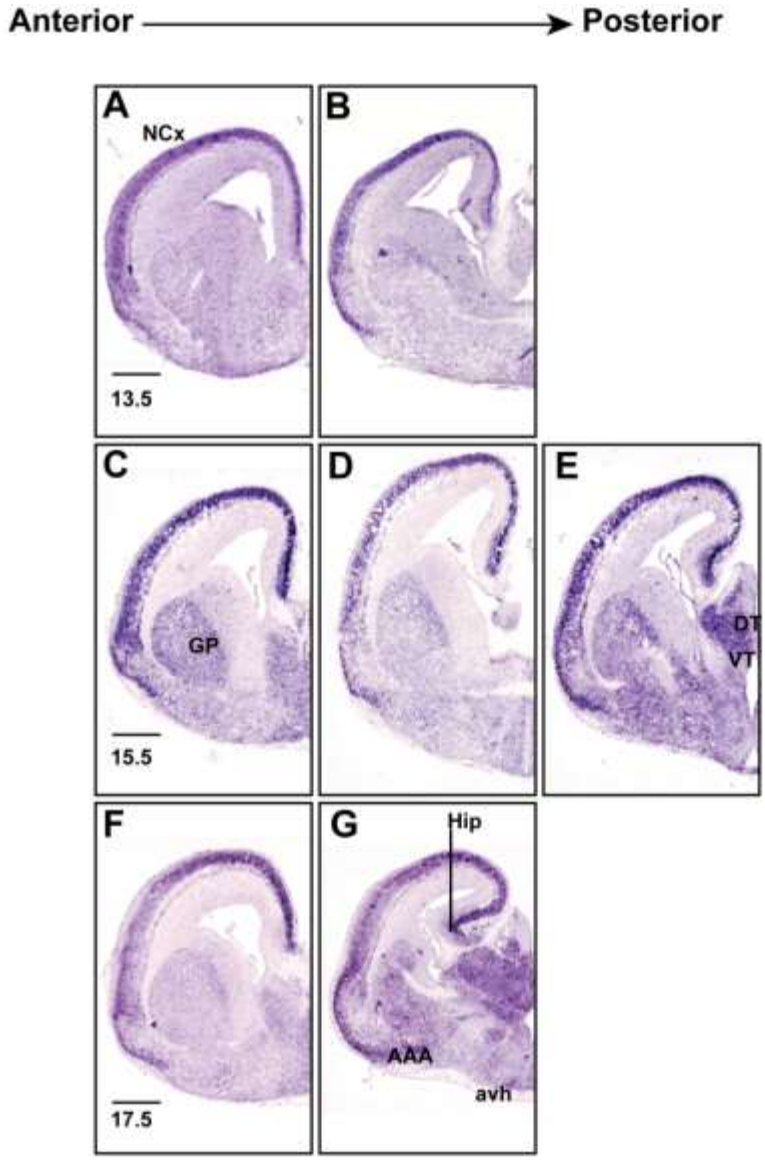


Figure 73. **Expression of *LINGO1* in the developing mouse brain**.
(A-G) In situ hybridization (ISH) analyses of coronal brain sections at E13.5 (A-B), E15.5 (C-E), E17.5 (F-G) using a digoxigenin-labelled *LINGO1* antisense probe on coronal brain sections of mouse embryos. A, C, F panels show anterior; B, D, G shows medial; E shows posterior sections of the brain, respectively. Abbreviations: AAA, Anterior amygdaloid area; avh,anterior ventral hypothalamic nucleus; DT,Dorsal Thalamus; GP, Globus Pallidus; Hip, Hippocampus; NCx, Neocortex; VT, Ventral Thalamus.Scale Bars: A-G, 200 μm.

LINGO1 expression increased during development and avoided the main proliferative regions at all the stages are analyzed. At E13.5, *LINGO1* was expressed in cortical plate (extending into the piriform cortex), subplate and in the developing hippocampus (Figure 73A, B). A faint expression was also observed in the thalamus and caudate

putamen at this stage (Figure 73AB). At E15.5 the expression of LINGO1 in the cortical plate, subplate and piriform cortex was consistent (Figure 73C-E). At this stage, expression at hippocampus and the whole basal ganglia became stronger, including the caudate putamen, the globus pallidus, the amygdaloid area and hipothalamic nuclei (Figure 73C-E). Strong expression at dorsal and ventral thalamus was also observed, mainly in differentiating fields (Figure 73E). At E17.5 strong expression in cortical plate and subplate still existed but in the most lateral-ventral part of the cortex the staining displayed a gradient from superficial high to deep-low (Figure 73F,G). Strong signal was also detected in the hippocampus, mainly in CA1 and CA3 regions. Caudate putamen, globus pallidus and anterior amygdaloid area expressed *LINGO1* along with anterior ventricular hypothalamic nucleus (Figure 73F-G). LINGO1 was also strongly expressed in the ventral and dorsal thalamus (Figure 73G).

4.7.2 Characterization of the neurons expressing LINGO1 in the developing mouse brain and human fetal brain.

I performed dual protocol of ISH and IF on coronal sections of developing mouse and human brain with CTIP2, SOX2 and TBR2, of which, and as previously mentioned only CTIP2 worked with this protocol.

Analysis of co localization of transcription factor CTIP2 with *LINGO1* in developing mouse brain

LINGO1 in mouse was expressed mainly in CP and separate stream in SP/IZ (Figure 74C) and in thalamus and GE. In neocortex expression of LINGO1 is much more intense in cingulated cortex (Figure 74I) than in dorsal cortex (Figure 74F). CTIP2 expression area perfectly overlaps with LINGO1 in mouse being expressed in CP (Figure 74BEH).

As in the mouse, in human neocortex *LINGO1* and CTIP2 both are expressed in CP area and thus expression area overlaps (Figure 75BCEF). However some scattered LINGO1 positive cells were also found in the ISVZ/OSVZ and IZ that may suggest specific functions of LINGO1 in the developing human cortex (Figure 75C, F).

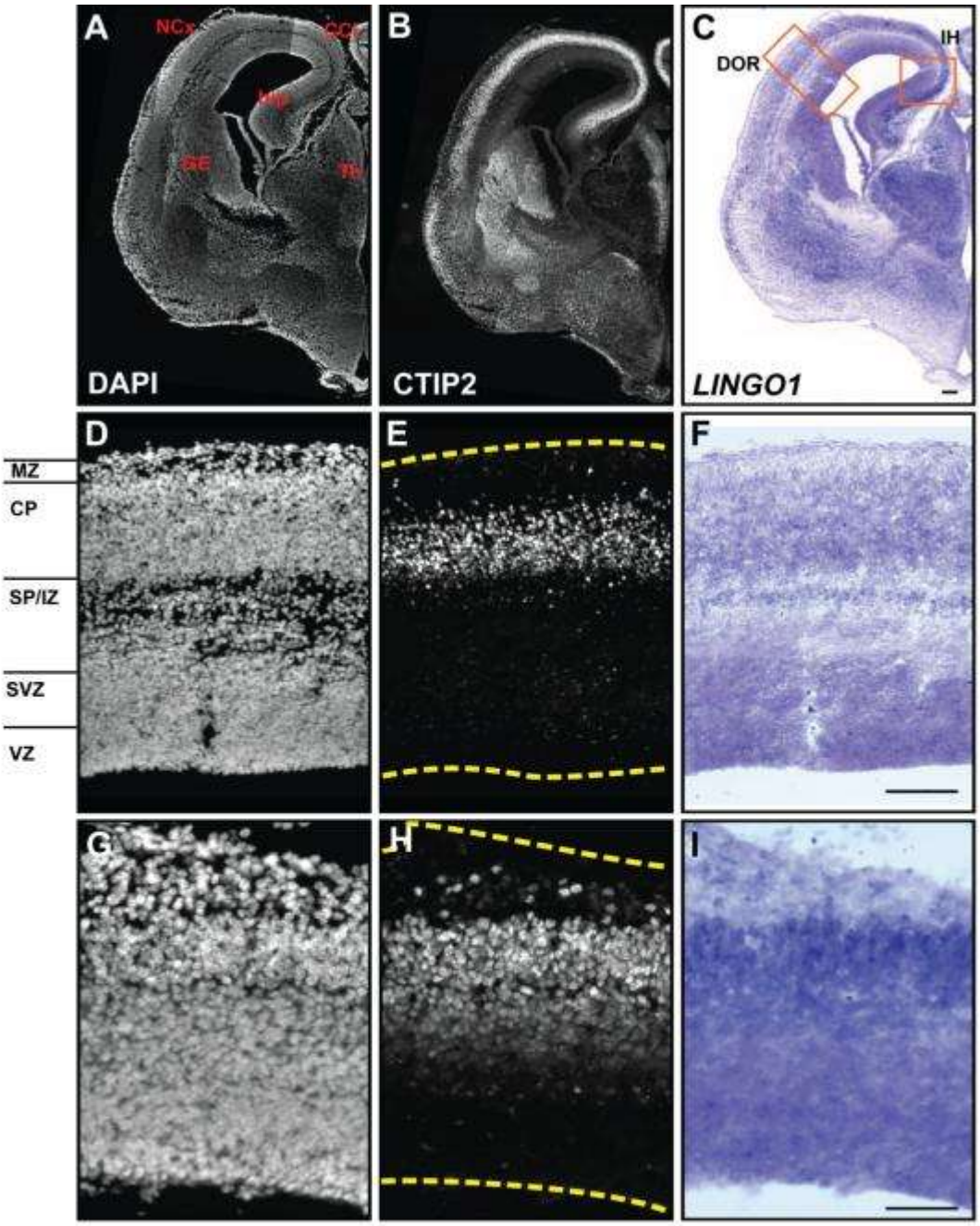


Figure 74. ***LINGO1*** **and CTIP2 dual ISH & IHC, performed on E16.5 mouse brain sections (A-C); Higher magnified view (20X) of neocortex region of C indicated by an orange box , IH is section D-F; DOR is section G-I**. Abbreviations: CP, Cortical plate; DOR, Dorsal; GE, Ganglionic Eminence; Hip, Hippocampus; IH, Inter hemisphere; IZ, Intermediate zone; LV, Lateral ventricle; MZ, Marginal zone; NCx, Neocortex; SP, Sub plate; SVZ, Subventricular zone; Th, Thalamus; VZ, Ventricular zone. Scale Bars: A-I: 100µm.

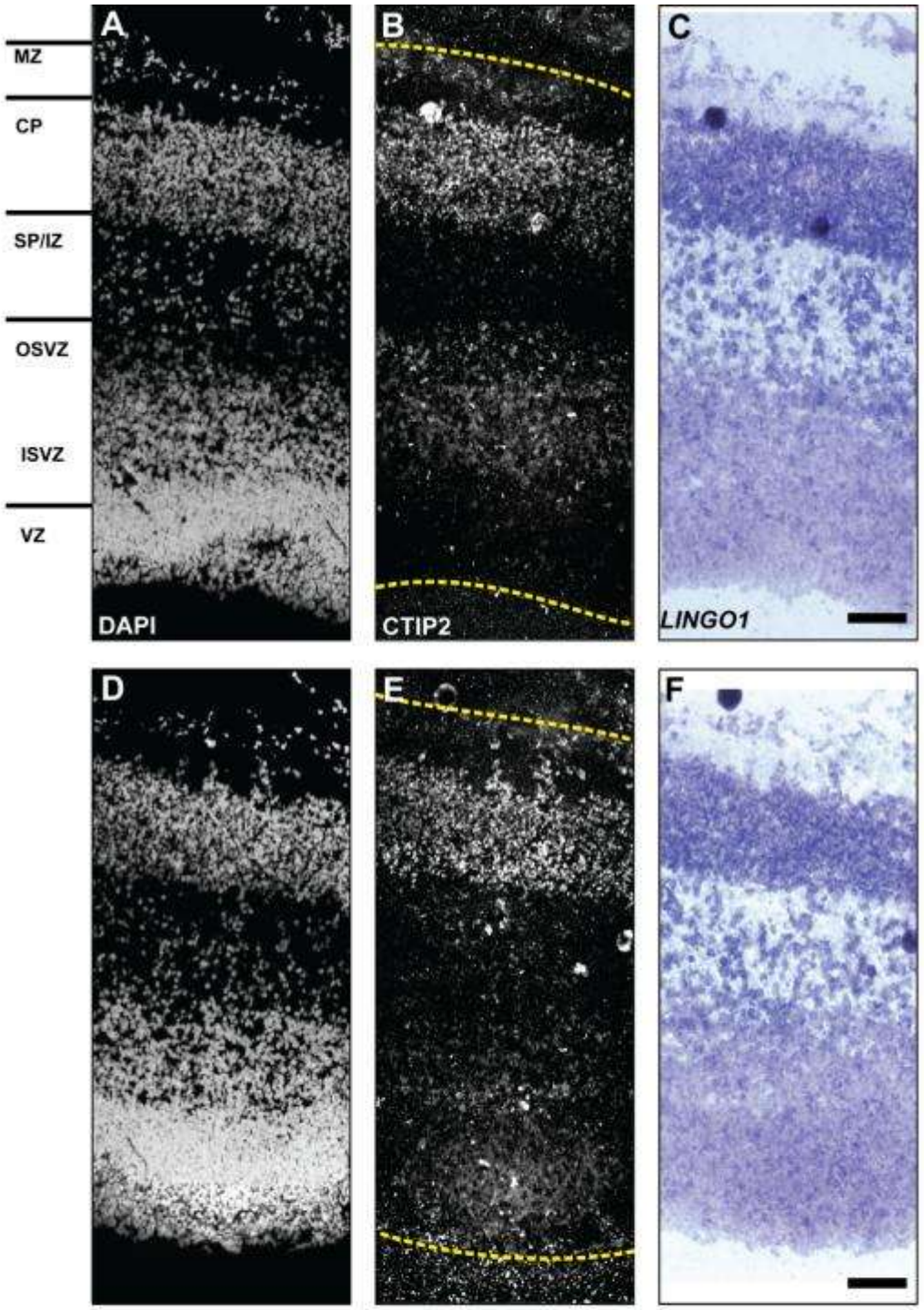


Figure 75. ***LINGO1*** **and CTIP2 dual ISH & IHC, performed on 15pcw human brain sections ; Higher magnified view (20X) of two neocortex region (A-C & D-F) showing expression regions of CTIP2 and** ***LINGO1***. Abbreviations: CP, Cortical plate; ISVZ, Inner subventricular zone; IZ, Intermediate zone; MZ, Marginal zone; OSVZ, Outer subventricular zone; SP, Sub plate; SVZ, Subventricular zone; VZ, Ventricular zone. Scale Bars: A-F: 100μm.

4.8.1 ISLR2 is located intracellular also not just in cytoplasmic membrane.

The secondary structure of ISLR2 predicts that it is a type I transmembrane protein and therefore must be mainly located ini the cytoplasmic membrane. Indeed, some earlier work suggested so but, in my experiments, I observed that ISLR2 is also located in intracellular compartments (proportionally even more than in the cytoplasmic membrane). I performed biotinylation assays and immunofluorescence experiments on HEK293T cells transfected with ISLR2-Myc using FLRT3-Myc as a positive control since its localization is well known to be at the cytoplasmic membrane.

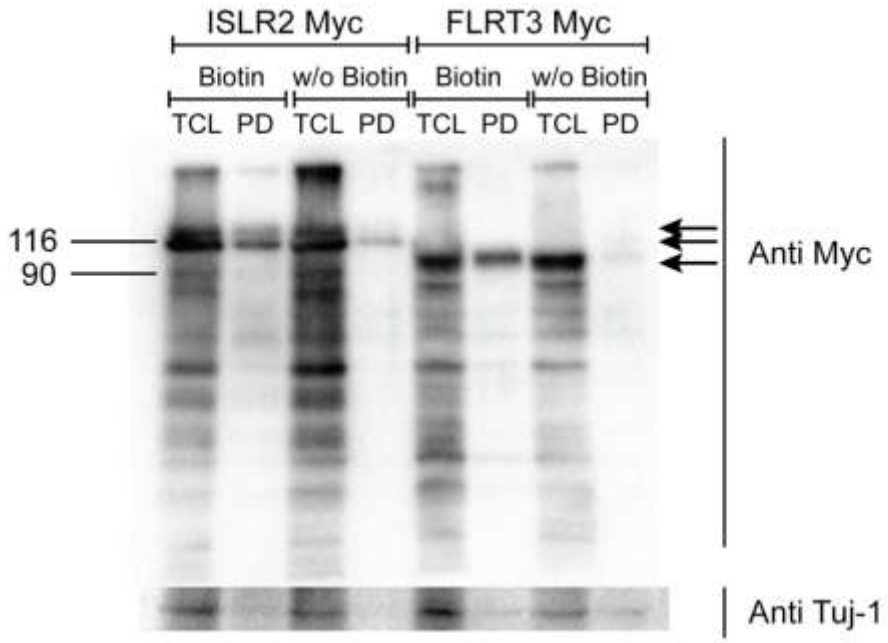


Figure 76. **Biotinylation Assay: Protein labeling on the cell surface.** HEK293T cells transfected with ISLR2-Myc and FLRT3-Myc and were treated with membrane-impermeable biotin. After solubilization, biotin-labeled proteins were precipitated with streptavidin beads. Precipitated proteins were then examined by western blot analysis using Myc and Tubulin -β III (TuJ-1) antibodies to assess the cell surface expression of proteins. The TuJ-1 blot is used to show a negative control for biotinylation as it is an intracellular protein.

For the biotinylation assay, surface proteins were first tagged with biotin, then cells were lysed and protein extracts precipitated with a Sulfo-NHS group (N-hydroxysuccinimide) and captured by streptavidin beads.

Precipitates were then analyzed by Western blot using antibodies against the myc epitope. The results showed that, respect to the total cell lysate, the amount of ISLR2 precipitated was much less in the case of ISLR2 than in the case of FLRT3 which suggests that a high proportion of the protein remains inside of the cell (Figure 76).

4.8.2 Immunofluorescent Assay

In order to visualize directly the distribution of ISLR2 within the cell, we performed an Immunofluorescence asay on transfected cells in different conditions of permeabilization and using tagged versions of ISLR2 at C- and N-terminus (Myc epitope tag in the intracellular part, and HA tag in the extracellular part). Thus, in the protocol 1 cells were first permeabilized before applying the antibodies so the total distribution of the protein (intracellular and surface compartments) could be visualized. The protocol 2 was designed to observe differences between inner and surface protein: first staining (primary and secondary antibodies) are applied without permeabilization and should only label the outer protein (anti-HA); the second staining will reveal the distribution of the total protein and is performed after permeabilization with the same primary antibody but with a secondary antibody having a different fluorofore. Protocol 3 is similar to protocol 2 with the only difference that primary antibody in first staining is previously pre-clustered with the secondary antibody and is applied to living cells. The clustering of the primary antibody together and the fact to "stimulate" living cells with these clusters, will improve the detection of the surface protein, even if there is little protein in membrane.

The results from these experiments show that in the positive control with FLRT3 most of the protein seems to be in the cytoplasmic membrane (Figure 77). In contrast, ISLR2 localization was mainly intracellular, although some part is located at the membrane (Figure 77). The intracellular pattern observed for ISLR2 seemed reticulated and most of it surrounding the nucleus (Figure 77).

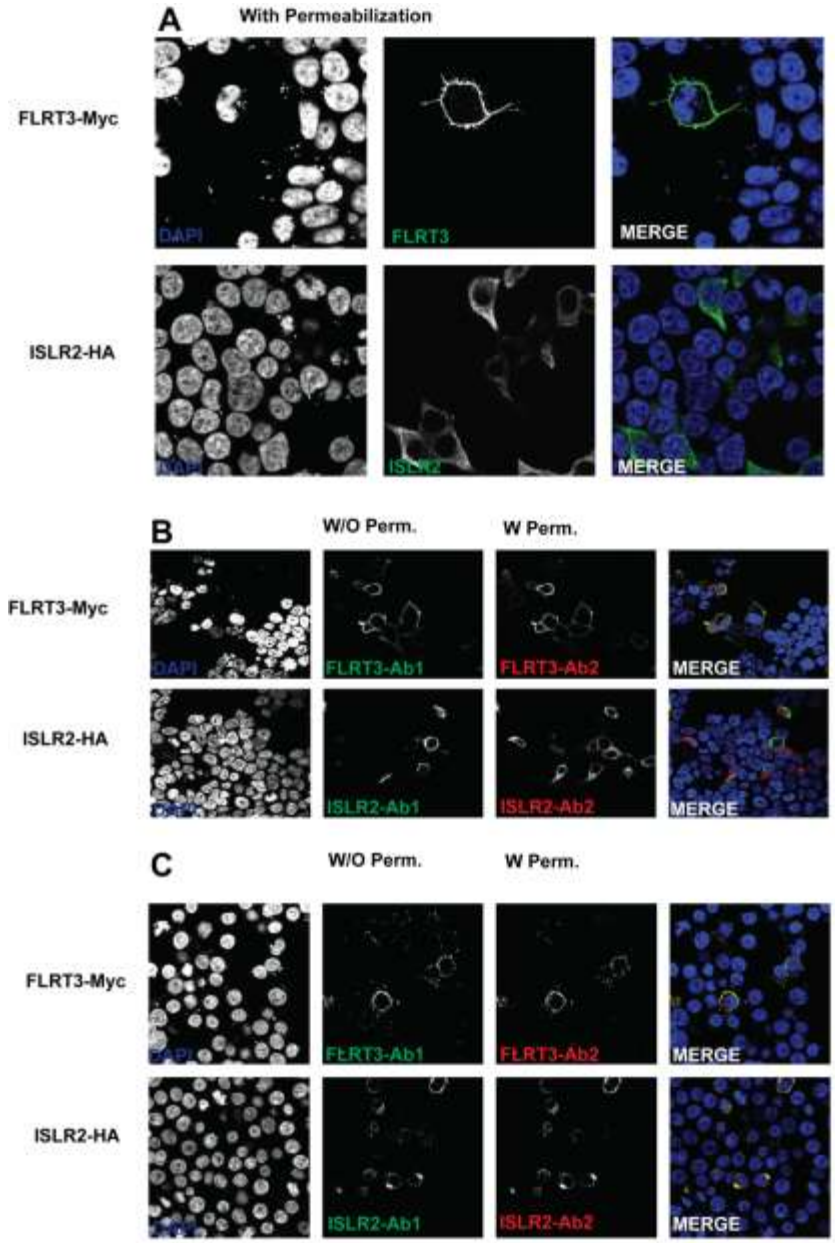


Figure 77. **Immunofluorescence assay for subcellular localization of ISLR2**.HEK 293T cells were transfected to overexpress either ISLR2 tagged with extracellular HA or FLRT3 with extracellular Myc, both with FLRT3-SP. A) Immunofluorescence was performed according to protocol 1 explained in M&M. ISLR2-HA showed an intracellular reticular pattern, in contrast to FLRT3 control, strongly expressed in the cytoplasmic membrane. B) Immunofluorescence was performed according to protocol 2 explained in M&M. In the first staining (green) without permeabilization (W/o perm) only the extracellular proteins were detected. In second staining (red) with permeabilization (W perm) both intracellular and extracellular proteins were detected. ISLR2 showed very little signal in extracellular, but high in intracellular. FLRT3 control shows both plasma membrane and intracellular localization. C) Immunofluorescence was performed according to protocol 3 explained in M&M. Islr2 was first clustered by the stimulation in living cells with anti-HA antibodies (bound to anti-rat Cy2, green). Cells were then fixed, permeabilized and incubated with anti-HA plus anti-rat Cy3 (red) in order to visualize the total protein. ISLR2 showed some extracellular localization.

5. DISCUSSION

5. DISCUSSION

Expression pattern of genes vary from highly localized, dense to widely spread or completely absent at different times and in different areas of the brain. Despite this heterogeneity, the expression pattern for a given gene is conserved among individuals and usually across similar species (human and mice, for instance) as suggested by previous microarray gene expression profiling studies (Caceres et al., 2003; Gu and Gu, 2003; Johnson et al., 2009; Miller et al., 2010; Oldham et al., 2008). Defining the temporal and spatial expression patterns of genes will be invaluable in understanding and identifying their contribution to neuronal fate, neural circuits, and brain function. Also, despite the conversation of gene expression, small subtle differences could have had an important impact on specific traits of human brain development compared to lissencephalic species like rodents that could contribute to our cognitive capabilities. In the present thesis work I hypothesized that genes encoding for eLRR-TMs would be potential molecular tools for the regulation of neuronal connectivity in mice and humans. Neocortical neurogenesis is inititated in anterolateral domains and it progresses posteromedially (McSherry and Smart, 1986), accounting for the uneven thickness of the neocortex along these tangential axes. Hence the present study was done on serial sections of brain from anterior to posterior and at different stages to analyse all variations of gene expression at different dimensions using *in situ* hybridization. To this end, we profiled the gene expression pattern of many members of eLRR-TM proteins in mouse embryonic brain sections at E13.5, E15.5 and E17.5 and human foetal brain sections at 15pcw and 22pcw. Analysis of these gene expression patterns consequently provided an extensive new data about the potential role of these genes in nervous system development and connectivity *in vivo*. Subsequently I analysed selected genes further.

5.1 Implications of gene expression pattern of ISLR2 in developing mouse and human brain.

ISLR2 is a *LIG* gene family transmembrane protein originally described as a mediator of axonal extension, branching, and guidance of somatosensory and spinal motor neurons (Mandai et al., 2009). In my analysis, I found the expression pattern of *ISLR2* to be

very spectacularly specific and widely distributed throughout the brain, not just neocortex. *ISLR2* has been reported to give strong signal in the nervous system during very early development stage (E10.5) also in mouse embryos (Homma et al., 2009).

In mouse, expression of *ISLR2* peaked at E13.5 and E15.5 stage and gradually decreased to some extent at E17.5, while no clear gradient of expression was observed from anterior to posterior. A clear transition was visible in expression of *ISLR2* at neocortex: at E13.5 expression was restricted mainly to CP while at E15.5 the expression became broader in CP and a separate stream appeared in SP which was persistent at later stage also. Another stream appeared in deep IZ/SVZ of neocortex at E15.5 extending towards hippocampus. In the thalamus, ISLR2 was expressed only in medial habenula and TRN with the second one extending into the IC and avoiding GP. A part from these regions an intense expression was observed in hypothalamus and POA. All the regions labelled by ISLR2 in brain are known guidepost areas for extending axons as well as for migrating neurons, suggesting strongly its role in axon guidance and neuronal migration. To analyse further the identity of these cells and understand the expression pattern of ISLR2 we performed double immunofluorescence with different markers including CTIP2, SATB2, SOX2, DCx, TuJ-1 and FLRT3. SATB2 and CTIP2 have been shown to play important roles in the establishment of cortico-cortical versus corticofugal connections, respectively (Arlotta P. Et al., 2005; Britanova et al., 2008). SATB2 is a matrix attachment region (MAR) interacting protein which generates callosal projection neurons in layers II-IV (Alcamo et al., 2008; Britanova et al., 2008), while CTIP2 encodes a C2H2-type zinc finger transcription factor, locates in layers V and VI and is required for the fasciculation and path finding of sub cortically projecting neurons (Arlotta et al., 2005; Britanova et al., 2008). Besides the neocortex CTIP2 has been reported previously to be expressed in other regions of the CNS, including the hippocampus, the olfactory bulb, and the striatum, from early embryonic stages of development (Leid et al., 2004; Arlotta et al., 2005). SATB2 is a repressor of CTIP2 and makes not only the callosal projection but also the subcortical connections (Alcamo et al., 2008). In my results I found ISLR2 colocalizes with CTIP2 and SATB2, suggesting a role for ISLR2 in corticofugal connections as well as callosal projection. This is confirmed by the expression of ISLR2 in axonal fibres in the IZ of the neocortex. These fibres could be either thalamocortical or corticofugal projections but since ISLR2 is not expressed in the ventral thalamus (the

major source of TCAs), my result suggest that ISLR2 is labelling mainly corticofugal axons. Also I didn't observe any expression of ISLR2 in ventral thalamus which is considered as major source of TCAs therefore reinforcing the idea that these fibres are corticofugal axons. ISLR2 is a transmembrane protein and therefore an interesting hypothesis about ISLR2 function is that it could mediate non-cell autonomous functions acting as a "ligand" for a yet unknown receptor. This is the case for instance for the FLRT family of LRR-TM proteins that can act as a ligand for Unc5 receptors in the regulations of SVZ IPCz in the developing mouse cortex (Yamagishi et al., 2011). SP has been defined as a transient zone below the CP and above IZ in developing cortex which contains residential subplate cells, and numerous other migrating cells and extending fibers through the region (Rakic, 1977; Bystron et al., 2008). Indeed it is also known that at early stages, subplate neurons (positive for ISLR2) are involved in thalamocortical axon pathfinding at the level of the intial areal targeting and pioneer the corticofugal pathway (Ghosh et al., 1990; Allendoerfer and Shatz, 1994; Molnar and Blakemore, 1995; Catalano and Shatz, 1998; Lopez-Bendito and Molnar, 2003). Another interesting region labelled by ISLR2 strongly was TRN, this structure has been compared to subplate as it contains largely transient cells, constitutes a temporary target for corticofugal projections and an important waiting compartment during development (Mitrofanis and Guillery, 1993; Lozsadi et al., 1996; Molnar and Cordery, 1999; Jacobs et al., 2007; Pinon et al., 2009). The thalamo-cortical projections and corticofugal projections extend towards each other early during development when the distances are minimal, however then they accumulate/wait in transient cell compartments of the subplate and thalamic reticular nucleus as shown in Figure 78 (Shatz and Rakic, 1981; Molnár and Cordery, 1999; Jacobs et al., 2007; Hanganu et al. 2002; Higashi et al. 2002; Moln´ar et al., 2003; Zhao et al. 2009). Here it's important to understand the "handshake hypothesis" as an explanation to how ascending thalamic axons navigate to their appropriate cortical targets with help from reciprocal descending cortical axons (Molnar and Blakemore, 1995). This hypothesis explains that thalamic axons and cortical axons find their proper pathway after doing a "handshake" near the internal capsule, thus guiding each other's growth over the distal end of their trajectories. However this hypothesis accounted only for the earliest corticofugal and thalamic projections and their interaction in internal capsule at time of crossing pallial-subpallial boundaries (Molnar and Blakemore, 1991). Thalamic reticular cells during development have been called the "subplate" of the thalamus (Mitrofanis and Guillery, 1993), and the

shared gene expression during development between subplate and thalamic reticular nucleus further supports these claims (Wang et al., 2011).

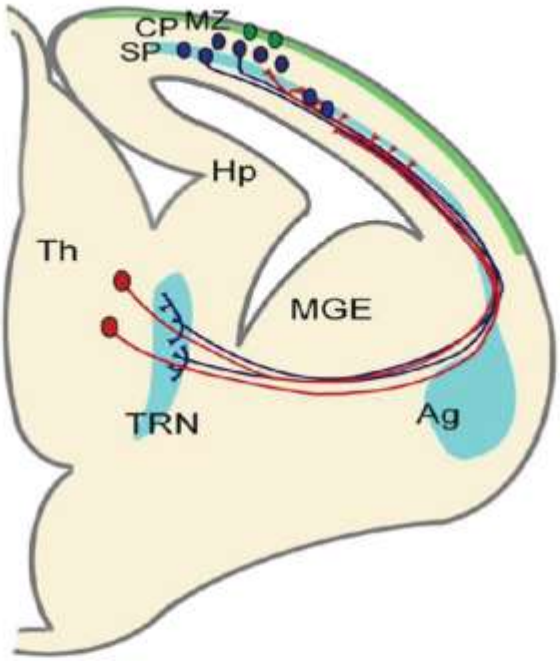


Figure 78 **Functional correlation between the developing thalamocortical projections, cortical SP zone, and TRN**. Corticofugal (blue) and thalamocortical (red) axons extend toward each other at early stages during embryonic development and reach close to their targets. However they both stop short of their ultimate targets and corticofugal projections from subplate and layer VI accumulate in the TRN and thalamocortical projections in SP respectively. Adapted from Montiel JF et al., 2011.

The expression pattern of *ISLR2* and Immunofluorescence clearly points its role in these two possible projections. Work done by Ginty's lab shows involvement of ISLR2 in mechanism underlying "handshake hypothesis" on encounter of TCAs and corticofugal axons (David Ginty, Personal Communication).

In human foetal brain, neocortex region it was very interesting to see that *CTIP2* expression pattern though mimicked that of mouse however unlike in mouse was also present in ISVZ apart from cortical plate, though it's known with doublelabelling of *CTIP2* with INPs (intermediate neuronal precursors) marker, *TBR2* (Hevner et al., 2006) that although *CTIP2* was detected in SVZ, it was not expressed by INPs.Although

in later stage (22pcw the labelling wasn't very prominent to suggest localization of *CTIP2* with *ISLR2* like in 15pcw neocortex.

Next, I tried to identify the nature of the neuronal subtype of the second stream visible in lower IZ/SVZ positive for ISLR2. One of the earliest transcription factors expressed in the developing CNS is Sox2 (Sex determining region of Y chromosome (Sry)-related high mobility group box2), a member of the extended Sox family (Gubbay et al., 1990; Koopman, 1999; Laudet et al., 1993; Sasai, 2001). Sox2 expression was restricted to the proliferating cell populations including neural stem and progenitor cells, glial precursors and proliferating astrocytes (Yaghoub MB et al., 2006). Sox2 controls the expression of several developmentally important genes (Sox2, Oct4, Nanog, nestin, δ crystalline, fibroblast growth factor 4, undifferentiated embryonic cell transcription factor 1 and F-box containing protein 15), hence, it plays a crucial role in embryonic development (Pevny and Lovell-Badge, 1997; Wegner, 1999; Kamachi et al., 2000; Avilion et al., 2003; Uchikawa et al., 2003, 2004; Catena et al., 2004; Miyagi et al., 2004; Wegner and Stolt, 2005). In mouse there was no colocalization at all in neocortex region between Sox2 and ISLRT2, ruling out role that ISLR2 was expressed by progenitor cells. What is the nature of these ISLR2+ neurons? Another ISLR2-strongly enriched area in the developing mouse brain was the POA which has been suggested as a novel source of GABAergic interneurons that migrate tangentially to their final destination in the cortex and hippocampus (Gelman et al., 2009). One explanation therefore would be that these ISLR2+ in the SVZ/IZ that we are referring as LCS (Lateral cortical stream) can be possible transient migration neurons towards the cortex as glutamatergic neurons (Teissier et al., 2010). The identity of neurons here and their migration path can be confirmed by further study with interneuron markers or in utero transplantation experiments. In contrast, in the human developing brain few neurons postive for ISLR2 overlapped with Sox2 in OSVZ region of neocortex, though ISLR2 was expressed mainly in CP but extended till OSVZ. In later stages, 22 pcw expression of SOX2 was distributed more widely reaching the IZ but the expression of ISLR2 was not so specific (probably beacause the bad quality of the sections at this stage) to conclude anything. For these later stages of the development of the human foetal brain, better sections and a more detailed marker study is needed to raise any conclusion.

To further define the neuronal subtype expressing ISLR2 I performed double labelling with DCX and TuJ-1 in mouse and human brain sections. DCX family proteins regulate filamentous actin structure in developing neurons (Fu X et al., 2013). DCX is a

developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons, that has been shown to be important for radial migration of cortical pyramidal neurons, contributing to layer formation (Francis F et al., 1999; Hirotsune et al. 1998; Meyer et al. 2002; Bai et al. 2003), and also playing a role in cortical interneuron migration (Friocourt et al., 2007). DCX is expressed in the brain throughout the period of corticogenesis in migrating and differentiating neurons and immunohistochemical studies show its localization in the soma and leading processes of tangentially migrating neurons, and in the axons of differentiating neurons (Francis F et al., 1999). On the other hand, TuJ1 (or neuron-specific class III beta-tubulin) is a type of tubulin thought to be expressed specifically during differentiation of neuronal cell types (Geisert E et al., 1989; Lee et al., 1990). Tubulins are the major building blocks of microtubules, which are structural components of the cytoskeleton and play important roles in cell structure maintenance, mitosis, meiosis, and intracellular transport, migration and axon guidance among others (refs). Accordingly, immunohistochemical staining of TuJ1 is found in cell bodies, dendrites, axons, and axonal terminations of differentiating neurons. One of the significant advantages associated with the use of immunohistochemical detection of TuJ1 is the degree to which it reveals the fine details of axons and terminations. We used neuron-specific class III beta-tubulin to study the distribution and morphology of immature neurons in the proliferative ventricular and subventricular zones of the developing neocortex. ISLR2 colocalizes with both DCX and TuJ1. Expression of DCX was bit more diffused and extended towards SVZ then ISLR2. Similarly TuJ-1 also colocalizes with ISLR2 in neocortex. LGE and most of the areas where ISLR2 is expressed concluding that this neurons are differentiating neurons.

Also as said earlier ISLR2 has role in guidance of sensory and spinal cord motor neurons (Mandai et al., 2009). My expression pattern goes in line with these findings and suggest that ISLR2 might have a wider role in axon guidance/outgrowth such as in the corticofugal axons. The recent findings that ISLR2 knock-out mice have deficits in the IC formation confirm this hypothesis (David Ginty, Personal communication). The intracellular role of ISLR2 is essentially unknown. Previous work has suggested ISLR2 to be localized in cell membrane but while analysing intracellular localization along with FLRT3 I observed that ISLR2 was localized not just in cell membrane but also in the cytoplasm. Three independent approaches supported this observation: in one hand,

immunofluorescence and byotinilization assay in transfected HEK293T cells; in the other, immunofluorescence with specific antibodies agains ISLR2 on tissue (developing cortex). These results suggest a complex signaling mechanism for ISLR2 i which it could act i) in the cell membrane as a receptor (cell-autonomous function) or as a ligand on opposing cells (non cell-autonomous function); ii) inside the cell as vesicle trafficking regulation.

5.2 Expression Profile of *FLRT* family and its implications

FLRT family is one of the eLRR-TM containing cell surface protein which has been most extensively studied in nervous system development (see Introduction section). However, the information available is just very recent and yet it's expected that many more functions are still to be unfolded including in the human nervous system. Overall *FLRT1*, *FLRT2* and *FLRT3* display distinct expression patterns where in some reginos are co-expressed and could therefore have redundant functions while in others they are complemetary suggesting specific roles in nervous system development.

FLRT1 expression pattern at E13.5 was interesting in regard that a very strong expression was observed in glial wedge region which gradually decreased during development. It is known that developing neocortical axons exit the hemisphere either laterally via the internal capsule or medially via the corpus callosum. The internal capsule acts as an intermediate target for corticofugal axons as suspected for ISLR2 which is supposed to meet there a binding partner that is important for the guidance of these axons (Metin et al., 1997; Richards et al., 1997; David Ginty, personal communication). Similarly, the glial wedge expresses a guidance activity that causes callosal axons to turn toward the midline (Shu T and Richards JL, 2001). The strong expression in glial wedge of *FLRT1* suggests its possible role in this guidance activity. FLRTs have been shown to act as ligands for Unc5 receptors (Karaulanov et al., 2009), although preferentially FLRT2 and FLRT3 show this binding reliably (Yamagishi et al., 2011). If FLRT1 acts as a guidance cue at the glial wedge, probably this is mediated by a receptor different from Unc5s. The expression pattern of FLRT1 in the neocortex

shows a gradual increase from E13.5 to E15.5 and then gradual decrease from E15.5 to E17.5. Apart from CP, a new stream of neurons develops at SVZ/VZ of neocortex which extends till the hippocampus. This expression pattern suggests a complex role for FLRT1 in regulating cortical neuron function that can range from cell fate and proliferation (in the SVZ/VZ) to neuron migration, differentiation and connectivity (in the CP). Interestingly, expression of *FLRT1* in the hippocampus shows a strong increase in expression across the stages which is maintained in the adulthood, in particular, in the CA1 region (reference or say "data not shown") indicating that FLRT1 may have a role in the development of the Schaffer Collateral Pathway that connects the CA3 with the CA1 region. It is interesting to observe that in the adult hippocampus FLRT2 co-localizes with FLRT1 in CA1 region while FLRT3 is specific for CA3 but not CA1. This expression pattern suggest a potential interesting role for FLRTs in the development of the hippocampal circuitry which may be relevant for learning and memory. Indeed, it has been recently shown that FLRT3 is important for excitatory synapse development in the hippocampus and therefore would be interesting to address these questions in tissue specific knock-out animals (O'Sullivan et al., 2012). *FLRT1*, as well as *FLRT2* and *FLRT3*, also showed similar strong labelling of TRN cells like *ISLR2* and could therefore play an important role as intermediate target for corticofugal and TC axons. As expected, *FLRT1* positive cells in the cortex showed co-staining with CTIP2 (in the CP, by comparing ISH with IF) as well as in the thalamus (at E16.5 in the mouse). In human neocortex at 15pcw *FLRT1* showed a similar expression pattern to mouse in CP and ISVZ/VZ. To verify the subtype of these cells we did labelling with SOX2 and TBR2 along with CTIP2, but SOX2 and TBR2 antibodies didn’t worked with this dual protocol of ISH and IF. It would be interesting and will provide more information about *FLRT1* role in human cortex development if FLRT1+ neurons could be better identified. As mentioned above, FLRT1 does not bind very well to Unc5 receptors suggesting it has different signalling mechanisms from FLRT2 and FLRT3 (Yamagishi et al., 2011). On the other hand it has been shown that the intracellular domain of FLRT1 is a substrate for FGFR and therefore it could be an important downstream signalling molecule for FGF during development. Nevertheless, the in vivo relevance of this observation and, in general, the in vivo role of FLRT1 needs to be addressed. The knock-out animals for *FLRT1* are available and are viable and fertile and do not display any obvious phenotype (data not shown).

The function of *FLRT2* has already been addressed by our group as a guidance cue for in vitro modulating axon projections and for in vivo neuron migration in the developing cortex (Yamagishi et al., 2011). At E15.5, in mouse *FLRT2* is expressed in CP which shows gradient from anterior to posterior. From here, the ectodomain of FLRT2 seems to be shed and act as a repulsive cue for Unc5D expressing IPCs in the SVZ (Yamagishi et al., 2011). This repulsiveness seems to be important for IPCs to stay in the SVZ and display the so-called "resting period" where IPCs, divide, change morphology (become multipolar) and can spread horizontally within the SVZ (reference). Before migration towards the CP, IPCs downregulate Unc5D and in this way become insensitive to the repulstive effect of FLRT2 and can migrate towards their final position whithin the CP (Yamagishi et al.,. 2011). In knock-out animals for *FLRT2* or *Unc5D* IPCs migrate prematurely from the SVZ and arrive faster to the CP (Yamagishi et al., 2011). Another interesting region where *FLRT2* is expressed is the kind of "corridor" that is formed between SVZ in the LGE and cp which became wider from E15.5 to E17.5. This expression pattern is very suggestive of a role for FLRT2 in controlling axon guidance (corticofugal and/or TCAs) or interneuron migration. Our group is currently addressing these two possibilities in vivo, in single *FLRT2* knock-out mice as well as in double knock-outs animals for *FLRT2* and *FLRT3*. *FLRT2* at E15.5 had quiet similar expression as *ISLR2* in the neocortex with stream of cells at SP, ham and strong signal at TRN but it disappears at E17.5. Also at stage E15.5 the expression observed at avh disappears at later stage. It was a pity that FLRT2 antibodies didn't work in human tissue, given the important role of FLRT2 in mouse development; its expression pattern in human brain would have been relevant.

FLRT3, like *FLRT1*, is also expressed in glial wedge at E13.5 in mouse. At this stage, expression in neocortex was restricted mainly to cingulate cortex unlike to the other two *FLRT* members. *FLRT3* was also found to be expressed in the boundary between LGE and MGE, close to the ventricle, presumably in the VZ. This expression pattern in the basal ganglia is very interesting and suggests a role for FLRT3 in interneuron cell fate specification, migration and/or perhaps a morphogenic role in the establishment of this LGE/MGE boundary. Our group is currently analyzing these possibilities using *FLRT3* knock-out animals. At E15.5 expression of *FLRT3* in neocortex became broader with cells labelled now in CP and IZ. A very interesting expression was observed in anterior ventricular hypothalamic nucleus and in thalamus. In this region, expression was very

strong in epithalamus, specially in the ventricular, proliferative, region. Our group has recently participated in the study of the role of this thalamic expression of FLRT3 in vivo and concluded that it is a key player in the axon guidance regulation of TCAs and the proper topographic distribution of these axons within the cortex (Leyva et al., 2014). In particular, this study showed that FLRT3 physically interacts with the axon guidance receptor Robo1 and modulates its signaling in the presence of Slit1. In this context Slit1 behaves as a permissive cue that does not trigger repulsion but an upregulation of DCC receptors at the surface of the neuron that in the presence of Netrin1 triggers attracton (Leyva et al., 2014). Both Slit1 and Netrin-1 are expressed in intermediate targets in the TCA pathway in a gradient rostral high to medial/caudal low, and need of FLRT3 to attract rostral TCAs to the rotral cortex to keep the topographic distribution of these axons (Leyva et al., 2014).

Expression in cortex eventually became stronger at E17.5 but maintained in CP and IZ. Also hippocampus showed a strong expression, particularly in the DG. This expression extends towards CA3 but is very low, or absent, in CA1. This pattern is maintained in the adulthood (Egea et al., 2008) and, as mentioned above, is complementary to FLRT1 and FLRT2 and may have important contributions for the establishment of hippocampal connectivity. A faint expression of *FLRT3* also appears in TRN at E17.5. In overall, the expression of *FLRT3* is very dynamic from one stage to another and this may reflect important roles in neuronal migration and axon guidance as demonstrated for TCA projections.

To understand further expression pattern of *FLRT* family, I tried to co-localize their expression with some markers suchs as PAX6, CTIP2, SOX2 and TBR2. For FLRT1, only CTIP2 immunoestaining worked after the *FLRT1* ISH (the antibodies against FLRT1 are not working properly). *FLRT1* expression overlaped with that of CTIP2 in the mouse CP indicating cells labelled by *FLRT1* are differentiating neurons. Similar results were obtained in humans suggesting similar roles for *FLRT1* in human and mouse neocortex. Transcription factor *PAX6* encodes a protein containing a paired domain and a homeodomain (Ton et al., 1991. 1992). *PAX6* labels the cells in the OSVZ of human and primate cortex and it has been suggested that *PAX6* cells in the OSVZ include both progenitor cells and postmitotic neurons (Fish, J.L et al., 2008; Gotz, M et al., 1998; Bayatti, N et al., 2008; Mo,Z and Zecevic, N, 2008) . In contrast, in rodents *PAX6* is expressed mainly by RG cells in the VZ and has been postulated to control

glutamatergic neuronal cell fate (Kroll &O'Leary, 2005; Gotz, M et al., 1998). Our PAX6 antiboy worked in human samples but not in mouse tissue while FLRT3 antibody behaved the opposite, so I could not compare the co-expression of the two in any of the two tissues. However, at least in the mouse, it seems as if FLRT3 and PAX6 should not co-localize. Since the expression pattern of PAX6 in human developing cortex is more complex it remains to be addressed whether FLRT3 could be importnat in these PAX6+ neurons. Glutamatergic projection neurons and their progenitors sequentially express *PAX6* followed by *TBR2* during development (Bulfone et al., 1999). *TBR2*/Eomes, T-box transcription factor is a known marker of intermediate progenitor cells (IPCs) committed to glutamatergic fate (Englund et al., 2005). Tbr2 is only transiently expressed during SVZ neurogenesis where directs transformation of RGC's into basal precursors and regulates neurogenesis in the cortical subventricular zone (Arnold, J.S et al., 2008; Sessa A. Et al., 2008). Tbr2 expression initiates between embryonic day 10 (E10) and E12 in a few differentiated preplate neurons including Cajal-Retzius cells but gets highest levels between E12 and E16 in SVZ IPCs coincident with the peak of murine cortical neurogenesis and decays after E17 (Englund et al. 2005; Molyneaux et al. 2007). The relevance of the Tbr2+ cells in the SVZ is highlighted by the fact that, in humans, a homozygous chromosomal translocation that disrupts *Tbr2* expression is associated with microcephaly, polymicrogyria, corpus callosum (CC) agenesis, cognitive deficits, and severe motor delay with hypotonia (Baala et al. 2007). Tbr2+ SVC IPCs have been also shown to dictate the migratory route and control the amount of subpallial GABAergic interneurons in the SVZ through a non-cell-autonomous mechanism involving Cxcl12 chemokine signaling (Sessa A., et al 2010). In the developing mouse cortex, my double immunoflurorescence Tbr2 and FLRT3 has revealed that FLRT3 is expressed in the IZ, in the region where SVZ neurons loose Tbr2 expression and start migrating to the CP. It would be interesting to address in vivo whether FLRT3 (like FLRT2 does) regulates the migration of the SVZ IPCs or the transition between bipolar and multipolar state.

5.3 Expression pattern of *AMIGO* and its biological implications

One family of three *LRRIG* proteins is the amphoterin-induced gene and open reading frame (*AMIGO*) subgroup, also known as the Alivin's (Kuja-Panula et al., 2003).

AMIGO1-3 have varied expression patterns with *AMIGO1* being ubiquitously expressed in brain without any specific pattern, *AMIGO2* being highly specific and *AMIGO3* showing a similar expression pattern as *AMIGO1*. *AMIGO1* has been reported to be present exclusively in nervous system, for instance in axonal tract fibres where it could function as a cell adhesion molecule that facilitates neuronal growth and defasciculation (Kuja-Panula et al., 2003). My analysis of *AMIGO1* expression at E13.5, E15.5 and E17.5 show a constant expression with no strong upregulation or downregulation. *AMIGO-1* labelled cells at neocortex, dorsal thalamus, hippocampus, ganglionic eminences but always in a very dispersed way. It is difficult to suggest any possible role for *AMIGO1* by its expression pattern and probably a comparative study simultaneously in human and mouse samples with different markers for different nervous system function like myelination with myelin specific marker α-CNPase as done in rat will provide better insight (reference of the paper where this myelination study was done).

AMIGO2 unlike *AMIGO1* labelled specifically cells in CP, GP, medial habenula and anterior dorsal hypothalamus nucleus. The expression pattern for *AMIGO2* was very interesting in the sense that it showed strong gradient during development. *AMIGO2* expression in medial habenula of epithalamus appears only in small region of medial sections but not in more anterior and posterior regions. This specific expression suggest an unwarranted role in axonal projections or fasciculation, provided AMIGO2 has similar functions as AMIGO1 (Kuja-Panula et al., 2003.Similar discrete expression is observed in hypothalamus but further work need to be performed to characterize the type of neurons that express AMIGO2 in this region as it has been suggested as neurogenic source (Kokoeva et al., 2005). Recent work suggests *AMIGO2* expression in the CA2-CA3 regions of the hippocampus might be relevant to promote survival and confer the particularly resistance of this area to neuronal injury and neurotoxicity (Laeremans et al., 2013).

AMIGO3 like *AMIGO1* is widespread in brain showing labelling in CP, hippocampus and other regions of the developing brain. The expression seems to be low although a faint gradient in CP from ventrolateral to dorsomedial can be observed. It is known that *AMIGO3* acts as co-receptor for NgR1/p75 in signalling pathway that inhibits axon growth in acute phase of adult central nervous system injury, suggesting a main role for AMIGO3 in adult brain (Ahmed et al., 2013). However, its role in normal conditions in any pathway is yet to be disclosed.

As suggested by earlier research, *AMIGO* family members may interact with each other. Also its is known

5.4 Expression pattern of *SALM* genes and possible biological implications

*SALM*s have been suggested to play a role in multiple aspects of neuronal differentiation. *SALM1*, *SALM2* and *SALM4* have conserved C-termini with a PDZ-domain binding consensus sequence. *SALM1* expression at early developmental stages was very faint but increased as development proceeded. At E15.5 cells mainly label lateral/ventral CP but avoided dorsal part of cortex. Interestingly at E17.5 they *SALM1* were strongly expressed in the dorsal cortex, mainly in the upper layers of the CP. At this stage, a faint expression in DT and hippocampus could also be observed. SALM1 has been reported to function in neurite outgrowth (Wang et al., 2006; Wang et al. 2008). In hippocampal neurons, SALM1 function has been shown to be regulated specifically in dendrites but not in axons and that the enrichment at the cell surface affects dendritic arborization (Seabold et al., 2011). This trafficking is regulated by intracellular motifs at the intracellular domain of the protein including the PDB binding motif (Seabold et al., 2011).

Comparatively to *SALM1*, *SALM2* showed stronger expression throughout the cortical plate and a thin stream of cells in subplate area. *SALM2* also labels hippocampus and thalamus. SALM2 has been reported to regulate the differentiation of excitatory synapses; when overexpressed, SALM2 increases the number of excitatory synapses and dendritic spines (Ko et al., 2006). As in the case of SALM1, overexpression of SALM2 enhanced neurite outgrowth (Wang et al., 2008). It would be therefore interesting to address if any of the expression patterns I observed correlates with these functions (hippocampus - excitatory synapse formation or thalamus - axon outgrowth/guidance).

SALM4 expression pattern in the developing brain gave a dynamic expression in CP very similar to *SAML2* with restriction of this expression to dorsal cortex at E17.5 stage, in particular in the more superficial layers of the CP. In E17.5 stage a faint expression in VZ suggest possible role in neurogenesis but that is unwarranted until a fine

characterization of these cells is performed. Hippocampus and dorsal thalamus also showed a faint staining of *SALM4*. As in the case of other SALM proteins, overexpression of SALM4 induced neurite branching, an effect that is mediated specifically in the case of SALM4 by the flotilin-1 signalling pathway (Swanwick et al., 2010).

*SALM*5 expression pattern is most interesting and strong of all members of *SALM* family with cells labelled in CP of neocortex which expression territory extends into the hippocampus and broadens during development showing a gradient within the CP from superficial-high to deep-low. At later stages, *SALM*5 also labels the basal ganglia (striatum, GP, amygdaloid area and preoptic area extended to hypothalamic area) and the thalamus,. Better characterization of all these positive regions will help us to understand the role of SALM5 in aspects of axon guidance (TCAs), neuron migration and differentiation (excitatory and inhibitory neurons). Owing to the interesting pattern of *SALM*5 I performed dual protocol of ISH followed by IF in human foetal brain sections for *SALM*5 probes to see the similarity and differences in expression pattern but, unfortunatelly, the probes didn't work very specifically.

Over all very little is known about the function of SALM proteins in the nervous system. However, the expression patterns presented in this thesis work together with the short list of published articles, suggest a role for SALMs probably in neuron differentiation during development (neurite outgrowth and defasciculation, for instance) and in early postnatal stages and adulthood, in synapse formation. It has been reported that SALM proteins act as cell adhesion molecules that are able to form heterometric and homomeric complexes suggesting that they expression must be considered as a whole (Seabold et al., 2008). In this regard, my expression pattern study is relevant because has revealed regions in the brain where these interactions might occur and be functional. Also, there is not available data addressing the phenotype of *SALM* knock-out animals, suggesting that they might have overlapping functions.

5.5 Expression pattern of *NLRR* genes and its biological implications

NLRR1 expression is restricted to very few regions in the developing brain, is very dynamic and overall, tend to be reduced during development. At E13.5, *NLRR1* labels the cells mainly in the hippocampus area and a very lateral-ventral region in the cortex

(CP). Interestingly, while development proceeds (E15.5) the expression from hippocampus reduces drastically suggesting a putative role for NLRR1 in the early morphogenesis of this region of the brain. In the cortex, *NLRR1* expression becomes broader in the CP and also can be detected in ventricular regions (SVZ) in the cortex and basal ganglia. At E17.5, expression of NLRR1 gets restricted to the dorsal cortex, mainly in the upper layers of the CP. These results suggest different roles for NLRR1 in nervous system development, related to differentiation/migration (staining in the cortex) and perhaps cell fate and proliferation (ventricular expression) as previously suggested (Buchanan and Gay, 1996). Related to this last function, N-MYC has been shown to promote cell proliferation through a direct transactivation of NLRR1 in neuroblastoma which induces a positive feedback enhancing EGF-mediated MYCN induction and accelerates tumor growth in vivo (Hossain et al., 2008; Hossain S et al., 2012).

NLRR2 expression is similar to *NLRR1* although is not as dynamic and levels remain constant during development. At early stages (E13.5) *NLRR2* is mainly expressed in the CP within the cortex but no expression was observed in the hippocampus region where *NLRR1* was very prominent. While development proceeds, cortical expression of NLRR2 becomes wider with a scattered expression pattern within the CP where it could play a redundant function to *NLRR1*. Expression in the thalamus and hippocampus at these stages becomes stronger, especially in the CA3 region. It would be interesting to follow *NLRR2* expression in the adult hippocampus; see if it is maintained and where exactly is located to hypothesize possible roles in hippocampal circuitry.

NLRR3 is the one we are interested most of the three members. As in the case of *NLRR1*, *NLRR3* is a good example of developmentally regulated gene expression. But in cotrast to *NLRR1*, *NLRR3* expression increased with development in all the areas of the brain. *NLRR3* labelled strongly cells in TRN and in later stages (at E17.5) we could also see staining in CP extending into the hippocampus and pyriform cortex, ventral and dorsal thalamus, basal ganglia and a faint stream in SP similar to *ISLR2* and *FLRT* family. In the CP, *NLRR3* expression is highest in the superficial layers. What was even more interesting was to detect *NLRR3*+ cells in the VZ/SVZ at later stages. We currently do not know the nature of these cells but they could be migrating interneurons or proliferating pyramidal neuron precursos. In contrast to NLRR1, NLRR3 has been shown to be negatively regulated by MYCN and its downregulation is associated with unfavourable outcome in neuroblastoma (Akter et al., 2011). These results indicate that

NLRRs play a role in cell proliferation and their expression in ventricular zones during brain development might be relevant for this function. After cortical brain injury in mouse increase in expression of NLRR-3 mRNA was observed suggesting role in pathophysiological response (Ishii et al., 1996)

5.6 Expression pattern of *LRIG* genes and its biological implications

Role of *LRIG* family in nervous system development is mainly unknown. *LRIG1* is having one of the most interesting expression patterns we identified during this screening with major expression areas to be restricted to the ventricular zone of mainly the neocortex, hippocampus and third ventricle. The overall expression gets reduced during development in accordance with the reduced number of progenitors with time. A faint expression, but that I think is meaningful, is visible in the CP as well (more prominent at E15.5). In human developing cortex, *LRIG1* expression was similar than in mice and a strong labelling was observed in the VZ confirming a possible role in neurogenesis. Previous work suggests that *LRIG1* regulates epidermal and epithelial stem cell homeostasis and is considered a tumor suppresor (Wong et al., 2012; Powell et al., 2012). The mechanisms of this function are not completely understood but it seems as if LRIG1 controls ErbB signalling in part by receptor degradation (Wong et al., 2012; Powell et al., 2012; Rafidi et al., 2013. The specific expression pattern of *LRIG1* in proliferative regions in the developing cortex suggests a similar role in the control of cell proliferation in this tissue that would be worth to address in vivo in knock-out animals.

LRIG2 and *LRIG3* have a completely different expression pattern compared to *LRIG1* and both label differentiating fields (CP, the hippocampus, ventral thalamus, pyriform cortex). In the case of *LRIG3*, the expression is very low during the developmental stages analyzed here while *LRIG2* expression was higher. Nevertheless, in the case of *LRIG3*, this expression may be meaningful since it has been reported to repess Netrin transcription during inner ear morphogenesis and migration (reference). In this tissue, *LRIG* genes have been shown to display redundant and independent functions which (Del Rio et al., 2013). Interestingly, in the case of LRIG1 and LRIG3 it seems that it exhibits interplay between the two proteins functioning in opposing directions: while LRIG1 negatively regulates ErbB signaling, LRIG3 does the contrary and stabilizes

ErbB receptors at the membrane (Rafidi et al., 2013). In our expression analysis during development *LRIG1* and *LRIG3* are not coexpressed suggesting that this interplay is not active in this tissue.

5.7 Expression pattern of *LINGO1* and its biological implications

LINGO1 is another candidate that showed an interesting expression pattern in the developing mouse brain and which I performed further characterization in human samples. In mouse, *LINGO1* expression becomes wider during development and labels cells in CP (extending toward the pyriform cortex and hippocampus), in the SP and in regions of the basal ganglia (cp/striatum, ventral thalamus and AAA). This expression pattern together with the co-localization between *LINGO1* and CTIP2 in the mouse and human cortex suggest a role of *LINGO1* mainly in neuron differentiation/migration or controlling, in a non-cell autonomous manner, the migration of interneurons or axons (for instance in the basal ganglia). LINGO1 has been traditionally studied in the adult nervous system because it is upregulated during nerve injury and because it has been thought to be one of the key molecules that prevent nerve regeneration (reference). LINGO1 form a multimeric complex with Nogo receptor (NgR1) and the neurotrophin recepor p75 conferring responsiveness to oligodendrocyte myelin glycoprotein and triggering axon retraction through the activation of the RhoA pathway (Mi et al., 2004). My ISH study shows a clear expression of LINGO1 during development yet the function of LINGO1 in these stages is less clear. It could be important for the oligodendrocyte differentiation and myelination of developing axons (Jepson et al., 2012; Mi et al., 2005) which could fit with the increase in the expression levels of the gene during development. On the other hand, recent findings have demonstrated that Nogo-A, through the receptor complex NgR-p75-LINGO1, regulates excitatory neuron migration in the developing cortex which will fit the expression pattern of LINGO1 I have detected in the CP (Mathis et al., 2010).

6. CONCLUSIONS AND OUTLOOK

6. CONCLUSIONS AND OUTLOOK

To date, the biological functions and mechanisms of eLRR-TM containing genes in nervous system development has just begun to be discovered and understood. In the present thesis work I have performed an ISH screening for several of these genes. In mouse this work covers more stages of development and thus more dynamics of change in expression during development however in human due to low accessibility to human samples the data acquired is more preliminary. In conclusion, the current work has shown the graded expression for most of these genes in the developing mouse and human nervous system and thus confirms our original hypothesis that they are important candidates to regulate nervous system development and connectivity. The information available in publications and in public databases about the expression pattern of these genes is uncompleted, especially during development. So, for most of the genes that I have analyzed, the information provided in the present work is novel and for others the systematic approach has provided a better view of how their expression change during development. I believe that the results of my screening will greatly facilitate the interpretation of the in vivo role of the eLRR-TM encoding genes analyzed in this work. And since they are transmembrane proteins, my results will not only provide clues about the cell autonomous functions of these genes, but also about possible non-cell autonomous functions, when they interact with opposing cells via other receptors.

Highlighted conclusions:

1) *ISLR2* is widely expressed in the developing brain including cortex (CP), hippocampus, dorsal thalamus, TRN and basal ganglia (not in GP). The areas of expression affect mainly differentiating fields (co-localization with DCX and beta-III tubulin) and do not include in most of the cases, the proliferative areas (no co-localization with Sox2).

2) ISLR2 colocalizes with CTIP2 in de developing cortex and also labels fibers in the IZ. The no expression in the ventral thalamus (major source of TCAs) suggests that ISLR2 is mainly expressed in corticofugal axons that could also regulate TCA projections as well by the "hand-shake" hypothesis in a non-cell autonomous manner.

3) In human developing cortex, as in the mouse, ISLR2 is expressed in the CP and colocalizes with CTIP2. In contrast to mouse, some scattered cells positive for ISLR2 are found in deeper layers (SP/IZ, OSVZ/ISVZ). This difference could be important for the mechanisms underlying the human cortical expansion.

4) ISLR2 colocalizes with SATB2 also in developing cortex suggesting a role in callosal projection and subcortical connections.

5) Both, in heterologous transfected cells as well as in neuronal tissue, ISLR2 is localized at the membrane as well as in intracellular compartments suggesting that its displays a complex signalling mechanism within the cell.

6) *FLRTs* are expressed during development in overlapping and independent patterns suggestive of redundant and specific functions. Expression of *FLRT2* and *FLRT3* in basal ganglia is suggestive of novel functions of these proteins in interneuron migration and/or axon guidance of the internal capsule axons (corticofugal and TCAs). Indeed *FLRT2* territory in LGE forms a corridor of no *FLRT2* expression that can be meaningful for these functions.

7) Expression of *LRIG1* was highly specific for the VZ while other two members were more widespread. This suggests that *LRIG1* might be involved in cell proliferation, cell fate or early migration of progenitors in the developing cortex.

8) *NLRR3* expression is upregulated during development and is expressed strongly in several places including thalamus, upper part of CP, VZ/SVZ region in the cortex and basal ganglia. The specific pattern and the multiple places where *NLRR3* is expressed suggest a multifunctional role of this gene in nervous system development.

Conclusions

Les funcions biològiques i els mecanismes d'acció dels gens que codifiquen per eLRR-TMs en el desenvolupament del sistema nerviós s'estan començant a descobrir i entendre. En el treball actual de tesi doctoral he portat a terme un *screening* per hibridació in situ per un grup d'aquests gens. En ratolí, aquest treball ha premés estudiar la dinàmica dels canvis d'expressió gènica durant varis estadis de desenvolupament; mentre que en humans i degut al limitat accés de mostres, l'estudi s'ha centrat en nomes un o dos estadis amb lo qual la informació obtinguda es preliminar. En conclusió, aquest treball ha mostrat expressió en el desenvolupament del cervell, tant humà com de ratolí, de la majoria dels gens seleccionats i per tant confirma la nostra hipòtesi original que aquests gens poden ser important candidats per regular la formació del sistema nerviós i la seva connectivitat. La informació de la que disposem en publicacions i dades de bases públiques sobre l'expressió gènica del gens eLRR-TM és incompleta, especialment durant el desenvolupament. Així, per la gran majoria dels gens que he analitzat, la informació obtinguda en aquest treball es nova i, per altres, l'aproximació sistemàtica que s'ha seguit ha permès tenir una millor idea de com la seva expressió gènica varia durant el desenvolupament. Crec que els resultats del meu treball seran de gran ajuda per interpretar el paper in vivo dels gens eLRR-TMs analitzats. I com l'anàlisi s'ha centrat en proteïnes transmembrana, els meus resultats no nomes aporten dades sobre la funció Autónomo dels gens estudiats, sinó també sobre possibles funcions no autònomes en las que les proteïnes eLRR-TMs actuen com a lligands en cèl·lules oposades.

Conclusions destacades:

1) *ISLR2* s'expressa de manera bastant general en el cervell en desenvolupament incloent l'escorça (CP), hipocamp, talem dorsal, TRN i ganglis basals (no in GP). Les àrees d'expressió afectades són principalment de diferenciació (co-localització amb DCX i beta-III tubulina) i no inclouen, en la majoria dels casos, àrees proliferatives (marcades amb Sox2).

2) ISLR2 co-localitza amb CTIP2 en l'escorça durant el desenvolupament i també marca fibres en la IZ. La no expressió en el talem ventral (d'on provenen la majoria de TCAs) suggereix que ISLR2 s'expressa principalment en axons corticofugals que poden també

regular les projeccions TCA mitjançant la hipòtesi "hand-shake" d'una manera no autònoma.

3) En l'escorça humana, com en ratolí, ISLR2 s'expressa en el CP i co-localitza amb CTIP2. A diferència del ratolí, algunes cèl·lules positives per ISLR2 es troben també en capes més profundes (SP/IZ, OSVZ/ISVZ). Aquesta diferencia potser rellevant en els mecanismes d'expansió cortical que te lloc en humans.

4) ISLR2 co-localitza amb SATB2 suggerint una funció en les projeccions del cos callós i conneccions subcorticals.

5) En cèl·lules heteròlogues transfectades i teixit neuronal, ISLR2 es localitza en la membrana plasmàtica així com en compartiments intracel·lulars suggerint que els mecanismes de senyalització són complexos.

6) El patró d'expressió de *FLRTs* mostra àrees on es solapen i àrees on s'expressen independentment el que suggereix que poden tenir funcions redundants i específiques. L'expressió de *FLRT2* i *FLRT3* en els ganglis basals suggereix noves funcions d'aquestes proteïnes en la migració d'interneurones i/o la guia axonal del axons de la capsula interna (corticofugals i TCAs). De fet el territori d'expressió de *FLRT2* en LGE forma un "passadís" de no expressió de *FLRT2* que pot ser significativa per aquestes funcions.

7) L'expressió de *LRIG1* es bastant específica i restringida al VZ mentre que els altres dos membres de la família mostren un patró d'expressió molt més ampli. Això suggereix que *LRIG1* pot estar implicat en la proliferació, la diferenciació i/o la migració primerenca dels progenitors neuronals.

8) L'expressió d'*NLRR3* està augmentada durant el desenvolupament i s'expressa de manera bastant clara en vàries regions incloent el tàlem, la part superficial del CP, la regió VZ/SVZ de l'escorça i els ganglis basals. Un patró tant específic i en regions tant diferents fa pensaren un paper multifuncional d'NLRR3 durant el desenvolupament del sistema nerviós.

Outlook

The expression screening performed in this thesis work was intended to identify genes encoding for eLRR-TM proteins that could play a role in nervous system development and connectivity and analyzed further by functional assays, in vitro and in vivo. Among the genes analyzed and according to their expression pattern I found interesting to study the functions of, specially, *ISLR2*, *NLRR3* and *LRIG1*. Also *FLRTs* are interesting genes and I have revealed expression patterns that were unnoticed before which indicate possible new functions in vivo. For FLRTs, our group has already a good battery of tools (including knock-out animals) to address their function in vivo, to which our group has already made important contributions during the last past years. My expression analysis has revealed interesting expression patterns of *FLRT2* and *FLRT3* in the basal ganglia and in the neocortex that may be relevant for interneuron tangential migration. This hypothesis is currently being addressed by members of our group.

For ISLR2, its expression patterns suggest a role in the neocortex in the projection of corticofugal axons that may affect as well, non-cell autonomously, the TCAs projections. Indeed, knock-out animals for ISLR2 display severe defects in the formation of the internal capsule that would fit with this hypothesis (David Ginty, personal communication). It would be interesting as well to use these *ISLR2* deficient mice and study its role in vivo in interneuron migration in cortex expansion and its co-expression with more specific layer markers to identify the neurons labelled by it since all the expression areas labelled by ISLR2 are nuclei for neuron migration. Screening of specific ligands is also required to further understand the working mechanism of ISLR2.

LRIG1 regulation of proliferation and its tumour suppressor function in vivo opens the possibility that it might be important for nervous system development as suggested by my expression pattern. So, LRIG1 putative role in neurogenesis, proliferation, early migration or cell fate of neurons during development is interesting for future investigation in genetically modified mouse models (already available).

Finally, another interesting candidate to study further according to my expression pattern is NLRR3 which showed a very specific expression pattern and was upregulated with development suggesting as well a role in neurogenesis during later stages of

development. NLRR3's known role in regeneration also requires more study seeing its extensive expression and to find its possible therapeutic use.

7. ANNEXURE

7. Annexure

7.1 Gain and loss-of-function approaches to study the function of ISLR2 and NLRR3

In order to study the function of ISLR2 and NLRR3 I took a loss-of-function approach (by downregulation) of *ISLR2* and *NLRR3* by specific shRNAs as well as a gain-of-function approach (by overexpression) where I cloned ISLR2 and NLRR3 full-length cDNAs obtained from Source Bioscience Lifesciences with IMAGE ID 6826287 and 5698788 respectively into pCAGIG vector. I used the RNAi consortium, Public TRC Portal website to design shRNAs and cloned five shRNAs for each gene. After cloning these shRNAs into FSV-si vector (provided by Dr. Mario Encinas) I performed lentivirus production and tested their knock-down efficiency by transient overexpression of ISLR2 and NLRR3 full length cDNAs Myc tagged (in pcDNA3) in HEK 293 cells that have been previously transduced with lentiviruses with the different shRNAs (Figure 79). Hence we selected two shRNAs each for ISLR2 and NLRR3 at the end with highest knockdown efficiency. Lentiviral vectors were prepared to assess the role of ISLR2 and NLRR3 down regulation in neuron differentiation in dissociated neuron cultures. The selected shRNAs were shuttled into a different vector pCA-β-EGFPm5 silencer3, 5605bp (kind gift from Dr.Matthieu Vermeren), in order to assess the function of ISLR2 and NLRR3 in vivo by ex vivo electroporation (see below).

7.2 Optimisation of *ex-vivo* Electroporation

I also optimised *ex vivo* electroporation to study role of ISLR2 and NLRR3 in neuronal migration during cortex development in an *in vivo* context. I electroporated cortical area of mouse embryo at E14.5 stage with empty pCAGIG vector as per the standard protocol. Figure 80 shows the results of an ex vivo electroporation with the empty pCAGIG vector. Since both ISLR2 and NLRR3 are expressed during mouse cortex development, my aim was to apply this technique to knock down any of these genes with the most efficient sequences selected in HEK293T cells to see any effect in

migration caused by ISLR2 and NLRR3 down regulation during development. After Electroporation I performed Brain slice culture of this Electroporated brains and cultured for 48 hours. After 48 hours I did Imunofluorescence to visualise GFP expressing neurons and their migration in neocortex.

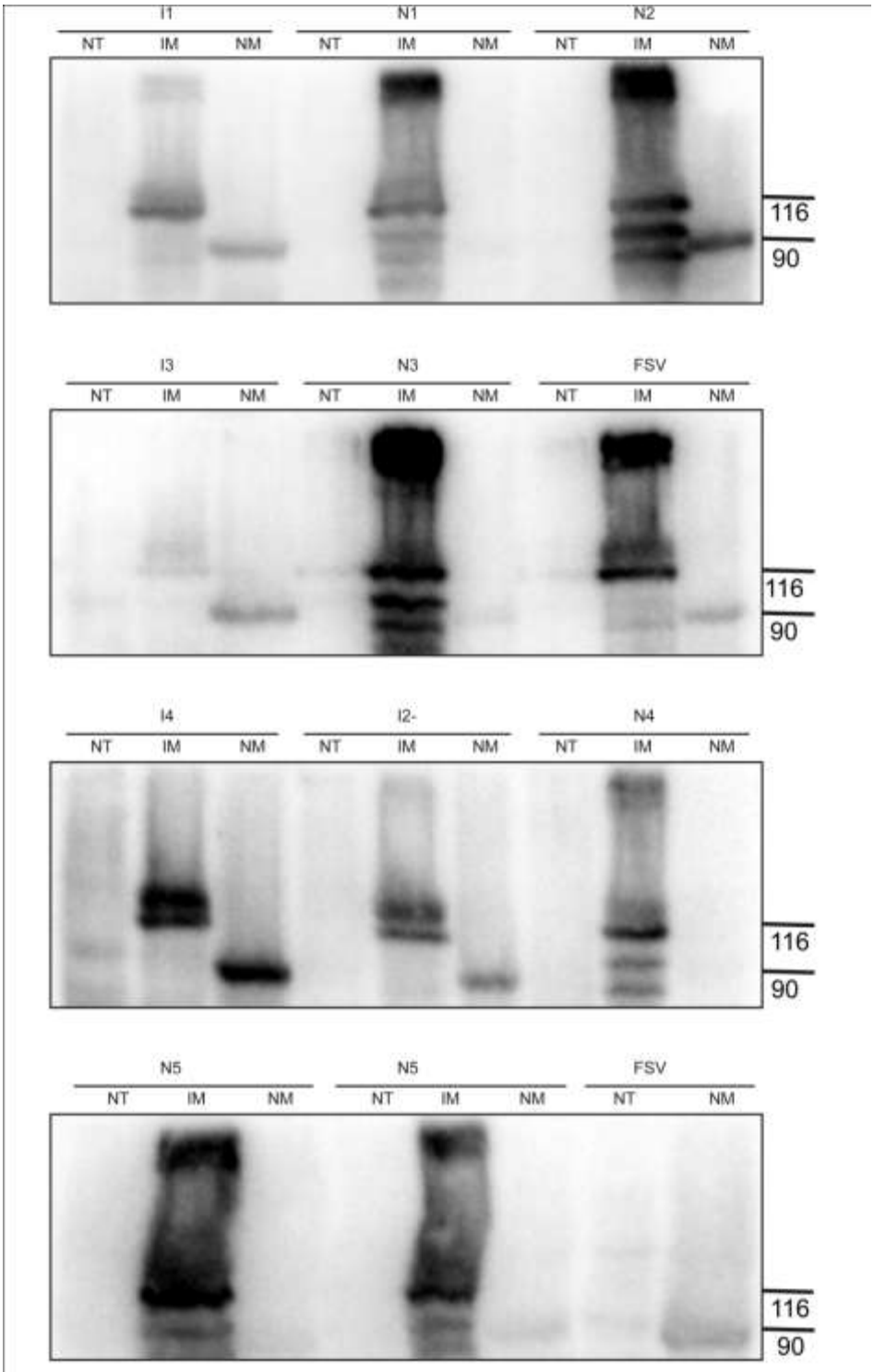


Figure 79. Knockdown efficiency of shRNAs. HEK 293T cells were infected with shRNA(I1-I4 and N1-N5) for 48 hours followed by transfection with ISLR2 Myc tagged, NLRR3 Myc tagged and Non transfected. Samples were analysed by westren blot with anti-Myc antibody. Results show efficient knockdown for four NLRR3 shRNA except for N2. Among ISLR2 shRNA only I3 and I1 doesn't show complete knockdown but change in expression of bands is prominent. Abbreviations: FSV, FSV-si vector; IM, ISLR2-Myc; I1-I4, shRNAs for ISLR2, NM, NLRR3-Myc; N1-N5, shRNAs for NLRR3; NT, Non transfected.

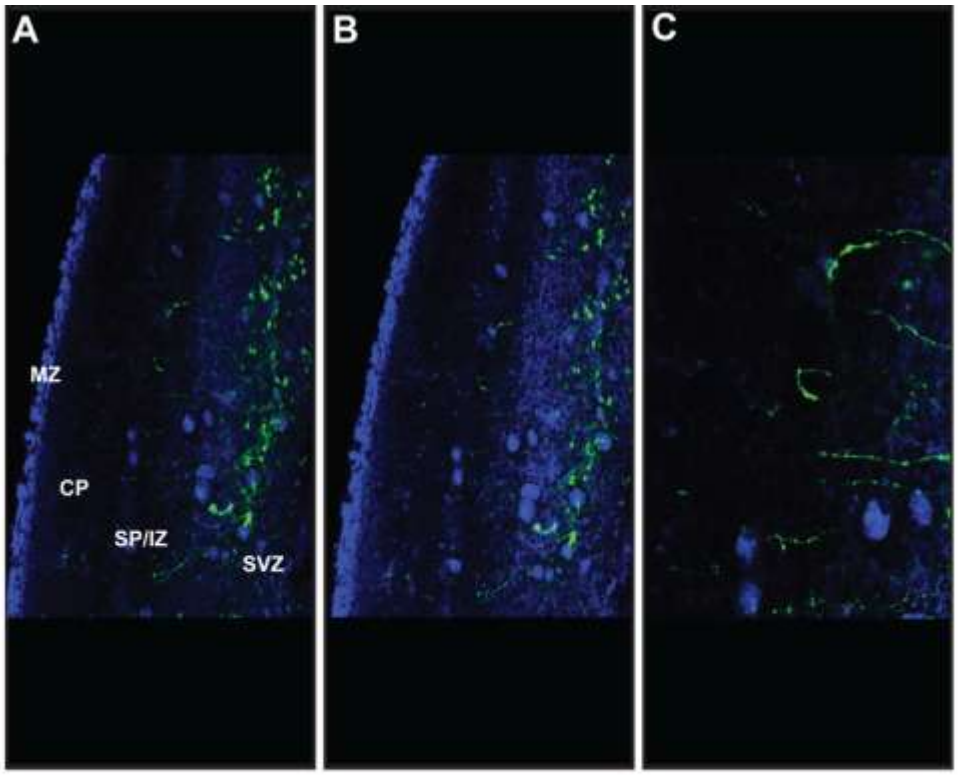


Figure 80. Expression of neurons in ex vivo electroporated cortex of mouse brain at E14.5 stage and performing Immunofluorescence after 48 hours brain slice culture stained with DAPI and anti-GFP (green), showing overexpression of pCAGIG vector and migration at different layers of cortex. A-B shows region of two cortical coronal sections, C shows higher magnified view of section A.

8. PUBLICATIONS AND MEETINGS

8. PUBLICATIONS AND MEETINGS

Analysis of the expression pattern of transmembrane proteins with extracellular leucine rich repeats (eLRRs) in the developing nervous system

Disha Chauhan, Pau Marfull, Sunsaku Homma, Carme Espinet and Joaquim Egea

Manuscript in preparation

9th FENS Forum of Neuroscience, Milan Italy, July 2014.

9. REFERENCES

9. REFERENCES

Abelson JF, Kwan KY, O'Roak BJ, Baek DY, Stillman AA, Morgan TM, Mathews CA, Pauls DL, Rasin M-R, Gunel M, et al (2005) Sequence variants in SLITRK1 are associated with Tourette's syndrome. *Science***310**: 317–20

Adams NC, Tomoda T, Cooper M, Dietz G, Hatten ME (2002) Mice that lack astrotactin have slowed neuronal migration. *Development***129**: 965–72

Ahmed Z, Douglas MR, John G, Berry M, Logan A (2013) AMIGO3 is an NgR1/p75 co-receptor signalling axon growth inhibition in the acute phase of adult central nervous system injury. *PLoS One***8**: e61878

Akins MR, Biederer T (2006) Cell-cell interactions in synaptogenesis. *Curr Opin Neurobiol***16**: 83–9

Alcamo EA, Chirivella L, Dautzenberg M, Dobreva G, Fariñas I, Grosschedl R, McConnell SK (2008) Satb2 regulates callosal projection neuron identity in the developing cerebral cortex. *Neuron***57**: 364–77

Allendoerfer KL, Shatz CJ (1994) The subplate, a transient neocortical structure: its role in the development of connections between thalamus and cortex. *Annu Rev Neurosci***17**: 185–218

Anderson SA, Eisenstat DD, Shi L, Rubenstein JL (1997) Interneuron migration from basal forebrain to neocortex: dependence on Dlx genes. *Science***278**: 474–6

Anderson SA, Marín O, Horn C, Jennings K, Rubenstein JL (2001) Distinct cortical migrations from the medial and lateral ganglionic eminences. *Development***128**: 353–63

Andrews W, Liapi A, Plachez C, Camurri L, Zhang J, Mori S, Murakami F, Parnavelas JG, Sundaresan V, Richards LJ (2006) Robo1 regulates the development of major axon tracts and interneuron migration in the forebrain. *Development***133**: 2243–52

Ang ESBC, Haydar TF, Gluncic V, Rakic P (2003) Four-dimensional migratory coordinates of GABAergic interneurons in the developing mouse cortex. *J Neurosci***23**: 5805–15

Angevine JB, Sidman RL (1961) Autoradiographic study of cell migration during histogenesis of cerebral cortex in the mouse. *Nature***192**: 766–8

Anton ES, Cameron RS, Rakic P (1996) Role of neuron-glial junctional domain proteins in the maintenance and termination of neuronal migration across the embryonic cerebral wall. *J Neurosci***16**: 2283–93

Anton ES, Kreidberg JA, Rakic P (1999) Distinct functions of alpha3 and alpha(v) integrin receptors in neuronal migration and laminar organization of the cerebral cortex. *Neuron***22**: 277–89

Anton ES, Marchionni MA, Lee KF, Rakic P (1997) Role of GGF/neuregulin signaling in interactions between migrating neurons and radial glia in the developing cerebral cortex. *Development***124**: 3501–10

Arlotta P, Molyneaux BJ, Chen J, Inoue J, Kominami R, Macklis JD (2005) Neuronal subtype-specific genes that control corticospinal motor neuron development in vivo. *Neuron***45**: 207–21

Arnold SJ, Huang G, Cheung AFP, Era T, Nishikawa S, Bikoff EK, Molnár Z, Robertson EJ, Groszer M (2008) The T-box transcription factor Eomes/Tbr2 regulates neurogenesis in the cortical subventricular zone. *Genes Dev***22**: 2479–84

Assadi AH, Zhang G, Beffert U, McNeil RS, Renfro AL, Niu S, Quattrocchi CC, Antalffy BA, Sheldon M, Armstrong DD, et al (2003) Interaction of reelin signaling and Lis1 in brain development. *Nat Genet***35**: 270–6

Austin CP, Cepko CL (1990) Cellular migration patterns in the developing mouse cerebral cortex. *Development***110**: 713–32

Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R (2003) Multipotent cell lineages in early mouse development depend on SOX2 function. *Genes Dev***17**: 126–40

Baala L, Briault S, Etchevers HC, Laumonnier F, Natiq A, Amiel J, Boddaert N, Picard C, Sbiti A, Asermouh A, et al (2007) Homozygous silencing of T-box transcription factor EOMES leads to microcephaly with polymicrogyria and corpus callosum agenesis. *Nat Genet***39**: 454–6

Bai J, Ramos RL, Ackman JB, Thomas AM, Lee R V, LoTurco JJ (2003) RNAi reveals doublecortin is required for radial migration in rat neocortex. *Nat Neurosci***6**: 1277–83

Bani-Yaghoub M, Tremblay RG, Lei JX, Zhang D, Zurakowski B, Sandhu JK, Smith B, Ribecco-Lutkiewicz M, Kennedy J, Walker PR, et al (2006) Role of Sox2 in the development of the mouse neocortex. *Dev Biol***295**: 52–66

Barrette B, Vallières N, Dubé M, Lacroix S (2007) Expression profile of receptors for myelin-associated inhibitors of axonal regeneration in the intact and injured mouse central nervous system. *Mol Cell Neurosci***34**: 519–38

Battye R, Stevens A, Jacobs JR (1999) Axon repulsion from the midline of the Drosophila CNS requires slit function. *Development***126**: 2475–81

Bayatti N, Moss JA, Sun L, Ambrose P, Ward JFH, Lindsay S, Clowry GJ (2008) A molecular neuroanatomical study of the developing human neocortex from 8 to

17 postconceptional weeks revealing the early differentiation of the subplate and subventricular zone. *Cereb Cortex***18**: 1536–48

Bayer S a, Altman J, Russo RJ, Dai XF, Simmons J a (1991) Cell migration in the rat embryonic neocortex. *J Comp Neurol***307**: 499–516

Beaubien F, Cloutier J-F (2009) Differential expression of Slitrk family members in the mouse nervous system. *Dev Dyn***238**: 3285–96

Behar TN, Li YX, Tran HT, Ma W, Dunlap V, Scott C, Barker JL (1996) GABA stimulates chemotaxis and chemokinesis of embryonic cortical neurons via calcium-dependent mechanisms. *J Neurosci***16**: 1808–18

Behar TN, Scott CA, Greene CL, Wen X, Smith S V, Maric D, Liu QY, Colton CA, Barker JL (1999) Glutamate acting at NMDA receptors stimulates embryonic cortical neuronal migration. *J Neurosci***19**: 4449–61

Behar TN, Smith S V, Kennedy RT, McKenzie JM, Maric I, Barker JL (2001) GABA(B) receptors mediate motility signals for migrating embryonic cortical cells. *Cereb Cortex***11**: 744–53

Bellion A, Baudoin J-P, Alvarez C, Bornens M, Métin C (2005) Nucleokinesis in tangentially migrating neurons comprises two alternating phases: forward migration of the Golgi/centrosome associated with centrosome splitting and myosin contraction at the rear. *J Neurosci***25**: 5691–9

Belvindrah R, Graus-Porta D, Goebbels S, Nave K-A, Müller U (2007) Beta1 integrins in radial glia but not in migrating neurons are essential for the formation of cell layers in the cerebral cortex. *J Neurosci***27**: 13854–65

Bielle F, Griveau A, Narboux-Nême N, Vigneau S, Sigrist M, Arber S, Wassef M, Pierani A (2005) Multiple origins of Cajal-Retzius cells at the borders of the developing pallium. *Nat Neurosci***8**: 1002–1012

Borrell V, Cárdenas A, Ciceri G, Galcerán J, Flames N, Pla R, Nóbrega-Pereira S, García-Frigola C, Peregrín S, Zhao Z, et al (2012) Slit/Robo signaling modulates the proliferation of central nervous system progenitors. *Neuron***76**: 338–52

Borrell V, Marín O (2006) Meninges control tangential migration of hem-derived Cajal-Retzius cells via CXCL12/CXCR4 signaling. *Nat Neurosci***9**: 1284–93

Bouvrée K, Larrivée B, Lv X, Yuan L, DeLafarge B, Freitas C, Mathivet T, Bréant C, Tessier-Lavigne M, Bikfalvi A, et al (2008) Netrin-1 inhibits sprouting angiogenesis in developing avian embryos. *Dev Biol***318**: 172–83

Britanova O, de Juan Romero C, Cheung A, Kwan KY, Schwark M, Gyorgy A, Vogel T, Akopov S, Mitkovski M, Agoston D, et al (2008) Satb2 is a postmitotic determinant for upper-layer neuron specification in the neocortex. *Neuron***57**: 378–92

Britto JM, Johnston LA, Tan S-S (2009) The stochastic search dynamics of interneuron migration. *Biophys J***97**: 699–709

Buchanan SGSC, Gay NJ (1996) Structural and functional diversity in the leucine-rich repeat family of proteins. *Prog Biophys Mol Biol***65**: 1–44

Budel S, Padukkavidana T, Liu BP, Feng Z, Hu F, Johnson S, Lauren J, Park JH, McGee AW, Liao J, et al (2008) Genetic variants of Nogo-66 receptor with possible association to schizophrenia block myelin inhibition of axon growth. *J Neurosci***28**: 13161–72

Bulfone A, Martinez S, Marigo V, Campanella M, Basile A, Quaderi N, Gattuso C, Rubenstein JLR, Ballabio A (1999) Expression pattern of the Tbr2 (Eomesodermin) gene during mouse and chick brain development. *Mech Dev***84**: 133–138

Bystron I, Blakemore C, Rakic P (2008) Development of the human cerebral cortex: Boulder Committee revisited. *Nat Rev Neurosci***9**: 110–22

Bystron I, Rakic P, Molnár Z, Blakemore C (2006) The first neurons of the human cerebral cortex. *Nat Neurosci***9**: 880–6

Cáceres M, Lachuer J, Zapala MA, Redmond JC, Kudo L, Geschwind DH, Lockhart DJ, Preuss TM, Barlow C (2003) Elevated gene expression levels distinguish human from non-human primate brains. *Proc Natl Acad Sci U S A***100**: 13030–5

Carić D, Gooday D, Hill RE, McConnell SK, Price DJ (1997) Determination of the migratory capacity of embryonic cortical cells lacking the transcription factor Pax-6. *Development***124**: 5087–96

Carim-Todd L, Escarceller M, Estivill X, Sumoy L (2003) LRRN6A/LERN1 (leucine-rich repeat neuronal protein 1), a novel gene with enriched expression in limbic system and neocortex. *Eur J Neurosci***18**: 3167–82

De Carlos JA, López-Mascaraque L, Valverde F (1996) Dynamics of cell migration from the lateral ganglionic eminence in the rat. *J Neurosci***16**: 6146–56

Catalano SM (1998) Activity-Dependent Cortical Target Selection by Thalamic Axons. *Science (80-)***281**: 559–562

Catena R, Tiveron C, Ronchi A, Porta S, Ferri A, Tatangelo L, Cavallaro M, Favaro R, Ottolenghi S, Reinbold R, et al (2004) Conserved POU binding DNA sites in the Sox2 upstream enhancer regulate gene expression in embryonic and neural stem cells. *J Biol Chem***279**: 41846–57

Caviness VS (1976) Patterns of cell and fiber distribution in the neocortex of the reeler mutant mouse. *J Comp Neurol***170**: 435–47

Caviness VS (1982) Neocortical histogenesis in normal and reeler mice: a developmental study based upon [3H]thymidine autoradiography. *Brain Res***256**: 293–302

Caviness VS (1992) Kallmann's syndrome--beyond "migration". *N Engl J Med***326**: 1775–7

Caviness VS, Sidman RL (1973) Time of origin or corresponding cell classes in the cerebral cortex of normal and reeler mutant mice: an autoradiographic analysis. *J Comp Neurol***148**: 141–51

Chae T, Kwon YT, Bronson R, Dikkes P, Li E, Tsai LH (1997) Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. *Neuron***18**: 29–42

Chen G, Sima J, Jin M, Wang K-Y, Xue X-J, Zheng W, Ding Y-Q, Yuan X-B (2008) Semaphorin-3A guides radial migration of cortical neurons during development. *Nat Neurosci***11**: 36–44

Chen Y, Aulia S, Li L, Tang BL (2006) AMIGO and friends: an emerging family of brain-enriched, neuronal growth modulating, type I transmembrane proteins with leucine-rich repeats (LRR) and cell adhesion molecule motifs. *Brain Res Rev***51**: 265–74

Chen Y, Hor HH, Tang BL (2012) AMIGO is expressed in multiple brain cell types and may regulate dendritic growth and neuronal survival. *J Cell Physiol***227**: 2217–29

Cooper J a (2013) Cell biology in neuroscience: mechanisms of cell migration in the nervous system. *J Cell Biol***202**: 725–734

Corbin JG, Nery S, Fishell G (2001) Telencephalic cells take a tangent: non-radial migration in the mammalian forebrain. *Nat Neurosci***4 Suppl**: 1177–82

Cousin B, Leloup C, Pénicaud L, Price J (1997) Developmental changes in integrin beta-subunits in rat cerebral cortex. *Neurosci Lett***234**: 161–5

Crandall JE, McCarthy DM, Araki KY, Sims JR, Ren J-Q, Bhide PG (2007) Dopamine receptor activation modulates GABA neuron migration from the basal forebrain to the cerebral cortex. *J Neurosci***27**: 3813–22

Cuzon VC, Yeh PW, Cheng Q, Yeh HH (2006) Ambient GABA promotes cortical entry of tangentially migrating cells derived from the medial ganglionic eminence. *Cereb Cortex***16**: 1377–88

D'Arcangelo G, Homayouni R, Keshvara L, Rice DS, Sheldon M, Curran T (1999) Reelin is a ligand for lipoprotein receptors. *Neuron***24**: 471–9

D'Arcangelo G, Miao GG, Chen SC, Soares HD, Morgan JI, Curran T (1995) A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature***374**: 719–23

Dehay C, Kennedy H (2007) Cell-cycle control and cortical development. *Nat Rev Neurosci***8**: 438–450

Denaxa M, Chan CH, Schachner M, Parnavelas JG, Karagogeos D (2001) The adhesion molecule TAG-1 mediates the migration of cortical interneurons from the ganglionic eminence along the corticofugal fiber system. *Development***128**: 4635–44

Denter DG, Heck N, Riedemann T, White R, Kilb W, Luhmann HJ (2010) GABAC receptors are functionally expressed in the intermediate zone and regulate radial migration in the embryonic mouse neocortex. *Neuroscience***167**: 124–134

Dickson BJ (2002) Molecular mechanisms of axon guidance. *Science***298**: 1959–1964

Dickson BJ, Keleman K (2002) Netrins. *Curr Biol***12**: R154–5

Dobyns WB, Truwit CL (1995) Lissencephaly and other malformations of cortical development: 1995 update. *Neuropediatrics***26**: 132–147

Dolan J, Walshe K, Alsbury S, Hokamp K, O'Keeffe S, Okafuji T, Miller SFC, Tear G, Mitchell KJ (2007) The extracellular leucine-rich repeat superfamily; a comparative survey and analysis of evolutionary relationships and expression patterns. *BMC Genomics***8**: 320

Dudanova I, Klein R (2013) Integration of guidance cues: parallel signaling and crosstalk. *Trends Neurosci***36**: 295–304

Dulabon L, Olson EC, Taglienti MG, Eisenhuth S, McGrath B, Walsh CA, Kreidberg JA, Anton ES (2000) Reelin binds alpha3beta1 integrin and inhibits neuronal migration. *Neuron***27**: 33–44

Van Eden CG, Mrzljak L, Voorn P, Uylings HB (1989) Prenatal development of GABA-ergic neurons in the neocortex of the rat. *J Comp Neurol***289**: 213–27

Edmondson JC, Liem RK, Kuster JE, Hatten ME (1988) Astrotactin: a novel neuronal cell surface antigen that mediates neuron-astroglial interactions in cerebellar microcultures. *J Cell Biol***106**: 505–517

Elias LA, Wang DD, Kriegstein AR (2007) Gap junction adhesion is necessary for radial migration in the neocortex. *Nature***448**: 901–907

Elias LAB, Kriegstein AR (2008) Gap junctions: multifaceted regulators of embryonic cortical development. *Trends Neurosci***31**: 243–50

Englund C, Fink A, Lau C, Pham D, Daza RAM, Bulfone A, Kowalczyk T, Hevner RF (2005) Pax6, Tbr2, and Tbr1 are expressed sequentially by radial glia,

intermediate progenitor cells, and postmitotic neurons in developing neocortex. *J Neurosci***25**: 247–51

Enkhbayar P, Kamiya M, Osaki M, Matsumoto T, Matsushima N (2004) Structural principles of leucine-rich repeat (LRR) proteins. *Proteins***54**: 394–403

Falconer DS (1951) Two new mutants, "trembler" and "reeler", with neurological actions in the house mouse (Mus musculus L.). *J Genet***50**: 192–205

Faulkner NE, Dujardin DL, Tai CY, Vaughan KT, O'Connell CB, Wang Y, Vallee RB (2000) A role for the lissencephaly gene LIS1 in mitosis and cytoplasmic dynein function. *Nat Cell Biol***2**: 784–91

Feng Y, Olson EC, Stukenberg PT, Flanagan LA, Kirschner MW, Walsh CA (2000) LIS1 regulates CNS lamination by interacting with mNudE, a central component of the centrosome. *Neuron***28**: 665–679

Fish JL, Dehay C, Kennedy H, Huttner WB (2008) Making bigger brains-the evolution of neural-progenitor-cell division. *J Cell Sci***121**: 2783–93

Flames N, Long JE, Garratt AN, Fischer TM, Gassmann M, Birchmeier C, Lai C, Rubenstein JL, Mar'in O (2004) Short- and long-range attraction of cortical GABAergic interneurons by neuregulin-1. *Neuron***44**: 251–261

Francis F, Koulakoff A, Boucher D, Chafey P, Schaar B, Vinet MC, Friocourt G, McDonnell N, Reiner O, Kahn A, et al (1999) Doublecortin is a developmentally regulated, microtubule-associated protein expressed in migrating and differentiating neurons. *Neuron***23**: 247–56

Friocourt G, Liu JS, Antypa M, Rakic S, Walsh CA, Parnavelas JG (2007) Both doublecortin and doublecortin-like kinase play a role in cortical interneuron migration. *J Neurosci***27**: 3875–83

Frotscher M (2010) Role for Reelin in stabilizing cortical architecture. *Trends Neurosci***33**: 407–14

Fu X, Brown KJ, Yap CC, Winckler B, Jaiswal JK, Liu JS (2013) Doublecortin (Dcx) family proteins regulate filamentous actin structure in developing neurons. *J Neurosci***33**: 709–721

Fukamachi K, Matsuoka Y, Ohno H, Hamaguchi T, Tsuda H (2002) Neuronal leucine-rich repeat protein-3 amplifies MAPK activation by epidermal growth factor through a carboxyl-terminal region containing endocytosis motifs. *J Biol Chem***277**: 43549–52

Geisert EE, Frankfurter A (1989) The neuronal response to injury as visualized by immunostaining of class III beta-tubulin in the rat. *Neurosci Lett***102**: 137–41

Ghosh A, Antonini A, McConnell SK, Shatz CJ (1990) Requirement for subplate neurons in the formation of thalamocortical connections. *Nature***347**: 179–81

Gleeson JG, Allen KM, Fox JW, Lamperti ED, Berkovic S, Scheffer I, Cooper EC, Dobyns WB, Minnerath SR, Ross ME, et al (1998) Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. *Cell***92**: 63–72

Goffinet AM (1979) An early development defect in the cerebral cortex of the reeler mouse. A morphological study leading to a hypothesis concerning the action of the mutant gene. *Anat Embryol (Berl)***157**: 205–16

Götz M, Stoykova A, Gruss P (1998) Pax6 Controls Radial Glia Differentiation in the Cerebral Cortex. *Neuron***21**: 1031–1044

Graus-Porta D, Blaess S, Senften M, Littlewood-Evans A, Damsky C, Huang Z, Orban P, Klein R, Schittny JC, Müller U (2001) Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. *Neuron***31**: 367–379

Gu J, Gu X (2003) Induced gene expression in human brain after the split from chimpanzee. *Trends Genet***19**: 63–5

Gubbay J, Collignon J, Koopman P, Capel B, Economou A, Münsterberg A, Vivian N, Goodfellow P, Lovell-Badge R (1990) A gene mapping to the sex-determining region of the mouse Y chromosome is a member of a novel family of embryonically expressed genes. *Nature***346**: 245–50

Guerrini R, Dobyns WB, Barkovich AJ (2008) Abnormal development of the human cerebral cortex: genetics, functional consequences and treatment options. *Trends Neurosci***31**: 154–62

Gupta A, Tsai L-H, Wynshaw-Boris A (2002) Life is a journey: a genetic look at neocortical development. *Nat Rev Genet***3**: 342–55

Hack I, Bancila M, Loulier K, Carroll P, Cremer H (2002) Reelin is a detachment signal in tangential chain-migration during postnatal neurogenesis. *Nat Neurosci***5**: 939–45

Hamburgh M (1963) Analysis of the postnatal developmental effects of "reeler," a neurological mutation in mice. A study in developmental genetics. *Dev Biol***8**: 165–185

Hanganu IL, Kilb W, Luhmann HJ (2002) Functional synaptic projections onto subplate neurons in neonatal rat somatosensory cortex. *J Neurosci***22**: 7165–76

Hatten ME (1999) Central nervous system neuronal migration. *Annu Rev Neurosci***22**: 511–539

Hatten ME (2002) New directions in neuronal migration. *Science***297**: 1660–3

Haydar TF, Wang F, Schwartz ML, Rakic P (2000) Differential modulation of proliferation in the neocortical ventricular and subventricular zones. *J Neurosci***20**: 5764–74

Heck N, Kilb W, Reiprich P, Kubota H, Furukawa T, Fukuda A, Luhmann HJ (2007) GABA-A receptors regulate neocortical neuronal migration in vitro and in vivo. *Cereb Cortex***17**: 138–48

Heng JI-T, Nguyen L, Castro DS, Zimmer C, Wildner H, Armant O, Skowronska-Krawczyk D, Bedogni F, Matter J-MM, Hevner R, et al (2008) Neurogenin 2 controls cortical neuron migration through regulation of Rnd2. *Nature***455**: 114–8

Herculano-Houzel S (2009) The human brain in numbers: a linearly scaled-up primate brain. *Front Hum Neurosci***3**: 31

Hevner RF, Hodge RD, Daza RAM, Englund C (2006) Transcription factors in glutamatergic neurogenesis: conserved programs in neocortex, cerebellum, and adult hippocampus. *Neurosci Res***55**: 223–33

Hiesberger T, Trommsdorff M, Howell BW, Goffinet A, Mumby MC, Cooper JA, Herz J (1999) Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron***24**: 481–9

Higashi S, Molnár Z, Kurotani T, Toyama K (2002) Prenatal development of neural excitation in rat thalamocortical projections studied by optical recording. *Neuroscience***115**: 1231–1246

Himanen J-P, Nikolov DB (2003) Eph receptors and ephrins. *Int J Biochem Cell Biol***35**: 130–4

Hirotsune S, Fleck MW, Gambello MJ, Bix GJ, Chen A, Clark GD, Ledbetter DH, McBain CJ, Wynshaw-Boris A (1998) Graded reduction of Pafah1b1 (Lis1) activity results in neuronal migration defects and early embryonic lethality. *Nat Genet***19**: 333–9

Hirotsune S, Takahara T, Sasaki N, Hirose K, Yoshiki A, Ohashi T, Kusakabe M, Murakami Y, Muramatsu M, Watanabe S (1995) The reeler gene encodes a protein with an EGF-like motif expressed by pioneer neurons. *Nat Genet***10**: 77–83

Homma S, Shimada T, Hikake T, Yaginuma H (2009) Expression pattern of LRR and Ig domain-containing protein (LRRIG protein) in the early mouse embryo. *Gene Expr Patterns***9**: 1–26

Hong W, Zhu H, Potter CJ, Barsh G, Kurusu M, Zinn K, Luo L (2009) Leucine-rich repeat transmembrane proteins instruct discrete dendrite targeting in an olfactory map. *Nat Neurosci***12**: 1542–50

Horesh D, Sapir T, Francis F, Wolf SG, Caspi M, Elbaum M, Chelly J, Reiner O (1999) Doublecortin, a stabilizer of microtubules. *Hum Mol Genet***8**: 1599–610

Horwitz AR, Parsons JT (1999) Cell migration--movin' on. *Science***286**: 1102–3

Hossain S, Ahmed MU, Alam S, Watanabe A, Harashima A, Yonekura H, Yamamoto H (2012a) Expressions and Roles of AMIGO Gene Family in Vascular Endothelial Cells. *Int J Biosci Biochem Bioinforma***2**: 1–5

Hossain S, Takatori A, Nakamura Y, Suenaga Y, Kamijo T, Nakagawara A (2012b) NLRR1 enhances EGF-mediated MYCN induction in neuroblastoma and accelerates tumor growth in vivo. *Cancer Res***72**: 4587–96

Huang EJ, Reichardt LF (2003) Trk receptors: roles in neuronal signal transduction. *Annu Rev Biochem***72**: 609–42

Ishii N, Wanaka A, Tohyama M (1996) Increased expression of NLRR-3 mRNA after cortical brain injury in mouse. *Mol Brain Res***40**: 148–152

Jacobs EC, Campagnoni C, Kampf K, Reyes SD, Kalra V, Handley V, Xie Y-Y, Hong-Hu Y, Spreur V, Fisher RS, et al (2007) Visualization of corticofugal projections during early cortical development in a tau-GFP-transgenic mouse. *Eur J Neurosci***25**: 17–30

Johnson MB, Kawasawa YI, Mason CE, Krsnik Z, Coppola G, Bogdanović D, Geschwind DH, Mane SM, State MW, Sestan N (2009) Functional and evolutionary insights into human brain development through global transcriptome analysis. *Neuron***62**: 494–509

Juliano RL (2002) Signal transduction by cell adhesion receptors and the cytoskeleton: functions of integrins, cadherins, selectins, and immunoglobulin-superfamily members. *Annu Rev Pharmacol Toxicol***42**: 283–323

Kajander T, Kuja-Panula J, Rauvala H, Goldman A (2011) Crystal structure and role of glycans and dimerization in folding of neuronal leucine-rich repeat protein AMIGO-1. *J Mol Biol***413**: 1001–15

Kajava A V (1998) Structural diversity of leucine-rich repeat proteins. *J Mol Biol***277**: 519–27

Kajava A V (2001) Review: proteins with repeated sequence--structural prediction and modeling. *J Struct Biol***134**: 132–44

Kalachikov S, Evgrafov O, Ross B, Winawer M, Barker-Cummings C, Martinelli Boneschi F, Choi C, Morozov P, Das K, Teplitskaya E, et al (2002) Mutations in LGI1 cause autosomal-dominant partial epilepsy with auditory features. *Nat Genet***30**: 335–41

Kamachi Y, Uchikawa M, Kondoh H (2000) Pairing SOX off: with partners in the regulation of embryonic development. *Trends Genet***16**: 182–187

Kanatani S, Yozu M, Tabata H, Nakajima K (2008) COUP-TFII is preferentially expressed in the caudal ganglionic eminence and is involved in the caudal migratory stream. *J Neurosci***28**: 13582–13591

Katayama K, Yamada K, Ornthanalai VG, Inoue T, Ota M, Murphy NP, Aruga J (2010) Slitrk1-deficient mice display elevated anxiety-like behavior and noradrenergic abnormalities. *Mol Psychiatry***15**: 177–84

Kerjan G, Dolan J, Haumaitre C, Schneider-Maunoury S, Fujisawa H, Mitchell KJ, Chédotal A (2005) The transmembrane semaphorin Sema6A controls cerebellar granule cell migration. *Nat Neurosci***8**: 1516–24

Khaitovich P, Kelso J, Franz H, Visagie J, Giger T, Joerchel S, Petzold E, Green RE, Lachmann M, Pääbo S (2006) Functionality of intergenic transcription: an evolutionary comparison. *PLoS Genet***2**: e171

Kidd T, Bland KS, Goodman CS (1999) Slit Is the Midline Repellent for the Robo Receptor in Drosophila. *Cell***96**: 785–794

Kim S, Burette A, Chung HS, Kwon S-K, Woo J, Lee HW, Kim K, Kim H, Weinberg RJ, Kim E (2006) NGL family PSD-95-interacting adhesion molecules regulate excitatory synapse formation. *Nat Neurosci***9**: 1294–301

King MC, Wilson AC (1975) Evolution at two levels in humans and chimpanzees. *Science***188**: 107–16

Kobe B, Deisenhofer J (1995) Proteins with leucine-rich repeats. *Curr Opin Struct Biol***5**: 409–416

Kobe B, Deisenhofer J (1993) Crystallization and preliminary X-ray analysis of porcine ribonuclease inhibitor, a protein with leucine-rich repeats. *J Mol Biol***231**: 137–40

Kobe B, Deisenhofer J (1994) repeat : a versatile binding motif. *Trends Biochem Sci***19**: 415–421

Kobe B, Kajava a V (2001) The leucine-rich repeat as a protein recognition motif. *Curr Opin Struct Biol***11**: 725–732

Kobe B, Kajava A V (2000) When protein folding is simplified to protein coiling: the continuum of solenoid protein structures. *Trends Biochem Sci***25**: 509–15

Kokoeva M V, Yin H, Flier JS (2005) Neurogenesis in the hypothalamus of adult mice: potential role in energy balance. *Science***310**: 679–83

Komuro H, Rakic P (1993) Modulation of neuronal migration by NMDA receptors. *Science (80-)***260**: 95–97

Koopman P (1999) Sry and Sox9: mammalian testis-determining genes. *Cell Mol Life Sci***55**: 839–56

Kornack DR, Rakic P (1995) Radial and horizontal deployment of clonally related cells in the primate neocortex: relationship to distinct mitotic lineages. *Neuron***15**: 311–21

Kriegstein A, Alvarez-Buylla A (2009) The glial nature of embryonic and adult neural stem cells. *Annu Rev Neurosci***32**: 149–84

Kriegstein A, Noctor S, Mart'inez-Cerdeño V, Martínez-Cerdeño V (2006) Patterns of neural stem and progenitor cell division may underlie evolutionary cortical expansion. *Nat Rev Neurosci***7**: 883–90

Kriegstein AR, Noctor SC (2004) Patterns of neuronal migration in the embryonic cortex. *Trends Neurosci***27**: 392–399

Kroll TT, O'Leary DDM (2005) Ventralized dorsal telencephalic progenitors in Pax6 mutant mice generate GABA interneurons of a lateral ganglionic eminence fate. *Proc Natl Acad Sci U S A***102**: 7374–9

Kuja-Panula J, Kiiltomäki M, Yamashiro T, Rouhiainen A, Rauvala H (2003) AMIGO, a transmembrane protein implicated in axon tract development, defines a novel protein family with leucine-rich repeats. *J Cell Biol***160**: 963–73

Lacy SE, Bönnemann CG, Buzney E a, Kunkel LM (1999) Identification of FLRT1, FLRT2, and FLRT3: a novel family of transmembrane leucine-rich repeat proteins. *Genomics***62**: 417–426

Laeremans A, Nys J, Luyten W, D'Hooge R, Paulussen M, Arckens L (2013) AMIGO2 mRNA expression in hippocampal CA2 and CA3a. *Brain Struct Funct***218**: 123–30

Lambert de Rouvroit C, Goffinet AM (2001) Neuronal migration. *Mech Dev***105**: 47–56

Larrivée B, Freitas C, Suchting S, Brunet I, Eichmann A (2009) Guidance of vascular development: lessons from the nervous system. *Circ Res***104**: 428–41

Laudet V, Stehelin D, Clevers H (1993) Ancestry and diversity of the HMG box superfamily. *Nucleic Acids Res***21**: 2493–2501

Laurén J, Airaksinen MS, Saarma M, Timmusk T (2003a) Two novel mammalian nogo receptor homologs differentially expressed in the central and peripheral nervous systems☆. *Mol Cell Neurosci***24**: 581–594

Laurén J, Airaksinen MS, Saarma M, Timmusk T õni. (2003b) A novel gene family encoding leucine-rich repeat transmembrane proteins differentially expressed in the nervous system☆☆Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under Accession Nos. AY182024 (human LRRT. *Genomics***81**: 411–421

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

Le TN, Du G, Fonseca M, Zhou Q-P, Wigle JT, Eisenstat DD (2007) Dlx homeobox genes promote cortical interneuron migration from the basal forebrain by direct repression of the semaphorin receptor neuropilin-2. *J Biol Chem***282**: 19071–81

Lee MK, Rebhun LI, Frankfurter A (1990) Posttranslational modification of class III beta-tubulin. *Proc Natl Acad Sci U S A***87**: 7195–9

Lee X, Yang Z, Shao Z, Rosenberg SS, Levesque M, Pepinsky RB, Qiu M, Miller RH, Chan JR, Mi S (2007) NGF regulates the expression of axonal LINGO-1 to inhibit oligodendrocyte differentiation and myelination. *J Neurosci***27**: 220–5

Leid M, Ishmael JE, Avram D, Shepherd D, Fraulob V, Dollé P (2004) CTIP1 and CTIP2 are differentially expressed during mouse embryogenesis. *Gene Expr Patterns***4**: 733–9

Leyva-Díaz E, del Toro D, Menal MJ, Cambray S, Susín R, Tessier-Lavigne M, Klein R, Egea J, López-Bendito G (2014) FLRT3 is a Robo1-interacting protein that determines Netrin-1 attraction in developing axons. *Curr Biol***24**: 494–508

Li G, Adesnik H, Li J, Long J, Nicoll RA, Rubenstein JLR, Pleasure SJ (2008) Regional distribution of cortical interneurons and development of inhibitory tone are regulated by Cxcl12/Cxcr4 signaling. *J Neurosci***28**: 1085–98

Li H, Chou S-J, Hamasaki T, Perez-Garcia CG, O'Leary DDM (2012) Neuregulin repellent signaling via ErbB4 restricts GABAergic interneurons to migratory paths from ganglionic eminence to cortical destinations. *Neural Dev***7**: 10

Lichtman JW, Smith SJ (2008) Seeing circuits assemble. *Neuron***60**: 441–8

Llorens F, Gil V, Iraola S, Carim-Todd L, Martí E, Estivill X, Soriano E, del Rio JA, Sumoy L (2008) Developmental analysis of Lingo-1/Lern1 protein expression in the mouse brain: interaction of its intracellular domain with Myt1l. *Dev Neurobiol***68**: 521–41

López-Bendito G, Cautinat A, Sánchez JA, Bielle F, Flames N, Garratt AN, Talmage DA, Role LW, Charnay P, Marín O, et al (2006) Tangential neuronal migration controls axon guidance: a role for neuregulin-1 in thalamocortical axon navigation. *Cell***125**: 127–42

López-Bendito G, Luján R, Shigemoto R, Ganter P, Paulsen O, Molnár Z (2003) Blockade of GABA(B) receptors alters the tangential migration of cortical neurons. *Cereb Cortex***13**: 932–42

López-Bendito G, Sánchez-Alcañiz JA, Pla R, Borrell V, Picó E, Valdeolmillos M, Marín O (2008) Chemokine signaling controls intracortical migration and final distribution of GABAergic interneurons. *J Neurosci***28**: 1613–24

Lozsádi DA, Gonzalez-Soriano J, Guillery RW (1996) The course and termination of corticothalamic fibres arising in the visual cortex of the rat. *Eur J Neurosci***8**: 2416–27

Lui JH, Hansen D V, Kriegstein AR (2011) Development and evolution of the human neocortex. *Cell***146**: 18–36

Lukaszewicz A, Savatier P, Cortay V, Giroud P, Huissoud C, Berland M, Kennedy H, Dehay C (2005) G1 phase regulation, area-specific cell cycle control, and cytoarchitectonics in the primate cortex. *Neuron***47**: 353–64

Magdaleno S, Keshvara L, Curran T (2002) Rescue of ataxia and preplate splitting by ectopic expression of Reelin in reeler mice. *Neuron***33**: 573–586

Majercak J, Ray WJ, Espeseth A, Simon A, Shi X-P, Wolffe C, Getty K, Marine S, Stec E, Ferrer M, et al (2006) LRRTM3 promotes processing of amyloid-precursor protein by BACE1 and is a positional candidate gene for late-onset Alzheimer's disease. *Proc Natl Acad Sci U S A***103**: 17967–72

Mandai K, Guo T, Hillaire CS, Meabon JS, Kanning KC, Bothwell M, St Hillaire C, Ginty DD (2009) LIG family receptor tyrosine kinase-associated proteins modulate growth factor signals during neural development. *Neuron***63**: 614–27

Marillat V, Cases O, Nguyen-Ba-Charvet KT, Tessier-Lavigne M, Sotelo C, Chédotal A (2002) Spatiotemporal expression patterns of slit and robo genes in the rat brain. *J Comp Neurol***442**: 130–55

Marín O, Rubenstein JL (2001) A long, remarkable journey: tangential migration in the telencephalon. *Nat Rev Neurosci***2**: 780–790

Marin O, Rubenstein JLR (2002) Patterning, Regionalization, and Cell Differentiation in the Forebrain. *In* J Rossant, PPL Tam, eds, Mouse Dev. *Elsevier*, pp 75–106

Marín O, Rubenstein JLR (2003) Cell migration in the forebrain. *Annu Rev Neurosci***26**: 441–83

Marín O, Valiente M, Ge X, Tsai L-H (2010) Guiding neuronal cell migrations. *Cold Spring Harb Perspect Biol***2**: a001834

Marín O, Yaron a, Bagri a, Tessier-Lavigne M, Rubenstein JL (2001) Sorting of striatal and cortical interneurons regulated by semaphorin-neuropilin interactions. *Science***293**: 872–875

Martínez-Cerdeño V, Noctor SC, Kriegstein AR (2006) The role of intermediate progenitor cells in the evolutionary expansion of the cerebral cortex. *Cereb Cortex***16 Suppl 1**: i152–61

Martini FJ, Valiente M, López Bendito G, Szabó G, Moya F, Valdeolmillos M, Marín O (2009) Biased selection of leading process branches mediates chemotaxis during tangential neuronal migration. *Development***136**: 41–50

Matsushima N, Tachi N, Kuroki Y, Enkhbayar P, Osaki M, Kamiya M, Kretsinger RH (2005) Structural analysis of leucine-rich-repeat variants in proteins associated with human diseases. *Cell Mol Life Sci***62**: 2771–2791

McSherry GM, Smart IH (1986) Cell production gradients in the developing ferret isocortex. *J Anat***144**: 1–14

Mesngon MT, Tarricone C, Hebbar S, Guillotte AM, Schmitt EW, Lanier L, Musacchio A, King SJ, Smith DS (2006) Regulation of cytoplasmic dynein ATPase by Lis1. *J Neurosci***26**: 2132–9

Métin C, Deléglise D, Serafini T, Kennedy TE, Tessier-Lavigne M (1997) A role for netrin-1 in the guidance of cortical efferents. *Development***124**: 5063–74

Meyer G (2002) Selective Expression of Doublecortin and LIS1 in Developing Human Cortex Suggests Unique Modes of Neuronal Movement. *Cereb Cortex***12**: 1225–1236

Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, Levesque M, Allaire N, Perrin S, Sands B, et al (2004) LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat Neurosci***7**: 221–8

Miller JA, Horvath S, Geschwind DH (2010) Divergence of human and mouse brain transcriptome highlights Alzheimer disease pathways. *Proc Natl Acad Sci U S A***107**: 12698–703

Mione MC, Cavanagh JF, Harris B, Parnavelas JG (1997) Cell fate specification and symmetrical/asymmetrical divisions in the developing cerebral cortex. *J Neurosci***17**: 2018–2029

Mitrofanis J, Guillery RW (1993) New views of the thalamic reticular nucleus in the adult and the developing brain. *Trends Neurosci***16**: 240–5

Miyagi S, Saito T, Mizutani K -i., Masuyama N, Gotoh Y, Iwama A, Nakauchi H, Masui S, Niwa H, Nishimoto M, et al (2004) The Sox-2 Regulatory Regions Display Their Activities in Two Distinct Types of Multipotent Stem Cells. *Mol Cell Biol***24**: 4207–4220

Miyata T, Kawaguchi A, Okano H, Ogawa M (2001) Asymmetric inheritance of radial glial fibers by cortical neurons. *Neuron***31**: 727–741

Miyata T, Ogawa M (2007) Twisting of neocortical progenitor cells underlies a spring-like mechanism for daughter-cell migration. *Curr Biol***17**: 146–51

Mo Z, Zecevic N (2008) Is Pax6 critical for neurogenesis in the human fetal brain? *Cereb Cortex***18**: 1455–65

Molnar Z (2003) Choreography of Early Thalamocortical Development. *Cereb Cortex***13**: 661–669

Molnár Z, Blakemore C (1995) How do thalamic axons find their way to the cortex? *Trends Neurosci***18**: 389–397

Molnár Z, Cordery P (1999) Connections between cells of the internal capsule, thalamus, and cerebral cortex in embryonic rat. *J Comp Neurol***413**: 1–25

Molnár Z, Garel S, López-Bendito G, Maness P, Price DJ (2012) Mechanisms controlling the guidance of thalamocortical axons through the embryonic forebrain. *Eur J Neurosci***35**: 1573–85

Molyneaux BJ, Arlotta P, Menezes JRL, Macklis JD (2007) Neuronal subtype specification in the cerebral cortex. *Nat Rev Neurosci***8**: 427–37

Montiel JF, Wang WZ, Oeschger FM, Hoerder-Suabedissen A, Tung WL, García-Moreno F, Holm IE, Villalón A, Molnár Z (2011) Hypothesis on the dual origin of the Mammalian subplate. *Front Neuroanat***5**: 25

Morante-Oria J, Carleton A, Ortino B, Kremer EJ, Fairén A, Lledo P-M (2003) Subpallial origin of a population of projecting pioneer neurons during corticogenesis. *Proc Natl Acad Sci U S A***100**: 12468–73

Morante-Redolat JM, Gorostidi-Pagola A, Piquer-Sirerol S, Sáenz A, Poza JJ, Galán J, Gesk S, Sarafidou T, Mautner V-F, Binelli S, et al (2002) Mutations in the LGI1/Epitempin gene on 10q24 cause autosomal dominant lateral temporal epilepsy. *Hum Mol Genet***11**: 1119–28

Morimura N, Inoue T, Katayama K, Aruga J (2006) Comparative analysis of structure , expression and PSD95-binding capacity of Lrfn , a novel family of neuronal transmembrane proteins. *Gene***380**: 72–83

Moya F, Valdeolmillos M (2004) Polarized increase of calcium and nucleokinesis in tangentially migrating neurons. *Cereb Cortex***14**: 610–618

Nadarajah B, Alifragis P, Wong ROL, Parnavelas JG (2002) Ventricle-directed migration in the developing cerebral cortex. *Nat Neurosci***5**: 218–24

Nadarajah B, Brunstrom JE, Grutzendler J, Wong RO, Pearlman AL (2001) Two modes of radial migration in early development of the cerebral cortex. *Nat Neurosci***4**: 143–50

Nadarajah B, Parnavelas JG (2002) Modes of neuronal migration in the developing cerebral cortex. *Nat Rev Neurosci***3**: 423–432

Nakai K, Kanehisa M (1992) A knowledge base for predicting protein localization sites in eukaryotic cells. *Genomics***14**: 897–911

Nam J, Mah W, Kim E (2011) The SALM/Lrfn family of leucine-rich repeat-containing cell adhesion molecules. *Semin Cell Dev Biol***22**: 492–498

Nasrallah IM, McManus MF, Pancoast MM, Wynshaw-Boris A, Golden JA (2006) Analysis of non-radial interneuron migration dynamics and its disruption in Lis1+/- mice. *J Comp Neurol***496**: 847–58

Nóbrega-Pereira S, Kessaris N, Du T, Kimura S, Anderson SA, Mar'in O (2008) Postmitotic Nkx2-1 controls the migration of telencephalic interneurons by direct repression of guidance receptors. *Neuron***59**: 733–745

Nóbrega-Pereira S, Marín O (2009) Transcriptional control of neuronal migration in the developing mouse brain. *Cereb Cortex***19 Suppl 1**: i107–13

Noctor SC, Flint AC, Weissman TA, Dammerman RS, Kriegstein AR (2001) Neurons derived from radial glial cells establish radial units in neocortex. *Nature***409**: 714–720

Noctor SC, Martínez-Cerdeño V, Ivic L, Kriegstein AR (2004) Cortical neurons arise in symmetric and asymmetric division zones and migrate through specific phases. *Nat Neurosci***7**: 136–44

O'Sullivan ML, de Wit J, Savas JN, Comoletti D, Otto-Hitt S, Yates JR, Ghosh A (2012) FLRT proteins are endogenous latrophilin ligands and regulate excitatory synapse development. *Neuron***73**: 903–910

Ogawa M, Miyata T, Nakajima K, Yagyu K, Seike M, Ikenaka K, Yamamoto H, Mikoshiba K (1995) The reeler gene-associated antigen on Cajal-Retzius neurons is a crucial molecule for laminar organization of cortical neurons. *Neuron***14**: 899–912

Okafuji T, Tanaka H (2005) Expression pattern of LINGO-1 in the developing nervous system of the chick embryo. *Gene Expr Patterns***6**: 57–62

Oldham MC, Konopka G, Iwamoto K, Langfelder P, Kato T, Horvath S, Geschwind DH (2008) Functional organization of the transcriptome in human brain. *Nat Neurosci***11**: 1271–82

Olk S, Zoidl G, Dermietzel R (2009) Connexins, cell motility, and the cytoskeleton. *Cell Motil Cytoskelet***66**: 1000–16

Ono T, Sekino-Suzuki N, Kikkawa Y, Yonekawa H, Kawashima S (2003) Alivin 1, a novel neuronal activity-dependent gene, inhibits apoptosis and promotes survival of cerebellar granule neurons. *J Neurosci***23**: 5887–5896

Pacary E, Heng J, Azzarelli R, Riou P, Castro D, Lebel-Potter M, Parras C, Bell DM, Ridley AJ, Parsons M, et al (2011) Proneural transcription factors regulate different steps of cortical neuron migration through Rnd-mediated inhibition of RhoA signaling. *Neuron***69**: 1069–1084

Paredes MF, Li G, Berger O, Baraban SC, Pleasure SJ (2006) Stromal-derived factor-1 (CXCL12) regulates laminar position of Cajal-Retzius cells in normal and dysplastic brains. *J Neurosci***26**: 9404–12

Parnavelas JG (2000) The origin and migration of cortical neurones: new vistas. *Trends Neurosci***23**: 126–31

Pasterkamp RJ, Kolodkin AL (2003) Semaphorin junction: making tracks toward neural connectivity. *Curr Opin Neurobiol***13**: 79–89

Pearce IA, Cambray-Deakin MA, Burgoyne RD (1987) Glutamate acting on NMDA receptors stimulates neurite outgrowth from cerebellar granule cells. *FEBS Lett***223**: 143–7

Pelvig DP, Pakkenberg H, Stark AK, Pakkenberg B (2008) Neocortical glial cell numbers in human brains. *Neurobiol Aging***29**: 1754–1762

Pevny LH, Lovell-Badge R (1997) Sox genes find their feet. *Curr Opin Genet Dev***7**: 338–344

Pilz DT, Matsumoto N, Minnerath S, Mills P, Gleeson JG, Allen KM, Walsh CA, Barkovich AJ, Dobyns WB, Ledbetter DH, et al (1998) LIS1 and XLIS (DCX) mutations cause most classical lissencephaly, but different patterns of malformation. *Hum Mol Genet***7**: 2029–37

Piñon MC, Jethwa A, Jacobs E, Campagnoni A, Molnár Z (2009) Dynamic integration of subplate neurons into the cortical barrel field circuitry during postnatal development in the Golli-tau-eGFP (GTE) mouse. *J Physiol***587**: 1903–15

Polleux F, Whitford KL, Dijkhuizen PA, Vitalis T, Ghosh A (2002) Control of cortical interneuron migration by neurotrophins and PI3-kinase signaling. *Development***129**: 3147–60

Des Portes V, Francis F, Pinard JM, Desguerre I, Moutard ML, Snoeck I, Meiners LC, Capron F, Cusmai R, Ricci S, et al (1998) doublecortin is the major gene causing X-linked subcortical laminar heterotopia (SCLH). *Hum Mol Genet***7**: 1063–70

Powell EM, Mars WM, Levitt P (2001) Hepatocyte growth factor/scatter factor is a motogen for interneurons migrating from the ventral to dorsal telencephalon. *Neuron***30**: 79–89

Pozas E, Ibáñez CF (2005) GDNF and GFRalpha1 promote differentiation and tangential migration of cortical GABAergic neurons. *Neuron***45**: 701–713

Preuss TM, Cáceres M, Oldham MC, Geschwind DH (2004) Human brain evolution: insights from microarrays. *Nat Rev Genet***5**: 850–60

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

Del R'io JA, Mart'inez A, Fonseca M, Auladell C, Soriano E, del Río JA, Martínez A (1995) Glutamate-like immunoreactivity and fate of Cajal-Retzius cells in the murine cortex as identified with calretinin antibody. *Cereb Cortex***5**: 13–21

Rabenau KE, O'Toole JM, Bassi R, Kotanides H, Witte L, Ludwig DL, Pereira DS (2004) DEGA/AMIGO-2, a leucine-rich repeat family member, differentially expressed in human gastric adenocarcinoma: effects on ploidy, chromosomal stability, cell adhesion/migration and tumorigenicity. *Oncogene***23**: 5056–67

Rafidi H, Mercado F, Astudillo M, Fry WHD, Saldana M, Carraway KL, Sweeney C (2013) Leucine-rich repeat and immunoglobulin domain-containing protein-1 (Lrig1) negative regulatory action toward ErbB receptor tyrosine kinases is opposed by leucine-rich repeat and immunoglobulin domain-containing protein 3 (Lrig3). *J Biol Chem***288**: 21593–21605

Rakic P (1982) Early developmental events: cell lineages, acquisition of neuronal positions, and areal and laminar development. *Neurosci Res Program Bull***20**: 439–51

Rakic P (1990) Principles of neural cell migration. *Experientia***46**: 882–91

Rakic P (1972) Mode of cell migration to the superficial layers of fetal monkey neocortex. *J Comp Neurol***145**: 61–83

Rakic P (1988) Specification of cerebral cortical areas. *Science***241**: 170–6

Rakic P (1995) Radial versus tangential migration of neuronal clones in the developing cerebral cortex. *Proc Natl Acad Sci U S A***92**: 11323–7

Rakic P (1977) Prenatal development of the visual system in rhesus monkey. *Philos Trans R Soc Lond B Biol Sci***278**: 245–60

Rakic P, Hashimoto-Torii K, Sarkisian MR (2007) Genetic determinants of neuronal migration in the cerebral cortex. *Novartis Found Symp***288**: 45–53; discussion 53–8, 96–8

Rakic P, Stensas LJ, Sayre E, Sidman RL (1974) Computer-aided three-dimensional reconstruction and quantitative analysis of cells from serial electron microscopic montages of foetal monkey brain. *Nature***250**: 31–4

Raper JA (2000) Semaphorins and their receptors in vertebrates and invertebrates. *Curr Opin Neurobiol***10**: 88–94

Reillo I, de Juan Romero C, García-Cabezas MÁ, Borrell V (2011) A role for intermediate radial glia in the tangential expansion of the mammalian cerebral cortex. *Cereb Cortex***21**: 1674–94

Rice DS, Curran T (1999) Mutant mice with scrambled brains: understanding the signaling pathways that control cell positioning in the CNS. *Genes Dev***13**: 2758–73

Richards LJ, Koester SE, Tuttle R, O'Leary DD (1997) Directed growth of early cortical axons is influenced by a chemoattractant released from an intermediate target. *J Neurosci***17**: 2445–58

Del Rio JA, Soriano E, Ferrer I (1992) Development of GABA-immunoreactivity in the neocortex of the mouse. *J Comp Neurol***326**: 501–26

Del Rio T, Nishitani AM, Yu W-M, Goodrich L V (2013) In vivo analysis of Lrig genes reveals redundant and independent functions in the inner ear. *PLoS Genet***9**: e1003824

Round J, Stein E (2007) Netrin signaling leading to directed growth cone steering. *Curr Opin Neurobiol***17**: 15–21

Russ AP, Wattler S, Colledge WH, Aparicio SA, Carlton MB, Pearce JJ, Barton SC, Surani MA, Ryan K, Nehls MC, et al (2000) Eomesodermin is required for mouse trophoblast development and mesoderm formation. *Nature***404**: 95–9

Sapir T, Elbaum M, Reiner O (1997) Reduction of microtubule catastrophe events by LIS1, platelet-activating factor acetylhydrolase subunit. *EMBO J***16**: 6977–6984

Sasai Y (2001) Roles of Sox factors in neural determination: conserved signaling in evolution? *Int J Dev Biol***45**: 321–6

Sasaki S, Mori D, Toyo-oka K, Chen A, Garrett-Beal L, Muramatsu M, Miyagawa S, Hiraiwa N, Yoshiki A, Wynshaw-Boris A, et al (2005) Complete loss of Ndel1 results in neuronal migration defects and early embryonic lethality. *Mol Cell Biol***25**: 7812–27

Schaar BT, McConnell SK (2005) Cytoskeletal coordination during neuronal migration. *Proc Natl Acad Sci U S A***102**: 13652–13657

Schmid RS, Shelton S, Stanco A, Yokota Y, Kreidberg JA, Anton ES (2004) alpha3beta1 integrin modulates neuronal migration and placement during early stages of cerebral cortical development. *Development***131**: 6023–31

Schwartz ML, Meinecke DL (1992) Early expression of GABA-containing neurons in the prefrontal and visual cortices of rhesus monkeys. *Cereb Cortex***2**: 16–37

Segura I, De Smet F, Hohensinner PJ, Ruiz de Almodovar C, Carmeliet P (2009) The neurovascular link in health and disease: an update. *Trends Mol Med***15**: 439–51

Sekine K, Honda T, Kawauchi T, Kubo K, Nakajima K (2011) The outermost region of the developing cortical plate is crucial for both the switch of the radial migration mode and the Dab1-dependent "inside-out" lamination in the neocortex. *J Neurosci***31**: 9426–39

Sessa A, Mao C-A, Colasante G, Nini A, Klein WH, Broccoli V (2010) Tbr2-positive intermediate (basal) neuronal progenitors safeguard cerebral cortex expansion by

controlling amplification of pallial glutamatergic neurons and attraction of subpallial GABAergic interneurons. *Genes Dev***24**: 1816–26

Sessa A, Mao C-AA, Hadjantonakis A-KK, Klein WH, Broccoli V (2008) Tbr2 directs conversion of radial glia into basal precursors and guides neuronal amplification by indirect neurogenesis in the developing neocortex. *Neuron***60**: 56–69

Shatz CJ, Rakic P (1981) The genesis of efferent connections from the visual cortex of the fetal rhesus monkey. *J Comp Neurol***196**: 287–307

Sheppard AM, Hamilton SK, Pearlman AL (1991) Changes in the distribution of extracellular matrix components accompany early morphogenetic events of mammalian cortical development. *J Neurosci***11**: 3928–42

Sheppard AM, Pearlman AL (1997) Abnormal reorganization of preplate neurons and their associated extracellular matrix: an early manifestation of altered neocortical development in the reeler mutant mouse. *J Comp Neurol***378**: 173–9

Shmelkov S V, Hormigo A, Jing D, Proenca CC, Bath KG, Milde T, Shmelkov E, Kushner JS, Baljevic M, Dincheva I, et al (2010) Slitrk5 deficiency impairs corticostriatal circuitry and leads to obsessive-compulsive-like behaviors in mice. *Nat Med***16**: 598–602, 1p following 602

Shu T, Richards LJ (2001) Cortical axon guidance by the glial wedge during the development of the corpus callosum. *J Neurosci***21**: 2749–58

Simon AM, Goodenough DA (1998) Diverse functions of vertebrate gap junctions. *Trends Cell Biol***8**: 477–483

Smart IHM, Dehay C, Giroud P, Berland M, Kennedy H (2002) Unique morphological features of the proliferative zones and postmitotic compartments of the neural epithelium giving rise to striate and extrastriate cortex in the monkey. *Cereb Cortex***12**: 37–53

Smith DS, Niethammer M, Ayala R, Zhou Y, Gambello MJ, Wynshaw-Boris A, Tsai LH (2000) Regulation of cytoplasmic dynein behaviour and microtubule organization by mammalian Lis1. *Nat Cell Biol***2**: 767–75

Solecki DJ, Govek E-EE, Tomoda T, Hatten ME (2006) Neuronal polarity in CNS development. *Genes Dev***20**: 2639–47

Solecki DJ, Model L, Gaetz J, Kapoor TM, Hatten ME (2004) Par6alpha signaling controls glial-guided neuronal migration. *Nat Neurosci***7**: 1195–1203

Sousa I, Clark TG, Holt R, Pagnamenta AT, Mulder EJ, Minderaa RB, Bailey AJ, Battaglia A, Klauck SM, Poustka F, et al (2010) Polymorphisms in leucine-rich repeat genes are associated with autism spectrum disorder susceptibility in populations of European ancestry. *Mol Autism***1**: 7

Stanco A, Szekeres C, Patel N, Rao S, Campbell K, Kreidberg JA, Polleux F, Anton ES (2009) Netrin-1-alpha3beta1 integrin interactions regulate the migration of interneurons through the cortical marginal zone. *Proc Natl Acad Sci U S A***106**: 7595–600

Stitt TN, Hatten ME (1990) Antibodies that recognize astrotactin block granule neuron binding to astroglia. *Neuron***5**: 639–49

Stossel TP, Condeelis J, Cooley L, Hartwig JH, Noegel A, Schleicher M, Shapiro SS (2001) Filamins as integrators of cell mechanics and signalling. *Nat Rev Mol Cell Biol***2**: 138–45

Stumm RK, Zhou C, Ara T, Lazarini F, Dubois-Dalcq M, Nagasawa T, Höllt V, Schulz S (2003) CXCR4 regulates interneuron migration in the developing neocortex. *J Neurosci***23**: 5123–30

Tabata H, Kanatani S, Nakajima K (2009) Differences of migratory behavior between direct progeny of apical progenitors and basal progenitors in the developing cerebral cortex. *Cereb Cortex***19**: 2092–2105

Tabata H, Nakajima K (2003) Multipolar migration: the third mode of radial neuronal migration in the developing cerebral cortex. *J Neurosci***23**: 9996–10001

Tabata H, Yoshinaga S, Nakajima K (2012) Cytoarchitecture of mouse and human subventricular zone in developing cerebral neocortex. *Exp brain Res***216**: 161–8

Takiguchi-Hayashi K, Sekiguchi M, Ashigaki S, Takamatsu M, Hasegawa H, Suzuki-Migishima R, Yokoyama M, Nakanishi S, Tanabe Y (2004) Generation of reelin-positive marginal zone cells from the caudomedial wall of telencephalic vesicles. *J Neurosci***24**: 2286–2295

Talamillo A, Quinn JC, Collinson JM, Caric D, Price DJ, West JD, Hill RE (2003) Pax6 regulates regional development and neuronal migration in the cerebral cortex. *Dev Biol***255**: 151–63

Tamamaki N, Fujimori KE, Takauji R (1997) Origin and route of tangentially migrating neurons in the developing neocortical intermediate zone. *J Neurosci***17**: 8313–8323

Tan SS, Breen S (1993) Radial mosaicism and tangential cell dispersion both contribute to mouse neocortical development. *Nature***362**: 638–640

Tan SS, Kalloniatis M, Sturm K, Tam PP, Reese BE, Faulkner-Jones B (1998) Separate progenitors for radial and tangential cell dispersion during development of the cerebral neocortex. *Neuron***21**: 295–304

Tanaka D, Nakaya Y, Yanagawa Y, Obata K, Murakami F (2003) Multimodal tangential migration of neocortical GABAergic neurons independent of GPI-anchored proteins. *Development***130**: 5803–13

Tanaka DH, Maekawa K, Yanagawa Y, Obata K, Murakami F (2006) Multidirectional and multizonal tangential migration of GABAergic interneurons in the developing cerebral cortex. *Development***133**: 2167–76

Tanaka DH, Yanagida M, Zhu Y, Mikami S, Nagasawa T, Miyazaki J, Yanagawa Y, Obata K, Murakami F (2009) Random walk behavior of migrating cortical interneurons in the marginal zone: time-lapse analysis in flat-mount cortex. *J Neurosci***29**: 1300–1311

Taniguchi H, Tohyama M, Takagi T (1996) Research report Cloning and expression of a novel gene for a protein with leucine-rich repeats in the developing mouse nervous system. *Brain Res Mol Brain Res***36**: 45–52

Teissier A, Griveau A, Vigier L, Piolot T, Borello U, Pierani A (2010) A novel transient glutamatergic population migrating from the pallial-subpallial boundary contributes to neocortical development. *J Neurosci***30**: 10563–10574

Tessier-Lavigne M, Goodman CS (1996) The Molecular Biology of Axon Guidance. *Science (80-)***274**: 1123–1133

Tiveron M-C, Rossel M, Moepps B, Zhang YL, Seidenfaden R, Favor J, König N, Cremer H (2006) Molecular interaction between projection neuron precursors and invading interneurons via stromal-derived factor 1 (CXCL12)/CXCR4 signaling in the cortical subventricular zone/intermediate zone. *J Neurosci***26**: 13273–8

Ton CCT, Hirvonen H, Miwa H, Weil MM, Monaghan P, Jordan T, van Heyningen V, Hastie ND, Meijers-Heijboer H, Drechsler M, et al (1991) Positional cloning and characterization of a paired box- and homeobox-containing gene from the aniridia region. *Cell***67**: 1059–1074

Tsai L-H, Gleeson JG (2005) Nucleokinesis in neuronal migration. *Neuron***46**: 383–8

Tsai M-L, Leung LS (2006) Decrease of hippocampal GABA B receptor-mediated inhibition after hyperthermia-induced seizures in immature rats. *Epilepsia***47**: 277–87

Tsuji L, Yamashita T, Kubo T, Madura T, Tanaka H, Hosokawa K, Tohyama M (2004) FLRT3, a cell surface molecule containing LRR repeats and a FNIII domain, promotes neurite outgrowth. *Biochem Biophys Res Commun***313**: 1086–1091

Uchikawa M, Ishida Y, Takemoto T, Kamachi Y, Kondoh H (2003) Functional Analysis of Chicken Sox2 Enhancers Highlights an Array of Diverse Regulatory Elements that Are Conserved in Mammals. *Dev Cell***4**: 509–519

Uchikawa M, Takemoto T, Kamachi Y, Kondoh H (2004) Efficient identification of regulatory sequences in the chicken genome by a powerful combination of embryo electroporation and genome comparison. *Mech Dev***121**: 1145–58

Valiente M, Marín O (2010) Neuronal migration mechanisms in development and disease. *Curr Opin Neurobiol***20**: 68–78

Vallender EJ, Mekel-Bobrov N, Lahn BT (2008) Genetic basis of human brain evolution. *Trends Neurosci***31**: 637–44

Walsh C, Cepko CL (1988) Clonally related cortical cells show several migration patterns. *Science***241**: 1342–5

Wang C-Y, Chang K, Petralia RS, Wang Y-X, Seabold GK, Wenthold RJ (2006) A novel family of adhesion-like molecules that interacts with the NMDA receptor. *J Neurosci***26**: 2174–83

Wang PY, Seabold GK, Wenthold RJ (2008) Synaptic adhesion-like molecules (SALMs) promote neurite outgrowth. *Mol Cell Neurosci***39**: 83–94

Wang WZ, Oeschger FM, Montiel JF, García-Moreno F, Hoerder-Suabedissen A, Krubitzer L, Ek CJ, Saunders NR, Reim K, Villalón A, et al (2011) Comparative aspects of subplate zone studied with gene expression in sauropsids and mammals. *Cereb Cortex***21**: 2187–203

Wegner M (1999) From head to toes: the multiple facets of Sox proteins. *Nucleic Acids Res***27**: 1409–20

Wegner M, Stolt CC (2005) From stem cells to neurons and glia: a Soxist's view of neural development. *Trends Neurosci***28**: 583–8

Weissman TA, Riquelme PA, Ivic L, Flint AC, Kriegstein AR (2004) Calcium waves propagate through radial glial cells and modulate proliferation in the developing neocortex. *Neuron***43**: 647–661

Welker WI (1990) The significance of foliation and fissuration of cerebellar cortex. The cerebellar folium as a fundamental unit of sensorimotor integration. *Arch Ital Biol***128**: 87–109

Wheldon LM, Haines BP, Rajappa R, Mason I, Rigby PW, Heath JK (2010) Critical role of FLRT1 phosphorylation in the interdependent regulation of FLRT1 function and FGF receptor signalling. *PLoS One***5**: e10264

Wichterle H, Garcia-Verdugo JM, Alvarez-Buylla A (1997) Direct evidence for homotypic, glia-independent neuronal migration. *Neuron***18**: 779–91

Wichterle H, Garcia-Verdugo JM, Herrera DG, Alvarez-Buylla A (1999) Young neurons from medial ganglionic eminence disperse in adult and embryonic brain. *Nat Neurosci***2**: 461–6

Wichterle H, Turnbull DH, Nery S, Fishell G, Alvarez-Buylla A (2001) In utero fate mapping reveals distinct migratory pathways and fates of neurons born in the mammalian basal forebrain. *Development***128**: 3759–71

Winberg ML, Noordermeer JN, Tamagnone L, Comoglio PM, Spriggs MK, Tessier-Lavigne M, Goodman CS (1998) Plexin A is a neuronal semaphorin receptor that controls axon guidance. *Cell***95**: 903–16

Wong K, Park HT, Wu JY, Rao Y (2002) Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr Opin Genet Dev***12**: 583–91

Woo J, Kwon S-K, Kim E (2009) The NGL family of leucine-rich repeat-containing synaptic adhesion molecules. *Mol Cell Neurosci***42**: 1–10

Wynshaw-Boris A, Gambello MJ (2001) LIS1 and dynein motor function in neuronal migration and development. *Genes Dev***15**: 639–51

Xie Z, Sanada K, Samuels BA, Shih H, Tsai LH (2003) Serine 732 phosphorylation of FAK by Cdk5 is important for microtubule organization, nuclear movement, and neuronal migration. *Cell***114**: 469–82

Yamagishi S, Hampel F, Hata K, Del Toro D, Schwark M, Kvachnina E, Bastmeyer M, Yamashita T, Tarabykin V, Klein R, et al (2011) FLRT2 and FLRT3 act as repulsive guidance cues for Unc5-positive neurons. *EMBO J***30**: 2920–33

Yau H-J, Wang H-F, Lai C, Liu F-C (2003) Neural development of the neuregulin receptor ErbB4 in the cerebral cortex and the hippocampus: preferential expression by interneurons tangentially migrating from the ganglionic eminences. *Cereb Cortex***13**: 252–64

Ybot-Gonzalez P, Cogram P, Gerrelli D, Copp AJ (2002) Sonic hedgehog and the molecular regulation of mouse neural tube closure. *Development***129**: 2507–2517

Yee KT, Simon HH, Tessier-Lavigne M, O'Leary DM (1999) Extension of long leading processes and neuronal migration in the mammalian brain directed by the chemoattractant netrin-1. *Neuron***24**: 607–22

Yoshida M, Assimacopoulos S, Jones KR, Grove EA (2006) Massive loss of Cajal-Retzius cells does not disrupt neocortical layer order. *Development***133**: 537–545

Yuste R, Peinado A, Katz LC (1992) Neuronal domains in developing neocortex. *Science (80-)***257**: 665–669

Zecevic N, Chen Y, Filipovic R (2005) Contributions of cortical subventricular zone to the development of the human cerebral cortex. *J Comp Neurol***491**: 109–22

Zhao C, Li K, Li J, Zheng W, Liang X, Geng A, Li N, Yuan X (2009) PKCdelta regulates cortical radial migration by stabilizing the Cdk5 activator p35. *Proc Natl Acad Sci U S A***106**: 21353–8

Zhu Y, Li H, Zhou L, Wu JY, Rao Y (1999) Cellular and molecular guidance of GABAergic neuronal migration from an extracortical origin to the neocortex. *Neuron***23**: 473–485

Zipursky SL, Wojtowicz WM, Hattori D (2006) Got diversity? Wiring the fly brain with Dscam. *Trends Biochem Sci***31**: 581–8